1995

Ca2+ signaling in porcine myometrial cells: Ca2+ channels, intracellular Ca2+ stores and guanine nucleotide-binding protein-coupled receptors

Ronghua ZhuGe
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Ca\(^{2+}\) signaling in porcine myometrial cells: Ca\(^{2+}\) channels, intracellular Ca\(^{2+}\)
stores and guanine nucleotide-binding protein-coupled receptors

by

Ronghua ZhuGe

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Signature was redacted for privacy.
In Charge of Major Work
Signature was redacted for privacy.
For the Major Department
Signature was redacted for privacy.
For the Graduate College

Iowa State University
Ames, Iowa

1995
Ca$^{2+}$ signaling in porcine myometrial cells: Ca$^{2+}$ channels, intracellular Ca$^{2+}$ stores and guanine nucleotide-binding protein (G protein)-coupled receptors

Ronghua ZhuGe

Major professor: Walter H. Hsu
Iowa State University

Fura-2 spectrofluorometry and imaging, and whole-cell patch-clamp techniques were used to characterize Ca$^{2+}$ channels and intracellular Ca$^{2+}$ stores for exploring signal transduction of the oxytocin receptor and the $\alpha_2A$-adrenergic receptor in freshly dispersed porcine myometrial cells. Two types of voltage-dependent Ca$^{2+}$ channels, i.e., L-type and T-type, were found in 8% of myometrial cells while in 92% of the cells, only L-type Ca$^{2+}$ channels were detected. The analysis of occurrence, voltage dependence and kinetics of Ca$^{2+}$ channels suggested that L-type channels were responsible for the delivery of Ca$^{2+}$ into cell upon membrane depolarization and T-type channel may be involved in uterine pacemaking. In addition to the Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ store, the myometrial cells contained the caffeine- and ryanodine-sensitive store. Ca$^{2+}$ release from the caffeine- and ryanodine-sensitive store in a fashion of quanta, in which quanta was defined as part of stores in 70% and the entire stores in 30% of cells. Activation of oxytocin receptors and $\alpha_2A$-adrenergic receptors induced an increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) through Ca$^{2+}$ release from intracellular stores and influx from extracellular environment. The contribution of influx and release was variable between two receptors: the release was a predominate component for oxytocin receptors while the influx is the major one for $\alpha_2A$-adrenergic receptors. The release process posed by activation of oxytocin receptors and $\alpha_2A$-adrenergic receptors was mediated by activation of Ins(1,4,5)P$_3$ receptors via a pertussis toxin (PTX)-insensitive G protein-phospholipase C-Ins (1,4,5)P$_3$ cascade. Oxytocin receptor-mediated influx was predominately attributed to opening of receptor-operated Ca$^{2+}$ channels, and to a lesser extent by L-type Ca$^{2+}$ channels. The capacitative Ca$^{2+}$ entry mechanism could not account for activation of these channels, instead, it required a concomitant formation of Ins(1,4,5)P$_3$ and depletion of
Ca\textsuperscript{2+} stores. On the other hand, the depletion of Ca\textsuperscript{2+} stores was not involved in \( \sigma_{2A} \)-adrenergic receptor-induced Ca\textsuperscript{2+} influx. Instead this influx was primarily mediated by opening of L-type Ca\textsuperscript{2+} channels which resulted from a decrease in cAMP through a PTX-sensitive G protein-adenylyl cyclase mechanism, or a direct coupling of the channels with a PTX-sensitive G protein.
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LIST OF ABBREVIATIONS

AR  adrenergic receptor
CaM  calmodulin
DHP  dihydropyridine
EGTA  [ethylenebis(oxyethylenenitrilo)] tetraacetic acid
Epi  epinephrine
Fura-2 AM  fura-2 acetoxymethyl ester
G protein  guanine nucleotide-binding protein
HBSS  Hank’s balanced salt solution
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I  current
I_{Ca}  Ca^{2+} current
I_{Ba}  Ba^{2+} current
IP_{3}  inositol 1,4,5-trisphosphate/Ca^{2+}-releasing channel
IP_{3R}  inositol 1,4,5-trisphosphate receptor
KRB  Krebs-Ringer bicarbonate solution
MLC  myosin light chain
MLCK  myosin light chain kinase
MLCP  myosin light chain phosphatase
OT  oxytocin
PG  prostaglandin
PKA  cAMP-dependent protein kinase
PKG  cGMP-dependent protein kinase
PLC  phospholipase C
PTX  pertussis toxin
ROC  receptor-operated Ca^{2+} channel
RyR  ryanodine receptor/Ca^{2+}-releasing channel
SR  sarcoplasmic reticulum
TG  thapsigargin
V  potential or voltage
VDCC  voltage-dependent Ca^{2+} channel
extracellular Ca\(^{2+}\) concentration
intracellular Ca\(^{2+}\) concentration
Ca\(^{2+}\)-induced Ca\(^{2+}\) influx
CHAPTER I  GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains five research papers preceded by a general introduction and a rationale, and followed by a general discussion, a summary and a list of references cited in the general introduction and discussion. The general introduction includes a research objective and a background and literature review. Chapters I and IV represent two papers previously published, and Chapters II, III and V correspond to manuscripts submitted for publication in American Journal of Physiology, the Journal of Pharmacological and Experimental Therapeutics and Journal of Biological Chemistry, respectively.

This dissertation contains a large part of the experimental results obtained by the author during the course of his graduate study under the supervision of his major professor, Dr. Walter H. Hsu.

Research Objective

The uterus is essential for the continuation of life by providing the embryo an optimum implantation and development environment and expelling the fetus at term. To be successful, uterine contractility must be exquisitely coordinated, i.e. relatively quiescent through gestation and highly active during labor. Coordinated contraction of the uterus is also necessary for the spacing of embryos within the uterine horns in polytocous animals and may facilitate sperm movement within the reproductive tract in animals and humans. Unfortunately, these roles are not always performed perfectly, as shown in cases of preterm labor in human medicine and stillbirth in animal husbandry. Preterm labor is considered as a major cause of neonatal handicap and mortality in human medicine, and stillbirth is the major episode of reproductive loss in pig industry. Although the underlying reasons for these diseases are not well understood, abnormal contraction coupling has been suggested as one of the major factors involved. Although advances have been made in our understanding of the reproductive process in the past two decades, there has been no significant reduction in the incidence of
preterm labor in human medicine. Many therapeutic treatments have been employed to
decrease the rate of stillbirths in the animals without understanding their effects on
uterine contraction during the parturient process. Therefore, it would be clinically and
scientifically significant to understand more about how uterine contraction is produced
and modulated.

Most information on uterine contractility has been obtained from studies
conducted in rodents and humans. There is a paucity of research performed in pigs
and other economically important animals. In terms of pigs, anatomical and
physiological studies have provided a relatively detailed description of the organization
and functions of the uterus. These studies have emphasized the phenomenon of
smooth muscle contractility as well as its modulation by agonists in vivo and in in vitro
multiple cell preparations. There is, however, little information available on the cellular
and molecular basis of contraction and its regulation.

Calcium ions (Ca\(^{2+}\)) play a central role in contraction coupling in smooth muscle
cells. A rise in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) induces the contraction of
smooth muscle, and external stimuli modulate the contraction by altering [Ca\(^{2+}\)]. The
purpose of this research was to identify and characterize the voltage-dependent Ca\(^{2+}\)
channels (VDCC) and intracellular Ca\(^{2+}\) stores, two major components of Ca\(^{2+}\)
homeostasis, in isolated single porcine myometrial cells. Because the myometrial
contraction is tightly regulated by hormonal and neuronal modulators, we also
investigated the cellular mechanisms (signal transduction) involved in the regulation of
Ca\(^{2+}\) signals by the oxytocin receptors and \(\alpha_{2A}\)-adrenergic receptors (AR), two major
guanine nucleotide-binding protein (G protein)-coupled receptors in porcine myometrial
cells. Ca\(^{2+}\) current, which reflects VDCC activity and [Ca\(^{2+}\)], was evaluated by whole-
cell patch-clamp technique and fluorescence spectrofluorometry and imaging technique,
respectively.
Background and Literature Review

Architecture of myometrium

Myometrium

The myometrium is the middle coat of the uterus, a hollow organ opening on one end in oviducts and the other end into the cervix and vagina. In animals, such as pigs, with bicornuate uteri, the myometrium is formed with an outer longitudinal muscle layer, running parallel to the long axis of the uterus with bundles of myometrial cells, and an inner circular muscle layer, perpendicular to the long axis with a diffuse arrangement of myometrial cells. These two layers are separated by a vascular plexus. In the simplex uterus of primates, the arrangement of myometrial cells is more complex and consists of three or four muscle layers. The myometrial cells are embedded in connective tissues consisting of collagen, elastin, glycoprotein and proteoglycans. These components facilitate the transmission of the contractile force generated by muscle cells. The intercellular space is also occupied by nerves, capillaries and fibroblasts. Contraction of the longitudinal layer would tend to shorten the uterus and constrict the lumen while contraction of the circular layer would constrict the uterine lumen only. Functional and structural studies indicate that the longitudinal layer is continuous with the circular layer. But the cells forming the muscle layer arise from different embryologic origins and possess differences in their phenotypes. The longitudinal muscle originates from the subserosal connective tissue, whereas the circular muscle is derived from the paranephric ducts (Lambert et al., 1990). The two layers exhibit marked differences in their responsiveness to stimulatory and inhibitory agents and innervation patterns. For example, circular muscle is primarily endowed with cholinergic nerves and the longitudinal muscle with adrenergic nerves in pigs (Taneike et al., 1991).

Myometrial cells

Myometrial cells are long and spindle-shaped. The size of cells ranges from 100 to 600 μm in length and 5 to 10 μm in diameter (Csapo, 1962). The size and number vary with the reproductive stages. During pregnancy, myometrial cells undergo hyperplasia (increase in cell number) and hypertrophy (cell division with an increase in
size). The largest myometrial cells are observed at term when they grow 2-5 times as long as at the time of embryo implantation (Afting and Elce, 1978; Shoenberg, 1977). These changes are thought to be influenced by steroid hormones (estrogen and progesterone) and the physical stretch of the uterine wall to accommodate the growth of the fetus.

Sarcolemma and sarcoplasmic reticulum

The unit membrane that encloses the muscle cells is usually referred to as the sarcolemma. In smooth muscle, the sarcolemma consists of the plasma membrane, basal lamina, and immediately adjacent connective tissue. The plasma membrane of myometrial cells has a trilaminar in appearance the same as in all eukaryotic cells, and is covered by a morphologically ill-defined coat termed the basal lamina. In analogy to other smooth muscles, the plasma membrane in myometrial cells can be divided into two structural expanses occupied by caveolae or by dense bands. The caveolae are the membrane-bounded vesicles that project inward from the cell surface, retaining luminal continuity with the extracellular fluid. They measure about 70 nm across and 120 nm in length (Gabella, 1981). More than one-third of the plasma membrane at the cell surface forms caveolae. This structure increases the surface membrane area, leading to a large surface area/volume ratio. The larger surface area facilitates the communication between cells and between the interior and external environment. For example, Ca^{2+}-ATPase, the enzyme that extrudes Ca^{2+} from the cytosol and maintains Ca^{2+} homeostasis, and inositol 1,4,5-trisphosphosphate receptor (IP_{3}R), a protein probably involved in Ca^{2+} entry through the plasma membrane, is localized in the membrane of the caveolae (Carafoli, 1991, Fujimoto et al., 1992). Also, electron probes detect a high concentration of Ca^{2+} inside the caveolae of smooth muscle (Popescu and Diculescu, 1975).

In the areas of the cell surface that are not occupied by caveolae, electron-dense material adheres to the inner side of the cell membrane and constitutes the so-called dense bands. They are about 0.2-0.4 \( \mu m \) in width and extend for 1-2 \( \mu m \) or more along the cell length; they are about 30-50 nm thick (Gabella, 1984). Dense bands are distributed all over the cell surface, occupying 30-50% of the cell profile in the middle portion of a cell and a greater percentage toward the ends of the cell (Gabella, 1981).
The results of immunohistochemistry indicate that α-actinin, a component of Z lines of skeletal muscle, vinculin and talin are localized in the dense bands (Schollmeyer et al., 1976). Thin (actin) filaments and intermediate filaments penetrate into the dense bands. It appears, therefore, that dense bands are involved in the transmission of force from the contractile apparatus to the cell membrane. The proteins α-actinin, vinculin and talin in dense bands provide the molecular link between actin filaments and transmembrane proteins.

The sarcoplasmic reticulum (SR) is an intracellular membrane system of tubules and cisternae. The SR occupies from 1.5 to 7.5% of the total cell volume in various smooth muscles (Hartshorne, 1987). As in other smooth muscle, myometrial cells contain both smooth and rough SR that appear to be continuous. Smooth SR is most frequently found in close apposition to the cell membrane, while rough SR are found near the nuclear poles, or among the myofilaments. Electron microscopy studies indicate that SR is one of the most important Ca²⁺ reservoirs. The concentration of Ca²⁺ in SR ranges from 5.3 mM to 15 mM in smooth muscles (Missiaen et al., 1992). Most of the Ca²⁺ within the SR is bound to the Ca²⁺-binding protein, calsequestrin. There is substantial experimental evidence indicating that SR is the organelle primarily responsible for the physiological regulation of cytoplasmic Ca²⁺ in smooth muscles (therefore, in this dissertation SR and intracellular Ca²⁺ store are used interchangeably). The regulation is mediated by two types of Ca²⁺-releasing channels, IP₃R and ryanodine receptor (RyR), and one type of Ca²⁺-ATPase. IP₃Rs and Ca²⁺-ATPase have been found but RyR is to be defined in myometrial cells (see below).

There is surface coupling between plasma membrane and SR. Large cisternae of smooth SR can be found immediately beneath and parallel to the cell membrane. In areas of caveolae this association is analogous to the T tubules of skeletal muscle. The gap between the plasma membrane and SR is only about 10 nm and is traversed by an electron opaque "bridge structure" resembling the "feet" of the triads of striated muscle (Devine et al., 1972; Broderick and Broderick, 1990). This structure may facilitate the release of Ca²⁺ from the SR following an action potential. The surface couplings may also be major sites of signal transduction.
Cell-cell coupling

The myometrial cells communicate with each other by means of gap junctions, which form as a result of interactions between intramembranous proteins, connexins, with surrounding cell membrane. Hemi-channels in one cell membrane are able to align themselves with similar structures in the adjacent membrane to form a pore connecting the cytoplasm of the two cells. This process provides sites for low-resistance electrical or ionic coupling between cells and provides a pathway for transport of metabolites (less than 1-1.7 kDa) directly between cells (Garfield, 1994). No current recording of the channel has been made in myometrial cells. In the lacrimal gland and cardiac cells the conductance of a single gap junction was shown to be 50-170 pS (Neyton and Trautman, 1985; Burt and Spray 1988).

A change in size and number of gap junctions is associated with reproductive stages. Studies of pigs (Thilander and Rodriguez-Martinez, 1989b, 1990), rats (Garfield et al., 1977), guinea pigs (Garfield et al., 1982), sheep (Garfield et al., 1979) and humans (Garfield and Hayashi, 1981) demonstrated that (1) gap junctions are absent or present in low numbers during most of pregnancy; (2) at the end of pregnancy, gap junctions increase in size and number; (3) the junctions are present in high numbers and of large size during labor; and (4) the gap junctions disappear within 24 hr after labor. In myometrial cells of cycling pigs, the number of gap junctions is low and the size is small (Thilander and Rodriguez-Martinez, 1989a). The change in gap junctions are attributed to estrogen and progesterone. In general, progesterone inhibits, whereas estrogen stimulates, gap junction formation. In sheep and rats, there is a good correlation among gap junction formation and an increase in estrogen, a decrease in progesterone, and an increase in the estrogen/progesterone ratio in maternal and fetal blood and in uterine tissues (Garfield et al., 1979; Garfield et al., 1982; Puri and Garfield, 1982). These types of dynamic changes in gap junctions have significant implications for maintenance and termination of pregnancy. The relative absence of gap junctions between myometrial cells throughout gestation may maintain pregnancy by limiting electrical communication between cells, thereby preventing coordinated contraction of the uterus. The formation of gap junctions in the myometrial cells may initiate or allow initiation of parturition by providing low resistance pathways between muscle cells, allowing rapid, synchronized spread of action potentials, leading
to well-coordinated contractions (Garfield, 1994).

The opening and closing of gap junctions in the myometrium can be regulated. Conductance of gap junctions is rapidly and reversibly reduced by an increase in intracellular pH and Ca\(^{2+}\) (Peracchia and Peracchia, 1980a, 1980b). Presumably, H\(^+\) and Ca\(^{2+}\) act on the same gating structure, but the sensitivity to pH is much greater than to Ca\(^{2+}\) because of higher H\(^+\) binding affinity. Hormones and neurotransmitters also modulate the junctional permeability. Elevated cAMP by isoproterenol reduces cell-cell diffusion and electrical coupling in rat myometrial cells (Garfield, 1994). In other cell types this has been demonstrated to be mediated by cAMP-dependent protein kinase (PKA)-induced phosphorylation of connexins (Saez et al., 1986). The regulation of cAMP on gap junctions may have physiological implication, since hormones such as relaxin and catecholamine, which change cAMP formation, could influence pregnancy and parturition by alteration of the gap junction.

Several other types of cell-to-cell coupling have been observed between the myometrial cells (Broderick and Broderick, 1990, Thilander and Rodriguez-Martinez, 1989a; 1989b). They include (1) simple apposition, in which the opposing cell membranes running parallel to each other are separated by approximately 15 nm; (2) interdigitation where cell projections are inserted into invaginations of another cell; and (3) intermediate junctions in which the coupling is formed between two dense bands in adjacent cells. The functions for these couplings are not clear. In pigs, the highest synchronized myometrial cell activity occurs in estrus when the gap junctions are few. In addition, a number of smooth muscles which exhibit propagated contraction do not contain gap junctions (Gabella, 1981; Daniel et al., 1976). It seems, therefore, that these couplings may function as low-resistance pathways for propagation of contraction.

Mechanism of smooth muscle contraction

Pattern of myometrium activity

In general, during most of pregnancy the myometrium is poorly coordinated with synchronous local contraction at low frequency and amplitude, and a relative unresponsiveness to stimulation by contractants. As term approaches, the pattern of the contractile activity undergoes a remarkable transformation: myometrium is highly
coordinated and displays synchronous contraction with an increase in frequency and amplitude, and becomes highly responsive to contractants. After labor, the activity of myometrium is quickly turned to the level during the pregnancy. In the estrous cycle, the myometrium show a highly coordinated contractility with the highest motility occurring during estrus and metestrus.

**Contractile proteins**

Major contractile proteins, actin, myosin and tropomyosin, have been found in myometrial cells from various species including pigs. In rat myometrium, the contents (mg/g weight) of these proteins are 22.5 ± 2.7, 23.9 ± 2.9 and 8.1 ± 2.0, respectively (Hartshore, 1987). In comparison, they are approximately 20.6 ± 3.4, 60.6 ± 11.9 and 16.0 ± 2.6 in the coronary artery (Hartshore, 1987), a tonic smooth muscle. It appears that the amount of myosin in (phasic) myometrial cells is considerably lower than in coronary (tonic) smooth muscle, and the amount of actin in myometrial cell tends to be higher. Arterial smooth muscle can develop more force than most other smooth muscles, and it is possible that this could be due, at least in part, to the higher myosin content.

Actin arranged in a double helix in situ forms the bone structure of thin filament which is about 7 nm in diameter. Thin filaments in myometrial cells are abundant and generally arrayed in irregular bundles paralleling the long axis of the cells, but the length of the bundles has not been determined. In addition to actin, smooth muscle thin filaments contain tropomyosin as do skeletal muscles, but no troponin although the tropomyosin retains the binding site for the troponin. Thin filaments can be associated with dense bands in sarcolemma and dense bodies, which contain α-actinin and are irregularly located in the cytoplasm.

The polymerization of myosin in smooth muscle in situ produces filaments approximately 15 nm in diameter, namely, thick filaments. These filaments are rod-shaped with an irregular profile in transverse section. In vascular muscle cells, they measure 2.2 μm in length (Ashton et al., 1975), longer than those (1.5 μm) in skeletal muscle. The number of thick filaments in myometrium seems to vary with the stage of the reproductive cycle. For example, in pigs the filaments are more numerous in the follicular phase and prepartum period. They become conspicuous during early
pregnancy and visible again in days 80-84 (Thilander and Rodriguez-Martinez, 1989a; 1989b; 1990).

Myosin consists of two globular head groups joined to a 150-nm-long tail. Associated with each globular head are two light chains: a 17 kDa chain and a 20 kDa chain. Myosin obtained from smooth muscle appears identical to that from striated muscle at the ultrastructure level but with at least two distinguishing features during assembly and sensitivity to phosphorylation. (1) Skeletal muscle myosin is assembled in bipolar arrangements, i.e., myosin head with the same direction in each half of the filaments, while in smooth muscle it is assembled in so-called side-polar arrangement, i.e., on one side of the filaments all the myosin heads project in the same orientation whereas on the other side they have the opposite orientation. This unidirectional arrangement permits actin to interact with myosin along the entire length of the thick filaments, which may explain the 10-fold greater shortening ability of smooth muscle compared to skeletal muscle. (2) The shape of isolated myosin in smooth muscles, unlike skeletal muscle myosin, is sensitive to the state of phosphorylation of the 20 kDa light chain, which builds the structural basis for contraction regulation in smooth muscle (see below).

A third type of myometrial filaments named intermediate filaments are 10 nm in diameter (Gabella, 1981). They are not components of the contractile apparatus but of the cytoskeleton. These filaments either are grouped into bundles, forming a core in the central part of the cell, or occur individually or in small groups among the myofilaments or in association with dense bodies and dense bands. The intermediate filaments associated with dense bodies and dense bands form a supporting framework that links actin and myosin filaments into an integrated mechanical network. This arrangement allows the uterus to generate force along its axis in any direction to accommodate the fetus in various positions and size during labor.

Ca$^{2+}$ dogma and myosin light chain (MLC) phosphorylation

The contractile proteins, thick filaments and thin filaments, in the uterus and other smooth muscles do not have the orderly array seen in skeletal and cardiac muscles. However, it is believed that the mechanisms of contraction are the same among them, i.e., the myosin filaments sliding past the actin filaments with no change
in the length of either type of filament, referred to as the sliding filament model (Huxley, 1969). \(Ca^{2+}\) is the trigger for the contraction of all three types of muscles. However, control of contraction of skeletal and cardiac muscle favors an thin filament-dependent mechanism while smooth muscle primarily relies on a thick filament-dependent mechanism. In skeletal and cardiac muscle, binding of \(Ca^{2+}\) with troponin relieves the tropomyosin blockage of the interaction of myosin heads with actin and initiates the actin-myosin crossbridge resulting in contraction. In contrast, as depicted in Fig. 1., a rise in \([Ca^{2+}]_i\), in smooth muscle results in the formation of an active complex among \(Ca^{2+}\), calmodulin (CaM), and myosin light chain kinase (MLCK). This complex catalyzes the transfer of the terminal phosphoryl group of MgATP\(^{2-}\) to serine 19 on each of the 20 kDa light chains of myosin. This phosphorylation reaction triggers the cycling of myosin crossbridges along the actin filaments, with development of force or shortening of the muscle associated with hydrolysis of ATP. Removal of \(Ca^{2+}\) from the cytosol results in dissociation of the \(Ca^{2+}\)-CaM-MLCK complex and regeneration of the inactive MLCK. Myosin light chain phosphatase (MLCP) dephosphorylates myosin and muscle relaxation occurs (Hartshorne, 1987, Allen and Walsh 1994).

The central role for \(Ca^{2+}\) and MLC phosphorylation in regulation of smooth muscle contraction has substantial experimental evidence. Some evidence is directly from studies in myometrial cells. (1) There is a correlation among contractants (such as OT, carbachol)-induced increase in \([Ca^{2+}]_i\), MLC phosphorylation and (initial) force development (Barany and Barany, 1990). (2) Relaxing agents, such as \(\beta\)-adrenergic agonists are believed to cause a decrease in \([Ca^{2+}]_i\), and a decrease in MLC phosphorylation (Barany et al., 1985). (3) Stretch-induced MLC phosphorylation and contraction is abolished by high EGTA treatment (Csabina et al., 1986). (4) Phosphatase induces relaxation of contracted myometrium containing saturated \(Ca^{2+}\) and CaM. This effect could be reversed by MLCK. (Haeberle et al., 1985), and (5) Spontaneous activity of the uterus is accompanied by cyclic phosphorylation and dephosphorylation. During spontaneous force development, phosphorylation increases, while during spontaneous relaxation phosphorylation decreases (Hanson and Lowy, 1963; Bretscher, 1984).
Ca\textsuperscript{2+} movements across membranes

In view of the primary role of Ca\textsuperscript{2+} in smooth muscle contraction, the most obvious way to control the contraction is to alter [Ca\textsuperscript{2+}]. The plasma membrane and SR represent the two most important membrane systems in cytosolic Ca\textsuperscript{2+} homeostasis. Both membranes form a barrier with an approximately 10,000-fold concentration gradient; therefore, an increase in permeability to Ca\textsuperscript{2+} leads to a marked increase in [Ca\textsuperscript{2+}]. The Ca\textsuperscript{2+} permeability of plasma membrane is primarily regulated by Ca\textsuperscript{2+} channels. Ca\textsuperscript{2+} channels can be viewed as macromolecular pores allowing the passage of Ca\textsuperscript{2+} from the extracellular space into cells and can be divided into two major categories: VDCC and voltage-independent Ca\textsuperscript{2+} channels or receptor-operated Ca\textsuperscript{2+} channels (ROC). Activation of VDCC is due to a change in membrane potentials, while ROC respond to membrane-mediated alternations other than a change in potential. The Ca\textsuperscript{2+} permeability in SR is controlled by two Ca\textsuperscript{2+}-releasing channels: IP\textsubscript{3}R and RyR; the former is activated by IP\textsubscript{3} formed by activation of receptors in plasma membrane and the latter may be activated by physical coupling with Ca\textsuperscript{2+} channels in the plasma membrane or Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism. (Berridge, 1993; McPherson and Campbell, 1993).

It has been known for a long time that low [Ca\textsuperscript{2+}], is required for cell function while high [Ca\textsuperscript{2+}], results in cell toxicity. In compensation for the high [Ca\textsuperscript{2+}], due to Ca\textsuperscript{2+} influx and release driven by the large electrochemical gradient, both the plasma membrane and SR possess Ca\textsuperscript{2+}-ATPase which expends metabolic energy to pump Ca\textsuperscript{2+} against the gradient. In addition, the plasma membrane embeds a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Since it is generally accepted that the Ca\textsuperscript{2+}-ATPase and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger adjust their activity according to [Ca\textsuperscript{2+}], and may not be primarily regulated by external stimuli, they will not be reviewed here. We will focus only on Ca\textsuperscript{2+} channels in the plasma membrane and Ca\textsuperscript{2+}-releasing channels in SR.

\textit{Ca\textsuperscript{2+} influx through the plasma membrane}

\textit{Voltage-dependent Ca\textsuperscript{2+} channels (VDCC)}

Electrophysiological and pharmacological studies have identified and characterized multiple types of VDCCs in different tissues, that is L-, N-, T- and P-types. Their basic characteristics are summarized in Table 1, which is a classification
Table 1. Classes of voltage-dependent calcium channels

<table>
<thead>
<tr>
<th>Channel types</th>
<th>L</th>
<th>N</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductance</td>
<td>25 pS</td>
<td>12-20 pS</td>
<td>8 pS</td>
<td>10-12 pS</td>
</tr>
<tr>
<td>Activation</td>
<td>High voltage</td>
<td>High voltage</td>
<td>Low voltage</td>
<td>Moderate high voltage</td>
</tr>
<tr>
<td>Inactivation</td>
<td>Slow</td>
<td>Moderate</td>
<td>Transient</td>
<td>Very slow</td>
</tr>
<tr>
<td>Location/function</td>
<td>Widespread, muscle, nerve</td>
<td>Neuronal transmitter release</td>
<td>Widespread, pacemaker</td>
<td>Neuronal, purkinje</td>
</tr>
<tr>
<td>Blockers</td>
<td>DHP, calciseptine, Conotoxin</td>
<td>Flunarizine?</td>
<td>Ψ-aga-IVA</td>
<td></td>
</tr>
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</table>

by the Ca\(^{2+}\) channel subcommittee of the International Union of Pharmacology receptor
omenclature committee (Spedding and Paoletti, 1992).

In human myometrial cells, Inoue et al. (1990) and Young et al. (1993) detected
two types of Ca\(^{2+}\) currents. One type was characterized by a large conductance (29
pS), while the other had a smaller conductance (12 pS). These conductance values are
similar to those obtained respectively for L-type and T-type channels in cardiac and
vascular smooth muscle cells (Missiaen et al., 1992).

The L-type Ca\(^{2+}\) channel is permeable to multiple divalent cations. In isolated
rat myometrial cells (Amédée et al., 1987), substitution by Ba\(^{2+}\) or Sr\(^{2+}\) for Ca\(^{2+}\) in the
physiological solution originated inward currents due to the passage of these ions
through the Ca\(^{2+}\) channel. Ba\(^{2+}\) was able to carry the largest current when compared
with Sr\(^{2+}\) and Ca\(^{2+}\), possibly because of its much weaker binding within the Ca\(^{2+}\)
channels. However, other divalent cations such as Cd\(^{2+}\) or Co\(^{2+}\) suppress L-type Ca\(^{2+}\)
currents. This channel is potentially and selectively influenced by dihydropyridine
(DHP) in myometrial cells. Nifedipine at micromolar concentrations abolishes the
current in myometrial cells (Amédée et al., 1987).

There is no information about ion permeability mediated by T-type channels in
myometrial cells. In some smooth muscles, the T-type channel has the same
permeability to Ba\(^{2+}\) and Ca\(^{2+}\) (Benham et al., 1987; Loirand et al., 1989). This
channel is relatively insensitive to Cd\(^{2+}\) and DHPs, and can be blocked by Ni\(^{2+}\).

The expression of myometrial Ca\(^{2+}\) channels seems to be species-dependent.
Human myometrial cells contain both L- and T-type Ca\(^{2+}\) channels (Young et al.,
1993; Inoue et al., 1990), but only one type of Ca\(^{2+}\) channel, i.e., L-type, is detected
in rat myometrial cells (Honore et al., 1989; Oyha and Sperelakis, 1989). In fact, a
non L- or T-type Ca\(^{2+}\) channel has been reported in rat myometrial cells (Rendt et al.,
1992). The influence of reproductive stages and steroid hormones on Ca\(^{2+}\) channels is
not well defined. Inoue and Sperelakis (1991) reported that the electrophysiological
properties and density of the L-type Ca\(^{2+}\) channels did not change during pregnancy in
rats. However, Mironneau (1994) found that the Ca\(^{2+}\) channel density significantly
increased during days 10 to 21 of gestation and between pregnant and nonpregnant
rats in spite of no changes in electrophysiological properties. The density of Ca\(^{2+}\)
channels was 9.2 ± 1.1 \(\mu A/\mu F\) at day 18 of gestation while it averaged 3.10 ± 0.70
\(\mu A/\mu F\) at day 10. In nonpregnant rats, the number was \(2.45 \pm 0.30 \mu A/\mu F\). Mershon et al. (1993) reported that there was a change in alternatively spliced forms of the L-type \(Ca^{2+}\) channel in the rat myometrium during labor. Furthermore, estrogen treatment has been shown to increase both the entry of \(Ca^{2+}\) and number of L-type channels in rat myometrium (Batra, 1987).

There is abundant evidence that L-type VDCC can be modulated via receptor-mediated mechanisms. In cardiac muscle, \(\beta\)-AR-induced activation of L-type \(Ca^{2+}\) channels is mediated by a direct G protein coupling with the channels or by PKA-mediated phosphorylation of the channels (Trautwein and Hescheler 1990). However, the modulation of L-type \(Ca^{2+}\) channels by contractants in myometrium, just as in other smooth muscles, is still puzzling and controversial. For example, OT has been reported to inhibit the VDCC in freshly dispersed rat myometrial cells (Inoue et al, 1992), but it increases the VDCC in cultured rat myometrial cells (Mironneau, 1994).

**Voltage-independent \(Ca^{2+}\) channels (Receptor-operated \(Ca^{2+}\) channels, ROC)**

In many smooth muscles, a variety of hormones and neurotransmitters activate ROCs, usually nonselective cation channels which are permeable to \(Na^+\), \(K^+\) and to a lesser extent to \(Ca^{2+}\) (Missien et al., 1992); but there is little evidence supporting the existence of ROC in myometrial cells. The only example is that ATP-activated channels, which play a important role in tonic modulation of vascular smooth muscle, were found in rat myometrial cells (Honore et al., 1989). ATP activates these channels dose-dependently. The permeability of these channels has little discrimination between \(Na^+\) and \(K^+\). The permeability to \(Ca^{2+}\) is to be determined. It was also observed that the response of this channel to ATP decreased as pregnancy progresses. Since diethylstilbestrol treatment facilitates the decrease in response, this decreased response to ATP may be due to an increase in estrogen levels (Mironneau, 1994).

**\(Ca^{2+}\) release from intracellular stores**

**Ins(1,4,5)P_3, (IP_3)-sensitive stores**

\(IP_3\), formed in response to external stimuli, mobilizes \(Ca^{2+}\) from intracellular \(Ca^{2+}\) stores by interacting with the \(IP_3\)R in the SR membrane (Berridge 1993). Several lines of evidence indicate that the myometrium contains \(IP_3\)-sensitive \(Ca^{2+}\) stores. The evidence includes 1) \(IP_3\) induces contractions in permeabilized myometrial preparations...
(Kanmura et al., 1988); 2) IP₃ stimulates the release of ⁴⁵Ca (Carsten and Miller, 1985); 3) IP₃ releases 45% of total stored Ca²⁺ and this release is inhibited by heparin in permeabilized rat myometrial cells (Sanborn et al., 1994); 4) IP₃R has been identified in the microsomal fraction isolated from human myometrium (Rivera et al., 1990) and the uterus expresses IP₃R mRNA (Nakagawa et al., 1994); and 5) IP₃-generating agonists, such as oxytocin and carbachol, increase [Ca²⁺], in human (Molnár and Hertelendy, 1990) and rat myometrial cells (Anwer and Sanborn, 1989).

**Caffeine-and ryanodine-sensitive stores**

RyR provides an additional principal Ca²⁺-release channel. Physiologically, this channel is activated by a direct interaction with a L-type Ca²⁺ channel in the transverse tubule of skeletal muscle, or by a CICR mechanism in cardiac muscle (McPherson and Campbell, 1993). Vascular and gastrointestinal smooth muscles contain RyRs with similar properties to skeletal and cardiac muscles (Herrmann-Frank et al., 1991; Xu et al., 1994). However, RyRs, or caffeine-sensitive Ca²⁺ stores in the myometrium are not consistently detected and mechanisms underlying the activation of this channel in smooth muscle cells are not understood. Several studies suggest that myometrial cells contain IP₃-insensitive stores (caffeine-sensitive stores). For example, caffeine induced the contraction of pregnant human myometrium (Izumi, 1994); IP₃ releases only part of Ca²⁺ accumulated in the intracellular stores in permeabilized cells (Sanborn et al., 1994). However, Ca²⁺ measurement studies indicated that rat myometrium does not have caffeine-sensitive Ca²⁺ stores (Arnaudeau et al., 1994), whereas human myometrium may contain atypical Ca²⁺ stores because ryanodine releases the intracellular Ca²⁺, but caffeine does not (Lynn et al., 1993).

**Myogenic contraction of myometrium**

In the absence of nervous and hormonal stimulation, myometrium displays regular spontaneous contractions. This feature is defined as myogenic contraction and appears to be due to active and passive electrical properties of muscle cells.
Resting membrane potential

The resting membrane potential is an important consideration because its change affects the spontaneous electrical activity of the muscle cells which is, in turn, responsible for the contractile events of the muscle. The resting membrane potential, determined by the ratio of the concentration and permeability of the two abundant ions $K^+$ and $Na^+$ in tissue, was around -55 mV with a range of -30 to -70 mV in myometrium from rats (Parkington and Coleman, 1990). Although values of resting membrane potential of myometrium in species other than rats are available, it is only in rats that an attempt has been made to determine the potential change among the reproductive stages. In longitudinal muscle of rats during pregnancy, the membrane potential increases from a nonpregnant level of -30 to -40 mV, reaching a maximum of -60 to -70 mV at midpregnancy, and then declines to approximately -50 mV at term (Kanda and Kuriyama, 1980). Permeability changes of the membrane to $K^+$ ions during pregnancy may cause a change in resting membrane potential (Kanda and Kuriyama, 1980; Kao 1977). Since the concentrations of estrogens increase markedly during the last two days of pregnancy in rats, while those of progesterone decrease, it has been speculated that changes in the steroid levels may contribute to the changes in membrane permeability to $K^+$ as parturition approaches. Indeed, progesterone administration to ovariectomized, estrogen-primed rats induces an increase in the permeability of the myometrial cell membrane to $K^+$, and progesterone injection on day 19 prolongs pregnancy and results in hyperpolarization (Kanda and Kuriyama, 1980).

Pacemaker activity

The characteristic electrical activity of the myometrium is spontaneous bursts of action potentials at irregular intervals. Many action potentials arise abruptly out of the resting membrane potential. However, some are preceded by a slow depolarization (Kao, 1977), which is similar to the pacemaker potential in the heart and those in other smooth muscles. The cell with the slow depolarization, thus, has been termed "pacemaker" or "pacesetter", while the other has been termed "pacefollower". Unlike the cells in the heart, pacemakers and pacefollowers in the myometrium are not anatomically distinct, that is, each myometrial cells is capable of becoming a pacemaker. This may explain the observation that pacemaker sites in a myometrial
preparation frequently shift from one area to another (Kuriyama and Csapo, 1961).

Spontaneous electrical activity in myometrium arises from pacemaker cells. The pacemaker potential leads to an action potential, which conducts to pacemakers as a result of spread of local circuit current, and triggers the regenerative permeability changes in the surrounding pacemakers. However, the ionic basis underlying the generation of pacemaker potential in myometrium has received scant attention. In rat longitudinal myometrium the pacemaker potential is associated with an increase in membrane resistance due to a decrease in K⁺ permeability (Kuriyama and Suzuki, 1976). However, in the same preparation, pacemaker potential disappears when external Na⁺ is reduced, suggesting that this potential results from an increase in membrane permeability to Na⁺ (Reiner and Marshall, 1975). But in cardiac muscle, the pacemaker depolarization appears to be associated with activation of inward current which is unselectively carried by K⁺ and Na⁺ (Difrancesco, 1993). Moreover, T-type Ca²⁺ channels may also be involved in pacemaker depolarization in excitable cells including smooth muscle (Huizinga et al., 1991). More investigations are needed to understand the pacemaker in myometrium.

**Action potential**

Action potentials observed in longitudinal myometrium of rats (Marshall, 1959) and rabbits (Kleinhaus and Kao, 1969), and in circular myometrium of guinea pigs (Parkington, 1984) and sheep (Parkington, 1983) display a simple time course consisting of a depolarizing upstroke followed by monotonic repolarization. However, in the circular myometrium of rats (Parkington, 1983) and mice (Osa, 1974) and in longitudinal myometrium of guinea pigs (Coleman and Parkington, 1987), complex action potentials occur that consist of a sustained phase of depolarization preceded by a spike and followed by a repolarization. Despite the similarity in form of the latter among the species, the mechanisms underlying these action potentials in different species may not be the same and are not clear. The ionic basis underlying the simple action potential, however, has been investigated in considerable detail. The inward current mediating the upstroke of this action potential seems to be carried by L-type Ca²⁺ channels similar to those in other smooth muscle cells (Parkington and Coleman, 1990). Early experiments indicated that Na⁺ channel is at least partially involved in the
inward current underlying the upstroke of the spike in longitudinal myometrium, because in Ca^{2+}-free medium the spike persists (Mironneau, 1973; Kao and McCullough, 1975). However, in the light of new findings that Na^+ can pass through L-type Ca^{2+} channels when Ca^{2+} is lower than 10^{-6} M in the medium (Hadley and Hume, 1987), the contribution of the Na^+ channels is probably small. The repolarization phase of the action potential is due to the movement of K^+ out of the cell through voltage-dependent as well as Ca^{2+}-dependent K^+ channels (Kao and McCullough, 1975).

The frequency of action potentials seems to correlate with the estrogen/progesterone ratio. In general, uteri display a high frequency of spontaneous action potentials during the follicular phase and during parturition, when the estrogen/progesterone ratio is high, while they exhibit a low rate of spontaneous action potentials in the luteal phase and during pregnancy when the ratio is low. This relationship probably arises mainly from estrogen-induced changes in membrane potential to a level which facilitates spontaneous depolarization by pacemaker cells. In addition, estrogen-induced stimulation of gap junction formation may enhance electrical coupling between cells (Riemer and Roberts, 1986). On the other hand, progesterone produces a hyperpolarization of membranes in myometrium from about -50 mV to about -65 mV, which would reduce excitability and impulse conduction (Riemer and Roberts, 1986).

Propagation of electrical activity

Synchronous and asynchronous patterns of contractile activity of uterine smooth muscles require coordination among billions of myometrial cells. Studies indicate that neither stimulation nor inhibition from the nervous or endocrine system can account for these patterns of contractions, suggesting that the contractile activity is myogenic in nature. It is known that propagation of action potentials is primarily responsible for the coordinate contraction coupling among the individual myometrial cells. Indeed, in longitudinal and circular muscles of pregnant myometrium, the inward Ca^{2+} current through VDCC during a single action potential initiates a twitch contraction. When action potentials are repetitively discharged for example, in the form of bursts, the contraction amplitude increases (Garfield, 1994). Contractions of
the uterus, therefore, are directly proportional to the quality and quantity of the action potential. Electrical activity propagates away from its site of origin to surrounding membrane regions by a local current flow. The propagation of current between cells is considered to occur by the same mechanism and involves the flow of ions between cells through channels that connect the cell interiors. Because gap junctions provide a low resistance pathway between cells (see above) they have been assumed to play a prominent role in this electrical event. Indeed, in many species, the presence of a large number of gap junctions between the myometrial cells during labor are associated with an increase in conduction velocity and a reduction in junctional resistance, thereby enhancing contractile activity. This is consistent with the notion that uterine inactivity and synchronous contractility are linked to the maintenance of pregnancy whereas coordinated synchronous contractions accompany and characterize labor.

Regulation of myometrium contraction by oxytocin

The contraction of any smooth muscle tissue may be modulated by myogenic, neurogenic, or hormonal mechanisms. There is a wealth of information that myometrial contractility is under dominant control by humoral mechanisms. Of the hormones, oxytocin (OT) has the greatest influence on the control of the myometrium.

Structure, biosynthesis and secretion

OT is a nonapeptide with an intramolecular disulfide bond between cysteine residues in positions 1 and 6 (Fig. 2). It was originally considered to be synthesized in the cell bodies of the magnocellular neurons in the supraoptic nuclei and paraventricular nuclei, and transported down the axons of their neurons to their ending in the posterior lobe of pituitary, i.e., neurohypophysis. Recent studies have shown that OT is also

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Figure 2. Sequence of oxytocin
synthesized in and secreted from various peripheral tissues including adrenal medulla, corpus luteum, ovary, placenta and uterus. A particularly intriguing finding is that concentration of OT and mRNA of OT in the epithelial layer of the endometrium of the rat uterus with concentrations were increased by 150-fold during pregnancy, exceeded hypothalamic mRNA by 70-fold at term, and fell dramatically after parturition (Lefebvre et al., 1992). OT gene is expressed in human uterus, amnion, chorion laeve and decidua at parturition (Chibbar et al., 1993). These results strengthen the notion that OT acts as an initiator of labor (see below).

Similar to vasopressin, OT is synthesized in the rough endoplasmic reticulum in the form of a prepropeptide consisting of 123 amino acids, of which 19 comprise the signal peptide. The signal peptide is immediately followed by the sequence of OT, and is separated by three amino acids -lys-lys-arg-, from the sequence of neurophysin I, the protein which is essential for the axonal transport of the hormone.

OT is released from nerve terminals of the neurohypophysis by a mechanism of exocytosis. Under normal physiological conditions, the secretion of OT is initiated by the influx of Ca^{2+} resulting from membrane depolarization subject to the arrival of action potentials upon stimulation of mammal gland or dilation of the cervix and vagina. The increase in [Ca^{2+}] causes the movement of secretory granules to the limiting membrane of the terminals, where they fuse and release their contents to the general circulation. The released OT reaches the peripheral target organs by transport in blood.

**Physiology and pharmacology**

**Uterine contractions induced by OT**

Stimulation of myometrial contraction was the first OT-dependent action discovered. This effect has been well illustrated in in vivo and in vitro studies in which OT increases the force, frequency, and duration of the contraction. OT represents the most potent contractant of uterus among agents known. The threshold concentration is 1 to 6 x 10^{-11} M for estrogen-primed rat uterus (Fitzpatrick and Bently, 1968) and about 1 to 2 x 10^{-10} M for term pregnant human myometrium (Fuchs and Fuchs, 1963), and 1 x 10^{-10} M for porcine myometrium (Yu et al., 1993). Electrophysiological studies indicated that the powerful stimulation of uterine contractions by OT is caused primarily through increasing the duration and repetition frequency of spike burst
discharges and increasing the spike discharge frequency within individual bursts (Garfield and Cole, 1989).

OT brings about an increase in milk flow (Ott and Scott, 1910). It is now known that this milk-ejection activity results from the contraction of myoepithelial cells. These contractile cells form a meshlike arrangement around the alveoli in the mammary glands and their contraction upon the stimulation by OT squeezes milk out of the alveoli into the ducts. The milk-ejection reflex is a classic neuroendocrine arc starting with stimulation of the nipple which allows activation of the OT neuron in supraoptic nuclei and paraventric nuclei, resulting in the release of OT into the circulation from the terminal of the neurophysis.

Initiation of labor

Despite the fact that OT is widely used to induce labor and to prevent postpartum hemorrhage, the role of endogenous OT in the initiation of labor has been controversial for many years. There are many studies favoring the idea that OT functions as a initiator of labor. For example, The number of OT receptors in the myometrium increases 40-100-fold in the hours preceding the onset of labor (Soloff et al., 1979), and the electrical stimulation of the pituitary stalk in rabbits toward the end of gestation promotes "normal" delivery within minutes (Lincoln, 1971), and in most animal species, labor can be induced with OT only within narrow time limits around the day of normal labor (Challis and Lye, 1994). It is apparent that OT released at term could initiate as well as promote parturition. Substantial evidence, however, opposes this notion: 1) the increase in plasma concentration of OT in the maternal circulation does not precede the onset of labor in humans or rats (Sellers et al., 1981; DeGeest et al., 1985); 2) in rats and humans normal parturition was unaffected in the absence of circulating OT in cases of experimental or clinical pituitary gland dysfunction (Phelan et al., 1978; Chard, 1977), and 3) administration of an OT antiserum or a specific OT-receptor antagonist to prepartum rats did not change the timing of parturition (Chan and Chen, 1992).

One possible explanation of the discrepancy between circulating OT levels and the onset of parturition is the specific response of myometrium to OT. Myometrial sensitivity to OT is enhanced dramatically immediately before and during parturition in
animals (Soloff, 1988). This effect is believed to be accomplished at least in part by: 1) an increase in concentration of OT receptor in myometrium; and 2) a decrease in relaxing pathway, suggesting that a rise in the concentrations of circulating OT would not be necessary to postulate a physiological importance for OT in the initiation of labor. The recent discovery that OT is synthesized locally in uterine tissue of the rat and human has added a new dimension to the controversy that has existed over the precise role of OT in relation to the initiation of labor. Rat uterine OT mRNA levels increased by more than 150-fold during gestation and exceeded hypothalamic OT mRNA by 70-fold at term, and OT mRNA levels in human amnion, chorion, and decidua increased significantly around the time of parturition (Lefebvre et al., 1992; Chibbar et al., 1993). These results suggest that the OT involved in initiating and maintaining parturition could be of uterine origin via a paracrine or autocrine mechanism. However, labor is a complicated process resulting from a complex interplay of multiple maternal and fetal factors. It is possible that different mechanisms exist in each species to initiate parturition. The precise roles of OT in the initiation of labor will require further investigation.

**Cellular mechanism (Signal transduction)**

The human OT receptor gene has been recently cloned and shown to encode a 388-amino-acid polypeptide (Kimura et al., 1992). Hydrophobicity plots suggest the presence of a seven transmembrane domain and a putative G protein interaction site in the OT receptor. Therefore, the OT receptor belongs to a G protein-coupled receptor superfamily. The signal transduction system for this receptor family consists of three distinct components, i.e., the receptor moiety, G protein and effectors.

G proteins represent an expanding family of signal coupling proteins that play key roles in many hormonal and neurotransmitter transduction processes in cells. G proteins act by transmitting the signal of receptor stimulation to effectors, which in turn produces the cellular response or generate second messengers. In general, the G proteins are heterotrimers with subunits of α, β and γ. The α-subunit differs among the membrane of the G protein family, thereby defining the G protein subtypes, whereas β-subunits and γ-subunits are quite homologous. In fact, βγ-complex of different G protein are functionally interchangeable. To date, 20 distinct α subunits have been
described. Based on the similarity of their amino acid sequences, they are divided into four subfamilies: $a_1$, $a_2$, $a_3$, and $a_4$. Five $\beta$ subunits and seven $\gamma$ subunits have so far been found. They can produce more than 30 different combinations.

The activation of a G protein is governed by a GTPase cycle (Fig. 3). In the resting state, a G protein has no contact with a receptor and an effector. The $\alpha$ subunit binds with GDP. When a hormone or a ligand binds with the receptor, the receptor is activated, which in turn causes G protein to exchange GDP for GTP. Certain bacterial toxins modify the G protein function by transferring ADP-ribose from NAD to specific amino acids within the $\alpha$-subunit. Pertussis toxin (PTX) covalently modifies a subset of $\alpha$-subunits ($\alpha_1$, $\alpha_2$, and $\alpha_3$) and inhibits the guanine nucleotide exchange by blocking the receptor-G protein coupling. Binding of GTP leads to a change in $\alpha$-subunit conformation that causes dissociation of the GTP-bound $\alpha$-subunit from the $\beta\gamma$-complex. The $\alpha$ subunit binds and activates effectors. The $\alpha$ subunit has been shown to play the critical, if not only, role for regulation of several effectors by their corresponding G proteins. There is, nonetheless, evidence that the $\beta\gamma$-complex could play an important role in regulation of certain effectors such as adenylyl cyclase and phospholipase $A_2$. Activation of G protein is terminated by a GTPase activity intrinsic to the $\alpha$ subunit. Binding of synthetic, nonhydrolyzable analogs of GTP such as GTP-$\gamma$S leads to persistent G protein activation. Cholera toxin-catalyzed modification of $\alpha_2$, the G protein mediating stimulation of cAMP formation, inhibits GTPase activity and leads to persistent activation by the natural ligand, GTP. Fluoride ions (in the form of an aluminum fluoride complex, ALF$_4^-$) also cause persistent activation of a G protein, since this complex binds adjacent to GDP and apparently mimics the $\gamma$-phosphate group of GTP.

Before the elucidation of the OT receptor structure, biochemical studies indicated that activation of the receptor by OT activates a G protein because ALF$_4^-$ mimics the response of OT and GTP, and decreases OT binding affinity in astroglial cell membrane (DiScala-Guenot and Strosser, 1992). Furthermore, the G protein coupled with OT receptor belongs to a PTX-insensitive G protein in myometrial cells in rats, humans and guinea pigs (Phaneuf et al., 1993; Marc et al., 1988). Recently this G protein has been found to be in the $G_a$ family, specifically, the $G_a$ and $G_{11}$, because the specific antibodies against $\alpha$-subunits of these two G proteins blocked OT-stimulated
phosphoinositide turnover in myometrial cells (Ku et al., 1995; Arnaudeau et al., 1994). This is consistent with the finding that the α subunits of PTX-insensitive G proteins, Ga and Ga₁₁, exist in mouse and human myometrial cells (Wilkie et al., 1991; Phaneuf et al., 1993). However, PTX evoked a marked inhibitory effect on OT-stimulated phosphoinositide turnover in rats (Anwer and Sanborn, 1989; Ruzycky and Crankshaw, 1988) and humans (Molnar and Heterlendy, 1990), suggesting that PTX-sensitive G protein also mediates the response to OT in the rat and human myometrium. In many other cell types, phosphoinositide turnover can also be regulated by PTX-sensitive G proteins. There is much evidence that these PTX-sensitive G proteins are G₁ and G₉ subtypes. However, efforts to demonstrate stimulation of phosphoinositide turnover by α-subunit of these G proteins have met with little or no success. The βγ-subunits of these proteins have recently been suggested as being responsible for phospholipase C (PLC) activation; however, there is no study testing this possibility in myometrial cells. Singh et al (1992) found that stimulation of phosphoinositide turnover by OT does not involve direct obligatory coupling to a PTX-sensitive G protein, rather indirect effects on PKA activity may contribute to the inhibition effect of PTX, suggesting an involvement of no βγ subunit in PTX-induced inhibition of response to OT in myometrial cells.

Ca²⁺ measurement studies demonstrated that OT increases [Ca²⁺]. It is known that under physiological conditions OT increases [Ca²⁺] by two mechanisms: stimulation of Ca²⁺ entry from the extracellular environment and mobilization of intracellular stored Ca²⁺ (Anwer and Sanborn 1989; Molnar and Hertelendy, 1990; Tasaka et al., 1991; Phaneuf et al., 1993). The release is generally considered to be mediated by activation of a receptor-G protein-PLC-IP₃ mechanism (Anwer and Sanborn 1989; Molnar and Hertelendy, 1990; Tasaka et al., 1991; Phaneuf et al., 1993), although findings from some studies suggest that this effect is due to the inhibition of Ca²⁺ extrusion and uptake by OT-induced depression of the Ca-ATPase in sarcolemma and sarcoplasmic reticulum (Magocsi and Penniston, 1991). Ca²⁺ enters the cell through Ca²⁺ channels. However, the Ca²⁺ channels involved are not well understood. The rise in [Ca²⁺], produced by OT may depend on external Ca²⁺ through L-type VDCC, because application of the Ca²⁺ channel blocker nicardipine reduces [Ca²⁺], to just 16% of OT control values in human myometrial cells (Tasaka et al., 1991). However, in rat myometrial cells the L-type VDCC blocker D-600 does not antagonize the OT-induced
increases in \([\text{Ca}^{2+}]\) (Anwer and Sanborn, 1989). Conflicts also exist in studies concerning the effects of OT on VDCC current. Mironneau (1994) observed that OT increased in cultured rat myometrial cells while Inoue et al. (1992) reported that OT decreased the \([\text{Ca}^{2+}]\) current in freshly dispersed rat myometrial cells. On the other hand, results of a study using \(^{45}\text{Ca}\) flux suggested that binding of OT to its receptor produced a rapid \([\text{Ca}^{2+}]\) influx through ROC (Batra 1986). The failure of D-600 to inhibit OT-induced increase in \([\text{Ca}^{2+}]\), (Anwer and Sanborn, 1989) and a lack of activation of OT on VDCC current in a patch-clamp study (Inoue et al, 1992) in myometrial cells provides further evidence that OT may stimulate ROC to increase \([\text{Ca}^{2+}]\). A very recent investigation showed that OT induced a biphasic increase in \([\text{Ca}^{2+}]\), with a spike and sustained phase; and the latter resulted partially from activation of ROC based on the inhibition by a VDCC blocker in rat myometrial cells (Arnaudeau et al, 1994). Shimamura et al (1994) directly recorded an OT-induced inward current which resulted from the opening of ROC. But whether this channel is involved in OT-induced \([\text{Ca}^{2+}]\) influx warrants further investigations.

In addition to inducing myometrium contraction by increasing \([\text{Ca}^{2+}]\), OT has been demonstrated to cause myometrium contraction by other mechanisms. OT can cause contraction of the uterus in an EGTA-containing medium. Although the amplitude is reduced by about 90% of that obtained in \([\text{Ca}^{2+}]\)-containing medium, these contractions persist for several hours. Also, OT induces contractions without a measurable increase in \([\text{Ca}^{2+}]\), or myosin light chain phosphorylation (Wray, 1993). These results suggest that activation of the OT receptor can trigger, in addition to a \([\text{Ca}^{2+}]\)-dependent mechanism, a \([\text{Ca}^{2+}]\)-independent mechanism, probably mediated through protein kinase C (Karibe et al., 1991) or a small G protein (Suga et al., 1993).

It has been demonstrated that OT stimulates the production of \(\text{PGE}_2\) and \(\text{PGF}_{2\alpha}\) from human and rat decidua, and that this effect is abolished by OT-receptor antagonists (Fuchs et al., 1982; Chan et al., 1982). Antagonists of PG have been reported to block OT-stimulated contraction of uterine strips from rabbits (Challis and Lye, 1994). Thus, OT-induced myometrial contraction in vivo, could be mediated by an indirect stimulation through increasing prostaglandin (PG) release.
Regulation of myometrium contraction by $\alpha_2$-adrenergic receptors

In contrast to hormonal modulation, we know less about the neuronal modulation of myometrial contraction. In spite of the fact that the uterus receives an extensive nerve supply, its physiological function in myometrial contraction still needs to be established. Here, we only review the adrenergic nerve with an emphasis on $\alpha_2$-adrenergic receptors (AR) of myometrial cells.

Adrenergic innervation and $\alpha_2$-AR

A considerable portion of innervation of the uterus consist of adrenergic nerves which supply the myometrium and uterus vascular smooth muscle. In most species, the adrenergic nerve supplying to the myometrium originates from cell bodies in the lumbar, mesenteric and paracervical ganglia. Concerning the nerve-muscle relationship, there is a low density of nerves to myometrial smooth muscle cells and nerves are associated with groups or bundles of myometrial cells.

Histochemical and pharmacological studies have suggested that there are differences in the distribution of adrenergic nerves in muscle layers among humans (Ottesen et al., 1982), rats (Hollingsworth, 1974), guinea pigs (Thorbert et al., 1977) and pigs (Thilander and Rodriguez-Martinez, 1989a; 1989b). The longitudinal layer is endowed predominantly with adrenergic nerves and the circular layer is predominantly innervated with cholinergic nerves. In addition, there is a regional difference in adrenergic innervation. Adrenergic nerve fibers are more abundant in the cornu than the corpus and $\alpha$-ARs are virtually absent from the cervix. Recently, functional studies in porcine myometrium confirm the above observations (Taneike et al., 1994).

The release of the neurotransmitter norepinephrine in the adrenergic nerve terminal modulates the contraction by interacting with adrenergic receptors in the plasma membrane of myometrial cells. The circulating catecholamines, epinephrine and norepinephrine, regulate the contraction in the same manner, suggesting that there are ARs existing in extrasynaptic areas. Both $\alpha$- and $\beta$-ARs have been found in myometrial cells. Activation of $\alpha$-ARs enhances the contraction and activation of $\beta$-AR decreases the contraction.

Radioligand binding assays demonstrated that $\alpha_1$- and $\alpha_2$-ARs are present in the myometrium of different species, including humans (Bottari et al., 1983), rats (Legrand
et al., 1993), guinea pigs (Arkinstall and Jones, 1988), rabbits (Falkay, 1990), ewes (Vass-Lopez et al., 1990b) and sows (Taneike et al., 1995). Based on functional and radio-ligand binding studies, \( \alpha \)-ARs can be further divided into four subtypes, i.e. \( \alpha_{2A} \), \( \alpha_{2B} \), \( \alpha_{2C} \) and \( \alpha_{2D} \). Ligand-binding studies indicated that 55% of \( \alpha \)-ARs are \( \alpha_{2A} \)-AR subtype and 45% are \( \alpha_{2B} \)-AR subtype in longitudinal muscle of rats (Legrand et al., 1993). In porcine myometrium, \( \alpha_{2A} \)-ARs are the major subtype of \( \alpha \)-ARs (Yang and Hsu, 1995c). This is confirmed by a function study in which catecholamine-induced contraction was mediated predominantly by \( \alpha_{2A} \)-ARs (Yang and Hsu, 1995d).

Steroid hormones profoundly influence the density of myometrial \( \alpha \)-ARs among species. In rabbits (Hoffman et al., 1981; Jacobson et al., 1987) and humans (Bottari et al., 1983, 1985) the density of myometrial \( \alpha \)-AR increases when the circulating estrogen concentrations are high. However, the density decreases in ewes (Vass-Lopez et al., 1990a and 1990b) and sows (Rexroad and Guthrie, 1983) in the same endocrine environment. On the other hand, the myometrial \( \alpha \)-AR concentration is high (Vass-Lopez et al., 1990a and 1990b) in the progesterone-treated or pregnant ewe. In contrast, in humans (Bottari et al., 1983 and 1985) and rabbits (Williams et al., 1976) the \( \alpha \)-AR density is decreased in the same endocrine environment. Moreover, myometrial \( \alpha \)-AR density increases greatly in mid-pregnancy, then declines abruptly at the end of pregnancy in rats and guinea pigs (Kyozuka et al., 1988; Legrand et al., 1993), whereas the \( \alpha \)-AR concentration increases in rabbits at term (Jacobson et al., 1987).

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The regional difference in the adrenergic innervation, the change in density of \( \alpha \)-AR to steroid hormones and during pregnancy suggest that \( \alpha \)-ARs play functional roles in reproductive processes. However, the exact roles have yet to be defined. One possibility is that \( \alpha \)-ARs regulate the contractility of the uterus. There is a consensus that activation of \( \alpha \)-AR by catecholamines induces myometrium contraction. Yet, there is considerable species difference in the role of \( \alpha \)-AR subtypes in catecholamine-induced contractions. In rodents and humans, \( \alpha_{1} \)-ARs are considered to be responsible for the contractile response to catecholamine, because prazosin, but not yohimbine, competitively inhibits the response (Hoffman et al., 1981; Riemer et al., 1987; Haynes
et al., 1993; Kaulenas et al., 1991). However, $\alpha_2$-ARs seem to be involved in the catecholamine-induced response in sheep, cattle and sows, since this response is inhibited by yohimbine (Marnet et al., 1987; Ko et al., 1990a; 1990b; Yang and Hsu, 1995a).

**Cellular mechanisms (Signal transduction)**

Three $\alpha_2$-ARs ($\alpha_{2a}$, $\alpha_{2b}$, and $\alpha_{2c}$) have been molecularly characterized to date. All of them contain the seven transmembrane domain structure, thereby belonging to the G protein-coupled receptor. It is clear that in many cell types activation of $\alpha_2$-AR results in an inhibition of adenyllyl cyclase activity, which is mediated through an inhibitory G protein (G). This is also true in myometrial cells. For example, the $\alpha_2$-agonist UK 14,304 inhibited basal cAMP production and the forskolin-stimulated increase in cAMP formation and this effect is markedly inhibited by PTX treatment in rabbit myometrium (Breuiller et al., 1990). Studies in pithed and anaesthetized animals have shown that $\alpha_2$-AR-mediated pressor responses are sensitive to PTX (Boyer et al., 1983), which indicated the probable involvement of G protein. However, there is no evidence in myometrium as to whether G protein is involved in $\alpha_2$-AR-mediated contraction.

In the presence of a VDCC blocker, or Ca\textsuperscript{2+}-free medium, $\alpha_2$-AR-mediated contraction was greatly reduced in porcine myometrium (Yang and Hsu, 1995b), indicating the importance of Ca\textsuperscript{2+} influx through VDCC in this contraction. In fact in vivo and in vitro studies have well demonstrated that an influx of extracellular Ca\textsuperscript{2+} through VDCC is necessary for the contraction mediated by $\alpha_2$-ARs in many other smooth muscles (Ruffolo et al., 1984; Timmermans et al., 1985). This conclusion is further confirmed by direct measurement of [Ca\textsuperscript{2+}], and recording of Ca\textsuperscript{2+} channel current in plasma membrane. For example, activation of $\alpha_2$-AR increases the [Ca\textsuperscript{2+}] in smooth muscle cells from the portal vein (Leprêtre and Mironneau, 1994) and rabbit tail vein (Li et al., 1993), and L-type Ca\textsuperscript{2+} current in smooth muscle from portal vein (Leprêtre and Mironneau, 1994). However, the cellular mechanism on $\alpha_2$-ARs mediating activation of VDCC has not been unequivocally defined. Leprêtre and Mironneau (1994) proposed that the $\alpha_{2A}$-AR couples to G protein to activate VDCC through activation of protein kinase C, because GF109203x, a protein kinase C
inhibitor, and a long time (24 h) treatment with phorbol dibutyrate blocked $\alpha_2$-AR-induced stimulation of the VDCC current. However, there is no evidence to date that $G_i$ can activate protein kinase C. Another recent study found that ATP-sensitive K$^+$ channel antagonists glibenclamide and disopyramide dose-dependently inhibited $\alpha_{2b}$-AR-mediated vasoconstriction, raising a possibility that $\alpha_{2b}$-AR is coupled with closure of ATP-sensitive K channels, leading to depolarization and constriction of vascular smooth muscle (Tateishi and Faber, 1995).

In preparations such as the canine saphenous vein (Guan et al., 1989), rabbit ear vein (Daly et al., 1990) and porcine myometrium (Yang, 1995), removal of extracellular Ca$^{2+}$ and application of a VDCC blocker only partially decreased the $\alpha_2$-adrenergic effects. These results suggest that DHP-insensitive Ca$^{2+}$ channels or Ca$^{2+}$ release from intracellular stores are implicated in $\alpha_2$-AR-mediated contraction. Another possibility is that activation of $\alpha_2$-AR sensitizes the contractile protein to intracellular Ca$^{2+}$ rather than an increase in [Ca$^{2+}$]. This mechanism has been proposed in $\alpha_2$-AR-mediated contraction of saphenous vein from rabbits (Aburto et al., 1993).
A rise in \([\text{Ca}^{2+}]\_i\) induces contraction of smooth muscle. Contraction coupling in smooth muscle, therefore, refers primarily to the cellular events that mediate a change in \([\text{Ca}^{2+}]\_i\), sufficient to regulate the activity of contractile proteins. In myometrial cells, information has accumulated on the regulation of \([\text{Ca}^{2+}]\_i\), and contraction coupling from studies on cell preparations from rodents and humans. However, there are few studies related to porcine and other species and little is known about basic characteristics of contraction coupling in porcine myometrium such as \(\text{Ca}^{2+}\) stores and their release, \(\text{Ca}^{2+}\) channels and \(\text{Ca}^{2+}\) influx.

One important method for the investigation of \(\text{Ca}^{2+}\) signal has been the voltage-clamp technique to record \(\text{Ca}^{2+}\) conductance in plasma membrane. The conductance is directly proportional with the activity of the ion channels. In a voltage-clamp experiment, the membrane potential is held constant and the transmembrane current required to maintain that potential is measured. From Ohm's law \((I = gV)\), it is apparent that if the potential \((V)\) is known and constant and the current \((I)\) is determined, one can obtain measurement of the total conductance \((g)\) of the membrane as a function of voltage and time.

Two-electrode voltage-clamp is traditionally assigned to measure the whole-cell current. It is not feasible, however, to apply it in cells like myometrial cells which are thin and small, because of the difficulty in penetrating two electrodes into them. The introduction of the patch-clamp technique by Neher and Sakmann (Hamill et al., 1981) made it possible to study ion channels in small cells. The patch-clamp technique is based on an electrical isolation of a small membrane (patch) from the rest of the cell membrane. To achieve this, the patch pipette is pressed against the cell membrane, and a slight negative pressure is applied to the pipette interior. Under appropriate conditions a tight seal between the cell membrane and the pipette develops. This seal, being referred to as a "giga-seal" (due to its resistance in the giga-ohm range) causes a very high resistance between the inside of the patch pipette and the surrounding bath solution. The giga-seal is essential in patch-clamp measurement because it greatly reduces the current noise of the recording. After obtaining giga-seal, three types of patch-clamp configurations can be achieved, i.e., cell-attached patch, whole-cell patch...
and inside-out or outside-out patch. Of the three configurations, the whole-cell configuration of patch-clamp has the advantage that intracellular solution can be controlled easily. In addition, channels with small conductance are more easily detected since the current measured is the sum of the amplitudes of currents from the population of channels expressed in the entire cell. The disadvantage of whole-cell patch-clamp is the tendency for currents (in particular for Ca$^{2+}$ currents) to diminish after a short time periods of time (run-down), as well as the dialysis of cytoplasmic constituents with the pipette solution leading to the loss of regulatory second messengers. As a remedy of the problems of whole-cell patch-clamp in Ca$^{2+}$ current recording, ATP and the Ca$^{2+}$ chelator EGTA are added to the pipette solution.

A variety of techniques such $^{45}$Ca$^{2+}$ uptake and efflux, aequorin luminescence, bis-azo absorbance dyes, and ion-specific electrodes have been developed to measure intracellular Ca$^{2+}$ in single cells. Because of their difficulty to use and their poor time resolution they are restricted in some laboratories (Cobbold and Rink 1987). The development of fluorescent probes has made the measurement of intracellular Ca$^{2+}$ considerably more accessible (Gryniewiez et al., 1985). Among the well-known Ca$^{2+}$ fluorescent probes are ratiometric dyes, which change their fluorescent properties after binding to free Ca$^{2+}$. The most popular dye, fura-2, is excited at two wavelengths: Ca$^{2+}$-free form at 380 nm and Ca$^{2+}$-binding form at 340 nm, and the emission of the two wavelengths is at 510 nm. Increasing [Ca$^{2+}$], results in an increase in the 340 nm/380nm ratio value. The advantage of ratiometric fluorescent dyes is that variabilities due to nonuniform dye concentrations, probe leakage, dye bleaching and cell thickness are canceled out during the ratio measurements, since these parameters have a similar effect on intensities at both wavelengths. Due to the high affinities for Ca$^{2+}$, this Ca$^{2+}$-chelating dye can reliably measure [Ca$^{2+}$], over the physiological agonist-induced range, from a resting level of approximately 100 nM to a maximal level of several micromolar. Also, the ratio measurement has a very high time resolution, down to the millisecond which is within the range of the Ca$^{2+}$ response to external stimuli. Therefore, this technique can record the Ca$^{2+}$ response in real time. By manipulation of experimental conditions, the relative contribution of extra- and intracellular Ca$^{2+}$ stores and of different Ca$^{2+}$ channels to contraction coupling can be addressed.
Until recently, the study of uterine contraction coupling has focused on the electrical and mechanical properties of smooth muscle at the levels of organ or isolated muscle strips. Morphologically, the myometrium consists largely of two cell types: smooth muscle cells and neurons. Although nerve fibers occupy only 1 to 2% of tissue mass, their presence restricts the use of functional probes, such as Ca\(^{2+}\)-channel blockers or high extracellular K\(^+\), that can stimulate or inhibit release of neurotransmitters (Makhlouf, 1987). In addition, the dense connective tissue matrix in the myometrium serves as a diffusion barrier to agents (Amédée et al., 1987). For electrophysiological studies, the size of the electrical coupling is too large for adequate control of membrane potential during voltage-clamp experiments of multiple myometrial cell preparation.

Addressing the concerns above, the studies performed on intact organ and isolated muscle strips have limitations in the interpretation of the contraction coupling mechanisms. Dispersed smooth muscle cells have been proposed as a useful approach to overcome the limitations because it reduces diffusion barriers to a minimum and eliminates coupling of muscle cells to each other and to neurons (Stemmer et al., 1992). Furthermore, these cells can be monitored by different biochemical and physiological approaches such as patch-clamp and Ca\(^{2+}\) measurement techniques. The studies performed in dispersed cells thus provide more precise information compared to mechanical studies. In Chapter III we developed a method to disperse single porcine myometrial cells whose basic functions were evaluated by whole-cell patch-clamp and Ca\(^{2+}\) measurement techniques.

Myometrial tissues display spontaneous action potentials and resultant contractions, and are therefore classified as phasic smooth muscle. The action potential in phasic smooth muscle is mediated by the VDCC. The VDCC has been found and characterized in rat and human myometrial cells, but there is no information about the VDCC in porcine myometrial cells. In chapter IV, we then identified and characterized the VDCC in prepartum porcine myometrial cells with regards to their voltage dependence, kinetics and pharmacology. This study is essential to understand the functional roles of VDCC in electrophysiological signaling as well as chemical signaling since they are frequent targets for modulation by hormones, neurotransmitters, and drugs in porcine myometrial cells.
In addition to Ca\(^{2+}\) influx across the cell membrane, Ca\(^{2+}\) release from intracellular stores is another fundamental process in Ca\(^{2+}\) homeostasis. Previous studies have revealed that myometrial cells from rodents and humans contain IP\(_3\)-sensitive stores. But the existence of caffeine-sensitive stores in myometrial cells is controversial. Rat myometrial cells do not have this type of store and human myometrial cells may contain a ryanodine-sensitive store which is insensitive to caffeine. In Chapter V, we, therefore, examined the existence of caffeine-sensitive stores in porcine myometrial cells. It has been shown that IP\(_3\) releases intracellular Ca\(^{2+}\) in quantal fashion. However, the information on how Ca\(^{2+}\) releases from caffeine-sensitive stores is sparse. Hence, we also studied possible Ca\(^{2+}\) release mechanisms from the caffeine-sensitive stores in porcine myometrial cells in this chapter.

The contractility of myometrium is regulated by neuronal and hormonal modulators through altering the Ca\(^{2+}\) moving processes. These modulators do not enter the cell, but instead, bind to receptors at the cell membrane and initiate a flow of information (signal transduction) that moves to the cell interior. Previous contractility and ligand binding experiments from our laboratory demonstrated that 1) activation of OT receptors and \(\alpha_2\)-ARs induced the contraction of porcine myometrium, 2) the \(\alpha_2\text{A}^+\) AR subtype was the predominant \(\alpha_2\)-AR, and 3) Ca\(^{2+}\) influx and release were involved in OT receptor- and \(\alpha_2\text{A}^+\)-AR-mediated contraction. However, the membrane and cellular events mediated by these two types of receptors to cause the contraction are unknown. We accordingly investigated the signal transduction of OT receptor and \(\alpha_2\text{A}^-\) AR in porcine myometrial cell in Chapter VI and VII, respectively. The study of signal transduction provides information not only for understanding the cellular mechanisms of contraction coupling of these two receptors, but also on exploring the possible targets of contraction regulation by therapeutic agents.
CHAPTER III  CHARACTERIZATION OF FRESHLY DISPERSED PORCINE MYOMETRIAL CELLS: EVIDENCE FOR VOLTAGE-DEPENDENT CA\(^{2+}\) CHANNEL AND REGULATORY RECEPTORS

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This study describes a method on the isolation of porcine myometrial cells with collagenase for patch-clamp study of Ca\(^{2+}\) currents and spectrofluorometry detection of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)])). When isolated, the myometrial cells showed the typical long cylinder shape. The length and diameter of myometrial cells were 505 ± 20 x 11 ± 0.5 μm (n = 40) in prepartum period and 265 ± 22 x 7 ± 0.4 μm (n = 40) in luteal phase. 90% of the cells were stained positively by immunocytochemistry with an antiserum against desmin and about 95% of the cells excluded trypan blue dye. The basal [Ca\(^{2+}\)], of myometrial cells in the luteal phase and prepartum period were 119 ± 12 (n = 30) and 154 ± 31 nmol l\(^{-1}\) (n = 48), respectively. In prepartum myometrial cells oxytocin (10\(^{-7}\) mol l\(^{-1}\)) and carbachol (10\(^{-4}\) mol l\(^{-1}\)) increased [Ca\(^{2+}\)], in a biphasic pattern with a sharp peak followed by a plateau. In the luteal phase cells adrenaline (10\(^{-7}\) mol l\(^{-1}\)) plus propranolol (10\(^{-6}\) mol l\(^{-1}\)) produced a biphasic increase of [Ca\(^{2+}\)]. However, in the absence of propranolol, the increase of [Ca\(^{2+}\)], by adrenaline was small. Prostaglandin F\(_{2\alpha}\) (10\(^{-6}\) mol l\(^{-1}\)) induced a monophasic increase of [Ca\(^{2+}\)], in luteal phase cells. By depolarizing the cells from -30 to +50 mV at the holding potential of -50 mV, Ca\(^{2+}\) currents were evoked with a threshold at -20 mV, reaching a maximum at +10 to +30 mV. Bay K 8644 (10\(^{-7}\) mol l\(^{-1}\)), an L-type Ca\(^{2+}\) channel agonist, and oxytocin (10\(^{-7}\) mol l\(^{-1}\)) enhanced Ca\(^{2+}\) currents by 166 ± 64% and 41 ± 10%, respectively, in prepartum cells. These results suggest that freshly dispersed porcine myometrial cells contain an intact membrane and possess functional voltage-dependent Ca\(^{2+}\) channels and receptors for major physiological

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regulators. Thus, porcine myometrial cells provide a useful model for the study of excitation-contraction coupling and the influence of physiological regulators in the myometrium.

INTRODUCTION

Calcium ion plays an obligatory role in smooth muscle excitation-contraction coupling. An increase in $[Ca^{2+}]_i$, from intracellular $Ca^{2+}$ store release and from extracellular $Ca^{2+}$ entry, triggers the calmodulin-dependent cascade inducing smooth muscle contraction. Physiologically active agents, such as oxytocin and catecholamine, modulate the excitation-contraction coupling by altering $[Ca^{2+}]_i$, through the release of $Ca^{2+}$ from the sarcoplasmic reticulum or the opening of $Ca^{2+}$ channels in the plasma membrane (Missiaen et al., 1992; Wray, 1993). The critical function of $Ca^{2+}$ in excitation-contraction coupling is demonstrated by the fact that changes in the contractile state of the muscle are often used as a bioassay for changes in $[Ca^{2+}]_i$ (Minneman, 1988). However, it is difficult to explore the cellular mechanisms of excitation-contraction coupling and its modulation by different agents in vivo or even in the intact muscle strips with regards to $Ca^{2+}$. The use of isolated single smooth muscle cells offers several advantages over the multicellular smooth muscle preparation, because single smooth muscle cells: 1) lack dense connective tissue matrix that may serve as a diffusion barrier to agents (Toro et al., 1986; Warshaw et al., 1986), 2) lack a synaptic terminal and varicosities that may contribute to the release of neurotransmitters during extracellular stimulations (Amédée et al., 1986), 3) lack gap junctions that form a functional syncytium (Garfield and Cole, 1989), complicating intracellular signal determination, and 4) can be used for patch-clamp (Hamill et al., 1981) and spectrofluorometry $Ca^{2+}$ determination techniques, as these two techniques have expanded our understanding of the mechanism of contraction and its modulations in smooth muscle cells.

Several reports describe the isolation of myometrial cells and characterization of electrophysiological and biochemical properties of freshly dispersed or cultured myometrial cells (Casey et al., 1984; Amédée et al., 1986; Boulet and Fortier, 1987; Kyozuka et al., 1987; Pressman et al., 1988; Ohya and Sperelakis, 1989; Phillippe et
Most of the studies, however, were performed in rodents. There is increasing evidence that the excitation-contraction coupling and uterine functions are species-specific. For example, $\alpha_1$-, but not $\alpha_2$-adrenoceptors, mediate an increase in myometrial contractions in rats (Digges, 1982), whereas in ewes (Marnet et al., 1987; Prud'Homme, 1988) and sows (Ko et al., 1990; Yang and Hsu, 1993), myometrial contractions are mediated by $\alpha_2$-, but not $\alpha_1$-adrenoceptors.

The primary objective of this study was to develop a freshly dispersed porcine myometrial cell model with functional voltage dependent Ca$^{2+}$-channels and receptors having intact signal transduction pathways. With this model one can address the modulation of myometrium contractility by hormonal and neural agents using Ca$^{2+}$ as a signal.

**MATERIALS AND METHODS**

*Isolation of porcine myometrial cells*

Porcine uteri were obtained from a local slaughter house and a surgical laboratory. Two different states, late (days 107-112) pregnancy and luteal phase of oestrous cycle were used, because these two states of porcine myometrium have responded to pharmacological contractants well (Yang and Hsu, 1993; Yu and Hsu, 1993). The pregnancy status of the uterus was determined by a previously described method (Evans and Sack, 1973), and the luteal phase was determined through visual inspection of the ovary containing corpora lutea, which at this stage were dark red to wine red (Arthur et al., 1989). The mid-portion of uterine horn was dissected and placed into Ca$^{2+}$- and Mg$^{2+}$-free Hank’s balanced salt solution (HBSS). The HBSS contains (in mmol l$^{-1}$): NaCl 137; KH$_2$PO$_4$, 0.66; KCl, 5.4; Na$_2$HPO$_4$, 0.35; dextrose, 4.2; Hepes, 10; phenol red, 11 mg/l (pH 7.4). After being transported to the laboratory, the tissue was rinsed in a Petri dish with HBSS and longitudinal muscle segments were excised. The segments were further minced into approximately 2 mm x 1 mm pieces and washed twice with HBSS. The minced tissue was placed in a 25 ml spinner flask (Bellco Glass, Vineland, NJ, USA) and incubated in HBSS containing 0.1% collagenase (352 U/mg, CLS II, Worthington Biochemical, Freehold, NJ, USA), and 0.2% bovine serum albumin (BSA) at 37 °C. The tissue segments were stirred
continuously at a rate of approximately 60 cycles/min to keep the undigested segments suspended. After the incubation with collagenase for 60 min and 20 min, respectively, the supernatant was discarded, because it contained debris, round stroma and endothelial cells. Fresh enzyme solution was added to the tissue suspension every 20 min, and the supernatant was collected and examined for the presence of myometrial cells, until desirable amounts of cells were harvested. The cells were washed and centrifuged at 50 x g, 25 °C, for 10 min, and were resuspended in a modified Krebs-Ringer bicarbonate solution (KRB) containing (in mmol l⁻¹): NaCl, 118; CaCl₂, 1.25; KH₂PO₄, 1.19; KCl, 4.74; MgSO₄, 0.5; dextrose, 8; Hepes, 10; 0.1% BSA (pH 7.4) for determinations. For obtaining the cells for the whole-cell Ca²⁺ current recordings, a modification of the above protocol was used to obtain cells with minimal contractions. The tissue segments were placed in a 20-ml scintillation vial with HBSS containing 0.5 mmol l⁻¹ MgCl₂, 0.1% collagenase, 0.1% BSA and 0.02% trypsin inhibitor (Sigma Chemical, St. Louis, MO, USA) and stirred gently at 37 °C for approximately 40 min. The supernatant was discarded and the pellet was transferred to HBSS. The pellet then was gently tritutrated with a fire polished plastic pipette and the cell suspension was placed in a culture dish with coverslips (3 mm x 3 mm) in the KRB and kept at 4 °C until use. The cells usually attached to the coverslip within 15 min and were used on the same day. Only the cells with bright and smooth appearance were used.

**Immunocytochemistry**

The cells were obtained in sterile condition from the sows on day 112 of pregnancy using the method described earlier, and were seeded into Petri dishes with histological slides. The slides were previously coated with poly-L-lysine. Waymouth MB752/1 (Sigma Chemical)-10% fetal bovine serum was used for cell culture along with 50 µg/ml gentamicin. The cells were incubated at 37°C with 5% CO₂ and 95% air and were fixed with 4% paraformaldehyde after growing for 2 days and stained for desmin by a modification of the avidin-biotin complex method (Hsu et al., 1981). Endogenous peroxidase activity was inhibited by incubation of the cells in 0.3% H₂O₂ for 30 min. After an incubation for 2 h with normal goat serum, the cells were incubated in primary anti-rabbit desmin monoclonal antibody (Sigma Chemical) at a
dilution of 1:100 for 24 h at room temperature. The secondary biotinylated anti-IgG serum (Vector Laboratories, Burlingame, CA, USA) was applied for 2 h at room temperature followed by an incubation with the avidin-biotin-horseradish peroxidase complex. The cells were washed between incubations with a solution containing (in mmol l⁻¹) NaCl, 154; KH₂PO₄, 43; KHPO₄ 23. Immunologically bound peroxidase was visualized by incubation for 18 min in 0.1 mol l⁻¹ sodium acetate containing 40 mg/100ml diaminobenzidine and 0.05% H₂O₂. The cells were finally lightly counterstained with neutral red, dehydrated, and mounted.

\[ [\text{Ca}^{2+}] \text{ determinations} \]

The cells (10⁶/ml) were incubated with 10⁻⁴ mol l⁻¹ Fura-2 acetoxyethyl ester (Molecular Probes, Eugene, OR, USA) for 45 min at 37 °C in KRB with gentle shaking. The cell mixture was then centrifuged at 50 x g for 10 min, resuspended in KRB (2 x 10⁶/ml) for at least 20 min before the determination. [Ca²⁺], was determined using a SLM8000 spectrofluorometer (SLM instruments, Urbana, IL, USA) as previously described (Hsu et al., 1991).

\[ \text{Ca}^{2+} \text{ current recordings} \]

The whole cell currents were recorded as described previously (Hamill et al., 1981) using an Axopatch-1D clamp amplifier (Axon instruments, Foster City, CA, USA). Patch pipettes (3-5 MΩ) were made on the micropipette puller (Narishige Instruments, Tokyo, Japan) using disposable glass pipettes (VWR Scientific, West Chester, PA, USA) and were polished on a custom-made microforge. The high-resistance seal between the edge of the electrode and the membrane was obtained by applying slight suction to the pipette after gently pushing onto the surface of the cell, which was viewed on a TV monitor. The liquid junction potentials were nullified before the formation of the high-resistance seal with an offset circuit. Access to the intracellular milieu was obtained by applying a negative pressure to the pipette. Ca²⁺ currents were evoked by step depolarization with 300 ms duration every 20 s from a holding potential of -50 mV and with a first step of -30 mV at a 10-mV increment. Linear resistance and capacitative currents were electronically compensated by P/N protocol with N = 4. Data were collected and analyzed using an IBM-PC computer with
analog-digital interface board and pClamp software 5.5 (Axon instruments, Foster City, USA). The patch pipette was filled with a solution containing (in mmol l⁻¹): Cs glutamate, 125; CsCl, 10; Mg-ATP, 2; Na₂EGTA, 10; Hapes, 10 (pH 7.2). The bath solution contained (in mmol l⁻¹): Choline chloride, 125; tetraethylammonium bromide, 20; 4-aminopyridine, 5; BaCl₂ 2; dextrose, 10; Hapes, 10 (pH 7.3). Agents were applied to the bath solution directly and all experiments were performed at room temperature.

**Drug preparations**

Adrenaline bitartrate, carbachol CI, oxytocin and propranolol HCl were from Sigma Chemical. Prostaglandin F₂α, tromethamine and Bay K 8644 were purchased from Upjohn (Kalamazoo, MI, USA) and Research Biochemical International (Natick, MA, USA), respectively. Carbachol and propranolol were dissolved in distilled water. Adrenaline and oxytocin were prepared in 0.1% ascorbic acid and KRB, respectively. Bay K 8644 was dissolved in anhydrous ethanol.

**Statistical analyses**

All values were expressed as mean ± SEM. Data were analyzed by Student’s paired t-test. The significance level was set at P<0.05.

**RESULTS**

**Morphology of porcine myometrial cells**

After dispersion, porcine myometrial cells appeared as long and thin cylinders under inverted microscopy (Fig. 1). The length and diameter of prepartum cells were 505 ± 20 μm and 11 ± 0.5 μm (n=40), and that of luteal phase cells were 265 ± 22 μm and 7 ± 0.4 μm (n=40). Using the cell isolation protocol for the Ca²⁺ current recordings, the smooth appearance indicated that most cells were in their relaxed state (Fig. 1.a). However, during the Ca²⁺ determination procedure many cells became contracted with a rippled surface and shorter in length (Fig. 1.b), compared to the relaxed cells for the patch-clamp technique (Fig. 1.a).
Fig. 1. Light phase-contrast micrographs of freshly dispersed smooth muscle cells from prepartum porcine uterus. Panel A shows relaxed smooth muscle cells for the patch-clamp study. Panel B shows variable contracted smooth muscle cells for Ca^{2+} determinations. Bars show 125 μm.
Verification and viability of porcine myometrial cells

Myometrial cells can be identified morphologically. Desmin, however, is a specific marker found in the cytoskeleton of muscle cells (Lazarides, 1982). In this study, 90% freshly dispersed cells were stained positively with the anti-desmin immunocytochemistry after a 2-day culture (Fig. 2), whereas negative controls failed to show the anti-desmin staining (data not shown). These results confirmed that freshly dispersed cells were smooth muscle cells. Compared to freshly dispersed cells, the cells after a 2-day culture were slightly more rounded and shorter. The desmin granules were seen in the cytosol of these cells. 95% of myometrial cells excluded trypan blue, thus they were viable with an intact cell membrane. At least 90% cells appeared bright under the microscope, further indicating positive viability (Pressman et al., 1988).

Responses of \([Ca^{2+}]\) to agonists

To determine if the isolated porcine myometrial cells were functioning, \([Ca^{2+}]\) determinations and \(Ca^{2+}\) current recordings were studied. The mean resting \([Ca^{2+}]\) values of freshly dispersed cells in luteal phase and prepartum period were 119 ± 12 (n = 30) and 154 ± 31 nmol l\(^{-1}\) (n = 48), respectively, using Fura-2 as the \(Ca^{2+}\) indicator. Oxytocin and carbachol caused an increase in \([Ca^{2+}]\), of prepartum cells in the presence of extracellular \(Ca^{2+}\) concentration of 1.25 mmol l\(^{-1}\) (Fig. 3). Oxytocin (10\(^{-7}\) mol l\(^{-1}\)) significantly increased \([Ca^{2+}]\), from a basal value of 153 ± 14 nmol l\(^{-1}\) to a peak of 244 ± 15 nmol l\(^{-1}\) followed by a plateau (192 ± 12 nmol l\(^{-1}\), taken at 3 min after oxytocin application, n = 9). Carbachol (10\(^{-4}\) mol l\(^{-1}\)) also significantly increased \([Ca^{2+}]\), from a basal value of 156 ± 15 nmol l\(^{-1}\) to a peak of 256 ± 10 nmol l\(^{-1}\), and a plateau of 201 ± 13 nmol l\(^{-1}\) (n = 4). Adrenaline in the presence and absence of propranolol as well as prostaglandin F\(_{2\alpha}\) caused an increase in \([Ca^{2+}]\) in the presence of extracellular \(Ca^{2+}\) concentration of 1.25 mmol l\(^{-1}\) (Fig. 4). After pretreatment with propranolol (10\(^{-6}\) mol l\(^{-1}\)) for 5 min, adrenaline (10\(^{-7}\) mol l\(^{-1}\)) significantly increased \([Ca^{2+}]\), from 120 ± 10 nmol l\(^{-1}\) to 195 ± 8 nmol l\(^{-1}\) and then maintained at a plateau of 154 ± 14 nmol l\(^{-1}\) (n = 5). Fig. 4a shows a representative tracing of these results. In the absence of propranolol (10\(^{-6}\) mol l\(^{-1}\)), adrenaline (10\(^{-7}\) mol l\(^{-1}\)) only slightly but significantly increased \([Ca^{2+}]\), from a basal value of 122 ± 6 to 148 ± 7 nmol l\(^{-1}\)(n = 5).
Fig. 2. Light micrograph of prepartum myometrial cells after 2 days in culture stained with desmin monoclonal antibody using immunocytochemistry. Bar shows 125 \( \mu m \).
Fig. 3. Effects of (a) $10^{-7}$ mol l$^{-1}$ oxytocin and (b) $10^{-4}$ mol l$^{-1}$ carbachol on $[\text{Ca}^{2+}]$, of freshly dispersed smooth muscle cells from prepartum porcine myometrium. Arrowheads show the addition of agents.
Fig. 4b shows a representative tracing of these results. Prostaglandin E_{2a} (10^{-6} \text{ mol l}^{-1}) caused a monophasic increase of [Ca^{2+}]] from a basal value of 119 ± 11 to 150 ± 8 nmol l^{-1} in luteal phase cells (P<0.05, n=3). Fig. 4c shows a representative tracing of these results. The [Ca^{2+}] responses to oxytocin and carbachol were similar in the luteal phase and prepartum cells (data not shown).

Properties of voltage-dependent Ca^{2+} channels and the influence by oxytocin

After blockade of K^{+} currents by high Cs^{+} (125 mmol l^{-1}) in the pipette solution, as well as tetraethylammonium and 4-aminopyridine in the bath solution, an inward current (Ba^{2+} current, I_{Ba}) was elicited by depolarizing from -30 to +50 mV from a holding potential of -50 mV (Fig. 5.a and Fig. 6.a) in the presence of 2 mmol l^{-1} Ba^{2+} in the bath solution. The currents were activated at the membrane potential of -20 mV and reached maximum at +10 to +30 mV. The maximal amplitude was approximately 100 - 500 pA (n = 12) in the luteal phase cells and 200 - 1500 pA (n = 20) in the prepartum cells. In the prepartum cells, Bay K 8644 (10^{-7} \text{ mol l}^{-1}) increased the peak amplitude by 166 ± 64% (n = 6, P<0.05, compared with the control tests), and Fig. 5 shows representative tracings of these results. The shape of I-V curve shifted to left after the Bay K 8644 administration, although the same shape of I-V curve also was observed. The Ca^{2+} current recovered after the washout. Oxytocin (10^{-7} \text{ mol l}^{-1}) increased the peak amplitude by 41 ± 10% (n = 7, P<0.05, compared with the control tests) after 3 min of application. Fig. 6 shows representative tracings of these results. The peak potential was not affected by oxytocin treatment. The Ca^{2+} currents also recovered after the washout.
Fig. 4. Effects of (a,b) adrenaline and (c) prostaglandin F₂α on the [Ca²⁺] of freshly dispersed smooth muscle cells from the luteal phase porcine myometrium. Arrowheads show the application of agents. Note that in (b) before the application of adrenaline (10⁻⁷ mol l⁻¹), cells were pretreated with propranolol (10⁻⁶ mol l⁻¹) for 5 min.
Fig. 5. Effect of Bay K 8644 on inward \( \text{Ba}^{2+} \) current (\( I_{\text{Ba}} \)) through \( \text{Ca}^{2+} \) channels in prepartum porcine myometrial cells. (a): (i) Control \( I_{\text{Ba}} \) elicited by 300 ms depolarization steps from -30 mV to +50 mV at the holding potential of -50 mV in the absence of Bay K 8644; (ii) \( I_{\text{Ba}} \) elicited by the same depolarization protocol in the presence of Bay K 8644 at the concentration of \( 10^{-7} \) mol l\(^{-1} \); and (iii) The \( I_{\text{Ba}} \) tracings obtained 5 min after the washout of Bay K 8644. (b): Correspondent I-V curve of peak \( I_{\text{Ba}} \) before (\( \Delta \)), after (\( \uparrow \)) application and washout (\( \diamond \)) of Bay K 8644.
Fig. 6. Effect of oxytocin on the $I_{ba}$ in prepartum porcine myometrial cells. The extracellular, pipette solutions and depolarization protocol were the same as in Fig. 5. (a): (i) Control $I_{ba}$ tracings in the absence of oxytocin; (ii) The $I_{ba}$ tracings obtained 3 min after the application of oxytocin ($10^{-7}$ mol l$^{-1}$); and (iii) The $I_{ba}$ tracings obtained 5 min after the washout of oxytocin. (b): The effect of oxytocin and its recovery on the I-V curve of peak $I_{ba}$: (a) before; (●) after oxytocin application and (▼) washout.
DISCUSSION

We described a method for the isolation of myometrial cells that can be manipulated as a population or single cells from porcine uteri during the luteal phase of the oestrous cycle and preparturition. The use of cultured cells appears to be an attractive solution for overcoming the limitations of the intact muscle preparation. However, slight variations in the isolation method and culture process may alter the cultured cell properties (Kocher et al., 1984; Boulet and Fortier, 1987). The changes in the property of the cultured myometrial cells may be partially attributed to the lack of sex steroids that regulate the uterine function in vivo. In fact, application of sex steroids makes cultured human myometrial cells responsive to contractants up to 7 days (Pressman et al., 1988), whereas lack of hormonal application makes rat myometrial cells lose the responsiveness to contractants in 36 hours (Amédée et al., 1986).

The long and thin cylinder morphological features observed in our isolated porcine myometrial cells appear to be very similar to those of the rat (Kyozuka et al., 1987) and human (Pressman et al., 1988) isolated myometrial cells and other smooth muscle cells (Warshaw et al., 1986) under light microscopy. The granular distribution of desmin was also consistent with the findings of Boulet et al. (1987) who observed that 90% of rabbit myometrial cells had desmin granules after the 4-day culture. The myometrial cells were easily identified by their morphology. Immunocytochemistry, however, has been useful in not only verifying the cells but also determining the percentage of smooth muscle cells in a population (Loeb et al., 1985), which is critical in the population [Ca^{2+}] determination, because the cells other than myometrial cells may show different response to contractants.

A major problem in the interpretation of such a study is that the dispersion process could damage the functional units, such as ion channels and receptors. In our study, we conclude that the freshly dispersed porcine myometrial cells were only minimally damaged by enzyme for the following reasons: 1) 95% of cells excluded trypan blue and had bright appearance, 2) low extent of Ca^{2+} indicator leakage as indicated by stable fluorescent ratio of 340/380, and 3) satisfactory responses to stimulants.
Because both electro-mechanical and pharmaco-mechanical coupling are of importance in excitation-contraction coupling, voltage-dependent Ca\(^{2+}\) channels and major contractant receptors were evaluated in the freshly dispersed porcine myometrial cells. Voltage-dependent Ca\(^{2+}\) channels are responsible for the upstroke of action potential to induce and maintain the long term myometrial contraction by mediating the Ca\(^{2+}\) entry from the external environment (Missiaen et al., 1992). The voltage-dependent Ca\(^{2+}\) channels in the murine and human myometrial cells are mainly L-type (Ohya and Sperelakis, 1989; Young et al., 1993), although a few T-type Ca\(^{2+}\) channels have also been identified (Young et al., 1993). The fact that Ca\(^{2+}\) currents are activated by depolarization and Bay K 8644 administration suggests that L-type Ca\(^{2+}\) channels exist in the porcine myometrium. The voltage-dependent Ca\(^{2+}\) channel is sensed not only by membrane depolarization but also by hormonal and neural stimulations. The voltage-dependent Ca\(^{2+}\) channel blocker, verapamil, greatly inhibited oxytocin-induced porcine myometrial contractions (Yu et al., 1993), supporting our findings that oxytocin stimulates the contractions by partly increasing [Ca\(^{2+}\)], through opening of these channels. However, our results contradicted to those obtained from rat myometrial cells, in which oxytocin inhibited Ca\(^{2+}\) currents (Inoue et al., 1992).

Myometrial contractility is spontaneously controlled by in vivo electrical activity and is highly regulated by neural and hormonal factors. Of the hormonal regulation, oxytocin and prostaglandin F\(_{2\alpha}\) are two potent stimulators for myometrial contractions. Several lines of evidence suggest that both of them induce contractions by increasing [Ca\(^{2+}\)], (Anwer and Sanborn, 1989; Mackenzie et al., 1990; Molnar and Hertelendy, 1990; Tasaka et al., 1991). Our present data suggested that oxytocin and prostaglandin F\(_{2\alpha}\) receptors and their signal transduction pathways were intact in the freshly dispersed porcine myometrial cells, because activation of these receptors by agonists increased [Ca\(^{2+}\)]. With regards to neural modulation, adrenergic and cholinergic nerves have a great influence on the myometrial contractility. Activation of \(\alpha\)-adrenoceptors and cholinergic receptors increases contractions, whereas activation of \(\beta\)-adrenoceptors promotes relaxation (Wray, 1993). Carbachol and adrenaline in the presence of a \(\beta\)-blocker propranolol increased [Ca\(^{2+}\)], suggesting that cholinergic, \(\alpha\)- and \(\beta\)-adrenoceptors were intact in this preparation.

Little information is in the literature concerning basic characteristics of
excitation-contraction coupling in the myometrium such as Ca\(^{2+}\) stores and their release, Ca\(^{2+}\) channels and Ca\(^{2+}\) entry. This prevents assessment of their regulation by physiological agents. In fact, even for the recognized major regulators of myometrial contraction, such as prostaglandins and oxytocin, surprisingly little is known about their mechanism of action at the cellular level (Parkington and Coleman, 1990). On the other hand, the patch-clamp technique and [Ca\(^{2+}\)], determination have greatly contributed to address the mechanism of excitation-contraction coupling and its regulation in cardiac and skeletal muscles, and other smooth muscle cells. These techniques should facilitate the study of mechanisms of excitation-contraction coupling and their regulation in the myometrium particularly in higher mammals.

In conclusion, we have developed a method to obtain freshly dispersed porcine myometrial cells that have integral structure. These cells express functional voltage-dependent Ca\(^{2+}\) channels and receptors for major contractants. These cells should be useful in the study of mechanisms of excitation-contraction coupling and their modulation by hormonal and neuronal agents with biochemical and electrophysiological approaches.

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CHAPTER IV  ROLES OF TWO TYPES OF CALCIUM CHANNELS IN MYOMETRIAL CELLS FROM PREPARTUM SOWS

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SUMMARY

Voltage-dependent Ca\textsuperscript{2+} channel currents were studied in freshly isolated prepartum porcine myometrial cells using the whole-cell patch-clamp technique. From a holding potential of -80 mV, 8% of the cells presented two types of Ca\textsuperscript{2+} currents; one type being activated at -60 mV and inactivated quickly with a single exponential (T-type) whereas the other required depolarization to -30 mV for activation and inactivated more slowly with two exponentials (L-type). However, 92% of the cells displayed only the L-type current. Nimodipine (1 \mu M) abolished the L-type current but did not inhibit the T-type current. Ni\textsuperscript{2+} (0.17 mM) abrogated the T-type current but failed to change the L-type current. The steady-state activation and inactivation for both types currents were voltage-dependent. Half-activation and -inactivation voltage was -25 mV and -51 mV for the T-type current; 11.2 mV and -10.3 mV for the L-type current. Superimposition of activation and inactivation curves showed a window current between -20 mV and +20 mV for the L-type and -60 mV and -20 mV for the T-type. Using the two-pulse protocol and Ba\textsuperscript{2+} replacement, we found that inactivation of the L-type current was dependent on both Ca\textsuperscript{2+} and membrane potential, while that of the T-type appeared to be dependent only on the membrane potential. The recovery from inactivation was described by a single exponential process for both L-type and T-type currents. In view of the occurrence, properties of voltage dependence and kinetics of these two Ca\textsuperscript{2+} channel currents, we suggested in prepartum porcine myometrial cells that L-type channels are responsible for depolarizing the membrane and delivering Ca\textsuperscript{2+} into the cytosol, whereas T-type channels may contribute to pacemaking action.
INTRODUCTION

Myometrium, a phasic smooth muscle, inherits the spontaneous action potential and consequently mechanical activity. It is well established that \( \text{Ca}^{2+} \) influx through dihydropyridine (DHP)-sensitive \( \text{Ca}^{2+} \) channels, usually defined as L-type, is responsible for the upstroke phase and/or plateau of the spontaneous action potential. However, the ion channels underlying initiation of the action potential (pacemaking) are less understood. Early studies suggested that decreased \( \text{K}^{+} \) conductance and increased \( \text{Na}^{+} \) conductance give rise to slow depolarization which precedes the action potential in myometrial cells (Kuriyama and Suzuki, 1976; Reiner and Marshall, 1975). However, recent patch-clamp studies showed that myometrial cells do not have \( \text{Na}^{+} \) channels before day 5 of pregnancy in rats, although the uterus shows spontaneous electrical and mechanical activity during the same period, suggesting that \( \text{Na}^{+} \) channels may not be a required element to initiate the action potential. In cardiac muscle, substantial evidence indicated that pacemaking depolarization is associated with activation of an inward current which is unselectively carried by \( \text{K}^{+} \) and \( \text{Na}^{+} \), but not with inactivation of an outward \( \text{K}^{+} \) current (Difrancesco, 1993). In phasic smooth muscle such as guinea-pig lymphatic vessels and portal vein spontaneous transient depolarization through \( \text{Ca}^{2+} \)-activated chloride channels has been suggested being a trigger for the action potential (Wang et al., 1992; Van Helden, 1993). It is likely that initiation of the action potential is not attributable to the decrease in \( \text{K}^{+} \) permeability in myometrial cells.

Excitable cells serving as pacemakers, such as the sino-atrial nodal cell (Hagiwara et al., 1988) and interstitial cell of the gastrointestinal tract (Lee and Sanders, 1993), express DHP-insensitive \( \text{Ca}^{2+} \) channels or T-type in addition to the L-type channels. The low-threshold of T-type channel leads to the hypothesis that this channel may be involved in pacemaking function. This is further supported by the evidence that the elimination of extracellular \( \text{Ca}^{2+} \) abolishes the spontaneous action potential in canine colon smooth muscle (Huizinga et al., 1991) and human myometrium (Kawarabayashi et al., 1986), and the blockade of T-type \( \text{Ca}^{2+} \) channels by \( \text{Ni}^{2+} \) slows the pacemaking depolarization rate in cardiac muscle (Hagiwara et al., 1988). The T-type \( \text{Ca}^{2+} \) channel has been reported to be present in human myometrial
cells (Inoue et al., 1990; Young et al., 1993). However, its characteristics have not been fully examined and the role of this channel is yet to be established in myometrial cells.

The patch-clamp studies on the Ca\(^{2+}\) channels in myometrial cells are performed mostly in preparations from rodents (Honore et al., 1989; Ohya and Sperelakis, 1989; Miyoshi et al., 1991; Rendt et al., 1992), few from humans (Inoue et al., 1990; Young et al., 1993) and none from other species. However, it is apparent that the population and activity of Ca\(^{2+}\) channels are species- and reproductive stage-dependent (Mironneau, 1994). In this study we attempted to define the population and voltage dependence, kinetic and pharmacological properties of Ca\(^{2+}\) channels in prepartum myometrial cells from pigs. We demonstrated that porcine myometrial cells contained L-type as well as T-type Ca\(^{2+}\) channels. The analyses on the occurrence, voltage dependence and kinetic of the currents suggested that the L-type functions primarily as a contributor in depolarizing the membrane and resultant increasing intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) while the T-type may be involved in the pacemaking function in the process of spontaneous action potential of myometrial cells.

**MATERIALS AND METHODS**

*Cell Isolation*

Single myometrial cells were isolated as previously described (ZhuGe et al., 1994). Briefly, longitudinal muscle segments were excised from porcine uteri between day 107 and day 112 of pregnancy as determined using a previously described method (Evans and Sack, 1973). The segments were minced and placed in a spinner flask and incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hank’s Balanced Salt Solution (HBSS) containing 0.1% collagenase (352 U/mg, CLS II, Worthington Biochemical, Freehold, NJ), 0.1% BSA and 0.05% trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) for approximately 40 min at 37° C. The supernatant was removed and the pellet transferred to HBSS. The pellet was then gently triturated with a fire-polished plastic pipette and the cell suspension was placed in a culture dish with coverslips in Krebs-Ringer Bicarbonate solution (KRB) kept at 4° C and used the same day. Only the cells
that appeared bright and smooth were used.

**Ca**<sup>2+</sup> Current Recordings

The whole-cell patch-clamp technique was applied to record Ca**2+** current (I<sub>ca</sub>) as described previously (ZhuGe et al., 1994) using a voltage-clamp amplifier (Axon 1D, Axon Instruments, Foster City, CA). Patch pipettes (3-5 MΩ) were made of disposable glass pipettes (VWR Scientific, West Chester, PA) by a two-stage pull and fire polished. The liquid junction potentials were nullified before the formation of gigaseals with an offset circuit. Access to the intracellular milieu was obtained by applying a negative pressure to the pipette. Linear resistance and capacitative currents were electronically compensated by P/4 protocol. Current were low-pass filtered at 1 KHz. The data were acquired and analyzed by an IBM computer with pClamp software version 5.5.1 (Axon Instruments). After obtaining gigaseals and rupturing the patch membrane, we observed that I<sub>ca</sub> gradually increased at the first 5-10 min dialysis period then was stable for 20-30 min, which was followed by a mild tendency of rundown. Therefore, treatments were applied and data were collected after the dialysis for 5-10 min in the present study. All experiments were performed at room temperature.

**Solutions**

The HBSS was composed of (in mM) NaCl, 137; KH<sub>2</sub>PO<sub>4</sub>, 0.66; KCl, 5.4; Na<sub>2</sub>HPO<sub>4</sub>, 0.35; dextrose, 4.2; HEPES, 10 and phenol red, 11 (pH 7.4). The KRB contained (in mM): NaCl, 118; CaCl<sub>2</sub>, 1.25; KH<sub>2</sub>PO<sub>4</sub>, 1.19; KCl, 4.74; MgSO<sub>4</sub>, 0.5; dextrose, 8; HEPES, 10 and BSA, 0.1% (pH 7.4). The pipette solution contained (in mM): Cs glutamate, 125; CsCl, 10; Mg-ATP, 2; EGTA, 10; HEPES, 10 (pH 7.2). The bath solution contained (in mM): Choline chloride, 125; tetraethylammonium bromide, 20; 4-aminopyridine, 5; CaCl<sub>2</sub> 2; dextrose, 10; HEPES, 10 (pH 7.3). When Ba<sup>2+</sup> was needed for the charge carrier, CaCl<sub>2</sub> in bath solution was substituted with equimolar BaCl<sub>2</sub>. All test agents were dissolved in water except that nimodipine was dissolved in ethanol and 4 μl if it was applied directly to the bath solution of 2 ml.. Data are presented as mean ± SE. The statistical significance was estimated by Student’s t test.
RESULTS

Effect of holding potential on $I_{\text{ca}}$

Manipulation of the holding potential (HP) is one of the useful approaches to separate different types of $I_{\text{ca}}$. We used this voltage protocol to examine the existence of two types of $I_{\text{ca}}$. The HP was first set in -80 mV to obtain a current/voltage plot (I/V plot), the potential then was increased to -50 mV to obtain a second I/V plot. Of all the cells tested, two types of responses were obtained. In 92% of the cells, the threshold of activation, the potential for peak current and the apparent reversal potential were not altered by the change in the HP. A representative of this response is shown in Fig.1A. In this cell, the threshold, peak and apparent reversal potentials were -20, +20 and +60 ~ +70 mV, respectively, in two situations. The only apparent difference appears to be a reduction in amplitudes for all potentials in the activation range (-30 to +50 mV) of $I_{\text{ca}}$ at the HP of -50 mV. In the other 8% cells, the change in HP from -80 mV to -50 mV affected the current. Fig. 1B shows the superimposed current tracings produced by three test potentials from two HPs and I/V plots for the same cell. When the HP was -80 mV, $I_{\text{ca}}$ was elicited at a test potential positive to -60 mV. The current evoked from HP of -80 mV were greater than those from -50 mv in the potential range between -30 to +40 mV. The two I-V plots almost overlapped at a more positive potential than +30 mV. The difference current which is probably the T type, began to activate at -60 mV, and reached peak at 0 mV and reversed at +50 mV. This current is inactivated quickly within 100 ms.

Effects of nimodipine, Ni$^{2+}$ and Cd$^{2+}$

In the majority cell types reported so far, the threshold for L-type $I_{\text{ca}}$ is usually -30 to -40 mV. However, L-type $I_{\text{ca}}$ can be activated near the resting membrane potential of -50 ~ -60 mV (Honore et al., 1989; Ganitkevich and Isenberg, 1990; Langton and Sander, 1993). The $I_{\text{ca}}$ altered by HP in some of porcine myometrial cells in the present study, therefore, could be resulted from two possibilities: 1) one population (L-type) with different voltage dependence or 2) two populations of Ca$^{2+}$ channels (L-type and T-type).
Fig. 1. Effect of changes in holding potential on $I_{ca}$. Superimposed traces (Aa, Ba) of currents elicited by 280 ms depolarizing pulses (amplitude indicated on each pair) from holding potentials of -80 mV (●) and -50 mV (◇), and current/voltage (I/V) plots (Ab, Bb) for $I_{ca}$ measured at the peak in correspondent cells in holding potential of -80 mV (●) and -50 mV (◇). Note in B the change in holding potential altered the current significantly. ▲ represented amplitude difference between two holding potentials.
The T-type and L-type $I_{Ca}$ could be separated by their pharmacological responses (Bean, 1985; Fox et al., 1987). DHPs such as nimodipine selectively block the L-type and Ni$^{2+}$ selectively blocks the T-type. The cells whose $I_{Ca}$ was changed by HP were subjected to nimodipine treatment. Nimodipine (1 $\mu$M) abolished the peak amplitude at the HP of -50 mV (Fig. 2A), indicating that nimodipine totally inhibited the L-type Ca$^{2+}$ current. However, at the HP of -80 mV, nimodipine (1 $\mu$M) only reduced the amplitude of peak currents with a residue which is quickly inactivated (Fig. 2B). Before nimodipine treatment, the inactivation of $I_{Ca}$ was best described by two exponentials with time constants of 19.5 ms and 137 ms. After application of nimodipine, $I_{Ca}$ left only with fast inactivation component with a time constant of 39 ms. These results suggested that nimodipine at the concentration of 1 $\mu$M specifically blocked the L-type $I_{Ca}$ but did not affect the T-type $I_{Ca}$ in porcine myometrial cells.

The effect of Ni$^{2+}$ on the T-type current was then studied in the cells with two types of $I_{Ca}$ in the presence of nimodipine (1 $\mu$M). Fig. 3 shows the current tracing obtained with depolarizing pulses from HP of -80 mV to 0 m and I-V plot in the presence and absence of Ni$^{2+}$. Ni$^{2+}$ reduced the peak current by 29 ± 7% (mean ± SE, n = 4) at 50 $\mu$M and abolished the current at 0.17 mM.

The effect of Ni$^{2+}$ on the L-type current was shown in Fig. 4. At 0.17 mM, Ni$^{2+}$ failed to change the current (data not shown). It reduced the current by 37 ± 5% at 0.5 mM. Complete blockade by Ni$^{2+}$ was achieved at the 1.7 mM. Cd$^{2+}$ was more potent in blocking the L-type $I_{Ca}$. It produced 65 ± 11% inhibition at the concentration of 0.17 mM and nearly abolished the current at 0.5 mM.

Steady-state inactivation and activation

Analysis of voltage-dependent activation and inactivation is one approach to differentiate the T- and L- type $I_{Ca}$. The voltage-dependence of steady-state activation is depicted in Fig. 5 for the T-type and Fig. 6 for the L-type. The activation ($I/I_{max}$) was estimated by normalizing the currents evoked by test potentials to the maximal current. The averaged data were fitted to the Boltzmann equation: $I/I_{max} = \{1 + \exp[(V_{1/2}-V)/k]\}^{-1}$ where $V_{1/2}$ is the half-activation potential and $k$ is the slope factor, a measure of the steepness of the voltage dependence of
Fig. 2. Effect of nimodipine on two components of $I_{Ca}$. Superimposed traces of currents elicited by 280 ms duration depolarizing step (10 mV) from holding potential of -80 mV (A) and -50 mV (B) in the absence (▲) and presence (△) of nimodipine (1 μM). The current evoked at the holding potential of -50 mV was abolished by nimodipine application, whereas the current obtained at the holding potential of -80 only partially reduced.
Fig. 3. Effect of Ni²⁺ on the T-type I_c. A, Superimposed current traces obtained in the absence (■) and presence of Ni²⁺ (50 μM, □; 0.17 mM, ▲). Ni²⁺ was applied in the presence of nimodipine (1 μM). The holding potential and test potential were -80 mV and 0 mV, respectively. B, I-V plots were obtained from the same cell in three conditions.
Fig. 4. Effect of Cd$^{2+}$ and Ni$^{2+}$ on the L-type I$_{Ca}$. Aa, Superimposed current traces obtained in the absence (○) and presence of Cd$^{2+}$ (0.17 mM, △; 0.5 mM, ▽). The holding potential and test potential were -50 mV and +30 mV, respectively; Ab, The I-V plots. Ba, Superimposed current traces obtained in the absence (●) and presence of Ni$^{2+}$ (0.5 mM, △; 1.7 mM, ▽). The holding potential and test potential were -50 mV and +10 mV, respectively; Bb, The I-V plots.
Fig. 5. Steady-state activation and inactivation of T-type $I_{Ca}$. The data were acquired in the presence of nimodipine (1 μM). Activation curve (○) was obtained by plotting the amplitudes of peak currents, normalized to maximal current obtained at test potential of 0 mV, against the test potential. Data were fitted with Boltzmann distribution equation $I/I_{\text{max}} = 1/(1 + \exp\left([V_{1/2} - V]/k\right))$ where $V_{1/2} = -25$ mV and $k = 10.4$ mV. Inactivation curve (○) was obtained by plotting the amplitudes of peak currents evoked by testing pulses, normalized to maximal current obtained at prepulse potential of -100 mV, against the prepulse potentials. Data were fitted by the Boltzmann equation $I/I_{\text{max}} = 1/(1 + \exp(V - V_{1/2})/k)$ where $V_{1/2} = -51$ mV and $k = 8.7$ mV. Insets show the pulse protocols. Data points are mean ± SE, n = 3.
Fig. 6. Steady state activation and inactivation of L-type $I_{Ca}$. The activation curve (●) was obtained by plotting the amplitudes of peak currents, normalized to maximal current obtained at test potential of $+20$ mV, against the test potential. The inactivation curve (○) was obtained by plotting the amplitudes of peak currents evoked by testing pulses, normalized to maximal current obtained at prepulse potential of $-100$ mV, against the prepulse potentials. Data were fitted same as in Fig. 5. For activation, $V_{1/2} = 11.2$ mV and $k = 11.9$ mV. For inactivation, $V_{1/2} = -10.3$ mV and $k = 10.8$ mV. Insets show the pulse protocols. Data points are mean ± SE, n = 12.
activations. By fitting, T-type current had a $V_{1/2}$ of -25 mV and $K$ of 10.4 mV. $V_{1/2}$ and $k$ for L-type current was 11.2 mV and 11.9 mV, respectively. The steady-state inactivation was characterized using the two-pulse protocol consisting of a long prepulse (5 s) and a 280 ms test pulse where the peak current is produced. The average values were fitted well by the following function: $\frac{I}{I_{\text{max}}} = \{1 + \exp[(V - V_{1/2})/k]\}$

where $V$ is the prepulse, $V_{1/2}$ is the potential required for half inactivation of the current, and $k$ is the slope factor. The inactivation was measured by the ratio of $I/I_{\text{max}}$ in which $I_{\text{max}}$ is the maximum current amplitude evoked during the test pulse after the most hyperpolarizing prepulse to -100 mV and $I$ is the currents evoked by test pulse. The inactivation curve were obtained by averaged data from 12 cells for L type current and 3 cells for T-type current. $V_{1/2}$ was -10.3 mV and -51 mV, and $k$ was 8.7 mV and 10.8 mV for L type and T type current, respectively. Inactivation is nearly complete at +30 mV and fully removed at - 60 mV for L type. For T-type current, the potential for full inactivation and removal of inactivation are -20 mV and -90 mV , respectively.

These data suggest that the activation and inactivation process appears to be strongly voltage-dependent. When the activation curve was superimposed on inactivation curve, the presence of a significant noninactivating component, "window" current, was noted between -20 mV and +20 mV for L-type. The window current is smaller in the T-type which was between -60 mV and -20 mV.

**Inactivation properties of L- and T-type of $I_{\text{Ca}}$**

When Ca$^{2+}$ was used as a charge carrier, the time course for inactivation of the L-type $I_{\text{Ca}}$ can be best described by the sum of two exponential. The fitting function was $I = I_o + I_1\exp(-t/\tau_1) + I_2\exp(-t/\tau_2)$ where $\tau_1$ and $\tau_2$ are the fast and slow time constants, $I_o$ and $I_1$ are the amplitude of the fast and slow components, and $I_2$ is the amplitude of the noninactivating component. $\tau_1$ was 23.3 ± 1.4 ms and $\tau_2$ was 125.6 ± 6.1 ms when the data were calculated from curve fits of the initial 250 ms from the test potential to evoke the peak current ($n = 19$, Fig. 7Aa). Replacement of Ca$^{2+}$ by Ba$^{2+}$ induced the inactivation of the L-type current to a single exponential with a time constant of 256.1 ± 30.6 ms ($n = 8$, Fig. 7Ab). The inactivation of the T-type $I_{\text{Ca}}$ was best fitted by one exponential with $\tau$ equalling to 31.9 ± 4.8 ms ($n = 7$, Fig. 8Aa) when Ca$^{2+}$ was the charge carrier. Substitution by Ba$^{2+}$ did not significantly change
the \( \tau \) (43.2 \pm 5.6, n=4, \text{Fig. 8Ab}).

To investigate the possible mechanisms for inactivation of Ca\(^{2+}\) channels, we applied a two-pulse protocol consisting of 1) a prepulse ranging from -40 to 100 mV for the L-type and -70 mV to +60 mV for the T-type, 2) a test pulse (280 ms in duration) to +20 mV in the L-type and 0 mV in the T-type, and 3) a resting interval of 15 ms, which is long enough to allow non-inactivated channels to deactivate, but is short enough to allow little recovery from inactivation.

The inactivation of the L-type \( I_{Ca} \) investigated by the two-pulse protocol was shown in Fig. 7B. When Ca\(^{2+}\) was the carrier, the currents produced by test pulse showed a U-shape against prepulse potentials, and the amplitude of the \( I_{Ca} \) was reversely proportional to that produced by prepulses, indicating that Ca\(^{2+}\)-dependent regulation is involved in inactivation of the L-type Ca\(^{2+}\) channel (Fig. 7Ba). When Ba\(^{2+}\) was the charge carrier, the inactivation of currents increased monotonically with prepulse depolarization and saturated after the prepulse potential producing the peak current was approached (Fig. 7Bb). These results suggested that inactivation of Ba\(^{2+}\) current was mediated by a purely voltage-dependent mechanisms without the involvement of a Ca\(^{2+}\)-dependent process.

When the two-pulse protocol applied to a cell with two types of \( I_{Ca} \) in the presence of 1 \( \mu \)M nimodipine, a plot of the current evoked by test pulse against the prepulse potential was obtained as shown in Fig. 8B. As the prepulse potential increased from -70 to 0 mV, the current amplitude of test pulse decreased. However, at potentials between 0 mV and +60 mV where the prepulse current decreased, the current of test pulse did not increase. The fact that lacking of the negative correlation between the current amplitudes produced by test pulse and that by prepulse indicated a purely voltage-dependent mechanism was involved in the inactivation of the T-type \( I_{Ca} \). Substitution of Ca\(^{2+}\) with Ba\(^{2+}\) did not change the amplitude or inactivation of current (data not shown) further confirmed no Ca\(^{2+}\)-dependent mechanism was involved in the T-type Ca\(^{2+}\) channel inactivation.

Recovery from inactivation

The recovery from inactivation was determined using a double-pulse protocol where identical 250 ms depolarizing pulses (\( P_1 \) and \( P_2 \)) to peak current voltages were
Fig. 7. Inactivation of L-type currents. A, Time course of decay phases of the currents elicited by a test pulse (+10 mV) from a holding potential of -50 mV with Ca$^{2+}$ (a) or Ba$^{2+}$ (b) as charge carrier. The currents were best fitted by $I = I_0 + I_1 \exp(-t/r_1) + I_2 \exp(-t/r_2)$ in a and $I = I_0 + I_1 \exp(-t/r)$ in b. The time constants were shown near the traces. B, Double pulse study on inactivation. Cells were depolarized with prepulse pulses (150 ms) to potentials ranging from -40 mV to +100 mV. After a brief return (15 ms) to holding potential, cells were depolarized to a test potential (+20 mV). Representative current traces obtained with Ca$^{2+}$ as charge carriers are shown in the left of B. The plots of peak current amplitudes evoked by prepulse pulses (●) and test pulse (○) against conditional potentials are shown in the right of B.
74

A
a

b

\[ \tau_f = 23.9 \text{ ms} \]
\[ \tau_s = 120.5 \text{ ms} \]

\[ \tau = 279.6 \text{ ms} \]
Fig. 8. Inactivation of T-type currents. A, Time course of decay phases of the currents elicited by a test pulse (0 mV) from a holding potential of -80 mV with Ca$^{2+}$ (a) or Ba$^{2+}$ (b) as charge carrier. The currents were best fitted by $I = I_0 + I_1\exp(-t/\tau)$. The time constants were shown near the traces. B, Double pulse study on inactivation. Cells were depolarized with prepulse pulses (150 ms) to potentials ranging from -70 mV to +60 mV. After a brief return (15 ms) to holding potential, cells were depolarized to a test potential (0 mV). Representative current traces obtained with Ca$^{2+}$ and Ba$^{2+}$ as charge carriers are shown in the left of B. The plots of peak current amplitudes evoked by prepulse pulses (●) and test pulses (○) against conditional potentials are shown in the right of B. Data were obtained in the presence of nimodipine (1 μM).
separated by increasing interpulse intervals ($\Delta t$). The recovery curve was expressed by plotting the current amplitude ratio ($P_j/P_i$) as a function of the interpulse duration. Representative current tracings elicited by this protocol for L- and T-type currents were shown in Fig 9Aa and 9Ba, respectively. Fig. 9Ab and 9Bb respectively show the mean data from 6 cells for the L-type and 3 cells for the T-type currents. The time course of recovery from inactivation was well described by a single-exponential process with time constants of $62 \pm 8$ ms for the L-type current and $145 \pm 23$ ms for the T-type current.

**DISCUSSION**

The present study demonstrated, using the whole-cell patch-clamp method that porcine myometrial cells posses two components of voltage-dependent Ca$^{2+}$ channel currents. One current was activated at -60 mV (low-threshold) and reached the peak around 0 mV. The other was activated around -30 mV (high-threshold) and displayed the peak around +20 mV. The high-threshold and low-threshold current in porcine myometrial cells can be respectively defined as L-type and T-type based on the following further observations: 1) The high threshold current inactivated slower than that of the low-threshold current. The decay phase of the former was long-lasting and is best described by the sum of two exponentials while the latter was transient and could be fitted by one exponential. 2) The steady-state inactivation and activation for the low-threshold current occurred at membrane potential more negatively than the high-threshold one, and 3) The high-threshold current was more sensitive to nimodipine and Cd$^{2+}$ while the low-threshold one was sensitive to Ni$^{2+}$ and resistant to nimodipine.

The presence of two different types of Ca$^{2+}$ currents have been shown in many smooth muscle and non-smooth muscle preparations (Benham et al., 1987; Loirand et al., 1987; Fox et al., 1987). However, the existence of two types of Ca$^{2+}$ channels is not well defined and seems specie-dependent in myometrial cells. Ohya and Sperelakis (1989) failed to detect the T-type $I_{Ca}$ in pregnant rat myometrial cells and claimed that this preparation contains only one type of Ca$^{2+}$ channel. However, two groups have recorded the T-type as well as the L-type current in whole-cell and single-channel levels.
Fig. 9. Recovery from inactivation of L- and T-type currents. A, The L-type currents were elicited by a paired double-pulse protocols where two depolarizing pulses (200 ms, +20 mV) were separated by increasing interpulse intervals (inset). a, Representative currents elicited by testing pulses with interpulse intervals ranging between 50 ms to 500 ms at a increment of 50 ms. b, Plot of ratios of P2/P1 against interpulse intervals. The data were fitted by a single exponential with recovery time constant of 145 ± 23 ms (mean ± SE, n=6). B, Representative T-type currents elicited by the double-pulse protocol (100 ms, 0 mV, inset) with interpulse intervals ranging between 100 ms to 800 ms at a increment of 100 ms (a). b, Plot of ratios of P2/P1 against interpulse intervals. The data were fitted by a single exponential with recovery time constant of 62 ± 8 ms (n=3).
A

\[ +20 \text{ mV} \quad P_1 \quad P_2 \]
\[ -50 \text{ mV} \quad \Delta t \]

![Graph showing current response to voltage pulses]

\[ 100 \text{ pA} \]
\[ 100 \text{ ms} \]

b

\[ \frac{I_{p_2}}{I_{p_1}} \]

![Graph showing ratio of currents over interpulse interval]

Interpulse interval (ms)
in pregnant myometrial cells from human (Inoue et al., 1991; Young et al., 1993). Further, Rendt et al. (1992) reported that the Ca^{2+} channel in nonpregnant rat myometrial cells is a non-L- or -T-type because the current was sensitivity to Ni^{2+}, but is activated and inactivated at a more positive potential than the T-type. The present findings suggested that Ca^{2+} current in porcine myometrial cells shows similarities and discrepancies to that in rat and human myometrial cell. For example, the L-type channel in all three species is highly sensitive to dihydropyridines. But the threshold for the L-type current in porcine myometrial cells was higher than that in rat and human myometrial cells in which they were approximately -50 mV (Honore et al., 1989; Inoue et al., 1990), and the potential producing the peak current and half-activation potential of T-type current were more negative in human myometrial cells where they were -69.7 mV and approximately -30 mV, respectively (Inoue et al., 1990).

In the present study, all cells displayed the L-type current, but only 8% of the cell showed the T-type current. It has been reported that only a small percentage of cells have the T-type current in many cell types including smooth muscle cells (Ganitkevich and Isenberg, 1990; Smirnov and Aaronson, 1992; Xiong et al., 1994). This phenomenon could reflect the heterogeneity in expression of the Ca^{2+} channel in same population of cells. It also might be related to the sensitivity to dispersed processes. For example, Alvarea and Vassort (1992) reported that the T-type current was detected in 91% of the cells isolated with collagenase plus 0.4 mg/ml trypsin, but only in 30% of those dissociated with 1.6 mg/ml pronase. In the present study the cells were dispersed with collagenase without trypsin. The dissociation procedure, therefore, may not be the reason for the result that only a small number of cells have the T-type current. It seems that this result was attributed to heterogeneously expressed Ca^{2+} channels in porcine myometrial cells.

The double pulse and Ba^{2+} substitution experiments showed distinct properties on the inactivation of these two Ca^{2+} currents. Firstly, the inactivation of the L-type current was fitted by a sum of two exponential functions whereas that of the T-type was best described by a single-exponential function. Secondly, the fast component of the inactivation of the L-type was removed when the charger carrier was switch from Ca^{2+} to Ba^{2+}. In contrast, the substitution of Ca^{2+} with Ba^{2+} had no effect on the inactivation of the T-type current. Finally, the inactivation level of the L-type current
was dependent on the amplitude of the Ca^{2+} current evoked by prepulse, i.e., U-shape relationship between the currents of testing pulse and potential of conditional pulse. However, this U-shape relationship was not found in the T-type current. Instead, we observed an L-shape relationship between them, i.e., the inactivation of the T-type current remained stable when the conditional pulse approached the positive membrane potential. These results indicated that inactivation of the T-type current was purely voltage-dependent. However, Ca^{2+}- and voltage-dependent mechanisms are involved in the L-type Ca^{2+} channel inactivation, more specifically, the Ca^{2+}-dependent mechanism may be responsible for fast inactivation and the voltage-dependent mechanism for slow inactivation. The underlying mechanisms for voltage-dependent inactivation of both Ca^{2+} channels are not clear. Obejero-Paz et al. (1991) reported that intercellular application of proteolytic enzymes specifically removes the voltage-dependent inactivation component of the L-type in A7r5 smooth muscle cell, suggesting that the "ball-and-chain" theory, a model proposed by Armstrong and Bezanilla (1977) to interpret the voltage-dependent inactivation of Na^+ and K^+ channels, may also operate in Ca^{2+} channels. However, the molecular motif involved in voltage-dependent inactivation of Ca^{2+} channels may be different from those in Na^+ and K^+ channels (Zhang et al., 1994).

The nature of Ca^{2+}-dependent inactivation of the L-type in smooth muscle is not fully understood. In the present study, Ca^{2+} currents were collected in the pipette solution containing 10 mM EGTA, in which [Ca^{2+}] would be extremely low. In other smooth muscle preparation, a more efficient Ca^{2+} chelator BAPTA reduced, but does not eliminate the Ca^{2+}-dependent inactivation of the L-type channel (Giannattasio et al., 1991; Yoshino et al., 1995). These findings suggest that 1) the Ca^{2+} source for inactivation comes from the entry through Ca^{2+} channels instead of the release from intracellular Ca^{2+} stores, and 2) Ca^{2+} acts at the inner cytosolic side of the channel in which it is inaccessible to EGTA and BAPTA. Single channel recordings from both native and reconstituted channels demonstrated that Ca^{2+} influx during the opening of Ca^{2+} channels changed the gating transition by acting on the cytosolic side of the channels (Yue et al., 1990; Haack and Rosenberg, 1994). Further study indicates that the site for Ca^{2+} binding is located in the \(a\) subunit of the channel (Neely et al., 1994).

The implication for having different inactivation mechanism of two Ca^{2+}
channels is unknown. Phasic smooth muscles contain a high density of L-type Ca\(^{2+}\) channels. These channels have a large conductance (~25 ps) and a "window" current during moderate depolarization. Also they are long-lasted activation. Therefore, the regenerative opening of the L-type produces bursts or slow wave of action potentials and concurrently delivers Ca\(^{2+}\) into the cytosol, resulting in a dramatic increase in [Ca\(^{2+}\)], to trigger the contraction. However, high [Ca\(^{2+}\)], is a toxic to cell. The Ca\(^{2+}\)-dependent inactivation of the L-type channel could provide an efficient negative feedback mechanism in association with Ca-activated K channel to terminate the action potential, thereby controlling Ca\(^{2+}\) influx and maintaining [Ca\(^{2+}\)], at an appropriate range. In comparison to the L-type Ca\(^{2+}\) channels in vascular smooth muscle (Giannattasio et al., 1991; Smirnov and Aaronson, 1992), this channel displays a more predominant Ca\(^{2+}\)-dependent inactivation process in porcine myometrial cells and other phasic smooth muscle (Yoshino et al., 1989; Vogalis et al., 1992; Yoshino et al., 1995), because when Ca\(^{2+}\) was the charge carrier in the latter, the relationship between the current of testing pulse and potential of conditional pulse was a full U-shape. In contrast, when Ba\(^{2+}\) was the charge carrier, the relationship between these two was a flat L-shape with minimal inactivation in currents of testing pulses. This might be a physiological relevant phenomenon in view of the fact that phasic smooth muscle displays action potential and resultant transient alternation in [Ca\(^{2+}\)], at higher amplitude, whereas the tonic smooth muscle displays graded changes in membrane potential and tonic change in [Ca\(^{2+}\)], at low amplitude. In fact, amplitudes of the L-type current in phasic smooth muscle in whole-cell patch-clamp studies are usually much higher than that in tonic smooth muscle (Langton and Standen, 1993; Ganitkevich and Isenberg, 1995). A stronger Ca\(^{2+}\)-dependent inactivation mechanism, therefore, favors the on-line regulation of Ca\(^{2+}\) influx in phasic smooth muscle cells.

The recovery from inactivation for the L-type and T-type current had a monoexponential time course when detected by a pulse evoking their peak current. This is consistent with the reports in other muscle preparations (Jmari et al., 1987; Akaike et al., 1998; Matsuda et al., 1990). The lack of difference in recovery between the L- and T-type current suggests that reversal of voltage-dependent inactivation may be of the rate-limiting step in the recovery of the Ca\(^{2+}\) channels. This stands in line with observations that higher buffering of [Ca\(^{2+}\)], did not significantly change the recovery of
In contrast to the substantial evidence that Ca^{2+} influx through opening of L-type channels produces the action potential and an increase in [Ca^{2+}], the functional implication of the T-type channel has not been well established. The present study and others indicated that the current through the T-type channel was smaller than those through the L-type channel. This is in agreement with studies showing that the T-type Ca^{2+} channel has a low conductance (~9 ps) and low density in smooth muscle (Yatani et al., 1987; Ganitkevich and Isenberg, 1991; Smirnov and Aaronson, 1992). These results suggest that the T-type channel may not be primarily involved in producing upstroke or plateau phase of action potential and increasing [Ca^{2+}]. It has been suggested that the T-type channel may be involved in the generation of pacemaking potential in cardiac muscle and initiation of slow wave action potential in colonic smooth muscle, because this channel is activated near the resting membrane potential and blockage of this channel by Ni^{2+} slows the pacemaking depolarization rate in cardiac cell (Hagihara et al., 1988) and the slow wave in colonic smooth muscle (Huizihga et al., 1991). The observation that there is a "window" current around -60 mV to -20 mV in the present study supports the idea that the T-type channel is involved in initiation of the action potential. The "window" current occurred near the resting membrane potential produces a tonic influx of Ca^{2+}, which could induce minor membrane depolarization despite the small amplitude of current, because smooth muscle cells have very high input resistances. For example 3.79 ± 0.58 GΩ (Matsuda et al., 1990) in coronary artery cells and 11.4 ± 1.3 GΩ in gallbladder smooth muscle (Shimada, 1993). In conjunction with the additional facts that 1) myometrium produces the spontaneous electrical activity, 2) only small percentage of cells contains the T-type channels in current study, and 3) elimination of extracellular Ca^{2+} abolishes the spontaneous action potential in human (Kawarabayashi et al., 1986) and porcine myometrium (Yang and Hsu, unpublished observation), it seems reasonable to hypothesize that the myometrial cells with T-type Ca^{2+} channels may function as pacemakers in uterus. The spontaneous action potential and resultant contraction can be proposed to form underlying the following sequence. The tonic influx of Ca^{2+} through the T-type channel around the resting membrane potential depolarizes to the threshold for regenerative opening of the L-type channel.

Ca^{2+} influx through the L-
type channel produces an action potential which propagates to and depolarizes adjacent cells, by the mediation of gap junctions. The L-type Ca\(^{2+}\) channels in these cells then are consequently activated and an action potential results. The Ca\(^{2+}\) entry during the action potential cause the contraction. Based on this hypothesis one would predict that the occurrence of cells containing the T-type channel can alter the frequency of uterine muscle contraction and the density of gap junctions between cells can modulate synchronous contractions. In fact other studies have shown that increasing in density of gap junctions is one of major elements for inducing synchronous contractions at term (Garfield, 1994). The frequency of spontaneous electrical and mechanical events of the uterus varies during the estrous cycle and pregnancy (Parkington and Coleman, 1990). It would be interesting to determine the occurrence of the T-type Ca\(^{2+}\) channel in different reproductive stages.

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REFERENCES


CHAPTER V  CAFFEINE- AND RYANODINE-SENSITIVE CALCIUM STORES IN PORCINE MYOMETRIAL CELLS: HETEROGENEITY OF ALL-OR-NONE CALCIUM RELEASE

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ABSTRACT

The mechanisms for Ca^{2+} release from caffeine-sensitive stores were investigated in freshly dispersed porcine myometrial cells utilizing the fura-2 method. Because the caffeine-sensitive Ca^{2+} store has not been detected in myometrium of mammals, we first determined the existence of this type of store in porcine myometrial cells. The evidence includes (1) caffeine(1-33 mM) induced a concentration-dependent increase in the intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in both the presence and absence of extracellular Ca^{2+}, and (2) although ryanodine alone (10 \mu M) failed to change [Ca^{2+}], it inhibited the response to caffeine in a use-, concentration- and time-dependent manner. In the cell suspension study, the amount of Ca^{2+} released by 10 mM caffeine was found to be inversely proportional to that by pre-administration of caffeine (1-33 mM). In the single cell study, about 30 % of cells responded to only a certain concentration of caffeine and the others responded to caffeine gradually. Thapsigargin, an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum, failed to increase [Ca^{2+}]. Pretreatment with thapsigargin inhibited the response to caffeine in a time- and concentration-dependent manner. These results suggest that in porcine myometrial cells (1) the Ca^{2+} released from the caffeine- and ryanodine-sensitive store is in an all-or-none manner through compartments of stores or the entire store of a cell, and (2) the release process is regulated by the luminal Ca^{2+} content of the stores.
INTRODUCTION

Intracellular Ca\textsuperscript{2+} stores play a crucial role in the regulation of the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) and subsequent Ca\textsuperscript{2+}-dependent cellular activities, e.g., contraction and secretion. Two Ca\textsuperscript{2+}-release channels are responsible for Ca\textsuperscript{2+} release from intracellular stores. One channel is physiologically regulated by inositol 1,4,5-trisphosphate (IP\textsubscript{3}) which is produced through a GTP binding protein-phospholipase C mechanism after activation of plasma membrane receptors (Berridge, 1993). The ryanodine receptors (RyR) provide an additional principal Ca\textsuperscript{2+}-release channel. Physiologically, this channel is activated by a direct interaction with voltage-dependent Ca\textsuperscript{2+} channels in the transverse tubule of skeletal muscle or by a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism in cardiac muscle (McPherson and Campbell, 1993). Many studies indicate that submaximal IP\textsubscript{3} releases only a fraction of IP\textsubscript{3}-sensitive stores, i.e. in a quantal or all-or-none fashion (Berridge, 1993). Two hypotheses have been proposed to explain the nature of the quanta: (1) Ca\textsuperscript{2+} stores consist of multiple compartments with different sensitivities to IP\textsubscript{3} (Muallem et al., 1989) or with heterogenous density of equally IP\textsubscript{3}-sensitive channels (Hirose and lino, 1994), and (2) the luminal Ca\textsuperscript{2+} content controls the sensitivity of IP\textsubscript{3} receptor (Ivrine, 1990). By comparison, the mechanisms on Ca\textsuperscript{2+} release from caffeine-sensitive stores are less understood. Only a few studies have addressed this issue, the results of which suggest that Ca\textsuperscript{2+} release from the caffeine-sensitive store is also in a quantal fashion (Cheek et al., 1993; Dettbarn et al., 1994).

Caffeine induces Ca\textsuperscript{2+} release from intracellular stores which is followed by contractions in smooth muscles (Missiaen et al., 1992), suggesting the existence of caffeine-sensitive Ca\textsuperscript{2+} stores. Biochemical and functional properties of partially purified RyRs from mammalian aortic and toad stomach smooth muscles (Herrmann-Frank et al., 1991; Xu et al., 1994) share many similarities to those of RyRs in skeletal and cardiac muscles. Yet, there is little information as to how Ca\textsuperscript{2+} is released from the caffeine-sensitive stores in smooth muscles. In addition, the existence of caffeine-sensitive stores in some smooth muscle preparations is controversial. One apparent example is myometrial cells. Rat myometrium does not have caffeine-sensitive Ca\textsuperscript{2+} stores (Arnaudeau et al., 1994), whereas human myometrium may contain an atypical...
type of RyRs, because ryanodine mobilizes intracellular Ca\(^{2+}\) but caffeine does not (Lynn et al., 1993). It was further stated in a recent review article that Ca\(^{2+}\) release from the sarcoplasmic reticulum of myometrial cells appears to be evoked by IP\(_3\), but not by caffeine (Wray 1993). However, Izumi (1994) reported that caffeine causes human myometrial contractions, which indirectly provides evidence for the existence of caffeine-sensitive Ca\(^{2+}\) stores in myometrial cells.

In the present study, freshly isolated porcine myometrial cells were used to investigate whether caffeine-sensitive stores exist. If so, what are possible mechanisms underlying Ca\(^{2+}\) release from these stores? Our present findings suggest that (1) porcine myometrium contains caffeine-sensitive Ca\(^{2+}\) stores that are sensitive to both caffeine and ryanodine, (2) Ca\(^{2+}\) is released from these stores in an all-or-none manner through compartments of the store or the entire store of a cell, and (3) this release process is modulated by the luminal Ca\(^{2+}\) content.

METHODS

Chemicals and materials

Caffeine and thapsigargin (TG) were from Sigma chemical (St. Louis, MO), ryanodine was from Calbiochem (La Jolla, CA), and fura-acetoxyethyl ester (AM) was from Molecular Probes (Eugene, OR).

Preparation of myometrial cells

Porcine uteri were obtained from a local slaughter house and the myometrial cells were isolated as previously described (ZhuGe et al., 1994). Briefly, longitudinal muscle segments were excised from porcine uteri in the luteal phase, identified by the presence of corpora lutea (Arthur et al., 1989). The segments were minced and placed in a spinner flask and incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hank’s Balance Solution containing 0.1% collagenase, 0.2% bovine serum albumin for 60 min and 20 min, respectively, at 37 °C. The supernatants in these two fractions were removed and replaced with fresh enzyme solution. The tissue was bathed for another 20 min and the supernatant, which contained myometrial cells, was collected. The cells were rinsed and centrifuged at 50 x g, 25 °C, for 10 min and resuspended in Krebs-Ringer
bicarbonate solution supplemented with 0.1% bovine serum albumin and 10 mM HEPES (KRB).

\[ [\text{Ca}^{2+}]_i, \text{measurement in cell suspension} \]

\[ [\text{Ca}^{2+}]_i \] of myometrial cells were measured as previously described (ZhuGe et al., 1994). The cells of \(2 \times 10^5/\text{ml}\) were gently shaken in KRB containing fura-2 AM 4 \(\mu\text{M}\) at 37 \(^\circ\text{C}\) for 45 min. The fura-2 loaded cells were centrifuged as above and diluted to \(2 \times 10^5/\text{ml}\) with KRB. Fura-2 fluorescence was monitored in a SLM8000 spectrofluorometer (SLM instruments, Urbana, IL) at excitation wavelengths of 340 nm and 380 nm and at an emission wavelength of 510 nm. The nominal \(\text{Ca}^{2+}\)-free environment was obtained by centrifugation (300 x g, 1 min) and the medium was replaced with the \(\text{Ca}^{2+}\)-free KRB supplemented with 0.1 mM EGTA immediately before \([\text{Ca}^{2+}]_i\) determination (fig. 1B and fig. 3). When cells were subjected to multiple treatments (fig. 2), then the nominal \(\text{Ca}^{2+}\)-free environment was obtained by adding 3 mM EGTA to cells in the \(\text{Ca}^{2+}\)-containing KRB to avoid damaging cells due to repeated centrifugations. All experiments were performed at 22-24 \(^\circ\text{C}\). After monitoring the effects of test agents, 4 mM EGTA was added to the cells followed by 0.2% Triton X-100, which permeabilized the cells. The resulting value of fluorescence represented minimal fluorescence. Maximal fluorescence was determined by adding 6 mM \(\text{CaCl}_2\) to the cuvettes to saturate the fura-2. Values for \([\text{Ca}^{2+}]_i\) were determined using the formula of Grynkiewicz et al. (1985).

\[ [\text{Ca}^{2+}]_i, \text{measurement in single cells} \]

Isolated cells were seeded on the coverslips of a custom-made petri dish and loaded with fura-2 by incubating with 2 \(\mu\text{M}\) fura-2 AM for 45 min at 37\(^{\circ}\text{C}\). The dishes containing the fura-2-loaded cells were mounted on the stage of an inverted fluorescence microscope (Carl Zeiss, NY). Fluorescent images were obtained by alternating excitation wavelengths at 340 nm and 380 nm. The emitted signal at 510 nm was collected every sec by a charged-coupled device camera and stored and processed by an Attofluor digital fluorescence imaging system (Atto instruments, MD). Ratios was calculated by pixel-for-pixel division of two background-corrected images of 340 nm and 380 nm. Noncell-containing area of the coverslip was imaged at each
wavelength as the correspondent background. Because of the difficulty in the calibration of [Ca\(^{2+}\)], in single cells (Jankowski et al., 1994), [Ca\(^{2+}\)], is expressed as a fluorescence ratio of 340/380 nm. The Ca\(^{2+}\)-free condition was obtained by replacing the Ca\(^{2+}\)-KRB with Ca\(^{2+}\)-free KRB.

**Data analysis**

Results are presented as mean ± S.E. Comparisons were made using the unpaired Student's t-test and significance level was set at P<0.05.

**RESULTS**

**Effect of caffeine on [Ca\(^{2+}\)], in cell suspension**

Caffeine (1 - 33 mM) induced a concentration-dependent increase in the [Ca\(^{2+}\)], of porcine myometrial cells from sows in the luteal phase of the estrous cycle (fig. 1). Caffeine induced these increases in both the presence and absence of extracellular Ca\(^{2+}\) with a maximal response observed at 10 mM (fig. 1). The EC\(_{50}\) values of caffeine were 2.6 ± 0.5 mM and 3.1 ± 0.4 mM (n = 5) in the absence and the presence of extracellular Ca\(^{2+}\), respectively. In the presence of extracellular Ca\(^{2+}\), the caffeine(10 mM)-induced increase in [Ca\(^{2+}\)], lasted >10 min. However, in the absence of extracellular Ca\(^{2+}\), the caffeine-induced increase in [Ca\(^{2+}\)], was transient (<2 min) and had a smaller amplitude than was observed in the presence of extracellular Ca\(^{2+}\). These results suggested that caffeine releases Ca\(^{2+}\) from intracellular stores as well as induces Ca\(^{2+}\) influx from the extracellular environment. We did not intend to examine Ca\(^{2+}\) influx in the present study. Instead, we concentrated on the characterization of caffeine-induced Ca\(^{2+}\) release mechanisms in this preparation.

**Effect of ryanodine on caffeine-induced increase in [Ca\(^{2+}\)], in cell suspension**

To investigate whether caffeine releases Ca\(^{2+}\) via activation of RyR, we studied the influence of ryanodine on the response to caffeine. Ryanodine alone (10\(^{-9}\) - 10\(^{-8}\) M) failed to alter [Ca\(^{2+}\)], of porcine myometrial cells during the 15-min exposure in both the presence and absence of extracellular Ca\(^{2+}\) in cell suspension (n = 3, data not shown). In other types of cells, ryanodine binds to Ca\(^{2+}\)-release channels and locks them in an
Fig. 1. Concentration-dependent increase in $[\text{Ca}^{2+}]_i$ by caffeine in freshly dispersed porcine myometrial cell suspension in the presence (A) and absence (B) of 1.25 mM extracellular $\text{Ca}^{2+}$. Arrow heads denote applications of caffeine in this figure and the following figures. Data shown are the representative of five experiments.
open subconductance state, preventing net Ca\(^{2+}\) accumulation and resulting in depletion of the Ca\(^{2+}\) store (Imagawa et al., 1987; Cheek et al., 1993). This effect is known to be use-dependent, thus requiring activation of RyR. To examine this feature, cells from the same longitudinal muscle were divided into four samples and were randomly assigned to the groups that were treated as follows: Group A was challenged directly with caffeine (10 mM) in the Ca\(^{2+}\)-containing KRB supplemented with 3 mM EGTA. Group B was pretreated with ryanodine (10 \(\mu\)M) for 6 min and centrifuged (300 x g, 1 min) and resuspended in Ca\(^{2+}\)-containing KRB, then exposed to caffeine as in group A 10 min later. Group C was pretreated with caffeine (10 mM) for 6 min and centrifuged (300 x g, 1 min) and resuspended in Ca\(^{2+}\) containing KRB, then challenged with caffeine as in group A 10 min later. Group D was pretreated with caffeine (10 mM) for 3 min after an incubation with ryanodine (10 \(\mu\)M) for 3 min. The cells were then centrifuged (300 x g, 1 min) and resuspended in Ca\(^{2+}\)-containing KRB for 10 min and finally exposed to caffeine as in group A. Fig. 2 shows the representative results of 3 experiments. Pretreatment of cells with ryanodine plus caffeine abolished the subsequent response to caffeine (fig. 2D). Pretreatment with ryanodine decreased the [Ca\(^{2+}\)] response to subsequent administration of caffeine (fig. 2B) by 21 \(\pm\) 4\% \((n=3, P<0.05, \text{comparing groups A and B})\). Pretreatment with caffeine also decreased the [Ca\(^{2+}\)] response to the subsequent caffeine administration (fig. 2C) by 34 \(\pm\) 7\% \((n=3, P<0.05, \text{comparing groups A and C})\). These results demonstrated that ryanodine inhibited Ca\(^{2+}\) release by caffeine in the use-dependent fashion.

To further characterize the effect of ryanodine on caffeine-induced increase in [Ca\(^{2+}\)], the cells were pretreated with variable concentrations of ryanodine (10\(^{-8}\) - 10\(^{-5}\) M) for 18 min or were treated with 10\(^{-5}\) M ryanodine for different times (2, 6, and 18 min). Fig. 3 shows that ryanodine inhibited the caffeine-induced Ca\(^{2+}\) release in a concentration- (A) and time- (B) dependent manner. Pretreatment with ryanodine (10\(^{-5}\) M) for 18 min abrogated the Ca\(^{2+}\) response to a maximal caffeine concentration of 10 mM. These observations suggested that: 1) caffeine acts on the caffeine- and ryanodine-sensitive store to release Ca\(^{2+}\), and 2) ryanodine blocks the response to caffeine by depleting this Ca\(^{2+}\) store.
Fig. 2. Inhibition of ryanodine on caffeine-induced [Ca$^{2+}$], increase in a use-dependent manner. Responses were obtained by applying caffeine (10 mM) in the Ca$^{2+}$-containing KRB supplemented with 3 mM EGTA after various pretreatments. A: Control response of cells to caffeine (10 mM). B: Cells were pretreated for 6 min in Ca$^{2+}$-containing KRB in the presence of ryanodine (10 μM) and centrifuged (300 x g, 1 min) and resuspended in the KRB for 10 min. C: Cells were incubated for 6 min in Ca$^{2+}$-containing KRB in the presence of caffeine (10 mM) and centrifuged and resuspended in the KRB for 10 min. D: Cells were pretreated with caffeine (10 mM) for 3 min after an incubation for 3 min with ryanodine (10 μM). The cells were then centrifuged and resuspended in the KRB for 10 min. Data shown are the representative of three experiments.
Fig. 3. Inhibition of ryanodine on caffeine-induced \([\text{Ca}^{2+}]\), increase in a concentration- and time-dependent manner. \([\text{Ca}^{2+}]\), was determined in the absence of extracellular \(\text{Ca}^{2+}\). A: Responses to caffeine (10 mM) after pretreatment with different concentrations of ryanodine for 18 min. B: Responses to caffeine (10 mM) after pretreatment with ryanodine (10 \(\mu\)M) for various times. The response to caffeine (10 mM) alone was considered as 100%. Values are mean ± S.E. from 3-5 independent experiments. *\(P<0.05\), compared with the caffeine alone group.
Graded $\text{Ca}^{2+}$ release to caffeine in cell suspension

In this experiment, we investigated the nature of $\text{Ca}^{2+}$ release from caffeine-sensitive stores. Fig. 4A shows $[\text{Ca}^{2+}]_i$ tracings from the cell suspension that was challenged with caffeine (1 mM - 33 mM), and was followed by 10 mM caffeine in the $\text{Ca}^{2+}$-free medium. Caffeine caused a dose-dependent release of intracellular $\text{Ca}^{2+}$. The subsequent application of 10 mM caffeine induced an additional release of intracellular $\text{Ca}^{2+}$. The released $\text{Ca}^{2+}$ by second administration of caffeine was inversely proportional to that by the first one. This relationship was further quantitatively presented in fig. 4B by plotting the peak $[\text{Ca}^{2+}]_i$, against caffeine concentration of the first administration.

Effect of caffeine on $\text{Ca}^{2+}$ release from single cells

The $\text{Ca}^{2+}$ release response to caffeine was characterized in two patterns at the single cell level. One of them was that upon cumulative administrations of caffeine (1-33 mM), cells responded to only a certain dose of caffeine. Fig. 5A shows two typical examples of this pattern. In the cell shown in the bottom tracing, caffeine at 1 and 3.3 mM failed to release intracellular $\text{Ca}^{2+}$, but 10 mM caffeine increased $[\text{Ca}^{2+}]_i$, as indicated by the rise of 340/380 ratios. Application of caffeine to 33 mM did not further release $\text{Ca}^{2+}$. In the upper tracing, 3.3 mM caffeine initiated the release and depleted the stores. Of the 73 cells examined in response to caffeine, 5 cells had an increase in $\text{Ca}^{2+}$ release at 3.3 mM, 12 cells at 10 mM and 3 cells at 33 mM.

The other 53 cells show another pattern of $\text{Ca}^{2+}$ release to caffeine. In these cells, after the response to a low concentration of caffeine was evoked, increasing caffeine to a higher concentration induced a further $\text{Ca}^{2+}$ release from these stores. As shown in fig. 5B, the initial concentrations of caffeine were 1 mM and 3.3 mM in bottom and upper tracings, respectively. Increased concentrations of caffeine caused further $\text{Ca}^{2+}$ release.

Effect of thapsigargin on caffeine-induced increase in $[\text{Ca}^{2+}]_i$ in cell suspension

The results of $\text{Ca}^{2+}$ efflux from microsomes and single channel recording from the planar lipid bilayer indicate that $\text{Ca}^{2+}$ modulates the RyR in a bell-shape with a maximum in the micromolar $\text{Ca}^{2+}$ concentration range (Meissner et al., 1986; Bull et
Fig. 4. Graded increase in Ca^{2+} release in cell suspension. A. Representative tracings on the effect of maximal caffeine after pre-stimulation with variable concentrations of caffeine. The pre-stimulation of caffeine (▲, 1-33 mM) induced a dose-dependent increase in [Ca^{2+}] , which was transient and returned to the basal level within 2 min. The maximal caffeine (▲, 10 mM) was then administrated which induced an additional increase in [Ca^{2+}] that was inversely proportional to the size of response to the pre-stimulation. B. Relationship of [Ca^{2+}] peaks of the first and second stimulations against first stimulation concentration of caffeine. ◦ and ○ denote the first and second application of caffeine, respectively. Data are mean ± S.E. (n = 4).
Fig. 5. Effects of caffeine on Ca$^{2+}$ release in single cells. [Ca$^{2+}$]$_i$ was measured in Ca$^{2+}$-free medium as described in Methods. A. Caffeine-induced the all-or-none Ca$^{2+}$ release from the whole cell. The cells were stimulated with accumulated caffeine at the concentration shown in the bottom bar. Note that the cells only responded to a particular concentration of caffeine, 3.3 mM in a and 10 mM in b. B. The caffeine-induced graded increase in Ca$^{2+}$ release. The protocol of experiment was the same as in A. The initiating concentrations are different with 1 mM in b and 3.3 mM in a.
al., 1993). To investigate the possibility regarding the modulation of Ca^{2+} on RyR in the intact smooth muscle cells, we examined the effect of thapsigargin (TG), an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum, on caffeine-induced increase in \([Ca^{2+}]_i\). Our previous findings indicated that TG did not induce an increase in \([Ca^{2+}]_i\) of porcine myometrium but progressively depleted the intracellular Ca^{2+} stores (ZhuGe et al., 1994). This depletion of Ca^{2+} store did not trigger the CICR process. Therefore, TG is a useful tool to determine the possible regulation of luminal Ca^{2+} concentration on RyR in this preparation. Fig. 6A shows the effect of TG on caffeine-induced Ca^{2+} release. TG (2 \mu M) inhibited the caffeine response time-dependently with \(t_{1/2}\) of 11 ± 1.5 min. Pretreatment with TG for 25 min abolished the response to caffeine. Moreover, TG (2 \mu M, 10 min) pretreatment caused a right-shift of the caffeine-induced dose-response curve with a decrease of EC_{50} about 3-fold (fig. 6B).

DISCUSSION

Our present findings showed that in porcine myometrial cells from sows in the luteal phase, caffeine induced a concentration-dependent increase in Ca^{2+} release and that ryanodine antagonized this effect of caffeine in a use-, concentration- and time-dependent manner. These findings provided evidence that: 1) porcine myometrium, like vascular and gastrointestinal smooth muscles (lino, 1989; Herrmann-Frank et al., 1991; Xu et al., 1994), may express RyRs as seen in skeletal and cardiac muscles, and 2) activation of these receptors by caffeine induces Ca^{2+} release from intracellular Ca^{2+} stores. Our observations were different from those of human myometrial cells, in which ryanodine, but not caffeine, releases Ca^{2+} from sarcoplasmic reticulum (Lynn et al., 1993). In addition, our findings were different from those of rat myometrial cells in which neither caffeine nor ryanodine affects \([Ca^{2+}]_i\), (Arnaudeau et al., 1994). These discrepancies may be attributed to the multiple effects of caffeine on \([Ca^{2+}]_i\). In addition to activating RyR, caffeine increases cAMP formation by inhibiting phosphodiesterase. As a result, it could promote Ca^{2+}-ATPase activity in sarcoplasmic reticulum (van Breemen and Saida, 1989) and Na^{+}-Ca^{2+} exchanger in the plasma membrane (Moore and Fay, 1993), leading to a decrease in \([Ca^{2+}]_i\). The net effect of caffeine on \([Ca^{2+}]_i\), accordingly, depends on the balance among these different
Fig. 6. Effect of thapsigargin (TG) on caffeine-induced increase in Ca$^{2+}$ release in cell suspension. Fura-2 loaded cells were pretreated with TG for variable times and then centrifuged (300 x g, 1 min) and resuspended in Ca$^{2+}$-free KRB. A. Responses to caffeine (10 mM) after pretreatment with TG (2 μM) for various times. The response to caffeine alone was considered as 100 %. * P<0.05 vs caffeine alone group. B. The response to caffeine (1-33 mM) in controls (o) and after pretreatment with TG (2 μM, o) for 10 min. Data are the net increase in [Ca$^{2+}$], over the basal level. * P<0.05 vs correspondent concentration.
A TG pretreatment time, min

% Increase in [Ca²⁺]
(Caffeine 10 mM = 100%)

0 5 10 15 20 25 30 35

TG pretreatment time, min

B

% Increase in [Ca²⁺], mM

0 10 20 30 40 50

Caffeine concentration, mM

0 5 10 15 20 25 30 35

% Increase in [Ca²⁺], mM

0 10 20 30 40 50

Caffeine concentration, mM

*
mechanisms. Myometrium from different species may have different sensitivities to cAMP (Izumi, 1994). Also, human myometrial [Ca²⁺], and contraction are not as responsive to cAMP as other types of smooth muscles (Word et al., 1991). It is possible, therefore, that different responses to caffeine among different species, or different smooth muscles, might be attributed to their heterogenous responses to cAMP.

In skeletal and cardiac muscles, ryanodine promotes the opening of RyR Ca²⁺-release channels at low concentrations (<10⁻⁵ M) but closes them at higher concentrations (>10⁻⁴ M) (Anserson et al., 1989; Bull et al., 1989). However, the effect of ryanodine on RyRs in smooth muscles is controversial. For example, ryanodine increases [Ca²⁺], in smooth muscle cells isolated from bovine coronary artery (Wagner-Mann et al., 1992) and inferior vena cava (Chen and van Breemen, 1993), but not in the cells isolated from rat tail artery (Alexander and Cheung, 1994). The increase in [Ca²⁺], by ryanodine has been attributed to Ca²⁺ release from caffeine-sensitive stores and a decrease in Ca²⁺ efflux through inhibition of Na⁺-Ca²⁺ exchange in the plasma membrane (Wagner-Mann et al., 1992). The Na⁺-Ca²⁺ exchanger is supposed to be negligible in regulation of Ca²⁺ homeostasis in myometrium (Wray, 1993), which seems to provide a simple explanation for failure of an increase in [Ca²⁺], by ryanodine in the present study. However, given the evidence that (1) ryanodine inhibited the caffeine-induced increase in [Ca²⁺], in a dose-, time- and use-dependant manner in the current study; (2) ryanodine induces the formation of an open subconductance state of RyR from toad smooth muscle cells (Xu et al., 1994); (3) ryanodine binds preferentially to the open state of RyR (Inui et al., 1988); and (4) Ca²⁺ sparks, due to spontaneous Ca²⁺ release through RyR, are modulated by ryanodine in cardiac muscle cells (Cheng et al., 1993). A more reasonable explanation for the different responses to ryanodine among smooth muscles could be proposed as follows: In the resting situation, RyRs spontaneously open and close at a rate which is tissue-dependent. In a tissue with relatively high open-close rate, ryanodine binds to and locks the channels at an open state, which could induce an increase in [Ca²⁺]. In a tissue with a low rate like porcine myometrium, ryanodine also binds to and locks the channels to cause Ca²⁺ release from the stores. However, the channels available are so few that the released Ca²⁺ was disposed of quickly through extrusion and
sequestration, preventing net changes in \([Ca^{2+}]\). Prolonged action of ryanodine in both cases could decrease and eventually deplete the store, thus the caffeine response would be abolished. Upon caffeine stimulation, the rate of opening of RyR \(Ca^{2+}\)-release channels is increased. More channels would be available for locking by ryanodine, leading to a rapid depletion of \(Ca^{2+}\) stores. The idea that heterogeneity in the spontaneous opening and closure of RyRs is supported by the evidence from the studies on molecular entities and function behaviors of RyRs in smooth muscles. For example, in contrast to inducing the formation of an open subconductance state of the channels in toad stomach smooth muscle, ryanodine fails to do so in aorta smooth muscle cells (Herrmann-Frank et al., 1991). Also, all three types of RyRs have been reported in smooth muscles (Marks et al., 1989; Hakamata et al., 1992; Xu et al., 1994). However, we cannot exclude the possibility that the differences in other components of \(Ca^{2+}\) homeostasis such as \(Ca^{2+}\)-ATPase contribute to the heterogenous responses to ryanodine. Further studies using confocal microscopy would provide more direct evidence with regards to the hypothesis of heterogeneity in random opening and closure of RyRs among smooth muscle cells from different organs and species.

In porcine myometrial cell suspension, a low concentration of caffeine released only a part of the caffeine-sensitive stores, since subsequent administration of a higher concentration of the caffeine induced a further increase in \([Ca^{2+}]\) in \(Ca^{2+}\)-free medium (fig. 4). This is similar to the finding of Cheek et al. (1993) who found that stimulation of chromaffin cells in suspension with low concentration of caffeine led to a partial depletion of caffeine-sensitive stores. This feature of caffeine-induced \(Ca^{2+}\) release is similar to that of \(IP_3\)-induced \(Ca^{2+}\) release from \(IP_3\)-sensitive stores, which has been defined as a quantal or all-or-none release. The nature of the quanta for \(IP_3\)-induced \(Ca^{2+}\) release has been the focus of many recent studies. Basically, two hypotheses have been proposed: first, the stores are arranged as discrete functional compartments with regard to the sensitivity to \(IP_3\) (Muallem et al, 1989; Hirose and lino, 1994); and second, the stores consist of homogenous compartments but the sensitivity of \(IP_3\) receptors is regulated by the luminal \(Ca^{2+}\) content (Irvine, 1990). In reconciliation with the above hypotheses, the compartments with heterogenous sensitivity to caffeine have been proposed to account for the all-or-none release from
the caffeine-sensitive store in chromaffin cells (Cheek et al., 1994). Yet, homogeneous stores with RyRs bearing a rapid intrinsic adaptation to Ca$$^{2+}$$ is the underlying mechanism for the quantal release from caffeine-sensitive stores in skeletal muscles (Györke and Fill, 1993; Dettbarn, et al., 1994). Our results did not provide clear evidence for the possibility of the all-or-none Ca$$^{2+}$$ release from compartments of Ca$$^{2+}$$ stores in porcine myometrial cells. However, The fact that TG inhibited the caffeine response time-dependently and decreased the EC$_{50}$ of the caffeine response markedly indicate that caffeine-induced Ca$$^{2+}$$ release is modulated by the luminal Ca$$^{2+}$$ content of Ca$$^{2+}$$ stores as TG decreased the luminal Ca$$^{2+}$$ content without causing the transient rise in [Ca$$^{2+}$$]. Consistent with these results, Cheek et al (1993) reported that an elevation in the luminal Ca$$^{2+}$$ of sarcoplasmic reticulum enhanced caffeine-induced Ca$$^{2+}$$ release from intact chromaffin cells. Therefore, regulation of RyR by luminal Ca$$^{2+}$$ content might be a mechanism for quantal Ca$$^{2+}$$ release from caffeine-sensitive stores in porcine myometrial cells.

One interesting finding of the present study was that caffeine released Ca$$^{2+}$$ in an all-or-none manner from the entire store of a cell, suggesting that caffeine-sensitive stores are not arrayed in compartmentalization in some of the porcine myometrial cells. Thus, a particular concentration of caffeine may trigger a complete emptying of the entire store. This type of response is also found in smooth muscle cells from the guinea-pig urinary bladder (Ganitkevich and Isenberg, 1992). The inactivation or desensitization of RyRs cannot be responsible for this phenomenon because the reconstituted RyRs in bilayers can not be inactivated (Sitsapesan and Williams, 1990). The possibilities for this to occur include: (1) Ca$$^{2+}$$ stores have homogenous sensitivity of RyRs to caffeine; and (2) caffeine-sensitive stores are luminal communicated. The latter seems to be probable since it has been demonstrated in hepatocytes that IP$_3$-sensitive stores are luminal communicated, which makes the almost entire store available for mobilization directly by IP$_3$ (Hajnóczky et al., 1994). Moreover, IP$_3$-generating agonists, carbachol and bradykinin, also induce the all-or-none release of the entire IP$_3$-sensitive stores in smooth muscles (lino et al., 1993; Marsh and Hill, 1993).

The present study and works by others (lino, 1989; Ganitkevich and Isenberg, 1992; Chen and van Breemen, 1993; Xu et al., 1994) indicate that smooth muscles
contain caffeine-sensitive Ca\(^{2+}\) stores. However, the physiological function of these stores needs to be elucidated. Earlier studies suggest that the caffeine-sensitive store is of less physiological importance because this store is smaller than the IP\(_3\)-sensitive store in smooth muscles when studied in skinned preparations (Lin, 1989). Ganitkevich and Isenberg (1992) argued, however, that the results from that type of preparation underestimate the role of caffeine-sensitive stores because the experimental conditions such as buffering of intracellular Ca\(^{2+}\) with 10 mM EGTA may suppress the physiological CICR mechanism. Indeed, by using more physiological conditions, such as experiments in intact cells, several studies demonstrated the role of caffeine-sensitive stores in the CICR mechanism of smooth muscles (Blatter and Wier, 1992; Ganitkevich and Isenberg, 1992). In view of the nature of CICR, caffeine-sensitive stores may be involved in amplifying the IP\(_3\)-generating agonist-induced Ca\(^{2+}\) signal and in inducing cytosolic Ca\(^{2+}\) oscillation and waves in smooth muscles (Missiaen et al., 1992). On the other hand, the fact that smooth muscles contain a bridging structure similar to that between the transverse tubule and the sarcoplasmic reticulum of skeletal muscles (Devine et al., 1972) with a correlated distribution of voltage-dependent Ca\(^{2+}\) channels and RyRs (Carrington et al., 1995), implies the possibility that in smooth muscle the existence of physical coupling of the Ca\(^{2+}\) channels and RyRs is physiologically involved in excitation-contraction coupling of skeletal muscle.

The finding from the present study for heterogeneity of caffeine-sensitive stores may imply another role for this type of Ca\(^{2+}\) stores in excitation-contraction coupling of smooth muscles, particularly in porcine myometrium. Synchronized contraction of smooth muscle is physiologically important for maintaining the normal function of hollow organs. A common role for each smooth muscle cell is to contract and generate force. However, individual smooth muscle cells may play different roles in the process of synchronized contractions. For example, some may be involved in the initiation of contractions or having a pacemaking function. Considering the essential functions of Ca\(^{2+}\) stores in excitation-contraction coupling of smooth muscles, the heterogeneity in mechanisms of Ca\(^{2+}\) release from caffeine-sensitive stores may be of great significance.
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CHAPTER VI  OXYTOCIN-INDUCED A BIPHASIC INCREASE IN \([\text{Ca}^{2+}]_i\) IN PORCINE MYOMETRIAL CELL FROM PREPARTUM SOWS: PARTICIPATION OF PERTUSSIS-SENSITIVE G-PROTEIN, INOSITOL-1,4,5-TRISPHOSPHATE-SENSITIVE CA\(^{2+}\) STORE AND VOLTAGE-DEPENDENT CA\(^{2+}\) CHANNELS

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**ABSTRACT**

This study investigated the underlying mechanisms of oxytocin (OT)-induced increases in intracellular Ca\(^{2+}\) concentrations (\([\text{Ca}^{2+}]_i\)) in acutely dispersed myometrial cells from prepartum sows. A dose-dependent increase in \([\text{Ca}^{2+}]_i\) was induced by OT (0.1 nM - 1 \(\mu\)M) in the presence and absence of extracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_o\)). \([\text{Ca}^{2+}]_i\) was elevated by OT in a biphasic pattern, with a spike followed by a sustained plateau in the presence of \([\text{Ca}^{2+}]_o\). However, in the absence of \([\text{Ca}^{2+}]_o\), the \([\text{Ca}^{2+}]_i\) response to OT became monophasic with a lower amplitude and no plateau, and this monophasic increase was abolished by the pretreatment with ionomycin, a Ca\(^{2+}\) ionophore. Administration of OT (1 \(\mu\)M) for 15 s increased inositol 1,4,5-trisphosphate (IP\(_3\)) formation by 61%. Pretreatment with pertussis toxin (PTX, 1 \(\mu\)g/ml) for 2 h failed to alter the OT-induced increase in \([\text{Ca}^{2+}]_i\) and IP\(_3\) formation. U-73122 (30 nM - 3 \(\mu\)M), a phospholipase C (PLC) inhibitor, depressed the rise in \([\text{Ca}^{2+}]_i\) by OT dose-dependently. U-73122 (3 \(\mu\)M) also abolished the OT-induced IP\(_3\) formation. Thapsigargin (2 \(\mu\)M), an inhibitor of the Ca\(^{2+}\)-ATPase in the endoplasmic reticulum, did not increase \([\text{Ca}^{2+}]_i\). However, it did time-dependently inhibit the OT-induced increase in \([\text{Ca}^{2+}]_i\). Nimodipine (1 \(\mu\)M), a voltage-dependent Ca\(^{2+}\) channel (VDCC) blocker,

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inhibited the OT-induced plateau by 26%. La\(^{3+}\)(1 mM), a nonspecific Ca\(^{2+}\) channel blocker, abrogated the OT-induced plateau. In whole-cell patch-clamp studies used to evaluate VDCC activities, OT (0.1 \(\mu\)M) increased Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) by 40% with no apparent changes in the current-voltage relationship. The OT-induced increase in \(I_{\text{Ca}}\) reached the maximum in 5 min and the increase was abolished by nimodipine (1 \(\mu\)M).

These results suggested that: 1) activation of OT receptors in porcine myometrium evokes a cascade in the PTX-insensitive G-protein-PLC-IP\(_3\) signal transduction, resulting in an increase in \([\text{Ca}^{2+}]_i\), 2) the OT-induced increase in \([\text{Ca}^{2+}]_i\), is characterized by a biphasic pattern, in which the spike is predominately contributed by the intracellular Ca\(^{2+}\) release from the IP\(_3\) sensitive-pool and to a lesser extent by Ca\(^{2+}\) influx, whereas the plateau is from increased Ca\(^{2+}\) influx, and 3) the influx is via VDCC and receptor-operated Ca\(^{2+}\) channels.

INTRODUCTION

In many nonexcitable cells, receptors coupling to heterotrimeric G protein are known to evoke a biphasic increase in intracellular Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)), characterized by an initial spike followed by a plateau phase (Putney, 1993). The spike is due to activation of phospholipase C (PLC) and its hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into a second messenger inositol 1,4,5-trisphosphate (IP\(_3\)), thereby releasing intracellular Ca\(^{2+}\) from the endoplasmic reticulum (ER). Although Ca\(^{2+}\) entry mechanisms are still a matter of dispute, depletion of intracellular Ca\(^{2+}\) store and IP\(_3\)/inositol 1,3,4,5-tetrakisphosphate (IP\(_4\)) has been considered to be the key signal for controlling this process (Irvine, 1992; Berridge, 1993; Putney, 1993). Evidence has been accumulated which suggests that agonists that elicit the increase in IP\(_3\) levels also induce a biphasic \([\text{Ca}^{2+}]_i\), increase in excitable cells in particular the smooth muscle (Murray and Kotlikoff, 1991; Kajita and Yamaguchi, 1993; Pacaud et al., 1993). However, information on mechanisms of this response with regards to Ca\(^{2+}\) entry cross the plasma membrane is not well-understood. It is likely that mechanisms of Ca\(^{2+}\) entry in excitable cells may be more complex that previously thought because voltage-dependent Ca\(^{2+}\) channels (VDCC) (Kajita and Yamaguchi, 1993), as well as receptor/second messenger-operated Ca\(^{2+}\) channels (ROC) are involved (Murray and
Oxytocin (OT) is a powerful stimulator of myometrial contractions and may serve as a physiological initiator of labor (Miller et al., 1993). OT receptors have a seven-transmembrane-domain structure (Kimura et al., 1992) and are coupled to a G-protein (Phaneuf et al., 1993). Activation of these receptors increases the generation of IP₃ and [Ca²⁺]ᵢ (Schrey et al., 1986; Marc et al., 1988; Anwer and Sanborn, 1989; Molnar and Hertelendy, 1990; Tasaka et al., 1991; Phaneuf et al., 1993). However, there is little information on the properties of OT-sensitive Ca²⁺ pools in the myometrium. It is not certain whether VDCC and/or ROC are involved in OT-induced increase in [Ca²⁺]ᵢ. For example, OT has been reported to activate VDCC in cultured rat myometrial cells (Mironneau, 1994), and to inhibit VDCC in freshly dispersed rat myometrial cells (Inoue et al., 1992).

It is generally accepted that receptors activating PLC are coupled to a Gq-protein, but the nature of the G-protein coupled to the OT receptor is controversial in the myometrium. It has been reported that the PTX-sensitive G-protein mediates OT-induced [Ca²⁺]ᵢ increase in the rat (Ruzychy and Crankshaw, 1988; Anwer and Sanborn, 1989) and human myometrium (Schrey et al., 1986; Molnar and Hertelendy, 1990). However, a significant fraction of the response to OT appears to be mediated by a PTX-insensitive G-protein in human myometrium (Phaneuf et al., 1993), and the response to OT appears to be mediated entirely by a PTX-insensitive G-protein in the guinea-pig myometrium (Marc et al., 1988). In the process of establishing an isolated porcine myometrial cell model, we observed that OT induced a biphasic increase in [Ca²⁺]ᵢ (ZhuGe et al., 1994). In the present study we characterized the dynamic profile of this response and signal transduction pathway of OT receptors in porcine myometrium.

**MATERIALS AND METHODS**

*Materials*

All chemicals were from Sigma Chemical Co. (St. Louis, MO), except that U-73122 was from Biomol Research Laboratory (Plymouth meeting, PA), nimodipine was from Research Biochemicals International (Natick, MA), collagenase
was from Worthington Biochemical (Freehold, NJ), and fura-2 acetoxymethylester (AM) was from Molecular Probes (Eugene, OR).

**Preparation of Myometrial Cells**

Single myometrial cells were isolated as previously described (ZhuGe et al., 1994). Porcine uteri between days 107 and 112 of pregnancy were obtained from a local packing plant and a surgical laboratory. The pregnancy status of the uterus was determined using a previously described method (Evans and Sack, 1973). Longitudinal myometrial segments were excised, minced and placed in a spinner flask. The minced tissue was incubated in \( \text{Ca}^{2+} \)- and \( \text{Mg}^{2+} \)-free Hank’s Balanced Salt Solution (HBSS) containing 0.1% collagenase, 0.2% BSA for 60 min and 20 min, respectively, at 37 °C. The supernatants in these two fractions were removed and replaced with fresh enzyme solution. The tissue was bathed for another 20 min and the supernatant, containing a substantial number of myometrial cells, was collected. This dispersion procedure was repeated several times until desirable amounts of cells were harvested. The cells were rinsed by centrifugation at 50 x g, 25°C, for 10 min and resuspended in Krebs-Ringer Bicarbonate solution with 0.1% BSA and 10 mM HEPES (KRB). The porcine myometrial cells prepared using the present method are minimally damaged by collagenase (ZhuGe et al., 1994).

**\( \text{Ca}^{2+} \) Measurements Using Fluorescence Photometry**

\([\text{Ca}^{2+}]\), was measured as previously described (ZhuGe et al., 1994). Myometrial cells were gently shaken in KRB containing 4 µM fura-2AM at 37 °C for 45 min. The fura-2 loaded cells were centrifuged as above and diluted to 2 x 10^5/ml with KRB. Fura-2 fluorescence was monitored in a SLM8000 spectrofluorometer (SLM instruments, Urbana, IL) with excitation wavelength of 340 nm and 380 nm and emission wavelength of 510 nm. In most experiments, \([\text{Ca}^{2+}]\), was performed immediately after centrifuging the cells for 1 min at 300 x g and resuspending the cell pellet in KRB. Under these conditions, there was negligible extracellular fura-2. After monitoring the effects of test agents, 4 mM EGTA (pH 7.4) was added to the cells followed by 100 µM digitonin, which lysed the cells. The resulting value of fluorescence represented minimal fluorescence. Maximal fluorescence was determined
by the addition of 4 mM CaCl₂ to the cuvette to saturate the fura-2. Values for [Ca²⁺],
were determined from the measured 340/380 ratios with the formula of Grynkiewicz et
al. (1985).

Measurement of IP₃ by Radioreceptor Assay

IP₃ was quantified by use of a competitive radioreceptor-binding assay kit
(Dupont, Boston, MA). 2 x 10⁵ cells/ml KRB were placed in polypropylene tubes and
incubated in a shaking water bath in the presence or absence of agents for 15 s at 37
°C. The reaction was terminated by addition of 20% (w/v) of ice-cold trichloroacetic
acid. The aqueous phase was extracted for IP₃ assay. Data were computed by
reference to a standard curve and expressed as fmol of IP₃/10⁶ cells.

Ca²⁺ Current Recordings

The whole-cell patch-clamp technique was used to record Ca²⁺ current as
described previously (ZhuGe et al., 1994). Patch pipettes (3-5 MΩ) were prepared of
disposable glass pipettes (VWR Scientific, West Chester, PA) by a two-stage pull and
fire polished. The liquid junction potentials were nullified before the formation of
gigaseals with an offset circuit. Access to the intracellular milieu was obtained by
applying a negative pressure to the pipette. Linear resistance and capacitative currents
were electronically compensated by P/N protocol with N = 4. Data were collected and
analyzed using an IBM-PC computer with analog-digital interface board and pClamp
software 5.5 (Axon instruments, Foster City, CA). The pipette solution contained (in
mM): Cs glutamate, 125; CsCl, 10; Mg-ATP, 2; EGTA, 10; HEPES, 10 (pH 7.2). The
bath solution contained (in mM): Choline chloride, 125; tetraethylammonium bromide,
20; 4-aminopyridine, 5; BaCl₂ 2; dextrose, 10; HEPES, 10 (pH 7.3). Agents were
applied to the bath solution directly and all experiments were performed at room
temperature.

Data Analysis

Data are presented as mean ± SE and were analyzed by the Student’s test for
paired and unpaired values. Tests were evaluated using a significance level of P<0.05.
Dose-dependent Elevation of \([\text{Ca}^{2+}]_i\) by Activation of OT Receptors

Fig. 1A shows the \([\text{Ca}^{2+}]_i\) in which the cells were challenged with OT (0.1 nM - 1 \(\mu\)M), in the presence (a) or absence (b) of 1.25 mM extracellular \(\text{Ca}^{2+}\) (\([\text{Ca}^{2+}]_o\)). In both situations, the increase in \([\text{Ca}^{2+}]_i\) was found to be saturable, with a maximum occurred at 0.1 \(\mu\)M OT. The EC\(_{50}\) values for OT were similar under both conditions (Fig. 1B): \((3.1 \pm 0.20) \times 10^{-9}\) M (\(n = 9\)) in the presence and \((2.8 \pm 0.16) \times 10^{-9}\) M (\(n = 4\)) in the absence of \([\text{Ca}^{2+}]_o\).

Sources for Increasing \([\text{Ca}^{2+}]_i\) by Activation of OT Receptors

In the presence of 1.25 mM \([\text{Ca}^{2+}]_o\), the increase of \([\text{Ca}^{2+}]_i\) by OT (0.1 \(\mu\)M) was characterized by an early spike followed by a sustained plateau lasting > 5 min (Fig. 1Aa). However, the plateau was not noticeable when the cells were exposed to OT < 0.1 \(\mu\)M. In a \(\text{Ca}^{2+}\)-free medium supplemented with 0.1 mM EGTA, OT (0.1 M) also evoked an increase in \([\text{Ca}^{2+}]_i\), but the magnitude of the spike was reduced to 74 \pm 8% (\(n = 9\)) and the \([\text{Ca}^{2+}]_i\) was restored to the basal level within 3 min (Fig. 1Ab). The time required to reach the peak \([\text{Ca}^{2+}]_i\) was similar in the presence and absence of \([\text{Ca}^{2+}]_o\). The sources \(\text{Ca}^{2+}\) for an increase in \([\text{Ca}^{2+}]_i\) activation of OT receptor was further examined in Fig. 2. EGTA (2 mM) abrogated the plateau established by OT (0.1 \(\mu\)M) (Fig. 2A). When the cells were challenged with ionomycin (0.1 \(\mu\)M) in the absence of \([\text{Ca}^{2+}]_o\), which induced a higher and longer increase in \([\text{Ca}^{2+}]_i\) to deplete the intracellular \(\text{Ca}^{2+}\) pool, OT (0.1 \(\mu\)M) failed to increase \([\text{Ca}^{2+}]_i\) (Fig. 2B). Thus, the peak resulted predominantly from the release of intracellular \(\text{Ca}^{2+}\), whereas the plateau was due to entry of extracellular \(\text{Ca}^{2+}\).

Effects of PTX on OT-induced \([\text{Ca}^{2+}]_i\) Elevation

To examine whether a PTX-sensitive G-protein is involved in the stimulatory effect of OT, we investigated the effect of PTX on the OT-induced rise in \([\text{Ca}^{2+}]_i\). Fig. 3 shows that PTX (1 \(\mu\)g/ml), incubated with cells for 2 h at room temperature, failed to alter the OT-induced increase in \([\text{Ca}^{2+}]_i\) in the absence (A) and presence (B) of 1.25 mM \([\text{Ca}^{2+}]_o\). In addition, increasing the incubation temperature to 37°C did not change
Fig. 1. Effect of OT on $[\text{Ca}^{2+}]_i$ in freshly isolated myometrial cells from prepartum sows. Cells were loaded with fura-2 for 45 min. A: $[\text{Ca}^{2+}]_i$ tracings induced by OT (0.1 nM - 1μM) in the presence (a) and absence of (b) of 1.25 mM $[\text{Ca}^{2+}]_o$. B: Dose response curves of OT-induced increase in peak $[\text{Ca}^{2+}]_i$, in the presence (o) and absence (●) of $[\text{Ca}^{2+}]_o$. Data are mean ± SE (n = 9). *, P<0.05, compared the two groups at the corresponding dose of OT.
Fig. 2. Contribution of the Ca^{2+} release and Ca^{2+} influx to OT-induced increase in [Ca^{2+}]_i in myometrial cells. A: EGTA (2 mM), being applied after establishing the plateau, reduced [Ca^{2+}]_i to basal level. B: Ionomycin (0.1 μM) induced intracellular Ca^{2+} release and blocked that of OT in the absence of [Ca^{2+}]_o. Data are the representative of four individual studies.
Fig. 3. Effect of PTX on the OT-induced $[\text{Ca}^{2+}]_i$ increase in myometrial cells. Cells were incubated for 2 h in the presence or absence of PTX (1 μg/ml). $[\text{Ca}^{2+}]_i$ was determined in the absence (A) or presence (B) of $[\text{Ca}^{2+}]_o$ 1.25 mM. Arrowheads show the application of OT (0.1 μM). Data are the representative of four (A) and seven (B) individual determinations.
the response to OT (data not shown).

**Effect of U-73122 on OT-induced [Ca\(^{2+}\)]\(_i\) Release**

U-73122 specifically inhibits PLC in a variety of cells (Bleasdale et al., 1989). Therefore, we examined if U-73122 specifically inhibited the Ca\(^{2+}\) release through inhibition of PLC. U-73122 (3 \(\mu\)M) abolished the OT-induced Ca\(^{2+}\) release but did not affect that induced by ionomycin (Fig. 4A), which bypasses the PLC-IP\(_3\) pathway. Fig. 4B shows further that U-73122 dose-dependently inhibited the stimulatory response to OT. IC\(_{50}\) of U-73122 to OT was (3.60 ± 0.23) \(\times\) 10\(^{-7}\) M (\(n=6\)).

**Effect of OT on IP\(_3\) Synthesis and its Influence by PTX and U-73122**

The basal level of IP\(_3\) was 61 ± 4.0 fmol/10\(^6\) cells over an incubation of 15 s. Stimulation by 1 \(\mu\)M OT resulted in a 61% increase in IP\(_3\). Pretreatment with U-73122 for 2 min, abolished the OT-induced increase in IP\(_3\) formation. However, pretreatment with PTX (1 \(\mu\)g/ml) for 2 h did not reduce OT-stimulated increase in IP\(_3\) (Fig. 5). PTX and U-73122 alone at the concentration studied failed to change IP\(_3\) levels of porcine myometrial cells (Data not shown).

**Effects of Thapsigargin (TG) Pretreatment on the OT-induced Ca\(^{2+}\) Release**

To characterize the intracellular pool of Ca\(^{2+}\) released by OT, we applied TG, a selective inhibitor of ER Ca\(^{2+}\)-ATPase, which can prevent the uptake of Ca\(^{2+}\) into the IP\(_3\)-sensitive Ca\(^{2+}\) pool (Thastrup et al., 1990). TG (2 \(\mu\)M) did not induce an apparent increase in [Ca\(^{2+}\)], in Ca\(^{2+}\)-free medium suggesting a low rate of Ca\(^{2+}\) leakage (basal release) into the cytosol. However the exposure to TG (2 \(\mu\)M) resulted in a progressive diminution of the amplitude of OT-induced Ca\(^{2+}\) transient over 45 min (Fig. 6).

**Effect of VDCC Blocker on OT-induced Increase in [Ca\(^{2+}\)], and Ca\(^{2+}\) Current**

To investigate the possibility that VDCC contributes to the influx, the VDCC blocker, nimodipine, was applied before OT in the [Ca\(^{2+}\)] measurement study. Nimodipine inhibited the OT-induced spike and plateau of [Ca\(^{2+}\)], by 11 ± 2\% and 26 ± 3\% (\(n=6\)), respectively (Fig. 7A). The effect of OT on VDCC was further evaluated by the whole-cell patch-clamp technique. Fig. 8A shows that OT increased the peak current of VDCC by 40 ± 6\% (\(n=12\)) with no apparent change in the current-voltage relationship. The time course of change of the current by bath application of OT is
Fig. 4. Effect of U-73122 on the release of intracellular Ca\textsuperscript{2+} by ionomycin and OT. 
$[\text{Ca}^{2+}]_i$ was determined in Ca\textsuperscript{2+}-free medium supplemented with 0.1 mM EGTA. A: U-73122 (3 µM) abolished OT (0.1 µM)-induced Ca\textsuperscript{2+} release, but did not inhibit that induced by ionomycin (0.1 µM). B: Dose-dependent inhibition of U-73122 on OT-induced Ca\textsuperscript{2+} release. Data are mean ± SE (n=6). *, P<0.05 vs. OT alone.
Fig. 5. Effects of PTX and U-73122 on OT-induced IP$_3$ formation in myometrial cells. Cells were exposed to PTX (1 μg/ml) and U-73122 (3 μM) for 2 h and 2 min, respectively, before the administration of OT (1 μM) for 15 s. Data are mean ± SE (n=3). Significant differences (P<0.05) between groups are designated by different letters.
Fig. 6. Time course effect of thapsigargin (TG) on the OT-induced Ca\textsuperscript{2+} release. Cells were incubated with TG (2 μM) in the presence of 1.25 mM [Ca\textsuperscript{2+}]\textsubscript{o}. [Ca\textsuperscript{2+}]\textsubscript{i} was measured in the Ca\textsuperscript{2+}-free medium supplemented with 0.1 mM EGTA. Data are mean ± SE (n=6).*, P<0.05 vs. OT alone.
Fig. 7. Effects of Ca\textsuperscript{2+} channel blockers on OT-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i}. [Ca\textsuperscript{2+}]\textsubscript{i} was determined in the presence of 1.25 mM [Ca\textsuperscript{2+}]\textsubscript{o}. A: The cells were challenged without and with nimodipine (Nim, 1 \textmu M) for 30 s before addition of OT 0.1 \mu M. B: La\textsuperscript{3+} (1 mM) applied before addition of OT blocked OT-induced Ca\textsuperscript{2+} plateau. Data are the representative of six (A) and four (B) experiments.
shown in Fig. 8B, in which the OT-elevated current reached a steady state in approximately 5 min; subsequent application of nimodipine (1 μM) rapidly abrogated the stimulatory effect of OT confirming the L-type VDCC nature.

**Effect of La**

Effect of La\(^{3+}\) on OT-stimulated Ca\(^{2+}\) Influx

The blockade of Ca\(^{2+}\) influx by La\(^{3+}\), a nonspecific Ca\(^{2+}\) channel blocker, was determined by adding the ion before OT application. We used a high concentration of 1 mM La\(^{3+}\) in these experiments as previously described in smooth muscle cells (Murray and Kotlikoff, 1991). La\(^{3+}\) alone caused a small decrease in [Ca\(^{2+}\)]. Addition of La\(^{3+}\) changed the OT-induced biphasic to monophasic Ca\(^{2+}\) increase (Fig. 7B).

**Effect of TG on Ca**

Effect of TG on Ca\(^{2+}\) Influx

In some types of nonexcitable cells, agonist-induced Ca\(^{2+}\) influx depends only on the depletion of an intracellular Ca\(^{2+}\) store, i.e., the capacitative mechanism (Putney, 1990). TG is a useful pharmacological tool for diagnosing the operation of the capacitative mechanism for Ca\(^{2+}\) influx (Takemura et al., 1989). Therefore, we investigated the role of intracellular Ca\(^{2+}\) stores in regulating Ca\(^{2+}\) entry with TG. Fig. 9 (trace a) shows that restoring extracellular Ca\(^{2+}\) in cell suspension induced an [Ca\(^{2+}\)] increase from 105 ± 4 nM to 144 ± 6 nM (n = 3). Pretreatment with TG (2 μM) for 45 min, did not enhance the further increase (Fig. 9, trace b). However, OT (0.1 μM) promoted the [Ca\(^{2+}\)] increase from 106 ± 4 to 179 ± 8 nM (n = 3, Fig. 9, trace c).

**Effect of U-73122 and TG Pretreatment on the OT-induced Increase in [Ca**

Effect of U-73122 and TG Pretreatment on the OT-induced Increase in [Ca\(^{2+}\)] in Ca\(^{2+}\)-Containing Medium

As demonstrated above, OT increased [Ca\(^{2+}\)], by promoting the release of intracellular Ca\(^{2+}\) store and entry of extracellular Ca\(^{2+}\). To examine the relationship between Ca\(^{2+}\) release and influx, we determined if inhibition of OT-induced Ca\(^{2+}\) release would affect OT-induced Ca\(^{2+}\) influx. In the presence of 1.25 mM [Ca\(^{2+}\)]\(_e\), U-73122 inhibited the OT-induced increase in [Ca\(^{2+}\)], dose-dependently and at 3 μM it abolished the response to OT, i.e., both the release and influx components disappeared (Fig. 10A). The relationship between the Ca\(^{2+}\) release and influx was further
Fig. 8. Effect of OT on Ca\textsuperscript{2+} current and its alteration by nimodipine (Nim). Aa: Peak Ca\textsuperscript{2+} current tracings elicited by 300 ms depolarization from -50 mV to +10 mV in the absence and presence of 0.1 μM OT. Ab: Voltage-current relationship of Ca\textsuperscript{2+} current before and after application of 0.1 μM OT. Peak current of control was considered as 100%. B: Time course effect of OT on Ca\textsuperscript{2+} current. Data points represent currents elicited by 300 ms steps from -50 mV to +10 mV at a interval of 20 s. Data are the representative of four experiments.
A

+10 mV 0.3 s
-50 mV

Control

OT 0.1 μM 200 pA
30 ms

B

Membrane potential, mV

-30 -20 -10 0 10 20 30 40 50

% of control peak current

0 20 40 60 80 100 120 140 160

Control

OT 0.1 μM

OT 0.1 μM Nim 1 μM Wash

Current, pA

-100 -200 -300 -400 -500

-600

0 2 4 6 8 10 12 14 16 18 20

Time, min
Fig. 9. Effect of TG on Ca\(^{2+}\) influx.  

a: Addition of 1.25 mM Ca\(^{2+}\) to cells in Ca\(^{2+}\) free medium increased [Ca\(^{2+}\)].  
b: Addition of 1.25 mM Ca\(^{2+}\) to cells pretreated with TG (2 μM) for 45 min did not enhance Ca\(^{2+}\) influx compared to a.  
c: Addition of 1.25 mM Ca\(^{2+}\) to cells after Ca\(^{2+}\) release by OT potentiated the Ca\(^{2+}\) influx compared to a.  
Data are the representative of three experiments.
Fig. 10. Effects of U-73122 and TG on OT-induced increase in $[\text{Ca}^{2+}]_i$ at 1.25 mM $[\text{Ca}^{2+}]_o$. A: U-73122 (b, 0.3 $\mu$M) reduced and (c, 3 $\mu$M) abolished the $[\text{Ca}^{2+}]_i$ response to OT (a, OT 0.1 $\mu$M alone), respectively. B: Pretreatment with 2 $\mu$M TG for 15 min (b) reduced and for 45 min (c) abolished the $[\text{Ca}^{2+}]_i$ response to OT (a, OT 0.1 $\mu$M alone), respectively. Data are the representative of four experiments.
evaluated by pretreatment with TG in Ca\textsuperscript{2+}-containing medium. Pretreatment with TG (2 \mu M) for 15 min inhibited the OT-induced increase in [Ca\textsuperscript{2+}], and for 45 min abolished OT-induced stimulatory effect in [Ca\textsuperscript{2+}], (Fig. 10B). These observations suggested that OT-induced Ca\textsuperscript{2+} influx depends on its release.

**DISCUSSION**

Data presented in the present study showed that OT increased [Ca\textsuperscript{2+}], of porcine myometrial cells in a dose-dependent manner in both the presence and absence of [Ca\textsuperscript{2+}]\textsubscript{o}. A mobilization of Ca\textsuperscript{2+} after OT application has been previously reported for human (Molnar and Hertelendy, 1990; Tasaka et al., 1991; Phaneuf et al., 1993) and rat (Anwer and Sanborn, 1989) myometrial cells. A novel observation in this study was that OT (0.1 \mu M) induced a biphasic increase in [Ca\textsuperscript{2+}], consisting of a spike followed by a plateau. This biphasic response of [Ca\textsuperscript{2+}], to OT was similar to that observed in many other cells, such as vascular and airway smooth muscles (Murray and Kotlikoff, 1991; Pacaud et al., 1993) in response to a variety of hormones. The ability of OT to elevate [Ca\textsuperscript{2+}], in the absence of [Ca\textsuperscript{2+}]\textsubscript{o} and its abolition by the ionomycin-induced depletion of Ca\textsuperscript{2+} stores suggested that the OT-induced Ca\textsuperscript{2+} spike is attributable to the release of Ca\textsuperscript{2+} from intracellular stores. In the absence of [Ca\textsuperscript{2+}]\textsubscript{o}, the amplitude of OT-induced spike in [Ca\textsuperscript{2+}], was reduced. This should not be due to the depletion of the intracellular Ca\textsuperscript{2+} store that was caused by a < 1 min exposure to a Ca\textsuperscript{2+}-free medium because the cells had a low Ca\textsuperscript{2+}-leakage as shown in the Fig. 6. These results suggested the OT-induced Ca\textsuperscript{2+} influx also contributes to the spike. However, the OT-induced plateau seems to be due entirely to the influx of Ca\textsuperscript{2+} from the extracellular source because it was abolished in the absence of [Ca\textsuperscript{2+}]\textsubscript{o} and by addition of EGTA. In the previous study, OT induced only a single transient contraction of porcine uterine strip in the absence of [Ca\textsuperscript{2+}]\textsubscript{o} and a sustained contraction in the presence of [Ca\textsuperscript{2+}]\textsubscript{o} (Yu et al., 1993), which was similar to those of norepinephrine in vascular smooth muscle (Nielsen et al., 1992) and acetylcholine in tracheal smooth muscle (Farley and Miles, 1978). In the present study, OT induced a transient and a prolonged increase in [Ca\textsuperscript{2+}], in the absence and presence of [Ca\textsuperscript{2+}]\textsubscript{o}, respectively. Although the time courses were not correlated precisely between contraction and
[Ca$^{2+}$], responses to OT, it is likely that the release of intracellular Ca$^{2+}$ triggers the transient contraction and that the Ca$^{2+}$ influx contributes to the maintenance of the sustained contractions. However, for prolonged contractions we cannot exclude the possibility that OT increases the sensitivity of contractile elements through Ca$^{2+}$-independent pathways (Matsuo et al., 1989) in addition to an increase in [Ca$^{2+}$].

In the present study, OT stimulated a rapid hydrolysis of PIP$_2$ into IP$_3$ in porcine myometrial cells which was consistent with the findings in other species (Schrey et al., 1986; Marc et al., 1988; Anwer and Sanborn, 1989; Molnár and Hertelendy, 1990; Tasaka et al., 1991; Phaneuf et al., 1993). This supports the view that PLC is coupled to the OT-induced increase in [Ca$^{2+}$], in the myometrium (Molnar and Hertelendy, 1990; Phaneuf et al., 1993). This view was further supported by the present findings that U-73122, a specific PLC inhibitor (Bleasdale et al., 1989), depressed both the IP$_3$ formation and Ca$^{2+}$ increase by OT. IP$_3$ is a second messenger which mediates the effects of a variety of hormones to release intracellular Ca$^{2+}$ from the ER (Berridge, 1993) and it releases Ca$^{2+}$ from microsomes in the rat myometrium (Carsten and Miller, 1985). In the present study, OT released Ca$^{2+}$ from the IP$_3$-sensitive pools in intact myometrial cells, because: 1) OT promoted the IP$_3$ formation and 2) TG, the ER Ca$^{2+}$ pump inhibitor which can deplete the IP$_3$-sensitive Ca$^{2+}$ pools (Thastrup et al., 1990), blocked OT-induced increase in [Ca$^{2+}$],. Our data did not favor the proposal that OT increases [Ca$^{2+}$], by inhibiting the Ca$^{2+}$-ATPase of the ER (Magocsi and Penniston, 1991) because OT released the intracellular Ca$^{2+}$ with a fast kinetics, and TG did not increase [Ca$^{2+}$],. Another interesting observation is that OT-sensitive Ca$^{2+}$ stores in the prepartum myometrium had a low leakage of Ca$^{2+}$ (Fig. 6). Our findings were consistent with the reports in A$_7f_5$ vascular smooth muscle cells showing that TG depletes intracellular Ca$^{2+}$ with $t_{1/2}$ of 18 min (Missiaen et al, 1992) and in rat aorta showing that pretreatment with TG for 1-3 h only partially inhibits phenylephrine-induced contraction (Low et al., 1991).

In the present study, PTX, at a high concentration of 1 $\mu$g/ml incubated for 2 h, failed to inhibit the increase of Ca$^{2+}$ and formation of IP$_3$ by OT. In our laboratory, the same PTX preparation at a lower concentration of 0.1 $\mu$g/ml abolished the $\alpha_2$-adrenergic receptor mediated decrease in insulin secretion from insulin-secreting cells (Chen and Hsu, 1994). These results suggested that a PTX-insensitive G-protein was
coupled to the OT receptor in porcine myometrial cells. Compelling evidence indicates a class of G-protein Gq, which does not contain the amino acid sequences necessary for PTX-induced ADP-ribosylation, mediates the receptor-activated hydrolysis of PIP2 by PLC in many tissues (Hepler and Gilman, 1992). Since Gaq and Ga11 are found in the mouse and human myometria (Wilkie et al., 1991; Phaneuf et al., 1993), it is likely that in porcine myometrial cells that a similar Gq family exists which mediates the IP3 and [Ca2+] responses to OT. Further studies are needed to characterize G-proteins in porcine myometrium.

Extracellular Ca2+ is required for the OT-induced increase in [Ca2+]. In the present study OT promoted the opening of L-type Ca2+ channels and nimodipine inhibited the OT-induced increase in VDCC current and [Ca2+]. These results suggested that VDCC is an essential component for OT-dependent increases in [Ca2+]. Our observation is in accord with the findings that nimodipine partially inhibits OT-induced increase in [Ca2+], in human myometrial cells (Tasaka et al., 1991), and OT increases the VDCC current in cultured rat myometrial cells (Mironneau, 1994). However, our data were not consistent with the reports that D-600, a VDCC blocker, fails to block OT-induced increase in [Ca2+], in rat myometrial cells (Anwer and Sanborn, 1989) and that OT depresses the VDCC current in freshly dispersed rat myometrial cells (Inoue et al., 1992). These discrepancies may reflect difference in the experimental condition and species used in the studies. The advantage of the present study was that the [Ca2+], determination and patch-clamp studies were performed using cells from the same tissues. Results from our present study with isolated cells also agreed with the study performed in isolated muscle strips, in which verapamil, a VDCC blocker, attenuated the OT-induced increase in contractions (Yu et al., 1993). However, Ca2+ entry through VDCC may not be the main source of Ca2+ influx induced by activation of OT receptors as shown in Fig. 7A. Our data on [Ca2+], provide evidence that the ROC is involved in the OT-induced Ca2+ influx. In fact, the ROC has been suggested to be involved in agonist-induced Ca2+ influx in several smooth muscles including myometrium (Batra, 1986; Murray and Kotlikoff, 1991; Pacaud and Bolton, 1991; Pacaud et al., 1993). Patch-clamp recordings of ROC currents, nevertheless, will be necessary to clarify the properties of these channels. In A7r5 vascular smooth muscle cells, vasopressin-induced Ca2+ influx is independent of
its release phase (Thibonnier et al., 1991). However, in our present study, the plateau phase of the increase in [Ca^{2+}], induced by OT depended on the spike phase of that in the porcine myometrium. This result combined with the fact that the time reaching the peak [Ca^{2+}] was similar in the presence and absence of [Ca^{2+}], indicated that the same signal transduction pathway, i.e, OT receptor-G-protein-PLC-IP_3, mediated both phases. IP_3 is a direct mediator for Ca^{2+} release (Berridge, 1993), but the signals for increasing Ca^{2+} influx following an increase in IP_3 remain to be elucidated. In nonexcitable cells current hypothesis indicates the following are involved in the stimulation of Ca^{2+} influx: 1) IP_3 and/or IP_4 with their receptors (Irvine, 1992; Luckhoff and Clapham, 1992); 2) diffusible messengers, such as Ca^{2+} influx factor (Randriamampita and Tsien, 1993), generated by depletion of IP_3 sensitive Ca^{2+} pool, and 3) unidentified small G-protein (Bird and Putney, 1993; Fasolato et al., 1993). In smooth muscle, depletion of Ca^{2+} stores has been suggested to induce Ca^{2+} influx (Pacaud and Bolton, 1991). The fact that the Ca^{2+} store depletion by TG did not enhance Ca^{2+} entry in the present study implicated that the signal for Ca^{2+} store depletion alone may not be sufficient to evoke the Ca^{2+} influx. Whereas pretreatment with U-73122 and TG, respectively, abolished the OT-induced Ca^{2+} influx suggesting that IP_3 could not be the sole signal for OT-induced Ca^{2+} influx. These observations may imply that in myometrial cells parallel signals from depletion of Ca^{2+} stores and production of IP_3/IP_4 are required for OT-induced Ca^{2+} influx. A similar mechanism has been proposed by Pacaud et al. (1993) in which norepinephrine-mediated Ca^{2+} entry in portal vein smooth muscle cells is attributed to the decrease in the Ca^{2+} content of the stores in addition to the increase in the concentrations of IP_3 and IP_4. Other possible mechanisms may include that the increased cytosolic Ca^{2+} (von Tscharner et al., 1986) or the components of downstream to Ca^{2+}, such as myosin light chain kinase (Nakanishi et al., 1994), are involved in the activation of the Ca^{2+} channels in plasma membrane. More studies are needed to explore the underlying mechanisms for OT-induced Ca^{2+} influx in myometrial cells.

In summary, OT elicited a biphasic Ca^{2+} increase in which the spike Ca^{2+} response was dependent on the intracellular Ca^{2+} release and, to a lesser extent, the Ca^{2+} influx. In addition, the plateau response was entirely dependent on the presence of extracellular Ca^{2+}. Our findings suggested that activation of OT receptors triggers a
PTX-insensitive G-protein (possibly a Gq)-PLC signal transduction pathway to release Ca\(^{2+}\) from IP\(_3\)-sensitive Ca\(^{2+}\) stores and to induce Ca\(^{2+}\) entry from the extracellular environment through VDCC and ROC in porcine myometrial cells.

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CHAPTER VII SIGNALING MECHANISMS ON $\alpha_2$-ADRENERGIC RECEPTOR-MEDIATED $\text{Ca}^{2+}$ INFLUX AND RELEASE IN PORCINE MYOMETRIAL CELLS

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ABSTRACT

Activation of $\alpha_2$-adrenergic receptors ($\alpha_2$-ARs) causes smooth muscle contractions by an increase in intracellular $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]). However, the cellular mechanisms mediating this effect are not well-understood. By using biochemical and electrophysiological approaches, we studied the mechanisms underlying the $\alpha_2$-AR-mediated increase in [Ca$^{2+}$], in freshly dispersed porcine myometrial cells. After the blockade of ARs with propranolol, epinephrine increased [Ca$^{2+}$], dose-dependently in both the presence and absence of extracellular Ca$^{2+}$. The rank order of $\alpha$ antagonists in inhibiting of [Ca$^{2+}$] response to epinephrine was yohimbine > WB4101 >> prazosin in both the presence and absence of extracellular Ca$^{2+}$, suggesting that epinephrine acts on $\alpha_2$ARs to increase Ca$^{2+}$ influx as well as Ca$^{2+}$ release from intracellular stores. Thapsigargin abolished the release but did not affect the influx. Pertussis toxin significantly inhibited the influx while failing to change the release. Nimodipine (1 $\mu$M), an L-type voltage-dependent Ca$^{2+}$ channel blocker, nearly abolished the influx. The peak increase in [Ca$^{2+}$], caused by epinephrine was reached within 20s of administration. At 20 s post-administration of epinephrine (1 $\mu$M), basal intracellular cAMP and forskolin-elevated cAMP concentrations decreased by 29 ± 10% and 28 ± 6% (mean ± S.E., n=4), respectively. Epinephrine (0.1 $\mu$M) enhanced the L-type Ca$^{2+}$ channel current by 36 ± 7%. Forskolin (10 $\mu$M) suppressed the Ca$^{2+}$ current by 26 ± 5%. Maximization of intracellular cAMP content by applying 8-Br-cAMP (100 $\mu$M) blocked the effect of epinephrine, but not Bay K 8644, on the current. U-73122, a phospholipase C inhibitor, reduced the Ca$^{2+}$ release by epinephrine and oxytocin dose-dependently but failed to inhibit the release by ionomycin. We concluded that activation of $\alpha_2$ARs induces Ca$^{2+}$ influx through
opening of L-type voltage-dependent Ca\(^{2+}\) channels as well as induces Ca\(^{2+}\) release from intracellular stores. These two processes may be mediated by two distinct signaling pathways: a pertussis toxin-sensitive G protein, assuming G\(_1\)/G\(_{0\alpha}\) couples to adenyllyl cyclase leading to a decrease in cAMP formation which results in activation of the Ca\(^{2+}\) channels; a pertussis toxin-insensitive G protein, possibly a G\(^{-}\), activates phospholipase C leading to Ca\(^{2+}\) release from intracellular stores.

INTRODUCTION

Alpha\(_2\)-adrenergic receptors (\(\alpha_2\)ARs) mediate a variety of cell functions including inhibitory effects, such as suppression of neurotransmitter and hormone release, and stimulatory effects, such as aggregation of platelet and contraction of smooth muscle. The \(\alpha_2\)-AR-mediated inhibitory effects are attributable to the activation of inhibitory G proteins (G\(_{i}\)/G\(_{0\alpha}\)), which then couple to various effectors including adenyllyl cyclase and ion channels (Limbird, 1988; Hille, 1992). However, the mechanisms by which \(\alpha_2\)ARs mediate stimulatory effects, especially the smooth muscle contraction, remain poorly understood. Activation of \(\alpha_2\)ARs has been shown to inhibit adenyllyl cyclase leading to a reduction in cAMP formation through the coupling of G\(_i\) in smooth muscles (Wu et al., 1988; Zhang et al., 1992). The rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\]) and subsequent smooth muscle contractions induced by \(\alpha_2\)AR activation depends on the Ca\(^{2+}\) influx via voltage-dependent Ca\(^{2+}\) channel (VDCC) through the activation of G\(_i\) (Leprêtre and Mironneau, 1994). Yet, the coupling mechanism as to how G\(_i\) activates the VDCC has not been established. Studies on contractility indicate that in many smooth muscle preparations, activation of \(\alpha_2\)ARs causes contractions in Ca\(^{2+}\) -free medium (Daly et al., 1990; Nielsen et al., 1992; Yang and Hsu, 1995a), which raises the possibility that \(\alpha_2\)ARs in addition to acting on the Ca\(^{2+}\) influx might also induce Ca\(^{2+}\) release by unknown mechanisms.

The cellular mechanisms for the contraction of smooth muscle upon the activation of \(\alpha_2\)ARs have been difficult to elucidate in both in vivo and in vitro isolated muscle strip studies, because \(\alpha_2\)ARs at different anatomical locations mediate the opposite effects on smooth muscle contractility. For example, activation of \(\alpha_2\)ARs in central nervous system leads to a reduction of the sympathetic tone and activation of
prejunctional $\alpha_2$ARs in sympathetic neurones results in a reduction in neurotransmitter release (Kobilka, 1992). In blood vessels the activation of $\alpha_2$ARs on endothelium releases the endothelium derived relaxing factors (Liao and Homey, 1994). All of the above three events tend to reduce the contractility and antagonize the direct effect of $\alpha_2$ARs in smooth muscles. In addition, many smooth muscle preparations contain more $\alpha_1$ARs than $\alpha_2$ARs, and both of them may couple to the same type of Ca$^{2+}$ channels (Nichols et al., 1989). These facts, therefore, produce the difficulty in demonstrating $\alpha_2$ mechanisms as well as highlight the danger inherent in making assumptions about mechanisms solely based on in vivo and in vitro contractility studies. It is apparent that the use of the single smooth muscle cell that has predominately $\alpha_2$ARs would be helpful in addressing cellular mechanisms underlying the $\alpha_2$AR-mediated smooth muscle contraction. The results of radioligand binding assay indicate that ARs in porcine myometrial cells are predominately $\alpha_1$ARs (Rexroad and Guthrie, 1983), more specifically $\alpha_{2A}$ subtype (Yang and Hsu, 1995b).

The main goal of this study is to investigate the cellular mechanisms by which $\alpha_2$ARs regulate Ca$^{2+}$ signal in porcine uterine smooth muscle. With Ca$^{2+}$ measurement and patch-clamp techniques, we found that activation of $\alpha_2$ARs induced Ca$^{2+}$ influx as well as Ca$^{2+}$ release from intracellular stores. The influx was attributed to a cAMP-dependent mechanism mediating by a $G/G_\alpha$ coupling, whereas a PTX-insensitive $G$ protein, likely a $G_\alpha$, was involved in the release process by activation of phospholipase C.

**EXPERIMENTAL PROCEDURES**

*Preparation of Myometrial Cells*

Single myometrial cells were isolated as previously described (ZhuGe et al., 1994). Briefly, longitudinal muscle segments were excised from porcine uteri of luteal phase as determined using a previously described method (Evans and Sack, 1973). The segments were minced and placed in a spinner flask and incubated in Ca$^{2+}$- and Mg$^{2+}$-free Hank’s Balanced Salt Solution (HBSS) containing 0.1% collagenase, 0.2% BSA for 60 min and 20 min, respectively, at 37°C. The supernatants in these two fractions were removed and replaced with fresh enzyme solution. The tissue was
bathed for another 20 min and the supernatant, containing a substantial number of myometrial cells, was collected. This dispersion procedure was repeated several times until desirable amounts of cells were harvested. The cells were rinsed by centrifugation at 50 x g, 25°C, for 10 min and resuspended in Krebs-Ringer Bicarbonate solution with 0.1% BSA and 10 mM HEPES (KRB).

**Ca²⁺ Measurements Using Fluorescence Photometry**

\([\text{Ca}^{2+}]_i\) was measured as previously described (ZhuGe et al., 1994). Myometrial cells were gently shaken in KRB containing 4 µM fura-2 acetoxymethylester (AM) at 37°C for 45 min. The fura-2 loaded cells were centrifuged as above and diluted to 2 x 105/ml with KRB. Fura-2 fluorescence was monitored in a spectrofluorometer with excitation wavelength of 340 nm and 380 nm and emission wavelength of 510 nm. Values for [Ca²⁺], were determined from the measured 340/380 ratios with the formula of Grynkiewicz et al. (1985). For experiments conducted in Ca²⁺-free condition, cell suspension was centrifuged (300 x g, 1 min) and replaced the medium with the Ca²⁺-free KRB supplemented with 0.1 mM EGTA immediately before [Ca²⁺], measurements.

**Ca²⁺ Current Recordings**

The whole-cell patch-clamp technique was used to record Ca²⁺ current as described previously (ZhuGe et al., 1994). Patch pipettes (3-5 MΩ) were prepared of disposable glass pipettes (VWR Scientific, West Chester, PA) by a two-stage pull and a fire polishing. The liquid junction potentials were nullified before the formation of gigaseals with an offset circuit. Access to the intracellular milieu was obtained by applying a negative pressure to the pipette. Linear resistance and capacitative currents were electronically compensated by P/4 protocol. Data were collected and analyzed using an IBM-PC computer with analog-digital interface board and pClamp software 5.5 (Axon instruments, Foster City, CA). The pipette solution contained (in mM): Cs glutamate, 125; CsCl, 10; Mg-ATP, 2; EGTA, 10; HEPES, 10 (pH 7.2). The bath solution contained (in mM): Choline chloride, 125; tetraethylammonium bromide, 20; 4-aminopyridine, 5; BaCl₂ 2; dextrose, 10; HEPES, 10 (pH 7.3). Agents were applied to the bath solution directly and all experiments were performed at room temperature.
Measurement of cAMP

The cell suspension (10^6 cells/ml) were incubated with propranolol (1 \mu M) for 5 min before the administration of epinephrine (Epi, 1 \mu M), forskolin (10 \mu M) and Epi plus forskolin, respectively, for 20 s at 37°C. The cAMP was determined using radioimmunoassay as described by Richards et al. (1979).

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO), except that U-73122 was from Biomol Research Laboratory (Plymouth meeting, PA), nimodipine was from Research Biochemicals International (Natick, MA), collagenase was from Worthington Biochemical (Freehold, NJ), and fura-2 AM was from Molecular Probes (Eugene, OR).

Data Analysis

Data are presented as mean ± S.E. and were analyzed by the Student’s test for paired and unpaired values. The significance level was set at P<0.05.

RESULTS

Effect of Epi on [Ca^{2+}]

Porcine myometrial cells contain \( \alpha \)ARs and \( \beta \)ARs, and activation of the latter has a negative effect on the contraction and [Ca^{2+}], (Yang and Hsu, 1993; ZhuGe et al., 1994). Therefore, when stimulated with Epi, the myometrial cells had been pretreated with propranolol (1 \mu M) for 5 min to block \( \beta \)ARs in the present study. In the presence of extracellular Ca^{2+} ([Ca^{2+}]), Epi (1-10 nM) increased the [Ca^{2+}] gradually to a plateau; up to 100 nM, it increased [Ca^{2+}], quickly to a peak then declined to a plateau during 5 mins of monitoring. In the absence of [Ca^{2+}], Epi ( \geq 100 nM) significantly increased [Ca^{2+}], which reached a peak then declined to the basal within 1 min (Fig. 1A). The peak times were about 20 s at a concentration of 100 nM in both cases. EC_{50} of Epi were 21 ± 4 nM and 27 ± 6 nM in the presence and absence of [Ca^{2+}], respectively (Fig.1B). In single cell Ca^{2+} imaging, [Ca^{2+}], profiles caused by Epi in the presence and absence of [Ca^{2+}], were similar to that in cell suspension study.
Fig. 1. Effects of Epi on [Ca\(^{2+}\)], in porcine myometrial cells. Panel A, [Ca\(^{2+}\)], tracings in response to Epi (0.1 \(\mu M\)) in the presence (upper tracing) and absence (bottom tracing) of [Ca\(^{2+}\)]\(_e\). Propranolol (1 \(\mu M\)) was present in this figure and the following figures to block the effect of Epi. Panel B, dose dependence in increase of [Ca\(^{2+}\)], by Epi. The basal [Ca\(^{2+}\)]\(_e\) was 112 ± 6 nM in the presence of [Ca\(^{2+}\)]\(_e\), and 89 ± 4 nM in the absence of [Ca\(^{2+}\)]\(_e\). Data are expressed as a percentage of increase over their own basal [Ca\(^{2+}\)]\(_e\), in presence (○) and absence (▼) of [Ca\(^{2+}\)]\(_e\), and represent the mean ± S.E. for six to nine separate determinations.
Fig. 2. Epi-induced increase in [Ca\textsuperscript{2+}], in single myometrial cells in the presence (A) and absence (B) of extracellular Ca\textsuperscript{2+}. The cells grown on coverslips were loaded with Fura-2 and imaged as described under "Methods" of Chapter 3. The cells were stimulated with 1 \mu M Epi as indicated by arrowheads in the presence of 1 \mu M propranolol. The 340nm/380nm ratios for each of cells were measured individually as a function of time and represented by separate traces on the graph.
A

\( [Ca^{2+}] = 1.25 \text{ mM} \)

\( \uparrow \)

Epi 1 \( \mu \text{M} \)

B

\( [Ca^{2+}] = 0 \)

\( \uparrow \)

Epi 1 \( \mu \text{M} \)
Therefore the \([Ca^{2+}]_i\) response to Epi was attributed to two processes: a \(Ca^{2+}\) influx from extracellular environment and a release from intracellular stores.

**Effects of \(\alpha\)AR antagonists on Epi-induced increase in \([Ca^{2+}]_i\).**

The radioligand binding study indicated that \(\alpha_2\)AR appears to be the predominantly \(\alpha_2\) in porcine myometrial cells. To investigate whether the \(\alpha_2\)ARs mediate Epi-induced increase in \([Ca^{2+}]_i\), we evaluated the effects of \(\alpha_2\)AR antagonists yohimbine (a nonsselective \(\alpha_2\)AR antagonist), WB 4101 (an \(\alpha_1\)AR + \(\alpha_2\)AR antagonist) and prazosin (an \(\alpha_1\) + \(\alpha_2\)AR antagonist) on this response (Ruffolo et al., 1991). The inhibitions by these antagonists of Epi-induced \([Ca^{2+}]_i\) change are depicted in Fig. 3. Yohimbine (1 nM-1 μM) and WB 4101 (1 nM-1 μM) dose-dependently inhibited the Epi (100 nM)-induced increase in \([Ca^{2+}]_i\) in the presence and absence of \([Ca^{2+}]_o\). The \(IC_{50}\) of yohimbine and WB 4101 to Epi were 17.2 ± 2.8 nM and 121 ± 23.2 nM in the presence of \([Ca^{2+}]_o\), and 21.8 ± 1.9 nM and 136 ± 14.2 nM in the absence of \([Ca^{2+}]_o\), respectively. However, prazosin did not reduce the Epi response significantly until a high concentration of 1 μM, which decreased the response by 21 ± 3% and 30 ± 2.2% in the presence and absence of \([Ca^{2+}]_o\), respectively. The rank order of inhibitory potency was yohimbine > WB4101 >> prazosin in both presence and absence of \([Ca^{2+}]_o\). In conjunction with results here and the ligand binding study (Yang and Hsu, 1995b), we suggest that in porcine myometrial cells, \(\alpha_2\)ARs mediate the Epi-induced increase in \(Ca^{2+}\) influx and release; and \(\alpha_1\)ARs, if any, are minimally involved in these responses to Epi.

**Effect of thapsigargin on Epi-induced increase in \([Ca^{2+}]_i\).**

In many cell types, \(Ca^{2+}\) influx and release are dependent events operated by \(Ca^{2+}\)-induced \(Ca^{2+}\) release mechanism (CICR) or \(Ca^{2+}\) release-triggered \(Ca^{2+}\) influx process (Berridge, 1993). It was apparent that CICR was not responsible for Epi-induced \(Ca^{2+}\) release, because Epi caused \(Ca^{2+}\) release in the absence of \([Ca^{2+}]_o\), a condition that does not allow \(Ca^{2+}\) influx to occur (Fig.1A). We then tested the possibility of the involvement of \(Ca^{2+}\) release-triggered \(Ca^{2+}\) influx mechanism by
Fig. 3. Effects of α₂AR antagonists on increase of [Ca²⁺], by Epi. Cells were pretreated with antagonists 5 min before Epi (0.1 μM) administration. [Ca²⁺], was determined in the presence (Panel A) and absence (Panel B) of [Ca²⁺]₀. The response to Epi was considered as 100%. Data are the mean ± S.E. from four (Panel A) and three (Panel B) separate determinations.
using thapsigargin (TG), a blocker of Ca\(^{2+}\) ATPase in sarcoplasmic reticulum, to deplete intracellular Ca\(^{2+}\) stores. As reported previously (ZhuGe et al. 1994), TG alone did not increase [Ca\(^{2+}\)], but it progressively inhibited the Epi-induced Ca\(^{2+}\) release and abolished this response after 15 min of the pretreatment (Fig. 4A). Based on this observation, we determined the effect of TG pretreatment for 15 min on Epi-induced increase in [Ca\(^{2+}\)]\(_i\) at the [Ca\(^{2+}\)]\(_o\) of 1.25 mM. Fig. 4B shows that TG pretreatment inhibited the peak by 56 ± 4% (n=4, P<0.05) but failed to change the plateau phase significantly which was measured at 5 min after the Epi application (Fig. 4B).

**Effects of pertussis toxin on \(\alpha_2\)AR-mediated Ca\(^{2+}\) influx and release**

Results in Fig.1A and Fig. 3B suggested that the Ca\(^{2+}\) influx and release induced by Epi were independent events, which might be mediated by different signaling mechanisms. Many studies indicate that the G protein may be a level for separating signal transduction pathways. Therefore, we hypothesized that the Ca\(^{2+}\) influx and release induced by Epi were due to functional coupling of \(\alpha_2\)ARs to two independent G proteins. To test this possibility, experiments were performed to determine the effect of pertussis toxin (PTX), a useful tool for differentiating the G proteins, on Epi-induced increase in [Ca\(^{2+}\)]. PTX (1 \(\mu\)g/ml), incubated for 2 h, significantly inhibited the Epi-induced increase in the peak and plateau of [Ca\(^{2+}\)], by 57 ± 3.5% and 76 ± 4% (n=6), respectively, in the presence of [Ca\(^{2+}\)]\(_o\) (Fig. 5A). However, it failed to alter the Epi-induced increase in [Ca\(^{2+}\)]\(_i\) in the Ca\(^{2+}\)-free medium (Fig. 5B, n=4). These data suggested that a PTX-sensitive G-protein is coupled to the Ca\(^{2+}\) influx while a PTX-insensitive G-protein to the Ca\(^{2+}\) release.

**The involvement of VDCC in \(\alpha_2\)-mediated Ca\(^{2+}\) influx**

Experiments on smooth muscle contractions from our laboratory (Yang and Hsu, 1995a) and others (Jim and Mathens, 1985; Stubbs et al., 1988) demonstrate that activation of \(\alpha_2\)ARs induces Ca\(^{2+}\) influx through promotion of VDCC opening. This possibility was further tested in the isolated porcine myometrial cells by pretreating cells with nimodipine (1 \(\mu\)M) before the Epi application. As shown in Fig. 6, nimodipine pretreatment significantly depressed the [Ca\(^{2+}\)]\(_i\) response to Epi with an inhibition of 59 ± 5% (n=4) in the peak and 82 ± 7% (n=4) in plateau, suggesting that the Epi-
Fig. 4. Effect of TG on Epi-induced increase in $[\text{Ca}^{2+}]_i$. Panel A, time dependence in inhibition of Epi-induced $\text{Ca}^{2+}$ release by TG. Cells were pretreated with TG in the presence of $[\text{Ca}^{2+}]_o$ for various times, were then subjected to centrifugation (300 x g, 1 min) immediately before $[\text{Ca}^{2+}]_i$ determination in the absence of $[\text{Ca}^{2+}]_o$. Panel B, TG inhibited the Epi-induced $\text{Ca}^{2+}$ release but exerted no significant inhibition in the influx. Cells were incubated without (upper tracing) and with TG (2 $\mu$M, bottom tracing) for 15 min. $[\text{Ca}^{2+}]_i$ was determined in the presence of $[\text{Ca}^{2+}]_o$. 
Fig. 5. Effect of PTX on Epi-induced increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined in the presence (Panel A) and absence (Panel B) of $[Ca^{2+}]_0$. Data are the representative of six (Panel A) and four (Panel B) experiments.
Fig. 6. Effect of nimodipine on Epi-induced Ca\(^{2+}\) influx. Cells were treated with nimodipine (Nim 1 \(\mu\)M, 1 min) before Epi (0.1 \(\mu\)M) stimulation. Data are the representative of four experiments.
induced Ca\textsuperscript{2+} influx is attributed to the opening of VDCC.

In an attempt to obtain more direct evidence, we examined the effect of Epi on VDCC current in a whole-cell patch-clamp study. VDCC in porcine myometrial cells is of primarily L-type (ZhuGe et al., 1994). Fig. 7 shows the effect of Epi on the L-type Ca\textsuperscript{2+} current. Application of 0.1 \mu M Epi into the bath increased the peak current by 36 ± 7% (n = 11, P < 0.05) and washout of Epi partially recovered the current, confirming the Epi response. The current-voltage plots in the absence and presence of Epi showed similar shape: the threshold voltage for the current and the voltage at which the maximum current was recorded were not altered by Epi. When cells were treated with PTX, Epi (0.1 \mu M) failed to increase the Ca\textsuperscript{2+} current (n = 3, data not shown).

Effect of Epi on intracellular cAMP concentration

Activation of \alpha_2-AR has been shown to inhibit adenlylyl cyclase activity leading to a reduction in intracellular cAMP concentration in smooth muscles (Wu et al., 1988; Zhang et al., 1992; Wright et al., 1995). However, there is a lack of evidence regarding the direct link between a reduction in intracellular cAMP concentration and activation of VDCC. To explore the possible link between these two events, cAMP content was detected after a 20 s stimulation of Epi, the time when the increase in [Ca\textsuperscript{2+}], reached maximum (Fig. 1). We speculated that if a decrease in cAMP content is the signal for activation of VDCC by \alpha_2-AR, this inhibition, thus, should be observed within 20 s following the Epi administration. The basal cAMP concentration was 59 ± 5 pmol/106 cells (n = 4). Forskolin (10 \mu M) increased the basal by 270 ± 24% (n = 4). Epi (1 \mu M) significantly inhibited both basal and forskolin-stimulated cAMP formation by 29 ± 10% and 28 ± 6% (n = 4), respectively.

Effect of cAMP on VDCC current

The data that parallel changes in increase of [Ca\textsuperscript{2+}], and reduction of cAMP by Epi suggested that a decrease in cAMP content is a possible mechanism for activation of VDCC by \alpha_2AR. To investigate this possibility further, we examined the effects of cAMP on VDCC current. Fig. 8 shows the time course effects of 8-Br-cAMP followed by application of Epi and an L-type VDCC activator Bay k 8644. The current was...
Fig. 7. Effect of Epi on the VDCC current. The currents were evoked by the step depolarizing pulses of 300 ms from the holding potential of -80 mV to a voltage between -70 mV to 60 mV with an increment of 10 mV. Ba²⁺ (5 mM) was used as the charge carrier. Epi (0.1 μM) increased the current at the voltages between -20 mV to +30 mV. Inset shows the peak current tracings before (circles) and during (opened triangles) the application of Epi (0.1 μM), and after the washout (closed triangles). Data are the representative of six experiments.
Fig. 8. Effect of 8-Br-cAMP on Epi- and Bay k 8644-induced increases in the Ca²⁺ current. The currents were evoked by a depolarization pulse from -80 mV to +10 mV with an interval of 20 s. The cell was pretreated with propranolol (1 μM, 5 min) before the application of 8-Br-cAMP. 8-Br-cAMP (100 μM) depressed the current and blocked the response to Epi (0.1 μM) but failed to affect the response to Bay K 8644 (1 μM) (n = 4). Inset shows the current tracings corresponding to small letters on the time course curve.
elicited by a depolarizing voltage from -80 mV to +10 mV at an interval of 20 s. 8-Br-cAMP (100 μM) decreased the current by 31 ± 4% (n=4). Subsequent application of Epi (0.1 μM) failed to increase the current. However, the application of Bay K 8644 (1 M) increased the current by 76 ± 12%. The effect of forskolin, an adenlyyl cyclase stimulator, on the VDCC current is shown in Fig. 9. Forskolin (10 μM) significantly reduced the peak current by 26 ± 5% (n=8) but did not change the current-voltage relationship. Forskolin's effect was totally recovered by a washout.

Effect of U73122 on α2A-AR-mediated Ca^{2+} release

The results in Fig. 4B suggested that a PTX-insensitive G protein was involved in the α2AR-mediated Ca^{2+} release. G₃ is the PTX-insensitive G protein that couples to Ca^{2+} release mechanism by a phospholipase C-IP₃ pathway (Hepler and Gilman, 1992). We then investigated the effect of phospholipase C inhibitor U73122 on α2AR-induced Ca^{2+} release. As shown in Fig. 10A, U73122 (3 μM) abolished Epi-induced Ca^{2+} release. The specificity of this action was examined by subsequent applications of oxytocin, which activates phospholipase C by a G₉₁₁ coupling (Ku et al., 1995), and ionomycin, a Ca^{2+} ionophore to release Ca^{2+} which bypasses the phospholipase C mechanism. As shown in Fig. 10A, U73122 also abolished the oxytocin-induced Ca^{2+} release but failed to inhibit the response to ionomycin. Effects of U73122 on Epi and oxytocin were dose-dependent with IC₅₀s of 520 ± 40 nM and 360 ± 30 nM, respectively (Fig. 10B).

DISCUSSION

The role of postjuntional α₂ ARs in modulation of smooth muscle contractions has been proposed for more than a decade (Yamaguchi and Kopin, 1980). However, the cellular mechanisms for this action have not been extensively studied until recently (Arturto et al., 1993; Leprêtre and Mironneau, 1994). The investigation of cellular mechanisms of α₂-AR-mediated contraction has been complicated by factors including 1) the lack of smooth muscle preparations which possess a high density of α₂ARs, and 2) the heterogeneous distribution and functions of α₂ARs (see Introduction). The findings of smooth muscle preparation with predominately α₂ARs
Fig. 9. Effect of forskolin on the Ca$^{2+}$ current. The depolarizing protocol was same as Fig. 6. Inset shows the peak current tracings elicited at the depolarizing voltage of 10 mV from the holding potential of -80 mV before (circles) and during (closed triangles) the application of forskolin (10 μM), and after the washout (filled triangles). Data are the representative of eight experiments.
Fig. 10. Effect of the phospholipase C inhibitor, U73122, on Epi-induced Ca\(^{2+}\) release. Panel A. The \([\text{Ca}^{2+}]_\text{i}\) tracings to consecutive applications of Epi, oxytocin and ionomycin and the alteration by U73122. Cells were treated without (a) and with U73122 (3 \(\mu\)M, b) for 90 s before the application of Epi. The \([\text{Ca}^{2+}]_\text{i}\) of cells was determined in the absence of \([\text{Ca}^{2+}]_\text{o}\). Panel B. The dose-dependent inhibition of U73122 on Epi- and oxytocin-induced Ca\(^{2+}\) release. The responses to Epi, oxytocin and ionomycin without the U73122 treatment were respectively considered as 100\%. Data are the mean ± S.E. (n = 4).
will certainly facilitate the elucidation of the mechanisms of the $\alpha_2$AR-mediated muscle contractions. Studies on contractility indicate that $\alpha_2$ARs rather than $\alpha_1$ARs mediate EPI-induced contraction in porcine myometrium (Yang and Hsu, 1995a). Studies on radioligand bindings of $\alpha_2$ARs indicate that these receptors in porcine myometrial cells belong to the $\alpha_{2A}$ subtype (Yang and Hsu, 1995b). The present functional study on the 

$[Ca^{2+}]_{i}$ confirmed and extended the evidence to support this conclusion because yohimbine, a nonselective $\alpha_2$AR antagonist, and WB 4101, an $\alpha_1$ and $\alpha_{2A}$AR antagonist, dose-dependently inhibited, while prasozin, an $\alpha_1$ and $\alpha_{2B}$AR antagonist, had negligible effect on the Epi response in the presence and absence of $[Ca^{2+}]_{i}$.

In vivo and in vitro contractility studies suggest that activation of $\alpha_{2A}$ARs promotes smooth muscle contractions by increasing the influx of $Ca^{2+}$ via opening of VDCC (Ruffolo et al., 1984; Timmermans et al., 1985; Yang and Hsu, 1995a). While this work was in progress, Lepretre and Mironneau (1994) reported direct evidence regarding this effect in smooth muscle cells from rat portal vein by measuring $[Ca^{2+}]_{i}$ and $Ca^{2+}$ currents. The results obtained herein were consistent with their findings, as the L-type $Ca^{2+}$ channel blocker nimodipine reduced the effect of Epi on $[Ca^{2+}]_{i}$, and Epi increased the L-type VDCC current. However, a novel finding made in the current study was about the coupling mechanism on $\alpha_{2A}$AR-induced opening of the VDCC.

Studies indicate that activation of $\alpha_2$ARs inhibits basal cAMP concentration (Stubbs et al., 1988) and forskolin-elevated cAMP formation (Fredholm et al., 1985; Wu et al., 1988; Zhang et al., 1992; Wright et al., 1995) in smooth muscles. However, the role of cAMP in modulation of VDCC has not been adequately investigated. Pertussis toxin inhibits the $\alpha_2$AR-induced increase in blood pressure in pithed rats, suggesting the possibility that inhibition of adenyllyl cyclase causing the decrease in cAMP formation may lead to the $\alpha_2$AR-mediated vascular contraction (Boyer et al., 1983) via the opening of VDCC. In the present study we evaluated more directly the role of cAMP on $\alpha_2$AR-mediated activation of VDCC in isolated cells. We intended to correlate the time relationship of $[Ca^{2+}]_{i}$, and cAMP changes caused by activation of $\alpha_2$ARs. Since $\alpha_{2A}$AR-induced $Ca^{2+}$ influx occurred within 20 s of Epi administration as indicated in $[Ca^{2+}]_{i}$ measurement (Fig. 1), then if the decrease in cAMP is the signal for activation of VDCC, this cAMP change should be detected within 20 s. Our results indicated that activation of $\alpha_2$ARs altered these two parameters in the correspondent time, suggesting
that the $\alpha_{2A}$AR may couple to a cAMP-dependent mechanism, leading to activation of the VDCC. This hypothesis was further confirmed by the evidence that (a) the maximization of intracellular cAMP action by 8-Br-cAMP blocked the Epi-induced increase VDCC current but not that of Bay K 8644 which directly opens the VDCC; (b) forskolin, which increases cAMP formation, inhibited the VDCC current.

In contrast to the consistent information with regards to $\alpha_{2A}$AR linking to the opening of the VDCC, there are discrepancies regarding whether $\alpha_{2A}$ARs also mediate the Ca$^{2+}$ release from intracellular stores. The use of either the VDCC blockers or exposure to Ca$^{2+}$-free medium result in abolition of $\alpha_{2A}$AR-mediated contractions in rat femoral vein (Stubbs et al., 1988) and canine saphenous vein (Jin & Matthews, 1985), but not in canine saphenous vein (Guan et al., 1989), rabbit ear vein (Daly et al., 1990), human subcutaneous resistance artery (Nilsen et al., 1992) or porcine myometrium (Yang and Hsu, 1995a) suggesting that activation of $\alpha_{2A}$ARs may release Ca$^{2+}$ from intracellular stores in these tissues. The present study provided the first direct evidence supporting this hypothesis, because Epi still induced an increase in the [Ca$^{2+}$], in Ca$^{2+}$-free medium and this increase was highly sensitive to yohimbine and WB 4101 but was resistant to prazosin. Our findings are distinct from the reports in which the increase in [Ca$^{2+}$], by activation of $\alpha_{2A}$ARs was solely from the Ca$^{2+}$ entry through VDCC in rat tail artery and rat portal vein smooth muscle cells (Li et al., 1993; Leprêtre and Mironneau, 1994). These discrepancies may be due to the difference in preparations, because those two preparations contain $\alpha_1$ARs and $\alpha_{2A}$ARs with $\alpha_1$ARs outnumbering $\alpha_{2A}$ARs (Templeton et al., 1989; Nasser et al., 1985; Digges and Summers, 1983), while porcine myometrial cells contain predominately $\alpha_{2A}$ARs with few $\alpha_1$ARs. Interestingly, in the rabbit saphenous vein, primarily an $\alpha_{2A}$AR preparation, the profile of [Ca$^{2+}$], produced by $\alpha_2$-agonist UK 14304 is similar to that in the present study (Arburto et al., 1993). Therefore, it seems that the $\alpha_2$AR-mediated release of intracellular Ca$^{2+}$ is tissue- and receptor concentration-dependent.

What are the mechanisms for $\alpha_{2A}$AR-mediated Ca$^{2+}$ release in smooth muscles? The ryanodine receptor is one of the intracellular Ca$^{2+}$-release channels and is activated by its physical coupling with VDCC in plasma membrane of skeleton muscle and by a CICR mechanism in cardiac muscles (McPhenson and Campbell 1993). There is no evidence for the possibility of releasing Ca$^{2+}$ by the physical coupling in smooth...
muscles although the foot-like structure similar to skeletal muscle has been found (Devine et al., 1972). However, many smooth muscles including porcine myometrial cells contain ryanodine receptors (Unpublished data), suggesting the existence of the CICR mechanism. It is likely that \( \alpha_{2a} \)ARs mediate \( \text{Ca}^{2+} \) release through the CICR mechanism. In the present study, activation of \( \alpha_{2a} \)ARs still increased the \( [\text{Ca}^{2+}]_i \), in the absence of \( [\text{Ca}^{2+}]_o \), and VDCC blocker only abolished the influx component but not the release component. Based on the aforementioned findings, we ruled out the involvement of CICR in \( \alpha_{2a} \)AR-mediated \( \text{Ca}^{2+} \) release.

In the present study, U73122 dose-dependently inhibited the \( \text{Ca}^{2+} \) release mediated by \( \alpha_{2a} \)ARs and oxytocin receptors. The oxytocin receptor couples to a phospholipase C-IP\(_3\) pathway in myometrial cells (Phaneuf et al., 1993, Ku et al., 1995). Furthermore, U73122 failed to reduce the \( \text{Ca}^{2+} \) response to ionomycin, which directly releases the intracellular \( \text{Ca}^{2+} \) and bypasses the phospholipase C-IP\(_3\) system. These data suggested that phospholipase C is involved in the \( \alpha_2 \)AR-mediated \( \text{Ca}^{2+} \) release mechanism. Our findings agree with those demonstrating that activation of cloned \( \alpha_2 \)ARs in COS cells (Cotecchia et al., 1990) and native \( \alpha_{2a} \)ARs in trachocytes (Liedtke, 1994) stimulate IP\(_3\) formation, respectively. However, \( \alpha_2 \)ARs could mediate the intracellular \( \text{Ca}^{2+} \) release by an additional process other than activation of phospholipase C. For example, in human erythroleukemia cells \( \alpha_2 \)ARs mediate the release of intracellular \( \text{Ca}^{2+} \) via a phospholipase C-independent mechanism (Michel et al., 1989).

The \( \alpha_{2a} \)AR belongs to a seven-transmembrane domain G protein coupled receptor superfamily. In most systems examined so far, the G protein coupled to \( \alpha_2 \)AR is PTX sensitive, i.e., \( G_i \) or \( G_s \) (Limbird, 1988). The PTX treatment inhibited the \( \alpha_{2a} \)AR-mediated increase in \( [\text{Ca}^{2+}]_i \) in \( \text{Ca}^{2+} \)-containing medium and blocked the \( \alpha_{2a} \)AR-mediated increase in VDCC current, suggesting that a PTX-sensitive G protein is involved in the \( \alpha_{2a} \)AR-mediated activation of VDCC in myometrial cells. This G protein may belong to the \( G_i/G_s \) family, as mastoparan, an activator of \( \alpha \) subunit of \( G_i/G_s \) (Higashilima et al., 1990) produces a similar response in VDCC to that by activation of \( \alpha_{2a} \)AR in rat portal vein myocytes (Leprétre and Mironneau, 1994), and \( G_s \) has been surprisingly found in human myometrial cells (Zumbihl et al., 1995). As discussed above, a cAMP-dependent mechanism via coupling of \( G_i \) seems to mediate the \( \alpha_{2a} \)AR-
mediated activation of the VDCC. Nevertheless, we can not exclude the possibility that G1/G2 directly couples to the L-type VDCC, since Go acting on N-type and L-type VDCCs has been shown in neurons (Mcfadzean et al., 1989; Sweeney and Dolphin, 1992). More studies are needed to establish the nature of the G protein responsible for \( \alpha_\text{AR} \)-mediated activation of VDCC in smooth muscles.

The \( \beta y \) subunit of G, has been shown to activate the phospholipase C (Park et al., 1993; Smrcha and Sternweis, 1993). It raises the possibility that this mechanism contributes to the \( \alpha_2\text{AR} \)-mediated Ca\(^{2+} \) release. If this is the case, one would expect the release is inhibited or abolished by PTX treatment. In the present study, however, PTX pretreatment failed to show an effect on [Ca\(^{2+} \)], in Ca\(^{2+} \)-free medium, suggesting that a PTX-insensitive G protein rather than \( \beta y \) subunit of G, is responsible for the \( \alpha_2\text{AR} \)-induced Ca\(^{2+} \) release. Several studies already demonstrated that myometrial cells contain the G\(_{\text{q}} \) family (Wilkie et al., 1991; Phaneuf et al., 1993). Therefore, we suggested that \( \alpha_2\text{ARs} \) may couple to a G\(_{\text{q}} \) to release Ca\(^{2+} \) from intracellular stores. Our results represent one of the first to suggest in a native cell type that \( \alpha_2\text{ARs} \) may couple to G\(_{\text{q}} \). A recent report indicates that \( \alpha_2\text{ARs} \) only couple to G, (Leprêtre and Mironneau, 1994). The possibility for this inconsistency may be related to the AR population and concentration. \( \alpha_2 \text{AR} \) is the minor receptor type responsible for the AR-mediated contraction in that preparation (Digges and Summers, 1983). In contrast, \( \alpha_2 \text{ARs} \) are the predominate receptor type in porcine myometrial cells from both radioligand binding and contractility studies (Rexroad and Guthrie, 1983; Yang and Hsu, 1993). The cells with high concentration of \( \alpha_2 \text{ARs} \) may have made the G\(_{\text{q}} \) coupled effect more distinct. Indeed, low concentrations of M\(_2 \) or 5-HT\(_{1A} \) receptors only cause coupling to G, and high concentration of them causes activation of G\(_{\text{q}} \) as well as G, (Ashkenaz et al., 1987; Fargin et al., 1989).

In summary, our present findings demonstrated that activation of \( \alpha_2\text{AR} \) increased [Ca\(^{2+} \)], which was predominately attributed to the Ca\(^{2+} \) influx and to a less extent, to Ca\(^{2+} \) release from intracellular stores in porcine myometrial cells. The influx was mediated by coupling of PTX-sensitive G protein, presumably G, or G\(_{\text{q}} \), to VDCC via a cAMP-dependent mechanism, although a direct coupling of G\(_{\text{q}} \)/G\(_{\text{o}} \) to VDCC could not be ruled out. A PTX-insensitive-G protein, likely a G\(_{\text{q}} \), coupled to phospholipase C leading to Ca\(^{2+} \) release from the intracellular stores.
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The major points pertaining to the results obtained in this dissertation have already been discussed in the Discussion section of each chapter in detail. This chapter will outline the major conclusions derived from the presented data and offer some speculative ideas related to the mechanisms underlying our experimental findings.

L-type and T-type Voltage-dependent \( \text{Ca}^{2+} \) Channels

Based on the properties of voltage dependence, kinetics, and pharmacology, we suggest that porcine myometrial cells of prepartum have two types of VDCC, the L-type and T-type, which have been found in many other smooth muscles and non-smooth muscles. Similar to other preparations, the L-type current in whole-cell recordings of porcine myometrial cells has a long duration and is activated at relatively high membrane potential. It is also activated and inactivated slowly. The T-type current is transient and is activated at low membrane potentials. It is activated and inactivated rapidly.

In addition to its voltage dependence nature, the L-type current is regulated by \( \text{Ca}^{2+} \) itself. The concomitant rise in [\( \text{Ca}^{2+} \)], induced by opening of the channel down-regulates or upon-regulates the activity of the channel. The down-regulation can be simply due to the depolarization which decreases the electrochemical driving force for \( \text{Ca}^{2+} \) influx. Direct binding of \( \text{Ca}^{2+} \) to the inside of the channel, however, seems to account for a unique process in \( \text{Ca}^{2+} \)-induced down-regulation of the channel. Recent studies suggest that the molecular motif related to this process is localized to a membrane-spanning segment S6 of the first repeat of the \( \alpha \) subunit, and to putative extracellular and cytoplasmic domains flanking (Zhang et al., 1994). Another possible mechanism for the down-regulation of the \( \text{Ca}^{2+} \) channel is a rise in [\( \text{Ca}^{2+} \)], which activates other types of ion channels, resulting in the repolarization of the membrane which closes the L-type \( \text{Ca}^{2+} \) channel. Several kinds of channels have been found to be activated by an increase in [\( \text{Ca}^{2+} \)]. These include \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) channels and non-selective cation channels (Amédée et al., 1990; Cole and Sanders, 1989). However, \( \text{Ca}^{2+} \)-activated channels, such as the \( \text{Ca}^{2+} \)-activated \( \text{Cl}^{-} \) channel found in
several smooth muscles tend to mediate membrane depolarization causing activation of L-type Ca\(^{2+}\) channels (Wang et al., 1992). Many agonists release intracellular Ca\(^{2+}\) which activates the above currents and alters membrane excitability and the activity of L-type Ca\(^{2+}\) channels. The up-regulation could be mediated by the activation of a calmodulin-dependent kinase II as a result of the increase in [Ca\(^{2+}\)], as demonstrated in gastric smooth muscle (McCarron et al., 1992).

It is generally agreed that L-type Ca\(^{2+}\) channels are responsible for the upstroke of the action potential and Ca\(^{2+}\) influx upon the membrane depolarization in smooth muscles. This conclusion is supported by the properties of this channel, which has a large conductance, a long-lasting opening and a high density in the cell. However, the physiological function of the T-type Ca\(^{2+}\) current has not been established. Possibilities were discussed in the Discussion section of Chapter IV. With regards to smooth muscle, T-type currents may play an important physiological role in the near resting membrane potential to regulate excitability in phasic smooth muscle and to control the tone in tonic smooth muscle because of their low threshold nature. Under normal physiological conditions, the myometrium displays spontaneous action potentials and contractions. Because only a small portion of cells contain the T-type Ca\(^{2+}\) channels in porcine myometrial cells, we propose that the cells expressing the T-type Ca\(^{2+}\) channels may function as a pacemaker of porcine myometrium.

To verify this hypothesis, it would be necessary to consider and evaluate the following two points: 1) find out the percentage of cells containing T-type channels in different reproduction stages and 2) study the effect of T-type channel blockers on spontaneous action potentials and contractions. It has been shown that the spontaneous action potential and contraction varies among the reproductive stages in vivo and in vitro. For example, the myometrial strips from follicular phase sows displayed the highest spontaneous contraction (Yang and Hsu, 1995a) while gap junctions are sparse in the same period (Thilander and Rodriguez-Martinez, 1989a). It is possible that the percentage of cells containing the T-type channels correlates with the frequency of spontaneous action potentials and contractions among the reproductive stages, i.e., the greatest the number T-type channels, the higher the frequency of spontaneous action potentials and contractions. In addition, it was found that the elimination of extracellular Ca\(^{2+}\) abolishes the spontaneous contractions in...
human myometrium and canine colonic smooth muscle (Kawarabayashi et al., 1986; Huizinga et al., 1991). In colonic smooth muscle, the Ca\(^{2+}\) channel responsible for this effect is probably the T-type (Huizinga et al., 1991). The present study suggests that T-type channels are involved as a pacemaker in the myometrium. Thus, the blockage of T-type channels is expected to reduce the frequency or amplitude of spontaneous action potentials in the myometrium. However, such a study is hampered by the lack of specific T-type Ca\(^{2+}\) channel blockers.

As discussed above, activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels depolarizes the membrane which induces the opening of L-type Ca\(^{2+}\) channels. This mechanism has been suggested to account for the initiation of action potentials in the portal vein (Wang et al., 1992) and lymphatic vessels (Van Helden, 1993). Activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels depends on the spontaneous increase in localized [Ca\(^{2+}\)]. However, the source for the increase in [Ca\(^{2+}\)], is not clear. T-type Ca\(^{2+}\) channels can be activated at the resting membrane potential. It is possible that Ca\(^{2+}\) entry through spontaneous opening of the T-type channels is the source for activating the Ca\(^{2+}\)-activated Cl\(^{-}\) channels. However, studies have proposed that Ca\(^{2+}\) released spontaneously from intracellular Ca\(^{2+}\) stores is the source for inducing activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channel. This notion is supported by evidence that cells have specific regions where the IP\(_3\)R and RyR seem to be particular sensitive to Ca\(^{2+}\) (Berridge and Dupont, 1994). These regions correlate with the (Ca\(^{2+}\)) hot spots and sparks observed by confocal microscopy (Cheng et al., 1993; Yao et al., 1995). However, the frequency of the spark was reduced in Ca\(^{2+}\)-free medium, indicating the importance of Ca\(^{2+}\) influx (through T-type Ca\(^{2+}\) channels?) on the spontaneous Ca\(^{2+}\) release.

**IP\(_3\)**- and Caffeine-sensitive Intracellular Ca\(^{2+}\) Stores

The findings that oxytocin increased both IP\(_3\) concentration and [Ca\(^{2+}\)], in porcine myometrial cells, suggest that they contain IP\(_3\)-sensitive Ca\(^{2+}\) stores. This is consistent with reports in myometrial cells from other species such as humans, rats and guinea pigs (Marc et al., 1988; Anwer and Sanborn, 1989; Molnár and Hertelendy, 1990). However, caffeine-sensitive stores have not been found in rat or human myometrial cells, despite the fact that caffeine causes the contraction of human uterine
strips (Izumi, 1994). The present study represents one of the first demonstrating the existence of caffeine sensitive Ca\textsuperscript{2+} stores in myometrial cells.

In many cell types, low concentrations of IP\textsubscript{3} release only a fraction of Ca\textsuperscript{2+} stores, i.e. in a quantal fashion (Muallem et al., 1989; Berridge, 1993). The present study found that Ca\textsuperscript{2+} released by caffeine also exhibited this characteristic in porcine myometrial cells, and this was consistent with studies in chromaffin cells and skeletal microsomes (Cheek et al., 1993; Dettbarn et al., 1994). Although at least two views on the mechanisms for quanta release have been proposed (Berridge, 1993), the structural basis for quanta is unknown. The nature of quanta release is an all-or-none fashion. The original idea is that Ca\textsuperscript{2+} stores consist of functional distinct compartments and the quanta is the compartment of Ca\textsuperscript{2+} stores. Interestingly, in a proportion of porcine myometrial cells the quanta can be defined as entirely caffeine-sensitive stores in the cell (Fig. 5A, Chapter V). A similar idea has been proposed for IP\textsubscript{3}-sensitive stores in smooth muscle cells from the trachea and intestine (Iino et al., 1993; Marsh and Hill, 1993). Recent studies using confocal microscopy have provided new insights on quanta. It has been observed that there are spontaneous Ca\textsuperscript{2+} spikes from the opening of RyRs in cardiac muscle and Ca\textsuperscript{2+} “puff” from opening of IP\textsubscript{3}R in oocytes (Cheng et al., 1993; Yao et al., 1995). The spike and puff seem to be a result of the opening of one or several RyRs and IP\textsubscript{3}Rs. Therefore the quanta could be defined as one or a cluster of Ca\textsuperscript{2+}-releasing channels.

The Ca\textsuperscript{2+} release from caffeine-sensitive stores is believed to induce contraction by physical coupling the VDCC on T tubules with RyR in SR of skeletal muscle, and by the CICR mechanism in cardiac muscle (Berridge, 1993; McPherson and Campbell, 1993). However, the role of caffeine-sensitive stores in smooth muscle is not established. The depolarization-induced rise in [Ca\textsuperscript{2+}], is contributed by the influx of extracellular Ca\textsuperscript{2+} through VDCC, but it is also likely that, as in cardiac muscle, the Ca\textsuperscript{2+} current carried by action potentials releases additional Ca\textsuperscript{2+} through CICR from Ca\textsuperscript{2+} stores. Indeed, the Ca\textsuperscript{2+}-buffer capacity of cytoplasmic Ca\textsuperscript{2+}-binding proteins exceeds the amount of Ca\textsuperscript{2+} carried by an action potential (Bond et al., 1984). Recent findings on the parallel distribution of VDCC and RyR in smooth muscle (Carrington et al., 1995) provide strong evidence that CICR is involved in amplification of Ca\textsuperscript{2+} signal by action potentials. This finding also suggests the possibility of physical coupling
between VDCC and RyR. However, some critical experiments are needed to make this conclusion. For example, one would need to demonstrate that depolarization without Ca\(^{2+}\) influx can induce Ca\(^{2+}\) release in smooth muscle.

Modulation of L-type Ca\(^{2+}\) Currents by G protein-coupled Receptors

The primary signal for gating L-type Ca\(^{2+}\) channels is depolarization of the plasma membrane, which induces conformational changes via specific voltage sensing region of these molecules. However, abundant information indicates that the L-type Ca\(^{2+}\) channels are modulated by G-protein coupled receptors (McDonald et al., 1994). In porcine myometrial cells, the fact that activation of OT receptors and \(\alpha_{2a}\)-ARs increases the L-type Ca\(^{2+}\) currents, supports this contention.

In porcine myometrial cells, activation of \(\alpha_{2a}\)-AR decreased intracellular cAMP concentrations, but increased L-type Ca\(^{2+}\) currents. On the other hand, elevation of the cAMP concentration by forskolin and application of cAMP analogue 8-Br-cAMP suppressed the current. The inhibition of Ca\(^{2+}\) current by cAMP has been shown in several other smooth muscles (Ishikawa et al., 1993; Droogmans et al., 1987). These results suggested that \(\alpha_{2a}\)-AR activates a cAMP-dependent mechanism to increase the activity of L-type Ca\(^{2+}\) channels. It is well established that cAMP-induced responses are mediated by phosphorylation of target protein by PKA. Whether this mechanism accounts for the inhibition of L-type Ca\(^{2+}\) channels by cAMP in smooth muscles, however, needs more studies. In fact, PKA-mediated phosphorylation of the L-type channel increases the channel activity in cardiac and skeletal muscle (Trautwein and Hescheler, 1990; Sculptoreanu et al., 1993). The deduced primary sequences of the \(\alpha_{1}\)-subunits of cardiac and smooth muscle L-type channel are >95% homogenous, and these subunits are sufficient to form voltage-activated channels in expression systems. Therefore, it seems reasonable to hypothesize that phosphorylation of L-type VDCC by PKA also activates the channel in smooth muscle. Indeed, 10 \(\mu\)M forskolin increases the Ca\(^{2+}\) current by nearly 100% in aortic A7r5 cells (Marks et al., 1990) and 40% in porcine coronary artery smooth muscle cells (Fukumitsu et al., 1990). Isoproterenol, which enhances cAMP formation, increases Ca\(^{2+}\) currents in trachea smooth muscle (Welling et al., 1992). In porcine myometrial cells, application of H89 (0.1-1 \(\mu\)M), a
PKA inhibitor, inhibited Ca\(^{2+}\) current (unpublished observation). These results suggest the possibility that inhibition of L-type VDCC by elevation of cAMP in some smooth muscle may be mediated by a PKA-independent mechanism. The following recent evidence suggests that cGMP-dependent protein kinase (PKG) is a promising candidate in mediating cAMP-induced inhibition of L-type Ca\(^{2+}\) channels in smooth muscle (Tang et al., 1992; Lincoln et al., 1990): 1) cAMP activates PKG in the biochemical assay, 2) The concentration ratio of cellular cAMP/cGMP is about 10, and 3) forskolin decreases [Ca\(^{2+}\)], in cells expressed with PKG and PKA, but increase [Ca\(^{2+}\)], in cells expressing PKA alone. The variation in response of L-type channels to cAMP among different smooth muscles may be explained by the difference in cAMP/cGMP or in their relative activity.

PTX treatment abolished the \(\alpha_{2a}\)-AR-mediated increase in the L-type current in porcine myometrial cells. This implies that \(\alpha_{2a}\)-AR may directly couple to VDCC. This hypothesis has been proposed by Ruffolo et al (1989) based on the results from in vivo studies. Indeed, there is evidence that G proteins directly regulates VDCC in other receptor systems including \(\beta\)-AR in cardiac muscle (McDonald et al., 1994). To fulfill the criteria for direct coupling of G-protein with VDCC, the following aspects have to be demonstrated in porcine myometrial cells: 1) GTP\(_{\gamma}S\) and AlF\(_4^-\), bypassing the receptor, mimic \(\alpha_{2}\)-AR-mediated activation of the Ca\(^{2+}\) channel, and 2) more importantly, \(G\) or its \(\alpha\) subunit (\(\alpha\)) prolongs the survival of excised Ca\(^{2+}\) channels and increases the activity of single Ca\(^{2+}\) channel incorporated into a planar lipid bilayer.

A different mechanism appears to be involved in the OT-induced increase of the L-type current. Activation of L-type Ca\(^{2+}\) current by agonist-producing IP\(_3\) has been found in several smooth muscle preparations. However, the underlying mechanisms for this effect is unknown. There is no evidence that IP\(_3\) or Ca\(^{2+}\) itself activates the L-type VDCC. The possible signal for this effect might be the down-stream of Ca\(^{2+}\). It has been demonstrated that activation of calmodulin-dependent kinase II enhances the L-type Ca\(^{2+}\) current in gastric smooth muscle (McCarron et al., 1992). It is possible that OT enhances the L-type VDCC through release of intracellular Ca\(^{2+}\) which activates the calmodulin-dependent kinase II in porcine myometrial cells. As discussed above, the rise in [Ca\(^{2+}\)], can induce the opening of Ca\(^{2+}\)-activated Cl\(^-\) channels, resulting in depolarization of the membrane and opening of L-type VDCC. It is also possible that
OT works through this process to increase the L-type current.

Voltage-independent Ca\(^{2+}\) Channels

The agonist-generating IP\(_3\) induces a biphasic increase in [Ca\(^{2+}\)]\(_i\) in many non-excitable cells. The initial rise results from a direct effect of IP\(_3\) on the IP\(_3\)R in intracellular Ca\(^{2+}\) stores. This release of intracellular Ca\(^{2+}\) is transient in nature and is usually followed by a more prolonged elevation of [Ca\(^{2+}\)]\(_i\), due to sustained Ca\(^{2+}\) entry across the plasma membrane. The sustained Ca\(^{2+}\) influx probably contributes to a prolonged effect, and to refill the intracellular Ca\(^{2+}\) store, which is a required process for the cell response to new stimulations. The present study found that OT produced a biphasic change in [Ca\(^{2+}\)]\(_i\), in porcine myometrial cells. A similar pattern of [Ca\(^{2+}\)]\(_i\), caused by agonist-generating IP\(_3\) has been reported in several other smooth muscles (Murrey and Kotlikoff, 1991; Kajita and Yamaguchi, 1993; Pacaud et al., 1993). It is clear from the present study and others (Pacaud et al., 1993) that the initial phase of [Ca\(^{2+}\)]\(_i\) change is ascribed to an IP\(_3\)-induced Ca\(^{2+}\) release the same as in non-excitable cells. However, the sustained phase could result from opening of VDCC, totally in the case of acetylcholine in trachea smooth muscle cell (Kajita and Yamaguchi, 1993) and partially in the case of OT in porcine myometrial cells. However, in most cases the sustained phase is attributed to the activation of voltage-independent Ca\(^{2+}\) channels. The results from the present work and the study (Pacaud et al., 1993) in portal vein on norepinephrine-induced activation of Ca\(^{2+}\) influx suggests that the depletion of Ca\(^{2+}\) stores and the formation of IP\(_3\) are the requirements for the opening of these voltage-independent Ca\(^{2+}\) channels. However, the nature of the channel is unknown in excitable cells. Others have found that agonist-generating IP\(_3\) activated nonselective cation channels in smooth muscles, For example, OT in rat myometrial cells (Shimamura et al., 1994), acetylcholine in intestinal smooth muscle (Pacaud and Bolton, 1991) and vasopressin in vascular smooth muscle (van Renterghem and Lazdunski, 1994). Whether this type of channel is responsible for the OT-induced Ca\(^{2+}\) influx needs to be defined. In non-excitable cells, at least three types of voltage-independent Ca\(^{2+}\) channels have been proposed to be responsible for the sustained phase (Ca\(^{2+}\) influx) (Fasolato et al, 1994): (1) ROCs that are independent of second
messenger and may be regulated by direct physical coupling with IP3R in the Ca2+ store. (2) Ca2+-store depletion-operated Ca2+ channels that are presumably regulated by a cytosolic Ca2+ influx factor released following depletion of Ca2+ from the intracellular Ca2+ stores, and (3) second messenger-operated Ca2+ channels that are regulated by IP3, IP4 and intracellular Ca2+ generated following receptor stimulation. Future work on OT- and agonist-induced Ca2+ influx in porcine myometrial cells and other excitable cells are needed to characterize the similar channel currents that exist in non-excitable cells.

Modulation of Ca2+ Release by G Protein-coupled Receptors

Pharmacomechanical coupling represents a physiologically important mechanism of contraction coupling in smooth muscle. Previous studies indicated that activation of OT receptors and α2A-ARs induced contraction of porcine uterine strips in Ca2+ free medium (Yang and Hsu, 1995a; Yu et al., 1995), implying the existence of pharmacomechanical coupling in this preparation. In the present study, we demonstrated that both receptors mediated Ca2+ release through a Gq-PLC-IP3 signal transduction, indicating the release of Ca2+ from intracellular stores is responsible for pharmacomechanical coupling of these two receptors in porcine myometrial cells. The agonist concentrations to produce the response were higher in the dispersed cells than that in the contraction study of muscle strips. This suggests that activation of these two receptors may activate a Ca2+-independent mechanism to evoke the contraction. Indeed, α7-AR increases the sensitivity of the contractile apparatus to Ca2+ in vascular smooth muscle (Aburto et al., 1993) and OT-induced contraction occurs without measurable increases in [Ca2+], or myosin light chain phosphorylation in rat myometrial cells (Matsuo et al., 1989). Another possibility is that gap junctions and the extracellular matrix in muscle strips facilitate the contraction response. But the discrepancy in responding doses between the two techniques could result simply from cell damage caused by the cell-dispersing process.

There are increasing numbers of reports that pretreatment with IP3 reduces Ca2+ release by subsequent application of caffeine and vice versa. In porcine myometrial cells, we observed that OT and caffeine dose-dependently inhibited the response to
each other, suggesting a complete overlap between IP₃- and caffeine-sensitive stores (unpublished results). This is consistent with reports in guinea pig ileal smooth muscle cells (Komori et al., 1993) and PC12 cells (Zacchetti et al., 1991). These results raise the possibility that IP₃R and RyR co-localize in Ca²⁺ stores or that the two types of Ca²⁺ store structurally and/or functional communicate. To clarify the precise relationship, immunolocalization of IP₃R and RyR in organelles is needed, which has been performed in few tissues. With this technique, it was found that in purkinje cells some organelles contain both IP₃R and RyR, while other possess one receptor type (Walton et al., 1991). It is apparent that caffeine-sensitive stores may be involved in amplifying responses to agonists either directly or indirectly to supply the Ca²⁺.

**G Protein: Signal Organizer**

It has been well established that heterotrimeric G proteins play an essential transducing role in linking cell-surface receptors to effector proteins at the plasma membrane. The G proteins transfer the external signals specifically. However, many studies have found that the specificity of G protein-mediated response is not universal. For example, multiple receptors activate one type of G protein (convergence) or one receptor activates more than one G protein (divergence). The results from the present study support this notion, because both α₂A-AR and OT receptor couple to a PTX-insensitive G-protein, probably a G₄ to activate PLC, and α₂A-AR couple to a PTX-sensitive G-protein in addition to a PTX-insensitive G protein. An apparent question arising from these data is how the cell achieves diversity and specificity in G-protein-mediated signal transduction. The answer to this question is not clear. With regards to α₂A-AR-mediated signal transduction in porcine myometrial cells, the interaction of receptors, G proteins and effectors may be regulated by the relative concentration of each component. It is possible that adenyllyl cyclase inhibition is limited by an enzyme or G protein, whereas PLC activation is limited by the receptor number, similar to the study on the porcine M₂ muscarinic receptors in CHO cells in which the stimulation of PLC was dependent on the receptor number, while inhibition of adenlyyl cyclase was similar regardless of the receptor number (Ashkenazi et al., 1987). Activation of different classes of G proteins by α₂-AR may also be due to the expression of receptor
subtypes or receptor subtype isoforms which cannot be distinguished pharmacologically. For example, an early study showed that an apparently homogenous population of prostaglandin E receptors couples to adenlyyl cyclase via a PTX-sensitive G protein and to PLC via a PTX-insensitive G protein in adrenal medulla (Negishi et al. 1989). A recent study suggests that this result is attributed to the expression of different splice variants of the EP3 receptor which exhibits the same affinity for prostaglandin E2 but couples to different effector systems, presumably by different G protein heterotrimers (Namba et al., 1993).

Selectivity can result from different affinities or efficacy by which individual G protein interacts with receptors and effectors. Compartmentalization of signaling components is probably another mechanism for signal transduction specificity. By confining a specific set of receptor, G-protein and effector to a local region of the cell membrane, potential interference between incoming signals can be minimized. There are several examples of cells including neurons (Strittmatter et al., 1990), polarized renal and enteral epithelium cells (Ercolani et al. 1990; van den Berghe et al. 1991) with asymmetrically distributed receptors or G proteins. Whether myometrial cells use this mechanism to segregate the signalling pathways remains to be clarified.

Physiological Implications of $\alpha_2$-Adrenergic Receptors

OT stimulates the contractility of myometrial cells both in vivo and in vitro, and the number and sensitivity of OT receptors increase as pregnancy progresses. This evidence indicates that OT functions as a physiological modulator of myometrium contraction. In contrast, although myometrium from species examined contain a high concentration of $\alpha_2$-AR (Digges, 1982), the role of $\alpha_2$-AR in myometrium has not been established. One possible function could be involved in the modulation of myometrium contractility. Interestingly, this action seems specie-dependent. In rodents (Kaulenas et al, 1991; Haynes et al., 1993; Hoffman et al., 1981) and humans (Digges, 1982), prasozin, rather than yohimbine, competitively decreases the response to catecholamine, therefore, $\alpha_1$-ARs, but not $\alpha_2$-ARs, are considered responsible for the contractile response to catecholamine in these species. However, increasing evidence indicates that $\alpha_2$-AR is the receptor subtype that mediates the contractile response to
catecholamines in cows (Ko et al., 1990a), ewes (Marnet et al., 1987; Prud'Homme, 1988), and sows (Ko et al., 1990b; Yang and Hsu, 1995d), because yohimbine competitively inhibited the contractile response to catecholamines or $\alpha_2$-AR agonist. The role of $\alpha_2$-AR in modulation of uterine contraction in sows is supported by the functional study on $[Ca^{2+}]$, in the present study. In fact, this notion has gained support from other studies. It is known that adrenergic nerves in myometrium undergo degeneration during pregnancy and disappear at term (Sporrong et al., 1981).

Although sparse, adrenergic nerves were still observed in porcine myometrium during pregnancy and parturition (Thilander and Rodriguez-Martinez, 1990). Moreover, the number of $\alpha_2$-ARs and the contractile response mediated by $\alpha_2$-ARs did not significantly decrease in porcine myometrium during pregnancy and at term when compared to that in cycling sows (Yang and Hsu, 1995c).

Although the contractile experiments failed to demonstrate the role of $\alpha_2$-ARs in catecholamine-induced contraction in rodents and humans, the physiological influence of steroid hormones on the number of $\alpha_2$-AR has been widely accepted among various species including rodents and humans. This leads to speculation that there should be a physiological role for $\alpha_2$-ARs in myometrium of rodents and humans. Several studies indicate that $\alpha_2$-AR activate the same signal transduction pathways in myometrial cells among species, that is, coupling with G protein leading to inhibition of adenylyl cyclase and a decrease in cAMP formation (Wu et al., 1988; Breuilier et al., 1990). It is also established that several types of receptors such as $\beta$-ARs and relaxin receptors mediate an increase in cAMP and relaxation of myometrium. The contractility of myometrium in vivo could be influenced by the balance between the contractile and relaxing pathways related to cAMP. It is possible that variations in cAMP content or the sensitivity of muscle to cAMP may contribute to whether an apparent contraction can be detected after the activation of $\alpha_2$-ARs. No data have indicated that there are differences in cAMP content among myometrium from various species. But there are studies suggesting that different sensitivities to cAMP may exist among myometrium from different species and between myometria and other smooth muscles (Word et al., 1990; Izumi, 1994). In addition, the $G_{\gamma}$, a G protein inducing the formation of cAMP, is expressed higher in pregnancy myometrium than that in myometrium at delivery (Europe-Finner et al., 1994). This may be an event determining the sensitivity of
muscle to cAMP. In other words, the tissue expressing a higher content of Gs could display a stronger response to agonists which alter the cAMP concentration. Therefore, it is possible that relaxing pathways are more overwhelming than the contractile system in myometrium from rodents and humans, which mask the contractile response mediated via $\alpha_2$-ARs. However, activation of $\alpha_2$-ARs could antagonize the relaxation caused by agonists such as endogenous catecholamine and maintain the contractility of myometrium at appropriate levels.

Another possible role for $\alpha_2$-AR may involve other cell functions rather than modulation of contraction. One striking change during pregnancy is that uteri (myometrial cells) undergo hyperplasia and hypertrophy, which is in part mediated by estrogens. On the other hand, the number of $\alpha_2$-AR is also increased by estrogens in several species (Jacobson et al., 1987; Bottari et al., 1985). Several lines of evidence suggest that expression of $\alpha_2$-AR is associated with an increased in adipose conversion, and is positively correlated with adipocyte size and adipose tissue mass (Saulnier-Blache et al, 1991). Recent studies show that the $\alpha_2$-AR-induced proliferation is linked to the mitogen-activated protein kinase pathways (Bouloumié et al., 1994). In considering the remarkable change in myometrial cells and high density of $\alpha_2$-AR during pregnancy, it can be reasonably speculated that $\alpha_2$-AR may be involved in hyperplasia and hypertrophy of the myometrium.
CHAPTER IX  GENERAL CONCLUSION

This study developed a procedure, using collagenase, to disperse single myometrial cells from the nonpregnant and pregnant pigs. The isolated cells were not only alive but also preserved the properties of intact muscle. The sizes of the cells were 505 ± 20 x 11 ± 0.5 μm in the prepartum period and 265 ± 22 x 7 ± 0.4 μm in the luteal phase of the estrous cycle. These cells can be used in many aspects of myometrial cell research such as studies of regulation of biomedical processes, electrophysiology, pharmacology and contraction.

The whole-cell patch-clamp technique was used to study voltage-dependent Ca^{2+} channels while Fura-2 spectrofluorometry and imaging were used to measure [Ca^{2+}], in the isolated myometrial cells. Compared to contraction studies, these two techniques have the advantage in directly evaluating the transportation and mobilization of Ca^{2+}, an essential messenger for excitation-contraction coupling in smooth muscle. By using these two techniques, the underlying mechanism on how external modulators regulate Ca^{2+}, and thereby contraction, can be investigated.

The basal [Ca^{2+}], of the cells in the luteal phase and the prepartum period were 119 ± 12 and 154 ± 31 nM, respectively. In 8% of the prepartum myometrial cells, two types of Ca^{2+} currents were detected: one type being activated at -60 mV and inactivated quickly with a single exponential (T-type) whereas the other required depolarization to -30 mV for activation and inactivated more slowly with two exponentials (L-type). However, in 92% of the cells only L-type currents were found. A DHP agonist, Bay K 8644 (0.1 μM), increased L-type current by about 2-fold, and the DHP antagonist nimodipine (1 μM) abolished L-type current without inhibiting the T-type current. Ni^{2+} (0.17 mM) abrogated the T-type current but failed to change the L-type current. The steady-state activation and inactivation for both types of currents were voltage-dependent. Half-activation and -inactivation voltages were -25 mV and -51 mV for the T-type current; 11.2 mV and -10.3 mV for the L-type current. Superimposition of activation and inactivation curve showed a "window" current between -20 mV and +20 mV for the L-type current and -60 mV and -20 mV for T-type current. Results of the two-pulse protocol and Ba^{2+} replacement suggested that inactivation of L-type current was dependent on both Ca^{2+} and membrane potential,
while that of the T-type appeared to be dependent only on the membrane potential. These results suggest that L-type currents are probably responsible for Ca\(^{2+}\) influx upon the stimulation of membrane depolarization while T-type current may be involved in pacemaking of the uterus.

In consistent with studies in other species, porcine myometrial cells were found to contain IP\(_3\)-sensitive Ca\(^{2+}\) stores. In addition, porcine myometrial cells contain caffeine-sensitive stores. Caffeine acted on RyR to release Ca\(^{2+}\) since ryanodine inhibited the response to caffeine in a use-, concentration- and time-dependent manner. The sensitivity of the RyR was modulated by the luminal Ca\(^{2+}\) content of the stores based on the observation that thapsigargin, the Ca\(^{2+}\)-ATPase inhibitor, attenuated the response to caffeine in a time- and concentration-dependent manner. The nature of Ca\(^{2+}\) release from the caffeine-sensitive store was in a fashion of quanta, that is, the low dose of caffeine only released a portion of the stores in a multiple cell preparation study. The results from single cell study suggested that the quanta can be defined as compartments of the stores in 70% of the cells, and the entire stores in 30% of the cells.

Activation of OT receptors and \(\alpha_{2A}\)-AR induces the contraction of porcine myometrium. The present study indicated that both contractions were mediated by an increase in \([\text{Ca}^{2+}]\). The Ca\(^{2+}\) was released from intracellular stores and entered from extracellular environment, as in both the presence and absence of extracellular Ca\(^{2+}\), increases in \([\text{Ca}^{2+}]\), were observed upon the stimulation of OT receptor and \(\alpha_{2A}\)-AR. The contribution of influx and release was different between the two agonists: release was the predominant pathway for the OT receptor while the entry was the major one for \(\alpha_{2A}\)-AR. The relationship of these two components seems different between the two types of receptors. Thapsigargin, which depletes the intracellular Ca\(^{2+}\) store, abolished the OT-induced increase in Ca\(^{2+}\) influx but failed to reduce the \(\alpha_{2A}\)-AR-mediated Ca\(^{2+}\) influx, suggesting that the influx and release were dependent events in OT receptor, but were independent in \(\alpha_{2A}\)-AR.

OT increased IP\(_3\) formation which was not affected by pretreatment with PTX but was abolished by the PLC inhibitor U73122. Moreover, PTX failed to alter and U73122 dose-dependently inhibited the OT-induced increase in \([\text{Ca}^{2+}]\). These results suggest that OT receptors are coupled to a PTX-insensitive G protein, probably a G\(_{s}\), to
activate PLC and generate IP$_3$, resulting in Ca$^{2+}$ release. On the other hand, the $\alpha_{2A}$-AR-mediated increase in Ca$^{2+}$ release was also inhibited by U73122 and was resistant to PTX, raising the possibility that $\alpha_{2A}$-AR has a ability to couple with a PTX-insensitive G protein, leading to activation of PLC and formation of IP$_3$. Therefore, it is apparent that OT receptors and $\alpha_{2A}$-ARs mediate Ca$^{2+}$ release from intracellular stores through the same mechanism. This was further supported by the results that thapsigargin time-dependently abolished OT receptor- and $\alpha_{2A}$-AR-mediated Ca$^{2+}$ release. The fact that the porcine myometrial cells contain a high density of $\alpha_{2A}$-AR is probably a reason for $\alpha_{2A}$-ARs coupling with PTX-insensitive G protein.

Ca$^{2+}$ influx upon the activation of OT receptors was predominately mediated by opening of ROC, and to a lesser extent of L-type Ca$^{2+}$ channels. The fact that thapsigargin alone did not induce Ca$^{2+}$ influx suggested that the capacitative Ca$^{2+}$ entry mechanism did not operate in porcine myometrial cells. However, thapsigargin as well as U73122 time-dependently inhibited OT-induced Ca$^{2+}$ influx. These results suggest that a concomitant formation of IP$_3$ and depletion of Ca$^{2+}$ stores were required for OT-induced Ca$^{2+}$ influx. In contrast to OT receptor-mediated Ca$^{2+}$ influx, depletion of Ca$^{2+}$ store by thapsigargin did not significant reduce $\alpha_{2A}$-AR-mediated Ca$^{2+}$ influx, indicating that distinct signaling mechanisms were involved in Ca$^{2+}$ release and influx caused by activation of $\alpha_{2A}$-AR. The $\alpha_{2A}$-AR-mediated Ca$^{2+}$ influx appears to result from the activation of L-type VDCC because nimodipine remarkably inhibited the influx, and activation of the receptor mediated L-type currents. Activation of $\alpha_{2A}$-AR reduced basal cAMP and forskolin-elevated the cAMP concentration, and forskolin suppressed L-type currents. Furthermore maximization of intracellular cAMP content blocked $\alpha_{2A}$-AR-mediated but not Bay 8644-induced increase in L-type currents. Pretreatment of PTX abolished $\alpha_{2A}$-AR-mediated Ca$^{2+}$ influx component and Ca$^{2+}$ currents. Therefore, it is proposed that $\alpha_{2A}$-AR couples to a pertussis toxin-sensitive G protein, assuming $G_i/G_o$, to inhibit the adenylyl cyclase, leading to a decrease in cAMP formation which results in activation of L-type VDCC and Ca$^{2+}$ influx. However, the results also imply that the activation of L-type channels may be mediated by a direct coupling with a G protein.

This study characterized the properties of VDCC and identified intracellular Ca$^{2+}$ stores in porcine myometrial cells. This information should help in understanding the electrophysiological and mechanical behavior of porcine myometrium. The
investigations of signal transduction of the OT receptor and α2A-AR provide the cellular basis for understanding their modulation of the contraction. In the future, in addition to studying the molecular basis on the function of Ca^{2+} regulatory units and coupling of signal transduction, it will be significant to evaluate the change of these regulatory units and components of signal transduction as the function of reproductive stages. It is believed that answers for them will produce the information on basic aspects of reproductive process, such as maintenance of pregnancy and parturition, and facilitate the development approaches in more efficient management of preterm labor in human medicine and stillbirths in animal industry.
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