A novel insulin from the bullfrog: its structure and function in protein secretion by hepatocytes

Jocelyn Jean Hulsebus
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Iowa State University, 1987
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UMI
A novel insulin from the bullfrog: Its structure and function in protein secretion by hepatocytes

by

Jocelyn Jean Hulsebus

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Ames, Iowa
1987
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GENERAL INTRODUCTION

**Rana catesbeiana**

*Rana catesbeiana*, the common bullfrog, is the largest of the North American Ranidae. They are widely distributed throughout the southern and central United States and can be found as far north as southern Minnesota (Wright and Wright, 1949). The natural habitat for this species is a body of permanent water such as a pond, lake, marsh or slow-moving stream (Blair, 1961). Due to the long winters in Iowa, bullfrogs usually do not emerge from hibernation until the later part of April. They do not begin to breed until late June and continue into early August (Barker, 1964; Carlander and Moorman, 1959).

*Rana catesbeiana* can remain tadpoles for 1-2 years and may reach sizes up to 40 grams with plentiful food supplies before completing metamorphosis. Bagnara and Kollros (1956) observed *Rana catesbeiana* near Iowa City and found that most tadpoles overwintered only once before entering metamorphic climax. It has been postulated that the length of time *Rana catesbeiana* remain tadpoles may be functions of the amount of food available and the ambient air temperature; but this has not been completely established.
Amphibian Metamorphosis

Anuran metamorphosis is a time of adaptive changes in the larva both internally, externally, and functionally. Throughout this period the larva changes from an aquatic tadpole to a terrestrial juvenile frog. Several attempts have been made to try and assess the biological significance of metamorphosis (Dodd and Dodd, 1976). Metamorphosis would enable predominantly terrestrial animals to lay their eggs in water, which is usually considered an advantage; it also enables an animal to use two different food sources during its life cycle (Dodd and Dodd, 1976). These different food sources allow both types of animals to grow and prosper without competing for food. One possible disadvantage for this type of metamorphosis would be the restriction of the anurans to a habitat that includes a body of water available during certain times of the year.

Taylor and Kollros (1946) have divided larval growth and metamorphosis into stages based on the emergence and progressive growth of the hind limb. The different larval stages are given Roman numeral designations. I-V are limb bud stages and signal the beginning of larval growth. VI-X are paddle stages and are a time of fast body growth, characterized by the development of a paddle-shaped hind limb. XI-XIX are foot stages. During the later stages the
animal reaches its largest size and the hind limb has become well-formed. The fore limbs are also completely formed but are located internally; they can be seen through the opercular window. The onset of metamorphic climax (XX-XXV) is signaled by the emergence of the fore limbs. Although it has taken 1-2 years for the tadpoles to reach stage XX, metamorphic climax will be completed in about 7-10 days. The changes that take place during climax are dramatic; the mouth is reformed and the tail is reabsorbed; internally the animal undergoes many structural and physiological changes to become the adult animal. Metamorphosis is very stressful, and many animals die before completing the process.

The actual trigger for the initiation of metamorphosis is still unknown. Hormones, as well as, environmental factors appear to be tightly coupled to regulate metamorphosis. The hormonal regulation of metamorphosis has been an area of endocrine study for many years (Platt, 1976; Frye, Brown, and Snyder, 1972; Etkin and Lehrer, 1960; Etkin, 1935; Gudernatsch, 1912). Thyroid hormones, triiodothyronine (T₃) and thyroxine (T₄), and prolactin are recognized as the primary hormones involved in regulating metamorphosis. T₃ and T₄ are functionally termed metamorphic hormones since they promote changes to more adult-like animals. Some of the physiological changes
attributed to regulation by these hormones are growth of the limbs, degeneration of the tail fin and muscle, calcification of the skeleton, degeneration of the gills, degeneration and reorganization of the digestive tract, degeneration and restructuring of the pancreas and induction of enzymes within the liver. The serum concentration of $T_3$ has been studied and shown to increase during climax when most of the previous changes occur. Conversely, prolactin is termed antimetamorphic. Prolactin promotes tail growth and appears to stimulate larval growth in general (Seki and Kikuyama, 1981; Frye, Brown, and Snyder, 1972). Although most functions of thyroid hormones and prolactin appear to be antagonistic, studies by Gona and Gona (1975) have actually shown that low levels of $T_3$ and $T_4$ are synergistic with prolactin. This synergistic effect may promote growth early in metamorphosis. The interactions of these hormones, whether antagonistic or synergistic, seem to involve osmoregulation within the tissues. Prolactin will stimulate the uptake of water, and $T_3$ and $T_4$ will stimulate dehydration (Platt, Christopher, and Sullivan, 1978; Platt and Li Cause, 1980; Platt and Hill, 1982). Serum prolactin has been shown to increase during climax (White and Nicoll, 1981) instead of decrease as proposed by Etkin (1935). This may imply that the main function of prolactin at climax
could be to keep the tissues from being severely dehydrated by the thyroid hormones.

The external changes that occur during metamorphic climax are dramatic and appear to be the primary changes that take place since they are so visible. Internally the changes are no less dramatic, being both physiological and biochemical in nature, and can be seen in all organ systems to varying degrees. In the tadpole the main respiratory organs are gills. During metamorphic climax the gills are reabsorbed and lungs, that are already present, take over the respiratory function (Dodd and Dodd, 1976).

The large coiled simple intestine is reabsorbed and a new more complex intestine and stomach are formed. In addition, intestinal enzymes such as alkaline phosphatase, peptidases or esterases become more prevalent after restructuring. Hydrogen ions are secreted into the newly formed stomach (Dodd and Dodd, 1976). These structural, enzymatic, and chemical changes are necessary for the difference in diet (primarily herbivore to primarily carnivore) that occurs between the tadpole and frog (Kaltenbach et al., 1979; Fox, 1981).

Externally, the liver does not appear to undergo overt changes in structure during larval life. Until climax, development of the liver is basically a simple expansion of the cell population with a corresponding increase in DNA
synthesis (Smith-Gill and Carver, 1981). Atkinson and Little (1972) have reported increased synthesis of DNA by tadpole livers. They concluded that the increase in DNA synthesis was not consistent with a fixed population of cells as reported by some researchers. DNA content reaches a maximum at climax and then the synthesis rate drops. At the same time, cell death and turnover rates increase, producing a net decrease in DNA content and cell number. The turnover of liver cells at climax may involve the replacement of larval cell types with adult cell types (Smith-Gill and Carver, 1981). Following climax, cell turnover rates decrease, and the new cell population expresses adult differentiated characteristics (Smith-Gill and Carver, 1981). Functionally, changes in the liver are associated with the adult metabolism and the migration from water to land. Tadpoles excrete about 90% of their nitrogenous waste as ammonia, but just prior to metamorphic climax urea becomes the main waste product (Munro, 1939; 1953; Brown et al., 1959). This shift from ammonia to urea is accomplished by the induction of urea cycle enzymes (Brown and Cohen, 1958; Brown et al., 1959; Balinsky et al., 1961). The induction of urea cycle enzymes depends on the presence of thyroid hormones and can be induced precociously by treating tadpoles with thyroid hormones. In addition, the liver is also responsible for manufacturing a number of
exported proteins, especially albumin. In tadpoles, plasma albumin levels are very low. These levels will increase two to four fold by the end of metamorphic climax and may continue to increase slightly into adulthood (Frieden, 1961; Feldhoff, 1971; Ledford and Frieden, 1973; Just et al., 1977). The increase of plasma albumin in the juvenile and adult animals is needed to help maintain the correct osmotic balance between the blood and tissues of the terrestrial animal (Frieden, 1961; Just et al., 1977). An increase in plasma albumin would also result in a greater ability to transport molecules in the blood (Frieden, 1961). Electrophoresis and autoradiography of serum from tadpoles treated with thyroid hormones showed an increased incorporation of labeled amino acids into the plasma proteins (Ledford and Frieden, 1973). Twelve hours after treatment with thyroid hormones, a relative increase in incorporation of labeled amino acids into albumin bands was seen when compared to untreated control animals (Ledford and Frieden, 1973). In adult frogs, insulin and estrogen have been shown to increase protein secreted from cultured liver cells into the medium (Stanchfield and Yager, 1978, 1979). In these cultured cells insulin was shown to increase the synthesis of albumin and estrogen the synthesis of vitellogenin. Penhos and Krahl (1963) have also shown that
insulin will stimulate perfused livers to increase incorporation of tritiated leucine into protein.

During larval development the pancreas grows continually until it reaches its largest size during the foot stages (Hulsebus and Farrar, 1985). At climax, the pancreas weight drops sharply (70%-80%) as the tissue is exposed to proteolytic degradation (Atkinson and Little, 1972; Leone et al., 1976; Fox, 1981; Hulsebus and Farrar, 1985). The fate of the pancreatic endocrine tissue and the role of its hormones, during climax, are not completely understood. The bullfrog beta cell volume appears to remain constant throughout the entire course of metamorphic change and also in the adult (Farrar and Hulsebus, 1987, submitted to Gen. Comp. Endocrinol.)

Amphibian Endocrine Pancreas

As in mammalian pancreas, the islet or endocrine tissue is scattered throughout the exocrine tissue and comprises only about 1%-2% of the total pancreas area (Lazarus and Volk, 1962; Falkmer and Östberg, 1977; Farrar and Hulsebus, 1987, submitted to Gen. Comp. Endocrinol.). Structurally, the adult amphibian islets are actually branching cords of cells intertwined between blood sinusoids rather than the round compact balls of tissues seen in mammals (Frye, 1964). The cording islet structure is thought to be absent from the pancreases of tadpoles prior to metamorphosis (Frye, 1964).
Larval islets are smaller than adult islets and appear as compact bundles.

Amphibian islets contain A, B, D, and pancreatic polypeptide cell types (Kaung and Elde, 1980; Khanna and Kumar, 1973; Hellman and Hellerstrom, 1962; Pollack et al., KU Med. Center, Kansas City, Kansas, 1987). These cells contain the peptide hormones glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. Very little is known about the function of these hormones in the amphibian. In anurans the ratio of A to B cells is about 1:1, and may roughly indicate the amount of hormone used by these animals (Falkmer and Ostberg, 1977). Kaung and Elde (1980) and Kaung (1981) have demonstrated glucagon and pancreatic polypeptide in the same cell in *Rana pipiens* using an immunocytochemistry stain. With their staining system, they have found some cells that contain only glucagon but no cells that contain only pancreatic polypeptide. Immunoperoxidase staining and electron microscopy studies by Tomita and Pollock (1981) did not show this combination of hormones in one cell type in the pancreas of *Rana catesbeiana*. In bullfrogs, glucagon and pancreatic polypeptide appear in two distinct cells. Insulin and somatostatin are not found with any other hormone in the amphibian islet cells. In mammals, B cells (insulin-containing) can be selectively destroyed with alloxan and
streptozotocin (Like and Rossini, 1976). Kerns and Farrar (1987) have shown that streptozotocin has little effect on the B cells of Rana catesbeiana adults and tadpoles. Even with high concentrations of this drug, the effect is at best minimal and transient. Neither serum nor pancreatic insulin-like immunoreactivity is decreased.

In mammals insulin is considered to be the major anabolic hormone (Gottschalk and Jarett, 1986) and will rapidly stimulate the uptake of glucose, amino acids, and ions into certain tissues, such as skeletal muscle. If insulin has a similar function in amphibians, it may be one of the important hormones of larval development. Studies have been done to try and assess the function of insulin in both the tadpoles and adults. deRoos and Parker (1985) administered insulin to adult bullfrogs and obtained non-detectable glucose levels. These low blood glucose levels appeared to have little effect on the animals. Houssay and Biasotti (1930) pancreatectomized toads and found that they would develop hyperglycemia. Frye (1965) pancreatectomized tadpoles and found that during the early stages of development they failed to develop hyperglycemia. Later staged tadpoles develop hyperglycemia, but its development is slow, suggesting that the regulation of glucose may not be the primary function for insulin in these animals. One of the acute problems of pancreatectomy is that in addition
to the loss of the hormones of the pancreas, all the digestive enzymes found in the exocrine pancreas are also lost. This immediately causes an upset in the ability of the animal to process food properly. Kerns and Farrar (1987) have tried to overcome this problem by using streptozotocin to selectively destroy the beta cells (insulin containing) cells of the pancreas. They have shown that even high doses of streptozotocin have little effect on these cells. Hulsebus and Farrar (1985) have shown that serum and pancreas extracts of *Rana catesbeiana* tadpoles and adults contain an insulin-like immunoreactivity (ILI). In the tadpoles the levels of ILI appear to increase in amount as both the body and pancreas grow in size. Both serum and pancreas amounts drop sharply during climax when the pancreas degenerates by about 80%. One possible function of ILI in the tadpole serum may be to stimulate anabolic processes during the time of rapid larval growth.

Functions of Insulin

Classically insulin is associated with the regulation of blood glucose levels. This appears to be the main function for this peptide hormone in mammals, but its function appears to vary widely throughout the vertebrate animal classes.
In the hagfishes (Class Agnatha), the effect of insulin differs depending on the study read. Matty and Gorbman (1978) have found a slight hyperglycemia following isletectomy and ten weeks fasting. This is in contrast to Falkmer and Matty (1966) where this operation had no effect on the blood sugar. One explanation for this discrepancy may be the difference in observation time, since the 1966 study was much shorter in duration (Eppling et al., 1980). Lampreys (Class Agnatha) will develop hyperglycemia following isletectomy and anti-insulin-sera injections. They will also develop hypoglycemia following injections of mammalian insulin (Falkmer and Matty, 1966; Plisetskaya and Leibushe, 1972; Plisetskaya et al., 1976). The onset of hyperglycemia and hypoglycemia is very slow, often taking several days to appear or disappear. Nevertheless, the data show that insulin can affect the glycemia of lampreys, but that a mammalian-like control is not present (Eppling et al., 1980).

Injections of crude extracts containing shark insulin were more effective than mammalian insulin in lowering blood glucose in sharks (Class Chondrichthyes) and the return to normal was more rapid following extract injection (Eppling, 1969). The American eel (Class Osteichthyes) showed no specific hyperglycemia following pancreatectomy (Lewis and Eppling, 1984). However, insulin injections in several
different varieties of eels showed increased incorporation of amino acids into body proteins (Thorpe and Ince, 1974; Inui and Yokote, 1975; Thorpe, 1976). If serum insulin levels are obtained by radioimmunoassay, the levels vary with the season and reproductive activity, as well as between different animals (Epple et al., 1980).

There is a difference in the response to insulin between the larval and adult anuran (Class Amphibia). Early larval animals will not respond to pancreatectomy by developing hyperglycemia but older tadpoles and adults will respond (Frye, 1965). deRoos and Parker (1985) have shown that injections of large doses of mammalian insulin will cause hypoglycemia in bullfrogs, but the duration of this condition is prolonged. Insulin injections, both high and low doses, can stimulate the incorporation of labeled amino acids into protein in Rana catesbeiana (Penhos and Krahl, 1962, 1963; Stanchfield and Yager, 1979).

Pancreatectomy in Alligator mississippiensis (Class Reptilia) resulted in a progressive increase in blood sugar and many animals survived for several weeks (Penhos and Ramey, 1973). When compared with other reptiles and anurans, the glycemic response to insulin is very slow and may be related to the slow metabolism of the alligator. Lizards and snakes (Class Reptilia) will respond to mammalian insulin by developing hypoglycemia. However, it
appears that lizards are more insensitive and the hypoglycemia that develops is less severe and of short duration (Miller, 1961; Penhos and Ramey, 1973). Very high glucose concentration caused a mammalian-like biphasic insulin release from lizard islets in vitro (Rhoten, 1974) and amino acids caused a monophasic release of insulin (Rhoten, 1973).

Pancreatectomy is very difficult in the Class Aves and results of this operation are highly variable and probably unreliable. In ducks, pancreatectomy resulted in hypoglycemia, normoglycemia, or hyperglycemia (Mialhe, 1958). Insulin injections provoke a mild hypoglycemia, however, the avian tissues are more responsive to avian than mammalian insulin in vivo (Hazelwood, 1973). Birds in general show a marked resistance to insulin and even very high levels of blood glucose fail to stimulate insulin release from the pancreas (Turner and Hazelwood, 1974). Insulin injections have a transitory effect on serum fatty acid concentration depending on the birds used. Little or no information is seen on the effect of insulin on protein synthesis in birds.

Pancreatectomy in the Class Mammalia results in severe hyperglycemia, glucosuria, depletion of glycogen stores, ketonuria, and breakdown of tissue proteins (Epple et al., 1980). These symptoms can be experimentally induced by alloxan or streptozotocin and can be prevented with
injections of exogenous insulin. Insulin is released from the pancreas in response to glucose, free fatty acids, and some amino acids such as leucine (Lacy, 1977).

The data suggest that the function of insulin varies widely throughout the animal classes. Pancreatectomy can be followed by diabetes mellitus, severe hypoglycemia, or no response (Epple et al., 1980). It seems that the control of amino acid levels may be a more ancient function, whereas the glucostatic function is more recently evolved and best developed in mammals (Epple et al., 1980).
Exhaustion of Dissertation Format

An alternative format was used in this dissertation. There are three sections after the General Introduction, each of the sections is an individual paper. The first section has been submitted to Endocrinology, the other two sections will be submitted for publication. Finally, a general summary and discussion of the dissertation is included. In the first section, the purification was performed by myself up the point of sequencing. Dr. Louisa Tabatabai performed the sequencing of the insulin molecule. The second section was performed mostly by myself and a small portion by Dr. Eugenia Farrar. The third section was performed entirely by myself. These three manuscripts were prepared by myself with assistance from my major professor, Dr. Eugenia Farrar, in editing and revision.
SECTION I:

PURIFICATION AND AMINO ACID SEQUENCE OF

BULLFROG (RANA CATESBEIANA) INSULIN

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ABSTRACT

Insulin was extracted and purified from the pancreas of the adult bullfrog, *Rana catesbeiana*, using Sephadex gel filtration and reverse phase high performance liquid chromatography (HPLC). A porcine insulin radioimmunoassay (RIA) was used to detect insulin immunoreactivity during purification. Twenty-five grams of pancreas tissue yielded 674 μg of pure insulin. Analysis of acid hydrolysates of the pure peptide showed that bullfrog insulin contained 52 amino acids. A and B chains were separated and their amino acid sequences determined. The amino acid sequence of bullfrog insulin is unique when compared to other known insulins. The amino terminus of the A chain has a two amino acid extension, not found in any other known insulin sequence. In addition, a methionine is substituted at B17 for the leucine seen in most mammal insulins. Most of the structurally important amino acids have been conserved, thus preserving the integrity of the three-dimensional molecule. The most notable are the six cysteines, the zinc binding site (B10, histidine), and nonpolar surface sequences involved in dimerization (B12-B16, B24-B25) and hexamerization (B14, B18, A13, A14).
INTRODUCTION

Insulin has been isolated and the primary structure determined for about 40 species including a variety of mammals, birds, reptiles, and fishes (Hallden et al., 1986; Kimmel et al., 1968; Kimmel et al., 1976; Lance et al., 1984). Although there is a high degree of sequence homology between the insulins, many have unique portions that affect the structure and function of the molecule. Amphibian insulin is the only insulin from a major class of vertebrate whose primary structure is unknown. Several lines of indirect evidence suggest that insulin is present in amphibians. Pancreatectomy results in hyperglycemia in several species (Penhos and Ramey, 1973). Mammalian insulin in large doses causes hypoglycemia, although most amphibians have comparatively low blood glucose levels and are very tolerant of hypoglycemia (Hanke and Neumann, 1972; deRoos and Parker, 1985). We have detected insulin-like immunoreactivity (ILI) in bullfrog, *Rana catesbeiana*, tadpoles and adults. The ILI in serum and pancreas increases during larval life, declines during metamorphosis, and increases again in juveniles and adults (Hulsebus and Farrar, 1985). In addition, Korns and Farrar (1987) found that bullfrog serum ILI concentration is positively correlated with blood glucose concentration. This study takes a first step toward understanding amphibian
insulin structure and function by purifying and sequencing the molecule.
MATERIALS AND METHODS

Adult bullfrogs were purchased from the Lemberger company of Oshkosh, Wisconsin. These animals were stored in large tanks with dripping water on a twelve hour light/dark cycle until used for experimentation. Animals were anesthetized in 0.5% tricaine. The pancreas was excised and frozen at -20°C until 25 grams of tissue were obtained for a purification preparation.

Extraction of the pancreas was by acid ethanol using the method of Pettinga (1958). The resulting extract was treated with absolute alcohol and anhydrous ethyl ether in a ratio of 1:4 by volume and was incubated overnight at -15°C. The white precipitate was removed by vacuum filtration and washed twice with anhydrous ether and once with cold acetone. The precipitate was dissolved in 20 ml of 0.1 M acetic acid for gel filtration.

Gel filtration was done at room temperature using Sephadex G-50 superfine purchased from Sigma. Two 2.5 X 90 cm columns were attached in series and eluted with 0.1 M acetic acid. Molecular weight standards (BSA, alpha-lactalbumin, porcine insulin, porcine glucagon with molecular weights of 66,000, 14,200, 6000, 3000, respectively) were used to determine the approximate elution volume of the hormone. Five ml fractions were collected and the protein absorbancy was measured at 276 nm.
The gel filtration fractions were diluted 1:250 with saline and analyzed for the presence of insulin immuno-reactivity using a clinical insulin radioimmunoassay (RIA) kit from Cambridge Diagnostics, Inc., Billerica, Mass. The kit uses a double antibody technique with porcine insulin standards and guinea pig anti-porcine insulin sera.

Reverse phase high performance liquid chromatography (HPLC) was performed with a Rainin instrument and a 0.46 x 25 cm Vydac C18 column. The gradient solutions consisted of 0.08% TFA (Sigma) in water for solvent A and 0.1% TFA in acetonitrile (HPLC grade, Fisher) for solvent B.

Amino acids were analyzed on a Beckman 121 MB analyzer after acid hydrolysis. Hydrolysis was done in evacuated sealed tubes with constant-boiling HCL for 20 hours at 110° (Kimmel et al., 1968; Kimmel et al., 1976; Lance et al., 1984)

A and B insulin chains from HPLC fraction 2 were cleaved by treatment with 2-mercaptoethanol. Cysteines were pyridylethylated with 4-vinyl pyridine (Fullmer, 1984) for identification during sequencing. The A and B chains were then separated by HPLC under the conditions as previously described. The samples were evaporated to dryness under a stream of nitrogen and dissolved in 60 ul of 0.1% TFA in 30% acetonitrile. A 30 ul aliquot was sequenced on a model 470A gas-phase protein sequenator (Applied Biosystems, Foster...
City, Ca), using a double coupling protocol. PTH-amino acids were determined by HPLC on a model 120A on-line PTH-amino acid analyzer. Picomol yields of the PTH-amino acids were calculated from the peak heights of known amounts of PTH-amino acid standards from a HPLC chromatogram run under identical conditions.
RESULTS

The elution pattern obtained from the gel filtration is shown in Figure 1. In the area of the porcine insulin and porcine glucagon standards, there are four peaks that could contain bullfrog insulin. Gel filtration peak 2 coelutes with the insulin standard and peak 4 with the glucagon standard. When the gel filtration fractions were diluted and assayed for insulin immunoreactivity, two peaks were found (Figure 1). The first peak is located at the approximate molecular weight of 11,000. This is probably bullfrog proinsulin since proinsulin will crossreact with the anti-insulin antibodies used. The second and largest peak of crossreactivity is centered over gel filtration peak 2, which coelutes with the insulin standard. The fractions under each of the four peaks were combined and flash evaporated.

The four gel filtration peaks were fractionated by reverse phase HPLC using an hydrophobic C18 column and a gradient of water/TFA and acetonitrile/TFA. With this gradient system most insulins are eluted from the column between 30% and 40% acetonitrile. Material from each of the HPLC peaks was acid hydrolyzed and analyzed for amino acid composition. The marker used for insulin was the presence of 6 cysteines.
Figure 1. Gel filtration of pancreas extract and immunoreactive insulin peaks using porcine RIA. Four major peaks (________) were obtained by gel filtration in the approximate molecular weight of the insulin standard. Two peaks (----------) of crossreactivity were seen with porcine anti-insulin sera.
Gel Filtration of Pancreas Extract
Sephadex G-50 SF
2.5 x 180 cm
Based upon the amino acid composition, gel filtration peak 1 contains an unknown peptide with no cysteines. Gel filtration peak 3 contains one major peptide that may be pancreatic polypeptide and contains no cysteines (personal communication Gail Pollock, KU Med. Center, Kansas City, Kansas). Gel filtration peak 4 contains 2 peptides. Peptide 1 has no cysteines and may be the C peptide. Peptide 2 has the same amino acid composition as human glucagon (personal communication Gail Pollock, KU Med. Center, Kansas City, Kansas).

When gel filtration peak 2 was fractionated on the HPLC, four peptide peaks were obtained (Figure 2). HPLC peak 2 contains 6 cysteines and 52 amino acids (Table 1). HPLC peak 1 is another unknown peptide. HPLC peaks 3 and 4 appear to be glucagon-like peptides based on their amino acid composition and number of 31 residues per polypeptide (personal communication Gail Pollock, KU Med. Center, Kansas City, Kansas).

When all HPLC peaks were assayed in the porcine RIA, only HPLC peak 2 from gel filtration peak 2 would crossreact in the assay. Based on the number of amino acids per polypeptide, the presence of 6 cysteines, and the cross-reactivity in the RIA, this molecule appears to be bullfrog insulin.
Figure 2. Reverse phase HPLC analysis of gel filtration peak 2. Four peaks were obtained. HPLC peak 2 is bullfrog insulin. The gradient line (-----) is shown.
Preparative HPLC of Bullfrog G50SF
.46 X 25 VYDAC C18
O.D. = 276nm
Flowrate 2 ml/min
Slope = 0.5% Acetonitrile/min
Table 1. Amino acid composition of bullfrog insulin

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52
The bullfrog insulin amino acid sequence was determined and is shown in Figures 3A and 3B. Both the A and B chain carboxy termini are still in doubt, therefore 51 of the 52 amino acids are shown. Our best estimation of the carboxy terminus is that A21 is asparagine and that the B29 arginine is terminal, based on the following rationale: 1) It seems unlikely that the A terminus would be cysteine; 2) A21 asparagine is invariable in all the other insulin species and is the only amino acid remaining in the composition when the other 51 amino acids are crossed off; and 3) B30 is missing or not terminal in several other species.

Bullfrog insulin contains all of the conserved areas that are important for the 3-dimensional structure of the molecule (Blundell et al., 1972). The most unique feature is a 2 amino acid extension on the amino terminus of the A chain. Bullfrog insulin also contains a methionine at B17 in the central portion of the chain. When methionine is present in other insulin B chains it is usually on either the amino or carboxy terminal ends. Bullfrog, unlike guinea pig or hagfish insulin, contains a histidine at B10 and should complex with zinc and be capable of forming a hexamer (Blundell et al., 1972).
Figure 3A. Amino acid sequence of the A chain of bullfrog insulin and comparison to other known insulins (Halden et al., 1986; Blundell et al., 1972; Blundell and Horuk, 1981; Horuk et al., 1979; Horuk et al., 1980)
<table>
<thead>
<tr>
<th>14 15 16 17 18 19 20 21 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYR-ASP-LEU-GLU-ASN-TYR-CYS</td>
</tr>
</tbody>
</table>

| Bullfrog | ASN | Glu, Pig, Rabbit, Dog, Sperm and Fin Whales, Monkey |
| GLN | ASN | Cattle |
| GLN | ASN | Sheep, Goat |
| GLN | ASN | Horse |
| GLN | ASN | Sperm Whale |
| GLN | ASN | Rat, Mouse, Syrian Hamster |
| HIS GLN | GLN | Cat |
| ASN GLN | MET SER | ASN | Guinea Pig |
| GLN | ASN | Coypu |
| GLN | GLN | ASN | Chinchilla |
| ASN GLN | LEU THR | ASN | Porcupine |
| GLN | ASN | Casiragua |
| GLN | ASN | Chicken, Turkey |
| GLN | ASN | Duck, Goose |
| GLN | ASN | Rattle Snake |
| GLN | ASN | Alligator |
| GLU | ASN | Colubrid Snake |
| PHE | GLN | ASN | Cod |
| PHE | GLN | ASN | Anglerfish |
| PHE | GLN SER | ASN | Tuna II |
| PHE | GLN SER | ASN | Toadfish II |
| PHE | GLN SER | ASN | Toadfish I |
| PHE | GLN | ASN | Bonito |
| PHE | GLU | ASN | Carp |
| PHE | GLY | ASN | Salmon |
| ASN | GLN | ASN | Dogfish |
| ASN | GLN | ASN | Hagfish |

Bullfrog insulin 1972; 1979;
**Figure 3B.** Amino acid sequence of the B chain of bullfrog insulin and comparison to other known insulins

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|
|   | PHE | PRO | ASN | GLN | TYR | LEU | CYS | GLY | SER | HIS | LEU | VAL | GLU | ALA | LEU | TYR | MET | VAL | CYS | GLY |
|   | VAL | HIS | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU |
|   | VAL | LYS | HIS | PRO | HIS | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU |
|   | VAL | SER | ARG | HIS | ASN | THR | SER | ARG | GLN | ASP | THR | SER | ARG | GLN | ASP | THR | SER | LYS | PHE | LEU | ILE |
|   | THR | VAL | SER | ARG | VAL | LYS | HIS | ASP | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | PHE | LEU | ILE |
|   | TYR | VAL | GLY | ARG | ALA | ALA | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS | HIS |
|   | ALA | ALA | ARG | ALA | ALA | ARG | ASP | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU |
|   | ALA | ARG | ALA | ARG | ALA | ARG | ASP | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU | LEU |
|   | MET | ALA | PRO | HIS | ALA | ALA | PRO | HIS | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU |
|   | VAL | ALA | ALA | HIS | ALA | PRO | HIS | ASP | LEU | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU |
|   | ALA | PRO | HIS | ALA | ALA | PRO | HIS | ASP | LEU | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU |
|   | ALA | ALA | PRO | HIS | ALA | ALA | PRO | HIS | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU |
|   | ASN | GLY | ALA | PRO | HIS | LEU | SER | HIS | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU | ASP | LEU |
|   | ALA | ALA | ALA | HIS | LEU | SER | HIS | ARG | THR | THR | GLY | HIS | LYS | ASP | ASN | ILE | ALA | LYS | ASP | ASN | ILE | ALA |
### Amino Acid Sequences and Corresponding Species

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</tr>
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<tr>
<td>GLU</td>
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<td>Rat II, Mouse II</td>
</tr>
<tr>
<td>ARG</td>
<td>Guinea pig</td>
</tr>
<tr>
<td>GLU</td>
<td>Coypu</td>
</tr>
<tr>
<td>HIS</td>
<td>Chinchilla</td>
</tr>
<tr>
<td>ASP</td>
<td>Porcupine</td>
</tr>
<tr>
<td>LYS</td>
<td>Casiragua</td>
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<tr>
<td>HIS</td>
<td>Chicken, Turkey</td>
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<td>ASP</td>
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<td>Rattle Snake</td>
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<tr>
<td>ASP</td>
<td>Colubrid Snake</td>
</tr>
<tr>
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</tr>
<tr>
<td>ASP</td>
<td>Anglerfish</td>
</tr>
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### Partial Sequences and Corresponding Species

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<td>ARG</td>
<td>Coypu</td>
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<tr>
<td>HIS-THR-LYS</td>
<td>Chinchilla</td>
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DISCUSSION

Extraction of 25 grams of bullfrog pancreas and subsequent purification produced 674 μg of insulin as calculated using its optical density and extinction coefficient (Hemerhorst and Stokes, 1986). The major portion of the purified insulin (630 μg) was saved for biological studies. The material is pure insulin as determined from its amino acid composition and amino acid sequence which is homologous to other insulins (Figures 3A and 3B). The sequence analysis of the two chains is in agreement with the amino acid composition, and this verifies the reliability of the analysis.

The amino acid sequence of bullfrog insulin is unique when compared to other known insulins. Assuming an Asn residue, the bullfrog insulin has 11 substitutions out of 50 residues with 2 additions and a deletion as compared to human or porcine insulin. It is more similar in number of substitutions to alligator insulin (7 out of 50) and chicken insulin (8 out of 50). In mammals the amino terminal glycine is on the surface of the molecule and associated with insulins' biological activity (Hallden et al., 1986; Blundell et al., 1972). If the amino group is removed or a bulky group is added to this glycine residue, the biological activity is greatly decreased. The reduced activity of proinsulin is thought to be at least partially
due to blocking of this terminus by the connecting C-peptide. Bullfrog insulin is the only insulin to date that shows a two amino acid extension of the amino terminus of the A chain. The extension on the A chain poses some interesting questions. Will this affect the biological activity and/or receptor binding of the insulin in the bullfrog? Is the amphibian receptor different from the mammalian? Does this insulin show decreased biological activity in a mammalian bioassay and/or receptor assay? We hope to address these questions in our lab in the future.

In addition to the amino terminal glycine of the A chain, A21 asparagine is also associated with the biological activity of the molecule in other insulins (Hallden et al., 1986; Blundell et al., 1972). This position is invariant in all the insulins to date. The probability is very high that the last amino acid at position A21 of the bullfrog insulin is asparagine.

Bullfrog insulin should form dimers and hexamers and bind zinc. The appropriate nonpolar surface sequences necessary for dimerization (B12-B16, B24, B25) and hexamerization (B14, B18, A13, A14) are conserved as compared to porcine insulin (Hallden et al., 1986; Blundell et al., 1972; Blundell and Horuk, 1981). While a methionine residue is substituted at B17 for the leucine seen in most mammalian insulins, the hydrophobicity of the region should be main-
tained. In addition, the zinc binding site (B10, histidine) is also conserved (Blundell et al., 1972; Blundell and Horuk, 1981).

The amino acids at A8, A9, A10 are part of the antibody binding site of mammalian insulins (Wilson, 1967). This is an area of high variability among the known insulins. It accounts for part of the difference in binding between heterologous insulins and antibodies. Bullfrog insulin is homologous in this region with chicken, turkey, and alligator insulins. It is probable that bullfrog insulin will crossreact better with antibodies to these insulins than with porcine anti-insulin antibodies. We hope to explore this possibility in the future.
REFERENCES


SECTION II

INSULIN STIMULATION OF PROTEIN SECRETION BY BULLFROG TADPOLE AND ADULT HEPATOCYTES

Jocelyn J. Hulsebus and Eugenia S. Farrar

Department of Zoology, Iowa State University
Ames, Iowa 50011
ABSTRACT

Bullfrog insulin has a two amino acid extension at the A1 position. Addition of a bulky group to this position on mammalian insulin results in a loss of biological activity. We compared the effects of bullfrog and porcine insulins on protein secretion by hepatocytes from bullfrog and tadpole livers. Hepatocytes were obtained by collagenase dispersion and were cultured in Waymouth's medium, a defined, serum-free medium. Tadpole hepatocytes were cultured in a 60% dilution of the adult Waymouth's to account for the difference in osmotic environment between tadpole and adult animals. After attachment, new medium, with or without hormone, was added. The cultures were incubated for 24 hrs and then the medium was removed. Total protein in the medium was determined colorimetrically and cellular DNA fluorometrically. Data were expressed as ug protein/ug DNA. Both hormones stimulated protein secretion in tadpoles and adults. However, adult cells, both treated and untreated, secreted more protein than tadpole cells. In addition, adult cells, but not tadpole cells, secreted more protein in response to bullfrog insulin than to porcine.
INTRODUCTION

Liver cell cultures have been used as a model system to test the effects of specific compounds on the synthesis and secretion of plasma proteins in mammals, birds, and frogs (Grieninger, 1983). By using this in vitro system, direct effects of individual compounds on the liver cells can be seen. Chick embryo hepatocytes and *Rana catesbeiana* (bullfrog) hepatocytes are well suited for this purpose since they can be maintained in a totally defined medium that does not contain serum (Granick et al., 1975; Stanchfield and Yager, 1978, 1979). Many plasma proteins are produced and secreted by the liver but, albumin is the major protein. Stanchfield and Yager (1979) have shown that *Rana catesbeiana* hepatocytes increase their secretion of albumin into the medium when bovine insulin is added, but an increase in protein synthesis, particularly albumin, has not been seen consistently in rat livers. Mortimore and Mondon (1970) have shown no insulin effect on secreted protein by perfused rat livers, while, Crane and Miller (1977) have shown an increased incorporation of labeled amino acids by hepatocytes in suspension culture with insulin treatment. Livers from diabetic rats synthesize and secrete fewer plasma proteins, and injections of insulin return the levels of plasma proteins to within the normal range (Green and Miller, 1960; Peavy et al., 1977). Serum
proteins increase dramatically in the metamorphosing bullfrog and probably contribute to their osmotic adjustment to a more terrestrial existence (Hernor and Freiden, 1980). Since larval bullfrogs have peak serum insulin-like immunoreactivity concentrations during prometamorphosis, insulin could be important in regulating plasma protein synthesis and secretion. This research describes the action of porcine insulin and bullfrog insulin, whose structure is very different from porcine (Hulsebus, Farrar, and Tabatabai, submitted to Endocrinology, 1987), on the total secreted protein from adult bullfrog hepatocytes in culture. In addition, it describes a modification of the culture system for tadpole hepatocytes, and tests the tadpole liver cells' abilities to secrete protein at different phases of development.
MATERIALS AND METHODS

Adult bullfrogs were purchased from the Lemberger Company of Oshkosh, Wisconsin and were kept in large tanks with dripping water. Frogs were not fed and were used within one week of shipment. Bullfrog tadpoles were seined from a sandpit north of Ames, Iowa and were kept in large tanks of dechlorinated water. Tadpoles were fed a prepared diet mixture of rabbit chow, agar, and gelatin (Hirschfeld et al., 1970). Tadpole developmental stages were determined based on the descriptions by Taylor and Kollros (1946). All animals were kept on a twelve hour light/dark cycle and were sacrificed between 10 am and 12 (noon). Animals were anesthetized in tricaine methane sulfonate (1%-adults, 0.5%-tadpoles) prior to use in experiments. Heparin (400 units/ml, adults-0.8 ml, tadpoles-0.1 ml) was injected into the ventricle of the heart prior to excision of the liver.

Excised bullfrog livers were cut into 2 mm cubes in 30% amphibian calcium/magnesium free balanced salt solution (BSS) as prepared by Stanchfield and Yager (1978). All culture medium reagents were purchased from Sigma. The liver cubes were rinsed in 100 ml of BSS for 30 minutes with agitation, and then transferred to a spinner flask containing 20 ml of Waymouth's medium (as prepared by Stanchfield and Yager, 1978) and 10 mg of crude collagenase.
Livers pieces were exposed to collagenase for approximately 3 hours at room temperature. Tadpoles were divided into developmental groups: premetamorphic (Taylor and Kollros stages I-XI), early prometamorphic (Taylor and Kollros stages XII-XV) and late prometamorphic (Taylor and Kollros stages XV-XIX). Tissue from several tadpole livers had to be pooled to obtain enough cells for each experiment. Eight premetamorphic and 6 prometamorphic livers were pooled. Livers were minced and dispersed by collagenase in a diluted form of Waymouth's medium. To account for the difference in osmotic environment between tadpole and adult cells, 3 concentrations of Waymouth's medium were tested (full strength, 60%, and 40%). Only cells plated in the 60% medium would attach to the culture dishes and actively secrete protein. All tadpole hepatocyte testing and cell dispersions were done in 60% Waymouth's medium. Hepatocytes were plated on poly-L-lysine (Sigma) coated culture dishes (35 mm) at an approximate density of 6.0 x 10^5 cells/cm². Cells were allowed to attach for 18-20 hours at 22-24°C in 2 ml of Waymouth's medium with no hormone added.

Porcine insulin (Sigma) and purified bullfrog insulin were added to the test culture dishes after attachment. Adult bullfrog culture dishes received either 3.5 ng/ml or 350 pg/ml, while tadpole culture dishes received only the
higher dose. Twenty-four hrs later the medium was removed and centrifuged at 2000 x g for 10 minutes to remove any cellular material. One ml of the spun medium was removed and placed in a 12 X 75 mm culture tube. One ml of 20% TCA was added to this aliquot, was mixed by vortexing, and was placed at 4°C overnight. The precipitated protein was collected by centrifugation, washed 2X with cold 5% TCA, and then dissolved in 0.5 ml of 0.2 N NaOH (Stanchfield and Yager, 1979). Aliquots were assayed for protein with a Coomassie Brilliant Blue dye reagent by the method of Read and Northcote (1981).

One ml of medium was added to the attached hepatocytes and the culture dishes were frozen at -20°C until assayed for DNA content. DNA concentrations were determined by a fluorometric method (Hinegardner, 1971). DNA was purchased from Sigma for use as a standard. To normalize the protein values and account for any differences in cell numbers/dish, DNA content of each dish was determined. Data were then expressed as ug protein/ug DNA.

ATP levels were determined on cultured cells (1.2 X 10⁶ cells/dish) which were rinsed twice with 22-24°C physiological saline and were frozen at -20°C in their culture dishes containing 1 ml of saline. Cells from 2 dishes were pooled and the ATP was measured by a modification of the firefly assay using a kit purchased from
Sigma. In the ATP assay, phosphoglycerate phosphokinase catalyzes the phosphorylation reaction of 3-phosphoglycerate to 1,3-diphosphoglycerate. A second enzyme, glyceraldehyde phosphate dehydrogenase, catalyzes the dephosphorylation of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate and the oxidation of NADH. A difference in absorbance results when NADH is oxidized to NAD and is a measure of the amount of ATP originally present.

All culture experiments were designed as randomized blocks. Cells from each experimental unit (1 spinner flask) were divided into 3 groups, control (untreated), bullfrog insulin treated, and porcine insulin treated. Each group contained 2-3 culture dishes. Data from the 2-3 culture dishes were averaged for each value. The number of replications for each experimental unit were, premetamorphic-7, early prometamorphic-9, late prometamorphic-3, and adults-6. Paired t tests were performed on the differences in protein secretion (hormone treated culture - control culture) and the level of significance determined. A probability level of 5% or less was considered significant.
RESULTS

In order to test the effects of dispersion and success of culture conditions, the hepatocytes' abilities to exclude dye and to maintain their ATP concentrations were assessed. Bullfrog hepatocyte viability, as determined by trypan blue exclusion, was between 90-98% immediately following dispersion. ATP concentrations were determined at 24, 48, and 72 hrs (Table 1). ATP remained at a constant level throughout the 72 hr period in either the presence or absence of insulin. Cells that failed to attach to the culture dishes had ATP levels that were three to five fold lower than the attached cells. Our results agree with those of Stanchfield and Yager (1979) who have shown that ATP levels increase after dispersion, level off at 24 hrs and then remain relatively constant for up to five days of culture.

Two doses of porcine and bullfrog insulin were tested in the bullfrog hepatocyte cultures and compared to untreated controls. The low dose (350 pg/ml) was estimated to be within the physiological range of insulin-like immunoreactivity in bullfrog serum (Hulsebus and Farrar, 1985). The high dose was 3.5 ng/ml and was above determined serum concentration. Both doses of the porcine and bullfrog insulin caused significant increases in total protein
Table 1. Adult ATP concentrations for 24, 48, and 72 hours. nMol ATP/2.4 X 10^6 cells/dish + S.D.

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<th>72hr</th>
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<td>10.2 + .25</td>
<td>9.9 + .20</td>
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<tr>
<td>Bullfrog Insulin (5)</td>
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<td>12.8 + .60</td>
<td>11.0 + .92</td>
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<tr>
<td>Porcine Insulin (5)</td>
<td>10.7 + .20</td>
<td>12.3 + .81</td>
<td>11.1 + 1.40</td>
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</tbody>
</table>

ATP levels for cells that failed to attach to the plate at 24 hours

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<tr>
<td>Porcine Insulin (3)</td>
<td>.34 + .02</td>
</tr>
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^Student's t-test was used for statistics.

^None of the above values were significant at the .05 level.

^Numbers in parentheses are number of replications.
Figure 1. Mean difference in protein secretion between treated and untreated adult bullfrog hepatocytes. For each pair of bars, the left bar is bullfrog insulin and the right bar is porcine insulin. Paired t-tests were done to determine the level of significance. Both doses of each insulin resulted in higher values (p < .001) than the controls. The difference in protein secretion between porcine and bullfrog insulin, for either the high or low dose, was significant (p < .05). The increase of protein secretion by the high dose, as compared to the low dose, for both bullfrog and porcine insulin was significant (p < .01). Number of replications=6
Effects of Insulins on Protein Secretion by Adult Hepatocytes

High and Low Doses of Insulin
Table 2. Mean total protein secretion from bullfrog hepatocytes in culture (μg pro/μg DNA ± S.E.) in response to bullfrog or porcine insulin

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<tr>
<th>Adults (6) Low Insulin Dose (350 pg/ml)</th>
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<th>Porcine Insulin</th>
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<td>86.0 ± 6.0</td>
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<th>Porcine Insulin</th>
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</thead>
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<td>7.2 ± 0.4</td>
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<td>6.7 ± 0.7</td>
<td>7.7 ± 0.6</td>
<td>7.4 ± 0.5</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Late Prometamorphic Tadpoles (3) (Insulin Dose-3.5ng/ml)</th>
<th>Control</th>
<th>Bullfrog Insulin</th>
<th>Porcine Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.8 ± 0.8</td>
<td>14.7 ± 0.5</td>
<td>11.4 ± 0.7</td>
</tr>
</tbody>
</table>

"Mean of experimental units. Each experimental unit contained 2-3 culture dishes.

"Numbers in parentheses are the number of replications for each experimental unit."
secretion (Figure 1 and Table 2), and the larger insulin dose caused greater effects than the smaller dose. In addition, there was a differential response between the porcine and bullfrog insulin. Both concentrations of bullfrog insulin caused increases in total protein secretion greater than that caused by the comparable doses of porcine insulin.

Tadpole cell viability, as determined by Trypan Blue exclusion, averaged 95%. The number of tadpole hepatocytes remained constant, undergoing neither extensive lysis nor growth, throughout the culture period as evidenced by the constant DNA content (7.2 ± 1.0 μg DNA/1.2 x 10⁶ cells/dish data not shown). Cellular content of DNA was typical of amphibian cells. ATP concentrations remained unchanged throughout the 24-48 hr culture period (Table 3). Tadpole dishes had the same number of cells but averaged 10 times less ATP than those from the adult. Insulin promoted hepatocyte protein secretion throughout larval life (Figure 2). All three tadpole groups, treated with insulin, had small but significant increases in protein secretion over that of the untreated controls. The late prometamorphic tadpoles secreted a significantly greater amount of protein in response to insulin than the two groups of earlier staged animals. No differential response between porcine and bullfrog insulin was seen in any tadpole group. The amount
Table 3. Tadpole ATP concentrations for 24 and 48 hours.

nMol of ATP/2.4 X 10^6 cells/dish + S.D. a, b

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Premetamorphic tadpoles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4)</td>
<td>1.07 + .14</td>
<td>1.11 + .05</td>
</tr>
<tr>
<td>Bullfrog Insulin (4)</td>
<td>1.14 + .08</td>
<td>1.17 + .10</td>
</tr>
<tr>
<td>Porcine Insulin (4)</td>
<td>1.09 + .09</td>
<td>1.11 + .08</td>
</tr>
<tr>
<td><strong>Prometamorphic Tadpoles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4)</td>
<td>.96 + .11</td>
<td>.91 + .09</td>
</tr>
<tr>
<td>Bullfrog Insulin (4)</td>
<td>1.19 + .13</td>
<td>1.23 + .16</td>
</tr>
<tr>
<td>Porcine Insulin (4)</td>
<td>1.09 + .09</td>
<td>1.11 + .15</td>
</tr>
</tbody>
</table>

a"Student's t-test was used for statistics.
b"None of the above values was significant at the .05 level.
c"Numbers in parentheses are numbers of replications.
Mean difference in protein secretion between treated and untreated bullfrog tadpole hepatocytes for three developmental stages. For each pair of bars, the left bar is bullfrog insulin and the right is porcine insulin. Porcine and bullfrog insulin increased protein secretion in all three developmental stages of tadpoles ($p < .001$) over untreated controls. The increase in total protein secretion during late prometamorphosis was significantly higher ($p < .05$) than the two previous developmental stages. No significant difference in protein secretion was seen at any stage between the two insulins. Number of replications: premetamorphic-7, early prometamorphic-9, late prometamorphic-3.
Effects of Insulins on Protein Secretion by Tadpole Hepatocytes

Stage of Development and treatment

Mean Difference ug prot./ug DNA
of protein secreted by tadpole liver cells was much less than the protein secreted by adult liver cells. For example, premetamorphic tadpole control hepatocytes secreted 6.5 ug protein/ug DNA/24 hrs, while adult control hepatocytes secreted 55 ug protein/ug DNA/24 hrs.
DISCUSSION

Stanchfield and Yager (1979) have shown that cultured bullfrog hepatocytes will respond to bovine insulin by increasing their secretion of total protein into the medium. They further determined that the major increase in any single protein was in albumin. We have shown that not only will porcine insulin stimulate total protein secretion, but that purified bullfrog insulin will cause an even larger increase in total protein secretion. The amount of response by the hepatocytes is directly related to the dose of insulin added to the culture medium and a bullfrog insulin concentration estimated to be within the physiological range of serum insulin concentration (350 pg/ml) significantly increases the total secreted protein over that secreted by untreated controls ($p < .01$). In addition to merely stimulating protein secretion, the hepatocytes showed a differential response to the two insulins. Bullfrog insulin was consistently more potent in eliciting a response from the hepatocytes. The ability to recognize and discriminate between the different insulins probably resides in the insulin receptor and its hormone binding site. Hulsebus and Farrar (submitted to Endocrinol, 1987) have shown that bullfrog insulin has a unique structure with a two amino acid extension on the amino terminus of the A chain. In mammals, this terminus is highly associated with the
biological activity of the molecule. Any manipulation of the A1 amino acid, such as, deletion of the amino acid or addition of a bulky group (Blundell et al., 1972) will significantly decrease the biological activity. It would appear that the bullfrog insulin receptor has compensated for this addition to its insulin, yet is still able to recognize and respond to porcine insulin that does not have the extension. The receptor fit may not be perfect for the porcine insulin since the overall biological response is less. The insulin stimulated increase in protein secretion does not appear to be dependent on an increase in cellular ATP. ATP levels remain constant in both the untreated and insulin treated cultures for 48 hrs (see also Stanchfield and Yager, 1979).

Tadpole cultures from all three developmental groups (premetamorphic, early prometamorphic and late prometamorphic) respond to both porcine and bullfrog insulin. The increase in total protein secretion by the tadpole hepatocytes was small (when compared to the adult response) but consistent. Tadpoles have low concentrations of albumin in their serum, and Stanchfield and Yager (1979) have shown that the major increase in adult hepatocyte protein secretion was due to an increase in secreted albumin. If insulin is only stimulating a slight increase in tadpole albumin secretion, this could account for the small
increases we observed. It is also possible that insulin could stimulate the secretion of small amounts of other proteins in the tadpole. For example, Nichol *et al.* (1986) have shown that insulin directly stimulates somatomedin production by cultured rat hepatocytes. It could be that insulin is stimulating tadpole hepatocytes to increase the production of somatomedin or other growth factors since the larva are rapidly growing. Just prior to metamorphosis, insulin could be stimulating the secretion of many plasma proteins that together contribute to the increased plasma protein concentration, and to the frogs improved tolerance of life on land. We did observe increased protein secretion, as well as increased insulin stimulation of secretion, in late prometamorphic tadpoles. All of these possibilities have yet to be explored.

None of the tadpole groups secreted more protein in response to bullfrog insulin than to porcine insulin at hormone concentrations of either 350 pg/ml (data not shown) or 3.5 ng/ml. The lack of differential response could be due to several possible mechanisms. First, the tadpole could have very few receptors. These receptors would be fully occupied by hormone and this occupation of the receptor would give a maximal response. Secondly, the post-receptor binding events could be incompletely developed or some enzymes could be totally lacking in the tadpole prior
to the major changes of metamorphosis. It would appear that the mechanism behind a differential hormone response has started to develop in the late prometamorphic animals. However, the apparent difference in stimulation by bullfrog and porcine insulin is not statistically significant. This could be due to the small number of animals in the late prometamorphic group.

The insulin stimulation of secretion is independent of cellular ATP concentration in tadpoles, as well as adults, since ATP concentration remains constant in the presence or absence of insulin. Tadpole hepatocyte ATP concentrations were about tenfold lower than those observed in adults. This could be related to the presence of relatively fewer numbers of mitochondria which are of a larval morphology in preclimax tadpoles (Smith-Gill and Carver, 1981). Not only are the tadpole mitochondria fewer in number, but they are also 7 times smaller in size than adult mitochondria and contain smaller amounts of the enzymes of the urea cycle and of oxidative phosphorylation, which increase during metamorphosis.

In summary (Figure 3), the ability of hepatocytes to respond to insulin is present in premetamorphic tadpoles, but the amount of total secreted protein is quite small until late prometamorphosis (just prior to climax). Tadpoles cannot differentiate between the heterologous
Figure 3. Composite graph of the mean differences in pro-secretion by bullfrog tadpole and adult hepatocytes treated with bullfrog and porcine insulin. For each pair of bars, the left bar is bullfrog insulin and the right is porcine.
Effects of Insulins on Protein Secretion by Bullfrog Hepatocytes

Stage of Development and Treatment

Mean Difference ug prot./ug DNA

- PRE
- E. PRO
- L. PRO
- AD-LO
- AD-HI
insulins by differential protein secretion. Adult hepatocytes respond to an insulin stimulus with a relatively large increase in protein secretion. Not only is the response much greater than the one seen in tadpoles, but the adult hepatocytes are able to differentiate between porcine and bullfrog insulin. Additional work needs to be done to determine: 1. if insulin stimulates mainly the secretion of specific proteins, such as albumin, or stimulates the secretion of most liver secreted proteins; 2. the mechanism of differential hormone response and how this mechanism changes with development.
REFERENCES


SECTION III

INCORPORATION OF $^{35}$S-METHIONINE AND $^{35}$S-CYSTEINE INTO SECRETED PROTEIN BY CULTURED BULLFROG TADPOLE AND ADULT HEPATOCYTES TREATED WITH BULLFROG AND PORCINE INSULIN

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Department of Zoology, Iowa State University
Ames, Iowa 50011
ABSTRACT

We have shown previously that bullfrog adult hepatocytes, both untreated and hormone treated, will secrete more protein than tadpole cells in culture. In addition, adult cells, but not tadpole cells, secreted more protein in response to bullfrog insulin than porcine. Stanchfield and Yager (1979) have shown that insulin will stop the preferential decline of albumin in cultured bullfrog adult hepatocytes. We tested the ability of cultured bullfrog hepatocytes to incorporate labeled amino acids into specific secreted proteins. \(^{35}\)S-methionine and \(^{35}\)S-cysteine were added to the medium of untreated and insulin (bullfrog and porcine) treated cultures and incubated for 12 hours. The medium was removed, dialyzed against water and then lyophilized. Samples were resolved by electrophoresis on SDS polyacrylamide linear gradient (12.5-20%) gels. Equal counts of radioactivity were loaded onto each well. The band patterns of secreted proteins from untreated and hormone treated cultures were detected by autoradiography and the autoradiograms were scanned using a laser densitometer. Insulin treatment did not preferentially induce specific proteins, it did induce a more generalized, equal increase in all the secreted proteins seen on the electrophoretic gels. The amount of albumin (expressed as percent of total) increased about 4
fold during late prometamorphosis, in both the control and insulin treated cultures. This change in albumin is a developmental change rather than an insulin induced change. Albumin (percent of total protein secreted) secretion by adults is only slightly higher than that of late prometamorphic tadpoles. The ability of insulin to specifically induce albumin synthesis and secretion from bullfrog hepatocytes may be masked by the long incubation time used in our experiments. Albumin could be induced rapidly, and this could be followed by a more generalized increase in all secreted proteins.
INTRODUCTION

Insulin is an important regulator of liver protein secretion in several vertebrate species. Hepatocytes from Rana catesbeiana tadpoles and adults increase their total protein secretion in response to both bullfrog and porcine insulin (Hulsebus and Farrar, Iowa State University, to be submitted to Gen. Comp. Endocrinol. 1987). Whether insulin causes preferential synthesis and secretion of specific proteins by bullfrog hepatocytes has not been completely determined. Stanchfield and Yager (1979) have shown that bovine insulin increases protein secretion from bullfrog hepatocytes over the secretion by untreated controls and prevents the preferential decline in albumin secretion seen over the 5 days of culture. Insulin may stimulate preferential secretion of albumin by other vertebrate cells. Insulin will stimulate the secretion of albumin, but not fibrinogen, from suspension cultures of rat hepatocytes (Crane and Miller, 1977), but has no effect on albumin secretion by perfused rat livers (John and Miller, 1969). Cultured chick embryo hepatocytes show a twofold increase in albumin synthesis when treated with insulin (Grieninger and Granick, 1975).

Insulin could be important in the stimulation of liver protein secretion during anuran metamorphosis. During larval development, albumin levels remain low in bullfrog
tadpoles but increase appreciably during late pro-
metamorphosis and in metamorphic climax (Frieden et al.,
1957; Ledford and Frieden, 1973). This increase in albumin
probably helps with osmoregulation during the adjustment to
terrestrial life (Just et al., 1977). Bullfrog tadpole
serum insulin-like immunoreactivity is highest during late
prometamorphosis when the increase in liver protein
secretion appears to begin (Hulsebus and Farrar, 1985).

Bullfrog insulin has a unique primary structure
(Hulsebus et al., Iowa State University, 1987, submitted to
Endocrinology). It has a two amino acid extension at the A1
position. This position, in mammal insulins, cannot be
added to without loss of biological activity. Bullfrog
insulin is more potent in stimulating protein secretion from
adult bullfrog hepatocytes. Whether this quantitative
difference in response is associated with qualitative
differences in the protein secreted in response to the two
hormones has not been determined. Our experiments compare
the differences in electrophoretic patterns of secreted
liver proteins between untreated and hormone (both bullfrog
and porcine insulin) treated hepatocyte cultures, and also,
the differences in the patterns between the adult and three
developmental stages of tadpoles.
MATERIALS AND METHODS

Adult bullfrogs were purchased from the Lemberger Company of Oshkosh, Wisconsin and were kept in large tanks with dripping water. Frogs were not fed and were used within one week of shipment. Bullfrog tadpoles were seined from a sandpit northeast of Ames, Iowa and were kept in large tanks of dechlorinated water. Tadpoles were fed a specially prepared lab diet of rabbit chow, gelatin, and agar (Hirschfeld et al., 1970). Developmental stages were determined based on the descriptions of Taylor and Kallros (1946). All animals were kept on a twelve hour light/dark cycle. Animals were anesthetized in tricaine methane sulphonate (1%-adults, 0.5% tadpoles) prior to use in experiments. Heparin (400 units/ml, adults-0.8 ml, tadpoles 0.1 ml) was injected into the ventricle of the heart prior to excision of the liver.

Excised bullfrog livers were cut into 2 mm cubes in 30% Amphibian calcium/magnesium free balanced salt solution (BSS) as prepared by Stanchfield and Yager (1978). All culture medium reagents were purchased from Sigma. The liver cubes were rinsed in 100 ml of BSS for 30 minutes with agitation, and then transferred to a spinner flask containing 20 ml of Waymouth's medium (as prepared by Stanchfield and Yager, 1978) and 10 ml of crude collagenase (Type V, Sigma). Tadpoles were divided into developmental groups:
premetamorphic (Taylor and Kollros stages I-XI), early pro-
metamorphic (Taylor and Kollros stages XII-XV), late pro-
metamorphic (Taylor and Kollros stages XV-XIX). Tissue from
several tadpole livers had to be pooled to obtain enough
cells for each experiment. Eight premetamorphic and 6 pro-
metamorphic livers were pooled. Livers were minced and
dispersed by collagenase in a diluted (60%) form of
Waymouth's medium. All tadpole hepatocyte testing and cell
dispersions were done in the 60% Waymouth's medium.
Hepatocytes were plated on poly-L-lysine (Sigma) coated
culture dishes (35 mm) at an approximate density of 6.0 x
10⁶ cells/cm². Cells were allowed to attach for 18 hours at
22-24°C in 2 ml of Waymouth's medium with no hormone added.

All culture experiments were designed as randomized
blocks. Cells from each experimental unit (1 spinner flask)
were divided into 3 groups, control (untreated), bullfrog
insulin treated, and porcine insulin treated. Each group
contained 2-3 culture dishes.

After attachment the culture medium was removed and
discarded. One ml of new medium, containing 10 u Ci/ml of
³⁵S methionine (specific activity = 1220 Ci/mmol) and ³⁵S-
cysteine (specific activity = 1210 Ci/mmol) purchased from
Amersham, was added to the culture dishes. Porcine insulin
(Sigma) or purified bullfrog insulin were added to the test
culture dishes. Adult bullfrog culture dishes received 350
pg/ml, while tadpole culture dishes received 3.5 ng/ml. Twelve hrs later the medium was removed and centrifuged at 2000 x g for 10 minutes to remove cellular debris. The medium was placed in a dialysis bag (molecular weight cutoff-3500) and dialyzed for 48 hrs with at least 3 changes of cold distilled water. The dialysate was placed in a 12 x 75 mm plastic culture tube, frozen at -70° C and then lyophilized.

The lyophilized samples were dissolved in electrophoresis sample buffer (59 mM Tris-PO₄, 10% glycerol, 2% SDS). Adult samples were dissolved in 150 ul of buffer and tadpoles samples in 75 ul. Aliquots were removed and diluted 1:1 with tris-HCl, pH-10.7, and analyzed for total radioactivity by thin-layer chromatography (TLA) according to a method by New England Nuclear and modified by Steinbeck and Roth (Iowa State University, submitted to The Journal of Immunology, 1987). Two and one-half microliters of diluted sample were spotted on 1 cm x 5 cm polysilic acid gel impregnated glass fiber TLC strips (ITLC-SA strips from Gelman) and allowed to dry. The strips were then placed in large scintillation vials containing 0.5 ml of chromatography solvent (30% methanol, 20% TCA, 10% acetic acid) for about 10 minutes or until the solvent front reached the top of the paper. Proteins remained precipitated at the origin and free amino acids moved to the
top of the strip with the solvent front. Strips were removed and the bottom 1.5 cm, containing the origin, was cut off and dried. The dried pieces were placed in the bottom of a scintillation vial and 5 ml of PPO-POPOP/toluene were added. The vials were counted in a Beckman scintillation counter for 5 minutes. Data were expressed as CPM/ul of electrophoresis buffer containing the protein sample.

SDS-PAGE electrophoresis was performed according to a modified Laemmlli procedure by Nilsen-Hamilton and Hamilton (1979). Slab gels (0.45 mm thick) with a linear gradient of 12.5% to 20% acrylamide/bisacrylamide and a 5% stacking gel were used. Equal amounts of radioactivity were loaded into each well of the gel. After staining/destaining, the gel was soaked in two changes of DMSO (30 minutes each), 20% PPO in DMSO (w/w) for three hours, cold water for 1 hour, and 25% methanol with 3% glycerol for 1 hour (Bonner and Laskey, 1974). The gel was dried onto filter paper. Radioactively-labeled proteins were located by autoradiography using Kodak X-Ray film (X-OMAT AR, GBX-2). Exposure time for the autoradiograms varied. High molecular weight protein bands were visible at 1 hour and low molecular weight bands were not visible before 8 hours.

Autoradiograms were scanned using a laser densitometer to determine the relative amount of labeled protein per
band. Each lane was scanned and the area under each peak determined. The percent of total secreted protein that was albumin was determined from these areas.
RESULTS

The amount of radioactivity per microliter of the samples in electrophoresis buffer is shown in Table 1. Early developmental staged tadpole hepatocytes have lower rates of synthesis and secretion of protein than adults, so the incorporation of labelled amino acids into protein is considerably lower. Late prometamorphic tadpole hepatocytes increase the amount of total secreted protein into the culture medium, and also the amount of labelled amino acids into protein over those seen in the two earlier stages.

Separation of the secreted proteins from adult bullfrog hepatocyte cultures are shown in Figure 1. Many of the newly-made proteins appeared in the upper portion (molecular weight above 66,000) of the gel. Relatively few proteins appeared in the low molecular weight portion of the gel. The protein band pattern remained essentially the same with or without hormone treatment. Frog albumin was identified by the following rationale: 1. It has a molecular weight of 68,000 and would run on the gels just above the 66,000 molecular weight standard; 2. Stanchfield and Yager (1979) immunoprecipitated albumin and the relative position of our band was the same; 3. The amount of protein in this band increased greatly in late prometamorphosis as would be expected of albumin. Based on the densitometer scans, albumin did not appear to specifically increase in response
Table 1. Incorporation of labelled amino acids into secreted protein by hepatocytes and albumin as a percent of total secreted protein. ($^{35}$S-methionine and $^{35}$S-cysteine)

<table>
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<th>Treatment</th>
<th>Adults CPM/ul ± S.D.</th>
<th>Albumin (%)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7768 ± 846 (6)^a</td>
<td>46 (3)^e</td>
</tr>
<tr>
<td>Bullfrog Insulin</td>
<td>11188 ± 2152 (6)</td>
<td>52 (4)</td>
</tr>
<tr>
<td>Porcine Insulin</td>
<td>8578 ± 492 (6)</td>
<td>50 (4)</td>
</tr>
<tr>
<td>Premetamorphic Tadpoles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1191 ± 98 (3)</td>
<td>11 (3)</td>
</tr>
<tr>
<td>Bullfrog Insulin</td>
<td>1343 ± 169 (3)</td>
<td>12 (3)</td>
</tr>
<tr>
<td>Porcine Insulin</td>
<td>1267 ± 126 (3)</td>
<td>12 (3)</td>
</tr>
<tr>
<td></td>
<td>Early Prometamorphic Tadpole</td>
<td>Late Prometamorphic Tadpoles</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Control</td>
<td>1288 ± 109 (3)</td>
<td>2337 ± 439 (3)</td>
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<td>Bullfrog Insulin</td>
<td>1725 ± 253 (3)</td>
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<td>1612 ± 172 (3)</td>
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"Numbers in parentheses is the number of replications.

"Numbers in parentheses is the number of lanes scanned by laser densitometer.

"Albumin percentages were obtained from densitometer scans of autoradiograms of polyacrylamide gels.
Figure 1. Incorporation of $^{35}$S-methionine and cysteine into secreted protein from bullfrog adult hepatocytes. Densitometer scans revealed no differences in the band pattern or the amount of labelled protein per band between untreated and hormone treated cultures. AD=adults, CT=control, BI=bullfrog insulin, PI=porcine insulin
to either insulin. Many of the newly made proteins appeared in the upper portion (molecular weight above 66,000) of the gel. Relatively few proteins appeared in the low molecular weight portion of the gel.

Premetamorphic tadpole cultured hepatocytes (Figure 2) secreted less protein than hepatocytes from adults. The number of proteins produced was similar to the adults but there were large differences in the concentration of protein in specific bands. Albumin, as well as the majority of the high molecular weight proteins, was produced in very small quantities in these animals. In addition, there was a low molecular weight protein band, of a molecular weight of approximately 8000, present in the tadpole medium that was absent in the adults. Insulin did not appear to stimulate or inhibit the secretion of any specific proteins.

Early prometamorphic tadpole culture medium (Figure 3) had essentially the same protein pattern as that from the premetamorphic tadpoles. As we have shown before, the amount of total secreted protein produced by these animals is about the same as those of the premetamorphic animals. The albumin band was very small and the additional band at approximately 8000 molecular weight was present. Insulin did not appear to stimulate or inhibit the secretion of specific proteins.
Figure 2. Incorporation of $^{35}$S-methionine and cysteine into secreted protein from bullfrog premetamorphic tadpole hepatocytes. Densitometer scans revealed no differences in the band pattern or the amount of labelled protein per band between untreated and hormone treated cultures. The amount of albumin (percent of total) is about 4 fold less than adults. A band of approximately 8000 MW is present in these tadpoles and not seen in adults. PRE=premetamorphic, CT=control, BI=bullfrog insulin, PI=porcine insulin.
PRE

<table>
<thead>
<tr>
<th>ct</th>
<th>bi</th>
<th>pl</th>
<th>ct</th>
<th>bi</th>
<th>pl</th>
</tr>
</thead>
</table>

Albumin

\[ 66,000 \]
\[ 45,000 \]
\[ 24,000 \]
\[ 8,000 \]
Figure 3. Incorporation of $^{35}$S-methionine and cysteine into secreted protein by bullfrog early and late prometamorphic tadpoles. Densitometer scans revealed no differences in the band pattern of the amount of labelled protein per band between untreated and hormone treated cultures. The amount of albumin (percent of total) increases about 4 fold between early and late prometamorphosis (12% to 40%). The amount of albumin (40%) in late prometamorphic tadpoles is similar to that seen in adults (48%). The band of approximately 8000 MW is still present in both early and late prometamorphic tadpoles. E.PRO=early prometamorphic, L.PRO=late prometamorphosis, CT=control, Bi=bullfrog insulin, PI=porcine insulin
The late prometamorphic tadpole hepatocytes showed a large increase in the amount of secreted albumin (Figure 3). Albumin secretion increased about 4 fold over that secreted by early prometamorphic animals and the percent of total protein that was albumin (40%) is nearly the same as the adult percentage (48%). The 8000 molecular weight band was still present. Insulin treatment caused no changes in the protein band pattern.
DISCUSSION

While, insulin treatment of bullfrog hepatocyte cultures did not induce preferential secretion of specific proteins, it did induce a more generalized, equal increase of all the secreted proteins seen on the electrophoretic gels. As we have shown previously, treatment of bullfrog adult and tadpole hepatocyte cultures with insulin (bullfrog and porcine) will cause an increase in the total amount of protein secreted in twelve hours. Stanchfield and Yager (1979) have shown that bullfrog hepatocytes treated with bovine insulin will cause total protein secretion to remain at higher levels than untreated controls throughout five days of culture. Albumin secretion was more insulin dependent than other proteins and preferentially declines without insulin. Several culture systems, using chick embryo hepatocytes and suspensions of rat hepatocytes, have shown specific increases in albumin secretion with insulin treatment (Grieninger and Granick, 1975; Crane and Miller, 1977). Peavy et al. (1978) treated rats with alloxan to induce diabetes. Production of albumin fell drastically and injections of insulin returned the blood albumin concentration to normal.

The failure of our bullfrog cultures to show an insulin-stimulated specific increase in albumin synthesis and secretion may be due to the physiological differences
between vertebrate liver cells. Insulin may not have this action in these animals. On the other hand, our experimental design would not have detected early, rapid insulin effects on albumin synthesis and secretion. If the action of insulin on protein synthesis and secretion were biphasic, albumin secretion could have been preferentially stimulated initially and then have been followed by a more generalized stimulation of secretion of other proteins. This type of biphasic response would be masked due to our long incubation time of 12 hours. Such a biphasic response has been observed in insulin treated chick embryo hepatocytes. Liang and Grieninger (1981) saw a rapid, but reversible, rise in albumin secretion in response to insulin treatment in their chick embryo hepatocyte cultures. The immediate response was not susceptible to treatment with actinomycin D, suggesting that insulin's immediate action was post transcriptional. However, over time actinomycin D could inhibit the action of insulin on albumin synthesis and secretion, suggesting that insulin could also initiate a transcriptional event. It is possible that the bullfrog liver cell response to insulin is also biphasic, but that under the conditions of our experiments could not be seen.

Developmental changes in hepatocyte protein secretion were primarily quantitative ones. The amount of protein secreted increased during late prometamorphosis and during
metamorphic climax when insulin stimulation of protein secretion increased as well (Hulsebus and Farrar, Iowa State University, to be submitted to Gen. Comp. Endocrinol., 1987). However insulin stimulation, under our experimental conditions, was of a general nature, influencing all proteins equally. One major secreted protein, of approximately 8000 MW, was present in tadpole culture medium but not adults. The identity of this protein has yet to be determined.

While insulin is clearly involved in the regulation of the amount of protein secreted by bullfrog liver cells, the direct regulatory action of insulin on protein secretion is still unknown. More work will be necessary to explain its action on protein synthesis in general and its possible specific action on the regulation of albumin synthesis and secretion.
REFERENCES


SUMMARY–DISCUSSION

Insulin is one of the most important metabolic hormones in the mammalian body. Although it has many varied functions in mammals, very little is known about the structure and function of insulin in lower vertebrates. We have shown (Hulsebus and Farrar, 1985) that bullfrog tadpoles and adults have an insulin-like immunoreactivity in both their serum and pancreas extracts. These animals have blood insulin concentrations that are similar to those found in humans but have blood sugars that are about 6 times lower. It would seem that with concentrations of this magnitude the hormone must have an active function or functions. Not only are the functions of insulin undetermined but, to date, no one has purified insulin from an amphibian.

One of the major aspects of this research is the purification and determination of the amino acid sequence of bullfrog insulin. Purified bullfrog insulin has both unique and conserved characteristics. As determined by the amino acid sequence, bullfrog insulin has all of the conserved areas that are important for the 3-dimensional structure of the molecule. The zinc binding site, β10 histidine, is present and bullfrog insulin may bind zinc. The appropriate nonpolar surface sequences necessary for dimerization and hexamerization are also conserved.
An important part of the antibody binding site of the insulin molecule are the amino acids A8-A10. When comparing all the known sequences of insulins, a high degree of variability is seen here. This can account for some of the differences in binding seen between insulin and a heterologous antibody. Bullfrog insulin is homologous in this area (A8-A10) with chicken, turkey, and alligator insulin. It could be possible for bullfrog insulin to bind with higher affinity to antibodies for these animals than with the porcine anti-insulin antibody we have previously used in the heterologous radioimmunoassay (Hulsebus and Farrar, 1985). It would be of great practical and financial importance if the chicken antibodies and hormones could be used for determining quantities of bullfrog insulin in a radioimmunoassay rather than bullfrog insulin. Bullfrog insulin, although easy to purify, is not produced in great quantity by the pancreas and less than 700 μg of pure insulin is obtained from 200 bullfrogs. Chicken insulin and anti-insulin antibodies can be obtained in large quantities more easily than bullfrog.

One of the first differences we discovered in the bullfrog insulin structure was that the number of amino acids is 52 instead of the more common number 51. The only other known insulin with 52 amino acids is hagfish. After sequencing, a very unique addition to the A chain was seen.
The amino terminal of the A chain has a two amino acid (LYS-PRO) extension. This is the only known insulin to date whose sequence has an addition at this location. Blundell et al. (1972) have shown that if the A1 glycine is deleted in mammalian insulin or if a bulky group is added, the biological activity of the molecule is greatly reduced. The reduced activity of proinsulin is thought to be at least partially due to blocking of this terminus by the C-peptide. This extension on the bullfrog insulin raises two questions. What is the effect of the extension on the biological activity, and how does it affect receptor binding? Comparisons of the receptor binding kinetics and the resulting bioactivity between insulins could help in resolving these questions. The experiments in this dissertation begin to investigate these questions by comparing the bioactivity of bullfrog and porcine insulin in a bullfrog hepatocyte culture system. We found that bullfrog insulin is more potent than porcine insulin in stimulating total protein secretion from cultured bullfrog hepatocytes. This suggests that a modification has taken place in the bullfrog insulin receptor to accommodate the amino acid extension on the bullfrog insulin. More studies, such as comparative receptor binding assays, would need to be done to strengthen this statement.
The mechanisms of action of bullfrog insulin in the hepatocyte in stimulating protein secretion are not completely known and need more investigation. We have not seen preferential secretion of any specific proteins, but have seen a generalized increase in all protein synthesis and secretion by bullfrog adult and tadpole hepatocytes. However, we can not rule out the possibility that insulin could cause a rapid increase in the synthesis and secretion of one or more specific proteins which was masked by the long labelling period. Short term labelling experiments need to be performed to see if there could be a biphasic response to insulin in the bullfrog hepatocytes as has been observed in chick embryo hepatocytes.

Insulin could have important functions during larval development and metamorphosis. The serum and pancreatic concentrations of insulin are increasing throughout larval life until just prior to metamorphic climax, but the hormone's function is not known. Blood glucose concentrations are low, and when mammalian insulin injections are given, the response is transient and slow. It appears that insulin does not regulate glucose acutely. Serum protein concentration, especially albumin, is low in premetamorphic and early prometamorphic tadpoles, but we have shown in this research that insulin can stimulate a small increase in protein secretion by the cultured
hepatocytes. During late prometamorphosis the pancreas starts to be degraded by proteolytic enzymes, yet insulin is still detectable and the hepatocytes become more responsive to insulin and secrete more protein than during any other tadpole developmental stage. It is interesting that even as the absolute amount of insulin is dropping, the hepatocyte responsiveness, as measured by protein secretion, is increased. Functionally insulin could be very important at this time in stimulating liver protein secretion. These proteins appear in the blood and are important in raising its osmotic concentration, an adaptation to life on land. In addition, insulin, during larval development, could be active in promoting glycogen storage. Storage of glycogen could be important for survival of the animal during metamorphic climax when it is not feeding. Whether bullfrog insulin is active in this process has yet to be determined.

Endocrine action during anuran metamorphosis is complex. Prolactin (PRL) and thyroid hormones (T_3 and T_4) are active in promoting total body growth and developmental changes toward the adult state. How insulin interacts with one or more of these in stimulating protein secretion by hepatocytes should be a promising area of investigation in the future.


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