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Electrophysiologic studies of hypothalamic adenosine-5'-triphosphate-sensitive potassium channels

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ABSTRACT

ELECTROPHYSIOLOGIC STUDIES OF HYPOTHALAMIC ADENOSINE-5'-TRIPHOSPHATE SENSITIVE POTASSIUM CHANNELS

by
Dinora Hernandez

Of particular interest in this study were the electrical phenomena involving currents from the ATP sensitive K channel in isolated hypothalamic ventromedial nucleus (VMN) neurons. These neurons may play an important role in the glucose-sensing system of the body. Rat VMN neurons were isolated. For neuronal isolation, the proteolytic enzyme pronase was used. However, pronase was too harsh. The use of papain, another proteolytic enzyme, led to better neuronal morphology. The perforated patch clamp technique was used to gain access to the cell's interior. For membrane pore formation, nystatin was used; but seal formation was difficult. Amphotericin was used instead, and this milder antibiotic worked better at seal formation. Lastly, Na/K currents obtained from isolated neurons were amplified, recorded, and analyzed.

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by
Dinora Hernandez

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ELECTROPHYSIOLOGIC STUDIES OF HYPOTHALAMIC ADENOSINE-5'-TRIPHOSPHATE-SENSITIVE POTASSIUM CHANNELS

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This thesis is dedicated to all the exceptional people who have unselfishly shared their knowledge and contributed to my learning and understanding. They include all the outstanding individuals that I encountered both at NJIT and at UMDNJ; my mother Martha Hernandez for her strength; and Barbaro J. Perez, for all his love.

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CHAPTER 1

INTRODUCTION

1.1 Cellular Physiology

The cellular membrane of a neuron forms a selective barrier to charged particles. Ion-specific channels allow the flow of current through the membrane in response to changes in membrane potential (E_m). Membrane potentials arise as a result of the concentration gradients of charged ions across the membrane. For instance, when a membrane becomes permeable to one ion species, the ions will diffuse to the area of least concentration and thus alter the charge separation across the membrane. Therefore, the membrane potential depends on the concentration gradient of the permeable ion and the permeability of the membrane to the ion. This relationship is quantitatively shown in the Goldman equation, which applies when E_M is not changing [Kandel et al., 1991]:

$$E_M = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o} \qquad (1)$$

where,

R = the gas constant $\left(8.314 \frac{Volt \cdot Couloumb}{^\circ K \cdot mol}\right)$

T = the absolute temperature ($^\circ K$)

F = Faraday's constant $\left(9.648 \cdot 10^4 \frac{Couloumb}{mol}\right)$

$[Na]_o$, $[K]_o$, $[Cl]_o$ = the extracellular concentration

$[Na]_i$, $[K]_i$, $[Cl]_i$ = the intracellular concentration

P = the permeability for each particular ion

As observed from this equation, the greater the concentration of a particular ion species and the greater its membrane permeability, the greater is its role in determining the membrane potential. The chemical force of diffusion is not the only force acting on the ions. There are more positive ions outside the cell relative to the inside. By convention, membrane voltage is measured with respect to the extracellular space; the inside of the cell is at a negative potential relative to the outside. If positively charged potassium ions diffuse out of the cell, they would meet other positively charged ions (Na ions) outside. These like charges repel each other. Therefore, the electrical potential required to balance the diffusion potential against the concentration gradient is referred to as the Nernst potential. The Nernst potential for an ion, E_{ion}, is [Hille, 1992]:

$$E_{ion} = \frac{RT}{z_{ion}F} \ln \frac{[C]_o}{[C]_i} \tag{2}$$

where z_{ion} = ionization index of the ion (z_{ion} = +1 for Na^+, K^+, -1 for Cl^-).

Thus, when the permeability to one particular ion is high, the Goldman equation reduces to the Nernst equation for that particular ion. For instance, since the permeability to K is greater than Na and Cl ($P_K >> P_{Cl}, P_{Na}$) at rest, as in neurons, the equation becomes (K_i=150mM and K_o=5mM):

$$E_K \approx \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i} = -90\text{mV} \tag{3}$$

Similarly, at the peak of the action potential, the membrane is much more permeable to Na^+ than to any other ion; thus, E_M approaches E_{Na}, the Nernst potential for Na (Na_o=150mM, Na_i=15mM):

$$E_{Na} \approx \frac{RT}{F} \ln \frac{[Na^+]_o}{[Na^+]_i} = +65\text{mV} \tag{4}$$

The Nernst Equation can be used to find the equilibrium potential. As observed from the Nernst Equation, the relative concentration of a particular ion inside and outside the cell will determine the polarity of the membrane. Thus, the membrane is said to be "depolarized" when the potential inside the cell increases, it becomes less negative. In excitable cells (neurons, muscle cells, etc.) depolarization opens voltage-sensitive channels to increase sodium permeability. This depolarization allows positive sodium ions to flow into the cell and cause an action potential. Conversely, the membrane is said to be "hyperpolarized" when the potential inside becomes more negative.

1.2 Potassium Channels

Potassium also contributes to the characteristic changes in the membrane potential during an action potential. Potassium channels open more slowly than the sodium channels. Opening potassium channels will draw the membrane potential closer to the potassium equilibrium potential [Hille, 1992]. Because the concentration of K^+ ions is greater inside the cell, the cell's interior is at a negative potential to balance the force of diffusion that drives K^+ ions out of the cell. Depolarization of the membrane potential is caused by a decrease in the intracellular K^+; the Nernst potential of the K^+ ion becomes less negative. Similarly, hyperpolarization of the membrane potential is caused by an increase in intracellular K^+ ions or an increase in K permeability.

There is a great variety of potassium channels found in cells. In general, open potassium channels stabilize the membrane potential of excitable cells, by hyperpolarizing the resting potential and keeping action potentials short [Hille, 1992]. Most potassium channels, such as the "delayed rectifiers", have voltage dependent gates that open after a delay in response to membrane depolarization. Other potassium channels, such as the "inward rectifiers" (K_{ir}), open and allow K^+ ions to enter only under hyperpolarization but not to exit under depolarization [Hille, 1992]. In this manner, allowing K^+ ions to move into the cell, K_{ir} channels prevent the membrane from becoming too hyperpolarized. In so doing, K_{ir} channels keep the membrane potential clamped near the potassium equilibrium potential. K_{ir} channels increase the refractory time and, therefore, decrease the number of action potentials that take place per unit time. Of particular interest in this study is a subtype of potassium channels known as the inward rectifier adenosine triphosphate (ATP) sensitive potassium (K_{ATP}) channel.

The K_{ATP} channels link bioenergetic metabolism to membrane excitability [Lazdunski, 1994]. These K_{ATP} channels are tissue specific and have different physiologic functions. They influence the excitability of mammalian heart [Noma, 1983, Kakei et al. 1984], skeletal muscle [Spruce et al., 1985], and neurons [Ashford et al. 1988]. The physiologic function of K_{ATP} channels has been well studied but is far from totally understood, in pancreatic beta cells [Cook and Hales, 1984, Rorsman and Trube, 1985].

1.3 K_{ATP} Channels in Pancreatic Beta-Cells

There are K_{ATP} channels on the β-cell membrane, which are responsible for the detection of plasma glucose concentration [Ashcroft, 1988]. Under normal conditions in β-cells glucose concentration is low and K_{ATP} channels are open and contribute to the resting membrane potential. Beta-cells respond to small variations of external glucose that change the intracellular ATP/ADP ratio [Lazdunski, 1994]. The pancreas does not need

insulin for the uptake of glucose. In this manner, the β-cell metabolism acts as an extracellular glucose sensor [Ashcroft et al., 1994].

When a meal is consumed, plasma glucose levels rise. The pancreatic β-cells immediately detect extracellular changes in plasma glucose levels and initiate glucose uptake. (See Figure 1.) This glucose uptake is facilitated by an insulin independent glucose transport GLUT-2 [Cotran et al., 1994]. The β-cell increases its metabolism of

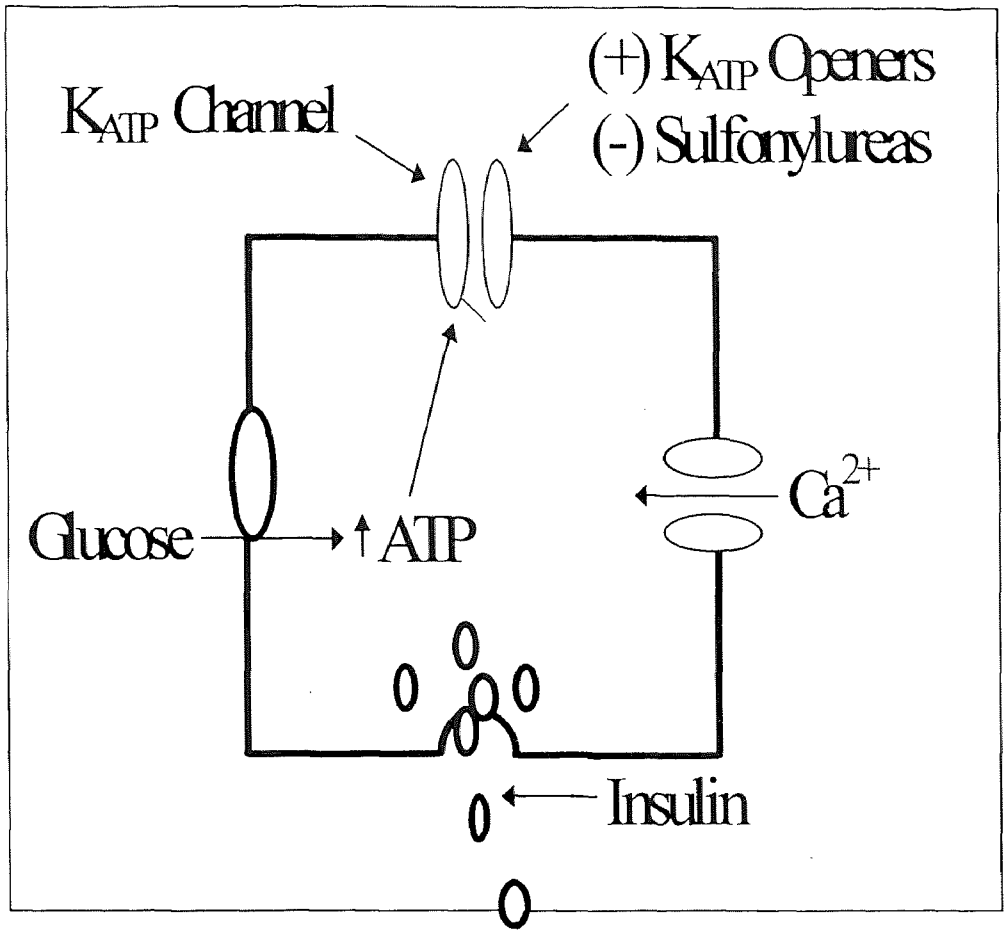

Figure 1 Schematic of the detection of plasma glucose levels by the pancreatic β-cell K_{ATP} channel. The rise of ATP, from glucose metabolism, causes the closing of K_{ATP} channels. This closing causes depolarization of the membrane causing the activation of voltage-dependent Ca^{2+} channels. This process initiates the exocytosis of secretory vesicles containing insulin. [Adapted from Katzung, 1995.]

glucose and thus increase the intracellular levels of ATP. High intracellular ATP levels cause the K_{ATP} channels to close. This closure causes K^+ ions to build up inside the cell and depolarizes the membrane. This depolarization in the cell activates the opening of voltage-dependent Ca^{2+} channels. The resulting Ca^{2+} influx causes an increase in intracellular Ca^{2+} concentration; and this process, in turn, initiates the exocytosis of secretory vesicles containing insulin. Conversely, periods of low glucose levels cause K_{ATP} channels to open and hyperpolarize the β-cell. This hyperpolarization stops the release of insulin.

The main function of insulin is to increase the rate of glucose transport into certain cells of the body. For instance, when insulin is released, muscle cells and adipose tissue are able to utilize glucose. However, the pancreas as well as the brain does not need insulin to take up glucose. There is a certain type of diabetes, in which there is a malfunction of glucose regulation as will be succinctly explained.

1.4 K_{ATP} Channels and Diabetes

Diabetes mellitus is impaired glucose utilization, which leads to various metabolic disorders. In the United States, there are about 10 million people with diabetes mellitus [Hadley, 1996]. In fact, diabetes mellitus ranks among the top ten causes of death in the Western nations. Diabetes is divided into two classes: primary diabetes mellitus, and secondary diabetes mellitus. Secondary diabetes is a rare type of diabetes; it includes hyperglycemia due to destruction of pancreatic islets affected by inflammatory pancreatic disease, surgery, tumors, certain drugs, iron overload, and certain acquired or genetic endocrine pathologies [Cotran et al., 1994].

Primary diabetes mellitus, is divided into two classifications. Insulin-dependent diabetes mellitus (IDDM), or type I diabetes, also known as juvenile-onset diabetes, accounts for about 20% of all cases of diabetes. Non-insulin-dependent diabetes mellitus

(NIDDM), or type II diabetes is also known as adult-onset diabetes and accounts for about 80% of the cases. Type II diabetes is further divided into obese, non-obese types and a third uncommon form, known as maturity-onset diabetes of the young [Cotran et al., 1994]. Type II diabetes is distinguished by both genetic and environmental factors that express themselves most often in obesity. That is, this disease is closely associated with diet [Mazze, 1995]. In fact, more than 85% of those with type II diabetes will be obese at the time of detection [Cotran et al., 1994]. In obesity there is decreased insulin sensitivity of tissues. Insulin resistance results from chronically elevated glucose levels seen in the obese individual. Hence, obese individuals may exhibit type II diabetes due to increased insulin resistance. However, the exact pathogenic mechanism of type II diabetes in relation to obesity is still not known.

Unlike Type I diabetics, who lack insulin, Type II diabetics are able to produce insulin. However, the insulin levels produced are less than that required to maintain glucose homeostasis [Cotran et al., 1994]. Type II diabetics are treated with sulfonylurea drugs such as tolbutamide. These drugs block K_{ATP} channels. That is, these agents mimic the actions of high intracellular levels of ATP and cause β-cell depolarization. Ultimately, insulin is secreted and blood sugar is lowered.

1.5 K_{ATP} Channels in the Nervous System

As mentioned before, in addition to the pancreas, there are K_{ATP} channels in other tissues. Studies from various types of central nervous system (CNS) neurons have shown the presence of K_{ATP} channels [Ashford et al., 1988]. It is speculated that the location of these K_{ATP} channels link cell excitability and metabolic state of the neuron. In addition, the brain's K_{ATP} channels might behave in a similar manner as the pancreatic β-cell K_{ATP} channels. The brain has a particular interest in sensing glucose, since it is its primary energy source [Skoloff, 1977]. There are neurons which directly sense and alter their firing rates as glucose availability changes [Oomura, 1974]. A preliminary hypothesis

developed by Mayer (1955) linked the brain with glucose sensing. This "glucostatic hypothesis" proposed that the brain senses changes in blood glucose levels, and any drop in plasma glucose triggers meal initiation. Let us discuss the role the brain plays in energy balance [Levin and Routh, 1996] in relation to glucose sensing.

1.6 Hypothalamic K_{ATP} Channels

Historically, the hypothalamus has been considered to play a key role in appetite regulation. In 1940, Hetherington and Ranson observed that lesions to the VMN led to excessive eating and obesity in rats. Further, experiments done by Anand and Brobeck, showed that stimulating the VMN caused hungry rats and cats to stop eating. Consequently, the VMN (see figure 2a & b) was regarded to play a principal role in satiety control. However, the VMN, in addition to playing a role in food intake regulation, has other functions. Other hypothalamic areas play important roles in energy regulation as well. For instance the paraventricular nucleus has been found to be important in feeding regulation [Aravich and Sclafani, 1983].

The VMN plays an important role in autonomic nervous system regulation. For example, the VMN has been found to be involved in stimulation of the sympathetic and inhibition of the parasympathetic systems [Steffens et al., 1988]. Interestingly, over eating and lower sympathetic activity have been shown in obese Zucker rats, a genetically obese animal model [Holt and York, 1989]. The VMN has also been found to be involved in the regulation of blood glucose and insulin levels. This regulation appears to be, mediated by catecholamines, such as norepinephrine (NE) [Steffens et al., 1988]. For example, injection of NE into the VMN leads to an increase of blood glucose [Chafetz et al.,1986, Steffens et al., 1981].

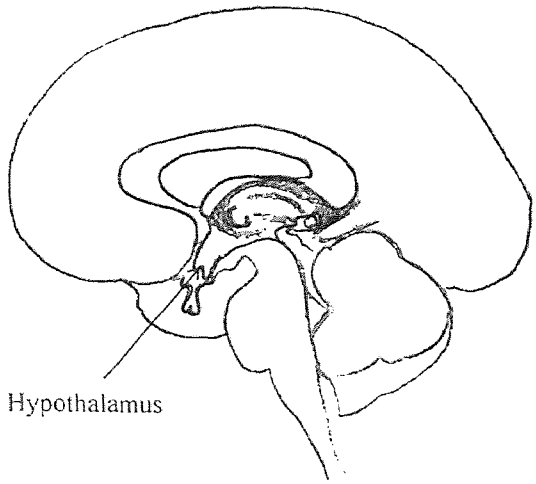

Figure 2a Sagittal schematic of the human brain, showing location of the hypothalamus

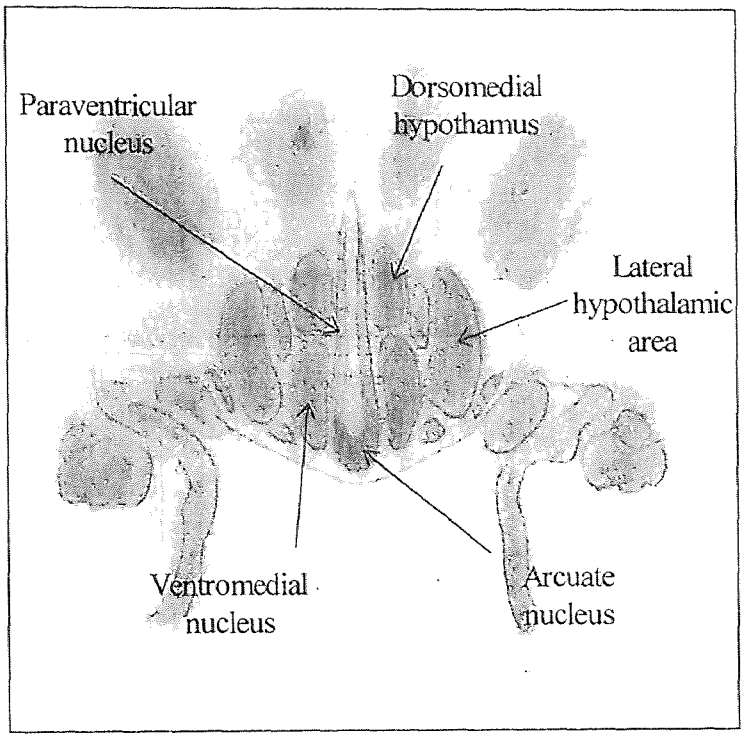

Figure 2b Hypothalamic frontal view sketch [Adapted from Kandel et al., 1991].

Ventromedial [Ono et. al., 1982] and lateral [Oomura et. al., 1974] nuclei of the hypothalamus respond to changes in circulating glucose concentration. Further, studies by Levin and Dunn-Meynel, 1995, show that intracarotid glucose infusion activate neurons in the paraventricular and ventromedial hypothalamic nuclei. This activation leads to the activation of the sympathetic nervous system [Levin, 1991]. Thus, VMN neurons are able to sense the extracellular glucose concentration [Ashford, 1990] in a manner similar to the pancreatic β-cell. It has been shown that glucose can depolarize these neurons through the closure of K_{ATP} channels [Ashford et al., 1990]. Thus, it is thought that K_{ATP} channels are inhibited as intracellular ATP concentrations in the VMH neurons rise in response to an increase in blood glucose [Ashford et. al., 1990].

CHAPTER 2

METHODS AND RESULTS

2.1 Introduction

Of interest in these studies were the electrical phenomena involving currents from the K_{ATP} channel in isolated hypothalamic ventromedial nucleus (VMN) neurons. These neurons are related to the glucose-sensing system of the body. The objective of this project was to gain experience utilizing electrophysiological techniques. Electrophysiology is a basic field of study that involves recording electric currents originating in living cells, analyzing, and explaining those currents. The environment had to be adequate to maintain the preparation healthy. Experience was gained in utilizing the patch clamp technique. The patch clamp technique required stable positioning of an electrode to provide a means to observe actual potential changes within the cell. Finally, the signals were amplified, recorded, and analyzed.

2.2 Animal Model

The rat is the animal model used in these studies of brain properties because of the great body of data available in the literature. For instance, there is a great number of relevant models, such as the diabetes induced model [Bonner-Weir et al., 1981, Rivero et al., 1991] and the genetically obese Zucker rat (fa/fa) model [Routh et al., 1990]. In addition, the reasonable size of rat brain allows the careful dissection of the VMN. The age of the rats used in these studies ranged in a narrow window of 11-18 days. During this interval, the brain was in a relatively undifferentiated state. However, since changes in obesity occur as early as 2 days of age [Moore et al., 1985] as well as in utero it can be said that the defect is present early on.

2.3 Cell Isolation

To minimize the animal's stress, newborn Sprague-Dawley rats (11-18 days old) were decapitated as quickly as possible using a small guillotine. The decapitation area and all instruments used were thoroughly cleaned, since rats have a keen sense of smell and become distressed at the smell of blood. The intact brain was immediately peeled out of the relatively fragile skull. The brain is very soft in nature, therefore, upon removal it was immersed in ice-cold oxygenated artificial cerebrospinal fluid to chill for a few minutes. The first priority was to keep the cells healthy, thus, the isolation procedure mimicked as close as possible, physiologic conditions. The artificial cerebrospinal fluid (ACSF) contained (in mM): 128 NaCl, 5 KCl, 1.24 NaH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 10 D-glucose. The pH was adjusted to 7.4 using HCl. All solutions were made with glass-distilled water. The ACSF had oxygen (95% O_2/5% CO_2) constantly bubbling through it, to maintain oxygenation of the brain. The chilled brain, was cut into a block containing the hypothalamic area, mounted on the cutting carriage of a vibratome (Campden Instruments LTD) with cyanoacrylate glue and bathed in oxygenated ACSF. About 4-6 coronal slices (400 μm thick) of the hypothalamus were placed in a beaker. The slices were maintained at room temperature in oxygenated ACSF for about 30 minutes before exposure to enzyme.

Each slice was digested with enzymes in a two step process. Initial time studies were performed to determine the optimal duration of enzymatic digestion, which varied depending on the litter and the size of the pup. If the isolated neurons contained more than one or two projections, then the time was increased. If no projections were seen then the enzyme concentration was decreased until satisfactory neurons were obtained. A viable neuron is characterized by a circular, translucent (bright), smooth surface of the cell body bearing preferably one or two axonal processes.

The following protocol has been used to isolate cells for patch clamp studies [Ye and Akaike, 1993, Ye and McArdle, 1995]. Each slice was incubated in pronase

(Calbiochem, San Diego, CA), 1mg/6ml in oxygenated ACSF at 32°C for 20 minutes. The slice was subsequently maintained in thermolysine (Calbiochem) in 1mg/6ml oxygenated ACSF for 20 minutes. This enzymatic protocol proved too harsh to the isolated neurons. In addition, experiments involving the K_{ATP} channel require that the neurons be maintained for longer periods of time. The isolation procedure is crucial, so a new enzymatic digestion protocol was implemented.

Studies done by Proks and Ashcroft, 1993, showed that the action of papain was faster and less destructive to the membrane. In support of this, Huetter et al., obtained the best structural conservation of dissociated cortical neurons using papain. Their preliminary studies showed that papain was superior for dissociating postnatal tissue compared to other enzymes [Huettner and Baughman, 1986]. Therefore, 5 mg of papain (Worthington), and 25 mg of L-Cysteine (Sigma) were weighed out in 5 ml ACSF and left at room temperature for about 30 minutes to ensure full enzyme activation. Each slice was incubated in this oxygenated papain containing solution at 35°C for 20 minutes. They were subsequently maintained for 30 minutes in oxygenated papain free ACSF containing 1 mg/ml ovomucoid trypsin inhibitor (Worthington), and 1 mg/ml Albumin, Bovine Fraction V (Sigma).

The slices recovered for 30 minutes in enzyme free oxygenated ACSF. A punch of the hypothalamic VMN (see Figure 3) was taken using a 500μm blunt needle. The punch was placed in a small (35mm x 10mm) Falcon 3001 culture dish in extracellular solution. This filtered bath solution contained (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 Hepes, pH 7.4 with 1N NaOH. Punches were subjected to gentle trituration using flame-polished Pasteur pipettes of decreasing internal diameter. The dispersed cells adhered to the bottom of the dish after 15 to 30 minutes. Neurons were observed under a microscope (Nikon Diaphot, Tokyo, Japan) equipped with phase contrast optics. The microscope was inverted, meaning the objective lens was underneath the dish, for top access of the electrode.

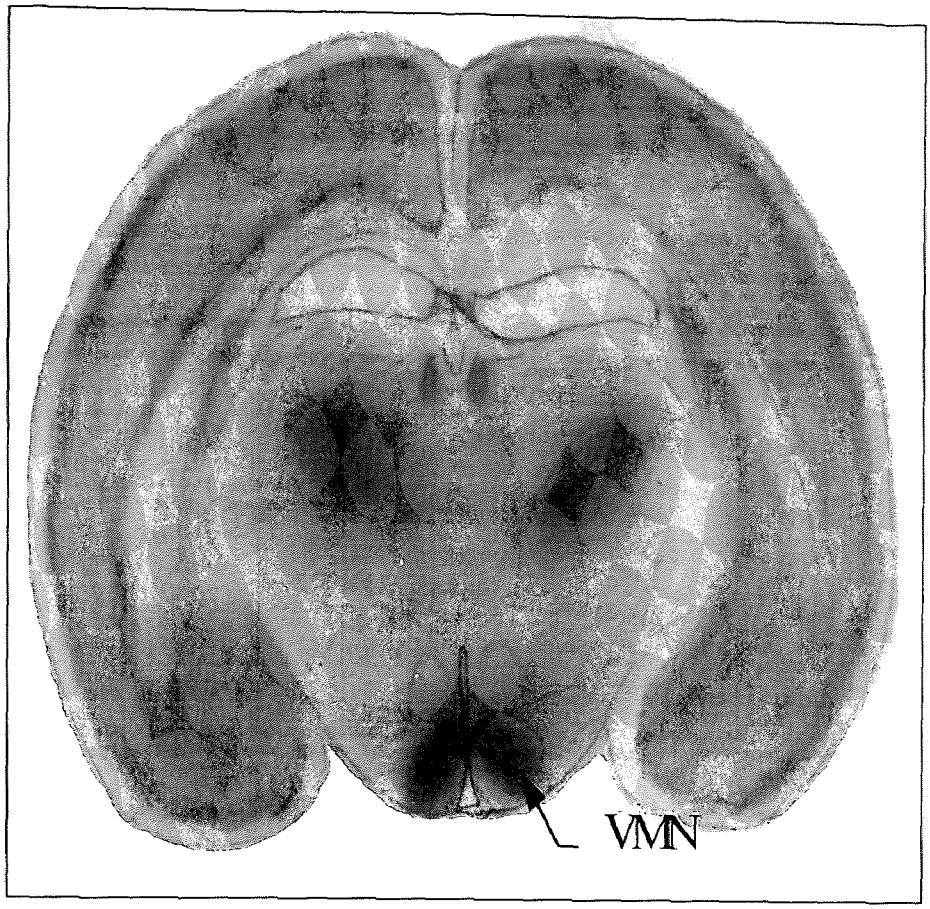

Figure 3 Coronal section sketch of a rat brain. The arrow indicates the location where the 500μm punch was taken [Adapted from Sherwood and Timiras, 1970].

2.4 Pipette Fabrication

Patch pipettes were made from 1.5-mm glass (World Precision Instruments, Inc.) capillaries. Two pipettes were obtained from each capillary after pulling on a two stage vertical electrode puller (David Kopf Instruments, Tujunga, CA,. Model 700C). This instrument contained nichrome heating coils. In the first pull, the capillary was centered with respect to the heating coil and enough current was applied to the coil to thin out the capillary at the middle. In the second pull the capillary was again centered with respect to the heating coil, and the thinned part became disconnected into two tapered ends. To

obtain pipettes of consistent properties, two blocks were used, with fixed pulling length for the two stages needed. Additionally, fixed heat settings were used, 22A, for the first pull. The second pull was done by trial and error until the desired pipette resistance was obtained. This heat setting turned out to be around 14.5A for pipette tip resistance between 3MΩ and 5 MΩ when filled with pipette solutions. These pipettes have opening diameters around 0.5 to 1 μm [Hamill et al., 1981]. The nichrome heating coils were replaced periodically to avoid inconsistencies in the pipette tip size.

Once the pipettes were pulled, they were fire-polished before use. Fire polishing is done to smooth the pipette tip for more secure seal formation. This step was observed under a microscope (MF-83, Narashige Scientific Instrument, Tokyo, Japan). Heat was supplied by a ∩ shaped platinum-iridium wire. This wire had a small (0.5 mm) glass ball at its vertex. The tip of the pipette was brought in proximity to the ball and current was applied to the wire for a few seconds. The fragile pipettes had to be kept as clean as possible. The pipettes were temporarily stored in a jar, which had a special cushion with indentations large enough to hold each pipette until needed.

2.5 Mechanical Setup

The pipette provides an electrical connection between the cytoplasm and a metal electrode that is connected to the amplifier, CV-3 headstage (Axon instruments). The metal electrode consisted of a silver/silver chloride (Ag/AgCl) interface, a silver wire coated with silver chloride. The preparation was grounded directly with a Ag/AgCl wire. The headstage was mounted on a three dimensional hydraulic micromanipulator (Narishige Co. LTD., Tokyo, Japan) used for highly precise manipulation. A coarse manipulator was used to rapidly position the solution filled pipette as close as possible to the cell. The micromanipulator was then used to accurately advance the pipette to contact the membrane of the cell.

An oscilloscope (Model 5103N, Tektronix Inc., Beaverton, Oregon), was used as a guide to know when the pipette was actually touching the cell. An oscilloscope displays the amplitude of voltage waveforms with respect to time coming from the amplifier. (See figure 4.) When the pipette touched the bath solution, a dominant signal was detected, corresponding to the positive voltage found in the bath. The electrode resistance was calculated using Ohm's law, $V = I \cdot R$. As the pipette approached the cell a change in voltage was detected, represented by an approximate 50% decline in amplitude of the signal. Once a patch was made, gentle suction was applied to form a seal and the amplitude on the oscilloscope drastically declined. At this point, the gain of the amplifier was increased to calculate the resistance of the seal. This step had to be performed very carefully, since the slightest movement during suction would excise a membrane patch from the cell.

The importance of having a mechanically stable set up is imperative to obtaining a successful seal. For this reason, the instruments were placed on an antivibration, air table (Micro-g Vibration Isolation System, Model 61-463, Technical Manufacturing Corp., Peabody, MA). This table consisted of a heavy slab resting on partially inflated pneumatic supports. Except for the microscope, all instruments were off the vibration isolation table. The manipulators could not drift or vibrate during recording. Thus, it was difficult to form a seal, for there was a drift in the pipette tip. The antivibration table was readjusted until every corner of the table top could easily move in every direction. However, the drift in the pipette tip persisted. It was then speculated that perhaps the micromanipulator was not working properly, primarily because the movements seen were in the micrometer range. Finally, it was found that the micromanipulator was working properly, thus, the drift originated in the macromanipulator. Once this was corrected a giga-ohm seal was formed.

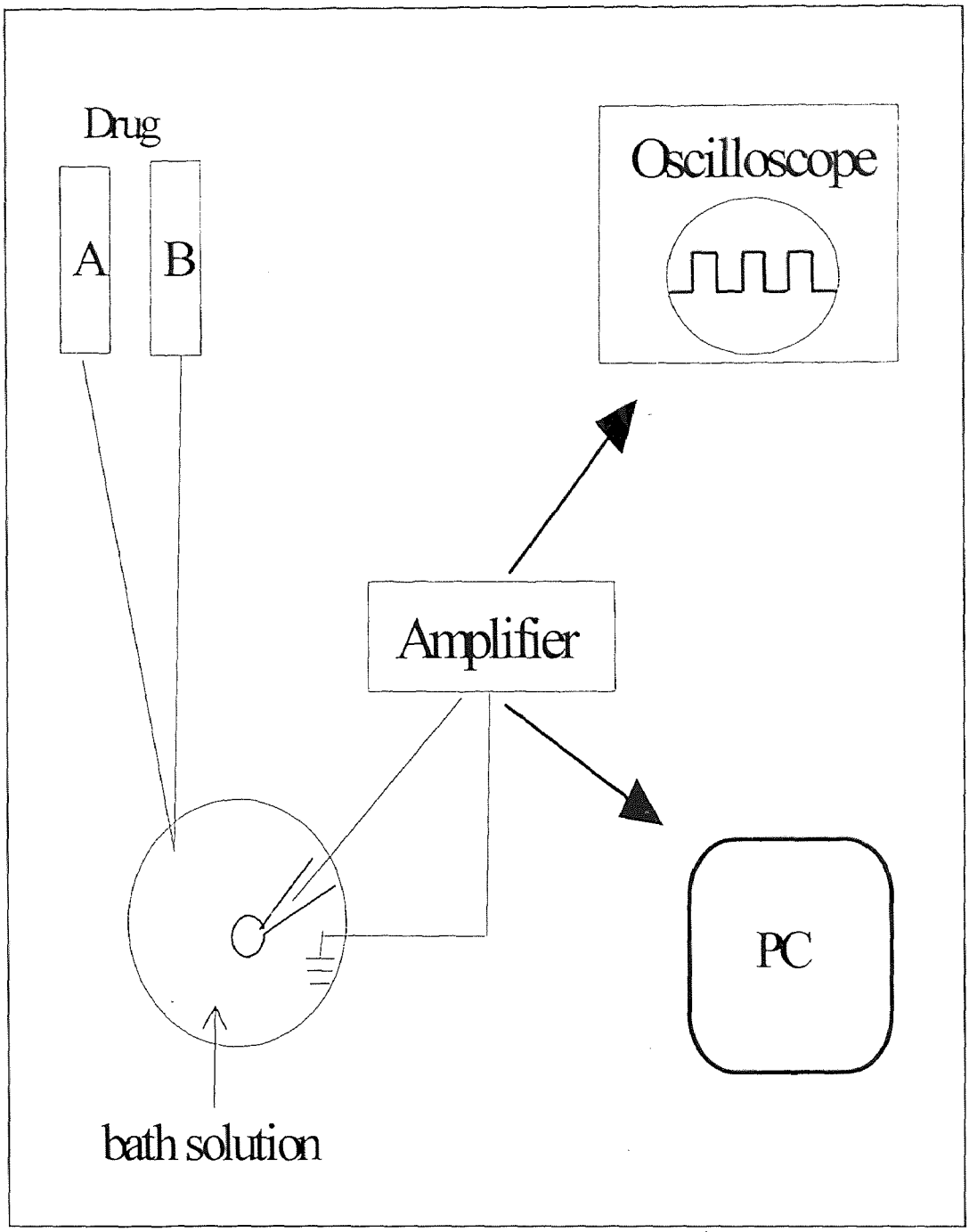

Figure 4 The electrophysiologic recording set up. The pipette provides an electrical connection between the cytoplasm and the metal electrode connected to the amplifier. The oscilloscope displays signals from the amplifier. Signals from the amplifier are stored in a PC for later analysis.

2.6 The Voltage Clamp Technique

Voltage clamp is a method in which the membrane voltage is controlled, and the current required to keep that voltage (V) is measured. The currents (I) that flow are proportional to the membrane conductance (G), i.e. the number of channels open. This relationship is shown by Ohm's law:

$$V = IR = \frac{I}{G} \tag{5}$$

Hamill et al. as well as Horn and Patlak developed the patch clamp "seal" formation recording technique. This technique consists of pressing a pipette tip against the surface of a cell, forming an electrical seal. This seal has high electrical resistance (giga (10^9) Ω) and is mechanically stable. In the cell attached conformation, although the membrane dimples (as shown in figure 5), the micropipette fails to penetrate. Thus, the membrane patch can be ruptured, by applying mild suction, keeping the pipette cell attached. In this whole cell recording conformation, the solution inside the pipette exchanges quickly with the intracellular side of the cellular membrane. Whole cell current recording measures the total ionic conductance in the cell membrane. Currents associated with depolarizing and hyperpolarizing steps can be recorded. The whole cell voltage clamp currents measured are suitable for studying the sum of many ionic currents flowing through individual channels. The whole cell method is used to quantify channels, in situations where there is one channel that dominates the electric characteristic of the membrane. A variation of the whole cell conformation is the perforated patch method, which enables whole cell measurements in a less invasive manner.

2.7 Perforated Patch

The perforated patch method consists of using nystatin to gain access to the interior of the cell. Nystatin is an antibiotic that forms pores in the cell's membrane. These nystatin transmembrane pores lower the electrical resistance to the cell's interior, providing better

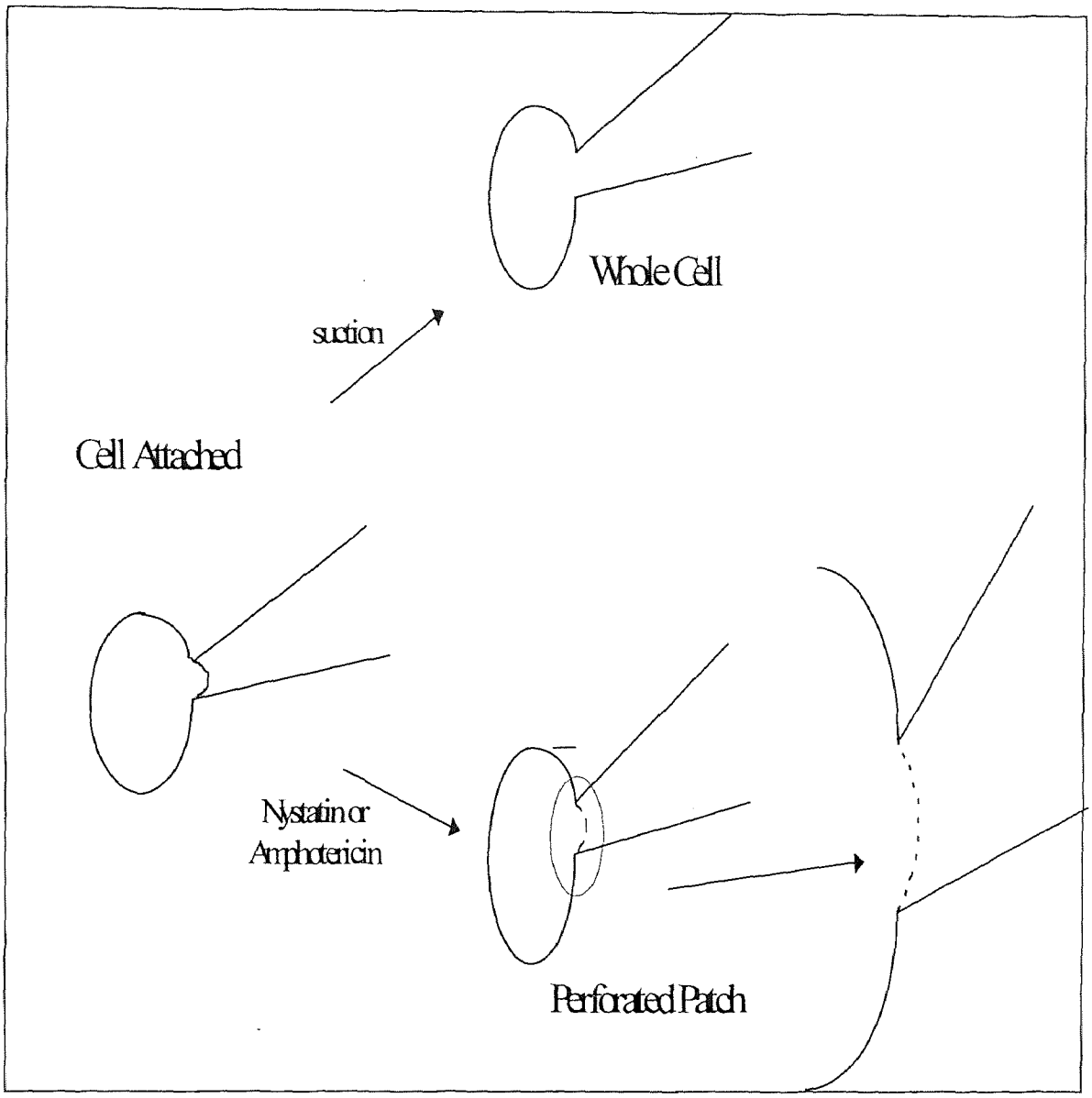

Figure 5 Schematic representation of the whole-cell and perforated patch recording configurations. A pipette is attached to the cell membrane. For whole cell recording, suction is applied to rupture the membrane. In the perforated patch configuration, nystatin or amphotericin is placed in the pipette solution to form pores in the membrane.

conservation of the cytoplasmic contents such as ATP. These pores, which are approximately 8 Å in diameter [Hille, 1992], do not allow large molecules to pass through their opening and are permeable only to monovalent cations and Cl^- ions. Therefore, whole cell recordings can be made without diluting required substances from the cell's cytoplasm. However, intracellular content manipulations can not be made (except for small ions). The K_{ATP} channel studied runs down. Run down refers to the loss of activity with time. By using the perforated patch method, current run down would decline, because ATP is not dialyzed away.

The nystatin stock solution contained 2 mg of nystatin dissolved into 2 ml of methanol. After covering the tube and vortexing it, nystatin solubilization was further enhanced by sonication for approximately 2 minutes in a bath sonicator. The final solution was yellow in color and slightly cloudy in appearance. To clear the solution a drop of HCl was added, then the pH was adjusted to 7.0-7.4 using 1N KOH. Since nystatin is susceptible to oxidation, the stock solution was frozen and used within two weeks.

Pipettes were filled with a solution that contained 10mg/ml nystatin stock solution in pipette solution. The pipette solution contained in mM: 55 KCl, 70 K_2SO_4, 7 $MgCl_2$, 10 HEPES, (buffered to pH = 7.4 with KOH). When using nystatin in the pipette filling solution, it was difficult to obtain a seal; apparently, nystatin interferes with seal formation. Therefore, amphotericin, a milder antibiotic was used as follows.

To prevent loosing activity due to prolonged storage and freezing, 3 mg of amphotericin B (Sigma) were weighted into small tubes and stored in the freezer. The stock solution of amphotericin was 60 mg/ml in 99.5 % DMSO. Since amphotericin is also susceptible to oxidation, the stock solution was used within one week. For recording, the patch pipette was filled with stock solution, diluted 250 times in pipette solution. The chances of getting a seal are greater in an antibiotic free solution. Therefore, the tip of the pipette was dipped in an antibiotic free solution and then back

filled with the pipette and amphotericin solution. After a seal was formed, the antibiotic diffused slowly to the tip to form pores. The bath had a volume of less than 0.5 ml and was perfused at about 2 ml/min. A small vacuum was used to reduce the fluid level, to minimize the immersion depth.

2.8 Proposed Protocol

The goal of this protocol was to determine the effects an experimental drug, BMS 180448-01 (see figure 6), had on the isolated VMN neurons. BMS 180448-01 is supposed to be a K_{ATP} channel opener (KCO). KCO agents open K channels that are sensitive to ATP. The mechanism of action of K_{ATP} openers is still unknown, however, they might have some therapeutic potential [Atwal, 1994]. Since, the interest lied in determining the effect of the drug on the K_{ATP} channel, the KCO would be applied to a closed channel state, to see if indeed, it opened the channel. In the absence of glucose (control) the channel is expected to be in the open state and an increase in channel activity would appear as an increase in current.

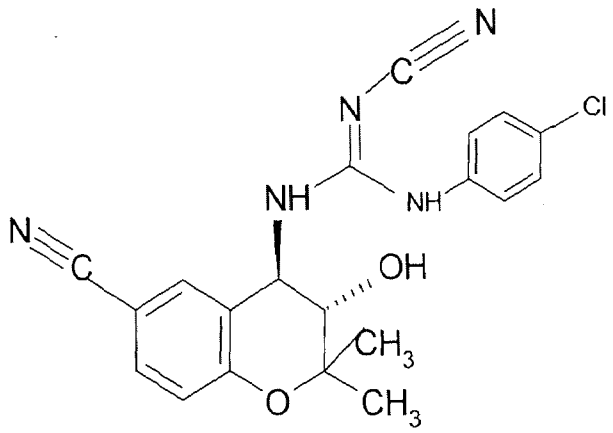

Figure 6 BMS-180448-01

Therefore, neurons would be isolated in glucose free solution and hyperpolarizing voltage steps would be introduced (described in the next section) to ensure that the channel was open. Conversely, glucose abolishes K_{ATP} channel activity and induces action potentials [Trube et al., 1986]. The bath would then be perfused with extracellular solution containing glucose. Thus, a decline in conductance would be seen. After the current changes were allowed to decline in activity, cromakalim, (see figure 7) a known KCO would be applied to the bath solution and hyperpolarizing voltage steps would be

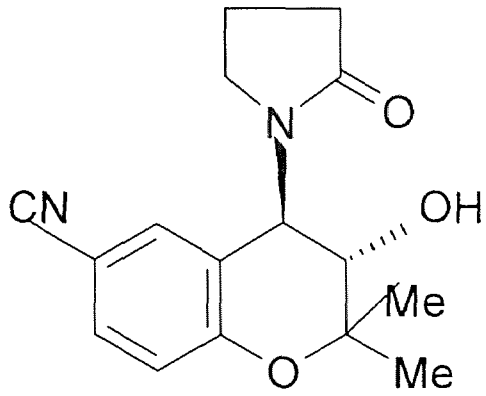

Figure 7 Cromakalim

applied again. Cromakalim is expected to open the channel, and the current activity is expected to rise. The bath would then be perfused with extracellular solution containing glucose, and a decline in activity was expected. Lastly, the experimental drug would be applied. If this drug was indeed a KCO, the channel would be expected to open. Thus, after applying the drug, hyperpolarizing voltage steps would be applied to see if the current amplitude changed. The negative potentials limit the number of channels open.

The data would be analyzed and BMS 180448-01 channel activity would be compared with that of cromakalim. There should be a decrease in K_{ATP} conductance seen when glucose is applied. The subtraction of glucose and glucose free currents will show the presence of K_{ATP} channels. As a final check, high concentrations of glibenclimide (100μmol) would be applied to shut off all K_{ATP} channels more reliably than glucose. This currents would then give an idea of how much of the whole cell current was due to K_{ATP}.

2.9 Data Collection and Analysis

Currents were amplified using the Axopatch-200/CV201 patch clamp amplifier (Axon Instruments, Inc., Foster City, CA). The reference potential for all measurements was the zero-junction potential between the solution in the pipette and the bath before forming the seal. The data was filtered and digitized (TL-3, Axon Instruments) at 5kHz with a 4-pole bessel filter. The data were stored on an IBM-PC and analyzed off line using Pclamp6 software.

Figure 8 shows membrane current records measured from isolated VMN neurons. The interior of a neuron was made electronically accessible with the perforated patch method, then it was voltage clamped and the membrane potential was changed in steps of 20mV. The step depolarization, from -70mV up to 110mV, changed the ionic permeability of the membrane. For instance, when the neuron was depolarized, there was a large inward current. This inward current reversed, giving rise to a large prolonged outward current. Thus, this depolarization initiated a current with Na and K components.

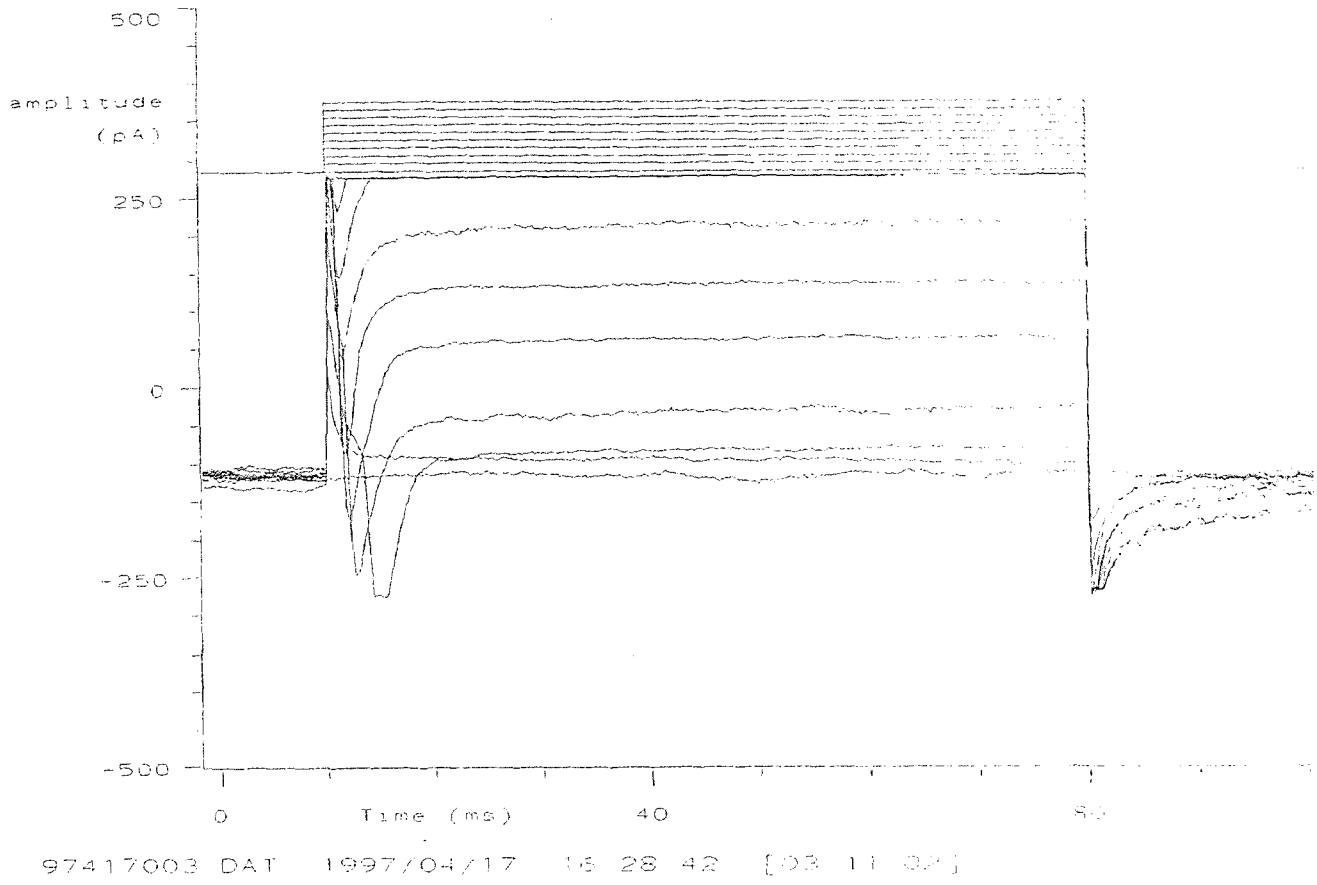

Figure 8 Family of voltage-clamp currents in isolated VMN neurons. A neuronal membrane is stepped under voltage clamp from a holding potential of -70mV to test pulse potentials ranging in 20mV steps from -70mV to 110mV.

The properties of Na and K currents were plotted in terms of current-voltage (I/V) relations. Figure 9 shows the peak transient Na current, obtained from each trace, as a function of the applied voltage potentials. The shape of the I/V relations between -50mV to -10mV reflects the voltage dependent opening of the Na channels. That is the slope of the Na curve changed steeply, at those specific potentials. In addition, figure 8 shows that

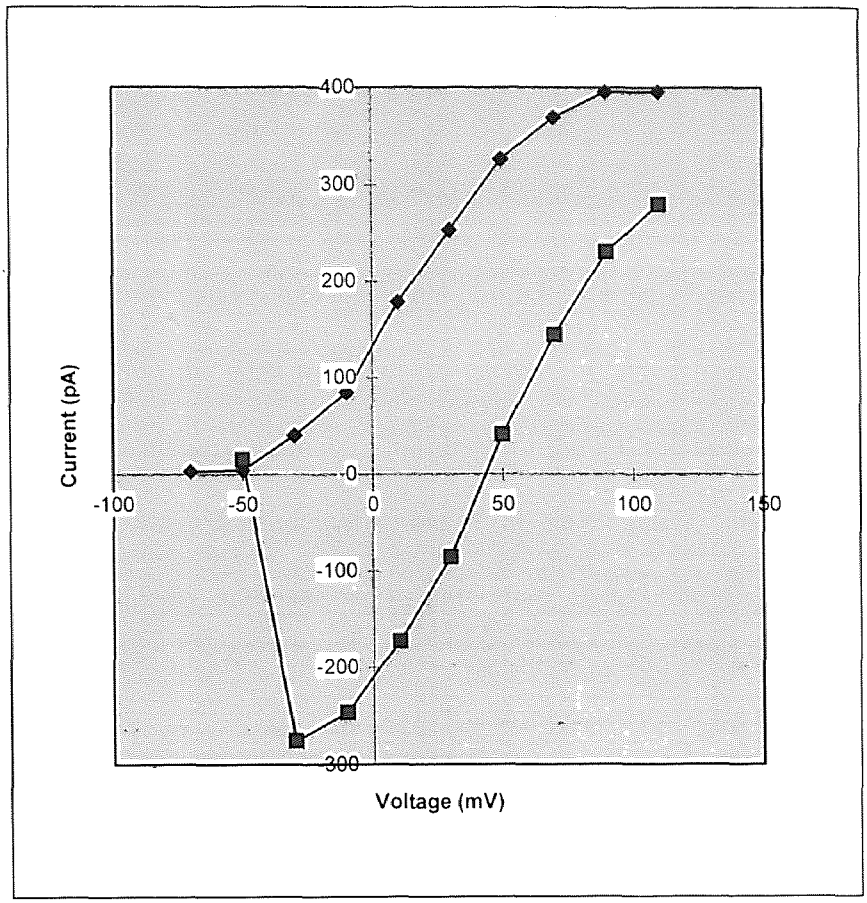

Figure 9 Na/K current-voltage relations of isolated VMN neurons. The VMN neuronal membrane potential is stepped under voltage clamp from the -70mV holding potential up to 110mV. Peak transient Na currents (■) from each trace were plotted against the voltage applied. Steady state currents (◆) are all outward as expected from the K channels. The shape of the I/V relations between -50mV and -10mV represent the voltage dependent opening of Na channels.

the early transient currents reverse their sign from inward to outward at around 48mV, as expected if they are carried by sodium ions. Specifically, at negative potentials to the sodium equilibrium potential (E_{Na}), there is an inward current, that is, Na ions rush into the cell. Whereas, at potentials positive to E_{Na}, the currents carried by Na ions are outward. Steady state values of membrane currents were determined by measuring data points within the last 5ms of the 200ms test pulse. These late currents are outward at all test potentials, as is expected from a current that is carried by K ions. Potassium ions have a reversal potential more negative than -60mV.

The membrane potential was stepped to test pulse potentials ranging in 10mV steps, from a holding potential of -60mV. Figure 11 shows whole-cell currents recorded in response to hyperpolarizing voltage steps to between -120mV and -70mV. These are steady state values of membrane currents determined by measuring data points within the last 5ms of the 200ms test pulse. Corresponding I/V relationships are given in figure 10. This figure shows an inward current, carried by K ions, under hyperpolarization. In addition this figure shows that there is a steep voltage dependence on hyperpolarization. Thus, the characteristics just described are typical of an inward rectifier. The reversal potential E_K would be approximated at a potential negative to -50mV, based on the behavior of the graph. Both current traces presented here have a small component of current before the applied depolarizing or hyperpolarizing pulses. This component of current is called "leakage current" [Hille, 1992], which is a voltage independent background conductance of undetermined ionic basis. To compensate for this, the leakage was subtracted from the actual current responses to obtain the I/V relation.

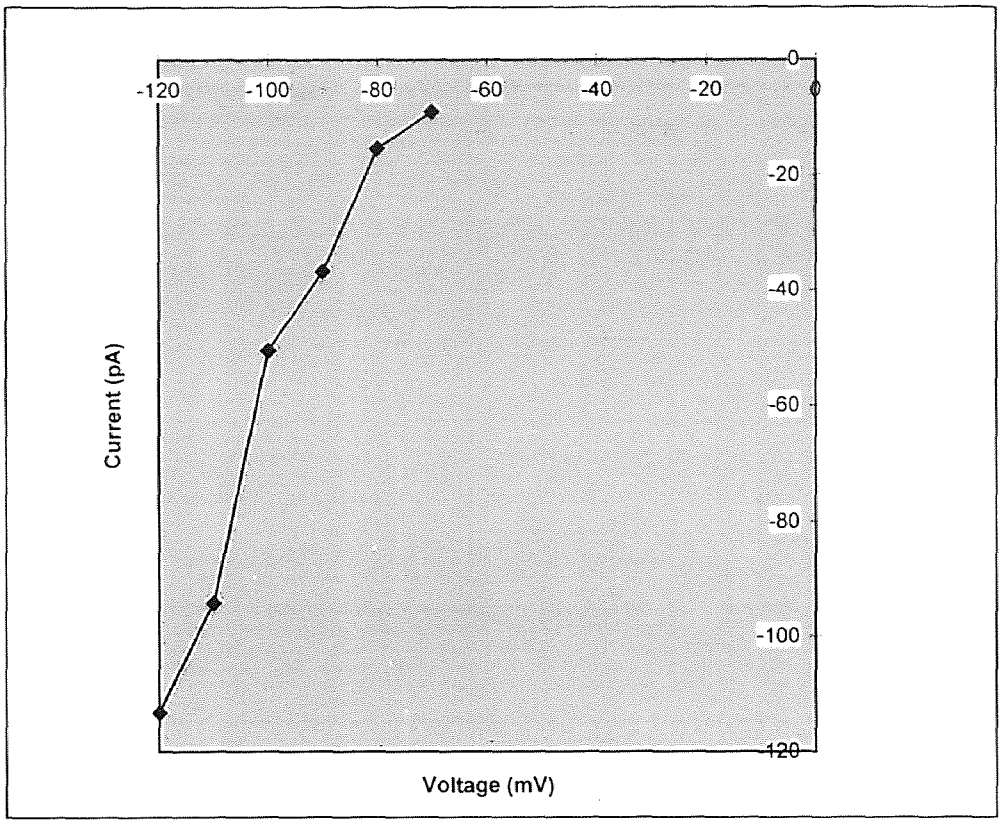

Figure 10 Steady state current/voltage (I/V) relationships for the whole cell currents shown in figure11.

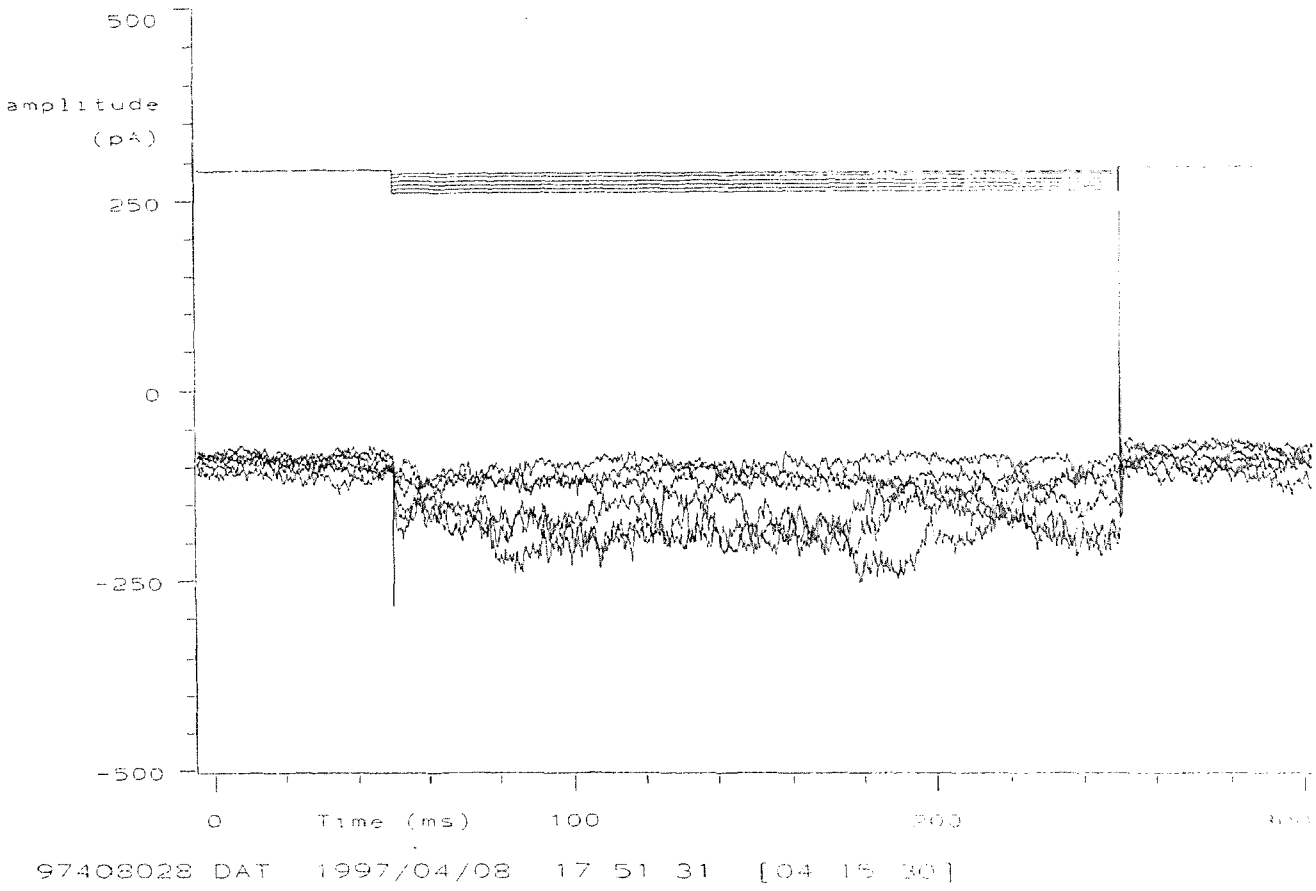

Figure 11 Family of hyperpolarizing voltage-clamp currents in isolated VMN neurons. Whole-cell currents recorded in response to voltage-clamp steps between -120mV and -70mV from a holding potential of -60mV, using the perforated-patch configuration.

CHAPTER 3

CONCLUSIONS AND RECOMENDATIONS

3.1 Conclusions

Of particular interest, was the measurement of the ATP sensitive K channel activity. These type of channels are located in the pancreatic β-cell and are responsible for the glucose sensing process. In addition, the K_{ATP} channel has been found in the cell membrane of neurons in the brain. Thus, a glucose sensing mechanism, similar to the pancreatic one, might be taking place in the brain.

Neurons were isolated from the VMN of the hypothalamus. At first the brain slices were subjected to enzymatic digestion using pronase and thermolysine, to isolate the neurons. However, the neurons obtained did not survive. Thus, a new enzymatic protocol was implemented, using the proteolytic enzyme papain followed by a trypsin inhibitor, BSA. Neurons isolated with papain had better morphology.

The perforated patch method was used to measure whole cell currents This technique allows membrane potential recording, voltage clamping, and intracellular content manipulation. This method consisted of using a pipette solution containing, the antibiotic, nystatin. Nystatin formed pores in the cellular membrane. These pores lowered the electrical resistance to the cell's interior and kept the cytoplasmic contents intact. Nystatin is a potent antibiotic and difficulties were encountered in seal formation. Therefore, amphotericin, a milder antibiotic was used, and resulted in better in seal formation.

In summary, experience was obtained in making solutions, isolating neurons, manufacturing pipettes and utilizing the amplifier to obtain neuronal currents. The main difficulty encountered in working with the K_{ATP} channel was in keeping the neurons alive long enough to run the intended experimental protocol. The isolation procedure

implemented here has been shown to work effectively for whole cell recording in other channels. In such experiments, the majority of channels studied respond to their agonists within seconds, therefore, an experiment can be done in 5-10 minutes. It is speculated that the K_{ATP} channel is metabolically activated. Thus, it takes approximately 15 minutes to determine whether the channel is a K_{ATP} channel in whole cell recordings.

The intended protocol consisted of determining the effects of a suspected channel opener, BMS-180448-01, on isolated VMN neurons. Initially, the neurons were isolated in a glucose free solution. Depolarizing voltage steps produced Na and K currents. Hyperpolarizing voltage steps elicited currents, which were expected to show an increase in K_{ATP} channel conductance. Glucose closes the K_{ATP} channel, therefore, a decrease in K_{ATP} channel conductance was expected, upon glucose application to the extracellular solution. Once the channels were closed by glucose, cromakalim would then be applied to the bath solution, to open the channel, and an increase in K_{ATP} channel conductance was expected. The procedure just described would be compared with BMS-180448-01. The effect of KCO's on the K_{ATP} channel would be studied That is current amplitude records between cromakalim and BMS-180448-01, would be compared. The subtraction of glucose free currents will show the presence of K_{ATP} channel. As a final check glibenclimide would be placed in the solution to completely shut off the channel. The currents obtained between glibenclimide and glucose would then be compared to determine the presence of the K_{ATP} channel in the cell membrane.

3.2 Recommendations for Future Work

In every area of research there is always room for improvement. The major difficulty encountered in utilizing this technique was keeping the preparation healthy for a relatively long time. Significant damage was produced to the cells by dissociating connective tissue through enzymatic digestion and through trituration. Other laboratories use the slice recording method to solve the isolation problems. For instance, Dr. Vanessa

H. Routh is currently working with this technique in Dr. Ashford's laboratory in Scotland. In tissue slice recording, the slices are placed in a special chamber to oxygenate and perfuse the slices. Disruption of the normal cellular environment is limited to the surface of the slice, thus keeping the slices closer to their original state than in the isolated neuron preparation. In addition, there is no trituration involved. On her return, Dr. Routh plans to implement the slice preparation for channel recordings to mitigate the difficulties encountered since working with the K_{ATP} channel.

Another difficulty encountered were the rats, since there was a narrow window to work with (between 11-18 days). In addition there were contamination concerns, since the pups acquired pin worms. It took a long time to decontaminate the animal facilities. The difficulties involved with animal models perhaps can be avoided by increasing the number of animals used or implementing tissue culture cells. However, in such manner there would be deviations from the actual physiological functions sought, that an animal model can provide best.

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