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Water reabsorption and urinary volume regulation are important processes in maintaining normal physiological function. Vasopressin (ADH) is the hormone that is responsible for regulating water homeostasis. We have been examining the mechanism whereby ADH enhances water permeability in renal epithelia. Our observations suggest that an integral component of the water reabsorptive process is the enzyme, protein kinase C (PKC). Using immunocytochemical techniques and enzyme assay procedures, we have identified PKC isozymes that translocate from the cytosol to the membrane during activation by ADH and PKC activators. We also report on an *in vitro* model of cultured kidney cells for measuring transepithelial fluid and electrolyte transport and have evaluated various filter supports for their transport characteristic limitations.

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INTRODUCTION

Water reabsorption and urinary volume regulation are important functions of the kidney and are critical for maintaining normal physiological function. Therefore, dysregulation of the kidney may lead to a variety of disease states including hypovolemia, hypertension and cardiovascular complications. During conditions of excessive heat and water deprivation the kidney is under the regulation of antidiuretic hormone (ADH), which increases the ability of the kidney to reabsorb fluid by acting on the collecting tubule and increasing its permeability to water. However, in extreme cases of water deprivation, the presence of ADH is not enough to maximize the reabsorbtive capacity of the kidney. Under these circumstances hypovolemia may become a medical emergency. Such actions could have devastating effects on the performance and effectiveness of soldiers during crucial sustained military operations in desert environments. The fundamental mechanism governing the transmembrane water transport process mediated by ADH receptors and its subcellular responses is little understood.

Vasopressin is known to increase osmotic water transfer across cortical renal tubular cells as well as renal model epithelia, including amphibian urinary bladder tissue (Bentley, 1958; DiBona, 1981; Hays, 1983; Mia et al., 1987). The increase in transepithelial aqueous flow induced by vasopressin occurs as a result of V_2 receptor interactions. The cascade of events initialized by the V_2 receptor cascade includes the activation of adenylcyclase, an increase in cAMP formation and the

activation of protein kinase A (PKA). These biochemical events are also believed to be accompanied by cellular ultrastructural changes. These submicroscopic structural changes are thought to include propagation of numerous microvilli on the membrane surface (Davis et al., 1974; DiBona, 1981; Mia et al., 1983, 1988; Mills and Malik, 1978; Spinelli et al., 1975). These alterations in the apical membrane surface are thought to result from the insertion of cytoplasmic water channels (aggrephores) following membrane fusion events (Chevalier et al., 1974; Kachadorian et al., 1978; Wade et al., 1981; Ausiello et al., 1987 and others). Such membrane fusion events are dependent very likely on the presence of cytosolic microtubules and microfilaments (Taylor et al., 1973; Hardy and DiBona, 1982; Hays et al., 1982; Pearl and Taylor, 1983; Mia et al., 1991).

Amphibian epithelia also contain an ADH V_1 receptor coupled to phosphoinositide metabolism and inositol phosphate release (Yorio et al., 1985; Yorio and Satumtira, 1989). Recently, it was demonstrated that mezerein (MZ), a non-phorbol activator of protein kinase C (PKC), increased transepithelial water flow when added to mucosal surface. The magnitude of water transport was less and occurred over a longer period of time than compared to ADH-stimulated tissues (Yorio and Satumtira, 1989). The increase in microvilli occurs as a result of the fusion of water channels with the apical membrane and is indicative of an increase in membrane surface area following hormone addition (Mia et al., 1991). What controls the cycling of water channels and their insertion into the membrane is uncertain. Our laboratory has demonstrated that renal epithelia, as well as amphibian

urinary bladder membranes, contain a vasopressin V_1 receptor which upon activation induces phosphoinositide breakdown and the formation of inositol triphosphate (IP_3), a modulator of intracellular ionic calcium and the release of diacylglycerol, an activator of protein kinase C (PKC). It has been proposed by our laboratory that part of the initiation of transepithelial water flow involves V_1 receptors and activation of PKC as shown by biochemical parameters and by immunogold detection of PKC isozyme subtypes (Mia et al., 1991, 1992). To test this hypothesis we have been using activators of PKC, such as phorbol esters and mezerein, as pharmacological tools to determine if PKC activation results in similar physiological responses as the hormone.

METHODS

Tropical toads, Bufo marinus, purchased from Carolina Biological Supply Company, Burlington, NC, or from NASCO, Ft. Atkinson, WI, were maintained at 23°C in an aquatic environment system irrigated with running tap water and were fed live crickets semi-weekly.

Experimental Protocol

Whole tissue

Intact urinary bladder sacs were excised surgically from doubly pithed toads and were placed in aerated Ringer's solution as described previously (Bentley, 1958; Mia et al., 1983, 1987). The composition of the Ringer's solution was as follows (in millimoles per liter): NaCl, 111; KCl, 3.35; $CaCl_2$ 2.7; $MgCl_2$, 0.5; $NaHCO_3$, 4.0 and glucose, 5.0. The pH of the solution was adjusted to 8.0 and the solution was

continuously aerated throughout the experimental procedures. An osmotic gradient was instituted by diluting the mucosal solution to a one-tenth of the Ringer's solution. Identical hemibladders were washed in Ringer's solution and were set up as 'sacs' for control and experimental tissues.

Cell culture

Toad urinary bladder epithelial cells were dissociated and collected under sterile conditions according to procedures described previously (Yorio et al., 1983; Yorio and Satumtira, 1989; Mia et al., 1988). During isolation procedures, all media contained antibiotics. These cells were washed and resuspended in Ca^{2+}, Mg^{2+}-free media and transferred to Falcon flasks and petri dishes containing microscope glass cover slips for growth. The culture flasks and petri dishes were retained in an incubator in a humidified 5% CO_2/air atmosphere at 28°C. The cell culture media consisted of a supplemented Quimby amphibian culture media (Gibco). The culture medium was changed three times/week and only primary confluent cultures were used. These tissues were being utilized in immunofluorescent studies for PKC enzymes, microfilaments and microtubule detection. We conducted experiments using intact toad urinary bladders and the A6 amphibian kidney cortical cells grown on supports to test the response of these cells following stimulation with ADH and mezerein (MZ)- an activator of PKC. These cells upon transfer to filter supports form confluent monolayers and tight cellular junctions. Scanning electron microscopic techniques were applied to evaluate the conformational changes of the apical surface of the toad urinary bladder and the A6 cells. The following treatments were used involving the

A6 cells SEM studies:

(a) Control Ringers + DMSO for 45 min and then 15 min in buffer

(b) In DMSO for 45 min and then 15 min in ADH

(c) In Mezerein for 45 min and then 15 min in buffer

(d) In Mezerein for 45 min and then 15 min in ADH

DMSO and ADH were added to the basal side of the membrane support while mezerein was added to the mucosal surface. Tissues were incubated for 60 min prior to fixation in 2% glutaraldehyde in PIPES buffer (0.02M) for 1 hr. Toad urinary bladder sacs were also stimulated using four different concentrations of mezerein (MZ), an activator of PKC, at 10^{-6}M, 10^{-7}M, 10^{-8}M and 10^{-9}M. These effects were compared to control tissues treated with vehicle (0.002% DMSO). A postfixation was carried out for 1 hr using 1% osmium tetroxide prior to dehydration. Fixed tissues were dehydrated in graded mixtures of ethanol and amyl acetate, and dried by critical point method using a CO_2 environment (Mia et al., 1987, 1988) or exchanges in a series of graded acetone or ethanol, and infiltrated in liquid Peldri II which was then cooled to a solid state by gradually dropping the temperature below 23°C (75°F). The sublimation process was then carried out using a freeze dryer or running fumehood. Critical point drying using Peldri II gave equally good or better results compared to results obtained by critical point drying by liquid CO_2. Pieces of dried toad bladder tissues and monolayer culture tissues were mounted on aluminum stubs using silver paint for electrical conductivity. The specimens were then coated with gold in argon environment using a sputter coater for scanning electron micro-

scopic observations. For TEM preparations, the minced tissues were dehydrated using exchanges of ethanol and propylene oxide to cast blocks in beam capsules using Luft's epon resin. Polymerization of the blocks was carried out in a oven at 60°C for 15 hr.

Immuno-Gold Cytochemistry

For immunocytochemical procedures using antibodies for protein kinase C (PKC), protein G-gold or A-gold probes, tissues were exposed to drugs and vehicle as before prior to fixation in 2% glutaraldehyde in PIPES buffer. Fixation was allowed to continue for 1 hr. These tissues, which received no postfixation with osmium tetroxide, were washed in deionized water, dehydrated through exchanges of ethanol and L.R. White and subsequently embedded in pure L.R. White resin using gelatin capsules. Tissue blocks were allowed to polymerize for 15 hr at 60°C in a vacuum oven. Ultrathin sections showing silver to light gold interference color were sliced using a diamond knife. These sections were collected on bare nickel grids. For indirect labeling of protein kinase C (PKC) enzymes using immunocytochemical procedures, each grid was floated on a drop of isotonic Ringer's solution containing specific monoclonal antibodies obtained from rabbits for each subspecies of protein kinase C (1/25 dilution) (Seikagaku America, Inc., Rockville, MD) for 2 hr in a moist chamber at room temperature. The grids were rinsed in deionized water and then floated on a drop of monodispersed protein A-gold or protein G-gold particles (1/25 dilution) (individually distributed gold particles attached to protein A or protein G)

in isotonic Ringer's solution for 2 hr in a moist chamber at room temperature. Air dried grids were stained with saturated uranyl acetate in 70% ethanol and lead citrate prior to TEM observations. To determine protein A-gold or protein G-gold nonspecific binding, tissues received only 0.1% BSA treatment (no PKC antibody) prior to protein A-gold or protein G-gold administration (Mia et al., 1991, 1992).

Drugs

8-Arginine vasopressin and mezerein were purchased from Sigma Chemical Co., St. Louis, MO. The concentration of ADH used in the experimental protocol was 10mU/ml and was added to the serosal side of the bladder. The concentration of mezerein used in the experiments was 10^{-6}M and was added to the mucosal side of the bladder. The mezerein was dissolved in a stock solution of DMSO to a concentration of 0.05M. The concentration of DMSO added was 0.002%.

Development of PKC Assay for cultured Epithelial Cells:

The immuocytochemical observations suggest that PKC isozymes are present in toad-urinary bladder preparations. We have developed a rapid simple protein kinase C (PKC) assay based on a mixed micelle method that can be routinely performed using tissue culture cells (Cammarata et al., In Press). Enzyme preparation was performed in membrane and cytosolic fractions respectively. The isolation of these fractions followed the sequence listed below.

PKC Determination

Cultured A6 cells are washed in serum-free medium. Cells are exposed to Ca^{2+}-Mg^{2+}-free PBS containing 0.25% trypsin + 1 mM EGTA for 5 min. Isolated cells are collected and resuspended in serum-containing or serum-free media. Cells are incubated with hormone and/or drugs for a predetermined time. The incubation is stopped by adding five-fold cold media and the cells are quickly centrifuged. Collected cells are homogenized in 2 ml of buffer (A) containing 20 mM Tris-HCl, pH = 7.5; 0.5 mM EGTA; and 25 ug/ml each of apotinin and leupeptin. Homogenate is centrifuged at 100,000 xg at 4°C for 30 min, yielding a soluble (cytosolic) and particulate (membrane) fraction. The enzyme in the membrane fraction is solubilized in 2 ml of buffer (A) supplemented with 0.5% Triton X-100 and homogenized. The homogenate is incubated on ice for 30 min, followed by centrifugation at 100,000 xg for 30 min. The soluble and membrane-bound extracted enzymes are partially purified by chromatography on DEAE-cellulose (DE52) after elution with 200 mM NaCl. The eluate from the DE52 column that contains enzyme is desalted by chromatography on Sephadex G25 and eluted with 20 mM Tris-HCl. The eluted soluble enzyme or membrane-bound extracted enzyme from the Sephadex G25 column was used for subsequent PKC analysis.

The PKC activity was followed by phosphorylation of its substrate (AC-MBP 4-14, a PKC substrate peptide purchased from Gibco) using ^{32}P-ATP measured at 30°C in an incubation volume of 50ul for 5 min. The assay mixture contained in final concentration: ATP, 20uM; $MgCl_2$, 10mM; $CaCl_2$, 200 uM; substrate, 25 uM;

phosphatidyl serine, 516 uM; 1-oleoyl-2-acetylglycerol (OAG), 344 uM; enzyme, 5-15 ug; Tris-HCl, 20 mM, pH = 7.5, with or without 20uM substrate-specific PKC inhibitor (PKC inhibitor peptide 19-36 from Gibco). About 500,000 cpm of ^{32}P-ATP was added to each test tube. The reaction was started by addition of ATP and stopped by taking 25ul of assay mixture and spotting onto phosphocellulose paper. Free ^{32}P-ATP and ^{32}-labelled substrate were separated by washing the paper with 0.85% of phosphoric acid for 5 min twice, followed by another two washes with distilled water. The radioactivity retained on the phosphocellulose paper after washing was determined by counting the paper in 10ml of scintillation liquid. PKC activity was determined by the difference between the activities in the absence and presence of the PKC substrate inhibitor. A unit of PKC activity is defined as incorporation of 1 umol of phosphate into the substrate per minute per mg protein. Proteins are determined using the method of Bradford.

Transepithelial water flow measurements of A6 cells grown on filter supports.

The morphological SEM observations suggested that A6 cells responded to both hormone and PKC activators in a similar fashion. To determine if these changes were associated with increases in membrane permeability and enhanced water transport, osmotic water flow was measured across A6 cells grown to confluency on Anocell and ICN Cellagen filter supports. Although there was an apparent morphological change following hormone addition, and previous data had shown an increase in adenylate cyclase activity, there was no increase in osmotic water flow. The reason for this lack of transepithelial water flow is unclear. We measured

radiolabelled water flow using ^3H-H_2O and testing the filter supports for their permeability. Our observations suggested that these commercial filters may be rate limiting for transepithelial water flow measurements. Please see attached manuscript that will be sent to the American Journal of Physiology for possible publication. We are also searching for different supports that may allow free diffusion of water.

Results

Apical views of the control tissues (vehicle treated) showed a uniform configuration of microridges over the entire surface (Fig. 1). The details of the control granular cells observed under SEM show a distribution of a microridge network throughout the cell surface and appear smooth. The cellular margins between granular cells similarly show uniform disposition with no visible occurrences of microvilli along the cellular attachments. The results of stimulation by four different concentrations of MZ were consistent with MZ-induction of the propagation of numerous microvilli over the granular cell surfaces. In some cases, the microvilli appeared to be crowded over the apical surface as well as on the cellular attachments (Figs. 2,3). The degree of crowding as well as the frequency of microvilli varied with the concentration of MZ applied to the mucosal surfaces of the urinary bladder sacs. The lower the concentration of MZ, especially at 10^{-9}M, the greater the number of microvilli appearing on the cell surface (Fig. 4). The extent of microvilli formation following a 5 min exposure to MZ at all four concentrations, appeared to be smaller in size compared to prolonged treatment with MZ for 60 min. This is similar to whar had been reported previously in toad urinary bladder (Mia et al., 1989). These

observations are similar to what is seen following treatment with antidiuretic hormone (ADH, vasopressin).

We also examined amphibian kidney cells in culture (A6 cells). Individual A6 cells, upon transfer onto ICN membrane supports or on Anocell filters, undergo rapid growth to confluency to form a continuous polarized monolayer. Figure 5 represents a surface view of such control cells (vehicle treated), grown on ICN Cellagen membrane supports, and which were only exposed to DMSO and buffer. The apical membrane surfaces of these cells, when viewed under the SEM, revealed the presence of a network of microridges along with distinct cellular tight junctions. Unstimulated A6 cells had a surface morphology very similar to that observed on the control toad urinary bladder tissues. Thus A6 cells may provide an ideal source of experimental tissue for an in vitro model for water and ion transport studies. Stimulation of the A6 cells by exogenous ADH for 15 min following exposure to vehicle (DMSO) for 45 min, resulted in the transformation of the apical microridges into numerous microvilli (Fig. 6). This hormone response was accompanied by some slight cellular swelling (Fig. 6). SEM pictures revealed that cells exposed to mezerein for 45 min, with a subsequent treatment in buffer for 15 min, had apical microridges transformed into numerous microvilli (Fig. 7). Subsequent buffer wash did not abolish the microvilli once they were formed due to the mezerein challenge. A6 cells, grown on ICN membrane supports, when exposed to mezerein for 45 min with an additional 15 min exposure to ADH, resulted in the expression of an enhanced number of microvilli over the apical membrane surfaces, cellular swelling, formation

of apical domes and caving of the cellular junctions (Fig. 8).

Immunogold detection of protein kinase C (PKC) isozymes was made using a specific monoclonal antibody to isozyme III and monodispersed protein A-gold or protein G-gold probes with a particle diameter of 10 nm or 20 nm. The protein A-gold particles were localized in diffused cytoplasmic bodies, as we have previously reported for protein G-gold probes, but the degree of intensity of labeling with protein A-gold probes is relatively more than that observed previously with protein G-gold probes. Figures 9 and 10 are micrographs showing the distribution of protein A-gold particles in clusters associated with PKC isozyme III in the cytosol (arrows) following stimulation of tissues with mezerein (MZ) for 60 min. In some instances, the cytoplasmic bodies in which PKC III isozyme is localized appear to be associated with a large number of microfilaments (Fig. 10, arrow). *Recently, Spudich* (1992) suggested an association of protein kinase C enzymes with meromyosins and microfilaments during cellular activation. Figure 11 illustrates the detection of PKC isozyme III in ADH stimulated tissues. It was observed that the distribution of protein A-gold or protein G-gold probes associated with PKC isozyme III was seen to be very similar to that observed with mezerein treated tissues. Gold particles associated with protein kinase C isozyme III, at times, appear at locations on the apical membrane surface (Fig. 12, arrow) as well as within microvilli (Fig. 13, arrow) possibly due to migration in association with aggrephores through the microvilli into the apical membrane likely for membrane fusion events. Expression of protein A- as well as protein G-gold particles over the dense bodies in the cytoplasm (Fig. 14, arrow) and on the apical

membrane domains (Fig. 15, arrow) indicated possible translocation of PKC enzymes to the apical membrane in a time course that was similar to the insertion of cytoplasmic water channels into the apical membrane following hormone addition (Mia et al., 1991). Control tissues exposed only to 0.1% BSA and protein A-gold or protein G-gold particles failed to show any labeling (Fig. 16). Expression of protein G-gold involving isozyme III (gamma) was seen to be localized in isolated patches rather than in discrete diffused bodies as seen with protein A-gold probes (Mia et al., EMSA, 1991).

V_1 versus V_2 interactions

Our laboratory has also shown that MZ can mimic vasopressin in mediating a water flow response, suggesting that activation of PKC may play a role in regulating transepithelial water flow. Recently, Teitelbaum and Strasheim (1990) demonstrated that rat inner medullary collecting tubule cells have a vasopressin V_1 receptor that is coupled to an increase in phosphoinositide hydrolysis, and that this response occurs at low hormone concentrations below that needed to increase adenylcyclase. These authors concluded that phosphoinositide hydrolysis may be involved in the initiation of the hydroosmotic response as the hormone can increase water flow at concentrations lower than that needed to increase cyclic AMP formation. Our laboratory has investigated the relationship between the cyclic AMP effects of vasopressin and its effects on phosphoinositide metabolism using the A6 amphibian kidney cell line (American Type Culture Collection). These cells, which

are derived from the cortical collecting tubule of the amphibian Xenapus laevis,can be grown on filter supports and the electrical properties of transepithelial potential difference (PD), short circuit current (Isc) and resistance (R) can be measured by mounting the filter support in a Lucite chamber. Under basal conditions the A6 cells establish a confluent monolayer on the filter support that has a PD of 37 mV and a resistance of almost 5 kohms (Table 1). Following the addition of vasopressin (ADH), a decrease in resistance and an increase in the Isc is seen. The increase in Isc appeared to be mediated through an increase in sodium transport as amiloride, a sodium channel blocker, inhibited the hormonal increase in transport. These results demonstrated that these cultured cells were responsive to vasopressin and that they probably had an active V_2 receptor coupled to adenylcyclase. To determine if this was indeed the case, we measured adenylcyclase activity in these cells using an adenine labeling method. Vasopressin (ADH) produced a dose-dependent (Fig.17) and time-dependent (Fig.18) increase in cyclic AMP formation indicating a functional adenylcyclase enzyme and a vasopressin coupled V_2 response (Fig.19). We also investigated the interaction between PKC and activation of adenylcyclase to determine if the V_1 receptor negatively regulated the V_2 response. PKC activation with MZ had no effect on the ability of vasopressin to stimulate cyclic AMP formation (data not shown). We have just begun to look at the presence of the V_1 receptor in these cells. This is being accomplished by measuring IP_3 formation and increases in intracellular calcium following hormone addition. The former measurement is obtained using radiolabelled myo-inositol to label the IP_3 while increases in intracellular

calcium are determined using a video imaging instrument (TCOM/UNT) and the molecular probe Fura-2AM.

We have also continued our interest in PKC activity in these renal epithelial cells. A PKC assay has been developed and was described in the methods above. The assay was linear with time (Fig. 20) and protein concentration (Fig. 21). In addition, optimum conditions were determined for phosphatidylserine and OAG addition (Fig. 22a,b) and for calcium (Fig. 23). In addition, we determined the maximum activation that was achieved with the PKC activators, mezerein and PMA (phorbol myristate acetate) (Fig. 24). Using this method we will begin to measure PKC activity in both the cytosol and membrane following stimulation of the cells with either ADH or mezerein (or PMA) activation. Time courses for PKC translocation (cytosol to membrane) will be determined under conditions of hormone treatment and osmotic water transfer. Pretreatment of the cells with PKC activators for extended periods of time will allow us to down regulate the PKC activity and to assess the involvement of PKC in the hormone mediated increase in water flow.

Epithelial cells in culture have become increasingly important "model" systems to study regulation of electrolyte and fluid transport. We have also been interested in developing an *in vitro* model to quantitate the water reabsorbtive response to antidiuretic hormone (ADH). Native epithelia, like kidney and toad urinary bladder epithelium, rest on their natural support. These supports represent a low restriction to the transepithelial movements of ions and fluid. Therefore, movement of ions and water across a preparation consisting of the epithelium with its connective tissue is

mainly dictated by the permeability properties of the epithelial layers. We have developed an *in vitro* tissue culture model of kidney cells (A6 cells) grown on filter supports. Although epithelia grown on permeable supports develop polarization and tight junctions as indicated by their electrical properties and morphology, the rate of transport of ions is usually lower than in the native tissue. This deficiency has been justified as due to the artificial conditions of the tissue culture environment. Despite the large numbers of electrophysiological studies with A6 cells, few have measured unidirectional Na or Cl fluxes across the filter supports without cells. A most puzzling finding has been the lack of net water flow under an osmotic gradient when cultured cells on filters are stimulated with ADH. We measure water flow responses to ADH and other agents across a variety of filter supports and found that the filter itself was rate limiting for transepithelial water flow measurements (See accompanying manuscript). Our findings suggest that investigators must be aware of the limitations of filter supports, as they are still imperfect replacements to the natural extracellular matrix on which epithelia perform their physiological functions We are in the process of testing other supports that more typically mimic the natural substrates.

REFERENCES

Ausiello, D.A., Skorecki, K.L., Verkman, A.S. and Bonvetre, J.V. 1987. Vasopressin signaling in kidney cells. *Kidney Int.*, 31, 527-529.

Bentley, P.J. 1958. The effects of neurohypophysial extracts on water transfer across the wall of the isolated urinary bladder of toad *Bufo marinus. J. Endocrin.*,17,

201-209.

Cammarata, P.R., Fan, W., Jin, Y. and Yorio, T. Protein kinase C activity and its relationship to myo-inositol uptake during hyperglycemic conditions in cultured bovine lens epithelial cells. Current Eye Res. (In Press).

Chevalier, J., Bourguet, J., Hugon, J.S. 1974. Membrane associated particles: distribution in frog urinary bladder epithelium at rest and after oxytocin treatment. *Cell Tiss. Res.*, **152**, 129- 140.

Davis, H.L., Goodman, B.P., Shuster, R.J., Rasmussen, H. and Martin, J.H. 1974. Effects of cytochalasin B on the response of toad urinary bladder to vasopressin. *J. Cell Biol.*, **63**, 986-997.

DiBona, D.R. 1981. Vasopressin action on the conformational state of the granular cell in the amphibian urinary bladder. I. Epithelial Ion and Water Transport. *J. Cardiovasc. Pharm.*, **8**, 523-528.

Hays, R.M. 1983. Alteration of luminal membrane structue by antidiuretic hormone. *Am. J. Physol.*, **245**, C289-C296.

Hays, R.M., Bourguet, J., Satir, B.H., Franki, N. and Rapaport, J. 1982. Retention of antidiuretic hormone-induced particle aggregates by luminal membranes separated from toad bladder epithelial cells. *J. Cell Biol.*, **92**, 237-241.

Hardy, M.A. and DiBona, R.D. 1982. Microfilaments and the hydro- osmotic action of vasopressin in toad urinary bladder. *Am. J. Physiol.*, **243**, C200-C204.

Kachadorian, W.A., Wade, J.B., Uiterwyck, C.C. and DiScala, V. A. 1977. Membrane structural and functional responses to vasopressin in toad bladder. *J.*

Membrane Biol., **30**, 381-401.

Mia, A.J., Tarapoom, N., Carnes, J. and Yorio, T. 1983. Alteration in surface substructure of frog urinary bladder by calcium ionophore, verapamil and antidiuretic hormone. *Tissue & Cell*, **15**, 737-748.

Mia, A.J., Oakford, L.X., Torres, L., Herman, C. and Yorio, T. 1987. Morphometric analysis of epithelial cells of frog urinary bladder. I. Effect of antidiuretic hormone, calcium ionophere (A23187) and PGE_2. *Tissue & Cell*, **19**, 437-450.

Mia, A.J., Oakford, L.X., Moore, T.M., Chang, P.H. and Yorio, T. 1988a. Morphometric analysis of epithelial cells of frog urinary bladder. II. Effect of ADH, Calcium ionophore (A23187) and verapamil on isolated dissociated cells. *Tissue & Cell*, **20**, 19- 33.

Mia, A.J., Oakford, L.X. and Yorio, T. 1988b. Effect of verapamil on cytoplasmic distribution of granules and microfilaments in amphibian urinary bladder. *Proc. Elect. Microscopic. Soc. Am.*, **46**, 324-325.

Mia, A.J., Oakford, L.X. and Yorio, T. 1989. Alterations in surface substructure and degranulation of subapical cytoplasmic granules by mezerein in toad urinary bladder epithelium. *Proc. Elect. Microscopic Soc. Am.*, **47**, 916-917.

Mia, A.J., Oakford, L.X. and Yorio, T. 1989. Modulation of cytoskeletal organization and cytosolic granule distribution by verapamil in amphibian urinary bladder. *J. Cell Biol.* **107**, 772.

Mia, A.J., Oakford, L.X. and Yorio, T., 1991, Role of PKC isozymes in water transport in toad urinary bladder.*Proc. Elect. Microscopic Soc. Am.* **49** 302-303.

Mia, A.J., Oakford, L.X., Thompson, P.D., Ning, Z.H. and Yorio, T., 1992. Role of PKC Isozyme III (alpha) in water transport in amphibian urinary bladder. Proc. Elect. Microscopic. Soc. Am.

,**50** 796-797.

Mills, J.J. and Malick, L.E. 1978. Mucosal surface morphology of the toad urinary bladder. Scanning electron microscopic study of the natriferic and hydro-osmotic response to vasopressin. *J. Cell Biol.*, **77**, 598-610.

Pearl, M. and Taylor, A. 1983. Actin filaments and vasopressin- stimulated water flow in a toad urinary bladder. *Am. J. Physiol.*, **245**, C28-C39.

Spinelli, F., Grosso, A. and DeSousa, R.C. 1975. The hydro- osmotic effect of vasopressin: a scanning electron-microscope study. *J. Membrane Biol.*, **23**, 139-156.

Spudich, J.A., 1992. Multifaceted aproaches to myosin structure and function in vitro and in vivo. *5th International Congress on Cell Biology, Madrid, Spain, 26th-31st July, 1992,* 13.

Taylor, A., Mamelak, M., Reaven, E., Maffly, R. 1973. Vasopressin: possible role of microtubules and microfilaments in its action. *Science*, **181**, 347-350.

Teitelbaum, I. and Strasheim, A. 1990. AVP stimulates adenylcyclase and phospholipase C in reciprocal fashion in cultured RIMCT cells. Am. J. Physiol. 259: C693-C696, 1990.

Wade, J.B., Stetson, D.L. and Lewis, J.A. 1981. ADH action: evidence for a membrane shuttle mechanism. *Ann. N.Y. Acad. Sci.*, **322**, 106-117.

Yorio, T., Henry, S., Hodges, D. and Caffrey, J.L. 1983. Relationship of calcium and prostaglandins in the antidiuretic hormone response. *Boichem. Pharmac.*, **32**, 113-118.

Yorio, T., Royce, R., Mattern, J., Oakford, L.X. and Mia, A.J. 1985. Inhibition of hydro-osmotic response to vasopressin and hypertonicity by phenothiazines and W7 calmodulin antagonis. *Gen. Pharmac.*, **164**, 347-353.

Yorio, T. and Satumtira, N. 1989. Contributon of the vasopressin V_1 receptor to its hydro-osmotic response.*Biol. Cell.* **66**, 7-12.

FIGURES

Figure 1. SEM of the control toad urinary bladder tissue showing network of microridges over the apical membrane of the granular cells. X15,000.

Figure 2. SEM of the apical membrane surface of the granular cells of the toad urinary bladder tissue showing propagation of small microvilli over the cell surface due to stimulation with MZ 10^{-6}M for 5 min. Tissue swelling is apparent compared to control. X15,000.

Figure 3. SEM of the apical membrane surface of the toad urinary bladder showing propagation of numerous small microvilli due to 5 min MZ 10^{-6}M treatment. X15,000.

Figure 4. SEM of the apical surface of the granular cells of the toad urinary bladder

tissue showing propagation of small microvilli over the membrane surface due to treatment with MZ 10^{-9}M for 5 min. Tissue swelling is not apparent compared to control. X15,000.

Figure 5. SEM of the control A6 cells grown on the ICN Cellogen membrane showing uniform distribution of microridges over the apical cell membranes. X8,500.

Figure 6. SEM of the A6 cells grown on the ICN membrane support showing propagation of microvilli over the apical cell membrane by ADH stimulation. X8,500.

Figure 7. SEM of the A6 cells grwon on the ICN membrane support showing propagation of microvilli over the apical cell membrane due to MZ challenge. X8,500.

Figure 8. SEM of the A6 cells grown on the ICN membrane support showing a large concentration of microvilli over the apical cell membrane due to stimulation with ADH and MZ. X8,500.

Figure 9. Localization of PKC isozyme III in MZ-treated toad urinary bladder tissue using protein A-gold (10nm) showing gold particles localized on the cytoplasmic body (arrow). X17,000.

Figure 10. Localization of PKC isozyme III in MZ-treated toad urinary bladder tissue

using protein A-gold (10nm) showing gold particles localized on the cytoplasmic body (arrow) which is associated with a large number of actin microfilaments. X60,000.

Figure 11. Localization of PKC isozyme III in ADH-treated toad urinary bladder tissue using protein A-gold (10nm) showing the distribution of gold particles in a discrete cytoplasmic body (arrow). X60,000.

Figure 12. Immunogold localization of PKC isozyme by protein G- gold particles over the apical plasma membrane (arrow) adjacent to a microvillus in the ADH-stimulated toad urinary bladder tissue. X120,000.

Figure 13. Immunogold localization of PKC isozyme III by protein G-gold showing the presence of gold particles within the microvillus (arrow) in the ADH-stimulated toad urinary bladder tissue. X39,000.

Figure 14. Immunogold localization of protein kinase C isozyme III in ADH-stimulated toad urinary bladder tissue using protein G-gold probes showing localization of gold particles (arrow) in the cytoplasm. X39,000.

Figure 15. Localization of PKC isozyme III in MZ-treated toad urinary bladder tissue using protein G-gold and showing PKC translocation to the apical plasma membrane

(arrow). X60,000.

Figure 16. Control toad urinary bladder section treated with 0.1% BSA showing no labeling with protein A-gold probes. X60,000.

Figure 17. Dose-response curve of vasopressin (ADH) effets on cyclic AMP formation in A6 cells. X60,000.

Figure 18. Time-response curve of vasopressin (ADH) effects on cyclic AMP formation in A6 cells as compared to control. X60,000.

Figure 19. Comparison of vasopressin (ADH) and *forskolin* effects on cyclic AMP formation in A6 cells as compared to control.

Figure 20. Time-dependent curves showing linear distribution of PKC activity in the membranes and in the cytosol.

Figure 21. Protein curve of PKC activity in the membranes.

Figure 22. Activation of PKC by OAG (a) and by PS (b).

Figure 23. A6 cells membrane Ca^{++} curve.

Other activities:

Under this research project, two African American undergraduate students, Pamler Thompson and Haile Yancy received training in cell biology and biomedicine. Pamler Thompson, graduated in May, 1992, with honors, Summa Cum Laude, and was accepted by The University of North Texas/Texas College of Osteopathic Medicine to begin her graduate studies leading to a Ph.D Degree in biomedicine. Haile Yancy, a Junior in biology at Jarvis Christian College, received further training in biomedicine under Health Career Opportunity Program at the Texas College of Osteopathic Medicine. These two students were replaced by two new undergrad uate students and have been receiving training in biomedical research and cell biology.

Equipment Purchased:

New equipment as approved by the Department of Army was purchased as follows:

Jarvis Christian College

1) Tissue Culture Incubator - for growing tissues

2) Tissue Culture Cabinet - for transfer of tissues under aseptic conditions

3) Autoclave - for sterilizing glass ware and media for culturing amphibian tissues for current research

4) Nikon Inverted Microscope - for examining tissue culture in flask

The laboratory on tissue culture is currently in operation involving culturing of amphibian A6 Kidney Cortical Cells.

Research Contributions

Published Absracts:

a) VASOPRESSIN-INDUCED MORPHOLOGICAL CHANGES IN CULTURED AMPHIBIAN A6 KIDNEY CELLS. FASEB J. 6(5): A957 (1992).

b) WATER PERMEABILITY RESPONSE TO VASOPRESSIN (AVP) OF A6 CELLS GROWN ON ANOCELL FILTER INSERTS. FASEB J. 6(5):A1194 (1992).

c) CHARACTERISTICS OF VASOPRESSIN V_1 AND V_2 ACTIONS IN CULTURED AMPHIBIAN A6 CELLS. *Proc. 5th International Congress on Cell Biology, Madrid,* Spain, 112, 1992.

d) ROLE OF PKC ISOZYME III ( ) IN WATER TRANSPORT IN AMPHIBIAN URINARY BLADDER Proc. Elec. Micr. Soc. Amer. (1992)

Full-length paper to be submitted:

a) Influence of filter supports on transport characteristics of cultured A6 kidney cell (To be submitted to the American Journal of Physiology - See accompanying manuscript)

Research in Progress:

a) Tissue blocks of experiments using the amphibian urinary bladders, stimulated by four different concentrations of mezerein (MZ), an activator of PKC, at 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}M, were sliced with a diamond knife for TEM studies of cytoplasmic fine structures to correlate with the SEM results reported earlier on project activities (8/1/91-10/31/91). The ultrathin sections, mounted on bare nickel grids, were stained with uranyl acetate and lead citrate for TEM observations.


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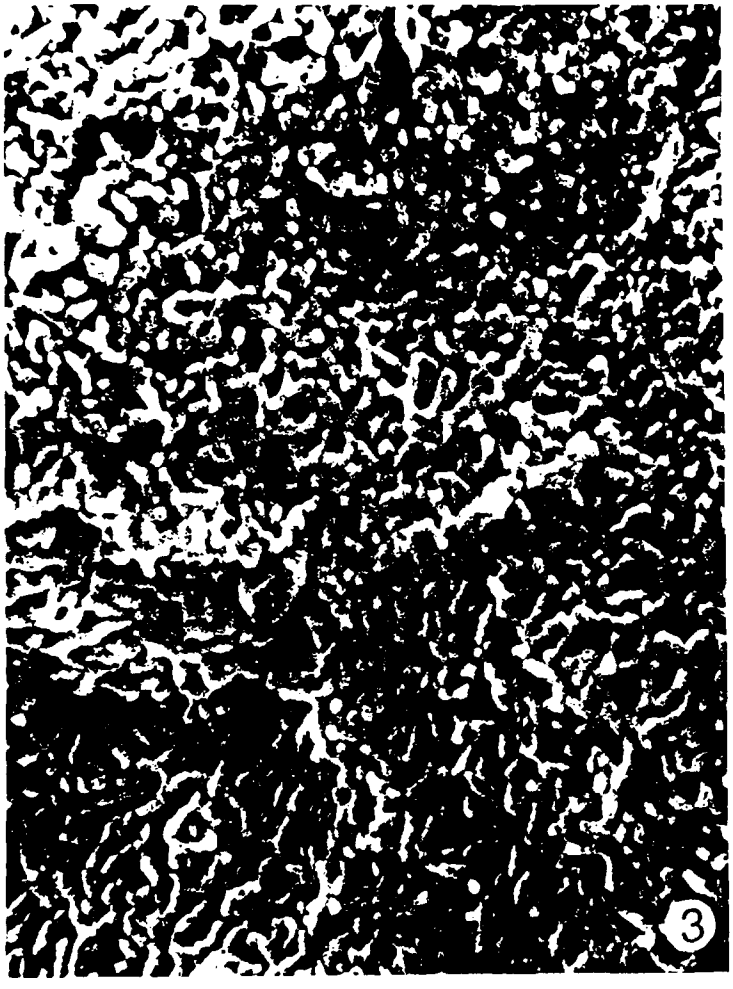
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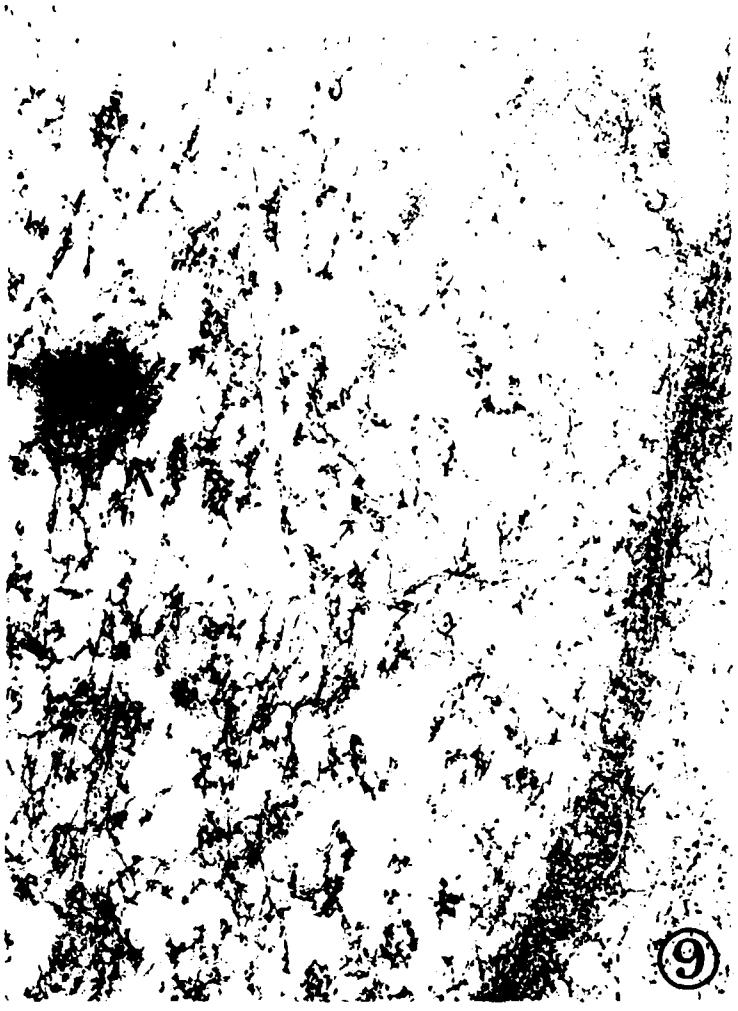
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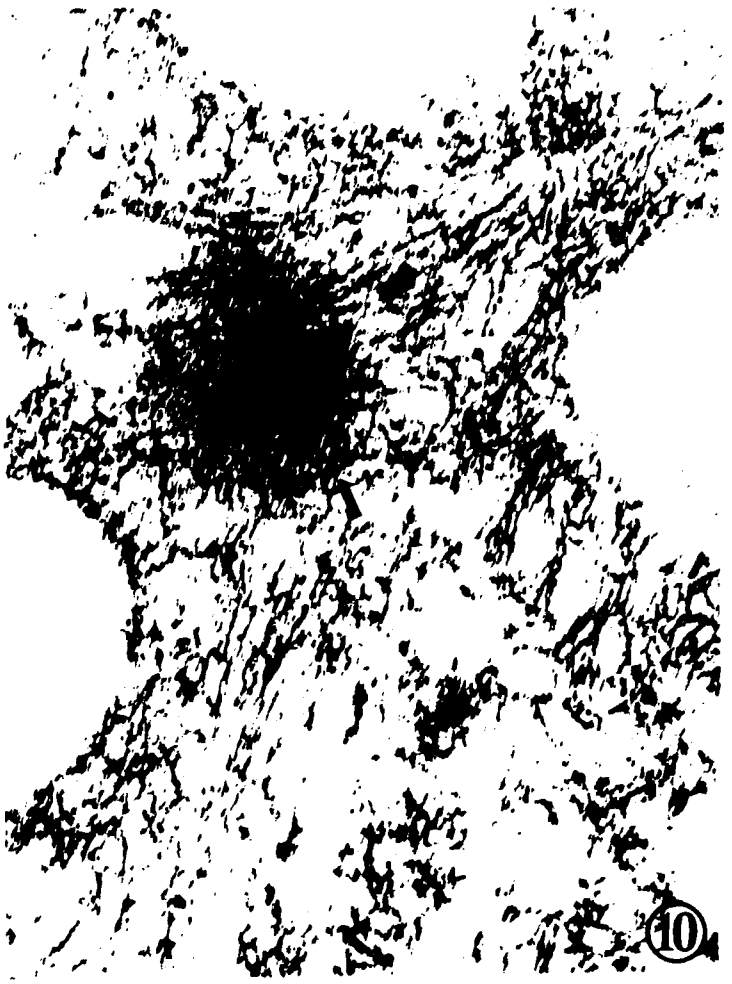
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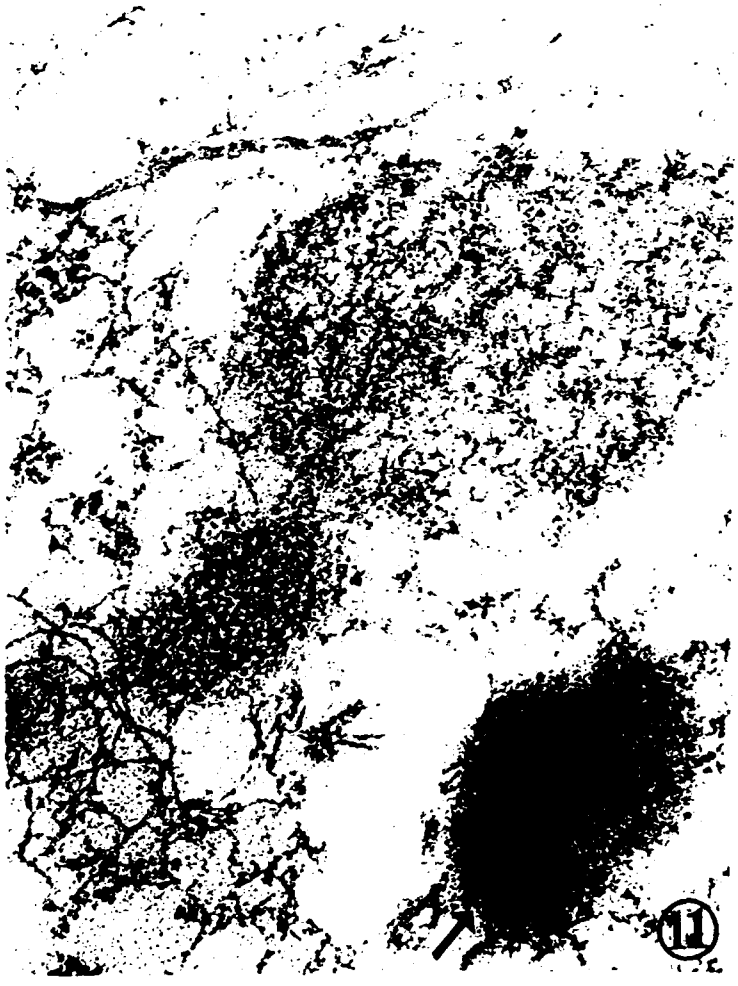
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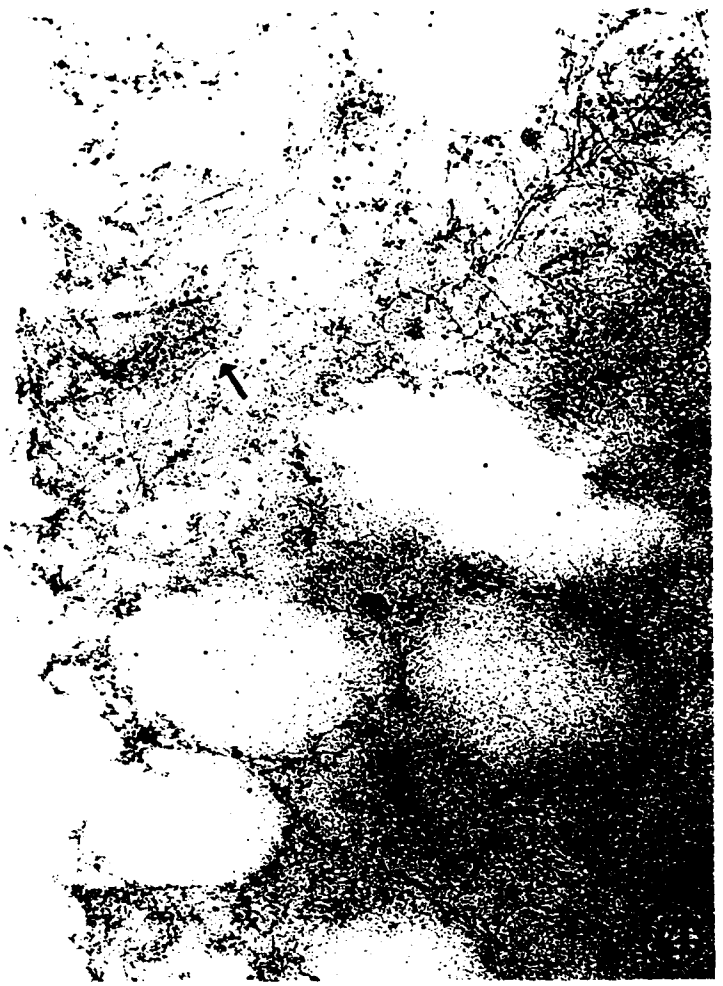

TABLE 1. Electrical Properties of A6 Cells Grown on Anocel Filters

TREATMENT	Potential Difference (PD- mV)	Short-Circuit Current (I_{sc}- $\mu A/cm^2$)	Resistance (R-$k\Omega \cdot cm^2$)
Control	37 ±5	8 ± 1	4.6
AVP (10 min)	31 ±4	11 ± 1*	2.7 *
AMILORIDE (30 min)	15 ±3**	3 ± .6**	4.9**
AMPHOTERICIN B (30 min)	28 ± 3***	24 ±18***	0.6***

The data represents the Mean ±S.E. of eight separate experiments. *p<.05 from control. **p<.05 from AVP. ***p<.05 from amiloride. The AVP concentration was 10 mU/ml; amiloride, 10^{-5}M; and amphotericin B, 10^{-5}M,

Figure 17

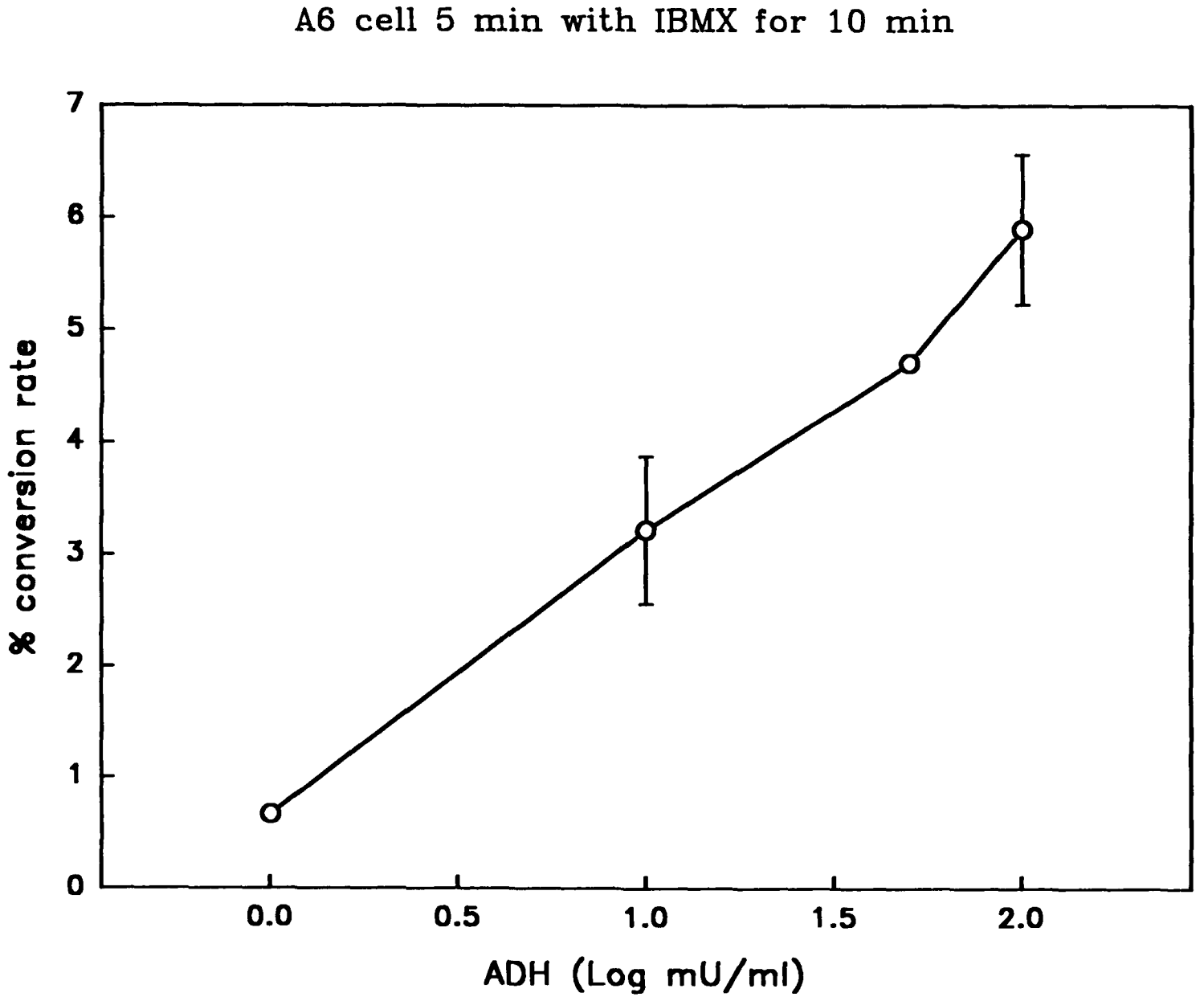
A6 cell 5 min with IBMX for 10 min
% conversion rate
0
1
2
3
4
5
6
7
0.0
0.5
1.0
1.5
2.0
ADH (Log mU/ml)

Figure 18

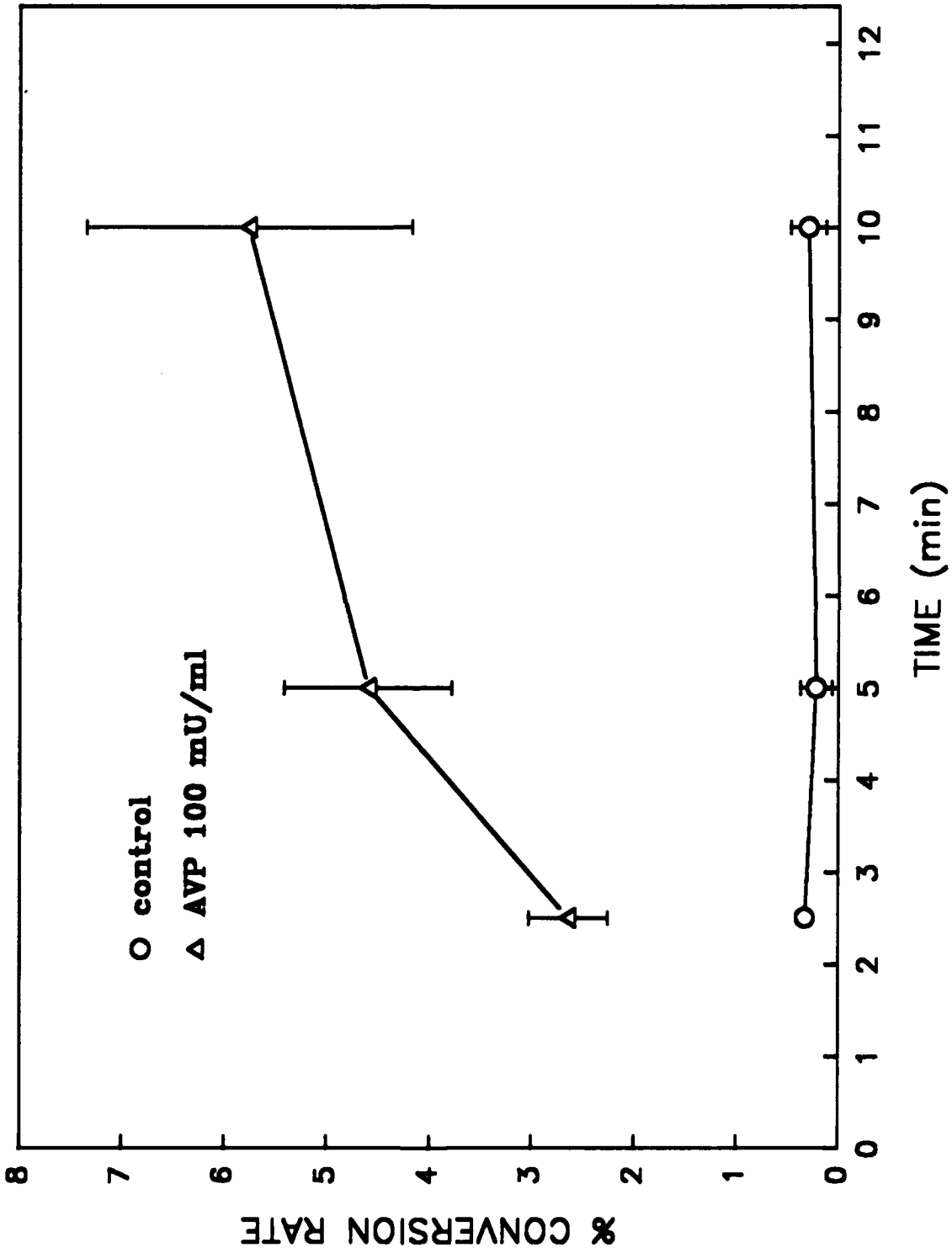
% CONVERSION RATE
TIME (min)
O control
Δ AVP 100 mU/ml
0
1
2
3
4
5
6
7
8
9
10
11
12

Figure 19

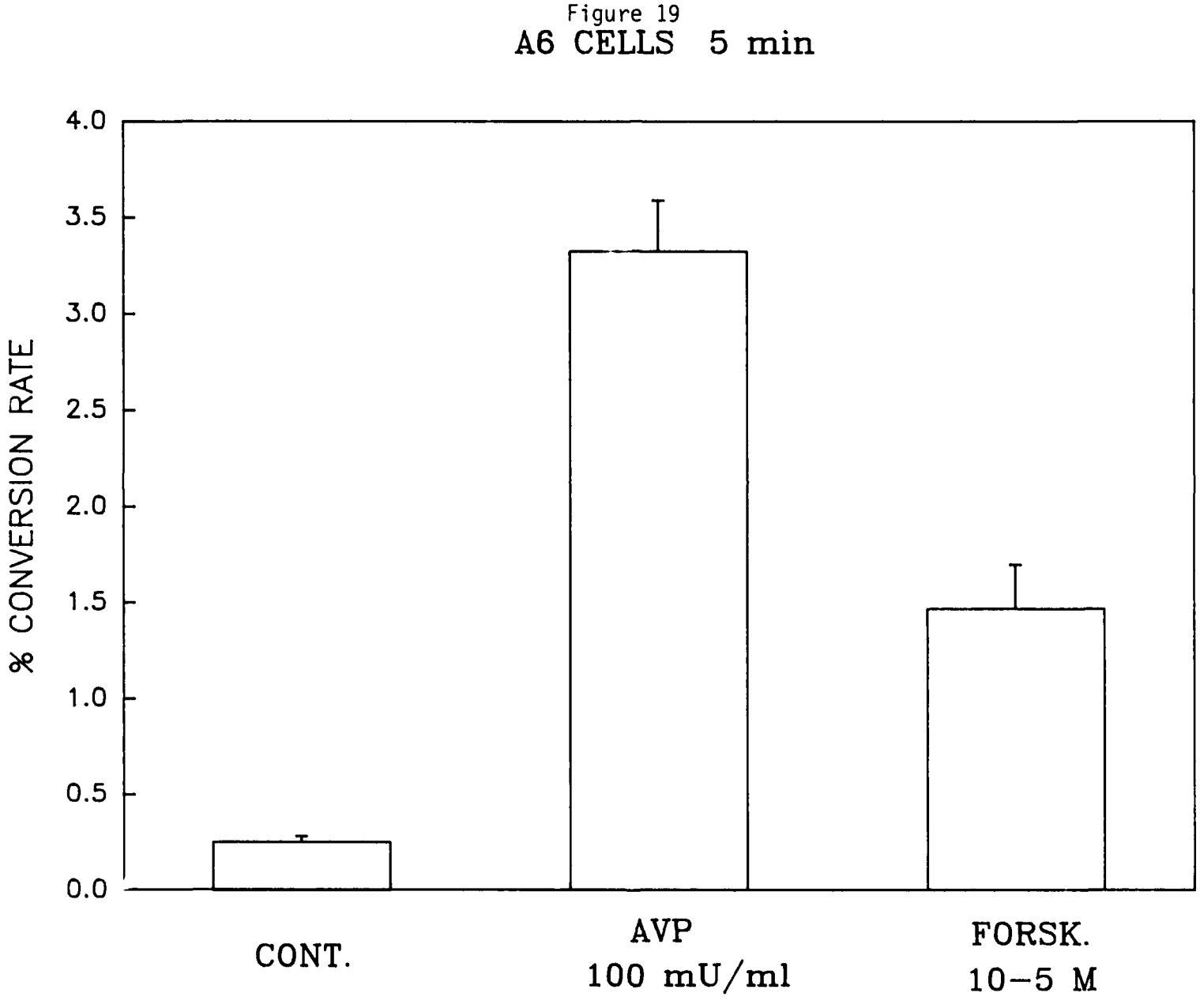
A6 CELLS 5 min
% CONVERSION RATE
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CONT.
AVP
100 mU/ml
FORSK.
10−5 M

Figure 20

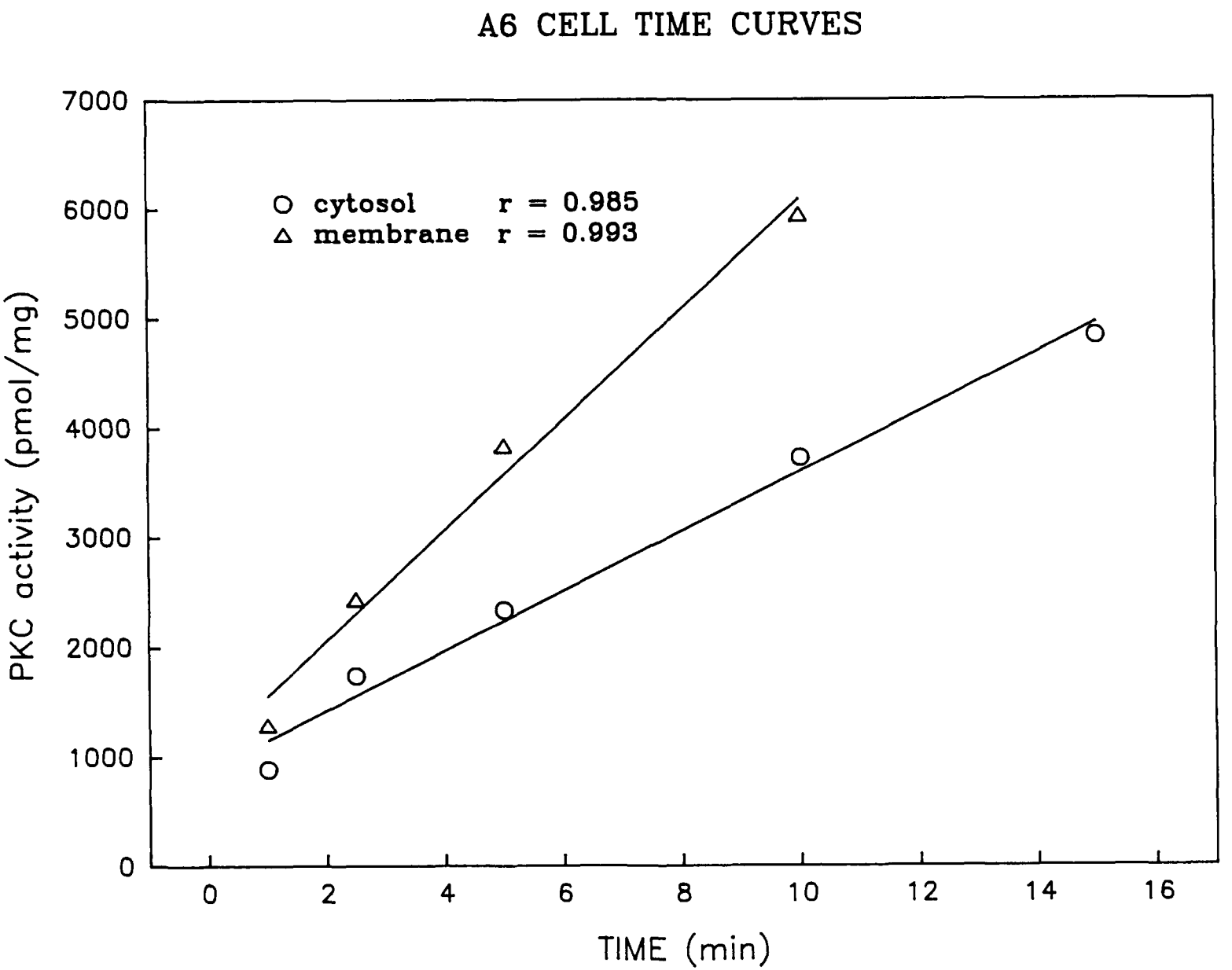
A6 CELL TIME CURVES
cytosol r = 0.985
membrane r = 0.993
PKC activity (pmol/mg)
TIME (min)
0
1000
2000
3000
4000
5000
6000
7000
0
2
4
6
8
10
12
14
16

Figure 21

A6 CELL MEMBRANE PROTEIN CURVE

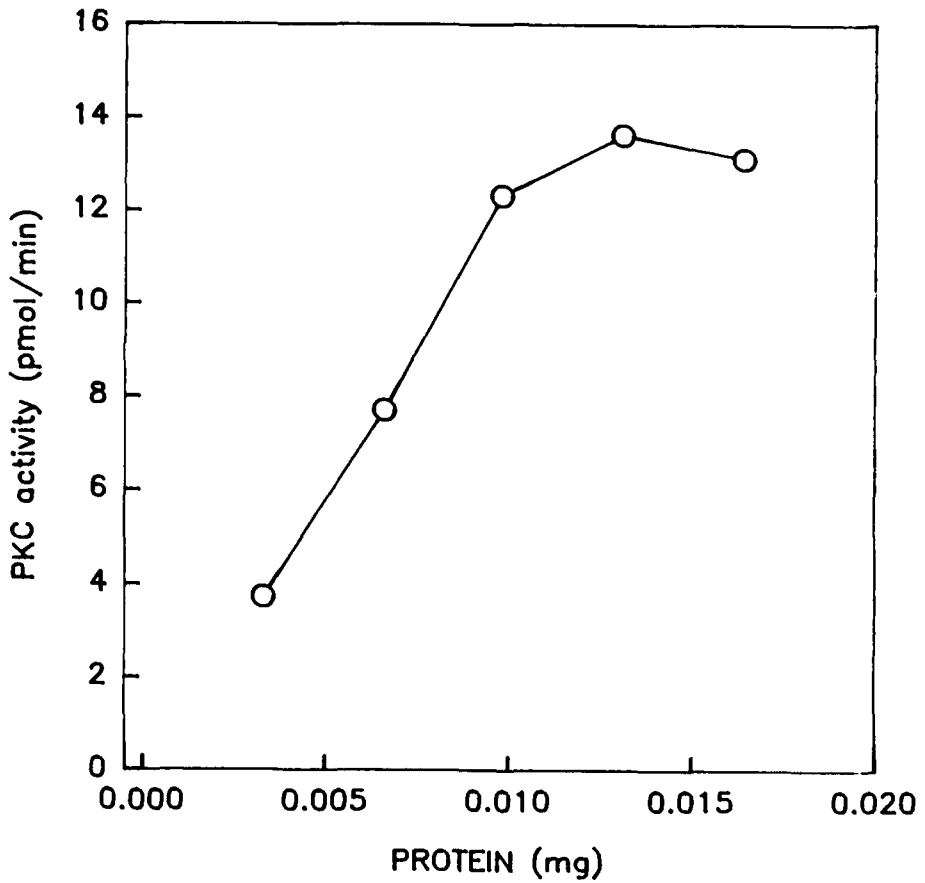

Figure 22a

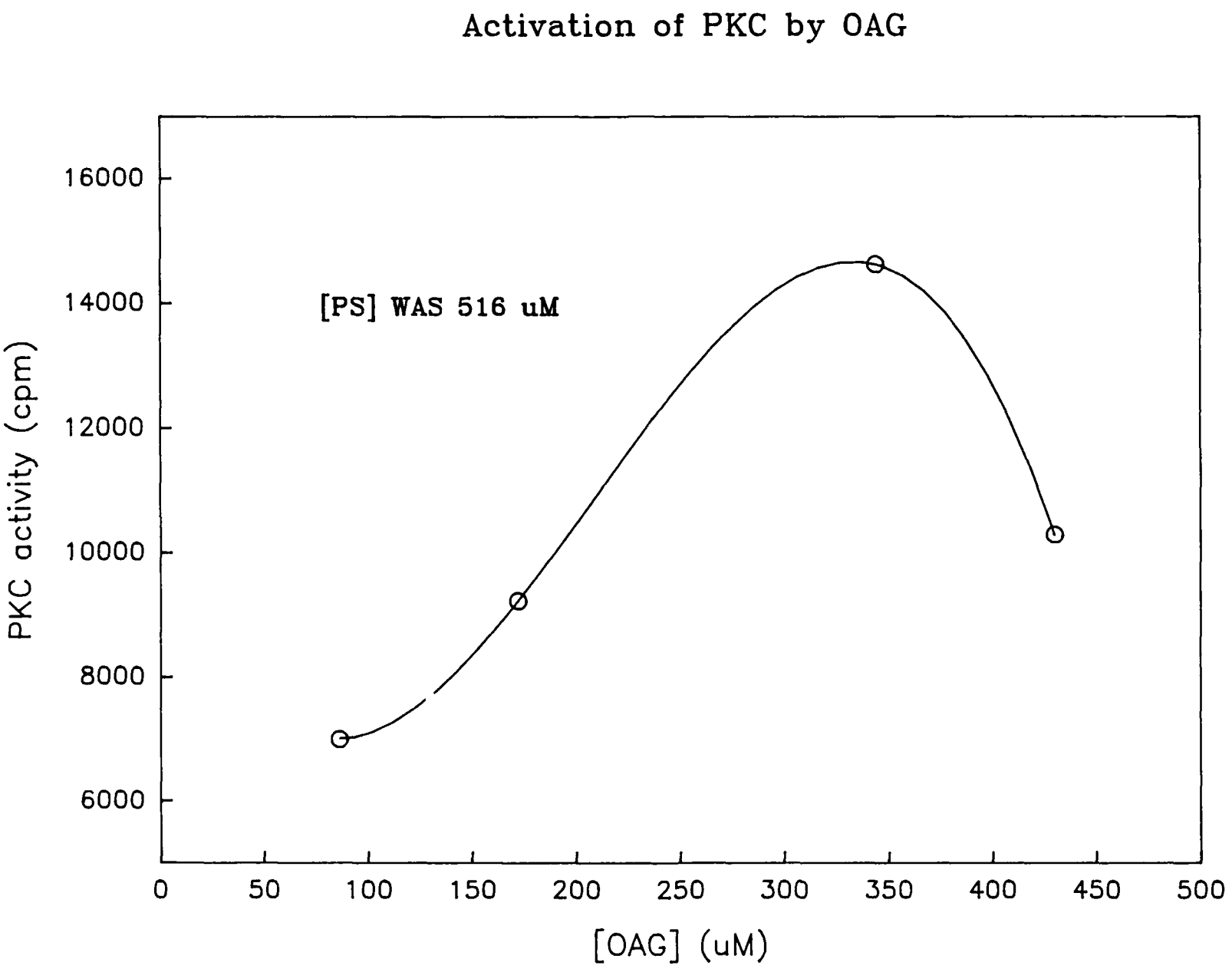
Activation of PKC by OAG
[PS] WAS 516 uM
PKC activity (cpm)
16000
14000
12000
10000
8000
6000
0
50
100
150
200
250
300
350
400
450
500
[OAG] (uM)

Figure 22b

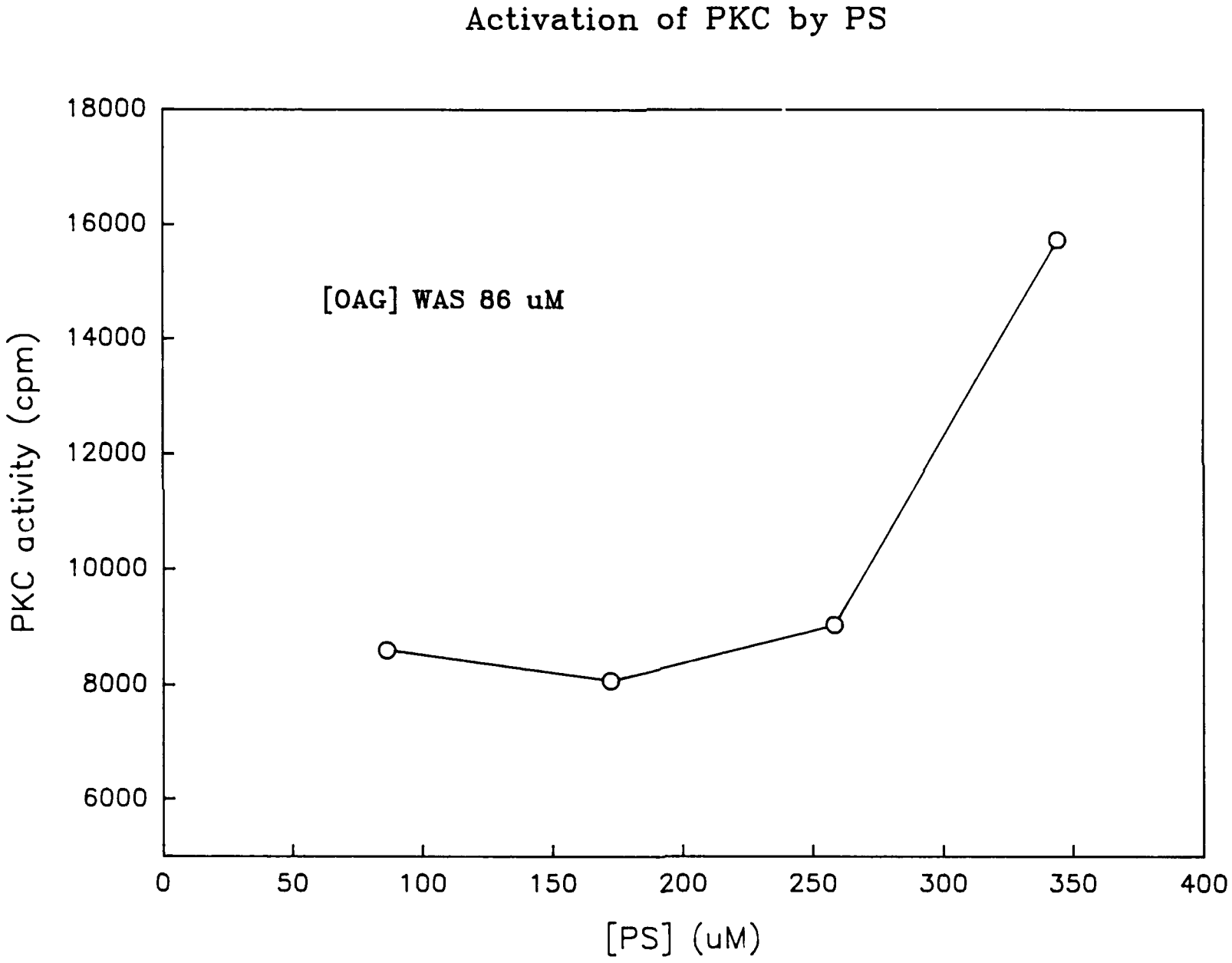
Activation of PKC by PS
[OAG] WAS 86 uM
PKC activity (cpm)
18000
16000
14000
12000
10000
8000
6000
0
50
100
150
200
250
300
350
400
[PS] (uM)

Figure 23

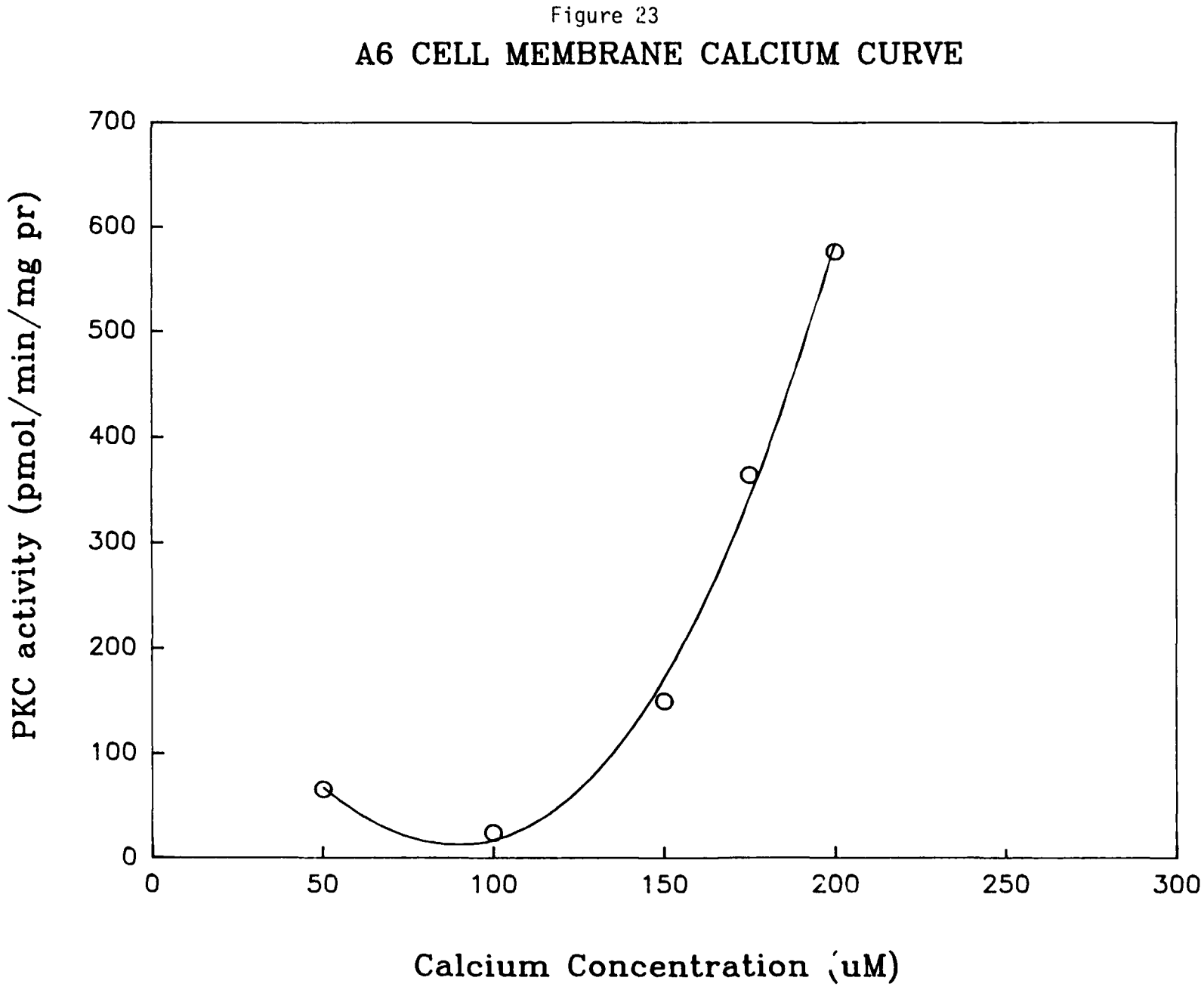
A6 CELL MEMBRANE CALCIUM CURVE
PKC activity (pmol/min/mg pr)
700
600
500
400
300
200
100
0
0
50
100
150
200
250
300
Calcium Concentration (uM)

Figure 24

A6 CELL MEMBRANE PMA AND MZ CURVES