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PRINCIPAL INVESTIGATOR: Dr. Abdul J. Mia

PI ADDRESS: Division of Science and Mathematics
Jarvis Christian College
Hawkins, Texas 75765

CO-PRINCIPAL INVESTIGATOR: Dr. Thomas Yorio

CO-PI ADDRESS: Department of Pharmacology
University of North Texas Health Science Center
Fort Worth, Texas 76107

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13. ABSTRACT (Maximum 200 words) Water reabsorption and urinary volume regulation are important processes in maintaining normal physiological function and vasopressin (ADH) regulates water reabsorption by the kidney in response to changes in plasma volume as may occur during dehydration. This process is particularly important to soldiers who are subject to excess heat and fluid deprivation in arid environments. We have been examining the mechanisms whereby ADH enhances water permeability in renal epithelia so we may find measures to enhance the kidney's responsiveness to ADH. Such regimens would maintain the water reabsorptive capacity of soldiers facing harsh conditions. Our observations suggest that an integral component of the water reabsorptive process by ADH is the cycling of water channels from the cytosol to the apical membrane. This cycling process involves exo- and endocytosis mechanisms. We have begun to examine membrane remodeling during hormone washout and a return to basal unstimulated conditions as a means to assess water channel cycling. In particular we were concerned with determining the role of protein kinase C (PKC) in this process and role of calcium mobilization.				
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Role of PKC During Apical Membrane Surface Remodeling In Mezerein Stimulated Toad Urinary Bladder

ABSTRACT

Vasopressin (ADH) promotes rapid osmotic water transfer in renal and toad urinary bladder epithelia by insertion of water channels into the apical plasma membrane. Water channels are then internalized through a complex process of endocytosis following down regulation or by withdrawal of hormone. Previously we reported on the role of endocytosis in apical membrane remodeling during retrieval of water channels following ADH withdrawal. The present study examined the effect of mezerein (MZ), an activator of protein kinase C (PKC), on the nature of apical membrane remodeling by endocytosis as compared to that produced by ADH. This study further evaluated the role of vasopressin V₁ receptor cascade in water channel recycling. Toad urinary bladder sacs were suspended at the end of glass tubes and subjected to an osmotic gradient. These sacs were then stimulated for 10 min to promote exocytosis coincident with enhanced water flow by adding MZ to the mucosal cavities. MZ was then removed by fresh buffer rinses, and the sacs were allowed to undergo endocytosis and apical membrane remodeling for 5, 10, 20, 30 and 60 min. At each specified time interval and at pre-buffer rinses, bladder sacs were fixed and processed for SEM, TEM, immunogold labeling and freeze fracture studies. Control tissues, during pre- or post-buffer rinses, showed little or no apparent apical membrane surface changes indicative of endocytosis. In contrast MZ stimulated tissues, during time-dependent retrieval periods, exhibited sequences of apical membrane internalization and subsequent apical membrane remodeling reminiscent of those observed for ADH challenged tissues. Although surface invaginations by endocytosis were induced in MZ stimulated tissues, their development was slower

than in ADH treated tissues. However, MZ stimulated tissues showed faster membrane recovery than the ADH stimulated tissues. Therefore, the incidence of endocytosis in MZ and ADH stimulated tissues reverses with time of retrieval. Counts of proposed endocytosis at 5 and 10 min into retrieval periods showed apparent increases in the number of endocytosed granular cells with over 31% and 45% in ADH stimulated tissues compared to no more than 3.50% of unstimulated control and 8% of MZ stimulated cells during the same corresponding time periods. This trend was reversed at 20 min post washout with almost 35% of the granular cells in MZ stimulated tissues with evidence of endocytosis, whereas less than 18% of cells in ADH challenged tissues had similar appearances. MZ stimulated tissues showed faster recovery with less than 24% and 10% at 30 and 60 min retrieval periods compared to over 27% and 23% in ADH challenged tissues during the corresponding retrieval periods. These results indicated that MZ, mimicking ADH actions, could induce surface membrane endocytosis, probably through the activation of PKC. Ultrathin sections, freeze fracture replicas and immunogold labeling of PKC isozyme I (γ) in toad urinary bladder indicated possible presence of putative water channels associated with coated vesicles. In addition, PKC III (α) was detected in particulate forms in ADH and MZ responsive A6 amphibian cultured kidney cells using immunofluorescent and FITC labeling techniques. Evidence for vasopressin mediated intracellular calcium mobilization came from video imaging observations. Intracellular calcium was monitored and measured in ADH responsive LLC-PK₁ cultured porcine kidney distal convoluted tubule cells, using Fura2-AM fluorescent dye. Vasopressin increased intracellular calcium partly through activation of oxytocin receptors. It is not clear if this effect functions to co-activate PKC isoforms (in conjunction with diacylglycerol) in enhancing transepithelial water flow.

INTRODUCTION

Eukaryotic cells often recycle intracellular membranes along with various resident proteins by processes of exo- and endocytosis to regulate a variety of cell functions (Wolfe, 1993). The process of exocytosis is essentially involved in the export of membranes and proteins from the cytosol to the apical membrane surface for fusion, whereas endocytosis is likely to recover all or portions of these cellular components in a reverse process into the cytosol. Endocytosis is thus an important cellular restoration process (Pearse and Crowther, 1987; Farquhar, 1985; Schmid, 1992) for cells to return to the normal state. The cycling of water channels in renal epithelia and in amphibian urinary bladder model membranes is thought to involve similar processes of exo- and endocytosis. Vasopressin (ADH) through V_1 and V_2 receptors induce exocytosis and enhances transepithelial water flow in concert with the appearance at the apical membrane surface of putative water channels. This apical membrane remodeling is accompanied by hormone activation of protein kinase A (PKA) and protein kinase C (PKC), in concert with propagation of apical microvilli from the normal phase of microridges (Mills and Malick, 1978; Spinelli et al., 1975; Mia et al., 1983, 1987 and others). The relationship between the ADH V_1 receptor and increased osmotic water flow is still not understood. Activation of the V_1 receptor results in an increase in intracellular calcium and activation of PKC. Mezerein (MZ), an activator of PKC, added to the mucosal surface of toad urinary bladders increased water flow similar to that observed with ADH (Yorio and Satumtira, 1989). This water flow response by MZ was also found to be correlated with the conversion of the normal phase of microridges into numerous microvilli over the apical membrane surface as seen by scanning electron microscopic (SEM) studies, indicating the ability of MZ to induce microvilli in a manner similar to what was observed following

stimulation of bladder tissues with ADH (Mia et al., 1989). This was also found to be accompanied by the activation of several PKC isozymes by MZ, as determined by immunoantibody and protein A-gold labeling techniques (Mia et al., 1991, 1992). Collectively, these observations suggested that the ADH V₁ receptor cascade, including PKC activation, could play an integral role in the insertion of water channels into the apical membrane by exocytosis (Mia et al., 1991).

The process of endocytosis in amphibian urinary bladder tissues has been described (Muller and Kachadorian, 1984; Masur et al., 1984, 1985; Harris et al., 1986; Coleman et al., 1987 and others) with demonstration of relative distribution of tubular vesicles (aggrephores, water channels) within the cytoplasm. However, little is known about the changes in surface topography, associated with induced endocytosis, under conditions that promote water channel retrieval following removal of ADH or MZ (Brown et al., 1990; Coleman et al., 1987; Mia et al., 1993a, 1993b, 1993c, 1994a). The toad urinary bladder serves as a powerful membrane model for ion and water transport processes because of its structural and functional similarities to the renal cortical collecting tubule (Bentley, 1958; DiBona, 1981 and others). Recently, we demonstrated that the apical membrane restoration process was initiated following withdrawal of ADH during normal washout (Mia et al., 1993a, 1993b, 1993c, 1994a). We also demonstrated that the membrane retrieval process continued till the apical membrane was restored to the pre-hormone unstimulated state. Therefore, similar studies were carried out to include the effect of MZ in the membrane restoration process during endocytosis following removal of MZ in an attempt to understand the role of PKC in membrane restoration (Mia et al., 1993c). We conducted time-course experimental studies, using the techniques of scanning and transmission electron microscopy (SEM, TEM) to correlate the changes in apical

membrane surface conformation by endocytosis during 5, 10, 20, 30 and 60 min retrieval periods following withdrawal of MZ.

In addition, PKC isozymes, upon activation, translocate from the cytosol to the particulate membrane for outward transport to the apical membrane surface as reported in oligodendrocytes (Asotra and Macklin, 1993) and MDBK renal cells (Simboli-campbell et al., 1993) respectively, using FITC immunofluorescent antibody labeling techniques. Previously, we reported localization of PKC isozyme subtypes I (γ), II (β) and III (α) in toad urinary bladders stimulated with ADH and MZ for 30 min and 60 min using the sensitive techniques of immunoantibody labeling and protein A-gold probes (Mia et al., 1991, 1992). We discovered the presence of gold particles in association with diffused cytoplasmic bodies, microvilli and in the apical membrane surface. Little is known about the role of PKC following stimulation of bladder sacs with hormone or PKC activators. Here, we report the localization of PKC isozyme I (γ) following stimulation of bladder tissues with MZ for 15 min using immunoantibody and protein A-gold labeling to correlate these results with the freeze fracture deep etching preparations. In addition, the presence of PKC isozyme α was detected in particulate forms in A6 amphibian kidney cultured cells using immunofluorescent antibody labeling and fluorescein isothiocyanate (FITC) following stimulation with ADH and MZ for 15 min. Collectively, these results and previous studies in our laboratory suggested that the effects of ADH on the activation of PKC appeared to involve vasopressin V_1 receptor activation. It is well established that stimulation of this receptor results in the breakdown of membrane polyphosphoinositides into inositol phosphates and diacylglycerol (Yorio and Satumtira, 1989). The inositol phosphate (IP_3) mobilizes intracellular calcium which then serves as second messenger function in activating enzymes essential in the

regulation of transepithelial water transport. The diacylglycerol activates PKC. In the current experiments we utilized video image analysis to ascertain the effects of vasopressin (ADH) on intracellular free calcium concentrations. In particular we attempted to determine the receptor responsible for the increase in calcium concentration mediated by ADH. In these studies, we used the fluorescent dye Fura-2AM to measure free intracellular calcium (Yorio et al., 1992) in LLC-PK₁ porcine kidney cortical cells, as these cells are known to be responsive to ADH and have been in use in various studies of membrane transport. This dye permeates the cell in its ester form. Once in the cell, esterases convert the dye to Fura-2 which is free to interact with calcium. The dye fluorescence is proportional to the free calcium in the cell. A ratio fluorescence technique using a dual excitation computer controlled video imaging allows monitoring of intracellular free calcium in real time.

METHODS

Tropical toads, *Bufo marinus*, purchased from Carolina Biological Supply Company, Burlington, NC, were maintained in an aquatic environment with free access to water and were fed live crickets biweekly.

Experimental Protocol:

Intact urinary bladder sacs, surgically excised from doubly pithed toads, were placed in aerated Ringer's solution (Mia et al., 1983, 1987). The composition of Ringer's solution was as follows (in millimoles per liter): NaCl, 111; KCl, 3.35; CaCl₂, 2.7; MgCl₂, 0.5; NaHCO₃, 4.0 and glucose, 5.0, with pH adjusted at 8.0. An osmotic gradient was instituted by diluting the mucosal solution to 1/10 of the Ringer's solution, and the solution was aerated continuously throughout the

experimental procedures.

SEM Techniques:

Toad hemibladder sacs, as placed in aerated isotonic Ringer's solution, were then suspended at the ends of glass tubes as shown in Figure 1, for experimental use with an osmotic gradient established between the mucosal and serosal sides of the toad bladder sac. The sacs were then stimulated individually by adding MZ to the mucosal cavity for 10 or 15 min. Following stimulation, the sacs were emptied, rinsed quickly with fresh buffer and then allowed to proceed for 5, 10, 20, 30 and 60 for induction of membrane internalization by endocytosis following similar procedures used for ADH stimulated tissues. This was noted as the retrieval period. The sacs were then emptied and fixed at each interval by plunging into 2% glutaraldehyde in PIPES buffer (0.02M). This fixative was then replaced with fresh solution of glutaraldehyde and allowed fixation for 1 hr at room temperature. A postfixation was carried out using 1% osmium tetroxide for 1 hr prior to dehydration. For SEM preparation, bladder sacs were placed in glass vials and processed through exchanges of graded acetone and liquified Peldri II for critical point drying. During final exchange, Peldri II was allowed to penetrate thoroughly into the tissues prior to solidify in closed vials by allowing the temperature to drop below 23°C. The solidified Peldri II was allowed to sublime in a running fume hood for critical point drying. The dried specimens were mounted on clean aluminum stubs using silver paint. This preparation was gold coated in argon environment in a sputter coater prior to surface viewing in the SEM.

Immuno-Gold Cytochemistry:

Paired toad urinary bladder tissues were exposed to either drugs or vehicle during experimental procedures prior to fixation in 2% glutaraldehyde in PIPES

buffer for 1 hr. These tissues received no postfixation with osmium tetroxide but rinses in PIPES buffer. Each tissue sample was minced into small pieces with a new razor blade prior to dehydration through exchanges of ethanol and L.R. White to cast into individual blocks in pure L.R. White resin using gelatin capsules. Polymerization was carried out in a vacuum oven in closed casules for 15 hr at 60°C. Tissue blocks were trimmed and sliced with a diamond knife for collecting on bare nickel grids. For indirect immunogold labeling of PKC isozymes γ , each grid was floated on a drop of 0.1% BSA made in isotonic Ringer's solution containing a monoclonal antibody of PKC isozyme γ IgG, in rabbit (1/25 dilution) for 2 hr at room temperature in a moist chamber. The grids were then exposed to protein A-gold probes (10nm with dilution at 1/25) for 2 hr at room temperature in a moist chamber for binding with anti-PKC isozyme. These grids received buffer rinses between treatments. Air dried grids were stained with saturated uranyl acetate in 70% ethanol and lead citrate and subjected to TEM observations. To determine the degree of non-specific binding with protein A-gold particles, the control grids were exposed to 0.1% BSA (no PKC antibody) or to a mixture of PKC I enzyme and PKC I antibody before exposing to protein A-gold particles. Control grids showing the presence of only few gold particles were rated to be relatively free of non-specific binding with gold probes.

Cell Culture:

The A6 amphibian kidney cultured cells have been found to be useful in vasopressin regulated polarized membrane flow studies (Verry et al., 1993). These cells, derived from the kidney cortical tissues of *Xenopus laevis*, were cultured on glass cover slips, which were placed in sterile plastic petri-dishes according to procedures described previously (Candia et al., 1993). A medium of NCTC-135 with

L-Glutamine, without Sodium Bicarbonate and with a 0.1 ml penicillin streptomycin additive was used per every 100 ml of medium to prevent contamination. This mixture was deemed as cell medium and was used to nourish the cells. These cells were grown for 5-7 days in an incubator at 28°C prior to use for experimental purpose. Cells forming a confluent monolayer on glass cover slips, were removed and rinsed in normal Ringer's solution for use. LLC-PK₁ cells, derived from porcine kidney cortex were similarly grown in tissue culture media. All culture media contained 20 ug/ml gentamycin, 10 Units/ml penicillin and 10 ug/ml streptomycin. Culture cells, washed and suspended in Ca²⁺, Mg²⁺-free media, and then transferred to falcon flasks, Anocell or ICN filter supports, or petri dishes containing microscope glass cover slips, as required. Cells were grown in tissue culture incubator in 5% CO₂/air in humidified environment at 37°C. These kidney cells were grown in sterile culture in GIBCO NCTC 109 culture media. With changes with fresh culture media three times a week, and following confluency in 2-3 days, cells were used for detection of intracellular calcium using video imaging analysis and Fura2-AM.

Immunofluorescent Microscopy Using FITC:

For immunofluorescent antibody labeling, A6 amphibian kidney cultured cells were grown as a monolayer on glass cover slips placed in sterile plastic petri-dishes. These cells were allowed to grow for 5-7 days to confluency prior to experimental intervention. The glass cover slips were then removed from the media, and rinsed in normal Ringer's solution for use. Control and experimental tissues were exposed to 0.002% DMSO, ADH 100mU/ml or MZ 10⁻⁶ M respectively for 15 min. They were then fixed without buffer rinses in 3% formaldehyde in PBS for 30 min, rinsed in PBS buffer, air dried and stored in the refrigerator. Air dried tissues were rinsed in buffer and then rendered permeable in cold methanol for 10 min (Yorio et

al., 1985). The permeablized cells were rinsed in PBS buffer, and then exposed to PKC antibody isozyme III (α) at 1/15 dilution for 1 to 2 hrs in a moist chamber at room temperature. Tissues received a thorough wash in the same buffer and treated with antibody IgG conjugated with fluorescein isothiocyanate (FITC; 1/16 dilution) for 2 hrs in the dark at room temperature. Cells were washed in PBS buffer and deionized water to mount on medium containing 9:1 glycerol and PBS buffer. Control cells received treatment only with 0.1% BSA and FITC. The preparations were examined in a Nikon Optiphot fluorescent microscope equipped with a narrow epifluorescent barrier filter. Photomicrographs were recorded on Kodak Tri-X film with film speed of ASA 400 and developed in a solution of Microdol-X developer.

Freeze Fracture Replica Preparations:

Freeze fracture studies were carried out using the whole toad urinary hemibladders which were set up as "sacs", and aerated continuously in isotonic Ringer's solution. The mucosal side of the bladder was filed with 1/10 concentration of Ringer's solution while the serosal side was bathed in isotonic Ringer's solution, thus establishing an osmotic gradient. Hormone or drug was added to the serosal side for experimental tissues and vehicle was added to controls. At various time intervals, the tissues were fixed by placing them in 2% glutaraldehyde in PIPES buffer. Fixation was allowed to proceed for 1 hr, then washed in PIPES buffer and cyroprotected with 30% glycerol in PIPES buffer. Pieces of tissues were placed on standard copper holders and plunged into liquid freon cooled to -190°C in a liquid nitrogen bath. Freeze fracture deep etching was performed in a Balzers 400T apparatus at -100°C with a knife pre-cooled with liquid nitrogen. The fractured surface was shadowed with evaporated platinum at an angle of 35° , and then coated with carbon at a 90° angle. The replicas were removed from the freeze fracture

apparatus and coated with 1% collodin in amyl acetate while still frozen. The collodin was allowed to dry and the tissues in the replicas were digested in 5.25% Na hypochloride. Washed replicas were then placed on bare copper grids for examination in the TEM.

Determination of Intracellular Calcium:

Free intracellular calcium was measured in ADH responsive LLC-PK₁ porcine kidney cultured cells, grown on glass cover slips using a dynamic video imaging system and fluorescent dye Fura2-AM. This dye upon exposure to cultured cells permeates into the cells and allows measurement of calcium. The epithelial cultured cells were allowed to incubate in Fura2-AM dye for 30 min for dye to penetrate, and then washed several times to remove extracellular medium. This preparation was then placed in a temperature controlled holder in place on the stage of a Nikon fluorescent microscope interfaced with video camera monitor and computer. The images as scanned for free calcium were recorded by computer software, and then free calcium values were calculated and determined using a ratio fluorescent technique.

Drugs:

8-Arginine vasopressin (ADH) and mezerein (MZ) were purchased from Sigma Chemical Co., St. Louis, MO. The concentration of ADH used in the experimental protocol was 100 mU/ml and was added to the serosal side of the bladder sac. The concentration of MZ used in the experiments was 10^{-6} M and was added to the mucosal side of the bladder. The MZ was dissolved in a stock solution of DMSO to a concentration of 0.05 M. The concentration of DMSO added was 0.002%.

RESULTS

SEM Studies:

We have used the technique of SEM to study membrane recycling in toad urinary bladders under a variety of experimental conditions, including following removal of ADH actions by a series of fresh buffer rinses (Mia et al., 1993a, 1993b, 1994). The current study was undertaken to evaluate the pattern of membrane retrieval by endocytosis following the addition and removal of MZ in urinary bladder tissues, as a means to assess the role of PKC in this process. Figures 2 and 3 are presented to illustrate the control and MZ stimulated toad bladder tissues. Control tissues during pre- or post-buffer rinses, regardless of the length of time, showed little or no visible signs of membrane internalization by endocytosis. Many scanning electron micrographs of control tissues representing each stage of retrieval at 5, 10, 20, 30 and 60 min were made to observe the relative distribution of endocytosis. Figures 4 and 5 represent two low power SEM images of control tissues at 20 and 60 min post washout periods, showing such microstructure disposition of the apical membrane surfaces, essentially dominated by the presence of a phase of microridges, and showing little or no signs of endocytosis. This phase of membrane profile was altered when MZ challenged bladder sacs were allowed to recover for 5 min following removal of MZ. A comparative morphometric analysis of endocytosis as induced by withdrawal of MZ was made at various retrieval time periods to correlate the relative distributions of endocytosis in MZ, ADH and control tissues. These results are presented in a graph (Fig. 6). These studies revealed no more than 3.50% of control granular cells showing signs of membrane invagination indicative of endocytosis (Mia et al, 1994). Figure 7 unveils the membrane profile at 5 min retrieval period with the first signs of endocytosis in the form of slight inpocketings

of the apical plasma membranes of the granular cells. At this time of retrieval, no more than 5% of granular cells showed membrane invagination, whereas ADH-stimulated tissues during corresponding retrieval period, showed over 31% of granular cells with endocytosis as represented in the graph (Fig. 6). Membrane retrieval by endocytosis appeared to progress with increased retrieval time, and therefore at 10 min retrieval period following MZ stimulation, the number of granular cells showing membrane retrieval was slightly over 7% in contrast to 46% of cells involved in endocytosis in ADH-stimulated tissues. Figure 8 is presented to unveil the membrane surface with distribution pattern of endocytosis at 10 min retrieval following withdrawal of MZ from the mucosal surface of the toad bladder. At this time some granular cells also showed a loss in the membrane microstructures (arrows), leaving a flattened membrane surface with no microridges and microvilli. This may represent a membrane transition state in granular cells undergoing invagination by endocytosis. Bladder sacs with 20 min into retrieval continued to show membrane transition, and showed a dramatic change in the number of cells involved in endocytosis. At 20 min retrieval period, close to 35% of granular cells showed endocytic involvement in MZ stimulated tissues as shown in the graph (Fig. 6) compared to 17.21% involved cells in ADH-stimulated tissues. In addition, few granular cells showed some degree of membrane restoration as shown in Figure 9 (arrows) by gradual narrowing of the surface openings. An enhanced view of the microstructure is presented in Figure 10 showing the gradual return of the membrane surface of the endocytosed pit with a gradual reconstruction of the flattened membrane surface into microridges.

The apical membrane retrieval in MZ stimulated bladder tissues is progressive like that seen with ADH-stimulated tissues. Figure 11 represents a global view of the

toad bladder sac containing a reduced number of invaginations at 30 min retrieval periods following withdrawal of MZ from the bladder sac. At this retrieval time, a number of cells showed recovery while other cells continued to exhibit slight surface invagination (Fig. 12), similar to 5 min retrieval following ADH removal, indicating that MZ treated cells undergo slow membrane remodeling. The membrane restoration can best be seen in enhanced SEM images as presented in figures 13 and 14 with membranes showing gradual return to a normal stage containing microridges from transitional phase lacking both microvilli and microridges. At this time of retrieval, following buffer rinses of MZ stimulated tissues, showed over 21% of cells with signs of endocytosis versus 27% in ADH-stimulated cells as shown in the graph (Fig. 6). At 60 min retrieval, both MZ- and ADH-stimulated tissues showed almost a complete restoration of the apical membranes in the granular cells (Figs, 15. 16) respectively, now showing the membrane surfaces like unstimulated control tissues (Figs. 2, 4). At 60 min retrieval period, only 9.47% of MZ stimulated cells showed signs of endocytosis in contrast to 23.12% involved cells in ADH-stimulated tissues. Experiments were also designed to test the hypothesis that continued permeability to water was dependent, in part, on the osmotic gradient for water flow. Bladders were set up as sacs as in Figure 1, and ADH was added after an initial baseline waterflow measurement. ADH was then removed from both bladder sacs, and the sacs were allowed to retrieve for a period of 2 hr while water flow was measured continuously. It was found that the experimental sac, with maintenance of osmotic gradient by periodically replacing fluid inside by fresh diluted buffer, did not maintain the water flow. On the contrary, the water flow returned to baseline faster than if the gradient was dissipated through ion and water flow changes (Fig. 17). In addition, there was little or no observable difference in membrane restoration in tissues which received

periodic adjustment of osmotic gradient from that which received no osmotic gradient adjustment during 60 min retrieval period. At 60 min, the percent of endocytosed cells in MZ-stimulated tissues was 9.47% as compared to 23.12% for ADH-stimulated tissues shown in the graph (Fig. 6).

Amphibian epithelia contain a ADH V_1 receptor coupled to phosphoinositide metabolism and ADH stimulates release of inositol trisphosphate (Yorio et al. 1985; Yorio and Satumtira, 1989). In addition, PKC isozymes, upon activation translocate to particulate membrane fractions for outward transport to the apical membrane as reported in oligodendrocytes and in renal tissues respectively (Asotra and Macklin, 1993; Simboli-Campbell et al., 1993). We also discovered the presence of several PKC isozymes in association with diffused cytoplasmic bodies and in the apical membrane in toad urinary bladder tissues stimulated with ADH and MZ for 30 and 60 min (Mia et al., 1991, 1992), suggesting a role of PKC in osmotic water transport, perhaps in the involvement of cycling of water channels. Here, we report the localization of PKC isozyme γ following stimulation of bladder tissues with MZ for 15 min using immunoantibody and protein A-gold labeling techniques. Figure 18 represents a control tissue exposed to monoclonal anti-PKC isozyme I antibody (γ) and protein A-gold (10nm) particles in which localization of few gold particles are viewed randomly distributed in the cytosol. To determine specific labeling, tissues treated with ADH for 15 min, exposed to a mixture of PKC enzyme I and anti-PKC I antibody, and protein A-gold particles to see that gold labeling was not greater than in control tissues primary antibody was omitted. Figure 19 represents such a treated tissue that was stimulated with ADH. There is a general lack of the presence of gold particles in this tissue, similar to that observed for control tissues which were exposed only to BSA with omission of primary antibody. Tissues challenged with MZ 10^{-6} M

for 15 min showed localization of gold particles in association with anti-PKC I (γ) predominantly surrounding the coated vesicles as shown in Figure 20 (arrow). It is uncertain whether the coated vesicles were released from the apical membrane during membrane restoration process. However, evidence indicates that this may be the case as several coated vesicles laced with gold particles appear to have migrated from the apical membrane into the cytosol as shown in Figures 21 (arrows). Coated vesicles encountered in TEM ultrathin sections and freeze fracture preparations are presented in Figures 22 and 23. These coated vesicles are distinctly different than the caveolae which are released from the membrane surfaces of the microfilament-rich cells present in toad urinary bladder sacs (Fig.24) and reported by Smart et al., (1993) in another type of tissue.

We also detected the presence of PKC isozyme III (α) in A6 amphibian kidney cultured cells grown as a monolayer on glass cover slips. Figure 25 represents a phase contract micrograph showing A6 cells on a glass cover slip. These cells received exposure to 0.002% DMSO for 15 min and served as control. Control cells, following fixation in 3% formaldehyde and then permeabilization in cold methanol, were exposed to 0.1% BSA and fluorescein isothiocyanate (FITC). These control cells showed the presence of diffused fluorescence with no detection of particulate structures (Fig. 26) Tissues exposed to ADH for 15 min, and then labeled with monoclonal anti-PKC isozyme III (α) and FITC, showed the presence of this isozyme in particulate forms (Fig. 27) indicating activation of PKC by ADH-stimulated bladder tissues. Similar treatment with MZ for 15 min also resulted in the detection of particulate structures (Fig. 28) as those seen with ADH-stimulated tissues. These results suggest a possible role of PKC in ADH actions, possibly as part of the overall water transport process.

Studies in our laboratory have suggested that the effects of ADH on the activation of PKC appear to involve ADH V_1 receptor activation. It has been well established that stimulation of this receptor results in the breakdown of membrane polyphosphoinositides into inositol phosphates and diacylglycerol. The inositol phosphate (IP_3) mobilizes intracellular calcium which then serves as a second messenger function in activating enzymes essential in the regulation of transepithelial water transport. The diacylglycerol activates PKC. In the current experiments we utilized video image analysis to ascertain the effects of ADH on intracellular calcium concentrations. We used the fluorescent dye Fura-2AM to measure free calcium using LLC-PK₁ cells grown in tissue culture. An SEM image (Fig. 29) depicts the surface profile of LLC-PK₁ cells as containing a scattered distribution of microridges with no apparent cellular tight junctions. As can be seen in Figure 30, 10^{-8} M AVP increased intracellular calcium in LLC-PK₁ cells. These cells are derived from the porcine kidney distal convoluted tubule, a known site for vasopressin action. Figures 31 and 32 compare the response of ADH to oxytocin, another peptide that has known antidiuretic effects in some kidney models. Oxytocin which also contracts smooth muscles by increasing intracellular calcium acts on oxytocin receptors since the increase in calcium by oxytocin appeared to be blocked by an oxytocin peptide antagonist (Fig. 33). Surprisingly, the effects of ADH on intracellular calcium were blocked by the oxytocin antagonist, suggesting that the increase in intracellular calcium by ADH may be mediated through an oxytocin receptor (Fig. 34). This is demonstrated quite readily in Figure 35, where ADH effects before and after the oxytocin antagonist are presented. The antagonist completely abolished the actions of ADH on calcium mobilization. Additional experiments are planned to test the actions of ADH and oxytocin in the presence of a vasopressin antagonist (V_1), to

determine if the receptor in the porcine kidney does not differentiate between these two peptides, oxytocin and vasopressin. Therefore we are still uncertain of the receptor that is coupled to the calcium signaling process in the kidney. Such answers await further experimentation.

DISCUSSION

Amphibian urinary bladder epithelia represent a remarkable cellular structure, and perhaps unique renal membrane model for experimental studies of water and ion transport dynamics (Gronowicz et al., 1980; Wade et al., 1981; Hays, 1983; Harris et al., 1986); Mia et al., 1983, 1994a and others). These and other studies indicated that the two plasma membranes (apical and basal) of toad urinary bladder like many other transport epithelia (Simmons 1992), which respond to ADH, readily undergo membrane transitions as a process to recycle water channels through exo- and endocytosis. We found the use of toad bladders indispensable in the investigation of membrane retrieval by endocytosis (Mia et al., 1993a, 1993b, 1993c, 1994a). The process of endocytosis in toad urinary bladder occurs following down regulation or withdrawal of hormone actions and when water flow dissipates with recovery of apical membrane water channels back into the cytosol. However, many key questions regarding the mechanism of water channel retrieval process by endocytosis remain unanswered. There has been numerous studies using the elegant techniques of SEM in elucidating the membrane surface dynamics involving exocytosis (Davis et al, 1974; Spinelli et al., 1975; Mills and Malick, 1978; Mia et al., 1983 and others). Observations of the apical membrane transition from microridges to microvilli in response to ADH challenge coincident with enhance osmotic water flow has been well documented. Therefore, the application of the technique of SEM in water transport studies is not new. What is new is the way we can manipulate the toad

urinary bladders to undergo endocytosis under controlled experimental conditions, and the ability to visualize the altered apical membrane surfaces at any time point by SEM (Mia et al., 1993a, 1994a). We can also induce apical membrane internalization by inducing endocytosis deliberately using various interval time periods following withdrawal of ADH or MZ. It is also anticipated that this *in vivo* toad urinary bladder membrane model of endocytosis could be utilized in isolating intact endocytosed coated pits, and perhaps water channels, for determination of their physical and chemical nature as well as their functional activity. Our observations of the apical surface changes associated with the process of endocytosis as induced by MZ appeared to be reminiscent of the ADH-stimulated tissues, suggesting that MZ can mimic the actions of ADH in the induction of surface changes indicative of endocytosis (Mia et al., 1994a, 1994b).

A good deal of literature is available on the chemistry, formation and dynamics of coated vesicles in tissues other than in amphibian urinary bladders (Hirokawa and Heuser, 1981; Brodsky, 1985; Pearse and Crowther, 1987; Heuser and Keen, 1988; Schmid, 1992). The presence of coated vesicles although reported in renal epithelia of Brattleboro rats following application of ADH, have not been described for other renal epithelia, like toad urinary bladders remained (Brown, 1988). Recently, we reported the presence of coated vesicles in ultrathin sections of ADH-stimulated toad urinary bladder tissue (Mia et al., 1994a). Their presence was substantiated in this tissue using the freeze fracture deep etching replica techniques as shown in Figure 23. The coated vesicles as seen in the toad urinary bladders appear in circular arrays surrounding a central membrane vacuole, and look different than those reported in other tissues. We examined numerous ultrathin sections in the TEM to detect the trilamellar knobbed bristles beneath the apical plasma membrane of the endocytosed bladder tissues as evidence of their origin, but found no evidence of their presence.

Therefore, it is not known how these coated vesicles were originated in the toad bladder tissues. In addition, we discovered the presence of several cytosolic putative coated vesicles, laced with gold particles in tissue treated with MZ for 15 min using PKC isozyme I (γ) antibody and protein A-gold particles. These vesicles show a distribution pattern in the cytoplasm suggestive of a process of migration from the apical membrane into the cytosol (Fig. 21). These observations suggest that these vesicles may have their origins at the apical membrane, likely in concert with the process of endocytosis during reverse membrane flow.

In our current series of studies of toad urinary bladder epithelia, we reported that priming of toad bladder tissues with ADH was prerequisite to enhancing the induction of endocytosis following withdrawal of hormone (Mia et al., 1993a, 1993b, 1994a). These results and previous studies on the process of MZ induced exocytosis (Mia et al., 1989) led us to undertake investigation on the phenomenon of endocytosis by tissue challenges with MZ, a non-phorbol activator of PKC, to correlate the role of the ADH V_1 receptor cascade in endocytosis during the retrieval process. PKC, a ubiquitous protein kinase, was found to be implicated in the regulation of diverse cellular functions in a variety of animal tissues (Yoshida et al., 1988). Amphibian epithelial tissue is known to contain ADH V_1 receptors coupled to phosphoinositide metabolism (Ausiello et al., 1987; Schlondorff and Satriano, 1985; Yorio et al., 1983). In addition, our recent experimental studies involving the actions of MZ on water transport processes (Yorio and Satumtira, 1989), and conformational changes in toad bladder epithelia (Mia et al., 1989), provided evidence for a role for PKC in ADH actions possibly in regulating water flow across the toad urinary bladder epithelia. We also reported localization of several PKC isozymes I (γ), II (β) and III (α) singularly, in distinct cytosolic diffused bodies, and at the apical membrane interface of toad urinary bladder granular cells, using specific PKC monoclonal

antibodies and protein A-gold particles in ultrathin sections (Mia et al., 1991, 1992). There are reports that PKC isozymes when activated translocate to intracellular cytoskeletal fractions in cardiac myocytes (Mochly-Rosen et al., 1990), oligodendrocytes (Asotra and Macklin, 1993) and in MDBK renal cells (Simboli-Capmbell et al., 1993). The present report includes localization PKC isozyme I (γ) in association of coated vesicles in MZ challenged toad urinary blader epithelia, using immunogold antibody labeling techniques, and PKC isozyme III (α) in A6 amphibian kidney cultured cells using immunoabtibody and FITC labeling techniques. These results suggest that various PKC isozymes may be involved in cycling of the putative water channels by exocytosis and coated vesicles by endocytosis following stimulation with ADH.

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KEY TO FIGURES

Figure 1. Diagrammatic presentation of the experimental set up of a toad urinary bladder sac for the purpose of measuring transepithelial water flow.

Figure 2. SEM of a control toad urinary bladder sac, retained in Ringer's solution for 10 min showing no evidence of endocytosis in the apical membrane surface. X3,000.

Figure 3. SEM of a toad urinary bladder challenged with MZ 10^{-6} M for 10 min showing no sign of endocytosis. X3,000.

Figure 4. SEM of a control toad urinary bladder retained under an osmotic gradient for 20 min showing no evidence of endocytosis. X3,000.

Figure 5. SEM of a control bladder sac retained under an osmotic gradient for 60 min showing little or no evidence of endocytosis. X700.

Figure 6. Graph represents the percentage of granular cells with endocytosis at 5, 10, 20, 30 and 60 min retrieval periods following withdrawal of ADH and MZ compared to control tissues.

Figure 7. SEM of a urinary bladder sac stimulated for 10 min with MZ and then retrieved for 5 min showing signs of endocytosis in the form of membrane invagination. X3,000.

Figure 8. Bladder tissue stimulated for 10 min with MZ and then retrieved for 10 min showing the presence of increased number of endocytosis. X3,000.

Figure 9. SEM of toad urinary bladder sac stimulated for 10 min with MZ and then retrieved for 20 min showing endocytosis as well as membrane restoration. X3,000.

Figure 10. Enhanced view of the toad bladder sac exposed to MZ for 10 min and then retrieved for 20 min showing the detail surface structure during gradual recovery of an endocytosed pit. X8,000.

Figure 11. A global view of the toad urinary bladder sac at 30 min retrieval following withdrawal of MZ showing few granular cells undergoing apical membrane

restoration. X1,260.

Figure 12. An enhanced view of the toad urinary bladder sac at 30 min retrieval following removal of MZ from the sac interior showing granular cells at various stages of membrane restoration process. X2,500.

Figure 13. MZ exposed bladder tissue for 10 min and then retrieved for 30 min showing the gradual restoration of the endocytosed granular cells. 3,600.

Figure 14. An endocytosed granular cell presented at higher magnification to show the detail microstructures during membrane restoration at 30 min retrieval following withdrawal of MZ X7,200.

Figure 15. A global SEM view of the toad urinary bladder sac showing almost complete restoration of the granular cells at 60 min retrieval following withdrawal of MZ. X1,000.

Figure 16. A comparative global SEM view of the toad urinary bladder sac showing almost complete restoration of the apical membranes at 60 min retrieval following withdrawal of ADH while maintaining an osmotic gradient. X1,000.

Figure 17. Shows the water flow response of bladder sac with intermittent adjustment of the osmotic gradient versus non-adjustment of osmotic gradient following hormone washout.

Figure 18. Control tissue exposed to anti-PKC I (γ) antibody and protein A-gold (10nm) particles showing few gold particles scattered over the cytoplasm. X67,000.

Figure 19. Tissue stimulated with ADH 100 mU/ml for 15 min and then labeled with PKC I enzyme and mixed with anti-PKC I, and protein A-gold probes to serve as an additional control. Presence of few gold particles indicates little or no specific binding with PKC isozyme I. X67,000.

Figure 20. Toad urinary bladder tissue stimulated with MZ for 15 min and then labeled with anti-PKC isozyme I antibody and protein A-gold particles. Gold

particles appear localized predominantly in association with coated vesicles indicating association of PKC isozyme I with coated vesicles (arrow). X33,750.

Figure 21. Toad urinary bladder tissue stimulated with MZ for 15 min and then labeled with anti-PKC isozyme I antibody and protein A-gold probes. Gold particles, associated likely with several coated vesicles (arrows), indicating the association of PKC isozyme I with coated vesicles and appear to show a pattern of migration from the apical membrane to the cytosol. X33,500.

Figure 22. Ultrathin section viewed in the TEM of coated vesicles (arrow) in the cytoplasm of a granular cell in toad urinary bladder following stimulation with ADH. X50,000.

Figure 23. Freeze fracture replica of an ADH-stimulated toad urinary bladder shows the presence of coated vesicles (arrow). X112,000.

Figure 24. Freeze fracture replica of toad urinary bladder tissue stimulated with ADH shows the presence of protocytosed caveolae (arrows) in a microfilament-rich cell. X67,000.

Figure 25. Phase contrast micrograph of A6 cells grown in monolayer on glass cover slip. X280.

Figure 26. Control A6 cells showing diffused fluorescence with no particulate formation upon exposure to FITC. X280.

Figure 27. A6 cells treated with 100 mU/ml ADH for 15 min, and then exposed to PKC III (α) antibody and fluorescein isothiocyanate (FITC) showing the presence of PKC isozyme III in particulate structures. X280.

Figure 28. A6 cells treated with $MZ10^{-6}$ M for 15 min, exposed to PKC III antibody and then to FITC showing PKC isozyme III in particulate structures as seen in ADH treated tissues. X700.

Figure 29. SEM of LLC-PK₁ cells showing scattered distribution of microridges over

the apical membrane. X3,500.

Figure 30. Intracellular calcium mobilization in LLC-PK₁ cells following dose additions of ADH and oxytocin.

Figures 31 and 32. Compares the calcium response of ADH to oxytocin.

Figure 33 and 34. Show the blocking of calcium by oxytocin.

Figure 34. The effect of the anti-oxytocin antagonist on the calcium effects of ADH and oxytocin.

Figure 35. Shows AVP (ADH) calcium effects before and after the anti-oxytocin antagonist.

OTHER MILESTONES

Full-length papers published and accepted:

- a. Candia, O., Mia, A.J. and Yorio, T. 1993. Influence of filter supports on transport characteristics of cultured A6 cells. *Am. J. Physiol.* 265 (Cell Physiol 34):C1479-C1488.
- b. Mia, A.J., Oakford, L.X. and Yorio, T. 1993. Surface membrane remodeling following removal of vasopressin in toad urinary bladder. *Tissue & Cell*, 26:189-201.
- c. Mia, A.J., Oakford, L.X. and Yorio, T. Protein Kinase C isoforms and vasopressin mediated water flow in toad urinary bladder. (In preparation).

Abstracts published and submitted:

- a. Mia, A.J., Gunter, S., Johnson, S., Franklin, J., Tuck, T., Gardner, D. and Yorio, T. 1993. Immunofluorescent detection of PKC alpha in A6 amphibian kidney cells. *Mol. Biol. Cell.* 4:3489a.
- b. Mia, A.J., Robinson, C., Bolden, A., Davidson, A.D., Oakford, L.X. and Yorio, T. 1993. Membrane remodeling by endocytosis in toad urinary bladders under

- sustained osmotic gradient. *Mol. Biol. Cell.* 4:4309.
- c. Robinson, C., Mia, A.J. and Yorio, T. 1993. Rate of water flow and membrane restoration during endocytosis in toad urinary bladders stimulated by vasopressin (ADH). *NIGMS Minority Programs Symposium* 1993:194.
 - d. Mia, A.J., Oakford, L.X., Davidson, A.D. and Yorio, T. 1994. Apical membrane restoration in toad urinary bladders during retrieval following protein kinase C activation. *FASEB J.* 8:A350.
 - e. Yorio, T and Mia, A.J. 1994 Protein Kinase C activity and vasopressin (ADH) action in toad urinary bladders. *FASEB J.* 8:A350.
 - f. Mia, A.J., Davidson, A.D., Robinson, C., Oakford, L.X. and Yorio, T. 1994. SEM studies of comparative membrane remodeling of endocytosis in toad urinary bladders following withdrawal of ADH and MZ. *MSA* 350-351.
 - g. Mia, A.J., Oakford, L.X. and Yorio, T. 1994. Possible association of PKC with coated vesicles in ADH-stimulated toad urinary bladders. *J. Cell Biol.* 1994.
 - h. Yorio, T., Baxter, N. and Mia, A.J. 1994. Vasopressin increase in intracellular calcium in culture renal epithelia may be mediated through oxytocin receptor. *J. Cell Biol.* 1994.

OTHER ACTIVITIES

Alvin Finkley, a chemistry major and Haile Yancy, a chemistry/biology major, supported by this grant for their career enrichment, graduated from Jarvis Christian College in May 1994. Haile Yancy has been accepted to enter into graduate school to pursue studies in biomedicine in Howard University, Washington, D.C. starting the fall semester 1994. He received full financial support from the University to undertake his graduate studies. Alvin Finkley plans to enter into the Graduate School of Biomedical Sciences at University of North Texas Health Science Center at Fort

Worth in January 1995. During the current year, three African-American undergraduate students, Julia Franklin, Sharla Hood and Crystal Woods received training in biomedical research and career enrichment involving animal tissue culture, immunofluorescent, bright field, scanning and transmission electron microscopy techniques. In addition, Kristi Henderson and Chasity Robinson, who received training under this research project, have been involved in further research at University of North Texas Health Science Center at Fort Worth under an MBRS project and at The University of Indiana, Bloomington, under a Howard Hughes Medical Research Grant, respectively.

Students continue to receive training using computer technology to develop their expertise in computer use in research and academic instruction.

Attendance at the Experimental Biology meeting - April, 1994.

Drs. Thomas Yorio and A.J. Mia attended the Experimental Biology Meeting at Anaheim along with two undergraduate students: Alvin Finkley and Haile Yancy. Alvin Finkley and Haile Yancy attended the meeting as guests of MARC/FASEB.

Attendance at the MSA Meeting - August, 1994

Drs. A.J. Mia and John F. Johnson and five undergraduate students which include: Julia Franklin, Annette Bolden, Susan Johnson, Crystal Woods and Tony Freeman attended the Annual MSA Meeting in New Orleans, LA., in which Drs. Mia, Yorio, Oakford and student Chasity Robinson presented a paper.

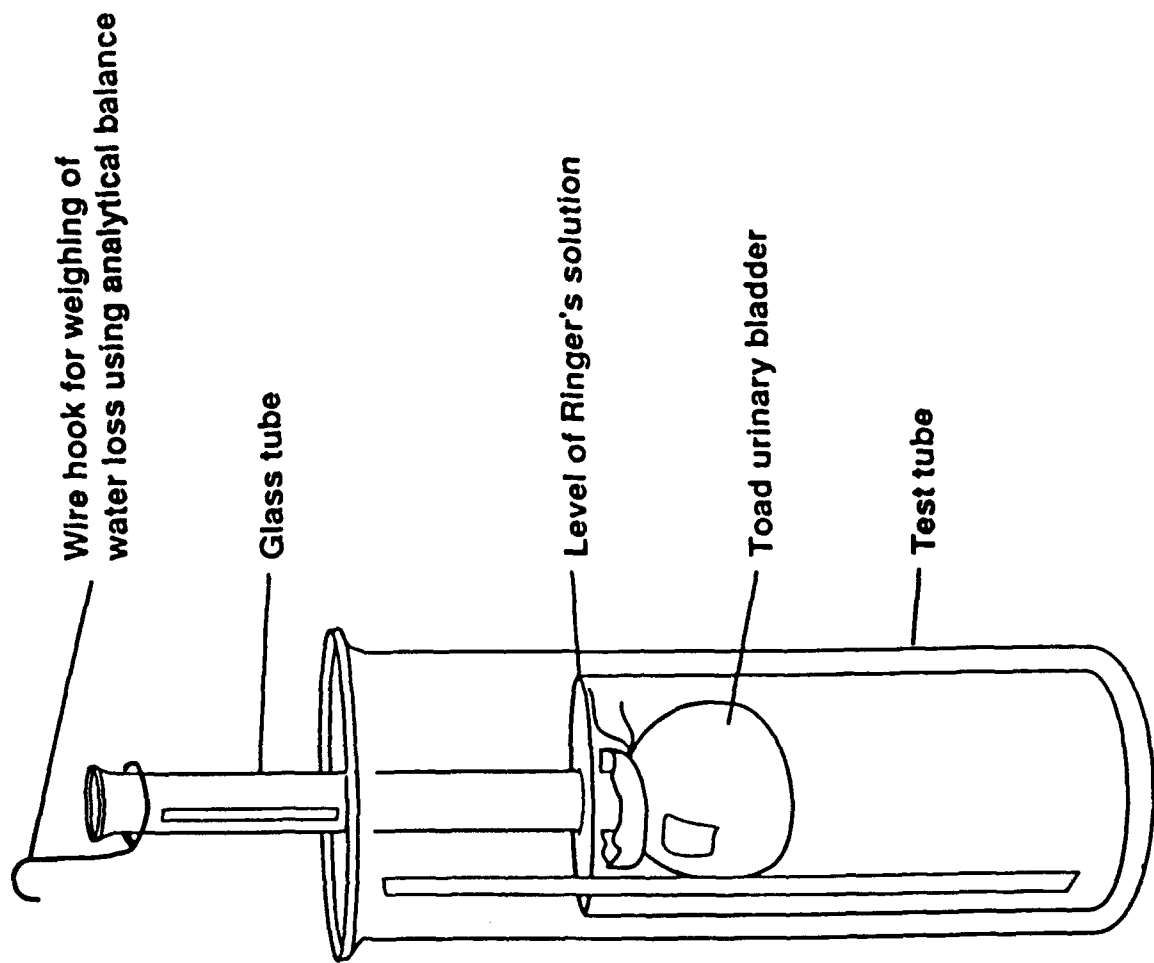
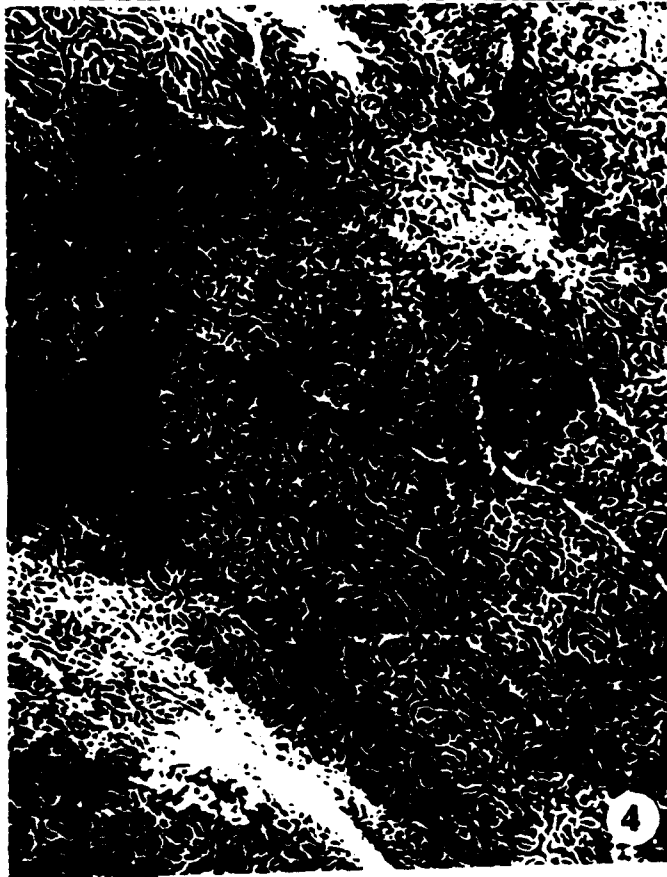
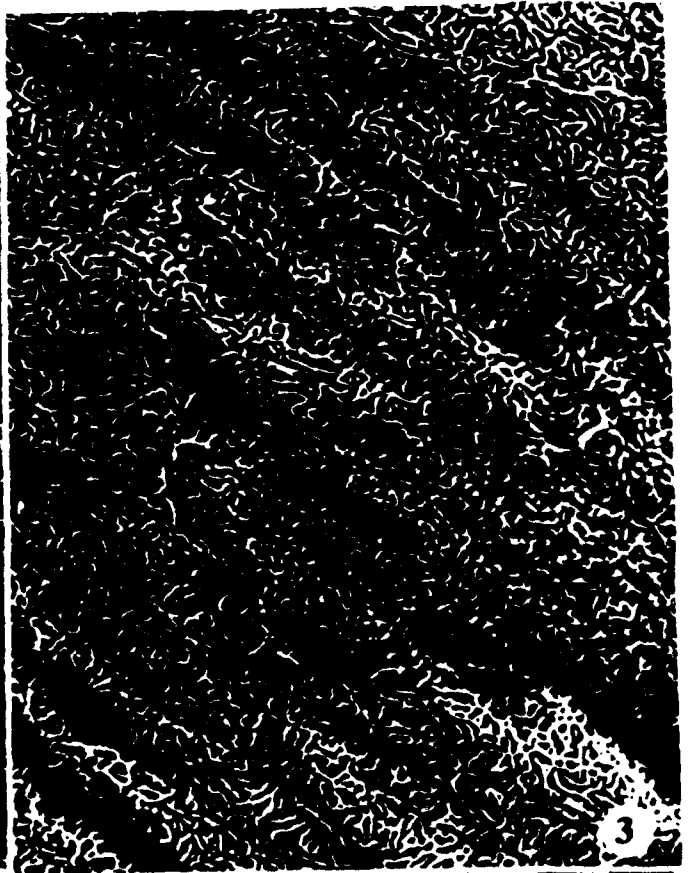
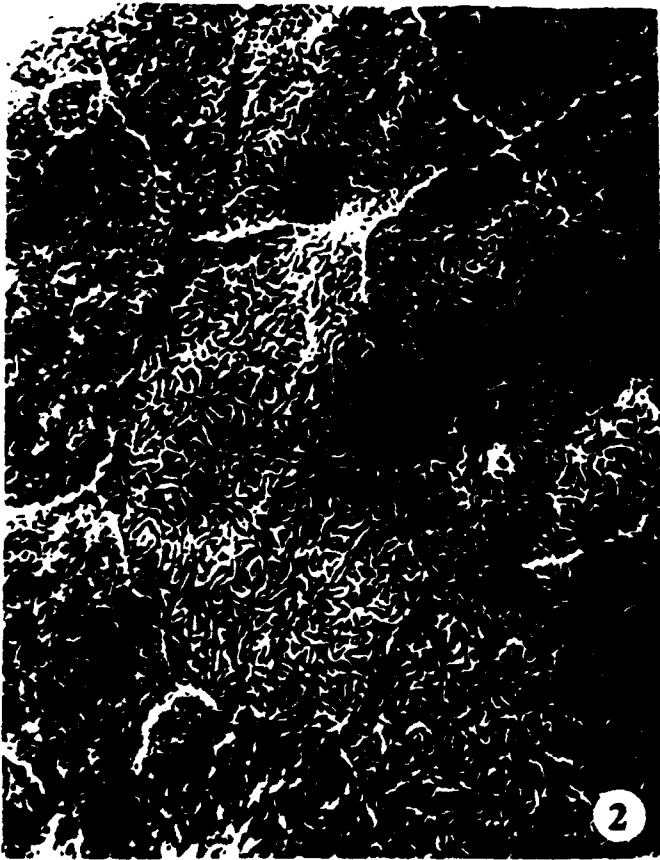


Figure 1. Illustrates the experimental set up of the toad urinary bladder sac as used.



Graph Represents the Percentage of Endocytosed Cells Stimulated by ADH and MZ Compared to Control Tissues

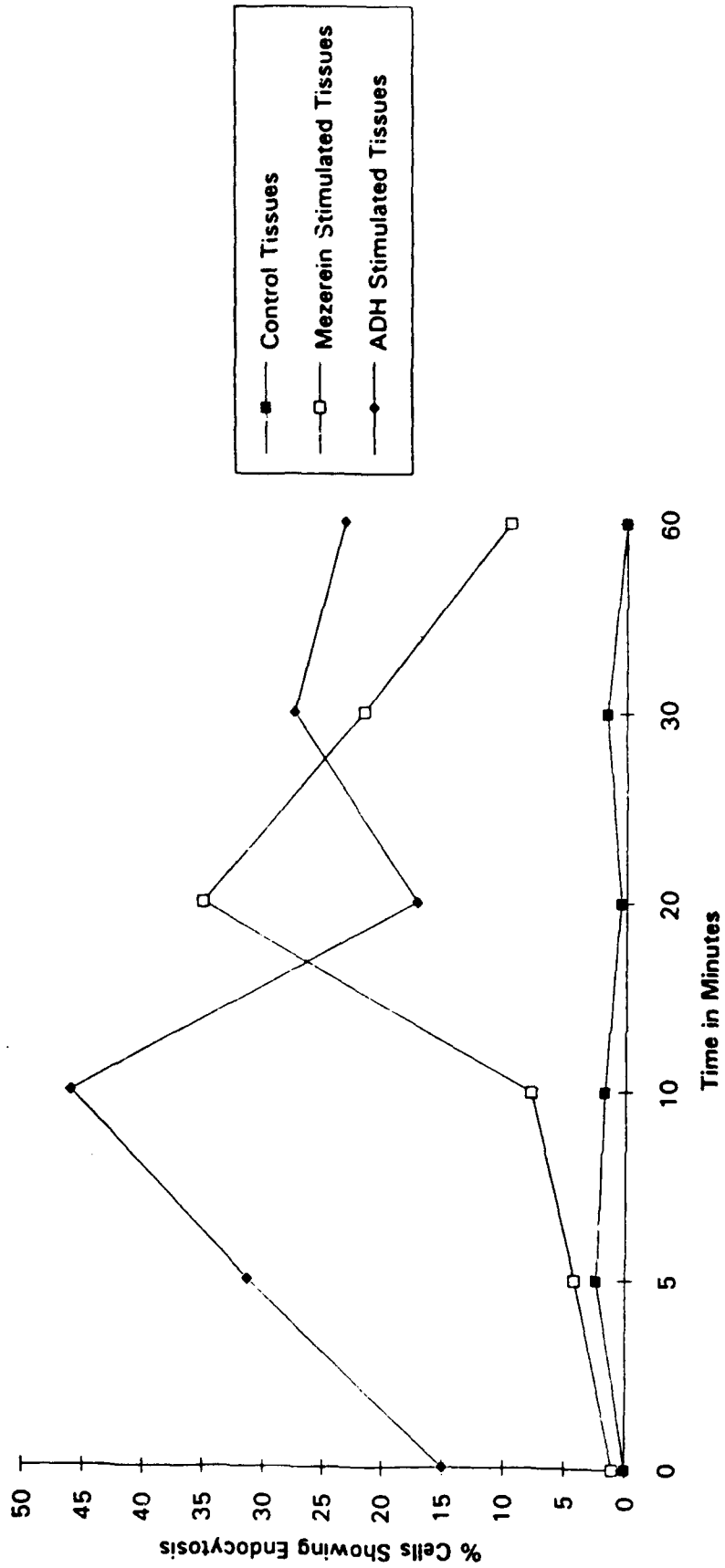
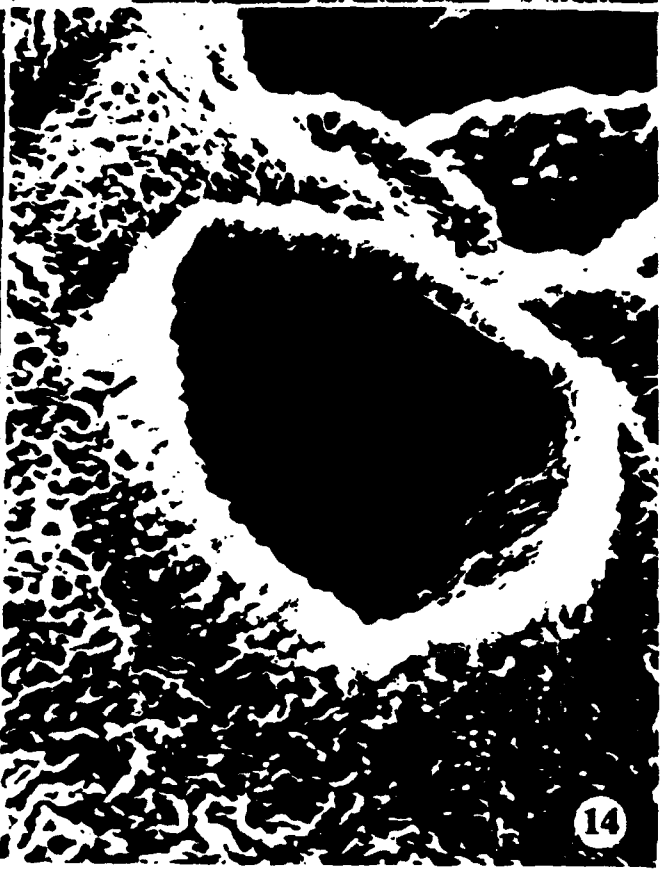


Figure 6







TOAD BLADDER WATER FLOW EXPERIMENT
effects of 20ul of adh into 20ml of ringer solution

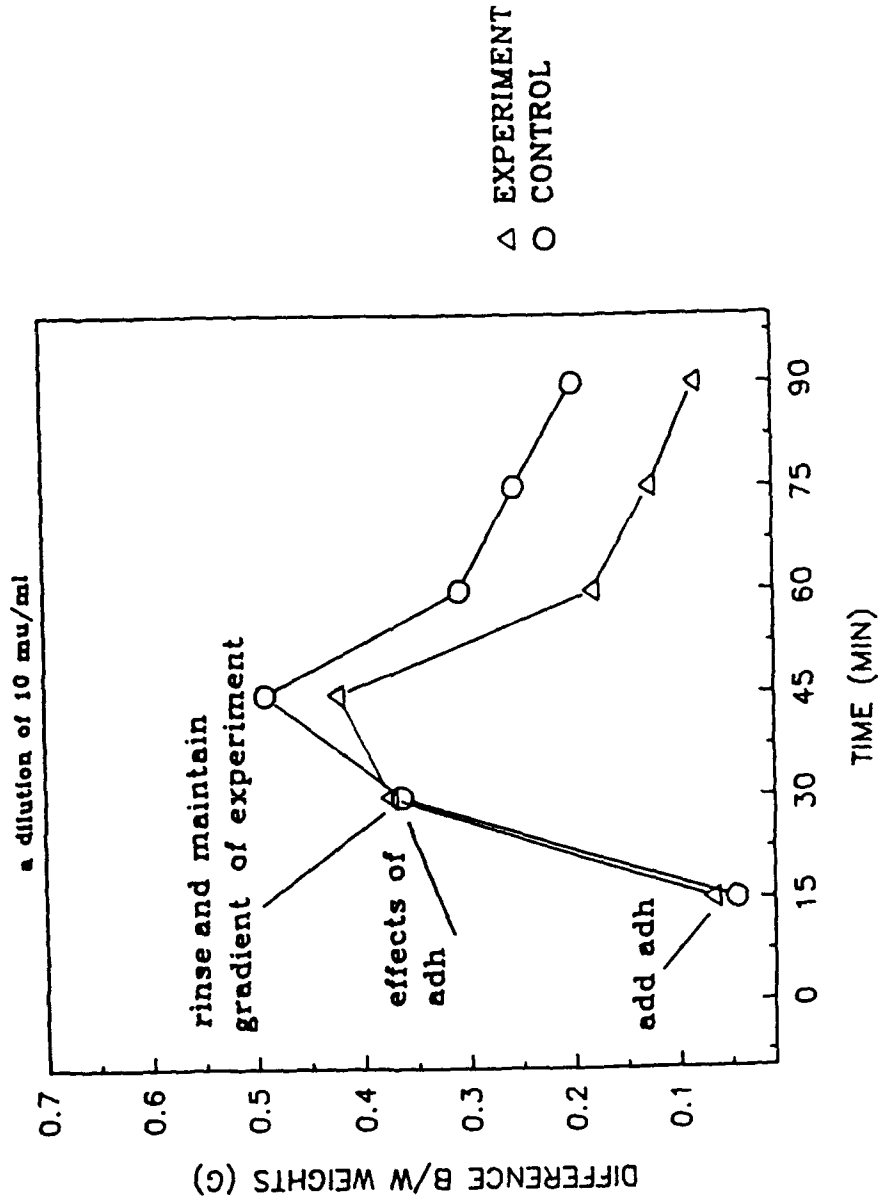
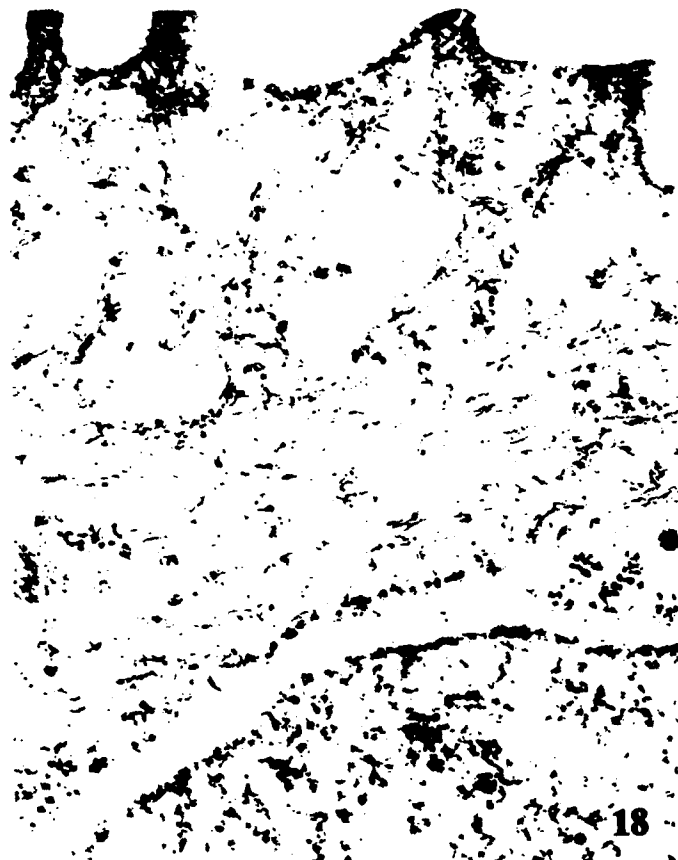


Figure 17



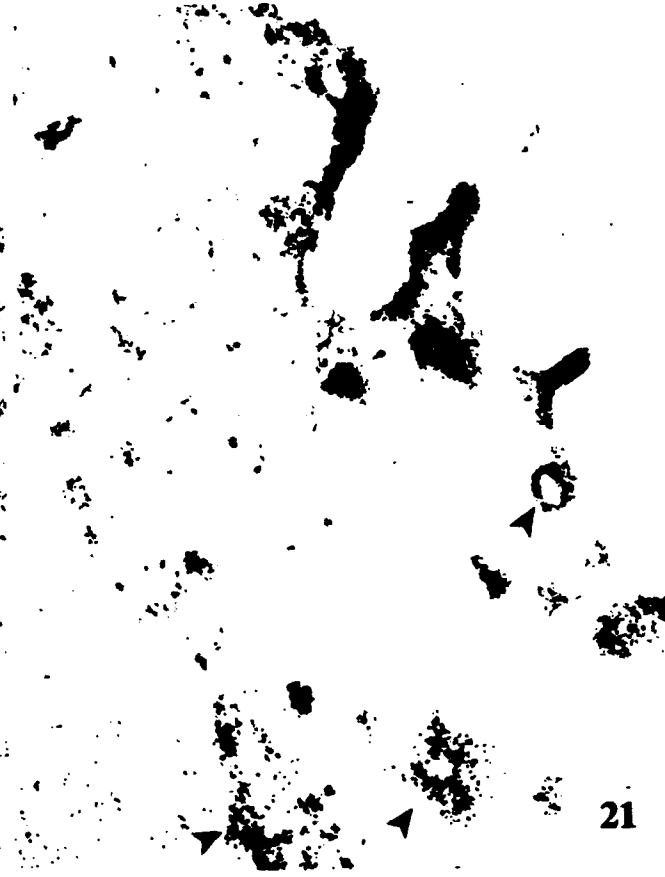
18



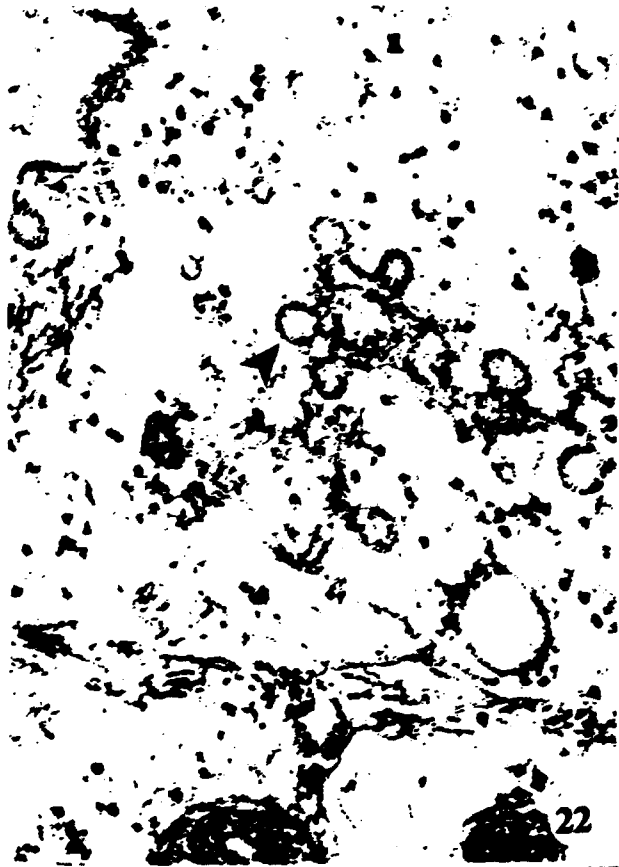
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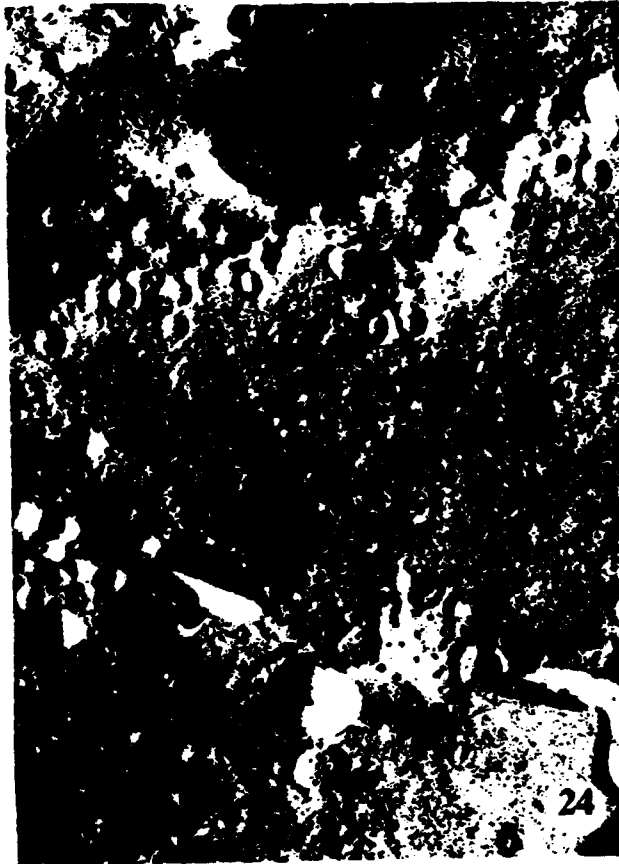
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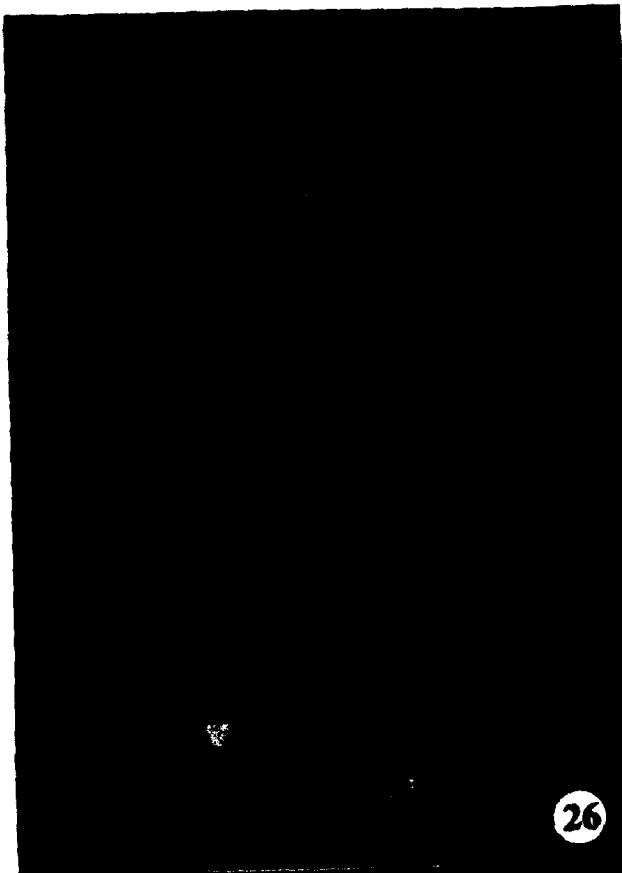
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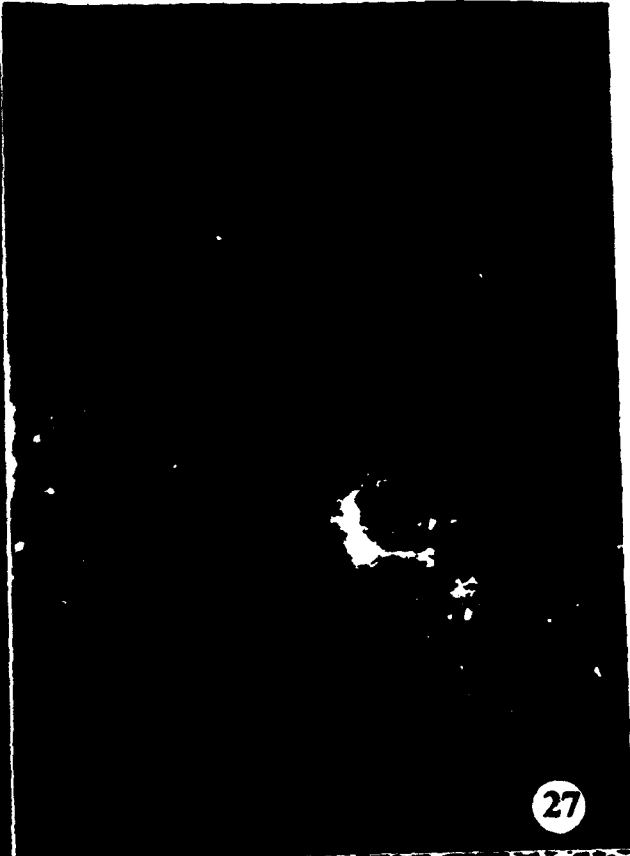
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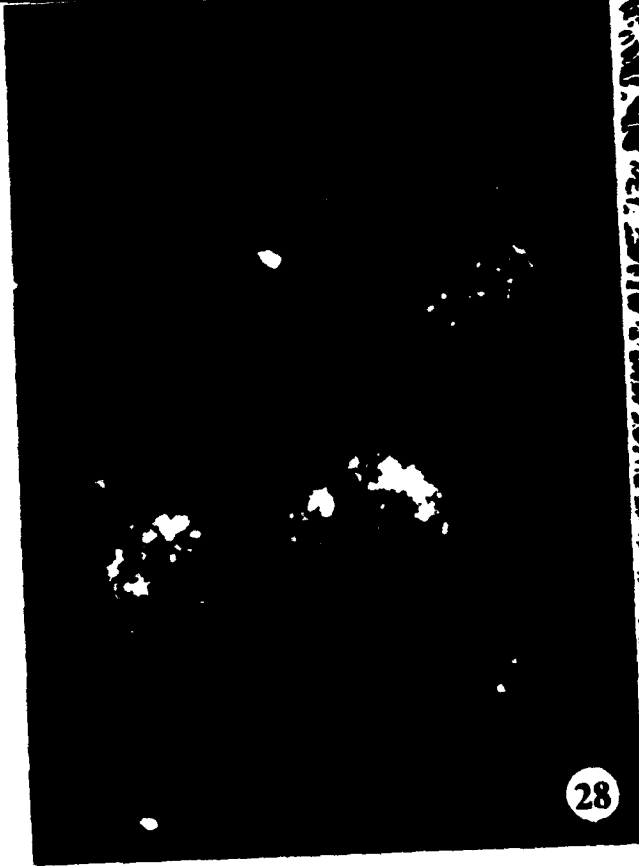
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