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## Title and Subtitle

Retrieval of Water Channels by Endocytosis in Renal Epithelia

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## Abstract

Combat crew effectiveness is crucial during sustained and demanding military operations. In particular, how soldiers cope with conditions of excess heat and fluid deprivation will ultimately impact on their ability to carry out their stated objectives. If the kidney should become damaged or obstructed, the kidney will fail to respond to the hormone vasopressin, which is responsible for regulating water balance. Understanding the specific mechanisms involved in the regulation of water balance by the kidney will provide information needed to design preventive measures for dealing with potential adverse conditions that may result in water deprivation and decreased renal responsiveness to vasopressin. The present study is designed to determine the cellular mechanisms involved in reduced fluid reabsorption and membrane reorganization following the removal of the actions of ADH. It is proposed that following removal of hormone, proteinaceous water channels, located in the apical membrane, are internalized through a process of endocytosis, delivered to the cytosol as a consequence of changes in intracellular calcium concentrations, PKC enzyme activation and reorganization of the microfilament/microtubule system. These studies may lead to new drug or non-drug treatment regimes that could be used to enhance the kidney's responsiveness to vasopressin or to maintain the level of water reabsorptive capacity of soldiers facing harsh environmental conditions of prolonged exposure to dehydration and water deprivation or impaired renal function from injury. Such preventive measures will be essential for maintaining the performance and combat effectiveness of our military forces.

## Subject Terms

Water flow; ADH (vasopressin); Endocytosis; Water Channels; Channel Retrieval; Membrane Retrieval

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# TABLE OF CONTENTS

Cover Page.................................................................................................................................. i
Report Documentation Page........................................................................................................ 1
Foreword....................................................................................................................................... 2
Table of Contents.......................................................................................................................... 3
Abstract......................................................................................................................................... 4
Introduction.................................................................................................................................... 5
Methods......................................................................................................................................... 7
Results........................................................................................................................................... 10
Discussion and Conclusions......................................................................................................... 17
References...................................................................................................................................... 21
Figures........................................................................................................................................... 26
Table............................................................................................................................................... 39
Appendix......................................................................................................................................... 40
Effect of Temperature on Apical Membrane Remodeling and Cytoplasmic Changes in ADH-stimulated Toad Urinary Bladders During Exo- and Endocytosis

Abstract

Previous ultrastructural studies demonstrated that ADH induced endocytosis during retrieval periods involved a large number of granular cells under preferable physiological conditions of 25°C. The objective of the current study was to determine if apical membrane remodeling as well as transepithelial water flow can be affected by lowering temperature to 15°C. This lower temperature may provide us with useful information on how renal epithelia regulate membrane permeability in homeo- and poikilothermic animals under variable environmental conditions. In particular, it may provide a means to monitor water channel recycling during such temperature changes. Control toad urinary bladders in the presence of an osmotic gradient at either 25° or 15°C show typical apical membrane surfaces with a predominance of microridge structure with no sign of membrane internalization when visualized by SEM. ADH-treated tissues during 15 min stimulation at 25° or 15°C responded with a propagation of apical microvilli. Few cells showed any signs of membrane retrieval (not more than 10% of granular cells) which correlated with control bladder tissues at 25° and 15°C. However, 15 min following removal of ADH, bladder tissues at 25° and 15°C showed surface invaginations involving over 44% and 80% of granular cells respectively. Although the ADH-challenged tissues at 15°C during a 15 min retrieval period revealed surface invaginations involving a large number of granular cells, these surface changes may not represent true events of endocytosis, as these invaginations appeared as shallow depressions with no loss of membrane microstructure as typified in ADH-stimulated tissues at 25°C during the same retrieval period. Therefore, the water channels in these tissues at the cold temperature likely remain intact in the apical
membranes without internalizing as endosomes into the cytosol; as a result, the rate of water flow in these tissues remains elevated compared to tissues held at room temperature. Ultrathin sections of the control and ADH-stimulated tissues at 25°C during exo- and endocytosis were unremarkable with respect to the cytoplasmic profiles. Alternatively, ADH-stimulated tissues during the same retrieval period at 15°C, showed extensive infoldings and compressions of the basolateral membranes associated with frequent invaginations. The lower temperature also resulted in the realignment of the organelles including the microfilaments and microtubules. However, at 30 min retrieval periods, ADH-stimulated tissues recover considerably showing a reduction in the number of shallow apical membrane invaginations involving fewer than 33% of granular cells. This may indicate that the membrane undergoes continuous remodeling even in cold temperature conditions at 15°C. In contrast, control tissues at this temperature and comparable retrieval period showed no more than 3% granular cells involved in membrane remodeling. These observations suggest that the apical membrane undergoes considerable reorganization following cessation of hormone action and that lowering the temperature may provide a means to monitor endocytosis and mechanisms responsible for water channel retrieval.

Introduction

Eukaryotic cells respond with membrane reconfiguration as part of a number of cellular processes. Exo- and endocytosis are two important cellular mechanisms that are involved in the regulation of enhanced transmembrane osmotic water flow and cycling of water channels in renal and toad urinary bladder granular epithelia following stimulation with vasopressin. Vasopressin induces exocytosis with insertion of water channels (Chevalier et al., 1974; Wade et al., 1981; Kachadorian et al., 1985; Hays et
al., 1987; Mia et al., 1989 and others) in concert with propagation of numerous microvilli over the apical membranes of the granular cells (Spinelli et al., 1975; Mills and Malick, 1978; Dratwa et al., 1979; LeFurgey and Tisher, 1981; Mia et al., 1983, 1987, 1988 and others). As a result the apical membrane undergoes conformational changes (DiBona, 1983) with an increase in apical membrane area (Palmer and Lorenzen, 1983) for enhanced water flow. Following cessation of hormone action or receptor down regulation, endocytosis restores the apical membrane and water channels are assimilated into the cytosol (Masur et al., 1972; Gronowicz et al., 1980; Muller and Kachadorian, 1984; Harris et al., 1986; Coleman et al., 1987; Wade et al., 1986; Ding et al., 1985; Mia et al., 1993, 1994a) and cells return to their control state. The cytoskeletal system, including the microfilaments and microtubules, is important to the water channel insertion process (Grosso et al., 1978; Taylor et al., 1973a; Muller et al., 1980; Hardy and DiBona, 1982; Pearl and Taylor, 1983 and others). Furthermore, microtubules have been implicated in temperature-dependent inhibition of transepithelial water flow in toad urinary bladder epithelia following stimulation with ADH (Taylor et al., 1973b, 1978). These and other studies on renal and toad urinary bladder epithelia contain little information on the effect of temperature on exo- and endocytosis involving apical membrane remodeling and changes in the cytoplasmic ultrastructure correlated with ADH-mediated water flow. Previously, we reported time-dependent experimental induction of endocytosis and apical membrane remodeling that corresponded with the rate of water flow at laboratory room temperature (25°C) in ADH-challenged toad urinary bladders (Mia et al., 1993, 1994a) with limited data at 15°C (Mia et al., 1995). Since endocytosis is a highly regulated process and is sensitive to temperature changes in the regulation of number and size of endocytosed vesicles (Romagnoli and Herzog, 1995), we conducted the current series of experiments at 25°
and 15°C to determine the effects of temperature on exo- and endocytosis in ADH-stimulated toad urinary bladder; and our data has been compared with our previous studies at room temperature (Mia et al., 1993, 1994a).

In addition, many cellular processes including the fusion of pinocytic vesicles and lysosomes in various mammalian tissues (Dunn et al., 1980; Haylett and Thilo, 1991) are temperature sensitive and inhibited selectively at temperatures below 17°C. Homeothermic as well as poikiloothermic animals, like amphibians, must adapt to changes in temperature rapidly during normal life cycles as well as during periods of hibernation. This adaptation must include the ability of the animals to osmoregulate. Experiments were conducted to analyze the temperature effects on endocytosis as well as the number and size of membrane invaginations by correlating the morphocytological changes observed in toad urinary bladders with the rate of osmotic water flow at 25°C and 15°C following stimulation with vasopressin and its subsequent removal.

Methods

Tropical toads, *Bufo marinus*, purchased from Carolina Biological Supply Company, Burlington, NC, were maintained in an aquatic environment irrigated with fresh supply of tap water, and were fed live crickets biweekly.

Experimental Protocol:

Urinary hemibladders were removed surgically from doubly-pithed toads, and set up as sacs at the ends of glass tubes and equilibrated in aerated normal Ringer's solution (Mia et al., 1994a) for 15 min at 25±1°C and 15±1°C in a thermostatically...
controlled water bath prior to experimental procedures. The composition of the 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. The Ringer's solution was continuously aerated throughout the experimental procedures. Paired hemibladder sacs were used as control and experimental tissues. An osmotic gradient was established between the serosal and mucosal sides using 1/10 dilution of Ringer's solution in the mucosal cavity. Vasopressin at a concentration of 100mU/ml was then added to the serosal bathing solution to stimulate the experimental bladder sacs for 15 min as in previous studies (Mia et al, 1993, 1994a). ADH stimulation for 10 or 15 min at room temperature induces maximum water flow as well as membrane fusion events with a limited membrane down regulation in toad urinary bladders (Ellis et al., 1980; Harris et al., 1986; Coleman et al., 1987; Muller and Kachadorian, 1984; Taylor and Marples, 1988; Mia et al., 1983, 1987, 1994a), whereas prolonged ADH stimulation ultimately causes spontaneous apical membrane remodeling and return to a state similar to reactivation (Mia et al, 1994b). Therefore, we have used 10 or 15 min ADH stimulation for most of our experiments including the current studies.

Following stimulation of bladder sacs with ADH, both control and experimental bladder sacs received two quick serosal buffer rinses at the appropriate temperature for withdrawal of hormone action, and the sacs were then allowed to retrieve for 15 or 30 min for endocytosis and apical membrane remodeling (Mia et al., 1994a) at 25°C and 15°C prior to tissue fixation. Before fixation osmotic water flow was measured gravimetrically at various time intervals according to Bentley (1988). For tissue fixation, the whole hemibladder sacs were removed from the ends of the glass tubes and quickly submerged into 2% glutaraldehyde at appropriate temperature and fixed for 1 hr. Following buffer rinses, postfixation was carried out using 1% osmium
tetroxide at room temperature for an additional hour. These tissues were then divided into samples for SEM and TEM preparations.

Tissue processing for SEM and TEM was carried out as described previously (Mia et al., 1987, 1994a). Briefly, for SEM, tissues were dehydrated through a series of acetone and liquified Peldri II for critical point drying. The dehydrated urinary bladder sacs were then submerged into fresh liquified Peldri II, and retained for 1-2 hrs for replacement of acetone with Peldri II. Peldri II with bladder sacs was allowed to solidify at a temperature below 23°C (75°F). Peldri II was then allowed to sublimate in a fume hood to complete critical point drying. These tissues were mounted on clean aluminum stubs and gold coated in an argon environment using a sputter coated for SEM observations. For comparative analysis, all SEM pictures were taken at X3,000 magnification with appropriate increase in the printing images. For TEM studies, fixed bladder sacs were buffer rinsed, minced into small pieces and then processed for embedding in epon for ultrathin sectioning (Mia et al., 1993, 1994a). Tissue blocks were polymerized overnight in an oven at 60°C. Ultrathin sections made with a diamond knife were collected on bare copper or nickel grids and exposed to uranyl acetate and lead citrate for staining and TEM studies (Mia et al., 1994a). All TEM pictures were made at X31,500, unless otherwise noted.
Drugs:

8-Arginine vasopressin (ADH) was purchased from Sigma Chemical Co., St. Louis, MO. The concentration of ADH used in the current series of experiments was 100 mU/ml and was added to the Ringer's solution on the serosal side.

Results

Previously, we reported a time-dependent experimental induction of endocytosis and changes in the rate of transcellular osmotic water flow in toad urinary bladders at 25°C (Mia et al., 1993, 1994a, 1994b). Since endocytosis is a highly regulated cellular process and is reported to be sensitive to temperature fluctuation, a series of experiments was completed to evaluate the chronological sequence of morphological events of endocytosis and apical membrane remodeling that occurs following the rate of water flow changes seen at 25° and 15°C for 15 or 30 minutes following removal of vasopressin. The microstructure changes of the apical membrane surface and the cytoplasm, associated with the effects of temperature and hormone, were assessed using the techniques of scanning and transmission electron microscopy (SEM, TEM). An SEM image from a control urinary hemibladder sac, fixed instantly in glutaraldehyde upon isolation from a doubly-pithed toad with no experimental manipulation, is presented in Figure 1. This procedure captures the apical membrane morphology in nearly its normal state showing a flat membrane surface configuration containing predominantly microridges. This predominant microridge structure was also shown in vitro in control bladder tissues set up as sacs using Ringer's solution with an imposed osmotic gradient. However, these bladder sacs showed some degree of swelling causing curvature at the apical membranes of the granular cells. Figures 2 and 3 represent the microstructure profiles of the control (no hormone) toad urinary bladder.
tissues retained in Ringer's solution with an imposed osmotic gradient for 15 min at 25° and 15°C, respectively. SEM observations of these control tissues showed the surface microstructure as predominantly composed of microridges (Figs. 2, 3) with no evidence of membrane surface invagination typically indicative of endocytosis. Complementary bladder tissue subjected to transmission electron microscopy (TEM) displayed cytoplasmic profiles composed of dense secretory granules, mitochondria and microfilaments with basolateral membranes containing slight indentations with intact desmosomes. Little or no apparent difference in cytoplasmic profile was found as was reported in earlier studies (Mia et al., 1987). Repeating this experimental procedure at 15°C with a 15 min endocytosis recovery period tends to induce no apparent cytoplasmic alterations involving subcellular organelles. Figure 4 represents a TEM image of a control tissue retained in Ringer's solution at 15°C during a 15 min retrieval period showing an overall cytoplasmic profile with evenly distributed organelles in the compact cytoplasm. The electron dense secretory granules (curved arrows) appear scattered within the cytoplasm mingled with mitochondria (arrowheads), rough ER cisternae (short arrows), golgi body (long arrow), a multivesicular body (b), microtubule and evenly distributed microfilaments. The slightly indented basolateral membranes with intact desmosomes (d) and nucleus (n) appear normal for the control toad urinary bladder tissues. In contrast, toad urinary bladder sacs stimulated with 100 mU/ml ADH for 15 min at 25° or 15°C when examined in the SEM showed propagation of numerous microvilli (arrows) over the apical membranes as presented in Figures 5 and 6 (arrows) respectively. At 25° or at 15°C during 15 min exocytosis, ADH-stimulated tissues may undergo some down regulation producing shallow depressions over the apical membranes and some separation of the basolaeral membranes of the granular cells. Figure 7 depicts an example of such apical membrane
depressions (arrows) and separations at the basolateral membranes of the granular cells exposed to ADH for 15 min at 15°C. Analysis of the apical membrane surfaces made from SEM micrographs representing both control (n=6 each) and ADH-stimulated (n=6 each) tissues at 25°C and 15°C (n=6) indicated no more than 10% of the granular cells showed evidence of surface membrane invaginations in ADH-challenged tissues during 15 min of treatment at 25°C or 15°C, whereas control tissues showed none. Osmotic water flow was significantly increased in ADH-stimulated tissues as compared to control tissues during this 15 min stimulation period (Table 1). These findings appear to correlate with results from previous studies showing no more than 15% of the granular cells with membrane invaginations at laboratory room temperature (Mia et al., 1994a). Ultrathin sections of tissues stimulated with ADH for 15 min at 15°C as examined in the TEM revealed a cytoplasmic composition containing a distribution of secretory granules, mitochondria and other subcellular organelles with evidence of separations of basolateral membranes (Fig. 8, arrows).

The above studies of hormone treatment and exocytosis were complemented with studies of endocytosis following washout at the corresponding temperatures and time periods of 25°C or 15°C for 15 and 30 min post washout. Both the control and the ADH-exposed tissues, following incubation for 15 min at 25°C or 15°C, received two quick serosal buffer rinses to remove ADH from the ADH-exposed tissues. These tissues were then allowed to retrieve for 15 or 30 min to allow for membrane remodeling. Figure 9 represents an example of SEM images showing invaginations at the apical surface with a loss of membrane microstructures in ADH-exposed tissues during the 15 min retrieval period at 25°C. In some cases, the invaginations involved the entire apical membrane surface with a formation of large cavities as shown in Figure 10. Ultrathin sections show considerable cellular reorientation producing large
inter- and intracellular cellular vacuoles (Fig. 11, v), with a displacement of the basolateral membranes causing separation and deep indentations at the basolateral membranes. However, the basolateral membranes were not found to be separated at points where desmosomes were present (Fig. 11, arrows). As a result of compression of the basolateral membranes, intracellular organelles, including the microfilaments, may become highly condensed within the cytoplasm (Fig. 11, arrowheads). However, the apical membrane disposition of the control toad bladder sacs at the corresponding temperature of 25°C during the same 15 min retrieval period, showed a predominant microridge structure very similar to the structure of the control tissues before washout (Figure 12). Control tissues at 15°C at the 15 min washout period showed some degree of apical membrane invagination (Fig. 13, arrow), while maintaining the predominant microridge structure. However, in some instances, control tissues showed some degree of membrane internalization during this 15 min retrieval period at 15°C (Fig. 14, arrows) indicating that low temperature had some effect on membrane remodeling. Whereas, control tissues at 25° or at 15°C during a 30 min retrieval period showed a general lack of invaginations indicative of endocytosis. In contrast, ADH-exposed urinary bladder sacs at 15°C at 15 min retrieval period showed a dramatic expression of membrane retrieval with membrane invaginations involving a large number of granular cells (Fig. 15). Here the apical membranes of the granular epithelial cells appear to have shallow apical membrane depressions without truly sequestering the apical membranes into large vacuoles or endosomes as appeared in tissues at 25°C (Mia et al., 1994a). Water flow in these tissues remained considerably elevated compared to other ADH-challenged tissues at 25°C (Table 1), indicating that the water channels may not have been internalized as endosomes during this washout period at the lower temperature. A closer SEM view further amplifies the shallow apical
membrane profiles with associated short microvilli and deep caving of the basolateral membranes (Fig. 16). Studies of the ADH-stimulated tissues at 15°C at the 15 min retrieval period showed that some granular cells underwent considerable reorientation and displacement of the cellular organelles from their normal distribution in the cells. Figure 17 represents a TEM image showing that the ADH-treated tissues at 15°C during 15 min retrieval period may show narrowing and elongation of the granular cells, and as a result, most cellular organelles including the rough ER and mitochondria are distribution longitudinally along the long axis of the cells and noticeable invaginations of the basolateral membranes are present (Fig. 17). Most secretory granules (arrows) as seen in this figure appear to have been displaced from the sub-apical region deep into the cytoplasm. In some cases, the cold effect on ADH-stimulated tissues at 15 min washout produced considerable alterations in the basolateral membranes showing extensive compressions and infoldings (Fig. 18, b). Additionally, microfilaments are seen to have compressed and clustered (arrows) between the compressed basolateral membranes. However, the sheer forces of compressions at the basolateral membranes failed to cause a complete separation of the adjacent cells at points of the desmosome attachments (Fig. 18, large arrows). These tissues also had an accumulation of a large number of electron dense secretory granules in some granular cells (arrows) as seen in Figure 19. Whereas, the control bladder tissues at 15°C at the 15 min washout period, showed a different cytoplasmic composition pattern with a scattered distribution of the cellular organelles including secretory granules (s), rough ER cisternae (r), and microfilaments (arrowheads) with slightly indented basolateral membranes and with intact desmosomes (Fig. 20, arrows).

We have also evaluated the apical membrane of the control and ADH-challenged toad urinary bladder tissues at 30 min following washout at 25° and 15°C. Figure 21
is an SEM image of control tissue at 25°C showing a large number of granular (arrows) as well as elevated goblet cells (arrowheads) with no apparent sign of invaginations at the apical membrane surface. The entire bladder surface appears intact with some tissue swelling expected with an imposed osmotic gradient whereas tissues treated with ADH at 25°C following a 30 min retrieval period show surface invaginations involving a large number of granular cells (Figure 22).

Control tissues retained at 25° and 15°C for 15 min showed no sign of apical membrane surface depression indicative of endocytosis. Whereas the ADH-exposed tissue at the same temperatures at 15 min showed nearly 10% of the granular cells with surface invaginations, indicating that even during ADH stimulation, some degree of membrane remodeling occurs that may reflect down regulation of the hormone response. Control tissues at 25° and 15°C during a 15 min washout period showed some apical membrane remodeling, although fewer than 6% and 11% cells, respectively, are involved. In contrast, ADH-challenged urinary bladder tissues at 25° and 15°C during the 15 min retrieval period showed a dramatic effect on membrane invagination, with 44% and 80% of cells showing membrane surface invaginations at 25°C and 15°C, respectively. The membrane surface collapse or invaginations appeared to be very shallow at 15°C showing no sign of membrane internalization. Therefore, the water channels in the ADH-challenged tissues at 15°C during the 15 min retrieval period may not have been internalized in the cytoplasm as endosomes. This may be a reason why we observed a sustained elevated water flow for tissues at 15°C even at 60 min post washout (see Table 1). SEM observations of the control tissues at 15°C during 30 min washout, revealed a general microstructure environment with only 2% of the granular cells showing signs of endocytosis at the apical membrane surface was comparable to control tissues observed at room temperature (25°C) during 30 min.
a similar washout period (Mia et al., 1994a). In contrast, ADH-challenged tissues at 25° or at 15°C for the 30 min washout period demonstrated considerable membrane surface invaginations involving a number of granular cells. The percent of granular cells showing the presence of invaginations over the apical membranes at 25°C was over 28% and at 15°C was over 33% in ADH-stimulated tissues versus less than 3% cells in comparable control bladder tissues. ADH-stimulated tissues at 15°C during this 30 min retrieval period showed vacuolated cytoplasmic features along with expanded rough ER cisternae and ruffled basolateral membranes as generally found associated with ADH-exposed urinary bladder tissues (Mia et al., 1987).
Discussion and Conclusions

The kidney plays a critical role in the reabsorption of water from the urinary side into the systemic circulation, and at times of thirst this is enhanced by antidiuretic hormone (ADH). The process of transcellular enhanced water reabsorption by ADH results from the insertion of water channels into the apical plasma membrane. This ADH response is seen in many ADH responsive renal tissues including the toad urinary bladder. Upon serosal stimulation of the toad bladder tissues with ADH, the apical membranes of the granular epithelial cells become highly water permeable following the membrane fusion of water channels during a process of exocytosis. Following exocytosis and, either the removal of the hormone or receptor down regulation, apical membrane undergoes spontaneous recovery in retrieving the water channels, including the plasma membrane components (aggrephores) by a process of endocytosis (Hays et al., 1994). A number of studies have been carried out to understand the process of endocytosis in toad urinary bladders by using fluid phase markers such as horseradish peroxidase (HRP) and fluorescent dextran as well as colloidal gold techniques with applications of transmission electron microscopy (Müller and Kachadorian, 1984; Harris et al., 1986, 1992; Ding et al., 1985; Coleman et al., 1989; Zeidel et al., 1992, 1993 and others). While these studies have contributed toward our understanding of the process of retrieval of water channels as related to endocytosis, little information was obtained about the time-dependent membrane remodeling that occurs following removal of the hormone. In addition, little is known about the phenomena of apical membrane restoration during endocytosis at lower temperatures (Mia et al., 1995). In our previous studies, we described the behavior of the apical membranes of toad urinary bladder granular epithelial cells under various time-dependent experimental conditions during exo- and endocytosis (Mia et al., 1983, 1987, 1993, 1994a, 1994b).
These studies were carried out at laboratory room temperature conditions of 25°C with or without stimulation by ADH. We observed that during hormone stimulation and exocytosis, ADH-stimulated tissues showed minimal signs of apical membrane remodeling in the form of surface invaginations (Mia et al., 1993, 1994a, 1994b). However, ADH-stimulated urinary bladder tissues, following washout, had extensive membrane surface invaginations indicative of surface remodeling. Transmission electron microscopic observations also confirmed the occurrence of large vacuolar compartments within the cytosol concurrent with the apical membrane internalization.

To determine if cold temperature could enhance our ability to monitor surface membrane remodeling and water channel endocytosis, we conducted a series of time-dependent experiments of ADH-challenged toad urinary bladder tissues under two different temperatures of 25° and 15°C.

Endocytosis is a highly regulated physiological process that plays a key role in the uptake of macromolecules from the external environment, membrane internalization and intracellular endosome transport (Hansen et al., 1991; Zeidel et al., 1992, 1993; Mia et al., 1994a and others) as well as clathrin-dependent endocytosis involving coated pits and vesicles (Rodman et al., 1990; Rothberg et al., 1990 and others). The process of endocytosis is also found to be temperature sensitive in which decreases in temperature results in the increase in the number and size of endocytic vesicles in thyrocites and in J774 cells in vitro (Romagnoli and Herzog, 1995). In our studies of toad urinary bladders, we observed a dramatic increase in the number of apical membrane invaginations involving a large population of granular cells both at 25° and 15°C during 15 min post washout retrieval periods in tissues that received ADH. The number of endocytosed invaginations at 25°C was over 44% versus 80% at 15°C, indicating an increase in the number of invaginations by almost two-fold at the lower
temperature. The number of invaginations was found to peak at 15 min post washout retrieval as 28% and 33% of the granular cells showed surface invaginations during a 30 min washout retrieval period for 25° and 15°C, respectively. Control tissues during a 15 min retrieval period at 25° and 15°C showed no more than 6% and 10% of granular cells with signs of membrane invaginations, respectively. Whereas during 30 min retrieval periods controls showed less than 3% involved granular cells both at 25° and 15°C. These results correlate with our previous studies at 25°C indicating that even lowering the temperature to 15°C neither arrested the process of apical membrane restoration nor greatly altered the pattern of the apical membrane surface remodeling leading to restoration of the apical membrane to nearly prehormone normal state at 60 min into retrieval, though the restoration process appeared to be somewhat slower at 15°C than at 25°C as observed at 30 min and 60 min retrieval periods.

Previous studies on endocytosis involving toad urinary bladders also indicated an increase in the number and dimensions of the endosomes as determined by HRP and FITC dextran at 10 and 30 min retrieval periods (Zeidel et al., 1992, 1993). This increase in the number of endosomes was also found to plateau at 30 min into retrieval. They also showed that at 30 and 60 min the endosomes were larger than the 10 min endosomes, whereas the control unstimulated tissues contained no endosomes. Furthermore, they found no functional water channels at 60 min presumably inactivated or degraded after 60 min following ADH withdrawal (Zeidel et al., 1993). Our current and previous SEM studies on endocytosis of toad urinary bladder apical membranes under various time-dependent experimental conditions (Mia et al., 1993, 1994a) also demonstrated almost a complete recovery of the apical membranes into a normal state following 60 min retrieval, with no more than 3% of the cells showing surface changes (Mia et al., 1994b). This may indicate that at 60 min following withdrawal of ADH,
water channels are largely internalized as endosomes leaving the apical membrane surface barely with any sign of membrane remodeling (Mia et al., 1994b).

SEM observations demonstrated an increase in the size of the apical membrane invaginations at different temperatures and retrieval periods. It was found that there was little difference in the size of invaginations between the control and ADH-stimulated tissues at 25°C during the 15 min post washout retrieval periods but at 30 min at the same temperature, we discovered over 34% increase in the size of invaginations in ADH-stimulated tissues compared to control tissues. We also found increases of over 40% and 146% in the size of invaginations in the ADH-stimulated tissues over control tissues at 25°C and 15°C at 15 and 30 min retrieval periods. Such a dramatic increase in the size of endosomes at the cold temperature was also reported on in vitro cultures of thyrocytes and J774 (Romagnoli and Herzog, 1995).

Our observations in the SEM and in the TEM using ultrathin sections revealed the collapse of the basolateral membranes due to extreme compression involving many granular cells during retrieval periods at 15°C in ADH-stimulated tissues. In addition, we also observed the assembly of a large number of microfilaments associated with basolateral membrane compression at 15°C. At this stage, we have not been able to determine the role of microfilaments and microtubules in the formation of apical membrane invaginations and the compression of the basolateral membranes at cold temperature. Displacement and compression of the microfilaments and likely microtubules along with compressed basolateral membranes may have contributed to the inhibition of water channel endocytosis that was observed at low temperature. Taylor et al., (1973a, 1973b, 1978) reported an involvement of microtubules in the temperature-dependent inhibition of water flow in colchicine-treated and ADH-stimulated toad urinary bladder tissues. They reported that inhibition of water flow by
colchicine was increased by 50% with lowering of the temperature by each 10 degree interval. Pearl and Taylor (1983) demonstrated an inhibition of water flow in ADH-stimulated toad urinary bladder tissues by cytochalasin B, and this inhibition was attributed to the loss of microfilament function caused by cytochalasin B. From these observations, one may infer that microtubules and microfilaments may have functional roles in the induction of apical membrane internalization associated with endocytosis; however, additional studies are needed.

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**Key to Figures**

Figure 1. SEM of control toad urinary bladder fixed immediately upon surgical removal with no experimental manipulation showing the flat surface image of the apical membrane with predominant distribution of microridges. X3,750.

Figure 2. SEM of control toad urinary bladder retained at 25°C for 15 min in Ringer's solution under an osmotic gradient showing predominant microridges over the apical membrane during exocytosis. X3,750.

Figure 3. SEM of control toad urinary bladder retained at 25°C for 15 min in Ringer's solution, buffer rinsed and then retrieved for 15 min showing the distribution of microridges over the apical membrane with no apparent sign of endocytosis. X3,750.

Figure 4. TEM of control toad urinary bladder granular epithelial cells retained at 15°C for min, followed by buffer rinses to allow for a 15 min recovery. TEM shows basolateral membranes with intact desmosomes (d), electron dense secretory granules
(curved arrows), mitochondria (arrowheads), rough ER cisternae (short arrows), golgi body (long arrow), a multivesicular body (m) within the dense cytoplasmic profile of these cells. X12,500.

Figure 5. SEM of toad urinary bladder stimulated with 100mU/ml ADH for 15 min at 25°C showing propagation of numerous short microvilli (arrows) over the apical membranes of the granular cells during exocytosis. X3,750.

Figure 6. SEM of toad urinary bladder stimulated with 100mU/ml ADH for 15 min at 15°C showing propagation of numerous microvilli (arrows) over the apical membranes of the granular cells during exocytosis. X3,750.

Figure 7. SEM of toad urinary bladder stimulated with 100mU/ml ADH for 15 min at 15°C showing the presence of shallow depressions (arrows) over the apical membrane of the granular cells during exocytosis. X3,750.

Figure 8. TEM of ADH-stimulated toad urinary bladder tissue at 15°C showing scattered distribution of cellular organelles along with slight separations of the basolateral membranes (arrows). X6,250.

Figure 9. SEM of toad urinary bladder stimulated with 100mU/ml ADH for 15 min at 25°C, buffer rinsed and allowed retrieval for 15 min showing invaginations over the apical membranes of the granular cells indicating possible endocytosis. X3,750.

Figure 10. SEM of toad urinary bladder stimulated with 100mU/ml ADH for 15 min at
25°C, buffer rinsed and then allowed to retrieve for 15 min showing large invaginations over the entire apical membranes of the granular cells during endocytosis. X3,750.

Figure 11. TEM of toad urinary bladder stimulated with 100mU/ml ADH at laboratory room temperature at 25°C, buffer rinsed and then retrieved for 20 min showing the presence of small and large inter- and intracellular vacuoles (v), condensed microfilaments (arrowheads), reorientation of the cells with a displacement of desmosomes (arrows). X6,250.

Figure 12. SEM of control toad urinary bladder retained in Ringer's solution at 25°C, buffer rinsed and then retrieved for 15 min showing predominant distribution of microridges over the apical membranes with no sign of membrane invagination and endocytosis. X3,750.

Figure 13. SEM of control toad urinary bladder retained in Ringer's solution for 15 min at 15°C, buffer rinsed and then retrieved for 15 min showing slight depressions (arrow) over the apical membranes during endocytosis. X3,750.

Figure 14. SEM of control toad urinary bladder retained in Ringer's solution for 15 min at 15°C, buffer rinsed and then retrieved for 15 min showing the induction of invaginations over the apical membranes due to cold temperature effect during endocytosis. X3,750.

Figure 15. SEM of toad urinary bladder stimulated with 100mU/ml ADH for 15 min at 15°C, buffer rinsed and then retrieved for 15 min showing invaginations and caving of
the basolateral membranes of the granular cells. X1,875.

Figure 16. An enhanced view of the Figure 15 showing detailed microstructure morphology of the apical membranes of the granular cells with invaginations and dwarf microvilli (arrows). X3,750.

Figure 17. TEM of toad urinary bladder tissue stimulated with 100mU/ml ADH at 15°C for 15 min, buffer rinsed and then allowed to retrieve for 15 min. Micrograph depicts cellular elongation with a caving in the basolateral membranes and a longitudinal distribution of cellular organelles including the rough ER, mitochondria with a displacement of the electron dense secretory granules (arrows) from the sub-apical region to deeper region of the cytoplasm. X6,250.

Figure 18. TEM of toad urinary bladder stimulated with ADH for 15 min at 15°C, followed by buffer rinses to allow 15 min recovery. Micrograph demonstrates the extensive infoldings of the basolateral membranes (b) with displacements of desmosomes (large arrows) and the clustering of the microfilaments (arrows) as a result of compression likely caused by ADH treatment at cold temperature at 15°C. X12,500.

Figure 19. TEM of toad urinary bladder stimulated with ADH at 15oC during 15 min retrieval period following buffer rinses showing an accumulation of a large number of electron dense secretory granules (arrows). X6,250.

Figure 20. TEM of control toad urinary bladder tissue retained at 15°C for 15 min, followed by buffer rinses to allow for a 15 min recovery. Figure shows the cytoplasmic
profile of the cells with a scattered distribution of microfilaments (arrowheads), secretory granules (s), rough ER cisternae (r) and basolateral membranes with slight infoldings and intact desmosomes (arrows). X25,000.

Figure 21. SEM of control toad urinary bladder retained in Ringer's solution for 15 min at 25°C, buffer rinsed and then retrieved for 30 min showing the global view with tissue swelling under osmotic gradient and no evidence of apical membrane surface invagination indicative of endocytosis. X500.

Figure 22. SEM of toad urinary bladder stimulated with ADH for 15 min at 25°C, buffer rinsed and then retrieved for 30 min showing the global view of apical membrane surface invaginations involving a large number of granular cells and making the cells porous. X500.

OTHER MILESTONES

Full-length papers accepted and submitted:


Abstracts published and submitted:
e) Nature of caveolae in the endothelial cells of toad urinary bladder. MSA 96.
g) Vasopressin-induced mobilization of intracellular calcium is linked to elevation of cyclic AMP (cAMP). Soc. Neurosci., in press, 1996.

OTHER ACTIVITIES
Attendance at the MSIP Meeting - November, 1995
Attendance at the Cell Biology Meeting - December, 1995
Attendance at the Experimental Biology Meeting - April, 1995
Attendance at the Minority Biomedical Symposium - November, 1995
Attendance at the MBRS Meeting - March, 1996
To be attended MSA Meeting - August, 1996
Training of Students

Three African-American undergraduate students at Jarvis Christian College have been receiving training in biomedical research and career enhancement under the supervision of Dr. Mia. Three of his students graduated from Jarvis in May, 1995. Susan Johnson upon graduation from Jarvis in May, 1996, with honors and Summa Cum Laude has been pursuing graduate study (Ph.D.) in biomedicine under the supervision of Dr. Yorio at the UNT Health Science Center at Fort Worth. Two additional African-American students from Jarvis who were trained with Dr. Mia are completing their first year of Ph.D. studies at the UNT Health Science Center at Fort Worth.
Table 1. Effect of temperature on osmotic water flow recovery following ADH treatment and washout.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Period I</th>
<th>Period II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hormone Treatment</td>
<td>WASHOUT</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>15 min</td>
</tr>
<tr>
<td>A. 25°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>104 ± 13 (12)</td>
<td>78 ± 29 (6)</td>
</tr>
<tr>
<td>ADH</td>
<td>851 ± 190 (12)</td>
<td>796 ± 59 (6)</td>
</tr>
<tr>
<td>B. 15°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>127 ± 15 (12)</td>
<td>79 ± 28 (6)</td>
</tr>
<tr>
<td>ADH</td>
<td>611 ± 95 (12)</td>
<td>870 ± 176 (6)</td>
</tr>
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</table>

The number of experiments are in parenthesis. Represented as means ± S.E.
APPENDIX
IS PROTEIN KINASE C ALPHA (PKCa) INVOLVED IN VASOPRESSIN-INDUCED EFFECTS ON LLC-PK1 PIG KIDNEY CELLS?

Adnan Dibas1, Abdul J. Mia2 and Thomas Yorio1.
Department of Pharmacology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX. 76107 and 2Division of Science and Mathematics, Jarvis Christian College, Hawkins, TX. 75765.

The involvement of protein kinase C (PKC) in vasopressin-induced effects on renal water reabsorption is still unresolved. Activation of PKC can be detected by its translocation from the cytosol (C) to the plasma membrane (PM). In LLC-PK1 cells, the redistribution of PKCa, a predominant isoform of PKC detected, was studied utilizing western blotting after stimulation with vasopressin. Vasopressin (100 mU/ml) failed to induce a translocation of PKCa from the C to the PM. By contrast, phorbol myristate acetate (PMA, 200 nM), a potent activator of PKC, induced a relocalization of PKCa from the C to the PM. After 2 hours of treatment of cells with PMA, PKCa was predominantly detected in the PM and absent from the C. These results suggest that the signal transduction pathway of vasopressin in LLC-PK1 cells does not involve PKCa activation and translocation.

The understanding of the signal transduction pathway of any peptide/hormone-induced processes is important to delineating the mechanisms and pathways involved in its action and regulation. Such understanding is crucial in analyzing pathological defective-mechanisms normally observed in patients. Unfortunately, the signal transduction pathways of many hormones including vasopressin are not fully elucidated. Vasopressin plays an important role in renal water reabsorption, and although it is known that it targets at least three different receptors, the details of its mechanism of action is still unclear (1).

Vasopressin V1 receptors are coupled to a phospholipase C system (2), in which vasopressin binding to V1 receptor induces inositol-1,4,5-trisphosphate (IP3) production, which mobilizes intracellular Ca2+ ([Ca2+]i), and releases diacylglycerol which is

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anticipated to activate protein kinase C (PKC). The receptor responsible for vasopressin actions in the renal tubule is the vasopressin V$_2$ receptor (3). Unlike the V$_1$ receptor, the V$_2$ receptor is coupled to an adenylyl cyclase enzyme where vasopressin stimulates cAMP production (4). In addition, a third receptor has been recently reported but shares no homology to V$_1$ or V$_2$ receptors and is termed VCAM (5). Vasopressin acting on this new receptor increases [Ca$^{2+}$]$_i$ through yet an unknown mechanism.

Although second messenger systems have been identified, the distal steps in the signal cascades are less defined. PKC has been suggested to be involved in regulating cell growth (6), amino-acid transport (7) and vasoconstriction (8) in kidney cells. However, a defined role of PKC in vasopressin-induced renal water reabsorption is not yet established. In this report, we tested the ability of vasopressin to induce the activation of PKC$\alpha$ and its translocation to the plasma membrane in LLC-PK$_1$ pig kidney cells, a widely used cell-line in characterizing vasopressin mechanisms. The results obtained, suggest that PKC$\alpha$ may not be involved in the signal transduction pathway of vasopressin in LLC-PK$_1$ cells.

MATERIALS AND METHODS:

Materials: Nitrocellulose membranes (0.2 μM) were from Schleicher & Schuell (Keene, NH). Aprotinin, leupeptin, soybean trypsin inhibitor and phenylsulfonylmethylfluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies against PKC$\alpha$ were purchased from Upstate Inc. (NY, NY).

Cell Culture: LLC-PK1 cells (passages 18-35) were subcultured in DMEM medium (Gibco Inc. Gaithersburg, MD) containing 10 % fetal bovine serum and antibiotics. Cells to be used in experiments were starved in serum-free medium overnight.

Isolation of cytosolic and plasma membrane fractions: Cells were washed twice in modified Krebs Hanks buffer (134 mM NaCl, 2.5 mM CaCl$_2$, 24 mM NaHCO$_3$, 5 mM KCl, 1.2 mM MgCl$_2$ and 25 mM Hepes, pH 7.4) to remove medium and incubated in fresh Krebs solution for 15-30 minutes. PMA (200 nM) or vasopressin were added to cells to initiate the start of stimulation. To terminate the reaction, cells were pelleted and the medium was discarded. Cells were then sonicated in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 17 μg/ml PMSF and 20 μg/ml soybean trypsin inhibitor).

The homogenate was centrifuged briefly to remove unbroken cells and nuclei. The resulting supernatant was centrifuged for 1 hour at 100,000 x g (4 °C). The resulting supernatant (cytosolic fraction, C) was removed and mixed with 1/4th volume of SDS-sample buffer (4X: 62.5 mM Tris-HCl, pH 6.8, 0.1 % (v/v) glycerol, 2 % SDS, 0.05 % 2-β-mercaptoethanol and
0.005 % (w/v) bromophenol blue). The remaining pellet was sonicated in fresh homogenization buffer and proteins were extracted by adding Triton-X 100 (1 %) and keeping the mixture on ice for 30 minutes. The mixture was further centrifuged at 30,000 x g for 30 minutes (4 °C). The resulting supernatant (plasma membrane fraction) was removed and mixed with SDS-sample buffer for gel electrophoresis.

**Western blotting:** Protein samples were separated on 7.5 % acrylamide as described (9). Gels were equilibrated in transfer buffer (192 mM glycine, 20 % methanol and 25 mM Tris-HCl, pH 8.3) for 15-30 minutes at room temperature and electroblotted on nitrocellulose membranes for 75 minutes at 100 volts using a Bio-Rad electroblotting unit. The membranes were dried at room temperature. Western blotting was performed using Tropix chemiluminescent Kit. Membranes were incubated with anti PKCa antibodies for 1.5 hours (1 μg/ml). The membranes were incubated with the secondary antibody, Goat anti-mouse, for 30 minutes (1:10,000).

**RESULTS:**

As shown in Fig. 1, vasopressin (100 mU/ml, at 3, 6, and 12 minutes) treatment failed to affect PKCa redistribution from the cytosol (C) to the plasma membrane (PM). Only PKCa isoform appeared to be present as antibodies against β or γ isoforms failed to be detected by western blotting (data not shown). Vasopressin failed to change PKCa cellular distribution under various times (2 minutes to 1 hour, data not shown). However, vasopressin at the same concentration increased \([Ca^{2+}]_i\) (as judged using the fluorescent Ca²⁺-sensitive dye fura-2, Fig. 2). Therefore, the increase in \([Ca^{2+}]_i\) appears to be a separate event that is not associated with PKC activation. This was also confirmed by the lack of IP₃ production upon vasopressin stimulation of LLC-PK₁ cells (Unpublished observations, Yorio et al.).

In contrast to the lack of effect of vasopressin on PKCa translocation, PMA (200 nM) induced a gradual translocation of PKCa from the C to the PM (15-120 minutes, Fig. 3). Similar treatment of cells with 4-α-phorbol-12, 13-didecanoate (4-α-PDD), an inactive form of phorbol esters that does not activate PKC, failed to induce any translocation response suggesting that the observed effect is not lipid-mediated (Fig. 4). Phorbol esters including PMA, are widely used to assess PKC translocation. Interestingly, the prolonged exposure to PMA resulted in down-regulation in membrane-bound PKC. This is due to ability of phorbol esters to promote translocation of calpain protease along
with PKC resulting in the degradation of PKC (10, 11). Inhibitors of calpain protease prolonged the amount of membrane-bound PKC.

DISCUSSION:

PKC activation and relocalization can be detected by at least three different methods, two direct and one indirect. The first direct method involves an assay of PKC activity in the cytosol and plasma membrane before and after stimulation (12). The second direct method utilizes western blotting to follow PKC redistribution (13). However, the indirect method assays the levels of phosphorylation of MARCKS protein, a specific substrate of PKC whereas, activation of PKC increased the levels of phosphorylated MARCKS rapidly (14).

Using the second direct method of PKC relocalization (western blotting), the present study suggests the lack of a role for PKCα in the vasopressin's signal transduction pathway in LLC-PK₁ kidney cells. A similar lack of PKC translocation has been reported in hepatocytes after vasopressin stimulation (15). Although vasopressin failed to induce PKC translocation in hepatocytes (15) and in LLC-PK₁ kidney cells, it induced PKC translocation in the anterior pituitary cells (14, 23), adipocytes (16) and platelets (17). Therefore, vasopressin-induced PKC translocation may be considered tissue/cell-specific.

PKC translocation is a key step in the signal transduction pathways of many hormone/agonist responses (e.g., insulin, thrombin and oxytocin). Examples of such translocation events are shown in table 1.

It has been suggested that PKC may play a role in regulating the responses to vasopressin in renal epithelia (18) or take part in its hydro-osmotic actions (19). The present study does not rule this out, but rather various isoforms of PKC may play important roles in hormone-induced responses depending on the site of action.

In conclusion, PKCα is unlikely involved in vasopressin-induced effects in LLC-PK₁ cells. However, a regulatory role for PKCα can not be totally eliminated. A regulatory role for PKC activation in the trafficking of water vesicles has been proposed in
the toad urinary bladder, a system that is well known to be regulated by vasopressin (20). Using phorbol esters, such a proposed role of PKC is currently under investigation in our laboratory.

ACKNOWLEDGMENT: This work was supported in part by a grant from USA Army (DAMD, 17-95-5086).

REFERENCES
Table no. 1

Agonists induced PKC translocation in different cell types.

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<tr>
<th>Effector</th>
<th>Cell type</th>
<th>Method of detection</th>
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<tr>
<td>Bile acids</td>
<td>Hepatocytes (12)</td>
<td>PKC activity assay (PAA)</td>
</tr>
<tr>
<td>Platelet activating factor</td>
<td>Feline tracheal epithelial cells (21)</td>
<td>PAA</td>
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<tr>
<td>formyl-Meth-Leu-Pro</td>
<td>Neutrophils (13)</td>
<td>Western blotting (WB)</td>
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<tr>
<td>Thrombin</td>
<td>Platelets (17)</td>
<td>WB</td>
</tr>
<tr>
<td>Insulin</td>
<td>Adipocytes (16) and skeletal muscles (22)</td>
<td>PAA and WB, respectively.</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Adipocytes (15)</td>
<td>PAA</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>Adipocytes (16), anterior pituitary (23) and platelets (17)</td>
<td>PAA</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>anterior pituitary (14)</td>
<td>MARCKS phosphorylation levels</td>
</tr>
<tr>
<td>Bombesin, endothelin and Bradykinin</td>
<td>Swiss 3T3 (24)</td>
<td>WB</td>
</tr>
<tr>
<td>1,2-dihydroxyvitamin D3</td>
<td>renal epithelial MDBK (25)</td>
<td>WB</td>
</tr>
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</table>
**Fig. 1:** Immunodetection of PKCα redistribution in the cytosolic (c) and plasma membrane (p) fractions of LLC-PK1 cells after stimulation with vasopressin (100 mU/ml).

Lane 1: control (c), lane 2: control (p), lane 3: vasopressin 3-minutes (c), lane 4: vasopressin 3-minutes (p), lane 5: vasopressin 6-minutes (c), lane 6: vasopressin 6-minutes (p), lane 7: vasopressin 12-minutes (c), and lane 8: vasopressin 12-minutes (p). Also, the effect of vasopressin was followed at shorter times (2 minutes) and after 15 and 60 minutes but no relocalization of PKCα was observed. The data are representative of at least 3 different experiments.
Fig. 2: Vasopressin (100 mU/ml) increased \([\text{Ca}^{2+}]_i\) in LLC-PK\(_1\) cells.

LLC-PK\(_1\) cells were loaded with fura-2 (1 \(\mu\)M) for 30-60 minutes followed by washing to remove excess fura-2. Cytosolic \(\text{Ca}^{2+}\) was calculated from the ratio of fura-2 fluorescence at excitation wavelengths of 340 nm and 380 nm according to equations described by Grynkiewicz et al. (26). Vasopressin induced a transient increase in \([\text{Ca}^{2+}]_i\) that declined to basal levels. The data are representative of a total of 30 cells studied. The time is given in minutes.
Fig. 3: Immunodetection of PKCα redistribution in the cytosolic (c) and plasma membrane (p) fractions of LLC-PK₁ cells after stimulation with PMA (200 nM).

Lane 1: control (c), lane 2: control (p), lane 3: PMA 15-minutes (c), lane 4: PMA 15-minutes (p), lane 5: PMA 2-hours, and lane 6: PMA 2-hours (p). There was a clear translocation of the enzyme from the cytosol to the plasma membrane. The data are representative of at least 3 different experiments.
Fig. 4: A comparison of the effects of PDD (200 nM) and PMA (200 nM) on PKC\(\alpha\) redistribution in LLC-PK\(_1\) cells after a treatment for 1 hour.

Lane 1: PDD (c), lane 2: PDD (p), lane 3: PMA (c), and lane 4: PMA (p). PDD failed to induce PKC\(\alpha\) translocation from the cytosol to the plasma membrane. The data are representative of 3 different experiments.
Mechanism of Vasopressin-Induced Increase in Intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_{i}$) in LLC-PK$_1$ Porcine Kidney Cells

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Running Title: Intracellular calcium in LLC-PK$_1$ cells

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Abstract

Analysis of the signal transduction cascade of vasopressin-induced increase in 
\([\text{Ca}^{2+}]_i\) in LLC-PK\textsubscript{1} cells was performed. Firstly, a comparison of the effect of 
vipressin on \([\text{Ca}^{2+}]_i\) in LLC-PK\textsubscript{1} cells to that produced in rat hepatocytes was 
performed (an intracellular mobilizing mechanism involving a \(V_1\)-receptor coupled to the 
production of inositol-1,4,5-trisphosphate (IP\textsubscript{3})). Secondly, the effect of known 
inhibitors of intracellular Ca\textsuperscript{2+} mobilization on vasopressin Ca\textsuperscript{2+}-response in LLC-PK\textsubscript{1} 
cells was studied. Vasopressin induced a transient increase in \([\text{Ca}^{2+}]_i\) in both LLC-PK\textsubscript{1} 
cells and hepatocytes. By contrast to the single \([\text{Ca}^{2+}]_i\) spike seen in LLC-PK\textsubscript{1} cells, 
vipressin induced an average of two to three \([\text{Ca}^{2+}]_i\) spikes in hepatocytes. The \(V_1\)- 
an antagonist (Pmp\textsuperscript{1}-O-Me-Tyr\textsuperscript{3}-Arg\textsuperscript{4}-vasopressin, 1 \(\mu\text{M}\)) abolished vasopressin Ca\textsuperscript{2+}- 
response in both cell-types. Inhibitors of intracellular Ca\textsuperscript{2+} mobilization, thapsigargin (5 
\(\mu\text{M}\)), BAPTA/AM (50 \(\mu\text{M}\)), and U73122 (1 \(\mu\text{M}\)) abolished the Ca\textsuperscript{2+}-response by 
vipressin in LLC-PK\textsubscript{1} cells. The results suggest that vasopressin-induced increase in 
\([\text{Ca}^{2+}]_i\) in LLC-PK\textsubscript{1} cells is mediated via a \(V_1\)-like receptor and involves the mobilization 
of intracellular Ca\textsuperscript{2+} through an IP\textsubscript{3}/thapsigargin-sensitive Ca\textsuperscript{2+}-pool.

Key words: \(V_1\)-receptor, thapsigargin, BAPTA/AM, PLC, U73122.
Vasopressin or antidiuretic hormone (ADH) is a nonapeptide produced by the brain and released from the neurohypophysis into the blood where it ultimately acts in the kidney to produce an antidiuretic action, and in the liver to regulate glucose metabolism (34, 8, respectively). In addition, it is transported into the brain, released from synapses and affects central regulatory processes including thermoregulation, cardiovascular homeostasis and learning (3).

Vasopressin can act on at least three different receptors. The vasopressin $V_1$ receptors are coupled to a phospholipase C system (PLC)(21), in which vasopressin binding to the $V_1$ receptor induces inositol-1,4,5-trisphosphate ($IP_3$) production that mobilizes intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$), and releases diacylglycerol which activates protein kinase C (PKC). Unlike the $V_1$ receptor, the $V_2$ receptor is coupled to an adenylyl cyclase enzyme where vasopressin stimulates cAMP production (15). Although it is believed that the $V_2$ receptor is the key receptor mediating vasopressin responses in the kidney, $V_1$ receptors have also been detected in renal tubules (7). In addition, a third receptor has been recently reported in the collecting tubules of rabbit, but shares no homology to $V_1$ or $V_2$ receptors and is termed VCAM (5). Vasopressin acting on this novel receptor increases $[Ca^{2+}]_i$ also by stimulating $IP_3$ production (6).

LLC-PK$_1$ kidney cells are widely used as a model to characterize vasopressin cellular mechanisms in the kidney (33). Weinberg et al. (32) has reported that vasopressin increased $[Ca^{2+}]_i$ in LLC-PK$_1$ cells. In the present studies, we employed two strategies to further characterize the mechanism of vasopressin-induced increase in $[Ca^{2+}]_i$ in LLC-PK$_1$ cells. The first strategy was to compare the $Ca^{2+}$-response of vasopressin in LLC-
PK1 cells to another cell-type where vasopressin-induced increase in [Ca\(^{2+}\)]\(_{i}\) has a well-understood mechanism. Therefore, rat hepatocytes were used since it has been shown that vasopressin-induced increase in [Ca\(^{2+}\)]\(_{i}\) was mediated via a V1-receptor involving the hydrolysis of phosphoinositide-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). The second strategy utilized the new inhibitors of intracellular calcium release mechanisms, thapsigargin (inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPase)(31) and BAPTA/AM, a membrane-permeable Ca\(^{2+}\)-chelator (16). Recently, both compounds have been described to be more efficient in testing the involvement of the intracellular Ca\(^{2+}\)-pool in agonists-induced increase in [Ca\(^{2+}\)]\(_{i}\), and therefore, were employed. The data obtained in the present report suggests that vasopressin-induced increase in [Ca\(^{2+}\)]\(_{i}\) is mediated by a V1-like-receptor and involves the release of Ca\(^{2+}\) from the intracellular stores.

METHODS

Materials. Phorbol myristate acetate (PMA) and arginine vasopressin were purchased from Sigma Chemical Co. (St. Louis, MO). Nifedipine, BAPTA/AM and thapsigargin were purchased from RBI (Natick, MA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR). V1-antagonist was purchased from Peninsula Laboratories (Belmont, MA).

Tissue Culture. Rat hepatocytes were a gift from Dr. Thomas Fungwe (Department of Biochemistry, UNTHSC). LLC-PK1 cells (passages 17-48) were maintained in DMEM medium supplemented with 44 mM NaHCO\(_3\), 10 % fetal bovine serum and antibiotics.
For Ca\(^{2+}\)-measurements, cells were subcultured onto 25 mm glass coverslips 1-2 days prior to experimentation. For cAMP assay, cells were plated onto multiwell coated dishes (3X4 each) 2 days prior to experiments.

**Intracellular Ca\(^{2+}\) Concentration Measurements.** All measurements were conducted using a Nikon Diaphot microscope (Tokyo, Japan), utilizing a dynamic single-cell video imaging technique using image-1FL Quantitative Fluorescence System (Universal Imaging, West Chester, PA). Hepatocytes and LLC-PK\(_1\) cells were loaded with fura-2/AM (5 \(\mu\)M) in a modified Krebs-Ringer solution (Krebs: 115 mM NaCl, 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 24 mM NaHCO\(_3\), 5 mM KCl and 25 mM Hepes, pH 7.4) for 30 minutes at 37 \(^\circ\)C. Cells were washed with 3 ml of Krebs to remove excess probe and \([Ca^{2+}]_i\) measurements determined by monitoring the ratio of fura-2 fluorescence at excitation wavelengths of 340 nm and 380 nm as described previously (19). Cytosolic Ca\(^{2+}\) was calculated according to equations described by Grynkiewicz et al. (12).

**cAMP Assay.**

**a. \(^3\)H-adenine Incorporation.** Cells were loaded with \(^3\)H-adenine (2 \(\mu\)Ci/ ml) for two hours in serum-free DMEM medium. After adenine incorporation, the medium containing excess \(^3\)H-adenine was replaced with a fresh serum-free DMEM containing 0.1 mM of the phosphodiesterase inhibitor, RO 20-1724. After incubation of cells for 10 min, cells were stimulated with vasopressin and the incubation was continued for another 10 min at 37 \(^\circ\)C. The reaction was terminated by aspirating the medium and adding 1 ml of a stop solution (5% tricholoacetic acid containing 0.5 mM cAMP).
b. Cyclic AMP Assay. Cyclic AMP was determined using the method of Salomon et al. (28). Thirty minutes after stopping the reaction, the stop solution from each well was aspirated and transferred to Dowex columns (AG50W-X8, Bio-RAD, Richmond, CA) and the eluate was collected into a scintillation vial. Three ml of distilled water was added to each column and the eluate containing $^3$H-ATP was collected in the same vials, mixed with 10 ml of Ecolumn scintillation cocktail (ICN, Costa Mesa, CA) and the radioactivity was counted. The Dowex columns were placed over alumina columns (A950-500, Neutral Brockman, Fisher, Fair Lawn N.J.) and 4 ml of distilled water was added to each column. This transfers the cAMP from Dowex columns to the alumina columns. After allowing both Dowex and alumina columns to go to a complete elution, the alumina columns were placed over another set of scintillation vials and 4 ml of a 0.1 M imidazole solution (pH = 7.5) was added to each column. The eluate containing both cold and $^3$H-cAMP was collected. Three hundred microliters of this eluate was saved for measurement of optical density and calculation of column efficiency. The column efficiency was calculated by measuring the absorbance of cold cAMP in stop solution (at 259 nm) before and after eluting from the column. Using these values, the counts for $^3$H-cAMP were normalized.

RESULTS

Vasopressin effects on $[\text{Ca}^{2+}]_i$ in LLC-PK₁ cells and rat hepatocytes.

Vasopressin (83 nM) induced an increase in $[\text{Ca}^{2+}]_i$ in LLC-PK₁ cells as judged by the increase in the fluorescence ratio of fura-2 (Fig. 1a). The increase in $[\text{Ca}^{2+}]_i$ was
transient where it peaked to 170.9 ± 10 nM (n = 16) and declined rapidly to basal levels (61.2 ± 2 nM, n = 39). In a series of separate experiments, the muscarinic agonist carbachol, was added immediately following the addition of vasopressin, and an additional increase in \([Ca^{2+}]_i\) was observed. The carbachol-induced increase in \([Ca^{2+}]_i\) was transient and similar to vasopressin’s response. \([Ca^{2+}]_i\) peaked and declined rapidly to basal levels (data not shown). Vasopressin at similar concentrations, also induced a transient increase in \([Ca^{2+}]_i\) in hepatocytes (Fig. 1b). However, multiple spikes of increased \([Ca^{2+}]_i\) were observed. This phenomenon has previously been reported in hepatocytes upon vasopressin stimulation (Rooney et al.)(26). Although vasopressin induced a transient increase in \([Ca^{2+}]_i\) in both cell types, only hepatocytes showed an oscillatory pattern. In order to verify the involvement of a \(V_1\)-like-receptor in vasopressin-induced effects on LLC-PK, cells, the effect of a \(V_1\) receptor antagonist (Pmp'-O-Me-Tyr\(^2\)-[Arg\(^8\)]-vasopressin) was evaluated. As shown in Fig. 2a, b, the \(V_1\) antagonist abolished vasopressin-induced increases in \([Ca^{2+}]_i\) in both LLC-PK, cells and hepatocytes, respectively.

Next, it was of interest to test whether the \(V_2\) receptor was also involved in vasopressin-induced increase in \([Ca^{2+}]_i\). Therefore, the effects of vasopressin on cAMP accumulation in LLC-PK, cells as well as the effect of cAMP on \([Ca^{2+}]_i\) were investigated. As shown in Fig. 3, vasopressin induced an increase in cAMP levels in LLC-PK, cells that was dose-dependent (EC\(_{50}\) = 25 nM). However, the addition of dibutyryl cAMP (up to 100 \(\mu\)M) had no effect on \([Ca^{2+}]_i\) (Fig. 4). The last observation
suggests that the V₂-receptor may not be involved in vasopressin-induced increase in [Ca²⁺]ᵢ in LLC-PKᵱ cells.

**Source of Ca²⁺ in vasopressin-induced increase in [Ca²⁺]ᵢ in LLC-PKᵱ cells.**

In the next series of experiments, the focus of the study was shifted to identify the source of Ca²⁺ involved in vasopressin-induced increase in [Ca²⁺]ᵢ. Thapsigargin (5-10 μM), a potent inhibitor of the endoplasmic reticulum Ca²⁺-ATPase (31), was used to deplete the intracellular Ca²⁺-pool. As illustrated in Fig. 5, thapsigargin induced a biphasic increase in [Ca²⁺]ᵢ where [Ca²⁺]ᵢ peaked at 1-2 minutes and declined to a lower Ca²⁺-level but, was maintained above basal levels. Thus, a new steady state of [Ca²⁺]ᵢ was reached. The addition of vasopressin (83 nM) failed to increase [Ca²⁺]ᵢ suggesting that vasopressin-induced increase in [Ca²⁺]ᵢ was due to a mobilization of intracellular Ca²⁺. The depletion of such a pool abolished the vasopressin Ca²⁺-response.

To further confirm the involvement of an intracellular Ca²⁺-pool in vasopressin-induced increase in [Ca²⁺]ᵢ, the effect of BAPTA/AM, an intracellular Ca²⁺-chelator was tested (16). As shown in Fig. 6, BAPTA/AM (50 μM) lowered basal [Ca²⁺]ᵢ and the subsequent addition of vasopressin had not effect on [Ca²⁺]ᵢ. Therefore, BAPTA/AM effect of chelating intracellular free Ca²⁺ abolished vasopressin-induced increase in [Ca²⁺]ᵢ.

To determine if extracellular calcium also contributed to the elevation of [Ca²⁺]ᵢ, nifedipine a potent inhibitor of extracellular Ca²⁺-influx by inhibiting voltage-sensitive
Ca\(^{2+}\)-channels (23), was added prior to vasopressin. However, the pre-treatment failed to suppress vasopressin-induced increase in \([Ca^{2+}]_i\) (data not shown).

Finally, to provide direct evidence for the involvement of an IP\(_3\)-dependent mechanism in vasopressin-\(Ca^{2+}\)-response, the effect of U73122, a potent inhibitor of phospholipase C actions (1) was tested. As shown in Fig. 7a, U73122 (1 \(\mu\)M) abolished vasopressin-induced increase in \([Ca^{2+}]_i\) in LLC-PK\(_1\) cells. Also, U73122 at a similar concentration, abolished vasopressin-induced increase in \([Ca^{2+}]_i\) in hepatocytes (Fig. 7b).

To determine the potential involvement of protein kinase C (PKC) in mobilization of intracellular \(Ca^{2+}\) in LLC-PK\(_1\) cells, the addition of phorbol myristate acetate (PMA, 200 nM) (activators of PKC) on \([Ca^{2+}]_i\) was tested. PMA had no effect on \([Ca^{2+}]_i\) (data not shown). Therefore, a role for PKC in vasopressin-induced increase in \([Ca^{2+}]_i\) was unlikely. It is noteworthy to report that okadaic acid, a phosphatase inhibitor (18), occasionally augmented vasopressin-induced increase in \([Ca^{2+}]_i\) suggesting the involvement of phosphorylated proteins that activate or potentiate the increase in \([Ca^{2+}]_i\) (data not shown). A summary of data is shown in table 1.

**DISCUSSION**

The present study has characterized the signal transduction cascade of vasopressin in LLC-PK\(_1\) cells, a widely used cell-line to analyze vasopressin-induced effects in the kidney. Based on the ability of a V\(_1\)-antagonist to abolish vasopressin-induced increases in \([Ca^{2+}]_i\) in LLC-PK\(_1\) cells as well as hepatocytes, the vasopressin-action appears to involve a V\(_1\)-like-receptor in LLC-PK\(_1\) cells. This conclusion is supported by the
observed inhibitory effects of U73122 (an inhibitor of PLC) on vasopressin-induced increases in $[\text{Ca}^{2+}]_i$. The present results also suggest that, the $V_2$-receptor although stimulated (cAMP production was increased in LLC-PK₁ cells), does not play a role in vasopressin-induced increase in $[\text{Ca}^{2+}]_i$. Nevertheless, this does not rule out a role for cAMP and its potential activation of cAMP-dependent protein kinase A (PKA) in regulating $\text{Ca}^{2+}$-homeostasis in LLC-PK₁ cells. We observed that when applying a pressure in the presence of dibutyryl-cAMP to LLC-PK₁ cells, a transient increase in $[\text{Ca}^{2+}]_i$ was observed, whereas applying the same pressure in the absence of dibutyryl-cAMP has no effect on $[\text{Ca}^{2+}]_i$ (unpublished observations, Dibas et al.). This phenomenon has been reported by Taniguchi et al. in rabbit collecting tubule cells as well (30). However, the nature of a pressure-sensitive channel is yet to be identified.

A similar lack of effect of cAMP on $[\text{Ca}^{2+}]_i$ has been previously reported in LLC-PK₁ cells (32), and other kidney cell-types including rat renal papillary and collecting tubule (14), rat glomular mesangial cells (2), perfused rabbit cortical thick ascending limb (21), and rabbit cortical collecting tubule (7). By contrast, cAMP-induced increases in $[\text{Ca}^{2+}]_i$ have also been reported for other kidney cell types including rat inner medullary collecting duct (weak response (28)), rat cortical duct, rat cortical thick collecting limb, rat outer medullary collecting duct (18), rat cortical collecting tubule (11), rabbit connecting tubules (3), rabbit proximal tubule (24), porcine renal cortical ascending limb (10), and the amphibian A6 kidney cell-line (20). There was no indication in these studies of a pressure-mediated effect for cAMP on $[\text{Ca}^{2+}]_i$. The ability of cAMP to increase $[\text{Ca}^{2+}]_i$ in different kidney cells types may be of a physiological significance.
However, further work is needed to elucidate the importance and the physiological relevance of cAMP-induced increase in \([Ca^{2+}]_i\) in different compartments of the kidney as well as to resolve the species-differences.

The source of calcium in mediating vasopressin-induced increase in \([Ca^{2+}]_i\) was intracellular. Thapsigargin, used to empty the intracellular \(Ca^{2+}\)-pools and BAPTA/AM, used to buffer the intracellular \(Ca^{2+}\)-pools, abolished vasopressin-induced increases in \([Ca^{2+}]_i\). The thapsigargin-induced increase in \([Ca^{2+}]_i\) was identical to that reported by Putney et al. (25) in parotid acinar cells as well as pancreatic β-cells (unpublished observations, Dibas et al.). These observations are important and suggest that LLC-PK₁ cells possess an intracellular \(Ca^{2+}\)-pool that is similar to other cell-types. However, the current data do not support a role for extracellular \(Ca^{2+}\) in vasopressin's \(Ca^{2+}\)-response.

The observed difference between the LLC-PK₁ cells and hepatocytes in the oscillatory \(Ca^{2+}\)-response may be due to several reasons. The first being the difference in the rate of down-regulation of vasopressin receptors in both cell types as well as the internalization of the peptide hormone. For example, Lutz et al. have shown that LLC-PK₁ cells have a unique mechanism for vasopressin peptide internalization that is different from the receptor-mediated endocytosis (17). In the same study, such a mechanism was absent from A10 smooth muscle cells (known to have a V₁ receptor). The second reason may be the level or isoform-type of PLC. Both cell types may have distinct feedback regulation that may limit the production of IP₃. A third possibility may reflect the differences in the efficiency of the \(Ca^{2+}\)-stores to maintain \(Ca^{2+}\)-homeostasis
in both cell types. In other words, the ability of each cell-type to balance the emptying and refilling of Ca$^{2+}$-stores.

In summary, the present study suggests that vasopressin-induced increase in [Ca$^{2+}$]$_i$ in LLC-PK$_1$ cells is mediated via a V$_1$-like-receptor and involves the mobilization of Ca$^{2+}$ from the intracellular stores. The study provides a model for analyzing the signal transduction pathway of specific agonists with an unknown mechanism of action by comparing its effects on other cell-types with well-characterized mechanisms. However, further research should be conducted to identify the kidney's V$_1$-like-receptor.

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Fig. 1a: Vasopressin (83 nM) increased \([\text{Ca}^{2+}]_i\) in LLC-PK\(_1\) cells.

LLC-PK\(_1\) cells loaded with fura-2 (5 \(\mu\)M) and treated with vasopressin at the time indicated with an arrow. As shown, vasopressin induced a transient increase in \([\text{Ca}^{2+}]_i\) that shaped in a peak but, \([\text{Ca}^{2+}]_i\) declined rapidly to basal levels. Traces of two different cells are shown.
Fig. 1b: Vasopressin (83 nM) increased $[\text{Ca}^{2+}]_i$ in rat hepatocytes.

Rat hepatocytes were loaded with fura-2 as described in Methods and were stimulated with vasopressin. Vasopressin induced an oscillatory pattern of increased $[\text{Ca}^{2+}]_i$. Traces of two different cells are shown.
Fig. 2a: The $V_1$-antagonist abolished vasopressin-induced increase in $[Ca^{2+}]_i$ in LLC-PK$_1$ cells.

LLC-PK$_1$ cells loaded with fura-2 were treated with the $V_1$-antagonist (1 μM) for 2-5 minutes prior to the addition of vasopressin (260 nM). However, vasopressin failed to increase $[Ca^{2+}]_i$. Traces of four different cells are shown.
Fig. 2b: The $V_1$-antagonist abolished vasopressin-induced increase in $[\text{Ca}^{2+}]_i$ in rat hepatocytes.

Rat hepatocytes loaded with fura-2 were treated with the $V_1$-antagonist (1 µM) for 2-5 minutes followed by the addition of vasopressin (260 nM), but no increase in $[\text{Ca}^{2+}]_i$ was observed. Traces of four different cells are shown.
Fig. 3: Vasopressin dose-dependently increased cAMP levels in LLC-PK₁ cells.

LLC-PK₁ cells were loaded with [³H]adenine as described in Methods. Vasopressin (1-100 nM) was added to cells (10 minutes incubation). ³H-ATP and ³H-cAMP accumulated in the cells were isolated by Dowex-alumina chromatography and quantitated. Results were normalized and expressed as percent of ATP converted to cAMP. Each value represents the MEAN ± SEM of at least three different determinations.
Fig. 4: Dibutyryl-cAMP, a membrane-permeable analog of cAMP, failed to increase $[\text{Ca}^{2+}]_i$ in LLC-PK$_1$ cells.

The addition of dibutyryl-cAMP (100 μM) to LLC-PK$_1$ cells had no effect on $[\text{Ca}^{2+}]_i$. However, the addition of vasopressin induced an increase in $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ measurements were determined by monitoring the ratio of fura-2 fluorescence at excitation wavelengths of 340 nm and 380 nm. Traces of two different cells are shown.
Fig. 5: Depletion of intracellular Ca\textsuperscript{2+}-pools with thapsigargin abolished vasopressin-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} in LLC-PK\textsubscript{1} cells.

Treatment of LLC-PK\textsubscript{1} cells with thapsigargin (TG, 5-10 µM) increased [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{i} was maintained above basal levels. The subsequent addition of vasopressin failed to increase [Ca\textsuperscript{2+}]\textsubscript{i}. Traces of four different cells are shown.
Fig. 6: Buffering of intracellular Ca$^{2+}$-pools with BAPTA/AM abolished vasopressin-induced increase in [Ca$^{2+}$]$_i$ in LLC-PK$_1$ cells.

The addition of BAPTA/AM (50 μM) lowered [Ca$^{2+}$]$_i$. The addition of vasopressin had no effect on [Ca$^{2+}$]$_i$. Traces of four different cells are shown.
Fig. 7a: U73122 (1 μM) abolished vasopressin-induced increase in [Ca$^{2+}$]$_i$ in LLC-PK$_1$ cells.

LLC-PK$_1$ cells were treated with the PLC inhibitor U73122 for 5-7 minutes prior to stimulation with vasopressin. However, vasopressin had no effect on [Ca$^{2+}$]$_i$ under these conditions. Traces of three different cells are shown.
Fig. 7b: U73122 (1 μM) abolished vasopressin-induced increase in [Ca$^{2+}$]$_i$ in rat hepatocytes.

U73122, a PLC inhibitor, abolished vasopressin-induced increase in [Ca$^{2+}$]$_i$ in rat hepatocytes. Traces of four different cells are shown.