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Vasopressin-induced activation of protein kinase C in renal epithelial cells

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Abstract

Recent studies indicate that the actions of arginine vasopressin (AVP) and other agonists that stimulate electrogenic sodium transport in renal epithelial A6 cells are linked to a Ca^{2+}-mobilizing signal transduction mechanism that involves generation of inositol trisphosphate. Since diacylglycerol is the other product in this pathway, studies were performed to determine the possible role of PKC in the stimulation of sodium transport. AVP induced a biphasic increase in diacylglycerol generation, characterized by an initial rapid rise and then a sustained elevation, and PKC activation, reflected by phosphorylation of a specific 80 kDa myristoylated alanine-rich PKC substrate (MARCKS). To determine the PKC isoform(s) involved in this process, immunoblot analysis was performed using antisera that recognize both classical PKC isoforms, XPKC-I and XPCK-II, cloned from *Xenopus* oocytes. The transcripts of both isoforms were expressed in the A6 cell. Since protein recognized by antisera was translocated from cytosol to the particulate fraction after exposure to AVP, one or both isoforms were activated in the A6 cell. Further studies showed that cyclohexyladenosine and insulin, additional agonists of sodium transport in A6 cells, also stimulated phosphorylation of MARCKS. These results argue that Ca^{2+}-dependent PKC is involved in the action of AVP, and that of other agonists, which stimulate sodium transport. © 1998 Elsevier Science B.V.

Keywords: A6 cell; Sodium transport; Protein kinase C; MARCKS; Vasopressin

1. Introduction

Electrogenic sodium transport by epithelial cells is limited by sodium conductance of the apical cell membrane, which is modulated by sodium channels [1–3]. Following the recent cloning of the subunit proteins that form this channel [4,5], important insights have been provided into its biochemical structure and topology [6]. The mechanism(s) that regulate agonist-stimulated upregulation of apical membrane conductance, however, is less well defined.

Recent studies have shown that multiple agonists, including AVP, stimulate electrogenic sodium transport in the A6 cell, derived from the kidney of *Xenopus laevis*, by a common signal transduction

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system [7–10]. This system involves turnover of phosphatidylinositol 4,5-bisphosphate (PIP_2) and release of Ca^{2+} from intracellular stores due to the action of inositol 1,4,5-trisphosphate. Since diacylglycerol (DAG), the natural activator of protein kinase C (PKC), is the second product of PIP_2 hydrolysis, it seemed possible that PKC was an active participant in upregulation of the system that mediates sodium transport. Using AVP as the model physiological agonist, the present study was performed to determine whether hormonal action involves PKC activation, and to examine which class of isoform(s) may be involved.

2. Experimental

2.1. Materials

Arginine vasopressin (AVP), phorbol 12 myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), protein A Sepharose CL4B and protease inhibitors were obtained from Sigma (St. Louis, MO). ^{32}[P]-orthophosphoric acid was supplied by Amersham (Arlington Heights, IL). Chelerythrine was from Alomone Laboratories (Jerusalem, Israel). The ECL kit was purchased from Dupont NEN (Boston, MA). Rabbit antiserum raised against a purified bovine brain MARCKS protein was kindly provided by Dr. Angus Nairn, Rockefeller University. Rabbit antisera directed against conserved regions in C1 and C2, or the kinase domain including C3 and C4 of rat brain PKC-γ [11] were kindly supplied by Prof. Yasuo Fukami, Kobe University, Kobe, Japan. The peptide sequences selected from rat brain PKC-γ were homologous with the sequences in XPKC-I and PKC-II cloned from *Xenopus* oocytes [12]. Full length cDNA for both isoforms were provided by Dr. H.F. Kung, NIH Cancer Institute. All other reagents were of highest grade available.

2.2. Cell culture

Experiments were performed on a clone of A6 cells (A6S2) derived from *X. laevis*. The methods employed for cell culture have been described elsewhere [8,9]. Briefly, experiments were performed on cells grown to confluence on porous Falcon membranes (Beckton-Dickenson, Bedford, MA) with an active surface area of 4.5 cm^2 after 10 to 14 days of subculture at 26°C, when these cells form a monolayer of fully differentiated epithelial cells with maximum electrical resistance. These conditions were the same as those employed in previous studies where sodium transport was measured.

2.3. Diacylglycerol measurement

AVP, 1 μM, was added to the basal media of monolayers. After various times of exposure to AVP, media was rapidly aspirated from both sides of monolayers, and the reaction was terminated by addition of 1 ml ice-cold methanol to the cells. The methanol was then transferred to a tube containing 1 ml of $CHCl_3$, and 0.8 ml of 0.2% SDS was added to solubilize the cells. The extract of cells was transferred to the tube containing the $CHCl_3$. Finally, any portion of extract still adherent to the membrane was collected in a second wash with 1 ml ice-cold methanol, which was also combined with the sample. Samples were vortexed for 30 s, placed on ice for 1 to 2 h, vortexed again after addition of another 1 ml of $CHCl_3$ and 1 ml of 0.2 M NaCl, and finally centrifuged for 5 min at $900 \times g$ to permit recovery of the lower phase containing lipids. The lipid extract was dried and stored under N_2 atmosphere at -70°C until the assay was performed.

Neutral lipids were extracted from the N_2 stored samples, as previously described [13,14], and DAG content was determined using a modification of the DAG kinase method of Preiss et al. [15].

2.4. ^{32}P-labelling and incubations

Cells were washed twice with a phosphate free amphibian Kreb's Ringer buffer (110 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.4) and incubated with 100 μCi/ml $^{32}P_i$ (specific activity, $\sim$200 mCi/mmol) for 3 h at 26°C. Previous studies have shown that ^{32}P, added in trace amounts, achieves equilibrium with the cellular pool of ATP within 2 h [16]. After labelling, cells were washed with serum-free culture medium three times and incubated with agonists as indicated in the figure legends. Reactions were terminated by replacing the medium with 0.8 ml

ice-cold lysis buffer (10 mM Tris, 10 mM NaCl, 20 mM NaF, 20 mM $NaPP_i$, 1 mM $ZnSO_4$, 0.5% Triton X100, 0.5 mg/ml saponin, pH 7.4) containing 2 mM AEBSF, 125 μM leupeptin, 100 μM chymostatin, and 25 μg/ml aprotinin. Cells were kept on ice for 5 min, scraped off with a rubber policeman and centrifuged for 1 min in a microcentrifuge. Supernatants were used to extract MARCKS by immunoprecipitation with anti-MARCKS antibody.

2.5. Isolation and identification of MARCKS

Previous studies in this laboratory demonstrated that MARCKS is present in the A6 cell [17]. Evidence was provided showing an 80-kDa protein, phosphorylated after exposure to PMA, isolated by the acid extraction method [18,19], and by immunoprecipitation with an antibody raised against MARCKS in bovine brain [20]. In addition, exposure to these bands to *S. aureus* V8 protease yielded a doublet of 13 and 9 kDa bands, characteristic of authentic MARCKS protein [20]. In the present study, MARCKS was isolated by immunoprecipitation after intact A6 monolayers grown on permeable membranes were exposed to physiological agonists, or PMA used as a positive control.

Aliquots of A6 cell lysates were pre-cleared in a suspension of Pansorbin cells (Calbiochem) and incubated overnight at 4°C with a 1:25 dilution of anti-MARCKS antiserum. After exposure to Protein A conjugated Sepharose-CLUB beads, samples were washed and boiled in SDS sample buffer. After centrifugation, the supernatant was applied to SDS-PAGE and autoradiography.

2.6. Northern blot analysis

Total RNA was isolated from A6S2 cells grown on porous cell culture membranes 10 days after seeding. Cells were lysed in TriZol reagent. Poly(A)$^+$ RNA was purified using an mRNA isolation system from Promega (Madison, WI), as described by the manufacturer. Poly(A)$^+$ RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred to a Nylon membrane (Hybond-N, Amersham, Arlington Heights, IL).

Two pUC18 plasmids, containing full length cDNAs of *X. laevis* PKC-I (2.1 kb) and PKC-II (2.2 kb) cDNA respectively, were kindly provided by Chen et al. [12]. The plasmids were propagated in XLI-Blue *E. coli* cells and isolated using a Maxiprep kit (Qiagen). The sequence of PKC inserts were confirmed. A 929-bp PCK-I *Xba*I (I-929 bp) fragment and a 666-bp PKC-II (1626–2292-bp) *Bam*HI fragment were isolated using Gel Extraction Kit (Qiagen), and radiolabeled with ^{32}P by random oligonucleotide-primed synthesis for Northern analysis. Hybridization conditions were 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution (1 × Denhardt solution is 0.1 g Ficoll 400, 0.1 g polyvinylpyrrolidone and 0.1 g bovine serum albumin in 500 ml H_20), 1% SDS and 100 μg/ml denatured salmon sperm DNA, at 42°C. Nylon membranes were washed in 2 × SSC containing 0.1% SDS at 42°C and exposed at −70°C.

2.7. Protein kinase C translocation studies

A6 cells grown as monolayers on Falcon membranes were stimulated with 300 nM PMA or 1 μM AVP for the indicated times at 26°C. After incubation, reactions were stopped by washing the cells quickly with 1 ml ice-cold 5 mM EGTA in PBS, pH 7.4. Buffer (200 μl) containing 60 mM Tris–HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 10 mM 2 mercaptoethanol and a cocktail of protease inhibitors, as described above, was then added to each culture cup. Cells were lysed by repeated freezing and thawing in dry ice and methanol for three cycles. Cytosolic and membrane fractions were separated by centrifugation for 5 min at 100 000 × g, in a refrigerated (Beckman Instruments) airfuge. Pellets were resuspended in 200 μl of the homogenization buffer containing 1% Triton X100. Aliquots of the cytosolic and membrane fractions were mixed with equal volumes of 2 × SDS sample buffer, boiled for 5 min, and processed for SDS-PAGE and immunoblotting with isoform specific antisera.

2.8. SDS-PAGE, immunoblotting and autoradiography

Slab gel electrophoresis was performed at constant current (30 mA) for approximately 5 h using a Laemmli buffer system. Gels were stained with either

Coomassie blue or silver stain, dried and autoradiographed to visualize ^{32}P orthophosphate-labeled proteins. For immunoblotting, gels were electrophoretically transferred onto nitrocellulose membranes using a BioRad wet transfer apparatus and the Towbin buffer system. Nitrocellulose membranes were stained with Ponceau Red to visualize the proteins. For immunostaining with PKC antibodies, blots were incubated as follows: 1% BSA in PBST, 1 h; PKC antibodies (1:1000 dilution) in 1% BSA and PBST, 2 h; PBST (PBS with Tween 20) for 15 min three times; HRPO-conjugated goat anti-rabbit IgG (H + L), 1:1000 dilution in 1% BSA and PBST; PBST for 15 min three times. An enhanced chemiluminescence (ECL) detection system was used according to the manufacturer's instructions to visualize the immunoreactive bands.

3. Results

3.1. AVP-stimulated production of diacyglycerol

Since previous studies demonstrated that the signal transduction mechanism mediating AVP stimulation of sodium transport in A6 cells involves PIP_2 hydrolysis and generation of inositol trisphosphate [9], we sought to determine whether diacylglycerol production was also stimulated. Fig. 1 demonstrates that a rapid increase in cellular DAG content occurred after addition of AVP, reaching a maximum level at approximately 5 to 10 s. The initial peak was followed by a second phase of sustained elevation for at least 30 min.

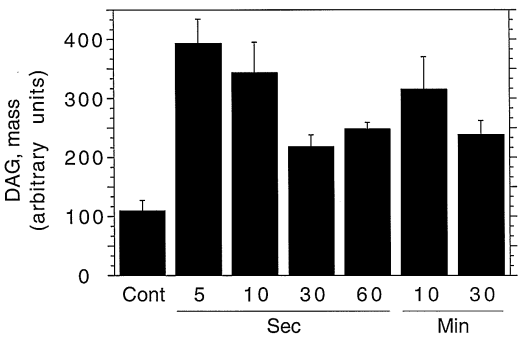

Fig. 1. Effect of AVP on generation of diacylglycerol (DAG): time-dependent elevation of A6 cell DAG content by 1 μM vasopressin. These data are representative of two separate experiments, each containing two samples at each time point. The DAG content was determined with Imagequant (3.1) software on a phosphoimager (Molecular Dynamics, Sunnyvale, CA), and data are expressed in arbitrary units, representing the integrated area of the $^{32}[P_i]$-labeled DAG samples. Values are mean ± range.

3.2. AVP-stimulated phosphorylation of MARCKS

After identification of MARCKS protein in A6 cells [17], we sought to determine whether vasopressin caused detectable PKC activation, using MARCKS phosphorylation as the marker of activation. After ATP pools of A6 cell monolayers were labeled with ^{32}P orthophosphate, cells were exposed to 1 μM AVP for various times. PMA, a pharmacological activator of PKC, was also used at 300 nM as a positive control. MARCKS was then immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

As shown in the autoradiograph in Fig. 2A, phosphorylation of the 80-kDa protein was increased both by PMA and AVP, suggesting that AVP indeed induced PKC activation. AVP-induced phosphorylation, presumably by PKC, was demonstrated within 1 min of agonist exposure and persisted for at least 1 h, corresponding to the time course of vasopressin stimulation of sodium transport [9]. By densitometry, phosphorylation was increased above control after exposure to PMA by 70%, and after exposure to AVP by 27%, 1 min; 27%, 10 min; 32%, 30 min; and 20% after 60 min. To verify the identity of the 80-kDa band obtained in AVP-treated samples as MARCKS protein, the 80-kDa bands from control and AVP-treated cells were reprocessed on 15% SDS-PAGE in the presence of *S. aureus* V8 protease. Fig. 2B shows that 13 and 9 kDa peptides, characteristic of authentic MARCKS proteins [20–22], were generated from both samples.

3.3. Expression of PKC-I and PKC-II in A6 cells

Since two isoforms that resemble classical PKC were identified in *Xenopus* oocytes, further experiments were performed to determine whether PKC-I and PKC-II were expressed in A6 cells. Northern blot analyses were performed with PKC-I and PCK-II cDNA probes. Fig. 3 shows that both PKC isoforms,

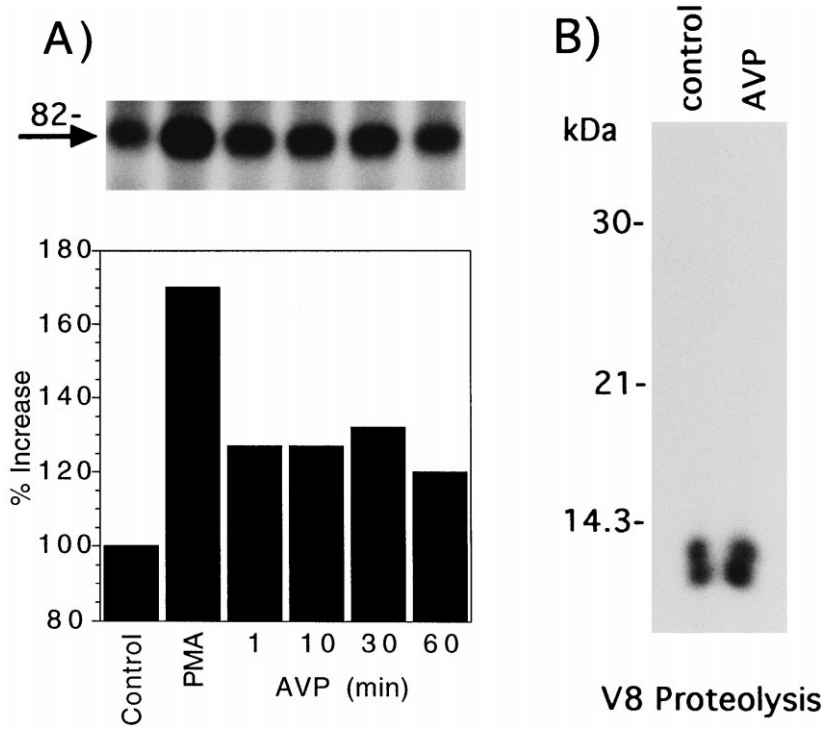

Fig. 2. Effect of AVP on MARCKS phosphorylation: (A) $^{32}[P_i]$-labeled A6 cells were stimulated with either PMA (300 nM, 5 min) or AVP (1 μM, for the times indicated). Cell lysates were immunoprecipitated with the MARCKS antibody and visualized by autoradiography following SDS PAGE. Arrow shows the position of the 80-kDa protein that is phosphorylated in response to either PMA and AVP. Note that AVP stimulated phosphorylation persists for 60 min. Data shown are representative of four experiments. (B) *S. aureus* V8 proteolysis of the 80 kDa protein phosphorylated in response to AVP yields the characteristic MARCKS pattern identical to that of the control protein.

corresponding to 2.1 kb (XPKC-I) and 2.2 kb (XPKC-II) were identified and therefore expressed in A6 cells.

3.4. AVP-stimulated translocation of PKC

Translocation of PKC from soluble to particulate portions of the cell characterizes the activation of some classes of PKC isoforms and may serve to identify the isoform(s) involved in an individual biological response. Immunoblotting experiments were therefore performed with antisera that recognize the two distinct isoforms of PKC identified in the A6 cell [11] to determine whether they are activated by AVP.

Fig. 4 shows that exposure of A6 cells to PMA resulted in an increase of an 80-kDa protein located in the particulate fraction as recognized by antisera raised against the C_2 region of classical isoforms in *Xenopus*. It is likely that the immunoreactive 80-kDa protein represented PKC because it migrated on SDS gels closely with rat brain PKC, used as a marker, and because it translocated to the particular fraction upon exposure to PMA. Similar results were obtained

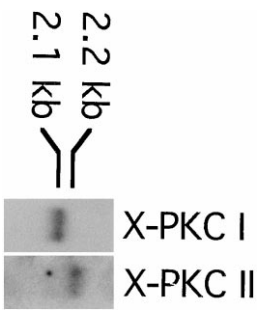

Fig. 3. Northern blot analysis of PKC-I and PKC-II expression in A6S2 cells. Expression of PKC-I and PKC-II in A6 cells was investigated by Northern blot. Molecular mass is indicated on the left. Membranes were hybridized using PKC-I and PKC-II cDNA probes and washed in 2× SSC containing 0.1% SDS.

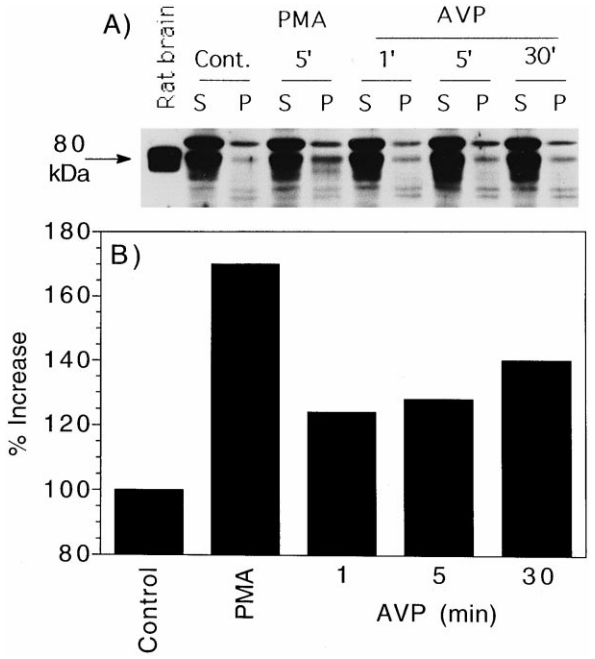

Fig. 4. Effect of AVP on PKC translocation: A6 cells were stimulated with 300 nM PMA or 1 μM AVP as indicated. Cell homogenates were separated into cytosolic (S) and particulate membrane (P) fractions as described in Section 2. (A) The immunoblot was probed with a C2 domain-specific PKC antibody that recognizes an 80-kDa protein in A6 cells co-migrating with the rat brain PKC (arrow). Note that both PMA and vasopressin stimulate the translocation of the 80-kDa protein from the cytosol to the membrane fraction. Data shown is representative of four experiments. (B) Densiometric analysis of the particulate membrane fraction that compares experimental conditions to control.

with antisera that recognize the C1 and kinase regions of PKC, although immunoreactions were less intense (data not shown).

Fig. 4 also demonstrates that AVP-induced PKC translocation from soluble to the particulate fraction, indicating PKC activation. The data shown are representative of one of four experiments. This response was evident at 1 min and persisted for at least 30 min, as shown in experiments involving MARCKS protein phosphorylation. Densitometric analysis showed that PMA increased the immunoreaction in the particulate fraction by 70%. AVP increased the immunoreaction in the particulate fraction by 24% at 1 min, 28% at 5 min and 40% at 30 min. This result therefore suggests that AVP activates at least one or both of the classical PKC isoforms identified in *Xenopus*; classical isoforms are characterized by a C_2 functional domain that is thought to represent the Ca^{2+} binding site, and includes isoforms α-, β- and -γ in mammals.

3.5. Action of cyclohexyladenosine (CHA) and insulin on PKC activation

Besides AVP, the adenosine analogue CHA, which preferentially binds to the A_1-receptor, and insulin stimulate electrogenic sodium transport in the A6 cell by a signal transduction mechanism that involves turnover of phosphatidylinositol 4,5-bisphosphate

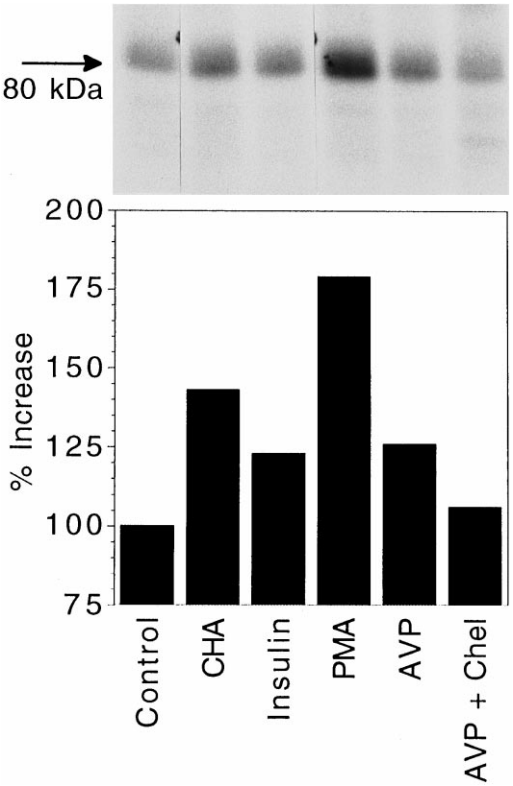

Fig. 5. Effect of other sodium transport agonists and a PKC antagonist on MARCKS phosphorylation: $^{32}P_i$-labeled A6 cells were stimulated for 5 min as follows: insulin, 1 μM; cyclohexyladenosine (CHA), 1 μM; AVP, 1 μM with or without 15 min preincubation with 5 μM chelerythrine (Chele); PMA, 300 nM. Phosphorylation and immunoprecipitation of MARCKS were carried out as described under Section 2. Arrow indicates the position of MARCKS protein. Data shown are representative of two experiments. Densitometric analysis of the depicted autoradiograph is shown, and is expressed as in Fig. 4.

[8,10]. Further studies were therefore, performed to determine whether these cell surface binding agonists also activated PKC, reflected by phosphorylation of MARCKS.

A representative autoradiograph and densitometric measurements are shown in Fig. 5. Compared to control, CHA and insulin increased phosphorylation of MARCKS by 43% and 23%, respectively. Phosphorylation increased by 79% with PMA and 26% with AVP, and were similar to the experiment shown in Fig. 2. Fig. 5 also shows that preincubation of cells with 5 μM of the highly specific PKC inhibitor chelerythrine abolished AVP-stimulated phosphorylation. Since chelerythrine was shown [9] to dose-dependently inhibit agonist-stimulated sodium transport in A6 cells with a $K_i \sim 1$ μM, a value similar to that observed with the isolated enzyme, these results argue that chelerythrine-induced inhibition of sodium transport was caused by PKC inhibition.

4. Discussion

The present study was designed to determine whether AVP activates PKC in the A6 cell, which has been used extensively as a model of distal nephron epithelial cells to study electrogenic sodium transport. A second messenger role for PKC in upregulation of sodium transport is supported by two previous findings: (1) AVP induced PIP_2 hydrolysis, reflected by the generation of inositol trisphosphate, and (2) inhibition of sodium transport dose-dependently by different classes of highly specific antagonists of PKC [9]. Since diacylglycerol, the natural agonist of PKC, is the second product of PIP_2 hydrolysis, an increase in diacylglycerol mass and PKC activation seemed likely.

The present study confirms an AVP-induced rise in diacylglycerol levels. The rapid initial increase in diacylglycerol levels has been shown in other cells to result from phosphatidylinositol breakdown [23]. In contrast, the sustained elevated levels for greater than 10 min may imply other sources for generation of diacylglycerol, such as phosphatidylcholine and phosphatidic acid (reviewed in Ref. [23]). Further studies using isotopically labeled phospholipids will be required to establish the origin of diacylglycerol at early and late phases of production and are currently underway.

MARCKS is a specific endogenous substrate for PKC [24]. The use of MARCKS as a phosphoreceptor therefore, provides a specific assay for the endogenous activity of PKC when activated by physiological agonists, in contrast to the traditional in vitro assay that employs histones that are not specific for PKC [25]. Moreover, AVP-induced phosphorylation of MARCKS shows that the 80-kDa protein is involved in the transduction pathway activated by this agonist. Since MARCKS is known to link F-actin to the plasma membrane and appears to regulate dynamic changes in the cytoskeleton [26], its phosphorylation by AVP suggests that the action of AVP may result in alterations of the cytoskeleton.

The demonstration that PKC is translocated from cytosol to a membrane fraction, in contrast, provides indirect evidence of PKC activation, but also can identify PKC-isoforms involved in a reaction when antibodies are raised against a class of isoforms or specific isoforms. The antibodies used in the present immunoblot experiments were reported to identify two different PKCs in *Xenopus* which, on the basis of structural and functional characteristics, resemble PKC α and β isoforms found in rat brain [11].

Exposure of A6 cells to AVP resulted in rapid phosphorylation of MARCKS protein in vivo within 1 min, corresponding to the rapid rise in IP_3, Ca, the onset of sodium transport observed in a previous study [9], and the AVP-induced increase in diacylglycerol levels in the present study. In addition, these studies show that increased levels of diacylglycerol and PKC activation both persisted for at least 30 to 60 min after exposure to AVP, suggesting that PKC activation may be involved in the sustained action of the hormone. In A6 cells, the AVP stimulated increase in sodium transport persists for several hours [9].

The increase in PKC-induced phosphorylation of MARCKS of about 25 to 30% corresponds to the range of phosphorylation previously reported in other intact cells using physiological stimuli, as judged by inspection of immunoblots or estimates made from densitometry [27,28]. The action of PMA caused about a twofold increase in phosphorylation, as in other reports when large doses are used [29]. The greater immunoreaction due to PMA may reflect

maximal stimulation of multiple isoforms of PKC, which, in contrast to the natural agonist diacylglycerol, are not rapidly downregulated [30].

The demonstration that exposure to AVP resulted in translocation of PKC to a membrane fraction suggests that one or both of the classical PKC-isoforms previously identified in the *Xenopus* oocyte and expressed in the A6 cell are involved in the transduction pathway activated by AVP. The antiserum used in this study reacts with the C_2 region of the enzyme, a region which confers Ca^{2+} sensitivity and one which is implicated in the capacity to translocate to membranes upon an increase in Ca^{2+}. These results, however, do not exclude a possible role for other PKC-isoforms in the transduction pathway.

Evidence derived from previous studies in this laboratory that suggest that PKC activation serves to upregulate sodium transport are contrary to earlier reports. Application of phorbol esters to the apical membrane of A6 cells [31], or phorbol esters or DAG analogues to the basolateral membrane of rabbit distal nephron [32], inhibited basal and AVP-stimulated sodium transport. More recent studies, however, did not detect an inhibition of sodium transport when cortical collecting tubules from DOC-treated rats were exposed to PMA or the synthetic DAG analogue OAG [33]. In other studies in contrast, phorbol esters and DAG analogues were reported to stimulate sodium transport in frog skin [34]. Interpretation of these studies, is unclear, however, since phorbol esters and DAG analogues stimulate multiple PKC isoforms that may have effects unrelated to the signal transduction pathway of interest. Moreover, their action, unlike that of native DAG, are not susceptible to feedback mechanisms that control dephosphorylation of PKC. [30]. Recent reports indicate that the physiological implications of phobol ester-induced biological responses should be interpreted with caution [35,36].

5. Conclusion

Together, these studies indicate that vasopressin and other agonists of electrogenic sodium transport cause activation of PKC in the A6 cell. They also indicate that chelerythrine-induced inhibition of sodium transport correlates with inhibition of PKC phosphorylation. Furthermore, they suggest that one or more calcium-dependent PKC isoforms cloned in *Xenopus* are messengers in the transduction pathway that subserves stimulation of hormone-stimulated sodium transport.

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