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Phorbol 12-myristate 13-acetate Down-Regulates Na,K-ATPase Independent of Its Protein Kinase C Site: Decrease in Basolateral Cell Surface Area

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The effect of protein kinase C (PKC) stimulation on the pump current (I_p) generated by the Na,K-ATPase was measured in A6 epithelia apically permeabilized with amphotericin B. Phorbol 12-myristate 13-acetate (PMA) produced a decrease in I_p carried by sodium pumps containing the endogenous *Xenopus laevis* or transfected *Bufo marinus* α1 subunits (~30% reduction within 25 min, maximum after 40 min) independent of the PKC phosphorylation site (T15A/S16A). In addition to this major effect of PMA, which was independent of the intracellular sodium concentration and was prevented by the PKC inhibitor bisindolylmaleimide GF 109203X (BIM), another BIM-resistant, PKC site-independent decrease was observed when the I_p was measured at low sodium concentrations (total reduction ~50% at 5 mM sodium). Using ouabain binding and cell surface biotinylation, stimulation of PKC was shown to reduce surface Na,K-ATPase by 14 to 20% within 25 min. The same treatment stimulated fluid phase endocytosis sevenfold and decreased by 16.5% the basolateral cell surface area measured by transepithelial capacitance measurements. In conclusion, PKC stimulation produces a decrease in sodium pump function which can be attributed, to a large extent, to a withdrawal of sodium pumps from the basolateral cell surface independent of their PKC site. This reduction of the number of sodium pumps is parallel to a decrease in basolateral membrane area.

INTRODUCTION

The requirement for sodium pump activity is highly variable between cell types and needs to be cell specifically adapted to varying physiological conditions. The intrinsic kinetic properties of the Na,K-ATPase play a central role. Indeed, the intracellular sodium concentration is generally rate limiting, since the $K_{0.5}$ for this ion normally lies above its intracellular concentration (de Weer, 1992; Verrey *et al.*, 1996). Other short- and long-term regulatory mechanisms also play an important role in the control of sodium pump function (for review, Bertorello and Katz, 1995; Ewart and Klip, 1995; Verrey *et al.*, 1996). For instance in kidney tubules, the number of sodium pumps per cell is nephron segment specific and is regulated in response to hormones and/or sodium load (Verrey *et al.*, 1996). Besides this long-term regulation which is based on changes in the total number of pumps per cell, other mechanisms regulate the cell surface expression of sodium pumps as shown for the down-regulation of the sodium pump current induced by PKC activation in *Xenopus laevis* oocytes (Vasilets *et al.*, 1990; Schmalzing *et al.*, 1991).

Finally, regulation of the function of individual pumps by noncovalent interactions and/or covalent modifications has been postulated. Over the past years, it has been demonstrated that the α subunit can be phosphorylated by PKC in vitro and in intact cells (Lowndes *et al.*, 1990; Bertorello *et al.*, 1991; Chibalin *et al.*, 1992; Beguin *et al.*, 1994; Feschenko and Sweadner,

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1995). In the case of the *Bufo marinus* α1 subunit (α1TBM), the PKC phosphorylation site has been mapped to the Thr-15 and Ser-16, close to the NH_2 terminus of the molecule (Beguin *et al.*, 1994). However, it is a matter of debate whether this phosphorylation plays a regulatory role in the Na,K-ATPase function. Using purified enzymes, Bertorello *et al.* (1991) showed that phosphorylation by PKC of shark rectal gland enzyme produces an inhibition of Na,K-ATPase activity, whereas Feschenko and Sweadner (1995) found no inhibition after phosphorylation of mammalian enzymes by PKC.

The effects of PKC activation in vivo on Na,K-ATPase function are also conflicting. Down-regulation was observed in kidney proximal tubules (Bertorello and Aperia, 1989; Satoh *et al.*, 1993), MDCK cells (Shahedi *et al.*, 1992), OK cells (Middleton *et al.*, 1993), and *X. laevis* oocytes (Vasilets *et al.*, 1990); no effect was found in rat kidney cortical collecting duct (Satoh *et al.*, 1993) and LLC-PK1 cells (Middleton *et al.*, 1993), and an increase in the sodium sensitivity was described in kidney proximal tubule (Féraille *et al.*, 1995). These discrepancies might be due to different conditions of PKC stimulation, the use of different techniques to measure the sodium pump function, and/or species- or cell type-specific differences (see DISCUSSION).

Transepithelial sodium reabsorption in the kidney cortical collecting duct has been shown to be decreased following PKC activation (Hays *et al.*, 1987), and the decrease in sodium channel activity induced by high intracellular sodium (feedback regulation; Frindt *et al.*, 1996), acetylcholine (Takeda *et al.*, 1994), or prostaglandin E_2 (Ling *et al.*, 1992) is mediated by PKC activation. A similar role for PKC in sodium channel regulation by sodium feedback and prostaglandin E_2 has been described in *X. laevis* A6 cells that are used as a model for cortical collecting duct principal cells (Ling and Eaton, 1989; Eaton *et al.*, 1995). A direct stimulation of PKC by phorbol esters has also been shown to inhibit amiloride-sensitive sodium transport and to activate Cl conductive pathways leading to Cl secretion across A6 epithelia (Yanase and Handler, 1986).

In this study, we show that not only the apical but also the basolateral sodium transport step mediated by the Na,K-ATPase is affected by PKC activation in A6 epithelia. Measurements of the sodium pump current (I_p) in epithelia formed by wt A6 cells expressing only the endogenous ouabain-sensitive Na,K-ATPase α1 subunit or by A6 cells stably expressing exogenous ouabain-resistant wt or PKC-site mutant Na,K-ATPase α1 subunits show that PMA induces a down-regulation of the Na,K-ATPase function which is not dependent on the PKC phosphorylation site of the α subunit. This down-regulation correlates with a decrease in the number of cell surface pumps. Measurements of fluid phase endocytosis and of the basolateral membrane capacitance indicate that this effect might be to a large extent mediated by an increase in the overall basolateral endocytotic activity, leading to a reduction of the basolateral surface area.

MATERIALS AND METHODS

Cell Culture

A6 cells from the A6-C1 subclone were cultured on permeable supports (Transwell polycarbonate filters, 0.4-μm pore size, 4.7 cm^2, Costar) as described earlier (Beron and Verrey, 1994). Experiments were performed with epithelia cultured for 15 to 23 days. The first 10 days, epithelia were maintained in bicarbonate-buffered medium supplemented with 10% fetal calf serum in 5% CO_2 atmosphere. Filter cultures were then transferred to serum-free *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid- (HEPES) buffered medium.

A6-C1 Cell Lines Expressing wt or PKC Mutant Na,K-ATPase α1TBM

Cell lines expressing the wt α1TBM subunit have been characterized previously (Beron *et al.*, 1995). The α1TBM subunit mutated in its N-terminal PKC phosphorylation site (amino acid residues T15 and S16 mutated to alanine; Beguin *et al.*, 1994) was subcloned into the eukaryotic expression vector pcDNAI (Invitrogen, San Diego, CA). Transfection with Lipofectamine reagent and selection of ouabain-resistant A6-C1 cells was as described (Beron *et al.*, 1995). Ouabain-resistant colonies were isolated by ring cloning. Culture conditions were as described for the A6-C1 cells. Ouabain at a concentration of 20 μM was always present for culture on plastic dishes. Sodium butyrate (2 mM) which was included in the HEPES-buffered medium enhanced the expression of transfected α1 subunits in some of the cell lines (Beron *et al.*, 1995).

Electrical Measurements of Ion Transports

Transepithelial electrical measurements were performed on filter cultures in a modified Ussing chamber using an automatic voltage clamp device connected to a MacLab/4e data recording unit and using the Chart software (AD Instruments, Castel Hill, Australia; Verrey, 1994). The transepithelial electrical resistance (R_{TE}) ($\Omega \times cm^2$) was calculated according to Ohm's law from the spontaneous transepithelial potential difference V_{TE} (mV) and the short circuit current I_{sc} ($\mu A \times cm^{-2}$; V_{TE} clamped to 0 mV). The effect of PKC on the transepithelial electrical parameters of intact epithelia was measured in serum-free HEPES-buffered culture medium. Transepithelial resistance was always $\geq 5000\ \Omega \times cm^2$. The PKC activator PMA or its inactive analogue 4α-phorbol 12-myristate 13-acetate (αPMA) was added to the apical and basolateral compartments. The cell monolayers were kept in open circuit configuration, and the I_{sc} was measured every minute.

The function of the sodium pumps was measured as sodium pump current (I_p) at defined sodium concentrations in epithelia apically permeabilized with amphotericin B (Beron *et al.*, 1995). Briefly, monolayers were washed on both sides and then apically permeabilized for 25 min at 28°C with 20 μg/ml amphotericin B (Fungizone, Life Technologies, Inc.) in sodium-free buffer containing 5 mM $Ba(OH)_2$, 25 mM HEPES, 98 mM potassium gluconate, 1 mM K_2HPO_4, 1 mM calcium gluconate, 1 mM $MgCl_2$, and 10 mM glucose, pH 7.35 (Beron *et al.*, 1995). The treatment with either 100 nM PMA, 100 nM αPMA (control phorbol ester) or vehicle (0.6% dimethyl sulfoxide) was initiated as indicated. The specific PKC inhibitor bisindolylmaleimide GF 109203X (BIM, 5 μM; Toullec *et al.*, 1991) or 0.5% dimethyl sulfoxide was given 15 min earlier. For measurements, filters were transferred into the Ussing chamber containing fresh sodium-free basolateral buffer, and the apical

buffer was replaced. The epithelia were maintained in short circuit conditions (V_{TE} clamped at 0 mV), and sodium pumps were activated by replacing defined volumes of apical and basolateral buffer with sodium-containing buffer (as above but with sodium gluconate and Na_2HPO_4 instead of potassium salts) to reach the desired final sodium concentration (5 or 20 mM sodium) or by complete buffer replacements (90 mM sodium). Ouabain (1.4 mM) was added to the basolateral compartment 4–5 min after sodium addition, and the decrease in I_{sc} induced within 1 min was taken as I_p. Control experiments showed that no cardiotonic steroid-sensitive I_p was measurable in the absence of sodium.

To test the effect of PKC on the function of hybrid pumps containing an exogenous, cardiotonic steroid-resistant α1 subunit (wt or PKC mutant), epithelia from transfected cell lines were permeabilized and treated as described above for the parental A6-C1 cell line. The decrease in I_{sc} observed within 1.5 min after addition of 20 or 50 μM strophanthidin was taken as I_p carried by the endogenous sodium pumps while that observed within 1 min after the subsequent addition of 1.4 mM ouabain was taken as I_p of hybrid pumps. These strophanthidin concentrations were chosen essentially to block the I_p carried by endogenous sodium pumps. Indeed, based on the inhibition curves described below, it could be calculated that 20 or 50 μM strophanthidin added to the basolateral compartment inhibited 98.8% or 99.5% of the current carried by the endogenous sodium pumps, respectively, and 15–18 or 30–36% of that carried by the hybrid pumps.

To determine the K_i of strophanthidin in A6-C1 cell lines expressing the transfected α1TBM subunits, apical permeabilization and induction of maximal I_p with 90 mM sodium were performed as described above. After equilibration, the I_p was inhibited by increasing concentrations of strophanthidin (1–250 μM) added to the basolateral compartment. Ouabain (2 mM) was given for complete inhibition. An equation describing a hyperbolic model for two independent binding sites was used to fit curves to the data (GraphPad Prism, GraphPad Software Inc.). The K_i and the amount of each binding site were derived from the curves (Beron *et al.*, 1995).

[^{3}H]Ouabain Binding

To test the effect of PKC stimulation on the number of ouabain-binding sites expressed at the cell surface, bindings of [^{3}H]ouabain were performed on A6-C1 monolayers cultured on filter supports. Permeabilization of the epithelia and treatment with PMA or αPMA was as described above for measurements of sodium pump current. The apical buffer was replaced 20 min after the beginning of permeabilization with potassium-free buffer containing 5 mM $Ba(OH)_2$, 25 mM HEPES, 98 mM sodium gluconate, 1 mM Na_2HPO_4, 1 mM calcium gluconate, 1 mM $MgCl_2$, and 10 mM glucose (pH 7.35) and supplemented with 20 μg/ml amphotericin B. The basolateral surface was washed twice with the same buffer but without ionophor. Filter cups were transferred to cluster wells with potassium-free buffer supplemented with 2 μM ouabain [20 nM [^{3}H]ouabain (23.0 Ci/mmol, New England Nuclear, Boston, MA) and 1.98 μM unlabeled ouabain] and incubated for 5 min at 28°C in the open circuit configuration. Preliminary experiments have shown that under these conditions the binding was saturating. PMA or αPMA was present on both sides of the epithelia during the binding. Nonspecific binding was measured in the presence of 2 mM unlabeled ouabain. The incubation was stopped by transferring the filter cups to ice-cold 0.8 times concentrated phosphate-buffered saline supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (0.8× PBS-CM) and replacing the apical solution with the same buffer. The basolateral side was rapidly washed twice, and the filters were excised with a razor blade and washed three times for 1 min with 3 ml of 0.8× PBS-CM on a horizontal shaker. The filters were then transferred to scintillation vials and extracted for 16 h in 9 ml of scintillation fluid (Emulsifier-Safe, Packard Instruments, Meriden, CT) before counting in a beta counter (Betamatic, Kontron Digicad, Munich Germany). Radioactivity bound in the presence of 2 mM unlabeled ouabain (less than 10% of total counts) was subtracted. Duplicates were made for each time point.

Cell Surface Biotinylation, Immunoprecipitation, and Streptavidin Blot

A6-C1 cells cultured on filter supports were permeabilized with amphotericin B in sodium-free buffer and treated as described above. The filters were transferred to ice-cold buffer containing 120 mM NaCl, 10 mM triethanolamine (pH 9.0), 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ and biotinylated with 1.1 mM succinimidyl-6-(biotinamide)hexanoate (NHS-LC-biotin, Pierce Chemical, Rockford, IL) according to published protocols (Beron and Verrey, 1994; Gottardi *et al.*, 1995). Cells were solubilized in lysis buffer (1% Triton X-100, 0.4% sodium deoxycholate, 66 mM EDTA, 50 mM Tris-HCl, pH 7.4, and supplemented with protease inhibitors) at 4°C (Beron and Verrey, 1994). Immunoprecipitation of the Na,K-ATPase β1 subunit from equal amounts of proteins and detection of cell surface-expressed molecules by streptavidin blot followed by chemiluminescence were performed as described previously (Beron and Verrey, 1994). The film images were digitalized using a video camera system (Bio-Print, Vilber Lourmat, Marne-La-Vallee, France), and signal densities were compared with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Uptake of Horseradish Peroxidase (HRP)

Uptake of HRP by basolateral fluid phase endocytosis was measured as described previously (Verrey *et al.*, 1993). Briefly, filter cultures were preincubated for 1.5 h in uptake medium (UM; HEPES-buffered culture medium supplemented with 0.5% bovine serum albumin; pH adjusted to 7.4) and treated with PMA, BIM + PMA, or vehicle for 25 min as described above. The filters were then placed on 200-μl drops of UM supplemented with 5 mg HRP/ml, and their apical surfaces were covered with 500 μl of UM. Drugs used for treatments were present on both sides during the 5-min uptake period. The whole procedure was at 28°C for test filters and at 4°C for determination of background activity. The uptake was stopped and the filters extensively washed in ice-cold buffer (0.8× PBS, 0.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.5% bovine serum albumin; pH adjusted to 4.5 and filtered just before use). Cell-associated HRP was extracted for 45 min at 4°C in 1% Triton X-100 and its activity measured using *o*-dianisidine (Sigma Chemical, St. Louis, MO) as substrate. Background activity determined in duplicate filters did not exceed 20% of the total activity and was subtracted.

Measurement of the Basolateral Membrane Capacitance

Basolateral membrane capacitance was estimated from the capacitive transient current measured in response to a voltage jump applied to the modified Ussing chamber (see above). The chamber was voltage clamped using a standard four-electrode voltage clamp configuration. The clamp loop response was optimized for the impedance of the chamber and the monolayer, as confirmed using a circuit simulation. Capacitive transients were acquired with the MacLab system (see above) running Scope V 3.4.1 software and sampling at 20 kHz. Voltage steps of 100 ms duration and 1 mV amplitude were applied to the preparation at 0.25-s intervals, and the transient response was averaged over 64 cycles for each determination. Amphotericin B (in sodium-free buffer, as for I_p measurements) was used to permeabilize the apical membrane in the chamber (at 26°C) 10 min before the addition of PMA or αPMA (100 nM) to allow the preparation to stabilize. This was confirmed by visually monitoring the stability of the baseline and time course of the capacitive transient. Determination of the capacitance was made at 5-min intervals by integrating the acquired current transient after baseline subtraction.

Statistics

Data are expressed as means ± SE. Variations between the different groups were evaluated by analysis of variance, and the significance of the difference between pairs was determined using the Newman-Keuls post test.

RESULTS

PKC Activation by Phorbol Ester Inhibits Amiloride-sensitive Sodium Reabsorption

In intact A6-C1 epithelia, PKC activation by PMA induced a rapid increase in transepithelial short circuit current (I_{sc}) which reached a maximum after approximately 4–5 min (Figure 1A) while αPMA was inactive (Figure 1B). Apical amiloride (50 μM), given 25 min after PMA addition, did not influence the I_{sc} (Figure 1A), in contrast to its inhibitory effect in untreated and αPMA-treated epithelia (Figure 1B). These effects of PMA were only partially prevented by the PKC inhibitor BIM at a concentration of 5 μM. However, at a higher concentration, BIM (15 μM) entirely prevented the PMA-induced amiloride-resistant I_{sc}, but per se it inhibited the amiloride-sensitive part of the I_{sc}.

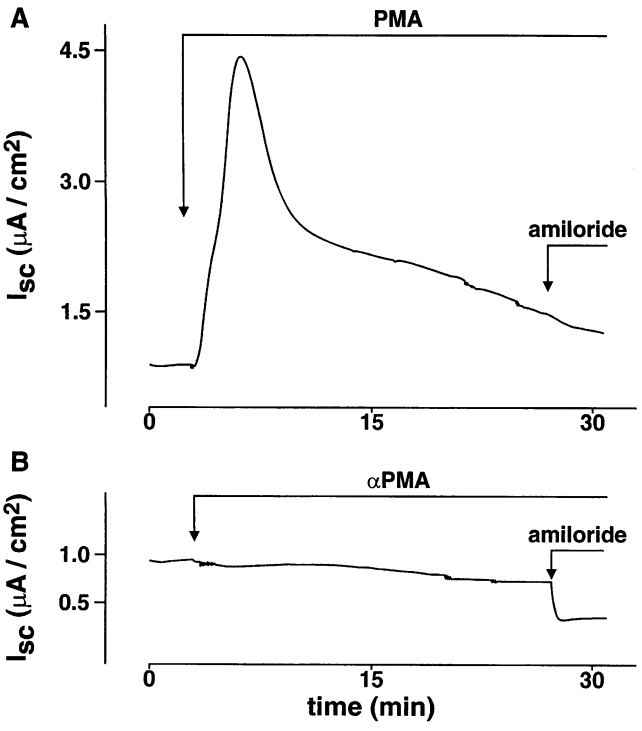

Figure 1. Effect of PMA on transepithelial short circuit current (I_{sc}) measured in intact A6-C1 epithelia. Monolayers of A6-C1 cells were cultured on filters for a total of 15–23 d, the last 5–13 d prior to the experiment in serum-free HEPES-buffered medium. The intact epithelia were maintained in short circuit configuration (V_{TE} clamped at 0 mV) during the recordings. A shows an example of treatment with PMA (10^{-7} M, given to both sides) and B with the control phorbol ester αPMA. Amiloride (5×10^{-5} M) was added to the apical medium. Note the transient increase in I_{sc} and the inhibition of its amiloride-sensitive component by PMA.

The lack of effect of amiloride on the PMA-induced I_{sc} indicated that this current was not via the amiloride-sensitive sodium channel and that, furthermore, PMA inhibited the preexisting amiloride-sensitive sodium transport. These observations correspond to those made by Yanase and Handler (1986) who concluded that activators of PKC inhibit sodium transport and stimulate an amiloride-resistant I_{sc}, which most likely represents basolateral to apical chloride transport.

PKC Activation Decreases the Sodium Pump Current (I_p) in A6 Epithelia

To test whether the basolateral sodium transport step via the sodium pump is also affected by PKC activation, the transport activity of sodium pumps was measured as ouabain-inhibitable pump current (I_p). These measurements were performed at defined intracellular sodium concentrations in A6-C1 epithelia, apically permeabilized with amphotericin B in the absence of sodium (recording shown in Figure 5A) as described (Beron *et al.*, 1995). As shown in Figure 2, PMA time dependently reduced the I_p induced with 90 mM sodium. A significant effect was detectable after 15 min and a maximum was reached after 40 min (40% reduction compared with control). Longer incubation periods did not further decrease the maximal I_p.

In subsequent experiments, treatments were routinely initiated at the same time as permeabilization. After 25 min, PMA reduced the maximal I_p by 29%, whereas no change was induced by the control phorbol ester αPMA (Figure 3A). BIM alone had no effect on the maximal I_p but completely reversed the effect of PMA. This result indicated that PMA decreases sodium pump current via its stimulatory action on PKC.

To evaluate whether PMA had an effect on the apparent sodium affinity of the sodium pumps, similar experiments were performed in the presence of 20 mM or 5 mM sodium (Figure 3, B and C). The absolute values of I_p induced in control epithelia by 20 mM or 5 mM sodium were 3.80 ± 0.10 μA/cm² and 0.40 ± 0.02 μA/cm², respectively. PMA significantly reduced these currents by 43 and 49%, whereas αPMA had no effect. BIM, which alone did not affect the current induced by 20 mM sodium, prevented the effect of PMA to a large extent, but did not abolish it, in contrast to its effect at 90 mM sodium. However, the decrease induced by PMA relative to the PMA + BIM condition was not sodium dependent (28.2%, 34.8%, and 29.5% at 90 mM, 20 mM, and 5 mM sodium, respectively). It can be concluded from these results that the major PKC effect (BIM-inhibitable PMA effect) on the I_p is independent of the intracellular sodium

concentration. In addition to this major sodium-independent effect, PMA appears to produce a further decrease in the I_p stimulated by low sodium concentrations, an effect which is resistant to 5 μM BIM.

Characterization of A6 Cell Lines Expressing Exogenous Sodium Pump α1 Subunits

PKC has been shown to phosphorylate the sodium pump α1 subunit at a N-terminal site (Beguin *et al.*, 1994). To test whether this phosphorylation is required for the observed down-regulation of the sodium pump transport activity, we established A6 cell lines stably expressing the cardiotonic steroid-resistant α1TBM (Jaisser *et al.*, 1992) which was mutated in its PKC phosphorylation site (T15A/S16A; Beguin *et al.*, 1994) and compared them with cell lines expressing the wt α1TBM subunit. These latter cell lines had previously been characterized by Western blotting and I_p inhibition experiments using the cardiotonic steroid strophanthidin (Beron *et al.*, 1995).

Figure 4 shows examples of I_p inhibition experiments performed on epithelia of untransfected A6-C1 cells and cells expressing wt and mutant α1TBM subunits. The concentration of strophanthidin required for half maximal inhibition (K_i) and the fraction of the I_p carried by the hybrid pumps containing the wt or mutant α1TBM subunit were estimated by curve fitting using an equation describing a model with two independent binding sites. In the cell line expressing the wt α1TBM subunit, the I_p carried by the hybrid pumps represented 26.3 ± 0.9% (n = 3) of the total current, whereas in the cell lines expressing the mutant α1TBM subunit it was 41.8 ± 3.0% (n = 4) and 18.8 ± 0.9% (n = 4). The K_i for strophanthidin of hybrid pumps containing the mutant α1TBM subunit was 115 ± 6 μM (n = 8, from three different cell lines). This value is close to but significantly different from the one obtained for pumps containing the wt α1TBM subunit (90 ± 5 μM, n = 3, $p < 0.05$) and to the previously reported K_i of the major sodium pump population of the *B. marinus* bladder cell line TBM-54 (82 ± 3 μM, n = 5, $p < 0.005$) which naturally expresses α1TBM (Beron *et al.*, 1995). These results demonstrate the presence of a second functionally active sodium pump population in the transfected cell lines and show that the apparent affinity of the hybrid sodium pumps for strophanthidin is nearly unchanged by the mutation of the PKC phosphorylation site.

PKC Activation Decreases the I_p Carried by Exogenous wt and Mutant Sodium Pumps

Using the cell lines expressing either a wt or mutant sodium pump α1TBM subunit, we could test whether the PKC phosphorylation site of the α1 subunit is required for down-regulation of sodium pump function. Epithelia of the different cell lines were treated and permeabilized as described above. After induction of a maximal pump current with 90 mM sodium the endogenous sodium pump population was inhibited with 20 or 50 μM strophanthidin. The hybrid pumps were then inhibited with 1.4 mM ouabain (Figure 5A and MATERIALS AND METHODS).

In the cell line expressing the wt α1TBM subunit (Figure 5, B and C), the current carried in control conditions by endogenous sodium pumps was 3.71 ± 0.59 $\mu A/cm^2$ and that carried by the hybrid pumps 0.99 ± 0.17 $\mu A/cm^2$ [note that the I_p measured for endogenous pumps contains also 15–36% of that carried by hybrid pumps and that the I_p measured for hybrid pumps contains 0.5–1.2% of that carried by endogenous pumps (see MATERIALS AND METHODS)]. PKC stimulation by PMA reduced the current carried by the endogenous pumps (Figure 5B) and hybrid pumps (Figure 5C) to a similar extent (35 ± 2% and 41 ± 3%, respectively). The control phorbol ester αPMA had no effect. The PKC inhibitor BIM alone had no effect but abolished that of PMA on the function of both pump populations.

In the cell lines expressing the mutant α1TBM subunit, the mean control values of the currents carried by the endogenous and the hybrid pumps were 4.20 ± 0.17 $\mu A/cm^2$ and 1.31 ± 0.66 $\mu A/cm^2$, respectively

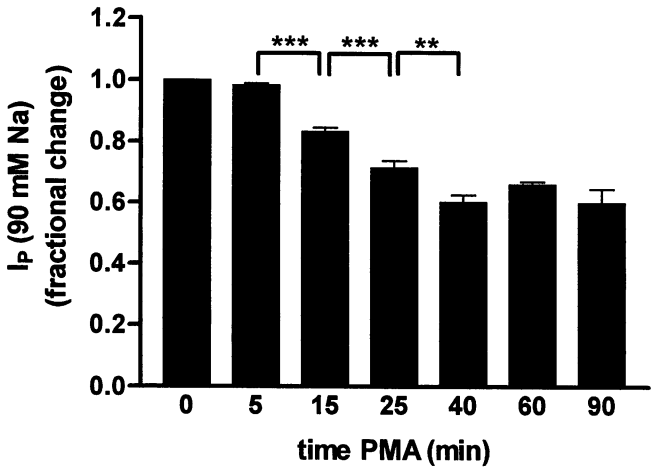

Figure 2. Time course of PMA effect on the sodium pump current (I_p) measured across A6-C1 epithelia apically permeabilized with amphotericin B. Permeabilization was for 25 min in sodium-free buffer. PMA (10^{-7} M) treatment was initiated as indicated. During the recordings, epithelia were maintained in short circuit conditions, and sodium pumps were activated by replacing the sodium-free buffer on both sides with buffer containing 90 mM sodium. Ouabain (1.4 mM) was added to the basolateral compartment 4–5 min after sodium addition, and the decrease in I_{sc} induced within 1 min was taken as I_p as shown in Figure 5A. The maximal I_p induced with 90 mM sodium in control epithelia was 8.45 ± 0.43 $\mu A/cm^2$. Bars represent the mean fractional changes ± SE from three to six experiments. Significant differences between consecutive time points are indicated (***, $p < 0.001$; **, $p < 0.01$).

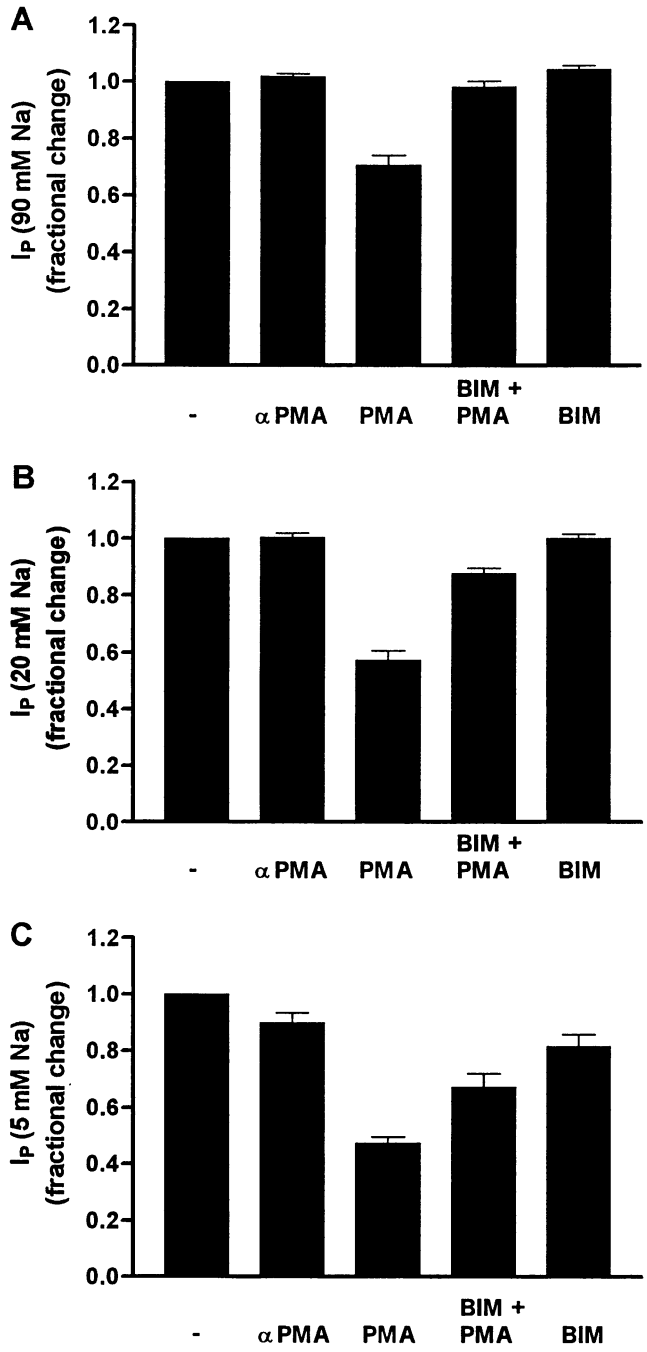

Figure 3. Sodium dependency of PMA effect on I_p across apically permeabilized A6-C1 epithelia. The measurements of the I_p were performed at the indicated sodium concentrations 25 min after the beginning of apical permeabilization and the concomitant addition of PMA (10^{-7} M), αPMA (10^{-7} M), or vehicle. BIM (5×10^{-6} M) was given 15 min earlier. The mean I_p for the control filters is indicated in RESULTS. Bars represent the mean fractional changes ± SE from four to six experiments. At all three sodium concentrations, the I_p elicited in the presence of PMA was significantly lower compared with the other four conditions ($p < 0.001$). The I_p in the PMA + BIM condition was lower than that in the control, αPMA, and BIM conditions at 20 mM sodium (B, $p < 0.01$) and lower than that in the control and αPMA conditions at 5 mM sodium (C, $p < 0.05$).

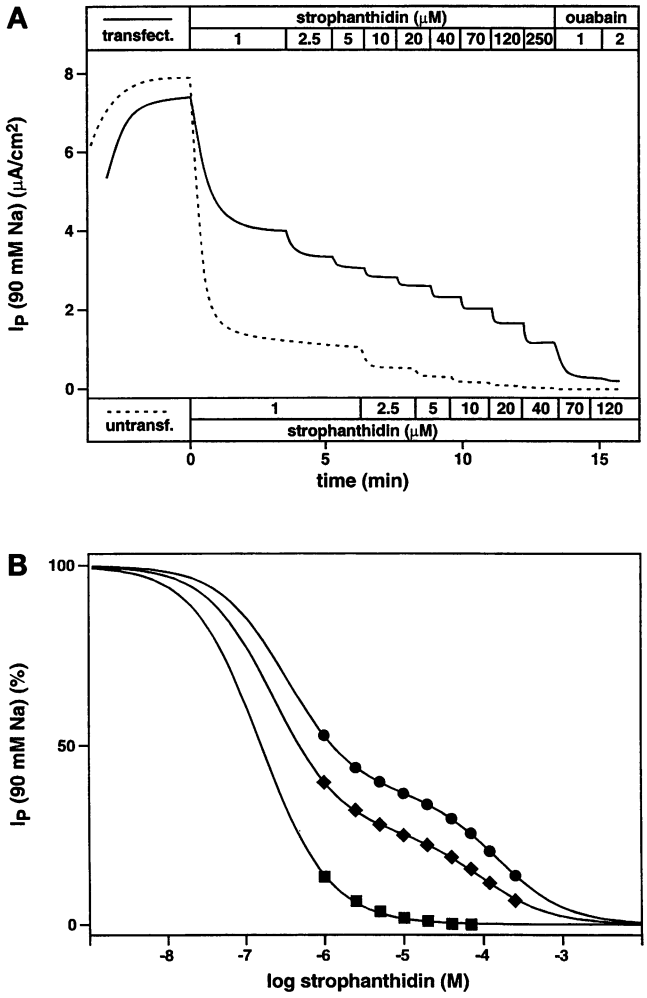

Figure 4. Dose dependence of the I_p inhibition by strophanthidin in epithelia formed by untransfected and transfected A6-C1 cells. The I_p was activated by 90 mM sodium as in Figure 2. Increasing concentrations of strophanthidin were added to the basolateral buffer. Ouabain (2 mM) was used to block the pumps containing the exogenous α subunit. (A) Examples of original recordings performed on epithelia from the untransfected A6-C1 cell line (- - -) and from a cell line expressing the PKC site mutant α1TBM (*Bufo marinus* Na,K-ATPase α1 subunit, ——). (B) Hyperbolic curves corresponding to one or two binding site(s) models were fitted to the experimental data (■, A6-C1 cells; ◆, cell line expressing the wt α1TBM; ●, cell line expressing PKC site mutant α1TBM). Mean K_i values are given in RESULTS.

(mean of eight experiments with three cell lines). PMA reduced the I_p (90 mM sodium) carried by the endogenous and the mutant hybrid pumps to a similar extent (22 ± 4% and 30 ± 2%), whereas αPMA had no effect. BIM prevented the effect of PMA and had no effect on the sodium pump function when added alone (Figure 5, D and E).

The sodium dependence of the PMA effect on I_p was tested for the sodium pumps with the mutant exogenous α subunits. The results were superim-

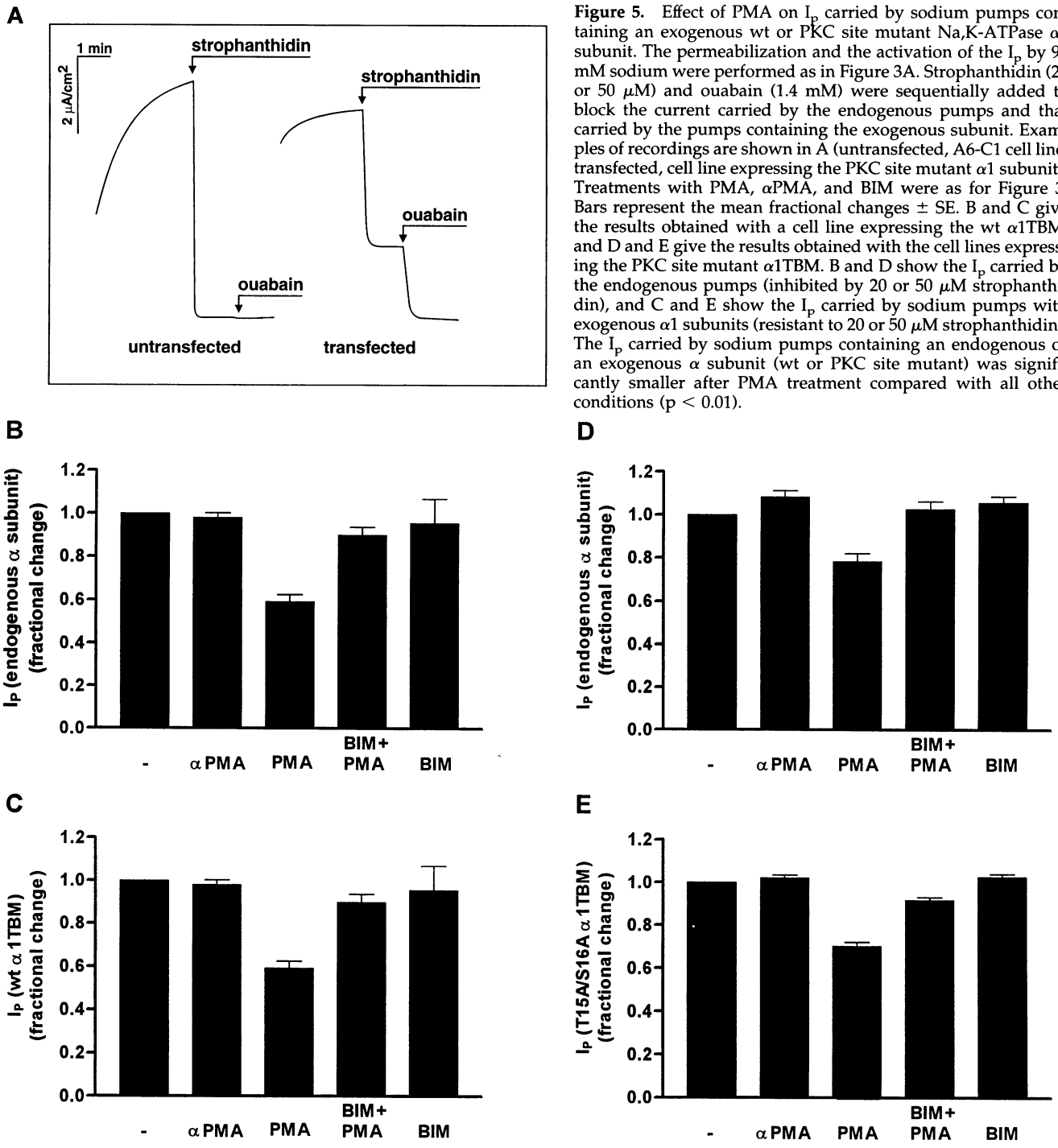

Figure 5. Effect of PMA on I_p carried by sodium pumps containing an exogenous wt or PKC site mutant Na,K-ATPase α1 subunit. The permeabilization and the activation of the I_p by 90 mM sodium were performed as in Figure 3A. Strophanthidin (20 or 50 μM) and ouabain (1.4 mM) were sequentially added to block the current carried by the endogenous pumps and that carried by the pumps containing the exogenous subunit. Examples of recordings are shown in A (untransfected, A6-C1 cell line; transfected, cell line expressing the PKC site mutant α1 subunit). Treatments with PMA, αPMA, and BIM were as for Figure 3. Bars represent the mean fractional changes ± SE. B and C give the results obtained with a cell line expressing the wt α1TBM, and D and E give the results obtained with the cell lines expressing the PKC site mutant α1TBM. B and D show the I_p carried by the endogenous pumps (inhibited by 20 or 50 μM strophanthidin), and C and E show the I_p carried by sodium pumps with exogenous α1 subunits (resistant to 20 or 50 μM strophanthidin). The I_p carried by sodium pumps containing an endogenous or an exogenous α subunit (wt or PKC site mutant) was significantly smaller after PMA treatment compared with all other conditions ($p < 0.01$).

posable with those shown in Figure 3 for wt A6-C1 cells, both for the pumps with the endogenous α subunit (inhibited by 20 μM strophanthidin) as well as for those with the mutated exogenous subunit (resistant to 20 μM strophanthidin).

The fact that the results obtained for the sodium pumps containing the PKC site mutant α1 subunit were almost identical to those obtained with untransfected A6-C1 cells (see above and Figure 3) demonstrates that the down-regulation of sodium pump function after PKC stimulation by phorbol ester is independent of the N-terminal PKC phosphorylation site in the Na,K-ATPase α1 subunit.

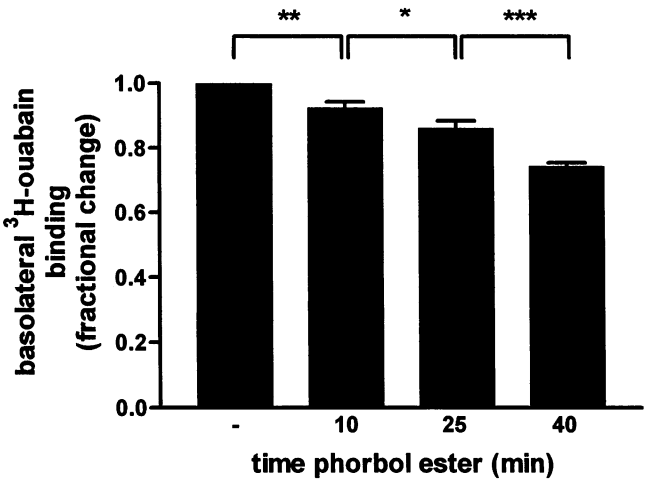

Figure 6. Time course of PMA effect on Na,K-ATPase cell surface expression measured by [^{3}H]ouabain binding. A6-C1 monolayers were cultured, apically permeabilized with amphotericin B, and treated with PMA as for the I_P measurements shown in Figs. 3A and 5. [^{3}H]ouabain bindings were performed as described in MATERIALS AND METHODS. Bars represent the mean fractional changes ± SE from three to four experiments. Significant differences between consecutive time points are indicated (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

PKC Activation by Phorbol Ester Decreases the Number of Cell Surface Expressed Sodium Pumps

We then tested the hypothesis that PKC activation by phorbol ester decreases the number of cell surface-expressed sodium pumps and therefore decreases the current carried by the sodium pumps. Epithelia of untransfected A6-C1 cells were treated and permeabilized as described above, and the number of ouabain-binding sites, corresponding to ouabain-binding-competent sodium pumps expressed at the cell surface, were measured by saturating [^{3}H]ouabain binding. Figure 6 shows that PMA induced a decrease in the number of binding sites which followed a parallel time course to that of the decrease in I_p (Figure 2) but the absolute level of which was lower. The inactive phorbol ester αPMA had no effect.

To test whether this decrease in ouabain-binding sites corresponded to a decrease in cell surface Na,K-ATPase molecules, we labeled basolaterally expressed proteins of apically permeabilized epithelia with the membrane-impermeant reagent NHS-LC-biotin. The β1 subunits of the A6-C1 sodium pumps were immunoprecipitated and biotinylated subunits were visualized by streptavidin blot (Figure 7A).

Figure 7B shows the quantification of several blots. After stimulation of PKC with PMA for 25 min, the number of cell surface-expressed sodium pumps was decreased by 20 ± 4%. Treatment of the cells with αPMA had no effect. The down-regulation of sodium pump cell surface expression by PMA was completely abolished by BIM, which had no effect when given alone.

These results indicate that the decrease in ouabain-binding sites induced by PMA represents a decrease in cell surface Na,K-ATPase molecules, as shown for the β1 subunit. Comparing the extent of PMA-induced decrease in I_p with that of Na,K-ATPase cell surface expression [e.g., at 25 min PMA: 29% I_p reduction in Figure 2 versus 17% decrease in cell surface Na,K-ATPase (mean of Figures 6 and 7)], it appears that approximately 60% of the PMA-induced decrease in sodium pump current could be mediated by a decrease in Na,K-ATPase cell surface expression.

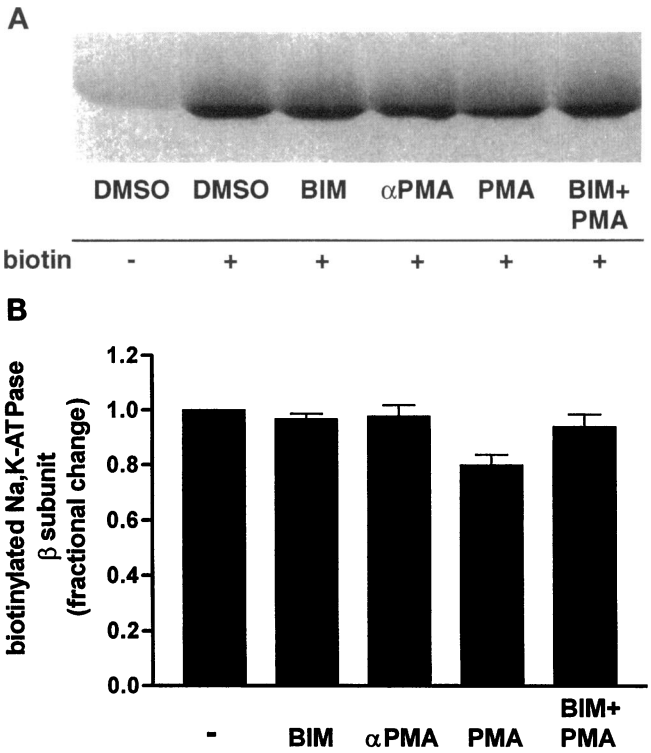

Figure 7. Effect of PMA on Na,K-ATPase cell surface expression measured by surface labeling. Epithelia were cultured, apically permeabilized with amphotericin B, and treated for 25 min with PMA, αPMA, and BIM as for the I_p measurements shown in Figs. 3A and 5. Labeling with NHS-LC-biotin, immunoprecipitation, streptavidin blot, signal detection and quantification are described in MATERIALS AND METHODS. (A) Streptavidin blots of the immunoprecipitated Na,K-ATPase β1 subunit which migrates as a compressed band just below the immunoglobulin heavy chain. (B) Quantification of the signals from streptavidin blots. Bars represent the means of three experiments ± SE. The signal for cell surface Na,K-ATPase β subunit was significantly lower after PMA treatment than in the control conditions ($p < 0.01$) or the other three conditions ($p < 0.05$).

PKC Activation by PMA Increases Basolateral Fluid Phase Endocytosis

To test whether the PMA-induced decrease in sodium pump cell surface expression could be linked to a general increase in basolateral endocytosis, the basolateral uptake of a fluid phase marker (HRP) into unpermeabilized A6-C1 epithelia was measured. As shown in Figure 8, activation of PKC by PMA increased the basolateral uptake of HRP 7.5-fold (5-min uptake period). This effect was completely abolished when the PKC inhibitor BIM was present. It can be concluded that PKC stimulation by PMA increases basolateral fluid phase endocytosis, and that this increase in membrane movement might lead to the observed decrease in cell surface expression of the sodium pump and, consecutively, mediate a large part of the down-regulation of sodium pump function.

PKC Activation Decreases the Basolateral Membrane Surface Area

To test whether the PKC-mediated increase in endocytosis would result in an overall decrease in the basolateral cell surface area, we estimated this surface area by electrical capacitance measurements of the apically permeabilized filter-cultured epithelia. Figure 9A shows the transepithelial current in response to a 1 mV step. The steady-state current represents the current necessary for maintaining the new basolateral membrane potential 1 mV above the preceding clamp level, and the transient current represents the current required to charge the basolateral membrane capacitance. The basolateral membrane capacitance, which provides a measure of membrane area assuming a constant intrinsic membrane capacitance (~1 μF/cm^2), was estimated by integrating the transient relaxation to give the membrane charge transfer induced by the voltage jump. The mean capacitance 10 min after initiation of permeabilization (time 0 in Figure 9B) was 66.1 ± 0.7 μF per 4.7-cm^2 filter culture (n = 8). This corresponds to a surface area of approximately 66 cm^2 per filter, which is ~14-fold the surface of the epithelia. Taking the number of 3.4 × 10^6 cells per filter culture (Beron and Verrey, 1994), one finds a mean basolateral surface area per cell of 19.4 pF ≅2 × 10^3 μm^2. PMA added 10 min after initiation of apical permeabilization produced a decrease in the basolat-

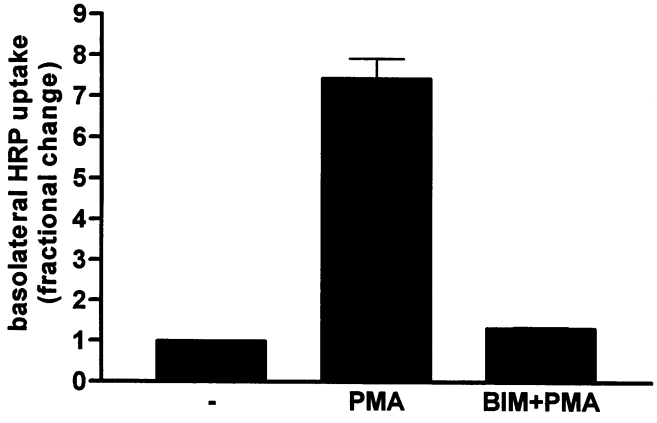

Figure 8. Effect of PMA on basolateral fluid phase endocytosis. Cell culture and treatment of intact epithelia with PMA and BIM were as for Figure 3. Basolateral uptake of the fluid phase marker HRP was at 28°C for 5 min as described in MATERIALS AND METHODS. Bars represent the means of five experiments ± SE. The uptake was significantly higher after PMA treatment than in the control and BIM + PMA conditions ($p < 0.001$).

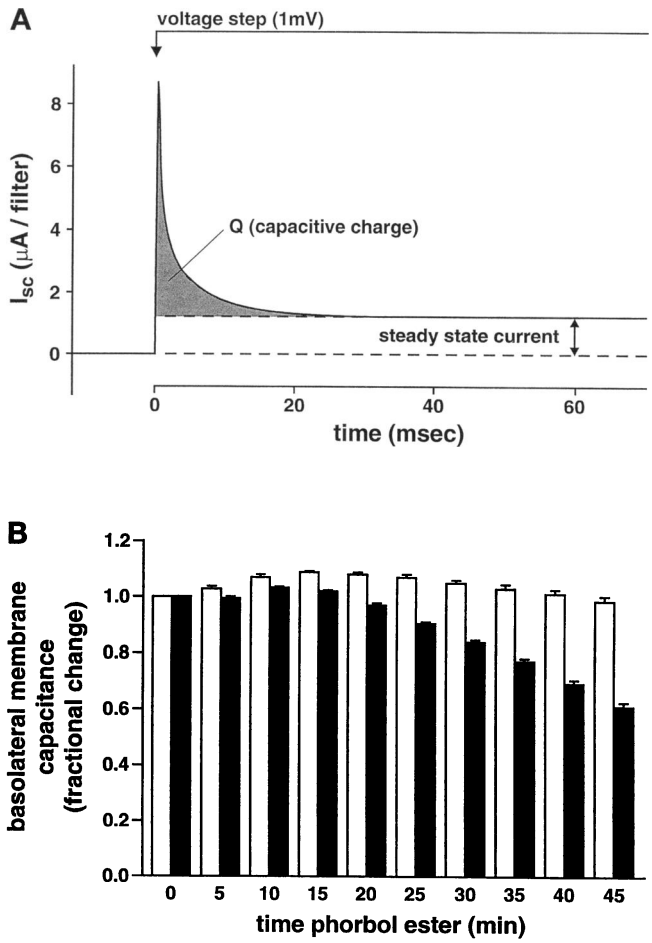

Figure 9. Effect of PMA on basolateral cell surface measured as membrane capacitance. Cell culture was as for I_p measurements. Apical permeabilization with amphotericin B was initiated 10 min before the addition of PMA or αPMA. An example of the averaged current produced by a voltage jump of 1 mV is shown in A. The area under the transient capacitative current relaxation (shaded) was measured every 5 min. Absolute values of capacitance are given in RESULTS. B shows a time course of the mean capacitance difference between control (αPMA, □) and PMA-treated (■) epithelia (n = 4). The decrease induced by the PMA treatment started to be significant 10 min after PMA addition ($p < 0.05$; $p < 0.001$ after 15 min).

eral membrane area (Figure 9B). This effect was prevented by 5 μM BIM which had no impact when given alone. The PMA-induced decrease in membrane area (Figure 9B, relative to the area measured in the presence of αPMA) had a similar time course and amplitude as the decrease in the number of sodium pumps expressed at the cell surface (Figure 6) such that the number of sodium pumps expressed per surface area apparently remained constant.

DISCUSSION

Effect of PKC Stimulation on Na,K-ATPase Function

The effect of PKC stimulation on Na,K-ATPase function in vitro and in vivo remains a matter of debate. The contradictory effects observed on Na,K-ATPase function might be due to several reasons. First, different parameters were measured in conditions which are not comparable. For instance, measurements of ^{86}Rb uptake or ATPase activity using cytochemistry were performed in conditions in which the intracellular sodium concentration was not controlled. Hence, the measured parameter reflects the intracellular sodium concentration,because of the steep sodium activation kinetics of the sodium pump (de Weer, 1992; Beron *et al.*, 1995). In other studies the ATPase activity was measured in disrupted cells, permitting control of the sodium concentration. However, a drawback of this latter approach is that the original cellular structure is lost and that some diffusible regulatory factors might be diluted. It is also possible that the disruption procedure does not provide equal accessibility for substrates to all sodium pumps (for review, Verrey *et al.*, 1996). Furthermore, measurements were performed in different cell types which might differ in the expression of PKC isoforms and other components of the involved signaling cascades. Finally, differences in experimental conditions during the PKC stimulation might modify the result by interfering with signaling cascades, as recently shown for the level of oxygen supply in isolated rat kidney proximal tubules (Féraille *et al.*, 1995).

In the present study, the sodium pump current (I_p) was measured in A6 epithelia, apically permeabilized with amphotericin B. This approach allows a direct measurement of the sodium pump transport activity in the original cellular context and at controlled intracellular sodium concentrations (Beron *et al.*, 1995). The preservation of the epithelial organization and the selectivity of the amphotericin B pores for monovalent ions prevent the loss of functionally important structures and of soluble cellular components such that most regulatory mechanisms are expected to be preserved. Using this method, we have previously shown that the sodium concentration required for half-maximal activation ($K_{0.5}$) of A6 cell sodium pumps is 20 mM and the Hill coefficient is 2.4 (with symmetrical buffers and the concentration of sodium plus potassium kept constant; Beron *et al.*, 1995).

Using this approach, we show now that PKC activation with PMA leads to a substantial decrease in the I_p at all tested intracellular sodium concentrations. Furthermore, the wt and PKC site mutant Na,K-ATPase α1TBM (Jaisser *et al.*, 1992; Beguin *et al.*, 1994) expressed in A6 cells are subjected to the same down-regulation, demonstrating that the PKC effect on the pump current is independent of the presence of the PKC phosphorylation site in the α1 subunit.

In parallel to this effect on the maximal I_p [measured at 90 mM intracellular sodium (Beron *et al.*, 1995)], PMA also decreases the level of the sodium pumps expressed at the basolateral surface of A6 cells, to an extent corresponding to approximately 60% of the I_p decrease (see RESULTS). This suggests that the decrease in sodium pump cell surface expression mediates a large part (~60%) of the decrease in maximal I_p. It has to be mentioned that at low intracellular sodium concentrations, PMA tended to decrease the I_p more than at 90 mM sodium. This additional effect was not prevented by 5 μM of the PKC inhibitor BIM which, in contrast, completely blocked the effect of PMA on Na,K-ATPase cell surface expression and on fluid phase endocytosis.

Effect of PKC Stimulation on Sodium Reabsorption

Several studies have shown that direct PKC stimulation and hormones acting via PKC stimulation decrease the activity of the amiloride-sensitive epithelial sodium channel and hence sodium reabsorption in the cortical collecting duct and A6 epithelia (Yanase and Handler, 1986; Hays *et al.*, 1987; Ling and Eaton, 1989; Ling *et al.*, 1992; Takeda *et al.*, 1994; Frindt *et al.*, 1996). In the present study, we confirm that PKC stimulation by PMA blocks the amiloride-sensitive short circuit current in A6 epithelia while it stimulates an amiloride-resistant I_{sc}, which most likely corresponds to Cl secretion (Figure 1; Yanase and Handler, 1986). These effects of PMA and their inhibition by PKC inhibitors are complex. Indeed, it appears that, besides the fact that the PKC activator PMA inhibits the amiloride-sensitive current, a large part of this current is also blocked by the PKC inhibitor BIM, as if a tonic action of PKC was required to maintain the activity of the amiloride-sensitive epithelial sodium channel. Furthermore, a higher concentration of BIM (15 μM) is required to block the PMA effect on Cl secretion than to block that on basolateral endocytosis. Similarly, part of the I_p inhibition by PMA at low sodium concentration is not prevented by 5 μM BIM. A possible interpretation is that PKC isoforms with differential sensitivities to inhibitors (Martiny-Baron *et al.*, 1993;

Wilkinson and Hallam, 1994; Wilborn and Schafer, 1996) play different roles in the control of transepithelial sodium transport across A6 epithelia.

In any case, the present study shows that PKC stimulation by PMA acts on sodium reabsorption across A6 epithelia not solely by decreasing the activity of apical epithelial sodium channels but also by coordinately decreasing the basolateral sodium pumping activity, an effect which is to a large extent mediated by a decrease in the number of cell surface sodium pumps.

PKC Stimulation Decreases Cell Surface Na,K-ATPase, Stimulates Endocytosis, and Decreases Cell Surface Area

The steady-state level of cell surface expression of a protein is determined by its rate of endo- and exocytosis. These rates are dependent on the overall cell-specific endo/exocytosis activity, the intrinsic affinity of a given protein for endocytosis and exocytosis sites (i.e., coated pits), and/or its exclusion from such sites through interaction with other proteins, for instance, with elements of the submembranous cytoskeleton. Our data exclude that the decrease in Na,K-ATPase cell surface expression observed after PKC stimulation is due to PKC-mediated phosphorylation of the α1 subunit which could modify a signal for endo/exocytosis or for anchoring to the cytoskeleton. On the other hand, our data suggest that an increase in the overall endocytosis activity is responsible for the decrease in Na,K-ATPase cell surface expression. Such an increase in endocytosis could lead to a decrease in Na,K-ATPase cell surface expression without a change in its apparent affinity for (coated) pits. Indeed, this would be the case if a steady proportion of the endocytosed Na,K-ATPase was excluded from recycling to the cell surface or if membrane recycling to the cell surface (exocytosis) was not increased to the same extent as endocytosis, leading to a decrease in basolateral surface area. This latter possibility is supported by previous experiments showing that the basolateral protein secretion (indicative of exocytotic activity) is only marginally increased by PMA (Verrey *et al.*, 1993).

Taking advantage of the apical permeabilization, we could measure the basolateral surface area as electrical capacitance by voltage clamp. This approach allowed us to show that indeed PMA treatment induces a decrease in the basolateral cell surface area which is proportional to the observed decrease in basolateral sodium pumps.

In summary, Na,K-ATPase down-regulation by PKC activation in A6 epithelia appears to be mediated to a large extent by an increase in basolateral endocytosis, which leads to a decrease in the basolateral membrane area and in Na,K-ATPase cell surface expression, independent of its PKC phosphorylation site.

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