Protein Kinase Regulation of a Cloned Epithelial Na⁺ Channel

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ABSTRACT We examined the regulation of a cloned epithelial Na⁺ channel (αβγrENaC) by protein kinase A (PKA) and protein kinase C (PKC). Experiments were performed in Xenopus oocytes and in planar lipid bilayers. At a holding potential of -100 mV, amiloride-sensitive current averaged -1,279 ± 111 nA (n = 7) in αβγrENaC-expressing oocytes. Currents in water-injected oocytes were essentially unresponsive to 10 μM amiloride. A 1-h stimulation of PKC with 100 nM of PMA inhibited whole-cell currents in Xenopus oocytes at 17.1 ± 1.8, and 22.1 ± 2.6% of control (n = 7), at holding potentials of -100 and +40 mV, respectively. Direct injection of purified PKC resulted in similar inhibition to that observed with PMA. Additionally, the inactive phorbol ester, phorbol-12-myristate-13-acetate, 4-O-methyl, was without effect on αβγrENaC currents. Pretreatment with the microtubule inhibitor colchicine (100 μM) did not modify the inhibitory effect of PMA; however, pretreatment with 20 μM cytochalasin B decreased the inhibitory action of PMA to <20% of that previously observed. In vitro-synthesized αβγrENaC formed an amiloride-sensitive Na⁺-selective channel when incorporated into planar lipid bilayers. Addition of PKC, diacyl-glycerol, and Mg-ATP to the side opposite that which amiloride blocked, decreased the channel’s open probability (P_o) from 0.44 ± 0.06 to 0.13 ± 0.03 (n = 9). To study the effects of PKA on αβγrENaC expressed in Xenopus oocytes, cAMP levels were elevated with 10 μM forskolin and 1 mM isobutyl-methyl-xanthine. This cAMP-elevating cocktail did not cause any stimulation of αβγrENaC currents in either the inward or outward directions. This lack of activation was also observed in oocytes preinhibited with PMA and in oocytes pretreated with cytochalasin B and PMA. Neither α-ENaC nor αβγrENaC incorporated into planar lipid bilayers could be activated with PKA and Mg-ATP added to either side of the membrane, as P_o remained at 0.63 ± 0.06 (n = 7) and 0.45 ± 0.05 (n = 9), respectively. We conclude that αβγrENaC is inhibited by PKC, and that αβγrENaC is not activated by PKA. Key words: PKA • PKC • ENaC • oocytes • PLB

INTRODUCTION

Apically located amiloride-sensitive Na⁺ channels are pivotal to the regulation of transepithelial Na⁺ transport. These Na⁺ channels are regulated by various hormonal and humoral factors, many of which act via second messenger activation of protein kinases. Two important protein kinases, namely, PKA and PKC, have been implicated in the regulation of amiloride-sensitive Na⁺ channels. For example, a variety of agents, such as antidiuretic hormone, forskolin, theophylline, and permeable hydrolyzable analogues of cAMP, have been used to increase intracellular levels of cAMP and to activate the PKA pathway. In a number of different epithelia, these agents consistently and markedly activate transepithelial Na⁺ transport via activation of apical Na⁺ channels (Els and Helman, 1991; Marunaka and Eaton, 1991; for review see Benos et al., 1995). This cAMP-mediated activation is widely considered a hallmark of amiloride-sensitive Na⁺ channels.

Activation of PKC has been shown by a variety of methods to inhibit transepithelial Na⁺ transport via inhibition of apical Na⁺ channel activity. Yanase and Handler (1986) demonstrated that PMA inhibits Na⁺ transport in turtle colon. Ling and Eaton (1989) showed by patch clamp of A6 epithelia that PKC activation inhibits open channel density. Silver et al. (1993) have demonstrated that elevation of Ca²⁺, and presumably activation of PKC, caused a decrease of Na⁺ channel activity (NPo) in the rat cortical collecting duct (CCD). Similarly, from measurements on a purified bovine papillary Na⁺ channel reconstituted into planar lipid bilayers, Oh et al. (1993) have confirmed these observations and have suggested that at least a compo-

1Abbreviations used in this paper: CFTR, cystic fibrosis transmembrane conductance regulator; DAG, diacyl-glycerol; g⁺mil, amiloride-sensitive inward slope conductance at -100 mV; g⁺mil, amiloride-sensitive outward slope conductance at +40 mV; I⁺mil, whole-cell current at a holding potential of -100 mV; I⁺mil, whole-cell current at a holding potential of +40 mV; I⁺mil, amiloride-sensitive current at 10 μM amiloride; I⁺mil, amiloride-insensitive current; I⁺mil, amiloride-sensitive current at -100 mV; I⁺mil, amiloride-sensitive current at +40 mV; IBMX, isobutyl-methyl-xanthine; I/V, current to voltage relationship; MPMA, phorbol-12-myristate-13-acetate, 4-O-methyl; Npo, total channel density; Fpo, open probability; rENaC, renal epithelial Na⁺ channel; τenkre, time constant for inhibition by PKC; Vrev, reversal potential or open circuit voltage.
The present work was carried out to determine the regulation of a cloned rat epithelial Na\(^{+}\) channel (rENaC) by PKC and PKA. These channels are formed by a multimeric combination of \(\alpha\), \(\beta\), and \(\gamma\) subunits. These associated proteins were initially cloned from colonic epithelia of rats fed a low Na\(^{+}\) diet (Canessa et al., 1993, 1994), but have now been expanded to include human, bovine, and *Xenopus* homologues (McDonald et al., 1994, 1995; Fuller et al., 1995; Puoti et al., 1995). ENaCs have been proposed as candidates for the channels that mediate Na\(^{+}\) transport in renal CCDs, because patch clamp studies of these channels expressed in *Xenopus* oocytes revealed that they form a small-conductance (5 pS), highly Na\(^{+}\)-selective channel with high amiloride-sensitivity and long open and close times, similar to the observed pharmacological and kinetic profiles of Na\(^{+}\) channels present in these epithelia (Palmer and Frindt, 1988). With the exception of a recent report by Stutts et al. (1995) describing the effect of PKA on \(\alpha\beta\gamma\)-rENaC expressed in MDCK cells, no data exist describing the regulation of \(\alpha\beta\gamma\)-ENaCs by protein kinases.

Channel function was studied in the *Xenopus* oocyte heterologous expression system and in planar lipid bilayers. We conclude that stimulation of PKC by 100 nM PMA causes an inhibition of \(\alpha\beta\gamma\)-rENaC expressed in *Xenopus* oocytes. This inhibition was also confirmed by direct injection of PKC. The largest part of the response to PMA was abolished by a 2-4-h pretreatment with the microfilament disrupter cytochalasin B, but not by the microtubule disrupter colchicine. Inhibition by PKC was also confirmed for \(\alpha\beta\gamma\)-rENaC reconstituted into planar lipid bilayers, where a direct inhibition of single-channel open probability (\(P_0\)) was observed after the addition of PKC, diacetyl-glycerol (DAG), and ATP. These observations suggest a dual mechanism of direct and indirect action of PKC. In contrast, there was no effect of forskolin and isobutylmethylxanthine (IBMX) on oocytes expressing \(\alpha\beta\gamma\)-rENaC, consistent with the lack of an effect of cAMP on Na\(^{+}\) transport in the rat colon (Bridges et al., 1984), the tissue from which ENaCs were originally cloned. Additionally, no effect of PKA-mediated phosphorylation on ENaC single channel activity was observed in planar lipid bilayers. These observations indicate that the channel formed by \(\alpha\beta\gamma\)-rENaC by itself is insensitive to PKA activation.

**Materials and Methods**

**RNA Synthesis**

The pSport plasmid (GIBCO BRL, Gaithersburg, MD), which contained either \(\alpha\), \(\beta\), or \(\gamma\)-rENaC (a generous gift from Dr. B. Rossier, University of Lausanne), was linearized with overnight incubation with Not I (Promega Corp., Madison, WI). Cystic fibrosis transmembrane conductance regulator (CFTR) contained in PBQ4.7, a generous gift of Dr. R. Frizzell (University of Alabama at Birmingham), was linearized overnight with EcoRV (Promega Corp.). The appropriate DNA was purified using the Geneclean kit (Bio 101, Inc., Vista, CA). Sense RNA was synthesized from purified plasmid DNA using T7 RNA polymerase for \(\alpha\beta\gamma\)-rENaC and for CFTR, according to the manufacturer’s instructions (Promega Corp.). RNA was in vitro synthesized in the presence of a methylguanosine cap analogue, \(\text{m}^4\text{G}’\text{ppp}’\text{G}\) (NEB, Beverly, MA), in threefold excess of GTP. This procedure has been reported to stabilize cRNA and thus enhance its translational efficiency (Kriegl and Melton, 1984; Melton et al., 1984). After two rounds of phenol/chloroform extraction and ethanol precipitation, RNA was quantitated by measuring OD at 260 nm and stored at \(-80^\circ\text{C}\).
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following equation:

\[ i = m \sum_{i=1}^{N} \xi_i \]

\[ P_o = \frac{1}{T-N}. \]

where \( \xi \) is an event dwell time, \( N \) is the number of channels (equals one), and \( m \) and \( T \) are the number of events and the duration of the analyzed record, respectively.

**In Vitro Translation**

For planar lipid bilayer experiments, \( \alpha \beta \gamma \gamma \alpha \) was in vitro translated using a nucleoside-treated rabbit reticulocyte cell lysate system in the presence of canine microsomal membranes (Promega Corp.) as previously described (Awayda et al., 1995). The translation reaction was carried out using \(~20 \mu Ci of [35S]methionine (Dupont-New England Nuclear, Boston, MA). The translation reaction was terminated and solubilized by the addition of 0.4% (final concentration) of Triton X-100. An equal volume of sample buffer (125 mM Tris, 20% glycerol, 0.01% bromophenol blue) was subsequently added, and the lysate containing de novo-synthesized protein was purified by passing through a gel filtration (G-75) Sephadex column (Pharmacia Biotech, Piscataway, NJ). The column fraction enriched in ENaC was determined via scintillation counting and autoradiography as described by Awayda et al. (1995) and by Ismailov et al. (1996). Liposomes were made by the addition of 10 µl of the column fraction enriched in \( \alpha \) alone or 10 µl of each fraction enriched in \( \alpha, \beta, \) and \( \gamma \) and 590 or 570 µl of reconstitution buffer (400 mM KCl, 5 mM Tris, 0.5 mM MgCl₂, pH 7.4) to Na⁺-evaporated phospholipids, containing 300 µg of diphytanoyl-phosphatidyl-ethanolamine, 200 µg of diphenylcarbamoyl-phosphatidyl-serine, and 150 µg of phosphatidylcholine. From the amount of incorporated [35S]methionine and the percent methionine composition of each ENaC subunit, we estimated that we were reconstituting \(~0.3-0.5 \mu g of each ENaC subunit into liposomes (i.e., a protein/lipid ratio of 0.0014-0.0023:1).

**Membrane Vesicles**

Oocyte membrane vesicles were prepared as previously described (Perez et al., 1994; Awayda et al., 1995). Briefly, 50 oocytes were homogenized in 0.7 ml of an ice-cold high potassium buffer containing protease inhibitors. The homogenate was overlaid on a sucrose density gradient of 

\[ 35,000 g, the interphase layer was collected and respun at 50,000 g. The pellet, which contained the membrane fraction, was resuspended and stored at \(-20^\circ C\) until use.

By convention, inward flow of cations is designated as inward current (negative current), and all voltages are reported with respect to ground or bath. Data are reported as mean ± SD, with the exception of single-channel open probabilities, which are reported as mean ± SEM. Significance was determined using Student's t test at the 95% confidence level.

**RESULTS**

Expression of \( \alpha \gamma \gamma \alpha \) in Xenopus oocytes resulted in the appearance of inward amiloride-sensitive currents. Indeed, for oocytes clamped at \(-100 \) mV, amiloride-sensitive currents \((i_{amiloride})\) averaged \(54 \pm 5 \) nA, \((n = 15\) oocytes, from five toads), and ranged from 21 to 113 nA. These small values of current were consistent with
those previously reported from the expression of the α subunit of ENaC (Canessa et al., 1993; Fuller et al., 1995). Because of the difficulties in resolving small nanoamp-level changes of current over the course of >1 h, we chose to examine the effect of PMA on oocytes expressing all three ENaC subunits. Under these conditions, the currents attributed to the presence of all three ENaC subunits are much larger (see below, and Canessa et al., 1994), and the time course of current changes can be easily followed out for extended periods even in oocytes with inherent baseline drift.

**Effect of PKC on Oocytes Expressing αβγ-rENaC**

Coinjection of cRNA encoding for the αβγ-rENaC subunits resulted in much larger amiloride-sensitive currents, and \( I_{\text{am}} \) averaged \(-1,936 \pm 214 \text{ nA} \) \((n = 59 \text{ oocytes from 22 toads)}\). This enhanced current was observed despite the 6-12-fold lower concentration of injected cRNA (see Materials and Methods). A representative whole-cell current trace for an oocyte expressing αβγ-rENaC is shown in Fig. 1 A. This trace was collected at the end of the control period, which usually lasted 30–60 min, depending on stability of the baseline. As shown in Fig. 1, B and C, amiloride reversibly inhibited the majority of current at all voltages. Amiloride was added at 10 μM for 5 min (after 3–4 min no further inhibition is observed, indicating that a saturating concentration of amiloride is equilibrated in the chamber). As expected, with the addition of a saturating concentration of a blocker such as amiloride \( (K_i \sim 70 \text{ nM, see Fuller et al. (1995), which is approximately two orders of magnitude less than the concentration used for complete block in this study}) \), the washout period was longer than the wash-in period. Usually, complete reversal of the effect of amiloride was on the order of 15–20 min and was largely dependent on the exchange rates of solutions through the chamber.

The current measured at different hyperpolarizing voltages was essentially all amiloride sensitive, and was highly reversible upon washout of amiloride (see Fig. 1, D and E). The \( I/V \) relationships of the amiloride-sensitive component before and after recovery from amiloride are shown in Fig. 1 F. Consistent with a previous report by Canessa et al. (1994), the current attributed to ENaC was slightly inwardly rectified in the range of \(-100 \text{ mV to } +40 \text{ mV}\). However, these investigators did not extend their measurements to voltages more depolarized than +40 mV. As observed in Fig. 1, amiloride-sensitive ENaC currents between +40 and +80 mV were slightly outwardly rectified and were reversibly blocked by amiloride, as the entire \( I/V \) relationship, before adding amiloride and after recovery from amiloride, was essentially unchanged (data representative of 11 experiments where reversibility of amiloride was tested). Indeed, amiloride-sensitive currents averaged \(-1,936 \pm 214, 268 \pm 53, \text{ and } 827 \pm 123 \text{ nA at } -100, +40, \text{ and } +80 \text{ mV, respectively} \) \((n = 59 \text{ oocytes})\).
of the corresponding I/V relationships in Fig. 2 indicates that the reversal potential was near +20 mV. This marked shift of $V_{rev}$ ($V_{rev}$ ranged from −5 to +25 mV in αβγ-rENaC-expressing oocytes versus a range from −35 to −60 mV in water-injected oocytes, and, furthermore, $V_{rev}$ in ENaC-expressing oocytes, after inhibition by 10 μM amiloride, was in the same range as that of water-injected oocytes) is consistent with the activation of an Na⁺ conductance. The magnitude of $V_{rev}$ never reached the expected equilibrium reversal potential for Na⁺, assuming an intracellular [Na⁺] of 5–10 mM (Kushner et al., 1989; Dascal, 1987). This has been attributed to an elevated intracellular [Na⁺] due to loading of oocytes expressing such high levels of an Na⁺-selective channel (Canessa et al., 1994). Thus, in αβγ-rENaC-expressing oocytes, the amiloride-sensitive currents measured on either side of this reversal po-
TABLE I

<table>
<thead>
<tr>
<th>Current, nA</th>
<th>Control1</th>
<th>Amiloride</th>
<th>Control2</th>
<th>PMA</th>
<th>E1/C2</th>
<th>Amiloride</th>
<th>E2/C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inward (I_{-100})</td>
<td>Mean: -1,757</td>
<td>-73</td>
<td>-1,279</td>
<td>-214</td>
<td>0.171</td>
<td>-60</td>
<td>0.050</td>
</tr>
<tr>
<td>± SEM</td>
<td>87</td>
<td>11</td>
<td>111</td>
<td>22</td>
<td>0.018</td>
<td>9</td>
<td>0.009</td>
</tr>
<tr>
<td>Outward (I_{+40})</td>
<td>Mean: +388</td>
<td>+29</td>
<td>+273</td>
<td>+57</td>
<td>0.221</td>
<td>+17</td>
<td>0.067</td>
</tr>
<tr>
<td>± SEM</td>
<td>35</td>
<td>4</td>
<td>34</td>
<td>5</td>
<td>0.026</td>
<td>2</td>
<td>0.012</td>
</tr>
</tbody>
</table>

n = 7; amiloride was added at 10 μM for 5 min before and after adding 100 nM PMA for 1 h. Control2 (C2) represents the value of current immediately before adding PMA, E1 represents the value of current 1 h after adding PMA, and E2 represents the value current after adding amiloride at the end of the experiment.

Potential are representative of inward and outward ENaC currents. We limited our analysis to the effects of drugs on inward and outward amiloride-sensitive currents at -100 (I_{-100}^{amil}) and +40 mV (I_{+40}^{amil}), respectively, because Xenopus oocytes are known to contain voltage- and time-dependent Ca²⁺ and Cl⁻ channels, which are activated at voltages >+60 mV (Dascal, 1987). Consequently, we also limited our report to effects on the amiloride-sensitive membrane slope conductance at these two voltages.

The effects of PMA on I_{-100} and I_{+40} are summarized in Table I. PMA decreased I_{-100} by 83% from a baseline value of -1,279 to -214 nA. This value is near that of I_{amil}, which averaged the amiloride-insensitive current, -60 nA. Similar results were observed for I_{+40}, which was inhibited by 78% of control and further inhibited by amiloride to 6.7% of control. Values of I_{amil} at either voltage were slightly decreased after the addition of PMA; however, the absolute values of these changes are negligible when compared with the values of amiloride-sensitive ENaC currents. The amiloride-insensitive currents were of similar magnitude to the background currents present in untreated water-injected oocytes at the same voltage (data not shown). Therefore, we conclude that the majority of whole-cell current at these two voltages is attributed to ENaC, and that expression of this channel does not alter the natural density of endogenous channels, at least between -100 and +40 mV. Moreover, these low leak values obviate the need for any leak subtraction. Nevertheless, reported values already have the amiloride-insensitive component subtracted.

The time course of inhibition of oocytes expressing αβγ-ENaC by 100 nM PMA is shown in Fig. 3. The inward amiloride-sensitive slope conductance at -100 mV (g_{amil}^{in}) decreased slowly from 15.9 ± 1.4 to 1.9 ± 0.5 μS (n = seven oocytes from three toads). Similarly, the outward slope conductance (g_{amil}^{out}) decreased from 6.3 ± 0.8 to 1.1 ± 0.2 μS (n = seven oocytes from three toads). After correction for a baseline drift, which was
present in this group of experiments, the time course of the decrease of both \( g_{i\text{amil}} \) and \( g_{o\text{amil}} \) could be approximated with monoeXponential kinetics with similar time constants (\( \tau_{\text{PKC}} \)) in the range of 24 min. PMA was slightly more efficacious at decreasing \( g_{o\text{amil}} \) than \( g_{i\text{amil}} \). After 1 h of PMA treatment, \( g_{i\text{amil}} \) and \( g_{o\text{amil}} \) approached the values of slope conductance of amiloride-treated oocytes, which averaged 0.9 ± 0.3 and 0.6 ± 0.1 \( \mu \text{S} \), respectively.

**Specificity of the PMA Effect**

To ascertain that the observed inhibition with PMA could be attributed to PKC activation, we examined the effect of MPMA, an inactive analogue of PMA, on oocytes expressing \( \alpha\beta\gamma\text{-ENaC} \). Shown in Fig. 4 is the time course of action of 100 nM MPMA. No significant effects were observed on either \( g_{i\text{amil}} \) and \( g_{o\text{amil}} \), even after 30 min. By contrast, after half an hour, PMA caused a 67% and a 53% decrease of \( g_{i\text{amil}} \) and \( g_{o\text{amil}} \), respectively. Thus, it appears that the PMA-mediated effect was likely due to its ability to activate PKC rather than a nonspecific lipophilic effect.

To rule out that inhibition of ENaC by PMA, and presumably PKC, was due to nonspecific retrieval of oocyte plasma membrane–containing ENaC, and any other membrane-bound proteins, we performed experiments on oocytes expressing wild-type CFTR. As shown in the representative example in Fig. 5, the cAMP-elevating mixture of 10 \( \mu \text{M} \) forskolin and 1 mM IBMX stimulated whole-cell currents in CFTR-injected oocytes >30-fold (mean current at −100 mV was 3.9 ± 0.7 \( \mu \text{A} \), \( n = 33 \) oocytes from nine toads). Comparable experiments in water-injected oocytes resulted in virtually no change of whole-cell currents (\( n = 15 \), nine toads). PMA treatment did not affect CFTR currents even after 45 min of

**Figure 4.** MPMA, a weak PKC analogue, does not inhibit amiloride-sensitive \( \alpha\beta\gamma\text{-ENaC} \) currents. A lack of effect was observed on both the inward (A) and outward (B) amiloride-sensitive conductances (\( n = 4 \) oocytes from two toads).
continuous incubation (Fig. 5 C). The time course of activation of CFTR with the cAMP cocktail and the subsequent effect of a PMA effect is shown in Fig. 5 D. In this group of five oocytes (two toads), it is clear that PMA does not have any detectable effects on \( \mathcal{I}_{-100} \). These data provide direct evidence that PMA (and presumably PKC) do not cause nonspecific inactivation of membrane channels by indiscriminate plasma membrane endocytosis (see Discussion) and suggest that inhibition of \( \alpha \beta \gamma \text{-ENaC} \) by PMA (and presumably PKC) occurs via specific inhibition of the channel itself.

To demonstrate further the link between PMA and PKC activation and ENaC inhibition, we recorded the whole-cell currents in ENaC-expressing oocytes before and after direct injection of PKC (see Materials and Methods). As shown in the representative example in Fig. 6, \( \mathcal{I}_{-100} \) decreased by 51% from \(-5.04\) to \(-2.46 \mu A\) 1 h after injection of PKC. In this configuration, amiloride-insensitive currents were only slightly higher than those in oocytes impaled with two electrodes only (\( \mathcal{I}_{\text{amil}} = -0.19 \pm 0.02 \mu A, n = 4 \)), indicating the absence of appreciable leak introduced by the third injecting electrode. This example was representative of four experiments from two toads where the average changes of \( \mathcal{I}_{-100} \) for oocytes injected with PKC and those injected with vehicle alone, are summarized in Fig. 6 C. The magnitude (\( \mathcal{I}_{-100} \) decreased by \( 47.3 \pm 1.9\% \) after 1 h) and the time course of these changes are analogous to the values of inhibition of ENaC (after correction of baseline drift) caused by the addition of PMA alone (see Fig. 3), providing further proof for the specificity of the actions of PMA.

**Role of the Cytoskeleton**

The observation of a relatively long \( \tau_{\text{PKC}} \) may be indicative of a specific endocytic trafficking of ENaC in response to PKC activation. To assess the extent, if any, of cytoskeletal involvement in the inhibition by PMA, experiments were carried out on oocytes pretreated with either 20 \( \mu \text{M} \) cytochalasin B or 100 \( \mu \text{M} \) colchicine. Cytochalasin B was added for 2-4 h, followed by a 30-min incubation with PMA. As shown in Fig. 7, the response to PMA was greatly blunted for both \( g_{\text{amil}}^{\text{in}} \) and \( g_{\text{amil}}^{\text{out}} \), which decreased by 2.6 and 0.9 \( \mu \text{S} \), respectively. As 30 min was greater than the \( \tau_{\text{PKC}} \) of the decrease of either \( g_{\text{amil}}^{\text{in}} \) or \( g_{\text{amil}}^{\text{out}} \), a much larger decrease of conductance was expected (see above) than was actually observed. This blunted response to PMA implicates a role of actin microfilaments in the PKC-mediated inhibition of \( \alpha \beta \gamma \text{-ENaC} \).
Figure 8. Direct inhibition of ENaC by PKC in planar lipid bilayers. Protein was obtained by in vitro translation as described in Materials and Methods. Both α-ENaC and αβγ-ENaC were equally inhibited by PKC. The data are representative of 13 α-ENaC and 9 αβγ-ENaC experiments from three translation reactions. Similar results were obtained for the ENaC channel obtained from plasma membrane vesicles of oocytes expressing the appropriate protein. Shown in A is a representative compressed and expanded scale trace for both α-ENaC and αβγ-ENaC under control conditions. The representative trace after inhibition by PKC is shown in B. The respective all-points amplitude histograms are shown in D and E. 10 μM amiloride was routinely added at the end of each experiment to block channel activity completely, as shown in C and F. Bilayer membrane potential was clamped to 100 mV. The time course of inhibition of single-channel activity with PKC could not be determined because of the transient stimulation of ENaC current observed after stirring of the chamber after the addition of drugs. This is likely attributed to the mechanical activation properties of ENaC (see Awayda et al., 1995; Ismailov et al., 1996).
rENaC. The PMA-induced inhibition of αβγ-rENaC was unaltered by a 4-5-h pretreatment with colchicine (n = three oocytes from two toads, data not shown). This result indicates the absence of microtubule involvement in the activation of PKC and subsequent inhibition of αβγ-rENaC.

Direct Effects of PKC on α-ENaC and αβγ-rENaC in Planar Lipid Bilayers

We previously described a protocol for studying in vitro-translated ENaCs in planar lipid bilayers (Awayda et al., 1995; Ismailov et al., 1996). Using this technique, we demonstrated that in vitro-translated α-ENaC and αβγ-rENaC form functional amiloride-sensitive Na⁺-selective channels. We used this technique to study the direct effects of PKC on ENaCs. Only membranes with a single channel were used in the present experiments. Consistent with previous reports, α-ENaC alone and αβγ-rENaC formed amiloride-sensitive Na⁺ channels with multiple conductance substates of 13 pS (Ismailov et al., 1996). These channels were both cation selective and had a high Na⁺ to K⁺ permeability ratio (10:1). Both channels were exquisitely sensitive to the diuretic amiloride, with apparent inhibitory equilibrium dissociation constant of 165 ± 20 nM (n = 8 from three different translation reactions for each of α-ENaC and αβγ-rENaC). As evident from the representative single channel records in Fig. 8 A, and the associated all-points histograms in D, the channel formed by α-ENaC alone resided predominantly in a main 40-pS conductance state, whereas the channel formed by αβγ-rENaC resided predominantly in its 13-pS subconductance state. Addition of a phosphorylation cocktail containing purified rat brain PKC (5 ng/ml), DAG (5 μM), and Mg-ATP (100 μM) directly inhibited both α-ENaC and αβγ-rENaC (Fig. 8, B and E). The PKC cocktail did not affect ENaC single channel conductance (main conductance or subconductance levels) but caused a decrease of α-ENaC main conductance state Po from 0.62 ± 0.08 to 0.14 ± 0.02 (n = 13, from three different translation reactions). Similar effects of the PKC cocktail were observed on in vitro-translated αβγ-rENaC incorporated into planar lipid bilayers (13-pS conductance), as the Po of the prevalent subconductance state decreased from 0.44 ± 0.06 to 0.13 ± 0.03 (n = 9, from three different translation reactions). This inhibition was not observed with DAG or ATP alone, and was only observed when all three components were added to the presumptive intracellular side of the incorporated channel, the side opposite to that blocked by amiloride. Subsequent addition of 10 μM amiloride completely abolished any channel activity for both α-ENaC and αβγ-rENaC (Fig. 8, C and F).

Lack of an Effect of PKA on Oocytes Expressing αβγ-rENaC

Despite the observation of PKC-mediated inhibition of ENaC, it is not clear whether ENaC alone can reconstitute all the properties of Na⁺ channels found in tight epithelia. The issue of regulation of ENaC by cAMP and PKA is central to this question. This question was addressed by assessing PKA regulation of ENaC at both the oocyte expression system and incorporation into planar lipid bilayers.

αβγ-rENaC-expressing oocytes were treated with 10 μM forskolin and 1 mM IBMX. This concentration of forskolin and IBMX is known to cause a robust and rapid (within minutes) activation of CFTR expressed in Xenopus oocytes via elevation of cAMP levels and subsequent activation of PKA (Cunningham et al., 1992; Drumm et al., 1991). A representative effect of forskolin and IBMX treatment on whole-cell currents on CFTR-expressing oocytes is shown in Fig. 5, A and B. This effect was rapid, with nearly 50% of the increase in whole-cell current occurring within 5 min of adding forskolin and IBMX.

On the other hand, there was no effect of the same cAMP-elevating cocktail on the amiloride-sensitive αβγ-rENaC currents in the same batch of oocytes. Indeed, the currents and the corresponding I/V relationship for the control oocyte (Fig. 9 A) could not be differentiated from the current and I/V relationship after treatment with forskolin and IBMX for 40 min (Fig. 9 B). As summarized in Fig. 10, this cAMP-elevating cocktail did not have any immediate or long term (40 min) effects on either g₅₅₅₅ or g₅₅ₐₖ. In this group of experiments, P₉₉₀₀ and P₉₅₀₀ were unchanged and averaged −1,143 ± 201 and 306 ± 58 nA (n = five oocytes from three toads), respectively.

To rule out the possibility that the lack of a cAMP activation of αβγ-rENaC in Xenopus oocytes is attributed to a spontaneously maximally activated channel, experiments were carried out by first inhibiting αβγ-rENaC current by a 1-h incubation with PMA followed by stimulation of cAMP levels with forskolin and IBMX. As seen in Fig. 11, there was no effect of cAMP activation in PMA-pretreated oocytes. This lack of an effect was observed on both g₅₅₅₅ and g₅₅ₛₖ. Moreover, no effect of cAMP on either of these conductances was also observed in three separate oocytes (from two toads) that were treated with forskolin and IBMX.
have been pretreated with cytochalasin B followed by PMA (data not shown). These results indicate that ENaC could not be activated by cAMP (and presumably PKA) in oocytes with an intact or a disrupted cytoskeleton, and that this lack of activation is also independent of the spontaneous rates of activation of these channels.

**Lack of Direct Effects of PKA on α-ENaC and αβγ-ENaC in Planar Lipid Bilayers**

The direct effects of PKA were assessed on α-ENaC and αβγ-ENaC incorporated into planar lipid bilayers. Addition of 1.85 ng/ml catalytic subunit of PKA and 100 μM Mg-ATP did not alter the $P_o$ for either α-ENaC or αβγ-ENaC obtained by way of in vitro translation (Fig. 12). $P_o$ remained at 0.63 ± 0.06 ($n = 7$, from three separate translation reactions) and 0.45 ± 0.05 ($n = 9$, from three separate translation reactions) for α-ENaC and αβγ-ENaC, respectively. In addition, the relative distribution of the main conductance state and all other subconductance states was unaltered by the addition of PKA and ATP for both of α-ENaC and αβγ-ENaC (Fig. 12, B and D). Only single channel membranes were used, and PKA and ATP were added sequentially to both sides of the bilayer membrane. The single channel behavior of α-ENaC and αβγ-ENaC obtained from oocyte vesicles was similar to that of those obtained by way of in vitro translation (see Awayda et al., 1995, and Ismailov et al., 1996, for further details). Additionally, ENaC channels from oocyte vesicles were not activated by PKA and ATP in planar lipid bilayers (data not shown). These concentrations of PKA and ATP are known to activate a variety of channels in planar lipid bilayers, including a purified bovine...
renal papillary channel (Ismailov et al., 1994). Thus, both αrENaC alone and αβγ-rENaC were refractory to stimulation by direct PKA phosphorylation.

D I S C U S S I O N

We have examined the effects of PKA and PKC on a recently cloned epithelial Na+ channel. Activation of PKC or its direct addition caused an inhibition of αβγ-rENaC in both *Xenopus* oocytes and planar lipid bilayers, similar to the observations made after stimulation of PKC in native and cultured high resistance Na+-transporting epithelia. On the other hand, we have not been able to show any effect of forskolin and IBMX on αβγ-rENaC expressed in *Xenopus* oocytes, nor was there any direct effect of PKA and ATP on αβγ-rENaC in planar lipid bilayers.

Comparison of ENaC in Oocytes and Bilayers

When incorporated into planar lipid bilayers, both αrENaC and αβγ-rENaC form amiloride-sensitive, Na+-selective channels. However, the channels formed by αrENaC display a larger conductance and a larger mean current than channels formed by αβγ-rENaC. This is consistent with prior observations from our laboratory (Awayda et al., 1995; Ismailov et al., 1996). This observation is in contrast with the amount of current present in oocytes expressing the α subunit alone versus oocytes expressing all three subunits. This discrepancy may be explained in terms of differences of total channel density (N_v) and P_o. First, N_v may be lower in oocytes expressing exogenous α-rENaC versus oocytes expressing exogenous αβγ-rENaC. Indeed, preliminary evidence from our laboratory using immunofluorescent quantification of the relative amounts of ENaC channels in oocytes expressing the α subunit versus oocytes expressing all three subunits is in agreement with this hypothesis (Awayda, M.S., A. Tousson, and D.J. Benos, unpublished observations). Second, α-rENaC P_o may be less than the αβγ-rENaC P_o when embedded in the oocyte plasma membrane. This hypothesis is also consistent with observations from patch clamp studies of ENaC-expressing oocytes (Ismailov et al., 1996). Thus, differences between αrENaC and αβγ-rENaC in oocytes may be accounted for by changes of both N_v and P_o.

A feature of Na+ channels that is often observed in some epithelia is a so-called channel "run down," where the amount of current attributed to the Na+ channel is initially high and decreases gradually. In many instances, a new steady state current is not reached until 2–3 h after short-circuiting of the epithelia (Els and Helman, 1991). The reason for this observation is not completely understood but may be attributed to phosphatase activation, Na+ self inhibition (Li and Lindemann, 1983), or autoregulatory changes of channel density (Els and Helman, 1991; Silver et al., 1993) subsequent to short-circuiting of epithelia. Additionally, this may be due to reequilibration of the intracellular ionic composition (including the Na+ concentration) subsequent to intracellular voltage perturbations as suggested by Kidder and Rehm (1970). This phenomenon was not observed for the single channel properties in planar lipid bilayers. This could be attributed to the fact that all of the above possibilities, including changes of N_v, are absent in an artificial bilayer environment. Moreover, this phenomenon, while markedly evident in some groups of oocyte expression experiments (see for example the control period in Fig. 4), was absent in other groups of oocyte expression experiments (see for example the control periods in Figs. 5, 8, and 11). Indeed, it was our experience that some groups of oocytes even underwent an upward drift of control, baseline, amiloride-sensitive ENaC current. We found no correlation between the direction of baseline drift and kinase regulation of ENaC in oocytes.

It is known that membrane lipid composition can alter channel properties (Frolich, 1979; Forte et al., 1981; Szabo and Busath, 1983; for review see Barrantes, 1993). Even Na+ channels embedded in the same membrane can display remarkably different properties when the channel is in direct contact with the intracellular milieu, as is the case for an Na+ channel recorded in the cell-attached patch configuration, or when the
Figure 12. Lack of a direct effect of PKA and ATP on the ENaC channel incorporated into planar lipid bilayers. Data are representative of seven α-rENaC and nine αβγ-rENaC experiments from three translation reactions. Similar results were obtained for the ENaC channel obtained from oocyte plasma membrane vesicles. A–F are the same as in the legend of Fig. 9, with the exception of the addition of PKA and ATP instead of PKC, DAG, and ATP.
channel is in contact with an artificial buffer, as is the case for an Na\(^+\) channel recorded in the excised patch configuration (Chinet et al., 1993). Thus, a straightforward comparison between a channel embedded in a biological membrane and the same channel embedded in an artificial membrane may not be feasible. However, the aim of this study was not to compare channel conductance and kinetic properties between the two systems directly, but, rather, to verify broadly, using two completely different techniques, the regulation of the same protein by two protein kinases.

**Inhibition by PKC**

Inhibition of epithelial Na\(^+\) transport by PKC has been implicated in the autoregulatory response of epithelia in response to varying external Na\(^+\) concentrations (Ling and Eaton, 1989). These investigators described a decrease of \(P_o\) of the Na\(^+\) channel in A6 epithelia to values near 0 with a 15–30-min incubation with PMA. Similar findings were observed by Mohrmann et al. (1987), who noted that PMA caused a maximal inhibition of 51% of \(^{22}\)Na\(^+\) uptake into LLC-PK1 cells after 10–15 min. Our observations are consistent with those previously reported. The time course of inhibition followed simple exponential kinetics, with a \(\tau_{PKC}\) near 24 min (Fig. 3). A possible explanation for the discrepancy of the time course of PKC inhibition between the results reported here and the aforementioned studies is temperature variability. Whereas Mohrmann et al. (1987) carried out their experiments at 37°C, and Ling and Eaton (1989) carried out their experiments at 23–24°C, our temperature ranged from 19 to 20°C. Additionally, the large size of oocytes, which would pose a large diffusion barrier compared with that of epithelial cells, may account for the slow effect of PMA. Alternatively, time course differences may be due to inherent differences in the expression system.

Whereas no reports exist on the effect of PKC or PKC agonists on the rat colon, the tissue from which the ENaCs were originally cloned, two groups have carried out experiments on isolated rat CCD, which is known to contain ENaC message and protein (Duc et al., 1994). According to Rouch et al. (1993), there is no effect of PMA or intracellular Ca\(^{2+}\) elevation on Na\(^+\) transport in the isolated rat CCD. These observations are not without controversy because Silver et al. (1993) reported a Ca\(^{2+}\)-induced decrease of Na\(^+\) transport in isolated rat CCD. These differences may be attributed to the fact that Rouch et al. (1993) have carried out experiments on deoxycorticosterone-treated rats fed a normal Na\(^+\) diet with isolated tubules further stimulated with AVP, whereas Silver et al. (1993) carried out their experiments on rats fed a low Na\(^+\) diet without deoxycorticosterone or AVP stimulation. Because ENaC expressed in *Xenopus* oocytes, incorporated into planar lipid bilayers, and endogenous to the rat colon is insensitive to PKA stimulation, our data should be more reasonably compared with the conditions of Silver et al. (1993), who provide indirect evidence for the inhibition of ENaC by PKC.

**Specificity of PMA**

Whereas PMA has been used in many systems to activate PKC, it was important to verify that the observed changes with PMA are not due to a generalized membrane trafficking event, but, rather, are due to a specific effect on ENaC. In the *Xenopus* oocyte system, PMA has been reported to inhibit the Na\(^+\)/K\(^+\) ATPase pump activity (Vasilets and Schwarz, 1992). This observation may be interpreted as a nonspecific effect of PMA in oocytes. However, this finding may also be interpreted as supportive of the role of PKC in inhibiting transepithelial Na\(^+\) transport by simultaneously down-regulating the Na\(^+\) channel and the pump. This is especially true given that PKC activation inhibits the Na\(^+\)/K\(^+\) ATPase endogenous to a variety of epithelial tissues (Wang et al., 1993; Shahedi et al., 1995). Furthermore, PMA does not inhibit every channel or transporter studied in the *Xenopus* oocyte expression system. For example, Fournier et al. (1993) have described a phorbol ester–induced stimulation of a P-type Ca\(^{2+}\) channel; Charpentier et al. (1995) used phorbol esters to activate a slow inward Na\(^+\) current endogenous to *Xenopus* oocytes; and Corey et al. (1994) found that PMA, via activation of PKC, caused a stimulation of the activity of a cloned γ-aminobutyric acid transporter. Thus, it is apparent that PMA does not have the same action on ENaC as other membrane-bound proteins expressed in *Xenopus* oocytes.

Three separate measures were taken to rule out nonspecific effects of PMA in our oocyte preparation. First, we used the inactive phorbol ester (which does not appreciably activate PKC) to demonstrate the lack of an effect on ENaC. Second, we demonstrated that activated CFTR, a membrane-bound channel protein, is not inhibited (or stimulated) by PMA. Third, we demonstrated that direct intracellular injection of PKC can mimic the inhibitory action observed with external addition of PMA. Therefore, we conclude that PMA is not exerting a nonspecific effect on ENaC in oocytes.

**Role of the Cytoskeleton**

One interpretation of the bilayer experiments (Fig. 8) is that PKC phosphorylation causes a direct inhibition of \(\alpha\)ENaC. However, in oocytes, the majority of inhibition by PMA is sensitive to treatment with cytochalasin B. This indicates that PKC may be acting via a dual direct and indirect mechanism of action. Another possibility is that treatment with cytochalasin B inhibits the activation of PKC itself and thus renders PMA ineffec-
In that respect, it is known that activated PKC translocates to the plasma membrane. This translocation process is not simply a matter of diffusion but, rather, an association of different PKC isoforms with different intermediate or microfilaments. Spudich et al. (1992) have reported a colocalization pattern between β-PKC and vimentin in rat basophilic leukemia cells. McBride et al. (1991) have reported colocalization of phospholipase C and α-PKC with actin filaments in rat embryo fibroblasts. Zalewski et al. (1990, 1991) have proposed that zinc is likely to control the distribution of membrane-bound PKC via an actin cytoskeletal interaction. Thus, either PKC translocation or translocation of an intermediate protein that is phosphorylated by PKC may be affected by disrupting the actin cytoskeleton with cytochalasin B. At this time we cannot rule out the possibility that PKC has a dual action on ENaCs: a direct mechanism, in addition to internalization of membrane-bound Na+ channels.

Lack of a PKA Effect

In contrast to the effects of PKC, most amiloride-sensitive Na+ channels are activated by PKA. This activation varies in terms of the magnitude of the elevated response and whether the elevation is sustained. Nevertheless, cAMP causes a two- to threefold activation of the macroscopic rates of Na+ transport in many epithelia. As seen from the results presented here, under no conditions could either α- or αβγ-ENaC be stimulated by PKA. This lack of activation was observed both in the oocyte and in the bilayer systems.

Three major explanations that may resolve the lack of PKA activation of ENaC can be offered. First, it is possible that ENaC is simply missing a PKA-transducing site. Indeed, such a site has been proposed for an Na+ channel purified in our laboratory, where a 300-kD protein that associates with a renal Na+ channel was found to be phosphorylated by PKA and ATP (Sariban-Sohrabiy et al., 1988; Oh et al., 1993). This protein could be a possible candidate for an ENaC-associated protein, since it is found in bovine papilla, Aβ, and toad bladder by different laboratories (Benos et al., 1987; Blazer-Yost and Reci-Pinto, 1994; Kleyman et al., 1989). A recent report by Stutts et al. (1995) indicates that αβγ-ENaC can be stimulated by PKA when expressed in stably transfected MDCK cells and NIH 3T3 fibroblasts. The cAMP-dependent activation was much larger for ENaC expressed in MDCK cells (~90%; see Stutts et al., 1995, Fig. 2) than in fibroblasts (~25%; see Stutts et al., 1995, Fig. 4). A possible interpretation of the discrepancy with our data is that ENaC transfected into these cells is associating with a protein that confers cAMP activation. This possibility is especially plausible in MDCK cells, a renal cell line that likely has all the machinery for cAMP-activated Na+ transport. This hypothesis would certainly be consistent with the finding that ENaC can be activated by PKA in the rat CCD (Rouch et al., 1993) but not in the rat colon (Bridges et al., 1984).

A second possibility that could explain the lack of a PKA activation of ENaC in planar lipid bilayers is that PKA activates ENaC in native epithelia by stimulating trafficking of channels to the apical membrane or by activating quiescent or dormant channels already present in the membrane. However, whereas this would explain the results of the planar lipid bilayer experiments, it fails to explain the results of the oocyte expression studies. It is unlikely that the oocyte expression system does not reproduce an altered trafficking effect of PKA on ENaC, as it is clear that CFTR can be activated by PKA in oocytes, and that CFTR in native epithelia has been shown to be a channel that is trafficked to the apical membrane in response to PKA activation (Tousson et al., 1996). Thus, this alternative appears unfeasible at the present time.

A third possibility that could explain the lack of a PKA effect is that these ENaC clones are unrelated to the Na+ channel in tight epithelia. This possibility is unlikely since many of the pharmacological and biophysical ENaC characteristics are remarkably similar to those of many native Na+ channels from tight epithelia; thus, it is dismissed without further discussion.

Conclusions

The present findings have two implications. First, based on the similarity of the diverse actions of PKC on ENaC and other native and cultured epithelia, it is likely that this channel represents the central core of epithelial Na+ channels. Second, it is likely that PKA must be acting on an as yet unidentified protein associated with the ENaCs. The widespread distribution of ENaCs in cAMP-responsive epithelia provides indirect evidence for these conclusions.

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