Differential Effects of Protein Kinase C on the Levels of Epithelial Na\textsuperscript{+} Channel Subunit Proteins\textsuperscript{*}

Regulation of epithelial Na\textsuperscript{+} channel (ENaC) subunit levels by protein kinase C (PKC) was investigated in A6 cells. PKC activation altered ENaC subunit levels, differentially decreasing the levels of both \(\beta\) and \(\gamma\), but not \(\alpha\)ENaC. Temporal regulation of \(\beta\) and \(\gamma\)ENaC by PKC differed; \(\gamma\)ENaC decreased with a time constant of 3.7 \(\pm\) 1.0 h, whereas \(\beta\)ENaC decreased in 13.9 \(\pm\) 3.0 h. Activation of PKC also resulted in a decrease in trans-epithelial Na\textsuperscript{+} reabsorption for up to 48 h. PMA activation of PKC resulted in negative feedback inhibition of PKC protein levels beginning within 4 h. Both \(\beta\) and \(\gamma\)ENaC levels, as well as transport tended toward pretreatment values after 48 h of PMA treatment. PKC inhibitors attenuated the effects of PMA on ENaC subunit levels and Na\textsuperscript{+} transport. These results directly show for the first time that PKC differentially regulates ENaC subunit levels by decreasing the levels of \(\beta\) and \(\gamma\) but not \(\alpha\)ENaC protein. These results imply a PKC-dependent, long term decrease in Na\textsuperscript{+} reabsorption.

Sodium homeostasis is essential to proper maintenance of total body water and electrolyte content, and thus, blood pressure control. The activity of luminal, epithelial Na\textsuperscript{+} channels (ENaC)\textsuperscript{1} is rate-limiting for trans-epithelial Na\textsuperscript{+} reabsorption across the renal collecting duct and other Na\textsuperscript{+} reabsorbing epithelium. Thus, understanding regulation of ENaC activity is relevant to physiology as well as to treating disease with associated fluid imbalance.

ENaC is a heterotetrameric channel complex composed of at least three homologous but distinct subunits: \(\alpha\), \(\beta\), and \(\gamma\) (1). Numerous results show that expression of ENaC subunit message and protein are differentially regulated in various tissues and species (reviewed by Refs. 2–4). For example, Masilamani and colleagues (5) recently showed in rat collecting duct principal cells that \(\alpha\)ENaC protein levels increase in response to steroid (Ref. 6; also refer to Fig. 1 of the present study). Besides aldosterone, Zentner et al. (7) recently showed in the rat parotid epithelial cell line, Pa-4, that expression of \(\alpha\)ENaC mRNA and possibly protein was decreased within 6 h by protein kinase C activation.

Activation of PKC decreases Na\textsuperscript{+} reabsorption across renal epithelium by affecting ENaC (8–11). Studies of single channel properties show that in amphibian, rat, and rabbit distal tubule cells, ENaC activity is decreased within 5–10 min after activation of PKC (12–15). A rapid initial decrease in ENaC open probability is, in part, responsible for the early change in activity; however, it appears that PKC may also subsequently affect the number of functional channels (14, 15). Although most studies are consistent with PKC decreasing ENaC open probability initially and then subsequently reducing channel number, Els et al. (16) showed with blocker-induced noise analysis in A6 cells that PKC activation, besides producing the initial decrease in open probability and a longer term decrease in channel number, might also lead to a small initial compensatory increase in channel number presumably in response to decreased open probability. Nevertheless, actions on channel open probability likely precede effects on number and both reduce overall sodium transport. In a provocative study, Ishikawa and colleagues (15) report biphasic actions of Ca\textsuperscript{2+}-dependent processes (e.g. activation of PKC) on repression of ENaC activity with time constants ranging between 1–2 and 100–160 min. The current results are consistent with the latter time constant representing a decrease in ENaC number.

Recently, Shimkets et al. (17) showed for the first time that PKC directly phosphorylates ENaC when this ion channel is overexpressed and PKC is substantially activated. It is unclear how PKC-mediated phosphorylation relates to ENaC kinetics and number and, thus, Na\textsuperscript{+} reabsorption. Moreover, the long term actions of PKC on transport and ENaC protein levels have not been studied.

Because ENaC subunit levels can be differentially regulated in response to various factors and PKC is known to affect ENaC activity, perhaps, in part, through regulation of channel number, we tested the hypothesis that PKC differentially regulates subunit protein levels. This is the first report directly showing that ENaC subunit protein levels are differentially affected by PKC with both \(\gamma\) and \(\beta\), but not \(\alpha\)ENaC, decreasing in response to kinase activation. The decrease in subunit levels is consistent with the long term actions of PKC on ENaC resulting in decreased Na\textsuperscript{+} channel number and, thus, transport.

MATERIALS AND METHODS

All experiments were performed on A6 cells (American Tissue Type Culture; passages 75–80). A6 cells were cultured, as described previously (14, 18), in medium (3 parts Coon's F-12 and 7 parts Leibovitz's L-15) modified for amphibian cells (104 mM NaCl, 25 mM NaHCO\textsubscript{3}, pH 7.4) and supplemented with fetal bovine serum (10%) and aldosterone (1.5 \(\mu\)M). High resistance (\(\sim\)2 K\Omega) A6 cell monolayers plated on permeable 3.8-cm\textsuperscript{2} inserts (0.02 \(\mu\)m Anopore membrane; Naïle NUNC Inter-

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\textsuperscript{§} To whom correspondence should be addressed: 7703 Floyd Curl Dr., Dept. of Physiology, University of Texas Health Science Center, San Antonio, TX 78229. Tel.: 210-567-4528; Fax: 210-567-4410; E-mail: stockand@UTHSCSA.edu.

\textsuperscript{1} The abbreviations used are: ENaC, epithelial Na\textsuperscript{+} channel(s); PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.
PKC Decreases γ and β but Not αENaC Protein Levels

A6 cells were models of the principal cell frequently used for the study of regulated Na\(^+\) reabsorption. These cells have been specifically used for investigating the contribution and modulation of ENaC during transport. A6 cell signal transduction and electrophysiological properties are believed to be similar to those of mammalian collecting duct principal cells. A6 cells express the epithelial Na\(^+\) channel in the luminal membrane with the thin channel’s gating kinetics and number influenced by cortico-steroids (2, 3, 10). In the current study, we used the A6 cell line to investigate the regulation of ENaC subunit levels by protein kinase C. We show directly for the first time with ENaC subunit-specific antibodies that activation of PKC with PMA results in a decrease in γ and β but not αENaC protein levels; γENaC decreased prior to βENaC. PKC also decreased Na\(^+\) reabsorption. Decreases in subunit levels and transport in response to PMA were reduced or eliminated with PKC inhibitors. Our results are consistent with activation of PKC resulting in long term (>4 h) depression of β and γENaC levels leading, in part, to a sustained (up to 48 h) decrease in Na\(^+\) reabsorption.

The Western blot analysis in Fig. 1 is consistent with A6 cells expressing all three ENaC subunits: α, β, and γ. It is well documented both biochemically and electrophysiologically that A6 cells contain typical, fully functional (heteromultimeric) ENaC (2, 3, 10). In fact, the cDNAs encoding these subunits have been cloned from A6 cells (20). More importantly for the current study, results in Fig. 1 show that we have antibodies that specifically recognize each of the ENaC subunits.

For Western blot analysis (representative blots shown in Fig. 1), each lane within a gel contained lysate with the same amount of total protein (~80 μg). The blots of A and C in Fig. 1 were probed with anti-αENaC antibody AB586 and anti-βENaC antibody AB592, respectively. The first and third lanes in these blots contained lysate harvested form A6 cells serum and aldosterone starved for ~72 h (~). The second and fourth lanes (+) had lysate from cells treated with aldosterone for >72 h. The right two lanes (+ pep) of A and C were probed with antibody preabsorbed with 0.1 mg/ml of the respective immunogens. The top blot of B was probed with AB586. This blot was
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stripped and reprobed with anti-γ-xENaC (lower blot of B, where – and – have the same meaning). The effects of aldosterone on α and γENaC in A6 cells were inconsistent with steroid clearly increasing αENaC in two of four experiments (an increase is shown in A, and no effect is shown in the top blot of B) and increasing γENaC in one of four experiments (refer to bottom blot of B). In contrast, long term exposure to aldosterone (1.5 μM; ≥ 72 h), as shown by the Western blot of C and summarized in D, consistently and significantly increased βENaC 20.8 ± 5.5-fold (seven of seven experiments). For all experiments, aldosterone-treated cells had significantly more Na⁺ transport. These blots suggest interesting characteristics for ENaC subunits in A6 cells: 1) the relative molecular weight of subunits are α < γ < β and 2) β and possibly α are often observed as doublets perhaps indicating glycosylation. Both subunits contain putative glycosylation sites and previously have been reported to be glycosylated (5, 6, 21). Moreover, β but neither γ nor αENaC subunit levels correlated well with the actions of aldosterone to increase transport suggesting that βENaC may be limiting for long term steroid-regulated reabsorption across A6 cells. However, these results are preliminary in this regard, and thus, this notion needs to be investigated further prior to making more definitive statements on this topic.

It is well documented in A6 cells that ENaC transcript and protein subunit levels are differentially regulated by aldosterone and other factors (reviewed in Refs. 2, 3, and 8). The results of Fig. 1 are consistent with findings we have published previously (6) showing that β (detected with an antibody distinct from the one used in the current study) but not α and γENaC protein levels are reproducibly increased in response to long term administration of aldosterone. Similarly, J. P. Johnson and colleagues also find in A6 cells that β but not α and γENaC protein is increased by aldosterone. It is unclear why these results differ from those of May et al. (21) in A6 cells and Masilamani et al. (5) in rat collecting duct principal cells showing that only αENaC is consistently increased in response to aldosterone. Nonetheless, our antibodies, as well as those of others, are useful tools for studying the regulation of ENaC subunit protein levels.

Although PKC is known to decrease ENaC activity (reviewed in Refs. 9, 10, and 22), the direct actions of PKC on ENaC subunit levels has not been investigated. In addition, a temporal correlation between PKC effects on ENaC subunit levels and Na⁺ reabsorption has not been established. Thus, we tested the hypothesis that PKC differentially regulates ENaC subunit levels and also temporally correlated effects on subunit levels with changes in transport.

The representative Western blots of Fig. 2A show that γ (bottom blot) and β (middle blot) but not αENaC (top blot) levels are decreased within 24 h in response to PMA (100 ng/ml) activation of PKC. This effect was not observed with inactive phorbol ester (4α-PMA; 100 ng/ml). The Western blots and bar graph of Fig. 2B and C summarize the actions of PMA at differing concentrations on β and γENaC subunit levels and also temporally correlated effects on subunit levels with changes in transport.

Our findings that γ and β but not αENaC subunit levels decrease in response to PKC activation are different than the results of Zentner et al. (7) in which PKC was found only to decrease αENaC transcript levels in this latter study. Also in this latter study, preliminary evidence was provided that PKC effects on αENaC transcript levels translated into decreased subunit protein expression. Thus, even though they have not directly examined subunit protein levels, it seems likely that there is a difference in the response to PMA of salivary gland cells and A6 cells. There are many possible explanations for the difference. The simplest would be that the complement of transcription factors present in salivary cells and A6 cells is different and that, therefore, regulation of gene expression is different. A second simple possibility is that the signaling pathways activated by PMA are different in the two cell types so that the relative activation of different transcription factors would be different. Interestingly, the time course of PKC action on ENaC subunits agrees with the results of Zentner et al. (7). Nonetheless, more research is necessary to understand the regulation of ENaC subunits in different epithelia.

Fig. 3 shows the time course of PMA actions on ENaC subunit and PKC levels. The representative blots of Fig. 3 (A–C) show that although αENaC levels (A) are not affected by PMA (100 ng/ml) for up to 96 h, βENaC (B) and γENaC (C) levels decrease prior to 24 h after activation of PKC. Both β and γENaC increase toward pretreatment levels 72 h after PMA addition. PMA also decreased PKC levels beginning 2–3 h after

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*J. P. Johnson, M. D. Rokaw, and R. S. Edinger, personal communication.
addition. PKC levels remained substantially depressed throughout the course of these experiments (*n* = 6). In contrast, 4α-PMA had no effect on PKC levels between 0–96 h (*n* = 4; not shown). These findings show that PMA addition to A6 cells does in fact activate PKC because phorbol activation of PKC is well known to cause self-inhibition via a cellular negative feedback loop resulting in decreased kinase expression.

The effects of PMA on ENaC subunit levels (A) and calculated current (B), and trans-monolayer resistances (C) and voltages (D) are summarized in Fig. 4. The mean ± S.E. (*n* ≥ 3) describing the relative (compared with time 0) density of each subunit at the indicated times are shown with the short dashed (○), long-dashed (○), and solid (●) regression lines fitting *α*, *β*, and γENaC, respectively. γENaC begins to decrease soon after PMA addition with a time constant of 3.7 ± 1.0 h. The decrease in βENaC levels is described by a time constant of 13.9 ± 3.0 h. The change in αENaC over 96 h was not significantly different from that at time 0 (*p* < 0.01). γENaC showed no effect on *α*, *β*, and γENaC at any time point (times detected = 2, 6, 8, 12, and 24 h; not shown). The decrease in both γ and βENaC were fit best by a single exponential, suggesting that PMA had only one major effect on ENaC protein levels. Currently, it is unclear whether the action of PMA results in a decreased subunit expression or increased subunit degradation. In the continued presence of PMA, presumably because of self-inhibition of PKC, *β* and γENaC subunits levels began to return to pretreatment levels after 24–48 h (with time constants of 32 ± 8.1 and 52 ± 10 h for *β* and γENaC, respectively, which are not statistically different from one another; *p* = 0.128). The levels of γ and βENaC at 96 h were significantly greater then the levels of these subunits at 24 h. Importantly, the increase toward pretreatment levels for γ and βENaC after 48 h demonstrate the reversibility of PKC actions on subunit levels. It is unclear why there is an extended lag time after PKC self-inhibition prior to β and γENaC levels returning toward pretreatment values. This observation needs to be more carefully examined but suggests some prolonged suppression of ENaC levels that is first triggered by PKC and then sustained after kinase down-regulation. Alternatively, the results imply that ENaC synthesis is quite slow.

The summary graph of relative (to time 0) Na⁺ current in 4B shows that PMA (100 ng/ml, ○) but not 4α-PMA (100 ng/ml, □) decreases trans-monolayer resistances (C) and voltages (D). Similar to current, the decrease in voltage in response to PMA was immediate. In contrast, the major decrease in resistance began 8–12 h and peaked 24 h after PMA treatment. After 48 h, monolayer voltages and resistances increased toward pretreatment levels. The decrease in γ and βENaC as well as Na⁺ reabsorption all substantially preceded the decrease in monolayer resistance and were completely reversible. In fact, the resistance of 3.2 ± 0.1 KΩ (*n* = 20) in the presence of PMA at 8 h (a time point where γ and βENaC, as well as transport were suppressed) was not different than the 3.5 ± 0.1 and 3.3 ± 0.1 KΩ for control (*n* = 10) and 4α-PMA (*n* = 10), respectively. The fact that changes in ENaC subunit levels and transport preceded those in resistance suggest that these earlier parameters change independent of resistance. The cause of the resistance decrease after 10 h is currently unclear. Simple measurements of transepithelial current cannot distinguish between a PMA-dependent change in the paracellular versus transcellular resistance. However, we believe that the decrease in monolayer resistance resulting from PMA treatment did not simply arise from a decrease in total protein expression or cell viability, because αENaC, as well as extracellular-regulated kinase and phosphatidylinositol 3-kinase (not shown; *n* = 3) levels were not effected by PMA treatment. It is interesting that the PMA-reduced Na⁺ transport began to return to pretreatment levels only after β and γENaC levels began to return to pretreatment values (>48 h after PMA treatment). This suggests that PKC decreases Na⁺ reabsorption at later time points via a decrease in ENaC levels, which are independent and possibly in addition to actions on ENaC open probability. It is reasonable to presume that the increases in both ENaC levels and transport result from self-inactivation of PKC.

It is unclear from the results of Shimkets *et al*. (17) showing...
PKC-mediated phosphorylation of ENaC whether this phosphorylation results in a decrease in open time, a decrease in the number of channels in the luminal membrane arising from either an increase in membrane recycling, or an increase in degradation. Moreover, it is unclear whether the actions of PKC on ENaC are direct or mediated through other molecules. Our results do not distinguish between either direct or indirect actions but do support a decrease in ENaC number after 3 h resulting from either a decrease in subunit synthesis or an increase in subunit degradation. It is interesting that May et al. (21) showed the half-lives of ENaC subunits to be about 1 h. If PKC inhibits ENaC subunit synthesis, as suggested by Zentner et al. (7), normal channel turnover in the face of suppressed synthesis could account for the decrease in both γ and βENaC. Thus, the current results, as well as those of others, support the hypothesis that the initial early actions of PKC on ENαC are to decrease sodium transport by decreasing open probability, and then, subsequently, PKC decreases sodium transport by reducing the number of ENaC subunits capable of forming channels. In other words, PKC appears to have a biphasic action to decrease ENaC transport activity measured as the product of the number of channels and the open probability (NPo), an early phase involving a decrease in ENaC activity resulting from a change in P1, and a later phase of action involving a decrease in the total cellular amount of ENaC (which alters N, the functional ENaC in the luminal membrane).

Fig. 5 (A and B) shows that the PKC inhibitor GF 109203X attenuates the effects of PMA on β and γENaC levels and Na+ reabsorption, respectively. These results are consistent with PMA-dependent activation of PKC resulting in both effects. GF 109203X (200 nM) was added simultaneously with PMA (100 ng/ml) to A6 cell monolayers with current and lysate being assessed after 24 h. As shown in the Western blot of Fig. 5A, both γ and βENaC were clearly present in cells treated with PMA plus GF 109203X but not PMA alone (n = 3). GF 109203X alone had no effect (n = 3, not shown). Relative transport (compared with time 0) was also protected by GF 109203X with 1.0 ± 0.1, 0.05 ± 0.02, and 0.3 ± 0.02 for control, PMA, and PMA plus GF 109203X, respectively (n = 6). It is interesting that GF 109203X blocked all effects of PMA on ENaC subunit levels but only attenuated PMA effects on transport by ∼30%. This observation is consistent with the idea that PMA affects both channel number and open probability at 24 h and that the effective dose of GF 109203X on these two parameters is different. Alternatively, the effective dose of GF 109203X to counter PKC inhibition of other transport proteins, such as the serosal Na+K+-ATPase, may be different, or PMA may have actions on transport independent of PKC. Shown in Fig. 5 (C and D) are the dose-dependent actions of the PKC inhibitor calphostin C on γENaC levels (n = 3) and transport (n = 6),
respectively, in the presence of PMA. Calphostin C alone had no effect on either parameter (not shown, \( n = 3 \)). PMA and calphostin C were added simultaneously to transport and subunit levels being assessed 24 h later. For the relative current graph (Fig. 5D) the actions of calphostin C on PMA-depressed transport (at 24 h) were normalized (against time 0) and then standardized to control (not shown). The action of calphostin C to reverse PMA-dependent suppression of \( \beta \) and \( \gamma \)ENaC levels was similar to its action on PMA-depressed transport. This finding is different from that of GF 109203X, suggesting that calphostin C inhibits PKC actions on open probability and number with a similar effective dose.

It is interesting that PKC inhibitors blocked both the early and latter actions of PMA on transport. The early actions are presumably manifested by changes in channel kinetics or number of channels in the luminal membrane with little dependence on total cellular ENaC levels (13–15). That we see a PKC-dependent decrease in ENaC subunit levels beginning 3 h after PMA addition strongly suggests that later actions of PMA on transport (>3 h) must result from a decrease in total cellular ENaC levels. Because this is the first biochemical characterization of PKA actions on ENaC subunit levels and no electrophysiological study has characterized the long term effects of PKA on ENaC kinetics, we are unable to definitely state the contributions or the temporal importance of either PKC-dependent changes in ENaC kinetics or levels to \( \mathrm{Na}^+ \) transport. However, our results are consistent with the hypothesis that PKC decreases \( \gamma \) and \( \beta \)ENaC subunit levels, which leads, in part, to a long term decrease in \( \mathrm{Na}^+ \) reabsorption.

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