The effect of rapamycin on single ENaC channel activity and phosphorylation in A6 cells

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Yue, Gang, Robert S. Edinger, Hui-Fang Bao, John P. Johnson, and Douglas C. Eaton. The effect of rapamycin on single ENaC activity and phosphorylation in A6 cells. Am J Physiol Cell Physiol 279: C81–C88, 2000.—Rapamycin and FK-506 are immunosuppressive drugs that bind a ubiquitous immunophilin, FKBP12, but immunosuppressive mechanisms and side effects appear to be different. Rapamycin binds renal FKBP12 to change renal transport. We used cell-attached patch clamp to examine rapamycin’s effect on Na+ channels in A6 cells. Channel NPF was 0.5 ± 0.08 (n = 6) during the first 5 min but fell close to zero after 20 min. Application of 1 µM rapamycin reactivated Na+ channels (NPF = 0.47 ± 0.1; n = 6), but 1 µM FK-506 did not. Also, GF-109203X, a protein kinase C (PKC) inhibitor, mimicked the rapamycin-induced reactivation in a nonadditive manner. However, rapamycin did not reactivate Na+ channels if cells were exposed to 1 µM FK-506 before rapamycin. In PKC assays, rapamycin was as effective as the PKC inhibitor; however, epithelial Na+ channel (ENaC) phosphorylation was low under baseline conditions and was not altered by PKC inhibitors or activators. These results suggest that rapamycin activates Na+ channels by binding FKBP12 and inhibiting PKC, and, in renal cells, despite binding the same immunophilin, rapamycin and FK-506 activate different intracellular signaling pathways.

epithelial sodium channel; amiloride-sensitive sodium channels; protein kinase C; single channels

Rapamycin is a macrolide antifungal agent. Like another drug, FK-506, it inhibits T cell activation and has been used as an immunosuppressant for prevention of rejection in organ transplantation (4, 24). Rapamycin and FK-506 interact with intracellular binding proteins or immunophilins, the FK-506 binding proteins (FKBPs) named for their molecular weights (FKBP12, FKBP56, FKBP25). The immunophilins are accessory proteins that interact with target molecules to induce active conformations of the targets. The immunosuppressive effects of both rapamycin and FK-506 are mediated by binding to the immunophilin and subsequent exposure of an effector region on the drugs that then inhibit downstream pathways (4, 24). Although structurally similar and binding the same set of immunophilins, rapamycin and FK-506 have different mechanisms of action and effects on T cell function. Both of the agents have high binding affinity to FKBP12; however, the rapamycin-FKBP12 complex activates p70 S6 kinase and inhibits interleukin-2 (IL-2)-induced T cell proliferation (3), whereas the FK-506-FKBP12 complex suppresses calcineurin and decreases IL-2 expression (4, 24). FKBP12 is a ubiquitous protein that is present in many cell types other than immune cells (12), thus many of the effects of rapamycin and FK-506 other than immunosuppression may be related to disruption of normal signal transduction in other cell types. In particular, rapamycin can have significant renal effects resulting in abnormalities of renal transport (15, 17).

Recently, we have shown that rapamycin inhibits endogenous protein kinase C (PKC) activity and increases Na+ transport of renal cells in culture (monolayers of A6 cells) (21, 22). Previous work from our laboratory has also shown that the activity of Na+ channels in A6 cells is increased when PKC is inhibited and decreased when PKC is stimulated (11). This suggests that rapamycin may stimulate the transepithelial Na+ transport by inhibition of PKC activity. In this work, we investigate the effect of rapamycin and FK-506 on the activity of single Na+ channels in A6 cells and the relationship to PKC activity.

MATERIALS AND METHODS

A6 cell preparation. A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 68th passage. All experiments were performed on passages 71–80 with no discernable variation among different passages. The cells were maintained in plastic tissue flasks (Corning, NY) at 27°C in a humidified incubator with 4% CO2 in air. The culture media was a mixture of Coon’s medium F-12 (3 parts) and Leibovitz’s medium L-15 (7 parts) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and 1.5 µM aldosterone for amphibian cells with 103 mM NaCl and 25 mM NaHCO3, pH 7.4. For patch-clamp experiments, the

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cells were subcultured on permeable, collagen-coated aluminosilicate supports (Nunc) attached to the bottoms of plastic rings. For PKC assay, the cells were subcultured on millipore inserts (Millipore).

**Patch recording.** A plastic ring containing an A6 cell monolayer was mounted in a recording chamber on an inverted microscope (Nikon). Both the apical side and basolateral side of the monolayer was bathed in amphibian saline solution containing (in mM): 96 NaCl, 3.4 KCl, 0.8 CaCl₂, 0.8 MgCl₂, and 10 HEPES at pH 7.4 (titrated with 1 N NaOH). A perfusion setup was used to exchange the apical bath solution to expose A6 cells to rapamycin, FK-506, or the PKC inhibitor GF-109203X.

For these experiments, we used the cell-attached patch-clamp methods following standard procedures (9). Patch pipettes were fabricated from TW 150 glass (World Precision) and fire polished to produce tip resistances of 5–10 MΩ when filled with amphibian saline solution. Experiments were performed at room temperature (22–23°C). Single-channel events from cell-attached patches were measured with an Axopatch 200 amplifier, low-pass filtered at 5 kHz, recorded on a digital video recorder (Sony), and then digitized at 500 Hz using a Scientific Solutions analog-to-digital converter and Pentium computer equipped with Axotape software (Axon). The convention for applied voltage to the apical membrane (−V_{pipette}) represents the voltage deflection from the patch potential (i.e., the resting membrane potential for cell-attached patches). Inward Na⁺ current (pipette to cell) is represented as downward transitions in single-channel records.

**Data analysis.** The data were subsequently transferred to a VAX computer (Computer Equipment) for single-channel analysis. The 5-min data records were low-pass filtered at 100 Hz using a software Gaussian filter and analyzed using locally developed software that closely follows the algorithms presented by Colquhoun and Sigworth (5). Although patches containing only a single channel are ideal for performing kinetic analysis, many cell-attached patches on A6 cells contain multiple Na⁺ channels (13, 18). N_P, the product of the number of channels and the open probability, a parameter that can be determined independent of knowledge of the number of channels within a patch, was used to measure the channel activity within a patch. N_P was calculated as the relative area under an all-points amplitude histogram and expressed as follows

\[ N_P = \sum_{i=1}^{N} i \cdot A_i \]

In the above equation, A is the area under Gaussian curve, N is the total number of observable channels in a patch, i is the number of channels, and P_o is the open probability of an individual channel in a patch.

An alternative but equivalent method for calculating N_P from single-channel records without making any assumptions about the total number of channels in a patch or the P_o of a single channel is given by

\[ N_P = \sum_{i=1}^{N} \frac{i \cdot t_i}{t} \]

where j is the total recording time, N_A is the apparent number of channels within the patch determined as the highest observable current level, i is the number of channels open, and t_i is the time during which i channels are open. If channels open independently of one another and the exact number of channels in a patch is known, then the P_o of a single channel can be calculated by dividing N_P by the number of channels in a patch. The total number of functional channels (N) in the patch was determined by observing the number of peaks detected in all-points amplitude histograms constructed from event records of adequate duration to provide 95% confidence of determining the correct N according to methods we have previously described (10, 13). The mean open time (t_o) of N channels can be calculated as follows

\[ t_o = \frac{N_P}{n^2} = \frac{\sum_{i=1}^{N} i \cdot t_i}{n^2} \]

where n is the total number of transitions between states during the total recording period (t) and the other parameters are the same as in Eq. 2. This value represents the average time the channel spends open (in any open state) and should not be confused with the mean residency time of the channel in a specific state (sometimes called the mean open time for the state). In patches with multiple channels, we determined the most likely value for N and then calculated values for mean open time (t_o) by measuring the duration of the events in which all N channels were open (t_N) and remembering that t_o = t_N/N. The difficulty with this method is the assumption that N can be determined accurately and that there are enough events with all channels open to obtain a meaningful distribution of intervals. To insure the accuracy of this approach, we only used patches in which we had >95% probability of having estimated the number of channels correctly (following the methods of Ref. 13 or in which t_o can be determined unambiguously (patches with no overlapping open events)). Nonetheless, this measure provides an easy way to distinguish whether experimental manipulations (e.g., rapamycin or PKC inhibitors) modify P_o by affecting the channel’s open states or closed states.

**PKC assay.** After exposure for 20 min to 0.1 mM phorbol 12-myristate 13-acetate (PMA), 1 µM rapamycin, 0.4 µM GF-109203X, or diluent, A6 cells were washed three times with ice-cold Ca²⁺-free PBS. Cells were then scraped from the filters using a rubber policeman into ice-cold PBS and centrifuged at 1,500 g for 5 min to pellet the cells. The cell pellet was resuspended in homogenization buffer containing 100 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 10 µM leupeptin, 10 µg/ml chymostatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM sodium vanadate, 0.5 µM okadaic acid, and 10% glycerol. The resulting suspension was centrifuged at 100,000 g for 1 h at 4°C. The supernatant represents the crude cytosolic fraction, and the pellet represents the crude membrane fraction. The crude membrane fraction was used for PKC assay. PKC activity was measured with a Spinzyme assay kit (Pierce) using fluorescent dye-labeled N-acetylated peptide sequence of myelin basic protein (Pierce) as a substrate (6). This is a specific substrate of α, β, and γ isozymes of PKC and a poor substrate for protein kinase A (23). Briefly, crude membrane fractions containing 10 µg of protein were mixed with reaction buffer to a final concentration of 2 mM ATP, 10 mM MgCl₂, 0.1 mM CaCl₂, 0.002% Triton X-100, 20 mM Tris, and 0.2 mM phosphatidylserine, pH 7.4, and allowed to incubate for 30 min at 30°C. Bound phosphorylated substrate was eluted using 0.1 M ammonium bicarbonate and 0.02% sodium azide, pH 8, and detected by measuring absorbance at 570 nm. To generate a standard curve, absorbance was measured in samples containing a
range of known activity (0–0.020 units per reaction) of PKC from rat brain. One unit of specific activity is defined as the amount that will transfer 1 nmol of phosphate to histone H1 per minute at 30°C.

**Subunit phosphorylation.** A6 cells grown as described above were fed with phosphate-free Ringer solution (75 mM NaCl, 1 mM CaCl2, 4.5 mM KCl, 8 mM NaHCO3, 5 mM glucose, pH 7.4) for 1 h. The solution was replaced with phosphate-free Ringer solution containing 200 mCi/ml [32P]PO4 to both the basal and apical sides and incubated at room temperature for 3 h. The cells were then either left untreated (control), treated with PMA (0.1 µM), or treated with rapamycin (1 µM). The cells were incubated 30 min and isolated in gentle lysis buffer (50 mM Tris·HCl, 1% Nonidet P-40, 76 mM NaCl, 10% glycerol, 2 mM EGTA, 5 mg/ml each of PMSF, antipain, leupeptin, and pepstatin A, pH 7.4) containing 0.2 mM Na2VO4 and 50 mM NaF. The cells were lysed on ice for 60 min. The lysates were spun at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and the protein concentration was determined. Equal amounts of proteins were immunoprecipitated using anti-α, anti-β, or anti-γ epithelial Na+ channel (ENaC) antibodies in gentle lysis buffer. Antibodies have been previously characterized for specificity and ability to immunoprecipitate individual subunits (14, 20, 26, 29). The anti-subunit antibodies were precipitated using GammaBind (Amersham Pharmacia). The immunoprecipitates were washed three times in gentle lysis buffer and separated on a 10% SDS-PAGE. Analysis was performed using Molecular Imager FX system using Quantity One software (Bio-Rad).

**Statistics.** The data are presented as mean ± SE. Paired or unpaired t-tests were used as appropriate to compare experimental groups. Results were considered significant when *P* < 0.05.

**RESULTS**

**Rapamycin increases the activity of single Na+ channels in A6 cells.** Typical single Na+ channel recordings from A6 cell patches are shown in Fig. 1A. In these records, as in many A6 channel recordings, the channel activity decreased with time. At the beginning of the recording period, multiple channel openings are observable (Fig. 1A, top). After 20 min of recording, there are only brief channel openings (Fig. 1A, middle), and after 30 min there are no observable openings (Fig. 1A, bottom left). This decrease in activity is common in A6 cell single Na+ channel recordings, even in cell-attached patches. However, these inactive channels are reactivated 10 min after application of 1 µM rapamycin in the apical bath solution (Fig. 1A, bottom right). The time required for this reactivation varies in different cells from 10 to 20 min. This result suggests that in A6 cells, rapamycin reverses whatever mechanism originally caused the loss of Na+ channel activity. The effect of rapamycin can be more easily seen in all-points amplitude histograms corresponding to the data records in Fig. 1A. The histograms show that there are at least three active channels (4 peaks in the histogram corresponding to 0, 1, 2, and 3 channels open at the beginning of recording (Fig. 1B), 1 active channel after 20 min (Fig. 1C), and 2 active channels 10 min after exposure of A6 cells to 1 µM rapamycin (Fig. 1D)). We calculated NP, from the histograms and plotted NP vs. time for six A6 cells before and after application of rapamycin in Fig. 2. In each of these six cells, NP significantly decreased after 20 min of continuous recording, but in every case, application of rapamycin...
increased activity, sometimes to levels above that of the initial activity.

**Rapamycin increases the activity of single Na\textsuperscript{+} channels by increasing \( P_o \) and channel mean open time.** If rapamycin is altering Na\textsuperscript{+} channel posttranslational regulatory pathways, then there should be specific effects on channel gating. Figure 3 shows the results of experiments similar to those in Fig. 2 in which channel activity is recorded for 5 min immediately after seal formation, again 20 min later, and then again 10 min after application of different specific treatments. Bars labeled A are a group of patches that are finally treated with 1 µM rapamycin. The results are consistent with the results of Fig. 2 with an initial high level of activity followed by a reduction after 20 min and a subsequent increase in activity after treatment with rapamycin (but not after treatment with saline alone: bars labeled D). However, analysis of the data shows that all of the change in channel activity is due to a change in open probability (top) and that the change in open probability is due to a change in the mean open time of individual channels (bottom). There was no statistically significant change in the mean closed time.

**The effect of rapamycin is mimicked by a PKC inhibitor.** Figure 3 also shows (in the bars labeled B) that exposure of A6 cells to 0.4 µM PKC inhibitor GF-109203X reversed the time-dependent loss of activity and increased \( P_o \) and mean open time of Na\textsuperscript{+} channels to the same extent as rapamycin. Additionally, rapamycin could not further stimulate channels after treatment with GF-109203X. This implied that rapamycin was acting by reducing PKC activity in a signaling pathway that normally inhibits the channel.

**The effect of rapamycin requires interaction with FKBP12.** The effect of rapamycin on Na\textsuperscript{+} channel activity could be entirely unrelated to its immunosuppressive effect. To test this possibility, we used FK-506 to competitively block the interaction of rapamycin with FKBP12. FK-506 had no effect on Na\textsuperscript{+} channel activity. On the other hand, Fig. 3 shows that if we perfused 1 µM rapamycin after A6 cells had been treated with 1 µM FK-506, rapamycin no longer reactivated Na\textsuperscript{+} channels (Fig. 3, bars labeled C). This implies that FK-506 interferes with the effect of rapamycin on Na\textsuperscript{+} channel activation.

**Rapamycin and GF-109203X inhibit PKC activity of A6 cells.** Although we had previously shown that rapamycin was capable of inhibiting PKC activity in A6 cells (21, 22), we wished to show that, indeed, under the explicit conditions of these experiments, rapamycin (and our PKC inhibitor) did inhibit cellular PKC activity. Figure 4 and Table 1 show the relative PKC activity of the membrane fraction of A6 cells. PMA (0.1 µM), a
known PKC activator, increased PKC activity by ~2.3-fold, whereas 1 µM rapamycin and 0.4 µM GF-109203X decreased the PKC activity to a similar extent by greater than threefold. This result indicates that rapamycin inhibits PKC activity almost as effectively as GF-109203X, a known PKC inhibitor.

Rapamycin and PMA do not alter the phosphorylation of the α-, β-, or γ-subunit of the Na⁺ channel. The results above show that rapamycin alters the activity of individual Na⁺ channels and the enzymatic activity of PKC. One obvious explanation of rapamycin's effects is that tonic PKC phosphorylation of the Na⁺ channel itself reduces channel open probability. In fact, all of the three subunits necessary to produce a functional channel have potential PKC phosphorylation sites in their intracellular domains (19) and, at least in heterologous expression systems, PMA stimulation of PKC promotes phosphorylation of the β- and γ-subunits (25). Therefore, we examined phosphorylation of the three subunits in untreated cells, cells treated with PMA, and rapamycin-treated cells. Figure 5A shows the results obtained after the cells were labeled for 3 h with [³²P]PO₄ (as described in MATERIALS AND METHODS). The cells were then either left untreated (control), treated with PMA (0.1 µM), or treated with rapamycin (1 µM) after which the cytosolic fractions from cell lysates were subjected to immunoprecipitation using anti-α, anti-β, or anti-γ ENaC antibodies. The immunoprecipitates were resolved on gels and the autoradiograms imaged on a phosphoimager. The images are shown in Fig. 5A and the relative densities given in Table 2. Because the same amount of cellular protein was immunoprecipitated in each

![Table 1. PKC activity in A6 cell lysates](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%Control</th>
<th>P (vs. Control)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 10.8</td>
<td></td>
</tr>
<tr>
<td>PMA (0.1 µM)</td>
<td>235.8 ± 13.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Rapamycin (1 µM)</td>
<td>31.8 ± 14.1</td>
<td>0.018</td>
</tr>
<tr>
<td>GF-109203X (0.4 µM)</td>
<td>20.8 ± 6.40</td>
<td>0.003</td>
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Values are means ± SE; n = 3 for all treatments. Effect of stimulators and inhibitors relative to control, PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

![Table 2. Relative phosphorylation of α-, β-, and γ-subunits with activators and inhibitors of PKC phosphorylation](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Subunit Density Relative to Control</th>
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<tbody>
<tr>
<td></td>
<td>α</td>
</tr>
<tr>
<td>Rapamycin (1 µM)</td>
<td>0.81 ± 0.05</td>
</tr>
<tr>
<td>PMA (0.1 µM)</td>
<td>0.94 ± 0.03</td>
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Values are means ± SD; n = 4 for all treatments.
case, the image suggests that there is little phosphorylation of γ and some background phosphorylation of the α and β subunits, but despite conditions that dramatically alter PKC activity in A6 cells (see Fig. 4), there was no significant difference in the level of phosphorylation of any of the subunits in response to either PMA or rapamycin. This result implies that in a cell system expressing native channels, PKC alters activity of the channels, but not by altering the phosphorylation of any of the subunits. Thus PKC must be phosphorylating (and rapamycin must be preventing phosphorylation of) a closely associated regulatory protein rather than one of the ENaC subunits. The low level of phosphorylation in the absence of PKC activators or inhibitors suggests that in native systems like A6 cells there may be little phosphorylation by PKC of native subunits.

DISCUSSION

Amiloride-sensitive ENaCs are the primary sites for discretionary regulation of Na⁺ reabsorption in the kidney. The understanding of this regulation has important implications for understanding the regulation of total body Na⁺ balance. Previous work has shown that PKC plays an important role in regulation of Na⁺ transport of A6 cells, a tissue culture model of distal nephron principal cells. Activators of PKC inhibit short circuit current, a measure of Na⁺ transport in A6 cell cultures (27). Patch-clamp experiments further demonstrated that activation of PKC with phorbol esters reduced the open probability of Na⁺ channels, whereas inhibition of PKC with d-sphingosine increased the open probability and number of active Na⁺ channels (11). Na⁺ channel proteins from a variety of cell types including A6 cells have been cloned and shown to consist of at least three homologous subunits (α-, β-, and γ-ENaC) (19), all of which have potential PKC phosphorylation sites in their intracellular domain (19), and two can be phosphorylated when expressed in Madin-Darby canine kidney (MDCK) cells (25). In addition, a biochemically purified preparation of Na⁺ channel protein from A6 cells and from bovine kidney consists of six nonidentical subunits with molecular mass ranging from 40 to 315 kDa (2). Two of these subunits (55 and 130 kDa) from bovine kidney are phosphorylated by PKC. When incorporated into planar lipid bilayers, the purified protein produces single-channel events of which open probability is reduced by PKC phosphorylation (16). These observations suggest that Na⁺ channels in A6 cells can be regulated by PKC either by direct phosphorylation of the channel protein or by phosphorylation of a regulatory protein closely associated with the channel.

Recently, Rokaw et al. (21, 22) reported that rapamycin treatment increased Na⁺ transport in the A6 cell monolayer in Ussing chambers. In the present study, we further characterized the effects of rapamycin on Na⁺ transport by examining the properties of single Na⁺ channels. Our data indicated that rapamycin and the PKC inhibitor GF-109203X but not the closely related immunosuppressive drug FK-506 increased the activity measured as $N_{P_o}$ of Na⁺ channels. This implies that the activity of Na⁺ channels in cell-attached patches is related to the activity of PKC and that one effect of rapamycin is to reduce PKC activity. In immune cells, rapamycin and FK-506 bind to the immunophilin FKBP12 and form a ligand-immunophilin complex. Because FKBP12 is present in the membrane fraction of A6 cells (21), rapamycin and FK-506 could also bind to the immunophilin in A6 cells and thereby mediate rapamycin’s effect on Na⁺ channels. This seems likely because, first, when A6 cells were pretreated with FK-506, rapamycin was unable to activate Na⁺ channels, and second, the stimulatory effect of rapamycin on transepithelial Na⁺ transport of A6 cells was blocked by the subsequent addition of 100-fold excess of FK-506 (22). We could not perform the same experiment while recording from single channels because rapamycin-stimulated Na⁺ channel activation lasts only from the time of application until ~30–50 min after patch formation when channel activity decreases via other mechanisms. Therefore, it is not practical to test if the effect of rapamycin is blocked by subsequent application of FK-506. It is the ligand-immunophilin complex and not the immunophilin or the ligand by itself that determines the specific downstream effect specific to each drug (4, 24). For example, FKBP12 alone has no effect on PKC activity except in the presence of rapamycin. PKC activity was significantly inhibited (21). Although binding to the same immunophilin, FK-506 did not affect channel activity. This difference may be partly explained by the X-ray diffraction study that provided different structures for the FK-506-FKBP12 complex and rapamycin-FKBP12 complex (4, 8) and is supported by the observations that the transduction events associated with the two drugs are different.

Because the amino acid sequence of FKBP is >97% identical to that of bovine endogenous protein C inhibitor 2 (7), the simplest explanation of our results is that FKBP12 by itself is a poor inhibitor of PKC, but after binding to rapamycin, the conformation of the FKBP12 changes to become a strong PKC inhibitor. This hypothesis is supported by our PKC assay experiments in which PKC activity in an A6 cell lysate is inhibited to the same extent by rapamycin as by a direct inhibitor of PKC, GF-109203X. The time course of rapamycin inhibition of PKC activity is similar to that of Na⁺ channel stimulation after rapamycin treatment, presumably reflecting the time necessary to form a stable rapamycin-FKBP12 complex. In addition, the fact that FK-506 did not change the PKC activity is consistent with electrophysiological observation that FK-506 did not alter the Na⁺ channel activity. However, FK-506 does strongly bind FKBP12, which is consistent with the ability of FK-506 to block the action of rapamycin, presumably by blocking the rapamycin binding site. Taken together, the present biochemical and patch-clamp studies indicate that rapamycin, but not FK-506, upregulates the amiloride-sensitive ENaCs and inhibits PKC activity of A6 cells through binding the immunophilin (FKBP12). Rapamycin and FK-506, although binding the same
immunophilin, appear to activate different downstream signal transduction pathways.

However, some questions remain. First, is the rapamycin-induced activation of FKBP12 to produce PKC inhibition a pharmacological effect or is there an endogenous rapamycin-like binding factor that can produce a physiologically relevant FKBP12-mediated PKC inhibition? Second, does PKC directly phosphorylate the Na⁺ channel itself or some other closely associated regulatory molecule? Our experiments and the literature provide us with no insight into the first question. There may be some information relevant to the second question. Awayda et al. (1) have examined the PKC regulation of Na⁺ channels cloned from rat colonic epithelial cells (α-, β-, and γ-rENaC). They found that the amiloride-sensitive Na⁺ channels of oocytes expressing rENaC were inhibited by PKC. In addition, PKC reduced the open probability of the in vitro translated rENaC protein after being reconstituted into planar lipid. There has also been a recent report that β- and γ-rENaC can be phosphorylated by PKC when the subunits are overexpressed in MDCK cells. There is, however, no evidence in A6 cells that any of the Na⁺ channel subunits are phosphorylated by PKC despite the presence of a potential PKC phosphorylation site in the α-xENaC amino acid sequence (19). In contrast, biochemical studies in our laboratory suggest that in vitro translated xENaCs are not phosphorylated by PKC and that channels are not phosphorylated by PKC in excised, inside-out patches from A6 cells (28). The results in this paper also show that a PKC activator, PMA, does not enhance and a PKC inhibitor, rapamycin, does not reduce the in vivo phosphorylation of any of the ENaC subunits in A6 cells. It will be interesting to investigate the target site for PKC phosphorylation and the pathways by which PKC regulates the apical Na⁺ channels of A6 cells.

Of course, the lack of effect of PKC on ENaC subunits in no way suggests that they might not be phosphorylated and regulated by other kinases. Figure 5, in which the same amount of cytosolic protein was immunoprecipitated to detect each subunit, does suggest that at least the α- and β-subunits are phosphorylated and that the γ-subunit is either weakly phosphorylated or not phosphorylated. The alternative explanation that it is present at only low copy number in the cells seems to be belied by Western blots on A6 cells in which β- and γ-subunits are present in approximately equal amounts with the α concentration about two times higher than either β or γ (14). Despite the lack of effect of PKC on subunit phosphorylation, the pattern of background phosphorylation is interesting. First, the phosphorylated subunits that are immunoprecipitated by the subunit-specific antibodies are all of higher molecular weight than the molecular weight predicted from the subunit sequences and, in fact, appear to correspond to the glycosylated form of the β- and γ-subunits even though the antibodies immunoprecipitate large amounts of the nonglycosylated form of the subunits as detected by Western blotting of the immunoprecipitates (14, 20, 26, 29). Second, there are two phosphorylated forms of α and both are relatively high molecular weight. This corresponds to the reports that the plasma membrane form of α runs at 150 and 180 kDa and the lower molecular weight forms are unprocessed, cytosolic forms that predominate in heterologous expression systems (14, 26, 29). Thus only the presumptively functional plasma membrane forms of the subunits appear to be phosphorylated at all (although not by PKC). It will be interesting to determine the source of the phosphorylation and whether it plays any role in the assembly of the subunits into a functional multimeric protein.

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