Rapamycin Inhibits Protein Kinase C Activity and Stimulates Na+ Transport in A6 Cells*

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Rapamycin and FK506 have unique cellular effects despite the fact that they bind to the same set of immunophilins, the FK506 binding proteins (FKBP). We have previously reported that rapamycin (RAP) stimulates sodium transport in A6 cells. FK506 did not stimulate sodium transport but did inhibit the stimulation seen in RAP-treated cells. Since FKBP12 has been shown to have sequence homology with an endogenous inhibitor of protein kinase C (PKC) and PKC inhibition has been shown to increase Na+ channel activity in A6 cells, studies to determine the effect of RAP on PKC activity and its relationship to sodium transport were performed. Here we report that RAP stimulates sodium transport, and the effect is not additive to that seen with a cell-permeant inhibitor of PKC and the effect is not additive to that seen with a cell-permeant inhibitor of PKCs and -b subtypes. RAP significantly inhibits endogenous PKC activity in A6 cells both in membrane and cytosolic preparations. There is a strong correlation between the degree of inhibition of PKC activity and the stimulation of sodium transport by RAP. Rapamycin has no effect on Na+/K+-ATPase activity over this time course. Purified recombinant FKBP12 with or without FK506 has no effect on PKC activity when incubated with a rat brain-derived PKC preparation of known activity. By contrast, RAP plus FKBP12 significantly inhibits PKC activity. RAP plus FKBP12 inhibits the PKC and not the -b subtype. The results demonstrate inhibition of PKC activity by RAP and not FK506 through its binding to FKBP12. The inhibition of PKC activity by RAP stimulates sodium transport in A6. The results therefore imply the existence of an endogenous RAP-like ligand which when bound to FKBP12 could regulate Na+ channel activity through this mechanism.

Tacrolimus (FK506), cyclosporin, and rapamycin (RAP) are known to have effects on T-cell activation and proliferation. These effects are mediated through the interaction of the drug with its binding protein or immunophilin. The immunophilins are divided into two broad classes, the FK506 binding proteins (FKBPs) and the cyclophilins, both of which are characterized by peptidyl-prolyl cis-isomerase (rotamase) activity. The FK506 binding proteins bind both FK506 and RAP and are named for their molecular weights (FKBP12, FKBP56, FKBP25). The cyclophilins bind the structurally distinct macrocyclic peptide cyclosporin. The immunosuppressive effects of these agents are not mediated solely by the drug themselves or by the immunophilin to which they bind. Rather the binding of drug to the immunophilin exposes an effector region on the drug which then inhibits downstream pathways (1, 2). At present it is know that two distinct drugs that bind to the same immunophilin can have drastically different effects. For example, although FK506 and RAP both bind with high affinity to FKBP12 only FK506 inhibits calcineurin (1, 2). In contrast, the RAP-FKBP complex has been found to inhibit cell proliferation by inhibiting p70S6 kinase activation (22).

A6 cells, derived from Xenopus laevis are a well characterized, high resistance epithelia with transport characteristics similar to the mammalian cortical collecting duct (3). Na+ channel activity is known to be increased in response to aldosterone (4, 23), insulin (5), and vasopressin (4, 6–9), each acting through unique pathways. In addition, inhibition of protein kinase C (PKC) activity has been shown to increase sodium channel activity in cell-attached patches of A6 (3). We have recently reported that RAP stimulates transepithelial sodium transport in A6 (10). The effect is seen within 15 min of addition and persists for 4 h. An excess of FK506 had no effect on basal transport but completely inhibited the RAP-induced effect. This suggested that the effect was mediated through a RAP-immunophilin complex (10), since both drugs bind to the same set of immunophilins.

In this regard, FKBP12 is known to have sequence homology to an endogenous PKC inhibitor I-2 (11). This suggested that RAP might inhibit PKC activity through its interaction with this immunophilin. RAP has been found to block a classical PKC (cPKC)-dependent and phosphatidylinositol 3-kinase-dependent stimulation of p70S6 kinase activity (12, 22). While RAP has no effect on phosphatidylinositol 3-kinase activity (12), the effect of RAP on cPKC activity has not been reported. The effect might be peculiar to the RAP-FKBP12 complex since it has been clearly demonstrated that FK506 does not have an effect on PKC activity (13). Studies were therefore performed to determine the effect of RAP on PKC activity and its relationship to sodium transport in A6.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A6 cells were grown as described previously in amphibian media in 10% fetal calf serum on millipore inserts (Millipore Corp.) and studied when they exhibited stable electrical resistance (10–14 days) (10, 23). Amiloride-sensitive short circuit current (Isc) was measured in a sterile in-hood modified Ussing chamber as described previously (10). Nystatin was added to the apical solution (100 units/ml) to permeabilize the apical membrane while the cells were in the short-circuited state. The peak of the subsequent increase in Isc was recorded (10) and is a measure of maximal Na+ /K+-ATPase activity.

**PKC Assay**—A6 cells were exposed to drug or diluent for the appropriate time and then washed three times with ice-cold calcium-free phosphate-buffered saline. Cells were then scraped from the filters using a rubber policeman into ice-cold phosphate-buffered saline and centrifuged at 1500 × g for 5 min to pellet the cells. The cell pellet was resuspended in homogenization buffer containing 100 mM Tris-HCl, 1
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Materials—N-myristoylated Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln, representing the pseudo-substrate sequence from cPKCα and β, was obtained from BIOMOL (Plymouth Meeting, PA). This is a highly specific inhibitor which is N-terminally myristoylated to allow membrane permeability (16, 17). RAP was obtained from BIOMOL and stored as a 2 mM solution in ethanol at −70 °C and diluted into serum-free media on the day of the experiment. Human recombinant FKBP12 from Escherichia coli (15) with or without 10 μM FK506 or 10 μM RAP or FK506 and RAP for 10 min at room temperature. Reaction buffer to a final concentration of 2 mM ATP, 10 mM MgCl2, 0.1 mM CaCl2, 0.02% Triton X-100, 20 mM Tris(hydroxymethyl)aminomethane, 0.2 mM phosphatidyl L-serine, pH 7.4, and allowed to incubate for 30 min at 30 °C. Bound phosphorylated substrate was eluted using 0.1 M ammonium HCO3 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 protein kinase C from rat brain. 1 unit of specific activity is defined as the amount that will transfer 1 nmol of phosphate to histone H1 per min at 30 °C. 1 μM RAP or FK506 affected exogenous PKC activity in the presence of exogenous FKBP12, PKC activity from rat brain of known activity (10 microunits) was incubated in the presence of 7 μg of purified recombinant FKBP12 from E. coli and with or without 10 μM FK506 or 10 μM RAP or FK506 and RAP for 10 min at room temperature. Reaction buffer to a final concentration of 2 mM ATP, 10 mM MgCl2, 0.1 mM CaCl2, 0.02% Triton X-100, 20 mM Tris(hydroxymethyl)aminomethane, 0.2 mM phosphatidyl L-serine, pH 7.4, and then added and allowed to incubate for 30 min at 30 °C, and PKC activity was determined. In parallel experiments, FKBP12 was incubated with PKCa or PKCB subtypes in the presence or absence of RAP, and PKC activity was determined as described above.

Western Blotting—This was performed as described previously (23). Crude cytosolic and crude membrane fractions were protein-matched and solubilized in sample buffer containing 5% SDS, 8% 2-mercaptoethanol, 0.5 mM EDTA, 10% glycerol, 0.025% bromphenol blue, pH 6.8. Samples were heated at 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis using 5% stacking gels and 15% separating gels. Rainbow colored molecular weight standards (Sigma) were run in adjacent lanes. For FKBP12 detection, samples were solubilized in sample buffer containing 10 mM N-dithiothreitol (DTT) and were heated overnight at 37 °C, and then for 20 min at 95 °C. Resolved proteins were transferred to nitrocellulose membranes as described previously (23), and transfers were overlaid with 1:1000 dilutions of specific primary antibody in 1% non-fat dried milk in phosphate-buffered saline (1% BLOTTO). Reactive proteins were detected with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in 1% BLOTTO and signal-detected by an enhanced chemiluminescence system (Amersham Corp.) and exposed to Kodak X-omat AR film (Eastman Kodak Co.).

Results—Data were analyzed using one-way analysis of variance on Number Cruncher statistical software (NCSS, Hintze, Kayville, UT). Data are expressed as means ± S.E. A p value of less than 0.05 was considered significant.

We have previously shown that 1 and 10 nM RAP stimulate transepithelial sodium transport in A6 cells and that this effect is blocked by a 100-fold excess of FK506 (10). To determine the dose-response relationship of this effect, cells were exposed to dilutions of RAP, and the short circuit current (Isc) at 1 h was measured and expressed as the % increase relative to control Isc. The Kd is approximately 10 nM. Sodium channel activity in A6 cells is known to be modulated by several hormones each acting through unique pathways (4). Vasopressin activates sodium channels through a PKA-associated phosphorylation (4, 6–9), and insulin stimulates Na+ transport through a mechanism dependent upon tyrosine kinase activity (5). Since the stimulation of sodium transport by insulin or vasopressin was similar to the time course seen with RAP, we considered the possibility that RAP might be acting through a common pathway. As shown in Fig. 2, the simultaneous addition of insulin and RAP resulted in an additive effect on sodium transport compared with when RAP was added alone. Cells exposed to vasopressin had a significant increase in sodium transport, and when RAP was added after 60 min to these cells, there was a further stimulation of transport. These data suggest that RAP stimulates sodium transport via an independent mechanism. Since RAP binds to FKBP12 which has been reported to have nearly identical sequence homology to an endogenous inhibitor of PKC (11, 13), the effect of RAP on stimulating sodium transport might be through the inhibition of PKC. To determine the effect of PKC inhibition on sodium transport, we exposed A6 cells to an N-myristoylated cell-permeant peptide pseudosubstrate sequence of the PKCa and β isoforms and measured the effect of ISc. As shown in Fig. 3, the PKC inhibitor stimulated sodium transport. When the PKC inhibitor and RAP were added simultaneously to A6 cells, no additional stimulation of ISc was noted. This suggested that the mechanism of action of RAP on stimulation of sodium transport was via inhibition.
PKC activity was measured in crude cytosolic and membrane fractions that were exposed to either PKC inhibitor, RAP, or phorbol 12-myristate 13-acetate (PMA) in vitro. PMA is known to stimulate PKC activity and inhibit sodium transport in these cells (18, 19). As shown in Table I, PKC activity was significantly inhibited by both the PKC inhibitor and RAP in the membrane fractions. PKC activity in the cytosol was significantly inhibited by RAP, although not by the PKC inhibitor. PMA stimulated PKC activity in both the membrane and cytosolic fractions. We next measured PKC activity in the crude cytosolic and crude membrane fractions from cells that were exposed for 1 h to either 50 nM PKC inhibitor, 1.2 μM RAP, 1.2 μM FK506, or 0.1 μM PMA. As shown in Table II, PKC activity is greatest in the membrane fractions of control cells. As expected, PKC activity was stimulated in the membrane fractions of RAP-treated cells and decreased in the cytosol (25). The PKC activity was significantly inhibited in both the cytosolic and membrane fractions of cells treated with the N-myristoylated PKC inhibitor. RAP inhibited PKC activity to the same degree as the PKC inhibitor in both the cytosolic and membrane fractions. FK506 had no effect on PKC activity in either the cytosol or the membrane fraction. (Cytosol: control, 8.8 ± 0.4; FK506, 8.9 ± 0.4 units; Membrane: control, 13.8 ± 0.30; FK506, 13.9 ± 0.4 units. n = 4). To determine if the effect of the agents on activity was due to changes in the association of the PKC Isoenzyme with the membrane and cytosolic fractions, the experiment shown in Fig. 4 was performed. PMA treatment resulted in a decrease in the amount of PKC Isoenzyme in the cytosolic fraction and an increase in the membrane fraction relative to control as has been described previously (35). Although RAP inhibits PKC activity in the membrane fraction (Table II), RAP did not inhibit the association of the PKC Isoenzyme with the membrane fraction (Fig. 4).

Since FK506 inhibits the stimulation of sodium transport by PKC, we measured the effect of a 100-fold excess of FK506 on the RAP-associated inhibition of PKC activity. FK506 prevented the RAP-associated inhibition of PKC activity (Table II). Since it has been shown that inhibition of PKC is associated

![Fig. 2. The stimulation of sodium transport by rapamycin is additive to stimulatory effects of insulin or vasopressin. The simultaneous addition of insulin and RAP resulted in an additive effect on sodium transport compared when RAP or insulin was added alone. Cells exposed to vasopressin had a significant increase in sodium transport, and when RAP was added after 60 min to these cells, there was a further stimulation of transport. These data suggest that RAP stimulates sodium transport via an independent mechanism. n = 6.](image1)

![Fig. 3. PKC inhibitor stimulates sodium transport in A6, and the effect is not additive to that seen with rapamycin alone. Since RAP binds to FKBP12 which has been reported to have nearly identical sequence homology to an endogenous inhibitor of PKC (11, 13), the effect of RAP on stimulating sodium transport might be through the inhibition of PKC. To determine the effect of PKC inhibition on sodium transport, we exposed A6 cells to an N-myristoylated cell-permeant peptide pseudosubstrate sequence of the PKCa and -β isoforms and measured the effect of I\textsubscript{\text{iso}}. PKC inhibitor stimulated sodium transport. When the PKC inhibitor and RAP were added simultaneously to A6 cells, no additional stimulation of I\textsubscript{\text{iso}} was noted. This suggested that the mechanism of action of RAP on stimulation of sodium transport was via inhibition of PKC activity. n = 4.](image2)
FKBP12 on the activity of PKC

cut state, the peak of the increase in Isc reflects maximal ATPase activity by two methods. When the apical membrane is exposed to 1 μM RAP, 10 μM PMA, or diluent for 1 h, and and protein-matched samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed using an antibody specific for the PKCα subtype. Reactive proteins were detected with a horseradish peroxidase-conjugated second antibody, enhanced chemiluminescence, and autoradiography. The PKCα protein is 80 kDa. CYTO, crude cytosolic preparation; MEM, crude membrane preparation. C, control; P, PMA; R, rapamycin. RAP did not block association of the PKCα subtype with the membrane fraction. n = 4 for each sample.

with activation of Na+/K+-ATPase in certain kidney cell lines (20, 21), we measured the effect of this agent on Na+/K+-ATPase activity by two methods. When the apical membrane is permeabilized to cations with nystatin while in the short-circuited state, the peak of the increase in Isc reflects maximal Na+/K+-ATPase activity (10). As can be seen in Fig. 7, cells exposed to 1 μM RAP for 1 h demonstrate a peak nystatin-associated current that is different than control. Next, A6 cells were exposed to 1 μM RAP or diluent for 1 h, and enzymatic Na+/K+-ATPase activity was measured in whole cell lysates. RAP had no effect on Na+/K+-ATPase activity in A6.

To determine if RAP inhibits PKC activity through its interaction with FKBP12, the following experiment was performed. Purified FKBP12 was incubated in the presence of either diluent, 10 μM RAP, 10 μM PMA, and 100 μM FK506 at room temperature for 10 min in the presence of 10 micromolars of PKC activity from rat brain. As shown in Table III, FKBP12 had no effect on PKC activity. By contrast, RAP, when incubated in the presence of FKBP12, significantly inhibited PKC activity. When RAP was incubated with a 10-fold excess of FK506 in the presence of FKBP12, PKC activity was increased toward control values. By contrast, FK506 + FKBP12 had no effect on PKC activity (control: 9.98 ± 0.02; PKBP12: 10.25 ± 0.33 micromolars, n = 4). We next measured the effect of drugs and FKBP12 on the activity of PKCα and -β isoenzymes. Purified FKBP12 was incubated in the presence of drugs and 8 micromolars of PKCα or PKC-β isoenzyme (one unit of PKCα or -β will transfer 1 nmol of phosphate to histone I per min at pH 7.4 at 30°C). RAP significantly inhibited the PKCα isoenzyme but not the PKC-β isoenzyme (PKCα activity: FKBP12 alone 7.77 ± 0.7; FKBP + RAP: 4.76 ± 0.8 micromolars, p < 0.02, n = 4. PKCβ activity: FKBP12 alone 7.3 ± 0.6; FKBP12 + RAP 6.9 ± 0.6 micromolars, p = not significantly different than FKBP12 alone; n = 4).

**Table II**

**RAP but not FK506 inhibits PKC activity in whole cells exposed to drug in vivo**

|            | Cytosol  | Membrane |
|------------|----------|----------|
| Control    | 7.59 ± 0.39 | 11.55 ± 0.5 |
| RAP        | 5.58 ± 0.14* | 7.06 ± 0.61* |
| PKC inhibitor | 5.59 ± 0.18* | 8.72 ± 0.34* |
| RAP/FK     | 6.86 ± 0.221 | 9.73 ± 0.20  |
| PMA        | 5.25 ± 0.35* | 14.99 ± 0.41* |

*p < 0.05 versus control.

*p < 0.0001 versus control.

**Fig. 4. Effect of rapamycin and PMA on the association of PKCα subtype with crude membrane or cytosolic fractions as quantified by Western blotting.** Crude membrane and cytosolic proteins were prepared from cells exposed to 1 μM RAP, 10 μM PMA, or diluent for 1 h, and protein-matched samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed using an antibody specific for the PKCα subtype. Reactive proteins were detected with a horseradish peroxidase-conjugated second antibody, enhanced chemiluminescence, and autoradiography. The PKCα protein is 80 kDa. CYTO, crude cytosolic preparation; MEM, crude membrane preparation. C, control; P, PMA; R, rapamycin. RAP did not block association of the PKCα subtype with the membrane fraction. n = 4 for each sample.

**Fig. 5. FKBP12 is found in both the cytosolic and membrane preparation.** Crude membrane and cytosolic proteins were prepared from cells exposed to diluent and solubilized in sample buffer containing containing 10 mM DTT. Samples were heated at 95 °C for 3 min before resolving on SDS-PAGE. Proteins were transferred and probed with FKBP12 antibody and detected with an enhanced chemiluminescence system and autoradiography. Migration of rainbow colored molecular weight markers are noted at the left. A, proteins from cytosolic fraction; B, proteins from membrane fraction. The FKBP12 is present in the cytosolic fraction. There are reactive proteins at 24 kDa in the membrane fraction. Lane C, membrane fractions were prepared as above but solubilized in sample buffer containing 10 mM DTT, heated overnight at 37 °C, and then heated at 95 °C for 20 min before resolving on SDS-PAGE. Proteins were transferred to nitrocellulose and probed with the FKBP12 antibody and detected as in A. Under stringent reducing conditions, FKBP12 is found in the membrane fractions. n = 2 for each sample.

**DISCUSSION**

RAP is a fungal macrolide which has recently been introduced as an immunosuppressant for use in solid organ transplantation (1, 2). Although RAP and FK506 are structurally similar and bind to a common set of immunophilins, the FK506 binding proteins, their mechanism of action and effects on T-cell function are remarkably dissimilar. It has been shown that it is the ligand-immunophilin complex and not the immunophilin or the ligand itself that determines the specific downstream effect peculiar to each drug (1, 2). Whereas the FK506-FKBP12 complex inhibits calcineurin-dependent lymphokine gene transcription and T-cell activation (1), the RAP-immunophilin complex inhibits cytokine-stimulated T-cell proliferation in part through the inhibition of both PKC-dependent and PKC-independent p70 kinase serine phosphorylations (22).

A6 cells conduct sodium in a vectorial fashion through apically localized Na+/K+-ATPase pumps. Sodium channel activity is known to be affected by several hormones including vasopressin (4, 6–9), insulin (5), and aldosterone (4, 23), each acting through different mechanisms. It has previously been shown that PKC stimulation inhibits Na+ transport (18, 19), and inhibition stimulates Na+ channel activity (5, 24). Since RAP stimulates sodium transport in A6 and FKBP12 has sequence homology to an endogenous PKC inhibitor (11), it seemed likely that the effect of RAP on sodium transport was occurring through inhibition of PKC by its interaction with FKBP12. Previous studies have failed to demonstrate an effect of FK506 on PKC activity (13); however, the effect of RAP on PKC activity has not been reported.

Here we demonstrate that RAP stimulates sodium transport.
RAP Inhibits PKC Activity in A6 Cells

in A6 with a $K_d$ of 10 nM (Fig. 1). This is similar to the dissociation constant for RAP binding to FKBP12 in human T-cells (1, 2). The effect is blocked by a 100-fold excess of FK506 (10). These results imply that the effect is mediated through a common FK binding protein. The effect was additive to that induced by either insulin or vasopressin. By contrast, a cell-permeant inhibitor of cPKCa and β-subtypes stimulates sodium transport in this cell line, and the simultaneous addition of RAP plus this inhibitor resulted in no additional increase in Na⁺ transport (Fig. 3). These results suggested that RAP was stimulating sodium transport via inhibition of PKC activity. PKC activity was measured in crude cytosolic and membrane fractions. As shown in Tables I and II, phorbol stimulates PKC activity in the membrane fractions. RAP and the cell-permeant PKC inhibitor inhibit PKC activity in membranes to the same extent. The PKC inhibitor inhibited PKC activity in the membrane fractions only. When intact A6 cells were exposed to these agents (Table II), the bulk of PKC activity was measured in the membrane fraction. There is a strong linear relationship between the inhibition of PKC activity and the stimulation of sodium transport by RAP. The numbers above each point represent the concentration of RAP used. 95% confidence intervals are shown above and below the regression line. $n$ = 4 for each point.

**Fig. 6.** The stimulation of sodium transport and inhibition of PKC activity by rapamycin are strongly related. To determine the relationship of the inhibition of PKC activity with the stimulation of sodium transport by RAP, the dose-response curves for the inhibition of PKC activity and the stimulation of sodium transport by RAP were compared as follows. A6 cells were exposed to serial dilutions of RAP or diluent ($1 \times 10^{-2}$-$1 \times 10^{-13}$ M), and $I_n$ was measured at 1 h and recorded as relative to control $I_n$ at 1 h. Cells were then scraped, and PKC activity was measured in the membrane fraction. There is a strong linear relationship between the inhibition of PKC activity and the stimulation of sodium transport by RAP. The **TABLE III**

**RAP-FKBP12 inhibits PKC activity**

To determine if RAP or FK506 in the presence of exogenous FKBP12 affected exogenous PKC activity, PKC activity from rat brain of known activity (10 microunits) was incubated in the presence of 7 μg of purified human recombinant FKBP12 from E. coli (15) (Sigma) with or without 10 μM RAP or 10 μM FK506 and RAP for 10 min at room temperature. Then reaction buffer to a final concentration of 2 mM ATP, 10 mM MgCl$_2$, 0.1 mM CaCl$_2$, 0.002% Triton X-100, 20 mM Tris(hydroxymethyl)aminomethane, 0.2 mM phosphatidyl-L-serine, pH 7.4, was added and allowed to incubate for 30 min at 30 °C, and PKC activity was determined. In parallel experiments, FKBP12 was incubated with PKCα or PKCβ subtypes in the presence or absence of RAP, and PKC activity was determined as described above. $n$ = 4.

shown a typical distribution of the FKBP’s to the nucleus and cytoplasm or attached to the cell cytoskeleton (33). The finding of FKBP12 in the crude membrane described here is not inconsistent with its association with the cytoskeletal elements. The requirement for stringent reducing conditions to resolve this protein in the membrane fractions supports the notion that it might be associated with other cellular proteins. There is evidence that the regulation of the amiloride-sensitive sodium channel is in part dependent upon its association with the actin cytoskeleton (34). Whether FKBP12 is associated with and has actions on the cytoskeleton will be the subject of future study. The finding that the degree of PKC inhibition is strongly correlated with the degree of stimulation of transepithelial sodium

**Fig. 7.** Rapamycin has no effect on enzymatic Na⁺/K⁺-ATPase activity or nystatin-stimulated short circuit current. Since it has been shown that inhibition of PKC is associated with activation of Na⁺/K⁺-ATPase in certain kidney cell lines (20, 21), we measured the effect of this agent on Na⁺/K⁺-ATPase activity by two methods. When the apical membrane is permeabilized to cations with nystatin while in the short-circuited state, the peak of the increase in $I_n$ reflects maximal Na⁺/K⁺-ATPase activity (10). As demonstrated by the two bars on the left side of this figure, cells exposed to 1 μM RAP for 1 h demonstrate a peak nystatin-associated current that is not different than control. RAP, rapamycin; CON, control; $n$ = 4. Next, cells were scraped from filters and Dounce-homogenized, and Na⁺/K⁺-ATPase activity is determined from the difference between $P_i$ released in control tubes and that released in identical samples containing 1 mM ouabain (10) as described in the text. Activity is measured in μmol P/mg protein/h as shown on the right axis. CON, control (2.0 ± 0.01); RAP, rapamycin (1.8 ± 0.08), $n$ = 4.
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Transport by RAP (Fig. 6) strongly suggests that RAP stimulates sodium transport via inhibition of PKC.

The time course of stimulation of sodium transport by RAP strongly suggests that this effect is a channel phenomenon rather than an effect on basolateral Na\(^+/K\)^-ATPase activity. Nevertheless, since PKC stimulation has been shown to inhibit Na\(^+/K\)^-ATPase activity in different renal cell lines (20), we measured Na\(^+/K\)^-ATPase activity by two methods in cells exposed to RAP at the peak of the sodium transport response. There was no effect of RAP on either nystatin-stimulated sodium transport or enzymatic Na\(^+/K\)^-ATPase activity. This provides strong support for the hypothesis that the effect of the PKC inhibition induced by RAP is through a sodium channel effect.

Finally, to determine if RAP inhibits PKC through interaction with FKBP12, we examined the effect of this protein on PKC activity (Table III). FKBP12 alone has no effect on PKC activity, but in the presence of RAP, PKC activity is significantly inhibited. This effect is partially reversed in the presence of a 10-fold excess of FK506 (Table III). The effect seems to be specific for the \(\alpha\) isoform of PKC since there is no effect on the activity of the \(\beta\) isoenzyme of PKC.

Taken together, these data demonstrate that RAP inhibits PKC activity in A6 cells through its interaction with FKBP12 and that inhibition of PKC is the mechanism by which RAP stimulates sodium transport. It thus seems likely that PKC exerts a tonic inhibitory effect on sodium channel activity as has been previously suggested (3, 26). The finding that FK506 has no effect on sodium transport or PKC activity, although it binds tightly to FKBP12, represents another example of the peculiar effects determined by the immunophilin-immunophili- lin ligand complex. The ability of a single immunophilin to present two immunosuppressive ligands to effectors associated with two distinct pathways raises the possibility that the immunophilins may function as general presenting molecules, by analogy to the way that major histocompatibility complex molecules present a large number of peptides to the polymorphic T-cell receptors (1). This concept implies the existence of endogenous immunophilin ligands which, through their binding to the same immunophilin, would either activate or inhibit some downstream pathway depending on the cellular need (1, 2).

PMA has been shown to stimulate phosphorylation of the 150- and 50-kDa subunits of the amiloride-sensitive sodium channel (26), and inhibition of PKC activity results in an increase in the open probability of the sodium channel in cell-attached patches (3, 24). It is likely that dephosphorylation of the channel itself or some channel regulatory protein results in an increase in channel opening. It is therefore interesting to postulate the existence of an endogenous RAP-like ligand that might ultimately regulate basal sodium channel activity through inhibition of PKC-associated phosphorylations. Identification of the relevant proteins that are regulated in this fashion will be the subject of future studies. Moreover, FKBP12 is associated with the ryanodine calcium channel (27–29) and is associated with calcium transients in human neutrophils (30). Since transepithelial sodium transport is dependent upon Na\(^+/Ca\)^2\(^+\) exchange (3, 31) and cPKC is dependent on intracellular Ca\(^2+\) concentration (32), this suggests that RAP and perhaps an endogenous Rap-like ligand might affect intracellular calcium as another means of altering sodium channel activity.

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REFERENCES

1. Schreiber, S. L. (1991) Science 251, 283–287
2. Clarky, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 56–61
3. Ling, E. N., and Eaton, D. C. (1989) Am. J. Physiol. 256, F1094–F1103
4. Shaeffer, J., and Hawk, A. (1992) Kidney Int. 41, 255–268
5. Matsumoto, P. S., Ohara, A., Duchatelle, P., and Eaton, D. C. (1990) Am. J. Physiol. 258, C246–C253
6. Ausiello, D. A., Skorecki, K. L., Verkman, A. S., and Bonventre, J. V. (1987) Kidney Int. 31, 521–529
7. Pratt, A. G., Bertorello, A. M., Ausiello, D. A., and Cantiello, H. F. (1993) J. Physiol. 465, C224–C235
8. Marunaka, Y., and Eaton, D. C. (1991) Am. J. Physiol. 260, C1071–C1084
9. Kleyman, T. R., Ernst, S. A., and Coupaye-Gerard, B. (1994) Am. J. Physiol. 266, F506–F511
10. Rokaw, M. D., West, M. E., Palevsky, P. M., and Johnson, J. P. (1996) Am. J. Physiol. 271, C194–C202
11. Goebel, M. G. (1991) Cell 64, 1051–1052
12. Chung, J., Grammer, T. C., Lemon, K. P., Kozlaukas, A., and Blenis, J. (1994) Nature 370, 71–74
13. Ruff, V., McGee, J. E., Yem, A. W., Deibel, M. R., and Leach, K. L. (1992) Immunol. Invest. 21, 259–273
14. Farrar, Y. J., Vanaman, T. C., and Slevin, J. T. (1991) Biochem. Biophys. Res. Commun. 180, 694–701
15. Siegelkier, J. J., Wiederecht, G., Greulich, H., Bouton, D., Hung, S. H. Y., Cryan, J., Hodges, P. J., and Sigal, N. H. (1990) J. Biol. Chem. 265, 21011–21015
16. Ward, N. E., and O’Brian, C. A. (1993) Biochemistry 32, 11903–11909
17. Eicholtz, T., deBont, D. B. A., deWidt, J., Liskamp, R. M. J., and Ploegh, H. L. (1993) J. Biol. Chem. 268, 1982–1986
18. Yanase, M., and Handler, J. S. (1986) Am. J. Physiol. 250, C517–C522
19. Benn, M. J., Awaday, M. S., Ismail, I. L., and Johnson, J. P. (1995) J. Membr. Biol. 143, 1–18
20. Middleton, J. P., Khan, W. A., Collinsworth, G., Hannun, Y. A., and Medford, R. M. (1993) J. Biol. Chem. 268, 15958–15964
21. Fleschenko, M. S., and Svedner, K. J. (1995) J. Biol. Chem. 270, 14072–14077
22. Calve, V., Crew, C. M., Vik, T. A., and Bierer, B. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7571–7575
23. Frindt, G., Palmer, L. G., and Windhager, E. E. (1996) Am. J. Physiol. 270, F271–F276
24. Nishizuka, Y. (1992) Science 258, 607–614
25. Oh, Y. H., Smith, P. R., Bradford, A., Keeton, D., and Benso, D. J. (1993) Am. J. Physiol. 265, C85–C91
26. Jayaraman, T., Brantilles, A.-M., Timmerman, A. P., Flesicher, S., Erjument-Bronge, H., Tempts, P., and Marks, A. R. (1992) J. Biol. Chem. 267, 9474–9477
27. Collins, J. H. (1991) Biochem. Biophys. Res. Commun. 178, 1288–1290
28. Brantilles, A. B., Onodrias, K., Scott, A., Kobrinsky, E., Onodriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks, A. R. (1994) Cell 77, 513–523
29. Bang, H., Muller, W., Hans, M., Brun, K., and Swandulla, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3435–3438
30. Taylor, A., and Windhager, E. E. (1979) Am. J. Physiol. 236, F505–F512
31. Nishizuka, Y. (1995) FASEB J. 9, 484–496
32. Ruff, V. A., Yem, A. W., Morris, P. L., Adams, L. D., Reardon, I. M., Deibel, M. R., and Leach, K. L. (1992) J. Biol. Chem. 267, 21285–21288
33. Pratt, A. G., Heltzman, E. J., Brown, D., Cunningham, C. C., Reisin, I. L., Kleyman, T. R., Mclaughlin, M., Jackson, G. R., Gydon, J., and Cantiello, H. F. (1996) J. Biol. Chem. 271, 18045–18055