LY-294002-inhibitable PI 3-kinase and regulation of baseline rates of Na\(^+\) transport in A6 epithelia

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Blocker-induced noise analysis of epithelial Na\(^+\) channels (ENaCs) was used to investigate how inhibition of an LY-294002-sensitive phosphatidylinositol 3-kinase (PI 3-kinase) alters Na\(^+\) transport in unstimulated and aldosterone-stimulated A6 epithelia. From baseline Na\(^+\) transport rates \((I_{Na})\) of 4.0 ± 0.1 (unstimulated) and 9.1 ± 0.9 \(\mu\text{A/cm}^2\) (aldosterone), 10 \(\mu\text{M}\) LY-294002 caused, following a relatively small initial increase of transport, a completely reversible inhibition of transport within 90 min to 33 ± 6% and 38 ± 2% of respective baseline values. Initial increases of transport could be attributed to increases of channel open probability \((P_o)\) within 5 min to 143 ± 17% (unstimulated) and 142 ± 10% of control (aldosterone) from baseline \(P_o\) averaging near 0.5. Inhibition of transport was due to much slower decreases of functional channel densities \((N_T)\) to 28 ± 4% (unstimulated) and 35 ± 3% (aldosterone) of control at 90 min. LY-294002 (50 \(\mu\text{M}\)) caused larger but completely reversible increases of \(P_o\) (215 ± 38% of control at 5 min) and more rapid but only slightly larger decreases of \(N_T\). Basolateral exposure to LY-294002 induced no detectable effect on transport, \(P_o\) or \(N_T\). We conclude that an LY-294002-sensitive PI 3-kinase plays an important role in regulation of transport by modulating \(N_T\) and \(P_o\) of ENaCs, but only when presented to apical surfaces of the cells.

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We have used a noninvasive pulse method of weak blocker-induced noise analysis to monitor the time-dependent changes of single-channel currents \( i_{Na} \), apical membrane functional channel densities \( N_{T} \), and channel open probabilities \( P_{o} \) that algebraically determine the net effect of LY-294002 on macroscopic rates of Na\(^+\) transport \( (I_{Na}) \). We observed and report that LY-294002 acts exclusively from the apical surface of the cells with entirely different \( P_{o} \) and \( N_{T} \) response times. Whereas \( P_{o} \) is increased almost immediately (~1 min), the decreases of \( N_{T} \) occurred relatively slowly over 60–90 min, resulting in a completely reversible inhibition of basal rates of Na\(^+\) transport in unstimulated and aldosterone-primed A6 epithelia.

**MATERIALS AND METHODS**

**Tissues.** A6 cells were used at passages 115 and 118 and grown in a humidified incubator at 28°C containing 1% CO\(_2\). The cells were transferred from their frozen state, seeded and grown on 75-cm\(^2\) plastic culture flasks (Costar, Cambridge, MA), and then subcultured on Transwell-Clear inserts (Costar) for at least 10 days to achieve confluence and complete development of their transepithelial transport inserts (Costar) for at least 10 days to achieve confluence and complete development of their transepithelial transport.

**Electrical measurements.** The methods of study of amiloride-sensitive ENaCs using blocker-induced noise analysis were identical to those of previous reports from our laboratory (4, 15, 20). The pulse method relies on the fact that weak Na\(^+\) channel blockers like 6-chloro-3,5-diamino-pyrazine-2-carboxamide (CDPC; 27,788-6; Aldrich Chemical, Milwauke, WI) interact with open channels, causing fluctuations of the channels between open and blocked states and thereby giving rise to blocker-induced current noise characterized by Lorentzian in power density spectra (PDS) (18, 20). The apical chambers were perfused continuously with solution containing 10 \( \mu \)M CDPC except during pulse intervals, when the CDPC concentration was increased to 30 \( \mu \)M. In these experiments, the 100 \( \mu \)M amiloride-insensitive currents measured at the ends of the experiments averaged near 0.1 \( \mu \)A/cm\(^2\). After subtraction of the amiloride-insensitive currents from the \( I_{sc} \) the macroscopic blocker or amiloride-sensitive Na\(^+\) currents at 10 \( (I_{Na}^{0}) \) and 30 \( \mu \)M CDPC \( (I_{Na}^{M}) \) were used in all calculations.

Current noise PDS were always measured in pairs at 10 and 30 \( \mu \)M CDPC, giving rise to blocker-induced Lorentzian and fractional inhibitions of the amiloride-sensitive Na\(^+\) currents that were used in calculation of \( i_{Na} \), \( P_{o} \), and \( N_{T} \) at the time points of measurement. The PDS were fit by nonlinear regression (TableCurve 2D; Jandel Scientific, San Rafael, CA) to a mathematical model consisting of low-frequency 1/f noise characterized by the coefficient \( S_{t} \) and the exponent \( \alpha \) as \( S_{t}/f^{\alpha} \); the blocker-induced Lorentzian characterized by its low-frequency plateau value \( (S_{0}) \) and corner frequency \( (f_{c}) \) as \( S_{0}/(1 + f_{c}/f)^{\alpha} \), and high-frequency amplifier noise characterized by the coefficient \( S_{f} \) and the exponent \( \beta \) as \( S_{f}/f^{\beta} \) (20).

The \( f_{c} \) at 10 and 30 \( \mu \)M CDPC at the sequential time points were fit to smooth curves as described previously (TableCurve 2D), thereby filtering out small uncertainties in estimation of the individual \( f_{c} \) (20). The on \( (k_{on}) \) and off \( (k_{off}) \) rate coefficients for the channel transitions between open and blocked states were calculated from the slopes of the rate constants of \( f_{c} \) at 10 and 30 \( \mu \)M CDPC, where \( 2\pi f_{c} = k_{on}B + k_{off}B \) and B is the blocker concentration. The blocker equilibrium constants \( (K_{B}) \) were calculated as \( K_{B} = k_{off}/k_{on} \).

**Single-channel currents.** \( i_{Na} \) were calculated at 10 \( \mu \)M CDPC with Eq. 1.

\[
i_{Na} = \frac{S_{o}}{P_{gain}} \left( \frac{2\pi f_{c}}{4\pi k_{on}B} \right)^{2}
\]

The original equation (25) was modified to include a power gain correction \( (P_{gain}) \). In contrast to frog skin, where the basolateral membrane capacitance \( (C_{b}) \) is far greater in value than the apical membrane capacitance \( (C_{a}) \) due to the functional coupling of multiple cell layers that markedly increase basolateral membrane area and capacitance \( (3) \), the basolateral membrane area in cell monolayers of A6 epithelia is far less in value in absolute terms and in terms relative to apical membrane area. Consequently, the fractional capacitance \( (C_{i}/C_{b} + C_{a}) \) is significantly less than unity in A6 epithelia compared with frog skin. The formal consequence for this area-related difference of capacitance is presented in Appendix A, leading to a correction for \( S_{o} \) by \( P_{gain} \) to account for attenuation of the Lorentzian power measured and the actual power originating within the apical membrane ENaCs. Accordingly, the current noise measured in short-circuit currents \( (I_{sc}) \) underestimates the actual Na\(^+\) current noise originating in the channels \( (I_{Na}) \). If \( C_{a} \) and \( C_{b} \) at very low audio frequencies are 1.38 and 20 \( \mu \)F/cm\(^2\), respectively, as we have determined with impedance measurements of A6 epithelia grown on Transwell-Clear inserts (T. G. Păunescu and S. I. Helman, unpublished observations), then \( P_{gain} \) is 0.875. We have assumed constancy of \( P_{gain} \) in all calculations, recognizing that small differences among tissues will lead to relatively small uncertainties in determination of the single-channel currents.
Open-channel densities. Open-channel densities ($N_o$) at 10 μM CDPC ($N_o^{10}$) were calculated as $I_{N_o}^{10}/I_{N_o}$, where the superscripts here and elsewhere indicate the blocker concentration. $N_o$ in the absence of blocker, was calculated as described previously (18, 20) with Eq. 2

$$N_o = N_o^{10} \left(1 + \frac{P_o B^{10}}{K_B}\right)$$

Channel open probabilities. During control and experimental periods, $P_o$ were calculated as described previously (20) with Eq. 3. $N_o^{B^{10}/B}$ represents the quotient of open-channel densities ($N_o^{B^{10}}/N_o^{B}$) determined at 10 (B1) and 30 (B2) μM CDPC

$$P_o = 1 - \frac{N_o^{B^{10}/B}}{B_o N_o^{B^{10}B} - B_1 K_B}$$

Because $N_o^{B^{10}} = I_{N_o}^{B^{10}}/I_{N_o}$, $N_o$ can be equated with the quotient $P_o B^{10}/B^{B^{10}/B}$, which represents the quotient of the fractional changes of macroscopic currents and the fractional changes of single-channel currents. Hence, open probability can be determined from the decreases of macroscopic rates of Na$^+$ entry into the cells caused by increasing the CDPC concentration from 10 to 30 μM and from the concurrent increases of single-channel currents that occur with hyperpolarization of apical membrane voltage. With small fractional changes of $I_{N_o}$, relatively smaller fractional changes of apical membrane voltage are expected together with equally smaller changes of $I_{N_o}$, $I_{N_o}$ were calculated with Eq. 7 (APPENDIX C).

Functional channel densities. $N_{T}$ were calculated as the quotient $N_o / P_o$. $N_T$ represents the total number of channels involved in apical membrane Na$^+$ entry into the cells that fluctuate between open and closed states of the channel. Channel densities are expressed in units of millions of channels per cm$^2$ of planar area or per 100 µm$^2$, approximating the planar area occupied by a single cell. It may be emphasized that channels or subunits of ENaCs that reside within the apical membranes in nonfunctional or quiescent states would not be detected.

Basolateral membrane resistance. The basolateral membrane resistance ($R_j$) was calculated at each time point with Eq. 5 (APPENDIX B).

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were performed with SigmaStat (Jandel Scientific) using paired or unpaired t-tests where appropriate. A P value <0.05 was considered significant.

RESULTS

LY-294002 inhibits Na$^+$ transport in unstimulated and aldosterone-pretreated tissues. Shown in Fig. 1 are typical strip-chart recordings of the $I_{sc}$ response to 10 or 50 μM LY-294002 added to the apical perfusion solution of either a control unstimulated tissue or a tissue that had been pretreated with aldosterone to stimulate its baseline rate of Na$^+$ transport. After the tissues were short-circuited in chambers, the $I_{sc}$ were allowed to stabilize for ~1.5 h before onset of a 2-h control period that was followed by a 90-min experimental period, during which time the tissues were exposed to LY-294002. Subsequently, LY-294002 was removed from the apical solution for 2.5 h, and the experiments were terminated after complete inhibition of blocker-sensitive Na$^+$ entry into the cells by 100 μM amiloride.

Examination of these records indicated that inhibition of transport by LY-294002 was preceded within 1 min by a relatively small but readily apparent stimulation of $I_{sc}$, especially in those tissues treated with 50 μM LY-294002 (Fig. 1C). Inhibition of $I_{sc}$ proceeded relatively slowly with quasi-exponential time constants of 20.2, 22.1, and 13.4 min for the experiments shown in Figs. 1A, 1B, and 1C, respectively. It can be observed that the response to LY-294002 was more rapid in unstimulated tissues treated with the higher 50 μM concentration of LY-294002 than with 10 μM LY-294002 in unstimulated and aldosterone-pretreated tissues. Inhibition of $I_{sc}$ by LY-294002 was completely reversible with recovery of $I_{sc}$ noticeably slower in those tissues treated with 50 μM LY-294002.

Also indicated in Fig. 1 are the absolute changes of $I_{sc}$ caused by increasing the CDPC concentration from 10 to 30 μM. The measurements of $I_{sc}^{10}$ and $I_{sc}^{30}$ were obtained from data acquired digitally (8 points/s) at high resolution that permitted determination of the fractional changes of amiloride-sensitive currents, $I_{sc}^{30/10}$, within 60–90 s after the CDPC concentration was increased to 30 μM. At these times, channels have redistributed between closed, open, and blocked states (20). PDS were acquired at 10 μM CDPC just before elevation of CDPC to 30 μM. PDS were also acquired at 30 μM CDPC during the last 2 min of the 2-min, 40-s pulse durations, thereby yielding the $f_o$ and $S_o$ of the Lorentzians at 10 and 30 μM CDPC.

Stimulation precedes inhibition of $I_{sc}$ caused by LY-294002. Summarized in Fig. 2 are the initial increases of $I_{sc}$ caused by LY-294002. The strip-chart digitized data (1 point/s) were normalized for the purpose of summarization to their zero time values at intervals of 25 s. LY-294002 (10 μM) caused relatively similar, small fractional increases of $I_{sc}$ (mean of 4–5%) within 1–2 min, despite differences in baseline rates of Na$^+$ transport in control and aldosterone-pretreated tissues. With 50 μM LY-294002, $I_{sc}$ was increased absolutely and fractionally more so than with 10 μM within 1–2 min (mean of 18%) before the $I_{sc}$ began to return toward zero time values. Within 5 min, $I_{sc}$ returned to zero time values and continued to decrease thereafter as indicated in Fig. 1.

Underlying the time-dependent changes of $I_{Na}$ are differential time- and concentration-dependent changes of $P_o$ and $N_T$ (see Time-dependent changes of $P_o$ and Time-dependent changes of $N_T$). The results of our analysis are summarized in Fig. 3 for unstimulated control and aldosterone-pretreated tissues challenged with 10 μM LY-294002 and in Fig. 4 for unstimulated tissues challenged with 50 μM LY-294002. The mean responses of the $I_{Na}$ have been reported previously for tissues challenged with 10 μM LY-294002 (5). The data presented in Fig. 4A indicate the average time course of inhibition of $I_{Na}$ and its reversibility in unstimulated tissues challenged with 50 μM LY-294002. Notably, the first time point of measurement after exposure to LY-294002 is at 5 min, so the initial stimulation of transport is not indicated in Figs. 3 and 4. During experimental periods, measurements of
The zero time $I_{Na}$ averaged $4.00 \pm 0.14 \mu A/cm^2$ in unstimulated tissues ($n = 5$) and was decreased by $10 \mu M$ LY-294002 to $1.33 \pm 0.25 \mu A/cm^2$, or to $33.1 \pm 5.8\%$ of zero time values, within 90 min. Despite aldosterone stimulation of $I_{Na}$ to $9.12 \pm 0.94 \mu A/cm^2$, $10 \mu M$ LY-294002 decreased $I_{Na}$ to $3.38 \pm 0.32 \mu A/cm^2$, or fractionally to $37.7 \pm 1.8\%$ of its elevated zero time values, and thus essentially to the same extent as was observed with unstimulated tissues. Unstimulated tissues challenged with $50 \mu M$ LY-294002 responded more quickly, as indicated above, from zero time $I_{Na}$ of $3.42 \pm 0.24 \mu A/cm^2$, reaching stable plateau values within 60 min and averaging $0.79 \pm 0.24 \mu A/cm^2$ at 90 min ($23.2 \pm 4.1\%$).

**Time-dependent changes of $I_{Na}$.** Our previous analysis had indicated that inhibition of transport could not be ascribed to changes of $I_{Na}$ when control or aldosterone-prestimulated tissues were challenged with $10 \mu M$ LY-294002 (5) or, as indicated in Fig. 4B, with $50 \mu M$ LY-294002. At 5 min, $I_{Na}$ was unchanged (5) or essentially unchanged (Fig. 4B) from zero time values. At 15 min and thereafter, inhibition of transport was accompanied by relatively small increases of $I_{Na}$ that were reversed on withdrawal of LY-294002 and return of $I_{Na}$ toward and above zero time values. Such behavior of $I_{Na}$ is expected and consistent with changes of fractional transcellular resistance [$R_a/(R_a + R_b)$], where apical membrane resistance ($R_a$) is increased (LY-294002 exposure) or decreased (LY-294002 withdrawn) relative to any change of basolateral membrane resistance ($R_b$) (see Changes of $R_b$ caused by LY-294002). Clearly, inhibition of $I_{Na}$ could not be due to changes of $I_{Na}$, and so inhibition of transport at the apical membranes of the cells was due to time-dependent decrease of $N_o$.

Zero time single-channel currents averaged near 0.4 pA in all groups of experiments. With single-channel conductance ($\gamma_{Na}$) equal to 5 pS, the absolute value of apical and basolateral membrane voltage would be $80 \ mV (i_{Na}/\gamma_{Na})$ at zero time and would have increased to $\sim 90 \ mV$ when $I_{Na}$ was near 0.45 pA during tissue exposure to LY-294002.
dent changes of
after withdrawal of LY-294002 from the apical solution. Comparison of the time-dependent changes of
LY-294002 for 90 min and allowed to recover
are means ± SE. Unstimulated tissues were treated with 10 µM LY-294002 (n = 5) or 50 µM LY-294002 (n = 5), and aldosterone-prestimulated tissues were treated with 10 µM LY-294002 (n = 7).

The f_i of the CDPC-induced Lorentzians and the blocker equilibrium constants (K_B) for CDPC were similar in value to those reported previously for A6 epithelia (4, 14, 15, 20) and were unchanged by LY-294002 (data not shown).

Time-dependent changes of P_o. LY-294002 caused changes of both P_o and N_T. However, the time courses and directions of change were entirely different. Figures 3A and 4C summarize the time-dependent changes of P_o. Within 5 min, the P_o were increased acutely by 10 µM LY-294002 (Fig. 3A) and more so by 50 µM LY-294002 (Fig. 4C). Zero time P_o averaged near 0.5 in unstimulated and aldosterone-prestimulated tissues challenged with 10 µM LY-294002. The increases of P_o were essentially the same [to 143 ± 17% (unstimulated) and to 142 ± 10% of control (aldosterone)], despite differences in baseline rates of \( I_{Na} \) that averaged 4.00 ± 0.14 and 9.12 ± 0.94 µA/cm², respectively, in unstimulated and aldosterone-prestimulated tissues (5). When tissues were challenged with 50 µM LY-294002, P_o was increased from 0.42 ± 0.06 to 0.81 ± 0.07, or to 215 ± 38.3%, at 5 min (Fig. 4C). From maximum increases observed at 5 min, the P_o returned slowly over 90 min toward zero time values. On withdrawal of LY-294002, P_o was reversed toward or below zero time values and was particularly rapid in those tissues challenged with 50 µM LY-294002.

It should be noted that P_o were calculated using the quotient \( \frac{I_{Na0}}{I_{Na}} = N_T \). Zero time values of \( I_{Na0} \) averaged near 0.82 in all groups of tissues. Zero time values of \( I_{Na0} \) averaged near 1.07 and remained essentially unchanged by LY-294002. Accordingly, the changes of P_o reflected the time-dependent changes of \( I_{Na0} \) because the K_B were not changed by LY-294002, as indicated above.

Time-dependent changes of N_T. Compared with P_o, the directional change, time course, and concentration dependence of P_o on LY-294002 were completely different (Figs. 3B and 4D). At 10 µM LY-294002 in unstimulated and aldosterone-prestimulated tissues (Fig. 3B), N_T decreased with quasi-exponential time constants of 16.9 and 19.6 min, and from zero time values of 24.0 ± 2.6 and 60.3 ± 9.0 channels/100 µm², respectively. Parenthetically, aldosterone stimulation of transport in these groups of experiments is due solely to increase of N_T and is the same as reported previously (20). At 90 min, N_T was decreased to 6.9 ± 1.3 (unstimulated) and 19.5 ± 2.3 (aldosterone) channels/100 µm², or to 28.4 ± 3.7% and 34.5 ± 3.1% of zero time values, respectively, irrespective of the 2.5-fold...
difference of zero time values. The limiting values of \( N_T \) derived from the exponential fits of data indicated that at infinite time, \( N_T \) would have stabilized at 28.1% (unstimulated) and 30.7% (aldosterone) of zero time values in tissues treated with 10 \( \mu M \) LY-294002.

Compared with tissues treated with 10 \( \mu M \) LY-294002, the \( N_T \) of tissues treated with 50 \( \mu M \) LY-294002 decreased more rapidly with a time constant of 10.9 min, reaching stable values within 60 min (Fig. 4D). From zero time values that averaged 29.4 ± 5.6 channels/100 \( \mu m^2 \), \( N_T \) was decreased to 4.3 ± 0.7 channels/100 \( \mu m^2 \) at 90 min, or to 15.3 ± 1.7% of zero time values.

**LY-294002 does not act from the basolateral surface of the tissues.** LY-294002 presented to the tissues at their basolateral surface was, surprisingly, completely without effect on Na⁺ transport (Fig. 5). The typical patterns of inhibition of \( I_{Na} \) elicited by apically applied LY-294002 at the same concentration (10 \( \mu M \)) were completely absent, as were the changes of \( i_{Na} \), \( P_o \), and \( N_T \). To the extent that apical and basolateral membrane permeabilities to LY-294002 are the same (per unit area) and to the extent that basolateral membrane surface area is far greater than apical membrane surface area, the actual concentration of intracellular LY-294002 should be far greater when LY-294002 is presented to the tissues from the basolateral solution. Consequently, the absence of detectable changes of transport is most surprising if it is assumed that the LY-294002 inhibitable PI 3-kinase is accessible equally well from the extracellular fluids bathing the apical and basolateral surfaces of the cells. Clearly, a simple cytoplasmic site(s) of action is precluded among schemes that may ultimately explain how LY-294002 causes both increase of \( P_o \) and decrease of \( N_T \) with entirely different time courses, but only when cells are presented with LY-294002 from the apical face of the cells.

**Changes of \( R_b \) caused by LY-294002.** Although not a major aim of these studies, it was of interest to know if apical LY-294002 caused changes of the basolateral membrane resistance of the cells. Calculations were carried out with Eq. 5 (APPENDIX B) and the results summarized as indicated in Fig. 6. Zero time \( R_b \) of unstimulated tissues averaged 9,028 ± 783 \( \Omega \cdot cm^2 \) (\( n = 15 \)), whereas those of aldosterone-prestimulated
tissues averaged $3,864 \pm 879 \, \Omega \cdot \text{cm}^2$ ($n = 7$). Thus steroid stimulation of transport by aldosterone was mediated not only by increases of apical membrane $N_T$ but also by increases of basolateral membrane conductance, due most likely to activation of a basolateral membrane $K^+$ conductance as suggested originally by Schultz (35). Whereas stimulation of transport by aldosterone was accompanied by decrease of $R_b$, inhibition of transport by LY-294002 was accompanied by a reversible increase of $R_b$ as indicated in Fig. 6A. When normalized to zero time values as shown in Fig. 6B, the fractional increases of $R_b$ were essentially the same in unstimulated and aldosterone-prestimulated tissues at 10 and 50 $\mu$M LY-294002. At 90 min, $R_b$ was increased about two- to threefold above zero time values. Notably, however, LY-294002-related increases of $R_b$ were delayed by at least 15 min from onset of the increases of $P_o$ reported above and the decreases of $N_T$ that were observed during this same time period. In this regard, it also remains curious that LY-294002 applied to the basolateral membrane is without effect on $R_b$, suggesting that changes of $R_b$ do not occur by direct interaction of LY-294002 with the basolateral membrane of the cells or by access to the cytosol from the basolateral surface of the cells.

**DISCUSSION**

We have examined the role of an LY-294002-sensitive PI 3-kinase in modulating basal (unstimulated) and aldosterone-prestimulated $\text{Na}^+$ transport in cell-cultured A6 epithelia derived from the renal distal tubule of *Xenopus laevis*. A noninvasive pulse method of weak blocker-induced noise analysis was used to determine whether inhibition of transport in response to LY-294002 was due to changes of $i_Na$, $N_T$, and/or $P_o$ that together determine the overall effect of the inhibitor on $\text{Na}^+$ transport.

Our analysis has revealed that apical exposure of A6 epithelia to LY-294002 causes, after a relatively small initial stimulation, a marked and completely reversible inhibition of both basal and aldosterone-prestimulated $\text{Na}^+$ transport due to slow time-dependent decreases of functional ENaC densities within the apical membranes of the cells. The initial stimulation of transport was due to considerably more rapid but quantitatively

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Fig. 5. Absence of change of $i_{Na_A}$ (A), $i_{Na_B}$ (B), $P_o$ (C), and $N_T$ (D) when tissues ($n = 5$) are presented with 10 $\mu$M LY-294002 applied at the basolateral membrane surface. Values are means $\pm$ SE. Compare these data with the data presented in Figs. 3 and 4.
smaller fractional increases of ENaC $P_o$. LY-294002 applied apically not only increased apical membrane resistance (decrease of ENaC $N_T$) but also caused, after a substantial delay, a reversible increase of basolateral membrane resistance. Similar qualitative changes were seen at 10 and 50 $\mu$M LY-294002. However, significant differences became apparent not only in the time course of change of the $P_o$ and $N_T$ but also in the sensitivity of the $P_o$ and $N_T$ to these concentrations of LY-294002.

The difference in sensitivity of the $P_o$ and the $N_T$ to LY-294002 is emphasized in Fig. 7. Near-maximal increases of $P_o$ were measured at 5 min. The response at 5 min expressed in absolute terms (Figs. 3 and 4) or normalized to zero time values (Fig. 7) indicated clearly that $P_o$ was concentration dependent in the range of 10–50 $\mu$M LY-294002. With $P_o$ averaging in the range of 0.4–0.5, the maximal increases of $P_o$ would not exceed a normalized increase of $2$- to $2.5$-fold, so the increases of $P_o$ at 50 $\mu$M LY-294002 are close to the maximum that can be elicited by LY-294002. Accordingly, regardless of mechanism, a half-maximal concentration of LY-294002 in the range of $15$–$20$ $\mu$M would be required to cause a 50% increase of $P_o$.

Maximal or near-maximal decreases of $N_T$ could be elicited by LY-294002 within 60 min at 50 $\mu$M and within 90 min at 10 $\mu$M (Figs. 3 and 4). Examination of the normalized decreases of $N_T$ (Fig. 7) indicated that $N_T$ was most sensitive to LY-294002 at concentrations $<10$ $\mu$M. The dependency of $N_T$ on LY-294002 could as a first approximation be reasonably characterized by an IC$_{50}$ in the range of $1$–$2$ $\mu$M LY-294002, which would be remarkably close to the IC$_{50}$ of 1.4 $\mu$M of the LY-294002 inhibitable PI 3-kinase reported by Vlahos et al. (40).

It is thus interesting to note that the response of $N_T$ to LY-294002 is most likely attributable to an LY-294002 inhibitable PI 3-kinase. This PI 3-kinase is involved directly at least in one of the steps or processes mediating regulation of apical membrane ENaC densities. Stimulation of transport by insulin and aldosterone is mediated by increases of $N_T$ (4, 20), and LY-294002 inhibits both the insulin- and aldosterone-mediated increases of transport (5, 31). It remains to be determined whether LY-294002 inhibits hormone-stimulated transport by preventing increases of $N_T$. If LY-294002 causes substantial increases of $P_o$ as reported here, then the LY-294002-mediated prevention of a threefold or greater stimulation of transport by aldosterone and/or insulin must be due, on quantita-
tive grounds, to loss of hormone-mediated increases of $N_P$.

It may be useful to note that we are not aware, at least from our own studies, of how the $P_o$ of ENaC is regulated in intact epithelial cells. It is thus, at this time, impossible to know how and why LY-294002 increases $P_o$ in reversible fashion. The concentrations of LY-294002 required to increase $P_o$ are substantially greater than those required to decrease $N_P$. Whether this action of LY-294002 on $P_o$ is a nonspecific effect or an effect mediated by yet another PI 3-kinase or a PI 3-kinase with a different IC$_{50}$ remains to be determined, among other possibilities. Notably, whatever the mechanism, it was readily apparent that the effect of LY-294002 on $P_o$ occurred rapidly (minutes) and reversibly.

Understanding the complex interactions that regulate the channel kinetics of ENaCs will require a detailed analysis of all the potential effectors in the regulatory pathway(s). Activation of PI 3-kinase is an early step in the phosphoinositide pathway, which typically includes downstream kinase cascades. Which of the effector pathways forms the physical link to ENaC via PI 3-kinase activation is unknown.

Interestingly, one of the kinases that is activated downstream of PI 3-kinase, the serum glucocorticoid-induced kinase (sgk), has recently been shown to be an aldosterone-induced protein (10, 29). Coexpression of sgk and ENaC in oocytes leads to increased Na$^+$ flux, suggesting that sgk can regulate channel activity. However, we have previously shown that aldosterone treatment has a direct effect on phosphatidylinositol phosphorylation, indicating the existence of another aldosterone-induced protein at or before the PI 3-kinase step, which is well before the sgk protein (5). Thus multiple components of the phosphoinositide pathway may be induced by aldosterone, whereas a constitutive level of activation of the pathway appears to be necessary for maintaining basal transport. The task of identifying each of the proteins, their functions as well as the number of steps in the complete pathway(s), remains a challenge for future investigations.

**Side action of LY-294002.** Our results with regard to the ineffectiveness of LY-294002 when applied to the basolateral surface were completely unexpected. LY-294002 acts exclusively from the apical surface of the epithelium, with no significant effects on $I_{Na}$, $N_P$, and $R_e$ when presented at the same concentration to the basolateral surface of the tissues. To our knowledge, this is a novel finding because this inhibitor is known to exhibit equal efficacy in whole cell assays using both adherent and suspended cells and in in vitro enzyme assays, suggesting that it is membrane permeable (40) (C. J. Vlahos, personal communication). The striking absence of response to basolateral LY-294002 is clearly not consistent with the action of a membrane-permeable inhibitor blocking the activity of a cytosolic enzyme.

Our finding suggests that the current view of PI 3-kinase as a cytosolic enzyme (at least of the LY-294002-inhibitable PI 3-kinase) that is translocated to the membrane on activation may be too simplistic. In many experiments, “cytosolic” simply means that the enzyme is found in the soluble fraction during subcellular fractionation procedures after cellular disruption. Likewise, “membrane bound” simply means associated with a membrane fraction during the fractionation procedure. Neither of these terms allows one to assess exactly where in the cell the enzyme may be found.

It is difficult to imagine that a single event such as the binding of the catalytic subunit of PI 3-kinase to a phosphorylated intermediate (e.g., insulin receptor substrate) will cause the enzyme to migrate through the cytoplasm of the cell to a very specific area of the cell membrane. Rather, it is easier to imagine that complexes of intermediates may reside in close proximity to the final effectors. To our knowledge, there have been no detailed studies attempting to localize PI 3-kinase within the cytosolic or membrane subcompartments. This task will be complicated by the number of isoforms of PI 3-kinase that may be localized in various compartments, respond to different stimuli, and have different substrates. Our results suggest that the PI 3-kinases that are responsible for basal as well as hormone-stimulated Na$^+$ transport are localized and compartmentalized in such a way that only apically applied LY-294002 is effective. Further investigations are required to resolve unequivocally whether LY-294002 can traverse the plasma membrane and to determine precisely the localization of the specific PI 3-kinase that is involved in the localization of membrane ion transport.

Our results do not prove, but are consistent with the idea, that LY-294002 prevents insertion and/or stimulates withdrawal of ENaCs from the apical plasma membrane of A6 cells. A similar effect was demonstrated for A-type potassium channels in hippocampal pyramidal neurons (41). In these cells, LY-294002 and wortmannin inhibition of PI 3-kinase was shown to cause decreases of membrane area and three different types of K$^+$ currents. Both wortmannin and LY-294002 induced a decrease of A-type K$^+$ channel density within the plasma membrane.

There are, however, a large number of studies that describe how PI 3-kinase plays a crucial role in endocytic and/or exocytotic phenomena (2, 6, 8, 9, 16, 23, 24, 33, 38) [see also reviews by Shepherd et al. (37) and De Camilli et al. (12) and references therein]. Our own data do not rule out the possibility that PI 3-kinase may be involved in withdrawal of channels from the apical membrane because, on balance, the steady-state apical membrane density of functional ENaCs will be determined by the rates of insertion and withdrawal. Analogously, PI 3-kinase inhibition was shown to induce a decrease in the endosomal recycling of intracellular NHE3 isoforms of the Na$^+$/H$^+$ exchanger to the cell membrane in AP-1 Chinese hamster ovary cells (23). On the weight of the available evidence cited above, our bias favors the view that PI 3-kinase is involved in shuttling of channels to and/or from the...
apical membrane of the cells. However, we cannot rule out the possibility that PI 3-kinase is involved in recruiting channels and/or subunits from quiescent or nonfunctional states that are resident within the apical plasma membranes of the cells.

Thus, in summary, it will be most interesting to resolve where and how LY-294002 inhibitable PI 3-kinase is involved in regulation of basal ENaC channel densities and to learn at what step or steps PI 3-kinase is required for the hormonal response to insulin and aldosterone in regulation of transepithelial absorption of sodium. Our findings suggest that LY-294002 inhibits a novel member of the PI 3-kinase family involved in regulation of epithelial sodium channels.

APPENDIX A

Correction of the Lorentzian $S_o$ for Power Gain Loss

Shown in Fig. 8 are the usual direct current (DC) and alternating current (AC) electrical equivalent circuits of short-circuited epithelia with apical ($R_a$ and $C_a$) and basolateral ($R_b$ and $C_b$) membrane slope resistances and capacitances. $E_o$ is the Thévenin electromotive force (EMF) of the basolateral membrane with values that exceed the potassium equilibrium potential difference due to the contribution of the current generated by the $\text{Na}^+$-$\text{K}^+$-ATPase pump current flowing through $R_b$ (17, 21). Because the electrical operating point of apical membrane ENaCs is far removed from equilibrium, the Thévenin $E_o$ is at or very near zero, so the apical membrane behaves electrically as a resistor paralleled by its capacitance.

$E = \text{Thévenin electromotive force (EMF)}$ of the basolateral membrane.

The basolateral membrane is modeled by its Thévenin electromotive force ($E_b$) and slope resistance ($R_b$). The DC apical membrane current carried into the cell is $I_{Na}^c$ where $I_{Na}^c = E_o/(R_a + R_b)$. The alternating current (AC) equivalent circuit (B) additionally contains apical ($C_a$) and basolateral ($C_b$) membrane capacitances (EMF’s behave as short-circuits in AC circuits). Conductance fluctuations of the channels give rise to sodium current noise ($I_{Na}^c$), $C_a$ and the parallel combination of $R_b$ and $C_b$ act as a frequency-dependent current divider. A fraction of $I_{Na}^c$ is shunted back to the apical solution, whereas the current flowing through the basolateral membrane appears as current noise in the short-circuit ($I_{Na}^sc$). Hence, $I_{Na}^c < I_{Na}^sc$.

In the presence of a $\text{Na}^+$ channel blocker, the conductance fluctuations of ENaCs between open and blocked states give rise to ac current noise ($I_{Na}^sc$). With $C_a$, the basolateral membrane conductance ($G_b$), and $C_b$ acting as a current divider, the short-circuit AC noise ($I_{Na}^sc$) is less than $I_{Na}^c$. Defining the noise current gain ($I_{gain}$) as

$$I_{gain} = \frac{I_{Na}^sc}{I_{Na}^c} = \frac{G_b + j\omega C_b}{G_a + j\omega(C_a + C_b)}$$

and the power gain as $P_{gain} = (I_{gain})^2$, the absolute value of $P_{gain}$ ($|P_{gain}|$) can be calculated as indicated in Fig. 9. For the purpose of calculation relevant to the study of A6 epithelia grown on Transwell-Clear substrates, we have calculated the power gain using DC capacitances of 1.38 and 20 $\mu$F/cm² for $C_a$ and $C_b$, respectively (T. G. Păunescu and S. I. Helman, unpublished observations). $|P_{gain}|$ approaches a limiting value of 0.875 with increasing frequency in the range of $\sim 0.1$–10 Hz that encompasses the range of $R_b$ encountered in our experiments (see RESULTS). Consequently, spectral density power levels arising from $\text{Na}^+$ channel Lorentzian current noise will be underestimated by $\sim 12.5\%$ at frequencies greater than $\sim 10$ Hz. For $R_b$ between 2,000 and 40,000 $\Omega \cdot \text{cm}^2$, our calculations indicate that the half-power point frequencies for transition between upper and lower bounds of $P_{gain}$ are 3.74 and 0.19 Hz, respectively.

It may be noted in particular for CDPC-induced noise that the corner frequencies of the Lorentzians range upward from $\sim 40$ Hz, so current noise power levels at these and higher frequencies are attenuated by the limiting value of $|P_{gain}|$, as indicated above. At much lower frequencies, $\sim 10$ Hz and less, and in the range of the $1/f$ noise that far exceeds in value the power levels of the plateau values of the Lorentzians, Lorentzian noise contributes negligibly to the total power measured. Accordingly, the $S_o$ values of the Lorentzians measured were corrected as indicated in Eq. 1 for attenuation of
current noise attributable to recirculation of Na\textsuperscript{+} current noise through the apical membrane capacitance.

APPENDIX B
Calculation of \( R_b \)

While tissues are short-circuited, the absolute values of apical and basolateral membrane voltages (\( V_a \) and \( V_b \), respectively) must be identical. If single-channel conductance of ENaCs (\( \gamma_{\text{Na}} \)) averages near 5 pS (100 mM apical Na\textsuperscript{+} concentration) (25), then membrane voltages can be estimated at every time point of our studies from the quotient \( i_{30/10}^\text{Na} / \gamma_{\text{Na}} \). For typical single-channel currents near 0.4 pA, \( V_a = V_b = 80 \text{ mV} \), which is in the range expected for a short-circuited tight epithelial cell with transepithelial transport of Na\textsuperscript{+} rate limited by the apical membrane ENaC resistance of the cell. Because \( V_i = E_b - I_{\text{sc}} R_b \), \( R_b \) can be calculated as

\[
R_b = \frac{E_b - (i_{30/10}^\text{Na} / \gamma_{\text{Na}})}{i_{30/10}^\text{Na}}
\]

As in frog skin and other tight Na\textsuperscript{+}-transporting epithelia (17, 21, 22, 26), the \( E_b \) of A6 epithelia has been determined to average near 110 mV both in untreated and aldosterone-pretreated epithelia and over at least a threefold difference in rates of Na\textsuperscript{+} transport (19). Although \( E_b \) is not strictly a constant, and to the extent that it can vary among tissues between \( \sim 100 \) and 120 mV, there is an \( \sim 20\% \) uncertainty in obtaining absolute values of \( R_b \) if \( E_b \) is assumed to be rate limited, as we have done in our calculations. To the extent that we are interested in measuring changes of \( R_b \), changes of \( R_b \) greater than \( \sim 20\% \) were considered significant.

APPENDIX C
Blocker Concentration-Related Changes of Single-Channel Currents

Inhibition of apical membrane Na\textsuperscript{+} entry by amiloride and other ENaC blockers causes hyperpolarization of apical membrane voltage as expected according to the DC electrical equivalent circuit shown in Fig. 8A. Changes in voltage are determined by the changes of fractional transcellular resistance \( R_a = R_{50/10} R_b / R_{\text{sc}} \) where \( V_i = V_b \) with reference to a grounded apical solution (19). Because changes of \( V_a \) are directly proportional to changes of \( i_{\text{Na}} \)

\[
\frac{\Delta V_a}{V_a} = -\frac{\Delta I_{\text{sc}} R_b}{i_{30/10}^\text{Na}} = \frac{\Delta i_{\text{Na}}}{i_{\text{Na}}}
\]

For the case in which CDPC concentration is increased from 10 to 30 \( \mu \text{M} \) with single-channel currents \( i_{30/10}^\text{Na} \) and \( i_{50/10}^\text{Na} \), respectively, the ratio \( i_{50/10}^\text{Na}/i_{30/10}^\text{Na} \) to be referred to as \( i_{\text{Na}} \), can be calculated with Eq. 7. Because \( i_{30/10}^\text{Na} = i_{30/10}^\text{Na} - 1 = \Delta i_{\text{Na}}/i_{\text{Na}} \), it can be shown that

\[
i_{30/10}^\text{Na} = 1 + (1 - I_{\text{sc}}^{50/10}) \frac{E_b}{I_{\text{sc}}^{50/10} \gamma_{\text{Na}}} - 1
\]

where \( I_{\text{sc}}^30/10 \) represents the ratio of short-circuit currents at 30 and 10 \( \mu \text{M} \) CDPC. Here, as above, our goal is to measure changes of \( P_e \). The assumption of constancy of \( E_b \) among tissues would result in small absolute errors (\( \sim 5\% \)) of \( i_{50/10}^\text{Na} \), and hence \( P_e \), but would not alter the outcome of calculations where the fractional changes of \( I_{\text{Na}}^{30/10} \) are considerably larger than those of \( I_{\text{Na}}^{50/10} \), as is the case for our experiments.

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REFERENCES


