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Dual Role of Prostaglandins (PGE_2) in Regulation of Channel Density and Open Probability of Epithelial Na^+ Channels in Frog Skin (*R. pipiens*)

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Abstract. Prostaglandins are important in signaling pathways involved in modulating the rates of Na^+ transport in a diverse group of tissues possessing apical membrane epithelial channels. PGE_2 is known to cause either stimulation, inhibition or transient stimulatory changes of Na^+ transport. We have continued our studies of frog skins that are known to respond to forskolin and PGE_2 with large steady-state increases of transport and have used noninvasive methods of blocker-induced noise analysis of Na^+ channels to determine their channel densities (N_T) and open probabilities (P_o). In the absence of exogenous hormones, baseline rates of Na^+ transport are especially high in scraped skins (*R. pipiens pipiens*) studied in the fall season of the year. Na^+ transport was inhibited by indomethacin and by removal of the unstirred layers of the corium (isolated epithelia) alone suggesting that PGE_2 is responsible for the sustained and elevated rates of transport in scraped skins. Changes of transport caused by indomethacin, forskolin or PGE_2 were unquestionably mediated by considerably larger changes of N_T than compensatory changes of P_o. Since cAMP caused no change of P_o in tissues pretreated with indomethacin, PGE_2 appears in this tissue to serve a dual role, increasing the steady state N_T by way of cAMP and decreasing P_o by unknown mechanisms. Despite appreciable PGE_2-related decreases of P_o, the net stimulation of transport occurs by a considerably greater cAMP-mediated increase of N_T.

Key words: Epithelia — Na^+ channels — Prostaglandin — Noise analysis — Adenosine 3′,5′-cyclic monophosphate

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Introduction

Prostaglandins acting as local hormones are important regulators of Na^+ transport in a wide variety of epithelial tissues including frog skins and renal cortical collecting ducts (CCDs). No single mechanism of action can explain the role of PGE_2 in regulation of Na^+ transport, in part because of the different responses to PGE_2 among tissues. PGE_2 causes inhibition of Na^+ transport in the rabbit CCD (Stokes & Kokko, 1977; Iino & Imai, 1978) but does not affect Na^+ transport in the rat CCD (Chen et al., 1991) or cultured cells derived from rabbit CCDs (Sonnenburg & Smith, 1988). At the other extreme, PGE_2 is among the most potent agents that stimulate Na^+ transport at least in tight epithelia such as amphibian skin (Ramwell & Shaw, 1970; Lote et al., 1974; Putnam, 1990; Putnam & Grubbs, 1990; Hall et al., 1976; Gerencser, 1978; Els & Helman, 1981).

The precise role(s) of PGE_2 in control of Na^+ absorption is not clear. It is generally believed that at least in the rabbit CCD and toad urinary bladder, PGE_2 modulates the response to other hormones, in particular those to vasopressin (AVP) (Schlondorff & Satriano, 1985; Sonnenburg & Smith, 1988). In rabbit CCDs as in toad urinary bladder, AVP causes a transient stimulation of Na^+ transport, and it has been suggested that the secondary inhibition of transport is mediated via a PGE_2-dependent process (Holt & Lechene, 1981). Thus, there are clear time-dependent stimulatory and inhibitory processes in Na^+ transport mediated by PGE_2, depending on the tissue studied.

It is generally accepted that the mechanism of action of PGE_2 involves changes in cAMP via activation of adenylate cyclase. It is well appreciated that intimate feedback relationships exist among the prostaglandins, cAMP, calcium and other signaling pathways in the

regulation of Na^+ transport (Breyer, 1991; Hébert, 1994). It is less well appreciated what roles prostaglandins subserve in setting and maintaining baseline rates of Na^+ transport at apical and basolateral membranes of the cells of a variety of epithelia that possess highly selective apical membrane Na^+ channels, like those of renal distal tubules.

Frog skin constitutes a model system where PGE_2 causes a sustained stimulation of Na^+ transport, free from complications that may arise secondary to increases of apical membrane water transport (Bentley & Main, 1972; Erlij et al., 1989) and/or K^+ secretion as in CCDs. Accordingly, the aim of this study was to determine whether prostaglandins, in the absence of AVP activation of cAMP, are involved in regulation of channel density (N_T) and/or open probability (P_o). We made use of noninvasive methods of weak channel blocker-induced noise analysis (Helman & Baxendale, 1990; Els & Helman, 1991) to study the changes of N_T and P_o in frog skin that cannot be studied by patch clamp.

Since PGE_2 is synthesized in the epithelial cells and released into the extracellular fluid (Hall et al., 1976; Erlij et al., 1986) its behavior will also depend on the unstirred layers that exist at the basolateral surfaces of the epithelium. In frog skin a large layer of subepithelial tissue, or corium, can be several hundred μm thick especially in the fall season of the year. Such unstirred layers restrict diffusion of endogenously produced prostaglandins from the extracellular spaces adjacent to the basolateral membranes to the bulk solution bathing the corium. Accordingly, with restricted diffusion, buildup of prostaglandin concentration within the extracellular spaces would lead to prostaglandin receptor binding at the basolateral membranes and thus self stimulation of transport. In this regard, we had observed that Na^+ transport in intact or scraped skins of fall frogs was consistently greater than in frogs studied at other times of the year confirming the now classical observations of Hall et al. (1976) that such preparations with substantial unstirred layers gave Na^+ transport rates considerably higher than isolated epithelia devoid of the corium that had been prepared from the same animals. It was known to Hall et al. that seasonal differences in baseline rates of transport correlated with cAMP and its response to prostaglandins. Accordingly, we studied both scraped and isolated epithelia with the idea that isolated epithelia at their reduced rates of transport would respond poorly to inhibition of prostaglandin synthesis despite their ability to generate prostaglandins but ineffectively owing to their rapid diffusion from basolateral extracellular spaces.

We report that PGE_2 causes two well-defined and opposing effects on the steady state or chronic properties of apical membrane Na^+ channels: one involved in large increases of channel density and the other involved in lesser but substantial decreases of channel open probability leading to sustained increases of Na^+ transport.

Materials and Methods

Tissues

Abdominal skins of *R. pipiens pipiens* (northern frogs) (Kons Scientific, Germantown, WI) were studied during the fall season of the year when steady-state short-circuit currents are predictably at their highest values. Two preparations to be referred to as scraped skins and isolated epithelia were used. Scraped skins were prepared as described previously (Helman, Cox & Van Driessche, 1983) by removing the majority of the corium (about 90%) but leaving a substantial component of unstirred layer (collagen) at the basolateral surface of the epithelium. Isolated epithelia were prepared by the collagenase method of Fisher, Erlij & Helman (1980) to remove the entire corium as evidenced by transmission electron microscopy (Richards, Dando & Els, 1989; Richards & Els, 1994) or by stereoscopic inspection of the tissues at the end of each experiment. Adenylate cyclase in such preparations has been localized exclusively to the basolateral membranes (Richards & Els, 1994) so that PGE_2-mediated increases of cAMP occur via receptors at the basolateral membranes.

Tissues were short-circuited continuously in edge damage-free chambers designed specifically for noise analysis (Abramcheck, Van Driessche & Helman, 1985) for at least 3 hr to allow the short-circuit currents to stabilize. The chambers were perfused continuously without recirculation of Ringer solution containing in mM, 100 NaCl, 2.4 $KHCO_3$, 2 $CaCl_2$ at a pH ~ 8.1 at flow rates of about 8 ml/min through chamber volumes of about 0.5 ml. This assured constancy of the composition of the solutions bathing the tissues and avoidance of buildup of concentrations of substances like the prostaglandins that are released from the tissues to the surrounding media.

Forskolin and PGE_2 were prepared as stock solutions in 10^{-2} M ethanol and diluted directly into the Ringer solution that perfused the basolateral chamber at final concentrations of 2.5 μM and 1 μM, respectively. Indomethacin was used at 50 μM to inhibit prostaglandin synthesis although it was previously found to cause a maximal inhibition of Na^+ transport at 5 μM (Els & Helman, 1981). All control and experimental solutions contained identical concentrations of ethanol (0.02%). Although a relatively low concentration of 2.5 μM causes a maximal stimulation of Na^+ transport, tissue levels of cAMP can be increased markedly by 25 μM forskolin to levels far greater than required to ''saturate'' the physiological response, namely Na^+ entry at the apical membranes of the cells (Els & Helman, 1991). Tissue cAMP was measured by radioimmunoassay in isolated epithelia in response to indomethacin and PGE_2 using methods described previously (Els & Helman, 1991).

Noise Analysis

Tissues were subjected to weak channel blocker-induced noise analysis (CDPC; 6-chloro-3,5-diamino-pyrazine-2-carboxamide) to measure single channel Na^+ currents (i_{Na}), channel densities (N_T) and channel open probabilities (P_o) with tissues transporting Na^+ at or near their spontaneous rates of transport (*see* Results). The method has been described in detail previously (Helman & Baxendale, 1990). Two experimental protocols were used.

Steady-State Staircase Experiments

After stabilization of the short-circuit currents (I_{sc}), all tissues were subjected first to staircase increases of CDPC at concentrations between 5 and 50 μM. Current noise power density spectra measured at each concentration yielded the low frequency plateau values (S_o) and the corner frequencies (f_c) of the Lorentzians from which blocker on (k_{ob}) and off (k_{bo}) rate coefficients were calculated from the linear relationships between the blocker concentration [B] and $2\pi f_c$. After characterizing the tissue in this way, CDPC was removed completely from the apical solution and the I_{sc} allowed to return to control values. Thereafter, tissues were treated with indomethacin (60–90 min) and treated further with either forskolin or PGE_2 (60–90 min) in the continued presence of indomethacin. Staircase analysis was repeated to determine the changes that occurred due to either indomethacin or the combined action of forskolin or PGE_2 and indomethacin. All experiments ended with addition of 100 μM amiloride to the apical solution for at least 30 min to determine the amiloride insensitive short-circuit current which in these tissues reflects Na^+ transport through blocker-insensitive Na^+ channels. This current was subtracted from all values of I_{sc} to give the macroscopic rates of Na^+ transport in the absence (I_{Na}) and the presence of blocker (I^B_{Na}, with the unequivocal assumption that this amiloride insensitive current was constant for the duration of an experiment. This assumption was acceptable when the amiloride-sensitive currents were considerably greater in value than the amiloride-insensitive currents which is normally the case for studies of amphibian skin where small changes of amiloride-insensitive current, if they occurred, would not alter the conclusions.

Single channel currents i^B_{Na} and open channel densities N^B_o as a function of [B] were calculated with Eqs. (1) and (2).

$$i^B_{Na} = \frac{S_o(2\pi f_c)^2}{4\, I^B_{Na}\, k_{ob}[B]} \tag{1}$$

$$N^B_o = (I^B_{Na} / i^B_{Na}) \tag{2}$$

The i^B_{Na} were extrapolated to zero [B] to give the corresponding blocker-independent values, i_{Na} and hence the $N_o = I_{Na}/i_{Na}$.

Since a 1:1 stoichiometry exists between the channel and the blocker as evidenced by linear dependency of the f_c on [B], and since the kinetics conform to a 3-state model of closed, open, and blocked states (Helman & Baxendale, 1990) in the absence of autoregulatory increases of channel density that accompany apical inhibition of Na^+ entry into the cells, the channel open probability as a function of [B] can be calculated as:

$$P^B_o = \frac{1 - (N^B_o / N_o)}{(N^B_o/N_o)(B/K_B)} \tag{3}$$

where open channel density in the absence and presence of [B] are, respectively, N_o and N^B_o. To the extent that autoregulatory increases of channel density (N_T) occur cumulatively with the time of inhibition of Na^+ entry, an apparent open probability referred to previously as β′ or P^B_o is expected to decrease with increasing [B] (Helman & Baxendale, 1990). Hence, extrapolation of P^B_o to zero [B], reflecting the absence of blocker and autoregulatory changes of channel densities gives rise to the P_o of unblocked channels.

It should be emphasized that it is usually assumed that the blocker-insensitive Na^+ currents as measured by maximal amiloride inhibition of Na^+ transport remain constant or essentially so for the duration of an experiment. It has also been assumed that autoregulatory increases of channel densities occur mainly if not solely by increase of blocker-sensitive channels. This latter stipulation has been supported by the observation of relatively small increases of i^B_{Na} with increasing [B] that are expected due to hyperpolarization of apical membrane voltage secondary to increases of fractional transcellular resistance (*see* Appendix). To the extent that autoregulatory increases of channel density include increases of both blocker-sensitive and blocker-insensitive recruitment of channels, the i^B_{Na} may appear to decrease with increasing [B] due to an overestimate of the blocker-sensitive i^B_{Na} used in calculation of i^B_{Na} (*see* Results).

Time Course Experiments

Time course experiments were done to determine the rates of change of single-channel currents (I^{20}_{Na}) and open channel densities (N^{20}_o) during control and experimental periods. Following return from control staircase noise analysis, 20 μM CDPC was introduced into the apical solution for the remainder of the experiments. The continuous presence of CDPC allowed spectra to be obtained at intervals of 5 min and hence allowed determination of the rates of change of I^{20}_{Na} and N^{20}_o caused by indomethacin, forskolin and PGE_2 in scraped skins and isolated epithelia. Since the k_{ob} is unchanged (*see* Results), the I^{20}_{Na} and N^{20}_o could be calculated as indicated above.

All experiments were done at room temperature. Summary data are reported as Means ± SEM. Statistical evaluations were done with SigmaStat (Jandel Scientific Software, San Rafael, CA) using paired or unpaired *t*-tests where appropriate with significance at $P < 0.05$.

Results

Time Course Experiments

Time-course experiments were carried out with scraped skins and isolated epithelia to test for their relative sensitivity to indomethacin. The baseline parameters prior to resolving the time-dependent changes of single-channel currents and open channel densities are summarized in Table 1. After subtracting the amiloride insensitive currents that averaged 1.88 and 0.71 μA/cm^2 for scraped and isolated epithelia, respectively, from the steady-state values of I_{sc}, it was clearly apparent that Na^+ transport rates were greater in scraped skins (44.71 μA/cm^2) than in isolated epithelia (16.26 μA/cm^2) despite the absence of exogenous hormonal stimulation (*see also* Fig. 3). Single channel currents were significantly higher by about 38% in isolated epithelia as was expected in tissues of lower transport rate and higher fractional transcellular resistance (fR_a) due to decrease of open channel density and hence hyperpolarization of apical membrane voltage (*see* Discussion and Appendix). N_o averaged 116 and 31.8 million channels/cm^2 (or 116 and 31.8 channels/cell assuming a million cells/cm^2) in scraped and isolated tissues. Channel open probability was markedly different, averaging 0.26 in scraped skins

Table 1. Baseline parameters of scraped skins and isolated epithelia used in time-course experiments

	I_{Na} $\mu A/cm^2$	i_{Na} pA	N_o $10^6/cm^2$	P_o	N_T $10^6/cm^2$
Scraped skins (6)	44.71 ± 3.39	0.39 ± 0.02	116 ± 12	0.26 ± 0.03	506 ± 94
Isolated epithelia (6)	16.26 ± 2.88	0.54 ± 0.04	31.8 ± 7.1	0.66 ± 0.06	55.8 ± 18.0
	k_{ob} rad/s μM	k_{bo} rad/s	K_B μM		
Scraped skins	7.29 ± 0.23	215.7 ± 5.0	29.8 ± 1.4		
Isolated epithelia	6.28 ± 0.23	215.1 ± 4.9	34.6 ± 2.0		

Values are Means ± SEM ($n = 6$). 1 μM PGE_2. $K_B = k_{bo}/k_{ob}$.

and 0.66 in isolated epithelia. Accordingly, the difference in rates of Na^+ transport between isolated epithelia and scraped skins was due to about a 10-fold difference in N_T that would have resulted in even greater differences in the rates of Na^+ transport if the P_o and i_{Na} had remained the same.

Despite the major differences between tissue preparations given above, the k_{ob} but not the k_{bo} was significantly different between scraped skins and isolated epithelia. k_{ob} averaged 7.21 ± 0.19 (scraped, $n = 16$) and 6.60 ± 0.17 (isolated, $n = 20$) rad/s μM ($P < 0.01$); k_{bo} averaged 230.4 ± 6.5 (scraped, $n = 16$) and 217.7 ± 3.4 (isolated, $n = 20$) rad/s ($P < 0.08$) indicating that the access time of CDPC to the blocker site was greater in scraped skins by about 10% while the residency time at the blocker site was the same in both scraped skins and isolated epithelia. Such changes will be compared with those caused by forskolin, PGE_2, and indomethacin (*see below*).

The time-dependent changes of Na^+ transport rates caused by indomethacin and either PGE_2 or forskolin are illustrated in Fig. 1. After an initial delay of about 10 min, indomethacin caused significant decreases of I_{sc} over 1 hr, but much more so in scraped skins than in isolated epithelia both in absolute and fractional terms. These preparations like those of intact skins (Els & Helman, 1981) were reversibly stimulated by exogenous PGE_2 with the rate of increase more rapid than the rate of decrease of transport following removal of PGE_2 from the basolateral solution.

Despite absence of exogenous hormonal stimulation, scraped skins like intact skins (Els & Helman, 1981, and references therein) were markedly sensitive to indomethacin indicating that baseline rates of transport were maintained by prostaglandin synthesis and by PGE_2-activated adenylate cyclase production of cAMP (Table 2) as in the studies of Hall et al. (1976). Subsequent challenge with forskolin activation of adenylate cylcase caused a sustained return of Na^+ transport to values somewhat above their pre-indomethacin control values. It will be apparent below that our experimental design in use of both forskolin and PGE_2 to stimulate transport following indomethacin inhibition of prostaglandin synthesis allowed us to distinguish between changes of P_o that could have been caused by either PGE_2 or cAMP.

The time-dependent changes of single-channel currents and open channel densities underlying the changes of macroscopic currents are summarized in Fig. 2. The changes in transport were paralleled by changes of open channel density and single channel currents to be expected (*see* Appendix). In isolated epithelia, PGE_2 increased I_{Na} within 20 min by 315% (Fig. 1) mainly through a 408% increase in N_o and a relatively small 22.1% decrease of i_{Na} (Fig. 2). Forskolin similarly caused large increases of N_o and relatively smaller decreases of i_{Na} from indomethacin depressed levels of Na^+ transport and N_o. The changes of N_o and i_{Na} with PGE_2 and forskolin were completely reversible.

Steady-State Staircase Experiments

It is appreciated that changes of open channel density can occur by either changes of P_o and/or N_T. Our previous studies with forskolin had indicated that while N_T was elevated dramatically by cAMP, P_o was also decreased significantly but to a much lesser degree than the increase of N_T (Els & Helman, 1991). As we could not distinguish between a cAMP-mediated change of P_o or change of P_o related to other factors like Ca^{++} or prostaglandins, it became of particular interest to know whether steady state changes of P_o were prostaglandin-related especially in view of the marked difference of P_o between scraped skins and isolated epithelia and their differential sensitivity to indomethacin.

Three groups of experiments were carried out with staircase protocols designed to allow us to differentiate between cAMP- and PGE_2-mediated effects on P_o and N_T. As indicated in Fig. 3, both scraped skins and isolated epithelia were treated first with indomethacin to inhibit basal rates of PGE_2 synthesis. This was followed by treatment of the tissues with either forskolin or PGE_2.

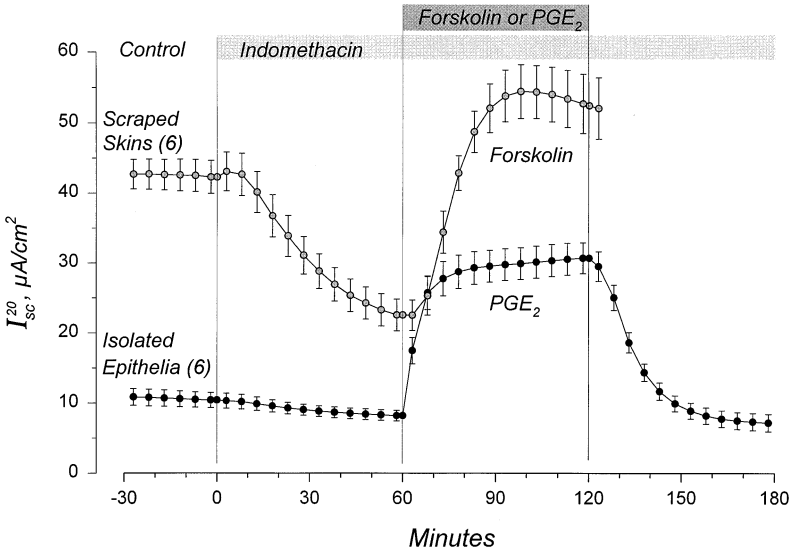

Fig. 1. Time-course experiments performed on scraped skins and isolated epithelia with 20 μM CDPC continuously present in the apical solution. After 60 min in 50 μM indomethacin, scraped skins were treated with 2.5 μM forskolin and isolated epithelia were treated with 1 μM PGE_2 added to the basolateral solution for a further 60 min (indomethacin continuously present). The effects of PGE_2 were completely reversible on washout of PGE_2. Effects of forskolin are reversible (Els & Helman, 1991). Values are Means ± SEM. $n = 6$.

Table 2. Assay for cAMP in isolated epithelia of frog skin

Control	Indomethacin	+PGE_2 (20 min)
2.22 ± 0.11	1.90 ± 0.02	3.06 ± 0.21

Indomethacin/control	PGE_2/Indomethacin
0.88 ± 0.01	1.62 ± 0.11

Indomethacin and PGE_2 caused significant changes of cAMP. Indomethacin caused a 40% decrease of cAMP in intact skins studied by Hall et al. (1976) which is considerably greater than the 12% decrease observed in isolated epithelia devoid of the corium. Maximum increase of transport occurs with a 62% increase of cAMP from its indomethacin pretreated state.

Forskolin alone causes maximum increases of transport with less than a 99% increase of cAMP (Els & Helman, 1991). Values are Means ± SEM ($n = 16$). cAMP in units of pmoles/cm^2; 50 μM indomethacin; 1 μM PGE_2.

The blocker-dependent changes of I^B_{Na}, S_o, $2\pi f_c$ and i^B_{Na} for all treatments are summarized in Fig. 3.

With CDPC as the channel blocker and $K_B = k_{bo}/k_{ob}$ near 30 μM, the I_{Na} were inhibited by relatively small increments at [CDPC] between 5 and 50 μM. S_o of the Lorentzians exhibited the usual biphasic dependence on [B] and the $2\pi f_c$ were linearly dependent on [B] in all treatment groups. As in our previous experiments (Els & Helman, 1991), forskolin caused no change of the on-rate, k_{ob}, but caused a consistent but small significant increase of the off-rate, k_{bo}. Similarly, PGE_2 caused a small increase of the k_{bo} with no change of the k_{ob} suggesting that increases of k_{bo} may be associated with increases of cAMP mediated by either forskolin or PGE_2. However, k_{bo} was unchanged by decreases of cAMP caused by indomethacin and indeed, k_{bo} was not different in scraped skins and isolated epithelia despite considerable differences of transport and cAMP. Accordingly, it remains unknown what factors are involved in determining the kinetics of blocker interaction with the channels. It should be stressed that the changes of rate coefficients are indeed small but easily measured and may ultimately provide information helpful in understanding the nature of the blocker site and the access of the blocker to its binding site.

Single-Channel Currents and Open-Channel Densities i^B_{Na} and N_o

Single-channel currents varied inversely with the magnitude of the macroscopic Na^+ transport rates as indicated in Fig. 3 as expected due to changes of fractional transcellular resistance, and hence apical membrane voltage (V_a) (Els & Helman, 1981). The mean i_{Na} ranged between 0.26 and 0.58 pA at I_{Na} between 44 and 10 μA/cm^2. As will be indicated in the Appendix, the magnitudes of the i_{Na} and their dependence on transport rate are consistent with channels exhibiting single-channel conductance near 5 pS and where changes of transport are due primarily, if not solely, to changes of the density of channels at the apical membranes of the cells.

Relatively small blocker concentration dependent increases of single channel currents are expected due to small increases of fractional transcellular resistance and apical membrane voltage (V_a) (Helman & Baxendale, 1990) when a weak channel blocker like CDPC causes small decreases of Na^+ transport. In general, this was observed in the present, as in past experiments. We did observe especially in tissues of lower transport rate that i^B_{Na} tended to decrease with increasing [B]. In control and indomethacin isolated epithelia, i^B_{Na} decreased on av-

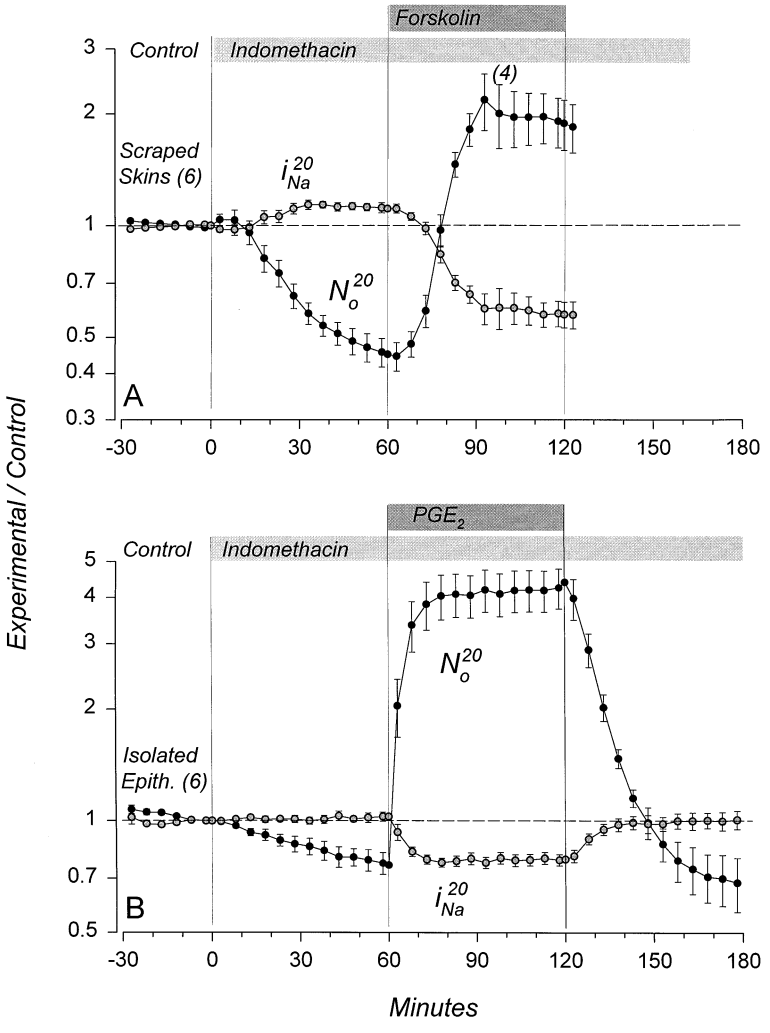

Fig. 2. Time-dependent changes to i_{Na} and N_o caused by forskolin in scraped skins and PGE_2 in isolated epithelia. The data of each experiment were normalized to the values of i_{Na} immediately before addition of indomethacin to the basolateral solution. Summary data are plotted semilogarithmically to better illustrate the relative changes of i_{Na} and N_o.

erage by about 7% at 50 μM CDPC.[1] It is unlikely that this is due to depolarization of V_a. Because i_{Na}^B are calculated on the assumption that blocker-insensitive macroscopic currents remain constant and since the S_o reports noise arising only from blocker-sensitive channels, it is possible that inhibition of transport is accompanied by increase of blocker-insensitive macroscopic currents that would lead to apparent decreases of i_{Na}^B. Although our suggestion and analysis of data will not be presented in detail here, extrapolation of the i_{Na}^B to zero [B] provided values of i_{Na} that were not dependent on autoregulatory increases of either blocker-insensitive or blocker-sensitive channels. Hence, the open channel density, N_o, in the absence of blocker was known from the quotient I_{Na}/i_{Na}.

A summary of the changes of N_o measured with staircase protocols is shown in Fig. 4. In all experimental groups, indomethacin decreased N_o. Both forskolin and PGE_2 stimulated N_o to values considerably greater than their pre-indomethacin control values. N_o ranged between 19.0 and 194 channels/100 μm^2 among the various states of transport in scraped and isolated tissues.

Differential Effects of Forskolin and PGE_2 on Channel Open Probability

P_o was determined by extrapolation of the P_o^B to zero [B] thereby taking into account autoregulatory increases of channel densities (Helman & Baxendale, 1990). The P_o of all untreated scraped and isolated epithelia averaged 0.31 and 0.56, respectively (Fig. 5*A*). With prostaglandin synthesis inhibited by indomethacin in scraped and

[1] Although relatively small blocker concentration dependent decreases of single channel currents were observed principally in tissues of lower rates of transport in the present series of experiments, similar changes of greater magnitude under other experimental conditions have been observed (Kizer, 1990).

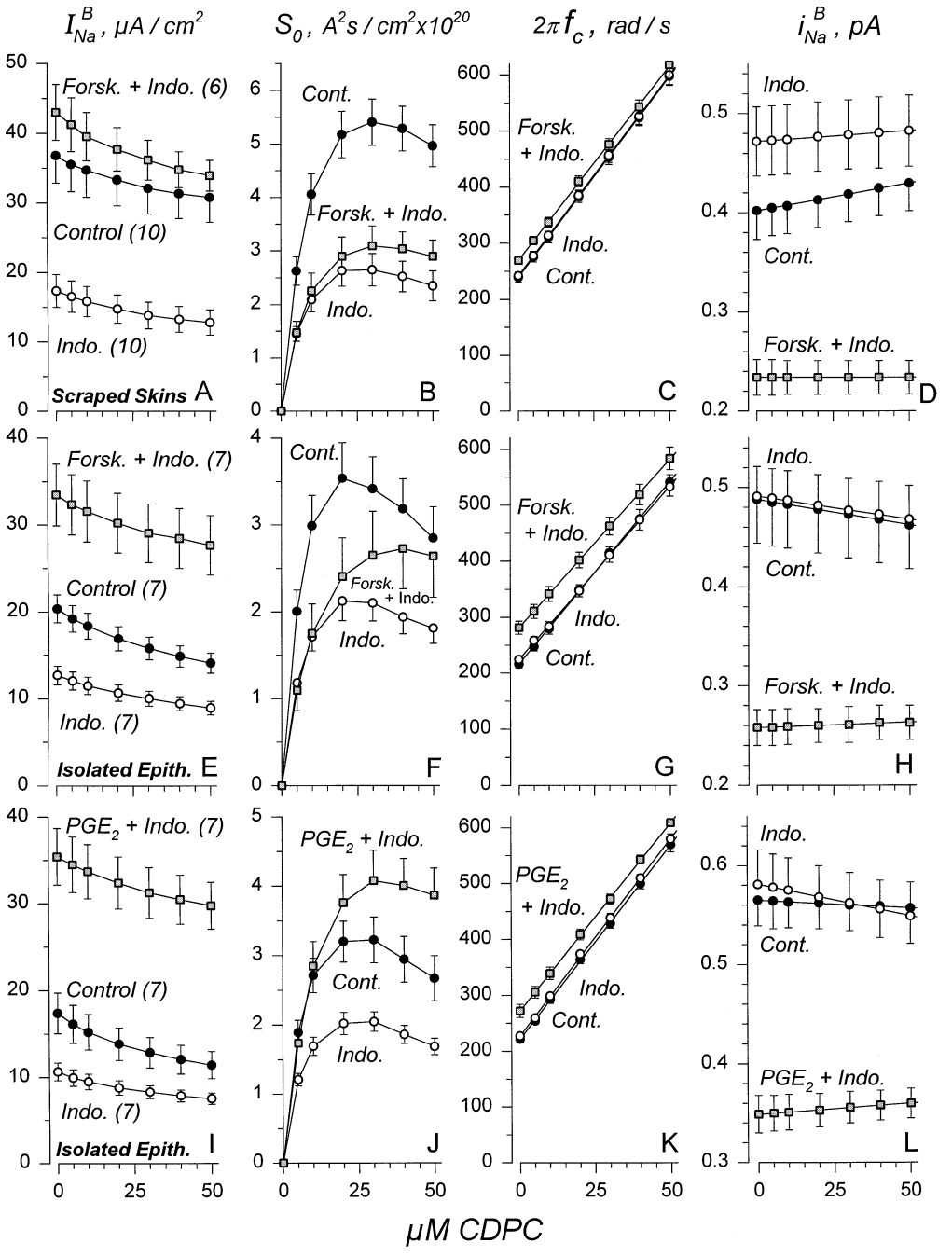

Fig. 3. Summary of blocker-dependent changes of I^B_{Na}, S_0, $2\pi f_c$ and i^B_{Na} in three groups of experiments. (i) Scraped skins were treated with indomethacin and then with forskolin (*panels A–D*). (ii) Isolated epithelia were treated with indomethacin and then with forskolin (*panels E–H*). (iii) Isolated epithelia were treated with indomethacin and then with PGE_2 (*panels I–L*). The format of data presentation is the same as before (Els & Helman, 1991; Helman & Baxendale, 1990). All panels show the dependency of the respective variables and parameters on the CDPC blocker concentration. Macroscopic currents (I_{Na}) and single channel currents (i_{Na}) in the absence of blocker are plotted on the ordinates for control and treated states of the tissues. CDPC on- and off-rate coefficients are determined from the slope and intercept at the ordinate of the corner frequency plots.

isolated epithelia, neither forskolin nor cAMP caused a change of P_o despite marked stimulation of channel density (Fig. 5*B–D*). In isolated epithelia, indomethacin caused no change of P_o suggesting no direct or ancillary effect of this drug alone on the P_o of the Na^+ channels. However, (i) when isolated epithelia were treated with exogenous PGE_2 (Fig. 5*D*), (ii) when PGE_2 synthesis was inhibited by indomethacin in scraped skins (Fig.

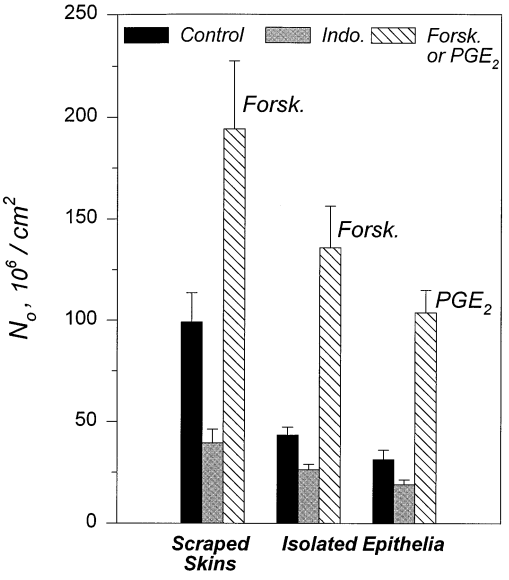

Fig. 4. Summary of changes of open-channel densities caused by forskolin and PGE_2 in indomethacin pretreated scraped and isolated tissues.

5*B*), and (iii) when drug untreated isolated epithelia were prepared from scraped skins thereby releasing them from the endogenous influence of PGE_2 (Fig. 5*A*), P_o was changed significantly. Indeed, PGE_2, independent of its action through cAMP, caused decrease of P_o. Such changes of P_o oppose those of cAMP-mediated increases of channel density in regulation of Na^+ transport. Because the changes of channel density are considerably greater than the changes of P_o, PGE_2 serves in the epithelium of frog skin mainly to cause steady state increases of transport that are blunted by the lesser but nevertheless important decreases of P_o.

Changes of Total Channel Densities (N_T) and cAMP

Summarized in Fig. 6 are the changes of N_T caused by indomethacin, forskolin and PGE_2 in scraped and isolated epithelia. Indomethacin in all experiments caused significant decreases of N_T. The decreases of N_T were considerably larger in the scraped skins of higher transport rate than in the lower transporting isolated epithelia. Both forskolin and PGE_2 caused large increases of N_T to comparable values in both tissue preparations consistent with the idea that both agents acting through cAMP caused maximal or saturable effects similar to those we reported previously at least for forskolin. N_T averaged near 450 channels/100 μm^2 at the highest rates of transport and downward to values near 60 channels/100 μm^2 at the lowest rates of transport encountered in the present studies (Fig. 6).

Our previous experiments had indicated that maximal stimulation of channel densities occurred with rather small increase of tissue cAMP, and our findings are similar to those reported by Hall et al. (1976). Comparable changes of cAMP were measured in the present series of experiments (Table 2) when isolated epithelia were treated with PGE_2 for 20 min corresponding to the time of near maximal stimulation of N_T. Indomethacin alone caused a significant decrease of cAMP in isolated epithelia, and we presume that the changes of cAMP in scraped skins would be even greater in our own tissues like those reported by Hall et al. (1976).

Discussion

We have been intrigued over many years by our own observations like those of Hall et al. (1976) that in the absence of exogenous hormonal stimulation, frog skins exhibit their highest steady state rates of Na^+ transport in the fall season of the year so that factors other than short-term acting exogenous hormones like AVP could not be invoked to explain seasonal differences in baseline rates of transport. Indeed, species differences exist since southern frog skins (*R. pipiens berliendieri*) that are never subjected to freezing temperatures, maintain exceedingly high rates of transport often exceeding 100 $\mu A/cm^2$ throughout the year that are never observed with northern *R. pipiens* (Els & Helman, 1981). Although frog skins in general respond to exogenous PGE_2 especially well after indomethacin inhibition of endogenous prostaglandin synthesis, it has been noted that baseline rates of Na^+ transport do not respond to indomethacin in some skins (Erlij et al., 1986; Bjerregaard & Nielsen, 1990), contrary to the observations of most investigators but due possibly to abnormally low rates of prostaglandin synthesis or abnormally high rates of diffusion of the prostaglandins through the unstirred layers of the corium at the times they were studied. In this regard, the vast majority of experiments with frog skin have been carried out with whole skins with their epithelium juxtaposed to large unstirred layers of the corium. Such unstirred layers act to impede diffusion of endogenously produced prostaglandins to the basolateral solution thereby allowing buildup of PGE_2 concentration in the vicinity of the prostaglandin receptors at the basolateral membranes of the cells.

Consequently, depending upon the rates of synthesis and release, and the rate of diffusion through unstirred layers to the basolateral solution, baseline rates of Na^+ transport can be modulated at least in part by the unstirred layers. In the context of our own experiments, it is likely that the differences of baseline rates of transport

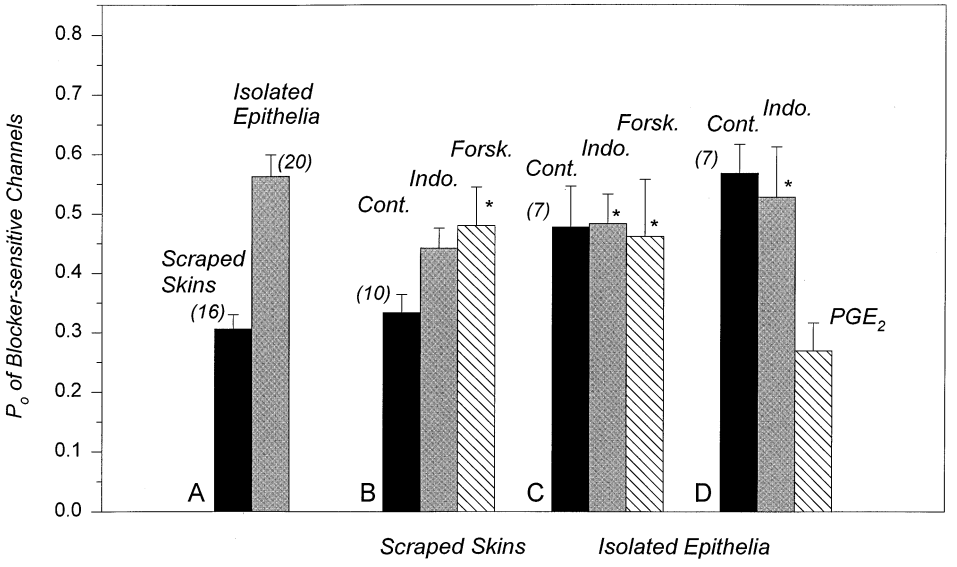

Fig. 5. Summary of (i) the difference of P_o in untreated scraped skins and isolated epithelia (*panel A*) and (ii) the changes of P_o caused by indomethacin, forskolin and PGE_2 in scraped and isolated tissues (*panels B, C,* and *D*). Differences of P_o between adjacent states of the tissues are significant ($P < 0.05$) except where indicated by an asterisk (*).

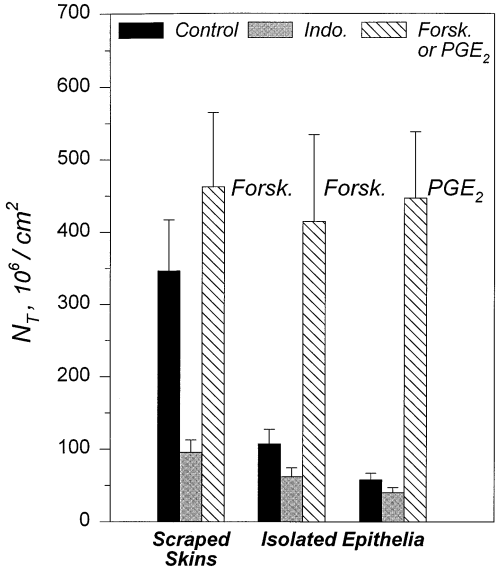

Fig. 6. Summary of the changes of N_T in scraped and isolated epithelia caused by indomethacin followed by either forskolin or PGE_2.

between scraped skins and isolated epithelia are not due to differences in the rates of PGE_2 synthesis, as the isolated epithelia were prepared from the same scraped skins where unquestionably indomethacin caused consistent and appreciable inhibition of Na^+ transport. In isolated epithelia of considerably lower baseline rates of transport, indomethacin caused significantly smaller fractional inhibitions of transport with the process of isolation causing no apparent changes to the PGE_2 receptors nor pathways involving cAMP activation of the channels. The simplest explanation for the differences of baseline rates of transport between tissue preparations in our own experiments appears to be differences alone in the unstirred layers at the basolateral membranes of the cells. We note that at other times of the year, when baseline rates of PGE_2 synthesis are presumably not as high as appears to be the case during the fall season, baseline rates of Na^+ transport appear not to be significantly different among isolated epithelia, scraped and whole skins of *R. pipiens pipiens.* Whether or not this seasonal difference reflects an adaptive mechanism in preparation for hibernation is not known, but it is tempting to speculate that such a mechanism may include an increase in prostaglandin metabolism and perhaps a more generalized increase in lipid metabolism.

The dominant influence of indomethacin, AVP, PGE_2 and forskolin in frog skin is to change the apical membrane conductance of the cells (Els & Helman, 1981). Whereas AVP increases apical channel density with insignificant changes of P_o, forskolin causes large increases of channel density with relatively small compensatory decreases of P_o (Els & Helman, 1991) leading to large steady state increases of open channel density suggesting a possible role for cAMP in regulation of P_o. In this regard we were particularly intrigued to observe that P_o was markedly different between isolated epithelia and scraped skins and that this might be due to either prostaglandin alone or to differences in PGE_2-adenylate cyclase-mediated intracellular cAMP. This led in design of the experiments to protocols to differentiate between the effects of PGE_2 and cAMP on P_o. In scraped skins

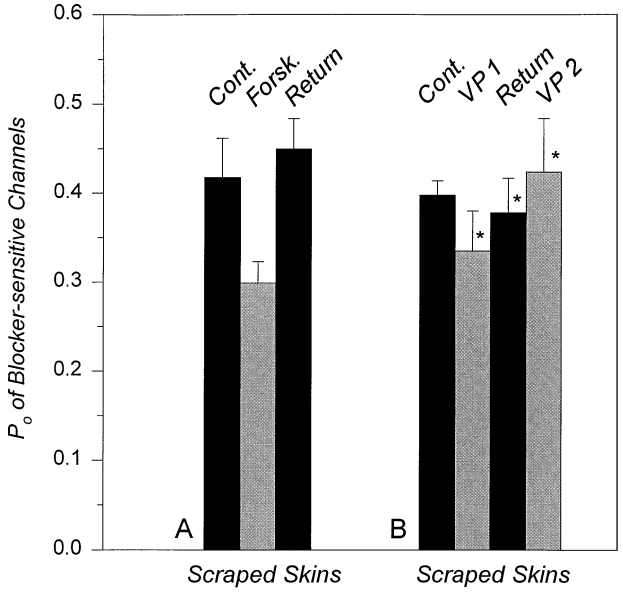

Fig. 7. Changes of P_o caused by forskolin and vasopressin in scraped skins studied previously (Els & Helman, 1991) and included here for comparison with the results of our present experiments. Forskolin caused a relatively small and reversible decrease of P_o. Vasopressin caused no significant change of P_o during two sequential repeat exposures to vasopressin.

indomethacin inhibition of PGE_2 synthesis and cAMP caused a 13% increase of P_o, and forskolin thereafter caused stimulation of transport and cAMP with no change of P_o. Similarly with isolated epithelia at reduced rates of transport and in the absence of PGE_2 stimulation of transport, and with assured indomethacin inhibition of PGE_2 synthesis, forskolin caused no change of P_o, suggesting that differences of P_o were due to the prostaglandin and not to changes of intracellular cAMP. When indomethacin pretreated tissues were challenged with PGE_2, P_o was decreased to 51% of its control. Thus, the steady-state 2-fold differences of P_o between isolated epithelia ($P_o = 0.306 \pm 0.024$) and scraped skins ($P_o = 0.563 \pm 0.037$) could be attributed to the prostaglandins and not to changes of cAMP.

These findings taken at face value would seem at odds with our previous observation that forskolin-mediated increase of cAMP caused a small but significant decrease of P_o (Fig. 7). It may be stressed that cAMP independence of P_o could only be demonstrated in our present studies in tissues where PGE_2 synthesis was already inhibited. In tissues with intact synthetic pathways for prostaglandins, cAMP is known to cause increase of intracellular Ca^{++}, and Ca^{++} in turn is known to stimulate both PLC and PLA_2 leading to increases of PGE_2 (Hébert, 1994). Consequently it is possible, but remains to be tested, that decrease of P_o caused by cAMP is related to Ca^{++}-mediated PGE_2 synthesis. Put in context at least for frog skin, there can be no question that the major influence of AVP and prostaglandins is to stimulate Na^+ transport by way of large increases of channel density. In comparison with the cAMP-mediated increases of channel density, the decreases of P_o that accompany stimulation of transport are in absolute terms important as modifiers of channel open probability, but minor insofar as their compensatory influence in inhibiting Na^+ transport.

Whether or not this steady-state effect of prostaglandins on P_o is important in other tissues such as rabbit and rat CCDs remains to be determined. To the extent that stimulation of channel density and decrease of open probability are compensatory, the time course of change of macroscopic rates of Na^+ absorption as measured by fluxes or short-circuit currents could reflect more complicated underlying time-dependent changes of P_o and N_T including transient and oscillatory behavior and those noted above that are tissue specific. Such time-course data are not yet available. In patch-clamp experiments of A6 epithelia, PGE_2 caused within 3 to 6 min large decreases of open channel density (~90%) by decrease of P_o (Kokko et al., 1994) with, however, no difference from control of P_o in tissues treated chronically with PGE_2. Such large initial decreases of P_o and open channel density ($N_o = NP_o$), must lead to large initial decreases of short-circuit currents that have never been observed at least in frog skins or toad urinary bladders. It is possible that in A6 epithelia as in rabbit CCDs, acute initial decreases of P_o are quantitatively greater than increases of N_T and that these initial decreases of P_o are not sustained as they are in frog skin. Nevertheless, it will remain an intriguing problem to sort out the similarities and differences among tissues including A6 epithelia as models of distal tubules of the kidney. As with the differences in response to PGE_2 in various tissues, A6 epithelia appear also to share considerable diversity, as Keeler and Wong (1986) have reported that PGE_2 stimulates Cl^- transport alone with no effect on Na^+ transport in A6 epithelia.

The question of how PGE_2 regulates P_o remains open. As noted by Breyer (1991), PGE_2 acts most likely at a post-cAMP step not involving adenylate cyclase. Since prostaglandins increase intracellular cAMP which in turn increases Ca^{++}, then Ca^{++} acting either directly on the channels and/or Ca^{++} acting through PLC and DAG on PKC may be surmised as a candidate for regulation of P_o. On this point our own preliminary experiments are not the same in A6 epithelia and frog skin (Els et al., 1995). Phorbol ester stimulation of PKC caused an initial decrease of P_o in A6 and a relatively small initial increase of P_o in frog skin, accompanied by changes of N_T while ionomycin in frog skin caused substantial initial increases of P_o (X. Liu, W.J. Els and S.I. Helman, *unpublished observations*).

Ordway, Singer & Walsh (1991) have pointed out that ion channels may be regulated directly and indirectly by fatty acids, and Blobe, Khan & Hannun (1995) have suggested that PKC may be a target itself for arachidonic acid. In view of the many differences among tissues as measured macroscopically, biochemically and biophysically, it would seem that tissues select among signaling pathways to accomplish their particular roles in modulating the rates of Na^+ absorption. How this is accomplished in regulation of P_o remains to be resolved.

The experiments were carried out while W. Els was on sabbatical leave from the University of Cape Town with support from his institution which is gratefully acknowledged. We thank Tracey Mencio and Dr. Victor Ramirez for their assistance in measurements of cAMP and A.L. Helman for assistance in the preparation of this manuscript. This work was supported by DK-30824 from the National Institutes of Health.

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Appendix

In intact sheets of epithelial cells, single-channel currents must change with changes of macroscopic rates of transport due to hyperpolarization or depolarization of apical membrane voltage. To the extent that changes of transport occur through changes of apical membrane conductance caused either by drugs or other regulatory processes, it is possible to predict the relationship between i_{Na}, N_o and the macroscopic rates of Na^+ transport (I_{Na}). The present series of experiments provided data that allowed us to test a simple relationship and thereby exclude the possibility that additional mechanisms other than apical membrane conductance contribute importantly to the regulation of Na^+ transport by cAMP and PGE_2 in frog skin.

Electrical considerations for a series arrangement of apical and basolateral membranes in their short-circuited state requires quite generally that:

$$I_{Na} = \frac{E_a + E_b}{R_a + R_b} \tag{4}$$

$$V_a = E_a(1 - fR_a) - E_b\, fR_a \tag{5}$$

where E_a and E_b are Thévenin emfs; R_a and R_b are the slope resistances of apical and basolateral membranes, respectively; and $fR_a = R_a/(R_a + R_b)$ is the fractional transcellular resistance. Since the *I-V* relationship of the apical membrane is distinctly nonlinear becoming essentially linear at physiological voltages in frog skin (Helman, 1979; Helman & Thompson, 1982), the E_a approaches values of zero. Equation (5) simplifies to: $V_a = -E_b\, fR_a$. Hence, changes of V_a are mediated principally by changes of E_b and/or fR_a in the range of V_a normally encountered under all conditions of our experiments. $i_{Na} = \gamma_{Na} V_a$ where γ_{Na} is the single channel slope conductance at physiological V_a. Hence:

$$i_{Na} = -\gamma_{Na}\, E_b\, fR_a \tag{6}$$

Substituting conductances G_b and $G_a = \gamma_{Na} N_o$ into Eq. (6), we arrive at:

$$i_{Na} = \gamma_{Na}\, E_b - (\gamma_{Na} R_b)\, I_{Na} \tag{7}$$

which is the equation of a straight line with slope m and intercept b. Accordingly, if E_b and R_b of the basolateral membranes is constant and γ_{Na} is constant, then i_{Na} is linearly related to the macroscopic rates of transport. The i_{Na} at the intercept would provide an estimate of the maximal i_{Na} when apical membrane voltage is maximally hyperpolarized by decrease of apical membrane conductance which occurs when open channel density and I_{Na} approach zero either spontaneously or by inhibition of apical Na^+ entry. With increasing open channel density and rates of transport, the resulting depolarization would cause decrease of i_{Na}.

Shown in Fig. 8*A* is the relationship between the mean i_{Na} and the mean I_{Na} measured in all groups of experiments and states of the tissues regardless of their treatment. Given the extremes of transport rates and i_{Na} and the nonpaired nature of the data, the data conformed remarkably well to a linear relationship. i_{Na} ranged between near 0.2 pA at I_{Na} of 50 $\mu A/cm^2$, increasing to 0.65 pA as I_{Na} approached zero. Regardless of how measured, by noise analysis or by patch clamp, i_{Na} invariably falls in this range (Helman & Kizer, 1990).

The E_b of the basolateral membranes measured in microelectrode experiments of *R. pipiens* bathed with the same Ringer solution as used in the present experiments averages near 120 mV (Helman, 1979; Helman & Thompson, 1982). As the intercept at the ordinate $i_{Na} = \gamma_{Na} E_b$, the γ_{Na} is near 5.4 pS which again is remarkably the same as measured for native Na^+ channels exhibiting extremely high selectivity for Na^+. From the slope of the line (−0.0081), the mean R_b is 1480

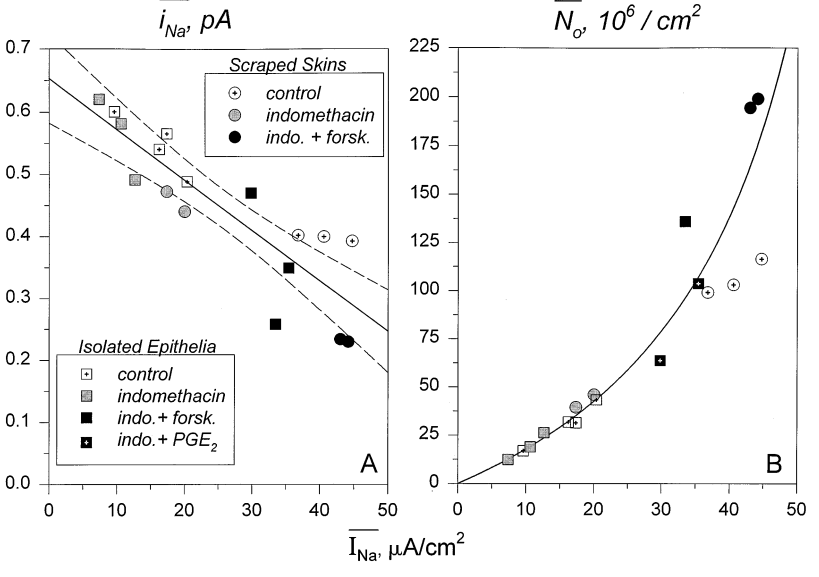

Fig. 8. Relationship between the i_{Na}, N_o and I_{Na} of all groups of experiments in their control, indomethacin, forskolin or PGE_2-treated states. Shown in *panel A* are the least square linear regression slope and the 95% confidence interval. The solid line of *panel B* was calculated with Eq. 8 using the slope and intercept determined from the data in *panel A.*

Ωcm^2 which also falls into the range of mean values that have been reported previously for *R. pipiens.*

If indeed, i_{Na} varies linearly with I_{Na}, then open channel densities vary nonlinearly with I_{Na}. As $I_{Na} = i_{Na} N_o$, and substituting $(-mI_{Na} + b)$ for i_{Na}, it follows that:

$$N_o = \left(-m + \frac{b}{I_{Na}}\right)^{-1} \quad (8)$$

Plotted in Fig. 8*B* are the mean values of N_o measured in all treatment groups and a solid line calculated with the values of *m* and *b* (Fig. 8*A*). Open-channel densities range upwards towards about 200 million/cm^2 at 45 $\mu A/cm^2$ or 200 channels/100 μm^2. At physiological rates of transport such low channel densities are adequate to support Na^+ transport in the frog skin and in CCDs where similar rates of Na^+ absorption have been measured. To the extent that the I_{Na}-i_{Na} relationship is linear, we infer at least for the conditions of the present studies that neither forskolin, indomethacin nor PGE_2 caused appreciable changes to the pumps or K^+ leaks at the basolateral membranes of the cells that could have appreciably affected the macroscopic changes of Na^+ transport at the apical membranes of the cells. Small effects cannot be ruled out. This conclusion is supported by the fact that the effects of PGE_2 are completely reversible despite prolonged exposure to either endogenously produced or exogenous PGE_2.

Note Added in Proof

Recent experiments using weak channel blocker-induced noise analysis indicate that PGE_2 stimulates the amiloride-sensitive short-circuit current in control and aldosterone-prestimulated A6 epithelia. As in frog skin, stimulation of Na^+ transport is due to large increases of N_T and relatively large compensatory decreases of P_o.

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