J. Membrane Biol. 85, 215–223 (1985)

The Journal of
Membrane Biology

Mechanism of Inhibition of Net Ion Transport Across Frog Corneal Epithelium by Calcium Channel Antagonists

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Summary. In the isolated bullfrog cornea, three calcium channel antagonists had dose-dependent inhibitory effects on the Cl-originated short-circuit current (SCC). Their order of decreasing potency was bepridil, verapamil and diltiazem. One millimolar diltiazem inhibited the SCC by 98% and subsequent incubation with the calcium ionophore A23187 had no restorative effect. Increasing the bathing solution Ca concentration from 0.05 to 15 mM, however, decreased diltiazem's inhibitory efficacy. This antagonist depolarized the intracellular potential difference V_{sc} from −54 to −18 mV (tear : reference) and the voltage divider ratio FR_o decreased from 0.58 to 0.30, suggesting an increase in basolateral membrane electrical resistance. Additional indication of a basolateral membrane effect by the drug was that preincubation with 10^{-5} M amphotericin B in Cl-free Ringer's did not eliminate the inhibitory effect of the drug on the Na- and K-elicited SCC. In the absence of amphotericin B in Cl-free Ringer's (SCC = 0), 1×10^{-3} M diltiazem depolarized the V_{sc} from −78 to −9 mV suggesting that the increase in basolateral membrane resistance was due to K channel blockade. Diltiazem (1×10^{-3} M) significantly decreased cyclic AMP content; however, isoproterenol in the presence of the drug increased cyclic AMP fourfold without having any restorative effect on the inhibited SCC. Therefore, the inhibition of the Cl-originated SCC resulting from an increase in basolateral membrane K resistance is not caused by a decline in cyclic AMP content. In plasma membrane-enriched fractions prepared from broken cell preparations of bovine corneal epithelium, 1×10^{-3} M diltiazem had no inhibitory effects on either Na,K-ATPase or Ca,Mg-ATPase activities. These latter effects further point to the selectivity of diltiazem as an inhibitor of K-channel activity, but do not preclude a Ca-channel blocker effect by the drug in the micromolar range.

Key Words calcium antagonists · cyclic AMP · frog cornea · ion transport · potassium conductance

Introduction

In the isolated frog corneal epithelium, the short-circuit current is nearly accounted for by a net transport of Cl (Zadunaisky, 1966). The remaining portion is attributed to net Na transport in the opposite direction from the tear to the stromal side bathing solution (Candia & Askew, 1968). The steps in active Cl transport are thought to include carrier-mediated coupled Na : Cl uptake across the basolateral membrane followed by Cl electrodiffusion across the Cl permselective apical membrane into the tears (Reuss et al., 1983). The driving force for the Na : Cl uptake is the inward-directed Na gradient across the basolateral membrane which is maintained by the Na : K pump. The basolateral membrane has an appreciable K conductance, which is partially inhibited by the cation barium.[1]

Net Cl transport is stimulated by increases in intracellular cAMP (Chalfie et al., 1972; Schaeffer et al., 1982; Reinach & Kirchberger, 1983). Similarly, it is thought that an increase in intracellular calcium activity results in enhancement of net Cl transport since the calcium ionophore A23187 stimulates this process (Candia et al., 1977). To further characterize the role of calcium as a regulator of ion transport in this tissue, we considered the effects of three calcium channel antagonists in this tissue. Each chosen agent represents a structurally different class of agents: verapamil, diltiazem and bepridil. These agents compete with calcium to inhibit slow inward calcium current of cardiovascular and smooth muscle, and inhibit stimulus-secretion coupling in a variety of other tissues. The drugs considered in our study are effective as calcium channel antagonists in the concentration range between 10^{-8} and 10^{-6} M, whereas at higher concentrations a variety of nonspecific effects have been documented. These nonspecific effects include inhibition of potassium, sodium and other ionic channels; interaction with adrenergic, cholinergic and opiate receptors; and alteration of phosphodiesterase, adenylate cyclase and Na : K pump activities (Janis & Scriabine, 1983; Triggle & Swamy, 1983).

[1] P. Reinach and W. Nagel. Interaction between apical and basolateral membrane ionic conductances during Cl transport in the frog corneal epithelium. (*Submitted for publication*)

Table 1. Composition of Ringer's solutions (mM)

Solute	NaCl	Cl Free	15 mM Ca	Low Ca	NaBS*	35 mM KNaBS*
NaCl	104	—	76.00	104	—	—
Na_2SO_4	—	54	—	—	—	—
NaBS*	—	—	—	—	104	104
KCl	2.5	—	2.5	2.5	—	—
K_2SO_4	—	1.25	—	—	—	—
KBS*	—	—	—	—	2.0	35
$CaCl_2$	1.0	—	15	0.05 stroma; 0.1 tears	—	—
Ca gluconate	—	1.0	—	—	6.0	6.0
$MgSO_4$	2.0	2.0	2.0	2.0	2.0	2.0
HEPES	2.0	2.0	2.0	2.0	2.0	2.0
Na_2HPO_4	0.46	0.46	0.46	0.46	—	—
glucose	26.0	26.0	26.0	26.0	5	5
sucrose		55.0	16.0	—	55	—

* BS = benzene sulfonate.

The results of the present study show that 1 to 6 $\times 10^{-4}$ M verapamil, 10^{-4} to 10^{-3} M diltiazem and 1 to 6 $\times 10^{-5}$ M bepridil inhibit net ion transport. These concentrations are higher than those needed for calcium channel blockade. One of these agents, diltiazem was studied further for its mechanism of action in the isolated frog corneal epithelium and was found to inhibit basolateral potassium conductance and to decrease 3′,5′-cyclic adenosine monophosphate content and perhaps Na:K pump activity. In a broken cell preparation of either bovine corneal epithelium or rabbit kidney cortex, however, diltiazem did not alter Na,K-ATPase activity. All of these results suggest that the primary action is an inhibition of basolateral membrane potassium conductance resulting in complete inhibition of net ion transport.

Materials and Methods

Transepithelial Electrical Measurements

Bullfrog (*Rana catesbeiana*) corneas were dissected and mounted in Ussing-Zerahn chambers as described previously (Candia, 1972). The solutions and their compositions are given in Table 1. Each solution had a pH of 8.2 and an osmolality of 220 to 230 mOsm.

The following drugs were used: dl-isoproterenol HCl, A23187, ouabain octahydrate, dl-propranolol HCl and 3-isobutyl, 1-methylxanthine, i.e., IBMX (Sigma Chemical Co., St. Louis, Mo.); amphotericin B (E.R. Squibb and Sons, Princeton, N.J.), bepridil HCl (McNeil Pharmaceutical, Spring House, Pa.), dl-verapamil HCl (Knoll Pharmaceuticals, Whippany, N.J.) and diltiazem HCl (Marion Laboratories, Kansas City, Mo.). A23187 and bepridil stock solutions were prepared in absolute ethanol and all other agents were dissolved in deionized water. Added volumes of stock solutions never exceeded a total of 100 μl in 5 ml of Ringer's. The transepithelial electrical parameters were measured as previously described (Candia & Schoen, 1978). Drugs were only added to corneas with short-circuit current (SCC) and transepithelial electrical resistance (R) values greater than 6 μA/cm^2 and 1.0 KΩ cm^2, respectively.

Intracellular Electrical Measurements

Corneas were mounted horizontally in a previously described chamber to measure the intracellular electrical parameters (Nagel, 1976). These parameters are the intracellular potential difference V_{sc} (referenced to tear) and the voltage divider ratio FR_o. V_{sc} was recorded with microelectrodes backfilled with 1.5 M KCl which had been prepared from capillary tubing using a Brown Flaming M-77 puller. These electrodes had input resistances between 30 and 80 M in the tissue's bathing solution and tip potentials of less than 5 mV. The microelectrodes were connected via a AgCl-coated Ag wire to a high input impedance ($\approx 10^{15}$ Ω) preamplifier (Analog Devices 515J) with negative capacitance compensation. The corneas were continuously short-circuited by means of an automatic clamping device (Frankenberger & Nagel, *in preparation*). Impalements of the corneal epithelium were done as previously described (Nagel & Reinach, 1980). V_{sc} is the apical membrane potential difference (referenced to tear) under short-circuit conditions, and FR_o is the voltage divider ratio, a measure of the relative apical membrane resistance, and is calculated from the quotient of the ratio, $\Delta V_o/\Delta V_t$. V_o and V_t are the changes in apical membrane and the open-circuit transepithelial potential, respectively, resulting from periodically perturbing the otherwise maintained short-circuit state to 20 mV for 100 to 250 msec at rates of 0.5 to 2 Hz to measure the transepithelial electrical conductance. The values of all the desired parameters including the microelectrode input resistance were continuously displayed on a six-channel strip chart recorder. The criterion used to validate a particular impalement was to see if the substitution of NaCl Ringer's on the tear side with Cl-free Ringer's resulted in a reversible and significant depolarization of V_{sc} as well as a substantial increase of FR_o.

3′,5′-Cyclic Adenosine Monophosphate

Cyclic AMP was measured by radioimmunoassay (New England Nuclear, Boston, Mass.). Corneas were de-endothelialized with a cotton swab, cut in half, trimmed of sclera and equilibrated

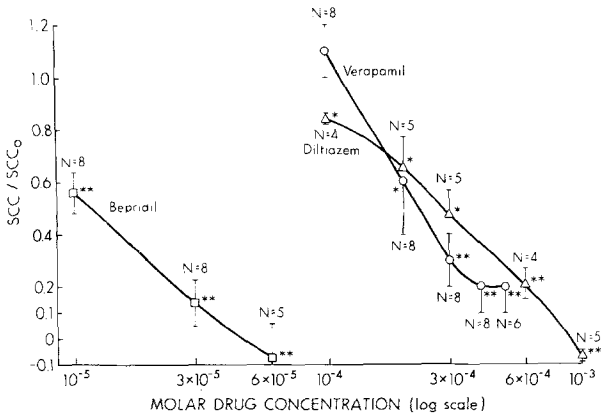

Fig. 1. Concentration response curves for bepridil, diltiazem and verapamil on the normalized Cl-originated SCC. Points are shown as means ± SEM for the numbers of indicated corneas. * $P < 0.05$; ** $P < 0.01$ with respect to control value

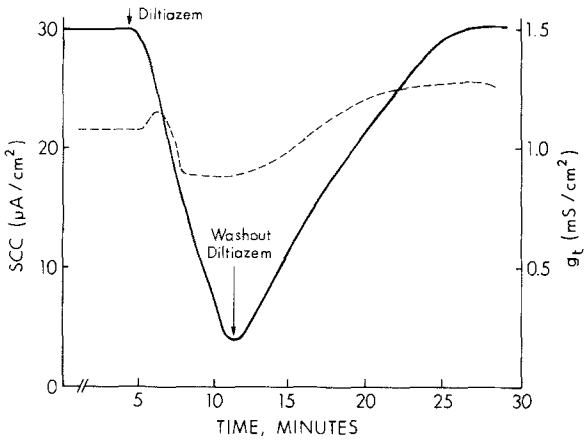

Fig. 2. Effects of 2×10^{-3} M diltiazem in the tear side bathing on the SCC and the transepithelial conductance, g_t

with gentle shaking for 15 min at room temperature in NaCl or Cl-free Ringer's. After equilibration the halves were transferred to the identical Ringer's containing 5×10^{-4} M IBMX for 5 min before adding diltiazem or distilled water (vehicle). The tissues were exposed to 0.3×10^{-3} M or 1×10^{-3} M diltiazem for 15 min and were immediately removed for homogenization. In some experiments after diltiazem preincubation, isoproterenol or distilled water was added for another 10 min. The tissues were then removed from their vials, ground in glass homogenizers containing 0.5 ml of 10% trichloroacetic acid (TCA) and centrifuged for 10 min at 18,000 × *g*. The supernatants were extracted four times with diethyl ether to remove TCA and lipids. The ether was evaporated at 70°C for 10 min and the samples were stored frozen.

Protein content of the tissues were measured by the method of Lowry et al. (1951). In all cases control and experimental determinations were done simultaneously for a particular condition. Cyclic AMP values were not corrected for recovery, but in initial assays recovery ranged from 99 ± 5.6% to 106 ± 4.3% in separate assays of 16 samples (mean ± SD). Cyclic AMP content was expressed as pmol cyclic AMP/mg protein.

Na,K-ATPase assay

Na,K-ATPase was measured as potassium-stimulated *p*-nitrophenyl phosphatase as described by Murer et al. (1976). Basolateral membrane-enriched fractions from bovine corneal epithelium and rabbit kidney cortex were prepared by the method of Kotagal et al. (1982). Corneal tissue was incubated for 3 hr in the presence and absence of 1×10^{-3} M diltiazem and the effects of the drug were compared to the control to obtain percent of inhibition. All reactions were linearly dependent on protein and period of incubation. The kidney preparation was richer in Na,K-ATPase activity and 15 min was an adequate incubation time.

Results

In NaCl Ringer's, the effects on the SCC of drugs representative of three structural classes of calcium antagonists are compared (Fig. 1). The SCC was inhibited by all of the drugs in a dose-dependent manner. Bepridil (EC-50 = 2.0×10^{-5} M) was most potent, and verapamil (EC-50 = 2.3×10^{-4} M) and diltiazem (EC-50 = 2.9×10^{-4} M) were less potent. At the highest inhibitory concentrations, the ratios of post-drug resistance to predrug resistance (R/Ro ± SEM) were 1.07 ± 0.26, 1.00 ± 0.40, and 1.26 ± 0.20 for bepridil, verapamil and diltiazem, respectively. In all cases, the inhibitory effect on the SCC was most rapid when the drugs were added to the tear side bathing solution, and no additional effect was observed when a given drug was added to both bathing solutions. Therefore, in all cases the effects of drug addition to the tear side were studied. Diltiazem was chosen for further study, because at doses as high as 2×10^{-3} M its inhibitory effect on the transepithelial electrical parameters were reversible. Figure 2 demonstrates the recovery of the SCC within 20 min after washout of 2×10^{-3} M diltiazem which suggests that the drug does not irreversibly affect cellular parameters.

To identify the cellular site of action of diltiazem, corneas bathed in Cl^- free Ringer's were preincubated with 10^{-5} M amphotericin B which eliminates the Cl permselectivity of the apical membrane (Candia et al., 1974). In this condition the SCC is accounted for by the algebraic difference between the net transepithelial Na and K fluxes mediated by basolateral Na : K pump activity (Candia et al., 1984). After amphotericin B preincubation (Fig. 3), diltiazem inhibited the SCC in a dose-dependent fashion, but the EC-50 was increased compared to that in corneas bathed in NaCl Ringer's (7.2×10^{-4} M *vs.* 2.8×10^{-4} M). The dose-response relationship in Cl-free Ringer's was significantly ($P < 0.05$) different from that in NaCl Ringer's, as determined by linear regression and analysis of covariance. Although active Cl transport is slightly more

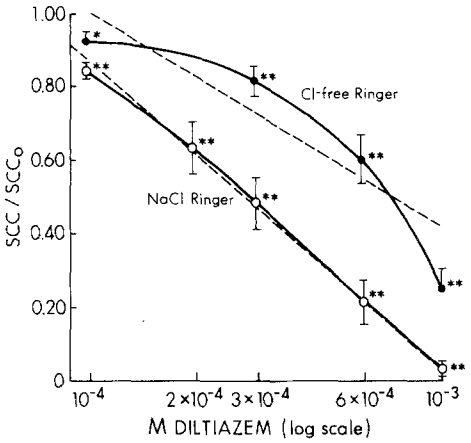

Fig. 3. Comparison of diltiazem dose-response curves in NaCl and Cl-free Ringer's on the normalized SCC. Broken lines were calculated by linear regression. Analysis of covariance indicates that the two curves are significantly different. Each point represents the mean value ± SEM for the indicated number of corneas. * $P < 0.05$; ** $P < 0.01$ with respect to predrug value

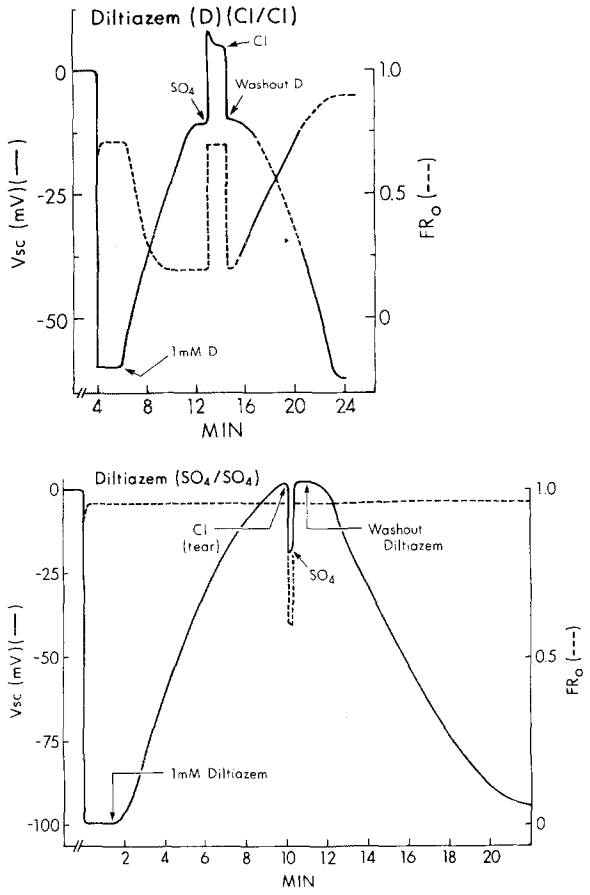

Fig. 4. Comparison of the effects of 1×10^{-3} M diltiazem on the intracellular electrical parameters V_{sc} is indicated by the solid line and FR_o by the broken line. NaCl Ringer's: upper panel; Cl-free Ringer's: lower panel. A loss of cellular impalement occurred during the period in the upper panel indicated by V_{sc} and FR_o having broken and solid lines, respectively. Validation of impalement is shown by temporary substitution of NaCl-Ringer's with Cl-Ringer's in the tear side bathing solution followed by return to NaCl-Ringer's

sensitive to the drug than net Na and K transport, the data suggests that a primary site of diltiazem action is at a transport-related site in the basolateral cell membrane.

The intracellular electrical parameters of short-circuited corneas were also characterized in NaCl and Cl-free Ringer's solution to further delineate the mode of interaction of diltiazem with the basolateral membrane. In typical experiments, the effects of diltiazem are compared under the two conditions (Fig. 4). It should be noted that unlike the transepithelial electrical measurements of Fig. 3, corneas bathed in Cl-free Ringer's were not prestimulated with amphotericin B. In NaCl Ringer's (upper panel), after a stable impalement had been obtained for 2 min, diltiazem superfusion resulted in a very large depolarization of the intracellular potential difference V_{sc} from 58 to 8 mV after 6 min with a large decrease of the voltage divider ratio from 0.70 to 0.22. Once the intracellular electrical parameters had stabilized, the validity of the impalement was evaluated by measuring the effects of Cl removal on these parameters.

In Cl-free Ringer's, diltiazem also had a large depolarizing effect on V_{sc} by about 100 mV without any change of FR_o. A short-term exposure on the tear side to NaCl Ringer's containing 1 mM diltiazem resulted in hyperpolarization of V_{sc} and a decrease of FR_o suggesting adequate impalement. The effect of diltiazem was reversible since less than 10 min after washout of diltiazem V_{sc} had nearly repolarized to its control value. The effects of diltiazem on the intracellular parameters are summarized in Table 2. Diltiazem depolarized V_{sc} both in the presence and absence of Cl, but had no effect on FR_o in Cl-free Ringer's. The lack of an effect on FR_o in Cl-free Ringer's is due to the fact that FR_o is nearly equal to one. In Cl-free Ringer's, basolateral resistance is approximately one-tenth of the apical membrane resistance and very large changes in basolateral membrane resistance would be required to influence FR_o.

The large depolarization of V_{sc} in NaCl and Cl-free Ringer's and the decrease of FR_o in NaCl Ringer's with diltiazem suggested an inhibition of basolateral membrane K conductance. This notion is supported by the fact that K is above electrochemical equilibrium in the corneal epithelium (Reuss et al., 1983). In addition, the basolateral membrane has an appreciable K conductance.[1] We attempted to demonstrate an alteration of basolateral membrane K conductance with diltiazem by measuring the effect of this drug on a K-originated current elicited in corneas by preincubation with 10^{-5} M amphotericin B. The tissue was bathed with a stromal-

Table 2. Effects of 1×10^{-3} M diltiazem on the intracellular electrical parameters of frog corneal epithelium under short-circuit conditions

	NaCl ($n = 6$)		Cl-free ($n = 6$)	
	V_{sc}	FR_a	V_{sc}	FR_a
Control:	-54 ± 6	0.58 ± 0.05	-78 ± 5	0.91 ± 0.03
Diltiazem:	-18 ± 5*	0.30 ± 0.07*	-9 ± 4*	0.90 ± 0.03

* Significantly different from corresponding control ($P < 0.05$, paired t-test).

Table 3. Effects of 2×10^{-4} M diltiazem on transepithelial electric parameters of corneas whose tear and stromal bathing solutions contained 35 and 2 mM K, respectively ($n = 5$)

	Control	Amphotericin B	Ouabain	Diltiazem
SCC, $\mu A/cm^2$	7.0 ± 2.1	16.7 ± 4.7	8.5 ± 2.1	7.9 ± 2.1*
R_t, $K\Omega \cdot cm^2$	3.2 ± 1.7	1.5 ± 0.3	1.3 ± 0.3	1.2 ± 0.3

* Significantly different from ouabain ($P < 0.05$, paired t-test).

directed K gradient (i.e., tear → stroma 35 : 2 mM K: *see* Table 1 for Ringer's composition). Despite an extremely small apical membrane K permeability, there is an appreciable K current of 7.0 $\mu A/cm^2$ due to the K conductance of the paracellular shunt pathway (Table 3). Incubation with amphotericin B eliminates the permselectivity of the apical membrane resulting in a greater than twofold increase of this current to 16.7 $\mu A/cm^2$. Under this condition the current is the sum of net Na translocation towards the stroma by the Na : K pump and net diffusion of K in the same direction through shunt and cellular leak pathways. The pump component of the current was eliminated with ouabain resulting in a 31% decline of the current to 8.5 $\mu A/cm^2$. The remaining current represented transcellular K movement across the basolateral membrane and K flow through the shunt. Diltiazem (2×10^{-4} M) had a maximal inhibitory effect on this flow. This concentration decreased the K-originated current flow across the basolateral membrane by 40% $\left(\text{i.e. } \frac{8.5 - 7.9}{8.5 - 7.0}\right)$. This degree of inhibition of basolateral K flow may be an underestimate of the drug's inhibitory effect since amphotericin B does increase shunt conductance as a function of time (Candia et al., 1984).

Calcium Dependency of Diltiazem on SCC

The calcium antagonists studied are known to inhibit calcium uptake into cardiovascular and other tissues and their specific effects demonstrate competitive kinetics with calcium (Fleckenstein, 1983; Janis & Scriabine, 1983) resulting in a decreased potency of the blocker with higher ambient calcium concentrations. Figure 5 demonstrates such a right-hand shift in the concentration response curve for three NaCl Ringer's solutions containing calcium ranging from 0.05 to 15 millimolar. The shift from low or control calcium to high calcium is significant ($P < 0.02$) as determined by linear regression and analysis of covariance.

On the other hand, the calcium ionophore A23187 (10^{-6} M), which stimulates net Cl transport by up to 220% (Candia et al., 1977), was incapable of reversing the diltiazem-inhibited SCC (Fig. 6). Even after A23187 pretreatment, the action of diltiazem on the SCC was unaffected (*data not shown*). Thus, the inhibitory action of diltiazem on the SCC is not a result of calcium uptake blockade.

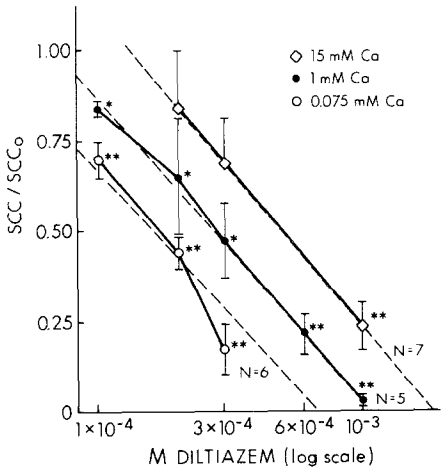

Fig. 5. Ca bathing solution dependency of inhibitory effects of diltiazem on normalized SCC. Broken lines were calculated by linear regression. Analysis of covariance indicates that the diltiazem dose response in low Ca or 1 mM Ca is significantly different from that in 15 mM Ca. Each point and bracket represents the mean ± SEM for the number of indicated corneas. * $P < 0.05$; ** $P < 0.01$ with respect to the control value

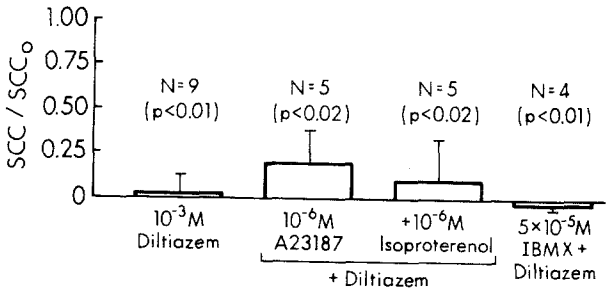

Fig. 6. Comparison of the effect of 1×10^{-3} M diltiazem on the normalized SCC with the effects of A23187, isoproterenol and IBMX on corneas pretreated with 1×10^{-3} M diltiazem. Significance is indicated with respect to the control period for each condition

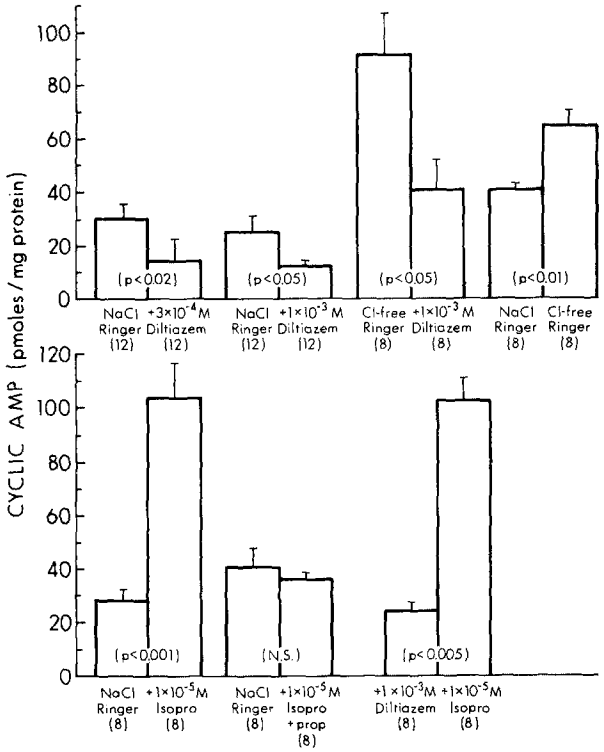

Fig. 7. Cyclic AMP content in paired frog corneal halves: isoproterenol (isopro) – propranolol (prop). All values are means ± SEM for the numbers shown in parentheses. The levels of significance are also indicated and were obtained using Student's *t*-test for paired variates. *P* values were calculated by comparing each of the experimental conditions to the control. N.S., not significant

The calcium dependency in Fig. 5, however, may be explained by an ability of extracellular calcium to stabilize potassium channels (Petersen & Maruyama, 1984) in the basolateral cell membrane, partially protecting them from diltiazem inhibition.

Absence of Reversal by Isoproterenol and IBMX

Agents which increase cellular cyclic AMP are known to stimulate the Cl-originated SCC (Chalfie et al., 1972; Beitch et al., 1974; Schaeffer et al., 1982; Reinach & Kirchberger, 1983). After a maximal inhibitory concentration of diltiazem (1×10^{-3} M), however, tissues failed to respond to either isoproterenol or IBMX (Fig. 6). This result suggests that the drug-induced inhibition of net Cl transport does not stem from an alteration of cyclic AMP turnover.

Cyclic AMP

At concentrations greater than those needed to inhibit calcium uptake, the calcium channel antagonists have been reported to have nonspecific effects which include alteration of cyclic AMP turnover (Levine et al., 1983). Both 3×10^{-4} and 1×10^{-3} M diltiazem decreased cyclic AMP content of corneas preincubated with IBMX (Fig. 7). Similarly, 1×10^{-3} M diltiazem had a significant inhibitory effect in Cl-free Ringer's, further indicating that the decrease in cyclic AMP is not a result of Cl transport inhibition. The high cyclic AMP content in the corneas bathed in Cl-free Ringer's did not appear to arise from an experimental error since their content was also elevated in another group of tissues with respect to the NaCl Ringer's control.

The responsiveness of corneas to isoproterenol is indicated by the greater than threefold increase in cyclic AMP content and this response was blocked in the presence of propranolol. Isoproterenol also elicited a fourfold increase in the cyclic AMP content of corneas preincubated with 1×10^{-3} M diltiazem. This effect suggests that the decrease in cyclic AMP content by diltiazem is not the primary mechanism for the drug's inhibition of active Cl transport.

Na,K-ATPase

Bovine corneal epithelial peelings were fractionated using sucrose density gradient centrifugation. In preliminary studies, we found that the band having a density between 1.14 to 1.16 g/ml contained maximal enrichment of Na,K-ATPase activity. Kidney cortex preparations were 60 ± 10% ouabain inhibitable ($n = 3$). The corneal preparations, however, were 21 ± 4% ouabain inhibitable ($n = 6$) indicating a lower density of Na,K-ATPase activity. Nevertheless, the three other isolated bands contained significantly less ouabain-inhibitable activity. In the most enriched Na,K-ATPase-containing band from either rabbit kidney cortex or bovine cornea, 1×10^{-3} M diltiazem had no significant effect on ATPase activity. In the corneal preparation the respective control and experimental values were 0.31 ± 0.05 and 0.34 ± 0.07 nmol Pi/mg protein/min ($n = 3$).

Discussion

In the submillimolar range three calcium channel antagonists effectively inhibited net transepithelial ion transport across the frog cornea. This inhibitory effect is probably not related to calcium channel blockade since, in tissues having calcium channel activity, these drugs maximally inhibit this process in the micromolar range (Janis & Scriabine, 1983). Furthermore, the inhibitory effect of diltiazem on the Cl-originated SCC was not reversed by the calcium ionophore A23187. Reversal would have been

expected if the SCC inhibition was a result of calcium uptake blockade by the drug (*cf.* Fig. 6).

Previous to this study, calcium channel blockers had been shown to inhibit a gamut of other processes at concentrations that are orders of magnitude higher than those used to inhibit calcium channel activity. Adrenergic, cholinergic and opiate receptors have binding constants of about 10^{-6} M for D-600 (Fairhurst et al., 1980; Jim et al., 1981). In the micromolar range, nimodipine and nicardipine competitively inhibit the calmodulin-sensitive and calmodulin-insensitive forms of cyclic AMP phosphodiesterase (Epstein et al., 1982). The same authors found that verapamil and nimodipine antagonize calmodulin stimulation of phosphodiesterase. In the submillimolar range, D-600, diltiazem and nifedipine inhibit cyclic AMP generation and also inhibit phosphodiesterase in the isolated toad bladder (Levine et al., 1983). Some calcium channel inhibitors are thought to stimulate Ca,Mg-ATPase activity and Ca uptake by isolated skeletal, cardiac muscle and sarcoplasmic reticulum (Colvin et al., 1982). We have found no effect of 1×10^{-3} M diltiazem on Ca,Mg-ATPase activity of plasma membrane-enriched fractions prepared from bovine corneal epithelium (*data not shown*). Variable effects of these agents have been reported on Na,K-ATPase activity including no change as well as stimulation of pump activity (Murphy et al., 1982; Pan & Janis, 1984). Verapamil also has been reported to inhibit K influx and efflux in cardiac Purkinje fibers (Posner et al., 1975).

In the frog corneal epithelium, the predominant factor responsible for the electrodiffusion of Cl across the apical membrane is the potassium diffusion potential across the basolateral membrane. Intracellular K is above electrochemical equilibrium and there is appreciable basolateral membrane K conductance resulting in K outflow which is large enough to account for most of the negativity of the intracellular compartment. Our first indication of a basolateral membrane effect on an ion transport-related pathway by diltiazem was that preincubation with amphotericin B did not eliminate the inhibitory effect of the drug on the SCC. The large depolarization of the V_{sc} regardless of the presence or absence of Cl is indicative of a decrease in K outflow across the basolateral membrane whose permselectivity for K is much larger than for Cl or Na.[1] Another indication of a basolateral membrane alteration by diltiazem is that FR_o in NaCl Ringer's decreased. Since net ion transport decreased, the only compatible explanation is an increase of the basolateral membrane resistance. FR_o did not change in Cl-free Ringer's since the basolateral membrane resistance increase was small relative to the much larger increase in apical membrane resistance resulting from removing Cl from the bathing solution. The inhibitory effects of diltiazem in the millimolar range of either the Cl- or Na^+K-originated SCC are explainable in terms of a decrease in basolateral membrane K conductance based on the drug's effects on the transepithelial and intracellular electrical parameters. At lower diltiazem concentrations these electrical parameters are unaffected by the drug; however, it is conceivable that Ca-channel activity is selectively inhibited. Accordingly, we cannot determine whether the inhibitory effects of diltiazem in the millimolar range are due to an inhibition of only K-channel or both Ca- and K-channel activity.

Despite the very large depolarizing effect of diltiazem on V_{sc} and the decrease in cyclic AMP content by the drug, these effects were reversible. Figure 2 shows the excellent reversibility of the inhibitory effects of the drug subsequent to washout. Isoproterenol markedly increased the cyclic AMP content of corneas preincubated with diltiazem (Fig. 7). These results indicate an effective tissue interaction without an irreversible alteration of cellular parameters. The large alteration of the electrical parameters and rapid reversibility of the electrical parameters suggests that diltiazem is extremely efficacious to blocking K-channel conductance through a weak type of interaction. These effects are in contradistinction with the K-channel blocker, Ba, whose effects on the electrical parameters of the cornea are smaller, but less readily reversible.[1]

All of our results show that the inhibitory effects of diltiazem on net ion transport are not the result of a primary effect on the Na : K pump. The time course of depolarization of V_{sc} with diltiazem was continuous and always much larger than that with ouabain; ouabain depolarizes V_{sc} by less than 10 mV.[1] In some tissues preincubated with ouabain, diltiazem still had a large depolarizing effect on V_{sc}. Furthermore, we could not measure any inhibitory effect of diltiazem on ouabain-inhibitable Na,K-ATPase activity in broken cell preparations of either bovine corneal epithelium or rabbit kidney cortex. The latter preparation is much richer in Na,K-ATPase activity than the former since ouabain inhibited enzymatic activity by about 60 and 20%, respectively. Finally the results of the K-gradient experiments (*cf.* Table 3) indicate two points: (1) the inhibitory effects of diltiazem and ouabain on the current were additive suggesting an alternate site of action for diltiazem than the pump, and (2) diltiazem inhibited a K-originated current arising in part from K efflux into the stroma across the basolateral membrane. In the turtle colonic mucosa, a similar type of protocol was used to demonstrate that Ba blocks a basolateral membrane K conductance (Kirk & Dawson, 1983).

Inasmuch as the primary effect of diltiazem is

an inhibition of a cyclic AMP-independent basolateral membrane K-conductance, secondary effects on the Na:K pump are also tenable with our results. In Cl-free Ringer's, after preincubation with amphotericin B, 1×10^{-3} M diltiazem had a larger inhibitory effect on the SCC than the K-channel blocker barium (Candia et al., 1984). We found that diltiazem inhibited the SCC by 78% whereas in the earlier study 1×10^{-3} M barium only inhibited the SCC by 18%. In the corneal epithelium, 1×10^{-3} M diltiazem is more effective than 2×10^{-3} M $BaCl_2$ in inhibiting the Cl-originated SCC.[1] Barium inhibited the Cl-originated SCC by 61%, whereas diltiazem decreased this current by about 100%. This larger inhibitory effect by diltiazem is explainable in terms of a greater suppression of basolateral membrane K conductance. The possibility also exists that the inhibitory effect by diltiazem on basolateral membrane K conductance is sufficient to have a corresponding secondary effect on Na:K pump activity.

In summary, our results show that the primary mechanism of inhibition of net ion transport by diltiazem is ascribable to the blockade of K channels in the basolateral membrane which may be stabilized by extracellular calcium. These channels are not regulated by intracellular cAMP since even though isoproterenol increased cyclic AMP-content in the presence of diltiazem this agonist had no restorative effect on the inhibited SCC. Finally, there is an indication that the inhibition of basolateral membrane K conductance may have a corresponding feedback effect on the Na:K pump.

This work was supported by a National Institutes of Health grant (EY 04795) to Peter Reinach and performed at Washington University, Department of Ophthalmology. We wish to thank Wolfram Nagel for his input regarding the effects of diltiazem on the electrical parameters. We also wish to thank Chris Blazynski and Ronald Walkenbach for their help in the measurement of cyclic AMP.

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Received 1 October 1984; revised 22 January 1985