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Effect of Ibuprofen and Rofecoxib Transport Parameters in the Frog Corneal Epithelium

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Abstract. Effects of cyclooxygenase (COX) inhibitors on transport parameters of the frog corneal epithelium were studied. Epithelial cells of the intact cornea were impaled with microelectrodes. Under short-circuit current (I_{sc}) conditions, 10^{-4} M ibuprofen (IBU) (non-specific COX inhibitor) or 5×10^{-5} M rofecoxib (COX-2 inhibitor) were added to the tear solution. With ibuprofen, I_{sc} decreased by 1.0 from 3.1 μA/cm^2; intracellular potential, V_o, depolarized by 14.2 from −56.9 mV; IBU did not affect the transepithelial conductance, g_t, or the apical membrane fractional resistance, fR_o. With rofecoxib, I_{sc} decreased by 0.9 from 4.3 μA/cm^2; V_o depolarized by 18 from −62.4 mV; g_t significantly increased by 0.03 from 0.37 ms/cm^2; and fR_o decreased by 12 from 50. Basolateral membrane K^+ and apical membrane Cl^- partial conductances were studied by the ion substitution method.

Depolarization of V_o by an increase in stromal K^+ from 4 to 79 mM was smaller with IBU (17.5 mV) or rofecoxib (19.2 mV) than without the inhibitors (29.1 and 29.3 mV, respectively). Depolarization of V_o, by a decrease in tear Cl^- from 81 to 8.1 mM, was abolished by the COX inhibitors. Decrease in I_{sc} and V_o can be explained by a decrease in the K^+ and Cl^- conductances. Experiments with amphotericin B ruled out a major effect of the inhibitors on the Na^+/K^+ ATPase pump.

Key words: Membrane potentials — Electrical resistance — Short-circuit current — Ibuprofen — Rofecoxib — Corneal epithelium (*Rana catesbeiana*)

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are inhibitors of cyclooxygenase (COX) enzymes. While their inhibition of COX-2, responsible for inflammation, is beneficial, their inhibition of COX-1, protective of stomach and renal function, can be deleterious (Vane & Botting, 1995; 1998). Spenney & Bhown (1977), using an in vitro preparation of the bullfrog gastric mucosa, found that aspirin increased initially the transepithelial potential (PD) and resistance, followed by a decline in PD towards zero. Miller et al. (1984) found that aspirin decreased the short-circuit current and PD when applied to the mucosal bathing solution. These effects were prevented or reversed by the use of prostaglandins. Sanders et al. (1985) found that aspirin decreased the electrical resistance of canine oxyntic cell monolayers when added to the acidified apical solution. Schmitt & Meves (1995) have found that prostaglandins modulate neuronal calcium channels. In recent years, Cuppoletti et al. have found an increase in Cl^- currents in ClC-2 Cl^- channels from human tissue by arachidonic acid and also by inhibition of cyclooxygenase with ibuprofen (Cuppoletti, Malinowska & Tewari, 1999; Cuppoletti et al., 2001; Tewari et al., 2000). Prostaglandins have been found to affect the osmotic water permeability in the frog and trout urinary bladder (Natochin et al., 1998).

Therefore, there is evidence that ibuprofen and other NSAIDs affect epithelia transport parameters, including amphibian epithelia. There is not much known concerning effects of these drugs on the intact tissue and, in particular, specific ion conductances. The frog corneal epithelium is an excellent model to test the biological effects of substances that affect epithelial transport, such as Cl^--secreting epithelia. The corneal epithelium actively transports Cl^- from stroma to tear. The primary active transport is the Na^+/K^+ ATPase pump located in the basolateral membrane (Candia, Bentley, & Cook, 1974; Carras-

quer et al., 1987). Other transporters that play an important role on Cl^- secretion are the Cl^- conductance located in the apical membrane (Nagel & Reinach 1980; Reuss et al. 1983; Nagel & Carrasquer, 1989) and the K^+ conductance (Candia et al., 1974; Carrasquer et al. 1987) and the NaCl symport located in the basolateral membrane (Zadunaisky, 1972; Frizzell, Field, & Schultz, 1979; Candia, 1982; Reuss et al., 1983; Nagel & Carrasquer, 1989). Also, effects on the corneal epithelium may be extrapolated to other epithelia, especially Cl^--secreting epithelia. If there is an inhibition of active transport in the corneal epithelium, the use of the microelectrode technique enables us to determine which of the transport parameters are affected. Such an approach is adopted in the study of the effects of the nonspecific COX inhibitor ibuprofen and the COX-2 inhibitor rofecoxib.

Materials and Methods

Bullfrogs (*Rana catesbeiana*) were kept in the laboratory at room temperature, with free access to water. Each animal was anesthetized by cooling, followed by decapitation. The eyes were removed and the corneas were excised. The corneas were mounted tear side up in a lucite chamber (Nagel, 1976; Nagel & Reinach, 1980). The tissue was supported by a copper grid with a radius of curvature slightly less than that of the in vivo cornea. An opening of 0.4 cm^2 communicated the upper (epithelial) chamber (0.2 ml) with the lower (stroma) chamber (0.3 ml). Note that the *stroma* (*stromal*) chamber or solution is used throughout the paper with reference to chamber or solution closest to the stroma. Both chambers were continuously perfused at a rate of about 5 ml/min to insure complete exchange in 5–10 sec. A slight negative hydrostatic pressure was applied to the lower chamber to help secure the cornea to the copper grid. Control (regular) solutions contained (in mM): Na^+, 102; K^+, 4.2; Ca^{2+}, 1; Mg^{2+}, 0.8; Cl^-, 81; SO_4^{2-}, 0.8; HCO_3^- 25; phosphate 1; and glucose 10. In experiments involving an increase in K^+ concentration, the control solutions had (in mM): Na^+ 27 and choline 75; then K^+ was substituted for choline in high-K^+ solutions. All solutions were gassed with 5% CO and 95% O_2. The pH of the solutions was 7.3–7.4.

Ibuprofen or rofecoxib was added to the tear solution to a final concentration of 10^{-4} M and 5×1^{-5} M respectively. Pilot experiments, in which the concentrations were below these values, showed at best minimal effects. When the drugs were added to the stromal solution up to a final concentration of 10^{-4} M, there were zero or minimal effects.

Two pairs of macroelectrodes and one microelectrode were used. One pair was used to measure the transepithelial potential difference. It comprised calomel electrodes connected via KCl bridges to within 0.5 mm of tissue surfaces. The other pair was used to send current. It comprised AgCl-coated Ag wire loop electrodes, 4 mm from the tissue on either side. The intracellular potential, V_o, was recorded with 3 M KCl-filled microelectrodes, which had an input resistance of 50–70 Mohm. Corneas were short-circuited using an automatic clamp device (Biomed. Inst., Germering, FRG), except for brief perturbations that lasted about 200 msec, during which the transepithelial potential was clamped at +10 mV (stroma side positive). These perturbations were repeated every 1–2 sec and were used for measurement of the transepithelial conductance, namely, $g_t = \Delta I_t/\Delta V_t$ where V_t and I_t are the transepithelial voltage and current, respectively. Another parameter of importance was the apical membrane fractional resistance, namely, $fR_o = R_o/(R_o + R_i) = \Delta V_o/\Delta V_t$ where R_o and R_i are the resistances across the apical and basolateral membranes, respectively. The values of short-circuit current (I_{sc}), g_t, fR_o, and V_o were recorded together with the microelectrode resistance on a multichannel strip chart recorder (Linseis, TYP 2065). I_{sc} is defined as positive when the direction of current is from tear to stroma via the tissue. Hyperpolarization of V_o is defined as an increase in the negativity of the intracellular potential. Depolarization is regarded as the opposite of hyperpolarization. Student's *t*-test with paired observations was performed to determine the level of significance when applicable.

Results

Effect of 10^{-4} M Ibuprofen or 5×10^{-5} M Rofecoxib in the Cornea Tear Solution

Figures 1*A* and 1*B* show the effects of ibuprofen and rofecoxib, respectively, when added to the tear solution with pH of 7.3 in both solutions. From 13 experiments for ibuprofen and 14 experiments for rofecoxib, the figures exhibit the mean values of I_{sc}, fR_o, g_t, and V_o plotted versus time, with zero being the time of addition of the drugs.

While Figs. 1*A* and 1*B* show the typical time course of the experiments, Table 1 presents numerical data of the mean control values and the mean changes of the parameters at 15 min after addition of the drugs for all experiments. The left two columns show the data for ibuprofen experiments: I_{sc} decreased by 1.2 from 3.5 $\mu A/cm^2$, control; V_o depolarized by 15.2 from −58.0 mV, control; fR_o and g_t were not statistically affected by the non-specific COX inhibitor. The right two columns show data for rofecoxib. The effects of rofecoxib are similar to those of ibuprofen with reference to the decrease in I_{sc} and depolarization of V_o. While fR_o and g_t were not affected by ibuprofen, they were affected to a small extent, but significantly, by rofecoxib, that is, fR_o decreased by 12 from a control value of 49.6 and g_t increased by 0.03 from 0.37 mS/cm^2.

Significant decreases in I_{sc} by the COX inhibitors could be due to inhibition of the following pathways: the Na^+-K^+ ATPase pump, the $Na^+/K^+/2Cl^-$ cotransporter or the K^+ conductance located in the basolateral membrane and/or the Cl^- conductance located in the apical membrane. Effects of the inhibitors on the K^+ and Cl^- conductances were determined using the ion substitution method.

Effects of 10^{-4} M Ibuprofen or 5×10^{-5} M Rofecoxib in the Tear Solution on the Response of V_o to a Change in Stromal K^+ Concentration from 4 to 79 mM and Tear Cl^- Concentration from 100 to 10 mM

The upper line of data of Table 2 presents the mean control values and the mean changes of V_o at 10 min after increasing stromal K^+ from 4 to 79 mM in six experiments. The lower line of data presents similar

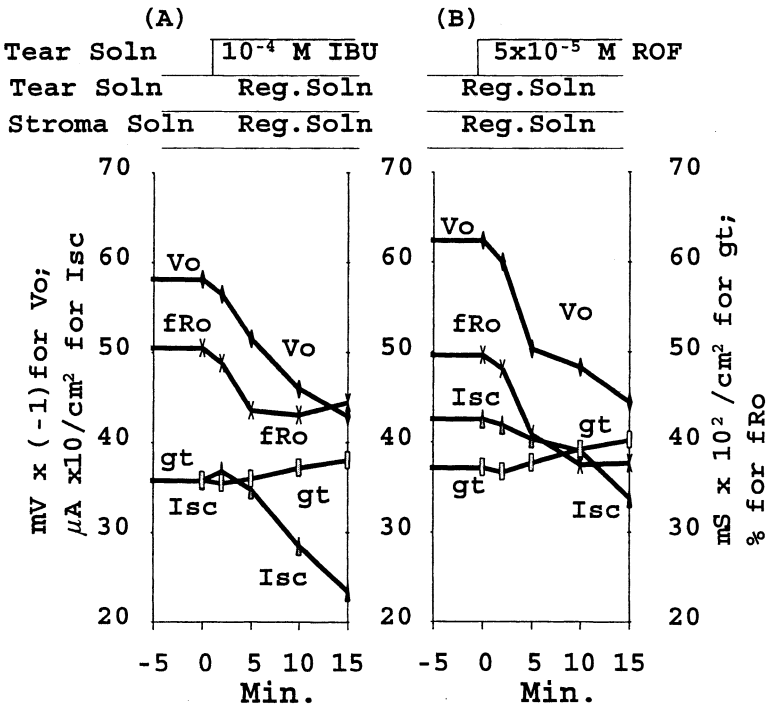

Fig. 1. Effect of 10^{-4} M ibuprofen (*A*) or 5×10^{-5} M rofecoxib (*B*) in the tear solution. The pH was 7.3 in the bathing solutions. Values are means from 13 experiments for ibuprofen and 14 experiments for rofecoxib. Short-circuit current, I_{sc}, in $\mu A/cm^2$; apical membrane fractional resistance, fR_o, unitless; transepithelial conductance, g_t, in mS/cm^2; intracellular potential, V_o, in mV; all parameters are plotted versus time. Zero time when ibuprofen or rofecoxib were added.

changes of V_o in which the tear Cl^- concentration was decreased from 81 to 8.1 mM in six experiments. The left two columns of Table 2 present the data obtained before ibuprofen and the right two columns of Table 2 present the data obtained with ibuprofen in the tear solution, at least for 30 min before changing the concentration of the ions. Before ibuprofen, the increase from 4 to 79 mM K^+ depolarized V_o by 28.7 from a control of −61.8 mV and, after ibuprofen, the increase from 4 to 79 mM K^+ depolarized V_o by 17.5 from a control of −36.1 mV. Before ibuprofen, the decrease from 81 to 8.1 mM Cl^- depolarized V_o by 11.0 from a control of −58.0 mV. With ibuprofen in the tear solution, there was no significant depolarization when the Cl^- concentration was changed. Data are compatible with a decrease of both the K^+ and the Cl^- conductances by ibuprofen.

Table 3 presents the data on rofecoxib. Like ibuprofen, rofecoxib decreased significantly the responses of V_o to concentration changes of stromal K^+ or tear Cl^-. Both COX inhibitors decreased the K^+ conductance in the basolateral membrane and the Cl^- conductance in the apical membrane.

In order to examine the Na^+/K^+ ATPase pump as the site of an inhibitor of I_{sc}, Candia et al. (Candia et al., 1974; Candia, Reinach, & Alvarez, 1984) devised a method by which the inhibitor is used in the presence of amphotericin B, in Cl^--free solutions. Amphotericin B, added to the tear solution, opens Na^+ and K^+ channels in the apical membrane of the corneal epithelium, resulting in an increase in the activity of the Na^+/K^+-ATPase and an increase in I_{sc} (Candia et al., 1974; 1984; Carrasquer et al., 1989, 1991). By removing Cl^- from the bathing media, the possible effect of the inhibitor on the NaCl cotransporter in the basolateral membrane or on the Cl^- conductance pathway in the apical membrane is eliminated. Therefore, to evaluate the possibility that ibuprofen inhibits the Na^+/K^+-ATPase pump, experiments were performed with amphotericin B in the tear solution.

Effect of 10^{-4} M Ibuprofen or 5×10^{-5} M Rofecoxib in the Tear Solution on I_{sc} and G_t in the Presence of 10^{-5} M Amphotericin B in the Tear Solution, in Cl^--Free Solutions

The left two columns of Table 4 show the typical effect of amphotericin B when added to the tear solution in Cl^--free solutions, that is, an increase in I_{sc} by 5.2 from zero $\mu A/cm^2$ and a significant increase in g_t by 0.07 from 0.15 mS/cm^2. The middle two columns of Table 4 show the effects of ibuprofen, with amphotericin B in the tear solution. Under these conditions, ibuprofen did not affect I_{sc} or g_t. The right two columns show effects of rofecoxib, with amphotericin B in the tear solution. With rofecoxib, there was a small but significant decrease in I_{sc} of 1.0 from 6.8 $\mu A/cm^2$ before the addition of rofecoxib. It appears that ibuprofen did not affect the Na^+/K^+-ATPase pump but that rofecoxib inhibited the pump to a small extent. An effect of rofecoxib on the apical membrane Na^+ or K^+ conductances, opened by amphotericin B, is unlikely, since the drug did not affect the change in I_{sc} induced by a change in tear Na^+ or K^+ (*data not presented*).

Table 1. Effects of adding 10^{-4} M ibuprofen (13 expts.) or 5×10^{-5} M rofecoxib (14 expts.) to tear solution

	Ibuprofen		Rofecoxib	
	Control	Δ = 15 min	Control	Δ = 15 min
I_{sc}	3.6 ± 0.2	−1.2 ± 0.1[a]	4.3 ± 0.6	−0.9 ± 0.4[b]
fR_o	50.5 ± 5.0	−6.2 ± 3.5[ns]	49.6 ± 2.5	−12.0 ± 3.0[a]
g_t	0.36 ± 0.02	0.02 ± 0.01[ns]	0.37 ± 0.02	0.03 ± 0.01[b]
V_o	−58.0 ± 2.2	15.2 ± 2.2[a]	−62.4 ± 2.1	18.0 ± 2.2[a]

Values are means ± SE. Control: values before the addition of the drugs. The other values are the changes obtained 15 min after addition of the drugs.
Units are: I_{sc}, μA/cm^2; fR_o, unitless; g_t, mS/cm^2; V_o, mV.
[a] $P < 0.01$; [b] $P < 0.05$; [ns] $P > 0.05$.

Table 2. Effects on V_o of changing stromal K^+ or tear Cl^- concentration without and with 10^{-4} M ibuprofen (IBU) in the tear solution

	V_o				n
	Without IBU		With IBU		
	Control	Δ = 10 min	Control	Δ = 10 min	
Stromal K^+ from 4 to 79 mM	−61.8 ± 3.4	28.7 ± 0.9[a]	−36.1 ± 2.0	17.5 ± 2.1[a]*	6
Tear Cl^- from 81 to 8.1 mM	−58.0 ± 3.4	11.0 ± 2.9[b]	−42.9 ± 2.9	1.9 ± 2.5[ns]*	5

Symbols and units as in Table 1. Control: values before change in ion concentration;
The other values are the changes obtained 10 min after the change in ion concentration.
*$P < 0.01$ when comparing the effects of changing tear K^+ in the presence versus absence of IBU. n, number of experiments.

Table 3. Effects on V_o of changing stromal K^+ or tear Cl^- concentration without and with 5×10^{-4} M rofecoxib in the tear solution

	V_o				n
	Without rofecoxib		With rofecoxib		
	Control	Δ = 10 min	Control	Δ = 10 min	
Stromal K^+ from 4 to 79 mM	−67.8 ± 2.3	27.2 ± 2.8[a]	−46.1 ± 2.9	16.0 ± 2.1[a]*	6
Tear Cl^- from 81 to 8.1 mM	−64.0 ± 4.2	6.4 ± 3.6[b]	−48.8 ± 4.0	1.5 ± 2.7[ns]*	5

Symbols and units as in Tables 1 and 2.

Discussion

The main effects of ibuprofen and rofecoxib were a decrease in I_{sc} and a depolarization of V_o. The four major pathways that contribute to I_{sc} in the corneal epithelium are the electroneutral NaCl cotransporter in the basolateral membrane (Zadunaisky, 1972; Frizzel et al., 1979; Candia, 1982; Reuss et al., 1983; Nagel & Carrasquer, 1989) and three electroconductive pathways, namely, the Na^+/K^+ ATPase and the K^+ conductance in the basolateral membrane (Candia et al., 1974; Carrasquer et al., 1985; 1987) and the Cl^- conductance in the apical membrane (Nagel & Reinach, 1980; Reuss et al., 1983; Nagel & Carrasquer, 1989). It should be noted that, with 4 mM (or greater) K^+ solutions, the Na^+/K^+ ATPase conductance is much smaller than the K^+ conductance (Carrasquer et al., 1985; 1987). An inhibition of any of the four pathways by ibuprofen could have been responsible for the decrease in I_{sc}. The depolarization of V_o suggests that the COX inhibitors affected one or more of the three conductive pathways: the Na^+/K^+ ATPase, the K^+ conductance and/or the Cl^- conductance. Inhibition of the Na^+/K^+ ATPase pump by ibuprofen is improbable, since it did not affect I_{sc} in the presence of amphotericin B in Cl^--free solutions. On the other hand, it appears that rofecoxib affected the Na^+/K^+ ATPase pump to some extent. However, the effects of ibuprofen and rofecoxib on I_{sc} and V_o can be best explained by a decrease in the K^+ conductance in the basolateral membrane and a decrease in the Cl^- conductance in the apical membrane.

The effects of ibuprofen and rofecoxib on the K^+ and Cl^- conductances were determined using the ion substitution method. An increase in stromal K^+ concentration results in a depolarization of V_o. If the COX inhibitors decreased the basolateral membrane

Table 4. Effects on I_{sc} and g_t of adding first amphotericin B (AmB), then ibuprofen (IBU) or rofecoxib to tear solution

	AMB, 5×10^{-5} M		IBU, 1×10^{-4} M		Rofecoxib, 5×10^{-5} M	
	Control (1)	Δ = 15 min	Control (2)	Δ = 15 min	Control (3)	Δ = 15 min
I_{sc}	-0.1 ± 0.2	5.2 ± 0.7^{a}	5.8 ± 0.8	-0.1 ± 0.4^{ns}	6.8 ± 0.8	-1.0 ± 0.2^{a}
g_t	0.15 ± 0.02	0.07 ± 0.01^{a}	0.27 ± 0.03	0.03 ± 0.02^{ns}	0.33 ± 0.02	0.01 ± 0.02^{ns}

Cl-free solution on both sides.
Symbols and units as in Table 1. Control (1): values before AmB addition. Control (2): values before IBU addition, with AmB in tear solution. Control (3): values before rofecoxib addition, with AmB in tear solution. The other values are the changes obtained 15 min after addition of respective drugs. 12 expts. for AmB; 6 expts. for IBU; 6 expts. for rofecoxib.

K^+ conductance, the change in V_o induced by an increase in stromal K^+ would be smaller with than without the drugs. This effect was observed.

In line with the above reasoning, when the Cl^- concentration was decreased in the tear solution, the depolarization of V_o occurred to a lesser extent in the presence than in the absence of the COX inhibitors. Therefore, not only the basolateral membrane K^+ conductance was decreased by the inhibitors, but also the apical membrane Cl^- conductance.

Since the effects are about the same with the COX-1/COX-2 inhibitor ibuprofen and the COX-2 inhibitor rofecoxib, it appears certain that precursors or products of cyclooxygenase 2 are involved in the preservation or disturbance of the basolateral membrane K^+ channel and the apical membrane Cl^- channel. Results are compatible with an accumulation of arachidonic acid or a depletion of prostaglandins post the cyclooxygenase effect, e.g., prostaglandin E. Preliminary studies indicate that archidonic acid may have similar effects to those of the COX inhibitors.

A finding that we cannot explain with present experiments is the evidence for a decrease in the K^+ conductance in the basolateral membrane and for a decrease in the Cl^- conductance in the apical membrane by the COX inhibitors, while the transepithelial conductance does not change with ibuprofen or even increases to a small degree with rofecoxib. Perhaps these inhibitors increase the conductance of the paracellular pathway.

In summary, with addition of the COX inhibitors ibuprofen and rofecoxib to the tear solution, there is a decrease in short-circuit current and intracellular potential of the frog corneal epithelium. These effects can be explained by a decrease of the apical membrane Cl^- and basolateral membrane K^+ conductances with little effect at most on the Na^+/K^+ ATPase pump.

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