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Fatty acids inhibit apical membrane chloride channels in airway epithelia

(ion channel/arachidonic acid/cyclooxygenase/lipoxygenase/cystic fibrosis)

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ABSTRACT **Apical membrane Cl^- channels control the rate of transepithelial Cl^- secretion in airway epithelia. cAMP-dependent protein kinase and protein kinase C regulate Cl^- channels by phosphorylation; in cystic fibrosis cells, phosphorylation-dependent activation of Cl^- channels is defective. Another important signaling system involves arachidonic acid, which is released from cell membranes during receptor-mediated stimulation. Here we report that arachidonic acid reversibly inhibited apical membrane Cl^- channels in cell-free patches of membrane. Arachidonic acid itself inhibited the channel and not a cyclooxygenase or lipoxygenase metabolite because (*i*) inhibitors of these enzymes did not block the response, (*ii*) fatty acids that are not substrates for the enzymes had the same effect as arachidonic acid, and (*iii*) metabolites of arachidonic acid did not inhibit the channel. Inhibition occurred only when fatty acids were added to the cytosolic surface of the membrane patch. Unsaturated fatty acids were more potent than saturated fatty acids. Arachidonic acid inhibited Cl^- channels from both normal and cystic fibrosis cells. These results suggest that fatty acids directly inhibit apical membrane Cl^- channels in airway epithelial cells.**

Transepithelial Cl^- secretion by airway epithelia is an important process that helps determine the quantity and composition of the respiratory tract fluid (1). Cl^- enters the cell at the basolateral membrane through a Na^+-coupled cotransporter and exits passively through apical membrane Cl^- channels. Regulation of apical Cl^- channels controls, in part, the rate of transepithelial secretion.

Three intracellular second messenger pathways that regulate apical membrane Cl^- channels and thereby Cl^- secretion have been identified. (*i*) cAMP stimulates Cl^- secretion and activates outwardly rectifying Cl^- channels by cAMP-dependent protein kinase (2, 3). (*ii*) Several secretory hormones increase cellular diacylglycerol levels (4); diacylglycerol activates protein kinase C (PKC), which has dual effects on the outwardly rectifying Cl^- channel. At low concentrations of Ca^{2+}, PKC activates the outwardly rectifying Cl^- channel; but, when the Ca^{2+} concentration is increased, PKC inactivates this same channel (5, 6). (*iii*) Several secretory hormones, such as bradykinin, increase cellular levels of calcium (7) and increase apical membrane Cl^- channel activity by a Ca^{2+}-dependent process (8).

In addition to these three second messengers, the inflammatory peptide bradykinin also stimulates arachidonic acid release (9) and synthesis of many arachidonic acid metabolites (10–12). Previous studies have shown that arachidonic acid can regulate ion channels (13–15). The purpose of this study was to determine if arachidonic acid regulates the outwardly rectifying Cl^- channel in airway epithelia.

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MATERIALS AND METHODS

Cell Culture. Airway epithelial cells obtained from dog and from normal and cystic fibrosis (CF) human airways were isolated and cultured as described (5). Cells were studied at room temperature 1–4 days after plating. Except where indicated otherwise, all experiments were performed on airway epithelial cells from dogs.

Electrophysiology. We used the excised inside-out patch-clamp technique as described (5, 16). A List model EPC7 amplifier (Medical Electronic, Darmstadt, F.R.G.) was used for current amplification and voltage clamping and a laboratory computer system (Indec System, Sunnyvale, CA) was used for data acquisition and analysis. Current was filtered at 1 kHz and sampled every 500 μs. The pipette (external) solution contained 140 mM NaCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes adjusted to pH 7.3 with NaOH. The bath (internal) solution contained 140 mM NaCl, 5 mM $MgCl_2$, and 10 mM Hepes adjusted to pH 7.3 with NaOH. Estimated internal free Ca^{2+} concentration was 1 μM (0.87 mM $CaCl_2$/1 mM EGTA), 100 nM (0.398 mM $CaCl_2$/1 mM EGTA), 10 nM (0.062 mM $CaCl_2$/1 mM EGTA), or <10 nM (no added $CaCl_2$ and 1 mM EGTA). Outward current is shown as an upward deflection; the current level when channels are closed is shown by the dashed lines. Cl^- channels were activated by membrane depolarization (up to +140 mV) (17), although we also occasionally observed channels activating spontaneously after excision (17). There was no difference in the effect of fatty acids on channels activated by the two means. After channel activation, the membrane voltage was held at −40 mV and stepped to +40 mV for 2 s every 4 s. All tracings are shown at +40 mV. Cl^- channels were identified by their current–voltage relationship (including conductance and outward recitification) and activation by depolarization. Open-state probability (P_o) was determined from amplitude histograms. Mean open times were determined during 2-s intervals once every 4 s by using half-amplitude crossing analysis (18). All substances were added by exchanging the internal (bath) solution.

Materials. Fatty acids (>99% pure, obtained from Nu Chek Prep, Elysian, MN) were stored at −80°C in chloroform. Immediately before use, they were dried under N_2, resolubilized in ethanol, and stored at 4°C. The maximum amount of ethanol added was 0.5%. In some experiments the buffer also contained albumin (fatty acid free, Sigma; fatty acids were removed with charcoal treatment as in ref. 18 and confirmed to be <0.005% by using a colorimetric assay). Addition of ethanol or albumin alone had no effect. Arachidonic acid metabolites (Cayman Biochemicals, Ann Harbor, MI) were stored at −20 or −80°C; immediately before use, the ethanol was evaporated under N_2 and the compound was dissolved in perfusion solution.

Abbreviations: CF, cystic fibrosis; PKC, protein kinase C; P_o, open-state probability.

To determine the free arachidonic acid concentration, we used the following equation:

$$\bar{v} = \frac{K_1[A] + 2K_1K_2[A]^2 + \cdots + 8K_1K_2\cdots K_8[A]^8}{1 + K_1[A] + K_1K_2[A]^2 + \cdots + K_1K_2\cdots K_8[A]^8},$$

where $\bar{v}$ is the molar ratio of bound fatty acid to albumin, K_1 through K_8 are the association constants for linoleic acid binding to fatty acid-free albumin, and [A] is the total unbound fatty acid concentration in molarity [from Spector *et al.* (19)]. The calculated free arachidonic acid concentration was 5 μM, with 421 μM arachidonic acid and 100 μM albumin, and 0.5 μM, with 198 μM arachidonic acid and 100 μM albumin.

RESULTS AND DISCUSSION

To determine if arachidonic acid regulates the outwardly rectifying secretory Cl^- channel, we used the single-channel patch–clamp technique (16) to obtain cell-free membrane patches from airway epithelial cells. We activated Cl^- channels with depolarization (≥120 mV, ref. 17). Fig.1 shows that addition of arachidonic acid to the internal cytosolic surface of the membrane patch inactivated Cl^- channels. A channel was considered inactivated when no channel openings were observed for at least 15 s of continuous recording, although in all cases in which longer recordings were performed (up to 5 min), the channel remained closed until arachidonic acid was removed. Inactivation occurred within 80 ± 16 s (mean ± SEM, n = 9) after addition of the fatty acid. In contrast, channels remained activated for more than 300 s in paired patches voltage clamped using the same protocol but not exposed to arachidonic acid (Table 1).

Because long-chain free fatty acids are poorly soluble in aqueous solution, it is difficult to be sure of their free concentration. Therefore, we used albumin as a carrier, to buffer and fix the concentration of arachidonic acid. At an arachidonic acid/albumin molar ratio of 4:1 (free arachidonic acid concentration of approximately 5 μM), Cl^- channels were also inactivated (Fig. 1 and Table 1). When the free arachidonic acid concentration was decreased by decreasing the arachidonic acid/albumin ratio to 2:1 (free concentration of approximately 0.5 μM), arachidonic acid did not inactivate Cl^- channels (Table 1). In two experiments where only single channels were observed in each patch, the open-state probability (P_o) did not significantly decrease upon addition of 0.5 μM arachidonic acid: P_o was 0.99 ± 0 and 0.96 ± 0.08 under control conditions and decreased to 0.87 ± 0.22 and 0.90 ± 0.21, respectively. Thus the Cl^- channel is inhibited at arachidonic acid concentrations similar to the K_m of the cyclooxygenase (5 μM) and lipoxygenase (3.4–28 μM) enzymes (20), suggesting that arachidonic acid may inhibit the Cl^- channel at concentrations achieved in the cell.

Arachidonic acid only inactivated Cl^- channels from the internal cytosolic surface of the patch. When we included 10 μM arachidonic acid in the external (pipet) solution, depolarization activated Cl^- channels in six of seven patches. In contrast, when arachidonic acid was in the internal solution, depolarization failed to activate Cl^- channels in eight patches (alternate patches were studied with internal or external arachidonic acid). For channels exposed to external arachidonic acid, the P_o was 0.82 ± 0.03 compared to a P_o of 0.89 ± 0.02 in control patches (n = 3). Moreover, none of these channels inactivated even when observed for more than 480 s (n = 3). However, subsequent addition of 10 μM arachidonic acid to the internal surface of the same patches inactivated the Cl^- channels in four of four patches tested; an example is shown in Fig. 2. The data suggest that arachidonic acid interacts with a site on the intracellular surface of the plasma membrane to inactivate the Cl^- channel. These results contrast with the effects of another amphiphilic molecule with a carboxyl group that inhibits airway epithelial Cl^- channels from either side of the plasma membrane, 5-nitro-2-(3-phenylpropylamino)benzoic acid (21).

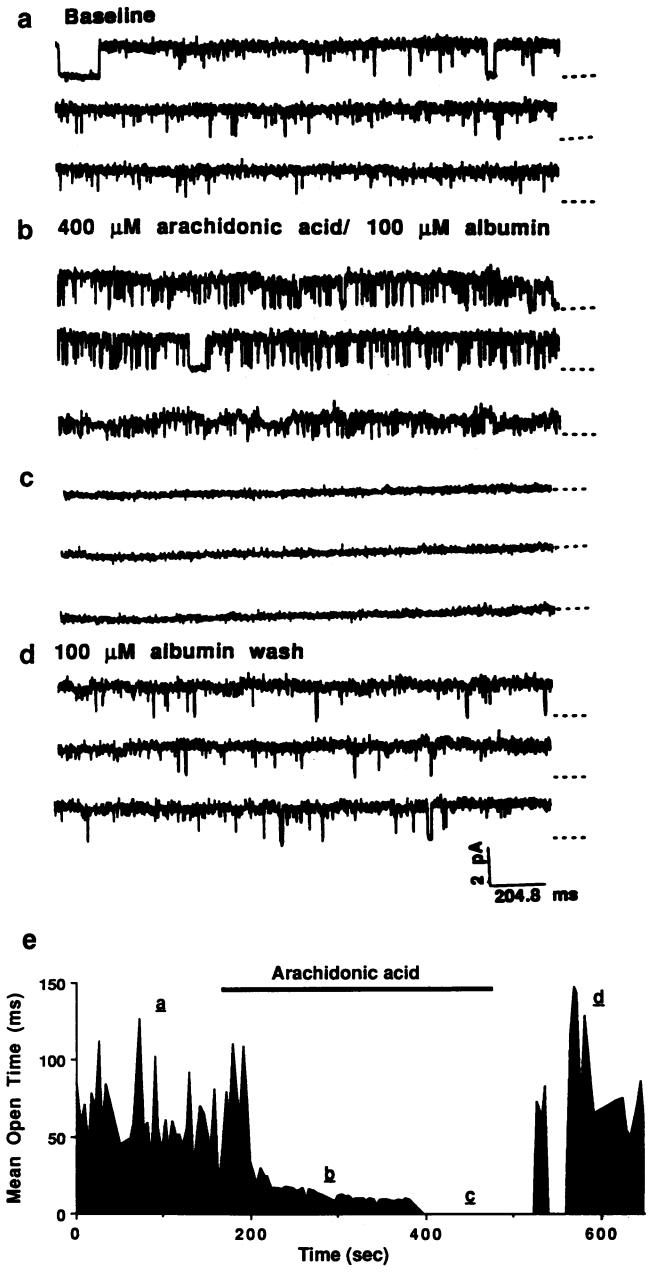

FIG. 1. Effect of arachidonic acid on a Cl^- channel. (*a*) Baseline, no additions. P_o was 0.92. (*b* and *c*) Arachidonic acid (400 μM) and 100 μM albumin (estimated 5 μM free arachidonic acid) added to the internal surface of the patch. Traces in *b* were obtained 130 s and traces in *c* were obtained 227 s after addition of arachidonic acid. (*d*) Albumin (100 μM) without arachidonic acid. P_o was 0.90. (*e*) Plot of mean open time vs. time for the same experiment. The regions labeled a, b, c, and d correspond to *a–d*. All tracings were obtained from the same membrane patch.

Prior to inactivation (defined as the channel remaining in the closed state), arachidonic acid often caused a rapid flickering between the open and closed state (Fig. 1*b*); as a result the mean open time decreased from 62.8 ± 3.9 to 11.6 ± 0.6 ms (Fig. 1 *b* and *e*) before complete closure (Fig. 1 *c* and *e*). In addition, current amplitude appeared to decrease sometimes, probably due to very rapid incompletely resolved

Table 1. Effect of fatty acids and arachidonic acid metabolites on Cl^- channels

Intervention	No. patches with channels inactivated by intervention/ no. total patches containing activated channels
Control	2/25
Arachidonic acid (20:4) (10 μM)	18/19*
Albumin (100 μM)	0/7
Arachidonic acid (400 μM)/albumin (100 μM)	7/7*
Arachidonic acid (200 μM)/albumin (100 μM)	0/6
Linoelaidic acid (18:2, trans) (40 μM)	4/5*
Myristoleic acid (14:1) (40 μM)	7/7*†
Oleic acid (18:1) (10 μM)	3/3*
$PGF_{2\alpha}$ (10 μM)	1/5
PGE_2 (10 μM)	1/5
LTB_4 (3–5 μM)	3/8
LTC_4 (10 μM)	3/8
LTD_4 (3–10 μM)	1/6
12-HETE (5–10 μM)	0/7
5-HPETE (10 μM)	0/4
15-HETE (10 μM)	0/4
Palmitic acid (16:0) (40 μM)	2/5
Myristic acid (14:0) (40 μM)	3/7
Caprylic acid (8:0) (100 μM)	1/7

Experiments were performed as described in legend of Fig. 1. Statistical significance was tested using χ^2 analysis for expected values ($n \geq 5$) and Fisher's exact test otherwise. Among those fatty acids and arachidonic acid metabolites in which inactivation did not occur (those without an *), no apparent change in channel kinetics was observed. PG, prostaglandin; LT, leukotriene; 12-HETE and 15-HETE, 12- and 15-hydroxyeicosatetraenoic acid, respectively; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid.

*$P < 0.05$ vs. control.

†Myristoleic acid did not always completely inactivate the channel, but in all seven cases it altered channel kinetics by causing flickering or an apparent decrease in amplitude (mean current always decreased by greater than 66%; for example, see Fig. 3).

transitions between the open and closed states (e.g., see the third trace in Figs. 1*b* and 3). These intermediate states are also observed during inactivation with 5-nitro-2-(3-phenylpropylamino)benzoic acid (21) and PKC (unpublished observation).

To determine if the effect of arachidonic acid was reversible, we again took advantage of the fatty acid binding sites present on albumin (19). Washing the cytosolic surface of the patch with buffer alone reactivated Cl^- channels on only one of eight occasions. However, when albumin (70–100 μM) was included in the wash buffer (Fig. 1*d*), Cl^- channels reactivated on seven of eight occasions in an average time of 176 ± 51 s.

The Ca^{2+} concentration bathing the cytosolic surface of the membrane determines the effect of PKC on the Cl^- channel (5) and may regulate other membrane-associated enzymes. However, changes of internal (cytosolic) Ca^{2+} concentration did not influence the ability of arachidonic acid to inhibit the Cl^- channel: channels were inactivated by 10 μM arachidonic acid when the internal Ca^{2+} concentration was <10 nM (3 of 3 cases), 100 nM (9 of 10 cases), 1 μM (6 of 6 cases), and 1 mM (2 of 2 cases).

Many of the cellular effects of arachidonic acid are mediated by cyclooxygenase and lipoxygenase metabolites (20). Previous studies of K^+ channels suggest that arachidonic acid metabolites can be generated in excised patches (22, 23). Therefore, we did several studies to determine whether metabolism of arachidonic acid is required for Cl^- channel inactivation. (*i*) We tested whether indomethacin (1 μM) and

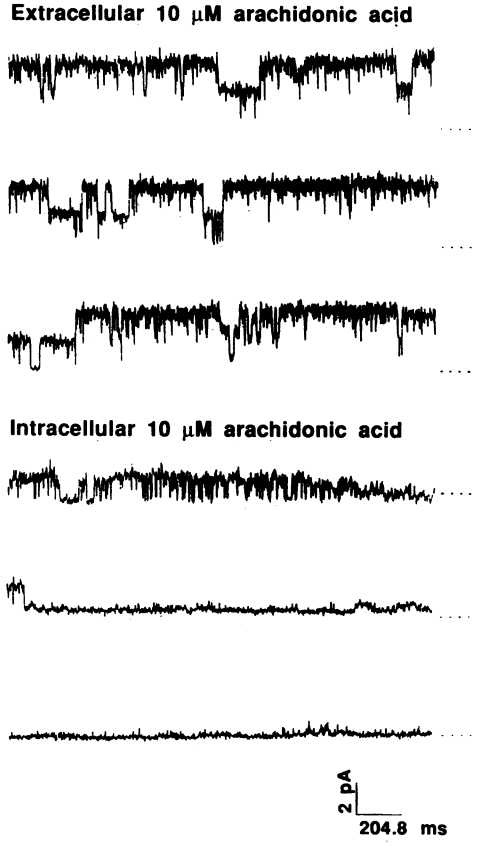

FIG. 2. Effect of arachidonic acid on the external and internal surface of a Cl^- channel. (*a*) Arachidonic acid (10 μM) was included in the external (pipette) solution prior to obtaining seal on the cell membrane. The Cl^- channel was activated with depolarization (+120 mV). Two channels were present in the patch. (*b*) Arachidonic acid (10 μM) added to the internal surface of the same membrane patch.

nordihydroguaiaretic acid (10 μM), which inhibit cyclooxygenase and lipoxygenase enzymes, respectively, prevent the arachidonic acid effect. During a 5-min incubation with these inhibitors, channels remained in the activated state, but, with subsequent addition of arachidonic acid (10 μM), channels inactivated in less than 2 min ($n = 5$).

(*ii*) We examined the regulatory effect of three fatty acids that are not substrates for the lipoxygenase or cyclooxygenase enzymes: linoelaidic acid, myristoleic acid, and oleic acid. Fig. 3 and Table 1 show that all three inhibited the Cl^- channel.

(*iii*) We tested the effect of a number of arachidonic acid metabolites. Airway epithelia have been shown to produce cyclooxygenase [prostaglandins E_2 and $F_{2\alpha}$ (9–11)] and lipoxygenase [leukotrienes D_4, C_4, and B_4; 12-hydroxyeicosatetraenoic acid (12-HETE); 5-hydroperoxyeicosatetraenoic acid (5-HPETE); and 15-hydroxyeicosatetraenoic acid (15-HETE) (11, 12, 24)] metabolites. None of these metabolites inactivated Cl^- channels as frequently as arachidonic acid (Table 1). Cytochrome P450 metabolites of arachidonic acid (20) are unlikely to mediate the effects of arachidonic acid because they require NAD(P)H, which was not present in the buffer. These data indicate that metabolism of arachidonic acid is not required to inactivate Cl^- channels and suggest that fatty acids may have a direct effect.

To explore the fatty acid structural requirements for Cl^- channel inactivation, we also tested the effect of three saturated fatty acids: palmitic acid (16:0), myristic acid (14:0), and caprylic acid (8:0) (Table 1). These fatty acids did not cause significant Cl^- channel inactivation. Myristoleic acid (14:1) inactivated a greater proportion of channels than

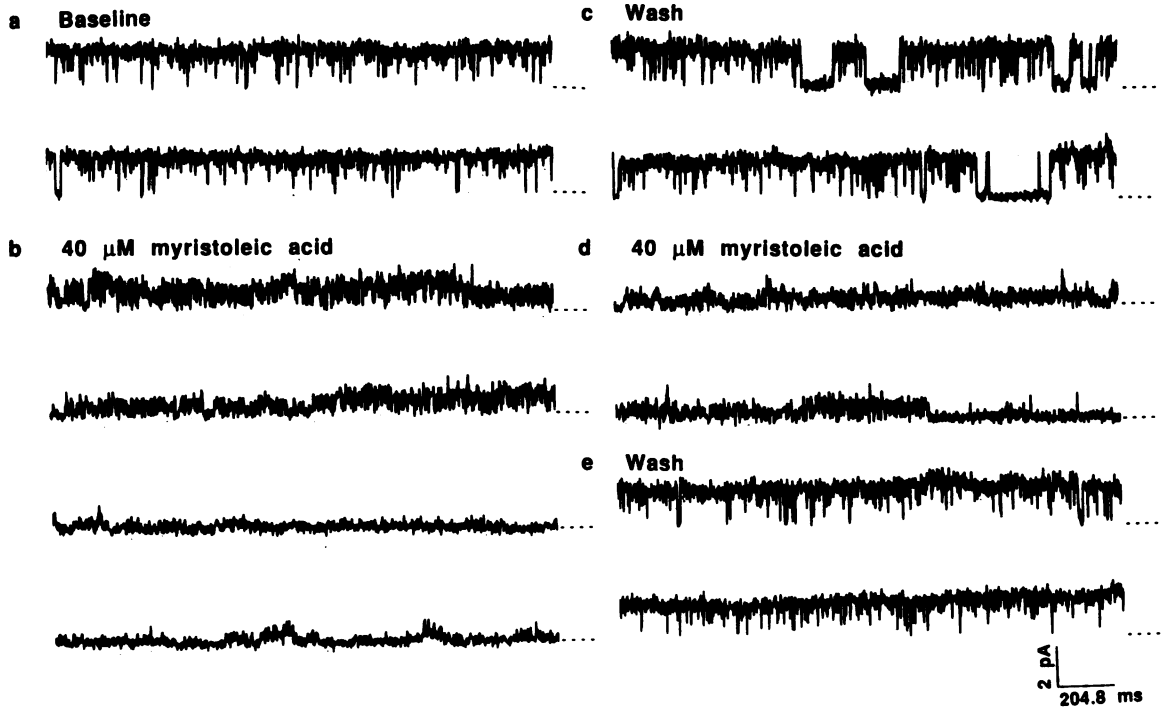

FIG. 3. Effect of myristoleic acid on a Cl^- channel. (*a*) Baseline, no additions (P_o = 0.93 and mean current was 2.02 ± 0.03 pA). (*b*) Myristoleic acid (40 μM) was added to the cytosolic surface of the patch (mean current was 0.63 ± 0.08 pA). (*c*) Control conditions after removing myristoleic acid "Wash" (P_o = 0.65 and mean current was 0.95 ± 0.05 pA). (*d*) Myristoleic acid (40 μM) (mean current was 0.60 pA). Because we were not able to determine P_o when the "apparent" current amplitude decreased, we report values of mean current. Although both myristoleic acid and arachidonic acid inhibited the channel, the myristoleic acid effects were readily reversible without the addition of albumin to the buffer, but reversal of arachidonic acid required albumin. All tracings were obtained from the same membrane patch.

myristic acid (14:0) ($P < 0.05$), suggesting that the presence of double bonds is important for inactivation.

Because regulation of Cl^- channels is defective in CF, we examined the effect of arachidonic acid on Cl^- channels from CF cells. Arachidonic acid inactivated CF channels (n = 3/3, one person), just as it did channels from normal human (n = 3/3, one person) and canine (n = 18/19) airway epithelial cells. A previous report (25) suggested that abnormal arachidonic acid metabolism occurs in CF lymphocytes (a lack of glucocorticoid-mediated inhibition of arachidonic acid release). One might speculate that elevated intracellular levels of arachidonic acid cause the abnormal Cl^- channel regulation in CF (2, 3, 5, 6, 26, 27). Although not tested directly, our observations suggest that this is not the case. We found that channels, in excised patches inactivated by arachidonic acid, could not be reactivated by depolarization (n = 8/8). In contrast, depolarization activates Cl^- channels in both normal and CF cells (17).

How do fatty acids inactivate the Cl^- channel? Our data indicate that inhibition is not dependent on fatty acid metabolism, phosphorylation (note that the internal solution did not contain ATP), or dephosphorylation (the effects were reversible and repeatable in the same patch). We considered the possibility that changes in membrane fluidity might be responsible. Cis-unsaturated fatty acids change a number of the physical properties of biological membranes, suggesting general changes in membrane lipid structure (28, 29). However, saturated fatty acids and trans-unsaturated fatty acids have either opposite effects or no effect (28, 29). As a result, it has been suggested that if protein function is affected by cis- but not trans-unsaturated fatty acids, the response may result from a change in the lipid structure. Our observation that linoelaidic acid (18:2, trans) inactivated the channel suggests that such changes in membrane lipid structure are not required for fatty acid inactivation of the Cl^- channel. The observation that arachidonic acid selectively inhibits from the intracellular surface of the Cl^- channel also argues against general changes in membrane structure.

Thus we speculate that fatty acids "directly" interact with the channel or an associated protein to alter its function. This conjecture is consistent with the observations that two enzymes, PKC (30, 31) and guanylate cyclase (32), are activated by arachidonic acid. In addition, fatty acids have been shown to regulate ion channels; fatty acids activate K^+ channels in gastric smooth muscle cells (13) and neonatal rat atria (14) and inhibit gap junctions in rat lacrimal glands (15). Furthermore, the general structure of a fatty acid resembles many of the synthetic Cl^- channel inhibitors (33).

We speculate that receptor-mediated increases of cellular arachidonic acid in the airway epithelia (G. Denning and M.J.W., unpublished observation) may have dual effects that serve to regulate Cl^- channels and hence the rate of transepithelial Cl^- secretion.

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