PKA and arachidonic acid activation of human recombinant ClC-2 chloride channels

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Tewari, Kirti P., Danuta H. Malinowska, Ann M. Sherry, and John Cuppoletti. PKA and arachidonic acid activation of human recombinant ClC-2 chloride channels. Am J Physiol Cell Physiol 279: C40–C50, 2000.—An HEK-293 cell line stably expressing the human recombinant ClC-2 chloride channel was used in patch-clamp studies to study its regulation. The relative permeability $P_{Cl}/P_{Cl}$ calculated from reversal potentials was $I_{Cl} > Cl^{-} = NO_{3}^{-} > SCN^{-} > Br^{-}$. The absolute permeability calculated from conductance ratios was $Cl^{-} = Br^{-} = NO_{3}^{-} > SCN^{-} > I^{-}$. The channel was activated by cAMP-dependent protein kinase (PKA), reduced extracellular pH, oleic acid (C:18 cis9, elaidic acid (C:18 trans9), arachidonic acid (AA; C:20 cisΔ5,8,11,14), and by inhibitors of AA metabolism, 5,8,11,14-eicosatetraynoic acid (ETYA; C:20 transΔ5,8,11,14), α-methyl-4-(2-methylpropyl)benzeneacetic acid (ibuprofen), and 2-phenyl-1,2-benziselenazol-3-[2H]-one (PZ51, ebselen). ClC-2 $Cl^{-}$ channels were activated by a combination of forskolin plus IBMX and were inhibited by the cell-permeant myristoylated PKA inhibitor (mPKI). Channel activation by reduction of bath pH was increased by PKA and prevented by mPKI. AA activation of the ClC-2 $Cl^{-}$ channel was not inhibited by mPKI or staurosporine and was therefore independent of PKA or protein kinase C activation.

pH-activated ion channels; gastric HCl secretion; lung chloride channels; cystic fibrosis; nonsteroidal anti-inflammatory agents; ibuprofen; ebselen; 5,8,11,14-eicosatetraynoic acid

IN EPITHELIAL AND NONEPITHELIAL tissues (2, 42) including stomach (3, 21, 38) and lung (1, 26, 36, 38), ClC-2 $Cl^{-}$ channels are widely distributed. Activation by voltage and pH have been documented in a variety of recombinant systems (2, 7, 15, 21, 38, 41). In contrast, IB3-1 cells, a bronchial epithelial cell line isolated from a cystic fibrosis patient that expresses the ClC-2 $Cl^{-}$ channel, and IB3-1 cells overexpressing human recombinant ClC-2, did not exhibit PKA-dependent activation of net $36Cl^{-}$ flux (36). The rat ClC-2 $Cl^{-}$ channel also did not exhibit PKA-activated $Cl^{-}$ currents (15). Precise details of previous attempts to demonstrate PKA activation of ClC-2 in cells have not appeared (15, 36). Differences in expression systems, the use of different activators of PKA, and the lack of use of phosphodiesterase inhibitors by others may underlie the reported lack of activation by PKA. In the present study, a cell-permeant form of the PKA inhibitor, myristoylated PKI (mPKI), was used to inhibit PKA and thus to determine whether there was endogenous PKA activation of $Cl^{-}$ currents. Forskolin was employed to activate adenylate cyclase, and IBMX was used to inhibit phosphodiesterases. The interdependence of PKA, pH, and AA effects was studied using PKA inhibitors and activators. The effect of the protein kinase C (PKC) inhibitor staurosporine was also studied.

AA and its metabolites serve as lipid first or second messengers (29) in regulation of ion channels. AA is released from membrane phospholipids through the action of phospholipase A1 and phospholipase C. Nonsteroidal anti-inflammatory agents (NSAIDS) such as ibuprofen (α-methyl-4-(2-methylpropyl)benzeneacetic acid) are inhibitors of cyclooxygenase 1 and 2 (27, 43, 46) and would be expected to increase levels of AA (23). ETYA (5,8,11,14-eicosatetraynoic acid, C:20 transΔ5,8,11,14) is a nonmetabolized analog of AA that inhibits cyclooxygenase and lipoxygenase (23). Ebselen is also an inhibitor of cyclooxygenase and lipoxygenase (10, 12, 44). These channels participate. In the present study, the human recombinant form of the ClC-2 $Cl^{-}$ channel was studied in a stably transfected HEK-293 cell line. HEK-293 cells have low endogenous $Cl^{-}$ currents and have been well characterized for electrophysiological studies of ClC $Cl^{-}$ channels (31).
drugs were tested to determine whether inhibition of metabolism of AA also increased Cl− currents.

Cis-unsaturated fatty acids including oleic acid (C:18 cisΔ9), AA (C:20 cisΔ5,8,11,14), and ETYA(C:20 transΔ5,8,11,14) and trans-unsaturated fatty acids including elaidic acid (C:18 transΔ9) activate K+ channels, whereas saturated fatty acids are without effect (32). The effects of these fatty acids on CIC-2 Cl− channels were examined to compare with the more thoroughly studied effects of fatty acids on K+ channels.

The present study confirms and extends studies of PKA and pH activation of the human CIC-2 Cl− channel using patch-clamp studies, reports expected differences between ion selectivity obtained from current measurements (15, 36, 42) and those determined from zero current reversal potentials (3, 21), and demonstrates for the first time that AA activates CIC-2 Cl− channels. The present study allows a comparison between the effects of AA and other fatty acids on CIC-2 Cl− channels and previous studies on other channels, including K+ channels (23, 32), and studies with cyclooxygenase and lipoxygenase inhibitors suggest new mechanisms of action of NSAIDs. These results may be important for understanding regulation of these channels in the physiological processes in which they participate.

MATERIALS AND METHODS

Transfection of HEK-293 cells. Frozen aliquots of HEK-293 cells were obtained from American Type Culture Collection (ATCC), thawed, and maintained at 37°C in 5% CO2 in MEM supplemented with 5% heat-inactivated horse serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were transfected with 15 μg of His- and T7-tagged human CIC-2 cDNA in the mammalian expression vector pcDNA3.1 using Lipofectamine for 5 h at 37°C in serum-free medium. Cells were returned to serum-containing medium, maintained for 2 wk in serum-containing medium, and then grown in the presence of 300 μg/ml G418. The surviving cells were then tested for expression of Cl− currents and CIC-2 mRNA. Mock transfected cells were prepared using the same vector but expressing a different protein. Positive cell lines were grown and kept frozen as stocks.

Measurement of whole cell Cl− currents. Currents were elicited by voltage-clamp pulses (1,500-ms duration) between +40 and −140 mV in 20-mV increments from a beginning holding potential of −30 mV. Currents were measured 50–100 ms after start of the pulse. The external solution was normal Tyrode solution containing (in mM) 135 NaCl, 1.8 CaCl2, 1 MgCl2, 5.4 KCl, 10 glucose, and 10 HEPES (pH 7.35 or 6.5, as indicated). The pipette solution was (in mM) 130 CsCl, 1 MgCl2, 5 EGTA, and 10 HEPES (pH 7.35). Freshly prepared AA, ETYA, and ibuprofen were dissolved in DMSO and diluted into the bath solution resulting in a final concentration of 1% DMSO. The free acid form of AA was used. This was shipped under inert gas. All precautions recommended by the manufacturer were taken to prevent oxidation of AA, including use of solutions in organic solvent, storage of stock solutions in organic solvent at −20°C in sealed containers, and protection from light. In some cases, DMSO was bubbled with nitrogen before preparation and immediate use of the solutions. All measurements with compounds in DMSO were compared with controls containing 1% DMSO alone. Pipettes were prepared from borosilicate glass and pulled by a two-stage Narkashige puller to give 1–1.5 MΩ resistance. Data was acquired with an Axopatch CV-4 headstage with a Digi- data 1200 digitizer and an Axopatch 1D amplifier. Data was analyzed using pCLAMP 6.04 software (Axon Instruments, Foster City, CA), Lotus 123 (Microsoft), and Origin (Microcal).

Statistical significance of the difference between two means was determined with the Student’s t-test using n, the number of cells. I-V (current-voltage relationship) curves were fit using the least-squares method to obtain slopes and reversal potentials. Shifts of reversal potential were used to calculate relative permeabilities according to the relationship (11)

\[
D_{rev,X} = E_{rev,X} - E_{rev,Cl} = \frac{RT}{zF} \ln \frac{P_X[X^-]}{P_{Cl^-}[Cl^-]}
\]

where \(E_{rev,Cl}\) is the reversal potential with normal bath Cl− and \(E_{rev,X}\) is the reversal potential upon near total substitution of bath Cl− with the test anion, and where \(R\) is the gas constant, \(T\) is absolute temperature, \(z\) is valance, and \(F\) is Faraday’s constant.

Materials. ETYA: C:20 transΔ5,8,11,14; mPKI: protein kinase A inhibitor 14−22 amide myristoylated Mvr-N-Gly-Ary-Thr-Gly-Ary-Ary-Asn-Ala-Ile-NH2, trifluoroacetate salt; AA: ETYA 20.4 (C:20 cisΔ5,8,11,14); oleic acid: cis 9-octadecanoic acid 18.1 (C:18 cisΔ9); palmitic acid; hexadecanoic acid; ibuprofen: alpha-methyl-4-(2-methylpropyl)benzeneacetic acid; and staurosporine were obtained from Calbiochem. Elaidic acid: trans 9 octadecanoic acid 18.1 (C:18 transΔ9), IBMX, ATP, HEPES, inorganic and organic salts were from Sigma Chemical. Sylgard Isomer 184 was from Dow Corning. HEK-293 cells were obtained from ATCC. MEM, Lipopectamine, and G418 were obtained from GIBCO. pcDNA3.1 was from Invitrogen. Borosilicate glass (#7052) was obtained from Garner Glass.

RESULTS

PKA and pH activation of human CIC-2 Cl− channels. Figure 1A shows typical whole cell Cl− currents of nontransfected HEK-293 cells in the basal state (left), with 5 μM forskolin plus 20 μM IBMX (center), and with subsequent reduction of bath pH from pH 7.35 to pH 6.5 (right). As summarized in Fig. 1E, there was no significant effect of forskolin plus IBMX or reduction of bath pH on Cl− currents of nontransfected cells. In contrast, Fig. 1B shows that typical whole cell Cl− currents of transfected cells in the basal state (left) are increased after treatment with forskolin plus IBMX (center) and further increased after subsequent reduction of the pH of the bath solution from pH 7.35 to pH 6.5 (right). As summarized in Fig. 1, D and E, forskolin plus IBMX significantly increased (P < 0.001) Cl− currents at −140 mV from −135 ± 14 pA to −569 ± 42 pA (n = 19). In a separate set of experiments to test the effect of extracellular pH, basal currents of −146 ± 35 pA were significantly increased (P < 0.001) to −499 ± 76 pA with forskolin plus IBMX at pH 7.4, and these currents further significantly increased (P < 0.001) to −811 ± 73 pA (n = 7) with subsequent reduction of bath pH to pH 6.5. Cl− currents of mock transfected cells were not increased by forskolin plus IBMX (−66 ± 17 pA vs. −73 ± 34 pA, n = 3), not shown.
Effect of pH on basal Cl\textsuperscript{−} currents. Figure 1B, left, shows typical Cl\textsuperscript{−} currents in transfected HEK-293 cells at pH 7.35 after treatment with forskolin + 20 μM IBMX (center), and after subsequent reduction of bath pH to pH 6.5 (right). These small pH effects are in contrast to the large effects of pH on forskolin plus IBMX activated channels (Fig. 1, D and E). The effect of bath pH on Cl\textsuperscript{−} currents after treatment with PKA inhibitors was therefore investigated to determine whether pH effects required prior activation.

Effect of pH with inhibition of PKA. Figure 1C, center and right, show typical Cl\textsuperscript{−} currents of cells treated with 0.4 μM mPKI and forskolin + 20 μM IBMX at pH 7.35 (●) and pH 6.5 (×), and cells treated with PKA activators + 0.4 μM mPKI at pH 7.35 (+) and pH 6.5 (−).
7.35 and was continued upon reduction of pH. As summarized in Fig. 1, D and E, 0.4 μM mPKI prevented activation by 5 μM forskolin plus 20 μM IBMX, and there was no significant increase in Cl\(^{-}\) currents upon reduction of pH when PKA was inhibited by mPKI. In this expression system, endogenous or exogenous activation of PKA is required for pH activation of ClC-2 Cl\(^{-}\) channels.

**Effect of sodium nitroprusside.** Sodium nitroprusside is an activator of guanylate cyclase. Ten micromolar sodium nitroprusside increased Cl\(^{-}\) currents in transfected cells at all tested holding potentials (Fig. 2A). Conductance ratios (absolute permeabilities) for the halides were Br\(^{-}\) (1.03 ± 0.02) = Cl\(^{-}\) (1.0) > I\(^{-}\) (0.68 ± 0.06); n = 3. Conductance ratios for the nonhalides were Cl\(^{-}\) (1.0) > SCN\(^{-}\) (0.85 ± 0.06) = NO\(_3\)\(^{-}\) (0.74 ± 0.22) > I\(^{-}\) (0.68 ± 0.06); n = 3. SO\(_4\)\(^{2-}\).

**Ion selectivity of human ClC-2 Cl\(^{-}\) channels from current measurements.** I-V curves for transfected cells treated with forskolin plus IBMX were generated with symmetrical Cl\(^{-}\) and with nearly bi-ionic substitution of bath Cl\(^{-}\) with test anions for three cells and are shown in Fig. 3A. Ratios of slopes and differences in reversal potentials (MATERIALS AND METHODS) after changing from Cl\(^{-}\) to test anions in the bath were used to determine absolute and relative permeabilities, respectively.
Currents were obtained in Cl\textsuperscript{−} and then with substitution of NaCl with the NaCl solutions (MATERIALS AND METHODS) and then with substitution of NaCl with the Na\textsuperscript{+} salt of the indicated anion in the bath. The ratios of the slopes of the I-V curve for Cl\textsuperscript{−} vs. test anions were used to determine the absolute permeabilities (conductance ratios) and the differences in the reversal potentials with substitutions of test anions were used to calculate the relative permeabilities, P\textsubscript{X}/P\textsubscript{Cl\textsuperscript{−}}. The summarized data for 3 cells are shown for control Cl\textsuperscript{−} (■), Br\textsuperscript{−} (○), NO\textsubscript{3}\textsuperscript{−} (▲), SCN\textsuperscript{−} (▼), I\textsuperscript{−} (●), and SO\textsubscript{4}\textsuperscript{2−} (+). B: an expanded view of the I-V curve for Cl\textsuperscript{−} (■), Br\textsuperscript{−} (○), and I\textsuperscript{−} (●). Data are plotted as means ± SE; n = 3 cells.

(0.3 ± 0.05) gave the lowest conductance ratio. The ratios of conductance are similar to those reported by others (7, 42). The relative permeabilities, P\textsubscript{X}/P\textsubscript{Cl\textsuperscript{−}} for the halides were: I\textsuperscript{−} (1.11 ± 0.04) > Cl\textsuperscript{−} (1.0) = Br\textsuperscript{−} (1.0); n = 3. P\textsubscript{I\textsuperscript{−}}/P\textsubscript{Cl\textsuperscript{−}} was significantly greater (P < 0.01) than for Cl\textsuperscript{−}. This is the same sequence for P\textsubscript{X}/P\textsubscript{Cl\textsuperscript{−}} that was previously reported for the native and recombinant rabbit channel (3, 21). P\textsubscript{X}/P\textsubscript{Cl\textsuperscript{−}} for the other anions anions were I\textsuperscript{−} > NO\textsubscript{3}\textsuperscript{−} = SCN\textsuperscript{−} ≈ Br\textsuperscript{−}. Figure 3B shows a portion of an I-V curve for a typical cell that was expanded to more clearly show shifts in reversal potentials for I\textsuperscript{−}, Cl\textsuperscript{−}, and Br\textsuperscript{−}.

Effect of AA. Figure 4, A and B, respectively show Cl\textsuperscript{−} currents of transfected cells before and after treatment with 1 μM AA. As shown in Fig. 4C, AA increased Cl\textsuperscript{−} currents at all holding potentials tested. The increase in Cl\textsuperscript{−} currents was dose dependent with a K\textsubscript{0.5} of 0.35 μM (Fig. 4D). As summarized in Fig. 4E for current measurements at −140 mV, 1 μM AA significantly increased (P < 0.01) Cl\textsuperscript{−} currents from −106 ± 15 pA to −2,639 ± 389 pA (n = 6). Cl\textsuperscript{−} currents of nontransfected cells were not affected by 1 μM AA (Fig. 4E). Currents at −140 mV in mock transfected cells were also not affected by 1 μM AA (−34 ± 11 pA vs. −27 ± 6.4 pA, n = 3), not shown.

Independence of PKA and AA effects. Figure 4E shows that Cl\textsuperscript{−} currents of mPKI-treated cells were also activated (P < 0.05) by 1 μM AA from −296 ± 23 pA to −2,311 ± 504 pA (n = 4), a current level that was not significantly different from that in the absence of mPKI (−2,639 ± 426 pA; Fig. 4E). Thus AA activation of ClC-2 Cl\textsuperscript{−} currents is independent of PKA action. Cl\textsuperscript{−} currents at −140 mV of nontransfected cells were not affected by 1 μM AA (Fig. 4E) or 0.4 μM mPKI (−81.8 ± 43 pA, n = 3), not shown.

Effect of PKC inhibition on AA effects. PKC has been implicated in some studies of AA effects on ion channels (23). AA activates PKC with a K\textsubscript{0.5} of ~100 μM (28). Control currents at −140 mV of −251 ± 52 pA were not significantly different after addition of 10 nM of the PKC inhibitor staurosporine, −394 ± 102 pA (n = 3). Subsequent treatment with 1 μM AA significantly increased (P < 0.001) currents to −1,736 ± 134 pA (n = 3; data not shown), a level not significantly different from the level of activation by 1 μM AA in the presence or absence of mPKI (data not shown). Inhibition of PKC and other protein kinases by staurosporine did not prevent activation by AA, suggesting that PKC is not involved in AA activation of ClC-2.

Effect of ETYA. ETYA is a nonmetabolized analog of AA that inhibits cyclooxygenase and lipoxygenase (23). ETYA increased Cl\textsuperscript{−} currents at all holding potentials tested (Fig. 5A). The effect of ETYA was dose dependent with a K\textsubscript{0.5} of 3 μM (Fig. 5B). Basal Cl\textsuperscript{−} currents of transfected cells at −140 mV were significantly increased (P < 0.01) from −300 ± 87 pA (n = 16) to −3,594 ± 410 pA (n = 4) with 10 μM ETYA (Fig. 5E), but currents of nontransfected cells were not affected (Fig. 5E). These results suggest that the effect of AA (or ETYA acting as an analog of AA) may be due to direct effects on the channel.

Effect of ibuprofen and ebselen. Ibuprofen is an inhibitor of cyclooxygenase 1 and 2 (27, 43, 46). As shown in Fig. 5C, ibuprofen activated Cl\textsuperscript{−} currents in transfected cells at all holding potentials tested. The effect of ibuprofen was dose dependent with a K\textsubscript{0.5} of 300 μM (Fig. 5D). As summarized in Fig. 5E, Cl\textsuperscript{−} currents at −140 mV significantly increased (P < 0.02) from −91 ± 12 pA (n = 16) to −518 ± 57 pA (n = 3) with 200 μM ibuprofen and increased (P < 0.05) to −3,101 ± 676 pA (n = 3) with 400 μM ibuprofen. There was no effect of ibuprofen on nontransfected cells (Fig. 5E). Ebselen (PZ51) is a peroxide scavenger that is structurally and chemically unrelated to the NSAIDS but that inhibits cyclooxygenase and lipoxygenase (12, 19). One hundred
Fig. 4. Effect of arachidonic acid (AA). Representative recordings of Cl⁻ currents of transfected cells before (A) and after (B) treatment with 1 μM AA are shown. C: I-V curves for AA at the indicated concentrations. D: the dose-response curve for the summarized data measured at -140 mV. E: the summarized data for transfected and nontransfected cells. Data in C–E are given as means ± SE; n = number of cells, shown in parentheses. AA was dissolved in DMSO, and all measurements of control and treated cells were carried out in 1% DMSO. *P < 0.01; **P < 0.05 with respect to control.
micromolar ebselen significantly increased \((P < 0.01)\) currents at \(-140\) mV from \(-130 \pm 32\) pA to \(-1,485 \pm 147\) pA \((n = 3, \text{not shown})\). This supports the view that inhibition of AA metabolism pathways can lead to activation of ClC-2 Cl\(^-\) channels.

**Effect of other fatty acids.** K\(^+\) channels have been shown to be activated by AA, ETYA, and other satu-
rated fatty acids (32). Oleic acid (100 μM), a cis-saturated C18 fatty acid, significantly increased \( P < 0.001 \) Cl⁻ currents at \(-140 \text{ mV from } -300 \pm 87 \text{ pA to } -2,120 \pm 310 \text{ pA (n = 4)}, \) not shown. Elaidic acid (100 μM), a trans-unsaturated C18 fatty acid, significantly increased \( P < 0.01 \) currents from \(-195 \pm 94 \text{ pA to } -1,891 \pm 433 \text{ pA (n = 4)}, \) not shown. Palmitic acid (100 μM), a saturated C16 fatty acid, did not affect currents \( (n = 3), \) not shown. CIC-2 Cl⁻ channels exhibit the same pattern of activation with fatty acids as the TREK-1 K⁺ channel (32). Oleic acid is an activator of PKC at these high concentrations (25), but elaidic acid does not affect PKC (39). The activation of CIC-2 by both cis- and trans-unsaturated C18 fatty acids, together with a lack of effect of staurosporine on AA activation, strengthens the argument that PKC is not involved in the activation by fatty acids. Effect of intracellular Ca²⁺. Ten micromolar ionomycin plus 2.8 mM Ca²⁺ failed to cause an increase in Cl⁻ currents in either nontransfected or transfected cells \( (n = 3), \) not shown. The lack of Ca²⁺ dependence has been previously noted for CIC-2 expressed in oocytes (3, 7, 21, 38, 41).

**DISCUSSION**

An HEK-293 cell line stably expressing human recombinant CIC-2 mRNA and function was used in patch-clamp studies to investigate the effects of PKA activators and inhibitors, pH, and AA. The relative permeability, \( P_X/\Phi_{Cl} \), from reversal potentials were \( \Gamma^- > Cl^- = NO_3^- = SCN^- > Br^- \). The absolute permeability from conductance ratios was \( Br^- > Cl^- = NO_3^- = SCN^- > \Gamma^- > SO_4^{2-} \). The channel was activated by a combination of forskolin plus IBMX, and this activation was inhibited by the cell-permeant form of the PKA inhibitor mPKI. The channel exhibited activation by pH that was increased with forskolin plus IBMX and abolished by mPKI. Sodium nitroprusside, an activator of guanylate cyclase, was shown to be an activator, but Cl⁻ current activation was inhibited by mPKI, suggesting crossover activation of PKA by cGMP. Increased intracellular Ca²⁺ was without effect on human CIC-2 Cl⁻ currents. AA at submicromolar concentrations activated Cl⁻ currents, and this activation was not inhibited by mPKI or staurosporine. Inhibition of cycloxygenase with ibuprofen, or both cyclooxygenase and lipoxygenase with ETYA or ebselen, mimicked the effect of AA.

Patch-clamp studies of transfected cells are widely used for studies of the regulation of ion channels. HEK-293 cells exhibit a low intrinsic Cl⁻ current and have been widely utilized for studies of ion channels, including the CIC-2 Cl⁻ channel (31). Previous studies of human CIC-2 in HEK-293 cells have employed a transient expression system. The stably transfected cell line used in the present studies gave reproducible Cl⁻ currents in the basal state and after treatment with various agents, facilitating comparisons among various treatments. The Cl⁻ currents in this cell line were activated by treatment with forskolin plus IBMX, as expected from the presence of numerous consensus phosphorylation sites on human CIC-2 and from previous studies of the rabbit (3, 21, 41) and human (38) recombinant CIC-2 channels reconstituted in planar lipid bilayers. Forskolin was combined with IBMX to inhibit phosphodiesterase action. Previous attempts in IB3-1 clonal cell lines transfected with human recombinant CIC-2 Cl⁻ channels failed to show 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate plus forskolin stimulated 36Cl⁻ efflux (36). Similar failures were reported when cAMP levels were raised in Xenopus laevis oocytes expressing human CIC-2 under conditions sufficient to stimulate cystic fibrosis transmembrane conductance regulator (CFTR) (15). In the former study, phosphodiesterase inhibitors were not used, and in the latter study, the agents or conditions employed were not reported. Inhibitors of PKA were employed in the present study to determine whether there was basal activation of the channels by endogenous PKA. The rationale for the use of the PKA inhibitor in the present study was similar to that used in recent studies of physiologically relevant cAMP stimulation of Ca²⁺ channel α-subunits in both Xenopus laevis oocytes and HEK-293 cells. The Ca²⁺ channel is fully phosphorylated in the basolateral state (33, 34) in both of these expression systems. It was, however, possible to study reversible regulation of Ca²⁺ channels by PKA after first inhibiting endogenous PKA with protein kinase inhibitors. The results with the cell-permeant PKA inhibitor, mPKI, shows that endogenous basal PKA activity is low in the HEK-293 cell line used here. The level of endogenous activation of PKA in the basal state was not established in other expression studies (7, 9, 31, 36, 42).

Block of PKA activation by mPKI did not affect subsequent AA activation. This shows that AA activation is independent of PKA activation and that myristoylated PKI did not have deleterious effects on expression of CIC-2 Cl⁻ currents. In contrast to the lack of mPKI effect on AA stimulation, prior treatment of the cells with mPKI in the presence of forskolin plus IBMX abolished Cl⁻ channel activation by reduced extracellular pH. In other studies where only pH activation of CIC-2 currents have been demonstrated (15, 36), one possibility is that endogenous basal activation by PKA or other activators may have been higher than in the present studies.

In our previous studies (3, 21), zero current reversal potentials were obtained from single channel studies under nearly bi-ionic conditions using the channel from native tissues or the recombinant channel expressed in oocytes and reconstituted into planar lipid bilayers. The reversal potential measurements were then used in the zero current form of the Goldman-Hodgkin-Katz equation (in Ref. 11) to determine the permeability ratios, \( P_X/\Phi_{Cl} \), (11), which were \( \Gamma^- > Cl^- > Br^- > NO_3^- \) (3, 21). In the present study, the relative permeability ratios were identical to those obtained in bilayer studies. The ratios of the slopes of the I-V curves (conductance ratios) gave ion selectivity as absolute ionic permeabilities (11) of \( Cl^- > Br^- > NO_3^- > SCN^- > \Gamma^- > SO_4^{2-} \). This sequence is similar to that found for rat CIC-2 by others (42). The difference be-
between permeability ratios defined by currents and permeability ratios determined by zero current reversal potentials is not unexpected. To quote Hille, it is “...a quite common finding that absolute permeabilities determined from conductances or sizes of currents do not agree with permeability ratios determined from reversal potentials” (11). This is because the two different measurements depend on different properties of the pore. Absolute permeabilities obtained from measurements of currents are sensitive to block and partial saturation, whereas those obtained from zero current reversal potentials are not.

AA effects on ion channels are sometimes mediated through the activation of PKC (23). PKC inhibition of recombinant rat ClC-2 currents in neurons has been demonstrated (40). ETYA inhibits PKC in vitro (28) and ETYA prevents activation of PKC in vivo by inhibition of the lipoxygenase pathway mediators that activate PKC in vivo (8, 30).

The cis-unsaturated C:18 fatty acid, oleic acid, activated ClC-2 and also activates PKC isozymes at high concentrations (K_{d}, 50 μM) (16, 25). The trans C:18 unsaturated fatty acid, elaidic acid, and the saturated C:16 fatty acid, palmitic acid, do not activate PKC (39). The activation of ClC-2 by elaidic acid suggests that PKC is not involved in fatty acid activation of ClC-2. In intact cells, PKC pathways can be activated by metabolites of palmitic acid, but these actions occur at the transcriptional level and require long periods of incubation. AA does not act through that pathway (35). Staurosporine did not affect activation of ClC-2 by AA. A similar lack of involvement of PKC in AA activation of a K⁺ channel was demonstrated using staurosporine (4). ETYA activates ClC-2 (present study) and inhibits PKC (28). Elaidic acid (present study) activates ClC-2 and is without effect on PKC (39). Staurosporine inhibits PKC without affecting AA activation. Taken together, these results demonstrate that activation of ClC-2 by fatty acids does not involve PKC.

The TREK-1 K⁺ channel responds similarly to AA, ETYA, and oleic, elaidic, and palmitic acids (32). This suggests a similar mechanism of action of fatty acids in activation of these two types of channels. Patel et al. (32) have suggested that activation of the TREK-1 K⁺ channel by fatty acids involves preferential insertion of negatively charged amphiphiles into the external leaflet of the bilayer, causing a change in the structure of the membrane. The structural change in the membrane activates the channel through a charged sensor region on the K⁺ channel. It is tempting to speculate that fatty acid activation of ClC-2 might occur through a similar mechanism.

AA and inhibitors of AA metabolism have been reported to inhibit or activate a large variety of ion channels (5, 13, 14, 20, 23, 24, 29, 32). AA may either bind directly to ion channels (23, 29) or indirectly affect ion channels through local modification of membrane structure (20, 32, 37). When the metabolism of endogenous AA was blocked by ETYA (23) or the cyclooxygenase inhibitor ibuprofen (27, 45, 46), the channel was activated, suggesting an indirect action of ibuprofen on channel activity.

An alternate possibility is that ibuprofen binds directly to the channel. NSAIDS, including flufenamic acid, mefenamic acid, and niflumic acid (but not ibuprofen) inhibit nonspecific cation channels (6). At least one compound that is chemically related to NSAIDS, 5-nitro-2-(3-phenylpropylamino)benzoic acid, potently inhibits Cl⁻ channels (45). All NSAIDS are structurally related because they fit into the binding groove of cyclooxygenase isozymes and exclude substrate from binding (18, 22). A different class of inhibitor of cyclooxygenase and lipoxygenase unrelated in structure to the NSAIDS was required to test the hypothesis that inhibition of AA metabolism was responsible for ClC-2 activation by ibuprofen. Cyclooxygenase requires hydroperoxides for initiation of the cyclooxygenase reaction. Peroxide scavengers such as glutathione peroxidase block the activity of cyclooxygenase 1 and 2 (19) and also block lipoxygenase (12, 44). Ebselen is a glutathione peroxide mimetic that has been previously shown to block cyclooxygenase and lipoxygenase activity by reducing the reactive oxygen species required for the activity of these enzymes (10). As such, ebselen is not an NSAID, acts by a different mechanism than ibuprofen, and inhibits the metabolism of AA. In the present studies, ebselen increased ClC-2 Cl⁻ channel activity, strengthening the argument that inhibition of AA metabolism will affect ClC-2 and other channels (23) such as K⁺ channels, (32) which are affected by AA and other fatty acids.

The activating effects of fatty acids are in sharp contrast to the inhibitory effect of AA on the 45-pS outward rectifier of normal airway and T84 cells (13, 14) and the inhibitory effect of ibuprofen on CFTR (5). Given that the ClC-2 channel is present in the lung and has been suggested to be an alternative pathway for Cl⁻ transport in the lung (1, 26, 38), this channel, unlike CFTR (5) or the outward rectifier (13, 14), may be activated during ibuprofen therapy of cystic fibrosis patients (17). AA activation of ClC-2 Cl⁻ channels may have other larger implications in NSAID treatment. The present results suggest that NSAID effects on ion channels may have physiological and pathophysiological consequences in addition to effects on pain and inflammation.

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