INTESTINAL CHLORIDE SECRETION is modulated by changes in cellular adenosine 3′,5′-cyclic monophosphate (cAMP) [e.g., vasoactive intestinal polypeptide (VIP)], guanosine 3′,5′-cyclic monophosphate (cGMP) [e.g., heat-stable enterotoxin of Escherichia coli], and Ca2+ (e.g., acetylcholine) in response to secretory agonists (3, 4, 7, 24). Adenosine is released by invading neutrophils and eosinophils during an inflammatory response and thus may act as a stimulus for diarrhea in intestinal inflammatory diseases (31, 32, 41). Although adenosine was shown to be a potent Cl− secretagogue, changes in these classical second messengers were not detected (6, 12, 32). However, adenosine was shown to increase cellular arachidonic acid (AA) levels in T84 cells, and its secretory effects could be blocked by inhibitors of both phospholipase A2 and 1,2-diacylglycerol (DAG) lipase (5, 6); both enzymes catalyze AA generation (36). More recently, VIP (cAMP) and E. coli heat-stable enterotoxin (cGMP) have been shown to increase AA in T84 cells (5), in addition to their effects on cAMP production. Inhibition of DAG lipase resulted in a partial inhibition of these Cl− secretory responses (5). These results suggest that AA may be an important second messenger in modulating Cl− secretion in intestinal diarrheal disease. However, the apical and basolateral ion channels responsible for carrying the secretory current in response to AA have not been identified.

AA is a well-known modulator of K+, Na+, Ca2+, and Cl− channels in a variety of tissues (33, 38). Importantly, fatty acids have been shown to modulate Cl− channels in secretory epithelia. AA was shown to inhibit an outwardly rectifying Cl− channel in airway epithelia (2, 17) as well as a volume-sensitive Cl− conductance in intestinal epithelium (23). Also, cAMP-mediated Cl− secretion is inhibited by AA in airway epithelia (34). However, these inhibitory effects are incongruent with the secretory response associated with elevated AA. In our companion paper (10), we demonstrate that the basolateral membrane K+ channel activated by Ca2+-dependent agonists (KCa) is potentially inhibited by AA. To our knowledge, neither an intestinal K+ conductance (GK) nor Cl− conductance has been shown to be upregulated directly by AA, as would be required if elevated AA stimulates Cl− secretion under some conditions. During the course of our studies on the role of AA in regulating KCa, we identified a large-conductance K+ channel that is activated by AA. We have characterized this channel’s K+ /Na+ selectivity, blocker sensitivity, and its regulation by fatty acids. We speculate that this channel may be important in carrying K+ across the basolateral membrane during Cl− secretory responses in which AA serves as a stimulatory second messenger.

METHODS

Cell culture T84 cells were grown in Dulbecco's modified Eagle's medium and Ham's F-12 (1:1) supplemented with 15 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES), 14 mM NaHCO3, and 10% fetal bovine serum. The cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. Patch-clamp experiments were performed on single cells plated onto glass coverslips 16–48 h before use.

Solutions. During inside-out patch-clamp recordings, the bath contained (in mM) 145 potassium gluconate, 5 KCl, 1 MgCl2, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 10 HEPES (pH adjusted to 7.2 with KOH). A free Ca2+ concentration of <10 nM was chosen for these experiments to eliminate the activity of the KCa we previously described in these cells (9). The pipette solution contained (in mM) 140 potassium gluconate, 5 KCl, 1 CaCl2, 1 MgCl2, and 10 HEPES (pH adjusted to 7.2 with KOH). For outside-out recordings, the bath contained 1 mM CaCl2 in the
absence of any added EGTA, whereas the pipette solution Ca\(^{2+}\) was buffered to <10 nM with EGTA (1 mM).

Single-channel recording. Single-channel currents were recorded using a List EPC-7 amplifier (Medical Systems) and recorded on videotape for later analysis as described previously (9). Pipettes were fabricated from KG-12 glass (Wilmad Glass). All recordings were done at a holding voltage of ~100 mV unless otherwise noted. The voltage is referenced to the extracellular compartment, as is the standard method for membrane potentials. Inward currents are defined as the movement of positive charge from the extracellular compartment to the intracellular compartment and are presented as downward deflections from baseline in all recording configurations.

Single-channel analysis was performed on records sampled at 8 kHz after low-pass filtering at 2 kHz. The average length of record analyzed to determine channel open probability (P\(_o\)) was 154 ± 4 s (n = 78). P\(_o\) was calculated, using Biopatch software (version 3.11; Molecular Kinetics), from the mean total current (I) divided by the single-channel current amplitude (i) and the maximum number of channels observed in a patch (N), such that P\(_o\) = I/N. The i was determined from the amplitude histogram of the current record, and N was determined from a visual inspection of the record after activation of the channel by a maximal concentration of AA (3–10 µM).

For determining mean open and closed times, an open-closed transition was considered valid if it remained in the state for at least two sample periods (250 µs). Event-duration histograms for both the open time (τ\(_o\)) and fast closed time (τ\(_c\)) were constructed by binning at the sampling rate (125 ms) from 1.0 to 6–15 ms (i.e., >4 times the time constant). To determine the burst duration (τ\(_{burst}\)) and the long closed times (τ\(_{c2}\) and τ\(_{c3}\)), the channel recordings were filtered at 100 Hz and sampled at 500 Hz to eliminate the contribution of τ\(_{c1}\) from the recordings. An open-closed transition was considered valid if it remained in the state for at least two sample periods (4 ms). For τ\(_{burst}\), the data were binned at 4-ms intervals for construction of the event-duration histogram. Because of the limited number of long closed events under control conditions, an exponential fit of the data could not be achieved from a single record. Therefore, the event-duration histograms for τ\(_{c2}\) and τ\(_{c3}\) was constructed after the concatenation of eight separate recordings with a P\(_o\) > 3% (average P\(_o\) = 0.08). The additional five single-channel recordings had P\(_o\) < 3% such that these recordings would contribute very few long closed events. The event-duration histogram was constructed by binning at 10-ms intervals. These event-duration histograms were then fit to exponential functions to determine the open (τ\(_o\)) and closed (τ\(_c\)) time constants.

Chemicals. All fatty acids were obtained from Sigma Chemical, made as >1,000-fold stock solutions in dimethyl sulfoxide (DMSO), and stored under N\(_2\) at −80°C. The fatty acids were dissolved to the final working concentration just before use, and all solutions were continuously bubbled with N\(_2\) during perfusion through the patch-clamp chamber. Charybdoxin (CTX) was made as a 10 µM stock solution in our bath solution for outside-out recording. 4-Aminopyridine (4-AP), quinine, glibenclamide, and trans-6-cyano-4-(N-ethylsulfonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane (293B) were made as a 1,000-fold stock solution in DMSO. 293B was a generous gift from Dr. Rainer Greger (Albert-Ludwigs-Universitat, Freiberg, Germany). Indomethacin and nordihydroguaiaretic acid (NDGA) were obtained from Biomol. Cell culture medium was obtained from GIBCO.

Data analysis. All data are presented as means ± SE, where n indicates the number of experiments. Statistical analysis was performed using the Student’s t-test. A value of P < 0.05 was considered statistically significant.

RESULTS

After excision of membrane patches from T84 cells into symmetric K\(^+\) in the absence of bath Ca\(^{2+}\) (~10 nM), a large-conductance channel was observed in ~10–15% of recordings. Single-channel currents for one such patch are shown in Fig. 1 in the absence of AA (A). The average current-voltage (I-V) relationship for eight recordings is shown in Fig. 1B. The channel displayed a linear I-V relation with an average conductance of 161 ± 3 pS (n = 8). The K\(^+\)/Na\(^+\) selectivity of this channel was assessed by equimolar substitution of Na\(^+\) for K\(^+\) in the bath solution; K\(^+\)-to-Na\(^+\) permeability ratio (P\(_k\)/P\(_n\)) was calculated from the Goldman-Hodgkin-Katz relation. Substitution of 100 meq Na\(^+\) for K\(^+\) resulted in a shift in the reversal potential of 26 ± 1 mV (n = 3). A shift of 27 mV is predicted for a perfectly K\(^+\)-selective electrode. These data yield a calculated K\(^+\)/Na\(^+\) selectivity of ~25:1. As is apparent from Fig. 1, this channel exhibited modest voltage dependence, with the P\(_o\) increasing at depolarizing potentials. In five patches, the P\(_o\) at −100 mV was 0.17 ± 0.02, and this increased to 0.59 ± 0.05 at +100 mV.

Effect of AA on K\(^+\) channel activity. The effect of AA on this large-conductance, Ca\(^{2+}\)-independent K\(^+\) channel is shown in Fig. 2A. Under control conditions, the channel opened infrequently. However, superfusion of AA (3 µM) resulted in a rapid increase in channel activity. The average concentration-P\(_o\) curve is shown in Fig. 2B. These data were fit to a Michaelis-Menten function with an apparent stimulatory constant (K\(_s\)) of 1.39 µM. At a maximally effective concentration (10 µM), AA increased the P\(_o\) from 0.04 ± 0.01 (n = 13) to 0.66 ± 0.03 (n = 4). On the basis of this AA-dependent activation, this channel will be referred to as K\(_{AA}\) throughout this paper.

As is apparent in Figs. 1 and 2, K\(_{AA}\) shows burst-type openings punctuated by long-lived closed states. To determined the kinetic state of the channel affected by AA, we constructed open and closed time event-duration histograms, as shown for one patch in Fig. 3, A–C. In the absence of AA, the open time (τ\(_o\)) is defined by a single exponential (2.4 ms), indicating a single open state (Fig. 3A). In 13 patches, τ\(_o\) averaged 2.3 ± 0.2 ms. The closed time (τ\(_c\)) of K\(_{AA}\) was described by three states: a short-lived closed state (τ\(_{c1}\)) and two long-lived closed states (τ\(_{c2}\) and τ\(_{c3}\)). As illustrated for one patch (Fig. 3B), the short-lived closed state was defined by a single exponential (τ\(_{c1}\) = 1.0 ms). In 13 patches, τ\(_{c1}\) averaged 1.3 ± 0.2 ms. Because of the limited number of long closed events in any one single-

1 If the closed-time histograms are fit starting at 0.625 ms (the limit of resolution using a filter cut-off frequency of 2 kHz), an additional exponential was required to fit the data that averaged 0.21 ± 0.02 ms (n = 19). Because this is below the limit of resolution filtering at 2 kHz, the first three bins were excluded from the fit such that we began binning at 1 ms. Because this is five times the rapid time constant, it will contribute little to the remaining fit.
channel recording, we are unable to determine \( \tau_{c2} \) and \( \tau_{c3} \) from a single patch. Therefore, we concatenated eight recordings that had a \( P_o \) of >3% (average \( P_o = 0.08 \); see M	extsc{ethods}). These data were fit by two exponentials (Fig. 3C), where \( \tau_{c2} = 14.9 \) ms and \( \tau_{c3} = 106 \) ms. These results indicate that \( K_{AA} \) is kinetically described by one open and three closed states in the absence of AA (see D	extsc{iscussion}).

The effect of AA on the open and closed times for one patch is illustrated in Fig. 3, D and E. Addition of 3 \( \mu \)M AA had no effect on the \( \tau_{c3} \) (Fig. 3D) of \( K_{AA} \) (2.6 ms). In 11 experiments, the \( \tau_{c2} \) averaged 2.2 \pm 0.2 ms after activation by AA (3 \( \mu \)M), which is not different from control. In contrast, AA induced a concentration-dependent decrease in mean closed time from 266 \pm 125 ms in control solutions to 2.6 \pm 1.0 ms in the presence of 3 \( \mu \)M AA (\( n = 11; P < 0.05 \)). As shown for one patch in Fig. 3E, \( \tau_{c2} \) was independent of AA concentration (1.2 ms). In 11 patches, \( \tau_{c1} \) averaged 1.5 \pm 0.1 ms, which is not different from control. Because of the limited number of long closed events in the presence of AA, we are unable to determine \( \tau_{c2} \) and \( \tau_{c3} \) in these experiments. These data demonstrate that AA increases the \( P_o \) of \( K_{AA} \) by reducing the long closed times (\( \tau_{c2} \) and \( \tau_{c3} \)) of the channel, thereby increasing the channel opening rate (see D	extsc{iscussion}).

The effect of AA on burst duration (\( \tau_{burst} \)) was determined after filtering of the data at 100 Hz to eliminate the fast closed events (\( \tau_{c1} \)) that punctuate the open channel burst (see M	extsc{ethods}). In one patch, \( \tau_{burst} \) was 16.7 ms (Fig. 4A), and this increased to 35.2 ms in the presence of 3 \( \mu \)M AA (Fig. 4B). In eight experiments, \( \tau_{burst} \) averaged 19.8 \pm 3.4 ms, and this was increased to 46.7 \pm 4.3 ms (\( P < 0.01 \)) in the presence of AA (3 \( \mu \)M).

**Effect of cyclooxygenase, lipoxygenase, and cytochrome P-450 inhibitors.** AA is rapidly metabolized by the cyclooxygenase-, lipoxygenase-, and cytochrome P-450-dependent oxidative pathways. Thus we determined whether inhibitors of these pathways would alter the ability of AA to activate \( K_{AA} \). Neither phenidone (250 \( \mu \)M), an inhibitor of both cyclooxygenase and lipoxygenase, a combination of cyclooxygenase (indo-

methacin; 1 \( \mu \)M) and lipoxygenase (NDGA; 2 \( \mu \)M) inhibitors, nor clotrimazole (1 \( \mu \)M), an inhibitor of cytochrome P-450, altered the ability of AA to activate the channel (data not shown). Thus these oxidative metabolites are not responsible for the increased gating observed.

**Effects of additional fatty acids.** We next determined whether the observed stimulatory effect of AA on \( K_{AA} \) was specific for AA or whether additional fatty acids would also modulate its activity. For these experiments, we used an additional cis-unsaturated fatty acid, linoleic acid (C\(_{18}\); cis, cis-\( \Delta^9,\Delta^12 \)), the trans-
unsaturated fatty acid elaidic acid (C\(_{18}\); trans-\( \Delta^9 \)), and a saturated fatty acid, myristic acid (C\(_{14}\)). The results of one experiment are shown in Fig. 5A. Elaidic acid (3 \( \mu \)M) failed to increase \( P_o \) above control levels. The subsequent addition of linoleic acid (3 \( \mu \)M) induced a distinct increase in channel \( P_o \) and the further addition of AA (3 \( \mu \)M) resulted in an additional activation. The data for these fatty acids plus myristic acid are summarized in Fig. 5B. Similar to elaidic acid, myristic acid failed to activate \( K_{AA} \), whereas linoleic acid was ~40% as effective as AA in activating the channel.

**Effects of \( K^+ \) channel blockers.** Although AA has been implicated in modulating \( Cl^- \) secretion in T84 cells, no blocker pharmacology has been defined for the \( G_K \) that...
participates in this secretory response. Because of the very low $P_o$ of this channel under control conditions, all of our blocker studies were carried out in the presence of 3 µM AA to activate the channel. In initial experiments, we determined whether Ba$^{2+}$ would block $K_{AA}$ from the cytoplasmic side in an inside-out patch. The results of one experiment are shown in Fig. 6. Initially, the patch voltage was held at $-80$ mV. Application of Ba$^{2+}$ (1 mM) to the inside of the channel resulted in a nearly complete inhibition of activity, which was subsequently relieved by changing the clamp potential to $+80$ mV. This voltage-dependent block is typical for Ba$^{2+}$ inhibition of $K_1$ channel currents (47).

To relate our findings to results from intact epithelia, it is more relevant to determine whether compounds that block $K_1$ channels in other systems will inhibit $K_{AA}$ from the outside of the membrane. Therefore, we determined the effects of several blockers during outside-out recordings. For these studies, AA was used to activate the channel similar to our inside-out recordings (data not shown). This indicates that $K_{AA}$ is activated by AA from the outside of the membrane also. The effect of Ba$^{2+}$ (3 mM) on $K_{AA}$ in an outside-out patch is shown in Fig. 7. Ba$^{2+}$ induced a voltage-dependent block of $K_{AA}$ from the extracellular side, inhibiting the channel at $-100$ mV while having no apparent effect at $+60$ mV. In four patches, 3 mM Ba$^{2+}$ reduced mean current ($I$) by $90 \pm 1\%$ at $-100$ mV (P < 0.01). Although cytoplasmic Ba$^{2+}$ blocked $K_{AA}$ by inducing a long-lived closed state (Fig. 6), the inhibition of $K_{AA}$ by extracellular Ba$^{2+}$ was accompanied by an apparent reduction in single-channel amplitude (i; Fig. 7), although this could not be clearly resolved at this high concentration of Ba$^{2+}$. Therefore, we determined the effect of 1 mM Ba$^{2+}$ in three additional patches. Ba$^{2+}$ (1 mM) reduced $P_o$ from $0.54 \pm 0.02$ to $0.25 \pm 0.02$ at $-100$ mV ($n = 3$), and
this was accompanied by an apparent reduction in i from 17.6 ± 0.6 to 14.9 ± 1.0 pA (n = 3; P < 0.05). These results suggest that Ba^{2+} blocks K_{AA} by interacting with two distinct binding sites from the intra- and extracellular side of the channel.

In addition to Ba^{2+}, we evaluated the effects of tetraethylammonium (TEA; 10 mM), CTX (50 nM), 293B (30 µM), 4-AP (4 mM), glibenclamide (300 µM), and quinine (300 µM) on channel activity in outside-out patches. TEA induced a small, voltage-dependent reduction in i. At −100 mV, i was reduced 21.8 ± 0.2% (P < 0.01), whereas at +100 mV, this inhibition was 7.2 ± 1.1% (n = 4; P < 0.01). We previously demonstrated that CTX was a potent blocker of K_{Ca} (inhibition constant = 3.5 nM) in both single-channel and Ussing chamber studies (9, 11). In contrast to this property of K_{Ca}, CTX (50 nM) failed to inhibit K_{AA} (data not shown). Lohrmann et al. (28) characterized a novel inhibitor of the cAMP-dependent K^{+} channel in rabbit colon, 293B. We recently demonstrated that this compound inhibited the cAMP-mediated Cl^- secretion across T84 monolayers while not affecting Cl^- secretion dependent on K_{Ca} (11). In three outside-out patches, 293B (30 µM) failed to inhibit K_{AA}, suggesting it is not the cAMP-activated K^{+} channel. Additionally, 4-AP (4 mM; n = 2), glibenclamide (300 µM; n = 3), and quinine (300 µM; n = 5) all failed to inhibit channel activity.

Effect of membrane stretch on K^{+} channel activity. During the course of our studies, we observed that negative pressure applied to the pipette during inside-out recordings resulted in the activation of a large-conductance channel that appeared to have identical characteristics to the channel activated by AA (Fig. 8, top). Therefore, we wished to determine whether this stretch-activated channel was in fact the same as the AA-dependent K^{+} channel we have characterized. Application of a submaximal concentration of AA (2 µM) to the same patch that was shown to possess the stretch-activated channel resulted in the activation of K_{AA} (Fig. 8, bottom), suggesting they are the same channel. In the presence of AA, application of negative pressure resulted in the further activation of the same channel rather than an additional conductance state. This result demonstrates that both AA and stretch are capable of activating this large-conductance, Ca^{2+}-independent K^{+} channel.

Effect of stretch-activated channel blockers on K_{AA}. The above results led us to determine whether the known inhibitors of stretch-activated ion channels, amiloride (25, 44) and Gd^{3+} (49), would block K_{AA} in excised, outside-out patches. For these experiments, we used AA to first activate the channel. Gd^{3+} (100 µM) had no effect on K_{AA} (n = 3; data not shown). In contrast, amiloride induced an apparent reduction in i at both +100 and −100 mV. The effect of 2 mM amiloride on K_{AA} in an outside-out patch is shown in Fig. 9. In this patch, i was reduced from 15.2 to 7.0 pA at +100 mV and from 17.0 to 3.6 pA at −100 mV. In four patches, amiloride (2 mM) reduced i at −100 mV from 18.5 ± 0.6 to 3.6 ± 0.1 pA (P < 0.001). This inhibition of i resulted in an 80 ± 3.5% reduction in I with no apparent reduction in channel P_o (control = 0.43 ± 0.04; amiloride = 0.43 ± 0.06). These results suggest that amiloride induces a voltage-dependent reduction in i of K_{AA}. Unfortunately, we were not able to routinely maintain patch seal integrity at positive voltages, so the voltage dependence of this inhibition could not be quantitatively determined.
from rabbit distal colon (8, 29). Similar to our findings, the 190-pS channel described by Burckhardt and Gogelein (8) from distal colonic crypts was observed in only 10% of patches and exhibited a similar blocker pharmacology; the channel was not sensitive to block by CTX, although extracellular Ba²⁺ did inhibit the channel. Thus a channel in native epithelium studied in vitro expresses many of the same characteristics as described here.

Mechanism of AA-induced activation. Our results demonstrate that the activation of KAA by AA is not due to an oxidative metabolite of AA; neither phenidone, indomethacin, NDGA, nor clotrimazole inhibited the AA-induced activation. Rather, we speculate that AA itself activates KAA. In addition, the levels of AA required to activate KAA (Kₐ = 1.4 µM) are likely to be physiologically relevant, since both cyclooxygenase and lipooxygenase have Michaelis constants of 3–5 µM for AA oxidation in vitro (36).

Based on event-duration analysis data, we demonstrate that KAA is minimally described by a single open state (O), a fast closed state (C₁), and two long-lived closed states (C₂, C₃). We predict a linear state diagram of the form

\[
\begin{align*}
C₃ & \xrightarrow{k_{3,2}} C₂ \xrightarrow{k_{2,1}} C₁ \xrightarrow{k_{1,o}} O \\
C₂ & \xrightarrow{k_{2,1}} C₁ \xrightarrow{k_{1,o}} O \\
C₁ & \xrightarrow{k_{1,o}} O \\
O & \xrightarrow{k_{o,1}} C₁ \\
\end{align*}
\]

The rate constant for the O→C₁ transition (k₀₁) can be directly determined from the inverse of the time constant (1/τ₀₁ = 435 s⁻¹). An initial approximation of the additional rate constants was obtained from their respective time constants. Two of these can be reliably approximated. The opening rate k₁,o (769 s⁻¹) can be determined from τ₁,o, since this transition constitutes the majority of closed events. This is especially true in the absence of AA, where P₀ is defined by the open time divided by the open plus fast closed times (τ₀/τ₀ + τ₁). τ₁,o is independent of AA concentration. In addition, we can approximate the C₁→C₂ transition rate from the burst duration analysis. In this case, C₁ was eliminated by filtering the data at a low frequency such that the transition rate of interest, O→C₂, can be determined from 1/τburst (50.5 s⁻¹). Using channel simulation software (Biopatch, version 3.11), we determined that these first approximations did not reliably predict channel behavior under control conditions; these rate constants predicted a P₀ of 0.35 (the channel records used to determine τ₀,o and τ₁,o had a P₀ of 0.08). With k₀,o, k₁,o, and k₁,2 held constant, the additional rate constants were determined based on their ability to reliably predict the observed channel behavior. The rate constants shown above predict a P₀ of 0.08, a τ₀ of 2.6 ms, and τ₁,o, τ₁,2, and τ₁,3 of 1.3, 15.8, and 200 ms, respectively. Eliminating events shorter than 4 ms (3 times τ₁,o) from the simulated data indicated a τburst of 31 ms. Thus this kinetic scheme reliably predicts the observed channel behavior.

We used this kinetic scheme to predict which state was being affected by AA. If AA interacts with C₃ to increase the opening rate k₃,2, then we can approximate
the AA-dependent on-rate ($K_d = k_{off}/k_{on}$) at 18 µM$^{-1}$s$^{-1}$. At 10 µM AA, this would predict a $P_o$ of 0.38. Similarly, with the assumption that AA interacts with $C_2$ to increase $k_{2,1}$ with a predicted on-rate of 36 µM$^{-1}$s$^{-1}$, our model predicts a $P_o$ of 0.42 at 10 µM AA. Neither of these accurately reflects the observed gating of $K_{AA}$. Because AA does not affect $C_1$ or the open state of the channel, this suggests that AA interacts with both $C_3$ and $C_2$. Using our model, we evaluated this possibility. If AA induced a 10-fold increase in both $k_{3,2}$ and $k_{2,1}$, this would predict a $P_o$ of 0.61, similar to that observed at 10 µM AA. In addition, this model accurately predicts the increased burst duration observed during AA stimulation (Fig. 4). In the simulated model, with 10 µM AA interacting with both $C_3$ and $C_2$, $t_{burst}$ increased to 45 ms. Finally, our results suggest that the on-rates for these two reactions must be different. Identical on-rates would predict a concentration-$P_o$ curve exhibiting negative cooperativity, whereas our data were best fit using a Hill coefficient of 1.4, i.e., slight positive cooper-
ativity. Thus we conclude that AA interacts with both long-closed states of the channel to increase the opening rate and hence $P_o$. Clapham et al. (48), studying an AA-activated $K_{a1}$ channel in cardiac tissue, predicted a similar kinetic scheme, comprising one open and three closed states. Similar to our findings, the open time of this channel was not affected by AA (48).

Although we are able to identify which kinetic state of the channel is being modulated by AA, our results do not allow us to discern whether AA is acting at the...
channel protein itself or interacting with a closely associated protein or with the membrane bilayer. Fatty acids are known to interact directly with purified protein kinase C (43) as well as with fatty acid binding proteins. Indeed, a putative fatty acid binding domain has been identified in the N-methyl-D-aspartate receptor (40). Thus a similar direct interaction with K$_{AA}$ is possible. We demonstrate also that stretch activates K$_{AA}$. Although a kinetic analysis has not been undertaken on the stretch-dependent activation, it appears qualitatively to be similar to that induced by AA, i.e., the channel clearly displays bursting behavior that is consistent with an open state punctuated by fast closed events (Fig. 8). This, coupled with an increased P$_o$, suggests that stretch decreases the long-closed time(s) of the channel. The K$_{AA}$ described in cardiac tissue is also activated by stretch; in both cases a similar kinetic scheme describes this activation (18). A similar kinetic has previously been described for stretch-activated channels of chick skeletal muscle (13) and amphibian proximal tubule (42) as well. In both cases, it was the interburst interval that was shortened to increase P$_o$ with no effect on r$_c$ or the fast closed time(s) (13, 42). This suggests a similar mechanism of action for these two modulators or that a common modulator, AA, activates the channel in both instances.

Effects of K$^+$ channel blockers on K$_{AA}$. We evaluated the effects of several known K$^+$ channel blockers on K$_{AA}$ activity, including CTX, 4-AP, 293B, TEA, glibenclamide, quinine, and Ba$^{2+}$. Of these, only TEA and Ba$^{2+}$ blocked the channel from the extracellular side. However, neither of these blocked with high affinity. TEA blocked in a voltage-dependent fashion, similar to its effect on KCa (9). Our results suggest K$_{AA}$ expresses two binding sites for Ba$^{2+}$. From the cytoplasmic side of the channel, Ba$^{2+}$ induced a long-lived, voltage-dependent closed state (Fig. 6) as previously described (47). In contrast, application of Ba$^{2+}$ to the extracellular side of the channel resulted in an apparent reduction in single-channel amplitude that was also voltage dependent (Fig. 7). Recently, Gray and co-workers (45) demonstrated a similar blocker pharmacology for Ba$^{2+}$ in epithelial cells from human vas deferens, i.e., cytoplasmic Ba$^{2+}$ induced long closed events, whereas extracellular Ba$^{2+}$ caused a voltage-dependent flickery block. Similarly, Hurst et al. (16) demonstrated that extracellular Ba$^{2+}$ induced both long- and short-lived blocked states in the Shaker K$^+$ channel. In both cases, these
results were interpreted as indicating the channel expressed two distinct Ba$^{2+}$ binding sites.

Effect of stretch-activated ion channel blockers on K$_{AA}$. Our results demonstrate that Gd$^{3+}$, a known inhibitor of nonselective, stretch-activated cation channels (49), had no effect on K$_{AA}$, suggesting it is unrelated to these channels. Amiloride has also been shown to inhibit both stretch-activated K$^+$ channels (44) and nonselective cation channels (25). We demonstrate that amiloride (2 mM) dramatically reduced the i of K$_{AA}$ (Fig. 9). This reduction in i by amiloride is similar to what has previously been reported for amiloride block of both the Xenopus oocyte nonselective cation channel and Lymnaea neuron K$^+$ channel (25, 44). Although our results on the voltage dependence of this block are preliminary, they suggest that the amiloride-induced reduction in i is voltage dependent, i.e., the block is partially relieved at positive voltages (Fig. 9). The amiloride block of the mechanosensitive channel in Xenopus oocytes was highly voltage dependent, being completely relieved by positive holding potentials (25), whereas the amiloride block of the K$^+$ channel of Lymnaea neurons was voltage independent (44). Thus our results fall between these two extremes. Our results with amiloride further support the notion that K$_{AA}$ and K$_{stretch}$ are indeed the same protein.

The elucidation of the role of K$_{AA}$ in intestinal Cl$^-$ secretion or volume regulation (see below) will require the identification of high-affinity blockers that distinguish between K$_{AA}$, K$_{Ca}$, and the cAMP-activated K$^+$ channel, K$_{cAMP}$. Lane et al. (25) have demonstrated a structure-activity relationship for the block of the Xenopus oocyte mechanosensitive channel by amiloride analogs. It will be important to determine whether high-affinity analogs can be identified for K$_{AA}$ in T84 cells, thus allowing its physiological role to be elucidated.

Does AA modulate intestinal Cl$^-$ secretion? Cl$^-$ secretion requires the coordinate regulation of both an apical Cl$^-$ conductance and basolateral G$_K$. Minimally, if AA is to modulate Cl$^-$ secretion, it must increase basolateral G$_K$, thus hyperpolarizing both membranes and increasing the driving force for Cl$^-$ exit across the apical membrane through constitutively open Cl$^-$ channels, a paradigm proposed previously for the mechanism of action of Ca$^{2+}$-dependent agonists (4). We have now characterized a K$^+$ channel that is potently activated by AA, K$_{AA}$. Recently, Barrett (5) demonstrated that the response of T84 cells to VIP and forskolin was attenuated by a DAG lipase inhibitor and that VIP increased AA. These results suggest that AA may be an important modulator of Cl$^-$ secretion in intestinal tissue. Although our results demonstrate that K$_{AA}$ has a distinct pharmacological profile from the cAMP-activated G$_K$ (it is not blocked by 293B, an inhibitor of K$_{cAMP}$), a selective blocker for K$_{AA}$ will be required to further explore its role in this response.

Inflammatory diseases of the intestine, including Crohn’s disease and ulcerative colitis, are characterized by the migration of eosinophils and neutrophils into the intestinal lumen (15). It is now known that both of these cell types release AMP, which is converted by an apical ecto-5’-nucleotidase to produce the secretagogue adenosine (32, 41). Several investigators found no increase in cAMP, cGMP, or Ca$^{2+}$ during an adenosine-mediated Cl$^-$ secretory response (6, 12, 32), suggesting a novel signaling pathway. Subsequently, Barrett and Bigby (6) demonstrated a tight correlation between changes in cellular AA generation and the resultant Cl$^-$ secretory response to apical adenosine, suggesting that AA may serve this role.

Ulcerative colitis is also characterized by elevated active kallikrein, which will release kinin (50). The kinins are known to activate phospholipase A$_2$ and therefore increase AA levels (1). Also, the proinflammatory cytokine tumor necrosis factor-α, which is thought to play a role in the pathogenesis of inflammatory bowel disease, was recently shown to potentiate the phospholipase A$_2$-stimulated release of AA (14, 27). Clearly then, the inflamed intestine is characterized by elevated levels of secretory agonists that likely increase cellular AA levels. In fact, the inflamed intestine is primed for an exaggerated response; the AA composition of phospholipids is increased in both Crohn’s disease and ulcerative colitis (37, 39). The oxidative conversion of AA to the eicosanoids is known to be a key step in the etiology of inflammatory bowel disease. Because the initial step in this cascade is the liberation of AA from phospholipids, and AA is a well-known modulator of ion channels in a variety of tissues (33, 38), including epithelia (2, 17, 34), it is likely that AA itself may play an important role in the diarrhea associated with inflammatory bowel disease. We speculate that K$_{AA}$ is the G$_K$ responsible for carrying K$^+$ current across the basolateral membrane during an AA-mediated Cl$^-$ secretory response.

Does K$_{AA}$ play a role in volume regulation? In addition to being activated by AA, we demonstrate that K$_{AA}$ is similarly activated by changes in membrane tension. Thus K$_{AA}$ may be activated by AA, the mechanical stimuli associated with haustral contractions required to move the colonic contents toward the rectum or with the regulatory volume decrease associated with cell swelling. The regulatory volume decrease response of jejenum (30) is associated with activation of a Ba$^{2+}$-sensitive G$_K$. In our studies, Ba$^{2+}$ inhibited the channel while other known K$^+$ channel blockers did not. Both AA and stretch have previously been shown to activate K$^+$ channels in smooth muscle (21), neurons (20), and cardiac tissue (18, 19). One hypothesis that has been proposed to link these two separate modulators is that the physical deformation of the patch during stretch activates a phospholipase causing the release of free AA and activation of the channel (18).

Differential modulation of two K$^+$ channels in the T84 cell line. In our companion paper we characterized the effect of AA on the basolateral membrane K$^+$ channel activated by Ca$^{2+}$-dependent agonists, K$_{Ca}$, in intestinal epithelia (10). Although K$_{Ca}$ was potently inhibited by AA (inhibition constant = 425 nM), K$_{AA}$ is potently activated (K$_i$ = 1.39 µM). Although K$_{Ca}$ was inhibited by fatty acids in general, the activation of K$_{AA}$ was very...
selective for \( \Delta 6 \)-unsaturated fatty acids, with AA being more effective than linoleic acid at the same concentration. The differential modulation of two distinct \( K^+ \) channels in the same cells has previously been described in cardiac myocytes (19) and stomatal guard cells (26). Thus the role that AA will play in the \( K^+ \) secretory response of the cell will depend on the preexisting regulatory context in which it acts.

In conclusion, we have characterized a large-conductance, \( \text{Ca}^{2+} \)-independent \( K^+ \) channel with a linear \( I-V \) relationship whose activity is increased by AA, stretch, and membrane depolarization. We speculate that this \( K^+ \) channel is important in the modulation of \( K^+ \) secretion by agonists employing AA as a second messenger (e.g., VIP, adenosine) during both physiological and pathophysiological responses. As such, this channel may represent a novel pharmacological target in the treatment of diarrheal disease. The development of selective blockers of this channel (perhaps amiloride analogs) will be required to confirm its role in the \( K^+ \) secretory response, particularly in inflammatory states.

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