

Arachidonic Acid Metabolites Alter G Protein-mediated Signal Transduction in Heart

Effects on Muscarinic K⁺ Channels

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ABSTRACT The muscarinic acetylcholine receptor (mAChR)-stimulated, inwardly rectifying K⁺ current ($I_{K[ACh]}$) was examined in single bullfrog atrial cells using the whole-cell patch clamp technique. $I_{K[ACh]}$ was activated either by bath addition of 1 μ M ACh or via activation of the G protein, G_k, with guanosine- γ -thiotriphosphate (GTP γ S). Arachidonic acid (AA) modulated $I_{K[ACh]}$ under both conditions. AA decreased mAChR-stimulated $I_{K[ACh]}$ and increased the rate of decay from the peak current (desensitization). In addition, AA affected GTP γ S-activated $I_{K[ACh]}$ by modulation of G_k. The effects of AA and its metabolites on G_k were assessed by examining their effects on both the basal rate of G_k activation by GTP γ S, and the mAChR-mediated increase in activation rate produced by nanomolar ACh. AA increased the basal rate of GTP γ S-mediated $I_{K[ACh]}$ activation, but reduced the ACh-induced augmentation of this rate. All of the effects of AA on GTP γ S-mediated $I_{K[ACh]}$ activation were produced by metabolites. A lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), decreased the basal and ACh-enhanced rate of $I_{K[ACh]}$ activation in both the presence and absence of exogenous AA. In contrast, indomethacin (INDO), a cyclooxygenase inhibitor, increased the basal rate of $I_{K[ACh]}$ activation by GTP γ S in both the presence and absence of exogenous AA, and reversed the effects of AA on the ACh-augmented basal rate. AA metabolites produced via lipoxygenase and cyclooxygenase pathways thus have opposing effects on the signal transduction pathway from mAChR to $I_{K[ACh]}$. We directly tested a lipoxygenase pathway metabolite, LTC₄, on GTP γ S-mediated $I_{K[ACh]}$ activation and found that it not only overcame the inhibitory effects of NDGA, but also increased both the basal and ACh-augmented rate of $I_{K[ACh]}$ activation. From these data, we propose that AA metabolites modulate the function of G_k by altering its kinetic properties.

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INTRODUCTION

Acetylcholine (ACh) binding to muscarinic acetylcholine receptors (mAChR) on atrial cells activates an inwardly rectifying potassium current, $I_{K[ACh]}$ (Garnier et al., 1978; Simmons and Hartzell, 1987). Although it is clear that a GTP-binding protein (G_k) mediates the coupling between mAChR and the ion channel (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985), the exact nature of the coupling remains elusive.

Activation of $I_{K[ACh]}$ by G_k has been suggested to involve the direct interaction of G_k with both mAChR and the ion channel. Initial experiments (Logothetis et al., 1987; Yatani et al., 1987) demonstrated that isolated, preactivated G proteins (or activated subunits) were capable of inducing $I_{K[ACh]}$ in excised patches of atrial cell membranes. Cytoplasmic second messengers were apparently not involved, since the channels were not activated in on-cell patches when ACh was applied to the cell, but were activated when ACh was included in the patch pipette during on-cell recording (Soejima and Noma, 1984).

The ability to apply isolated G protein subunits to the cytoplasmic face of excised membrane patches has produced some interesting insights into G protein-mediated signal transduction. Both isolated α (for review, see Brown and Birnbaumer, 1988) and $\beta\gamma$ (for review, see Neer and Clapham, 1988) subunits have been shown to produce $I_{K[ACh]}$ -like currents. The mode of action of the isolated subunits was thought to be the same: insertion of either the α or $\beta\gamma$ subunits into the patch membrane, and direct interaction with the $I_{K[ACh]}$ channel protein.

Although the initial reports of $\beta\gamma$ -mediated activation of $I_{K[ACh]}$ assumed a direct interaction of the subunits with the $I_{K[ACh]}$ channel (Logothetis et al., 1987), it now seems clear that the $\beta\gamma$ subunits are mediating at least some of their effects via activation of phospholipase A_2 (PLA₂). G proteins are involved in the activation of PLA₂ (for review, see Burch, 1989), and it has been suggested that in some systems, activation is mediated by $\beta\gamma$ subunits (Jelsema and Axelrod, 1987). Antibodies to PLA₂ block the ability of $\beta\gamma$ subunits to activate $I_{K[ACh]}$ in excised patches (Kim et al., 1989), and bath application of arachidonic acid (AA), blockers of AA metabolism, and 5-lipoxygenase metabolites are all capable of altering $I_{K[ACh]}$ single channel activity (Kim et al., 1989; Kurachi et al., 1989). Pertussis toxin did not block the ability of AA metabolites to modulate $I_{K[ACh]}$ (Kurachi et al., 1989), nor were inhibitors of AA metabolism able to block the GTP γ S-mediated activation of $I_{K[ACh]}$, suggesting that α subunits mediate $I_{K[ACh]}$ activation in the absence of the $\beta\gamma$ pathway. Taken together, the results of experiments with exogenously applied G protein subunits suggested that $I_{K[ACh]}$ may be modulated by complex interactions between the α subunit and products of $\beta\gamma$ -activated metabolic pathways.

To dissect out the effects of AA and its metabolites on each of the components of the normal activation cascade, we used a kinetic approach which can directly assess the functional state of G_k (Breitwieser and Szabo, 1988). The key feature of the method is the use of high concentrations of hydrolysis-resistant GTP analogues at fixed ratios with GTP (e.g., for GTP γ S/GTP > 20/1), which results in the irreversible activation of every G_k molecule that releases its bound GDP. This process is directly reflected in the appearance of $I_{K[ACh]}$. Under these special conditions, the

rate of $I_{K[ACh]}$ activation is likely to correspond to the rate-limiting step in the receptor-independent activation of G_k , namely, GDP release (Breitwieser and Szabo, 1988; Szabo and Otero, 1989). The kinetic approach proved to be a remarkably reproducible and sensitive assay of the state of G_k , and amplified the effects of AA and its metabolites. Our results suggest that AA can modulate both the rate of mAChR-independent G_k activation and the ability of ACh to increase this rate. AA metabolites thus set the "tone" of the mAChR- α_k - $I_{K[ACh]}$ pathway. A preliminary account of these results has appeared (Scherer and Breitwieser, 1990).

METHODS

Solutions

HEPES-buffered Ringer solution contained (in millimolar): NaCl, 90; KCl, 2.5; MgCl₂, 5; CaCl₂, 2.5; and HEPES, 20; pH 7.4. For the electrophysiological experiments, 5 μ M TTX and 0.5 mM CdCl₂ were added to block Na⁺ and Ca²⁺ currents, respectively. Other additions to the superfusing solution are noted in the figures. Stock solutions of AA (20 mM, in ethanol) were stored in aliquots under nitrogen at -20°C. Fresh aliquots were used daily. The sulfidopeptide leukotriene C₄ (LTC₄) was purchased in ethanol and stored at -80°C until the day of the experiment. Aliquots were diluted into HEPES-buffered Ringer and used within 4 h. Stock solutions of indomethacin (INDO) in ethanol and nordihydroguaiaretic acid (NDGA) in dimethylsulfoxide were made fresh daily. Neither solvent (maximal concentration, 3.0%) had any effect on GTP γ S- or ACh-stimulated $I_{K[ACh]}$ (data not shown). The intracellular solution contained (in millimolar): K⁺ aspartate, 80; KCl, 30; HEPES, 5; K⁺ EGTA, 1; GTP, 0.05; MgATP, 5; pH 7.4. When present, GTP γ S was added to the intracellular solution as the Li salt.

All salts were reagent grade. All chemicals and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO) except HEPES (Ultral) and AA (Calbiochem Corp., San Diego, CA), and LTC₄ (Cayman, Ann Arbor, MI).

Cell Dissociation

Isolated atrial cells from the bullfrog *Rana catesbeiana* were obtained as described by Fischmeister and Hartzell (1986) with minor modifications. Briefly, the heart was removed, rinsed in HEPES-buffered Ringer, and then perfused with 25 ml Ca²⁺-free Ringer solution (in millimolar): NaCl, 88.4; KCl, 2.5; MgCl₂, 1.8; NaHCO₃, 23.8; NaH₂PO₄, 0.6; and Na⁺ pyruvate, 2.5; fatty acid-free bovine serum albumin, 1 mg/ml; and MEM vitamins and essential amino acids, 1 μ l/ml, equilibrated with 5% CO₂ at 28°C. The heart was then perfused (at 28°C) with 25 ml of a recirculating dissociation solution (Ca²⁺-free Ringer solution with 5 mM creatine, 50 U/ml penicillin, and 50 μ g/ml streptomycin) containing 10 mg trypsin (bovine pancreas; Boehringer Mannheim Biochemicals, Indianapolis, IN) and 62.5 mg collagenase B (*Clostridium histolyticum*, lot 144702; Boehringer Mannheim Biochemicals). After 60–90 min the heart was placed in dissociation medium without enzymes and the atrium dissected. Cells were released by gentle agitation of the atrium in dissociation medium for 30–45 min. The myocytes were then diluted twofold with storage solution (Ca²⁺-free Ringer solution with 0.9 mM CaCl₂; 5 mM glucose; 1 μ l/ml MEM vitamins and essential amino acids; 50 U/ml penicillin; and 50 μ g/ml streptomycin). Cells remained viable for 6–8 h after isolation.

Electrophysiology

Currents were measured by the whole-cell patch clamp technique (Hamill et al., 1981) as previously described (Breitwieser and Szabo, 1985, 1988). Patch electrodes were fabricated from square bore glass (Glass Co. of America, Millville, NJ) on a Flaming Brown micropipette puller (Sutter Instrument Co., San Rafael, CA) and were used without firepolishing. Junction potentials were not corrected. Voltage pulses were generated with a computer-loaded Arbitrary Waveform generator (model 75; Wavetek, San Diego, CA). The protocol for monitoring $I_{K[ACh]}$ used sequential 250-ms steps to -90 , -125 , and -5 mV from the holding potential of -85 mV (with return to the holding potential between steps). Steady-state current-voltage relationships were determined by assessing the average current at the end of 250-ms voltage steps. Pulse protocols were applied to the cell continuously from the moment of patch rupture. A 5-mV hyperpolarizing step from the holding potential was included in all pulse protocols to allow continuous monitoring of the pipette series resistance, which was not electronically compensated. Currents were measured with a patch clamp amplifier (EPC-7; List, Darmstadt-Eberstadt, FRG) and monitored with an oscilloscope (model 310; Nicolet Instrument Corp., Madison, WI). Signals were converted to digital signal via a PCM-1 instrumentation adaptor (Medical Systems Corp., Greenvale, NY) and stored on VCR tape (VHS, model AG-1230; Panasonic, Secaucus, NJ). Rates were estimated by recording the analogue signal on either an Astromed (model Z100; Astromed, West Warwick, RI) or Gould recorder (model 220; Gould Inc., Cleveland, OH). All experiments were performed at room temperature (22 – 24°C).

RESULTS

AA Alters Both the Magnitude and Time Course of $I_{K[ACh]}$ Activation

ACh induces an inwardly rectifying K^+ current in isolated bullfrog atrial myocytes, as illustrated by the steady-state current-voltage relationship shown in Fig. 1 A. To assess whether AA could activate whole-cell $I_{K[ACh]}$ to a level comparable to that obtained with ACh, we superfused bullfrog atrial cells with $20 \mu\text{M}$ AA. AA produced minimal activation of $I_{K[ACh]}$ (Fig. 1 B): in this particular experiment, there was a 3-pA increase in outward current at -5 mV.

Also illustrated in Fig. 1 B is the response of the myocyte to ACh in the continued presence of AA. After a 2-min exposure to $20 \mu\text{M}$ AA, stimulation with $1 \mu\text{M}$ ACh elicited a smaller current than that seen in response to ACh in the absence of AA (compare Fig. 1, A and B). Since AA also altered the kinetics of $I_{K[ACh]}$ decay after ACh application (see below), the mean ACh-induced $I_{K[ACh]}$ in the presence of AA was assessed 30 s after the peak response and was 37.8 ± 4.0 pA (SEM; $n = 17$) at -5 mV. This is less than half of the steady-state $I_{K[ACh]}$ observed in the absence of AA (88.1 ± 8.1 pA; $n = 32$).

AA modified the kinetics of decay of the current induced by ACh. Illustrated in Fig. 2 A is the control time course of decay of $I_{K[ACh]}$ at -5 mV in response to $1 \mu\text{M}$ ACh. Application of ACh induced a peak $I_{K[ACh]}$ which decreased to $57 \pm 5\%$ ($n = 4$) of peak within 1 min. There was, in addition to the decrease in magnitude of the peak current in the presence of $20 \mu\text{M}$ AA, a more marked time-dependent decline in $I_{K[ACh]}$ (Fig. 2 B). In this representative experiment, the peak $I_{K[ACh]}$ in the presence of AA was lower than control, and $I_{K[ACh]}$ decayed to $<20\%$ of peak current within 1

min. $I_{K[ACh]}$ after 1 min of 1 μ M ACh in the presence of 20 μ M AA was only $30 \pm 6\%$ of the corresponding peak $I_{K[ACh]}$ also measured in the presence of AA ($n = 4$).

The inhibitory effects of AA on $I_{K[ACh]}$ induced by ACh may be due to either (a) a direct block of the open $I_{K[ACh]}$ channel by AA or a metabolite, or (b) an effect of AA on mAChR and/or G_k . The next section uses GTP γ S, a hydrolysis-resistant GTP analogue, to examine whether AA can block the $I_{K[ACh]}$ channel directly.

AA Does Not Block Open $I_{K[ACh]}$

Including 1 mM GTP γ S in the electrode solution induces a slow, mAChR-independent activation of $I_{K[ACh]}$, which reaches a maximum, steady-state $I_{K[ACh]}$ in ~ 3 min (Breitwieser and Szabo, 1988). Addition of 1 μ M ACh does not induce additional

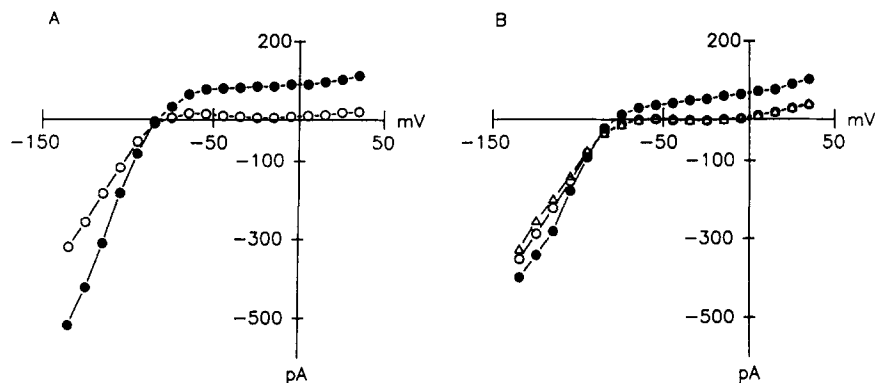


FIGURE 1. Effect of arachidonic acid on the steady-state current-voltage relationship of atrial cells. Steady-state currents were measured at the end of 250-ms voltage pulses from a holding potential of -85 mV. Traces were not corrected for series resistance across the patch pipette: at the highest applied potentials, the voltage offset amounted to <3 mV. The patch pipette contained control intracellular solution, and the superfusing Ringer solution contained 5 μ M TTX and 0.5 mM CdCl₂ to block Na⁺ and Ca²⁺ currents, respectively. *A*, Control: background K⁺ current (open circles) and current after 30 s superfusion with a Ringer solution containing 1 μ M ACh (solid circles). *B*, Arachidonic acid: background K⁺ current (open circles), current after 90 s exposure to 20 μ M AA (open triangles) and current after 30 s superfusion of 1 μ M ACh in the continued presence of 20 μ M AA (solid circles).

current once the steady-state $I_{K[ACh]}$ has been reached. Furthermore, unlike $I_{K[ACh]}$ induced by ACh in the presence of GTP, the current generated in the presence of GTP γ S does not decay after removal of ACh. A representative time course showing the slow, GTP γ S-mediated generation of $I_{K[ACh]}$ and parameters used to calculate the basal activation rate are illustrated in Fig. 3. The basal rate of GTP γ S-mediated $I_{K[ACh]}$ activation is calculated by dividing the absolute rate (picoamperes per minute) of current increase during the linear, initial rate portion of $I_{K[ACh]}$ activation by the final steady-state current, $I_{K[ACh]ss}$ (picoamperes).

Also illustrated in Fig. 3 is the lack of effect of 20 μ M AA on $I_{K[ACh]}$ once GTP γ S-dependent activation of G_k , and hence $I_{K[ACh]}$, has occurred. This suggests that the inhibition by AA of $I_{K[ACh]ss}$ activated by ACh in the presence of GTP

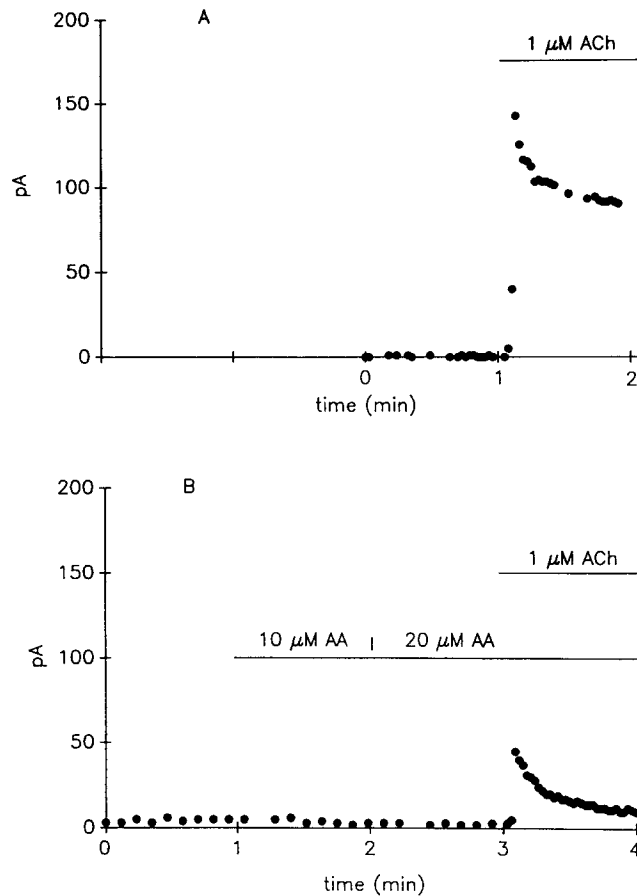


FIGURE 2. Effect of AA on the time course of $I_{K[ACh]}$ decay in response to ACh. $I_{K[ACh]}$ was monitored by measuring the current at the end of 250-ms voltage pulses to -5 mV, from a holding potential of -85 mV. Plots are aligned to allow comparison of the time course after ACh application. Data are plotted from the time of patch rupture. The pipette contained control intracellular solution. *A*, Time course of $I_{K[ACh]}$ decay after exposure to $1 \mu\text{M}$ ACh. *B*, Time course of $I_{K[ACh]}$ decay after exposure to $1 \mu\text{M}$ ACh in the presence of $20 \mu\text{M}$ AA. The cell was superfused sequentially with Ringer solution containing first 10 and then $20 \mu\text{M}$ AA. $I_{K[ACh]}$ was then elicited by switching to a Ringer solution containing both $20 \mu\text{M}$ AA and $1 \mu\text{M}$ ACh.

(Fig. 2 *B*) is not due to a direct block of the open $I_{K[ACh]}$ channel by AA, and further implies that AA must produce its effect by modulating mAChR, G_k , and/or their interactions.

AA and Its Metabolites Modify the Rate of GTP γ S-mediated $I_{K[ACh]}$ Activation

The physiological effects of AA can be complex since AA is rapidly metabolized via two major routes: the cyclooxygenase and lipoxygenase pathways. Blocking either pathway results in both reduced production of metabolites via the blocked pathway

and enhanced production of metabolites via the unblocked pathway. We therefore examined the effects on $I_{K[ACh]}$ activation rates of blocking either the cyclooxygenase (with INDO) or lipoxygenase (with NDGA) pathways. We summarize our conclusions in Fig. 4 to aid in an examination of the data. As illustrated, cyclooxygenase and lipoxygenase pathway metabolites have opposing effects on $I_{K[ACh]}$ activation. The rate of $I_{K[ACh]}$ activation is the result of a balance between the inhibitory actions of cyclooxygenase metabolites and the stimulatory effects of lipoxygenase metabolites, including LTC₄. Thus, blocking production of lipoxygenase metabolites with NDGA inhibits $I_{K[ACh]}$ activation, while blocking production of cyclooxygenase metabolites with INDO potentiates $I_{K[ACh]}$ activation. In this section, the rate of $I_{K[ACh]}$ activation

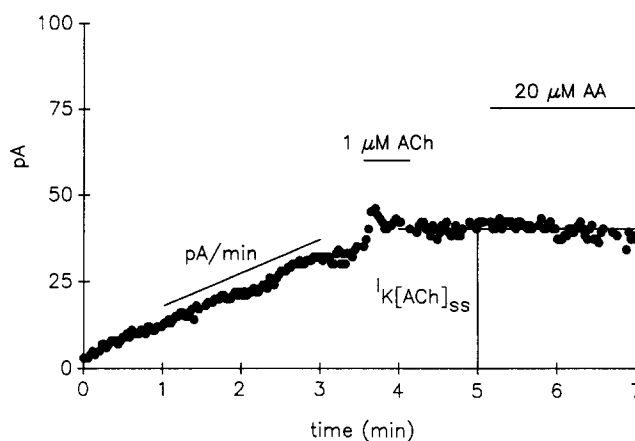


FIGURE 3. Effect of AA on $I_{K[ACh]}$ activated by GTP γ S. 1 mM GTP γ S (20/1 ratio with GTP) was added to the control intracellular solution. Generation of $I_{K[ACh]}$ by GTP γ S was monitored from the moment of patch rupture by periodic 250-ms voltage steps from the holding potential (-85 mV) to -5 mV. Maximal $I_{K[ACh]}$ was assessed by switching to a Ringer solution containing $1 \mu\text{M}$ ACh, which resulted in a peak current that decayed to a steady-state level ($I_{K[ACh]}_{ss}$). The rate of activation of $I_{K[ACh]}$ was calculated from the slope of the change in current with time (picoamperes per minute) normalized to $I_{K[ACh]}_{ss}$ (picoamperes). For this cell, the basal activation rate was 0.22 min^{-1} . After $I_{K[ACh]}_{ss}$ was reached, ACh was washed from the bath to test for complete irreversibility of $I_{K[ACh]}$ and then the superfusing solution was switched to a Ringer solution containing $20 \mu\text{M}$ AA, as indicated in the figure.

in the presence of GTP γ S is used to isolate the G_k - $I_{K[ACh]}$ interaction and to examine the effect of AA and its metabolites on the rate of GDP release by G_k .

The rate of $I_{K[ACh]}$ activation induced by hydrolysis-resistant guanine nucleotides (GXP) depends only on the ratio of GXP/GTP for GXP concentrations >0.1 mM. At high ratios ($\geq 20/1$ for GTP γ S) the maximal basal $I_{K[ACh]}$ activation rate has been shown to be equivalent to the rate of release of GDP from G_k (Breitwieser and Szabo, 1988; Szabo and Otero, 1989), and is of the order of 0.3 min^{-1} . The results of the previous section demonstrate that AA does not alter $I_{K[ACh]}$ once G_k has been fully activated by GTP γ S, implying that the channel itself is not blocked by AA. Thus, it is possible to use the rate of $I_{K[ACh]}$ activation to assess whether AA or its

metabolites can affect the activation of G_k by $GTP\gamma S$, or the interaction of G_k - $GTP\gamma S$ with the channel. The tabulated data are illustrated in Fig. 5. With a ratio of 20/1 of $GTP\gamma S/GTP$ (1 mM $GTP\gamma S/50 \mu M$ GTP), the $I_{K[ACh]}$ activation rate was $0.28 \pm 0.02 \text{ min}^{-1}$ (SEM; $n = 18$), as has been shown previously (Breitwieser and Szabo, 1988). In the presence of $20 \mu M$ AA this rate was significantly increased to $0.47 \pm 0.03 \text{ min}^{-1}$ ($n = 24$). Similar results were obtained in the presence of $10 \mu M$ atropine (control, 0.27 ± 0.03 , $n = 3$; $20 \mu M$ AA, 0.44 ± 0.05 , $n = 5$), implying that AA does not produce its effect by binding to the agonist site of mAChR. While an effect of AA on unoccupied mAChR cannot be ruled out, it is more likely that the ability of AA to increase the basal rate of $I_{K[ACh]}$ activation by $GTP\gamma S$ may be ascribed to either an effect of AA (or its metabolites) on the release of GDP from G_k or to an enhancement of the ability of activated G_k to interact with the channel.

Also illustrated in Fig. 5 are the effects of INDO and NDGA on the basal rate of $I_{K[ACh]}$ activation under control conditions, and in the presence of either $20 \mu M$ AA or $10 \mu M$ LTC_4 . INDO ($10 \mu M$) increased the rate of $I_{K[ACh]}$ activation in both the absence and presence of exogenously applied AA. In contrast to the effect of INDO, NDGA ($10 \mu M$) reduced the rate of $I_{K[ACh]}$ activation by $\sim 30\%$. NDGA also blocked the increase in the basal rate determined in the presence of $20 \mu M$ AA. It should be

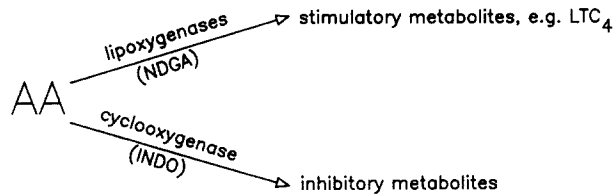


FIGURE 4. Major pathways for AA metabolism. See text for details.

pointed out that NDGA reduced the magnitude of $I_{K[ACh]}$ elicited by either ACh or $GTP\gamma S$ (data not shown). However, since the rate of $GTP\gamma S$ -mediated $I_{K[ACh]}$ activation was always normalized to the final ACh-stimulated $I_{K[ACh]_{ss}}$ in the same cell (as illustrated by the control activation rate in Fig. 3), the rates are directly comparable with those measured in the absence of NDGA.

These data suggest that normal cellular AA metabolism must contribute to the ability of G_k to release GDP and become activated. This is suggested by both the effects of NDGA and INDO on basal $I_{K[ACh]}$ activation rates in the absence of exogenous AA, and by the fact that the effects of the inhibitors are the same in the presence of exogenous AA. Furthermore, the data imply that metabolites produced by both the cyclooxygenase and lipoxygenase pathways modulate G_k : lipoxygenase metabolites appear to enhance the rate of G_k activation, while cyclooxygenase metabolites appear to inhibit it.

To confirm this hypothesis, we examined the effects of a 5-lipoxygenase metabolite, LTC_4 , on the rate of $I_{K[ACh]}$ activation by $GTP\gamma S$, in the presence and absence of either NDGA or INDO. LTC_4 was chosen for two reasons: (a) it had been shown to produce $I_{K[ACh]}$ single channel activity (Kim et al., 1989; Kurachi et al., 1989) and (b) it is the sulfidopeptide leukotriene that gives rise to a variety of further metabolites,

allowing isolation of one branch of the 5-lipoxygenase metabolite cascade. 10 μM LTC₄ in the absence of ACh had only a minor effect on whole-cell $I_{\text{K}[\text{ACh}]}$ in the presence of GTP (<2 pA at -5 mV), but it greatly accelerated the basal rate of GTP γ S-mediated $I_{\text{K}[\text{ACh}]}$ activation, as illustrated in Fig. 5. Significantly, NDGA did not block the stimulatory effect of LTC₄. INDO on the other hand acted synergistically with LTC₄, producing a rate of $I_{\text{K}[\text{ACh}]}$ activation 10-fold higher than that seen under control conditions. These results are consistent with products of AA metabolism mediating opposing effects on the rate of GTP γ S-mediated $I_{\text{K}[\text{ACh}]}$ activation, and hence on the rate of GDP release from G_k.

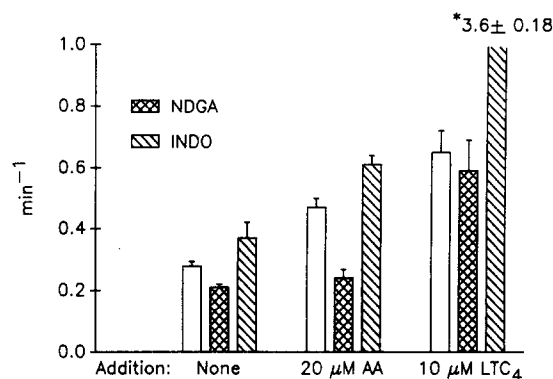


FIGURE 5. Effect of AA, metabolites, and blockers on the basal rate of $I_{\text{K}[\text{ACh}]}$ activation. The basal rate of $I_{\text{K}[\text{ACh}]}$ activation was measured as illustrated in Fig. 3, using an intracellular solution containing 1 mM GTP γ S. Immediately upon patch rupture the superfusing solution was changed to a Ringer solution containing the illustrated mixes of control, 20 μM AA, or 10 μM LTC₄, plus the indicated inhibitors (either

10 μM NDGA or 10 μM INDO). Bars indicate the mean \pm SEM of four or more observations. The bar indicating the mean rate of activation in the presence of LTC₄ + INDO (3.6 \pm 0.18 min^{-1}) was truncated for clarity. The rate of $I_{\text{K}[\text{ACh}]}$ activation in the presence of either 20 μM AA or 10 μM LTC₄ was significantly greater than the control basal rate ($P < 0.01$). The blockers, NDGA or INDO, significantly altered the corresponding rate in the absence of blocker ($P < 0.01$), except for the effect of NDGA in the presence of LTC₄ (which was not significantly different from the rate in the presence of LTC₄ alone). The rate in the presence of AA + NDGA was not significantly different from the rate in the presence of NDGA alone.

AA and Its Metabolites Modulate the mAChR-induced Increase in the Rate of GTP γ S-mediated $I_{\text{K}[\text{ACh}]}$ Activation

Nanomolar concentrations of ACh, which do not activate appreciable $I_{\text{K}[\text{ACh}]}$ in the absence of GTP γ S, lead to a marked acceleration of the rate of GTP γ S-mediated $I_{\text{K}[\text{ACh}]}$ activation (Breitwieser and Szabo, 1988). This is consistent with the notion that mAChR activation enhances the rate of GDP release from G_k, and reflects the mAChR-G_k interaction. Fig. 6 illustrates the action of 5 nM ACh on the rate of activation of $I_{\text{K}[\text{ACh}]}$ at high GTP γ S/GTP (20/1). The current was sampled at -5 mV from a holding potential of -85 mV starting immediately after breaking into the cell. GTP γ S (1 mM) in the pipette quickly began to diffuse into the cell and $I_{\text{K}[\text{ACh}]}$ began to activate at the mAChR-independent rate. The superfusing solution was then switched to one containing 5 nM ACh, causing an abrupt increase in the rate of $I_{\text{K}[\text{ACh}]}$ activation. $I_{\text{K}[\text{ACh}]_{\text{ss}}}$ was then assessed by switching to a Ringer solution

containing 1 μM ACh. In this cell, 5 nM ACh had already induced the maximal $I_{\text{K[ACh]}}$ and 1 μM ACh thus had no further effect. Both the basal and the ACh-augmented rates were normalized to this maximal $I_{\text{K[ACh]ss}}$. In this particular cell the rates were 0.23 and 5.07 min^{-1} , respectively. We examined the effects of AA and its metabolites on the ACh-induced increase in the rate of activation of $I_{\text{K[ACh]}}$. AA, metabolites, and/or blockers were present from the moment of patch rupture, and various ACh concentrations were applied as described above.

We first examined the effects of LTC_4 on ACh-augmented $I_{\text{K[ACh]}}$ activation, since its effects on the basal rate of $I_{\text{K[ACh]}}$ activation were purely stimulatory. Confirming a previous study (Breitwieser and Szabo, 1988), ACh over the range from 1 to 10 nM produced an enhancement of the rate of GTP γ S-mediated $I_{\text{K[ACh]}}$ activation. LTC_4 , which stimulated the basal rate of $I_{\text{K[ACh]}}$ activation by GTP γ S, also enhanced the

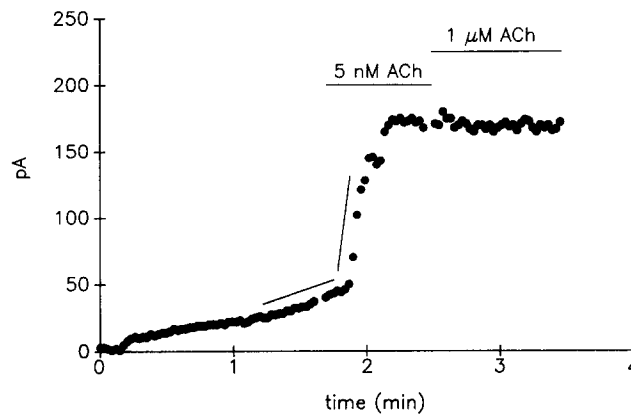


FIGURE 6. Effect of ACh on the activation of $I_{\text{K[ACh]}}$ by GTP γ S. The intracellular solution contained 1 mM GTP γ S. After a basal rate of $I_{\text{K[ACh]}}$ activation was observed, the superfusing solution was switched to one containing 5 nM ACh, as indicated by the bar. The rate of $I_{\text{K[ACh]}}$ activation increased abruptly. $I_{\text{K[ACh]ss}}$ was determined by addition of 1 μM ACh, which did not significantly increase the current. The basal rate of activation for this cell was 0.23 min^{-1} and the rate in the presence of 5 nM ACh was 5.07 min^{-1} .

ACh-mediated increase in this rate (Fig. 7). This effect of LTC_4 was not altered by the presence of 10 μM NDGA, although NDGA by itself virtually prevented the activation of $I_{\text{K[ACh]}}$, even in the presence of 10 nM ACh. The ability of LTC_4 to overcome the block by NDGA suggests that NDGA is specifically inhibiting metabolism via lipoxygenases (and preventing production of a required activating metabolite), rather than interacting nonspecifically with either mAChR or the channel.

The effects of exogenously applied AA are more complex than those of LTC_4 , since both stimulatory and inhibitory metabolites are produced, as suggested by the results in Fig. 5. To simplify an examination of the data, the effects of NDGA and INDO on the ACh-augmented rate of $I_{\text{K[ACh]}}$ activation in the presence of exogenous AA are displayed separately.

20 μM AA (Fig. 8 A) produced a rightward shift in the activation curve as compared with the control (in contrast to its stimulatory effect on mAChR-independent $I_{\text{K}[\text{ACh}]}$ activation rates in Fig. 5). 10 μM NDGA, as discussed above, virtually blocked the enhancement of $I_{\text{K}[\text{ACh}]}$ activation rate produced by ACh, and AA was not able to overcome this block. These results suggest that inhibitory metabolites (cyclooxygenase pathway) have a greater effect on the ACh-mediated rate of $I_{\text{K}[\text{ACh}]}$ activation than on the mAChR-independent basal rates.

10 μM INDO (Fig. 8 B), by blocking utilization of exogenously applied AA via the cyclooxygenase pathway, produced an ACh-augmented rate of $I_{\text{K}[\text{ACh}]}$ activation which was not significantly different from control. Neither INDO + AA nor INDO alone was able to shift the ACh-mediated activation curve to the level of LTC_4 ,

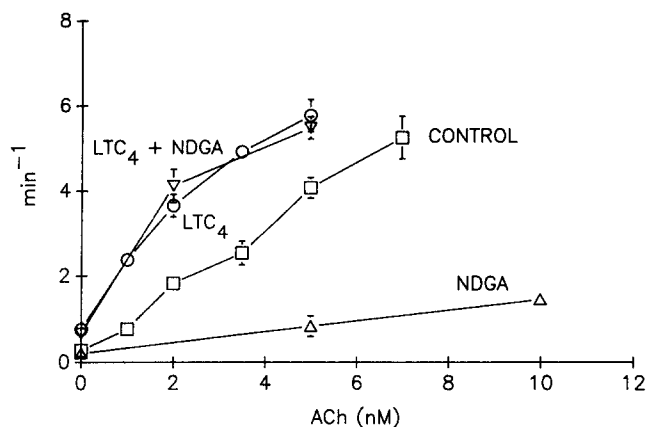


FIGURE 7. LTC_4 modulation of the ACh-induced increase in $I_{\text{K}[\text{ACh}]}$ activation rate. The ACh-augmented rate of $I_{\text{K}[\text{ACh}]}$ activation was measured as described in Fig. 6. LTC_4 and/or NDGA were present from the time of patch rupture. After a basal rate of $I_{\text{K}[\text{ACh}]}$ activation was observed, the superfusing solution was switched to one containing the indicated concentration of ACh in the continued presence of LTC_4 and/or NDGA, as indicated. The control curve was determined as in Fig. 6 without the addition of LTC_4 or NDGA. Each value represents the mean \pm SEM of four or more determinations. Squares, control; circles, 10 μM LTC_4 ; triangles, 10 μM NDGA; inverted triangles, 10 μM LTC_4 + 10 μM NDGA.

suggesting that the normal ACh-mediated augmentation of the $I_{\text{K}[\text{ACh}]}$ activation rate (in the absence of any exogenous lipid metabolites or blockers) is limited by cellular production of stimulatory metabolites, such as LTC_4 . The ACh-induced augmentation of the $I_{\text{K}[\text{ACh}]}$ activation rate in the absence of exogenous AA metabolites represents the maximal rate at which LTC_4 or its further metabolites can be produced. Only when exogenous LTC_4 is added to the superfusing solution do the activation curves shift to the left of control, implying that cellular metabolism, under these conditions, is rate limiting. The results further suggest that significant levels of the inhibitory metabolite(s) are not produced unless exogenous AA is provided. An excess of AA saturates the lipoxygenase pathways and results in the production of

metabolites via the cyclooxygenase pathway, which apparently has a lower affinity for AA than the lipoxygenase pathway.

DISCUSSION

In this paper we present evidence that AA metabolites modulate the ACh-stimulated K^+ channel of bullfrog atrial cells by altering the function of the signal-transducing, GTP-binding protein, G_k , which couples mAChR to the ion channel. AA ($20 \mu\text{M}$) is able to elicit a modest activation of $I_{K[ACh]}$ under whole-cell recording conditions (2–4

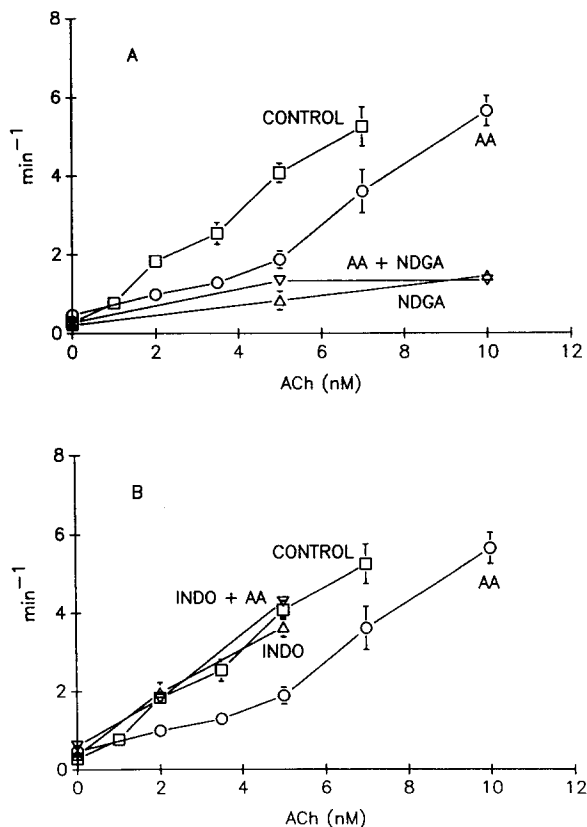


FIGURE 8. AA modulation of the ACh-induced increase in $I_{K[ACh]}$ activation rate. The effect of AA, INDO, and NDGA on the ACh-induced increase in $I_{K[ACh]}$ activation rate was measured as described in Fig. 7. Each value represents the mean \pm SEM of four or more observations. The control rate of ACh-induced increase in $I_{K[ACh]}$ activation rate (squares) is replotted from Fig. 7 for comparison. *A*, Effect of AA and/or NDGA on the dose response curve to ACh. Squares, control; circles, $20 \mu\text{M}$ AA; triangles, $10 \mu\text{M}$ NDGA; inverted triangles, $20 \mu\text{M}$ AA + $10 \mu\text{M}$ NDGA. *B*, Effect of AA and/or INDO on the dose-response curve to ACh. Squares, control; circles, $20 \mu\text{M}$ AA; triangles, $10 \mu\text{M}$ INDO; inverted triangles, $20 \mu\text{M}$ AA + $10 \mu\text{M}$ INDO.

pA, compared with 88.1 pA for $1 \mu\text{M}$ ACh), although the major effect of AA metabolites appears to be in modulating the cellular response to ACh. ACh, in the presence of AA, elicits a smaller peak $I_{K[ACh]}$ and the current decays to significantly lower steady-state levels, suggesting that AA metabolites may modulate $I_{K[ACh]}$ desensitization.

Metabolism of AA Is Required

Modulation of ion channel function by AA can proceed via either a direct mechanism (Kim and Clapham, 1989; Ordway et al., 1989; Vacher et al., 1989) or through

the production of metabolites (Piomelli et al., 1987; Kim et al., 1989; Kurachi et al., 1989). To assess the contribution of each of these possibilities to the effects of AA on $I_{K[ACh]}$ activation, we (a) examined the effects of the metabolic inhibitors NDGA and INDO on the response to AA, and (b) tested whether AA metabolites could overcome the blocking effects of the inhibitors.

Virtually all of the effects of AA on $I_{K[ACh]}$ activation are mediated by AA metabolites. Both inhibitory and stimulatory metabolites are produced (as discussed below; see also Fig. 4). The effect of AA on the basal activation rate was blocked by NDGA to a level not statistically different from that found in the presence of NDGA alone (0.24 min^{-1} [AA + NDGA] vs. 0.21 min^{-1} [NDGA alone]). If the block of the basal rate of activation of $I_{K[ACh]}$ by NDGA results in a residual rate that is due to direct effects of AA, then this effect is saturated by cellular AA levels, since addition of $20 \mu\text{M}$ exogenous AA did not significantly increase this rate, suggesting that all of the effects of AA are due to metabolites.

Lipoxygenase Metabolites Increase the Rate of $I_{K[ACh]}$ Activation

NDGA blocks lipoxygenases, and when added to the bath in the presence or absence of exogenous AA, decreases both the basal and ACh-augmented rate of $I_{K[ACh]}$ activation. NDGA reduced the amplitude of $I_{K[ACh]}$ activated via either ACh (+GTP) (Scherer and Breitwieser, unpublished observations) or GTP γ S (see Fig. 9 A). All of the effects of NDGA are reversed by the 5-lipoxygenase product, LTC₄. These results strongly suggest that products of the 5-lipoxygenase pathway, specifically sulfidopeptide leukotrienes, enhance the rate of G_k activation. The specific metabolites of NDGA-blockable pathways that modulate G_k are not known since NDGA blocks not only 5-lipoxygenase, but also 12-lipoxygenase and epoxygenases (Needleman et al., 1986; Capdevila et al., 1988). Although we find that LTC₄ reverses the effect of NDGA, other leukotrienes or metabolites may be equally effective, since LTC₄ breaks down readily to LTD₄, LTE₄, etc.

Cyclooxygenase Metabolites Decrease the Rate of $I_{K[ACh]}$ Activation

INDO, a cyclooxygenase inhibitor, consistently increased both the basal rate of $I_{K[ACh]}$ activation in either the absence or presence of exogenously applied AA, and reversed the inhibitory effect of AA on the ACh-mediated augmentation of the basal activation rate. These results are consistent with the notion that a metabolite of the cyclooxygenase pathway slows the rate of G_k activation. A complication is that diversion of AA into the lipoxygenase pathways may occur upon cyclooxygenase blockade (Aehringhaus et al., 1984), thus producing more of the stimulatory metabolites. Two of our results rule out diversion of AA into the stimulatory lipoxygenase pathway as the sole explanation for the effects of INDO and argue for the production of an inhibitory metabolite: first, INDO reverses the inhibition of the ACh-augmented $I_{K[ACh]}$ activation rate mediated by exogenously applied AA; and second, INDO greatly potentiates the effect of exogenously applied LTC₄. The cyclooxygenase metabolite(s) that inhibit $I_{K[ACh]}$ activation are unknown since prostaglandins, thromboxanes, and 12-hydroxy-heptadecatrienoic acid are all produced via this pathway. Experiments to identify the cyclooxygenase pathway metabolites that inhibit G_k activation are underway.

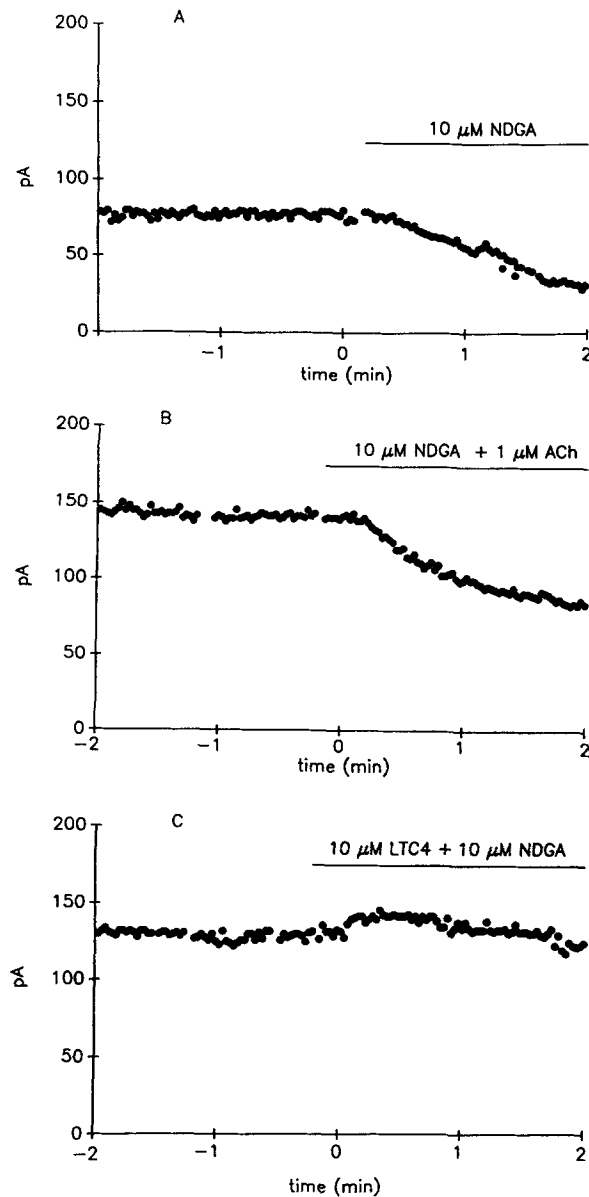


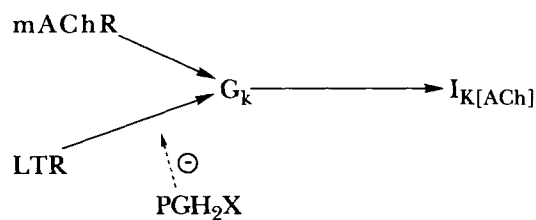
FIGURE 9. Effect of NDGA on GTP γ S-activated $I_{K[ACh]}$. $I_{K[ACh]}$ was activated by 1 mM GTP γ S in the intracellular solution; maximal $I_{K[ACh]}$ was obtained by a brief pulse of 1 μ M ACh, which was then washed from the bath. Illustrated is the effect of switching to a Ringer solution containing NDGA plus various other effectors on the GTP γ S-activated current. A, 10 μ M NDGA; B, 10 μ M NDGA plus 1 μ M ACh; C, 10 μ M LTC₄ plus 10 μ M NDGA.

Mechanism of Action of AA Metabolites

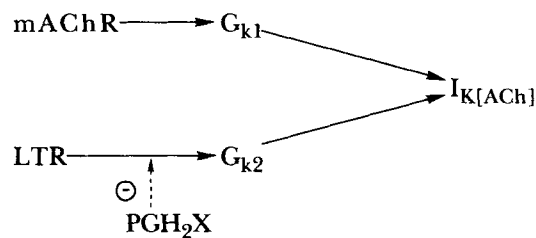
Our data would suggest that both lipoxygenase and cyclooxygenase products of AA modulate $I_{K[ACh]}$ through G_k . Three configurations of a receptor-G protein-effector signal-transducing pathway could account for our data, as diagrammed below, with LTR representing a leukotriene receptor, LT representing an unspecified leukotriene, and PGH₂X representing one or more of the metabolites produced from prostaglandin H₂ (PGH₂) via the cyclooxygenase pathway, which negatively modulate

the effects of LTR. Model 1 assumes mAChR and LTR converge at G_k , with both receptors capable of increasing the rate of GDP release from G_k . Model 2 differs from model 1 in that mAChR and LTR interact with distinct G proteins, both of which are capable of activating $I_{K[ACh]}$, and thus the two pathways converge at the level of the channel. Model 3 states that metabolites of AA alter the kinetic properties of G_k and affect its interactions with mAChR and/or the ion channel.

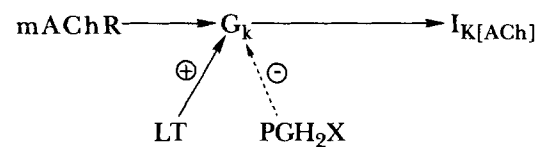
Models 1 and 2 assume that the relevant LTR is coupled to a G protein. Although [³H]LTD₄ binding in lung is modulated by guanine nucleotides, binding of LTC₄ is not, suggesting that not all leukotriene receptors are coupled to effectors via G



Model 1



Model 2



Model 3

proteins (Hogaboam et al., 1983). If the relevant LTR is coupled to a G protein, it cannot be G_k , since the effect of leukotrienes on single-channel $I_{K[ACh]}$ activation was not blocked by pertussis toxin (PTX) treatment (Kurachi et al., 1989), which interferes with the interaction of receptors with G_k (Pfaffinger et al., 1985). Model 1 is thus ruled out. Model 2 is still plausible if it is assumed that G_{k2} is not sensitive to PTX, and hence is not uncoupled from LTR by PTX treatment.

Agonists for G protein-coupled receptors act to enhance GDP release from inactive G proteins. Once the G protein becomes activated with GTP γ S, removal of agonist (e.g., ACh) is without effect (Breitwieser and Szabo, 1985, 1988). Similarly,

removal of lipoxygenase products by addition of NDGA should be without effect if the leukotrienes are acting only by increasing the rate of GDP release from G_k via a classical receptor–G protein interaction. Fig. 9 illustrates the test of these predictions. In all three panels, $I_{K[ACh]}$ was maximally activated by GTP γ S. NDGA was able to block the GTP γ S-activated $I_{K[ACh]}$ (Fig. 9 A). Addition of ACh with NDGA did not overcome the effect of NDGA (Fig. 9 B). LTC $_4$, however, if added at the same time as NDGA, was able to prevent the inhibition of the GTP γ S-activated $I_{K[ACh]}$ (Fig. 9 C), and indeed, resulted in a slight increase in the level of GTP γ S-activated current. These results further rule out models 1 and 2, since in both cases one would expect uncoupling of all of the receptors from their respective G proteins once the G proteins had been activated by GTP γ S.

Model 3 is the most parsimonious explanation of our data. We suggest that both lipoxygenase and cyclooxygenase metabolites directly modulate the functional state of G_k . These metabolites produce their effects on both the inactive, GDP-bound form of G_k and on α_k -GTP γ S, suggesting that the α subunit is modified. Modulation of GTP γ S-activated $I_{K[ACh]}$ by AA metabolites requires that either (a) the channel-associated α_k -GTP γ S can still be modulated by AA metabolites or (b) there is a finite amount of dissociation of α_k -GTP γ S from the ion channel during steady-state current activation. The ability of LTC $_4$ to increase steady-state, GTP γ S-activated $I_{K[ACh]}$ (Fig. 9 C) suggests it is able to promote α_k -GTP γ S – channel association:



implying that both unassociated α_k -GTP γ S and ion channels exist under steady-state conditions. LTC $_4$ could thus increase the steady-state, GTP γ S-activated $I_{K[ACh]}$ by shifting the equilibrium in favor of channel \cdot α_k -GTP γ S. This has implications for the physiological activation of $I_{K[ACh]}$ by ACh. As illustrated in Fig. 2 A, peak $I_{K[ACh]}$ desensitizes to the steady-state current within the first 60 s after ACh application, implying that not all of the available ion channels are active during the steady state. Thus, both the net ability of G_k to activate $I_{K[ACh]}$ and desensitization may, in part, be the result of alterations in the balance of inhibitory and stimulatory AA metabolites with time after mAChR activation.

It is necessary to rule out direct modifications of either the ion channel or mAChR by AA metabolites. Several lines of evidence suggest that the ion channel is not directly blocked or modulated by AA metabolites. Fig. 3 suggests that the GTP γ S-activated current is not directly modulated by AA, and the results of Fig. 9, A and C suggest that NDGA does not produce its effects by directly blocking the ion channel, since LTC $_4$ is able to overcome the blocking effects.

Our results rule out the mAChR as the major locus of AA metabolite action. While AA decreases the binding of the muscarinic antagonist [3 H]quinuclidinylbenzilate to cardiac (Rauch et al., 1989) or synaptic membranes (Aronstam et al., 1977), it has also been reported to increase binding of the muscarinic agonist carbamylcholine (Baron and Kloog, 1984). Our results exclude an effect of AA or its metabolites at the agonist-binding site of mAChR binding, since AA has the same effect(s) on the rate of $I_{K[ACh]}$ activation in the absence or presence of atropine (10 μ M). In addition, the effects of AA metabolites and blockers on the ACh-augmented

rate of $I_{K[ACh]}$ activation can be predicted from their effects on the basal rate of G_k activation, suggesting that mAChR is interacting normally with an altered G_k . Finally, AA metabolites affect the GTP γ S-activated $I_{K[ACh]}$ (Fig. 9) under conditions in which mAChR is uncoupled from G_k . Thus, although we cannot at this time rule out a minor effect of AA and/or its metabolites on mAChR, our data would suggest that the primary site of action for AA and its metabolites is at the level of G_k .

It is of interest to speculate about the ways in which G_k could be modified by AA and its metabolites. There have been reports of phosphorylation of G_i in response to protein kinase C activation (Katada et al., 1985). In addition, there is evidence that hydroperoxy acids decrease the activity of the calcium-calmodulin protein kinase II (Piomelli et al., 1989). Therefore, it is possible that a tonic level of phosphorylation of G_k is modulated by AA and its metabolites. It is also possible that some other type of dynamic covalent modification of G_k occurs in response to the balance of stimulatory and inhibitory metabolites of AA that are present in the membrane. Myristoylation (Buss et al., 1987; James and Olson, 1989a), palmitoylation (James and Olson, 1989b) and farnesylation (Casey et al., 1989; Hancock et al., 1989) have all been described as regulated modifications of eukaryotic proteins, including *ras* (Willumsen et al., 1984; Magee et al., 1987). Such lipid-mediated covalent modifications may be the reason that cloned G_i subtypes are much less potent at promoting activation of $I_{K[ACh]}$ than biochemically purified, native G proteins (Yatani et al., 1988). Alternately, some type of noncovalent interaction of AA metabolites or further products with G_k may occur.

Comparison with Excised Patch Results

Our data confirm and extend the results of Kim et al. (1989) and Kurachi et al. (1989): both groups have reported that AA and various lipoxygenase metabolites are capable of stimulating single-channel $I_{K[ACh]}$, although not to the same extent as GTP γ S. Our results confirm the submaximal stimulation of $I_{K[ACh]}$ by AA at the whole-cell level: 20 μ M AA induced only 2–4 pA of $I_{K[ACh]}$, compared with 88.1 pA inducible by ACh. The inability of AA to maximally activate whole-cell $I_{K[ACh]}$ in the absence of ACh, the attenuation of ACh-induced $I_{K[ACh]}$ by AA (Fig. 2), and the influence of AA and metabolites on the rate of G_k activation (e.g., Fig. 5) suggest that AA and metabolites play a modulatory role in $I_{K[ACh]}$ activation. Both Kurachi et al. (1989) and Kim et al. (1989) have demonstrated stimulatory effects of lipoxygenase metabolites. Kurachi et al. (1989) concluded that these metabolites either enhance GDP/GTP exchange of G_k in a receptor-independent manner, or that they couple directly to the channel via membrane receptors interacting with PTX-insensitive G proteins. Our results demonstrate that lipoxygenase metabolites increase the GDP release rate from G_k and thus modulate $I_{K[ACh]}$. Our data also provide an explanation for the observation of Kurachi et al. (1989) of the slight stimulatory effect of INDO on single-channel $I_{K[ACh]}$. Since cyclooxygenases are present in endoplasmic reticulum (Needleman et al., 1986), one might expect a greater contribution of inhibitory metabolites in the whole-cell recording configuration. Our whole-cell data define a distinct inhibitory role for cyclooxygenase products in modulating $I_{K[ACh]}$ and suggest that $I_{K[ACh]}$ is dynamically regulated by the balance of stimulatory and inhibitory AA metabolites.

Physiological Implications

We have shown that metabolites of AA specifically modulate $I_{K[ACh]}$ and suggest that they act by altering the kinetic properties of G_k . Stimulation of mAChR results in activation of $I_{K[ACh]}$, inhibition of adenylate cyclase, and stimulation of phosphatidylinositol metabolism. All of these effects are mediated by G proteins and thus may be subject to modulation by AA. Physiological responses to ACh such as alterations in heart rate, contractility, and conduction velocity may also be affected by AA.

The results we have obtained in the presence of exogenously applied AA may be more relevant to pathological states, in which large amounts of AA and metabolites are produced. For example, exogenously generated leukotrienes have been shown to alter cardiac performance. Leukotrienes (LTC_4 and LTD_4) are produced in isolated heart preparations in response to antigenic stimulation (Aehringhaus et al., 1983; Levi et al., 1985; Yacoub and Piper, 1988) or during myocardial infarction (Evers et al., 1985). Although all of the leukotrienes produced during pathophysiological states may not be produced by the myocytes, leukotrienes do impinge directly on cardiac muscle, probably through specific receptors (Hogaboom et al., 1985), resulting in a negative inotropic effect (Hattori and Levi, 1984; Levi et al., 1985; Bjornsson et al., 1987). Our data would suggest that part of the mechanism by which leukotrienes reduce myocardial contractility may be modulation of $I_{K[ACh]}$, and possibly other second messenger pathways.

There also appears to be a defined role for AA metabolism under nonpathological states. The effects of NDGA and INDO on $I_{K[ACh]}$ activation rates in the absence of exogenous AA suggest that cellular metabolism of AA contributes to the normal functioning of G_k and of the signal transduction pathway, which ultimately activates $I_{K[ACh]}$. Since prostaglandins are produced by isolated hearts in response to muscarinic receptor stimulation (Jaiswal and Malik, 1988), it is likely that modulation of $I_{K[ACh]}$ by AA metabolites occurs as a normal consequence of mAChR stimulation. Indeed, lipoxygenase products of AA metabolism may be necessary for full expression of $I_{K[ACh]}$, since $I_{K[ACh]}$ is greatly attenuated by NDGA. Finally, AA release resulting from activation of phospholipase A_2 or phospholipase C plus diacylglycerol lipase (Burch, 1989) via other receptor systems may modulate $I_{K[ACh]}$ indirectly by altering G_k responsiveness to mAChR stimulation.

We thank Dr. Dan Raben for helpful discussions and Leora Hamosh for excellent technical assistance.

This work was supported by a Grant-in-Aid from the American Heart Association with funds contributed in part by the American Heart Association, Texas Affiliate, Inc., and NIH grant HL-41972 to G.E.B. R.W.S. is supported in part by a fellowship from the American Heart Association, Maryland Affiliate.

Original version received 8 February 1990 and accepted version received 17 May 1990.

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