Leukotriene C_4 Modulation of Muscarinic K^+ Current Activation in Bullfrog Atrial Myocytes

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ABSTRACT The effects of leukotriene C_4 (LTC_4) on activation of muscarinic acetylcholine receptor (mAChR)–stimulated, inwardly rectifying K^+ current ($I_{K[ACh]}$) were examined in single bullfrog atrial myocytes using the whole-cell patch clamp technique. LTC_4 produced a reversible, concentration-dependent increase in steady-state, guanosine-γ-thiotriphosphate (GTPγS)-activated $I_{K[ACh]}$, with a $K_{0.5}$ of 3.1 μM. LTC_4 also increased the rate of GTPγS-mediated $I_{K[ACh]}$ activation, both in the absence and presence of 1 nM ACh, with comparable $K_{0.5}$ values of 4.7 μM under basal conditions and 4.9 μM in the presence of 1 nM ACh. LTC_4 did not alter the relative affinities of the G protein, G_k, for GTPγS and GTP. We hypothesize that all of the effects of LTC_4 on the kinetics of G_k-mediated $I_{K[ACh]}$ activation are produced at a common site with a $K_{0.5}$ of 3–5 μM. The effects of LTC_4 on $I_{K[ACh]}$ activation are fully reversible in the presence of GTPγS. Under physiological conditions (i.e., intracellular GTP), 10 μM LTC_4 increased the ACh-activated peak $I_{K[ACh]}$. Inhibitors of cellular LTC_4 production, including 5,8,11,14-eicosatetraynoic acid, baicalein, cinnamyl-3,4-dihydroxy-α-cyanocinnamate, and α-pentyl-4-(2-quinolinylmethoxy)-benzene methanol, greatly attenuated ACh-dependent $I_{K[ACh]}$ activation, preventing activation of peak, and producing a lower steady-state $I_{K[ACh]}$ (when compared with the control response in the same cell). Addition of exogenous LTC_4 was able to overcome the effects of LTC_4 synthesis inhibitors, restoring both the peak and steady-state $I_{K[ACh]}$ responses. Although the mechanism of LTC_4-mediated modulation of $I_{K[ACh]}$ activation is not known, our results suggest that endogenously produced lipoxygenase metabolites of arachidonic acid, specifically LTC_4, are involved in the physiological process of $I_{K[ACh]}$ activation.

INTRODUCTION

Acetylcholine (ACh) binding to atrial muscarinic receptors (mAChR) induces activation of an inwardly rectifying potassium current, $I_{K[ACh]}$. A pertussis toxin–sensitive G

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protein, G_k, couples mAChR to the $K_{[ACh]}$ channel. A variety of arachidonic acid metabolites, including leukotrienes, induce $I_{K[ACh]}$ activity at the single channel level (Kim, Lewis, Graziadei, Neer, Bar-Sagi, and Clapham, 1989; Kurachi, Ito, Sugimoto, Shimizu, Miki, and Ui, 1989). Receptor-mediated arachidonic acid liberation and subsequent metabolism via the 5-lipoxygenase pathway has been implicated in the activation of $I_{K[ACh]}$ via both α-adrenergic (Kurachi, Ito, Sugimoto, Shimizu, Miki, and Ui, 1991) and platelet activating factor receptors (Nakajima, Sugimoto, and Kurachi, 1991) in mammalian atrial myocytes. It has been suggested that mAChR-mediated release of arachidonic acid occurs via direct βγ subunit activation of phospholipase A_2 (PLA_2; Kim et al., 1989; Clapham, 1990), although more recent experiments suggest that βγ subunits are able to activate the $K_{[ACh]}$ channel despite the presence of inhibitors of arachidonic acid release and/or metabolism (Ito, Tung, Sugimoto, Kobayashi, Takahashi, Katada, Ui, and Kurachi, 1992).

Both lipoxygenase and cyclooxygenase metabolites of arachidonic acid modulate whole-cell $I_{K[ACh]}$ (Scherer and Breitwieser, 1990), with the most pronounced stimulatory effect produced by the leukotriene LTC_4. LTC_4 increases both the rate of guanosine-γ-thiotriphosphate (GTPγS)–mediated activation of $I_{K[ACh]}$ and the GTPγS-mediated rate of $I_{K[ACh]}$ activation in the presence of low concentrations (1–10 nM) of ACh, suggesting that its effects are additive with those of muscarinic agonists. The magnitude of steady-state $I_{K[ACh]}$ (60 s after ACh application, following densensitization of peak current) is slightly increased by 10 μM LTC_4 in the presence of nordihydroguaiaretic acid (NDGA, a 5- and 12-lipoxygenase inhibitor) when G_k was persistently activated by GTPγS (Scherer and Breitwieser, 1990), suggesting the possibility that some of the effects of LTC_4 can be localized to either GTPγS-activated G_k, the muscarinic K^+ channel itself, or their interaction (since mAChR was uncoupled from the pathway).

In this report, we explore more fully the role of LTC_4 in $I_{K[ACh]}$ activation, to determine whether the effects are relevant to the physiological mechanism of mAChR-mediated $K_{[ACh]}$ channel activation. We find that all of the LTC_4-mediated effects are half-maximal at 3–5 μM, and that both the rate of G_k activation and the magnitude of GTPγS-activated steady-state $I_{K[ACh]}$ are enhanced by LTC_4. Since blockers of LTC_4 biosynthesis decrease the magnitude of ACh-stimulated $I_{K[ACh]}$, we suggest that the presence of LTC_4 is required for maximal mAChR-mediated $I_{K[ACh]}$ activation.

METHODS

Cell Preparation

Dissociated atrial myocytes were obtained from the bullfrog *Rana catesbeiana* with a collagenase/trypsin perfusion procedure as previously described (Scherer and Breitwieser, 1990). The heart was removed, rinsed in HEPES-buffered Ringers solution, and perfused with Ca^{2+}-free Ringers solution (mM): 88.4 NaCl, 2.5 KCl, 1.8 $MgCl_2$, 23.8 $NaHCO_3$, 0.6 NaH_2PO_4, 2.5 pyruvate, 1 mg/ml fatty acid–free bovine serum albumin, and MEM vitamins and essential amino acids (1 μl/ml, 100X stock; Sigma Chemical Co., St. Louis, MO), equilibrated with 5% CO_2 at 28°C. The heart was then perfused for 60 min with a recirculating dissociating solution (25 ml of Ca^{2+}-free Ringers containing 5 mM creatine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.4 mg/ml trypsin (Boehringer Mannheim Corp., Indianapolis, IN), and 2.3–2.5 mg/ml collagenase B

(*Clostridium histolyticum;* Boehringer Mannheim Corp.). Myocytes were released from the dissected atria by agitation and diluted twofold with Ca^{2+}-free Ringers plus 0.9 mM $CaCl_2$, 5 mM glucose, 1 μl/ml MEM vitamins and essential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin, and stored in suspension at room temperature until use (within 6–8 h).

Solutions

Bath solution (HEPES-buffered Ringers solution) contained (mM): 90 NaCl, 2.5 KCl, 5 $MgCl_2$, 2.5 $CaCl_2$, and 20 HEPES, pH 7.4. To block Ca^{2+} and Na^+ currents, 500 μM $CdCl_2$ and 5 μM tetrodotoxin were added, respectively. The intracellular solution contained (mM): 80 K^+ aspartate, 30 KCl, 1 EGTA, 0.05 Na_2GTP, 5 MgATP, and 5 HEPES, pH 7.4. When present, GTPγS (1 mM) was added as the Li salt. Stock solutions of 5,8,11,14-eicosatetraynoic acid (ETYA; Calbiochem-Novabiochem Corp., La Jolla, CA or Cayman Chemical Co., Inc., Ann Arbor, MI), 5,6,7-trihydroxyflavone (baicalein; BIOMOL Research Labs Inc., Plymouth Meeting, PA), cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC; BIOMOL Research Labs Inc.), and α-pentyl-4-(2-quinolinylmethoxy)-benzenemethanol (L-655,238; BIOMOL Research Labs Inc.) in ethanol, or leukotrienes C_4 (glycine, *N*-[*S*-[1-(4-carboxy-1-hydroxybutyl)-2,4,6,9-pentadecatetraenyl]-*N*-L-γ-glutamyl-L-cysteinyl]-, [*R*-[*R**,*S**-(*E,E,Z,Z*)]]), B_4 (6,8,10,14-eicosatetraenoic acid, 5,12-dihydroxy-, [*S*-[*R**,*S**-(*E,Z,E,Z*)]]), or D_4 (glycine, *N*-[*S*-[1-(4-carboxy-1-hydroxylbutyl)-1,4,6,9-pentadecatetraenyl-L-cysteinyl]-[*R*-[*R**,*S**-(*E,E,Z,Z*)]]) (Cayman Chemical Co., Inc.) in either ethanol or 50:50 PBS/ethanol were stored at −80°C. Ethanol concentrations in the final extracellular solutions did not exceed 3%, and were without effect in control experiments.

Electrophysiology

Whole-cell patch clamp (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) of atrial myocytes was performed, with minor modifications, as previously described (Scherer and Breitwieser, 1990). Electrodes were fabricated of square bore glass (1.0 mm o.d.; Glass Co. of America, Millville, NJ) on a Flaming Brown micropipette puller (Sutter Instrument Co., Novato, CA) and were used without fire-polishing. Tip potentials were corrected, but junction potentials were not. Voltage clamp protocols were generated with a computer-driven arbitrary waveform generator (model 75; Wavetek, San Diego, CA), or with PClamp software (version 5.5.1; Axon Instruments, Inc., Foster City, CA). The protocol for monitoring $I_{K[ACh]}$ utilized sequential 250-ms voltage steps to −90, −125, and −5 mV from a 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 to various potentials. Pulse protocols were applied to the cell continuously from the moment of patch rupture. A 5-mV hyperpolarizing pulse from the holding potential was included in all protocols to allow continuous monitoring of the pipette series resistance, which was not electronically compensated (this procedure provides a measure of the access of pipette solutions to the interior of the cell; Breitwieser and Szabo, 1988). Currents were measured with a List EPC-7 patch clamp amplifier (List, Darmstadt-Eberstadt, Germany) and monitored with a model 310 oscilloscope (Nicolet Instrument Corp., Madison, WI). Signals were digitized with a PCM-1 instrumentation adaptor (Medical Systems Corp., Greenvale, NY) and stored on VCR tape. Rates of $I_{K[ACh]}$ activation were estimated by recording the analog signal on an Astromed recorder (model Z100; Astro-Med, Inc., West Warwick, RI) or by linear fits to digitized PClamp files. Data are reported as mean ± SEM. Dose–response relationships to the equations noted in the text were fit by least-squares minimization using the Marquardt-Levenberg algorithm (NFIT; Island Products, Galveston, TX). All experiments were performed at room temperature (22–24°C).

RESULTS

Effect of LTC_4 on GTPγS-activated Steady-State $I_{K[ACh]}$

A variety of arachidonic acid metabolites, including leukotrienes, modulate activation of the muscarinic K^+ current, $I_{K[ACh]}$, in atrial myocytes (Kim et al., 1989; Kurachi et al., 1989; Scherer and Breitwieser, 1990). In particular, LTC_4 (10 μM in the presence of 10 μM NDGA, a 5- and 12-lipoxygenase inhibitor), produces a slight increase in steady-state, GTPγS-activated $I_{K[ACh]}$ (Scherer and Breitwieser, 1990). Because the LTC_4-mediated increase in steady-state GTPγS-activated $I_{K[ACh]}$ may result from a novel regulatory mechanism, we examined the properties of this effect of LTC_4. Pipette solutions contained 1 mM GTPγS. A brief pulse of 1 μM ACh was used to activate peak $I_{K[ACh]}$, which declined to steady-state $I_{K[ACh]}$ ($I_{K[ACh]ss}$), and persisted despite removal of ACh from the bath solution, as is illustrated for the control experiment in Fig. 1 *A*. Illustrated in Fig. 1, *B* and *C*, are the effects of bath application of 1 and 10 μM LTC_4, respectively, on GTPγS-activated $I_{K[ACh]ss}$. LTC_4 reversibly elicits additional current, which is not affected by 10 μM atropine, but is blocked by 0.5 mM Ba^{2+} (data not shown). The current–voltage relationship of the LTC_4-elicited current is qualitatively similar to the steady-state current–voltage relationship of $I_{K[ACh]}$ in the same cell (Fig. 1 *D*, relationships plotted for the cell illustrated in Fig. 1 *C* at the times denoted by the various symbols), suggesting that LTC_4 increases $I_{K[ACh]}$ rather than activating a distinct K^+ current. This interpretation is further supported by the fact that LTC_4 has been shown to activate $K_{[ACh]}$ channels in cell-attached and excised patches (Kim et al., 1989; Kurachi et al., 1989; Clapham, 1990).

The LTC_4-mediated increase in $I_{K[ACh]ss}$ occurs rapidly, and upon removal of LTC_4 the current rapidly returns to the control level of GTPγS-mediated steady-state $I_{K[ACh]}$ (Fig. 1, *B* and *C*). The reversibility of this effect suggests that LTC_4 does not act via a G protein–coupled receptor (which would mediate a persistent increase in $I_{K[ACh]ss}$ under these experimental conditions). The increase in $I_{K[ACh]}$ induced by LTC_4 is saturable (Fig. 2). The line through the data points represents the fit to a single site model, with a $K_{0.5}$ of 3.1 μM. The LTC_4-mediated increase in $I_{K[ACh]}$ saturates at 155% of the control, GTPγS-activated $I_{K[ACh]ss}$. Comparison of the increase in steady-state $I_{K[ACh]}$ achieved by 10 μM LTC_4 (143 ± 22% of control $I_{K[ACh]ss}$, $n = 10$), 10 μM LTB_4 (109 ± 11% of control $I_{K[ACh]ss}$, $n = 5$), and 10 μM LTD_4 (109 ± 25% of control $I_{K[ACh]ss}$, $n = 5$) suggests specificity for the structure of LTC_4. Neither a lipophilic analogue (LTB_4) nor an analogue with a truncated peptidyl group (LTD_4) is able to mimic the LTC_4 effect.

ETYA, which inhibits both cyclooxygenases and lipoxygenases (Tobias and Hamilton, 1979; Bokoch and Reed, 1981), has no effect on GTPγS-activated $I_{K[ACh]ss}$ at concentrations as high as 10 μM. Fig. 3 illustrates a typical experiment, in which $I_{K[ACh]}$ was first persistently activated in the presence of GTPγS, and then ETYA (10 μM) was applied. The lack of ETYA effect on GTPγS-activated $I_{K[ACh]ss}$ suggests that cellular production of LTC_4 does not contribute significantly to the maintenance of GTPγS-activated $I_{K[ACh]ss}$. This result is not specific to ETYA, since other inhibitors of LTC_4 synthesis such as baicalein (a lipoxygenase and leukotriene biosynthesis inhibitor; Sekiya and Okuda, 1982; Kimura, Okuda, and Arichi, 1987), and

L-655,238 (a 5-lipoxygenase activating protein [FLAP] inhibitor; Evans, Leveille, Mancini, Prasit, Therien, Zamboni, Gauthier, Fortin, Charleson, Macintyre et al., 1991) also have no effect on GTPγS-activated steady-state $I_{K[ACh]}$. ETYA does, however, modulate the GTPγS-mediated rate of $I_{K[ACh]}$ activation (all experiments done with a pipette solution containing 1 mM GTPγS and 50 μM GTP): control, 0.27 ± 0.01 min^{-1} (n = 6); 1 μM ETYA, 0.16 ± 0.01 min^{-1} (n = 7), suggesting an

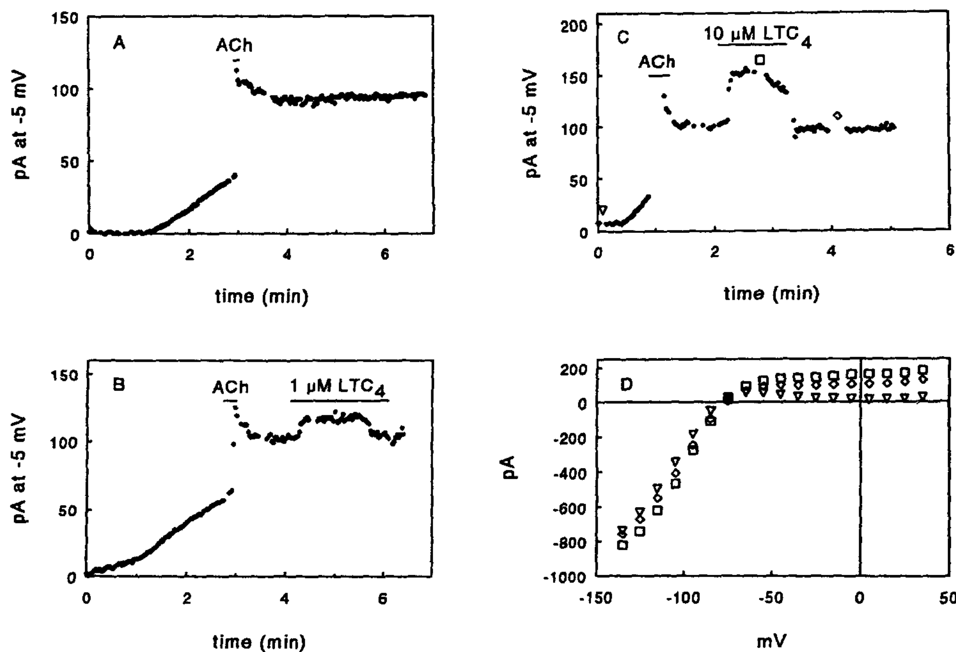

FIGURE 1. Effect of LTC_4 on GTPγS-activated $I_{K[ACh]ss}$. $I_{K[ACh]}$ was activated in the presence of 1 mM GTPγS and 0.05 mM GTP. A brief (10–15 s) exposure to 1 μM ACh was used to induce rapid, irreversible activation of $I_{K[ACh]}$. Plotted in *A–C* are the time-dependent changes in the averaged magnitude of outward current at −5 mV at the end of 250-ms voltage steps from a holding potential of −85 mV. (*A*) Washout of ACh under control conditions results in stable $I_{K[ACh]ss}$. (*B*) After washout of ACh, 1 μM LTC_4 was added to the superfusing Ringers solution, resulting in an increase in outward current. Washout of LTC_4 resulted in a return to the control level of $I_{K[ACh]ss}$. (*C*) Same protocol as described in *B*, with 10 μM LTC_4. (*D*) Current–voltage relationships for the experiment illustrated in *C*, taken at the times indicated by the symbols: ▽, before activation of $I_{K[ACh]}$; □, GTPγS-activated $I_{K[ACh]ss}$ in the presence of 10 μM LTC_4; ◇, GTPγS-activated $I_{K[ACh]ss}$. The cell was held at −85 mV and stepped to the various test potentials for 250 ms. Average current at the end of each pulse is plotted.

involvement of endogenous lipid metabolites during activation. The effect of ETYA on the GTPγS-mediated rate of $I_{K[ACh]}$ activation is overcome by LTC_4: 1 μM ETYA + 10 μM LTC_4, 0.46 ± 0.03 min^{-1} (n = 3), which is significantly higher than the control rate, and almost the same as the rate obtained in the presence of 10 μM LTC_4 alone, 0.53 ± 0.05 min^{-1} (n = 4). The absence of an effect of ETYA on steady-state GTPγS-activated $I_{K[ACh]}$ and the attenuation by ETYA of the GTPγS-

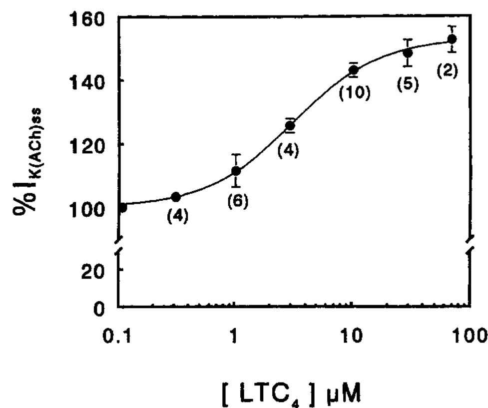

FIGURE 2. Dose response for LTC_4-mediated increase in GTPγS-activated $I_{K[ACh]ss}$. Experiments were performed as illustrated in Fig. 1, *B* and *C*. Plotted versus the LTC_4 concentration in the superfusing Ringers is the % $I_{K[ACh]ss}$ = ($I_{K[ACh]}$ in LTC_4)/(control $I_{K[ACh]ss}$) × 100. Data are plotted as mean ± SEM (except for 70 μM LTC_4, which is plotted as the average ± range for $n = 2$). The number of independent determinations is indicated in parentheses. Data were fitted by nonlinear least-squares minimization to the following equation: $B + \max/(1 + K/[LTC_4])$, with best fit parameters B = 98.04%; max = 56.75%; K = 3.06 μM.

mediated $I_{K[ACh]}$ activation rate suggest that the primary role for LTC_4 under these experimental conditions (i.e., in the presence of GTPγS) is promotion of G_k activation.

LTC_4 Modulation of GTPγS-mediated Activation of $I_{K[ACh]}$

LTC_4 (10 μM) increases the rate of GTPγS-mediated activation of $I_{K[ACh]}$ in both the absence and presence of ACh (Scherer and Breitwieser, 1990). To establish whether these effects are mediated by LTC_4 at the same site that modulates $I_{K[ACh]ss}$, we determined the concentration dependence of the LTC_4-mediated increases in $I_{K[ACh]}$ activation rates.

Fig. 4 *A* illustrates a typical experiment used to measure the rate of GTPγS-mediated $I_{K[ACh]}$ activation in the presence of LTC_4. Pipette solutions contained 1 mM GTPγS. Immediately upon achieving the whole-cell configuration, 70 μM LTC_4 was added to the bath solution until a linear rate of $I_{K[ACh]}$ activation was observed. 1 μM ACh was then superfused to fully activate $I_{K[ACh]}$ (note that the ACh solution did *not* contain LTC_4). The linear, initial rate of $I_{K[ACh]}$ activation was normalized to $I_{K[ACh]ss}$ determined in the absence of LTC_4 (i.e., the control, GTPγS-mediated

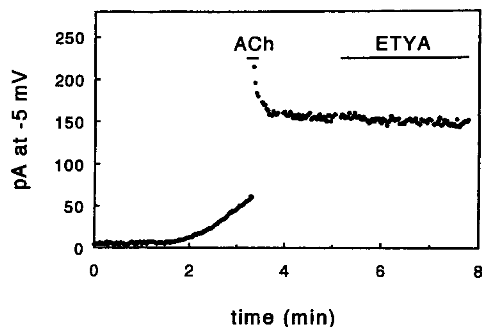

FIGURE 3. Effect of ETYA on GTPγS-activated $I_{K[ACh]ss}$. Pipette contained 1 mM GTPγS. 1 μM ACh was applied briefly (10 s) to fully activate $I_{K[ACh]ss}$ which persisted upon ACh washout. 10 μM ETYA was then superfused.

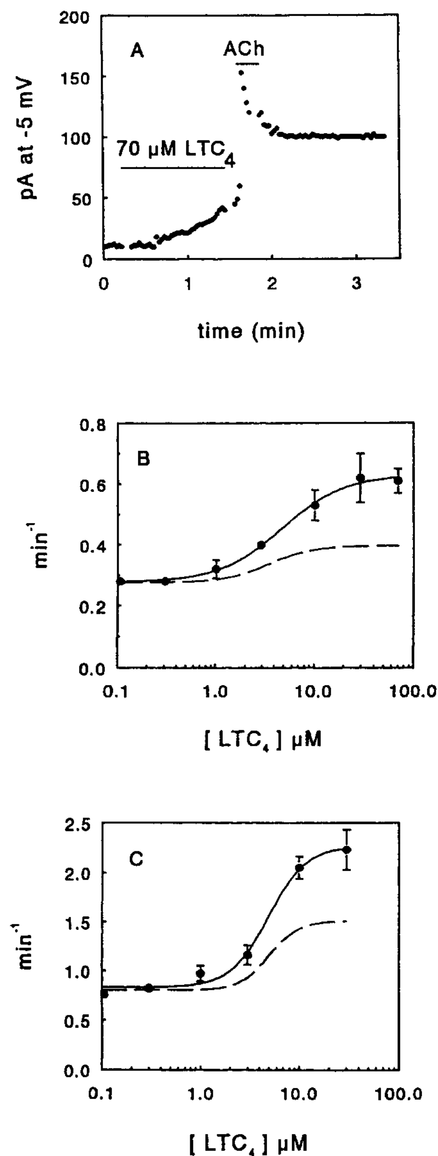

FIGURE 4. Effect of LTC_4 on basal and ACh-dependent GTPγS-mediated rates of $I_{K[ACh]}$ activation. Pipette solutions contained 1 mM GTPγS and 0.05 mM GTP (20:1 ratio). (*A*) Effect of LTC_4 (70 μM) on the basal rate of $I_{K[ACh]}$ activation. Immediately upon patch rupture, the superfusing solution was switched from control Ringers to one containing 70 μM LTC_4. After an observable initial rate was obtained, the superfusing solution was switched to one containing 1 μM ACh (but no LTC_4). Washout of ACh resulted in a stable GTPγS-activated $I_{K[ACh]ss}$. (*B*) Concentration dependence of LTC_4 enhancement of the GTPγS-mediated $I_{K[ACh]}$ activation rate. Experimental protocol illustrated in *A*. The LTC_4 concentration is plotted versus the normalized rate of $I_{K[ACh]}$ activation (min^{-1}), calculated as: initial rate (pA/min)/$I_{K[ACh]ss}$ (pA). Data were fitted (*solid line*) to the equation: $B + max/[1 + (K/[LTC_4])^n]$, with $B = 0.28\ min^{-1}$; max = 0.353 min^{-1}; $K = 4.74$ μM; $n = 1.4$. (*C*) Concentration dependence of LTC_4 enhancement of the GTPγS-mediated $I_{K[ACh]}$ activation rate in the presence of 1 nM ACh. Experimental protocol as in *A*, with both LTC_4 and 1 nM ACh in the superfusing solution. Data were fitted (*solid line*) with the same equation as in *B*, with $B = 0.83$ min^{-1}; max = 1.44 min^{-1}; $K = 4.88$ μM; $n = 2.3$. Data plotted in both *B* and *C* represent mean ± SEM, with at least four independent experiments at each concentration of LTC_4. Dashed lines in both *B* and *C* are the relationships obtained when the rate of $I_{K[ACh]}$ activation is calculated by normalization to the LTC_4-enhanced $I_{K[ACh]ss}$ (obtained by multiplying $I_{K[ACh]ss}$ in each experiment by the fold increase in $I_{K[ACh]ss}$ produced by that dose of LTC_4, calculated from the fit in Fig. 2).

$I_{K[ACh]ss}$). The LTC_4 dose response for the basal, mAChR-independent, GTPγS-mediated rate of $I_{K[ACh]}$ activation is illustrated in Fig. 4 *B*. The data were fitted with the Hill equation, with a $K_{0.5}$ of 4.7 μM and a Hill coefficient of 1.4.

A dose response for LTC_4 in the presence of 1 nM ACh was also determined, and is

illustrated in Fig. 4 *C*. Experiments were as in Fig. 4 *A*, except that the cell was exposed to a solution containing both the test concentration of LTC_4 and 1 nM ACh. The rate of $I_{K[ACh]}$ activation in the absence of LTC_4 under these conditions was 0.76 ± 0.03 min^{-1} ($n = 5$), which is consistent with the enhanced rate of $I_{K[ACh]}$ activation mediated by 1 nM ACh (Breitwieser and Szabo, 1988). The data of Fig. 4 *C* were fitted with the Hill equation, with a $K_{0.5}$ of 4.9 μM and a Hill coefficient of 2.3. The similarities in the estimated $K_{0.5}$ for LTC_4 mediation of both the increase in GTPγS-activated $I_{K[ACh]ss}$ (Fig. 2) and for enhanced GTPγS-mediated $I_{K[ACh]}$ activation rates (in the absence [Fig. 4 *B*] and presence of 1 nM ACh [Fig. 4 *C*]) suggest a common site of LTC_4 action.

The GTPγS-mediated rate of $I_{K[ACh]}$ activation is normalized to the steady-state $I_{K[ACh]}$ obtained in each cell, which has been considered to reflect the rate of G_k activation (Breitwieser and Szabo, 1988). The rates of GTPγS-mediated $I_{K[ACh]}$ activation in the presence of LTC_4 were also determined in this manner (Fig. 4). This is an overestimate of the effect of LTC_4 on the rate of G_k activation, however, since LTC_4 can increase the steady-state GTPγS-activated $I_{K[ACh]}$ (Fig. 2) independent of an effect on G_k activation (i.e., when all of G_k has been persistently activated by GTPγS). The two effects of LTC_4 (i.e., on G_k activation and on steady-state GTPγS-activated $I_{K[ACh]}$) can be separated, however, since (*a*) the effect of LTC_4 on $I_{K[ACh]ss}$ can be independently determined (Fig. 2); (*b*) the effect of LTC_4 on $I_{K[ACh]ss}$ is rapidly reversible (Fig. 1, *B* and *C*); and finally, (*c*) $I_{K[ACh]ss}$ determined in the absence of exogenous LTC_4 is not a function of continued, endogenous LTC_4 production (Fig. 3). Plotted as dashed lines in Fig. 4, *B* and *C*, are the dose–response relationships obtained by normalizing to the LTC_4-modulated $I_{K[ACh]ss}$ (calculated from the dose–response relationship in Fig. 2). Estimated in this manner, the LTC_4-mediated increases in $I_{K[ACh]ss}$ and in G_k activation rate each account for ~50% of the effect of LTC_4 on the rates of GTPγS-mediated $I_{K[ACh]}$ activation.

Effects of LTC_4 on G_k Activation Rates

G_k activation incorporates a number of kinetically distinct steps, including release of GDP, binding of GTP (or GTPγS), dissociation into a guanine nucleotide triphosphate–bound α_k plus $\beta\gamma$, and diffusion of activated subunits to the $K_{[ACh]}$ channel. Any or all of these steps may be modulated by LTC_4. We had previously assumed that the limiting rate of $I_{K[ACh]}$ activation was due to the slow, basal rate of GDP release from the inactive heterotrimeric G_k (Breitwieser and Szabo, 1988), but it is possible that this step is not rate limiting under all conditions. LTC_4 might affect the rate of GTPγS-mediated $I_{K[ACh]}$ activation not by modulating the rate of GDP release, but by altering the relative GTPγS/GTP affinities of the nucleotide-free form of G_k, i.e., increasing the affinity of G_k for GTPγS relative to GTP. To assess this possibility, we determined the effect of 3 μM LTC_4 on the rates of $I_{K[ACh]}$ activation in the presence of various GTPγS/GTP concentration ratios. The rates of activation in the presence of LTC_4 were determined as illustrated in Fig. 4 *A*, normalized to $I_{K[ACh]ss}$ measured in the absence of LTC_4. The GTPγS/GTP dose–response relationships are illustrated in Fig. 5. The GTPγS/GTP ratio that mediates the half-maximal $I_{K[ACh]}$ activation rate is 1.5 in both the presence and absence of 3 μM LTC_4, suggesting that LTC_4 does not alter the relative affinities of G_k for GTP and GTPγS. Although it is possible that

LTC_4 may alter the diffusion of subunits to the channel, this possibility seems unlikely since lipid analogues such as LTB_4 and LTD_4 did not mimic the effect, although they might be expected to produce comparable changes in membrane fluidity. Thus the most likely steps for LTC_4 modulation of G_k activation rates are (*a*) the rate of GDP release or (*b*) the rate of dissociation of G_k subunits, once guanine nucleotide triphosphate has bound. These two steps are not distinguishable under our experimental conditions, and we thus lump them together into the term "G_k activation," which is clearly modulated by LTC_4.

LTC_4 Modulation of GTP-mediated Activation of $I_{K[ACh]}$

The results of the previous sections suggest that LTC_4 both enhances the rate of G_k activation and increases GTPγS-activated $I_{K[ACh]ss}$. It is likely, therefore, that GTP-dependent $I_{K[ACh]}$ activation should likewise be enhanced. We examined both the effects of exogenously applied LTC_4 and blockers of endogenous LTC_4 production on the activation of $I_{K[ACh]}$ by ACh in the presence of intracellular GTP.

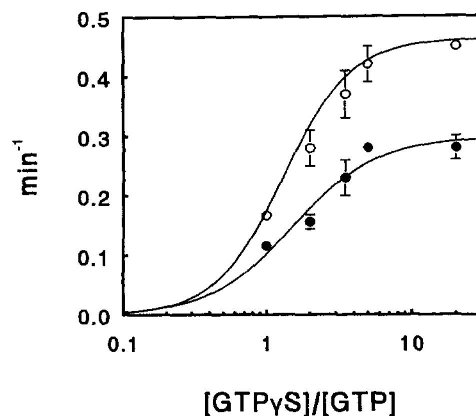

FIGURE 5. Effect of LTC_4 on dependence of $I_{K[ACh]}$ activation rate on GTPγS/GTP concentration ratio. Experiments as illustrated in Fig. 1 *A* were performed with different GTPγS/GTP concentration ratios in the absence (*filled circles*) and presence (*open circles*) of 3 μM LTC_4. The normalized rate of $I_{K[ACh]}$ activation (normalized in both cases to the $I_{K[ACh]ss}$ in the absence of LTC_4) is plotted versus the [GTPγS]/[GTP] ratio. Data points represent the mean ± SEM for at least four independent experiments at each [GTPγS]/[GTP] ratio. Actual concentrations were: 20:1, 1 mM GTPγS/50 μM GTP; 5:1, 1 mM GTPγS/200 μM GTP; 3.5:1, 368 μM GTPγS/105 μM GTP; 2:1, 100 μM GTPγS/50 μM GTP; 1:1, 50 μM GTPγS/50 μM GTP. Data for both curves were fitted to the equation: $\max/\{1 + [K/([GTP\gamma S]/[GTP])]^n\}$. Best fit parameters were: (control) max = 0.3 min^{-1}, K = 1.48 μM, n = 1.43; (3 μM LTC_4) max = 0.46 min^{-1}, K = 1.45 μM, n = 1.6.

A double ACh application protocol was developed to allow each cell to serve as its own control, as illustrated in Fig. 6 *A*. ACh (1 μM) was applied for 2 min, followed by a return to control Ringers solution for at least 4 min. 1 μM ACh was then applied a second time in the absence (Fig. 6 *A*) or presence of various modulators (Fig. 6, *B–F*). In the control experiment (Fig. 6 *A*) both peak $I_{K[ACh]}$ and $I_{K[ACh]ss}$ (assessed 90 s after the peak) of the second application of ACh were indistinguishable from the first ([$peak_2/peak_1$]%: 99.8 ± 1.3%; [SS_2/SS_1]%: 97.3 ± 2.7%; n = 6), confirming that a 4-min control period between ACh applications was sufficient to allow complete recovery from desensitization.

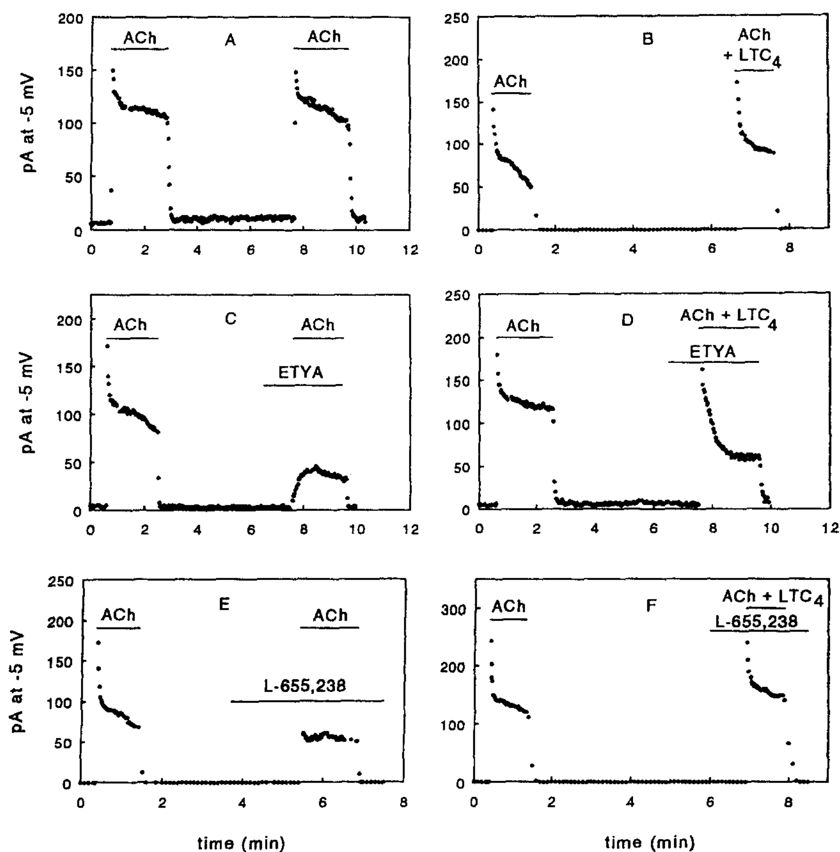

FIGURE 6. Effect of LTC_4 on ACh-dependent $I_{K[ACh]}$ activation. Two applications of 1 μM ACh (separated by a 4–5-min wash period in control Ringers solution) were applied to each cell, to allow each cell to serve as its own control. The second ACh exposure was accompanied by various test compounds. (*A*) Control experiment, demonstrating that the 5-min wash period between ACh applications is sufficient to fully restore both peak $I_{K[ACh]}$ and $I_{K[ACh]ss}$. (*B*) Effect of 10 μM LTC_4. The second application of ACh was accompanied by 10 μM LTC_4. (*C*) Effect of 10 μM ETYA. 10 μM ETYA was superfused 1 min before and during the second ACh application. (*D*) LTC_4 can eliminate the block of $I_{K[ACh]}$ produced by ETYA. 10 μM ETYA was superfused 1 min before and during the second ACh application, which was accompanied by 10 μM LTC_4. (*E*) Effect of 5 μM L-655,238. 5 μM L-655,238 was superfused 2 min before and during the second 1-μM ACh application. (*F*) LTC_4 can eliminate the block of peak $I_{K[ACh]}$ produced by L-655,238. 5 μM L-655,238 was superfused 1 min before and during application of both 1 μM ACh and 10 μM LTC_4.

When 10 μM LTC_4 is applied coincident with the second ACh challenge, there is a significant increase in the instantaneous peak $I_{K[ACh]}$: [peak$_2$(LTC_4)/peak$_1$(control)]%: 122 ± 6.7% (n = 5). There is greater variability in the steady-state $I_{K[ACh]}$ activated in the presence of ACh plus 10 μM LTC_4 when compared with that activated by 1 μM ACh alone: [SS_2(LTC_4)/SS_1(control)]% is 114 ± 22% (n = 5), but there appears to be a slight stimulatory effect of 10 μM LTC_4.

Since exogenously applied LTC_4 enhances peak $I_{K[ACh]}$ activated in the presence of ACh, we determined whether endogenous LTC_4 production might be responsible for activation of peak $I_{K[ACh]}$ under control conditions. The ability of ACh to elicit $I_{K[ACh]}$ in the presence of 10 μM ETYA (which blocks endogenous production of leukotrienes from free arachidonic acid) or 5 μM L-655,238 (which prevents activation of 5-lipoxygenase activating protein [Evans et al., 1991]) was examined. The results, illustrated in Fig. 6, *C* and *E*, suggest that block of endogenous LTC_4 production severely limits the ability of ACh to activate $I_{K[ACh]}$. In the presence of either 10 μM ETYA or 5 μM L-655,238, ACh is able to activate $I_{K[ACh]}$, albeit without activation of an instantaneous peak $I_{K[ACh]}$ and with a greatly attenuated $I_{K[ACh]ss}$. ETYA (at 10 μM) produces a slowing of the rate of $I_{K[ACh]}$ activation by ACh, while low concentrations of L-655,238 (3–5 μM) block activation of peak $I_{K[ACh]}$ without an effect on the initial rate of $I_{K[ACh]}$ activation. High concentrations of L-655,238 (10–20 μM) mimic the effects of ETYA, both blocking peak $I_{K[ACh]}$ activation and slowing the rate of activation in response to 1 μM ACh.

Both ETYA and/or L-655,238 may cause block of peak $I_{K[ACh]}$ activation by a variety of potential mechanisms: (*a*) direct block of the $K_{[ACh]}$ channel; (*b*) nonspecific perturbations of the myocyte membrane which interferes with signal transduction; or (*c*) block of LTC_4 synthesis. Direct block of the $K_{[ACh]}$ channel does not occur, since neither ETYA (Fig. 4) nor L-655,238 (data not shown) blocks GTPγS-activated $I_{K[ACh]ss}$. Nonspecific perturbations of the myocyte membrane that affect the mAChR-mediated signal transduction pathway can be ruled out by determining whether the effects of ETYA and/or L-655,238 are mitigated by exogenously applied LTC_4. When the second application of ACh includes both ETYA and LTC_4 (Fig. 6 *D*) or L-655,238 and LTC_4 (Fig. 6 *F*), the time course and magnitude of $I_{K[ACh]}$ resembles the response in the presence of ACh plus LTC_4 (Fig. 6 *B*), suggesting that the effects of ETYA and L-655,238 can be attributed to block of endogenous LTC_4 production.

Other inhibitors of LTC_4 synthesis, including CDC and baicalein, also block activation of peak $I_{K[ACh]}$ in response to application of 1 μM ACh by the double pulse protocol illustrated in Fig. 6. All of these compounds inhibit activation of peak $I_{K[ACh]}$, with more variable effects on steady-state $I_{K[ACh]}$ (60–90 s after peak), as illustrated in Table I. The commonality of the effects with a variety of LTC_4 synthesis inhibitors strongly supports a role of endogenous arachidonic acid metabolites, specifically LTC_4, in activation of peak $I_{K[ACh]}$.

DISCUSSION

LTC_4 Modulation of $I_{K[ACh]}$ Activation

We have used a variety of experimental strategies to investigate the role of LTC_4 in modulating activation of the muscarinic K^+ channel of bullfrog atrial myocytes. We find that LTC_4 enhances GTPγS-mediated $I_{K[ACh]}$ activation by increasing both the apparent rate of G_k activation (either GDP release from G_k or dissociation of the activated G_k complex into α_k^{GTP} and βγ subunits) and the ability of activated G_k (α_k^{GTP} and/or βγ subunits) to activate the $K_{[ACh]}$ channel. LTC_4 does not alter the relative affinities of G_k for GTP and GTPγS. All of the effects of LTC_4 on $I_{K[ACh]}$ activation are produced with a $K_{0.5}$ of 3–5 μM, and we hypothesize that they are

mediated by LTC_4 binding to a common site. Kinetic cooperativity is apparent in the enhancement of the rate of GTPγS-mediated $I_{K[ACh]}$ activation, presumably since both LTC_4-mediated increases in G_k activation rate and G_k-mediated activation of the $K_{[ACh]}$ channel (as assessed by LTC_4-mediated increases in GTPγS-activated $I_{K[ACh]ss}$) contribute to the overall rate of $I_{K[ACh]}$ activation. Block of cellular LTC_4 biosynthesis with a variety of inhibitors of either 5-lipoxygenase (ETYA, CDC, baicalein) or FLAP (L-655,238) results in a greatly attenuated response to ACh under control (intracellular GTP) conditions, i.e., absence of peak and decreased $I_{K[ACh]ss}$. Exogenously applied LTC_4 can restore normal responsiveness to ACh in the presence of LTC_4 biosynthesis inhibitors, suggesting that the inhibitors are affecting $I_{K[ACh]}$ activation by reducing the cellular availability of LTC_4.

TABLE I

Effect of Various Inhibitors of LTC_4 Synthesis on the Activation of Peak and Steady-State $I_{K[ACh]}$

Compound	$(Peak_2/peak_1)\%$	$(SS_2/SS_1)\%$
10 μM ETYA	53 ± 6.6 (4)	77.5 ± 5.9 (4)
3 μM L-655, 238	47 ± 2.4 (4)	94 ± 3 (4)
5 μM L-655, 238	40 ± 3.2 (4)	82 ± 3 (4)
10 μM CDC	57 ± 7 (5)	83 ± 7 (5)
10 μM baicalein	51 ± 5.2 (6)	75 ± 4.4 (6)

The double ACh application protocol was used (as illustrated in Fig. 6). The compounds indicated in the table were present during the second application of 1 μM ACh. For those compounds that inhibit activation of peak current, $peak_2$ is defined as the maximum activatable $I_{K[ACh]}$. $(Peak_2/peak_1)\%$ is defined as the ratio of the peak $I_{K[ACh]}$ produced in response to 1 μM ACh plus the compound of interest to the control peak $I_{K[ACh]}$ in response to 1 μM ACh in the same cell × 100. $(SS_2/SS_1)\%$ is the ratio of the steady state (60–90 s after the peak) of $I_{K[ACh]}$ in the presence of 1 μM ACh plus the compound of interest to that observed under control conditions in the same cell × 100.

Characterization of LTC_4 Receptors in Atrial Myocytes

Leukotriene receptors have been identified in a variety of tissues, and appear to fall into two distinct signaling classes (Piper, 1984; Crooke, Mong, Clark, Hogaboom, Lewis, and Gleason, 1987; Cristol, Provencal, and Sirios, 1989). LTB_4 and $LTD_4(LTE_4)$ receptors have been well characterized, and are coupled to cell processes by G proteins. Activation of LTB_4 or $LTD_4(LTE_4)$ receptors results in increases in intracellular Ca^{2+}, IP_3, and activation of protein kinase C (Saussy, Sarau, Foley, Mong, and Crooke, 1989; Snyder and Fleisch, 1989; Bouchelouche and Berild, 1991). In contrast, biological effects of LTC_4 have been identified in numerous cell types, including modulation of leutinizing hormone secretion (Hulting, Lindgren, Hokfelt, Eneroth, Werner, Patrono, and Samuelsson, 1985; Kiesel, Przylipiak, Habenicht, Przylipiak, and Runnebaum, 1991; Dan-Cohen, Sofer, Schwartzman, Natarajan, Nadler, and Naor, 1992) and cardiac contractility (Hattori and Levi, 1984; Herman, Heller, Canavan, and Herman, 1988; Robleto, Reitmeyer, and Herman, 1988; Herman, Heller, and Herman, 1990), and activation of Ca^{2+} channels in

response to epidermal growth factor (Peppelenbosch, Tertoolen, den Hertog, and de Laat, 1992), although little is known about the signal transduction mechanism(s). Activation of cardiac LTC_4 receptors causes changes in cardiac contractility under conditions in which conversion of LTC_4 to $LTD_4(LTE_4)$ is prevented and alterations in cell Ca^{2+} or IP_3 are not observed (Herman et al., 1988; Chiono, Heller, Andazola, and Herman, 1991). LTC_4 binding is not affected by GTP (Hogaboom, Mong, Wu, and Crooke, 1983; Mong, Wu, Scott, Lewis, Clark, Weichman, Kinzig, Gleason, and Crooke, 1985; Cristol et al., 1989), suggesting that the receptor does not belong to the G protein–coupled superfamily.

Binding sites for LTC_4 are widely distributed (Cristol et al., 1989), although a biological activity associated with LTC_4 binding is not always apparent. Careful analysis of LTC_4 binding sites suggests that some apparent LTC_4 "receptors" may represent cytoplasmic enzymes such as glutathione transferase (Sun, Chau, Spur, Corey, Lewis, and Austen, 1986; Sun, Chau, and Austen, 1987). Recent characterizations of LTC_4 receptors have used isolated membranes and parallel assays for enzymatic binding sites, as well as serine borate to prevent LTC_4 metabolism to $LTD_4(LTE_4)$ (Herman et al., 1988; Chiono et al., 1991). It remains clear that LTC_4 receptors represent a distinct class with potentially novel signaling mechanisms.

Binding studies in membranes isolated from bullfrog ventricle (Chiono et al., 1991) indicated a single class of LTC_4 binding sites with K_d of 34 nM and a B_{max} of 52 pmol/mg protein (in the presence of 45 mM L-serine borate), with a decrease in affinity of two orders of magnitude in the absence of serine/borate. LTC_4 binding was not antagonized by up to 10 μM LTD_4, 30 μM LTE_4, or GTPγS. Our data in bullfrog atrial myocytes are consistent with the characteristics of LTC_4 receptors in membranes of bullfrog heart. LTC_4-mediated modulation of $I_{K[ACh]}$ activation does not involve a G protein–mediated pathway, since the LTC_4 effect is rapidly reversible upon washout of the lipid, even in the presence of GTPγS. Since our electrophysiological experiments were performed in the absence of serine borate, the $K_{0.5}$ for modulation of $I_{K[ACh]}$ activation (3–4 μM) is in line with that observed by Chiono et al. (1991). In addition, neither LTB_4 nor LTD_4 was able to mimic the effect of LTC_4, suggesting that the responses are specifically initiated by activation of LTC_4 receptors.

Mechanism of LTC_4-mediated $I_{K[ACh]}$ Modulation

The increasing complexity of the mechanism of $I_{K[ACh]}$ activation, via muscarinic (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985), α_1-adrenergic (Kurachi et al., 1989; Braun, Fedida, and Giles, 1992), adenosine (Kurachi, Nakajima, and Sugimoto, 1986), platelet activating factor (Nakajima et al., 1991; Ramos-Franco, Lo, and Breitwieser, 1993), and calcitonin gene-related peptide (Kim, 1991) receptors suggests that multiple signal transduction pathways (and distinct identities of coupling G proteins) are possible. In addition, both α_k and $\beta\gamma$ subunits may play multiple roles in $I_{K[ACh]}$ activation (Breitwieser, 1991), including potential direct interaction with the $K_{[ACh]}$ channel (Birnbaumer, Abramowitz, and Brown, 1990; Birnbaumer, 1992; Ito et al., 1992), activation of phospholipases (Clapham, 1990), and potential participation in desensitization via $\beta\gamma$ binding to β-adrenergic receptor kinase or the muscarinic receptor kinase homologue (Haga

and Haga, 1992; Pitcher, Inglese, Higgins, Arriza, Casey, Kim, Benovic, Kwatra, Caron, and Lefkowitz, 1992). A region of the α_i subunit NH_2 terminus has been implicated in the interaction with $\beta\gamma$, and may also be involved in modulation of the GDP release rate during G protein activation (Osawa, Khanasekaran, Woon, and Johnson, 1990; Dhanasekaran, Osawa, and Johnson, 1991), and thus the stability of the interaction between α_k and $\beta\gamma$ is also a potential site for modulation.

The results of our experiments suggest that some of the kinetic properties of the muscarinic receptor-G_k-$K_{[ACh]}$ channel signal transduction pathway are altered by LTC_4 to produce enhanced activation of the $K_{[ACh]}$ channel. Furthermore, our results suggest that cellular biosynthesis of LTC_4 may contribute to the process of $I_{K[ACh]}$ activation; i.e., LTC_4 is required for the rapid, ACh-mediated activation of $I_{K[ACh]}$ under physiological (GTP) conditions, but is not required for maintenance of steady-state $I_{K[ACh]}$ in the presence of either GTP or GTPγS.

The mechanism(s) by which LTC_4 mediates its effects on G_k activation cannot be resolved from the present experiments, but it is possible that either α_k or $\beta\gamma$ or their interaction with each other or the $K_{[ACh]}$ channel may be altered by LTC_4. We suggest that these latter participants in the signal transduction pathway are most likely to be affected, since the response to LTC_4 can be observed in the absence of mAChR stimulation, or after all of G_k has been persistently activated by GTPγS. Since LTC_4 receptors have not been well characterized (and no selective antagonists exist), we cannot distinguish between direct interaction of LTC_4 with proteins in the $K_{[ACh]}$ channel signal transduction pathway, or mediation of the effects by a potential LTC_4 receptor and novel signal transduction mechanism. Further experiments are required to fully define the role(s) for arachidonic acid metabolites in the modulation of $I_{K[ACh]}$ activation, and the potential contributions of cellular lipid metabolism to regulation of other G protein–dependent processes.

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