Leukotriene C₄ Modulation of Muscarinic K⁺ Current Activation in Bullfrog Atrial Myocytes

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ABSTRACT The effects of leukotriene C₄ (LTC₄) on activation of muscarinic acetylcholine receptor (mACHR)–stimulated, inwardly rectifying K⁺ current (IₖmAChR) were examined in single bullfrog atrial myocytes using the whole-cell patch clamp technique. LTC₄ produced a reversible, concentration-dependent increase in steady-state, guanosine–y-thiotriphosphate (GTPγS)-activated IₖmAChR, with a K₀.₅ of 3.1 μM. LTC₄ also increased the rate of GTPγS-mediated IₖmAChR activation, both in the absence and presence of 1 nM ACh, with comparable K₀.₅ values of 4.7 μM under basal conditions and 4.9 μM in the presence of 1 nM ACh. LTC₄ did not alter the relative affinities of the G protein, G₄₅, for GTPγS and GTP. We hypothesize that all of the effects of LTC₄ on the kinetics of G₄₅-mediated IₖmAChR activation are produced at a common site with a K₀.₅ of 3–5 μM. The effects of LTC₄ on IₖmAChR activation are fully reversible in the presence of GTPγS. Under physiological conditions (i.e., intracellular GTP), 10 μM LTC₄ increased the ACh-activated peak IₖmAChR. Inhibitors of cellular LTC₄ 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 IₖmAChR activation, preventing activation of peak, and producing a lower steady-state IₖmAChR (when compared with the control response in the same cell). Addition of exogenous LTC₄ was able to overcome the effects of LTC₄ synthesis inhibitors, restoring both the peak and steady-state IₖmAChR responses. Although the mechanism of LTC₄-mediated modulation of IₖmAChR activation is not known, our results suggest that endogenously produced lipoxygenase metabolites of arachidonic acid, specifically LTC₄, are involved in the physiological process of IₖmAChR activation.

INTRODUCTION

Acetylcholine (ACh) binding to atrial muscarinic receptors (mACHR) induces activation of an inwardly rectifying potassium current, IₖmAChR. A pertussis toxin–sensitive G
protein, G_{\alpha}, 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 \alpha-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 \beta subunit activation of phospholipase A_{2} (PLA_{2}; Kim et al., 1989; Clapham, 1990), although more recent experiments suggest that \beta 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-\gamma-thiotriphosphate (GTP\gammaS)-mediated activation of I_{K_{ACh}} and the GTP\gammaS-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 desensitization of peak current) is slightly increased by 10 \mu M LTC_{4} in the presence of nordihydroguaiaretic acid (NDGA, a 5- and 12-lipoxygenase inhibitor) when G_{\alpha} was persistently activated by GTP\gammaS (Scherer and Breitwieser, 1990), suggesting the possibility that some of the effects of LTC_{4} can be localized to either GTP\gammaS-activated G_{\alpha}, 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 \mu M, and that both the rate of G_{\alpha} activation and the magnitude of GTP\gammaS-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_{2}PO_{4}, 2.5 pyruvate, 1 mg/ml fatty acid–free bovine serum albumin, and MEM vitamins and essential amino acids (1 \mu M/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 3 mM creatine, 50 U/ml penicillin, 50 \mu g/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 Ca²⁺-free Ringers plus 0.9 mM CaCl₂, 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 MgCl₂, 2.5 CaCl₂, and 20 HEPES, pH 7.4. To block Ca²⁺ and Na⁺ currents, 500 μM CdCl₂ and 5 μM tetrodotoxin were added, respectively. The intracellular solution contained (mM): 80 K⁺ aspartate, 30 KCl, 1 EGTA, 0.05 Na₂GTP, 5 MgATP, and 5 HEPES, pH 7.4. When present, GTP₆S (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-a-cyanocinnamate (CDC; BIOMOL Research Labs Inc.), and α-pentyl-4-(2-quinolinylmethoxy)-benzenemethanol (L-655,238; BIOMOL Research Labs Inc.) in ethanol, or leukotrienes C₄ (glycine, N-[S-[1-(4-carboxy-l-hydroxybutyl)-2,4,6,9-pentadecatetraenyl]-L-L-'y-glutamyl-L-cysteinyl]-[R-[R*,S*-(E,E,Z,Z)]], B₄ (6,8,10,14-eicosatetraenoic acid, 5,12-dihydroxy-, [S-[R*,S*-(E,E,Z,Z)]], or D₄ (glycine, N-[S-[1-(4-carboxy-1-hydroxybutyl)-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 $I_{\text{K}_{\text{ACh}}}$ 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 $I_{\text{K}_{\text{ACh}}}$ 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 (NFT; Island Products, Galveston, TX). All experiments were performed at room temperature (22–24°C).
RESULTS

Effect of LTC₄ on GTPγS-activated Steady-State IKₐChₐ

A variety of arachidonic acid metabolites, including leukotrienes, modulate activation of the muscarinic K⁺ current, IKₐChₐ, in atrial myocytes (Kim et al., 1989; Kurachi et al., 1989; Scherer and Breitwieser, 1990). In particular, LTC₄ (10 μM in the presence of 10 μM NDGA, a 5- and 12-lipoxygenase inhibitor), produces a slight increase in steady-state, GTPγS-activated IKₐChₐ (Scherer and Breitwieser, 1990). Because the LTC₄-mediated increase in steady-state GTPγS-activated IKₐChₐ may result from a novel regulatory mechanism, we examined the properties of this effect of LTC₄.

Pipette solutions contained 1 mM GTPγS. A brief pulse of 1 μM ACh was used to activate peak IKₐChₐ, which declined to steady-state IKₐChₐ (IKₐChₐss), 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 LTC₄, respectively, on GTPγS-activated IKₐChₐss. LTC₄ reversibly elicits additional current, which is not affected by 10 μM atropine, but is blocked by 0.5 mM Ba²⁺ (data not shown). The current–voltage relationship of the LTC₄-elicited current is qualitatively similar to the steady-state current–voltage relationship of IKₐChₐ 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 LTC₄ increases IKₐChₐ rather than activating a distinct K⁺ current. This interpretation is further supported by the fact that LTC₄ has been shown to activate KₐCh channels in cell-attached and excised patches (Kim et al., 1989; Kurachi et al., 1989; Clapham, 1990).

The LTC₄-mediated increase in IKₐChₐss occurs rapidly, and upon removal of LTC₄ the current rapidly returns to the control level of GTPγS-mediated steady-state IKₐChₐ (Fig. 1, B and C). The reversibility of this effect suggests that LTC₄ does not act via a G protein–coupled receptor (which would mediate a persistent increase in IKₐChₐss under these experimental conditions). The increase in IKₐChₐ induced by LTC₄ is saturable (Fig. 2). The line through the data points represents the fit to a single site model, with a Kₐ₅₀ of 3.1 μM. The LTC₄-mediated increase in IKₐChₐ saturates at 155% of the control, GTPγS-activated IKₐChₐss. Comparison of the increase in steady-state IKₐChₐ achieved by 10 μM LTC₄ (143 ± 22% of control IKₐChₐss, n = 10), 10 μM LTB₄ (109 ± 11% of control IKₐChₐss, n = 5), and 10 μM LTD₄ (109 ± 25% of control IKₐChₐss, n = 5) suggests specificity for the structure of LTC₄. Neither a lipophilic analogue (LTB₄) nor an analogue with a truncated peptidyl group (LTD₄) is able to mimic the LTC₄ effect.

ETYA, which inhibits both cyclooxygenases and lipoxygenases (Tobias and Hamilton, 1979; Bokoch and Reed, 1981), has no effect on GTPγS-activated IKₐChₐss at concentrations as high as 10 μM. Fig. 3 illustrates a typical experiment, in which IKₐChₐ was first persistently activated in the presence of GTPγS, and then ETYA (10 μM) was applied. The lack of ETYA effect on GTPγS-activated IKₐChₐss suggests that cellular production of LTC₄ does not contribute significantly to the maintenance of GTPγS-activated IKₐChₐss. This result is not specific to ETYA, since other inhibitors of LTC₄ 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 involvement of endogenous lipid metabolites during activation. The effect of ETYA on the GTPγS-mediated rate of $I_{K(ACh)}$ activation is overcome by LTC₄; 1 μM ETYA + 10 μM LTC₄, 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 LTC₄ alone, 0.55 ± 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-
mediated $I_{K(ACh)}$ activation rate suggest that the primary role for LTC$_4$ under these experimental conditions (i.e., in the presence of GTP$\gamma$S) is promotion of $G_k$ activation.

**LTC$_4$ Modulation of GTP$\gamma$S-mediated Activation of $I_{K(ACh)}$**

LTC$_4$ (10 $\mu$M) increases the rate of GTP$\gamma$S-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 GTP$\gamma$S-mediated $I_{K(ACh)}$ activation in the presence of LTC$_4$. Pipette solutions contained 1 mM GTP$\gamma$S. Immediately upon achieving the whole-cell configuration, 70 $\mu$M LTC$_4$ was added to the bath solution until a linear rate of $I_{K(ACh)}$ activation was observed. 1 $\mu$M 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, GTP$\gamma$S-mediated...
**Figure 4.** Effect of LTC4 on basal and ACh-dependent GTPyS-mediated rates of \( I_{K(ACh)} \) activation. Pipette solutions contained 1 mM GTPyS and 0.05 mM GTP (20:1 ratio). (A) Effect of LTC4 (70 uM) 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 uM LTC4. After an observable initial rate was obtained, the superfusing solution was switched to one containing 1 uM ACh (but no LTC4). Washout of ACh resulted in a stable GTPyS-activated \( I_{K(ACh)} \). (B) Concentration dependence of LTC4 enhancement of the GTPyS-mediated \( I_{K(ACh)} \) activation rate. Experimental protocol illustrated in A. The LTC4 concentration is plotted versus the normalized rate of \( I_{K(ACh)} \) activation (min^{-1}), calculated as: initial rate \((\mu A/min) / I_{K(ACh)ss} \) (pA). Data were fitted (solid line) to the equation: \( B + \text{max}/[1 + (K/[LTC4])]^n \), with \( B = 0.28 \text{ min}^{-1} \); \( \text{max} = 0.353 \text{ min}^{-1} \); \( K = 4.74 \text{ uM} \); \( n = 1.4 \). (C) Concentration dependence of LTC4 enhancement of the GTPyS-mediated \( I_{K(ACh)} \) activation rate in the presence of 1 nM ACh. Experimental protocol as in A, with both LTC4 and 1 nM ACh in the superfusing solution. Data were fitted (solid line) with the same equation as in B, with \( B = 0.83 \text{ min}^{-1} \); \( \text{max} = 1.44 \text{ min}^{-1} \); \( K = 4.88 \text{ uM} \); \( n = 2.3 \). Data plotted in both B and C represent mean ± SEM, with at least four independent experiments at each concentration of LTC4. Dashed lines in both B and C are the relationships obtained when the rate of \( I_{K(ACh)ss} \) activation is calculated by normalization to the LTC4-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 LTC4, calculated from the fit in Fig. 2).

\( I_{K(ACh)ss} \). The LTC4 dose response for the basal, mAChR-independent, GTPyS-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 uM and a Hill coefficient of 1.4.

A dose response for LTC4 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 LTC4 and 1 nM ACh. The rate of \( \text{I}_K^{(\text{ACh})} \) activation in the absence of LTC4 under these conditions was 0.76 ± 0.03 min\(^{-1}\) (\( n = 5 \)), which is consistent with the enhanced rate of \( \text{I}_K^{(\text{ACh})} \) 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 \( \mu \text{M} \) and a Hill coefficient of 2.3. The similarities in the estimated \( K_{0.5} \) for LTC4 mediation of both the increase in GTP\( \gamma \)-S-activated \( \text{I}_K^{(\text{ACh})} \) (Fig. 2) and for enhanced GTP\( \gamma \)S-mediated \( \text{I}_K^{(\text{ACh})} \) activation rates (in the absence [Fig. 4 B] and presence of 1 nM ACh [Fig. 4 C]) suggest a common site of LTC4 action.

The GTP\( \gamma \)S-mediated rate of \( \text{I}_K^{(\text{ACh})} \) activation is normalized to the steady-state \( \text{I}_K^{(\text{ACh})} \) obtained in each cell, which has been considered to reflect the rate of \( \text{G}_k \) activation (Breitwieser and Szabo, 1988). The rates of GTP\( \gamma \)S-mediated \( \text{I}_K^{(\text{ACh})} \) activation in the presence of LTC4 were also determined in this manner (Fig. 4). This is an overestimate of the effect of LTC4 on the rate of \( \text{G}_k \) activation, however, since LTC4 can increase the steady-state GTP\( \gamma \)-S-activated \( \text{I}_K^{(\text{ACh})} \) (Fig. 2) independent of an effect on \( \text{G}_k \) activation (i.e., when all of \( \text{G}_k \) has been persistently activated by GTP\( \gamma \)S).

The two effects of LTC4 (i.e., on \( \text{G}_k \) activation and on steady-state GTP\( \gamma \)-S-activated \( \text{I}_K^{(\text{ACh})} \)) can be separated, however, since (a) the effect of LTC4 on \( \text{I}_K^{(\text{ACh})} \) can be independently determined (Fig. 2); (b) the effect of LTC4 on \( \text{I}_K^{(\text{ACh})} \) is rapidly reversible (Fig. 1, B and C); and finally, (c) \( \text{I}_K^{(\text{ACh})} \) determined in the absence of exogenous LTC4 is not a function of continued, endogenous LTC4 production (Fig. 3). Plotted as dashed lines in Fig. 4, B and C, are the dose–response relationships obtained by normalizing to the LTC4-modulated \( \text{I}_K^{(\text{ACh})} \) (calculated from the dose–response relationship in Fig. 2). Estimated in this manner, the LTC4-mediated increases in \( \text{I}_K^{(\text{ACh})} \) and in \( \text{G}_k \) activation rate each account for ~50% of the effect of LTC4 on the rates of GTP\( \gamma \)S-mediated \( \text{I}_K^{(\text{ACh})} \) activation.

Effects of LTC4 on \( \text{G}_k \) Activation Rates

\( \text{G}_k \) activation incorporates a number of kinetically distinct steps, including release of GDP, binding of GTP (or GTP\( \gamma \)S), dissociation into a guanine nucleotide triphosphate–bound \( \alpha \) plus \( \beta \gamma \), and diffusion of activated subunits to the \( \text{K}_{(\text{ACh})} \) channel. Any or all of these steps may be modulated by LTC4. We had previously assumed that the limiting rate of \( \text{I}_K^{(\text{ACh})} \) activation was due to the slow, basal rate of GDP release from the inactive heterotrimeric \( \text{G}_k \) (Breitwieser and Szabo, 1988), but it is possible that this step is not rate limiting under all conditions. LTC4 might affect the rate of GTP\( \gamma \)-S-mediated \( \text{I}_K^{(\text{ACh})} \) activation not by modulating the rate of GDP release, but by altering the relative GTP\( \gamma \)S/GTP affinities of the nucleotide-free form of \( \text{G}_k \), i.e., increasing the affinity of \( \text{G}_k \) for GTP\( \gamma \)S relative to GTP. To assess this possibility, we determined the effect of 3 \( \mu \text{M} \) LTC4 on the rates of \( \text{I}_K^{(\text{ACh})} \) activation in the presence of various GTP\( \gamma \)S/GTP concentration ratios. The rates of activation in the presence of LTC4 were determined as illustrated in Fig. 4 A, normalized to \( \text{I}_K^{(\text{ACh})} \) measured in the absence of LTC4. The GTP\( \gamma \)S/GTP dose–response relationships are illustrated in Fig. 5. The GTP\( \gamma \)S/GTP ratio that mediates the half-maximal \( \text{I}_K^{(\text{ACh})} \) activation rate is 1.5 in both the presence and absence of 3 \( \mu \text{M} \) LTC4, suggesting that LTC4 does not alter the relative affinities of \( \text{G}_k \) for GTP and GTP\( \gamma \)S. Although it is possible that
LTC₄ may alter the diffusion of subunits to the channel, this possibility seems unlikely since lipid analogues such as LTB₄ and LTD₄ did not mimic the effect, although they might be expected to produce comparable changes in membrane fluidity. Thus the most likely steps for LTC₄ modulation of Gₖ activation rates are (a) the rate of GDP release or (b) the rate of dissociation of Gₖ 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 "Gₖ activation," which is clearly modulated by LTC₄.

**LTC₄ Modulation of GTP-mediated Activation of Iₐ₄**

The results of the previous sections suggest that LTC₄ both enhances the rate of Gₖ activation and increases GTP₇S-activated Iₐ₄. It is likely, therefore, that GTP-

![Figure 5](image)

**Figure 5.** Effect of LTC₄ on dependence of Iₐ₄ activation rate on GTP₇S/GTP concentration ratio. Experiments as illustrated in Fig. 1 A were performed with different GTP₇S/GTP concentration ratios in the absence (filled circles) and presence (open circles) of 3 μM LTC₄. The normalized rate of Iₐ₄ activation (normalized in both cases to the Iₐ₄ in the absence of LTC₄) is plotted versus the [GTP₇S]/[GTP] ratio. Data points represent the mean ± SEM for at least four independent experiments at each [GTP₇S]/[GTP] ratio. Actual concentrations were: 20:1, 1 mM GTP₇S/50 μM GTP; 5:1, 1 mM GTP₇S/200 μM GTP; 3.5:1, 368 μM GTP₇S/105 μM GTP; 2:1, 100 μM GTP₇S/50 μM GTP; 1:1, 50 μM GTP₇S/50 μM GTP. Data for both curves were fitted to the equation: max/[1 + ([K]/([GTP₇S]/[GTP]))^n]. Best fit parameters were: (control) max = 0.3 min⁻¹, K = 1.48 μM, n = 1.43; (3 μM LTC₄) max = 0.46 min⁻¹, K = 1.45 μM, n = 1.6.

dependent Iₐ₄ activation should likewise be enhanced. We examined both the effects of exogenously applied LTC₄ and blockers of endogenous LTC₄ production on the activation of Iₐ₄ by ACh in the presence of intracellular GTP.

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 Iₐ₄ and Iₐ₄₃₃ (assessed 90 s after the peak) of the second application of ACh were indistinguishable from the first ([peak₂/peak₁]%: 99.8 ± 1.3%; [SS₂/SS₁]%: 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 LTC4 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 LTC4. The second application of ACh was accompanied by 10 μM LTC4. (C) Effect of 10 μM ETYA. 10 μM ETYA was superfused 1 min before and during the second ACh application. (D) LTC4 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 LTC4. (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) LTC4 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 LTC4.

When 10 μM LTC4 is applied coincident with the second ACh challenge, there is a significant increase in the instantaneous peak $I_{K_{ACh}}$: [peak2(LTC4)/peak1(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 LTC4 when compared with that activated by 1 μM ACh alone: [SS2(LTC4)/SS1(control)]% is 114 ± 22% (n = 5), but there appears to be a slight stimulatory effect of 10 μM LTC4.
Since exogenously applied LTC4 enhances peak $I_{K(ACh)}$ activated in the presence of ACh, we determined whether endogenous LTC4 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 LTC4 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 $I_{K(ACh)}$. 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 LTC4 synthesis. Direct block of the K$_{ACh}$ channel does not occur, since neither ETYA (Fig. 4) nor L-655,238 (data not shown) blocks GTP$_{Y}$-activated $I_{K(ACh)}$. 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 LTC4. When the second application of ACh includes both ETYA and LTC4 (Fig. 6 D) or L-655,238 and LTC4 (Fig. 6 F), the time course and magnitude of $I_{K(ACh)}$ resembles the response in the presence of ACh plus LTC4 (Fig. 6 B), suggesting that the effects of ETYA and L-655,238 can be attributed to block of endogenous LTC4 production.

Other inhibitors of LTC4 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 LTC4 synthesis inhibitors strongly supports a role of endogenous arachidonic acid metabolites, specifically LTC4, in activation of peak $I_{K(ACh)}$.

**DISCUSSION**

**LTC4 Modulation of $I_{K(ACh)}$ Activation**

We have used a variety of experimental strategies to investigate the role of LTC4 in modulating activation of the muscarinic K$^+$ channel of bullfrog atrial myocytes. We find that LTC4 enhances GTP$_{Y}$S-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 $\alpha_k^{GTP}$ and $\beta\gamma$ subunits) and the ability of activated $G_k$ ($\alpha_k^{GTP}$ and/or $\beta\gamma$ subunits) to activate the K$_{ACh}$ channel. LTC4 does not alter the relative affinities of $G_k$ for GTP and GTP$_{Y}$S. All of the effects of LTC4 on $I_{K(ACh)}$ activation are produced with a $K_{0.5}$ of 3–5 μM, and we hypothesize that they are
mediated by LTC4 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 LTC4-mediated increases in $G_{o}$ activation rate and $G_{o}$-mediated activation of the $K_{(ACh)}$ channel (as assessed by LTC4-mediated increases in GTPγS-activated $I_{K(ACh)ss}$) contribute to the overall rate of $I_{K(ACh)}$ activation. Block of cellular LTC4 biosynthesis with a variety of inhibitors of either 5-lipoxygenase (ETYA, CDC, baikalein) 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 LTC4 can restore normal responsiveness to ACh in the presence of LTC4 biosynthesis inhibitors, suggesting that the inhibitors are affecting $I_{K(ACh)}$ activation by reducing the cellular availability of LTC4.

**Table I**

| Compound          | $\text{(Peak}_{2}/\text{peak}_{1})/\%$ | $\text{(SS}_{2}/\text{SS}_{1})/\%$ |
|-------------------|--------------------------------------|----------------------------------|
| 10 μM ETYA        | 53 ± 6.6 (4)                          | 77.5 ± 5.9 (4)                   |
| 5 μM L-655,238    | 47 ± 2.4 (4)                          | 94 ± 3 (4)                       |
| 5 μM L-655,238    | 40 ± 5.2 (4)                          | 82 ± 3 (4)                       |
| 10 μM CDC         | 57 ± 7 (5)                            | 83 ± 7 (5)                       |
| 10 μM baikalein   | 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, $\text{peak}_{1}$ is defined as the maximum activatable $I_{K(ACh)}$; $\text{(Peak}_{2}/\text{peak}_{1})/\%$ 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 x 100. $\text{(SS}_{2}/\text{SS}_{1})/\%$ 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 x 100.

**Characterization of LTC4 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). LTBr and LTD4(LTE4) receptors have been well characterized, and are coupled to cell processes by G proteins. Activation of LTBr or LTD4(LTE4) 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 LTC4 have been identified in numerous cell types, including modulation of leutinizing hormone secretion (Hulting, Lindgren, Hokfelt, Eneroth, Werner, Patrono, and Samuelsson, 1985; Kiesel, Przylipiak, Habe- nicht, Przylipiak, and Runnebaum, 1991; Dan-Cohen, Sofer, Schwartzman, Natara- jan, 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 LTC₄ receptors causes changes in cardiac contractility under conditions in which conversion of LTC₄ to LTD₄(LTE₄) is prevented and alterations in cell Ca²⁺ or IP₃ are not observed (Herman et al., 1988; Chiono, Heller, Andazola, and Herman, 1991). LTC₄ 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 LTC₄ are widely distributed (Cristol et al., 1989), although a biological activity associated with LTC₄ binding is not always apparent. Careful analysis of LTC₄ binding sites suggests that some apparent LTC₄ “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 LTC₄ receptors have used isolated membranes and parallel assays for enzymatic binding sites, as well as serine borate to prevent LTC₄ metabolism to LTD₄(LTE₄) (Herman et al., 1988; Chiono et al., 1991). It remains clear that LTC₄ 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 LTC₄ binding sites with Kₐ of 34 nM and a Bₘₐₓ 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. LTC₄ binding was not antagonized by up to 10 μM LTD₄, 30 μM LTE₄, or GTPγS. Our data in bullfrog atrial myocytes are consistent with the characteristics of LTC₄ receptors in membranes of bullfrog heart. LTC₄-mediated modulation of IK[ACh] activation does not involve a G protein–mediated pathway, since the LTC₄ 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 Kₐₐₐ for modulation of IK[ACh] activation (3–4 μM) is in line with that observed by Chiono et al. (1991). In addition, neither LTB₄ nor LTD₄ was able to mimic the effect of LTC₄, suggesting that the responses are specifically initiated by activation of LTC₄ receptors.

Mechanism of LTC₄-mediated IK[ACh] Modulation

The increasing complexity of the mechanism of IK[ACh] activation, via muscarinic (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985), α₁-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 α₅ and βγ subunits may play multiple roles in IK[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 βγ binding to β-adrenergic receptor kinase or the muscarinic receptor kinase homologue (Haga
and Haga, 1992; Pitcher, Inglese, Higgins, Arriza, Casey, Kim, Benovic, Kwarat, Caron, and Lefkowitz, 1992). A region of the \( \alpha_i \) subunit NH\(_2\) terminus has been implicated in the interaction with \( \beta_y \), 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 \( \alpha_i \) and \( \beta_y \) 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\([\text{ACH}]\) channel signal transduction pathway are altered by LTC\(_4\) to produce enhanced activation of the K\([\text{ACH}]\) channel. Furthermore, our results suggest that cellular biosynthesis of LTC\(_4\) may contribute to the process of K\([\text{ACH}]\) activation; i.e., LTC\(_4\) is required for the rapid, ACh-mediated activation of K\([\text{ACH}]\) under physiological (GTP) conditions, but is not required for maintenance of steady-state K\([\text{ACH}]\) in the presence of either GTP or GTP\(\gamma\)S.

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 \( \alpha_i \) or \( \beta_y \) or their interaction with each other or the K\([\text{ACH}]\) 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\(_4\) has been persistently activated by GTP\(\gamma\)S. 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\([\text{ACH}]\) 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 K\([\text{ACH}]\) activation, and the potential contributions of cellular lipid metabolism to regulation of other G protein-dependent processes.

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