Mechanism of Muscarinic Receptor–induced K⁺ Channel Activation as Revealed by Hydrolysis-resistant GTP Analogues

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ABSTRACT The role of a guanine nucleotide–binding protein (Gₖ) in the coupling between muscarinic receptor activation and opening of an inwardly rectifying K⁺ channel [I_k(M)] was examined in cardiac atrial myocytes, using hydrolysis-resistant GTP analogues. In the absence of muscarinic agonist, GTP analogues produced a membrane current characteristic of I_k(M). The initial rate of appearance of this receptor-independent I_k(M) was measured for the various analogues in order to explore the kinetic properties of I_k(M) activation. We found that I_k(M) activation is controlled solely by the intracellular analogue/GTP ratio and not by the absolute concentrations of the nucleotides. Analogues competed with GTP for binding to Gₖ with the following relative affinities: GTP[y]S > GTP > GppNHp > GppCH₂p. At sufficiently high intracellular concentrations, however, all GTP analogues produced the same rate of I_k(M) activation. This analogue-independent limiting rate is likely to correspond to the rate of GDP release from inactive, GDP-bound Gₖ. Muscarinic receptor stimulation by nanomolar concentrations of acetylcholine (ACh), which do not elicit I_k(M) under control conditions, catalyzed I_k(M) activation in the presence of GTP analogues. The rate of Gₖ activation by ACh (k_ACh) was found to be described by the simple relationship k_ACh = 8.4 X 10⁸ min⁻¹M⁻¹[ACh] + 0.44 min⁻¹, the first term of which presumably reflects the agonist-catalyzed rate of GDP release from the Gₖ·GDP complex, while the second term corresponds to the basal rate of receptor-independent GDP release. Combined with the estimated Kₐ₅ of the I_k(M)[ACh] dose-effect relationship, 160 nM, this result also allowed us to estimate the rate of Gₖ·GTP hydrolysis, k_cat, to be near 135 min⁻¹. These results provide, for the first time, a quantitative description of the salient features of G-protein function in vivo.

INTRODUCTION

Vagal stimulation slows the heart rate and decreases the force of myocardial contractions by releasing the neurotransmitter acetylcholine (ACh) from para-
sympathetic nerve endings (Noble, 1975). At the cellular level, these effects are mediated by muscarinic ACh receptors localized in the cardiac sarcolemma. Voltage-clamp studies of frog atrium have revealed that stimulation of muscarinic receptors affects at least two transmembrane currents: it activates an inwardly rectifying K+ current, designated here as $I_{K(M)}$, and inhibits the slow inward Ca2+ current induced by $\beta$-adrenergic agonists, $I_{B}$ (Giles and Noble, 1976; Momose et al., 1982, 1984; Breitwieser and Szabo, 1985; Fischmeister and Hartzell, 1986). There is a significant delay between muscarinic ACh receptor activation and the appearance of an effect on these membrane currents (Purves, 1976; Hill-Smith and Purves, 1978; Hartzell, 1980; Nargeot et al., 1982). This slow time course of $I_{K(M)}$ activation has prompted several models that include rate-limiting steps beyond the initial binding of ACh (Pott and Pusch, 1979; Nargeot et al., 1982).

Recent studies of atrial myocytes revealed a cascade of events between muscarinic ACh receptor activation and $I_{K(M)}$ channel opening. In particular, intracellular application of hydrolysis-resistant GTP analogues was found to induce an antagonist-resistant, persistently activated $I_{K(M)}$, which suggests that channel opening is mediated by a receptor-activated guanine nucleotide-binding protein, which we have designated as $G_{k}$ to denote that it opens a K+-selective channel (Breitwieser and Szabo, 1985). This hypothesis was corroborated by the observation that channel opening is abolished when GTP is removed from the cytoplasmic aspect of the membrane (Pfaffinger et al., 1985). The pathway from muscarinic receptor stimulation to K+ channel activation is thus likely to involve the interaction of at least three membrane proteins, namely, the receptor, $G_{k}$, and the K+ channel itself.

Single-channel studies of isolated membrane patches have suggested that cytoplasmic second messengers are not required for channel activation (Kurachi et al., 1986a, b). More recently, isolated G-proteins were observed to activate $I_{K(M)}$-like channels in ripped-off patches of cardiac cell membranes (Codina et al., 1987; Logothetis et al., 1987; Yatani et al., 1987). Although a membrane-bound intermediate cannot be ruled out at present, the mode of activation of $I_{K(M)}$ by $G_{k}$ appears to be novel in that the G-protein activates the effector directly, instead of acting indirectly via cytoplasmic intermediates, as is the case for Ca2+ channels (Kameyama et al., 1985).

The major features of the signal transduction pathway for $I_{K(M)}$ activation have been inferred mainly from biochemical studies using broken-cell preparations (for reviews see Stryer and Bourne, 1986; Nathanson, 1987; Spiegel, 1987; Dunlap et al., 1987), so there are as yet few quantitative data concerning the mode of operation of this system in vivo. We have previously shown that isolated bullfrog atrial cells exhibit all of the characteristics of a fully coupled system, in which muscarinic receptor stimulation leads to G-protein–dependent K+ channel activation (Breitwieser and Szabo, 1985). We report here that we found it possible to dissociate the receptor from the rest of the pathway, allowing a direct investigation of G-protein–channel interactions. Specifically, we have used several hydrolysis-resistant GTP analogues to examine, in quantitative detail, the control of channel activation at the level of the G-protein, as well as the mod-
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A preliminary account of some of these results has been presented (Breitwieser and Szabo, 1987).

METHODS

Dissociation of Cells

The bullfrog, Rana catesbeiana, was used. The heart was removed and rinsed in perfusion solution, containing (millimolar): 110 NaCl, 5.4 KCl, 1.0 MgCl₂, and 10 HEPES, pH 7.2 (equilibrated with 100% O₂). The dissociation of cells was performed at 30°C by the method of Mitra and Morad (1985), using 230 U/ml collagenase (type 4194, Worthington Biochemical Corp., Freehold, NJ) and 0.5–1.0 U/ml protease (type XIV, Sigma Chemical Co., St. Louis, MO). Atrial cells were obtained by dissecting the heart after digestion and gently shaking the atrium in a modified perfusion solution containing 0.2 mM CaCl₂ and 10 mM lactic acid. Cells could be stored for at least 8 h in this solution, provided that it was gently agitated in order to prevent aggregation of the myocytes.

Solutions

The composition of the HEPES-buffered Ringer's solution was (millimolar): 90 NaCl, 2.5 KCl, 5.0 MgCl₂, 2.5 CaCl₂, 20 HEPES, and 10 glucose, pH 7.4. Tetrodotoxin (5 µM) and CdCl₂ (0.5 mM) were routinely added to the extracellular solution to block the fast Na⁺ and background Ca²⁺ currents, respectively. Occasionally, phenol red (0.25 mM) was added to alternate extracellular solutions in order to monitor the time course and completeness of solution changes. ACh was added to the HEPES-buffered Ringer's solution from a stock prepared daily. The standard intracellular solution contained (millimolar): 60 K⁺ aspartate, 50 KCl, 0.5 EGTA, 5.0 HEPES, and 1.0 ATP, as the Mg salt, pH 7.4 with KOH. Guanine nucleotides and/or metabolic blockers were added to this solution as the Li⁺ or K⁺ salts. Corresponding concentrations of Li⁺ alone had no effect on the ionic currents. Whenever the total concentrations of added guanine nucleotide exceeded 2 mM, the MgATP concentration was increased to 5 mM. Although 5 mM MgATP had no direct effect on ionic currents, it did increase the ability of the cell to tolerate high concentrations of hydrolysis-resistant guanine nucleotides by minimizing guanine nucleotide-induced contraction. All salts were reagent grade. HEPES was Ultrl grade from Calbiochem-Behring Corp., La Jolla, CA; GTP₃S [guanosine-5'-O-(3-thiotriphosphate)] and GppCH₂p [guanylyl(B,γ-methylene)-diphosphate] were from Boehringer Mannheim, Indianapolis, IN. GppNHp (guanylyl-imidodiphosphate), metabolic blockers, and all other nucleotides were obtained from Sigma Chemical Co.

Electrophysiology

The whole-cell recording configuration of the patch-clamp technique (Hamill et al., 1981) was used. Cells were allowed to settle in the recording chamber and then superfused with extracellular solution at a rate of 0.5–1.0 ml/min. All experiments were performed at room temperature (22–24°C). A valve, mounted near the recording chamber, allowed rapid extracellular solution changes. Control experiments, using solutions containing different K⁺ concentrations, indicated that under optimal conditions, the solution bathing the myocyte could be exchanged within 200 ms. Patch pipettes were fabricated from square-bore glass (Glass Co. of America, Millville, NJ) by a single pull of a vertical puller (700D, David Kopf Instruments, Tujunga, CA) and had resistances between 3 and 7 MΩ when filled with the standard intracellular solution. Membrane currents were mea-
Command pulses for the voltage-clamp protocols were applied to the bathing solution by a custom-designed, programmable waveform generator, and involved steps to various potentials, with a return to the holding potential between test potentials. Pulse protocols were applied to the cells from the moment of patch disruption to allow continuous monitoring of the cell currents. A 5-mV voltage step was included in most pulse protocols to allow continuous monitoring of pipette series resistance and cell membrane capacitance. For most experiments, we did not compensate for the effects of pipette series resistance, since these were found to be negligible. However, the effect of a linear series resistance reversing at 0 mV was taken into account for the current-voltage (I-V) relationships of Figs. 2 and 4. Current and voltage signals were recorded on FM magnetic tape at 3~i.p.s., corresponding to a bandwidth of 1,250 Hz, using a Racal 4D (Sarasota, FL) recorder. The data were then either displayed on a chart recorder for direct analysis or digitized at 12-bit resolution for computer-assisted analysis.

Calculation of \( I_{K_{\text{M}}} \) Activation Rates

The cell currents at \(-5\) mV were monitored continuously from the time of patch disruption. In the presence of GTP analogues (GXP), a slow linear increase in the outward current was usually observed within 1 min after patch disruption. The time required to reach complete activation of \( I_{K_{\text{M}}} \) depended upon the GXP/GTP ratio, and ranged from 3 to 15 min in the absence of ACh. In most experiments, the current increase was monitored for at least 3 min, and then a saturating dose of ACh (1 \( \mu \)M) was superfused to gauge the maximal \( I_{K_{\text{M}}} \) obtainable in the cell. The linear initial rates of the current increase were measured from chart recordings, normalized to the maximal, ACh-induced steady state \( I_{K_{\text{M}}} \), and are presented as the normalized activation rate (per minute). The cells were not selected for similarities in size (cell capacitance varied from 80 to 150 pF) or pipette resistance (although the pipette resistance was <7 M\( \Omega \)); the only normalization criterion used was the response to 1 \( \mu \)M ACh at the end of each experiment.

RESULTS

Characteristics of \( I_{K_{\text{M}}} \) in Bullfrog Atrial Cells

Preliminary experiments were designed to establish the salient features of the muscarinic receptor-induced inwardly rectifying K\(^+\) current, identified here as \( I_{K_{\text{M}}} \), in enzymatically isolated atrial myocytes of the bullfrog. Particular emphasis was placed on the characterization of the features of \( I_{K_{\text{M}}} \) that permit it to be distinguished from the background K\(^+\) current, \( I_{K_{\text{L}}} \). Fig. 1 illustrates typical effects of ACh on K\(^+\) currents. The upper panel demonstrates the effect of ACh on the cell response to voltage steps to \(-135\) and \(-45\) mV (duration 250 ms, with a return to the holding potential of \(-85\) mV for 250 ms between steps), allowing an assessment of changes in inward and outward current. In the absence of muscarinic agonist, the time-independent inwardly rectifying background K\(^+\) current, \( I_{K_{\text{L}}} \), contributes a significant inward current at hyperpolarizing potentials and a very small outward current at \(-45\) mV (Cleeman, 1981; Hume and Giles, 1983; Momose et al., 1983). Addition of 1 \( \mu \)M ACh results in
an increase in both inward and outward currents. Previous studies (Momose et al., 1982, 1984) have shown this current to be carried by K$^+$. In contrast with $I_{K1}$, the current elicited by ACh is time dependent (Iijima et al., 1985; Simmons and Hartzell, 1987), and settles to a steady value only after several hundred milliseconds. The effects of ACh are fully reversible upon washout of the ACh-containing Ringer's solution (the control and post-ACh traces are superim-

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**Figure 1.** Effects of ACh on K$^+$ currents in voltage-clamped bullfrog atrial cells. Upper panel: current response to 250-ms voltage steps to $-135$ and $-45$ mV from a holding potential of $-85$ mV. Upward deflections indicate outward currents. Application of 1 μM ACh caused an increase in the inward current at $-135$ mV and the outward current at $-45$ mV. Washout of ACh resulted in the return of membrane currents to control levels. The line indicates the zero-current level. The bath solution contained tetrodotoxin (5 μM) and CdCl$_2$ (0.5 mM). The pipette contained the standard intracellular solution, as described in the methods. Lower panel: time course of the membrane currents induced by 1 μM ACh. The currents at the end of each 250-ms pulse to $-5$ (upper trace) and $-135$ mV (lower trace), along with the current at the holding potential, $-85$ mV (middle trace), are plotted. Bath and pipette solutions as in the upper panel. Note the partially resolved decay of $I_{KOM}$ (desensitization) following ACh application. The line indicates the zero-current level.
posed). Additional features of the ACh-induced current are evident when the same experiment is continuously monitored by plotting the steady state current at the end of each 250-ms voltage step. This is shown in the lower panel of Fig. 1 for test pulses to -5 and -135 mV; the current at the holding potential (-85 mV) is plotted as well. Rapid bath application of ACh produced a current that decayed in time (e.g., desensitized) to ~67 ± 5.6% (n = 8) of the maximum peak current. In a manner similar to that observed in mammalian atrial myocytes (Carmeliet and Mubagwa, 1986), the current did not desensitize completely, but remained stable for minutes. Washout of ACh results in a very rapid return to background current levels (90–10%; decay time, <300 ms). Fig. 2 illustrates the effect of ACh on the steady state I-V relationship. In the 10–300-nM concentration range, ACh causes a progressive, scaled increase in both the inward and outward current. Note, however, that the shape of the I-V relationship for the current induced by ACh, although inwardly rectifying, differs from that of the background K⁺ conductance, I_{K1}. Thus, for example, at depolarizing potentials relative to the K⁺ equilibrium potential, I_{Km} is large and rather inde-
pendent of $V_m$, while $I_{k1}$ tends to become vanishingly small. This is most clearly seen by comparing the control $I-V$ relationship of Fig. 2 (pure background current, $I_{k1}$) with that of pure $I_{k(M)}$ shown in Fig. 4 (obtained from the data of Fig. 2 by subtraction of the background component from the net steady state current observed in the presence of 1 nM ACh). Further analysis of the data in Fig. 2 revealed that the shape of the $I_{k(M)}-V$ relationship is the same for all ACh concentrations, even under desensitizing conditions (e.g., ACh concentrations >300 nM). The magnitude of $I_{k(M)}$ is, of course, determined by the ACh concentration. For submicromolar concentrations of ACh, for which desensitization is minimal, the magnitude of the response (e.g., the scale factor necessary to superimpose $I_{k(M)}-V$ relations) can be approximated by a simple Michaelis-Menten-type dose-effect relationship with $K_{0.5} = 159$ nM. Desensitization interferes with the proper description of the dose-effect relationship for $I_{k(M)}$ above 1 nM ACh.

**Receptor-independent, Persistent Activation of $I_{k(M)}$ by Hydrolysis-resistant GTP Analouges**

Biochemical studies in a variety of isolated membrane preparations have demonstrated that hydrolysis-resistant GTP analogues (GXP) can bind to and irreversibly activate G-proteins even in the absence of receptor stimulation (Rodbell, 1975; Schramm and Rodbell, 1975; Codina et al., 1984; Kahn and Gilman, 1984; Katada et al., 1986; Wong and Martin, 1986). If a similar process of G activation occurs in intact atrial cells, then one should observe a corresponding receptor-independent activation of $I_{k(M)}$ whenever these analogues are injected into the cell. Experiments that confirm this hypothesis are shown in Fig. 3. Addition of GppNHp to the control electrode solution results in a slow, time-dependent increase in a current with properties characteristic of $I_{k(M)}$ (upper panel). The time dependence of the current in response to a voltage step is similar to that observed for $I_{k(M)}$ in Fig. 1. Moreover, the $I-V$ relationship for GXP-induced current is the same as the $I-V$ relationship derived from a control cell in which ACh was used to induce the current (Fig. 4). The absolute currents have been plotted for both cells, and the solid curve has been drawn through the data for the control cell. In this example, the $I-V$ relationships for the two cells coincide (the cells had roughly the same membrane area, as judged by the input capacitances). In other cases, while the magnitudes of the currents were different, the shapes of the $I-V$ relationships were identical when properly scaled. Two "fingerprint" properties of $I_{k(M)}$ are thus shared by the channel induced by the hydrolysis-resistant GTP analogue. A third property, its relation to the muscarinic ACh receptor, can be verified indirectly. If $I_{k(M)}$ is in fact activated by GXP, then ACh should not produce any additional current in a cell that has attained maximal stimulation by analogue. Fig. 4 also illustrates this point: bath application of 1 nM ACh to the cell in which the inwardly rectifying K + current was fully induced by GXP, in this case GTPγS, yielded no further increase in current, which implies that the GXP-induced current and $I_{k(M)}$ are identical. Note also that applications of atropine, either after the current increase is complete
Figure 3. Effect of intracellular GppNHp on K⁺ currents in bullfrog atrial cells. Upper panel: the cell was continuously stepped to −135 and −45 mV from a holding potential of −85 mV, with a 300-ms return to the holding potential between test voltage pulses. The patch pipette contained, in addition to the standard intracellular solution, 100 μM GppNHp. Lower panel: time dependence of the current increase in response to intracellular GppNHp. Same cell as in the upper panel. The steady state responses at the end of each 300-ms pulse are plotted for −45 (upper, outward current trace), −135 (lower, inward current trace), and −85 mV (near zero current). Atropine (0.5 mM) addition to the bath solution had no effect on the current. The lines at the left in both panels indicate the zero-current level.

The time course of GXP-induced current activation (lower panel, Fig. 3) illustrates that the current develops slowly, over the course of several minutes. The rate of this receptor-independent activation of $I_{K(M)}$ has been measured under a variety of experimental conditions in order to establish the various biochemical steps involved in the channel activation process in intact cells.
Roles of Endogenous GTP in the Activation of $I_{\text{K(M)}}$

The effect of intracellular GTP depletion on the muscarinic receptor-mediated regulation of ion channels has only recently been appreciated (Pfaffinger et al., 1985). Early studies of $I_{\text{K(M)}}$ in a variety of cardiac tissue and cell preparations, as well as the experiments in Figs. 1 and 2, were done in the absence of added GTP (Soejima and Noble, 1984; Momose et al., 1984; Giles and Noble, 1976; Garnier et al., 1978). In spite of this, ACh produced a large $I_{\text{K(M)}}$. Repeated exposure of cells to 1 μM ACh at 10-min intervals revealed no substantial decrease in the level of ACh-elicited current for times up to 30 min (data not shown). Moreover, GTP in the electrode solution, at concentrations up to 1 mM, had no effect on the maximal current elicited by ACh. The known involvement of $G_{\text{k}}$ in receptor-channel coupling in bullfrog atrial cells, as revealed by the effects of hydrolysis-resistant GTP analogues, suggests that these cells can main-

![Figure 4. Comparison of $I$-$V$ relationships for the receptor-dependent $I_{\text{K(M)}}$ and the receptor-independent, GTP analogue-induced $I_{\text{K(M)}}$. The steady state (at the end of 250-ms voltage steps from a holding potential of $-85$ mV) $I$-$V$ relationships for two cells are compared (the contribution of the background $I_{\text{k}}$ current was subtracted): a control cell (triangles), in which $I_{\text{K(M)}}$ was induced by addition of 1 μM ACh to the bath solution (the pipette contained standard intracellular solution), and a cell in which $I_{\text{K(M)}}$ was induced by including 200 μM GTPγS in the standard intracellular solution (circles), and the same cell, to which 1 μM ACh was applied, after the current increase owing to GTPγS was complete (squares). The traces were corrected for series resistance.](image)

tain a cellular level of GTP that allows maximal activation of $I_{\text{K(M)}}$, despite some loss of nucleotide to the patch pipette. We tested this possibility by examining the effects of a variety of metabolic inhibitors on the ability of ACh to elicit $I_{\text{K(M)}}$. The bullfrog atrial cell proved to be remarkably resistant to guanine nucleotide depletion to the low levels required to completely block $I_{\text{K(M)}}$.

We found only two rather harsh treatments that could achieve a cellular GTP depletion sufficient to completely prevent the appearance of $I_{\text{K(M)}}$ upon ACh stimulation of the muscarinic receptor. One of these required removal of ATP from the pipette solution and inclusion of the following metabolic inhibitors: 10 mM AppNHp (adenylyl-imidodiphosphate, a hydrolysis-resistant ATP analogue that competitively inhibits ATP synthesis), 100 μM Ap5A [P$^1$P$^3$-di(adenosine-5')-pentaphosphate, an adenylate kinase inhibitor], and 100 μM trypan blue (an inhibitor of nonspecific nucleotide transphosphorylation). Glucose was removed and 2 mM cyanide was added to the bath solution to uncouple mitochondrial
transport. The effect of this potion of metabolic inhibitors is illustrated in Fig. 5. For this experiment, \( I_{\text{K(M)}} \) was assessed by stepping the voltage to \(-5\) mV for 250 ms from the holding potential \((-85\) mV). \( 1 \mu\text{M} \) ACh was applied to the cell briefly at 6, 11, and 13 min after patch disruption. The progressive decrease in \( I_{\text{K(M)}} \) shown by the upper three traces clearly demonstrates the decay in the ability of ACh to elicit \( I_{\text{K(M)}} \) until, at 13 min, the agonist is without effect, as indicated by the near coincidence of this trace with the ACh-free control trace (bottom). Each of the inhibitors in the mixture, when used alone, in experiments lasting up to 30 min, could reduce but not abolish the stimulation of \( I_{\text{K(M)}} \) by ACh. This suggests that a variety of independent pathways exist for the maintenance of adequate cell nucleotide levels in adult atrial cells. Block of all the ATP synthetic and ATP/GTP transphosphorylation pathways, as well as loss of existing GTP pools to the electrode, are required for a full block of \( I_{\text{K(M)}} \).

A second condition that produces significant block of the ACh-dependent \( I_{\text{K(M)}} \) is the inclusion of \( 1 \mbox{mM GDP}\beta\text{S} \) [guanosine-\(5'\)-O-(2-thiodiphosphate)] in the standard pipette solution. Fig. 6 illustrates the time course of the decrease in ACh-stimulated \( I_{\text{K(M)}} \), as monitored by the steady state current at the end of 250-ms pulses to \(-5\) mV from \(-85\) mV, the holding potential. In this experiment, ACh was added to the extracellular solution as soon as a stable current was observed after access to the cell interior, so that the effect of electrode solution entry into the cell could be observed. The ACh-induced current decayed to very low levels within 3–4 min of cell dialysis. It is likely that the GDP\beta\text{S}-mediated inhibition of \( I_{\text{K(M)}} \) is a result of two distinct processes, one arising from block of cellular GTP synthesis by GDP\beta\text{S} substituting for GDP, and a second arising from direct competition of GDP\beta\text{S} with the remaining cellular GTP for the binding site on Gk (Eckstein et al., 1979; Iyengar et al., 1980; Ho et al.,

![Figure 5. GTP dependence of the cell response to ACh: effect of metabolic inhibitors. Membrane current responses to a voltage step to \(-5\) mV from a holding potential of \(-85\) mV are shown. \( 1 \mu\text{M} \) ACh was applied at 6 (upper trace), 11 (next trace), and 13 (trace near control) min after access to the cell interior. The electrode contained: 50 mM KCl, 60 mM K\(^+\) aspartate, 0.5 mM EGTA, 5 mM HEPES, 10 mM AppNHp, 100 \(\mu\text{M} \) Ap5A, and 100 \(\mu\text{M} \) trypan blue, pH 7.4. The Ringer's solution contained: 90 mM NaCl, 0.6 mM KCl, 5 mM MgCl\(_2\), 2.5 mM CaCl\(_2\), 20 mM HEPES, and 2 mM KCN, pH 7.4. The line at the left indicates the zero-current level.](https://example.com/figure5.png)
These two mechanisms combine to provide rapid inhibition of the ACh-induced current.

**GTP Modulation of Receptor-independent Activation of \( I_{K(M)} \) by GTP Analogues**

An initial examination of the receptor-independent activation of \( I_{K(M)} \), using electrode solutions that contained only hydrolysis-resistant GTP analogues and ATP, revealed a considerable variability in the rate at which \( I_{K(M)} \) appeared. In the previous section, we demonstrated that significant amounts of GTP may remain in the cell even when the cell is continuously dialyzed. As GTP is expected to be a competitive inhibitor of the receptor-independent activation process, which is a direct reflection of the binding of the hydrolysis-resistant GTP analogue to the activation site on \( G_k \), variations in the cellular GTP concentration are expected to alter the activation rate. Thus, uncontrolled, variable levels of GTP remaining in the cell may explain the variability in the rate of \( I_{K(M)} \) activation. We tested this possibility by including various known concentrations of GTP in the electrode solution, which contained a fixed concentration of GXP.

As long as the concentration of GTP in the pipette is above the steady state level of residual GTP in the cell, the electrode solution should effectively clamp the GXP/GTP ratio. We found that under these circumstances, the rates of \( I_{K(M)} \) activation were in fact much more reproducible. A typical experiment of this kind is shown in Fig. 7. The cell was monitored continuously, and the rate of appearance of the outward current at \(-5\) mV was determined. At the end of the experiment, 1 \( \mu \)M ACh was added to the bath in order to allow the rate of receptor-independent activation to be normalized to the maximal steady state \( I_{K(M)} \) observable in the same cell.

We found that the rate of \( I_{K(M)} \) activation depends only upon the GXP/GTP ratio, regardless of the absolute concentrations of GXP and GTP used to produce the ratio. Thus, for example, 500 \( \mu \)M GppNHp with 50 \( \mu \)M GTP gave essentially the same activation rate as 2 mM GppNHp with 200 \( \mu \)M GTP, 0.25 \( \pm \) 0.06 min\(^{-1}\) (\( n = 3 \)) and 0.28 \( \pm \) 0.03 min\(^{-1}\) (\( n = 6 \)), respectively. Fig. 8 summarizes the effect of adding GTP to pipette solutions containing 0.1, 0.5, 1, and 2 mM GppNHp. The rates of receptor-independent \( I_{K(M)} \) activation increase.
as a function of the GppNHp/GTP ratio and saturate near 0.3 min⁻¹. The data are well described by the Hill equation, $R/R_{max} = \frac{[GXP/GTP]}{[K_{0.5} + [GXP/GTP]]^n}$, with the following parameters of the best fit (solid line): $R_{max} = 0.29$ min⁻¹, $K_{0.5} = 3.26$, and $n = 1.7$. In order to determine the sensitivity of this process to guanine nucleotide structure, we examined the ability of two additional hydrolysis-resistant GTP analogues, GTPγS and GppCH₂p, to support receptor-independent activation of $I_{k(M)}$. As shown in Fig. 9, all three GTP analogues are capable of receptor-independent activation of the channel. Although the GTP analogues activate $G_{\kappa}$ at widely different GXP/GTP ratios, all three produced essentially the same maximal activation rate. The relative effectiveness of the guanine nucleotides for $G_{\kappa}$ activation are: GTPγS > GTP > GppNHp > GppCH₂p.

**Figure 7.** Time dependence of receptor-independent activation of $I_{k(M)}$. Membrane currents measured at the end of 250-ms pulses to $-5$ mV from a holding potential of $-85$ mV are plotted as a function of time. In addition to the standard intracellular solution, the patch pipette contained 2 mM GTPγS and 0.1 mM GTP. 1 μM ACh was added to the bath at the end of the experiment. The line at the left indicates the zero-current level.

**Figure 8.** Rate of activation of $I_{k(M)}$ depends only on the GppNHp/GTP concentration ratio and not on the absolute concentrations. The rates of activation of $I_{k(M)}$ (normalized to the maximum steady state current in 1 μM ACh) are plotted against the GppNHp/GTP concentration ratio in the patch pipette. The ratios were produced with GppNHp concentrations of: 0.1 (open squares), 0.5 (filled squares), 1.0 (open circles) and 2.0 (filled circles) mM. Each point represents the average of two to six experiments. The solid line is a nonlinear least-squares fit of the data to the Hill equation, as described in the text.
These data suggest that a common process limits the rate of $I_{K(M)}$ activation when the receptor is bypassed. This rate-limiting process should occur before guanine nucleotide binding, since it is independent of guanine nucleotide structure, and should be independent of receptor–G-protein interaction, since it is seen in the absence of receptor activation. A likely candidate is the step at which GDP dissociates from $G_k$. The validity of this contention was assessed by examining the effects of muscarinic agonist on the rate of persistent $I_{K(M)}$ activation in the presence of hydrolysis-resistant GTP analogues.

**Catalytic Effect of Agonist on the Rate of Persistent Channel Activation**

Biochemical studies have demonstrated that the basal GTPase activity of G-proteins is enhanced by muscarinic receptor stimulation (Onali et al., 1983; Haga et al., 1985; Kurose et al., 1986). The increased turnover rate results from receptor-mediated enhancement of the rate of release of GDP from the inactive, GDP-bound G-protein (Higashijima et al., 1987a). The limiting rate of receptor-independent activation of $I_{K(M)}$, common to all of the GTP analogues in the present studies, is likely to be a reflection of the low basal rate of GDP release from $G_k$. If ACh increases the GDP “off” rate by way of receptor activation, then ACh must also increase the rate of appearance of $I_{K(M)}$, provided that the cell is flooded with hydrolysis-resistant GTP analogue. This is demonstrated in Fig. 10. The cell was internally perfused with an electrode solution containing 1.0 mM GppNHp (20:1 ratio with GTP). After 100 s of equilibration time, 5 nM ACh was added to the bath, and $I_{K(M)}$ developed rapidly. Maximal $I_{K(M)}$ was induced within 25 s, as evidenced by the lack of further current stimulation upon addition of 1 μM ACh to the bath at the end of the experiment. Note that this is in
sharp contrast to the effect of 5 nM ACh on GXP-free, control cells, where it produces no measurable current (data not shown here, but see Fig. 2).

In order to determine the specificity of the effects of muscarinic receptor stimulation, the effect of a subthreshold ACh concentration on the rate of $I_{K(M)}$ activation was assessed at various GppNHp/GTP ratios (Fig. 11, filled circles). The ACh concentration was 2 nM to provide conditions under which only GXP-induced activation of $I_{K(M)}$ would be observed, without a background, GTP-induced increase in current. The receptor-independent activation curve for $I_{K(M)}$ (e.g., no ACh) is redrawn from Fig. 8 for comparison (open circles). It is evident that 2 nM ACh greatly accelerates the rate of channel activation at all ratios of GppNHp/GTP. The maximum activation rate is increased from 0.32 min$^{-1}$ (in the absence of ACh) to 2.46 min$^{-1}$. Moreover, the rate of activation relative to that of the receptor-free rate was increased by essentially the same factor for all
GppNHp/GTP ratios, which indicates that ACh has no effect on the relative affinity of G\textsubscript{k} for GTP and GppNHp. This is best illustrated by the theoretical lines, which differ only by a scale factor and were drawn to the best combined fit of the Hill equation with \( K_{0.5} = 3.9 \), \( n = 1.5 \), and \( R_{\text{max}} = 2.46 \text{ min}^{-1} \) for 2 nM ACh and \( R_{\text{max}} = 0.32 \text{ min}^{-1} \) for no ACh.

If ACh affects the rates of \( I_{K(M)} \) activation independently of analogue structure, then, at a given ACh concentration, other hydrolysis-resistant GTP analogues should support the activation of \( I_{K(M)} \) to the same extent (provided that the GXP/GTP ratios are high enough to produce the maximal activation rate for each analogue). The test of this expectation is shown for GppNHp and GTP\textgammaS in Fig. 12. The figure plots the activation rates in the presence of 0–5 nM ACh for both analogues, at a GXP/GTP ratio of 20:1, which is large enough for any GTP effects to be negligible. At least for this limited concentration range, the rate of \( I_{K(M)} \) activation depends linearly on ACh concentration. The data for both GTP analogues fall on the same line. That is, the maximal rate of \( I_{K(M)} \) activation depends only upon the concentration of ACh. This result supports the idea that muscarinic receptor activation results in an increased rate of GDP release from G\textsubscript{k}, which is mirrored directly by channel activation at high analogue/GTP ratios.

**DISCUSSION**

Bullfrog atrial cells proved to be particularly well suited for quantitative studies of G\textsubscript{k}-coupled muscarinic ACh receptor-induced \( I_{K(M)} \) activation in vivo. These enzymatically isolated cells are electrophysiologically simple (Hume and Giles, 1981; Moore et al., 1986), noncultured, and fully differentiated, and they exhibit normal pharmacological responses (Hume and Giles, 1983; Momose et
al., 1984) and have a well-developed ability to maintain their metabolism in the face of a significant loss of metabolites to the patch pipette. This accounts, in part, for the resiliency of receptor-dependent \( I_{\text{K(M)}} \) activation even when guanine nucleotides are not included in the pipette solution.

**G-Protein Involvement in Muscarinic Receptor Coupling to \( I_{\text{K(M)}} \)**

The requirement of guanine nucleotide for the process of \( I_{\text{K(M)}} \) activation was first demonstrated by the ability of GppNHp to permanently activate \( I_{\text{K(M)}} \) in bullfrog atrial myocytes (Breitwieser and Szabo, 1985) and by the ability of GTP-free pipette solutions to inhibit agonist-dependent channel activation in cultured embryonic chick atrial cells (Pfaffinger et al., 1985). In adult frog atrial myocytes, adequate removal of intracellular GTP in the latter type of experiment required the use of a mixture of metabolic inhibitors in addition to large patch pipettes. The slow time course of nucleotide depletion in these cells suggests the existence of several mechanisms for the maintenance of adequate intracellular GTP levels and/or the presence of intracellular nucleotide storage compartments, which must be depleted (e.g., with mitochondrial uncouplers) in order to achieve an observable inhibition of ACh-induced \( I_{\text{K(M)}} \). The affinities of G-proteins for GTP and the GTP analogues used in this study have been estimated from binding and activation studies to be in the 50–500-nM range (Cassel and Selinger, 1976; Pfeuffer and Eckstein, 1976; Birnbaumer et al., 1980; Northup et al., 1982; Hudson and Fain, 1983; Sunyer et al., 1984; Sternweis and Robishaw, 1984; Brandt and Ross, 1985; Kelleher et al., 1986; Yamanaka et al., 1986). Evidently, adult cardiac myocytes can maintain intracellular GTP concentrations above these levels despite continuous dialysis. In summary, the requirement for cellular GTP, as well as the ability of nonhydrolyzable GTP analogues to affect \( I_{\text{K(M)}} \) activation, provide strong evidence for the intimate involvement of a G-protein in the normal activation process of \( I_{\text{K(M)}} \) in intact cells.

**Estimation of the Steady State Cellular GTP Concentration**

An estimate of the steady state cellular GTP concentration in an internally dialyzed cell under whole-cell patch-clamp conditions is of prime importance in this study for several reasons. First, it provides a basis for understanding the paradox of a GTP-dependent process that can occur in the nominal absence of GTP. Second, it provides an estimate of a concentration below which the pipette solution cannot be relied upon to clamp the cell GTP concentration, and thus it sets limits on the cell nucleotide ratios that can consistently and reproducibly be achieved. Third, it represents one of the only attempts to estimate the cellular GTP concentration existing under the actual experimental conditions of whole-cell patch clamp.

An upper limit to the cell GTP concentration under whole-cell clamp conditions can be obtained from the data of Fig. 8 on GppNHp-mediated receptor-independent activation of \( I_{\text{K(M)}} \). The rates of receptor-independent activation of \( I_{\text{K(M)}} \) were maximal in the 0.5–2.5-mM concentration range, and were independent of the absolute concentration in the absence of added GTP in the pipette solution (GppNHp/GTP ratio of \( \infty \)). This implies that the steady state cellular
GTP concentration must be <50 μM, since it would otherwise slow the rate of channel activation by 0.5 mM GppNHp. For GppCH₂p, in contrast, the rate of receptor-independent activation of \( I_{K(m)} \) was lower than maximal in the same concentration range, and depended upon the absolute concentration of GppCH₂p in the pipette. The rate obtained with 1 mM GppCH₂p alone was 0.18 ± 0.04 min⁻¹ \((n = 4)\), well below the maximal rate of 0.3 min⁻¹. Addition of 25 μM GTP to the 1 mM GppCH₂p pipette solution did not significantly reduce the rate, which remained 0.18 ± 0.02 min⁻¹ \((n = 2)\). Higher GppCH₂p concentrations (2.0–2.5 mM) were required to approach the maximal receptor-independent activation rate (Fig. 9). The fact that 1 mM GppCH₂p produced virtually the same activation rate in the presence or absence of 25 μM GTP implies that the addition of GTP to the pipette solution had little effect on the steady state cellular GTP concentration, which therefore must be on the order of 25 μM. Taken together, these data imply that the steady state cellular GTP concentration under whole-cell patch clamp is in the 25–50-μM range. Given the above-mentioned high affinity (50–500 nM) of purified G-proteins for GTP, these concentrations are sufficient to fully activate the G-protein in our cells, explaining thereby both the observation of an ACh-induced \( I_{K(m)} \) when GTP is absent from the pipette solution and the inability of additional GTP in the pipette to enhance the ACh-induced \( I_{K(m)} \).

**Basal, Receptor-independent Turnover of \( G_\alpha \) In Vivo**

Under certain conditions, namely at high GXP/GTP ratios, GTP analogues elicit an inwardly rectifying K⁺ current. By several criteria, the analogue-induced current is identical to \( I_{K(m)} \). First, in agreement with a recent report of Simmons and Hartzell (1987), the time dependence of the current after a voltage step is found to be similar for both currents. Second, the \( I-V \) relationships for the two currents are identical. Finally, the two effects are not additive; ACh can elicit a current only if the analogue-activated current is not fully activated.

Theoretical and experimental considerations indicate that the concentration of small solutes like GTP and its analogues would be essentially uniform in the cell within 2 min after the rupture of the membrane under the patch pipette (Kameyama et al., 1985; Lapointe and Szabo, 1987). We have nevertheless circumvented any possible diffusional limitations imposed by the geometry of these cells and eliminated the contribution of endogenous GTP to the rate of \( I_{K(m)} \) activation by utilizing fixed ratios of nucleotides at concentrations well above the expected cellular concentrations and in vast excess over the measured affinities of the G-proteins for the nucleotides (as discussed above). Restricted diffusion to the far reaches of the cell may initially produce a gradient of absolute concentrations within the cell, but at each point in the cell, the ratio GXP/GTP should be the same. Thus, the use of fixed GXP/GTP ratios in the patch pipette eliminates any possible contribution of concentration gradients and examines only the effect of the competition of two guanine nucleotide substrates on the function of the G-protein, as evidenced by the similar rates of \( I_{K(m)} \) activation observed for a range of cell sizes.

All of the GTP analogues produced a persistent, receptor-independent acti-
vation of $I_{\text{KM}}$ that was stable for extended periods of time ($\geq 30$ min in our experiments), which indicates that the pools of activated G-protein produced by GTPγS, GppNHp, and GppCH2p are not inactivated by hydrolysis or loss of the GTP analogue. This observation is in agreement with the results of single-channel measurements on excised patches of membrane. GTPγS-induced activation of $I_{\text{KM}}$ channels persisted after GTPγS was washed out of the medium bathing the cytoplasmic side of the cell membrane (Kurachi et al., 1986c; Logothetis et al., 1987; Yatani et al., 1987), which suggests that the "off" rate of GXP over the time course of our experiments is essentially zero, and therefore the distinct relative binding affinities or the ability to compete with GTP of the various analogues must represent a phenomenon associated with the "on" step of GXP binding to $G_\alpha$. Moreover, our results suggest that activated receptor does not catalyze nucleotide exchange on activated $G_\alpha$. If this process were operative, ACh application to a cell in which $I_{\text{KM}}$ was fully activated at low GXP/GTP ratios would shift the G-proteins from the predominantly GXP-bound to the GTP-bound state so that a subsequent washout of ACh would reduce $I_{\text{KM}}$. Contrary to this expectation, ACh does not remove the persistence of $I_{\text{KM}}$ (data not shown). Taken together, these data indicate that hydrolysis-resistant GTP analogues trap $G_\alpha$ in a permanently activated uncoupled pool, whose size can be assessed from the magnitude of the $I_{\text{KM}}$ produced.

The effects of the GTP analogues reveal a basal, receptor-independent turnover of $G_\alpha$ in vivo. Our observation of identical maximal rates of $I_{\text{KM}}$ activation for the three GTP analogues suggests that basal G-protein turnover is limited by a process that precedes nucleotide binding, namely, the release of GDP from inactive, GDP-bound $G_\alpha$. This result is consistent with kinetic studies of GTPase activity on solubilized $G_\alpha$ (Higashijima et al., 1987a). At high GXP/GTP concentrations, practically all of the spontaneously released GDP from inactive $G_\alpha$ would be replaced by hydrolysis-resistant analogue, so that the rate of appearance of $I_{\text{KM}}$ would be a direct measure of the rate of GDP release. We found this rate to be near 0.3 min⁻¹. It is in surprisingly good agreement with the rate of GDP release measured in solubilized preparations of several G-proteins, 0.03–0.3/min (Higashijima et al., 1987a–c).

When only GTP is present in the cell, the basal rate of $G_\alpha$ turnover does not produce a measurable $I_{\text{KM}}$. Evidently, the rate of GTP hydrolysis is high enough to preclude the development of a significant pool of GTP-bound $G_\alpha$. The rate of GTPase activity ($k_{\text{cat}}$) measured either in isolated membranes or solubilized preparations of G-proteins is on the order of 2 min⁻¹ (Higashijima et al., 1987a). Since this rate is approximately an order of magnitude higher than the rate of basal GDP release, it would be sufficient to account for the lack of receptor-independent channel activation by GTP. Later in this discussion, we will show that $k_{\text{cat}}$ is in fact likely to be much larger in vivo.

GTP competes with the analogue for binding to $G_\alpha$, as evidenced by the decreased rates of $I_{\text{KM}}$ activation when the GTP/GXP concentration ratio is increased in the cell. The finding that the degree of competition depends only on the concentration ratio and is independent of the absolute levels of the guanine nucleotides demonstrates that the competition is a linear, concentration-
independent process. Note, however, that there is a large difference in the effectiveness with which the different analogues compete with GTP for binding to Gk; from the data in Fig. 9, the concentration ratios for half-maximal competition with GTP are 0.55, 3.25, and 44.1 for GTPγS, GppNHp, and GppCH₂p, respectively. A similar sequence of activation has been observed for transducin (Yamanaka et al., 1986). Since all of the guanine nucleotides should diffuse to the binding site of Gk at approximately the same rate, the specificity of binding cannot be explained by a diffusion-limited "on" rate for the nucleotide-Gk reaction. Rather, it is likely that a transient, nucleotide-specific equilibrium is established with the binding site, followed by a conformational change that locks the nucleotide into the binding site. The dissociation of Gk into subunits may be concomitant with this process.

**Mechanism of Muscarinic Receptor–catalyzed I_{K,M} Activation**

Muscarinic receptor stimulation induces an increase in the rate of I_{K,M} activation, which, in the presence of hydrolysis-resistant analogues, can be observed at normally subthreshold agonist concentrations. A simple model that can account for all of our observations is shown in Fig. 13. It incorporates the normal cycle for Gk activation by GTP, as well as the steps that occur in the presence of hydrolysis-resistant GTP analogues. It is instructive to examine the cycle that occurs under normal conditions (upper pathway). GkGDP is the predominant form of Gk in the absence of ACh, and there is a slow rate of receptor-independent GDP release, followed by binding of GTP. In the presence of ACh, the rate of GDP release, k(ACh), increases. At low concentrations of ACh, there is a linear dependence of k(ACh) on ACh concentrations: k(ACh) = 8.84 × 10⁶ min⁻¹M⁻¹[ACh] + 0.44 min⁻¹. The agonist-induced increase in GDP release from Gk results in a decrease in the steady state level of inactive, GDP-bound Gk, and a concomitant rise in the active, GTP-bound form. Pool sizes will depend upon the relative rates of GDP release (k(ACh)) and G·GTP hydrolysis (k_cat); maximal concentrations of ACh will shift the pools of Gk to the activated form, resulting in maximal channel activation.

There are two indirect ways in which the value of k_cat can be estimated for cardiac myocytes. Washout of ACh following maximal stimulation of I_{K,M} results
in a very rapid decrease in the current ($I_{K(M)}$ decays from 90 to 10% of the maximal current within 300 ms, which is at the limit of the solution-change capabilities of our present flow system). If the fall of $I_{K(M)}$ during washout reflects the fall in the concentration of the GTP-bound G-protein, then our washout data suggest that $k_{cat} > 200 \text{ min}^{-1}$. It is also possible to estimate $k_{cat}$ by extrapolating the data of Fig. 12 to the concentration of ACh that produces half-maximal activation of $I_{K(M)}$, 160 nM, and presumably of the Gk pool as well. If the extrapolation is valid, then for 160 nM ACh, $k_{ACh} = 135 \text{ min}^{-1}$. Since at this concentration the rate of Gk activation must equal the rate of its inactivation by GTP hydrolysis, we estimate $k_{cat}$ to be 135 min$^{-1}$. Both of these estimates are nearly two orders of magnitude larger than the value reported for $k_{cat}$ in solubilized preparations of brain Gm, $\sim 2 \text{ min}^{-1}$. The faster rate of GTP hydrolysis in cardiac myocytes may be a result of tissue or species specificity. There is the more likely possibility, however, that $k_{cat}$ in vivo is larger than that in solubilized systems, as would be expected, for example, if the GTP hydrolysis rate were influenced by Gk-effector interactions.

GTP analogues (illustrated in the lower branch of Fig. 13) interact with Gk in a manner identical to GTP, except that during the time course of our experiments, they can be considered nonhydrolyzable ($k_{cat} = 0$). This results in a buildup of activated GkGXP, and an irreversibly activated population of channels. In addition, GXP acts as a drain on the GkGDP pool, and even in the absence of ACh, there will be a time-dependent shift in the distribution of Gk forms, until the entire population becomes GXP-bound. GTP and GXP compete for G(,), the nucleotide-free form of Gk, and thus, at fixed concentrations of both GTP and GXP, there will be flow through both pathways. When all of Gk is in the permanently activated GkGXP pool and the GkGDP pool is depleted, receptor activation (which catalyzes GDP release from GkGDP) should be without effect. This was indeed observed: ACh had no effect on $I_{K(M)}$ after full activation of the system by GTP analogues.

The independence of the GTP and GXP pathways is demonstrated in experiments in which receptor-independent activation of $I_{K(M)}$ by GXP was not allowed to reach completion at the time of ACh application. Rapid bath application of a high ACh concentration (1 nM) under these conditions produces an additional increment in $I_{K(M)}$ characterized by a peak current decaying to the steady state maximal $I_{K(M)}$ in a manner similar to that observed in the absence of GXP (e.g., desensitization; see Fig. 7). Under the simplest circumstances, the transient in $I_{K(M)}$ produced by ACh (upper limit, Fig. 13) should be reduced in magnitude but not otherwise altered, as the pool of GkGDP available for activation by ACh is reduced by its conversion to GkGXP. That this expectation is indeed observed is demonstrated in Fig. 14, which plots the relaxation amplitude (e.g., the ratio of the peak to steady state receptor- [ACh-] induced increment in current) as a function of the degree of receptor-independent activation of $I_{K(M)}$ at the time of ACh application. The relaxation amplitudes are seen to be independent of receptor-independent activation; that is, the size of the agonist-dependent transient seems to directly reflect the size of the GkGDP pool. Although the mechanism of desensitization of muscarinic receptor-mediated $I_{K(M)}$ is not under-
stood, these results would suggest that the transient of $I_{K(M)}$ activation either derives from a receptor-mediated phenomenon or is a result of the rapid activation of the system. The cardiac muscarinic receptor has been shown to undergo agonist-dependent phosphorylation (Kwatra and Hosey, 1986), and this might alter the process of channel activation. Alternatively, desensitization may occur at $G_{G}$. Protein kinase C-mediated phosphorylation of $G_{G}$ has been reported to block the ability of muscarinic agonists to inhibit adenylate cyclase (Bauer and Jakobs, 1986; Bell and Brunton, 1986). If $G_{G}$ is phosphorylated upon muscarinic receptor stimulation, its ability to activate $I_{K(M)}$ may also be altered. Finally, the observed desensitization may arise from a property of the $I_{K(M)}$ channel itself: its rapid activation, induced by a sudden increase in the pool of activated $G_{G}$, could produce a transiently longer-lived and/or more conductive open channel state. While our data do not allow us to discriminate among these possibilities, they provide the rational basis for further experiments concerning the mechanism(s) of desensitization.

Although there is as yet no consensus on either the biochemical identity of the G-protein responsible for $K^{+}$ channel activation or the precise mechanism of the $G_{G}$ activation process, this uncertainty does not affect the interpretation of our experimental results, since we have defined $G_{G}$ functionally and measured the activation and turnover of $G_{G}$ in vivo. In summary, our experiments provide a quantitative understanding of the overall features of the $G_{G}$-mediated coupling between muscarinic receptor activation and $I_{K(M)}$ channel opening in vivo. In addition, these results demonstrate that it is possible to study the kinetics of G-protein activation in intact cells, which is essential for a detailed understanding of the physiological mechanism(s) regulating G-protein functions.
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REFERENCES

Bauer, S., and K. H. Jakobs. 1986. Phorbol ester treatment impairs hormone- but not stable GTP analog-induced inhibition of adenylate cyclase. FEBS Letters. 198:43-46.

Bell, J. D., and L. L. Brunton. 1986. Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. Withdrawal of GTP-dependent inhibition. Journal of Biological Chemistry. 261:12036-12041.

Birnbaumer, L., T. L. Swartz, J. Abramowitz, P. W. Mintz, and R. Iyengar. 1980. Transient and steady state kinetics of the interaction of guanyl nucleotides with the adenylyl cyclase system from rat liver plasma membranes. Interpretation in terms of a simple two-state model. Journal of Biological Chemistry. 244:3542-3551.

Brandt, D. R., and E. M. Ross. 1985. GTpase activity of the stimulatory GTp-binding regulatory protein of adenylate cyclase G. Accumulation and turnover of enzyme-nucleotide intermediates. Journal of Biological Chemistry. 260:266-272.

Breitwieser, G. E., and G. Szabo. 1985. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. Nature. 317:538-540.

Breitwieser, G. E., and G. Szabo. 1987. GTP prevents receptor-independent basal activation of I_{AC} by non-hydrolyzable GTP analogs. Biophysical Journal. 51:265a. (Abstr.)

Carmeliet, E., and K. Mubagwa. 1986. Desensitization of the acetylcholine-induced increase of potassium conductance in rabbit cardiac Purkinje fibres. Journal of Physiology. 371:239-255.

Cassel, D., and Z. Selinger. 1976. Catecholamine-stimulated GTpase activity in turkey erythrocyte membranes. Biochimica et Biophysica Acta. 452:538-551.

Cleeman, L. 1981. Heart muscle. Intracellular potassium and inward-going rectification. Biophysical Journal. 36:303-310.

Codina, J., J. D. Hildebrandt, L. Birnbaumer, and R. D. Sekura. 1984. Effects of guanine nucleotides and Mg on human erythrocyte N and N, the regulatory components of adenylyl cyclase. Journal of Biological Chemistry. 259:11408-11418.

Codina, J., A. Yatani, D. Grenet, A. M. Brown, and L. Birnbaumer. 1987. The α subunit of the GTP binding protein G₃ opens atrial potassium channels. Science. 236:442-445.

Dunlap, K., G. G. Holz, and S. G. Rane. 1987. G proteins as regulators of ion channel function. Trends in Neuroscience. 10:241-244.

Eckstein, F., D. Cassel, H. Lefkowitz, M. Lowe, and Z. Selinger. 1979. Guanosine 5'-O-(2-thiodiphosphate): an inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. Journal of Biological Chemistry. 254:9829-9834.

Fischmeister, R., and H. C. Hartzell. 1986. Mechanism of action of acetylcholine on calcium current in single cells from frog ventricle. Journal of Physiology. 376:183-202.

Garnier, D., J. Nargeot, C. Ojeda, and O. Rougier. 1978. The action of acetylcholine on background conductance in frog atrial trabeculae. Journal of Physiology. 274:381-396.

Giles, W., and S. J. Noble. 1976. Changes in membrane currents in bullfrog atrium produced by acetylcholine. Journal of Physiology. 261:109-123.
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Haga, K., T. Haga, A. Ichiyama, T. Katada, H. Kurose, and M. Ui. 1985. Functional reconstitution of purified muscarinic receptors and inhibitory guanine nucleotide regulatory protein. Nature. 316:731–733.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 391:85–100.

Hartzell, H. C. 1980. Distribution of muscarinic acetylcholine receptors and presynaptic nerve terminals in amphibian heart. Journal of Cell Biology. 86:6–20.

Higashijima, T., K. M. Ferguson, M. D. Smigel, and A. G. Gilman. 1987a. The effect of GTP and Mg²⁺ on the GTPase activity and the fluorescent properties of Gα. Journal of Biological Chemistry. 262:757–761.

Higashijima, T., K. M. Ferguson, P. C. Sternweis, E. M. Ross, M. D. Smigel, and A. G. Gilman. 1987b. The effect of activating ligands on the intrinsic fluorescence of guanine nucleotide-binding regulatory proteins. Journal of Biological Chemistry. 262:752–756.

Higashijima, T., K. M. Ferguson, P. C. Sternweis, M. D. Smigel, and A. G. Gilman. 1987c. Effects of Mg²⁺ and the βγ-subunit complex on the interactions of guanine nucleotides with G proteins. Journal of Biological Chemistry. 262:762–766.

Hill-Smith, I., and R. D. Purves. 1978. Synaptic delay in the heart: an ionophoretic study. Journal of Physiology. 279:31–54.

Ho, R.-J., Q.-H. Shi, and J. Ruiz. 1986. Conditional inhibition of forskolin-activated adenylate cyclase by guanosine diphosphate and its analog. Archives of Biochemistry and Biophysics. 251:148–155.

Hudson, T. H., and J. N. Fain. 1983. Forskolin-activated adenylate cyclase. Inhibition by guanylylimidodiphosphate. Journal of Biological Chemistry. 258:9755–9761.

Hume, J. R., and W. Giles. 1981. Active and passive electrical properties of single bullfrog atrial cells. Journal of General Physiology. 78:18–43.

Hume, J. R., and W. Giles. 1983. Ionic currents in single isolated bullfrog atrial cells. Journal of General Physiology. 81:153–194.

Iijima, T., H. Irisawa, and M. Kameyama. 1985. Membrane currents and their modification by acetylcholine in isolated single atrial cells of the guinea-pig. Journal of Physiology. 359:458–501.

Iyengar, R., J. Abramowitz, M. Bordelon-Riser, A. Blume, and L. Birnbaumer. 1980. Regulation of hormone-receptor coupling to adenylyl cyclase. Effects of GTP and GDP. Journal of Biological Chemistry. 255:10312–10321.

Kahen, R. A., and A. G. Gilman. 1984. ADP-ribosylation of G, promotes the dissociation of its α and β subunits. Journal of Biological Chemistry. 259:6235–6240.

Kameyama, M., F. Hofmann, and W. Trautwein. 1983. On the mechanism of β-adrenergic regulation of the Ca channel in the guinea-pig heart. Pflügers Arch. 405:285–293.

Katada, T., M. Oinuma, and M. Ui. 1986. Mechanisms for inhibition of the catalytic activity of adenylate cyclase by the guanine nucleotide-binding proteins serving as the substrate of islet-activating protein, pertussis toxin. Journal of Biological Chemistry. 261:5215–5221.

Kelleher, D. J., L. W. Dudyce, G. F. Wright, and G. L. Johnson. 1986. Ability of guanine nucleotide derivatives to bind and activate bovine transducin. Molecular Pharmacology. 30:603–608.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986a. Acetylcholine activation of K⁺ channels in cell-free membrane of atrial cells. American Journal of Physiology. 251:H681–H684.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986b. On the mechanism of activation of mus-
carinic K⁺ channels by adenosine in isolated atrial cells: involvement of GTP-binding proteins. *Pflügers Archiv.* 407:264-274.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986c. Role of intracellular Mg²⁺ in the activation of muscarinic K⁺ channel in cardiac atrial cell membrane. *Pflügers Archiv.* 407:572-574.

Kurose, H., T. Katada, T. Haga, K. Haga, A. Ichiyama, and M. Ui. 1986. Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *Journal of Biological Chemistry.* 261:6423-6428.

Kwatra, M. M., and M. M. Hosey. 1986. Phosphorylation of the cardiac muscarinic receptor in intact chick heart and its regulation by a muscarinic agonist. *Journal of Biological Chemistry.* 261:12429-12432.

Lapointe, J. Y., and G. Szabo. 1987. A novel holder allowing internal perfusion of patch-clamp pipettes. *Pflügers Archiv.* 410:212-216.

Lefkowitz, R. J., and M. G. Caron. 1986. Regulation of adrenergic receptor function by phosphorylation. *Journal of Molecular and Cellular Cardiology.* 18:885-895.

Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. 1987. The βγ subunits of GTP-binding proteins activate the muscarinic K⁺ channel in heart. *Nature.* 325:321-326.

Mitra, R., and M. Morad. 1985. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *American Journal of Physiology.* 249:H1056-H1060.

Momose, Y., G. Szabo, and W. Giles. 1982. Acetylcholine-induced outward current in single bullfrog atrial cells. *Biophysical Journal.* 37:328a. (Abstr.)

Momose, Y., G. Szabo, and W. Giles. 1983. An inward rectifying K⁺ current in bullfrog atrial cells. *Biophysical Journal.* 41:311a. (Abstr.)

Momose, Y., W. Giles, and G. Szabo. 1984. Acetylcholine-induced K⁺ current in amphibian atrial cells. *Biophysical Journal.* 45:20-22.

Moore, L. E., R. B. Clark, E. F. Shibata, and W. R. Giles. 1986. Comparison of steady-state electrophysiological properties of isolated cells from bullfrog atrium and sinus venosus. *Journal of Membrane Biology.* 89:131-138.

Nargeot, J., H. A. Lester, H. J. M. Birdsell, J. Stockton, N. H. Wassermann, and B. F. Erlanger. 1982. A photoisomerizable muscarinic antagonist. Studies of binding and of conductance relaxations in frog heart. *Journal of General Physiology.* 79:657-678.

Nathanson, N. M. 1987. Molecular properties of the muscarinic acetylcholine receptor. *Annual Review of Neuroscience.* 10:195-236.

Noble, D. 1975. The Initiation of the Heart Beat. Oxford University Press, London. 103-106.

Northup, J. K., M. D. Smigel, and A. G. Gilman. 1982. The guanine nucleotide activating site of the regulatory component of adenylate cyclase. Identification by ligand binding. *Journal of Biological Chemistry.* 257:11416-11423.

Onali, P., M. C. Olianas, J. P. Schwartz, and E. Costa. 1983. Involvement of a high-affinity GTPase in the inhibitory coupling of striatal muscarinic receptors to adenylate cyclase. *Molecular Pharmacology.* 24:380-386.

Pfaffinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille. 1985. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature.* 317:536-538.

Pfeuffer, T., and F. Eckstein. 1976. Topology of the GTP-binding site of adenyl cyclase from pigeon erythrocytes. *FEBS Letters.* 67:354-358.

Pot, L., and H. Pusch. 1979. A kinetic model for the muscarinic action of acetylcholine. *Pflügers Archiv.* 383:79-77.
Purves, R. D. 1976. Function of muscarinic and nicotinic acetylcholine receptors. *Nature*. 261:149–151.

Rodbell, M. 1975. On the mechanism of activation of fat cell adenylate cyclase by guanine nucleotides. An explanation for the biphasic inhibitory and stimulatory effects of the nucleotides and the roles of hormones. *Journal of Biological Chemistry*. 250:5826–5834.

Schramm, M., and M. Rodbell. 1975. A persistent active state of the adenylate cyclase system produced by the combined actions of isoproterenol and guanylyl imidodiphosphate in frog erythrocyte membranes. *Journal of Biological Chemistry*. 250:2232–2237.

Sibley, D. R., R. H. Strasser, J. L. Benovic, K. Daniel, and R. J. Lefkowitz. 1986. Phosphorylation/dephosphorylation of the β-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proceedings of the National Academy of Sciences*. 83:9408–9412.

Simmons, M. A., and H. C. Hartzell. 1987. A quantitative analysis of the acetylcholine-activated potassium current in single cells from frog atrium. *Pflügers Archiv*. 409:454–461.

Soejima, M., and A. Noma. 1984. Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Archiv*. 400:424–431.

Spiegel, A. M. 1987. Signal transduction by guanine nucleotide binding proteins. *Molecular and Cellular Endocrinology*. 49:1–16.

Sternweis, P. C., and J. D. Robishaw. 1984. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *Journal of Biological Chemistry*. 259:13806–13813.

Stryer, L., and H. R. Bourne. 1986. G proteins: a family of signal transducers. *Annual Review of Cell Biology*. 2:391–419.

Sunyer, T., J. Codina, and L. Birnbaumer. 1984. GTP hydrolysis by pure N, the inhibitory regulatory component of adenylyl cyclases. *Journal of Biological Chemistry*. 259:15447–15451.

Wong, S. K. F., and B. R. Martin. 1986. Activation of rat liver adenylate cyclase by guanosine 5' (β, γ-imido) triphosphate and glucagon. Existence of reversibly and irreversibly-activated states of the stimulatory GTP-binding protein. *Biochemical Journal*. 233:845–851.

Yamanaka, G., F. Eckstein, and L. Stryer. 1986. Interaction of retinal transducin with guanosine triphosphate analogues: specificity of the γ-phosphate binding region. *Biochemistry*. 25:6149–6153.

Yatani, A., J. Codina, A. M. Brown, and L. Birnbaumer. 1987. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein Gs. *Science*. 235:207–211.