Regulation of Ca\(^{2+}\) Current in Frog Ventricular Cardiomyocytes by Guanosine 5’-Triphosphate Analogues and Isoproterenol

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ABSTRACT Calcium currents (I\(_{ca}\)) were measured in frog ventricular myocytes using the whole-cell patch clamp technique and a perfused pipette. To gain insight into the role of G proteins in the regulation of I\(_{ca}\) in intact cells, the effect of internal perfusion with hydrolysis-resistant GTP analogues, guanylyl 5’-imidodiphosphate (GppNHp) or guanosine 5’-thiotriphosphate (GTP\(_{yS}\)), on I\(_{ca}\) stimulated by isoproterenol (Iso) or forskolin (Forsk) was examined. Significant differences were observed between the effects of the two GTP analogues. Internal perfusion of GppNHp resulted in a near-complete (~80%) and irreversible inhibition of Iso-stimulated I\(_{ca}\). In contrast, internal perfusion with GTP\(_{yS}\) resulted in only a partial (~40%) inhibition of Iso- or Forsk-stimulated I\(_{ca}\). The fraction of the current not inhibited by GTP\(_{yS}\) remained persistently elevated after the washout of Iso but declined to basal levels upon washout of Forsk. Excess internal GTP or GppNHp did not reduce the persistent I\(_{ca}\). Internal adenosine 5’-thiotriphosphate (ATP\(_{yS}\)) mimicked the GTP\(_{yS}\)-induced, persistent I\(_{ca}\). GppNHp sometimes induced a persistent I\(_{ca}\), but only if GppNHp was present at high concentration before Iso exposure. Inhibitors of protein kinase A inhibited both the GTP\(_{yS}\)- and ATP\(_{yS}\)-induced, persistent I\(_{ca}\). We conclude that: (a) GTP\(_{yS}\) is less effective than GppNHp in inhibiting adenylyl cyclase (AC) via the inhibitory G protein, G\(_i\); and (b) the persistent I\(_{ca}\) results from a long-lived G\(_s\)-GTP\(_{yS}\) complex that can activate AC in the absence of Iso. These results suggest that different hydrolysis-resistant nucleotide analogues may behave differently in activating G proteins and imply that the efficacy of G protein–effector molecule interactions can depend on the GTP analogue with which the G protein is activated.

INTRODUCTION Many ion channels are regulated both directly and indirectly by G proteins (Levitan, 1988). One common approach for studying G protein involvement in ion channel...
regulation has been to perfuse cells internally with hydrolysis-resistant GTP analogues to stably activate G proteins. Relatively little quantitative information exists, however, on the effects of these analogues on ionic currents, differences between analogues, or complications resulting from activation of multiple types of G proteins in cells. Because the cardiac Ca current is a prime model for ion channel modulation by neurotransmitters, we have used internal perfusion of cardiac myocytes with nucleotide analogues to provide quantitative information about how G proteins function in the intact cells.

β-Adrenergic receptor stimulation increases the cardiac calcium current (I_{Ca}), and muscarinic acetylcholine (ACh) receptors decrease I_{Ca} stimulated by β-adrenergic agonists (reviewed by Hartzell, 1988). β-Adrenergic agonists stimulate adenylyl cyclase (AC), activate the cAMP-dependent phosphorylation pathway, and increase I_{Ca} (reviewed by Trautwein and Hescheler, 1990), whereas ACh reduces the phosphorylation via inhibition of AC (Fischmeister and Hartzell, 1986; Hescheler, Kameyama, and Trautwein, 1986; Fischmeister and Shrier, 1989).

Studies in cell-free and reconstituted systems have demonstrated that the regulation of AC involves two species of G proteins, a stimulatory G protein (G_s) linked to the β-adrenergic receptor and an inhibitory G protein (G_i) linked to the muscarinic cholinergic receptor. β-Adrenergic stimulation of AC is thought to involve hormone-stimulated exchange of GTP for bound GDP on the G_s protein and subsequent dissociation of G_s and G_i subunits (Gilman, 1987). The GTP-ligated α subunit then binds AC and stimulates its catalytic activity. Hydrolysis of GTP and reassociation of α and β subunits terminates the G protein’s catalytic activity. The mechanism of G_i-mediated inhibition of AC is complex. It is not clear whether inhibition involves merely the binding and neutralization of α by β subunits released from G_i (reviewed by Gilman, 1987; Ross, 1989), or whether α or β might directly inhibit AC (see discussion in Pfeuffer and Helmreich, 1988). More recent experiments have provided evidence for direct actions of both α (Wong, Federman, Pace, Zachary, Evans, Pouyssegur, and Bourne, 1991) and β (Tang and Gilman, 1991) on AC.

Since reconstituted systems differ in important ways from intact cells (including protein stoichiometries and tissue sources, as well as disruption of subcellular compartmentalization, ion gradients, and transmembrane potentials), we have studied the G protein regulation of AC in intact cells utilizing internal perfusion of hydrolysis-resistant analogues of GTP and voltage clamp measurements of ionic currents in frog ventricular myocytes (Parsons, Lagrutta, White, and Hartzell, 1991). A similar approach was used in the pioneering work of Breitwieser and Szabo (1988) to study G protein turnover in intact atrial cells. They measured the muscarinic K channel, which is thought to be directly gated by a G protein, and thus were able to infer rates of activation and deactivation of the G protein. The cardiac Ca channel, which is phosphorylated by a cAMP-dependent kinase, provides a rapid assay for AC activity in intact cells. However, cAMP-dependent phosphorylation of the Ca channel involves a cascade of intracellular reactions, of which there may be several rate-limiting steps (Frace, Méry, Fischmeister, and Hartzell, 1993) that make difficult a similar detailed quantitative kinetic analysis of the G proteins that regulate AC. Nevertheless, the frog cardiomyocyte is an excellent model for studying G protein
regulation of AC in intact cells in the steady state because: (a) the Ca current is a defined function of intracellular cAMP (Fischmeister and Hartzell, 1987; White and Hartzell, 1988); (b) cAMP-dependent phosphorylation is the only stimulatory mechanism for β-adrenergic stimulation of I_{Ca} (Hartzell, Méry, Fischmeister, and Szabo, 1991; Parsons et al., 1991); and (c) the cAMP-dependent stimulation of I_{Ca} is amazingly stable during intracellular dialysis (Fischmeister and Hartzell, 1986).

In our previously published work (Parsons et al., 1991), we observed that guanylyl 5'-imidodiphosphate (GppNHp) inhibited I_{Ca} stimulated by either isoproterenol (Iso) or low concentrations of forskolin (Forsk). We suggested that this inhibition resulted from the inhibition of AC by activation of a Gi-like protein via basal nucleotide exchange in the absence of muscarinic agonist. In this article, we compare the effects of guanosine 5’-thiotriphosphate (GTPγS) and GppNHp to further characterize the activation of G_{s}. We report that although GppNHp completely inhibited Iso-stimulated I_{Ca}, GTPγS only partially inhibited I_{Ca} and also induced a persistent I_{Ca} resulting from a long-lived G_{s}-GTPγS complex that activated AC in the absence of Iso. Unlike Gi, which is activated by GppNHp in the absence of a muscarinic agonist (Parsons et al., 1991), the activation of G_{s} depends on β-adrenergic stimulation.

Preliminary reports of this work have appeared (Parsons and Hartzell, 1991, 1992).

METHODS

Preparation

Bullfrogs (Rana catesbeiana) were killed by decapitation and double pithing. Ventricular myocytes from freshly dissected hearts were isolated by enzymatic and mechanical dissociation and kept in maintenance media as previously described (Hartzell and Simmons, 1987).

Solutions and Cell Perfusion

During electrophysiological recordings of I_{Ca}, cells were bathed in and superfused with 115 mM NaCl, 20 mM CsCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Na pyruvate, 5 mM glucose, 0.3 μM tetrodotoxin, and 10 mM HEPES at pH 7.4. During the course of an experiment, the cell under analysis was exposed to various test compounds (ACh and Iso from Sigma Chemical Co., St. Louis, MO; Forsk from Calbiochem-Novabiochem Corp., La Jolla, CA; and Sotalol provided by Ken Minneman, Emory School of Medicine, Atlanta, GA) by positioning it in front of flowpipes containing superfusion solution supplemented with these chemicals. Cells were usually perfused internally with 118 mM CsCl, 5 mM K₂EGTA, 2.8 mM Na₂K₂ATP, 4.04 mM MgCl₂, 5 mM Na₂ creatine phosphate, and 10 mM K-PIPES at pH 7.15. The internal solution was changed by a system permitting continuous perfusion of the patch pipette (Fischmeister and Hartzell, 1987). Internal solution was supplemented with various test compounds (GTPγS, GppNHp, and GTP from Boehringer Mannheim Corp., Indianapolis, IN or Calbiochem-Novabiochem; adenosine cyclic 3',5'-(R₃)-phosphorothioate, kindly provided by Ira Cohen, SUNY Stony Brook, Stony Brook, NY; and Wiptide (the cAMP-dependent protein kinase inhibitor peptide, PKI(1-22)), Peninsula Labs., Inc., Belmont, CA). When Li₄ATPγS was included in the internal perfusion solution, Na₂K₂ATP was omitted. For guanine nucleotide concentrations <50 μM, the nucleotide was added to standard internal solution without correction for Mg²⁺ chelation. For higher guanine nucleotide concentrations, solutions were constructed to maintain free [Mg²⁺] constant at 1 mM (White and Hartzell, 1988), assuming the same stability constants for GppNHp and GTPγS as for GTP.
Figure 1. Effects of internal perfusion with GTPγS on Iso-stimulated \(I_{Ca}\) in frog cardiac myocytes. (A) Effect of 50 \(\mu M\) GTPγS on \(I_{Ca}\) elevated by 0.3 \(\mu M\) Iso. Each square represents net inward current elicited by a 400-ms voltage pulse from -80 to 0 mV. The cell was first exposed to 0.3 \(\mu M\) Iso for the period indicated by the horizontal line and was perfused with standard internal solution unless indicated otherwise. When \(I_{Ca}\) stabilized, internal 50 \(\mu M\) GTPγS was added as indicated. The Iso-stimulated \(I_{Ca}\) was partially inhibited. (B) Current traces of \(I_{Ca}\) obtained under control conditions, in the presence of Iso, and in the presence of Iso after...
In most experiments, GTPyS was used without repurification. However, commercially available GTPyS was often significantly contaminated with 260-nm absorbing material (presumably nucleotides). For some experiments, GTPyS or GppNHp was repurified to >95% by HPLC as previously described (Parsons et al., 1991).

Recording Protocols

Electrophysiological recordings were conducted using the whole-cell configuration of the patch clamp technique as previously described (Fischmeister and Hartzeil, 1987). $I_{Ca}$ was defined as the net inward current elicited by voltage pulses from -80 to 0 mV for 400 ms. Changes in series resistance during experiments were monitored by continued observation for abrupt changes in the amplitude or kinetics of the $I_{Ca}$ transient. If either was observed, then series resistance was usually recalculated from the capacitive transients associated with small voltage steps away from the holding potential or the experiment was terminated.

Data Analysis and Statistics

Calcium current densities were calculated by dividing $I_{Ca}$ by cell capacitance and assuming 1 µF = 1 cm$^2$. Data are expressed as mean ± standard error with $n$ equal to the number of cells studied. The time course of stimulation of $I_{Ca}$ by Iso was described by a parameter, $t_w$, which was defined as the time after initial stimulation with Iso when the magnitude of $I_{Ca}$ was equal to 63.2% of the difference between the basal $I_{Ca}$ and maximal Iso-stimulated $I_{Ca}$.

RESULTS

Inhibition of Ca Current by Intracellular GTPyS

GTPyS-mediated inhibition of Iso-stimulated $I_{Ca}$. We previously reported (Parsons et al., 1991) that GppNHp inhibited Iso-stimulated $I_{Ca}$ nearly completely. To determine whether similar results were obtained with other hydrolysis-resistant analogues of GTP, cells were internally perfused with GTPyS. The cell in Fig. 1, A and B was first perfused with a solution lacking any added guanine nucleotides and then exposed to 0.3 µM Iso. After $I_{Ca}$ stabilized, internal 50 µM GTPyS produced a partial decrease in the current (Fig. 1, A and B). On average, 0.3 µM Iso resulted in an 11.7 ± 1.1-fold ($n = 39$) stimulation of $I_{Ca}$ and inhibition by 50 µM GTPyS resulted in a persistent $I_{Ca}$ after GTPyS. Cell was exposed to 0.3 µM Iso and then perfused internally with 50 µM GTPyS. The current was only partially inhibited. After the inhibition reached an apparent steady state, Iso was washed out (at 20 min). The current remained unchanged for 5 min of washing out Iso. In control cells not exposed to GTPyS, the Iso response washed out completely in the same time period (open squares). (E) Summary of effects of internal perfusion with GTPyS on Iso-stimulated $I_{Ca}$. $I_{Ca}$ density ($µA/cm^2$) is plotted for several experimental conditions using 0.1–10 µM Iso and 50 µM GTPyS. Each bar represents the mean $I_{Ca}$ density value for the number of cells tested under each condition. Error bars are SEM.
42.2 ± 3.8% (n = 15) decrease in Iso-stimulated $I_{\text{Ca}}$ (Table IA, 1 and 2). Note that "percentage decrease" is the mean of the decreases observed in each individual cell. These values differ slightly from the decrease calculated from $I_{\text{Ca}}$ densities in Table I. Exposure to 100-fold greater concentrations of Iso did not reverse the partial

### TABLE I

Effects of GTP Analogues on $I_{\text{Ca}}$ in Frog Ventricular Myocytes

| External solution | Internal solution | $I_{\text{Ca}}$ density $\mu\text{A/cm}^2$ | n |
|-------------------|------------------|----------------------------------|---|
| **A. Effect of GTP$\gamma$S on Iso-stimulated $I_{\text{Ca}}$** |
| 1 Con | Con | 2.4 ± 0.2 | 39 |
| 0.3 μM Iso | Con | 26.5 ± 2.2 | 39 |
| 2 Con | 0.3 μM Iso | 29.7 ± 3.7 | 15 |
| 0.3 μM Iso | 50 μM GTP$\gamma$S | 18.7 ± 3.2 | 15 |
| Wash | 50 μM GTP$\gamma$S | 16.4 ± 2.1 | 15 |
| Wash | 50 μM GTP$\gamma$S + PKI* | 5.5 ± 0.6 | 6 |
| 3 0.3 μM Iso | Con | 22.4 ± 0.8 | 4 |
| 0.3 μM Iso | 500 μM GTP$\gamma$S | 12.9 ± 1.1 | 4 |
| Wash | 500 μM GTP$\gamma$S | 13.7 ± 0.3 | 3 |
| 4+ 0.3 μM Iso | Con | 12.3 ± 2.1 | 4 |
| 0.3 μM Iso | 50 μM GTP$\gamma$S | 22.2 ± 1.0 | 4 |
| **B. Effect of GTP$\gamma$S on Forsk-stimulated $I_{\text{Ca}}$** |
| 1 Con | Con | 2.9 ± 0.3 | 30 |
| 5 μM Forsk | Con | 23.6 ± 2.3 | 30 |
| 2 0.3-3 μM Forsk | Con | 29.6 ± 2.6 | 17 |
| 0.3-3 μM Forsk | 50 μM GTP$\gamma$S | 15.6 ± 1.6 | 17 |
| 3 3 μM Forsk | CON | 18.3 ± 2.6 | 4 |
| 3 μM Forsk | 500 μM GTP$\gamma$S | 12.7 ± 2.1 | 4 |
| 4+ 1-3 μM Forsk | Con | 12.6 ± 3.2 | 4 |
| 1-3 μM Forsk | 50 μM GTP$\gamma$S | 23.1 ± 6.7 | 4 |
| **C. Effect of ACh on Forsk-stimulated $I_{\text{Ca}}$ in the presence of GTP$\gamma$S** |
| 3 μM Forsk | 50 μM GTP$\gamma$S | 15.5 ± 2.3 | 5 |
| 3 μM Forsk + 10 μM ACh | 50 μM GTP$\gamma$S | 14.6 ± 2.0 | 5 |
| **D. Effects of GTP$\gamma$S applied before stimulation of $I_{\text{Ca}}$ with Iso** |
| Con | 50 μM GTP$\gamma$S | 4.2 ± 0.6 | 13 |
| 0.3 μM Iso | 50 μM GTP$\gamma$S | 29.0 ± 4.4 | 13 |
| Wash | 50 μM GTP$\gamma$S | 28.4 ± 4.7 | 12 |
| 10 μM ACh | 540 μM GTP$\gamma$S | 26.0 ± 4.4 | 9 |
| **E. Effects of ATP$\gamma$S applied before stimulation of $I_{\text{Ca}}$ with Iso** |
| Con | 2.5 mM ATP$\gamma$S | 4.3 ± 0.6 | 6 |
| 0.3 μM Iso | 2.5 mM ATP$\gamma$S | 18.7 ± 3.3 | 6 |
| Wash | 2.5 mM ATP$\gamma$S | 18.1 ± 3.5 | 6 |
| Wash | 2.5 mM ATP$\gamma$S + PKI§ | 7.5 ± 2.7 | 6 |
| **F. Effects of GppNHp on Iso stimulation of $I_{\text{Ca}}$** |
| 0.3 μM Iso | Con | 26.5 ± 2.2 | 39 |
| 0.3 μM Iso | 50 μM GppNHp | 6.6 ± 1.2 | 13 |
| 0.3 μM Iso | 500 μM GppNHp|| | 14.5 ± 2.5 | 21 |

*15 μM Wiptide or 0.1-1.0 mM (Rp)cAMPS. 
*Cells in which GTP$\gamma$S had stimulatory effects on either Iso, or Forsk-stimulated $I_{\text{Ca}}$. 
§16 μM Wiptide. 
||GTP analogue internally perfused before stimulation with Iso.
inhibition of Iso-stimulated ICa (n = 6) (data not shown). Similar incomplete inhibition was obtained with 500 μM internal GTPγS (Fig. 1 C) (Table I A, 3).

GTPγS inhibited Iso-stimulated ICa in most experiments (see Figs. 1 and 2). However, in four cells 50 μM GTPγS produced a transient inhibition followed by a stimulation of 2.0 ± 0.3-fold (Table I A, 4). The Iso-stimulated ICa density before internal GTPγS in these four cells was less than half that of the group inhibited by internal GTPγS (Table I A, 2 and 4), suggesting that the ability to respond to Iso was not the same in these cells.

GTPγS blocks the effects of GppNHp. The 42% inhibition of Iso-stimulated ICa by GTPγS contrasts with the ~80% decrease in ICa caused by internal perfusion of GppNHp during Iso stimulation of ICa (Parsons et al., 1991). However, replacing GTPγS with internal GppNHp for >25 min resulted in no additional decrease in ICa (Fig. 1 C). This finding suggests that the partial inhibition of Iso-stimulated ICa by GTPγS was not due to the incomplete activation of G1 by GTPγS.

Persistent stimulation of ICa. After ICa was partially inhibited by GTPγS, the current that was not inhibited remained elevated even when Iso was washed out (Fig. 1 D). This is also observed in Fig. 1 C after internal perfusion with both GTPγS and GppNHp. We will term this current that is stimulated above basal levels in the absence of β-adrenergic agonist “persistent ICa,” although it should be noted that this current exhibits the same voltage-dependent properties and inactivation as the basal current. In other words, the amplitude of the current is persistently stimulated above basal levels, but the current still requires depolarization to be activated. The effects of Iso and GTPγS on ICa are summarized in the bar graph in Fig. 1 E.

To define the mechanism of GTPγS-mediated inhibition of stimulated ICa, the effect of GTPγS on ICa was studied under three experimental conditions: (a) basal or unstimulated ICa; (b) ICa elevated by direct activation of AC via Forsk (Hartzel and Fischmeister, 1987); and (c) ICa stimulated by activation of protein kinase A with internal perfusion of cAMP.

Effect of GTPγS on basal ICa. We previously reported that GppNHp had no effect on basal ICa (Parsons et al., 1991). Similar results were observed with GTPγS. Cells were internally perfused for at least 10 min in the absence of Iso or Forsk with either 50 μM GTPγS or 50 μM GTP or a control internal solution containing no added guanine nucleotides. Under all three conditions a similar approximately twofold increase in basal ICa was observed. This runup is probably due to equilibration of the pipette solutions (Cs+, Mg2+, etc.) with the cell. These results support the suggestion that GTPγS has no effect on ICa in the absence of the activation of AC.

GTPγS-mediated inhibition of Forsk-stimulated ICa. The cell in Fig. 2 A was first perfused with a solution lacking any added guanine nucleotides and then exposed to 3 μM Forsk. When 50 μM GTPγS was added internally, ICa steadily decreased to an intermediate level (Fig. 2 A). On average, 3 μM Forsk resulted in a 9.4 ± 0.9-fold stimulation of ICa (n = 30) and 50 μM GTPγS resulted in a 46.6 ± 4.7% decrease (n = 17) in 3 μM Forsk-stimulated ICa (Fig. 2 A; Table I B, 1 and 2). Because Forsk stimulates the activity of AC in the absence of activation of G1 (Seamon and Daly, 1986), these results suggest that even in the absence of G1 activation, GTPγS can inhibit ICa.
FIGURE 2. Effect of internal GTPγS on Forsk-stimulated $I_{\text{Ca}}$. (A) Partial inhibition of 50 µM Forsk-stimulated $I_{\text{Ca}}$ by GTPγS. The cell was exposed to 3 µM Forsk and internally perfused with 50 µM GTPγS. $I_{\text{Ca}}$ steadily decreased, but reached the steady-state level, which was elevated above basal $I_{\text{Ca}}$. (B) Partial inhibition of Forsk-stimulated $I_{\text{Ca}}$ by 500 µM GTPγS. The cell was exposed to 3 µM Forsk and internally perfused with 500 µM GTPγS. $I_{\text{Ca}}$ steadily decreased, but reached the steady-state level, which was elevated above basal $I_{\text{Ca}}$. Subsequent washout of Forsk resulted in a rapid decline in the $I_{\text{Ca}}$. (C) Summary of effects of internal perfusion with GTPγS on Forsk-stimulated $I_{\text{Ca}}$. $I_{\text{Ca}}$ density (µA/cm²) is plotted for several experimental conditions using 3 µM Forsk and 50 µM GTPγS. Each bar represents the mean $I_{\text{Ca}}$ density value for the number of cells tested under each condition. Error bars are SEM.
A similar incomplete inhibition of 3 μM Forsk-stimulated $I_c_a$ was also observed with 500 μM GTPγS (Fig. 2 B). On average, 500 μM GTPγS resulted in a 31.6 ± 4.6% decrease ($n = 4$) in 3 μM Forsk-stimulated $I_c_a$ (Table I B, 3). However, upon washout of Forsk the elevated $I_c_a$ rapidly returned to basal levels. On average, 90.8 ± 2.2% of the initial Forsk-stimulated $I_c_a$ reversed upon washout of Forsk after internal perfusion with GTPγS (Fig. 2 B). This is in contrast to the finding with Iso and GTPγS (Fig. 1, C or D) in which >60% of the initial Iso-stimulated $I_c_a$ remained persistently elevated after the washout of Iso. Results are summarized in Fig. 2 C.

Some variation in the effect of GTPγS on Forsk-stimulated $I_c_a$ was observed. The cells depicted in Fig. 2, A and B, and 3 A exhibit the typical response. However, in some cells transient inhibition and sometimes stimulation of the Forsk-stimulated $I_c_a$ was observed. 4 of 21 cells (19%) that had been stimulated with Forsk (0.3–3 μM) and then internally perfused with GTPγS exhibited an increase in $I_c_a$. The average percentage increase in the Forsk-stimulated $I_c_a$ was 78.8 ± 37.2% ($n = 4$). As we observed with the atypical responses of Iso-stimulated $I_c_a$ to GTPγS (Table I A, 4), the mean amplitude of the Forsk-stimulated $I_c_a$ obtained before internal perfusion with GTPγS was less for cells in which GTPγS was stimulatory than for cells in which GTPγS was inhibitory (Table I B, 4).

$GTPγS$ blocks the effects of ACh. We examined the ability of ACh to further inhibit Forsk-stimulated $I_c_a$ that had been incompletely inhibited by GTPγS. In Fig. 3 A, 3 μM Forsk stimulated $I_c_a$ ~15-fold. $I_c_a$ was then briefly inhibited by a 3-min exposure to 20 μM ACh. After washout of ACh, internal perfusion of GTPγS (50 μM) partially inhibited the Forsk-stimulated $I_c_a$. After ~15 min of internal GTPγS, a second 3-min exposure to 20 μM ACh had only very small inhibitory effects on $I_c_a$. On average, ACh had no effect on Forsk-stimulated $I_c_a$ that was not inhibited by GTPγS (average 3.4 ± 7.8% decrease, $n = 5$) (Fig. 3 B, Table I C). Like the Forsk-stimulated $I_c_a$ that was partially inhibited by GTPγS in Fig. 2, the ACh-resistant current was not persistently activated either. $I_c_a$ rapidly returned to control levels after the removal of Forsk. On average, 90.2 ± 5.2% ($n = 5$) of the initial Forsk-stimulated $I_c_a$ reversed upon washout of Forsk after internal perfusion with 50 μM GTPγS and exposure to ACh (Fig. 3 B). These experiments demonstrate that after internal GTPγS, the ACh-resistant Forsk-stimulated $I_c_a$ does not remain elevated in the absence of Forsk.

Effects of GTPγS on cAMP-stimulated $I_c_a$. We examined the possibility that GTPγS might be inhibiting $I_c_a$ at a site beyond AC. For example, activation of a phosphodiesterase (PDE) would increase the degradation of cAMP and result in a decrease in $I_c_a$. However, in none of the cells studied did GTPγS result in inhibition of cAMP-stimulated $I_c_a$ ($n = 7$). We observed either no change or a small stimulation of cAMP-elevated $I_c_a$ after internal GTPγS. These results do not support the hypothesis that GTPγS-mediated inhibition of Iso or Forsk-stimulated $I_c_a$ is via the stimulation of a PDE.

Taken together these results suggest that GTPγS activates an inhibitory G protein whose primary site of action is AC, because the inhibitory effects on $I_c_a$ were observed only under conditions that activate AC (e.g., Iso or Forsk, but not basal or cAMP). Similar results and conclusions were reported for GppNHp (Parsons et al., 1991).
Persistent Ca Current Induced by GTPγS

As described above, the fraction of $I_{Ca}$ that was not inhibited by GTPγS remained persistently stimulated after washout of Iso (Fig. 1). This is in contrast to the results after internal GTPγS with Forsk-stimulated $I_{Ca}$. A persistent $I_{Ca}$ was not observed with internal GTPγS after the washout of Forsk (Figs. 2 and 3). Regardless of the degree of inhibition of Iso-stimulated $I_{Ca}$ by GTPγS, the noninhibited $I_{Ca}$ always remained elevated after washout of the β-agonist (Fig. 1). On average, only a 7.3 ± 2.5% ($n = 15$) decrease in $I_{Ca}$ followed the washout of Iso, leaving a persistent $I_{Ca}$ that was 9.0 ± 1.4-fold greater than basal $I_{Ca}$ (Table I A, 2). Sotalol (5 μM), a β-adrenergic antagonist, did not significantly reduce the persistent $I_{Ca}$ (4.2 ± 4.0% decrease, $n = 5$) (see Fig. 6 A). Similar results were obtained with 500 μM GTPγS (Table I A, 3).

We hypothesize that because the α subunits of G proteins do not hydrolyze GTPγS
at a significant rate (reviewed by Ross, 1989), the inhibition of Iso-stimulated \( I_{Ca} \) was due to the irreversible activation of an inhibitory G protein, and the persistent \( I_{Ca} \) was due to irreversible activation of a stimulatory G protein. Consistent with this hypothesis, persistent \( I_{Ca} \) was unchanged by prolonged (>20 min) washing out of GTP\( \gamma \)S with excess GTP (Fig. 4A). In Fig. 4A, \( I_{Ca} \) was stimulated to \( \sim 3.3 \) nA by 0.1 \( \mu \)M Iso. Internal perfusion with 50 \( \mu \)M GTP\( \gamma \)S reduced \( I_{Ca} \) to \( \sim 2 \) nA. After the washout of Iso, \( I_{Ca} \) remained elevated for >10 min. Subsequent perfusion with 500

\[ \text{FIGURE 4. Irreversible activation of persistent } I_{Ca} \text{ by GTP}\gamma\text{S. (A) Effects of GTP on persistent } I_{Ca} \text{ induced by GTP}\gamma\text{S. The cell was sequentially exposed to 0.01 and 0.1 Iso, resulting in an increase in } I_{Ca}. \text{ Internal perfusion with 50 } \mu \text{M GTP}\gamma\text{S partially inhibited } I_{Ca} \text{ and subsequent washout of Iso revealed persistent } I_{Ca}. \text{ Internal perfusion with 500 } \mu \text{M GTP did not reverse the persistent } I_{Ca}. \text{ The dotted line denotes a small time-dependent rundown of } I_{Ca}. (B) Effects of GppNHp on persistent } I_{Ca} \text{ induced by GTP}\gamma\text{S. The cell was stimulated with 0.3 } \mu \text{M Iso and internally perfused with 50 } \mu \text{M GTP}\gamma\text{S. Washout of Iso resulted in a persistent } I_{Ca} \text{ which was unchanged by prolonged internal perfusion with 500 } \mu \text{M GppNHp. (C) Effects of ACh on persistent } I_{Ca} \text{ induced by GTP}\gamma\text{S. } I_{Ca} \text{ density (\( \mu \text{A} / \text{cm}^2 \)) is plotted for several experimental conditions using 0.1–10 } \mu \text{M Iso, 50 } \mu \text{M GTP}\gamma\text{S, and } 10–20 \mu \text{M ACh. Each bar represents the mean } I_{Ca} \text{ density value for the number of cells tested under each condition. Error bars are SEM.} \]
μM GTP failed to reduce significantly the persistent $I_{Ca}$. Fig. 4B depicts a similar experiment except that GTPγS was washed out with a 10-fold excess of GppNHP. GTPγS partially inhibited the Iso-stimulated $I_{Ca}$; however, $I_{Ca}$ remained elevated upon washout of Iso. Subsequent internal perfusion with 500 μM GppNHP for ~20 min failed to reduce the persistent $I_{Ca}$. On average, GppNHP after GTPγS produced an insignificant decrease in $I_{Ca}$ of only 3.0 ± 1.4% ($n = 7$). This is in contrast to the ~80% decrease in $I_{Ca}$ caused by internal perfusion of GppNHP during Iso stimulation of $I_{Ca}$ (Parsons et al., 1991). These findings are consistent with the idea that the GTPγS-induced persistent $I_{Ca}$ is due to the irreversible activation of $G_{i}$.

GTPγS-mediated persistent $I_{Ca}$ not inhibited by ACh. The persistently stimulated $I_{Ca}$ that remained in the absence of Iso was unaffected by application of 10 μM ACh. On average, after prolonged internal perfusion with GTPγS, ACh produced an insignificant decrease (3.1 ± 3.3%, $n = 5$) of the persistent $I_{Ca}$. These effects are summarized in Fig. 4C.

GTPγS-induced persistent Ca current depends on protein kinase A. In frog cardiomyocytes, stimulation of $I_{Ca}$ by Iso is thought to be mediated exclusively by cAMP-dependent protein kinase (Hartzell et al., 1991; Parsons et al., 1991; Hartzell and Fischmeister, 1992). Thus, we predicted that AC was the site of action of the irreversibly activated G protein. To test this hypothesis, we examined the effect of inhibitors of cAMP-dependent protein kinase on the persistent $I_{Ca}$ induced by GTPγS. After the stimulation of $I_{Ca}$ by 0.3 μM, the $I_{Ca}$ was partially inhibited by internal perfusion with 500 μM GTPγS. The remaining $I_{Ca}$ was persistent after the removal of Iso, but the elevated $I_{Ca}$ was almost completely reversed by 16 μM Wiptide.

**Figure 5.** Effects of peptide inhibitors of protein kinase A on persistent $I_{Ca}$ induced by GTPγS. After the stimulation of $I_{Ca}$ by 0.3 μM, the $I_{Ca}$ was partially inhibited by internal perfusion with 500 μM GTPγS. The remaining $I_{Ca}$ was persistent after the removal of Iso, but the elevated $I_{Ca}$ was almost completely reversed by 16 μM Wiptide.

Persistent Ca current is enhanced by GTPγS before Iso. In the preceding experiments, GTPγS was perfused during Iso exposure. Quantitatively different results were obtained when GTPγS was perfused before Iso exposure. After internal perfusion with 50 μM GTPγS for >10 min, subsequent exposure to 0.3 μM Iso
resulted in a 7.8 ± 1.4-fold (n = 13) increase in $I_{Ca}$ (Table I D). After removal of Iso, a large persistent $I_{Ca}$ remained (Fig. 6 A). In the presence of GTPγS, $I_{Ca}$ decreased 18.7 ± 24.0% (n = 12) upon washout of Iso and was 7.5 ± 1.4-fold larger than the unstimulated $I_{Ca}$ (Table I D, Fig. 6 B). ACh (10 μM) or sotalol (5 μM) did not significantly inhibit the persistent $I_{Ca}$ that remained (Fig. 6 A). On average, the persistent $I_{Ca}$ was only negligibly reduced by ACh (8.2 ± 2.3%, n = 9) (Table I D) or

![Graph A]

**Figure 6.** Effects of prior internal perfusion with GTPγS on $I_{Ca}$. (A) Persistent $I_{Ca}$ after internal perfusion of GTPγS before stimulation with Iso that is resistant to β-antagonists and muscarinic agonists. The cell was internally perfused with 50 μM GTPγS and basal $I_{Ca}$ increased slightly. Exposure to 0.3 μM Iso elevated $I_{Ca}$, which remained persistently increased after washout of Iso. Subsequently, either 5 μM sotalol or 10 μM ACh resulted in only negligible inhibition of the persistent $I_{Ca}$. The rapid decrease in $I_{Ca}$ after exposure to 5 μM sotalol of a control cell is superimposed (open squares). (B) Summary of effects of prior internal perfusion with GTPγS on $I_{Ca}$. $I_{Ca}$ density is plotted for several experimental conditions using 0.3 μM Iso, 50 μM GTPγS, and 10 μM ACh.

by sotalol (3.3 ± 0.4%, n = 3). In contrast, under control conditions (in the absence of GTPγS), near-complete inhibition of Iso-stimulated $I_{Ca}$ by ACh (Fischmeister and Hartzell, 1986) or sotalol (open squares, Fig. 6 A) was observed. $I_{Ca}$ stimulated by Iso was 1.8 times larger when the cell was perfused with GTPγS before Iso exposure than when the cell was perfused with GTPγS during Iso exposure (cf. Table I, A and D).
Persistent Ca Current Induced by ATPγS

Because GppNHp almost completely inhibits Iso-stimulated \( I_{Ca} \), we have suggested that the predominant effect of internal GppNHp in frog ventricular myocytes is to activate the inhibitory G protein, \( G_i \) (Parsons et al., 1991). In contrast, it appears that the effects of GTPγS involve both \( G_i \) and \( G_s \) because GTPγS produces only incomplete inhibition and persistence of the noninhibited current. The difference between the effects of GppNHp and GTPγS could be explained by the fact that GTPγS can serve as a substrate for nucleoside diphosphate kinase (NDPK), whereas GppNHp cannot (Heidbüchel, Callewaert, Vereecke, and Carmeliet, 1990). To explore whether the observed differences between the internal perfusion with GTPγS and GppNHp were due to the thiophosphate donor property of GTPγS, another thiophosphate nucleotide, ATPγS, was perfused internally.

Internal perfusion with ATPγS resulted in a persistent \( I_{Ca} \) after Iso stimulation. The cell in Fig. 7 A was internally perfused with 2.5 mM ATPγS for > 10 min before stimulation with 0.3 nM Iso. Upon exposure to Iso, \( I_{Ca} \) increased >10-fold over ~25 min (\( t_e = 10.1 \) min). The average rate of increase in Iso-stimulated \( I_{Ca} \) with internal 2.5 mM ATPγS was \( t_e = 8.4 \pm 1.6 \) min (\( n = 6 \)). This was similar to the rate of Iso stimulation in the presence of internal 50 μM GTPγS (\( t_e = 5.2 \pm 0.7 \) min, \( n = 13 \)), but was much slower than the increase with 50 μM GTP (\( t_e = 1.3 \pm 0.1 \) min, \( n = 3 \)). Upon stabilization of the current and washout of Iso in the presence of ATPγS, \( I_{Ca} \) remained elevated. On average the Iso-stimulated persistent \( I_{Ca} \) in the presence of ATPγS was \( 4.8 \pm 1.2 \)-fold (\( n = 6 \)) greater than the unstimulated \( I_{Ca} \). Washout of Iso reduced \( I_{Ca} \) only \( 4.3 \pm 3.0\% \) (Table I E).

Effects of ATPγS on basal Ca current. In the presence of Iso, both GTPγS and ATPγS, but not GppNHp (Parsons et al., 1991), regularly induced an \( I_{Ca} \) that remained elevated above basal levels after the washout of the agonist. These findings suggested that transfer of the thiophosphate from the nucleoside thiophosphate (transphosphorylation) contributed to the development of the persistent \( I_{Ca} \). These data raise the question of whether the putative transphosphorylation required the presence of Iso. Cells were internally perfused for > 10 min in the absence of Iso with either 2.5 mM ATPγS (no added ATP), 50 μM GTP, or a control solution containing no added guanine nucleotides. Under all three conditions a similar approximately twofold increase in basal \( I_{Ca} \) was observed (Fig. 7 B). This runup of \( I_{Ca} \) is probably due to equilibration of the pipette solution (Cs+, Mg2+, etc.) with the cell. We have shown that internal perfusion of either GppNHp (Parsons et al., 1991) or GTPγS (Table I D) resulted in no significant increase in basal \( I_{Ca} \). These results show that the putative transphosphorylation step does not lead to the persistent activation of \( G_s \) at a significant rate in the absence of Iso.

ATPγS-induced persistent Ca current depends on protein kinase A. Protein kinase A inhibitors blocked the persistent \( I_{Ca} \) induced by internal perfusion with ATPγS. The cell in Fig. 7 C was internally perfused with 2.5 mM ATPγS for > 10 min before stimulation with 0.3 μM Iso. \( I_{Ca} \) increased with a \( t_e \) of 15.2 min. Upon washout of Iso, a persistent \( I_{Ca} \) remained. The dotted line in Fig. 7 C depicts the predicted rate of \( I_{Ca} \) rundown. A similar rundown of ~25 pA/min was observed in two other cells under
the same conditions. Persistent $I_{Ca}$ was subsequently inhibited by internal perfusion with 1 mM $(R_p)$cAMPS ($n = 6$, Table 1 E).

Persistent $Ca$ current induced rarely by GppNHp. A persistent $I_{Ca}$ was rarely observed with internal GppNHp, and only under conditions where a high concentration of GppNHp (500 $\mu$M) was present for a long period of time before Iso
application. (This is in contrast to the previously described experiments with GppNHp [Parsons et al., 1991] in which internal perfusion of the GTP analogue was initiated after exposure to Iso and during the steady-state stimulation of $I_{Ca}$ to β-agonist.) When GppNHp was perfused before Iso exposure, there was considerable variability in the subsequent Iso response. In some cells, stimulation by Iso was essentially abolished (Fig. 8 A). Most often Iso stimulation resulted in a persistent $I_{Ca}$ that was reduced in magnitude compared with control Iso responses (Fig. 8 B). However, in other cells Iso stimulated a large persistent $I_{Ca}$ (Fig. 8 C). The stimulatory response of $I_{Ca}$ to Iso was highly variable with this experimental paradigm as compared with internal perfusion of GppNHp during stimulation with Iso (Fig. 8 D; cf. ISO before GppNHp versus GppNHp before ISO). Despite the variation, on average $I_{Ca}$ was approximately two times larger with GppNHp before exposure to Iso than Iso before GppNHp (Table 1 F). In contrast to these results just described with 500 μM GppNHp, persistent $I_{Ca}$ was never observed with 50 μM GppNHp.

**DISCUSSION**

Internal perfusion of frog ventricular myocytes with hydrolysis-resistant analogues of GTP was used to examine the role of G proteins in the autonomic modulation of cardiac Ca channels. In our previous studies we showed that GppNHp activated an inhibitory G protein via basal nucleotide exchange in the absence of muscarinic agonists and virtually completely inhibited Iso- or Forsk-stimulated $I_{Ca}$ (Parsons et al., 1991). In this article we showed that GTPγS only incompletely inhibited Iso stimulation of $I_{Ca}$ and that the portion of the current that was not inhibited was persistently stimulated even in the absence of Iso. These results suggest that the two analogues of GTP differ in their ability to mediate inhibition of $I_{Ca}$ and to induce persistent $I_{Ca}$ after the washout of Iso.

Although the molecular identity of the G proteins involved in the effects that we
have observed is not known, for simplicity of discussion we will designate the G protein involved in Iso stimulation of \( I_{Ca} \) as \( G_s \) and the G protein involved in inhibition of \( I_{Ca} \) as \( G_i \). We feel this latter designation is justified because pertussis toxin blocks the inhibitory effects of ACh on \( I_{Ca} \) in cardiomyocytes from chick (Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985), guinea pig (Hescheler et al., 1986), and frog (Nakajima, Wu, Irisawa, and Giles, 1990). Furthermore, antibodies against the COOH terminus of mammalian \( G_s \) and \( G_i \) subunits recognize bands of appropriate molecular weights on Western blots of frog heart membrane homogenates (A. M. Spiegel, T. D. Parsons, and H. C. Hartzell, unpublished observations).

**Hydrolysis-resistant Analogues of GTP Activate \( G_i \)**

The similarity of the inhibitory effects of ACh, internal GppNHp, and GTP\(_7\)S on Iso- and Forsk-stimulated \( I_{Ca} \) suggests that all three agents act through the same mechanisms, although there are quantitative differences. Neither ACh (Fischmeister and Hartzell, 1986), GppNHp (Parsons et al., 1991), nor GTP\(_7\)S has effects on basal \( I_{Ca} \). Furthermore, ACh (Fischmeister and Shrier, 1989), GppNHp (Parsons et al., 1991), and GTP\(_7\)S inhibit Iso-stimulated \( I_{Ca} \) in what appears to be a noncompetitive manner; e.g., up to a 100-fold excess of Iso does not reverse the inhibition. Both ACh and GppNHp are thought to inhibit Iso-stimulated \( I_{Ca} \) by inhibition of AC via a mechanism involving \( G_i \) (Fischmeister and Hartzell, 1986; Hartzell and Fischmeister, 1987; Hartzell, 1988; Parsons et al., 1991). Thus, we assume that the inhibitory effects of GTP\(_7\)S are mediated via the irreversible activation of \( G_i \).

The ability of internal GTP\(_7\)S to antagonize the Iso stimulation of \( I_{Ca} \) in frog cardiac cells has been observed by other investigators (Fischmeister and Shrier, 1989; Nakajima et al., 1990). Nakajima et al. (1990) demonstrated complete inhibition of \( I_{Ca} \) stimulated by 1 \( \mu \)M Iso when the cell was previously perfused with 500 \( \mu \)M GTP\(_7\)S or when GTP\(_7\)S was perfused after Iso. Fischmeister and Shrier (1989) reported that exposure of cells to Iso in the presence of GTP\(_7\)S induced responses that varied significantly from cell to cell, which we have also observed. We do not have an explanation for the differences in the amount of inhibition observed by us and Nakajima et al. (1990).

The lack of effect of hydrolysis-resistant nucleotide analogues on basal \( I_{Ca} \) in frog is somewhat different from what has been reported for mammalian cardiac cells. GppNHp (Heschler et al., 1986) and GTP\(_7\)S (Shuba, Hesslinger, Trautwein, McDonald, and Pelzer, 1990) in guinea pig ventricular myocytes cause an increase in basal \( I_{Ca} \). The inhibition of \( I_{Ca} \) by ACh is also different in guinea pig (Heschler et al., 1986) than it is in frog cardiomyocytes (Fischmeister and Shrier, 1989): in guinea pig the inhibition appears to be competitive with Iso, whereas in frog it appears to be noncompetitive.

**Mechanisms of Partial Inhibition of Stimulated \( I_{Ca} \)**

Internal perfusion with GTP\(_7\)S resulted in only partial inhibition of Iso- or Forsk-stimulated \( I_{Ca} \) as compared with near-complete inhibition by ACh (Hartzell and Fischmeister, 1986) or GppNHp (Parsons et al., 1991). Furthermore, exposure to ACh after a steady-state partial inhibition had been produced by GTP\(_7\)S resulted in no additional inhibition. This incomplete inhibition by GTP\(_7\)S and the apparent
functional uncoupling of the muscarinic receptor from inhibition of AC could, in principal, be due either to incomplete activation of $G_i$ or to the relative inability of GTPyS-ligated $G_i$ to inhibit AC.

It seems unlikely that these effects can be explained by incomplete activation of $G_i$ by GTPyS. After Iso- or Forsk-stimulated $I_{Ca}$ has been partially inhibited by GTPyS, no additional inhibition was produced by (a) 10-fold higher concentrations of GTPyS, (b) GppNHp, or (c) ACh. However, both GppNHp and ACh can completely inhibit Iso-stimulated $I_{Ca}$ in the absence of GTPyS. This suggests that the incomplete inhibition of stimulated $I_{Ca}$ produced by GTPyS is not due to incomplete activation of $G_i$ by GTPyS.

We propose that the incomplete inhibition of Iso- and Forsk-stimulated $I_{Ca}$ by GTPyS involves a reduced ability of $\alpha_i(GTPyS)$ to inhibit AC, as compared with $\alpha_i(GppNHp)$ or $\alpha_i(GTPyS)$. $\alpha_i(GTPyS)$ could have a relatively lower affinity for AC or be less efficacious at inhibiting AC than $\alpha_i(GppNHp)$. This would result in partial inhibition of Iso- or Forsk-stimulated $I_{Ca}$ and a reduced responsiveness to ACh due to less inhibition per activated $G_i$. Thus, although GTPyS has a higher affinity than other GTP analogues for $G_i$ (Northup, Smigel, and Gilman, 1982), $G_i$ (Bokoch, Katada, Northup, Ui, and Gilman, 1984), transducin (Kelleher, Dudycz, Wright, and Johnson, 1986), and $G_K$ (Breitwieser and Szabo, 1988), the activity of the G protein–nucleotide complex may depend on the nucleotide bound and may be unrelated to the affinity of the nucleotide for the G protein.

The decreased ability of $\alpha_i(GTPyS)$ to inhibit $I_{Ca}$ could theoretically be due to a greater activation of $G_i$ by GTPyS than by GppNHp and competition between the effects of $G_i$ and $G_s$ on AC. If the potency or efficacy of GTPyS is greater than GppNHp at activating $G_s$, a higher ratio of activated $G_s$ to $G_i$ would occur and $G_i$ may not be able to inhibit AC completely. We discount this hypothesis because the stimulatory effect of Forsk is incompletely inhibited by GTPyS to the same extent as the effect of Iso. Because Forsk stimulates AC via a mechanism that apparently does not require $G_s$ (Seamon and Daly, 1986), it is unlikely that the incomplete inhibition of Forsk-stimulated $I_{Ca}$ by GTPyS occurs because GTPyS activates more $G_s$ than GppNHp does. The observation that Forsk stimulation of $I_{Ca}$ reversed immediately upon washout of Forsk (even in the presence of GTPyS) verifies the assumption that $\alpha_i(GTPyS)$ had not formed. Similarly, no difference in the ability of ACh to inhibit either Iso- or Forsk-stimulated $I_{Ca}$ after internal GTPyS was observed. This line of reasoning suggests that different ratios of $G_i/G_s$ activated by GTPyS or GppNHp cannot explain the differences in inhibition mediated by these analogues. The idea that GppNHp does not significantly activate $G_s$ because of its relatively lower affinity for $G_s$ is excluded by the observation that increasing the GppNHp concentration does not decrease the amount of inhibition produced by GppNHp, as would be expected if increasing GppNHp were able to activate $G_s$ (Parsons et al., 1991). Thus, we conclude that a role for $G_s$ in the incomplete inhibition of stimulated $I_{Ca}$ or in the GTPyS-mediated uncoupling of the muscarinic ACh receptor from inhibition-stimulated $I_{Ca}$ is unlikely.

In conclusion, internal perfusion of frog cardiomyocytes with GTPyS or GppNHp results primarily in the irreversible activation of $G_i$ via basal nucleotide exchange.
However, the ability of $\alpha_{i(GTPyS)}$ to inhibit AC appears to be reduced compared with $\alpha_{i(GppNHp)}$.

Incomplete Inhibition and Persistence Do Not Necessarily Share a Common Mechanism

The fraction of $I_{Ca}$ that was not inhibited by GTPyS remained persistently stimulated after the washout of $\beta$-adrenergic agonists. A similar large persistent $I_{Ca}$ was only very rarely observed with internal GppNHp. An obvious question that arises is: Are incomplete inhibition and persistence related? We will argue that persistence and poor inhibition are due to different mechanisms. Above, we have proposed that the ability of $\alpha_{i(GppNHp)}$ to inhibit AC was greater than that of $\alpha_{i(GTPyS)}$ and that the degree of inhibition depends on the nucleotide bound to the G protein. Below, we will argue that GTPyS induces a persistent $I_{Ca}$ whereas GppNHp rarely does, because GTPyS can activate $G_{s}$ by a pathway that is not utilized by GppNHp. Thus, whereas partial inhibition is caused by different efficacies of analogue-liganded $\alpha_{i}$ subunits, persistence is due to the ability of GTPyS to preferentially activate $G_{s}$.

This conclusion is both surprising and seemingly contradictory. However, it is supported by the fact that partial inhibition and persistence can be separated. For example, internal GTPyS only incompletely inhibits Forsk-stimulated $I_{Ca}$, but after washout of Forsk, $I_{Ca}$ is not persistent. Conversely, exposure to Iso after internal GTPyS usually produces a large persistent $I_{Ca}$ in the apparent absence of partial inhibition. Subsequent internal perfusion with GppNHp after development of persistent $I_{Ca}$ has no effect. Since all our data as well as those of Breitwieser and Szabo (1985, 1988) show a rapid basal nucleotide exchange on $G_{i}$, it is very likely that $G_{i}$ is fully liganded with GTP analogue under these conditions. Thus, persistence is not simply due to incomplete activation of $\alpha_{i}$ (as discussed above). Obviously, however, persistence would not be observed unless GTPyS also was relatively ineffective at producing inhibition.

Nature of Persistent $I_{Ca}$

Possible mechanisms. The persistent $I_{Ca}$ is probably due to persistent stimulation of AC by $G_{s}$ and not to some other effect of GTPyS. The strongest evidence was that blockers of protein kinase A largely inhibited the persistent $I_{Ca}$. This demonstrates that the persistent $I_{Ca}$ is dependent on continuing protein kinase activity, suggesting that persistent activation of $I_{Ca}$ is due to persistent stimulation of AC.

Breitwieser and Szabo (1985) observed persistent stimulation of $I_{Ca}$ by Iso in frog atrial myocytes perfused with GppNHp. We rarely observed a large persistent $I_{Ca}$ with GppNHp. However, when high concentrations of GppNHp were present for a long time before Iso application, a persistent $I_{Ca}$ was sometimes observed. The GppNHp-induced persistent $I_{Ca}$ was also variable in magnitude and often small in comparison to the larger, more consistently observed persistent $I_{Ca}$ with GTPyS.

The observation that the persistent $I_{Ca}$ is larger after internal perfusion with GTPyS than with GppNHp suggests that the activation of $G_{s}$ by GTPyS must be somehow different from the activation by GppNHp. The additional observation that ATPyS mimics GTPyS in the development of the persistent $I_{Ca}$ leads us to favor the idea that GTPyS acts via an additional pathway to activate $G_{s}$. GTPyS and ATPyS can...
be used as substrates for the synthesis of GTPγS from cellular GDP by NDPK, whereas GppNHp cannot.

The ability of ATPγS to produce a persistent $I_{Ca}$, as did GTPγS, was consistent with the idea that GTPγS was activating $G_{s}$ to produce a persistent $I_{Ca}$, but the fact that ATPγS might substitute for ATP and result in thiophosphorylation of the Ca channel complicated the interpretation of the ability of ATPγS to induce a persistent $I_{Ca}$. This presumably would result in the generation of a population of Ca channels that are resistant to dephosphorylation by phosphatases and are persistently activated (see Trautwein and Hescheler, 1990). (Rp)cAMPS, an inhibitor of protein kinase A, blocked the majority of the persistent $I_{Ca}$ in our experiments. This finding implied that: (a) thiophosphorylation of the Ca channel was not the major source of persistent $I_{Ca}$; (b) despite replacement of ATP with ATPγS in the pipette, sufficient levels of intracellular ATP were maintained by cellular metabolism (creatine phosphate was in the internal solution and glucose and pyruvate were in the bathing solution); and (c) ATP rather than ATPγS acted as a preferential substrate for protein kinase A in the phosphorylation of the Ca channel or the thiophosphorylated channel can be rapidly dephosphorylated.

**Proposed model.** We propose that persistent $I_{Ca}$ is produced by GTPγS but not by GppNHp because guanine nucleotide triphosphates have restricted access to $G_{s}$ and because guanine nucleotides are provided preferentially to $G_{i}$ by the enzyme NDPK (Fig. 9). We suggest that NDPK transfers the γ-phosphate of a nucleoside-5′-triphosphate outside the $G_{s}$ compartment to a nucleoside-5′-diphosphate within the compartment to activate $G_{s}$. This enzyme would serve to maintain a high local concentration of guanine nucleotide for $G_{s}$ activation when GTP or GTPγS was present, but would not do so with GppNHp because GppNHp is not a substrate for NDPK. GppNHp can enter this compartment, as evidenced by the fact that

![Figure 9: Model of activation by GTP analogues of G, in intact cardiomyocytes. NDP, nucleoside diphosphate; NDPK, nucleoside diphosphate kinase; ISO, isoproterenol; β-R, β-adrenergic receptor; M-R, muscarinic receptor; AC, adenyl cyclase. NDPK in the presence of agonist provides a pathway for the irreversible activation of $G_{s}$ that is available to ATPγS and GTPγS, but not GppNHp. Conversely, GTPγS or GppNHp, but not ATPγS, can irreversibly activate $G_{s}$, even in the absence of agonist. The small dashed arrow shows that the proposed subcellular compartmentalization $G_{s}$ is not a rigid barrier and that GppNHp can sometimes support the irreversible activation of $G_{s}$. Omitted from the diagram for simplicity is the rest of the well-established cAMP-dependent phosphorylation cascade that regulates $I_{Ca}$ in cardiac cells.](image-url)
prolonged perfusion of high concentrations of GppHNp does produce persistent \( I_{Ca} \) (Fig. 8). We do not necessarily envision this compartment as a defined physical entity, but rather as a virtual compartment created by the enzymatic funneling of selected substrates to the site of nucleotide exchange. In contrast, either GTP analogue can irreversibly activate \( G_i \) even in the absence of agonist; thus no such compartmentalization appears to exist for \( G_i \). Under certain restricted conditions, however, adenine nucleotides can activate \( G_i \) via conversion to guanine nucleotides via NDPK (Otero, Breitwieser, and Szabo, 1988; Szabo and Otero, 1990).

Other studies have proposed a regulatory function for enzymes located in close proximity to cardiac ion channels that act to control the local cytoplasmic environment of these channels. Weiss and Lamp (1989) demonstrated that glycolytic enzymes in close association with the ATP-sensitive K channel can act as a preferential source of ATP. A similar type of subcellular compartmentalization has been proposed for membrane-bound NDPK in the activation of the muscarinic K channel. In the absence of GTP, muscarinic K channel activity can be restored in excised patches of atrial myocytes by cytosolic exposure to several nucleosides including ATP and ATP\(\gamma\)S, but not AMP-PNP, an ATP analogue with an imido-linked terminal phosphate. Heidbuechel and colleagues have proposed that under physiological conditions NDPK acts as a local intracellular buffer of GTP (Heidbuechel et al., 1990). Further experiments will be needed to determine whether or not the transphosphorylation mechanism reported here plays a role in the physiological activation \( G_s \) by Iso and GTP.

Alternative interpretations. We believe that the ability of GTP\(\gamma\)S to induce a persistent \( I_{Ca} \) is best explained by a functional subcellular compartmentalization of \( G_s \) created by NDPK described above. However, another mechanism that could explain the ability of GTP\(\gamma\)S to induce a large persistent \( I_{Ca} \) is that \( \alpha_s(GppNHp) \) is more effective at stimulating AC than \( \alpha_s(GppNHp) \). This hypothesis has proved difficult to address because of our inability to define experimental conditions in which GppNHp reproducibly produces a large persistent \( I_{Ca} \). However, there are some data that support this suggestion that \( \alpha_s(GppNHp) \) has low efficacy in stimulating AC (Parsons et al., 1991). After inhibition of Forsk-stimulated \( I_{Ca} \) by GppNHp, exposure to Iso had no stimulatory effect on \( I_{Ca} \). However, after Iso, reexposure to Forsk produced a dramatic increase in \( I_{Ca} \). This increase was due to an \( \sim 100 \)-fold increase in the EC\(_{50} \) for Forsk. We interpret the rescue of Forsk-stimulated \( I_{Ca} \) by GppNHp to be due to Iso stimulating the loading of \( G_i \) with GppNHp. This activation of \( G_i \) increases the affinity of AC for Forsk, but \( \alpha_s(GppNHp) \) alone is incapable of stimulating the catalytic activity of AC.

Several other potential sites for the regulation of \( I_{Ca} \) by G proteins may exist in cardiomyocytes. Internal perfusion of hydrolysis-resistant GTP analogues could in theory activate a multitude of different G proteins that might result in a persistent \( I_{Ca} \). We will consider two possible alternatives to AC as sites of action for irreversibly activated G proteins. Several authors have suggested that \( G_s \) may have a direct effect on the channel (Yatani, Codina, Imoto, Reeves, Birnbaumer, and Brown, 1987; Imoto, Yatani, Reeves, Codina, Birnbaumer, and Brown, 1988; Yatani, Imoto, Codina, Hamilton, Brown, and Birnbaumer, 1988; Yatani and Brown, 1989). However, we believe that cAMP-dependent phosphorylation is the major regulatory
pathway of $I_{Ca}$ in these cells. This is supported by the observation that PKI completely inhibits the Iso-stimulated $I_{Ca}$ (Hartzell et al., 1991; Parsons et al., 1991). Furthermore, PKI also inhibited the majority of the persistent $I_{Ca}$. Thus, we do not believe that the direct action of G$_s$ on the Ca channel has a significant role in the development of the persistent $I_{Ca}$.

G protein-mediated inhibition of PDE has been suggested to underlie the stimulatory effects of glucagon on frog cardiomyocytes (Méry, Brechler, Pavoine, Pecker, and Fischmeister, 1990). Our observation that the internal GTP$y$S can sometimes further increase $I_{Ca}$ previously activated by internal cAMP is consistent with this claim. However, we do not believe that the irreversible inhibition of a PDE mediates the persistent $I_{Ca}$ described here because the persistent $I_{Ca}$ is only observed with Iso-stimulated $I_{Ca}$ and not with Forsk-stimulated $I_{Ca}$. If the effects of GTP$y$S were mediated by a PDE, the persistent $I_{Ca}$ would be expected after any transient increase in intracellular cAMP in the presence of GTP$y$S. However, a persistent $I_{Ca}$ has not been observed after internal GTP$y$S and subsequent washout of either Forsk or cAMP (Parsons, T.D., R.E. White and H.C. Hartzell, unpublished observations).

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REFERENCES

Bokoch, G. M., T. Katada, J. K. Northup, M. Ui, and A. G. Gilman. 1985. Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Journal of Biological Chemistry. 290:3550-3567.

Breitwieser, G., 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., and G. Szabo. 1988. Mechanism of muscarinic receptor-induced K$^+$ channel activation as revealed by hydrolysis-resistant GTP analogues. Journal of General Physiology. 91:469-493.

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.

Fischmeister, R., and H. C. Hartzell. 1987. Cyclic guanosine 3′,5′-monophosphate regulates the calcium current in single cells from frog ventricle. Journal of Physiology. 387:453-472.

Fischmeister, R., and A. Shrier. 1989. Interactive effects of isoprenaline, forskolin and acetylcholine on Ca current in frog ventricular myocytes. Journal of Physiology. 417:213-239.

Fricke, A. M., P.-F. Méry, R. Fischmeister, and H. C. Hartzell. 1993. Rate-limiting steps in the β-adrenergic stimulation of cardiac calcium current. Journal of General Physiology. 101:337-353.

Gilman, A. 1987. G proteins: transducers of receptor-generated signals. Annual Review of Biochemistry. 56:615-649.

Hartzell, H. C. 1988. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. Progress in Biophysics and Molecular Biology. 52:165-247.

Hartzell, H. C., and R. Fischmeister. 1986. Opposite effects of cyclic GMP and cyclic AMP on calcium current in single heart cells. Nature. 232:273-275.
Hartzell, H. C., and R. Fischmeister. 1987. Effect of forskolin and acetylcholine on calcium current in single isolated cardiac myocytes. Molecular Pharmacology. 32:639-645.

Hartzell, H. C., and R. Fischmeister. 1992. Direct regulation of cardiac Ca channels by G proteins: neither proven nor necessary? Trends in Pharmacological Sciences. 13:380-385.

Hartzell, H. C., P. F. Mery, R. Fischmeister, and G. Szabo. 1991. Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. Nature. 351:573-576.

Hartzell, H. C., and M. A. Simmons. 1987. Comparison of effects of acetylcholine on calcium and potassium currents in frog atrium and ventricle. Journal of Physiology. 389:411-422.

Heidbuchel, H., G. Callewaert, J. Vereecke, and E. Carmeliet. 1990. ATP-dependent activation of atrial muscarinic K⁺ channels in the absence of agonist and G-nucleotides. Pflügers Archiv. 416:213-215.

Hescheler, J., M. Kameyama, and W. Trautwein. 1986. On the mechanism of muscarinic inhibition of the cardiac Ca current. Pflügers Archiv. 407:182-189.

Imoto, Y., A. Yatani, J. F. Reeves, J. Codina, L. Birnbaumer, and A. M. Brown. 1988. α-Subunit of Gs directly activates cardiac calcium channels in lipid bilayers. American Journal of Physiology. 255:H722-H728.

Kelleher, D. J., L. W. Dudycz, G. E. Wright, and G. L. Johnson. 1986. Ability of guanine nucleotide derivatives to bind and activate bovine transducin. Molecular Pharmacology. 30:663-668.

Levitan, I. B. 1988. Modulation of ion channels in neurons and other cells. Annual Review of Neuroscience. 11:119-136.

Méry, P.-F., V. Brechler, C. Pavoine, F. Pecker, and R. Fischmeister. 1990. Glucagon stimulates the cardiac Ca current by activation of adenylyl cyclase and inhibition of phosphodiesterase. Nature. 345:158-161.

Nakajima, T., S. Wu, H. Irisawa, and W. Giles. 1990. Mechanism of acetylcholine-induced inhibition of Ca current in bullfrog atrial myocytes. Journal of General Physiology. 96:865-885.

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.

Otero, A. S., G. E. Breitwieser, and G. Szabo. 1988. Activation of muscarinic potassium currents by ATPγS in atrial cells. Science. 242:443-445.

Parsons, T. D., and H. C. Hartzell. 1991. Neurotransmitter regulation of Ica in frog ventricular myocytes is inhibited by GTPγS. Biophysical Journal. 59:553a. (Abstr.)

Parsons, T. D., and H. C. Hartzell. 1992. Characterization of persistent Ica in frog ventricular myocytes induced by GTPγS. Biophysical Journal. 61:285a. (Abstr.)

Parsons, T. D., A. Lagrutta, R. E. White, and H. C. Hartzell. 1991. Regulation of Ca current in frog ventricular cardiomyocytes by 5′-guanylylimidodiphosphate and acetylcholine. Journal of Physiology. 432:593-620.

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

Pfeuffer, T., and E. J. Helmreich. 1988. Structural and functional relationships of guanosine triphosphate binding proteins. Current Topics in Cell Regulation. 29:129-216.

Ross, E. M. 1989. Signal sorting and amplification through G protein-coupled receptors. Neuron. 3:141-152.

Seamon, K. B., and J. W. Daly. 1986. Forskolin: its biological and chemical properties. Advances in Cyclic Nucleotide and Protein Phosphorylation Research. 20:1-150.

Shuba, Y. M., B. Heslingter, W. Trautwein, T. F. McDonald, and D. Pelzer. 1990. Whole-cell calcium current in guinea-pig ventricular myocytes dialysed with guanine nucleotides. Journal of Physiology. 424:205-228.
Szabo, G., and A. S. Otero. 1990. G protein mediated regulation of K+ channels in heart. *Annual Review of Physiology.* 52:293–305.

Tang, W.-J., and A. G. Gilman. 1991. Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science.* 254:1500–1503.

Trautwein, W., and J. Hescheler. 1990. Regulation of cardiac L-type calcium current by phosphorylation and G proteins. *Annual Review of Physiology.* 52:257–274.

Weiss, J. N., and S. T. Lamp. 1989. Cardiac ATP-sensitive K+ channels. Evidence for preferential regulation by glycolysis. *Journal of General Physiology.* 94:911–935.

White, R. E., and H. C. Hartzell. 1988. Effects of intracellular free magnesium on calcium current in isolated cardiac myocytes. *Science.* 239:778–780.

Wong, Y. H., A. Federman, A. M. Pace, I. Zachary, T. Evans, J. Pouyssegur, and H. R. Bourne. 1991. Mutant α subunits of Gα inhibit cyclic AMP accumulation. *Nature.* 351:63–65.

Yatani, A., and A. M. Brown. 1989. Rapid β-adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science.* 245:71–74.

Yatani, A., J. Codina, Y. Imoto, J. P. Reeves, L. Birnbaumer, and A. M. Brown. 1987. A G protein directly regulates mammalian cardiac calcium channels. *Science.* 238:1288–1292.

Yatani, A., Y. Imoto, J. Codina, S. L. Hamilton, A. M. Brown, and L. Birnbaumer. 1988. The stimulatory G protein of adenylyl cyclase, Gs, also stimulates dihydropyridine-sensitive Ca channels. *Journal of Biological Chemistry.* 263:9887–9895.