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 (GTPγS), 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 GTPγS resulted in only a partial (~40%) inhibition of Iso- or Forsk-stimulated I_{Ca}. The fraction of the current not inhibited by GTPγS 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 (ATPγS) mimicked the GTPγS-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 GTPγS- and ATPγS-induced, persistent I_{Ca}. We conclude that: (*a*) GTPγS 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-GTPγS 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

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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 $\alpha_{(GTP)}$ and $\beta\gamma$ subunits (Gilman, 1987). The GTP-liganded α subunit then binds AC and stimulates its catalytic activity. Hydrolysis of GTP and reassociation of $\alpha_{(GDP)}$ and $\beta\gamma$ 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_s by $\beta\gamma$ subunits released from G_i (reviewed by Gilman, 1987; Ross, 1989), or whether α_i or $\beta\gamma$ might directly inhibit AC (see discussion in Pfeuffer and Helmreich, 1988). More recent experiments have provided evidence for direct actions of both α_i (Wong, Federman, Pace, Zachary, Evans, Pouyssegur, and Bourne, 1991) and $\beta\gamma$ (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 G_i-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 G_i, 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 $MgCl_2$, 1.8 mM $CaCl_2$, 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 K_2EGTA, 2.8 mM Na_2K_2ATP, 4.04 mM $MgCl_2$, 5 mM Na_2 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'-(R_p)-phosphorothioate, kindly provided by Ira Cohen, SUNY Stony Brook, Stony Brook, NY; and Wiptide (the cAMP-dependent protein kinase inhibitor peptide, $PKI_{(5-22)}$), Peninsula Labs., Inc., Belmont, CA). When Li_4ATPγS was included in the internal perfusion solution, Na_2K_2ATP was omitted. For guanine nucleotide concentrations < 50 μM, the nucleotide was added to standard internal solution without correction for Mg^{2+} chelation. For higher guanine nucleotide concentrations, solutions were constructed to maintain free [Mg^{2+}] constant at 1 mM (White and Hartzell, 1988), assuming the same stability constants for GppNHp and GTPγS as for GTP.

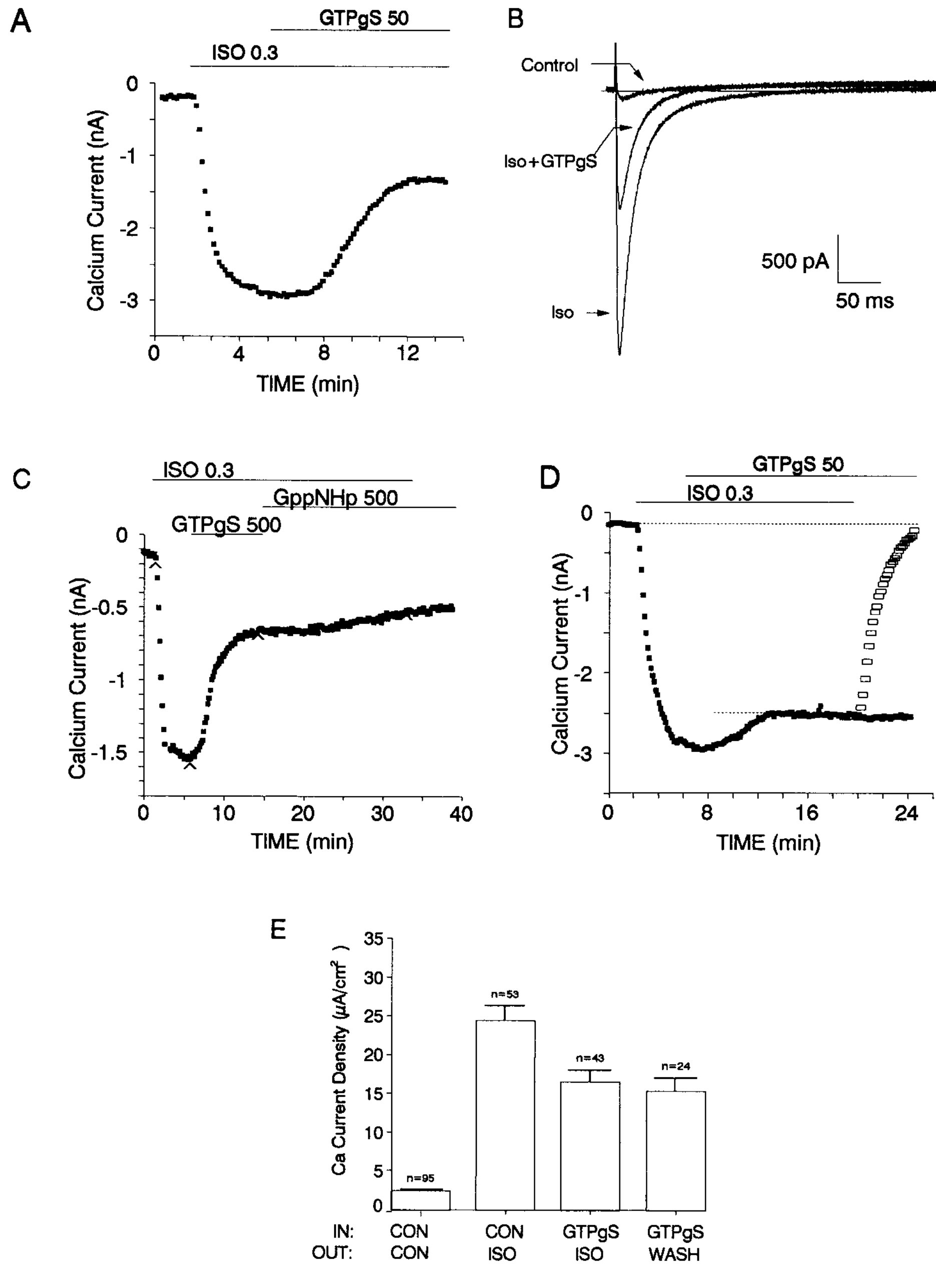

FIGURE 1. Effects of internal perfusion with GTPγS on Iso-stimulated I_{Ca} in frog cardiac myocytes. (*A*) Effect of 50 μM GTPγS on I_{Ca} elevated by 0.3 μM Iso. Each square represents net inward current elicited by a 400-ms voltage pulse from −80 to 0 mV. The cell was first exposed to 0.3 μM Iso for the period indicated by the horizontal line and was perfused with standard internal solution unless indicated otherwise. When I_{Ca} stabilized, internal 50 μM 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, GTPγS was used without repurification. However, commercially available GTPγS was often significantly contaminated with 260-nm absorbing material (presumably nucleotides). For some experiments, GTPγS 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 Hartzell, 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 cm². 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_e, 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 GTPγS

GTPγS-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 GTPγS. 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 GTPγS 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 GTPγS resulted in a

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internal perfusion with GTPγS from the experiment depicted in *A*. Current traces were not leak subtracted. Capacitive transient was blanked for 1.5 ms. (*C*) Effect of 500 μM GTPγS on I_{Ca} elevated by 0.3 μM Iso. The cell was first exposed to 0.3 μM Iso and then internally perfused with 500 μM GTPγS after I_{Ca} stabilized. After the partial inhibition of the Iso-stimulated I_{Ca}, 500 μM GppNHp was added internally. Neither internal GppNHp nor washout of Iso significantly changed the partial inhibition of the Iso-stimulated I_{Ca} by GTPγS. (*D*) Persistent I_{Ca} after GTPγS. Cell was exposed to 0.3 μM Iso and then perfused internally with 50 μM GTPγS. 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 GTPγS, the Iso response washed out completely in the same time period (*open squares*). (*E*) Summary of effects of internal perfusion with GTPγS on Iso-stimulated I_{Ca}. I_{Ca} density (μA/cm²) is plotted for several experimental conditions using 0.1–10 μM Iso and 50 μM GTPγS. 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_{Ca} (Table I *A*, *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_{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_{Ca} in Frog Ventricular Myocytes

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

*16 μM Wiptide or 0.1–1.0 mM (R_p)cAMPS.

‡Cells in which GTPγS had stimulatory effects on either Iso, or Forsk-stimulated I_{Ca}.

§16 μM Wiptide.

‖GTP analogue internally perfused before stimulation with Iso.

inhibition of Iso-stimulated I_{Ca} ($n = 6$) (data not shown). Similar incomplete inhibition was obtained with 500 μM internal GTPγS (Fig. 1 *C*) (Table I *A, 3*).

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

Persistent stimulation of I_{Ca}. After I_{Ca} 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 I_{Ca}," 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 I_{Ca} are summarized in the bar graph in Fig. 1 *E*.

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

Effect of GTPγS on basal I_{Ca}. We previously reported that GppNHp had no effect on basal I_{Ca} (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 I_{Ca} was observed. This runup is probably due to equilibration of the pipette solutions (Cs^+, Mg^{2+}, etc.) with the cell. These results support the suggestion that GTPγS has no effect on I_{Ca} in the absence of the activation of AC.

GTPγS-mediated inhibition of Forsk-stimulated I_{Ca}. 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, I_{Ca} steadily decreased to an intermediate level (Fig. 2 *A*). On average, 3 μM Forsk resulted in a 9.4 ± 0.9–fold stimulation of I_{Ca} ($n = 30$) and 50 μM GTPγS resulted in a 46.6 ± 4.7% decrease ($n = 17$) in 3 μM Forsk-stimulated I_{Ca} (Fig. 2 *A;* Table I *B, 1* and *2*). Because Forsk stimulates the activity of AC in the absence of activation of G_s (Seamon and Daly, 1986), these results suggest that even in the absence of G_s activation, GTPγS can inhibit I_{Ca}.

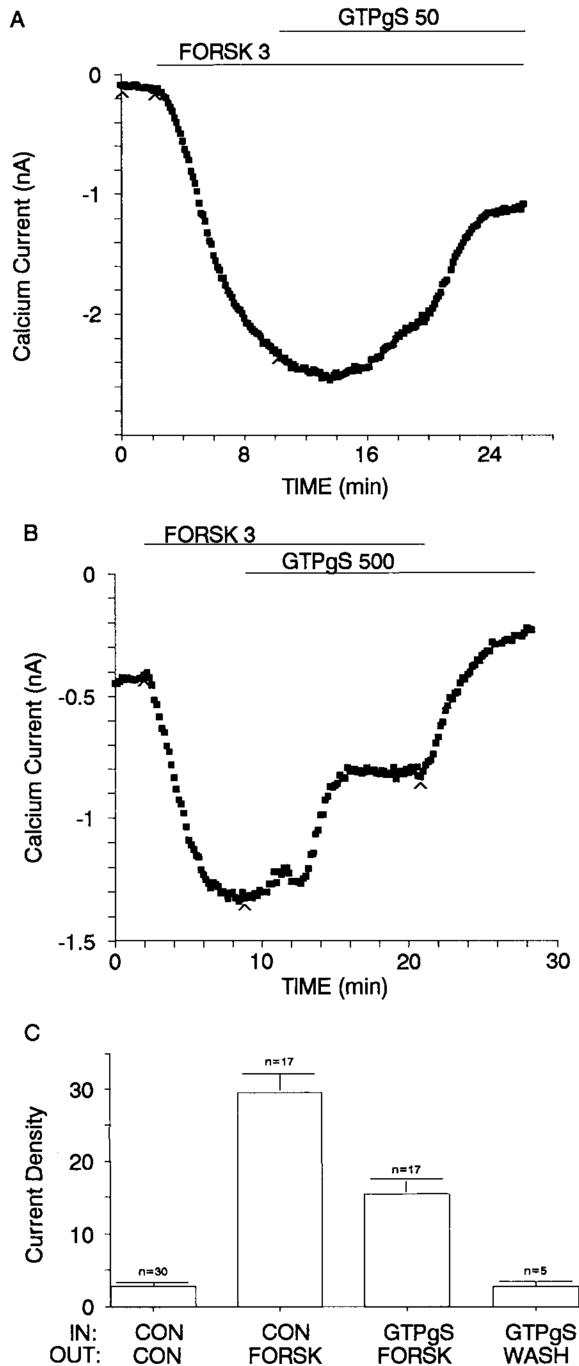


FIGURE 2. Effect of internal GTPγS on Forsk-stimulated I_{Ca}. (*A*) Partial inhibition of 50 μM Forsk-stimulated I_{Ca} by GTPγS. The cell was exposed to 3 μM Forsk and internally perfused with 50 μM GTPγS. I_{Ca} steadily decreased, but reached the steady-state level, which was elevated above basal I_{Ca}. (*B*) Partial inhibition of Forsk-stimulated I_{Ca} by 500 μM GTPγS. The cell was exposed to 3 μM Forsk and internally perfused with 500 μM GTPγS. I_{Ca} steadily decreased, but reached the steady-state level, which was elevated above basal I_{Ca}. Subsequent washout of Forsk resulted in a rapid decline in the I_{Ca}. (*C*) Summary of effects of internal perfusion with GTPγS on Forsk-stimulated I_{Ca}. I_{Ca} density (μA/cm^2) is plotted for several experimental conditions using 3 μM Forsk and 50 μM GTPγS. Each bar represents the mean I_{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_{Ca} 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_{Ca} (Table I *B, 3*). However, upon washout of Forsk the elevated I_{Ca} rapidly returned to basal levels. On average, 90.8 ± 2.2% of the initial Forsk-stimulated I_{Ca} 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_{Ca} 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_{Ca} 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_{Ca} 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_{Ca}. The average percentage increase in the Forsk-stimulated I_{Ca} was 78.8 ± 37.2% ($n = 4$). As we observed with the atypical responses of Iso-stimulated I_{Ca} to GTPγS (Table I, *A, 4*), the mean amplitude of the Forsk-stimulated I_{Ca} 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_{Ca} that had been incompletely inhibited by GTPγS. In Fig. 3 *A*, 3 μM Forsk stimulated I_{Ca} ~15-fold. I_{Ca} 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_{Ca}. After ~15 min of internal GTPγS, a second 3-min exposure to 20 μM ACh had only very small inhibitory effects on I_{Ca}. On average, ACh had no effect on Forsk-stimulated I_{Ca} 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_{Ca} that was partially inhibited by GTPγS in Fig. 2, the ACh-resistant current was not persistently activated either. I_{Ca} rapidly returned to control levels after the removal of Forsk. On average, 90.2 ± 5.2% ($n = 5$) of the initial Forsk-stimulated I_{Ca} 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_{Ca} does not remain elevated in the absence of Forsk.

Effects of GTPγS on cAMP-stimulated I_{Ca}. We examined the possibility that GTPγS might be inhibiting I_{Ca} 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_{Ca}. However, in none of the cells studied did GTPγS result in inhibition of cAMP-stimulated I_{Ca} ($n = 7$). We observed either no change or a small stimulation of cAMP-elevated I_{Ca} after internal GTPγS. These results do not support the hypothesis that GTPγS-mediated inhibition of Iso or Forsk-stimulated I_{Ca} 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_{Ca} 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

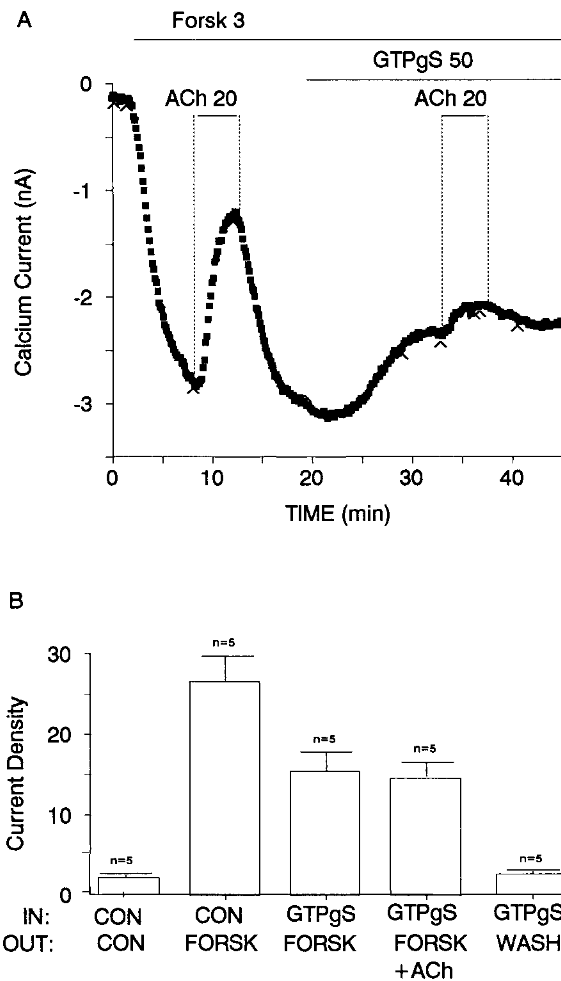


FIGURE 3. Effect of ACh on GTPγS-mediated partial inhibition of Forsk-stimulated I_{Ca}. (*A*) ACh does not augment GTPγS-mediated partial inhibition of Forsk-stimulated I_{Ca}. The cell was exposed to 3 μM Forsk and then to 3 μM Forsk plus 20 μM ACh. I_{Ca} increased in the presence of Forsk and was then inhibited by the addition of ACh. After washout of the ACh, the Forsk-stimulated I_{Ca} recovered and the cell was internally perfused with 50 μM GTPγS. After stabilization of the partial inhibition, a second brief application of ACh resulted in only a small amount of inhibition. (*B*) Summary of effects of ACh on GTPγS-mediated inhibition of Forsk-stimulated I_{Ca}. I_{Ca} density (μA/cm^2) is plotted for several experimental conditions using 3 μM Forsk, 10–20 μM ACh, and 50 μM GTPγS. Each bar represents the mean I_{Ca} density value for the number of cells tested under each condition. Error bars are SEM.

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 GTPγS with excess GTP (Fig. 4 *A*). In Fig. 4 *A*, I_{Ca} was stimulated to ~3.3 nA by 0.1 μM Iso. Internal perfusion with 50 μM GTPγS reduced I_{Ca} to ~2 nA. After the washout of Iso, I_{Ca} remained elevated for >10 min. Subsequent perfusion with 500

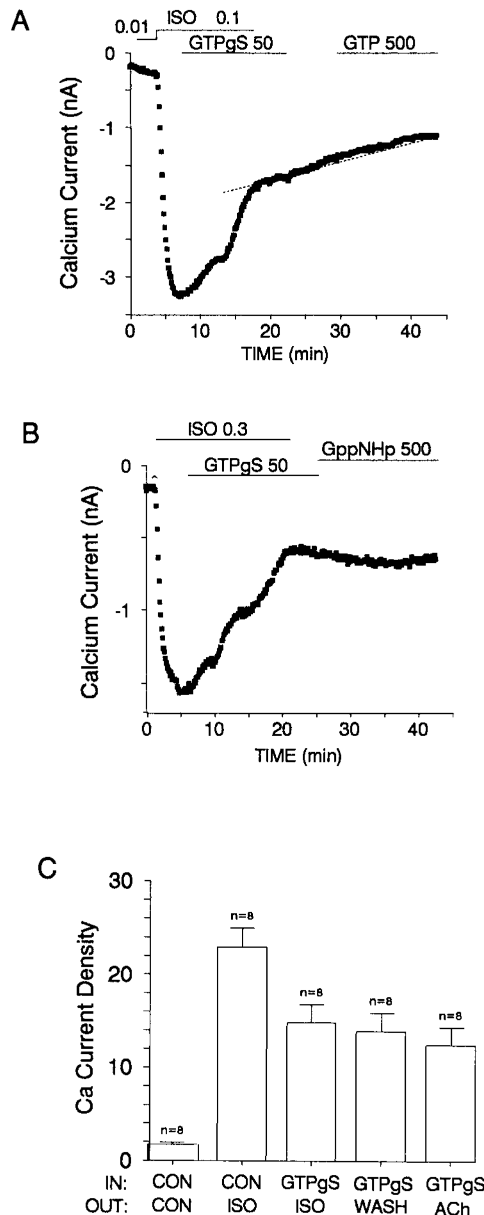


FIGURE 4. Irreversible activation of persistent I_{Ca} by GTPγS. (*A*) Effects of GTP on persistent I_{Ca} induced by GTPγS. The cell was sequentially exposed to 0.01 and 0.1 Iso, resulting in an increase in I_{Ca}. Internal perfusion with 50 μM GTPγS partially inhibited I_{Ca} and subsequent washout of Iso revealed persistent I_{Ca}. Internal perfusion with 500 μM GTP did not reverse the persistent I_{Ca}. The dotted line denotes a small time-dependent rundown of I_{Ca}. (*B*) Effects of GppNHp on persistent I_{Ca} induced by GTPγS. The cell was stimulated with 0.3 μM Iso and internally perfused with 50 μM GTPγS. Washout of Iso resulted in a persistent I_{Ca} which was unchanged by prolonged internal perfusion with 500 μM GppNHp. (*C*) Effects of ACh on persistent I_{Ca} induced by GTPγS. I_{Ca} density (μA/cm^2) is plotted for several experimental conditions using 0.1–10 μM Iso, 50 μM GTPγS, and 10–20 μM ACh. Each bar represents the mean I_{Ca} 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. 4 *B* 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_s.

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. 4 *C*.

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

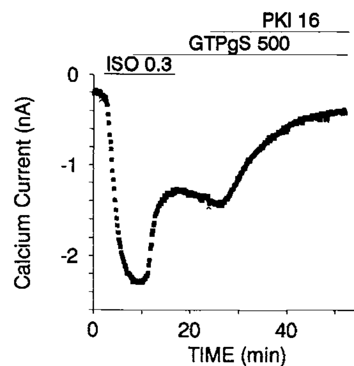


FIGURE 5. Effects of peptide inhibitors of protein kinase A on persistent I_{Ca} induced by GTPγS. After the stimulation of I_{Ca} by 0.3 μM, the I_{Ca} was partially inhibited by internal perfusion with 500 μM GTPγS. The remaining I_{Ca} was persistent after the removal of Iso, but the elevated I_{Ca}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

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} (Fig. 5). The cell in Fig. 5 was stimulated with 0.3 μM Iso and internally perfused with 500 μM GTPγS, and then Iso was washed out. Subsequent internal perfusion with 16 μM Wiptide inhibited the persistent I_{Ca}. Similar results were obtained with another protein kinase A inhibitor, (R_p)cAMPS, which also blocked persistent I_{Ca} (Table I *A, 2*). Similar results were obtained with 0.1 or 10 μM Iso (n = 4). These experiments support the hypothesis that persistent I_{Ca} results from the formation of a long-lived α_s(GTPγS) that continues to activate AC after the removal of Iso.

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

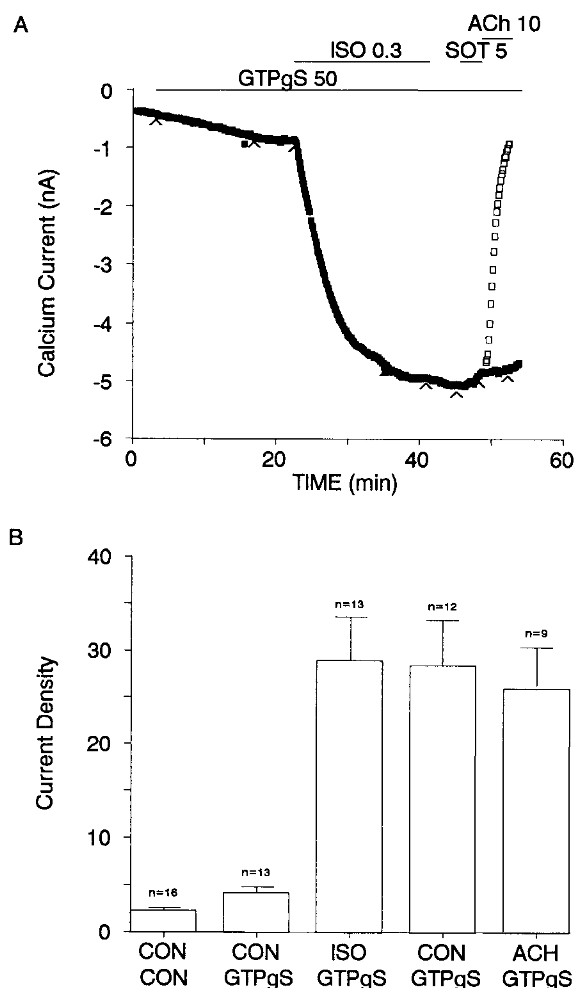


FIGURE 6. Effects of prior internal perfusion with GTPγS on I_{Ca}. (*A*) Persistent I_{Ca} after internal perfusion of GTPγS before stimulation with Iso that is resistant to β-antagonists and muscarinic agonists. The cell was internally perfused with 50 μM GTPγS and basal I_{Ca} increased slightly. 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 μM 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 ± 1.2–fold (n = 6) greater than the unstimulated I_{Ca}. Washout of Iso reduced I_{Ca} only 4.3 ± 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^+, Mg^{2+}, 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

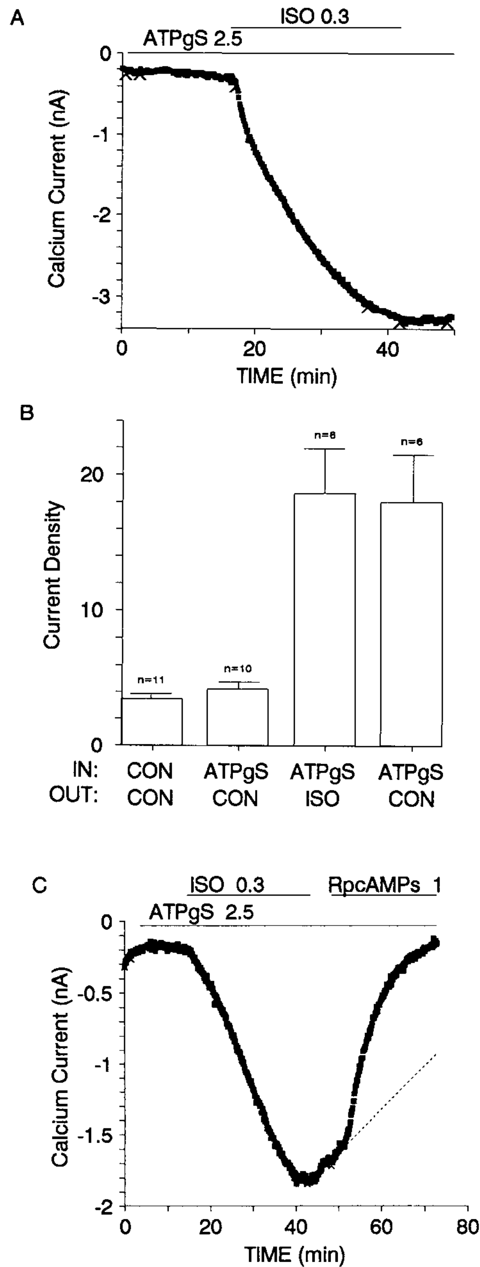
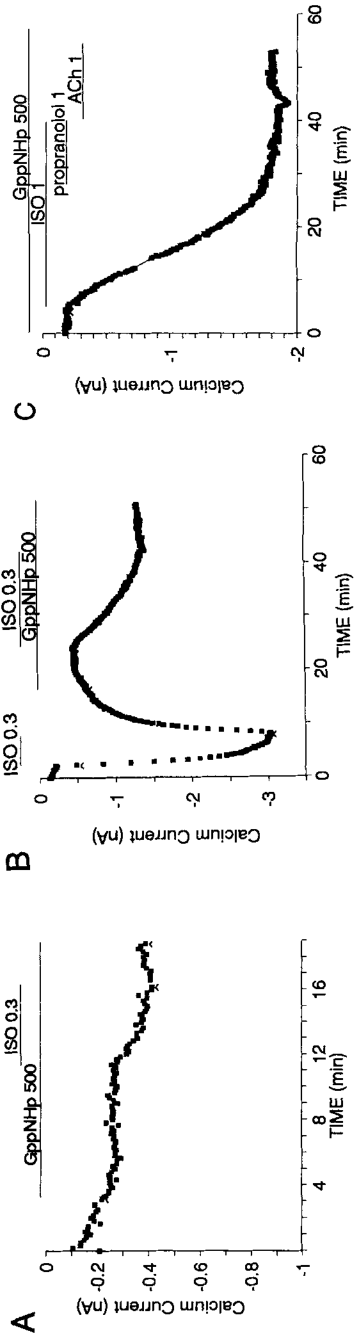
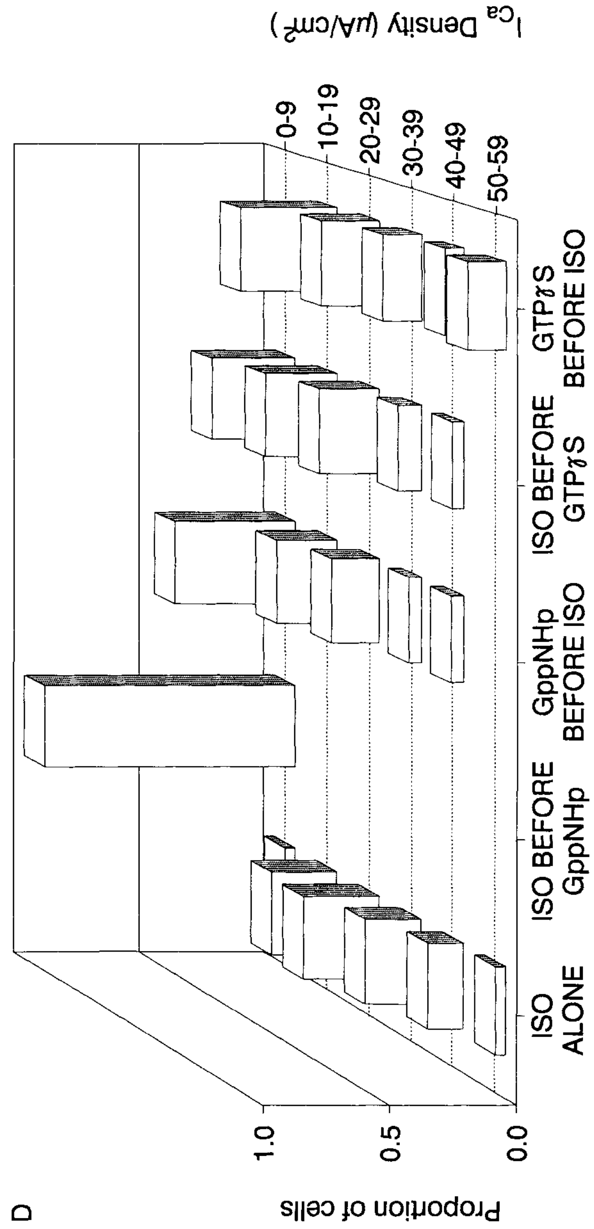


FIGURE 7. Effects of ATPγS on Iso-stimulated I_{Ca}. (*A*) Persistent I_{Ca} after internal ATPγS and Iso. The cell was internally perfused with 2.5 mM ATPγS and basal I_{Ca} was not significantly changed. Exposure to 0.3 μM Iso elevated I_{Ca}. The I_{Ca} remained persistently increased after washout of Iso. (*B*) Summary of the effects of ATPγS on Iso-stimulated I_{Ca}. I_{Ca} density is plotted for several experimental conditions using 0.3 μM Iso and 2.5 mM ATPγS. (*C*) Effect of (R_p)cAMPS on persistent I_{Ca} induced by ATPγS. After internal perfusion with 2.5 mM ATPγS, I_{Ca} was stimulated by 0.3 μM. Removal of Iso revealed a persistent I_{Ca} that was slowly running down (see dotted line). (R_p)cAMPS (1 mM) markedly accelerated the decrease in I_{Ca} and returned I_{Ca} to basal levels.

the same conditions. Persistent I_{Ca} was subsequently inhibited by internal perfusion with 1 mM (R_p)cAMPS (n = 6, Table I *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 μM) was present for a long period of time before Iso


A
GppNHp 500
ISO 0.3
Calcium Current (nA)
TIME (min)
B
ISO 0.3
ISO 0.3
GppNHp 500
Calcium Current (nA)
TIME (min)
C
GppNHp 500
ISO 1
propranolol 1
ACh 1
Calcium Current (nA)
TIME (min)


D
Proportion of cells
ISO ALONE
ISO BEFORE GppNHp
GppNHp BEFORE ISO
ISO BEFORE GTPγS
GTPγS BEFORE ISO
I_Ca Density (μA/cm²)
0-9
10-19
20-29
30-39
40-49
50-59

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 I *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

FIGURE 8. (*opposite*). Effects of prior internal perfusion with GppNHp on Iso stimulation of I_{Ca}. (*A*) Internal perfusion with GppNHp before Iso exposure blocks Iso response. After internal perfusion with 500 μM GppNHp, Iso (0.3 μM) produced a small increase in I_{Ca}. The increase in I_{Ca} was not reversed by washout of Iso. (*B*) Internal perfusion with GppNHp before Iso exposure reduces Iso response. The cell was exposed to 0.3 μM Iso, which resulted in an increase in I_{Ca} which was reversed upon washout of Iso. The cell was then internally perfused with 500 μM GppNHp and exposed again to 0.3 μM Iso. The second response to Iso was only 50% as large as the first. However, I_{Ca} remained elevated after the washout of Iso. (*C*) Large persistent I_{Ca} after internal perfusion of GTPγS before stimulation with Iso. After internal perfusion with 500 μM GppNHp, Iso (1 μM) produced a large increase in I_{Ca}. This I_{Ca} was not blocked by 1 μM propranolol or 1 μM ACh. (*D*) Stimulation of I_{Ca} by Iso in the presence and absence of GppNHp. Histograms of I_{Ca} density are plotted for five experimental conditions: stimulation with 0.3 μM Iso in the presence of control intracellular solutions (*Iso alone*); internal perfusion with 50 μM GppNHp during stimulation with 0.3 μM Iso (*Iso before GppNHp*); stimulation with 0.3 μM Iso after internal perfusion of 500 μM GppNHp (*GppNHp before Iso*); internal perfusion with 50 μM GTPγS during stimulation with 0.3 μM Iso (*Iso before GTPγS*); and stimulation with 0.3 μM Iso after internal perfusion with 50 μM GTPγS (*GTPγS before Iso*).

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 α 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-resistent Analogues of GTP Activate G_i

The similarity of the inhibitory effects of ACh, internal GppNHp, and GTPγS 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 GTPγS has effects on basal I_{Ca}. Furthermore, ACh (Fischmeister and Shrier, 1989), GppNHp (Parsons et al., 1991), and GTPγS 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 GTPγS are mediated via the irreversible activation of G_i.

The ability of internal GTPγS 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 μM Iso when the cell was previously perfused with 500 μM GTPγS or when GTPγS was perfused after Iso. Fischmeister and Shrier (1989) reported that exposure of cells to Iso in the presence of GTPγS 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 GTPγS (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 GTPγS 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 GTPγS resulted in no additional inhibition. This incomplete inhibition by GTPγS 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 GTPγS-liganded G_i to inhibit AC.

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

We propose that the incomplete inhibition of Iso- and Forsk-stimulated I_{Ca} by GTPγS involves a reduced ability of $\alpha_{i(GTP\gamma S)}$ to inhibit AC, as compared with $\alpha_{i(GTP)}$ or $\alpha_{i(GppNHp)}$. $\alpha_{i(GTP\gamma S)}$ 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 GTPγS has a higher affinity than other GTP analogues for G_s (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 α_i(GTPγS) to inhibit I_{Ca} could theoretically be due to a greater activation of G_s by GTPγS than by GppNHp and competition between the effects of G_i and G_s on AC. If the potency or efficacy of GTPγS 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 GTPγS 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 GTPγS occurs because GTPγS 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 GTPγS) verifies the assumption that $\alpha_{s(GTP\gamma S)}$ had not formed. Similarly, no difference in the ability of ACh to inhibit either Iso- or Forsk-stimulated I_{Ca} after internal GTPγS was observed. This line of reasoning suggests that different ratios of G_s/G_i activated by GTPγS 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 GTPγS-mediated uncoupling of the muscarinic ACh receptor from inhibition-stimulated I_{Ca} is unlikely.

In conclusion, internal perfusion of frog cardiomyocytes with GTPγS or GppNHp results primarily in the irreversible activation of G_i via basal nucleotide exchange.

However, the ability of $\alpha_{i(GTP\gamma S)}$ 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 GTPγS remained persistently stimulated after the washout of β-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 $\alpha_{i(GTP\gamma S)}$ and that the degree of inhibition depends on the nucleotide bound to the G protein. Below, we will argue that GTPγS induces a persistent I_{Ca}, whereas GppNHp rarely does, because GTPγS 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 α_i subunits, persistence is due to the ability of GTPγS 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 GTPγS only incompletely inhibits Forsk-stimulated I_{Ca}, but after washout of Forsk, I_{Ca} is not persistent. Conversely, exposure to Iso after internal GTPγS 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 α_i (as discussed above). Obviously, however, persistence would not be observed unless GTPγS 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 GTPγS. 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 GTPγS.

The observation that the persistent I_{Ca} is larger after internal perfusion with GTPγS than with GppNHp suggests that the activation of G_s by GTPγS must be somehow different from the activation by GppNHp. The additional observation that ATPγS mimics GTPγS in the development of the persistent I_{Ca} leads us to favor the idea that GTPγS acts via an additional pathway to activate G_s. GTPγS and ATPγS 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). (R_p)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

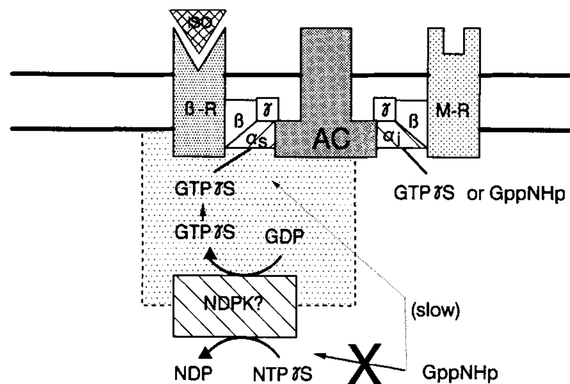


FIGURE 9. Model of activation by GTP analogues of G_s in intact cardiomyocytes. *NDP*, nucleoside diphosphate; *NDPK*, nucleoside diphosphate kinase; *ISO*, isoproterenol; *β-R*, β-adrenergic receptor; *M-R*, muscarinic receptor; *AC*, adenylyl cyclase. NDPK in the presence of agonist provides a pathway for the irreversible activation of α_s that is available to ATPγS and GTPγS, but not GppNHp. Conversely, GTPγS or GppNHp, but not ATPγS, can irreversibly activate α_i, 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 α_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.

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_s 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

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; Heidbüchel et al., 1990; 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 ATPγS, but not AMP-PNP, an ATP analogue with an imido-linked terminal phosphate. Heidbüchel and colleagues have proposed that under physiological conditions NDPK acts a local intracellular buffer of GTP (Heidbüchel 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 GTPγS 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 GTPγS to induce a large persistent I_{Ca} is that $\alpha_{s(GTP\gamma S)}$ 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 ~100-fold increase in the EC_{50} for Forsk. We interpret the rescue of Forsk-stimulated I_{Ca} by Iso to be due to Iso stimulating the loading of G_s with GppNHp. This activation of G_s 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 GTPγS 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 GTPγS were mediated by a PDE, the persistent I_{Ca} would be expected after any transient increase in intracellular cAMP in the presence of GTPγS. However, a persistent I_{Ca} has not been observed after internal GTPγS and subsequent washout of either Forsk or cAMP (Parsons, T.D., R.E. White and H.C. Hartzell, unpublished observations).

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