Complex Regulation of Calcium Current in Cardiac Cells
Dependence on a Pertussis Toxin–sensitive Substrate, Adenosine Triphosphate, and an α_1-Adrenoceptor

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Abstract

We investigated regulation of the cardiac L-type calcium channel by intracellular ATP and by α_1-adrenergic agonism using single adult guinea pig ventricular cells and the whole-cell patch clamp method. Inclusion of 5 mM ATP in the patch clamp pipette prevented calcium current rundown but did not increase the maximal magnitude of the slow inward calcium current (I_{Ca}). During β_1-adrenergic blockade with 10 μM (−)-propranolol, cells preincubated with 1 μg/ml pertussis toxin for 2–5 h exhibited a rapid twofold increase in I_{Ca} after rupture of the membrane patch when 5 mM ATP was present in the patch clamp pipette. In the absence of ATP, the increase in I_{Ca} did not occur. In pertussis toxin–treated cells, 100 μM (−)-phenylephrine inhibited the augmentation of I_{Ca}. This inhibitory effect was blocked by 100 nM terazosin, a selective α_1-antagonist. The inhibitory effect of α_1-adrenergic agonism was not mediated by cAMP-dependent phosphodiesterase since incubation with 100 μM (−)-phenylephrine did not augment the activity of this enzyme. We conclude that regulation of the L-type calcium channel in cardiac cells is complex, and is dependent on a pertussis toxin–sensitive substrate, ATP, and an α_1-adrenergic receptor. The marked increase in I_{Ca} after pertussis toxin treatment in the presence of ATP indicates significant inhibition of I_{Ca} by a pertussis toxin substrate, presumably the guanine nucleotide inhibitory protein (G_i) in the basal state. The inhibitory action of (−)-phenylephrine in pertussis toxin–treated cells is consistent with modulation of I_{Ca} by an α_1-adrenergic receptor not coupled to G_i. (*J. Clin. Invest.* 1990. 85:950–954.) adenosine triphosphate • α_1-adrenoceptor • calcium current • pertussis toxin

Introduction

In cardiac cells the slow inward calcium current (I_{Ca})[1] or L-type current is involved in action potential generation, activation of the pacemaker current in nodal tissue, and the initiation of contraction (1). There is abundant evidence that this current is modulated by norepinephrine and acetylcholine released at nerve endings (2, 3). In dorsal root tissue, norepinephrine inhibits the calcium-dependent action potential by means of a reduction of I_{Ca} by the neurotransmitter. There is indirect evidence that this antagonist effect occurs via an α-adrenergic receptor coupled to a G protein, probably the guanine nucleotide inhibitory protein (G_i), resulting in activation of protein kinase C through the phosphatidylinositol pathway (4–6). Although the antagonist effect of α-adrenergic stimulation is well established in neuronal cells, the influence of α_1-adrenergic agonism on the cardiac I_{Ca} is controversial; both an increase (7) or no effect have been reported (8–10). Recent observations in our laboratory indicate that in neonatal rat ventricular myocytes, the α_1-adrenergic receptor acting via a pertussis toxin–sensitive substrate, presumably G_i, is linked to inhibition of cAMP metabolism (11). Based on these preliminary data, we sought to determine whether α_1-adrenergic agonism had a similar effect on the cardiac I_{Ca} and if so, whether a pertussis toxin–sensitive G protein is a component of this signal transduction mechanism. We here report that (*a*) pretreatment of adult guinea pig heart cells with pertussis toxin results in marked augmentation of I_{Ca}; (*b*) this increase occurs only in the presence of exogenous ATP; and (*c*) stimulation of the α_1-adrenoceptor prevents the increase in I_{Ca} produced by pertussis toxin.

Methods

Isolation of single ventricular cells. Single ventricular cells were freshly isolated from male guinea pigs weighing 250–350 g by a modification of the procedure used by Silver et al. (12). Isolated cells were incubated in minimal essential medium at 37°C until voltage clamp experiments were begun; the experiments were then carried out at an ambient temperature of 22–25°C.

Voltage clamp experiments. Voltage clamp experiments were performed in the whole-cell configuration with the single-pipette patch clamp method (13) using an Axopatch-1B patch clamp system (Axon Instruments, Inc., Burlingame, CA). Pipettes were fabricated from 1.8-mm o.d. glass tubing (Microstar, Radnoti Glass, Monrovia, CA) using a microprocessor-based patch pipette puller (PC-84, Sutter Instruments, San Rafael, CA). The pipettes had an internal tip diameter of 2–2.5 μm after slight fire-polishing and a resistance of 1.5–2.0 MΩ after back-filling with the experimental internal solution. 1–4-GΩ seals between the pipette tip and the cell membrane were achieved in an external solution containing (in millimolar): NaCl 137, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, Hepes 10, and glucose 10 at pH = 7.4 (titrated with HCl). The internal pipette solution contained (in millimolar): KCl 140, $MgCl_2$ 2, Hepes 10, $CaCl_2$ 1, EGTA 11, and K_2ATP 5 at pH = 7.2 (titrated with KOH). For the ATP-free internal solution, KCl was

1. *Abbreviations used in this paper:* G_i and G_s, guanine nucleotide inhibitory or stimulating protein; I_{Ca}, slow inward calcium current.

increased to 150 mM. The fast inward sodium current was inactivated by holding the membrane potential at −40 mV. Maximum peak calcium current was measured from zero current level. Voltage clamp steps were applied at 0.5 Hz. After series resistance compensation the residual series resistance was reduced to ∼ 1 MΩ (average reduction of 74%). With a mean maximal calcium current of 3,000 pA, the maximal voltage drop across the residual uncompensated series resistance error was small (∼ 3 mV).

Pertussis toxin–catalyzed ADP-ribosylation. ADP-ribosylation was performed by a modification of the method described by Morris and Bilezikian (14). Dithiothreitol-activated pertussis toxin (2.5 μg) was incubated with membrane protein (200 μg per sample) for 2 h at 37°C in a 100-μl reaction mixture containing $MgCl_2$, 2.5 mM; thymidine, 20 mM; ATP, 1.0 mM; EDTA, 1 mM; K_3PO_4, 250 mM; and Tris-HCl, 75 mM at pH 7.4; GTP, 100 μM; [^{32}P]NAD, 5 μCi; NADP, 5 mM; and NAD, 100 μM. The reaction was terminated by the addition of 10 mM NAD in 50 mM Tris-HCl and 4 mM EDTA at pH 7.4 and centrifuged at 7,000 g for 5 min. The pellet was then dissolved and incubated for 5 min at 95°C in a 20-μl solution containing Tris-HCl, 20 mM; EDTA, 1 mM; dithiothreitol, 1 mM; and 1% cholate at pH 7.4. A 30-μl solution containing 10 mM N-ethylmalemide was then added and the resultant mixture was maintained at room temperature for 15 min. SDS-PAGE was carried out using the method of Laemmli (15).

Inactivation of G_i. Inactivation of G_i was accomplished by ADP-ribosylation with pertussis toxin. Freshly isolated guinea pig ventricular cells were incubated with 1 μg/ml of pertussis toxin and minimal essential medium for 2–5 h at 37°C. Before any voltage clamp experiments were performed the pertussis toxin–treated cells were superfused with external solution containing 10 μM (−)-propranolol for at least 10 min.

Measurement of phosphodiesterase activity. Cyclic AMP phosphodiesterase activity was measured as previously described (16, 17). Single ventricular myocytes were enzymatically isolated by the method described above from eight guinea pigs. The average yield of clear rod-shaped cells was 46±14% (mean±SD). Cells were incubated in minimal essential medium and 10 μM (−)-propranolol for 2 h at 37°C with (a) 100 μM (−)-phenylephrine. (b) 1 μg/ml of pertussis toxin, and (c) 100 μM (−)-phenylephrine and 1 μg/ml of pertussis toxin before phosphodiesterase activity was determined. Each determination was performed in triplicate.

Results

ATP and cardiac calcium current. Initial experiments were directed toward evaluating the stability of the preparation in the presence and the absence of ATP since it is well known that inclusion of millimolar amounts of ATP in the suction pipette prevents I_{Ca} rundown during single-cell voltage-clamp experiments (18). The ability of ATP to prevent I_{Ca} rundown is illustrated in Fig. 1, a and b. When 5 mM ATP was present in the pipette, maximum I_{Ca} amplitude in response to a depolarizing step to 0 mV remained stable for at least 38 min after rupture of the membrane patch. This figure also illustrates a more important feature for the present study, i.e., there was no increase in the magnitude of I_{Ca} even when exogenous ATP was readily available. This lack of effect of ATP on I_{Ca} is illustrated in Fig. 1 c, which demonstrates that in the basal state the maximum I_{Ca} recorded in the presence and the absence of 5 mM ATP was not significantly different. In 31 cells, the mean maximum I_{Ca} recorded in the absence of ATP in the patch pipette was 921±258 pA (mean±SD), whereas mean maximum I_{Ca} recorded from 25 cells in the presence of 5 mM ATP in the patch pipette was 961±231 pA.

Tonic inhibition of calcium current. In cardiac myocytes direct application of cAMP stimulates I_{Ca} (19–21). Since adenylyl cyclase activity is under the dual control of the gua-
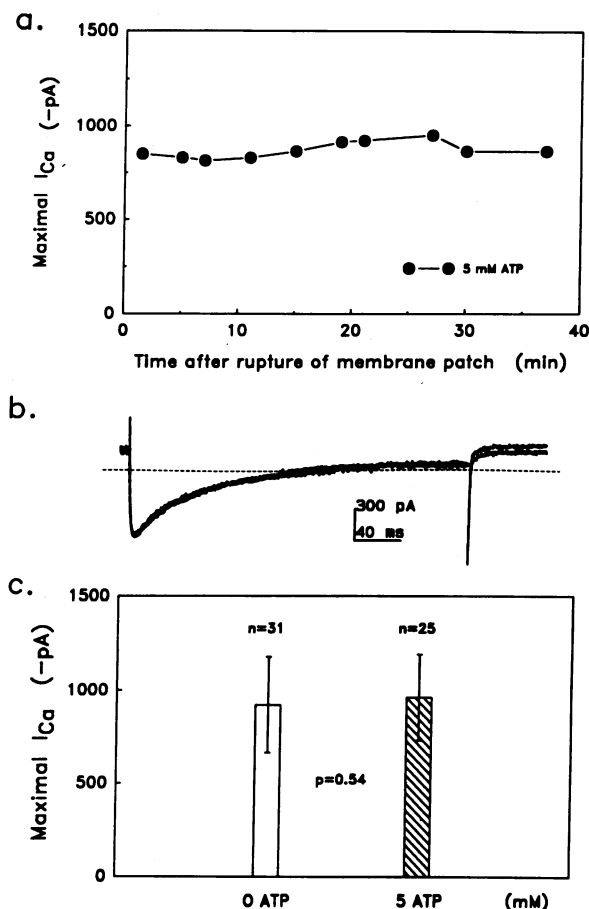
Figure 1. Effect of ATP on the cardiac calcium channel. (a) Inclusion of 5 mM ATP in the pipette prevented calcium current rundown. The maximal peak calcium current evoked by a step depolarization from −40 to 0 mV remained stable for at least 38 min. Cell CTRL01. (b) Superimposed current recordings in response to a clamp step from a holding potential of −50 mV to a membrane voltage of −10 mV obtained 5 and 37 min after achieving the whole-cell clamp configuration. The interrupted line represents zero current level. (c) Effect of ATP on the magnitude of the cardiac calcium channel. Inclusion of 5 mM ATP in the pipette solution did not augment the magnitude of calcium current. The basal maximal peak calcium current was similar with or without ATP in the pipette solution. Error bars represent ±SD.

nine nucleotide stimulatory protein (G_s) and one or more inhibitory G proteins (termed for simplicity G_i), one possible explanation for the lack of effect of ATP on the I_{Ca} is "tonic" inhibition of adenylyl cyclase activity in the basal state by one or more forms of G_i. In the presence of such tonic inhibition, cAMP formation would not be increased despite adequate levels of ATP in the pipette, thereby preventing augmentation of I_{Ca}. To test this hypothesis we examined the effects of pertussis toxin on I_{Ca}. Pretreatment with pertussis toxin inhibited the subsequent ability of the toxin to ADP-ribosylate a 40-kD band on SDS-PAGE (Fig. 2). In the presence of 5 mM ATP in the pipette, cells pretreated with pertussis toxin demonstrated a large surge of I_{Ca} which reached a peak of 236% above control ∼ 5 min after membrane patch rupture (Fig. 3, a and b). The current then gradually declined over the next 35 min. This is in sharp contrast to the control cell shown in Fig. 1, a and b, in which ATP had no appreciable effect on the current magnitude over time. In five cells pretreated with pertussis toxin the
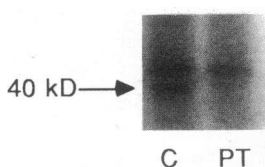
Figure 2. Autoradiogram of an SDS-PAGE experiment demonstrating ADP-ribosylation of a 40-kD band (*arrow*) by pertussis toxin and inhibition of ADP-ribosylation by pertussis toxin pretreatment. (*Left lane*) A representative preparation of guinea pig single ventricular myocytes incubated for 2 h with pertussis toxin in the presence of [^{32}P]NAD. *C*, control. (*Right lane*) Identical membrane preparation obtained from intact cells which had been pretreated with 1 μg/ml pertussis toxin (*PT*) for 5 h. This experiment was repeated three additional times with similar results.

mean maximum I_{Ca} was 1,520±906 pA at 0.5±0.1 min after rupture of the membrane patch to achieve the whole-cell voltage clamp configuration (Fig. 3 *c*). It increased to 3,028±1,829 pA at 7±1 min. After 23±2 min the current amplitude decreased to 1,644±729 pA. The augmentation of I_{Ca} in pertussis toxin–treated cells occurred only if exogenous ATP was available in the patch pipette. In the absence of exogenous ATP there was no enhancement or variation in I_{Ca} amplitude with time (Fig. 3 *a*).

Effect of α_1-agonism on the augmented calcium current. We asked whether an α_1-adrenergic agonist could modulate I_{Ca} in our system and whether this effect depends on the presence of G_i and ATP. As shown in Fig. 4, with 5 mM ATP in the pipette, 100 μM (−)-phenylephrine in the presence of 10 μM (−)-propranolol totally inhibited the rise in I_{Ca} produced by pertussis toxin. This effect was completely reversed by 100 nM terazosin, a selective α_1-adrenergic antagonist (Fig. 4). To determine whether the inhibition of I_{Ca} was the result of α_1-adrenergic augmentation of ATP hydrolysis (22), we measured cAMP-dependent phosphodiesterase activity after incubation of cardiac myocytes with 100 μM (−)-phenylephrine and 10 μM (−)-propranolol under conditions similar to those of the whole-cell voltage clamp experiments. The cAMP-dependent phosphodiesterase activities for control, (−)-phenylephrine, pertussis toxin, and (−)-phenylephrine plus pertussis treatment were 24.9±6.7, 25.5±7.4, 32.6±10.5, and 28.5±10.0 p mol/min per mg protein, respectively (mean±SD, $n = 8$). When compared to control, incubation with 100 μM (−)-phenylephrine did not increase cAMP-dependent phosphodiesterase activity. In addition, none of the other two treatments significantly altered the enzyme activity (one-way analysis of variance with repeated measures; $F = 2.41$, $P > 0.05$, $n = 8$).

Discussion

Occupation of β-adrenergic receptors by norepinephrine results in coupling of these receptors to G_s, leading to activation of adenylyl cyclase and subsequently of cAMP-dependent protein kinase which is involved in calcium channel phosphorylation; cholinergic agonism inhibits this response (2, 3, 23). Calcium channel phosphorylation enhances I_{Ca} either by an increase in the number of functionally available channels (2, 24) or by an increase in the probability that an individual channel will open at any given voltage (25). A family of GTP-sensitive proteins modulates the interaction between stimulatory and inhibitory cell membrane receptors and a variety of second messengers, including cAMP, inositol trisphosphate, protein kinase C, and diacylgylcerol (26, 27). The influence of G proteins on the calcium current is complex and depends on the cell studied (for a review, see Rosenthal et al. [27]). Direct
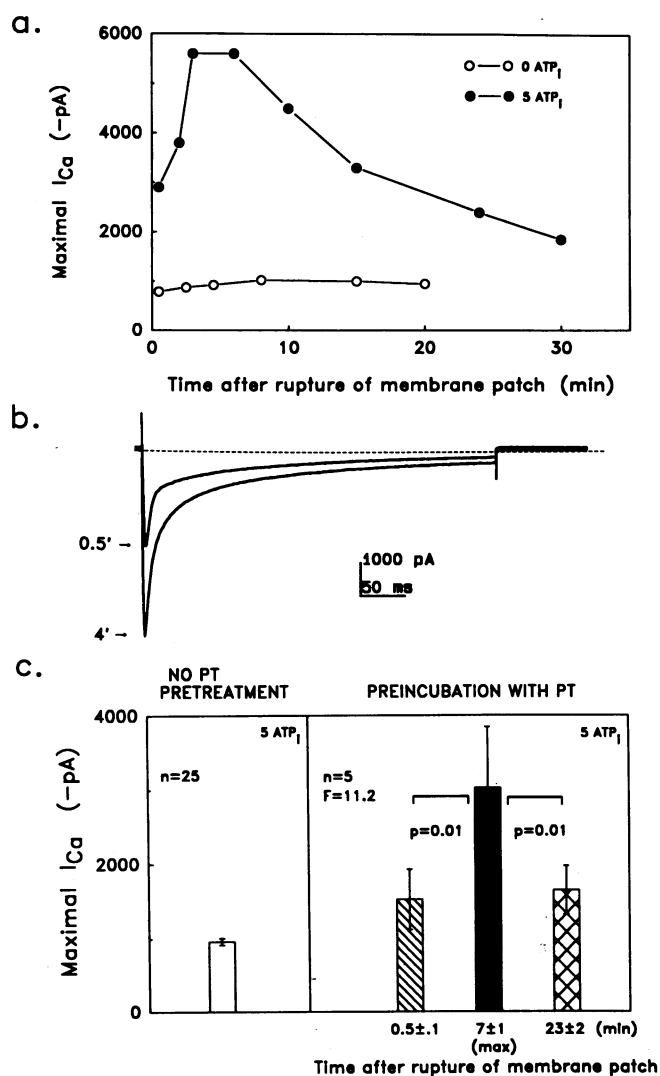
Figure 3. Effect of ATP on I_{Ca} in pertussis toxin–treated cells. (*a*) In cells preincubated with pertussis toxin, the presence of 5 mM ATP in the pipette resulted in a large surge of I_{Ca} which reached a peak at about 5 min after rupture of the membrane patch to achieve the whole-cell voltage clamp configuration (●). Cell PT01. The current then gradually declined over the next 35 min. After preincubation with pertussis toxin, without ATP in the pipette, the magnitude of the maximal peak current remained constant and unaugmented (○). Cell PT02. (*b*) Current recordings obtained from a pertussis toxin–treated cell in response to a depolarization from −40 to 0 mV at 0.5 and 4 min after rupture of membrane patch. The interrupted line represents zero current level. Cell PT01. (*c*) The mean maximal peak I_{Ca} magnitude of 5 cells at 7 min was significantly larger than that at 0.5 and 23 min after establishing the whole-cell clamp configuration. At 23 min I_{Ca} returned to the initial level in most cells. Statistical comparisons were performed using one-way analysis of variance with repeated measures. The magnitude of the maximal peak I_{Ca} from 25 cells not treated with pertussis toxin recorded with 5 mM ATP in the internal solution is included on the left for comparison. PT, pertussis toxin. Error bars represent ±SE.

effects of G_s on the calcium channel have also been reported (28).

Pertussis toxin has been used as a probe to irreversibly ADP-ribosylate G_i, including G_i in cardiac tissue (3) and G_0, a 39-kD guanine nucleotide binding protein prevalent in neural tissue (29). Our data indicate that, in the presence of ATP in
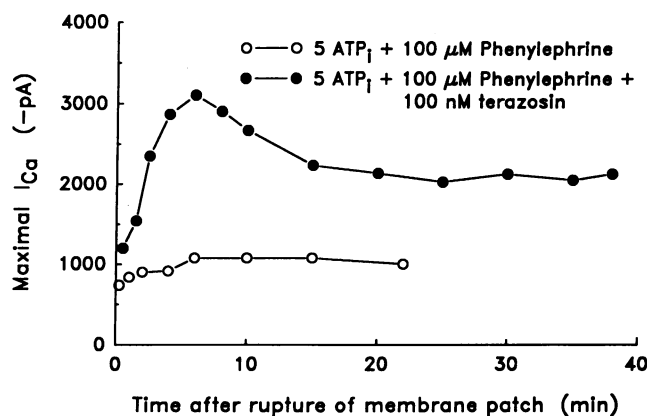
Figure 4. Effect of α_1-adrenergic stimulation on I_{Ca} in pertussis toxin–treated cells. In the presence of 100 μM (−)-phenylephrine and 10 μM (−)-propranolol in the external solution and 5 mM ATP in the internal solution, there was no increase in I_{Ca} (O). Cell PT05. This inhibition is an α_1-adrenergic effect because it was reversed when 100 nM terazosin, a selective α_1-adrenergic antagonist, was added to the external solution together with 100 μM (−)-phenylephrine (●). Cell PT10. These experiments were repeated four times with similar results. 5 ATP$_i$, 5 mM ATP in the pipette solution.

the pipette, incubation with pertussis toxin results in a large surge of I_{Ca} after establishment of the whole-cell voltage clamp configuration, an observation which has not been previously reported. As indicated in Fig. 3 *a*, the increase in I_{Ca} did not occur in the absence of exogenous ATP in the pipette. These data are in sharp contrast to the control cells (Fig. 1, *a* and *c*) in which ATP had no appreciable effect on the magnitude of I_{Ca}. Although Hescheler et al. (3) noted a modest 60% increase in I_{Ca} after pertussis toxin treatment, these investigators did not report a large surge in I_{Ca} with time nor did they test the effects of ATP. Our observations are consistent with the possibility that tonic inhibition of adenylyl cyclase activity by G_i or by a similar protein that is ADP-ribosylated by pertussis toxin prevents excessive calcium channel opening in the basal state and thereby acts as a homeostatic mechanism. These data do not exclude the possibility that G_i or another protein acting as a pertussis toxin substrate bypasses the adenylyl cyclase-cAMP cascade and interacts directly with the calcium channel.

Fig. 3 illustrates another significant finding. Despite a continuous supply of exogenous ATP via the pipette, the peak I_{Ca} declined rapidly after an initial surge. At the end of 23 min, it reached the same level as at 0.5 min. Bean et al. (24) observed a similar decline in I_{Ca} despite continuous β-adrenergic stimulation with isoproterenol in frog ventricular heart cells. One possible explanation for this decline is that the increase in cAMP resulting from incubation with a β-adrenergic agonist activates intracellular phosphodiesterase, thereby accelerating the breakdown of cAMP in an autoregulatory manner (30).

In contrast to the preliminary report of Yatani et al. (31), in which a direct effect of G_s on I_{Ca} in guinea pig atrial myocytes was noted, our observations indicate that an adequate level of ATP is a necessary prerequisite for pertussis toxin to act in an intact cell system. Our data suggest that this effect is mediated by a mechanism requiring channel phosphorylation. Our results also are in agreement with those of Armstrong and Eckert (32), who demonstrated that the dihydropyridine sensitive class of voltage-activated calcium channels must be phosphor

ylated for the channels to open when the membrane is depolarized.

In many systems, inhibition of adenylyl cyclase activity is mediated by an α-adrenergic receptor, particularly the α_2-adrenergic receptor (33). The guinea pig heart is richly endowed with α_1-adrenergic receptors (34), and we have recently reported in neonatal rat ventricular myocytes that agonist occupation of the α_1-adrenergic receptor inhibits cAMP formation through G_i, an effect which is prevented by pertussis toxin pretreatment (11). We wondered whether a similar effect could be demonstrated on I_{Ca}. Our data indicate that I_{Ca} is under the control of the α_1-adrenergic receptor in cardiac cells and that this inhibitory effect does not involve a pertussis toxin–sensitive substrate. These observations are in marked contrast to those of other investigators who were unable to demonstrate an effect of α_1-adrenergic agonists on the slow inward calcium current in ventricular preparations from adult guinea pig (8), cat (9), or rat tissue (10). However, our data are consistent with those of Iwakura et al. (35), who in a preliminary report noted that calcium transients as measured by the indicator fura-2 was diminished by treatment with norepinephrine in the presence of propranolol after KCl depolarization.

In most systems in which the calcium channel has been studied, hormone modulation via G_i or G_0 appears to be inhibitory (3, 20). The mechanism of α_1-adrenergic receptor inhibition of I_{Ca} that we observed at present is unclear. We considered the possibility that (−)-phenylephrine activated phosphodiesterase (22) leading to a decrease in cAMP and hence in calcium channel phosphorylation. However, we were unable to demonstrate such a (−)-phenylephrine-induced alteration of phosphodiesterase activity in our myocyte preparation. An additional possibility that requires further investigation is activation by α_1-adrenergic agonism of a hitherto unidentified G protein which either inhibits adenylyl cyclase activity or directly affects the calcium channel. It also is known that α_1-adrenergic occupation stimulates the production of diacylgylcerol which in turn activates protein kinase C, which can either augment (36) or inhibit (6, 37) I_{Ca} in a variety of neuronal tissues. On the other hand, in feline ventricular cells, phorbol 12-myristate 13-acetate, a putative protein kinase C activator, had no effect on I_{Ca} (9).

Although the mechanism of action of α_1-adrenergic agonism remains to be resolved, our findings clearly indicate that in cardiac cells the processes controlling calcium homeostasis require an adequate cellular supply of ATP, are likely under the "tonic" influence of a pertussis toxin sensitive substrate, and are subject to control by the α_1-adrenergic receptor via a signal transduction mechanism not coupled to a pertussis toxin–sensitive substrate. These observations have implications for understanding abnormalities that may occur under pathophysiological conditions, such as myocardial ischemia and congestive heart failure, where alterations in α_1-adrenergic receptors, ATP levels, and G protein function are known to occur (38–40).

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