On the Role of Phosphatase in Regulation of Cardiac L-Type Calcium Current by Cyclic GMP

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ABSTRACT

Does cGMP, via protein kinase G, inhibit cAMP-stimulated Ca\(^{2+}\) current (I_{Ca(L)}) in mammalian ventricular myocytes by phosphorylating the calcium channel at a site different from that acting on by cAMP or by dephosphorylating the calcium channel through phosphatase(s)? We tested these possibilities in guinea pig ventricular myocytes superfused with Tyrode’s solution (35°C) and dialyzed with adenosine 5′-O-(3-thiotriphosphate) ([ATP\(\gamma\)S]pip). ATP\(\gamma\)S is a kinase substrate but thiophosphorylated proteins are not phosphatase substrates. With 5 mM [ATP\(\gamma\)S]pip, I_{Ca(L)} increased gradually over 20 to 25 min and then rapidly in the presence of 3-isobutyl-1-methylxanthine. 8-Bromo-cGMP (8-Br-cGMP; 1 mM) did not inhibit I_{Ca(L)} significantly (−3 ± 11.8%, n = 21) in contrast to results with ATP dialysis (Imai et al., 2001). Similar results were obtained with 0.1 mM carbachol (CCh). I_{Ca(L)} increased after longer dialysis (≥40 min) with ATP\(\gamma\)S; again, 8-Br-cGMP had no effect. Also, isoproterenol (ISO) did not stimulate and CCh, alone or in the presence of ISO, did not inhibit I_{Ca(L)}. Block of CCh effect by ATP\(\gamma\)S, although consistent with cGMP action in muscarinic inhibition, could be explained by guanosine 5′-O-(3-thiotriphosphate) (GTP\(\gamma\)S) formation from ATP\(\gamma\)S via nucleoside diphosphate kinase. GTP\(\gamma\)S uncouples muscarinic and β-adrenoceptors from intracellular effectors. Failure of 8-Br-cGMP to reduce I_{Ca(L)} irreversibly excludes calcium channel phosphorylation as an inhibitory mechanism. We propose that cGMP inhibits I_{Ca(L)} by activating phosphatase(s) in guinea pig ventricular myocytes.

Extrinsic regulation of heart function usually involves opposing actions by the autonomic nervous system. The yin-yang hypothesis placed the postjunctural actions in the context of intracellular messengers, cAMP and cGMP, and their respective protein kinases having opposite effects on cellular processes (Watanabe and Besch, 1975). One target, the L-type calcium channel, has received considerable attention because the cAMP-activated protein kinase (PKA) increases basal current (Jiang et al., 2000). The PKG inhibitor KT5823 or replacement of serine by alanine at a consensus site (S533A) for PKG-dependent phosphorylation prevented inhibition by cGMP. In the α\(_{1c}\) component of the L-type channel, PKA phosphorylates a serine residue 1928 (Gao et al., 1997). Thus, antagonistic modulation of I_{Ca(L)} by cyclic nucleotides could be referable to phosphorylation of different regulatory sites on the α\(_{1c}\) subunit.

Activation of a phosphatase was excluded as a mechanism for inhibition by cGMP/PKG (Méry et al., 1991; Sumii and Sperelakis, 1995). This conclusion is at odds with the observation that acetylcholine (ACh), which increases cGMP, opposed PKA-dependent phosphorylation of proteins in guinea pig ventricular myocytes (Gupta et al., 1994). This was attributed to activation of protein phosphatase (PP). Okadaic acid, an inhibitor of PP, prevented dephosphorylation of proteins (Gupta et al., 1994) and inhibition of isoproterenol (ISO)-stimulated I_{Ca(L)} by ACh (Herzig et al., 1995). The phosphatase hypothesis was

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ABBREVIATIONS: PKA, protein kinase A; I_{Ca(L)}, L-type calcium current; ATP\(\gamma\)S, adenosine 5′-O-(3-thiotriphosphate); GTP\(\gamma\)S, guanosine 5′-O-(3-thiotriphosphate); CCh, carbachol; IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; PKG, protein kinase G; PP, protein phosphatase; ACh, acetylcholine; PDE, phosphodiesterase; 8-Br-cGMP, 8-bromo-cGMP; KT5823, (8S,10R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolol[1,2,3-fg;2′,3′-f]pyrrolo[3,4-\(\gamma\)]1,4-benzodiazocine-10-carboxylic acid methyl ester.
strengthened by evidence that inhibition of \(I_{\text{Ca(L)}}\) by carbachol (CCh) or 8-Br-cGMP was antagonized by dialysis with 300 mM okadaic acid (Sakai et al., 1999). As such, the results resemble those seen in GH4C1 cells where atrial natriuretic peptide increased a maxi-K channel current by activating a phosphatase through a cGMP/PKG pathway (White et al., 1993). Okadaic acid prevented maxi-K current stimulation by atrial natriuretic peptide. When \(Ba^{2+}\) (White et al., 1993). Okadaic acid prevented maxi-K current stimulation by atrial natriuretic peptide. When \(Ba^{2+}\) current through \(\alpha_{1c}\) subunits expressed in human embryonic kidney cells is elevated by forskolin-induced activation of adenyl cyclase, phosphatase inhibition by okadaic acid potentiates this current and the phosphorylation of serine 1928 (Gao et al., 1997).

We evaluated the phosphatase activation hypothesis for cGMP action on \(I_{\text{Ca(L)}}\). Our experiments were done with ATP\_S replacing ATP in the pipette solution [ATP\_S]_pip; 8-Br-cGMP was used to activate PKG. ATP\_S is a kinase substrate; the thiophosphorylated substrate resists phosphatase action (Yount, 1975). With 3 mM [ATP\_S]_pip, ISO increased \(I_{\text{Ca(L)}}\) more than with ATP and the stimulant effect persisted when ISO was removed (Kameyama et al., 1985; Sipido et al., 1995). PKA and PKG phosphorylate the L-type calcium channel at different sites. With ATP\_S present, we assume that if the effect of thiophosphorylation by PKA on \(I_{\text{Ca(L)}}\) cannot be reversed by activation of PKG, the antagonistic interaction in ATP occurs by dephosphorylation of the site phosphorylated by cAMP/PKA. On the other hand, if cGMP can oppose the effect of cAMP on \(I_{\text{Ca(L)}}\), in the presence of ATP\_S, the antagonism most likely occurs by thiophosphorylation of a different regulatory site on the \(\alpha_{1c}\) subunit.

Materials and Methods

**Ventricular Myocyte Isolation.** Single ventricular myocytes were isolated from the hearts of guinea pigs (250–450 g) anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally) and anaesthetized with heparin (1000 IU, i.p.). The heart was perfused according to the Langendorff technique with modified Tyrode’s solution containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 0.33 mM NaH\(_2\)PO\(_4\), 10 mM HEPES, and 20 mM glucose (pH 7.4 with NaOH). The procedures are the same as we reported previously (Imai et al., 2001). After collagenase and protease disrupted the extracellular matrix, the enzymes were washed out by perfusion with 50 ml of recovery solution containing 130 mM potassium aspartate, 5 mM K\(_2\)ATP, 5 mM HEPES, and 20 mM glucose; pH was adjusted to 7.4 with KOH. The venousides were removed, and the cells were dispersed in recovery solution and kept at 4°C for at least 1 h. A cell suspension droplet was placed in a chamber (500-μl volume) on the stage of an inverted microscope. After 10 min, superfusion began with Tyrode’s solution (2 ml/min) containing 10 mM glucose and 10 mM CaCl\(_2\) (35°C).

**Electrophysiology.** The patch rupture-whole cell voltage clamp technique used an EPC 7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Voltage commands and data acquisition were obtained with pClamp software (version 5.5; Axon Instruments, Union City, CA) and a Labmaster TL-1 interface (Axon Instruments). The pipette filling solution for glass electrodes (i.d., 1.1 mm; o.d., 1.3 mm) contained 135 to 140 mM cesium aspartate, 7 mM NaCl, 3.0 mM MgCl\(_2\), 3.5 or 5 mM ATP\_S, 10 mM EGTA, and 10 mM HEPES; pH 7.3 (with CsOH). In some experiments, the pipette solution also contained 1 mM GTP. The electrode resistance was 1 to 3 MΩ. Series resistance could be compensated up to 70% to values between 1 and 2.5 MΩ.

Membrane voltage was stepped from −80 to −40 mV for 300 ms to inactivate the fast Na\(^+\) and T-type Ca\(^{2+}\) currents. A second voltage step to +10 mV for 300 ms elicited \(I_{\text{Ca(L)}}\). The clamp protocol was repeated every 10 s.

**Drugs and Application.** Drugs were applied to myocytes by superfusion by gravity from a reservoir. The applied solutions were warmed to provide an experimental temperature of 35°C. CCh, ISO, 8-Br-cGMP, and 3-isobutyl-1-methylxanthine (IBMX) were prepared fresh daily from aqueous stock solutions.

**Data Analysis.** With Cs\(^+\)-rich pipette solution to block membrane K\(^+\) currents, \(I_{\text{Ca(L)}}\) is taken as peak current minus end-of-pulse current at +10 mV. Measurements are reported as mean ± S.E.M. The statistical significance of mean differences was determined by Student’s t test. \(p \leq 0.05\) was considered statistically significant.

**Results**

8-Br-cGMP inhibits \(I_{\text{Ca(L)}}\) augmented by ISO or IBMX. At 0.1 mM, 8-Br-cGMP inhibited \(I_{\text{Ca(L)}}\) in 0.1 mM IBMX by 34 ± 3.6% and in 10 mM ISO by 32 ± 7.1% (Imai et al., 2001). Accordingly, one would expect 0.1 mM 8-Br-cGMP to suppress cAMP-stimulated \(I_{\text{Ca(L)}}\) by about one-third. In the following experiments, a 10-fold greater concentration of 1 mM 8-Br-cGMP was used.

The results of a test with 8-Br-cGMP on IBMX-stimulated \(I_{\text{Ca(L)}}\) are shown in Fig. 1. The L-channel current was −0.7 nA upon patch rupture and gradually increased to −1.4 nA after 25 min, during which time the cell had been dialyzed with [ATP\_S]_pip of 5 mM. In this experiment, the pipette solution also contained 1 mM GTP. Addition of 0.1 mM IBMX to the superfusion fluid markedly increased \(I_{\text{Ca(L)}}\) to −4.6 nA at 30 min. When 1 mM 8-Br-cGMP was present for 5 min with IBMX, \(I_{\text{Ca(L)}}\) remained at −4.6 nA. Washout of 8-Br-cGMP ensued and \(I_{\text{Ca(L)}}\) remained elevated at −4.6 nA up to 40 min. This result is characteristic of that observed in a total of 21 cells in which 8-Br-cGMP was tested on cAMP-elevated \(I_{\text{Ca(L)}}\). In three of these cells, 10 mM ISO was used to stimulate \(I_{\text{Ca(L)}}\), and the results are included with those obtained with IBMX because the outcome was the same. Overall, the initial \(I_{\text{Ca(L)}}\) just before addition of cAMP-elevating agent was −1.0 ± 0.13 nA and it increased to −2.6 ± 0.27 nA (\(n = 21\)). On average, 1 mM 8-Br-cGMP changed cAMP-elevated \(I_{\text{Ca(L)}}\) by −3 ± 11.8% (\(n = 21\)), which is not significantly different from 0. 8-Br-cGMP reduced \(I_{\text{Ca(L)}}\) by −16 ± 5.6% (\(n = 8\)) in cells dialyzed with ATP\_S for <25 min, and by 5 ± 18.7% (\(n = 13\)) in cells dialyzed for >25 min (\(p = 0.2\)). Importantly, if 8-Br-cGMP reduced IBMX-stimulated \(I_{\text{Ca(L)}}\), the effect was transient.

There were two cells in which we did not observe this pattern; an example is shown in Fig. 2. The L-channel current increased from −1.4 to −2.8 nA during the first 10 min of dialysis; the pipette solution was the same as for the cell in Fig. 1. Addition of 0.1 mM IBMX at 10 min had a small stimulant effect on \(I_{\text{Ca(L)}}\) that eventually reached −3 nA at 16 min. When 1 mM 8-Br-cGMP was added, \(I_{\text{Ca(L)}}\) diminished rapidly over the next 4 min to −0.4 nA. The current did not change appreciably over the ensuing 15 min when 8-Br-cGMP and then IBMX were washed out. This result resembles that reported by Méry et al. (1991) in which cGMP irreversibly reduced cAMP-stimulated \(I_{\text{Ca(L)}}\). However, it is exceptional because it occurred in only 2 of the 23 cells tested and cannot be distinguished from rundown. In both instances, IBMX had been added at about 10 min after patch rupture and 8-Br-cGMP tested 5 min later. The time of IBMX addition ranged from 6 to 28 min with an average of 16 ± 2.
min in the 21 cells where the effect of 8-Br-cGMP was suppressed.

Inhibition of ISO- or IBMX-stimulated $I_{\text{Ca,L}}$ by CCh has been reported by many investigators. We found that 0.1 mM CCh inhibited ISO-stimulated $I_{\text{Ca,L}}$ by 77.9% and IBMX-stimulated $I_{\text{Ca,L}}$ by 6.3% (Imai et al., 2001). However, in cells dialyzed with ATP$_S$, CCh had little effect when tested against IBMX-stimulated $I_{\text{Ca,L}}$. An example is shown in Fig. 3. In this cell, $I_{\text{Ca,L}}$ was -0.2 nA upon patch rupture and increased to -0.9 nA during the next 20 min as the cell was dialyzed with [ATP$_S$]$_{\text{pip}}$ of 5 mM and [GTP]$_{\text{pip}}$ of 1 mM. Again, 0.1 mM IBMX, added at 20 min, promptly increased $I_{\text{Ca,L}}$ to -5 nA (Fig. 3). Current did not change upon addition of 0.1 mM CCh to the bath fluid for 5 min. When CCh was removed from the IBMX-containing solution, $I_{\text{Ca,L}}$ remained at -5 nA through 40 min.

Overall, 0.1 mM CCh reduced cAMP-elevated $I_{\text{Ca,L}}$ by $-32 \pm 9.9\%$ ($n = 12$). Although this is substantially less than that observed with ATP in the pipette solution (see above), it is greater than the inhibition observed with 8-Br-cGMP in separate experiments. However, when both were tested in the same six cells against IBMX-stimulated $I_{\text{Ca,L}}$, CCh reduced current by $-29 \pm 13.3\%$ (32 $\pm$ 5.2 min) and 8-Br-cGMP reduced it by $-21 \pm 9.7\%$ (26 $\pm$ 7.1 min). In nine cells, we obtained stable recordings during dialysis with [ATP$_S$]$_{\text{pip}}$ of 3.5 to 5 mM for 45 min. Under these conditions, tests with 1 mM 8-Br-cGMP

![Diagram](image1.png)

**Fig. 1.** 8-Br-cGMP fails to inhibit IBMX-stimulated $I_{\text{Ca,L}}$ in a ventricular myocyte dialyzed with 5 mM ATP$_S$. The pipette solution also had 1 mM GTP. Ordinate, $I_{\text{Ca,L}}$ in nanoamperes; abscissa, time in minutes. $I_{\text{Ca,L}}$ was 0.7 nA at patch rupture and slowly increased to -1.4 nA at 25 min. IBMX (0.1 mM), added at 25 min, caused $I_{\text{Ca,L}}$ to increase rapidly to 4.6 nA at 30 min. Subsequent addition of 1 mM 8-Br-cGMP negligibly affected IBMX-stimulated $I_{\text{Ca,L}}$. Horizontal lines indicate the time and duration of drug additions. Individual current traces recorded at A to E are shown above with current calibration in the right margin.

![Diagram](image2.png)

**Fig. 2.** An example of an exceptional result when IBMX-stimulated $I_{\text{Ca,L}}$ is “irreversibly” reduced by 8-Br-cGMP. Ordinates and abscissa are as in Fig. 1. L-channel current increased from -1.4 nA at patch rupture to -2.7 nA at 10 min. IBMX (0.1 mM) increased $I_{\text{Ca,L}}$ to -3 nA by 16 min, and 8-Br-cGMP (1 mM) promptly reduced current to -0.4 nA, which is below the level seen at patch rupture. Washout of 8-Br-cGMP at 21 min with IBMX still present did not cause any further change of $I_{\text{Ca,L}}$. See text for details.
Stimulated ICa(L) by either ligand (data not shown). ICa(L) by 8-Br-cGMP unlike the results from rat ventricular myocytes (Méry et al., 1991; Sumii and Sperelakis, 1995). A similar conclusion was reached from guinea pig ventricular myocytes (reviewed in Herzig et al., 1991). Those reporting cGMP/PKG-induced activation of PP are opposed ISO-stimulated ICa(L), but not basal ICa(L), in guinea pig ventricular myocytes (Hescheler et al., 2001). Stimulation of ICa(L) by 8-Br-cGMP was attributed to activation of PKA. Although PKG was excluded as an effector of 8-Br-cGMP in single atrial myocytes (Vandecasteele et al., 2001), it may have been the target in experiments with human atrial trabecular contractions (Nawrath et al., 1995; Flesch et al., 1997). No information is available on effectors of 8-Br-cGMP in human ventricle, which conceivably differ from those in atrium (Vandecasteele et al., 2001).

8-Br-cGMP could sometimes inhibit ICa(L) in cells dialyzed for ≥25 min with ATPγS. However, the inhibition was reversible, a finding inconsistent with cGMP/PKG causing irreversible inhibition via thiophosphorylation of the L-type calcium channel in rat ventricular myocytes (Méry et al., 1991). In only 2 of 23 cells, 8-Br-cGMP addition was accompanied by a sustained decrease of ICa(L) (Fig. 2). Although this could be an example of irreversible inhibition, it cannot be distinguished from current rundown. Sufficient time should be allowed for diffusion of ATPγS from the pipette solution and for replacing ATP for participation in thiophosphorylation reactions including those at the L-type calcium channel.

ATPγS interfered with inhibition of ICa(L) by 8-Br-cGMP favors the participation of PKG-activated PP in the cGMP signaling pathway. Dialysis with either PP1 or PP2A opposed ISO-stimulated ICa(L), but not basal ICa(L) in guinea pig ventricular myocytes (Hescheler et al., 1987). Okadaic acid inhibits PP1 and PP2A with the latter being more sen-

**Discussion**

With PDEs inactivated by IBMX, we assume that there are two pathways for inhibition of ICa(L) by 8-Br-cGMP, namely, phosphorylation of the α1c subunit that antagonizes the stimulant effect of cAMP/PKA and/or activation of PP that dephosphorylates the α1c subunit. Can 8-Br-cGMP or CCh suppress Ca2+ current through L-type channels in the presence of ATPγS, which allows kinases to thiophosphorylate substrates that resist phosphatase action? Irreversible suppression by 8-Br-cGMP would indicate that thiophosphorylation of the channel had occurred at a site different from that acted upon by cAMP/PKA (Méry et al., 1991; Sumii and Sperelakis, 1995). Alternatively, failure of 8-Br-cGMP to suppress ICa(L) in ATPγS would indicate that the thiophosphorylated channel was resistant to phosphatase. Our results are consistent with the PP hypothesis for inhibition by cGMP of current through L-type channels in guinea pig ventricular myocytes.

Neurotransmitter regulation of ICa(L) by reversible phosphorylation-dephosphorylation reactions is well known (McDonald et al., 1994; Herzig and Neumann, 2000). Serine 1928 of the α1c subunit has been reported to be an essential residue for phosphorylation by PKA (Gao et al., 1997). Our results point to the possibility that the site for phosphatase action on the α1c subunit is the same. This conclusion differs from that of others in several ways. Those who report that cGMP irreversibly inhibits ICa(L) by phosphorylation of the channel did experiments in rat ventricular myocytes (Méry et al., 1991; Sumii and Sperelakis, 1995). A similar conclusion emerged from experiments with mouse ventricular myocytes (Klein et al., 2000). We did not observe suppression of basal ICa(L) by 8-Br-cGMP unlike the results from rat ventricular myocytes (Sumii and Sperelakis, 1995). Our experiments and those reporting cGMP/PKG-induced activation of PP are from guinea pig ventricular myocytes (reviewed in Herzig and Neumann, 2000). Different species have distinct regulatory mechanisms for cGMP-dependent regulation of ICa(L). In amphibian ventricular myocytes, cGMP stimulated PDE, which reduces cAMP concentration (reviewed in Méry et al., 1997). This pathway is not prominent in mammalian ventricular myocytes and would be inoperable in our experiments because IBMX inhibits all PDEs. In human atrial trabeculae, 8-Br-cGMP had a negative inotropic effect (Nawrath et al., 1995; Flesch et al., 1997) but increased ICa(L) in single myocytes (Vandecasteele et al., 2001). Stimulation of ICa(L) by 8-Br-cGMP was attributed to activation of PKA. Although PKG was excluded as an effector of 8-Br-cGMP in single atrial myocytes (Vandecasteele et al., 2001), it may have been the target in experiments with human atrial trabecular contractions (Nawrath et al., 1995; Flesch et al., 1997). No information is available on effectors of 8-Br-cGMP in human ventricle, which conceivably differ from those in atrium (Vandecasteele et al., 2001).

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![Figure 3](https://example.com/fig3.png)
cGMP Activates Phosphatase to Inhibit Cardiac Current


L-type calcium current and contraction of guinea pig ventricular myocytes. *J Pharmacol Exp Ther* 279:1274–1281.


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