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A comparative study of the effects of three guanylyl cyclase inhibitors on the L-type Ca^{2+} and muscarinic K^+ currents in frog cardiac myocytes

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1 To investigate the participation of guanylyl cyclase in the muscarinic regulation of the cardiac L-type calcium current (I_{Ca}), we examined the effects of three guanylyl cyclase inhibitors, 1H-[1,2,4]oxidiazolo[4,3-a]quinoxaline-1-one (ODQ), 6-anilino-5,8-quinolinedione (LY 83583), and methylene blue (MBlue), on the β-adrenoceptor; muscarinic receptor and nitric oxide (NO) regulation of I_{Ca} and on the muscarinic activated potassium current $I_{K,ACh}$, in frog atrial and ventricular myocytes.

2 ODQ (10 μM) and LY 83583 (30 μM) antagonized the inhibitory effect of an NO-donor (S-nitroso-N-acetylpenicillamine, SNAP, 1 μM) on the isoprenaline (Iso)-stimulated I_{Ca} which was consistent with their inhibitory action on guanylyl cyclase. However, MBlue (30 μM) had no effect under similar conditions.

3 In the absence of SNAP, LY 83583 (30 μM) potentiated the stimulations of I_{Ca} by either Iso (20 nM), forskolin (0.2 μM) or intracellular cyclic AMP (5–10 μM). ODQ (10 μM) had no effect under these conditions, while MBlue (30 μM) inhibited the Iso-stimulated I_{Ca}.

4 LY 83583 and MBlue, but not ODQ, reduced the inhibitory effect of up to 10 μM acetylcholine (ACh) on I_{Ca}.

5 MBlue, but not LY 83583 and ODQ, antagonized the activation of $I_{K,ACh}$ by ACh in the presence of intracellular GTP, and this inhibition was weakened when $I_{K,ACh}$ was activated by intracellular GTPγS.

6 The potentiating effect of LY 83583 on Iso-stimulated I_{Ca} was absent in the presence of either DL-dithiothreitol (DTT, 100 μM) or a combination of superoxide dismutase (150 u ml^{-1}) and catalase (100 u ml^{-1}).

7 All together, our data demonstrate that, among the three compounds tested, only ODQ acts in a manner which is consistent with its inhibitory action on the NO-sensitive guanylyl cyclase. The two other compounds produced severe side effects which may involve superoxide anion generation in the case of LY 83583 and alteration of β-adrenoceptor and muscarinic receptor-coupling mechanisms in the case of MBlue.

Keywords: Methylene blue; LY 83583; ODQ; guanylyl cyclase; cardiac myocytes; patch-clamp; L-type calcium current; muscarinic activated potassium current; acetylcholine; nitric oxide; isoprenaline; cyclic nucleotides; phosphorylation

Introduction

It is well established that acetylcholine (ACh) regulates several ionic currents and second messenger pathways in the whole heart at well as in the isolated cardiac myocytes (reviewed in Lindemann & Watanabe, 1991; Fischmeister & Méry, 1996). Some of these modulations are involved in the muscarinic regulation of cardiac contractility. For instance, the binding of ACh to the cardiac muscarinic M_2 receptor leads to the activation of the inhibitory guanosine 5′-triphosphate (GTP)-binding protein, G_i, which in turn directly activates the K^+ channels underlying the muscarinic K^+ current, $I_{K,ACh}$. This short cascade contributes to the negative chronotropic effect of ACh. Activation of the G_i protein by ACh also leads to the inhibition of cardiac adenylyl cyclase activity. This mechanism is responsible for the muscarinic antagonism of the β-adrenoceptor activation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) synthesis. Since the elevation of cyclic AMP accounts for all of the β-adrenoceptor stimulation of the L-type Ca^{2+} current (I_{Ca}) and the Cl^- current (I_{Cl}) (Hartzell, 1991; Hwang *et al*., 1992), the inhibitory effect of ACh on the β-adrenoceptor stimulation of these currents is often interpreted as a result of the decrease in cyclic AMP production (Fischmeister & Méry, 1996). Indeed, the stimulation of I_{Ca} and I_{Cl} by exogenous cyclic AMP is resistant to ACh, suggesting that this agonist does not act on a mechanism located downstream from cyclic AMP production (Hartzell & Fischmeister, 1986; Hescheler *et al*., 1986; Nakajima *et al*., 1990; Tareen *et al*., 1991; Hwang *et al*., 1992). However, other studies suggest that the anti β-adrenoceptor effect of ACh involves the activation of a phosphatase (Gupta *et al*., 1993; Herzig *et al*., 1995) or the stimulation of a phosphodiesterase (PDE) (Kubalak *et al*., 1991).

ACh and other muscarinic agonists are also known to elevate cardiac guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in various species (reviewed in Lindemann & Watanabe, 1991; Fischmeister & Méry, 1996). This pathway is of physiological interest since both exogenous cyclic GMP and guanylyl cyclase activation by NO-donors or atrial natriuretic peptides can modulate cardiac contractility and I_{Ca} (Méry *et al*., 1993; Levi *et al*., 1994; Whaler & Dollinger, 1994; Han *et al*., 1995; Those *et al*., 1995). However, the involvement of cyclic GMP in the muscarinic regulation of cardiac ionic currents or contractility is still a matter of debate. L-Arginine analogues, which are competitive inhibitors of NO-synthase (NOS), were found to antagonize the inhibitory effect of ACh on the β-adrenoceptor stimulation of I_{Ca} in some studies (Han *et al*., 1995; Kitakaze *et al*., 1995; Han *et al*., 1996; Kelly *et al*., 1996) but not in others (Kennedy *et al*., 1994; Nawrath *et al*., 1995; Méry *et al*., 1996, see also Zakharov *et al*., 1996). Cyclic GMP was also suggested to be involved in the muscarinic inhibition of cardiac contractility and ionic currents, based on the findings that the effects of ACh can be antagonized by methylene blue

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(MBlue) and LY 83583, two guanylyl cyclase inhibitors (reviewed in Lindemann & Watanabe, 1991; Fischmeister & Méry, 1996; Kelly *et al*., 1996). The ability of these compounds to inhibit guanylyl cyclase activity was assessed in intact myocytes exposed to NO-donors such as 3-morpholinosydnonimine (SIN-1), sodium nitroprusside (SNP) or S-nitroso-N-acetylpenicillamine (SNAP). Indeed, MBlue and LY 83583 antagonize the regulation of I_{Ca} by these NO-donors (Méry *et al*., 1993; Levi *et al*., 1994; Whaler & Dollinger, 1994; Han *et al*., 1995). However, LY 83583 and MBlue also release superoxide anions (Barbier & Lefebvre, 1992; Marczin *et al*., 1992) which may affect proteins other than guanylyl cyclase and result in some undesirable side effects (Mironov & Lux, 1991; Clemo *et al*., 1992; Lüond *et al*., 1993; Mayer *et al*., 1993; Han *et al*., 1995) with unknown consequences on the regulation of cardiac I_{Ca}. Recently, a new guanylyl cyclase inhibitor has appeared, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), which unlike the two other inhibitors is not a superoxide anion generator (Garthwaite *et al*., 1995). Moreover, ODQ, unlike for example LY 83583 (Clemo *et al*., 1992), inhibits selectively the NO-sensitive of guanylyl cyclase (Garthwaite *et al*., 1995; Cellek *et al*., 1996; Schrammel *et al*., 1996). This confers on ODQ the potential capacity of decipher the involvement of cyclic GMP in the muscarinic regulation of I_{Ca}.

To obtain an insight into the mechanisms of action of MBlue, LY 83583 and ODQ at the single cell level, in the present study we have compared the effects of these compounds on the β-adrenoceptor, muscarinic receptor and NO regulation of I_{Ca} in frog isolated ventricular myocytes. The choice of this preparation was based on the observations that (1) cardiac I_{Ca} is more sensitive to NO-donors in frogs than in mammals (Méry *et al*., 1993; Whaler & Dollinger, 1995), and (2) the effects of NO-donors on I_{Ca} are not contaminated by and endogenous production of NO in frog ventricular myocytes (Méry *et al*., 1996). To examine the specificity of action of these compounds, we also examined their effects on $I_{K,ACh}$ in frog atrial myocytes. A preliminary account of some of these results has appeared (Hove-Madsen *et al*., 1996).

Methods

Electrophysiology

Frogs (*Rana esculenta*) were decapitated and double pithed. Ventricular cells were enzymatically dispersed by a combination of collagenase (Yakult, Japan) and trypsin (Type XIII, Sigma Chemical Co.) as described previously (Fischmeister & Hartzell, 1986; Méry *et al*., 1993). At the end of this procedure, the atria were transferred and minced carefully in a Ringer solution containing BSA (1 mg ml^{-1}), protease type XIV (0.3 to 0.45 mg ml^{-1}, Sigma Chemical Co.) and 800 μM $CaCl_2$. The pieces of tissue were slowly stirred in this medium at 30°C until release of isolated atrial myocytes (20–35 min). The supernatant, containing the isolated cells was then diluted in the K^+-containing solution (see below). Both ventricular and atrial myocytes were kept at 4°C until use (2–48 h following dissociation).

The whole-cell configuration of the patch-clamp technique was used to record the L-type calcium current (I_{Ca}) and K^+ currents on Ca^{2+}-tolerant cells (Fischmeister & Hartzell, 1986; Li *et al*., 1994). Most data were obtained with a routine protocol which was composed of a depolarizing pre-pulse to −50 mV (50 or 400 ms duration) followed by a test pulse to 0 mV (400 ms duration), elicited every 8 s from a holding potential of −80 mV (see Figures 6–7). I_{Ca} and $I_{K,ACh}$ were recorded separately in different experiments since the peak I_{Ca} current at 0 mV is contaminated by $I_{K,ACh}$ when both currents are active (Li *et al*., 1994). Thus, I_{Ca} was recorded under conditions where K^+ currents were blocked by replacing all intracellular and extracellular K^+ ions with Cs^+ (Fischmeister & Hartzell, 1986). Under these conditions, the time-dependent current measured as 0 mV can be attributed entirely to the L-type calcium channels (Fischmeister & Hartzell, 1986; Argibay *et al*., 1988). The pre-pulse to −50 mV was omitted in some experiments in ventricular cells where only I_{Ca} was studied. The experiments were done at room temperature and in a given experiment, the temperature did not change by more than 2°C.

Solutions for patch-clamp recordings

External Cs^+-containing solution contained (in mM): NaCl 107, HEPES 10, CsCl 20, $NaHCO_3$ 4, NaH_2PO_4 0.8, $MgCl_2$ 1.8, $CaCl_2$ 1.8, D-glucose 5, sodium pyruvate 5 and tetrodotoxin 3×10^{-4}; pH 7.4 adjusted with CsOH. In the external K^+-containing solution, CsCl was substituted for 2.5 mM KCl and pH adjusted with NaOH. Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 250 μm inner diameter capillary tubings flowing at a rate of ≈ 10 μl min^{-1} (Fischmeister & Hartzell, 1986).

The electrodes (0.6–2.0 MΩ) used to record I_{Ca} were filled with the internal Cs^+-containing solution which contained (in mM): CsCl 119.8, EGTA (acid form) 5, $MgCl_2$ 4, Na_2-phosphocreatine 5, Na_2ATP 3.1, Na_2GTP 0.42, $CaCl_2$ (pCa 8.5) 0.062 and HEPES 10; pH 7.1 adjusted with CsOH. In the internal K^+-containing solution, CsCl was substituted for 102 mM KCl and pH adjusted with KOH. The internal GTPγS containing medium was obtained by substituting GTPγS for GTP. Drug-containing solutions were then applied to the interior of the cell by a system that permitted perfusion of the patch-electrode with different solutions (Hartzell & Fischmeister, 1986; Méry *et al*., 1993).

Data analysis

During patch-clamp experiments, the maximal amplitude of the whole-cell I_{Ca} was measured as previously described (Fischmeister & Hartzell, 1986; Argibay *et al*., 1988). Muscarinic K^+ current $I_{K,ACh}$ was measured as the change of the steady-state end pulse current at 0 mV after the application of ACh (Li *et al*., 1994). Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was made possible by programming a PC-compatible 486/50 microcomputer in Assembling language (Borland) to determine, for each membrane depolarization, peak and steady-state current values (Fischmeister & Hartzell, 1986).

The results are expressed as mean ± s.e.mean. Differences between means were tested for statistical significance by Student's *t* test. In the text, the 'basal' condition for I_{Ca} refers to the absence of cyclic AMP-dependent phosphorylation of the calcium channels (Fischmeister & Méry, 1996). The individual I_{Ca} current traces shown in Figures 1–5 have been truncated so that only the first 125 ms following the beginning of the depolarizing pulse to 0 mV are displayed to emphasize the activation and inactivation kinetics of the whole-cell I_{Ca}. Each individual current trace at 0 mV is preceded by 10 ms of its corresponding holding current at −80 mV.

Drugs

6-Anilino-5,8-quinolinedione (LY 83583) was a gift from E. Lilly (Indianapolis, IN, U.S.A.). S-nitroso-N-acetylpenicillamine (SNAP) was from Calbiochem (La Jolla, CA, U.S.A.) or Tocris-Cookson (Bristol, U.K.). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) was from Tocris-Cookson. Superoxide dismutase (SOD, from bovine erythrocytes) and catalase (from bovine liver) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Tetrodotoxin (TTX) was from Latoxan (Rosans, France). All other drugs were from Sigma Chemical Co. Stock solutions were prepared as follows: LY 83583, 30 mM in ethanol; ODQ, 10 mM in dimethylsulphoxide (DMSO) or 100 mM in ethanol. These stock solutions were kept at 4°C or −20°C during the day and fresh stock solutions were made daily. A stock solution of SNAP (1 mM in physiological solution) was prepared at the beginning of each experiment. All solutions

were prepared by dilution to the desired concentration in the physiological solution at the beginning of each experiment.

Results

Effects of guanylyl cyclase inhibitors on the inhibitory effect of SNAP on I_{Ca}

We have shown previously that activation of the soluble guanylyl cyclase by the NO-donors SIN-1 and SNP does not modify the basal L-type calcium current (I_{Ca}) in frog cardiac myocytes. However, similar to exogenously applied cyclic GMP, these NO-donors regulate I_{Ca} which had been previously stimulated by isoprenaline, forskolin or intracellular cyclic AMP (Méry *et al*., 1993). Here, we found similar results with another NO-donor, SNAP, a nitrosothiol which was also shown to activate cyclic GMP production in cardiac myocytes (Kojda *et al*., 1996). Indeed, SNAP had no effect on basal I_{Ca} at a concentration of up to 100 μM (90.0±4.8% of the basal level, mean±s.e.mean, $n=3$) but addition of 1 μM SNAP to a cell which had been exposed to isoprenaline (Iso, 20 nM) produced a large inhibitory effect on I_{Ca} (Figure 1). As illustrated in the three individual experiments of Figure 1A–C, the basal I_{Ca} amplitude was enhanced several fold by the exposure of the frog ventricular myocyte to 20 nM Iso, a β-adrenoceptor agonist. On average, I_{Ca} density increased from 3.15±0.31 to 14.47±1.31 pA pF^{-1} ($n=19$). Addition of 1 μM SNAP in the presence of Iso reduced the β-adrenoceptor-mediated stimulation of I_{Ca} by ~60% on average, to a mean current amplitude of 6.21±0.85 pA pF^{-1} ($n=19$, Figure 1A–C). We then examined the effects of LY 83583, methylene blue (MBlue) and ODQ, three potential guanylyl cyclase inhibitors, on the inhibitory effect of SNAP on I_{Ca}. As shown in Figure 1A and C, addition of either LY 83583 (30 μM, Figure 1A) or ODQ (10 μM, Figure 1C) in the continuing presence of Iso and SNAP antagonized the inhibitory effect of the NO-donor on I_{Ca}. However, in marked contrast with the two other inhibitors, application of MBlue (30 μM) further reduced I_{Ca} (Figure 1B). On average (Figure 1D), the rank order of potency of the three guanylyl cyclase inhibitors in reversing the inhibitory effect of SNAP on I_{Ca} was ODQ > LY 83583 > > MBlue.

Effects of guanylyl cyclase inhibitors on the β-adrenoceptor-mediated stimulation of I_{Ca}

The above experiments suggest that ODQ and LY 83583, but not MBlue, reversed the inhibitory effect of SNAP on I_{Ca} because of their inhibitory effect on guanylyl cyclase. However, since these experiments required the presence of Iso, a possibility remained that these compounds were acting directly on the β-adrenoceptor-mediated cascade, independently of their effect on guanylyl cyclase. To examine this hypothesis, we investigated the effects of LY 83583, ODQ and MBlue, at the same concentrations as used above, on the response of I_{Ca} to Iso alone. In the experiments shown in Figure 2A and 2B, I_{Ca} was increased ~7 fold by a non saturating concentration of Iso (20 nM). Surprisingly, addition of 30 μM LY 83583 on top of the Iso stimulation resulted in an additional increase in I_{Ca} (Figure 2A) while addition of 30 μM MBlue resulted in a substantial decrease in I_{Ca} (Figure 2B). Figure 2C shows a summary of the results of a number of similar experiments, including 5 experiments with ODQ. In all 36 experiments, 20 or 200 nM Iso was used to enhance I_{Ca} from a mean basal amplitude of 2.47±0.17 pA pF^{-1} to an amplitude of 13.46±1.33 pA pF^{-1}. Addition of MBlue (30 μM) significantly reduced, while application of LY 83583 significantly increased, the β-adrenoceptor-mediated stimulation of I_{Ca}. Unlike the two other compounds, ODQ (at 10 μM) had no effect on the Iso-stimulated I_{Ca} (−0.34±13.56%, $n=5$, Figure 2C). However, it should be mentioned that LY 83583 had an atypical effect on 4 out 23 experiments, in which the drug clearly inhibited the Iso response (by 41.42±17.6%, $n=4$). The effects of MBlue and LY 83583 were not observed at lower drug concentrations (3 μM MBlue, $n=3$; 0.1–10 μM LY 83583, $n=5-7$) and were not accompanied by any significant change in the voltage-dependence of I_{Ca} amplitude or inactivation ($n=3-4$, data not shown).

Effects of guanylyl cyclase inhibitors on the cyclic AMP cascade

The efficacy of ODQ to reverse the inhibitory effect of SNAP on I_{Ca}, together with the absence of effect of ODQ in the presence of Iso alone, suggests that this drug acts as a true inhibitor of the NO-sensitive guanylyl cyclase. It also suggests that this guanylyl cyclase is not significantly active in frog ventricular myocytes in the absence of exogenous NO. Therefore, the stimulating and inhibitory effects of LY 83583 and MBlue, respectively, on the β-adrenoceptor-mediated stimulation of I_{Ca} are probably mediated by other mechanisms. To get some insights into these mechanisms, we tested the effects of all three compounds on an I_{Ca} which had been previously stimulated by forskolin, a direct adenylyl cyclase activator. As shown in Figure 3A, superfusion of a frog ventricular myocyte with 0.1 μM forskolin mimicked the stimulant effect of Iso on I_{Ca} (mean increase: from

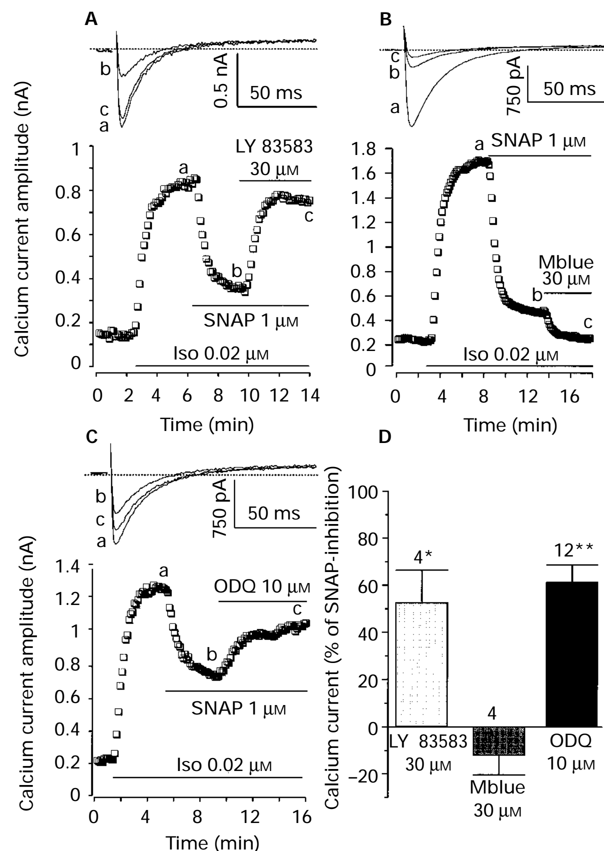

Figure 1 Effects of guanylyl cyclase inhibitors on the SNAP-inhibition of I_{Ca}. (A), (B) and (C) Individual experiments performed in isolated ventricular myocytes initially superfused with control external Cs^+ solution and dialysed with control intracellular Cs^+ solution. Each symbol corresponds to a measure of I_{Ca} at 0 mV, obtained every 8 s (see Methods). As indicated by the solid lines, cells were then superfused with isoprenaline (Iso, 20 nM), SNAP (1 μM) and LY 83583 (30 μM) in (A), methylene blue (MBlue, 30 μM) in (B), or ODQ (10 μM) in (C). The upper parts in (A)–(C) show the first 125 ms of individual I_{Ca} current traces recorded at the times indicated by the corresponding letters on the main graphs. The dotted lines indicate the zero-current level. (D) A summary of several similar experiments as in (A), (B) and (C). The amplitude of the effects of the guanylyl cyclase inhibitors were normalized with respect to the amplitude of the inhibitory effect of SNAP (1 μM) on the Iso (20 nM)-stimulated I_{Ca}. The columns indicate the means±s.e.mean of the number of experiments indicated above. Significant differences from the SNAP level are indicated as: $^*P<0.05$; $^{**}P<0.01$.

3.09 ± 0.47 pA pF^{-1} to 8.37 ± 1.37 pA pF^{-1}, $n = 15$). Addition of LY 83583 (30 μM) in the presence of forskolin resulted in a further increase in I_{Ca}. The effect of LY 83583 was reversible and reproducible. Figure 3C shows a summary of the results of 11 similar experiments with 30 μM LY 83583. LY 83583 induced $\approx$40% significant increase in the response of I_{Ca} to forskolin, an effect which was somewhat similar to the effect of the drug on the response of I_{Ca} to Iso (Figure 2A and C). ODQ (10 μM, $n = 6$) had no effect under similar conditions (Figure 3C). However, surprisingly, MBlue (30 μM) had also no significant effect on I_{Ca} in the presence of forskolin ($n = 7$, Figure 3C). To examine if the stimulant effect of LY 83583 was taking place downstream from adenylyl cyclase activation, the effect of the drug was examined on an I_{Ca} which was enhanced by intracellular dialysis with cyclic AMP. In the experiment shown in Figure 3B, intracellular application of 5 μM cyclic AMP induced a $\approx$4 fold stimulation of I_{Ca}. While addition of 30 μM LY 83583 to the intracellular solution had no effect on I_{Ca}, extracellular application of the drug induced a clear increase in the current amplitude. The results of several similar experiments are summarized in Figure 3D. Intracellular application of 5–10 μM cyclic AMP increased I_{Ca} density from 2.22 ± 0.31 to 11.56 ± 2.50 pA pF^{-1} ($n = 12$). Under these conditions, extracellular application of 30 μM LY 83583 led to a significant further increase in the response of I_{Ca} to cyclic AMP. This suggests that the stimulant effect of LY 83583 occurs at a step distal from cyclic AMP production in the β-adrenoceptor-mediated cascade. Like in the presence of forskolin, MBlue (30 μM, $n = 5$) and ODQ (10 μM, $n = 3$) had no significant effect on the response of I_{Ca} to intracellular cyclic AMP (Figure 3D). However, ODQ completely antagonized (by 108.89 ± 6.04%, $n = 3$) the inhibitory effect of 10 μM SNAP (38.68 ± 16.0% inhibition) on I_{Ca} stimulated by 5 or 10 μM cyclic AMP (data not shown). Since MBlue reduced the response of I_{Ca} to Iso but had no effect when the β-adrenoceptor was bypassed by either forskolin or cyclic AMP, it is likely that MBlue acts at some step(s) located between the β-adrenoceptor and the adenylyl cyclase.

Effects of guanylyl cyclase inhibitors on the muscarinic regulation of I_{Ca}

MBlue and LY 83583 have often been used to demonstrate the participation of guanylyl cyclase activity in the muscarinic regulation of cardiac contractility and I_{Ca} (Levi *et al.*, 1994;

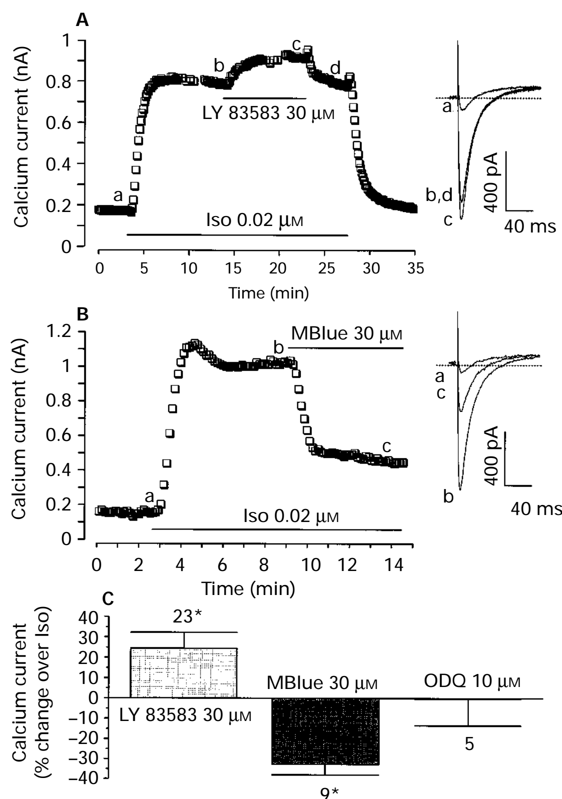

Figure 2 Effect of guanylyl cyclase inhibitors on isoprenaline (Iso)-stimulated I_{Ca}. (A) A frog ventricular myocyte was initially superfused with control Cs^+ solution and dialysed with control Cs^+ solution. Additions of Iso (20 nM) and LY 83583 (30 μM) are indicated by the solid lines. (B) Similar experiment except that methylene blue (MBlue, 30 μM) was added in the extracellular solution. On the right, the insets show the first 125 ms of individual I_{Ca} current traces recorded at the times indicated by the corresponding letters on the main graphs. The dotted lines indicate the zero-current level. (C) Summary of the effects of the guanylyl cyclase inhibitors on the Iso (20 and 200 nM)-stimulated I_{Ca}. The data were normalized with respect to the amplitude of the Iso-stimulated I_{Ca}. The columns indicate the means $\pm$ s.e.mean of the number of experiments indicated. Significant differences from the Iso level are indicated as: *$P < 0.01$.

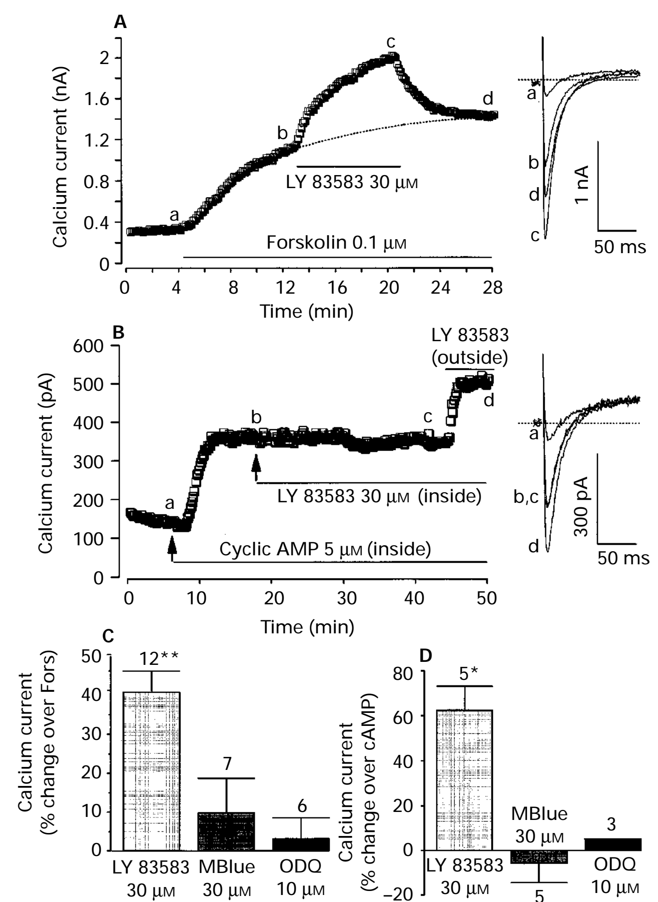

Figure 3 Effects of guanylyl cyclase inhibitors on forskolin- or cyclic AMP-stimulated I_{Ca}. In (A) and (B) frog ventricular myocytes were initially superfused with control Cs^+ solution and dialysed with control Cs^+ solution. In (A) the cell was superfused with forskolin (0.1 μM), on the top of which LY 83583 (30 μM) was added, as indicated by the solid line. In (B) internal dialysis with 5 μM cyclic AMP was started at the first arrow and continued throughout the rest of the experiment. LY 83583 30 μM was added in the intracellular solution at the time indicated by the second arrow. Addition of LY 83583 (30 μM) in the extracellular solution was performed as indicated by the solid line. On the right, the insets show the first 125 ms of individual I_{Ca} current traces recorded at the times indicated by the corresponding letters on the main graphs. The dotted lines indicate the zero-current level. (C) and (D). A summary of the effects of guanylyl cyclase inhibitors on I_{Ca} previously enhanced either by forskolin (0.1 μM) in (C), or cyclic AMP (5 or 10 μM) in (D). The data were normalized with respect to the amplitude of the stimulated I_{Ca}. The columns indicate the means $\pm$ s.e.mean of the number of experiments indicated. Significant differences from the stimulated level are indicated as: *$P < 0.0$, **$P < 0.01$.

Mubagwa *et al.*, 1994; and see refs in Kelly *et al.*, 1996). We thus designed specific experiments to examine whether the above side-effects of these compounds participate in their anti-muscarinic properties. A typical muscarinic inhibition of I_{Ca} is illustrated in the experiment of Figure 4A. As low as 10 nM ACh was able to antagonize almost completely the stimulating effect of a saturating concentration (30 μM) of Iso. However, application of 30 μM MBlue in the continuing presence of Iso and ACh quickly reversed most of the muscarinic inhibition of I_{Ca}. The anti-muscarinic effect of MBlue was mimicked by neither LY 83583 (30 μM) nor ODQ (10 μM). The results of several similar experiments are summarized in Figure 4B. In these experiments, Iso (30 μM) had a maximal stimulating effect, increasing I_{Ca} density from 1.80 ± 0.17 pA pF^{-1} to 13.06 ± 1.69 pA pF^{-1}, and addition of 10 nM ACh reduced the Iso-stimulated I_{Ca} to 3.12 ± 0.52 pA pF^{-1} ($n=13$). MBlue (30 μM) was the most potent anti-muscarinic agent since it antagonized almost completely the ACh-induced inhibition of I_{Ca}. By comparison, LY 83583 (30 μM) had a modest but significant effect, and ODQ (10 μM) failed to rescue the Iso-stimulated I_{Ca} from inhibition by ACh. In some experiments, exposure of the cells to 10 μM ODQ was prolonged for as long as 30 min but it still failed to alter the ACh inhibitory effect. Thus the rank order of potency of the guanylyl cyclase inhibitors to reverse the inhibitory effect of ACh on I_{Ca} was exactly opposite to that observed in the presence of SNAP.

Previous biochemical studies have shown that 10 nM of ACh may be too low to stimulate cyclic GMP production in cardiac preparations (Lindemann & Watanabe, 1991). We thus repeated the experiments with a higher ACh concentration (10 μM). However, in this case, we used a combination of Iso (1 μM) and forskolin (2 μM) to stimulate I_{Ca} in order to boost the adenylyl cyclase and prevent complete inhibition of cyclic AMP synthesis by ACh (Fischmeister & Shrier, 1989). Indeed, to regulate I_{Ca} in frog ventricular myocytes, cyclic GMP requires the presence of cyclic AMP (Hartzell & Fischmeister, 1986). In the experiment shown in Figure 5A, the combination of Iso and forskolin produced a large stimulation of I_{Ca}. Application of 10 μM ACh reduced by only $\approx 50\%$ this stimulation. Thus, adenylyl cyclase was not completely blocked by ACh and sufficient cyclic AMP was present in the myocyte. Superfusion of the myocyte with MBlue (30 μM) abolished the inhibitory effect of 10 μM ACh. LY 83583 (30 μM) also antagonized the effect of ACh on I_{Ca}, while 10 μM ODQ had no effect. The results of several similar experiments are summarized in Figure 5B. On average, the combination of 1 μM Iso and 2 μM forskolin enhanced I_{Ca} density from 3.92 ± 0.46 pA pF^{-1} to 38.70 ± 3.27 pA pF^{-1}, and I_{Ca} density decreased to 20.56 ± 2.52 pA pF^{-1} after addition of 10 μM ACh ($n=27$). In these experiments, external application of either MBlue (30 μM) or LY 83583 (30 μM) significantly reduced the muscarinic inhibition of I_{Ca}. These compounds were ineffective when used at concentrations below 3 μM ($n=2-3$), but at 10 μM, MBlue and LY 83583 reduced, respectively, by $50.72\pm14.24\%$ ($n=5$, $P<0.001$) and $25.93\pm3.83\%$ ($n=13$, $P<0.01$) the muscarinic inhibition of I_{Ca}. However, ODQ (10 μM) had no effect on the muscarinic inhibition of I_{Ca}, ruling out the participation of NO-sensitive guanylyl cyclase in these effects (Figure 5B).

Effects of guanylyl cyclase inhibitors on the muscarinic activation of $I_{K,ACh}$

Recently, Han *et al.* (1995) found that MBlue, but not LY 83583, abolished the muscarinic activation of $I_{K,ACh}$ in rabbit sinoatrial myocytes. This result raised the possibility that MBlue could act as a muscarinic receptor antagonist or interfered with the activation by the receptor of the G protein. For this reason, we examined the effect of the three guanylyl cyclase inhibitors on $I_{K,ACh}$ in frog atrial myocytes. As shown in Figure 6A, the steady-state currents recorded at -80, -50, and 0 mV were rapidly increased (within <8 s corresponding to the time interval between two consecutive pulses) in the outward direction upon application of 3 μM ACh. In this series of experiments, the end pulse current at 0 mV had a mean density of 1.62 ± 0.14 pA pF^{-1} ($n=31$), and was increased to 6.24 ± 1.57 pA pF^{-1} ($n=7$) and 8.84 ± 1.01 pA pF^{-1} ($n=18$) by 3 μM and 10 μM ACh, respectively. Like in rabbit sinoatrial cells (Han *et al.*, 1995), addition of 30 μM MBlue in the presence of ACh resulted in a total suppression of the slowly decaying $I_{K,ACh}$ (Figure 6A). This effect developed with similar kinetics as the activation of $I_{K,ACh}$ by ACh. The inhibitory effect of MBlue was reversed upon washout of the drug and $I_{K,ACh}$ recovered within <8 s from its inhibition, to an amplitude which was higher than the level of $I_{K,ACh}$ recorded just before application of MBlue (Figure 6A). Figure 6B summarizes the results of several similar experiments. In the absence of ACh, MBlue (30 μM) had no significant effect on the steady-state end pulse current recorded at any membrane potential (1.71 ± 0.54 pA pF^{-1} at 0 mV, $n=3$). However, MBlue produced a dose-dependent inhibition of the effect of ACh on $I_{K,ACh}$, with a threshold concentration of ≈ 3 μM and a complete block occurring at 30 μM (Figure 6B). The inhibitory effect of MBlue on $I_{K,ACh}$ was independent of membrane potential ($n=6$,

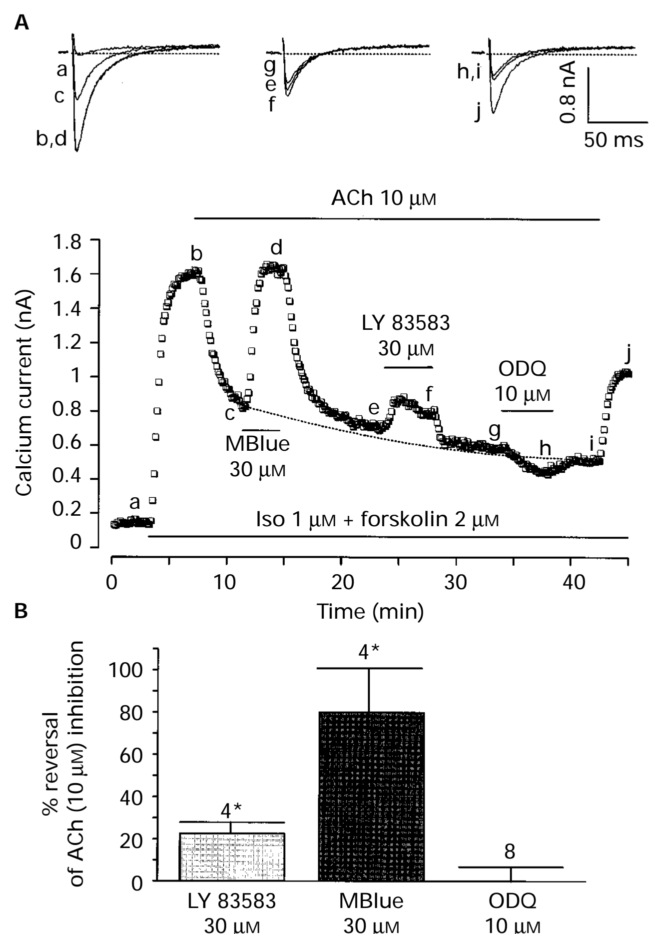

Figure 4 Effects of guanylyl cyclase inhibitors on the inhibition of I_{Ca} by a low concentration of acetylcholine (ACh). In (A), a frog ventricular cell was initially superfused with control Cs^+ solution and dialysed with control Cs^+ solution. For the periods indicated by the solid lines, the cell was successively superfused with methylene blue (MBlue, 30 μM), LY 83583 (30 μM) and ODQ (10 μM), in the presence of isoprenaline (Iso, 30 μM) and ACh (10 nM). The upper part of (A) shows the first 125 ms of individual I_{Ca} current traces recorded at the times indicated by the corresponding letters on the main graph. The dotted lines indicate the zero-current level. (B) A summary of the effects of guanylyl cyclase inhibitors on I_{Ca} in the presence of Iso (30 μM) plus ACh (10 nM). The data were normalized with respect to the amplitude of the muscarinic inhibition of I_{Ca}. The columns indicate the means$\pm$s.e.mean of the number of experiments indicated. Significant differences from the ACh level are indicated as: *$P<0.05$; **$P<0.01$.

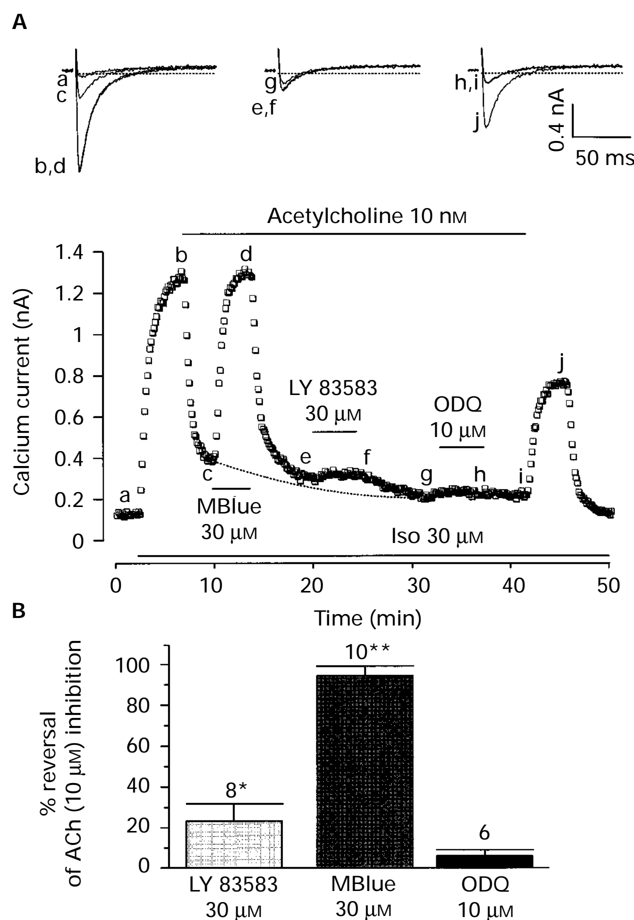

Figure 5 Effects of guanylyl cyclase inhibitors on the inhibition of I_{Ca} by a high concentration of acetylcholine (ACh). In (A), a frog ventricular cell was initially superfused with 10 μM ACh in the presence of isoprenaline (Iso, 1 μM) plus forskolin (2 μM) as indicated. MBlue (30 μM), LY 83583 (30 μM) or ODQ (10 μM) were added to the Iso + forskolin + ACh solution during the periods shown by the solid lines. The upper part of (A) shows the first 125 ms of individual I_{Ca} current traces recorded at the times indicated by the corresponding letters on the main graph. The dotted lines indicate the zero-current level. (B) A summary of the effects of guanylyl cyclase inhibitors on I_{Ca} in the presence of Iso (1 μM) plus forskolin (2 μM) and ACh (10 μM). The data were normalized with respect to the amplitude of the muscarinic inhibition of I_{Ca}. The columns indicate the means ± s.e.mean of the number of experiments indicated. Significant differences from the ACh level are indicated as: $^*P<0.05$; $^{**}P<0.01$.

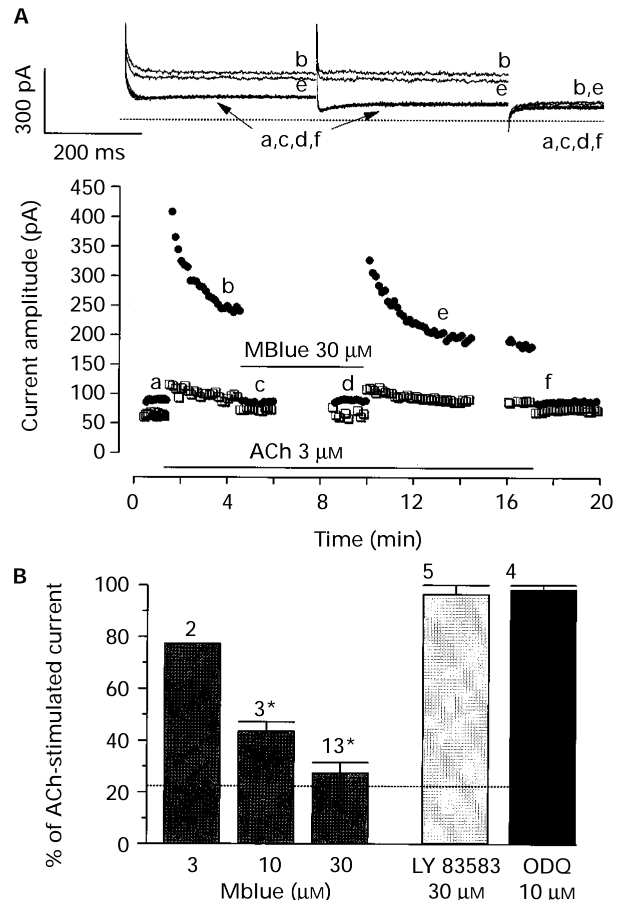

Figure 6 Effects of guanylyl cyclase inhibitors on the activation of $I_{K,ACh}$ by acetylcholine (ACh). (A) A frog atrial myocyte was initially superfused with control K^+ solution and internally dialysed with control K^+ solution. Each symbol is the measurement of the steady-state current at −80 mV (squares), or 0 mV (circles). Applications of ACh (3 μM) in the absence or presence of methylene blue (MBlue, 30 μM) are indicated by the solid lines. (Top) Individual current traces recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. (B) A summary of the effects of LY 83583, ODQ and of three concentrations of MBlue on $I_{K,ACh}$ in the presence of 3 or 10 μM ACh. The amplitude of the end pulse current at 0 mV in the presence of MBlue plus ACh was normalized with respect to the amplitude of the current in the presence of ACh alone. In (B), the dotted line indicates the mean basal level of the steady-state current at 0 mV, in the absence of any drug. The columns indicate the means ± s.e.mean of the number of experiments indicated. Significant differences from the ACh level are indicated as: $^*P<0.01$.

data not shown). As shown in Figure 6B, this anti-muscarinic effect of MBlue was not mimicked by either LY 83583 (30 μM) or ODQ (10 μM). Thus, MBlue, but not LY 83583 and ODQ, antagonizes the muscarinic activation of $I_{K,ACh}$.

Effect of MBlue on the GTPγS activation of $I_{K,ACh}$

To examine whether this inhibition takes place at the level of the receptor or at the level of the G protein, we examined the effects of MBlue on the activation of $I_{K,ACh}$ by intracellular dialysis with guanosine-5′-O-(γ-thiotriphosphate) (GTPγS). This hydrolysis resistant analogue of GTP leads to a maximal activation of $I_{K,ACh}$ even though the muscarinic receptor is free of agonist. In the experiment shown in Figure 7A, the patch pipette was filled with a GTPγS containing solution and the current recording was started just after rupture of the patch membrane. A spontaneous activation of $I_{K,ACh}$ was observed, due to the transfer of GTPγS to the G protein. When the current reached steady-state, application of ACh had a negligible additional effect, which was indicative of full activation of $I_{K,ACh}$ by GTPγS. However, addition of 30 μM MBlue inhibited the GTPγS-stimulated current by ≈40%. The results of several similar experiments are summarized in Figure 7B. In these experiments, the end pulse current at 0 mV had a mean density of 6.37 ± 0.64 pA pF^{-1} ($n=18$), after 7.48 ± 0.85 min dialysis with GTPγS. This current was similar in amplitude to the $I_{K,ACh}$ activated by a maximal concentration of ACh, in the presence of intracellular GTP. Application of ACh (10 μM) after the GTPγS-stimulated $I_{K,ACh}$ reached steady-state resulted in a small and irreversible additional increase in this current, to a density of 7.01 ± 0.73 pA pF^{-1} ($n=14$). MBlue induced a dose-dependent inhibition of the GTPγS-stimulated $I_{K,ACh}$ (Figure 7B), and the inhibition was similar whether ACh was present or not (data not shown). The maximal inhibitory effect observed at 30 μM MBlue was on average ≈20%, i.e. about 4 fold smaller than that seen under the normal activation of $I_{K,ACh}$ by ACh and GTP (Figure 6B). Thus, about 75% of the antagonistic effect of MBlue on the muscarinic activation of $I_{K,ACh}$ takes place before G protein activation, i.e. most likely at the muscarinic receptor level.

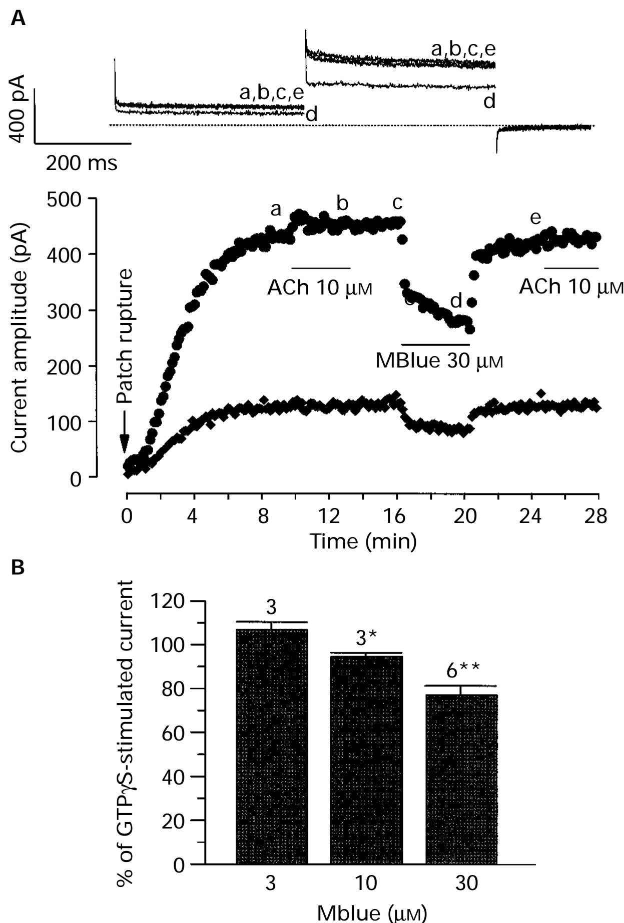

Figure 7 Effects of guanylyl cyclase inhibitors on the GTPγS-activation of $I_{K,ACh}$. (A) Currents were recorded immediately after the rupture of the patch membrane in a frog atrial myocyte superfused with control Cs^+ solution and internally dialysed with internal GTPγS-containing K^+ solution. Each symbol is the measurement of the steady-state current at −50 mV (diamonds) or 0 mV (circles). ACh (10 μM) and MBlue (30 μM) were applied to the cell as indicated by the solid lines. (Top) Individual current traces recorded at the times indicated by the corresponding letters on the main graph. The dotted line indicates the zero-current level. (B) A summary of the effects of MBlue on the GTPγS-activated $I_{K,ACh}$, in the absence of ACh. The amplitude of the end pulse current at 0 mV in the presence of MBlue was normalized with respect to the amplitude of the current in the presence of GTPγS alone. The columns indicate the means±s.e.mean of the number of experiments indicated. Significant differences from the GTPγS level are indicated as: $*P<0.05$; $**P<0.01$.

Role of superoxide anion in the effects of MBlue and LY 83583

MBlue and LY 83583 are superoxide anion generators (Barbier & Lefebvre, 1992; Marczin *et al*., 1992). Thus, one may question whether the release of superoxide anions by these compounds accounts for some of the side effects described above. In a series of experiments, MBlue and LY 83583 were applied in the presence of either dithiothreitol (DTT), a reducing agent, or a combination of superoxide dismutase (SOD) and catalase which removes superoxide anions. At 100 μM, DTT had no effect on the inhibitory effect of 30 μM MBlue on ACh-evoked $I_{K,ACh}$ ($n=3$). Similarly, SOD (150 u ml^{-1}) plus catalase (100 u ml^{-1}) did not change the antagonistic effect of MBlue on either ACh-evoked $I_{K,ACh}$ ($n=2$) or the ACh-inhibition of I_{Ca} ($n=2$). However, the potentiation of the Iso-stimulated I_{Ca} by LY 83583 (30 μM) was found to be sensitive to the reducing agents. In 5 ventricular myocytes, I_{Ca} was enhanced from a basal level of 2.44 ± 0.24 pA pF^{-1} to 8.32 ± 1.33 pA pF^{-1} in the presence of 20 nM Iso, and LY 83583 (30 μM) induced an additional $38.76\pm3.98\%$ ($P<0.01$) increase in the Iso-stimulated I_{Ca}. However, in the presence of 100 μM DTT, which by itself increased the Iso-response by $98.32\pm13.69\%$ ($P<0.001$), LY 83583 induced a strong inhibition of I_{Ca} to a level which was at $85.64\pm6.02\%$ of the Iso-stimulated level in the absence of DTT (different from the mean Iso and Iso plus DTT amplitudes at the 0.05 and 0.001 level, respectively). These experiments suggest that DTT prevents the stimulating effect of LY 83583 on the β-adrenoceptor cascade and unveils a strong inhibitory effect which was only occasionally observed in the absence of DTT (see above). However, this interpretation is complicated by the fact that DTT had a strong stimulating effect on its own on the Iso-stimulation of I_{Ca}.

The effect of a combination of SOD (150 u ml^{-1}) and catalase (100 u ml^{-1}) on the response of the frog ventricular myocyte to LY 83583 were examined in 5 other experiments. Addition of SOD and catalase potentiated the Iso (20 nM)-stimulated I_{Ca} by $32.86\pm8.71\%$ ($P<0.001$), an effect which was somewhat smaller than the effect of 30 μM LY 83583 in these cells ($62.14\pm4.48\%$ increase over Iso, $P<0.001$). However, the two stimulating effects were not additive, since the combination of LY 83583 with SOD/catalase increased I_{Ca} by $53.63\pm19.86\%$ over the Iso-stimulated level ($P<0.05$). Altogether, these data suggest that superoxide anion is involved in the stimulating effect of LY 83583 on I_{Ca}. Superoxide anion may also be involved in the anti-muscarinic effect of LY 83583, since DTT (100 μM) reduced the antagonizing effect of LY 83583 on the ACh inhibition of I_{Ca} ($n=2$, data not shown).

Discussion

This study describes multiple effects of guanylyl cyclase inhibitors on I_{Ca} and $I_{K,Ach}$, two ionic currents which play a major role in the control of cardiac excitability. As summarized in Table 1, each compound had a unique profile of action and only ODQ behaved in a manner which was consistent with its inhibitory action on guanylyl cyclase.

We have demonstrated earlier that the inhibitory effect of NO-donors on I_{Ca} in frog ventricular myocytes is a result of cyclic GMP production (Méry *et al*., 1993) and activation of the cyclic GMP-stimulated PDE (PDE2) (Hartzell & Fischmeister, 1986; Méry *et al*., 1995). Like other NO donors, SNAP stimulates guanylyl cyclase activity in cardiac myocytes and is able to inhibit I_{Ca} when used in the micromolar range of concentrations (Kojda *et al*., 1996, and this study). Since we found here that ODQ (i) had no effect on basal I_{Ca}, (ii) did not interfere with the stimulation of I_{Ca} by either Iso, forskolin or cyclic AMP, (iii) did not modify the muscarinic activation of $I_{K,ACh}$, but only (iv) antagonized the SNAP-induced inhibition of I_{Ca}, we conclude that ODQ inhibits efficiently the NO-sensitive guanylyl cyclase in frog ventricular myocytes and exerts no additional side effects in our experimental conditions. These results are in agreement with previous studies on the effects of ODQ on cyclic GMP and cyclic AMP pathways (Garthwaite *et al*., 1995; Cellek *et al*., 1996).

Like ODQ, LY 83583 antagonized the inhibitory effect of SNAP on the β-adrenoceptor stimulation of I_{Ca}. This compound is a superoxide anion generator (Barbier & Lefebvre, 1992; Marczin *et al*., 1992) and has been shown to affect the NO pathway in several ways, including through inhibition of the NO-sensitive guanylyl cyclase (Schmidt *et al*., 1985; Mülsch *et al*., 1988). For instance, LY 83583 has been shown to antagonize the regulation of cardiac cell volume by atrial natriuretic factor (ANF), presumably through the blockade of the NO-insensitive guanylyl cyclase (Clemo *et al*., 1992). However, for several reasons, guanylyl cyclase inhibition was unlikely to be the predominant mechanism of action of LY 83583 in our study. First, unlike ODQ and MBlue, LY 83583 consistently potentiated the stimulating effects of endogenous or exogenous cyclic AMP on I_{Ca} (see also Zakharov *et al*., 1996). Second, this effect was observed when the compound was applied extracel-

Table 1 Summary of the effects of guanylyl cyclase inhibitors on I_{Ca}

	Isoprenaline	*Forskolin*	*Cyclic AMP*	*Iso+SNAP*	*Iso+ACh*	*Iso-Fors+ACh*
LY 83583	+/−	+	+	+	+	+
Methylene blue	−	NS	NS	−	++	++
ODQ	NS	NS	NS	++	NS	NS

+ or − refer to the effects of the compounds on the amplitude of I_{Ca}. Iso, isoprenaline; SNAP, S-nitroso-N-acetylpenicillamine; Fors, forskolin.

luarly, but not when the drug was introduced directly into the cytoplasm via the patch pipette which should facilitate its action on guanylyl cyclase. Third, DTT reduced the stimulating effect of LY 83583 on I_{Ca}, although DTT has previously been shown to potentiate the inhibitory effect of LY 83583 on guanylyl cyclase activity (Mülsch *et al*., 1988). LY 83583 has also been found to stimulate I_{Ca} in the presence of ACh (Mubagawa *et al*., 1994; Han *et al*., 1995; 1996; this study). However, guanylyl cyclase inhibition does also not provide a satisfactory mechanism for this effect, since (i) LY 83583 exerted its effect at a nanomolar concentration of ACh which was probably too low to activate the guanylyl cyclase (Lindemann & Watanabe, 1991) and (ii) the effect of LY 83583 was not increased when ACh was used at micromolar concentrations.

The other superoxide anion generator, MBlue, was found to antagonize almost equally well the effects of Iso and ACh on I_{Ca}. However, guanylyl cyclase inhibition was unlikely to be responsible for this effect, since MBlue did not antagonize the inhibitory effect of SNAP on I_{Ca}. Previous biochemical studies have demonstrated that MBlue is a weak inhibitor of NO-sensitive guanylyl cyclase (Mayer *et al*., 1993). Furthermore, we found here that MBlue had dramatic effects on the muscarinic receptor activation of $I_{K,ACh}$, an effect which is clearly not mediated by the cyclic GMP/NO-pathway (reviewed in Fischmeister & Méry, 1996, and see Han *et al*., 1995). Most likely, the effects of MBlue take place at the level of the muscarinic receptor and β-adrenoceptor. Indeed, MBlue had no effect on forskolin-stimulated I_{Ca} and its inhibitory effect on $I_{K,ACh}$ was greatly reduced in the presence of intracellular GTPγS. Binding experiments are needed to identify more precisely the mechanisms of interaction of MBlue with membrane receptors in cardiac myocytes.

MBlue or LY 83583 have been used in previous studies to show that guanylyl cyclase participates in the muscarinic regulation of mammalian heart function (reviewed in Fischmeister & Méry, 1996; Keely *et al*., 1996). These include the regulation of cardiac inotropism and chronotropism and, at the level of the isolated myocyte, the regulation of I_{Ca}. Our study demonstrated that MBlue and LY 83583 may exert several deleterious side effects which are unrelated to inhibition of guanylyl cyclase (see also Schmidt *et al*., 1985; Mironov & Lux, 1991; Barbier & Lefebvre, 1992; Marczin *et al*., 1992; Lüönd *et al*., 1993; Mayer *et al*., 1993). Since ODQ appears to be the most selective tool among the three potential guanylyl cyclase inhibitors tested so far, it should be used in mammalian myocytes to clarify the functional consequences of cyclic GMP production inthe cardiac effects of ACh. In frog ventricular myocytes, ODQ was unable to modify the inhibitory effect of ACh on I_{Ca}, suggesting that NO-sensitive guanylyl cyclase is not involved in the muscarinic regulation of I_{Ca} in this preparation. In a recent study of ours, we have shown that NO-synthase activity also does not play a significant role in the muscarinic regulation of I_{Ca} and contractility in the frog ventricle (Méry *et al*., 1996). Thus, a putative coupling between muscarinic receptors and cyclic GMP production remains to be established in frog ventricular myocytes. Nevertheless, our studies indicate that the muscarinic regulation of I_{Ca} and contraction is best explained by the inhibition of adenylyl cyclase in frog ventricular myocytes (Méry *et al*., 1996; Jurevicius & Fischmeister, 1996). Further studies will help to clarify whether this conclusion can be generalized to other species and/or cardiac cell types. Until now, contradictory results have appeared in the literature on that issue, with data in favour (Han *et al*., 1995; Kitakaze *et al*., 1995; Han *et al*., 1996; Keely *et al*., 1996) and others against (Kennedy *et al*., 1994; Nawrath *et al*., 1995; Zakharov *et al*., 1996) a determinant role of cyclic GMP/NO-pathways in the muscarinic regulation of mammalian heart contractility and I_{Ca}.

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