Journal of Physiology (2002), **542.3**, *pp.* 711–723
DOI: 10.1113/jphysiol.2002.023341

www.jphysiol.org

Nitric oxide signalling by selective β_2-adrenoceptor stimulation prevents ACh-induced inhibition of β_2-stimulated Ca^{2+} current in cat atrial myocytes

Elena N. Dedkova, Yong Gao Wang, Lothar A. Blatter and Stephen L. Lipsius

Loyola University Chicago, Stritch School of Medicine, Department of Physiology, Maywood, IL 60153, USA

The present study determined the effects of acetylcholine (ACh) on the L-type Ca^{2+} current ($I_{Ca,L}$) stimulated by β_1- or β_2-adrenergic receptor (AR) agonists in cat atrial myocytes. When isoproterenol (ISO; 0.1 μM) plus the β_2-AR antagonist ICI 118,551 (ISO-β_1-AR stimulation) or 0.1 μM fenoterol, a β_2-AR agonist (FEN-β_2-AR stimulation) increased $I_{Ca,L}$, ACh (1 μM) inhibited $I_{Ca,L}$ by −60 ± 4 and −63 ± 6 %, respectively. When ISO plus the β_1-AR antagonist atenolol (ISO-β_2-AR stimulation) or 1 μM zinterol (ZIN-β_2-AR stimulation) increased $I_{Ca,L}$, ACh-induced inhibition of $I_{Ca,L}$ was significantly smaller, at −21 ± 3 and −24 ± 3 %, respectively. L-N^5-(1-iminoethyl)-ornithine (L-NIO, 10 μM), an inhibitor of nitric oxide (NO) synthase, enhanced ACh-induced inhibition of $I_{Ca,L}$ when stimulated by ZIN-β_2-ARs, but not when stimulated by ISO-β_1-ARs or FEN-β_2-ARs. Haemoglobin (50 μM), a NO scavenger, also enhanced ACh-induced inhibition when $I_{Ca,L}$ was stimulated by ZIN-β_2-ARs, but not when stimulated by FEN-β_2-ARs. ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs was not affected by 10 μM 1H-[1,2,4] oxadiazolo[4,3-a] quinoxaline-1-one (ODQ) a guanylate cyclase inhibitor, but was significantly enhanced by 500 μM reduced glutathione or 100 μM dithiothreitol, agents that act as sinks for *S*-nitrosylation. ACh-induced inhibition was smaller when $I_{Ca,L}$ was stimulated by spermine/NO, a NO donor, than by milrinone, a phosphodiesterase type III inhibitor. ISO (ISO-β_1/β_2-AR stimulation) increased $I_{Ca,L}$ and even though ISO releases NO, ACh prominently inhibited $I_{Ca,L}$. This inhibitory effect of ACh was enhanced by L-NIO. Stimulation of ZIN-β_2-ARs increased intracellular NO, whereas ISO-β_1-ARs or FEN-β_2-ARs failed to increase intracellular NO. These results indicate that in atrial myocytes, NO released by selective β_2-AR stimulation prevents ACh-induced inhibition of $I_{Ca,L}$ stimulated by β_2-ARs. NO acts via a cGMP-independent, *S*-nitrosylation mechanism. Although FEN acts via β_2-ARs, it fails to stimulate G_i-/NO signalling and preferentially stimulates G_s-/adenylate cyclase signalling, similar to β_1-ARs. These findings indicate that NO signalling modulates muscarinic receptor inhibition of atrial function stimulated by β_2-ARs.

(Resubmitted 25 April 2002; accepted 17 May 2002)
Corresponding author S. L. Lipsius: Loyola University Chicago, Stritch School of Medicine, Department of Physiology, Maywood, IL 60153, USA. Email: slipsiu@lumc.edu

In general, acetylcholine (ACh) elicits an accentuated inhibition of cardiac chronotropic and inotropic activities that are stimulated by β-adrenergic receptor (β-AR) stimulation. ACh exerts its inhibitory effects through stimulation of M_2 muscarinic receptors coupled via G_i-proteins to inhibit adenylate cyclase. It has been proposed that nitric oxide (NO) signalling activated by ACh may also participate in the inhibitory effects of ACh, although this idea remains controversial (Han *et al.* 1998; Vandecasteele *et al.* 1999; Belevych & Harvey, 2000; Godecke *et al.* 2001). Although ACh inhibits stimulation by β-ARs, cardiac muscle contains different subtypes of β-ARs and each subtype exhibits significantly different signal transduction mechanisms (Steinberg, 1999; Xiao *et al.* 1999). For example, β_1-ARs act exclusively via G_s-proteins coupled to adenylate cyclase to catalyse the synthesis of cAMP, which in turn activates protein kinase (PK)A signalling. In contrast, in several animal species (Xiao *et al.* 1995; Kuschel *et al.* 1999; Wang *et al.* 2002), including humans (Kilts *et al.* 2000), β_2-ARs couple to a variety of signalling pathways via both G_s- and G_i-proteins. In cat atrial myocytes, β_2-ARs couple via G_s-proteins to adenylate cyclase and via G_i-proteins to phosphatidylinositol 3′-kinase (PI-3K) signalling to release intracellular NO (Wang *et al.* 2002). In cardiac muscle, NO signalling operates primarily via two basic biochemical mechanisms: (1) stimulation of guanylate cyclase/cGMP production and (2) nitrosylation of sulfhydryl groups on the cysteine residues of various proteins (Broillet, 1999). It is now recognized that *S*-nitrosylation (cGMP-independent) reactions can modulate a wide variety of cellular functions (Broillet, 1999; Stamler *et al.* 2001). Together, these

considerations raise the question of how stimulation via different β-AR subtypes and agonists respond to inhibition by muscarinic receptor stimulation. In neonatal rat ventricular myocytes, muscarinic receptor stimulation inhibits cAMP accumulation and positive inotropic responses elicited by β_1-ARs but not by β_2-AR stimulation (Aprigliano *et al.* 1997). The purpose of the present study was therefore to determine whether there are differential inhibitory effects of ACh on $I_{Ca,L}$ when $I_{Ca,L}$ is prestimulated by either β_1- or β_2-AR agonists in adult cat atrial myocytes and, if so, the nature of the underlying mechanism.

METHODS

Adult cats of either gender were anaesthetized with sodium pentobarbital (50 mg kg^{-1}; I.P.). Once anaesthetized, a midsternal thoracotomy was performed and the heart rapidly excised. The heart was mounted on a Langendorff perfusion apparatus and atrial myocytes were dispersed by digestion with collagenase (type II, Worthington Biochemical), as reported previously (Wu *et al.* 1991). No discernable differences were noted between left and right atrial myocytes. Cells used for studies were transferred to a small tissue bath on the stage of an inverted microscope (Nikon Diaphot) and superfused with a Hepes-buffered, modified Tyrode solution containing (mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, Hepes 5, glucose 11, titrated with NaOH to pH 7.4. Solutions were perfused by gravity and experiments were performed at 35 ± 1°C. Atrial myocytes selected for study were elongated and quiescent. Voltage and ionic currents were recorded using a nystatin (150 μg ml^{-1})-perforated-patch (Horn & Marty, 1988) whole-cell recording method (Hamill *et al.* 1981). This method minimizes dialysis of intracellular constituents with the internal pipette solution and thereby preserves the physiological milieu and second-messenger signalling pathways. The internal pipette solution contained (mM): caesium glutamate 100, KCl 40, $MgCl_2$ 1.0, Na_2-ATP 4, EGTA 0.5, Hepes 5, titrated with KOH to pH 7.2. In addition to Cs^+ in the pipette solution, 5 mM CsCl was added to all solutions to block K^+ conductances. A single suction pipette was used to record voltage (bridge mode) or ionic currents (discontinuous voltage-clamp mode) using an Axoclamp 2A amplifier (Axon Instruments). Computer software (pCLAMP; Axon Instruments) was used to deliver the voltage protocols and to acquire and analyse data. $I_{Ca,L}$ was activated by depolarizing pulses from a holding potential of −40 to 0 mV for 200 ms every 10 s. Peak $I_{Ca,L}$ amplitude was measured with respect to steady-state current and not compensated for leak currents. Increases in peak $I_{Ca,L}$ amplitude induced by β-AR stimulation were determined with respect to basal $I_{Ca,L}$ amplitude. Changes in $I_{Ca,L}$ amplitude induced by ACh were determined as a percentage of the changes in $I_{Ca,L}$ amplitude induced by each β-AR agonist (see legend to Fig. 1).

Direct measurements of intracellular NO concentration ($[NO]_i$) were obtained by incubating cells with the fluorescent NO-sensitive dye 4,5-diaminofluorescein (DAF-2) (Kojima *et al.* 1998; Nakatsubo *et al.* 1998; Wang *et al.* 2002). Experiments were performed at room temperature. Cells were exposed to the membrane-permeant DAF-2 diacetate (5 μM; Calbiochem, San Diego, CA, USA) for 10 min at room temperature in standard Tyrode solution. Cells were subsequently washed for 10 min in Tyrode solution containing 100 μM L-arginine. DAF-2 fluorescence was excited at 480 nm. Emitted cellular fluorescence was recorded at 540 nm. Single-cell fluorescence signals were recorded with a photomultiplier tube (model R2693, Hamamatsu) by masking individual cells with an iris positioned in the emission path. Changes in cellular DAF-2 fluorescence intensities (F) in each experiment were normalized to the level of fluorescence recorded prior to stimulation (F_0), and changes in $[NO]_i$ are expressed as F/F_0. In the experiments designed to measure $[NO]_i$, solutions contained 100 μM L-arginine. Cells were field stimulated at 1 Hz.

Selective stimulation of β_1-ARs or β_2-ARs was accomplished as follows: 0.1 μM isoproterenol (isoprenaline; ISO), a mixed β_1-/β_2-AR agonist, in the presence of 0.01 μM atenolol, a selective β_1-AR antagonist (ISO-β_2-AR stimulation); 0.1 μM ISO plus 0.01 μM ICI 118,551, a selective β_2-AR antagonist (ISO-β_1-AR stimulation); 1 μM zinterol (ZIN), a selective β_2-AR agonist (ZIN-β_2-AR stimulation); 0.1 μM fenoterol (FEN), another selective β_2-AR agonist (FEN-β_2-AR stimulation). Previous work has shown that the stimulatory effects of ISO on $I_{Ca,L}$ are abolished by the combined exposure to atenolol plus ICI 118,551 and that BRL 37344, a specific β_3-AR agonist, has no effect on $I_{Ca,L}$ (Wang *et al.* 2002).

Drugs used in this study include ISO, acetylcholine chloride, FEN, atenolol, haemoglobin, L-N^5-(1-iminoethyl)ornithine (L-NIO), 1H-[1,2,4] oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), reduced glutathione (GSH), dithiothreitol (DTT), spermine/NO (SNO), milrinone (all from Sigma); Rp-cAMPs (LC Laboratories); ZIN (provided by Bristol-Myers Squibb, Princeton, NJ, USA) and ICI 118,551 (provided by AstraZeneca, Wilmington, DE, USA).

In general, results were obtained in cells isolated from the same hearts studied under control and test conditions. Data from two groups of cells were analysed using Student's unpaired t test with significance at $P < 0.05$. Data from multiple groups were analysed using a one-way analysis of variance (ANOVA) and Bonferroni test at $P < 0.05$.

The animal procedures used in this study were performed in accordance with the guidelines of the Animal Care and Use Committee of Loyola University Medical Center.

RESULTS

Figure 1*A*–*D* shows the effects of ACh on $I_{Ca,L}$ pre-stimulated by various β-AR agonists. Each panel shows selected recordings of peak $I_{Ca,L}$ obtained at different times (labelled a–c) during each experiment and consecutive measurements of peak $I_{Ca,L}$ throughout each experiment. ISO-β_1-AR stimulation increased $I_{Ca,L}$ (256 %) and the addition of ACh prominently inhibited $I_{Ca,L}$ (−61 %; Fig. 1*A*). ISO-β_2-AR stimulation also increased $I_{Ca,L}$ (282 %), and yet ACh elicited a much smaller inhibition of $I_{Ca,L}$ (−19 %; Fig. 1*B*) compared with the effects of ACh on $I_{Ca,L}$ stimulated by ISO-β_1-ARs. Likewise, ZIN-β_2-AR stimulation increased $I_{Ca,L}$ (182 %), and once again ACh elicited a relatively small inhibition of $I_{Ca,L}$ (−22 %; Fig. 1*C*). FEN-β_2-AR stimulation also increased $I_{Ca,L}$ (233 %; Fig. 1*D*). However, unlike the response of $I_{Ca,L}$ stimulated by either ISO-β_2-ARs or ZIN-β_2-ARs, ACh markedly inhibited $I_{Ca,L}$ (−58 %) stimulated by FEN-β_2-ARs. The effects of FEN to stimulate $I_{Ca,L}$ were abolished by 0.1 μM ICI 118,551 (control, 320 ± 47 % *vs.* + ICI, 5 ± 9 %; $n = 4$), and abolished by 50 μM Rp-cAMPs, a specific inhibitor of cAMP-dependent PKA activity (control, 201 ± 43 % *vs.* + Rp-cAMPs, 4 ± 7 %;

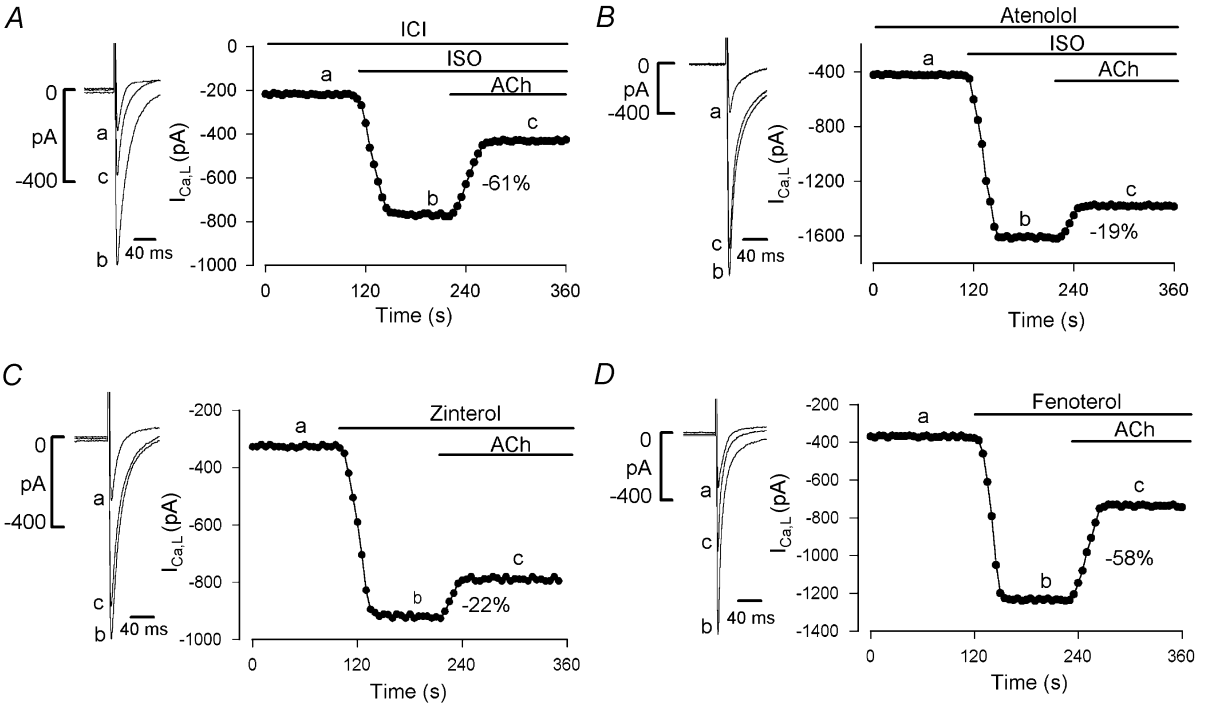

Figure 1. Effects of 1 μM ACh on L-type Ca^{2+} current ($I_{Ca,L}$) stimulated by various β-adrenergic receptor (AR) agonists

Effect of 1 μM ACh on $I_{Ca,L}$ stimulated by isoproterenol (ISO)-β_1-ARs (*A*), ISO-β_2-ARs (*B*), zinterol (ZIN)-β_2-ARs (*C*) and fenoterol (FEN)-β_2-ARs (*D*). Each panel shows original traces of peak $I_{Ca,L}$ during each phase (a–c) of the experiment and a graph of consecutive measurements of peak $I_{Ca,L}$ throughout the experiment. ACh-induced inhibition of $I_{Ca,L}$ was relatively large when $I_{Ca,L}$ was prestimulated by ISO-β_1-ARs (*A*; −61 %) or FEN-β_2-ARs (*D*; −58 %), and significantly smaller when $I_{Ca,L}$ was prestimulated by ISO-β_2-ARs (*B*; −19 %) or ZIN-β_2-ARs (*C*; −22 %). The percentage inhibition of $I_{Ca,L}$ induced by ACh was determined as the change in $I_{Ca,L}$ induced by ACh (b − c) in relation to the stimulated change in $I_{Ca,L}$ induced by the β-AR agonist (b − a) using the formula: % = (b − c)/(b − a) × 100. ICI = ICI 118,551, a selective β_2-AR antagonist.

$n = 5$; data not shown). These latter findings confirm that FEN stimulates $I_{Ca,L}$ specifically via β_2-AR-mediated activation of cAMP/PKA activity.

Figure 2*A* summarizes the ability of each β-AR agonist to stimulate $I_{Ca,L}$ (open bars) and the ability of ACh to inhibit each β-AR agonist-stimulated $I_{Ca,L}$ (hatched bars). Each β-AR agonist induced a prominent increase in $I_{Ca,L}$ amplitude. Note that stimulation of $I_{Ca,L}$ by FEN-β_2-ARs was essentially of the same order of magnitude as that induced by ISO-β_2-AR stimulation. ACh-induced inhibition of $I_{Ca,L}$ was largest when $I_{Ca,L}$ was stimulated by either ISO-β_1-ARs (−60 ± 4 %; $n = 16$) or FEN-β_2-ARs (−63 ± 6 %; $n = 12$). These values were not significantly different from each other. The inhibitory effects of ACh were smallest when $I_{Ca,L}$ was stimulated by either ISO-β_2-ARs (−21 ± 3 %; $n = 11$) or ZIN-β_2-ARs (−24 ± 3 %; $n = 23$). Again, these values were not different from each other. However, the

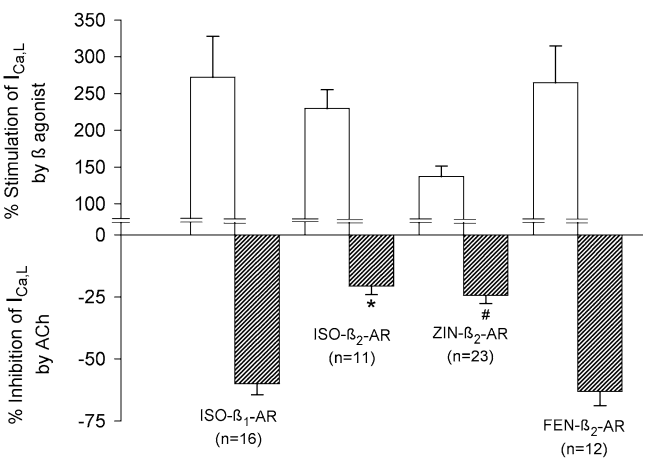

Figure 2. Summary of the effects of each β-AR agonist to stimulate $I_{Ca,L}$ (open bars) and ACh-induced inhibition of $I_{Ca,L}$ prestimulated by each β-AR agonist (hatched bars)

Each β-AR agonist stimulated $I_{Ca,L}$. ACh-induced inhibition of $I_{Ca,L}$ was largest when $I_{Ca,L}$ was stimulated by ISO-β_1-ARs or FEN-β_2-ARs, and significantly smaller when $I_{Ca,L}$ was stimulated by ISO-β_2-ARs or ZIN-β_2-ARs. Note that the ability of ACh to inhibit $I_{Ca,L}$ was not significantly different when $I_{Ca,L}$ was stimulated by either ISO-β_1-ARs or FEN-β_2-ARs. The numbers in parentheses indicate the number of cells studied. *$P < 0.05$ comparing ISO-β_2-AR *vs.* ISO-β_1-AR or FEN-β_2-AR responses. #$P < 0.05$ comparing ZIN-β_2-AR *vs.* ISO-β_1-AR or FEN-β_2-AR responses.

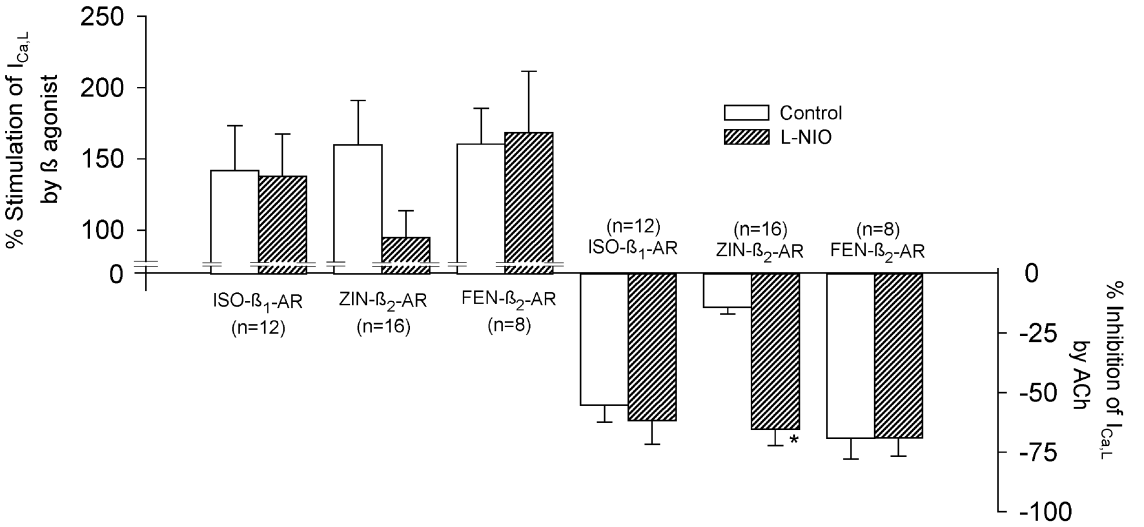

Figure 3. Summary of the effects of each β-AR agonist to stimulate $I_{Ca,L}$ and ACh-induced inhibition of $I_{Ca,L}$ prestimulated by each β-AR agonist in the absence (open bars) and presence (hatched bars) of 10 μM L-N^5-(1-iminoethyl)ornithine (L-NIO)

L-NIO had no significant effect on ISO-β_1-AR- or FEN-β_2-AR-induced stimulation of $I_{Ca,L}$, and L-NIO attenuated ZIN-β_2-AR-induced stimulation of $I_{Ca,L}$. Likewise, L-NIO had no significant effect on ACh-induced inhibition of $I_{Ca,L}$ stimulated by either ISO-β_1-ARs or FEN-β_2-ARs, but L-NIO significantly enhanced the ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs. The numbers in parentheses indicate the number of cells studied. $^*P < 0.05$.

inhibitory effects of ACh on $I_{Ca,L}$ stimulated by either ISO-β_2-ARs or ZIN-β_2-ARs were significantly smaller than the inhibitory effects of ACh when $I_{Ca,L}$ was stimulated by either ISO-β_1-ARs or FEN-β_2-ARs. It should be noted that the magnitude of the inhibitory effect of ACh on $I_{Ca,L}$ was not dependent upon the basal (prestimulated) $I_{Ca,L}$ amplitude or the absolute current level to which $I_{Ca,L}$ was stimulated by a given β-AR agonist. Moreover, when $I_{Ca,L}$ was stimulated via β_2-ARs using either ISO or ZIN, ACh-induced inhibition of $I_{Ca,L}$ was approximately of the same order of magnitude as the effects of ACh to inhibit basal (unstimulated) $I_{Ca,L}$ (Wang & Lipsius, 1995; Wang *et al.* 1998). This suggests that ACh does not exert any significant inhibition of $I_{Ca,L}$ that can be attributed to the stimulation of $I_{Ca,L}$ by either ISO-β_2-ARs or ZIN-β_2-ARs.

Previous results indicate that in cat atrial myocytes, intracellular NO release is regulated differentially by stimulation of different β-AR subtypes (Wang *et al.* 2002). We therefore examined whether the NO that is activated by β-AR stimulation influences ACh-induced inhibition of stimulated $I_{Ca,L}$. The data shown in Fig. 2 indicate that the ability of ACh to inhibit $I_{Ca,L}$ is essentially the same when $I_{Ca,L}$ is stimulated by either ISO-β_2-ARs or ZIN-β_2-ARs. Therefore, because of its greater selectivity for β_2-ARs, of

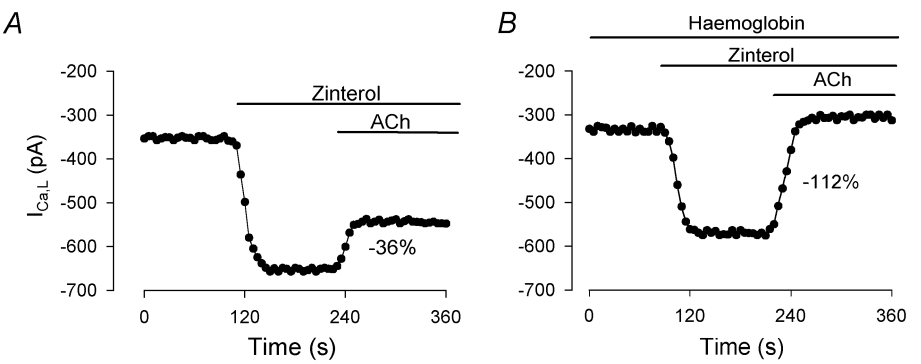

Figure 4. ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs in the absence (*A*) and presence (*B*) of 50 μM haemoglobin

A, in the absence of haemoglobin, ACh induced a typical inhibition of $I_{Ca,L}$ (−36 %) stimulated by ZIN-β_2-ARs. *B*, in another atrial cell, the presence of haemoglobin enhanced the ACh-induced inhibition of $I_{Ca,L}$ (−112 %) stimulated by ZIN-β_2-ARs. Data from these two cells were selected to illustrate that the effects of ACh cannot be attributed to significant differences in basal $I_{Ca,L}$ or ZIN-β_2-AR-stimulated $I_{Ca,L}$ amplitudes.

the two agonists only ZIN was used in the following experiments. We tested the effects of ACh on $I_{Ca,L}$ stimulated by ISO-β_1-ARs, ZIN-β_2-ARs or FEN-β_2-ARs in the absence and presence of 10 μM L-NIO, an inhibitor of endothelial NO synthase (eNOS; Rees *et al.* 1990). The graph in Fig. 3 summarizes both the stimulatory effects of each β-AR on $I_{Ca,L}$ (upper bars) and the inhibitory effects of ACh on $I_{Ca,L}$ stimulated by each agonist (lower bars) in the absence (open bars) and presence (hatched bars) of L-NIO. L-NIO alone had little effect on stimulation of $I_{Ca,L}$ induced by ISO-β_1-AR or FEN-β_2-AR stimulation, indicating that NO signalling does not participate in the effects of these agonists to stimulate $I_{Ca,L}$. However, L-NIO attenuated the stimulation of $I_{Ca,L}$ induced by stimulation of ZIN-β_2-ARs (ZIN, 160 ± 31 % *vs.* ZIN + L-NIO, 95 ± 19 %). These findings are consistent with the idea that ZIN acts via NO signalling to partially stimulate $I_{Ca,L}$ (Wang *et al.* 2002). Moreover, these results agree with previous findings that ZIN-β_2-ARs stimulate $I_{Ca,L}$ via two cAMP-dependent signalling pathways; G_s-/adenylate cyclase signalling and G_i-/PI-3K/NO signalling (Wang *et al.* 2002). L-NIO had little effect on ACh-induced inhibition of $I_{Ca,L}$ stimulated by either ISO-β_1-ARs or FEN-β_2-ARs. However, L-NIO significantly enhanced ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs (ACh, −14 ± 3 % *vs.* ACh + L-NIO, −65 ± 7 %; $P < 0.001$). Note that in the presence of L-NIO, ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs was similar in magnitude to that obtained when $I_{Ca,L}$ was stimulated by ISO-β_1-ARs or FEN-β_2-ARs. These findings are consistent with the idea that NO signalling induced by ZIN-β_2-ARs is responsible for the inability of ACh to effectively inhibit $I_{Ca,L}$ stimulated by ZIN-β_2-ARs. In contrast, stimulation of ISO-β_1-ARs or FEN-β_2-ARs does not induce NO signalling, and therefore the inhibitory effects of ACh are unaffected by inhibition of eNOS. This latter finding also indicates that NO signalling is not involved in ACh-induced inhibition of $I_{Ca,L}$ stimulated by these β-AR agonists.

To examine further the role of endogenous NO release, we tested the effects of haemoglobin, a potent NO scavenger, on ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs. Our previous studies have shown that in cat atrial myocytes extracellular haemoglobin abolishes the regulation of $I_{Ca,L}$ mediated by endogenous NO signalling (Wang *et al.* 1998). As shown in Fig. 4*A*, in the absence of haemoglobin, 1 μM ACh elicited a typical inhibition of $I_{Ca,L}$ (−36 %) stimulated by ZIN-β_2-ARs. In another atrial cell from the same heart (Fig. 4*B*), the presence of 50 μM haemoglobin markedly enhanced the ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs (−112 %). In this experiment, the presence of haemoglobin allowed ACh to completely inhibit the ZIN-β_2-AR-mediated stimulation of $I_{Ca,L}$ as well as some basal $I_{Ca,L}$. The data shown in Fig. 4*A* and *B* were chosen from two cells in which the amplitudes of initial basal $I_{Ca,L}$ and the ZIN-β_2-AR-stimulated $I_{Ca,L}$ were approximately the same in the absence and presence of haemoglobin. This was done to illustrate that the greater inhibitory effect of ACh on $I_{Ca,L}$ in the presence of haemoglobin was not due to any differences in basal $I_{Ca,L}$ or ZIN-stimulated $I_{Ca,L}$ amplitude. In total, the ACh-induced

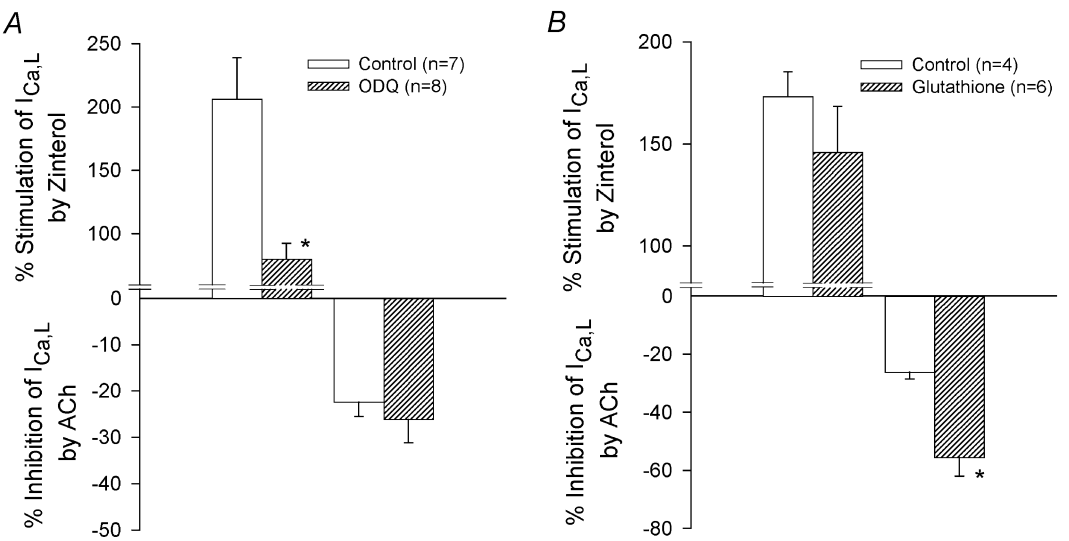

Figure 5. ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs in the absence (open bars) and presence (hatched bars) of 1H-[1,2,4] oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; *A*) and glutathione (*B*)

A, compared with control, 10 μM ODQ attenuated the stimulation of $I_{Ca,L}$ by ZIN-β_2-ARs. However, ODQ did not significantly affect the ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs. *B*, compared with control, 500 μM glutathione had no significant effect on $I_{Ca,L}$ stimulated by ZIN-β_2-ARs. However, glutathione significantly enhanced ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs. *$P < 0.05$.

inhibition of ZIN-stimulated $I_{Ca,L}$ was significantly larger in the presence (−85 ± 9 %; $n = 8$) than in the absence (−36 ± 5 %; $n = 5$) of haemoglobin ($P < 0.05$). In contrast, additional experiments showed that ACh-induced inhibition of $I_{Ca,L}$ stimulated by FEN-β_2-ARs was not significantly different in the absence (−58 ± 11 %; $n = 4$) or presence (−54 ± 3 %; $n = 4$) of 50 μM haemoglobin (data not shown). These findings further establish that stimulation of ZIN-β_2-ARs but not FEN-β_2-ARs acts via NO signalling to prevent ACh-induced inhibition of $I_{Ca,L}$.

A primary target of NO is guanylate cyclase (and thus the production of cGMP). Therefore, to determine the potential role of downstream cGMP signalling, we tested the effects of ACh on $I_{Ca,L}$ stimulated by ZIN-β_2-ARs in the absence and presence 10 μM ODQ, an inhibitor of guanylate cyclase (Brunner *et al.* 1996). Our previous work has shown that in cat atrial myocytes, ODQ inhibits NO signalling mechanisms that are mediated via cGMP (Wang *et al.* 1998). As shown in Fig. 5*A*, ODQ alone significantly decreased ZIN-mediated stimulation of $I_{Ca,L}$ (ZIN, 206 ± 33 % *vs.* ZIN + ODQ, 80 ± 12 %). Again, this is consistent with previous findings that the stimulatory effects of ZIN on $I_{Ca,L}$ are mediated partially via NO-cGMP-induced inhibition of phosphodiesterase (PDE) type III with the resulting increase in cAMP (Wang *et al.* 2002). However, there was no significant difference between the inhibitory effects of ACh on ZIN-stimulated $I_{Ca,L}$ in the absence (−22 ± 3 %) or presence (−26 ± 5 %) of ODQ. These findings suggest that NO signalling induced by ZIN is operating to prevent the effects of ACh via a cGMP-independent mechanism.

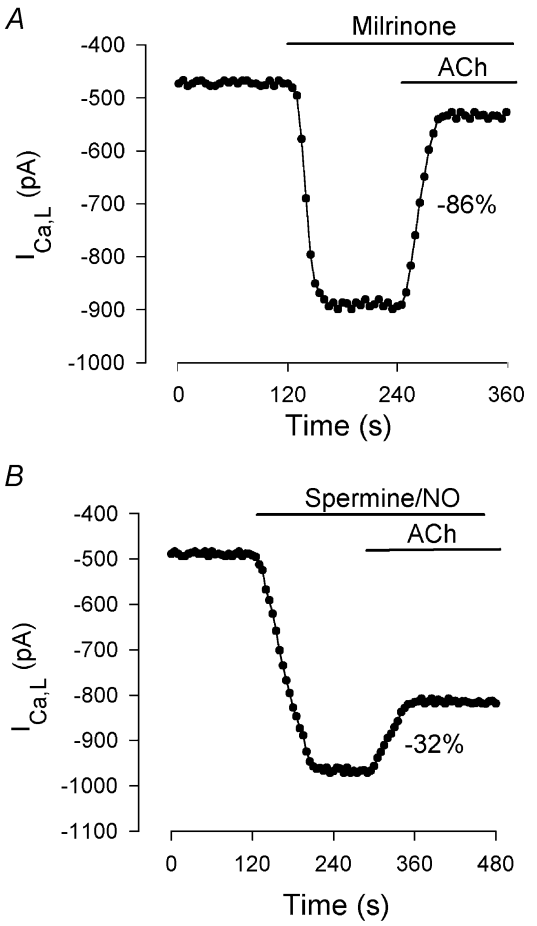

Figure 6. ACh-induced inhibition of $I_{Ca,L}$ when $I_{Ca,L}$ is stimulated by either 5 μM milrinone (*A*) or 100 μM spermine/nitric oxide (NO; *B*)

Graphs show consecutive measurements of peak $I_{Ca,L}$ amplitude throughout each experiment. During stimulation of $I_{Ca,L}$ by milrinone, 1 μM ACh induced a prominent inhibition of $I_{Ca,L}$ (−86 %). In another atrial cell from the same heart, although spermine/NO elicited a comparable stimulation of $I_{Ca,L}$, exposure to 1 μM ACh elicited a markedly smaller inhibition of $I_{Ca,L}$ (−32 %).

Alternatively, NO can alter a wide variety of cellular functions via *S*-nitrosylation (cGMP-independent) reactions (Broillet, 1999; Stamler *et al.* 2001). To examine this possibility, we tested the effects of ACh to inhibit $I_{Ca,L}$ stimulated by ZIN-β_2-ARs in the absence and presence of reduced glutathione (GSH), a sink for *S*-nitrosylation by NO. Exposure to 500 μM GSH alone had no effect on basal $I_{Ca,L}$ and no significant effect on ZIN-stimulated $I_{Ca,L}$ (Fig. 5*B*). However, as shown in Fig. 5*B*, GSH significantly enhanced ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs (ACh, −26 ± 2 % *vs.* ACh + GSH, −56 ± 6 %; $P < 0.05$). Similar results were obtained with 100 μM dithiothreitol (DTT), another agent that acts as a sink for *S*-nitrosylation (ACh, −20 ± 2 %, $n = 5$, *vs.* ACh + DTT, −57 ± 3 %, $n = 8$; $P < 0.05$). These findings suggest that the NO released by ZIN-β_2-AR stimulation is acting primarily via an *S*-nitrosylation mechanism to prevent ACh-induced inhibition of $I_{Ca,L}$.

In the following experiments, we tested the ability of ACh to inhibit $I_{Ca,L}$ when $I_{Ca,L}$ was stimulated by exogenous NO (i.e. without β_2-AR stimulation). In cat atrial myocytes, inhibition of PDE activity by milrinone elicits a prominent activation of cAMP-dependent stimulation of $I_{Ca,L}$ (Wang & Lipsius, 1995). In both cat (Wang *et al.* 1998) and human (Kirstein *et al.* 1995) atrial myocytes, NO stimulates $I_{Ca,L}$ via increases in endogenous cAMP generated by cGMP-mediated inhibition of PDE III. Therefore, $I_{Ca,L}$ was stimulated by inhibiting PDE III using either 100 μM SNO, an NO donor, or 5 μM milrinone, a specific PDE III inhibitor. As shown in Fig. 6*A*, when $I_{Ca,L}$ was stimulated by milrinone (89 %), ACh elicited a prominent inhibition of $I_{Ca,L}$ (−86 %). However, in another atrial myocyte from the same heart (Fig. 6*B*), when $I_{Ca,L}$ was stimulated by SNO (98 %), ACh-induced inhibition was markedly smaller (−32 %). In total, stimulation of $I_{Ca,L}$ elicited by SNO (98 ± 8 %; $n = 8$) and milrinone (112 ± 5 %; $n = 8$) was similar. However, ACh-induced inhibition of $I_{Ca,L}$ was significantly smaller when $I_{Ca,L}$ was stimulated by SNO (−29 ± 5 %) compared with milrinone (−73 ± 8 %; $P < 0.001$). These experiments could be interpreted as

further evidence that NO is responsible for preventing the inhibitory effects of ACh. However, an alternative explanation is that ACh is simply not able to effectively inhibit the stimulatory effects of NO signalling on $I_{Ca,L}$ (see Discussion).

So far, the results indicate that NO signalling prevents the ACh-induced inhibition of $I_{Ca,L}$ selectively stimulated by ISO-β_2-ARs or ZIN-β_2-ARs. In addition, ACh-induced inhibition of ISO-β_1-AR stimulation is prominent because this signalling pathway fails to release NO. This raises the question of whether the NO released by ISO-β_2-ARs can prevent the ability of ACh to inhibit ISO-β_1-AR stimulation. This question was approached by testing the effects of ACh on $I_{Ca,L}$ stimulated by ISO, a mixed β_1/β_2-AR agonist. Our previous work has shown that ISO elicits NO release via activation of β_2-ARs (Wang *et al.* 2002). As expected, 0.1 μM ISO markedly increased $I_{Ca,L}$ (213 %), and 1 μM ACh prominently inhibited $I_{Ca,L}$ (−57 %; Fig. 7*A*). Based on the present results, this prominent ACh-induced inhibition of $I_{Ca,L}$ stimulated by ISO-β_1/β_2-ARs is due primarily to the effect of ACh on ISO-β_1-AR signalling. Moreover, as shown in Fig. 7*B*, in another atrial myocyte, exposure to 10 μM L-NIO attenuated ISO-β_1/β_2-AR stimulation of $I_{Ca,L}$ (127 %). This confirms previous findings that ISO acts via NO signalling to partially stimulate $I_{Ca,L}$ (Wang *et al.* 2002). In addition, ACh-induced inhibition of $I_{Ca,L}$ was enhanced (−78 %) when NO release was blocked. As summarized in Fig. 7*C*, compared with the control, L-NIO decreased stimulation of $I_{Ca,L}$ by ISO-β_1/β_2-ARs (ISO, 355 ± 69 % *vs.* ISO + L-NIO, 183 ± 21 %), and ACh-induced inhibition of $I_{Ca,L}$ was significantly enhanced (ACh, −45 ± 5 % *vs.* ACh + L-NIO, −74 ± 5 %; $P < 0.002$). These findings suggest that the NO released by ISO-β_2-AR stimulation does not prevent the ability of ACh to inhibit ISO-β_1-AR stimulation. Moreover, they support the idea that NO acts preferentially to prevent ACh-induced inhibition of $I_{Ca,L}$ stimulated by β_2-ARs. In addition, the fact that ACh could still inhibit β_1-AR stimulation even

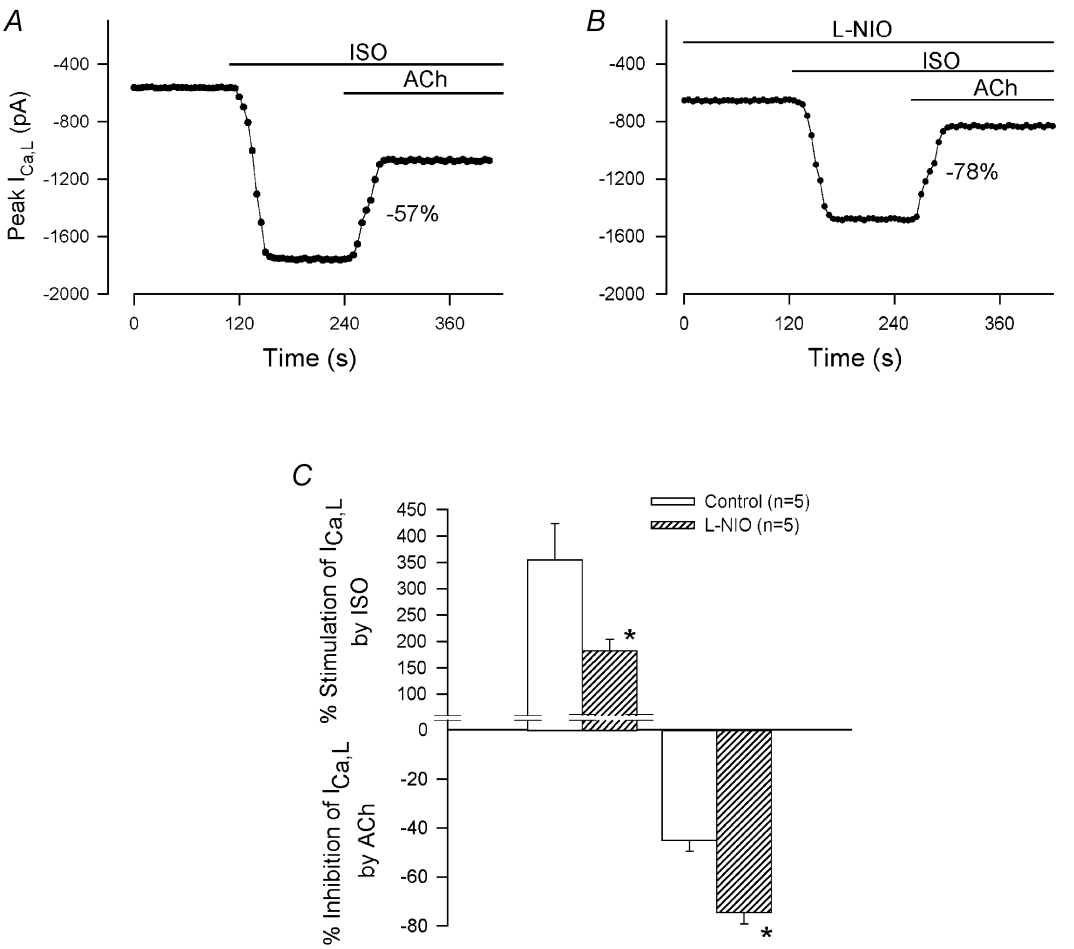

Figure 7. Effects of ACh on ISO-stimulated $I_{Ca,L}$ in the absence (*A*) and presence (*B*) of 10 μM L-NIO

A, in the absence of L-NIO, ACh elicited a prominent inhibition of ISO-stimulated $I_{Ca,L}$ (−57 %). *B*, in another atrial myocyte from the same heart, L-NIO attenuated ISO-induced stimulation of $I_{Ca,L}$ and enhanced ACh-induced inhibition of ISO-stimulated $I_{Ca,L}$ (−78 %). *C*, summary of the data shows that L-NIO significantly decreased ISO-induced stimulation of $I_{Ca,L}$ and significantly enhanced ACh-induced inhibition of ISO-stimulated $I_{Ca,L}$. *$P < 0.05$.

though ISO releases NO, makes it unlikely that NO is acting to inhibit muscarinic receptor function.

The present experiments also indicate that although FEN acts via β_2-ARs, it preferentially activates a signalling pathway similar to that activated by β_1-ARs (i.e. G_s-/adenylate cyclase). If this is correct, then the combined stimulation of FEN-β_2-ARs plus ZIN-β_2-ARs should behave like ISO, a mixed β_1/β_2-AR agonist. In other words, ACh should elicit a prominent inhibition of FEN-induced stimulation of $I_{Ca,L}$ even though ZIN-β_2-AR stimulation releases NO. In fact, simultaneous exposure to 0.1 μM FEN plus 1 μM ZIN increased $I_{Ca,L}$ (188 ± 75 %), and ACh prominently inhibited $I_{Ca,L}$ (−47 ± 6 %, $n = 2$; data not shown). In cells from the same heart, L-NIO attenuated the stimulation of $I_{Ca,L}$ by FEN + ZIN (107 ± 26 %) and enhanced the ACh-induced inhibition of $I_{Ca,L}$ (−75 ± 7 %; $n = 3$).

The present results indicate that different β-AR subtypes, as well as different β-AR agonists acting on the same β_2-AR subtype, differentially regulate NO release. To examine this idea directly, we measured endogenous $[NO]_i$ to determine the effect of each type of β-AR agonist. The effect of 10 μM ZIN-β_2-AR stimulation was to increase $[NO]_i$ (Fig. 8*A*), whereas 1 μM ISO-β_1-AR (Fig. 8*B*) and 1 μM FEN-β_2-AR stimulation (Fig. 8*C*) each failed to increase $[NO]_i$. Previous work has shown that ISO alone releases NO and that the ability of ISO and ZIN to release NO is abolished by inhibition of eNOS (L-NIO) or by blocking β_2-ARs (ICI 118,551; Wang *et al.* 2002). Note also that although the concentrations of FEN and ISO used in these experiments were 10 times higher than those used in the electrophysiology experiments, they failed to release NO. Figure 8*D* summarizes the effects of each β-AR agonist on $[NO]_i$. Additional experiments showed that simultaneous exposure to FEN + ZIN increased $[NO]_i$ essentially the same as ZIN-β_2-AR stimulation alone ($n = 3$; data not shown). Together, the present findings support the idea that the differential ability of ACh to inhibit $I_{Ca,L}$ depends upon whether the β-AR agonist used to stimulate $I_{Ca,L}$ is capable of releasing NO.

DISCUSSION

In general, stimulation of muscarinic receptors elicits an accentuated inhibition of β-AR stimulation of the heart. The present results indicate that in cat atrial myocytes, the magnitude of ACh-induced inhibition of β-AR-stimulated $I_{Ca,L}$ differs significantly depending upon which β-AR

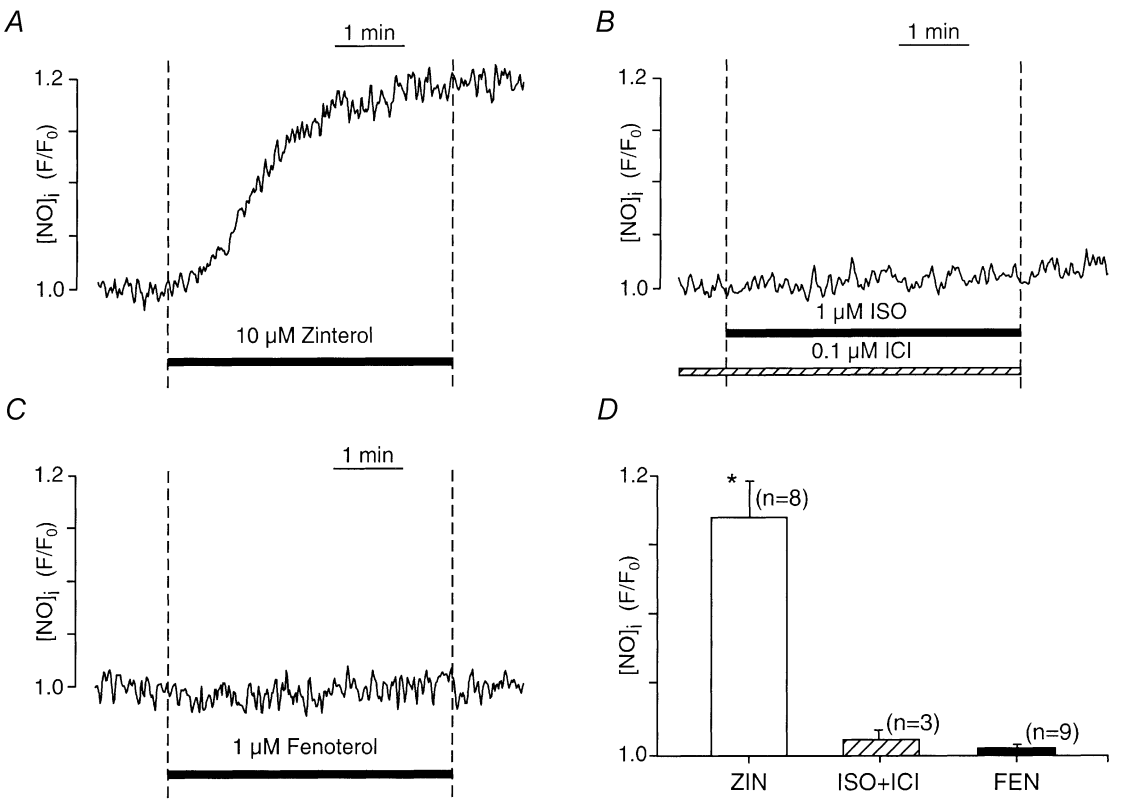

Figure 8. Measurements of intracellular NO ($[NO]_i$) in response to stimulation by ZIN-β_2-ARs (*A*), ISO-β_1-ARs (*B*) and FEN-β_2-ARs (*C*)

A, stimulation of ZIN-β_2-ARs increased $[NO]_i$. *B*, stimulation of ISO-β_1-ARs failed to increase $[NO]_i$. *C*, stimulation of FEN-β_2-ARs also failed to increase $[NO]_i$. *D*, summary showing the effects of each β-AR agonist on $[NO]_i$. ZIN-β_2-AR (open bar); ISO-β_1-AR (hatched bar); FEN-β_2-AR (filled bar). Numbers in parentheses indicate the number of cells tested. * $P < 0.05$.

subtype and agonist stimulates $I_{Ca,L}$. In general, ACh induces a significantly greater inhibition when $I_{Ca,L}$ is stimulated by β_1-AR agonists compared with β_2-AR agonists. These findings basically agree with those reported in neonatal rat ventricular myocytes in which β_1-AR stimulation of cAMP and inotropic activity is more susceptible than β_2-AR stimulation to inhibition by muscarinic receptor stimulation (Aprigliano *et al.* 1997). A major finding of the present study is that in adult atrial myocytes, the mechanism responsible for these disparate effects of ACh-induced inhibition is the release of NO by selective β_2-AR stimulation. In other words, the magnitude of ACh-induced inhibition correlated directly with whether a specific β-AR agonist released NO. Indeed, ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs or ISO-β_2-ARs were comparably small, and as shown here and in our previous work (Wang *et al.* 2002), both of these β_2-AR agonists release NO. On the other hand, ACh-induced inhibition of $I_{Ca,L}$ was prominent when $I_{Ca,L}$ was stimulated by either ISO-β_1-ARs or FEN-β_2-ARs, and both of these agonists fail to release NO. It is worth emphasizing that the inhibitory effects of ACh on $I_{Ca,L}$ differed markedly when $I_{Ca,L}$ was stimulated via the same β_2-AR subtype but by different β_2-AR agonists. The fact that stimulation of ZIN-β_2-ARs releases NO and FEN-β_2-ARs does not release NO strongly supports the idea that the primary mechanism responsible for the differential inhibitory effects of ACh is not stimulation of β_2-ARs *per se*, but rather whether a particular β_2-AR agonist releases NO. The functional role of endogenous NO release was further demonstrated by the findings that inhibition of NO release (L-NIO) enhanced the ability of ACh to inhibit $I_{Ca,L}$ stimulated by ZIN-β_2-ARs but not when $I_{Ca,L}$ was stimulated by either ISO-β_1-ARs or FEN-β_2-ARs. Likewise, when NO signalling was prevented by haemoglobin, ACh-induced inhibition of $I_{Ca,L}$ was enhanced when $I_{Ca,L}$ was stimulated by ZIN-β_2-ARs but not when stimulated by FEN-β_2-ARs. We therefore conclude that NO release by specific β_2-AR agonists is responsible for preventing the inhibitory effects of ACh on β_2-AR stimulation of $I_{Ca,L}$.

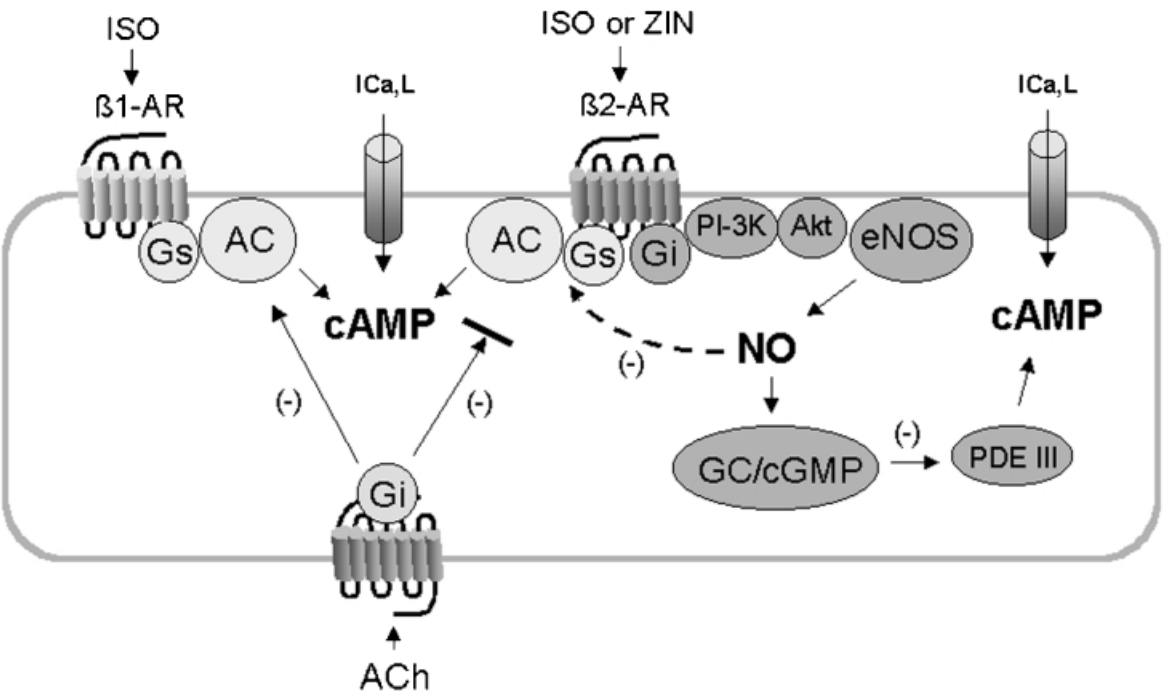

Figure 9. Schematic diagram of the proposed signalling mechanisms responsible for muscarinic receptor-mediated inhibition of β_1-AR- and β_2-AR-stimulated $I_{Ca,L}$

ISO stimulates both β_1-ARs and β_2-ARs via G_s-adenylate cyclase (AC)/cAMP signalling to increase $I_{Ca,L}$. In addition, ISO or ZIN act via β_2-ARs and G_i-/PI-3K/Akt signalling to activate endothelial nitric oxide synthase (eNOS) and NO release (Wang *et al.* 2002). NO acts via guanylate cyclase (GC)/cGMP-inhibition of phosphodiesterase (PDE) III to raise endogenous cAMP and stimulate $I_{Ca,L}$. ACh acts via muscarinic receptors coupled to G_i-protein to inhibit AC activated by β_1-AR/G_s-signalling. However, ACh is unable to effectively inhibit β_2-AR/G_i-NO signalling. In addition, NO released by β_2-AR stimulation acts locally via *S*-nitrosylation to decrease β_2-AR/G_s-/AC signalling. As a result, β_2-AR signalling is due primarily to G_i-NO signalling, which is not effectively inhibited by ACh. Not shown in this diagram is the effect of FEN. Although FEN acts via β_2-ARs, it preferentially stimulates only the G_s-/AC signalling pathway and therefore does not release NO. As a result, β_2-AR/G_s-/AC signalling is not depressed and therefore ACh-induced inhibition of $I_{Ca,L}$ stimulated by FEN is prominent, similar to that obtained with stimulation of $I_{Ca,L}$ via β_1-ARs.

Our previous work indicates that ZIN-β_2-AR stimulation acts via two parallel signalling pathways to generate cAMP-dependent stimulation of $I_{Ca,L}$; G_s-/adenylate cyclase signalling and G_i-/PI-3K/NO signalling (Wang *et al.* 2002; see Fig. 9). The present results indicate that when $I_{Ca,L}$ was stimulated by ZIN-β_2-ARs, NO released via the G_i-signalling pathway prevented ACh-induced inhibition of $I_{Ca,L}$ stimulated by the G_s-signalling pathway (Fig. 9). Thus, when ZIN-β_2-AR-mediated NO signalling was blocked, the enhanced inhibitory effect of ACh now resulted from inhibition of the remaining G_s-/adenylate cyclase signalling. This is consistent with the prominent ACh-induced inhibition of β_1-AR stimulation, which is mediated exclusively via G_s-/adenylate cyclase signalling. The present results also indicate that NO released by β_2-AR stimulation preferentially prevents the ability of ACh to inhibit β_2-AR but not β_1-AR stimulation. Thus, ISO stimulates both β_1- and β_2-ARs and releases NO via β_2-AR signalling (Wang *et al.* 2002). Even though ISO releases NO, ACh elicited a prominent inhibition of ISO-stimulated $I_{Ca,L}$. Based on the present study, this prominent effect of ACh is mediated primarily via inhibition of β_1-AR signalling. This idea is supported by the finding that when NO signalling was blocked the inhibitory effects of ACh were enhanced, presumably via the additional ACh-induced inhibition of β_2-AR signalling. In addition, the fact that ACh could elicit a prominent inhibition of ISO-stimulated $I_{Ca,L}$ even though ISO releases NO suggests that NO is not exerting a general inhibition of muscarinic receptor function. Rather, the fact that NO preferentially prevents ACh-induced inhibition of β_2-AR signalling and not β_1-AR signalling suggests that endogenous NO released by β_2-ARs acts locally near the β_2-AR to modulate the inhibitory effect of muscarinic receptor signalling (Fig. 9). Our previous work also has shown that in atrial myocytes, NO signalling either induced by β_2-ARs or by exposure to exogenous NO acts locally to regulate ACh-induced activation of K^+ channels (Wang *et al.* 2002). Both of these findings are consistent with local regulation by NO signalling (Dittrich *et al.* 2001). In addition, β_2-ARs (but not β_1-ARs) and eNOS are both localized within caveolae (Steinberg & Brunton, 2001), providing a possible substrate for the present findings that β_2-AR, but not β_1-AR function may be regulated locally by NO signalling.

Several of the present results indicate that FEN acts specifically via β_2-ARs to activate a cAMP signalling pathway similar to β_1-ARs. First, the effects of ISO-β_1-AR and FEN-β_2-AR stimulation were essentially the same (i.e. both increased $I_{Ca,L}$ to the same extent, both failed to activate NO release and both of their stimulatory effects on $I_{Ca,L}$ were prominently inhibited by ACh). In addition, FEN-mediated stimulation of $I_{Ca,L}$ was abolished by specific blockade of β_2-ARs, and by specific inhibition of cAMP-dependent PKA activity. As mentioned earlier, β_1-ARs act exclusively via G_s-/adenylate cyclase signalling. In cat atrial myocytes, β_2-ARs act via G_s-/adenylate cyclase signalling and via G_i-/PI-3K signalling to release NO (Wang *et al.* 2002). This explains why β_1-AR stimulation fails to release NO. Moreover, the present results lead to the conclusion that FEN is unable to stimulate NO release because it fails to activate G_i-protein signalling and acts preferentially to stimulate G_s-/adenylate cyclase signalling. The present findings also show that when $I_{Ca,L}$ was stimulated simultaneously by FEN + ZIN, ACh-induced inhibition was prominent even though NO was released by ZIN. This suggests that FEN-β_2-AR signalling is not regulated locally by the NO released by ZIN-β_2-ARs. Similar results were obtained when $I_{Ca,L}$ was stimulated by ISO, a mixed β_1/β_2-AR agonist. The idea that FEN stimulates a β_2-AR exclusively via G_s-/adenylate cyclase signalling can be explained by either: (1) two separate subclasses of β_2-ARs or (2) preferential activation of G_s- over G_i-protein signalling via a single class of β_2-ARs. The latter idea is consistent with agonist-directed trafficking of receptor signalling (Kenakin, 1995). In general, agonist trafficking of receptor responses is defined as the ability of different agonists, acting on a single receptor that couples to more than one G-protein, to preferentially activate a particular G-protein signalling pathway. The underlying theory is that different types of agonist can induce and/or select different receptor conformations (see also Kukkonen *et al.* 2001). This phenomenon has been reported for a variety of receptor-signalling mechanisms, some of which include 5-HT receptor coupling to phospholipases C and A_2 (Berg *et al.* 1998), differential activation of G_s- and G_i-signalling by different cannabinoid receptor agonists (Bonhaus *et al.* 1998), and differential coupling of α_{2A}-ARs to G_s- and G_i-mediated regulation of adenylate cyclase (Brink *et al.* 2000).

A primary target of NO signalling is guanylate cyclase (and thus the production of cGMP). The present results, however, indicate that ACh-induced inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs was unaffected by blocking guanylate cyclase with ODQ. Alternatively, reduced glutathione or dithiothreitol, two agents that act as a sink for *S*-nitrosylation by NO, significantly enhanced ACh-induced inhibition of $I_{Ca,L}$ when stimulated by ZIN-β_2-ARs. These findings suggest that NO is preventing the inhibitory effects of ACh primarily via a cGMP-independent, *S*-nitrosylation reaction. Because *S*-nitrosylation can modulate a wide variety of cellular functions (Broillet, 1999; Stamler *et al.* 2001) its specific site of action remains unclear. However, as mentioned earlier, the fact that ACh can prominently inhibit ISO-stimulated $I_{Ca,L}$ even though ISO releases NO, makes it unlikely that muscarinic receptor signalling is the site of NO action. Alternatively, NO can act via

S-nitrosylation to decrease β_2-AR/G_s-signalling (Adam *et al.* 1999) and to inhibit adenylate cyclase activity (McVey *et al.* 1999). Therefore, based on the present data, we propose the following: stimulation of β_2-ARs acts via both G_s- and G_i-signalling pathways. ACh is unable to effectively inhibit the β_2-AR/G_i-pathway that acts via NO/cGMP/cAMP signalling (Fig. 6). Moreover, NO released via β_2-AR/G_i-signalling acts via *S*-nitrosylation to inhibit the β_2-AR/G_s-/adenylate cyclase signalling pathway (Fig. 9). Therefore, the cAMP signalling pathway activated by β_2-AR stimulation is mediated primarily via G_i-/NO signalling. As a result, ACh is unable to effectively inhibit β_2-AR stimulation of $I_{Ca,L}$ (Fig. 9). However, when NO signalling is blocked, β_2-AR stimulation is now mediated primarily via G_s-adenylate cyclase signalling, which is effectively inhibited by muscarinic receptor stimulation.

In the present experiments, the inhibitory effects of ACh were determined as a percentage of the $I_{Ca,L}$ amplitude stimulated by each β-AR agonist. By this method, a smaller or larger β-AR-stimulated increase in $I_{Ca,L}$ amplitude could result in a larger or smaller calculated percentage inhibition induced by ACh. Several results, however, argue against the idea that this can account for the effects of ACh presented here. For example, ZIN elicited the smallest stimulation of $I_{Ca,L}$ and yet ACh exerted the smallest (rather than the largest) inhibition of $I_{Ca,L}$ stimulated by ZIN-β_2-ARs (Fig. 2). Conversely, stimulation of either ISO-β_1-ARs or FEN-β_2-ARs elicited the largest stimulation of $I_{Ca,L}$ and yet the effects of ACh also were largest (rather than smallest) when $I_{Ca,L}$ was stimulated by these agonists. Although ODQ significantly decreased ZIN-mediated stimulation of $I_{Ca,L}$, the inhibitory effects of ACh were not different in the presence or absence of ODQ (Fig. 5). On the other hand, stimulation of $I_{Ca,L}$ by SNO and milrinone were similar, and yet the effects of ACh were significantly smaller when $I_{Ca,L}$ was stimulated by SNO compared with milrinone (Fig. 6). We also selected data in which the stimulated amplitude of $I_{Ca,L}$ between two different cells was approximately the same and yet the effects of ACh in the presence of haemoglobin were markedly enhanced compared with those in absence of haemoglobin (Fig. 4). Finally, the relationship between $I_{Ca,L}$ amplitudes stimulated by each β-AR agonist and the percentage changes induced by ACh showed no significant correlation.

Functionally, the present results suggest that the NO released by specific β_2-AR agonists prevents ACh-induced inhibition of β_2-AR stimulation. Of course, noradrenaline is the primary neurotransmitter of sympathetic nerve activity and it acts primarily via β_1-AR signalling, with limited β_2-AR activity. In addition, the heart, including cat atria (Hedberg *et al.* 1980), exhibit a lower density of β_2-ARs than β_1-ARs, although the density of β_2-ARs in human atria is higher than in ventricular muscle (Stiles *et al.* 1983). β_2-AR signalling may therefore be a normal second line of sympathetic cardiac support. However, the relative contribution of different receptor subtypes to β-AR signalling in the heart is not static. In cat atrial myocytes, stimulation of integrin-mediated signalling dramatically decreases β_1-AR and increases β_2-AR signalling mechanisms, which regulate $I_{Ca,L}$ (Wang *et al.* 2000). In addition, the failing human heart exhibits a pronounced decrease in the ratio of β_1:β_2-ARs (Bristow *et al.* 1986). These considerations make it possible that under various pathological conditions, β_2-AR signalling assumes significantly greater importance. Moreover, evidence is accumulating that β_2-AR stimulation is beneficial. For example, β_2-AR stimulation acts via G_i-/PI-3K signalling to inhibit apoptosis (Communal *et al.* 1999; Chesley *et al.* 2000), while β_1-AR stimulation promotes apoptosis of cardiac myocytes (Communal *et al.* 1999). Interestingly, stimulation of muscarinic receptors inhibits the ability of β_1-AR stimulation to induce apoptosis (Communal *et al.* 1999). Endogenous NO release also exerts cardioprotective effects (Ping *et al.* 1999). It should be noted that in cat atrial myocytes, stimulation of β_2-ARs acts via G_i-/PI-3K signalling to elicit endogenous NO release (Wang *et al.* 2002). We therefore speculate that the mechanisms presented here may prevent muscarinic receptor stimulation from inhibiting the potentially beneficial effects of β_2-AR stimulation, while the potentially deleterious effects of tonic or chronic β_1-AR stimulation may be inhibited by muscarinic receptor stimulation. As a corollary, this mechanism may contribute to the beneficial effects of high vagal tone on the heart. In addition, the finding that FEN acts via β_2-ARs to preferentially activate a β_1-AR-like signalling pathway may be relevant to the development of pharmacological β_2-AR agonists that can preferentially activate specific signalling mechanisms.

REFERENCES

Adam, L., Bouvier, M. & Jones, T. L. Z. (1999). Nitric oxide modulates β_2-adrenergic receptor palmitoylation and signaling. *Journal of Biological Chemistry* **274**, 26337–26343.

Aprigliano, O., Rybin, V. O., Pak, E., Robinson, R. B. & Steinberg, S. F. (1997). β_1- and β_2-adrenergic receptors exhibit differing susceptibility to muscarinic accentuated antagonism. *American Journal of Physiology* **272**, H2726–2735.

Belevych, A. E. & Harvey, R. D. (2000). Muscarinic inhibitory and stimulatory regulation of the L-type Ca^{2+} current is not altered in cardiac ventricular myocytes from mice lacking endothelial nitric oxide synthase. *Journal of Physiology* **528**, 279–289.

Berg, K. A., Maayani, S., Goldfarb, J., Scaramellini, C., Leff, P. & Clarke, W. P. (1998). Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Molecular Pharmacology* **54**, 94–104.

Bonhaus, D. W., Chang, L. K., Kwan, J. & Martin, G. R. (1998). Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *Journal of Pharmacology and Experimental Therapeutics* **287**, 884–888.

Brink, C. B., Wade, S. M. & Neubig, R. R. (2000). Agonist-directed trafficking of porcine α_{2A}-adrenergic receptor signaling in Chinese hamster ovary cells: I-isoproterenol selectively activates G_s. *Journal of Pharmacology and Experimental Therapeutics* **294**, 539–547.

Bristow, M. R., Ginsberg, R., Umans, V., Fowler, M., Minobe, W., Rasmussen, R., Zera, P., Menlove, R., Shah, P., Jamieson, S. & Stinson, E. B. (1986). β1- and β2-adrenergic-receptor subpopulation in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective β1-receptor down-regulation in heart failure. *Circulation Research* **59**, 297–309.

Broillet, M. C. (1999). S-Nitrosylation of proteins. *Cellular and Molecular Life Sciences* **55**, 1036–1042.

Brunner, F., Schmidt, K., Nielsen, E. B. & Mayer, B. (1996). Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. *Journal of Pharmacology and Experimental Therapeutics* **277**, 48–53.

Chesley, A., Lundber, M. S., Asai, T., Xiao, R. P., Ohtani, S., Lakatta, E. G. & Crow, M. T. (2000). The β_2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G_i-dependent coupling to phosphatidylinositol 3′-kinase. *Circulation Research* **87**, 1172–1179.

Communal, C., Singh, K., Sawyer, D. B. & Colucci, W. S. (1999). Opposing effects of β_1- and β_2-adrenergic receptors on cardiac myocyte apoptosis. *Circulation* **100**, 2210–2212.

Dittrich, M., Jurevicius, J., Georget, M., Rochias, F., Fleischmann, J., Hescheler, J. & Fischmeister, R. (2001). Local response of L-type Ca^{2+} current to nitric oxide in frog ventricular myocytes. *Journal of Physiology* **534**, 109–121.

Godecke, A., Heinicke, T., Kamkin, A., Kiseleva, I., Strasser, R. H., Decking, U. K. M., Stumpe, T., Isenberg, G. & Schrader, J. (2001). Inotropic response to β-adrenergic receptor stimulation and anti-adrenergic effect of ACh in endothelial NO synthase-deficient mouse hearts. *Journal of Physiology* **532**, 195–204.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Han, X., Kubota, I., Feron, O., Opel, D. J., Arstall, M. A., Zhao, Y. Y., Huang, P., Fishman, M. C., Michel, T. & Kelly, R. A. (1998). Muscarinic cholinergic regulation of cardiac myocyte $I_{Ca\text{-}L}$ is absent in mice with targeted disruption of endothelial nitric oxide synthase. *Proceedings of the National Academy of Sciences of the USA* **95**, 6510–6515.

Hedberg, A., Minneman, K. P. & Molinoff, P. B. (1980). Differential distribution of beta-1 and beta-2 adrenergic receptors in cat and guinea-pig heart. *Journal of Pharmacology and Experimental Therapeutics* **212**, 503–508.

Horn, R. & Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145–159.

Kenakin, T. (1995). Agonist-receptor efficacy II: agonist trafficking of receptor signals. *Trends in Pharmacological Sciences* **6**, 232–238.

Kilts, J. D., Gerhardt, M. A., Richerdson, M. D., Sreeram, G., MacKensen, G. B., Grocott, H. P., White, W. D., Davis, R. D., Newman, M. F., Reves, J. G., Schwinn, D. A. & Kwatra, M. M. (2000). β_2-adrenergic and several other G protein-coupled receptors in human atrial membranes activate both G_s and G_i. *Circulation Research* **87**, 705–709.

Kirstein, M., Rivet-Bastide, M., Hatem, S., Benardeau, A., Mercadier, J. J. & Fischmeister, R. (1995). Nitric oxide regulates the calcium current in isolated human atrial myocytes. *Journal of Clinical Investigation* **95**, 794–802.

Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y. & Nagano, T. (1998). Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Analytical Chemistry* **70**, 2446–2453.

Kukkonen, J. P., Nasman, J. & Akerman, K. E. O. (2001). Modelling of promiscuous receptor-G_i/G_s-protein coupling and effector response. *Trends in Pharmacological Sciences* **22**, 616–622.

Kuschel, M., Zhou, Y. Y., Cheng, H., Zhang, S. J., Chen, Y., Lakatta, E. G. & Xiao, R. P. (1999). G_i protein-mediated functional compartmentalization of cardiac β_2-adrenergic signaling. *Journal of Biological Chemistry* **274**, 22048–22052.

McVey, M., Hill, J., Howlett, A. & Klein, C. (1999). Adenylyl cyclase, a coincidence detector for nitric oxide. *Journal of Biological Chemistry* **274**, 18887–18892.

Nakatsubo, N., Kojima, H., Kikuchi, K., Nagoshi, H., Hirata, Y., Maeda, D., Imai, Y., Irimura, T. & Nagano, T. (1998). Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins. *FEBS Letters* **427**, 263–266.

Ping, P., Takano, H., Zhang, J., Tang, X. L., Qiu, Y., Li, R. C., Banerjee, S., Dawn, B., Balafnova, Z. & Bolli, R. (1999). Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circulation Research* **84**, 587–604.

Rees, D. D., Palmer, R. M. J., Schulz, R., Hodson, H. & Moncada, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *British Journal of Pharmacology* **101**, 746–752.

Stamler, J. S., Lamas, S. & Fang, F. C. (2001). Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* **106**, 675–683.

Steinberg, S. F. (1999). The molecular basis for distinct β-adrenergic receptor subtype actions in cardiomyocytes. *Circulation Research* **85**, 1101–1111.

Steinberg, S. F. & Brunton, L. L. (2001). Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annual Review of Pharmacology and Toxicology* **41**, 751–773.

Stiles, G. L., Taylor, S. & Lefkowitz, R. J. (1983). Human cardiac beta-adrenergic receptors: subtype heterogeneity delineated by direct radioligand binding. *Life Sciences* **33**, 467–473.

Vandecasteele, G., Eschenhagen, T., Scholz, H., Stein, B., Verde, I. & Fischmeister, R. (1999). Muscarinic and β-adrenergic regulation of heart rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase. *Nature Medicine* **5**, 331–334.

Wang, Y. G., Dedkova, E. N., Steinberg, S. F., Blatter, L. A. & Lipsius, S. L. (2002). β_2-Adrenergic receptor signaling acts via NO release to mediate ACh-induced activation of ATP-sensitive K^+ current in cat atrial myocytes. *Journal of General Physiology* **119**, 69–82.

Wang, Y. G. & Lipsius, S. L. (1995). Acetylcholine elicits a rebound stimulation of Ca^{2+} current mediated by pertussis toxin-sensitive G protein and cAMP-dependent protein kinase A in atrial myocytes. *Circulation Research* **76**, 634–644.

Wang, Y. G., Rechenmacher, C. E. & Lipsius, S. L. (1998). Nitric oxide signaling mediates stimulation of L-type Ca^{2+} current elicited by withdrawal of acetylcholine in cat atrial myocytes. *Journal of General Physiology* **111**, 113–125.

Wang, Y. G., Samarel, A. M., & Lipsius, S. L. (2000). Laminin binding to β_1-integrins selectively alters β_1- and β_2-adrenoceptor signalling in cat atrial myocytes. *Journal of Physiology* **527**, 3–9.

Wu, J., Vereecke, J., Carmeliet, E. & Lipsius, S. L. (1991). Ionic currents activated during hyperpolarization of single right atrial myocytes from cat heart. *Circulation Research* **68**, 1059–1069.

Xiao, R. P., Cheng, H., Zhou, Y. Y., Kuschel, M. & Lakatta, E. G. (1999). Recent advances in cardiac β_2-adrenergic signal transduction. *Circulation Research* **85**, 1092–1100.

Xiao, R. P., Ji, X. & Lakatta, E. G. (1995). Functional coupling of the β_2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Molecular Pharmacology* **47**, 322–329.

Acknowledgements

We thank Ms Holly Gray for her technical assistance with these experiments. Financial support was provided by National Institutes of Health grants HL27652 and HL63753 (S.L.L.) and HL51941 and HL62231 (L.A.B.).