β_2-Adrenergic Receptor Signaling Acts via NO Release to Mediate ACh-induced Activation of ATP-sensitive K^+ Current in Cat Atrial Myocytes

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ABSTRACT In atrial myocytes, an initial exposure to isoproterenol (ISO) acts via cAMP to mediate a subsequent acetylcholine (ACh)-induced activation of ATP-sensitive K^+ current ($I_{K,ATP}$). In addition, β-adrenergic receptor (β-AR) stimulation activates nitric oxide (NO) release. The present study determined whether the conditioning effect of β-AR stimulation acts via β_1- and/or β_2-ARs and whether it is mediated via NO signaling. 0.1 μM ISO plus ICI 118,551 (ISO-β_1-AR stimulation) or ISO plus atenolol (ISO-β_2-AR stimulation) both increased L-type Ca^{2+} current ($I_{Ca,L}$) markedly, but only ISO-β_2-AR stimulation mediated ACh-induced activation of $I_{K,ATP}$. 1 μM zinterol (β_2-AR agonist) also increased $I_{Ca,L}$ and mediated ACh-activated $I_{K,ATP}$. Inhibition of NO synthase (10 μM L-NIO), guanylate cyclase (10 μM ODQ), or cAMP-PKA (50 μM Rp-cAMPs) attenuated zinterol-induced stimulation of $I_{Ca,L}$ and abolished ACh-activated $I_{K,ATP}$. Spermine-NO (100 μM; an NO donor) mimicked β_2-AR stimulation, and its effects were abolished by Rp-cAMPs. Intracellular dialysis of 20 μM protein kinase inhibitory peptide (PKI) abolished zinterol-induced stimulation of $I_{Ca,L}$. Measurements of intracellular NO ($[NO]_i$) using the fluorescent indicator DAF-2 showed that ISO-β_2-AR stimulation or zinterol increased $[NO]_i$. L-NIO (10 μM) blocked ISO- and zinterol-induced increases in $[NO]_i$. ISO-β_1-AR stimulation failed to increase $[NO]_i$. Inhibition of G_i-protein by pertussis toxin significantly inhibited zinterol-mediated increases in $[NO]_i$. Wortmannin (0.2 μM) or LY294002 (10 μM), inhibitors of phosphatidylinositol 3′-kinase (PI-3K), abolished the effects of zinterol to both mediate ACh-activated $I_{K,ATP}$ and stimulate $[NO]_i$. We conclude that both β_1- and β_2-ARs stimulate cAMP. β_2-ARs act via two signaling pathways to stimulate cAMP, one of which is mediated via G_i-protein and PI-3K coupled to NO-cGMP signaling. Only β_2-ARs acting exclusively via NO signaling mediate ACh-induced activation of $I_{K,ATP}$. NO signaling also contributes to β_2-AR stimulation of $I_{Ca,L}$. The differential effects of β_1- and β_2-ARs can be explained by the coupling of these two β-ARs to different effector signaling pathways.

KEY WORDS: electrophysiology • ion channels • cardiac • PI-3K signaling • G-protein–coupled receptor

INTRODUCTION

In general, autonomic nerve activity regulates cardiac function in a reciprocal manner. However, less understood are the mechanisms by which β-adrenergic receptor (β-AR)* stimulation influences the effects of subsequent muscarinic receptor stimulation. Previous work from this laboratory (Wang and Lipsius, 1995) has shown that in atrial myocytes, an initial exposure to isoproterenol (ISO) conditions the cell, such that a subsequent acetylcholine (ACh) exposure elicits a potentiated increase in K^+ conductance. The additional ACh-activated K^+ conductance exhibits inward rectification and is blocked by glibenclamide (GLIB), identifying it as ATP-sensitive K^+ current ($I_{K,ATP}$). Therefore, these findings indicate that after β-AR stimulation, subsequent muscarinic receptor stimulation elicits activation of two separate K^+ conductances: ACh-activated K^+ current ($I_{K,ACh}$) and $I_{K,ATP}$. Functionally, this mechanism may contribute to enhanced cholinergic inhibition of atrial function following β-AR stimulation. The conditioning effects of ISO are dependent on cAMP-dependent PKA signaling (Wang and Lipsius, 1995). However, because ISO is a nonselective β-AR agonist, it is not clear whether the effects of ISO are mediated via β1- and/or β_2-AR subtypes. Both types of β-ARs are present in cat atrial myocytes (Wang et al., 2000), and each type exhibits significantly different signal transduction mechanisms (Steinberg, 1999; Xiao et al., 1999b). Although β_1-ARs are more abundant than β_2-ARs, the proportion of β_2- to β_1-ARs may be greater in atrial than ventricular muscle (Buxton et al., 1987). In human atrial muscle, the relative proportion of β_2- to β_1-ARs has been reported at 20:80 (Brodde et al., 1983) and as high as 50:50 (Robberecht et al., 1983). β_1-ARs act ex-

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*Abbreviations used in this paper: ACh, acetylcholine; β-AR, β-adrenergic receptor; GLIB, glibenclamide; $I_{K,ACh}$, ACh-activated K^+ current; $I_{K,ATP}$, ATP-sensitive K^+ current; ISO, isoproterenol; NO, nitric oxide; PDE, phosphodiesterase; PI-3K, phosphatidylinositol 3′-kinase; PKI, PKA inhibitory peptide; PTX, pertussis toxin; SNO, spermine-NO.

clusively via G_s-proteins coupled to adenylate cyclase to catalyze the synthesis of cAMP, which in turn activates PKA. β_2-AR signaling appears more diverse: β_2-ARs couple to both G_s- and G_i-proteins (Xiao et al., 1995, 1999a; Kilts et al., 2000). Moreover, in contrast to β_1-ARs, several studies indicate that β_2-AR signaling acts locally to regulate L-type Ca^{2+} current ($I_{Ca,L}$) via cAMP/PKA activity, and is uncoupled from nonsarcolemmal regulatory proteins (Xiao and Lakatta, 1993; Xiao et al., 1994; Altschuld et al., 1995; Skeberdis et al., 1997; Zhou et al., 1997; Kuschel et al., 1999). These findings are consistent with the idea that β-AR regulation of $I_{Ca,L}$ can result from local or compartmentalized changes in cAMP (Hohl and Li, 1991; Jurevičius and Fischmeister, 1996), and that β-adrenergic regulation may correlate more closely with particulate rather than global cAMP levels (Hohl and Li, 1991). Much less is known about β_2-AR signaling in atrial muscle. In contrast to ventricular muscle, β_2-AR stimulation in human atrial muscle appears to exert global rather than local regulation of cellular functions (Kaumann et al., 1996).

Another important consideration is that in cardiomyocytes, β-AR stimulation activates NO production (Kanai et al., 1997; Sterin-Borda et al., 1998; Balligand, 1999). However, the role of NO signaling in heart depends on a variety of factors such as NO concentration, concurrent β-adrenergic stimulation, tissue type, and animal species (Balligand, 1999). In cat (Wang et al., 1998) and human (Kirstein et al., 1995) atrial myocytes, NO acts via cGMP-mediated inhibition of phosphodiesterase (PDE) type III activity to enhance cAMP-dependent stimulation of $I_{Ca,L}$. Therefore, the purpose of the present study was twofold: first, to determine whether β_1- and/or β_2-ARs are responsible for mediating ACh-induced activation of $I_{K,ATP}$; and second, to determine whether the conditioning effect of β-AR signaling is mediated via NO signaling. The results indicate that even though both β_1- and β_2-ARs stimulate cAMP signaling, only β_2-AR–stimulated cAMP mediated via NO signaling induces ACh to activate $I_{K,ATP}$. The differential effects of β_1- and β_2-ARs to regulate ion channel function may be explained by the coupling of different β-AR subtypes to different effector signaling pathways.

MATERIALS AND METHODS

Atrial myocytes were dispersed from adult cat atria using Langendorff perfusion and collagenase (type II; Worthington Biochemical) digestion as previously reported (Wu et al., 1991). No discernible differences were noted between left and right atrial myocytes. Cells used for electrophysiological studies were transferred to a small tissue bath (0.3 ml) on the stage of an inverted microscope (Nikon Diaphot) and superfused with a HEPES-buffered modified Tyrode solution containing the following (in mM): 145 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, and 11 glucose, and titrated with NaOH to a pH of 7.4. Solutions were perfused by gravity and heated to 35 ± 1°C. Atrial myocytes selected for study were elongated and quiescent. Voltage and ionic currents were recorded using a nystatin (150 μg/ml)-perforated patch (Horn and 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 physiological milieu and second messenger signaling pathways. The internal pipette solution contained the following (in mM): 100 potassium glutamate, 40 KCl, 1.0 $MgCl_2$, 4 Na_2-ATP, 0.5 EGTA, and 5 HEPES, and titrated with KOH to pH 7.2. 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, Inc.). Computer software (Pclamp; Axon Instruments, Inc.) was used to deliver voltage protocols, acquire, and analyze data. The effects of ACh on K^+ conductance were studied as previously described (Wang and Lipsius, 1995). In brief, an atrial cell was treated with two consecutive exposures to ACh (ACh_1 and ACh_2) separated by a 6-min recovery period in ACh-free Tyrode solution (see Fig. 1 A). Changes in total membrane conductance were assessed by imposing voltage-clamp ramps (40 mV/s) between −130 and +30 mV before, during, and after each ACh exposure. Voltage ramps offer the advantage of a rapid method for measuring peak ACh-induced currents throughout the voltage range. In general, experimental interventions, such as exposure to ISO, zinterol, or spermine-NO were imposed during the recovery period between ACh_1 and ACh_2. In this way, we determined the effect of each intervention on ACh-induced K^+ conductances by comparing the response to ACh_2 in relation to ACh_1. Measurements of K^+ conductance were obtained at −130 and +30 mV. The effects of each ACh exposure on K^+ conductance was fully reversible (see Fig. 1 A). Previous work indicates that the control currents do not affect the measurement of relative changes in K^+ conductances induced by ACh_2 in relation to ACh_1 (Wang and Lipsius, 1995). Therefore, in this study, ACh-induced K^+ currents were measured without subtraction of control currents. Control experiments indicate that an initial 30-s exposure to ACh followed by a 6-min recovery period has no effect on the response to a second ACh exposure (Wang and Lipsius, 1995). Therefore, any changes in K^+ conductance elicited by ACh_2 in relation to ACh_1 are attributed to the experimental intervention imposed during the recovery interval. Previous work (Wang and Lipsius, 1995) indicates that Ca^{2+} influx via $I_{Ca,L}$ during β-AR stimulation enhances ACh-induced activation of $I_{K,ATP}$. Therefore, $I_{Ca,L}$ was activated during the interval between ACh exposures by depolarizing voltage pulses from a holding potential of −40 to 0 mV for 200 ms every 10 s. In some experiments, $I_{Ca,L}$ was studied alone by replacing potassium glutamate with cesium glutamate in the pipette solution and adding 5 mM CsCl to the external solutions to block K^+ conductances. In other experiments, $I_{Ca,L}$ was recorded using a ruptured patch recording method to dialyze the cell interior with PKA inhibitors. PKA inhibitors were allowed to diffuse into the cell for ~5 min before recordings were performed. In these experiments, the internal pipette solution contained the following (mM): 100 cesium glutamate, 40 CsCl, 1 $MgCl_2$, 4 NaATP, 0.5 EGTA, 10 HEPES, and titrated with CsOH to pH 7.2. Unless stated otherwise, zinterol was tested in the presence of 0.01–0.1 μM atenolol to ensure β_2-AR stimulation. Cells were exposed to receptor antagonists for ~4 min before exposure to agonists. Inhibition of G_i-protein was achieved by incubating cells in pertussis toxin (PTX; 3.4 μg/ml; ≥3 h; 36°C) and confirmed by inhibition of ACh-activated $I_{K,ACh}$.

Direct measurements of intracellular NO ($[NO]_i$) were obtained by incubating cells with the fluorescent NO-sensitive dye 4,5-diaminofluorescein (DAF-2; Kojima et al., 1998; Nakatsubo et al., 1998). Experiments were performed at room temperature.

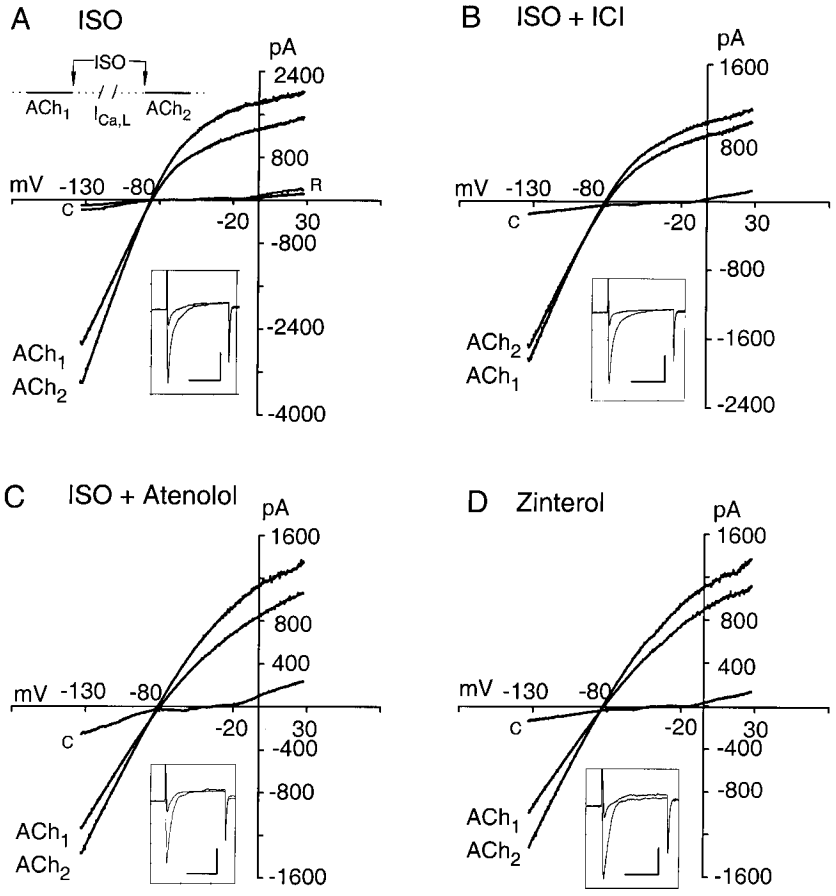

FIGURE 1. Current-voltage relationships showing the effects of 0.1 μM ISO (A–C) and 1 μM zinterol (D) on ACh_2-induced K^+ conductance and $I_{Ca,L}$ (insets). (A) ISO increased $I_{Ca,L}$ and mediated a potentiated increase in ACh_2-induced K^+ conductance compared with ACh_1. (B) In the presence of 0.01 μM ICI 118,551, a β_2-AR antagonist, ISO increased $I_{Ca,L}$, but failed to potentiate ACh_2-induced K^+ conductance. (C) In the presence of 0.01 μM atenolol, a β_1-AR antagonist, ISO increased $I_{Ca,L}$ and potentiated ACh_2-induced K^+ conductance. (D) Zinterol increased $I_{Ca,L}$ and potentiated ACh_2-induced K^+ conductance. c; control K^+ conductance before ACh_1. r; recovery after washout of ACh_2. (insets) $I_{Ca,L}$ calibration bars indicate 250 pA, 100 ms.

Cells were exposed to the membrane-permeant DAF-2 diacetate ([DAF-2 DA] = 5 μM; Calbiochem) for 10 min at room temperature in 1 ml 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 (F480). Emitted cellular fluorescence was recorded at 540 nm. Single cell fluorescence signals were recorded with a photomultiplier tube (model R2693; Hamamatsu Corp.) 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 before stimulation (F_o), and changes in $[NO]_i$ are expressed as F/F_o. In the experiments designed to measure $[NO]_i$, solutions contained 100 μM L-arginine. L-arginine was omitted when L-NIO was used to block NO synthase.

Drugs in this study include all of the following: isoproterenol, acetylcholine chloride, glibenclamide, atenolol, PK inhibitor (PKI), spermine-NO, L-N^5-(1-iminoethyl)ornithine (L-NIO), 1H-[1,2,4,]oxadiazolo[4,3-α]quinoxaline-1-one (ODQ), Wortmannin, LY294002, pertussis toxin (Sigma Chemicals); Rp-cAMPs, 4,5-diaminofluorescein diacetate (DAF-2 DA; Calbiochem); zinterol (provided by Bristol-Myers Squibb), and ICI 118,551 (provided by AstraZeneca).

In general, results were obtained in cells from the same hearts studied under control and test conditions. Data from two groups of cells were analyzed using unpaired *t* test with significance at $P \leq 0.05$. Data from multiple groups were analyzed using a one-way analysis of variance (ANOVA) followed by a *t*-Newman-Keuls test at $P \leq 0.05$.

RESULTS

β-Adrenergic Receptor Subtypes

Fig. 1 A shows a typical experiment in which an atrial myocyte was treated with two consecutive 30-s exposures to 10 μM ACh separated by a 6-min recovery period. During the recovery period, the cell was exposed to 0.1 μM ISO, a nonselective β_1/β_2-AR agonist, and $I_{Ca,L}$ was activated by voltage-clamp pulses (MATERIALS AND METHODS). As expected, ISO-β_1/β_2-AR stimulation elicited a marked increase in peak $I_{Ca,L}$ above basal levels (+258%; Fig. 1 A, inset). Both ACh_1 and ACh_2 exposures elicited an increase in K^+ conductance. However, after exposure to ISO, ACh_2 induced a potentiated increase in K^+ conductance compared with ACh_1. As summarized in Fig. 2, ISO-β_1/β_2-AR stimulation increased $I_{Ca,L}$ by 266 ± 78%, and ACh_2 increased K^+ conductance compared with ACh_1 by 31 ± 10% (at −130 mV) and 25 ± 3% (at 30 mV), respectively ($n = 4$; $P < 0.05$). These findings demonstrate that ISO elicits a conditioning effect that potentiates ACh_2-induced K^+ conductance as previously reported (Wang and Lipsius, 1995). The potentiated ACh_2-induced K^+ conductance has been identified as $I_{K,ATP}$ (Wang and Lipsius, 1995).

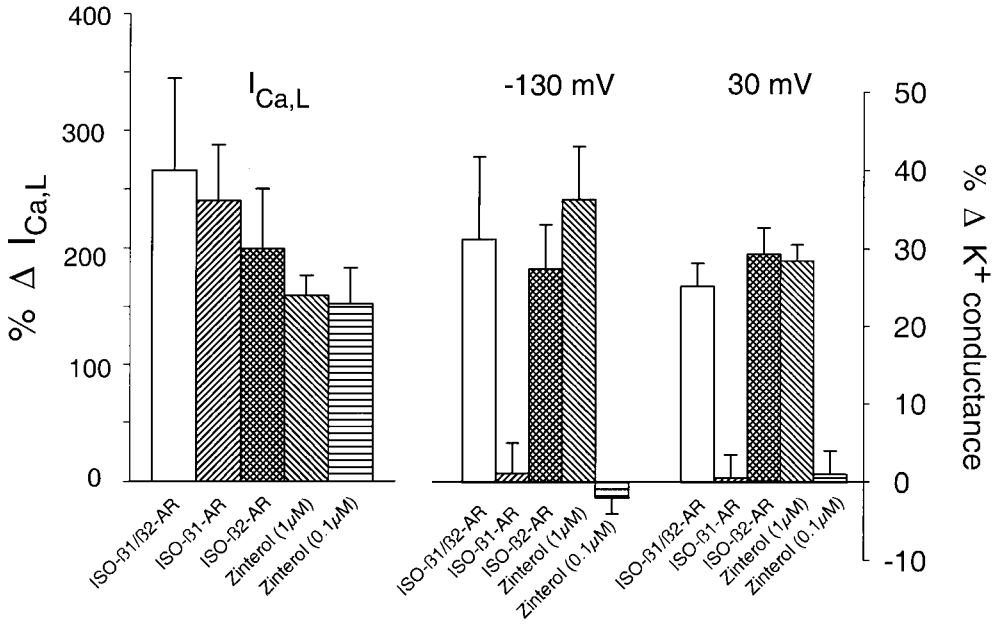

FIGURE 2. Summary of the effects of ISO (0.1 μM) and zinterol (0.1 and 1 μM) on $I_{Ca,L}$ and ACh_2-induced K^+ conductances measured at −130 and 30 mV. ISO-β_1/β_2-AR, ISO-β_1-AR or ISO-β_2-AR stimulation each increased $I_{Ca,L}$ and only ISO-β_1-AR stimulation failed to potentiate ACh_2-induced K^+ conductances. In addition, even though 0.1 and 1 μM zinterol increased $I_{Ca,L}$, the lower concentration of zinterol (0.1 μM) failed to potentiate ACh_2-induced K^+ conductance. (left ordinate) Percent change in $I_{Ca,L}$ above basal levels. (right ordinate) Percent change in K^+ conductance elicited by ACh_2 compared with ACh_1.

To determine whether the conditioning effect of ISO was mediated via β_1-ARs, the same protocol was repeated by testing 0.1 μM ISO in the presence of 0.01 μM ICI 118,551, a selective β_2-AR antagonist (O'Donnell and Wanstall, 1980). As shown in Fig. 1 B, ISO-β_1-AR stimulation increased $I_{Ca,L}$ markedly (372%, inset), but failed to elicit a potentiated increase in ACh_2-induced K^+ conductance. In fact, in this experiment, ACh_2 elicited an increase in K^+ conductance that was slightly smaller than ACh_1. As summarized in Fig. 2, ISO-β_1-AR stimulation increased $I_{Ca,L}$ (239 ± 48%), whereas ACh_2 failed to elicit a potentiated increase in K^+ conductance compared with ACh_1 (1 ± 4% at −130 mV, and 0.5 ± 3%, 30 mV; n = 8). In other words, ACh_1 and ACh_2 exposures induced essentially the same increase in K^+ conductance, indicating that ISO did not affect $I_{K,ACh}$. Similar results were obtained when the concentration of ISO was raised to 1 μM (in the presence of 0.1 μM ICI 118,551), i.e., stimulation of $I_{Ca,L}$ (395%) without potentiation of ACh_2-induced K^+ conductance (n = 3).

To determine whether the effects of ISO are mediated via β_2-AR signaling, we tested the effects of ISO plus 0.01 μM atenolol, a selective β_1-AR antagonist. Fig. 1 C shows that ISO-β_2-AR stimulation increased $I_{Ca,L}$ (248%, inset) and mediated a potentiated increase in ACh_2-induced K^+ conductance. As summarized in Fig. 2, ISO-β_2-AR stimulation increased $I_{Ca,L}$ by 199 ± 50% and ACh_2 increased K^+ conductance compared with ACh_1 by 27 ± 6% (−130 mV) and 29 ± 3% (30 mV; $P < 0.05$; n = 7). Additional experiments showed that 10 μM glibenclamide, an inhibitor of $I_{K,ATP}$, abolished the effect of ISO-β_2-AR stimulation to potentiate the ACh_2-induced K^+ conductance (−2 ± 9% at −130 mV, and 0.5 ± 5% at 30 mV) without affecting stimulation of $I_{Ca,L}$ (220 ± 58%; n = 4; not shown). This finding is consistent with ACh_2-induced activation of $I_{K,ATP}$, as previously reported (Wang and Lipsius, 1995). The combined presence of β_1-AR (0.1 μM atenolol) and β_2-AR (0.1 μM ICI 118,551) antagonists abolished the effects of 0.1 μM ISO to both stimulate $I_{Ca,L}$ (20 ± 5%) and mediate ACh-activated $I_{K,ATP}$ (4 ± 10% at −130 mV, and 0 ± 3% at 30 mV; n = 3). Further experiments showed that 0.01 μM BRL 37344, a β_3-AR agonist (Gauthier et al., 1999) failed to stimulate $I_{Ca,L}$ or mediate ACh-activated $I_{K,ATP}$ (n = 5; not shown).

To further establish that selective β_2-AR signaling is responsible for the conditioning effect, we tested zinterol, a selective β_2-AR agonist. As shown in Fig. 1 D, 1 μM zinterol increased $I_{Ca,L}$ (273%; inset) and mediated ACh_2-induced activation of $I_{K,ATP}$. As summarized in Fig. 2, zinterol increased $I_{Ca,L}$ by 159 ± 17% and increased ACh_2-induced K^+ conductance compared with ACh_1 by 36 ± 7% (−130 mV) and 28 ± 5% (30 mV; $P <$ 0.05; n = 16). To confirm that zinterol acted via β_2-AR stimulation, 1 μM zinterol was tested in the presence of 0.01 μM ICI 118,551. Interestingly, this relatively low concentration of ICI 118,551 abolished the effects of zinterol to mediate ACh_2-activated $I_{K,ATP}$ ([control] 23 ± 3% vs. [ICI] −12 ± 6% at −130 mV; and [control] 31 ± 5% vs. [ICI] −1 ± 3% at 30 mV) but only attenuated zinterol-induced stimulation of $I_{Ca,L}$ ([control] 193 ± 84 vs. [ICI] 160 ± 43%; not shown). Raising the concentration of ICI 118,551 to 1 μM also abolished zinterol-induced stimulation of $I_{Ca,L}$ ([control] 180% vs. [ICI] 0.2%; n = 6; not shown). These latter findings indicate that the sensitivity of β_2-ARs to mediate stimulation of $I_{Ca,L}$ is greater than to mediate ACh-activated $I_{K,ATP}$. This idea was explored further by testing a lower

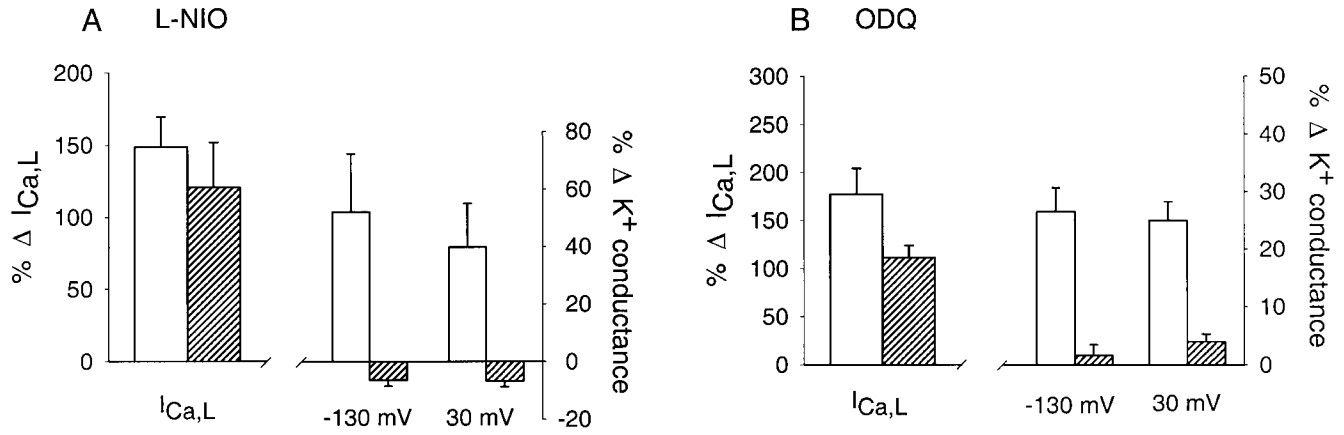

FIGURE 3. Effects of 1 μM zinterol on $I_{Ca,L}$ and ACh_2-induced K^+ conductances in the absence (open bars) and presence (hatched bars) of 10 μM L-NIO (A), an inhibitor of NO synthase, and 10 μM ODQ (B), an inhibitor of soluble guanylate cyclase. L-NIO (A) and ODQ (B) each attenuated zinterol-induced stimulation of $I_{Ca,L}$ and abolished the potentiation of ACh_2-induced K^+ conductances. Ordinates are the same as in Fig. 2.

(0.1 μM) zinterol concentration. As summarized in Fig. 2, 0.1 μM zinterol induced a typical increase in $I_{Ca,L}$ (152 ± 31%), but failed to elicit a potentiated increase in ACh_2-induced K^+ conductance compared with ACh_1 (−2 ± 2% at −130 mV, and 1 ± 3% at 30 mV; $n = 6$). Together, these results indicate that although stimulation of β_1- or β_2-ARs both increase $I_{Ca,L}$ markedly, only β_2-AR signaling selectively mediates ACh_2-induced activation of $I_{K,ATP}$. In addition, the effects of β_2-AR stimulation to mediate ACh-induced activation of $I_{K,ATP}$ and stimulate $I_{Ca,L}$ exhibit different signaling sensitivities. Finally, β_3-ARs do not participate in the conditioning effect of β-AR stimulation.

Nitric Oxide Signaling

β-AR stimulation can induce NO release in cardiac myocytes (Kanai et al., 1997). Therefore, we sought to determine whether the conditioning effect of β_2-AR stimulation is mediated via NO signaling by testing zinterol in the presence of 10 μM L-NIO, an inhibitor of constitutive NO synthase (Rees et al., 1990). The graph in Fig. 3 A shows that in the absence of L-NIO (open bars), zinterol elicited a typical increase in $I_{Ca,L}$ (150 ± 21%) and a potentiated increase in ACh_2-induced K^+ conductance (52 ± 20% at −130 mV, and 40 ± 15% at 30 mV). In a second group of cells from the same hearts, in the presence of L-NIO (Fig. 3 A, hatched bars), zinterol-induced stimulation of $I_{Ca,L}$ was attenuated (121 ± 31%), whereas ACh_2-induced activation of $I_{K,ATP}$ was abolished (−6 ± 2% at −130 mV, and −7 ± 2% at 30 mV) ($n = 12$; $P < 0.05$).

To determine whether NO signaling acts via cGMP, we tested zinterol in the presence of 30 μM ODQ, an inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995). The graph in Fig. 3 B shows that compared with control cells, ODQ also attenuated zinterol-induced stimulation of $I_{Ca,L}$ ([control] 177 ± 27% vs. [ODQ] 111 ± 13%) and abolished ACh_2-induced activation of $I_{K,ATP}$ ([control] 27 ± 4% vs. [ODQ] 2 ± 2% at −130

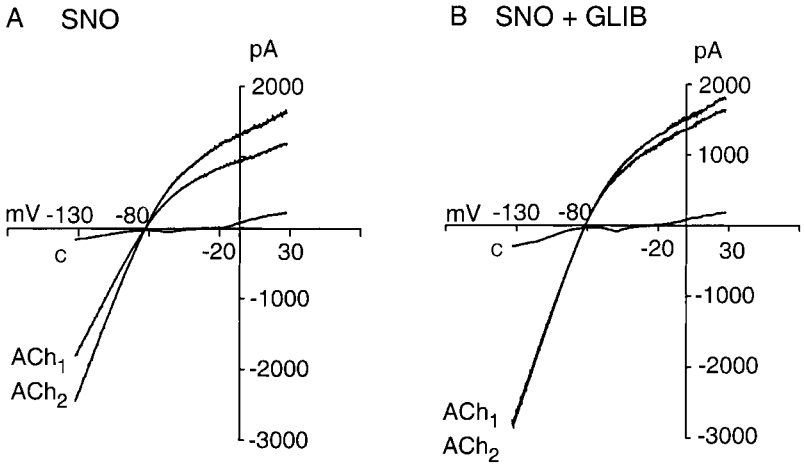

FIGURE 4. Current-voltage relationships showing the effects of 100 μM spermine-NO (SNO) on ACh-induced K^+ conductances in the absence (A) and presence (B) of 10 μM glibenclamide (GLIB). (A) SNO elicited a potentiated increase in ACh_2-induced K^+ conductance. (B) GLIB blocked the effect of SNO to mediate a potentiated increase in ACh_2-induced K^+ conductance. In the experiment shown, at positive voltages, ACh_2-induced K^+ conductance was slightly smaller than ACh_1. c; control K^+ conductance before ACh_1.

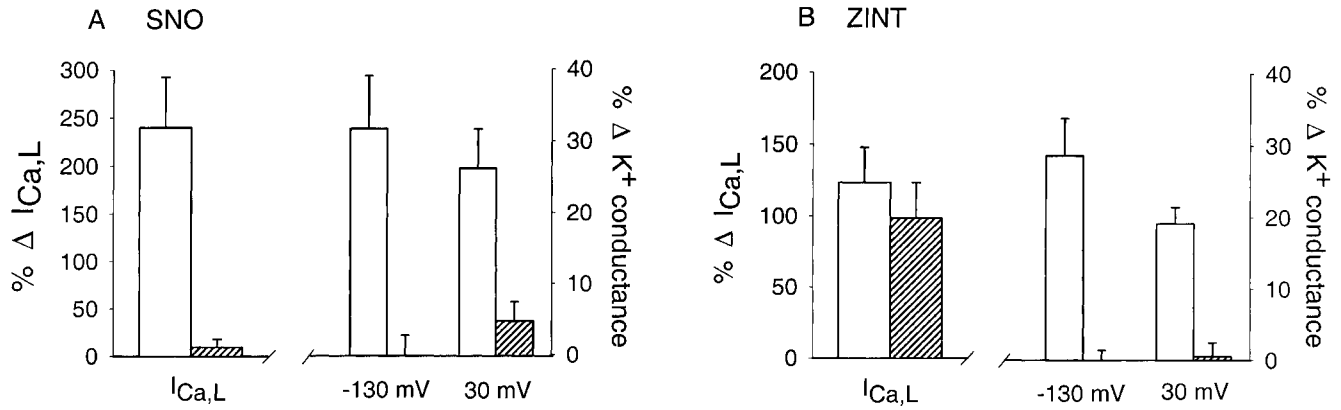

FIGURE 5. Effects of spermine-NO (SNO; A) and zinterol (ZINT; B) on $I_{Ca,L}$ and ACh_2-induced K^+ conductances in the absence (open bars) and presence (hatched bars) of 50 μM Rp-cAMPs. (A) Rp-cAMPs abolished the effects of 100 μM SNO to both increase $I_{Ca,L}$ and to mediate a potentiated increase in ACh_2-induced K^+ conductance. (B) Rp-cAMPs attenuated the effect of 1 μM zinterol to increase $I_{Ca,L}$ and abolished the effect of zinterol to mediate a potentiated increase in ACh_2-induced K^+ conductance. Ordinates are the same as in Fig. 2.

mV; and [control] 25 ± 3% vs. [ODQ] 4 ± 1% at 30 mV) ($n = 13$; $P < 0.05$).

If the conditioning effect of β_2-AR stimulation results from NO signaling, then exogenous NO should qualitatively mimic the effects of β_2-AR stimulation. Spermine-NO (SNO) spontaneously releases NO without the production of other biologically active byproducts or intermediates (Maragos et al., 1991). 100 μM SNO stimulated basal $I_{Ca,L}$ as previously reported (Wang et al., 1998), and mediated a potentiated increase in ACh_2-induced K^+ conductance compared with ACh_1 (Fig. 4 A). In a total of six cells, SNO increased $I_{Ca,L}$ by 240 ± 52% and potentiated ACh_2-induced K^+ conductance by 32 ± 7% (−130 mV) and 26 ± 5% (30 mV; $P < 0.05$). Moreover, 10 μM GLIB had no significant effect on SNO-induced stimulation of $I_{Ca,L}$, but as shown in Fig. 4 B, it abolished ACh_2-induced potentiation of K^+ conductance ([control] 32 ± 5% vs. [GLIB] −1 ± 2% at −130 mV; and [control] 30 ± 7% vs. [GLIB] −6 ± 7% at 30 mV) ($n = 8$; $P < 0.05$). Note that at positive voltages, ACh_2-induced K^+ conductance was slightly smaller than that induced by ACh_1. These findings demonstrate that exogenous NO mimics the effects of β_2-AR stimulation by stimulating $I_{Ca,L}$ and mediating ACh_2-induced activation of $I_{K,ATP}$. Together, these results indicate that β_2-AR stimulation acts via NO-cGMP signaling to mediate ACh_2-induced activation of $I_{K,ATP}$. In addition, NO signaling contributes to β_2-AR stimulation of $I_{Ca,L}$.

In cat atrial myocytes, NO-cGMP signaling enhances cAMP by inhibiting PDE type III activity (Wang et al., 1998). To determine whether β_2-AR–stimulated NO signaling acts via cAMP to mediate ACh-induced activation of $I_{K,ATP}$, we tested the effects of 100 μM SNO and 1 μM zinterol in the absence and presence of 50 μM Rp-cAMPs, an inhibitor of cAMP-dependent PKA activity (Van Haastert et al., 1984). In these experiments, cells were externally superfused with Rp-cAMPs. The graph in Fig. 5 A shows that compared with control cells (open bars), Rp-CAMPs abolished SNO-induced stimulation of both $I_{Ca,L}$ ([control] 240 ± 52% vs. [Rp-cAMPs] 9 ± 8%) and ACh_2-activated $I_{K,ATP}$ (32 ± 7% vs. 0 ± 3% at −130 mV, and 26 ± 5% vs. 5 ± 3% at 30 mV; $P < 0.05$). Interestingly, when the same experiment was performed with zinterol, compared with control responses (open bars), Rp-cAMPs only attenuated zinterol-induced stimulation of $I_{Ca,L}$ ([control] 123 ± 24% vs. [Rp-cAMPs] 98 ± 25%) while completely blocking ACh_2-induced activation of $I_{K,ATP}$ (29 ± 5% vs. 0 ± 1% at −130 mV; and 19 ± 2% vs. 1 ± 2% at 30 mV) ($n = 14$; $P < 0.05$). These results indicate that inhibition of cAMP-dependent PKA by Rp-cAMPs effectively abolishes NO-mediated signaling. Moreover, NO signaling stimulated by β_2-ARs acts via cAMP to mediate ACh-induced activation of $I_{K,ATP}$ and contributes to stimulation of $I_{Ca,L}$. The fact that inhibition of PKA abolished SNO-induced stimulation of $I_{Ca,L}$ but only attenuated β_2-AR stimulation of $I_{Ca,L}$ suggests that β_2-AR signaling regulates $I_{Ca,L}$, in large part, via a mechanism that is independent of NO signaling.

In other words, after blockade of NO signaling, β_2-AR stimulation still stimulates $I_{Ca,L}$ by at least 100% above basal levels. This NO-independent effect of β_2-AR stimulation could still be mediated by cAMP. It may not be inhibited by Rp-cAMPs because external application of Rp-cAMPs cannot reach high enough levels intracellularly to compete with cAMP and/or to access a separate cAMP compartment. To address this issue, we determined the effect of zinterol to regulate $I_{Ca,L}$ using a ruptured patch method to dialyze the cell interior with either 100 μM Rp-cAMPs or 20 μM cAMP-dependent PKA inhibitory peptide (PKI). This method allows better access to intracellular compartments and achieves a higher concentration of inhibitor intracellularly. Cells dialyzed without (control) and with drugs

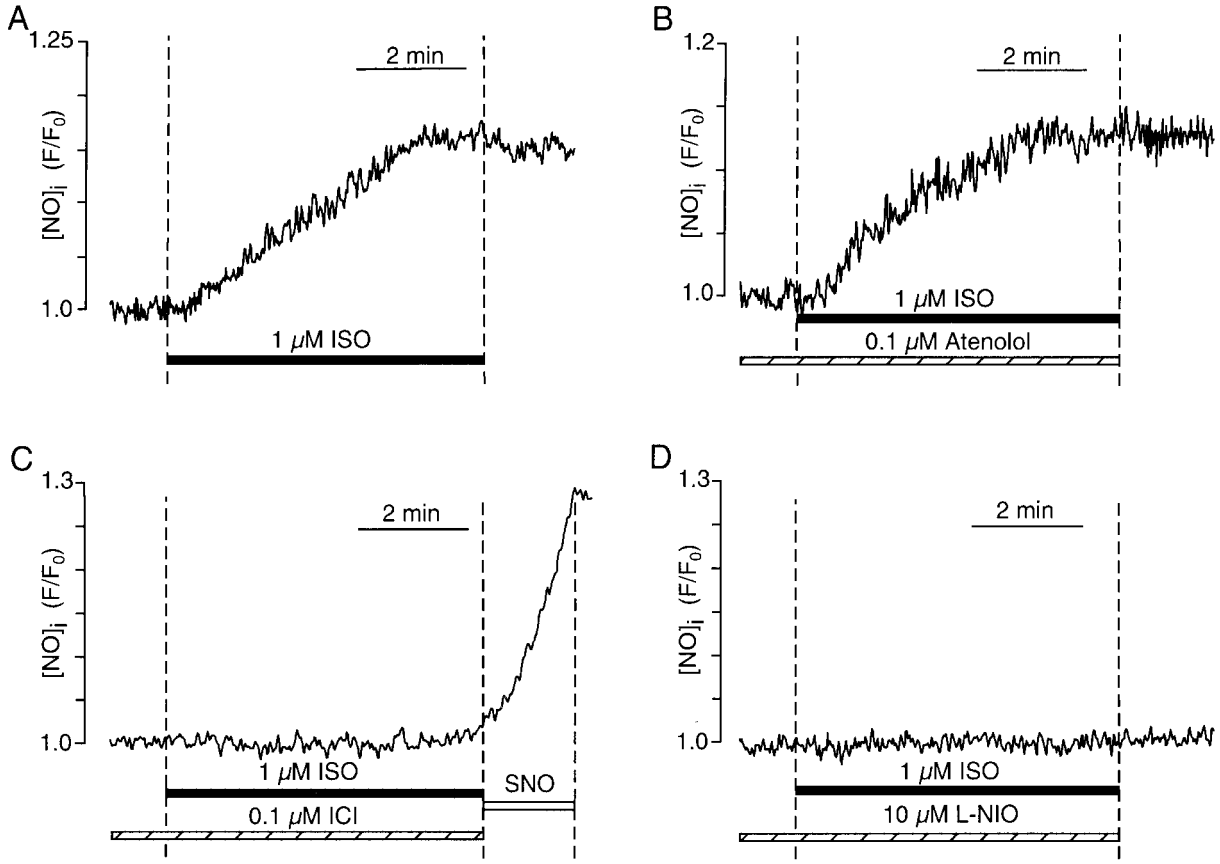

FIGURE 6. Effects of 1 μM ISO on $[NO]_i$. (A) ISO-β_1/β_2-AR stimulation increased $[NO]_i$. (B) ISO-β_2-AR increased $[NO]_i$. (C) ISO-β_1-AR had no effect on $[NO]_i$, although 100 μM SNO elicited a prominent increase in $[NO]_i$. (D) L-NIO blocked ISO-β_1/β_2-AR effects on $[NO]_i$.

were compared. Intracellular Rp-cAMPs elicited a significantly greater inhibition of 1 μM zinterol-induced stimulation of $I_{Ca,L}$ ([control] 140 ± 32% vs. [Rp-cAMPs] 54 ± 11%) ($n = 10$; $P < 0.05$; not shown). In addition, intracellular PKI essentially abolished the effect of 1 μM zinterol to stimulate $I_{Ca,L}$ ([control] 188 ± 17% vs. [Rp-cAMPs] 18 ± 6%; $n = 6$; $P < 0.05$) and almost completely blocked 0.1 μM ISO-induced stimulation of $I_{Ca,L}$ ([control] 176 ± 27% vs. [PKI] 25 ± 6%) ($n = 6$; $P < 0.05$; not shown). These findings indicate that β_2-AR stimulation of $I_{Ca,L}$ is mediated entirely via cAMP. Therefore, the β_2-AR–mediated stimulation of $I_{Ca,L}$ that remains after blockade of NO signaling is mediated via cAMP. Together, the present findings suggest that β_2-ARs generate cAMP via two different signaling pathways; NO-dependent and NO-independent.

Intracellular Nitric Oxide Release

In the following experiments, we used the NO indicator DAF-2 to directly visualize changes in intracellular NO concentration ($[NO]_i$). Fig. 6 (A–D) shows the effects of 1 μM ISO (β_1/β_2-AR stimulation, Fig. 6 A), 1 μM ISO in the presence of 0.1 μM atenolol (β_2-AR stimulation, Fig. 6 B), 1 μM ISO in the presence of 0.1 μM ICI 118,551 (β_1-AR stimulation, Fig. 6 C), and 1 μM ISO in the presence of 10 μM L-NIO (Fig. 6 D). In each experiment, the cells were field-stimulated at 1 Hz. ISO alone (Fig. 6 A) and ISO-β_2-AR stimulation (Fig. 6 B) both increased $[NO]_i$. ISO-β_1-AR stimulation (Fig. 6 C) failed to increase $[NO]_i$. However, exposure to 100 μM SNO elicited a prominent increase in $[NO]_i$, indicating that the NO indicator was functional (Fig. 6 C). ISO-mediated stimulation of $[NO]_i$ was abolished by pretreatment with 10 μM L-NIO (Fig. 6 D). These findings indicate that ISO acts via β_2-ARs, but not β_1-ARs, to activate NO release. As shown in Fig. 7 (A–D) the effects of β_2-AR stimulation on $[NO]_i$ was further examined by testing zinterol. Fig. 7 A shows that field stimulation (FS, arrow) alone had no effect on $[NO]_i$. During field stimulation, however, exposure to 10 μM zinterol increased $[NO]_i$ (Fig. 7 A). Zinterol-mediated increases in $[NO]_i$ were abolished by blocking β_2-ARs with ICI 118,551 (Fig. 7 B) or by blocking NOS with 10 μM L-NIO (Fig. 7 C). Fig. 7 D shows the effects of three different zinterol concentrations (10, 1, and 0.1 μM) on $[NO]_i$. Zinterol induced a dose-dependent increase in $[NO]_i$. The graph in Fig. 8 (A and B) summarizes the effects of 1 μM ISO and 10 μM zinterol on $[NO]_i$ (Fig. 8 A) and the

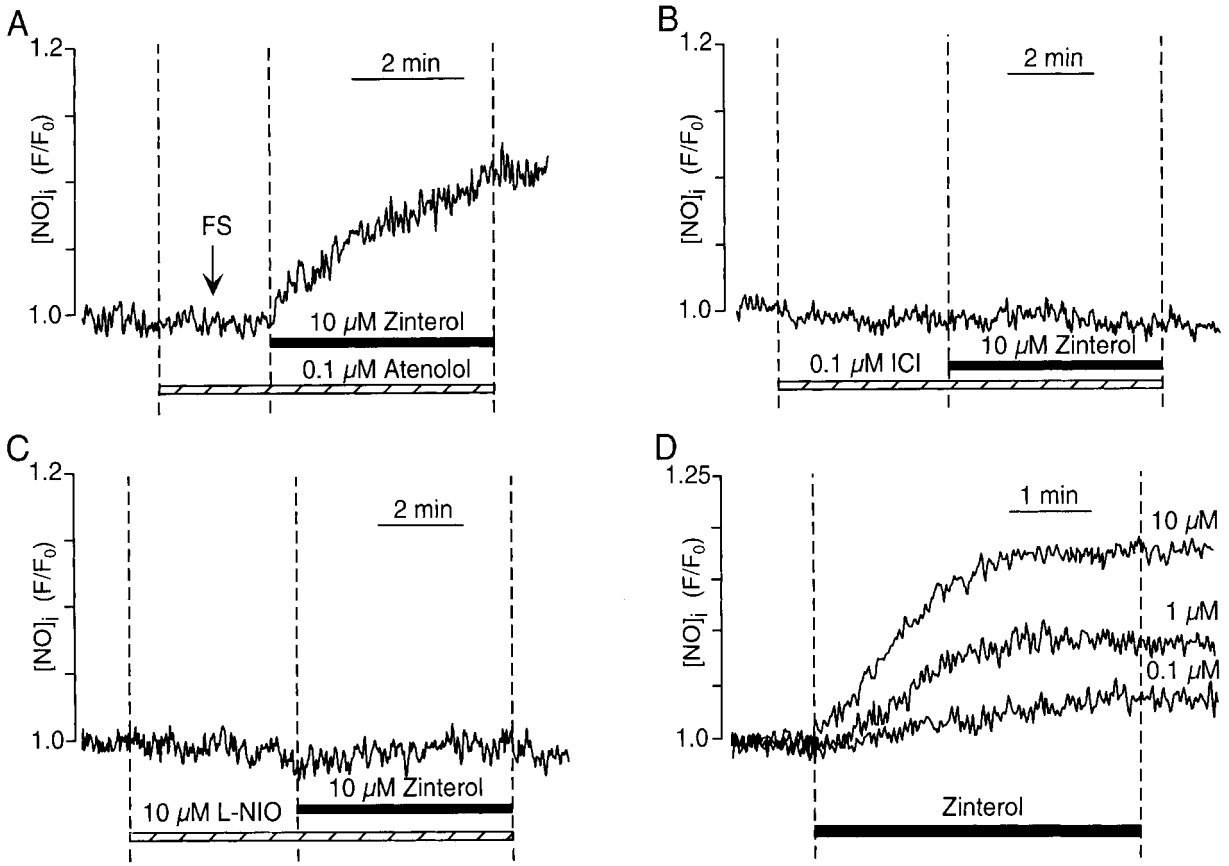

FIGURE 7. Effects of zinterol on $[NO]_i$. (A) Field stimulation (FS, arrow) had no effect on $[NO]_i$. However, during field of stimulation, exposure to 10 μM zinterol in the presence of 0.1 μM atenolol, increased $[NO]_i$. (B) Pretreatment with 0.1 μM ICI 118,551 blocked the effect of zinterol to increase $[NO]_i$. (C) Pretreatment with 10 μM L-NIO blocked the effect of zinterol to increase $[NO]_i$. (D) Zinterol (10, 1, and 0.1 μM) elicited a dose-dependent increase in $[NO]_i$.

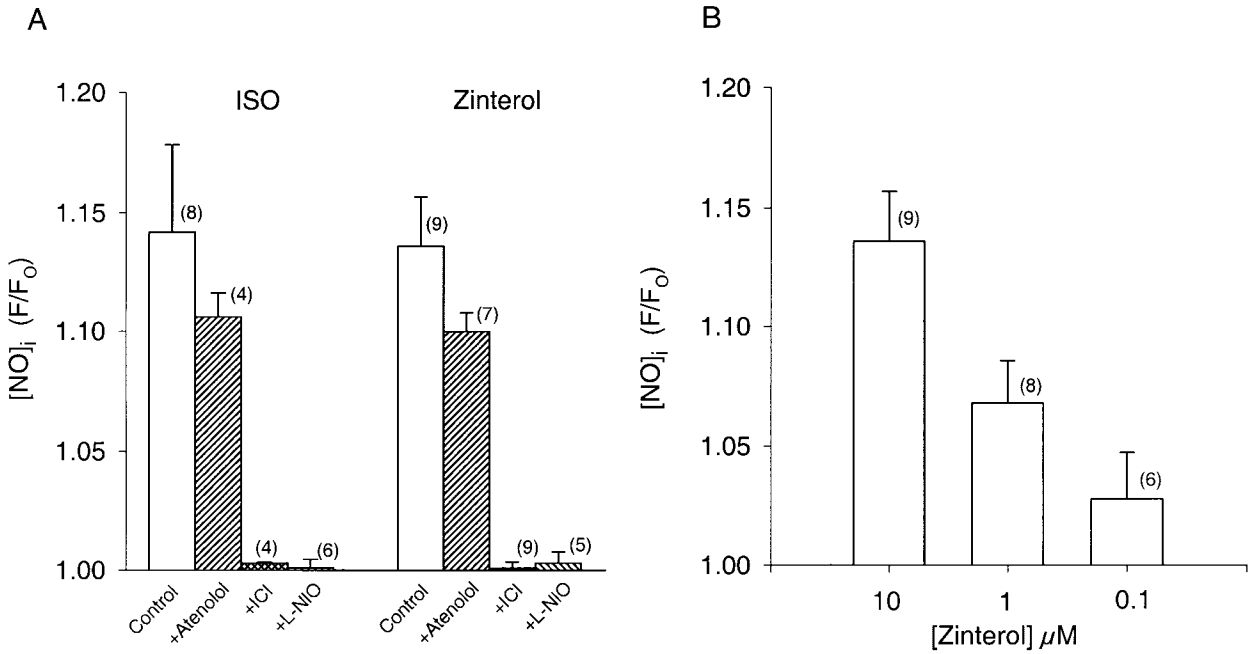

FIGURE 8. Summary of the effects of 1 μM ISO and 10 μM zinterol on $[NO]_i$. (A) The effects of ISO and zinterol to increase $[NO]_i$ (control) were not significantly affected by β_1-AR block (0.1 μM atenolol), but the effects of both agonists were abolished by β_2-AR block (ICI 118,551) or inhibition of NO synthase (L-NIO). (B) The effects of three different zinterol concentrations (10, 1, and 0.1 μM) on $[NO]_i$. The numbers in parentheses indicate the number of cells tested.

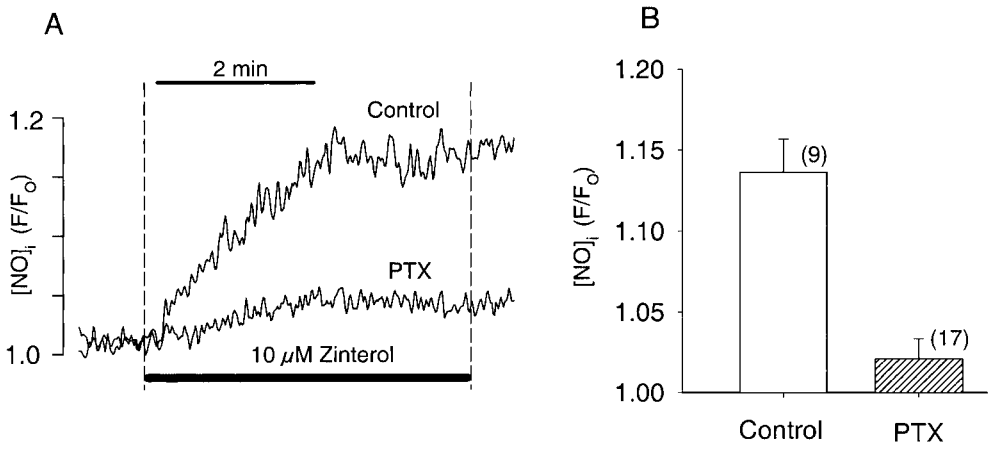

FIGURE 9. Effect of pertussis toxin (PTX) on zinterol-mediated stimulation of NO. (A) Under control conditions, 10 μM zinterol elicited a typical increase in $[NO]_i$. In another cell incubated in PTX, zinterol-induced increase in $[NO]_i$ was markedly decreased. (B) Graph summarizing the effects of 10 μM zinterol on control and PTX-treated cells. The numbers in parentheses indicate the number of cells tested.

dose-dependent effects of zinterol (Fig. 8 B). Both ISO and zinterol increased $[NO]_i$ (open bars). Although each response was slightly decreased by 0.1 μM atenolol, compared with control, the differences were not statistically significant. This concentration of atenolol may have exerted some β_2-AR blockade. Nevertheless, the effects of ISO and zinterol were abolished by β_2-AR blockade (0.1 μM ICI 118,551) or inhibition of NOS (10 μM L-NIO). Fig. 8 B summarizes the dose–response for the three different zinterol concentrations tested. At 0.1 μM zinterol, only three out of six cells elicited a small increase in $[NO]_i$. Further experiments showed that the β_3-AR agonist BRL 37344 (0.01 μM) failed to increase $[NO]_i$ (not shown). Together, these findings are consistent with the present electrophysiological findings that stimulation of β_2-ARs, but not β_1-ARs or β_3-ARs, act via NO signaling to mediate ACh-induced activation of $I_{K,ATP}$ and to stimulate $I_{Ca,L}$.

Finally, we sought to gain further insight into the signaling pathway through which β_2-ARs stimulate NO production and mediate ACh-induced activation of $I_{K,ATP}$. We determined whether the effect of β_2-ARs to release NO is mediated via G_i-protein by testing cells incubated in PTX (MATERIALS AND METHODS). Fig. 9 A shows the effect of 10 μM zinterol on a control cell and second cell obtained from the same heart incubated in PTX. Under control conditions, zinterol elicited a typical increase in $[NO]_i$. In the PTX-treated cell, zinterol-induced stimulation of NO production was markedly decreased. The graph in Fig. 9 B summarizes the effects of zinterol to increase $[NO]_i$ in control (1.1 ± 0.02; $n = 9$) and PTX-treated cells (1.0 ± 0.01; $n = 17$), and shows that compared with control, pretreatment with PTX significantly ($P < 0.001$) inhibited zinterol-induced stimulation of NO. These findings suggest that β_2-ARs act via G_i-protein to stimulate NO release.

In endothelial cells, stimulation of phosphatidylinositol 3′-kinase (PI-3K) signaling activates AKt (PKB)-mediated phosphorylation of eNOS, resulting in the production of NO (Dimmeler et al., 1999; Fulton et al., 1999). To examine the role of PI-3K, we incubated atrial cells in 0.2 μM Wortmannin, an inhibitor of PI-3K, for ~30–45 min. In a control cell (Fig. 10 A), 1 μM zinterol mediated a typical ACh_2-induced activation of $I_{K,ATP}$. In another cell from the same heart (Fig. 10 B), pretreatment with Wortmannin abolished the effect of zinterol to mediate ACh_2-induced activation of $I_{K,ATP}$. Comparing control ($n = 3$) and Wortmannin-treated cells ($n = 4$) showed that Wortmannin (wort) abolished the potentiated increase in ACh_2-induced K^+ conductance ([control] 37 ± 8% vs. [wort] 0.3 ± 1% at −130 mV; and [control] 36 ± 11% vs. [wort] 4 ± 4% at 30 mV) ($P < 0.05$). Additional experiments showed that 10 μM LY294002 (LY), a specific PI-3K inhibitor that acts via a different mechanism than Wortmannin (Vlahos et al., 1994), also abolished the potentiated increase in ACh_2-induced K^+ conductance ([control] 18 ± 2% vs. [LY] −0.1 ± 2% at −130 mV; and [control] 24 ± 4% vs. [LY] −7 ± 1% at 30 mV) ($n = 8$; $P < 0.05$; not shown). Furthermore, as shown in Fig. 10 C, in a control cell, 10 μM zinterol increased $[NO]_i$ and in a cell pretreated with Wortmannin, the zinterol-mediated increase in $[NO]_i$ was abolished. As summarized in Fig. 10 D, compared with control (1.2 ± 0.5; $n = 4$), Wortmannin (1.0 ± 0.2; $n = 3$) or LY294002 (0.9 ± 02; $n = 5$; $P < 0.05$) abolished zinterol-mediated increases in $[NO]_i$. These findings indicate that β_2-ARs act via PI-3K signaling to activate NO production that, in turn, mediates ACh-activated $I_{K,ATP}$.

DISCUSSION

Our previous work indicates that in cat atrial myocytes, an initial exposure to ISO acts via cAMP signaling to mediate a subsequent ACh-induced activation of $I_{K,ATP}$ (Wang and Lipsius, 1995). The present findings extend our previous work by elucidating several new underlying mechanisms: (1) the conditioning effect of β-AR stimulation acts selectively via β_2-ARs rather than the predominant β_1-AR signaling pathway; (2) β_2-ARs act via G_i-protein and PI-3K coupled to NO-cGMP-cAMP signaling to mediate ACh-induced activation of $I_{K,ATP}$;

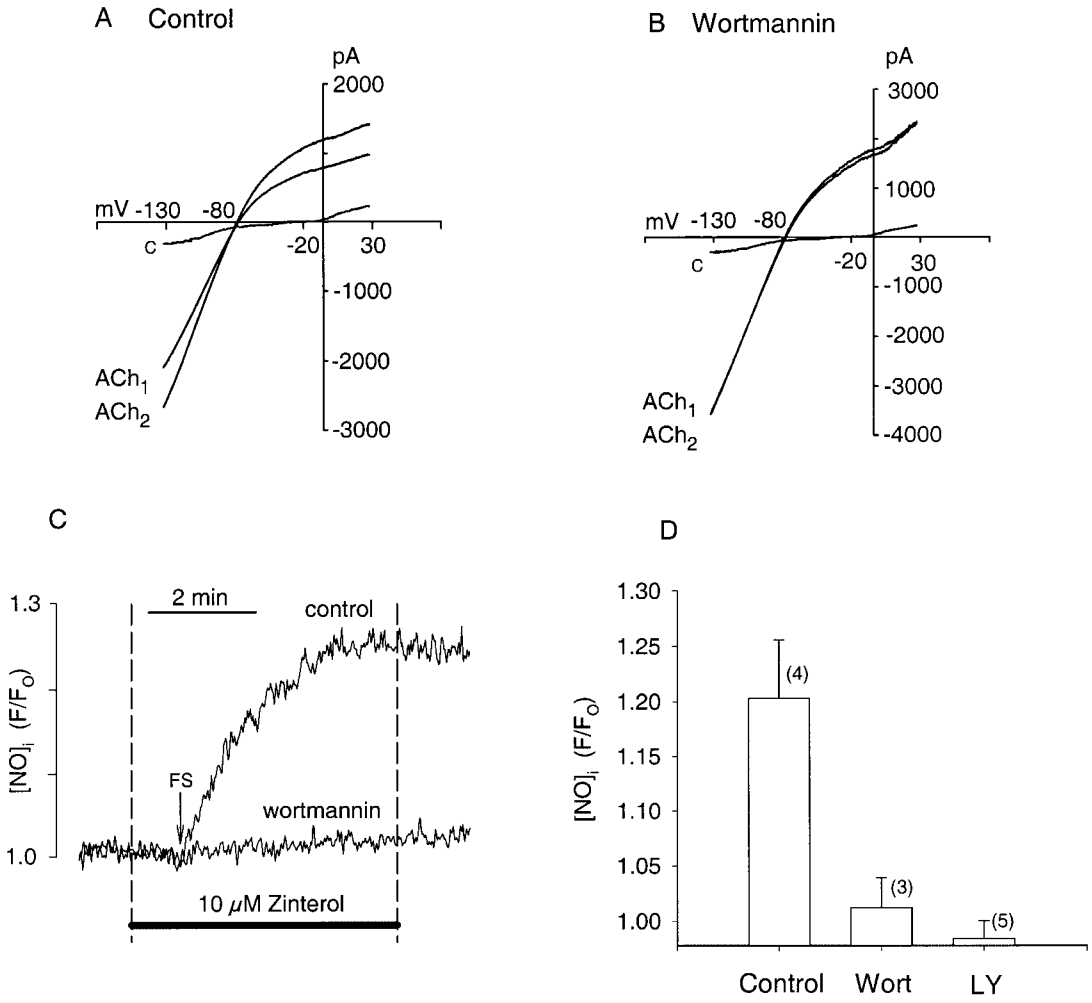

FIGURE 10. Effects of Wortmannin, an inhibitor of PI-3K, on ACh-induced activation of $I_{K,ATP}$ (A and B) and production of $[NO]_i$ (C and D). (A) Under control conditions, after exposure to 1 μM zinterol, ACh_2 induced a typical potentiated increase in K^+ conductance. (B) In another cell, pretreatment with 0.2 μM Wortmannin abolished the potentiated response to ACh_2. (C) Under control conditions, 10 μM zinterol stimulated $[NO]_i$. In another cell, pretreatment with 0.2 μM Wortmannin abolished zinterol-mediated stimulation of $[NO]_i$. (D) Summary graph showing that compared with control, pretreatment with either 0.2 μM Wortmannin (wort) or 10 μM LY294002 (LY) blocked zinterol-induced stimulation of $[NO]_i$. The numbers in parentheses indicate the number of cells tested.

and (3) NO signaling contributes to β_2-AR stimulation of $I_{Ca,L}$. In addition, to the best of our knowledge, this is the first report in adult atrial myocytes to demonstrate direct measurements of $[NO]_i$, and that G_i-protein and PI-3K signaling are coupled to NO production.

Perhaps one of the most surprising findings of the present study is that although β_1- and β_2-AR subtypes both stimulate cAMP, only cAMP stimulated by β_2-ARs mediated ACh-activated $I_{K,ATP}$. Moreover, only cAMP generated via NO signaling could mediate ACh-activated $I_{K,ATP}$. The fact that β_2-AR–mediated stimulation of $I_{Ca,L}$ and ACh-activated $I_{K,ATP}$ could be regulated, to a large extent, independently of one another suggests that β_2-ARs act via two different signaling pathways. Indeed, partial blockade of PKA activity (Rp-cAMPs), or β_2-ARs (ICI 118,551), or complete block of NO signaling (L-NIO or ODQ) abolished ACh-activated $I_{K,ATP}$, whereas β_2-AR stimulation of $I_{Ca,L}$ persisted. Similarly, low concentrations of zinterol stimulated $I_{Ca,L}$ without inducing ACh-activated $I_{K,ATP}$. The ability of β_2-ARs to stimulate $I_{Ca,L}$ without mediating ACh-activated $I_{K,ATP}$ is essentially the same as the response to β_1-AR stimulation. Because β_1-ARs are exclusively coupled via G_s-proteins to adenylate cyclase (Barr et al., 1997), it seems reasonable to assume that the β_2-AR signaling pathway that acts independently of NO signaling also is mediated via G_s-adenylate cyclase. This is supported by the present findings that inhibition of cAMP-dependent PKA (dialysis with PKI) abolished β_2-AR–mediated stimulation of $I_{Ca,L}$. Therefore, we conclude that β_2-ARs stimulate cAMP via two distinct signaling pathways: (1) G_s-protein coupled directly to adenylate cyclase, which is NO-independent; and (2) NO-cGMP–mediated inhibition of PDE type III activity (Fig.11). The latter pathway is consistent with

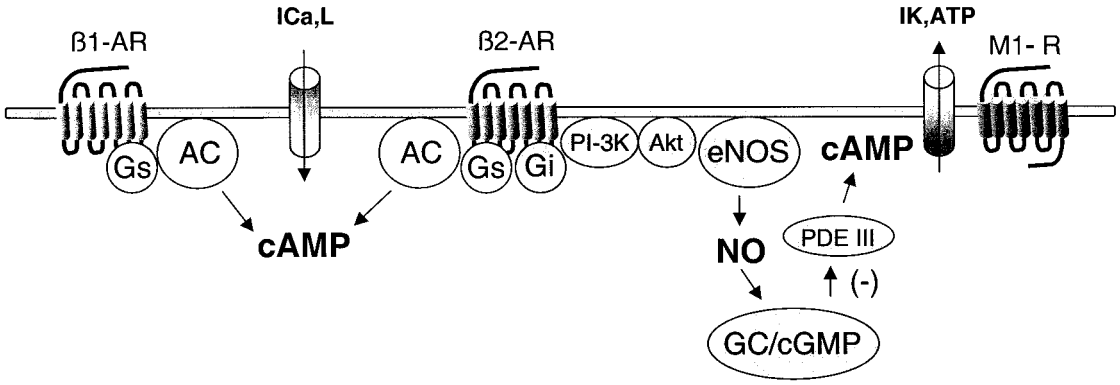

FIGURE 11. Schematic diagram summarizing the signaling pathways coupled to both β_1- and β_2-ARs in cat atrial myocytes. β_1-ARs couple exclusively via G_s-protein to adenylate cyclase (AC) to stimulate cAMP synthesis. β_2-ARs also couple via G_s-protein to AC to stimulate cAMP. Whether both β_1- and β_2-ARs are coupled to the same G_s-AC remains to be determined. β_2-ARs also couple via G_i-protein and PI-3K/Akt signaling to constitutive NO synthase (eNOS) and production of NO. NO stimulates guanylate cyclase (GC) to elicit cGMP-induced inhibition of PDE III, which raises cAMP generated by endogenous AC activity. Compartmentalized pools of cAMP generated via different β_1- and β_2-AR–mediated signaling pathways target regulation of L-type Ca^{2+} channels (ICa,L) and ACh-activated ATP-sensitive K^+ channels (IK,ATP). M1-R indicates subtype 1 muscarinic receptor.

our previous work in cat atrial myocytes, which indicates that NO signaling stimulates $I_{Ca,L}$ via cGMP-mediated inhibition of PDE type III (Wang et al., 1998).

The present work further indicates that stimulation of G_s-adenylate cyclase by either β_1- or β_2-ARs generates a pool of cAMP that is unable to mediate ACh-induced activation of $I_{K,ATP}$. In other words, stimulation of G_s-adenylate cyclase and NO signaling generate distinct compartments of cAMP. It is now well established that compartmentation of signaling molecules by scaffolding proteins can localize signaling mechanisms to specific intracellular sites (Couet et al., 1997; Okamoto et al., 1998; Steinberg and Brunton, 2001). For example, caveolin contained within caveolae membranes acts to anchor several important signaling components such as β_2-ARs, Gα subunits, eNOS, and isoforms of adenylate cyclase and PKC. In contrast, β_1-ARs are thought to be largely excluded from caveolae (Steinberg and Brunton, 2001). Moreover, other signaling components that exist in fixed spacial domains, such as A-kinase anchoring proteins, PKA regulatory subunits, phosphodiesterases, and phosphoprotein phosphatases, target cAMP signaling (Steinberg and Brunton, 2001). Indeed, Jurevičius and Fischmeister (1996) demonstrated that in frog ventricular myocytes, β-AR stimulation by ISO acts locally to stimulate $I_{Ca,L}$ via local elevation of cAMP, and that local PDE activity targets cAMP signaling to the channel. In the present study, the fact that exogenous NO, which is expected to raise global NO levels, mimicked the effects of β_2-receptor–mediated stimulation suggests that NO signaling targets specific PDE III activity colocalized with PKA to target cAMP locally generated by endogenous adenylate cyclase activity (Fig. 11). This is consistent with localized regulation by NO signaling (Dittrich et al., 2001). Moreover, inhibition of phosphodiesterase activities reduces the local response to β-adrenergic stimulation (Hohl and Li, 1991; Jurevičius and Fischmeister, 1996). This may explain why NO signaling induces ACh-activated $I_{K,ATP}$ as well as some stimulation of $I_{Ca,L}$. It is interesting to note that unlike β_1-AR stimulation, forskolin, a direct stimulator of adenylate cyclase, or 8-CPT-cAMP, a membrane-permeant analogue of cAMP, both elicit ACh-induced activation of $I_{K,ATP}$ equivalent to that of β_2-AR stimulation (Wang and Lipsius, 1995). Apparently, by raising intracellular cAMP to unphysiologically high levels, these agents probably flood restricted signaling compartments obscuring the delicate regulatory mechanisms that normally exist within the cell. This interpretation is supported by the findings that local application of ISO elicits local stimulation of $I_{Ca,L}$, whereas a similar exposure to forskolin stimulates $I_{Ca,L}$ throughout the cell (Jurevičius and Fischmeister, 1996).

In the present study, only β_2-AR signaling activated NO release. In rat ventricular myocytes both, β_1- and β_2-ARs stimulate NO release with β_1-ARs being more effective than β_2-ARs (Kanai et al., 1997). In rat atria, ISO stimulates NOS activity and the production of cGMP (Sterin-Borda et al., 1998), although different β-AR subtypes were not studied. The present results also indicate that stimulation of β_3-ARs failed to stimulate $I_{Ca,L}$ or mediate ACh-activated $I_{K,ATP}$, suggesting that this β-AR subtype is not involved in the conditioning effect of β-AR stimulation. In various animal species, including human (Gauthier et al., 1999), stimulation of β_3-ARs in ventricular muscle decreases contractility and in humans is presumably mediated via activation of an NOS pathway (Gauthier et al., 1998). The lack of β_3-AR response in cat atria may be species-dependent and/or due to differences between atrial and ventricular muscle. NO sig-

naling also exerts both negative as well as positive effects on β-adrenergic stimulation depending on various factors such as NO concentration, tissue type, and animal species (Balligand, 1999). The present results show that inhibition of NO signaling attenuated β_2-AR–induced stimulation of $I_{Ca,L}$, indicating that NO contributes to the stimulatory effects of β_2-AR signaling. This is consistent with the effects of NO signaling in both cat (Wang et al., 1998) and human (Kirstein et al., 1995) atrial muscle to stimulate $I_{Ca,L}$ via cGMP-mediated inhibition of PDE type III and elevation of cAMP.

The present results also demonstrate that β_2-ARs stimulate NO production via PTX-sensitive G_i-protein, which is consistent with reports that β_2-ARs are coupled to both G_s- and G_i-proteins (Xiao et al., 1995, 1999a; Kilts et al., 2000). In addition, the ability of β_2-ARs to mediate both stimulation of NO and ACh-induced activation of $I_{K,ATP}$ was abolished by blocking PI-3K signaling with either Wortmannin or LY294002. In endothelial cells, Akt is a downstream effector of PI-3K signaling, and can phosphorylate eNOS and stimulate production of NO (Fulton et al., 1999). Inhibition of PI-3K/Akt signaling or mutation of Akt sites on eNOS prevents activation of eNOS (Dimmeler et al., 1999). In fact, after submission of the present study, Vila Petroff et al. (2001) reported, in rat ventricular myocytes, that stretch-induced release of endogenous NO is mediated via PI-3K/Akt signaling. Moreover, in rat neonatal ventricular myocytes, β_2-AR stimulation protects from apoptosis via PI-3K/Akt signaling (Chesley et al., 2000). PI-3K/Akt signaling and protection from apoptosis were prevented by inhibition of G_i-protein (PTX), indicating that β_2-ARs act via G_i- to mediate PI-3K/Akt signaling. Therefore, we conclude that in cat atrial myocytes β_2-ARs are coupled via G_i-protein and PI-3K/Akt signaling to eNOS and the production of NO (Fig. 11). This would explain the present finding that β_1-ARs, which couple exclusively to G_s-protein, fail to mediate NO release. In addition, activation of PI-3K/Akt signaling is associated with enhanced cell survival (Kennedy et al., 1997; Datta et al., 1999), and NO signaling is a key mechanism in the cardioprotection conferred by ischemic preconditioning (Ping et al., 1999). Therefore, we speculate that β_2-AR stimulation acts via NO signaling to exert cardioprotective and/or antiapoptotic effects.

The effect of ISO to mediate ACh-activated $I_{K,ATP}$ is enhanced by Ca^{2+} influx via $I_{Ca,L}$ and is dependent on Ca^{2+} uptake and release from the SR (Wang and Lipsius, 1995). Based on these findings, we previously proposed that the conditioning effect of β-AR stimulation depended on cAMP to stimulate Ca^{2+} handling. However, this idea is difficult to reconcile with the present findings, which show that only cAMP generated exclusively via β_2-AR–mediated NO signaling is capable of inducing ACh-activated $I_{K,ATP}$. In other words, β_1-AR stimulation of cAMP would certainly be expected to stimulate Ca^{2+} handling, and yet it fails to mediate ACh-induced activation of $I_{K,ATP}$. Therefore, it appears that local NO-cAMP signaling must target additional sites that are more intimately related to cholinergic regulation of ATP-sensitive K^+ channels.

The present findings indicate that stimulation of the NO-dependent signaling pathway through which β_2-ARs mediate ACh-activated $I_{K,ATP}$ is less sensitive than the NO-independent (G_s-) signaling pathway through which β_2-ARs primarily regulate $I_{Ca,L}$. NO production may need to reach a critical threshold before it can raise cAMP concentrations sufficiently. This is supported by the present finding that although low concentrations of zinterol (0.1 μM) were capable of generating small amounts of $[NO]_i$, they failed to mediate ACh-induced activation of $I_{K,ATP}$. A similar argument could not explain the inability of ISO-β_1-AR stimulation to elicit ACh-activated $I_{K,ATP}$ or stimulate $[NO]_i$ because 1 μM ISO should have maximally stimulated β_1-ARs (Marsh and Smith, 1985). Functionally, the lower sensitivity of β_2-AR–mediated NO signaling indicates that this mechanism is probably invoked in response to relatively high levels of β-AR stimulation. As a result, β_2-AR–mediated NO signaling would augment stimulation of $I_{Ca,L}$ and, at the same time, condition the cell for subsequent enhanced cholinergic inhibition of atrial function via ACh-induced activation of $I_{K,ATP}$. Estimates indicate that ~1 nS/cell or <1% of the available conductance of ATP-sensitive K^+ channels is sufficient to shorten action potential duration by 50% (Nichols and Lederer, 1991). In the present study, $I_{K,ATP}$ activated by ACh represents an additional K^+ conductance of ~2–4 nS/cell (at 0 mV). This should elicit a profound shortening in action potential duration, resulting in a strong negative inotropic response and rapid termination of prior β-AR stimulation. This rapid termination may provide some protection from Ca^{2+} overload that could result from β-adrenergic-induced Ca^{2+} influx.

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