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Role of G Proteins in α_1-Adrenergic Inhibition of the β-Adrenergically Activated Chloride Current in Cardiac Myocytes

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SUMMARY

α_1-Adrenergic receptor stimulation can inhibit the Cl^- current activated by β-adrenergic receptor agonists in guinea-pig ventricular myocytes. We investigated the role of G proteins in mediating this type of α-adrenergic response. The combined α- and β-adrenergic agonist norepinephrine (NE) activated the Cl^- current with an EC_{50} value of 53 nM. Preincubation of myocytes with PTX decreased the EC_{50} value for NE activation of the Cl^- current to 5.9 nM, and addition of the α_1-adrenergic receptor antagonist prazosin did not cause any further change in sensitivity to NE. These results suggest that the α_1-adrenergic inhibition of β-adrenergic responses is mediated through a PTX-sensitive G protein. However, PTX pretreatment also increased the sensitivity of the Cl^- current to the selective β-adrenergic agonist isoproterenol (Iso), which indicates that the PTX treatment increases the sensitivity to β-adrenergic stimulation alone and that this could account for the PTX-induced change in sensitivity to NE. Consistent with this idea, the selective α_1-adrenergic receptor agonist methoxamine was still able to inhibit the Cl^- current activated by Iso in PTX-treated myocytes. However, the sensitivity to methoxamine was significantly decreased. In control cells, the Cl^- current activated by 30 nM Iso was inhibited by methoxamine with an EC_{50} value of 8.3 μM, but in PTX-treated cells, the EC_{50} value was 284 μM. The EC_{50} for methoxamine inhibition was similarly increased when the Cl^- current was activated by 300 nM Iso. These data suggest that the effects of PTX on α_1-adrenergic responses can actually be explained by changes in the sensitivity to β-adrenergic stimulation. To verify the role for a G protein in mediating the inhibitory α_1-adrenergic response, we examined the effect of methoxamine on the Cl^- current activated in cells dialyzed with the nonhydrolyzable GTP analogue guanosine-5′-*O*-(3-thio)-triphosphate. Pre-exposure to methoxamine resulted in an attenuated response upon subsequent exposure to Iso alone. We conclude that α_1-adrenergic inhibition of β-adrenergic responses is mediated by a G protein-dependent mechanism that appears to be PTX-insensitive.

The sympathetic nervous system exerts much of its influence on cardiac function by altering the activity of various ion channels. Many of the effects on ion channel function are mediated by the neurotransmitter NE, which can activate both α- and β-adrenergic receptors. In cardiac myocytes, β-adrenergic receptor stimulation is known to enhance the L-type Ca^{2+} current and delayed rectifier K^+ current and to activate the Cl^- current conducted by the cardiac isoform of the CFTR (1). These effects of β-adrenergic receptor stimulation on ion channels are mediated through the stimulation of adenylate cyclase, production of cAMP, and subsequent activation of protein kinase A (1).

α-Adrenergic receptor stimulation has also been reported to affect the activity of various cardiac ion channels. These effects include stimulation of the delayed rectifier K^+ current, as well as inhibition of the inward rectifier, transient outward, and ACh-activated K^+ currents (2–5). Unlike β-adrenergic responses, the mechanisms involved in α-adrenergic responses are not as well understood. α-Adrenergic receptor stimulation is known to activate PLC and PLA_2 (6, 7). PLC and PLA_2 are linked to several different signaling pathways, including those involved in activation of PKC, as well as production of AA. Both PKC and AA have been implicated in α-adrenergic regulation of cardiac ion channels (8, 9).

In addition to exerting autonomous effects, α-adrenergic receptor stimulation can also regulate ion channel function indirectly, by antagonizing β-adrenergic responses. In fact, it has been demonstrated that activation of α_1-adrenergic receptors contributes to the net effect of NE by limiting its response to β-adrenergic receptor stimulation (10, 11). This α_1-adrenergic effect is the result of inhibition of the β-adrenergic pathway at a point before G protein-dependent activa-

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ABBREVIATIONS: NE, norepinephrine; PTX, pertussis toxin; Iso, *R*-(−)-isoproterenol (+)-bitartrate; KHB, Krebs-Henseleit buffer; CFTR, cystic fibrosis transmembrane conductance regulator; ACh, acetylcholine; PLC, phospholipase C; PKC, protein kinase C; PLA_2, phospholipase A_2; AA, arachidonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

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tion of adenylate cyclase. In fact, this inhibitory effect seems to be specific for the β-adrenergic receptor, because α_1-adrenergic agonists, such as methoxamine, do not antagonize cAMP-dependent responses activated by H_2-histamine receptor stimulation (11). However, very little is known about the exact signaling pathway that mediates this inhibitory α_1-adrenergic response. Although the response is affected by PTX, it has not been determined whether α_1-adrenergic inhibition is mediated by a PTX-sensitive G protein (11).

In the present study, we investigated the role that G proteins play in α_1-adrenergic inhibition of the β-adrenergic responses, looking specifically at the regulation of the cAMP-regulated Cl^- current. The PTX-sensitivity of the α_1-adrenergic response seems to be caused by an indirect effect of PTX on β-adrenergic responses. We provide evidence that α_1-adrenergic inhibition of the β-adrenergically activated Cl^- current does indeed involve a G protein-dependent mechanism, but the G protein involved does not seem to be PTX-sensitive.

Materials and Methods

Cell isolation. Ventricular myocytes were isolated using a modification of a method previously described (12). Briefly, hearts were excised from anesthetized adult Hartley guinea-pigs of either sex and subjected to coronary perfusion via the aorta with KHB containing 120 mM NaCl, 4.8 mM KCl, 1.5 mM $CaCl_2$, 2.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose. The buffer's pH was maintained at 7.35 by bubbling with 95% O_2/5% CO_2 at 37°. Immediately after removal, the heart was perfused with normal Ca^{2+}-containing KHB for 5 min. The heart was then perfused with Ca^{2+}-free KHB for a further 5 min, after which time collagenase (type B; Boehringer Mannheim, Indianapolis, IN) was added to achieve a final concentration of 0.5–0.7 mg/ml. After 45 min of digestion, the ventricles were cut down, minced, rinsed free of collagenase, and then reintroduced to Ca^{2+}-containing KHB. Gentle trituration freed individual cells from the tissue for use in experiments on the day of isolation only.

Data acquisition and analysis. The cAMP-regulated Cl^- current was recorded using the conventional whole-cell configuration of the patch-clamp technique (13). Microelectrodes were pulled from borosilicate glass capillary tubing (Corning 7052, Garner Glass, Claremont, CA) and had resistances between 0.5 and 1.5 MΩ when filled with the following intracellular solution: 130 mM glutamic acid, 5 mM HEPES, 5 mM EGTA, 20 mM tetraethylammonium chloride, 5 mM MgATP, 0.1 mM Tris-GTP; the pH was adjusted to 7.1 with CsOH. The control extracellular solution contained: 140 mM NaCl, 5.4 mM CsCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM HEPES, and 11 mM glucose; the pH was adjusted to 7.4 with NaOH. Currents were recorded using an Axopatch 200 voltage-clamp amplifier (Axon Instruments, Foster City, CA) and an IBM-compatible computer with a TL-1–125 interface and pCLAMP software (Axon Instruments). The bath was grounded with a 3 M KCl/agar bridge; no compensation was made for junction potentials.

Cells were placed on a 0.5 ml chamber under an inverted microscope, where they were superfused with a control solution at a rate of 1 to 2 ml/min. Solutions for all experiments were maintained at 37°. Using a fast-flow system described previously (14), the extracellular solution bathing the cell from which currents were being recorded could be changed in <1 sec. The Cl^- current was isolated by blocking all K^+ channels with Cs- and/or tetraethylammonium-containing intra- and extracellular solutions. L-type Ca^{2+} channels were blocked by adding 1 μM nisoldipine to all extracellular solutions. Na^+ and T-type Ca^{2+} channels were inactivated by using a holding potential of −30 mV. The time courses of changes in Cl^- conductance were monitored by applying 100-msec voltage steps to +50 mV once every 3 sec. Current-voltage relationships were recorded by applying 100-msec voltage steps from the holding potential of −30 mV to test potentials from −120 mV to +50 mV in 10 mV increments. The Cl^- current was defined as the agonist-induced difference current obtained by subtracting current traces recorded in the absence of drug from those recorded in the presence of drug(s). Current magnitude was taken as the average measured over a 15-msec span at the end of each 100-msec step. The Cl^- conductance was calculated by linear regression of the current-voltage relationship positive to the reversal potential.

To determine the concentration dependence of drug induced effects, cumulative concentration-response relationships were performed, and data were fit to the following equation:

$$G_{Cl} = \frac{G_{max} - G_{min}}{1 + \left(\frac{EC_{50}}{[drug]}\right)^n} + G_{min}$$

where G_{Cl} is the relative magnitude of the Cl^- conductance measured in the presence of a given drug concentration, G_{max} is G_{Cl} measured in the presence of a maximally stimulating concentration of Iso or NE alone, G_{min} is the minimum value of G_{Cl}, EC_{50} is the concentration of drug at which G_{Cl} is 50% of G_{max}, and n is the slope of the relationship. For each cell, G_{max} was determined, and all Cl^- conductance measurements were normalized to that value. Fitting was accomplished using a nonlinear least-squares curve-fitting routine (SigmaPlot, Jandel Scientific, San Rafael, CA). Results are reported as mean ± standard error. Statistical comparisons between 2 groups were conducted using Student's t test, and where comparisons involved several groups of cells One-way analysis of variance and the Bonferroni t test were used (SigmaStat, Jandel Scientific).

Drugs and reagents. Most compounds were prepared as stock solutions so that the desired final concentration was achieved by 1:1000 dilution with the external control solution. Unless otherwise noted, all drugs were purchased from Research Biochemicals Inc. ACh, Iso, methoxamine hydrochloride, NE, and GTPγS (Sigma Chemical, St. Louis, MO) were prepared in distilled water. Prazosin hydrochloride was initially dissolved in dimethylsulfoxide (Sigma); the concentration of dimethylsulfoxide in the final solution was 0.01%. For experiments in which prazosin was used, cells were incubated in a solution containing 1 μM prazosin for at least 1 hr before, as well as during, patch clamp experiments. Nisoldipine (a gift from Miles Laboratories, Natick, MA) was prepared as a stock solution in polyethylene glycol; the concentration of polyethylene glycol in the final solution was 0.05%. Ascorbic acid (50 μm; Sigma) was added to all solutions containing Iso or NE to prevent oxidative degradation.

For some experiments, cells were further treated with 2 μg/ml PTX (List Biological Laboratories, List Biochemicals, Campbell, CA) for 3 hr at 37°. The muscarinic receptor agonist ACh can inhibit the Cl^- current activated by Iso, and this effect can be blocked by PTX (15). Therefore, in groups of cells pretreated with PTX, we only included those that exhibited a lack of inhibition by ACh.

Results

PTX blocks the α-adrenergic component of the NE response. We have previously demonstrated that prazosin, an α_1-adrenergic receptor antagonist, increases the sensitivity of the cAMP-regulated Cl^- current to NE, a combined α- and β-adrenergic receptor agonist (11). This indicates that although NE is able to activate the cAMP-regulated Cl^- current through the activation of β-adrenergic receptors, the ability of NE to also activate α-adrenergic receptors contributes to the net effect by actually limiting the β-adrenergic response. Furthermore, preliminary experiments have suggested that this inhibitory effect exerted by α-adrenergic receptor activation may be mediated by a PTX-sensitive G

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protein. If this is true, then the sensitivity of the Cl^- current to activation by NE should be increased in myocytes treated with PTX, and in PTX-treated myocytes, the sensitivity to NE should not be affected by prazosin.

To begin testing this hypothesis, we first characterized the response to NE under control conditions. Cells were exposed to increasing concentrations of NE. NE concentrations of >10 nM were necessary to activate the Cl^- current, and maximal activation required concentrations of >100 nM (Fig. 1A). The NE-activated current exhibited properties expected for a current conducted by the cardiac isoform of CFTR: time independence (Fig. 1B), outward rectification, and a reversal potential near the predicted Cl^- equilibrium potential (Fig. 1C). In cells treated with PTX, the properties of the current activated by NE were identical to those observed under control conditions. However, the sensitivity to NE was significantly increased. The Cl^- current was activated by concentrations as low as 3 nM NE, and the current was maximally activated at 30 nM NE (Fig. 2).

Comparing concentration response relationships obtained from several cells demonstrates that PTX-treatment decreased the EC_{50} for NE activation of the Cl^- current from 53 ± 7.1 to 5.9 ± 1.3 nM (Fig. 3). This represents a statistically significant ($p < 0.001$) increase in the sensitivity to NE. However, adding an α_1-adrenergic receptor antagonist did not cause any further increase in the sensitivity to NE (Fig. 3). In the presence of 1 μM prazosin, NE activated the Cl^- current with an EC_{50} value of 5.7 ± 1.1 nM in PTX treated myocytes (Fig. 3). This contrasts results obtained in non-PTX treated myocytes, in which 1 μM prazosin significantly increased the sensitivity to NE (11).

Effects of PTX on α- and β-adrenergic responses. The fact that prazosin did not affect the response to NE in PTX-treated cells is consistent with the idea that α_1-adrenergic

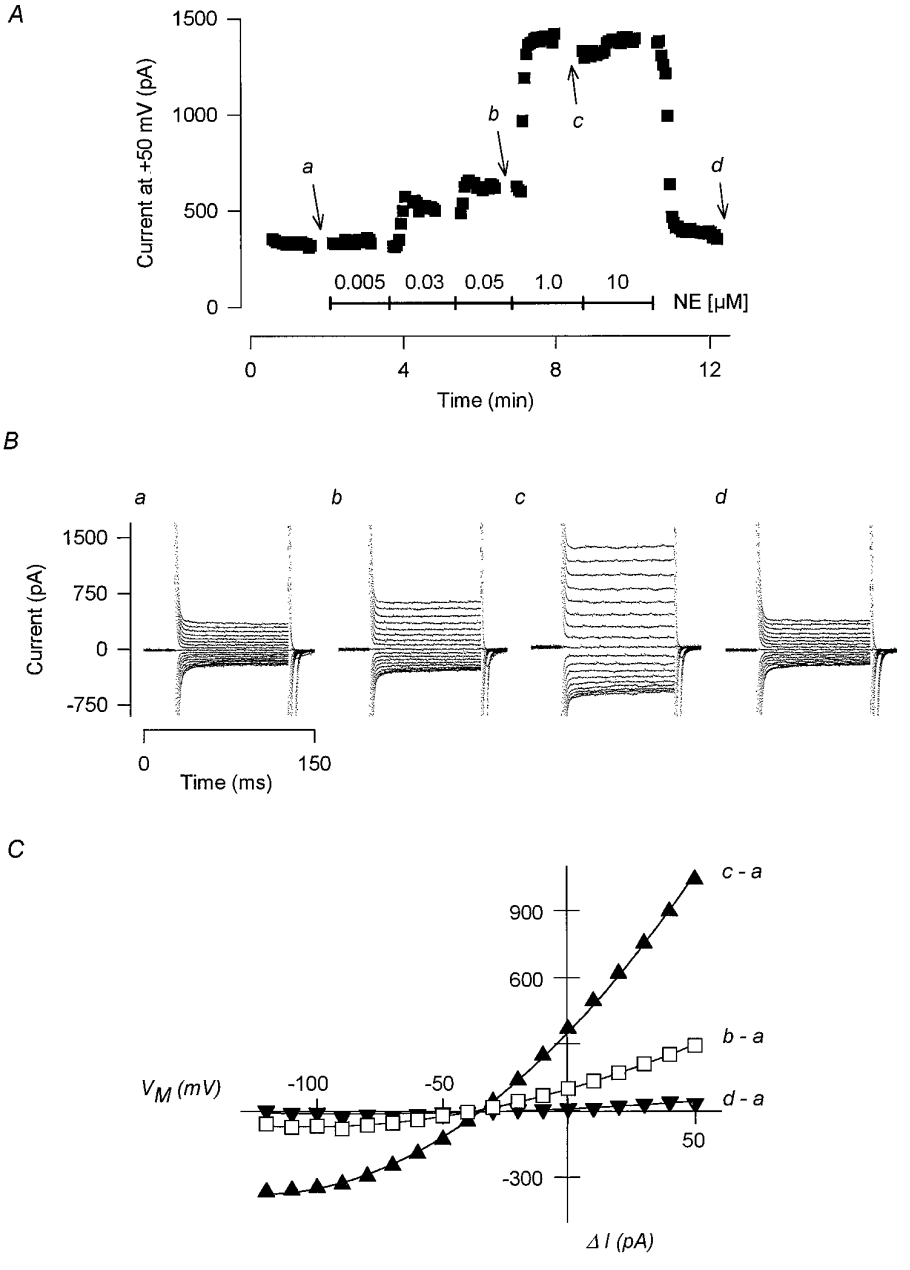

Fig. 1. Norepinephrine activates the CFTR Cl^- current in a concentration-dependent manner. A, Time course of changes in membrane current recorded during 100-msec voltage-clamp steps to +50 mV applied every 3 sec. B, Membrane currents recorded at time points in the protocol illustrated in A. Currents were elicited by 100-msec voltage-clamp steps to membrane potentials between −120 and +50 mV in 10-mV increments. C, Membrane potential (V_M)-dependence of difference current (ΔI) obtained by subtracting currents recorded under control conditions (*a*) from currents recorded in the presence of 50 nM NE (*b*), a maximally stimulating concentration of NE (*c*), and after washout of NE (*d*). This experiment is representative of the control data averaged in Fig. 3.

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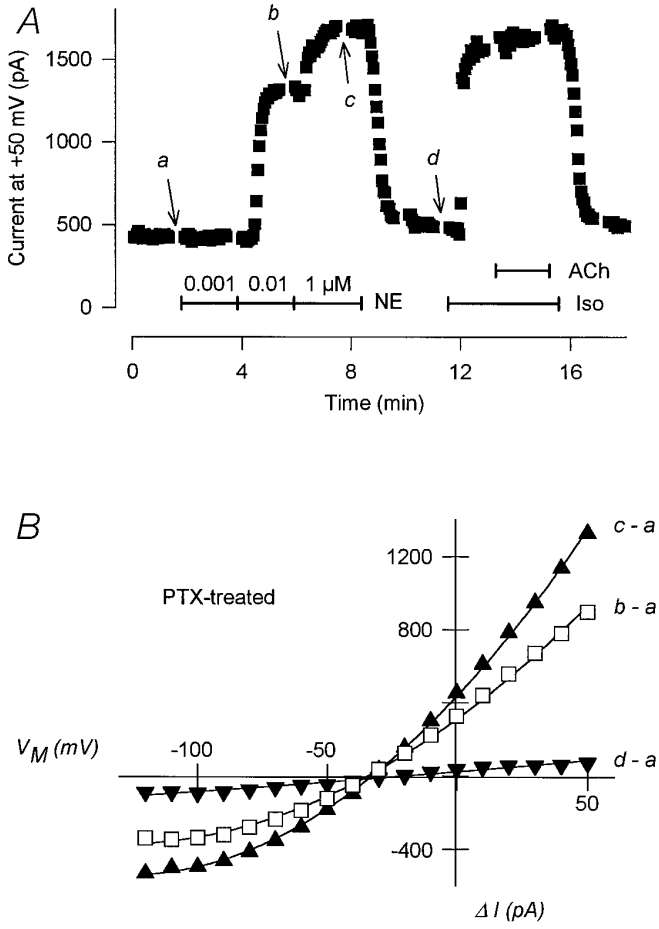

Fig. 2. Exposure to PTX increases the sensitivity of the Cl^- current to NE. A, Time course of changes in membrane current. The lack of response to muscarinic stimulation by 1 μM ACh confirms that PTX-treatment was effective. B, Membrane potential (V_M)-dependence of difference current (ΔI) obtained by subtracting currents recorded under control conditions (*a*) from currents recorded in the presence of 10 nM NE (*b*), a maximally stimulating concentration of NE (*c*), and after washout of NE (d). This experiment is representative of the PTX data averaged in Fig. 3.

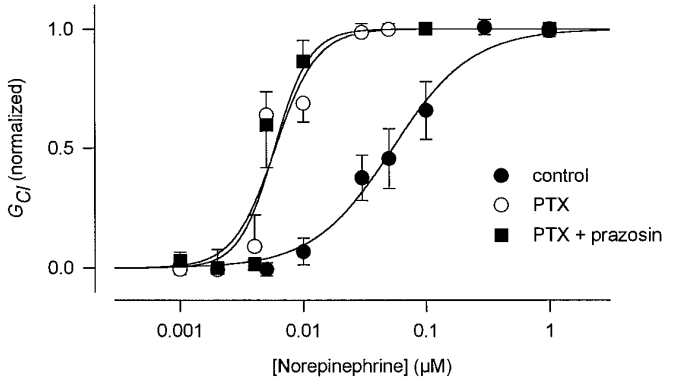

Fig. 3. The effect of PTX and prazosin on the sensitivity of the Cl^- current to NE. Concentration-response relationship for cells exposed to increasing concentrations of NE in the absence of PTX or prazosin (●), cells exposed to PTX alone (○) and cells exposed to both PTX and the α_1-adrenergic receptor antagonist prazosin (■). The Cl^- conductance (G_{Cl}) measured at each concentration was normalized to a maximally stimulating concentration of NE in the same cell. Data were fit to a logistic equation using a non-linear, least-squares curve-fitting routine (see Materials and Methods). ●, average of 5 to 14 experiments; ○ and/or ■, average of 5 to 7 experiments. In PTX-exposed cells, only those cells that exhibited a lack of inhibition to 1 μM ACh were included.

inhibition of β-adrenergic responses is mediated through a PTX-sensitive G protein. However, an alternative explanation is that PTX-treatment actually increased the sensitivity to β-adrenergic stimulation, without blocking the inhibitory α_1-adrenergic response.

To test this alternative explanation for the effect of PTX on the response to NE, we determined what effect, if any, PTX treatment had on the sensitivity of the response to Iso, a selective β-adrenergic receptor agonist. In untreated myocytes, the threshold for activation of the cAMP-regulated Cl^- current was approximately 1 nM, and the current was maximally activated by Iso concentrations of >30 nM. An example of the concentration dependence of the Iso response in an untreated myocyte is illustrated in Fig. 4A. In PTX-treated myocytes, the threshold for activation of the Cl^- current was approximately 0.1 nM, and the current was maximally activated by Iso concentrations of as low as 3 nM. An example of the concentration dependence of the Iso response in a PTX-treated myocyte is illustrated in Fig. 4B. Comparing concentration response relationships obtained from several cells demonstrates that PTX-treatment decreased the EC_{50} for Iso activation of the Cl^- current from 5.0 ± 0.05 to 1.4 ± 0.2 nM (Fig. 4C). This represents a statistically significant ($p < 0.005$) increase in the sensitivity to Iso.

The fact that PTX increases β-adrenergic receptor sensitivity raises the question of whether α_1-adrenergic inhibition of β-adrenergic responses is really mediated by a PTX-sensitive G protein. To investigate this question, we examined the concentration dependence of the response to methoxamine, a selective α_1-adrenergic receptor agonist, in PTX-treated myocytes. In these experiments, the Cl^- current was first activated by 30 nM Iso alone. After this, cells were exposed to increasing concentrations of methoxamine in the continued presence of Iso. As illustrated in the example in Fig. 5A, methoxamine was still able to completely inhibit the Iso-activated Cl^- current. In untreated cells, methoxamine inhibited the Iso-activated Cl^- current with an EC_{50} value of 8.3 ± 1.4 μM (Fig. 5C). In PTX-treated cells, methoxamine was still able to completely inhibit the Iso-activated current, but in this case, the EC_{50} value was 284 ± 44.0 μM (Fig. 5C). Although PTX treatment did not block the response to methoxamine, it did significantly ($p < 0.001$) decrease the sensitivity to methoxamine.

These results suggest that if the α_1-adrenergic response is mediated by a PTX-sensitive G protein, it cannot be completely inhibited by PTX, or more than one type of G protein is involved. Alternatively, the shift in the sensitivity to methoxamine could be caused by the PTX-induced increase in the sensitivity to β-adrenergic receptor stimulation. However, this would have to mean that the antagonistic interaction between α_1- and β-adrenergic stimulation is functionally competitive. To determine whether or not this is true, we investigated what effect increasing the concentration of Iso used to activate the Cl^- current had on the sensitivity of the response to methoxamine in cells that were not treated with PTX. In these experiments, we increased the concentration of Iso to 300 nM. After activation of the Cl^- current, cells were then exposed to increasing concentrations of methoxamine in the continued presence of Iso. Fig. 5B shows the protocol used

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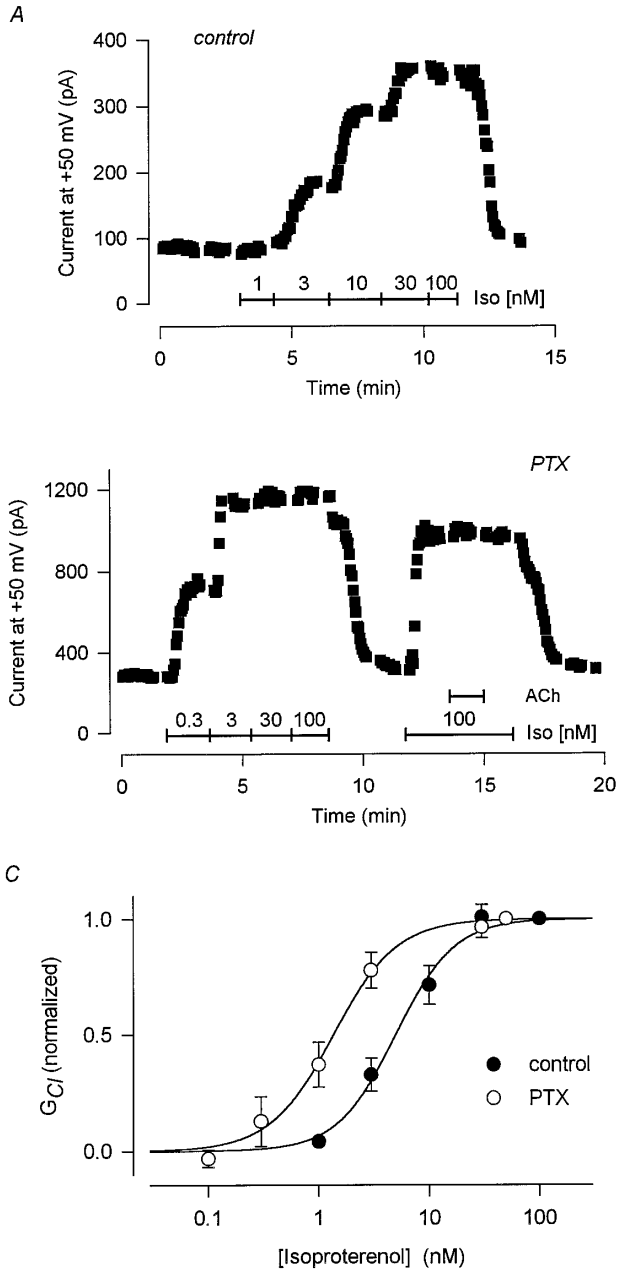

Fig. 4. Exposure to PTX increases the sensitivity of the Cl^- current to β-adrenoceptor activation. A, Time course of changes in membrane current in a control cell showing approximate half-maximal activation at a concentration of 3 nM Iso and maximal activation at 30 nM Iso. B, Time course of changes in membrane current in a cell pretreated with PTX showing approximate half-maximal activation at a concentration of 0.3 nM Iso and maximal activation of the current at 3 nM Iso. The lack of response to muscarinic stimulation by 10 μM ACh confirms that PTX-treatment was effective. C, Concentration-dependent activation of the Cl^- current by Iso in the absence (●) and presence of PTX (○). ●, average of 7 experiments; ○, average of 5 to 8 experiments. In PTX-exposed cells, only those cells that exhibited a lack of inhibition to 10 μM ACh were included in the data. The Cl^- conductance (G_{Cl}) measured at each concentration was normalized to a maximally stimulating concentration of Iso in the same cell. The individual experiments illustrated in A and B are representative of the averaged data shown in C.

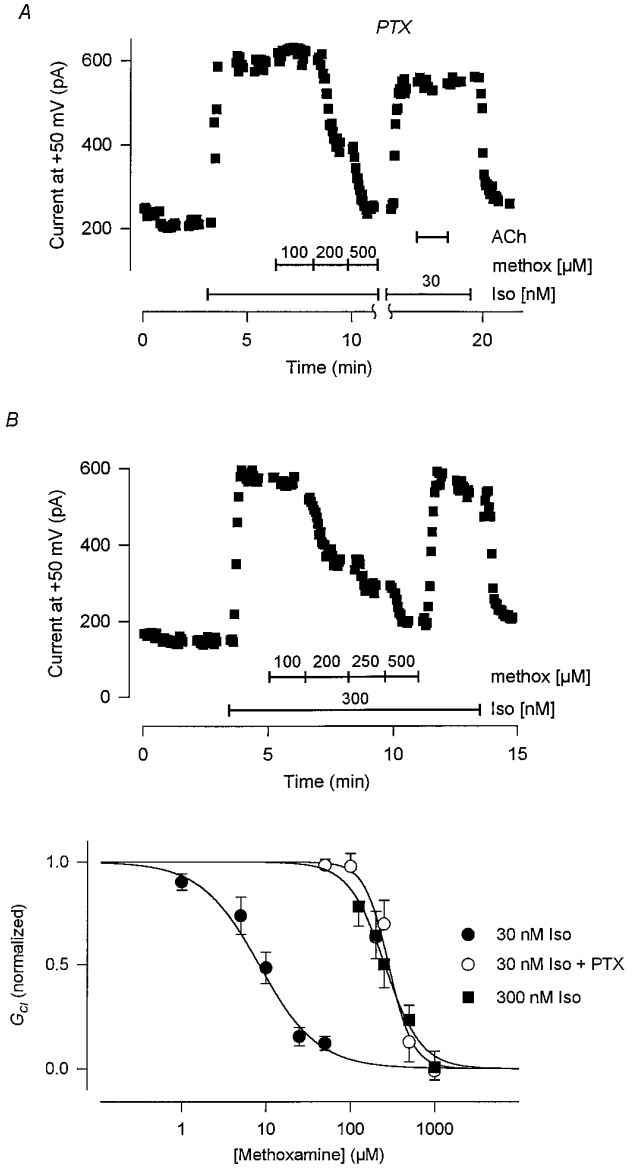

Fig. 5. Methoxamine completely inhibits the current activated by 30 nM Iso in the presence of PTX, and the current activated by 300 nM Iso in the absence of PTX. A, Time course of changes in membrane current activated by 30 nM Iso showing inhibition at concentrations of methoxamine > 100 μM in cells exposed to PTX. The lack of response to muscarinic stimulation by 10 μM ACh confirms that PTX-treatment was effective. B, Time course of changes in membrane current activated by 300 nM Iso showing inhibition at concentrations of methoxamine >100 μM. Note the similarity in the sensitivity to methoxamine in this cell with that shown in A. C, Concentration-dependent inhibition of the Cl^- current activated by 30 nM Iso (●), 300 nM Iso (■), and 30 nM Iso in the presence of PTX (○). ●, average of 4 to 5 experiments; ○, average of 4 to 7 experiments. In PTX-treated cells, only those cells that exhibited a lack of inhibition to 10 μM ACh were included in the data. ■, average of 5 to 11 experiments. The Cl^- conductance (G_{Cl}) measured at each concentration was normalized to G_{Cl} activated by a maximally stimulating concentration of Iso in the same cell. The individual experiments illustrated in A and B are representative of the averaged data shown in C.

in one cell. Under these conditions, methoxamine completely inhibited the Cl^- current with an EC_{50} value of 255 ± 34.0 μM. Comparing the methoxamine concentration-response relationships obtained when the Cl^- current was activated by either 30 or 300 nM Iso (Fig. 5C) clearly illustrates that increasing the level of β-adrenergic receptor stimulation significantly ($p < 0.001$) decreases the sensitivity to methoxamine.

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G protein-dependence of α-adrenergic response. The data presented up to this point suggest that the α_1-adrenergic inhibition of β-adrenergic responses is not mediated through a PTX-sensitive G protein. To verify that the α_1-adrenergic response is indeed mediated through a G protein-dependent mechanism, the effect of methoxamine was investigated in cells dialyzed with a pipette solution containing 100 μM GTPγS. In these experiments, cells were sequentially exposed to 1 mM methoxamine, 30 nM Iso, and 3 μM forskolin. It has previously been demonstrated that α_1-adrenergic receptor stimulation can inhibit the Cl^- current activated by β-adrenergic receptor stimulation with Iso, but it cannot inhibit the current activated by direct stimulation of adenylate cyclase with forskolin (10, 11). Therefore, if the α_1-adrenergic response is mediated through a G protein, and that G protein is irreversibly activated by GTPγS during exposure to methoxamine, subsequent activation of the Cl^- current by Iso should be attenuated, but activation of the Cl^- current by forskolin should not.

In cells that were not pre-exposed to methoxamine, 30 nM Iso irreversibly activated the Cl^- current, and subsequent exposure to a maximally stimulating concentration of forskolin had no further effect (Fig. 6, A and B). The lack of additional response to 3 μM forskolin indicates that Iso had maximally activated the Cl^- current. However, in cells that were exposed to methoxamine first, exposure to 30 nM Iso still irreversibly activated the Cl^- current, but subsequent exposure to forskolin resulted in further activation of the current (Fig. 6, C and D). The additional response to forskolin indicates that Iso had not maximally activated the Cl^- current, and it also supports the idea that forskolin responses are less sensitive to inhibition by α_1-adrenergic receptor stimulation. In eight cells that were not pre-exposed to methoxamine, the magnitude of the Cl^- conductance activated by Iso was 94.5 ± 4.4% of that recorded in the presence of forskolin in the same cell (Fig. 6, A and B). In cells that were pre-exposed to methoxamine, the response to Iso was 58.3 ± 9.4% of the forskolin response (9 cells; $p < 0.005$).These results suggest that pre-exposure to methoxamine in the presence of GTPγS had irreversibly activated the inhibitory α_1-adrenergic path-

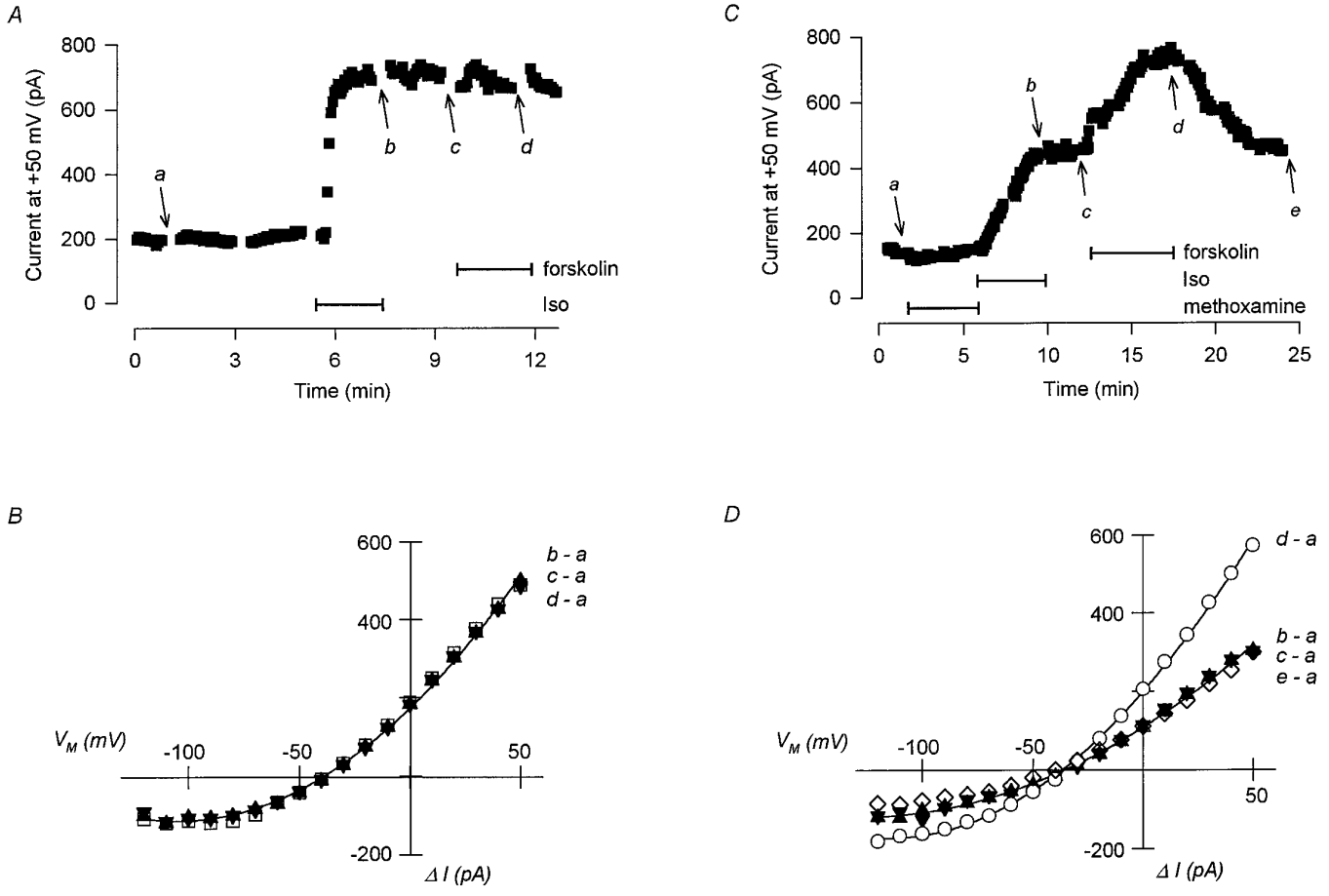

Fig. 6. α_1-Adrenergic inhibition of the β-adrenergically activated Cl^- current involves a G protein-dependent mechanism. A, Time course of changes in membrane current during which a cell dialyzed with 100 μM GTPγS is sequentially exposed to 30 nM Iso and 3 μM forskolin. This cell was not exposed to methoxamine. Note that the response to Iso is irreversible and the magnitude of the current elicited by 30 nM Iso is similar to that elicited by forskolin. B, Membrane potential (V_M)-dependence of difference current (Δ*I*) obtained by subtracting currents recorded under control conditions (*a*) from currents recorded in the presence of 30 nM Iso (*b*), after washout of Iso (*c*), and in the presence of 3 μM forskolin (*d*) as indicated in A. C, Time course of changes in membrane current during which a cell dialyzed with 100 μM GTPγS is sequentially exposed to 1 mM methoxamine, 30 nM Iso and 3 μM forskolin. Exposure to methoxamine alone activates no current, and subsequent exposure to Iso irreversibly activates a current that is only approximately 50% of the magnitude of the current elicited by a maximally stimulating concentration of forskolin. D, Membrane potential (V_M)-dependence of difference current (Δ*I*) obtained by subtracting currents recorded under control conditions (*a*) from currents recorded in the presence of 30 nM Iso (*b*), after washout of Iso (*c*), in the presence of 3 μM forskolin (*d*), and after washout of forskolin (*e*) as indicated in C. Similar results were obtained in eight control cells and nine methoxamine-exposed cells. See text for details.

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way, because subsequent exposure to Iso in the absence of methoxamine did not fully activate the Cl^- current.

Discussion

The purpose of the present study was to investigate the role of G proteins in α_1-adrenergic inhibition of β-adrenergic responses in cardiac myocytes. PTX prevents receptor-dependent activation of the G proteins G_i and G_o, and it has been shown to antagonize the ability of α_1-adrenergic receptor agonists to inhibit Iso-mediated responses (11). This suggests that α_1-adrenergic inhibition of β-adrenergic responses may involve one of these PTX-sensitive G proteins. This conclusion is also consistent with our results demonstrating that PTX increases the sensitivity of the cAMP-regulated Cl^- current to activation by NE (Fig. 3). NE is an agonist at both α- and β-adrenergic receptors, and the net response to NE is a balance between the inhibitory and stimulatory effects of α- and β-adrenergic receptor stimulation, respectively (11). Therefore, the increase in NE sensitivity could be explained if PTX were blocking the α_1-adrenergic component. However, this interpretation is complicated by the fact that, in cardiac myocytes, PTX can increase the sensitivity to β-adrenergic receptor stimulation (16). We confirmed this by demonstrating that PTX increased the sensitivity of the cAMP-regulated Cl^- current to activation by Iso (Fig. 4C). Therefore, it is likely that the increase in sensitivity to NE in PTX-treated myocytes is caused, at least in part, by an increase in sensitivity to β-adrenergic receptor stimulation.

The fact that PTX increases the sensitivity to β-adrenergic receptor stimulation raises the question of whether α_1-adrenergic inhibition of β-adrenergic responses really involves a PTX-sensitive G protein at all. We found that PTX-treatment did not prevent the ability of methoxamine to inhibit the Iso-activated Cl^- current, although it did significantly shift the concentration-dependence of the methoxamine response (Fig. 5C). The shift in the sensitivity to methoxamine could be explained by the PTX-induced increase in sensitivity to β-adrenergic receptor stimulation, but only if the sensitivity to α_1-adrenergic responses depends on the level of β-adrenergic stimulation. We verified this point by demonstrating that increasing the concentration of Iso used to activate the Cl^- current increased the concentration of methoxamine needed to inhibit the response (Fig. 5C). Therefore, the inability of PTX to block the response to methoxamine is consistent with the idea that the α_1-adrenergic response does not involve a PTX-sensitive G protein.

If the α_1-adrenergic response does not involve a PTX-sensitive G protein, one question (which our results do not directly address) is why prazosin had no effect on the sensitivity to NE in PTX treated cells (Fig. 3). This might be explained by the dependence of the α_1-adrenergic response on the level of β-adrenergic stimulation. By increasing the relative degree of β-adrenergic receptor stimulation at any given concentration of NE, the contribution of α_1-adrenergic receptor stimulation may no longer be great enough to produce an inhibitory effect in PTX-treated myocytes. However, this does not rule out the possibility that the α_1-adrenergic response involves a G protein that is partially inhibited by PTX. Similarly, we cannot say that the PTX-induced shift in methoxamine sensitivity (Fig. 5C) is caused solely by a change in β-adrenergic responsiveness.

Another question worth considering is whether inhibition of the Cl^- current by high concentrations of methoxamine in cells treated with PTX (Fig. 5A) or in cells where the Cl^- current was activated by high concentrations of Iso (Fig. 5B) can be explained by an antagonistic effect of this drug at the β-adrenergic receptor. It has been reported that methoxamine is able to inhibit agonist binding to β-adrenergic receptors in cardiac tissue (17), but the dissociation constant predicted by those binding studies is > 250 μM. If we assume that methoxamine is acting as an antagonist at β-adrenergic receptors (18), the minimum EC_{50} values for inhibition of the Cl^- current activated by 30 nM Iso, 300 nM Iso, and 30 nM Iso in the presence of PTX should have been 1.8 mM, 15 mM, and 5.6 mM, respectively. These concentrations of methoxamine are orders of magnitude greater than the EC_{50} values we obtained, indicating that it is highly unlikely that methoxamine inhibition of the Iso-activated Cl^- current is caused by antagonism at the β-adrenergic receptor under any of our experimental conditions.

The ability of PTX to block a receptor mediated response is often used as direct evidence for the role of G proteins. In light of our data questioning the idea that PTX directly blocks α_1-adrenergic inhibition of β-adrenergic responses, another way to illustrate the role of G proteins is to demonstrate that the response can be maintained by nonhydrolyzable GTP analogues. We did this by showing that a brief exposure to methoxamine results in sustained inhibition of β-adrenergic responses in the presence of GTPγS (Fig. 6, C and D). This clearly demonstrates the role of G proteins in mediating α_1-adrenergic inhibition of β-adrenergic responses.

Significance. Our conclusion from the present study is that α_1-adrenergic inhibition of β-adrenergic responses involves a G protein that is resistant to the effects of PTX. In the heart, α-adrenergic receptors are coupled to at least two different PTX-insensitive G proteins, G_q and G_h (19). Both of these G proteins are known to activate PLC. Activation of PLC is associated with the production of inositol triphosphate and diacylglycerol. Inositol triphosphate activates Ca^{2+}-dependent signaling pathways and diacylglycerol activates PKC. However, it has been previously demonstrated that α_1-adrenergic inhibition of β-adrenergic responses occurs under conditions where intracellular Ca^{2+} is buffered (10, 11). The α_1-adrenergic response is also neither blocked by inhibition of PKC nor mimicked by activation of PKC with phorbol esters (11). Thus, the present study suggests that the inhibitory effect of α_1-adrenergic receptor activation is mediated by a novel signaling mechanism. One possibility may involve the activation of PLA_2 and subsequent production of AA. Consistent with such an hypothesis is the fact that exogenous AA can inhibit β-adrenergic stimulation of the L-type Ca^{2+} current in frog ventricular myocytes (20).

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