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Modulation of N-type calcium currents by presynaptic imidazoline receptor activation in rat superior cervical ganglion neurons

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Presynaptic imidazoline receptors ($R_{i\text{-}pre}$) are found in the sympathetic axon terminals of animal and human cardiovascular systems, and they regulate blood pressure by modulating the release of peripheral noradrenaline (NA). The cellular mechanism of $R_{i\text{-}pre}$-induced inhibition of NA release is unknown. We, therefore, investigated the effect of $R_{i\text{-}pre}$ activation on voltage-dependent Ca^{2+} channels in rat superior cervical ganglion (SCG) neurons, using the conventional whole-cell patch-clamp method. Cirazoline (30 μM), an $R_{i\text{-}pre}$ agonist as well as an α-adrenoceptor (R_α) agonist, decreased Ca^{2+} currents (I_{Ca}) by about 50% in a voltage-dependent manner with prepulse facilitation. In the presence of low-dose rauwolscine (3 μM), which blocks the α_2-adrenoceptor ($R_{\alpha2}$), cirazoline still inhibited I_{Ca} by about 30%, but prepulse facilitation was significantly attenuated. This inhibitory action of cirazoline was almost completely prevented by high-dose rauwolscine (30 μM), which blocks $R_{i\text{-}pre}$ as well as $R_{\alpha2}$. In addition, pretreatment with LY320135 (10 μM), another $R_{i\text{-}pre}$ antagonist, in combination with low-dose rauwolscine (3 μM), also blocked the $R_{\alpha2}$-resistant effect of cirazoline. Addition of guanosine-5′-*O*-(2-thiodiphosphate) (2 mM) to the internal solutions significantly attenuated the action of cirazoline. However, pertussis toxin (500 ng ml^{-1}) did not significantly influence the inhibitory effect of cirazoline. Moreover, cirazoline (30 μM) suppressed M current in SCG neurons cultured overnight. Finally, ω-conotoxin (ω-CgTx) GVIA (1 μM) obstructed cirazoline-induced current inhibition, and cirazoline (30 μM) significantly decreased the frequency of action potential firing in a partly reversible manner. This cirazoline-induced inhibition of action potential firing was almost completely occluded in the presence of ω-CgTx. Taken together, our results suggest that activation of $R_{i\text{-}pre}$ in SCG neurons reduced N-type I_{Ca} in a pertussis toxin- and voltage-insensitive pathway, and this inhibition attenuated repetitive action potential firing in SCG neurons.

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Calcium ion (Ca^{2+}) influx through voltage-dependent Ca^{2+} channels plays an important role in the regulation of the intracellular Ca^{2+} concentrations in neurons. An increase in the cytoplasmic Ca^{2+} concentration triggers various physiological events, such as changes in gene transcription, membrane excitability and neurotransmitter release. Thus, modulation of voltage-dependent Ca^{2+} channels directly regulates the extent of Ca^{2+} entry and supply, which, in turn, are significant means of controlling neuronal function. In the sympathetic nervous system, N-type Ca^{2+} channels are important for the regulation of noradrenaline (NA) release in various animal species (el-Din & Malik, 1988; Hirning *et al.* 1988; Clasbrummel *et al.* 1989; Lipscombe *et al.* 1989; Pruneau & Angus, 1990) and also in the human heart atrium (Göthert & Molderings, 1997; Molderings *et al.* 2000). In addition, many neurotransmitter receptors, such as α_2-adrenergic (Galvan & Adams, 1982; Lipscombe *et al.* 1989; Song

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et al. 1989; Schofield, 1990; Beech *et al.* 1992), muscarinic (Wanke *et al.* 1987; Song *et al.* 1989; Beech *et al.* 1991; Bernheim *et al.* 1991), adenosine (Zhu & Ikeda, 1993), prostaglandin E_2 (Ikeda, 1992), somatostatin (Ikeda & Schofield, 1989; Beech *et al.* 1991; Shapiro & Hille, 1993) and neuropeptide Y, have been known to suppress N-type Ca^{2+} channels (for review, see Hille, 1994) and thus modulate NA release at nerve terminals in rat superior cervical ganglion (SCG) neurons (Toth *et al.* 1993; Koh & Hille, 1997).

Early reports showed that imidazoline derivatives are related to adrenaline and have a potency to regulate blood pressure (Hartmann & Isler, 1939), suggesting that their properties are attributed to α-adrenergic agonistic or antagonistic mechanisms. However, after clonidine and other imidazolines were found to lower blood pressure by acting at non-adrenoceptor sites in the brainstem (Bousquet *et al.* 1984), many functional and radioligand binding studies have supported the existence of α-adrenoceptor (R_α)-independent imidazoline-binding receptors in various organs (Ernsberger *et al.* 1987; Escriba *et al.* 1999; Piletz *et al.* 1999). Imidazoline receptors have been subclassified into at least two major groups (I_1 or I_2 receptors) based largely upon ligand selectivity and subcellular distribution. The I_1 receptors are mainly located in the ventrolateral medulla and are known to play a role in the exertion of central control over vascular tone (Ernsberger & Haxhiu, 1997; Bousquet & Feldman, 1999; Bruban *et al.* 2001). The I_2 receptors are found in atrial appendages, vascular smooth muscle cells, carotid bodies, and prostate and cerebral cortices (Renouard *et al.* 1993; Regunathan *et al.* 1995; Youngson *et al.* 1995; Molderings *et al.* 1997). To date, no clear functional role has been discovered for these I_2 receptors, although they are thought to be associated with eating behaviour in rats (Polidori *et al.* 2000) and various mental disorders, such as major depression (Meana *et al.* 1993; Sastre *et al.* 1995) and Alzheimer's disease (Ruiz *et al.* 1993). More recently, a non-I_1/non-I_2 new presynaptic-modulating imidazoline receptor has been found in blood vessels and hearts of rabbits, rats, guinea-pigs and humans (Molderings & Göthert, 1995, 1998; Likungu *et al.* 1996; Molderings *et al.* 1997). This presynaptic imidazoline receptor ($R_{i\text{-}pre}$) is located in the sympathetic axon terminal and regulates blood pressure by modulating peripheral NA release (Göthert *et al.* 1999). However, the precise mechanism of $R_{i\text{-}pre}$-induced inhibition of NA release in the sympathetic nerve terminals has not yet been elucidated.

It is possible that $R_{i\text{-}pre}$-induced inhibition of NA release in peripheral sympathetic nerve terminals may be mediated through the inhibition of Ca^{2+} currents (I_{Ca}), resulting in decreased intracellular Ca^{2+} concentrations. To investigate this possibility, we tested the effect of $R_{i\text{-}pre}$ activation on I_{Ca} in SCG neurons. Our results provide evidence that activation of $R_{i\text{-}pre}$ significantly inhibited N-type I_{Ca} ($I_{Ca\text{-}N}$) acting through a voltage- and PTX-independent pathway in rat SCG neurons.

Methods

This study was conducted in accordance with the the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Preparation of SCG neurons

Superior cervical ganglion neurons were enzymatically dissociated according to a previously described, modified method (Schofield & Ikeda, 1988). Briefly, adult (200–300 g) male Sprague–Dawley rats were anaesthetized with enflurane and decapitated. Ganglia were dissected from the lateral side of the carotid artery bifurcation and placed in cold Dulbecco's phosphate buffer saline (D-PBS). The ganglia were then desheathed, cut into small pieces, and incubated in Earle's balanced salt solution (EBSS) containing 0.7 g l^{-1} collagenase type D (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 0.25 g l^{-1} trypsin type I (Roche Molecular Biochemicals) at 35°C for 45 min in a shaking water bath. After incubation, ganglia were dispersed into single neurons by vigorous shaking of the culture flask. The solution was centrifuged at 100 ***g***, and the neurons were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S; all from Invitrogen, Carlsbad, CA, USA). The neurons were plated on poly-L-lysine-coated 12 mm glass coverslips and incubated in a humidified incubator with 95% air, 5% CO_2. Neurons were used within 12 h after plating. If necessary, neurons were incubated with 500 μg l^{-1} PTX for 14–18 h.

Electrophysiology

Calcium current was recorded using conventional whole-cell techniques. Electrode resistance varied from 3 to 5 MΩ when filled with internal solution. We performed measurements using an Axopatch 200A patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA). Voltage and current commands and digitization of membrane voltages and currents were controlled using a Digidata 1322A interfaced with Clampex 9.2 of the pClamp software package (Molecular Devices) on an IBM-compatible computer. We analysed data using Clampfit 9.2 (Molecular Devices) and Prism 4.0 (GraphPad, San Diego,

CA, USA). Currents were low-pass filtered at 2 kHz using the four-pole Bessel filter in the amplifier. Membrane capacitance values were taken from automatically calculated recordings by pClamp 9.2 software. Action potentials were recorded in the current-clamp mode. Membrane potential measurements were low-pass filtered at 10 kHz. Only cells with resting membrane potential less than −50 mV were included in the analysis. Multiple independently controlled syringes served as reservoirs for a gravity-driven fast drug perfusion system. Switching between solutions was accomplished by manually controlled valves. All experiments were conducted at room temperature.

Solutions and drugs

The solution used for whole-cell experiments has been previously described (Park *et al.* 2001). The internal (pipette) solution contained the following (mM): 120 *N*-methyl-D-glucamine, 20 tetraethylammonium hydroxide (TEA-OH), 11 EGTA, 1 $CaCl_2$, 10 Hepes, 4 Mg-ATP, 0.3 Na_2-GTP and 14 creatine phosphate and was titrated to pH 7.4 with methanesulfonic acid (CH_3SO_3H). The external (bath) solution contained the following (mM): 140 CH_3SO_3H, 10 Hepes, 10 $CaCl_2$ and 15 glucose and was titrated to pH 7.4 with TEA-OH. The external solution for current clamp contained the following (mM): 143 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 Hepes; the solution was adjusted to pH 7.4 with NaOH. The pipette solution used for current-clamp recordings contained the following (mM): 113 potassium gluconate, 30 KCl, 1.2 $MgCl_2$, 4 MgATP, 0.4 Na_2GTP, 10 phosphocreatine, 10 Hepes and 0.05 EGTA; the solution was adjusted to pH 7.2 with KOH.

The EBSS, DMEM, FBS and P/S were purchased from Gibco (Carlsbad, CA, USA). Cirazoline, ω-conotoxin (ω-CgTx) GVIA and LY320135 were purchased from Tocris Cookson Inc. (Bristol, UK). All other drugs were purchased from Sigma-Aldrich Chemicals. All drugs were dissolved in distilled water as stock solutions (1–100 mM).

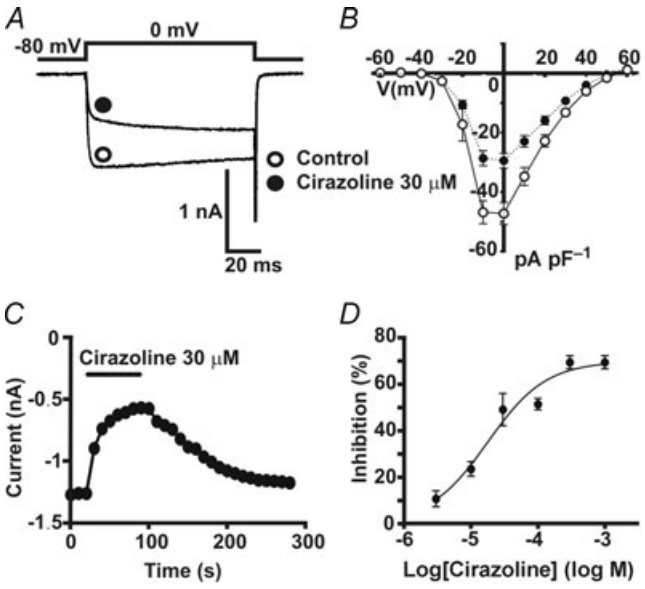

Figure 1. The effect of cirazoline on calcium current (I_{Ca}) of rat superior cervical ganglion (SCG) neurons
A, representative traces in the presence (●) and absence (○) of 30 μM cirazoline. The I_{Ca} was evoked by 100 ms depolarizing step pulses up to a test potential of 0 mV from a holding potential of −80 mV. After the current amplitude had stabilized, 30 μM cirazoline was applied to the cell from a micropipette that was placed close to the cell. *B*, *I–V* relationship curve of I_{Ca} measured 10 ms after the onset of the depolarizing pulses in the absence (○) and presence (●) of 30 μM cirazoline. *C*, time course of cirazoline-induced blockage of I_{Ca}. *D*, concentration–response curve for cirazoline-induced I_{Ca} inhibition. Inhibition (%) was determined as $(1 - I_{drug}/I_{control}) \times 100\%$. To reduce the effects of desensitization, only one concentration was tested per neuron. Each point represents the mean ± S.E.M. for the indicated number of neurons.

Data analysis

Data are presented as the means ± S.E.M., with the number of experiments given within parentheses. The concentration–response curves of cirazoline and NA for I_{Ca} inhibition were calculated by fitting to a single-site binding isotherm with least-squares non-linear regression using Prism 4.0 (GraphPad). We used unpaired Student's *t* tests to compare two groups. One-way ANOVA was used to compare multiple groups with Tukey's *post hoc* test. Differences were considered to be significant at $P < 0.05$.

Results

Inhibition of I_{Ca} by cirazoline

We tested whether cirazoline, a putative R_{i-pre} agonist (Göthert *et al.* 1999; Molderings *et al.* 2002), modulated I_{Ca} in SCG neurons. Calcium current was evoked by 100 ms depolarizing step pulses to a test potential of 0 mV from a holding potential of −80 mV. The average membrane capacitance of SCG neurons was 27 ± 5 pF ($n = 58$). Application of 30 μM cirazoline resulted in a significant, reversible inhibition of I_{Ca} by 49 ± 8% ($n = 5$; Fig. 1*A* and *C*). Cirazoline inhibited I_{Ca} over a potential range from −40 to +40 mV according to the current–voltage (*I–V*) relationship (Fig. 1*B*). We generated concentration–response curves for NA-induced I_{Ca} inhibition (Fig. 2*C*). The degree of inhibition was estimated as the ratio of decreased current to control current elicited by test pulses at 0 mV, starting from −80 mV. The concentration at which cirazoline inhibited I_{Ca} in SCG neurons by 50% was about 30 μM (Fig. 1*A* and *D*).

Inhibition of I_{Ca} by NA

Some imidazoline derivatives, such as BDF 6143, clonidine, idazoxan and cirazoline, have been reported to bind equally well to $R_{\alpha 2}$ and $R_{i\text{-}pre}$ (Göthert *et al.* 1999; Molderings *et al.* 2002). Thus, it was necessary to compare the effect of cirazoline with that of NA, which inhibits $I_{Ca\text{-}N}$ exclusively through the α_2-adrenergic receptor (Schofield, 1990, 1991). We found that 1 μM NA rapidly and reversibly inhibited I_{Ca} by 49 ± 1% ($n = 5$; Fig. 2*A* and *B*), consistent with previous reports (Schofield, 1990, 1991; Ehrlich & Elmslie, 1995; Ikeda, 1996). The effect of NA was concentration dependent, and the dose at which NA inhibited I_{Ca} by 50% in SCG neurons was about 1 μM (Fig. 2*A* and *C*). We therefore performed our experiments using 30 μM cirazoline or 1 μM NA to achieve the same level of I_{Ca} inhibition (49 ± 8%, $n = 5$ *versus* 49 ± 1%, $n = 5$; Figs 1*A* and 2*A*).

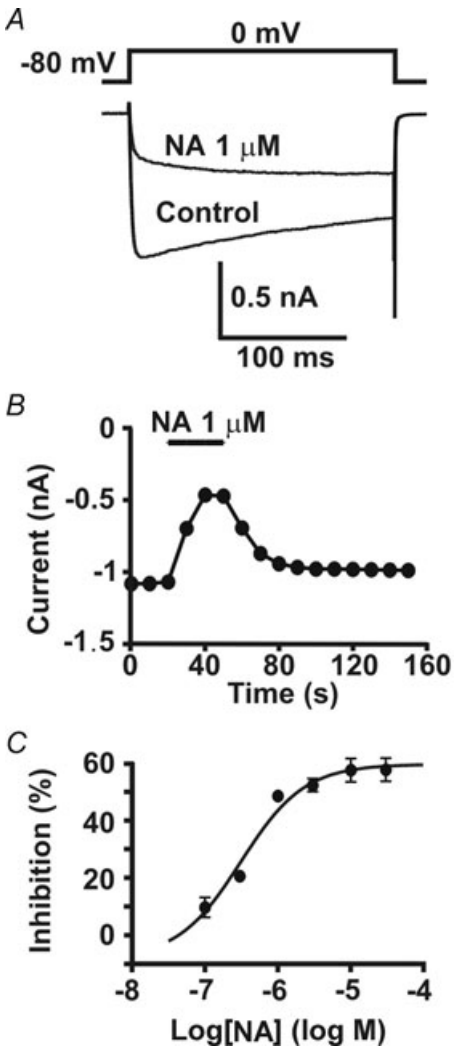

Figure 2. The effect of noradrenaline (NA) on I_{Ca} of rat SCG neurons
A, representative traces in the absence and presence of 1 μM NA. The I_{Ca} was evoked as described in Fig. 1*A*. *B*, time course of NA-induced I_{Ca} inhibition. *C*, concentration–response curve for NA-induced I_{Ca} inhibition. Inhibition (%) was determined as described in Fig. 1*D*. Only one concentration was tested per neuron. Each point represents the mean ± S.E.M. for the indicated number of neurons.

Characteristics of NA-induced I_{Ca} inhibition

As previously reported (Elmslie *et al.* 1990), NA (1 μM) rapidly inhibited I_{Ca} in SCG neurons (48 ± 7%, $n = 6$; Fig. 3*A*), and NA-induced Ca^{2+} inhibition displayed the hallmarks of voltage-dependent inhibition, namely kinetic slowing and prepulse facilitation or relief of current inhibition by conditioning depolarizing pulses. Prepulse facilitation, which is defined as the ratio of the postpulse to prepulse current amplitude, increased from 1.10 ± 0.02 to 1.68 ± 0.12 ($n = 6$) after NA application (Fig. 3*A*). This NA-induced I_{Ca} inhibition was almost completely blocked (8 ± 2.7%, $n = 7$; Figs 3*B* and 4) by pretreatment with 3 μM rauwolscine, a $R_{\alpha 2}$ antagonist with $K_i = 4.6$ nM (Uhlén *et al.* 1998). Rauwolscine pretreatment (3 μM) also nearly completely prevented the NA-induced increase of prepulse facilitation that increased only from 1.08 ± 0.02 to 1.15 ± 0.02 ($n = 7$; Fig. 3*B*).

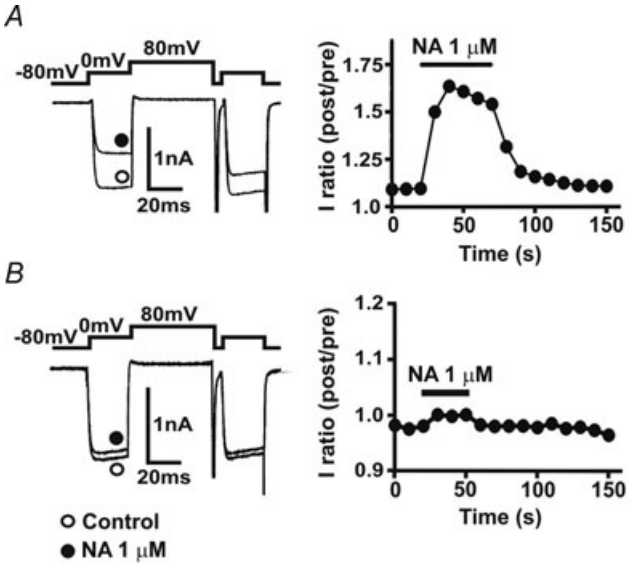

Figure 3. Characteristics of NA-induced I_{Ca} inhibition in rat SCG neurons
A, representative traces of NA (1 μM)-induced I_{Ca} inhibition (left panel) and NA-induced increase (right panel). The I_{Ca} was evoked every 10 s by a double-pulse voltage protocol consisting of two identical test pulses (0 mV from a holding potential of −80 mV) separated by a large depolarizing conditioning pulse of +80 mV. Prepulse facilitation was calculated by the ratio of the postpulse to prepulse current amplitudes (post/pre) measured isochronally at 10 ms after the start of the test pulse. *B*, representative traces of the effect of low-dose rauwolscine (3 μM) on NA-induced I_{Ca} inhibition (left panel) and increase (right panel). Note that 3 μM rauwolscine almost completely prevented NA-induced I_{Ca} inhibition.

Characteristics of cirazoline-induced I_{Ca} inhibition

We investigated the characteristics of cirazoline-induced I_{Ca} inhibition in rat SCG neurons (Fig. 5). Cirazoline (30 μM) inhibited I_{Ca} in rat SCG neurons (45 ± 3%, $n = 7$; Figs 4 and 5*A*). Prepulse facilitation increased from 1.05 ± 0.02 to 1.44 ± 0.06 ($n = 7$) upon cirazoline treatment (Fig. 5*A*, right panel). In the presence of 3 μM rauwolscine, which completely blocked $R_{\alpha 2}$, cirazoline still inhibited I_{Ca} by 33 ± 2% without noticeable increase of prepulse facilitation (increase from 1.12 ± 0.03 to 1.16 ± 0.07, $n = 7$; Fig. 5*B*). This $R_{\alpha 2}$-resistant component of cirazoline-induced I_{Ca} inhibition was almost completely blocked by pretreatment with 30 μM rauwolscine (7 ± 0.6%, $n = 8$, $P < 0.01$, cirazoline with 3 μM rauwolscine *versus* cirazoline with 30 μM rauwolscine, one-way ANOVA, Tukey's *post hoc* test; prepulse facilitation from 1.09 ± 0.03 to 1.05 ± 0.04, $n = 8$; Figs 4 and 5*C*). In addition, in the presence of 3 μM rauwolscine and 10 μM LY320135, which also antagonizes $R_{i\text{-}pre}$ (Molderings *et al.* 1999, 2002), cirazoline-induced I_{Ca} inhibition was also significantly attenuated (12 ± 1%, $n = 8$, $P < 0.01$, cirazoline with 3 μM rauwolscine *versus* cirazoline with 10 μM LY320135, one-way ANOVA, Tukey's *post hoc* test; prepulse facilitation from 1.16 ± 0.07 to 1.13 ± 0.07, $n = 8$; Figs 4 and 5*D*). These results suggest that cirazoline inhibited I_{Ca} mainly through activation of $R_{i\text{-}pre}$ in a voltage-independent manner.

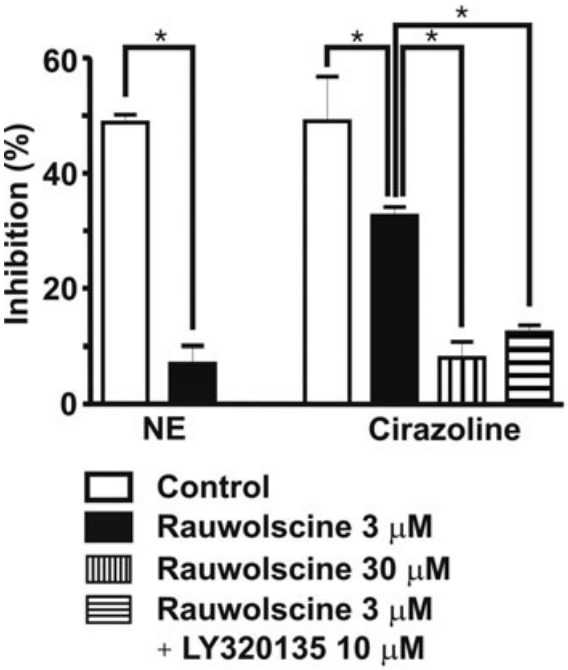

Figure 4. Effects of rauwolscine and LY320135 on I_{Ca} inhibition induced by NA and cirazoline
Inhibition (%) was calculated using the amplitudes of currents determined isochronally 10 ms after the start of the prepulse. The *x*-axis labels represent agonists used in I_{Ca} inhibition. The key represents the antagonists used in pretreatment. Cells that had not been pretreated served as a control. Data are presented as means + S.E.M. From left to right, $n = 6$, 7, 7, 7, 8 and 8. Multiple comparisons were made among rauwolscine-pretreated cirazoline groups using one-way ANOVA, with Tukey's *post hoc* test. Otherwise, unpaired Student's *t* test was used to compare two groups. *$P < 0.01$.

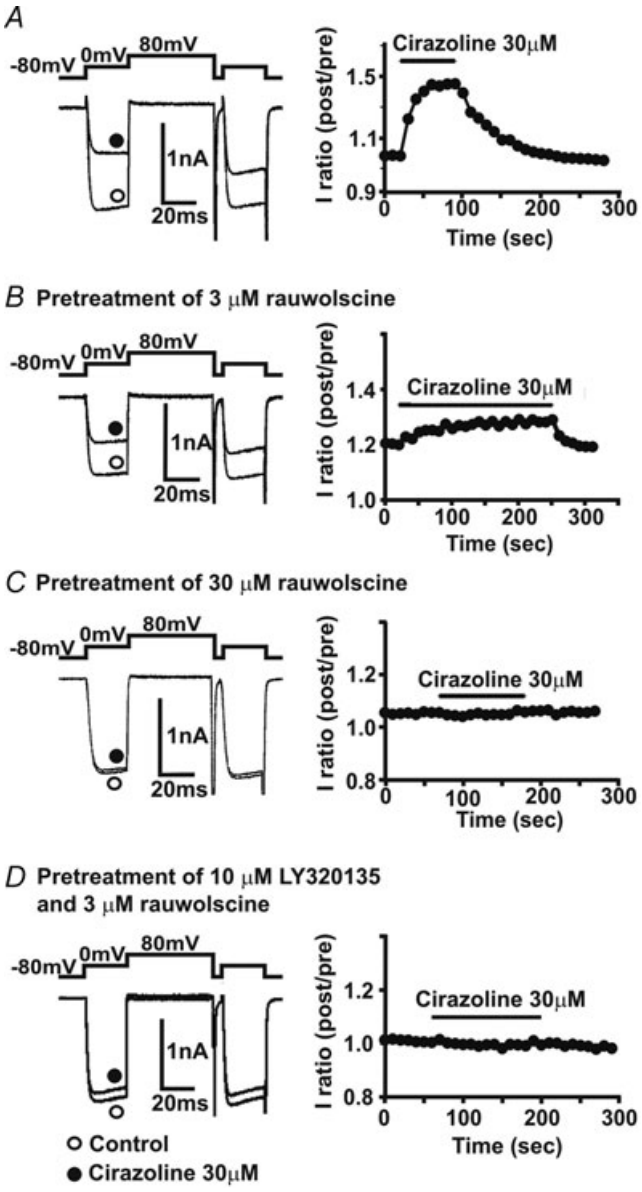

Figure 5. Characteristics of cirazoline-induced I_{Ca} inhibition in rat SCG neurons
A, representative traces of cirazoline (30 μM)-induced I_{Ca} inhibition (left panel) and cirazoline-induced increase (right panel). Evocation of I_{Ca} and calculation of prepulse facilitation were performed as in Fig. 3. *B*, representative traces showing the effects of low-dose rauwolscine (3 μM) on cirazoline-induced I_{Ca} inhibition (left panel) and increase (right panel). Note that cirazoline still inhibited I_{Ca} significantly in the presence of 3 μM rauwolscine. *C*, representative traces of the effects of high-dose rauwolscine (30 μM) on cirazoline-induced I_{Ca} inhibition (left panel) and increase (right panel). *D*, representative traces of the effects of LY320135 (10 μM) and rauwolscine (3 μM) on cirazoline-induced I_{Ca} inhibition (left panel) and increase (right panel).

Modulation of ω-CgTx GVIA-sensitive I_{Ca} by cirazoline

Consistent with previous results (Plummer *et al.* 1989; Regan *et al.* 1991; Mintz *et al.* 1992), rat SCG neurons displayed large I_{Ca}, with about 80% attributed to ω-CgTx GVIA-sensitive N-type Ca^{2+} channels ($76 \pm 4\%$, $n = 5$; Fig. 6*A* and *B*). We therefore tested whether N-type Ca^{2+} channels were modulated by cirazoline using an ω-CgTx GVIA occlusion experiment. We used 30 μM cirazoline in the presence of 1 μM ω-CgTx GVIA. Cirazoline-induced I_{Ca} inhibition was almost completely occluded by application of ω-CgTx GVIA ($6 \pm 1.4\%$, $n = 5$; Fig. 6), suggesting that cirazoline modulates mainly ω-CgTx GVIA-sensitive N-type Ca^{2+} channels in rat SCG neurons.

Effects of GDPβS and PTX on cirazoline-induced I_{Ca} inhibition

We determined the involvement of G proteins in cirazoline-induced I_{Ca} inhibition using GDPβS, a hydrolysis-resistant GDP analogue known to prevent G protein activation (Holz *et al.* 1986; Jeong & Wurster, 1997). The presence of GDPβS (2 mM) in the internal solution significantly prevented the I_{Ca} inhibition produced by NA (1 μM) (from $48 \pm 1\%$, $n = 7$ to $5 \pm 1.5\%$, $n = 5$; Fig. 7*C*). In a similar manner, cirazoline (30 μM)-induced I_{Ca} inhibition was nearly completely blocked by dialysis of GDPβS (2 mM) into the internal solution (from $49 \pm 8\%$, $n = 5$ to $5 \pm 1.9\%$, $n = 7$; Fig. 7*A* and *C*).

To elucidate the nature of G protein coupling between $R_{i\text{-}pre}$ and I_{Ca}, SCG neurons were incubated for 16–18 h in a medium containing PTX (500 ng ml^{-1}; Schofield, 1991; Shapiro *et al.* 1994*b*; Park *et al.* 2001). Pretreatment with PTX strongly attenuated the NA-induced I_{Ca} inhibition

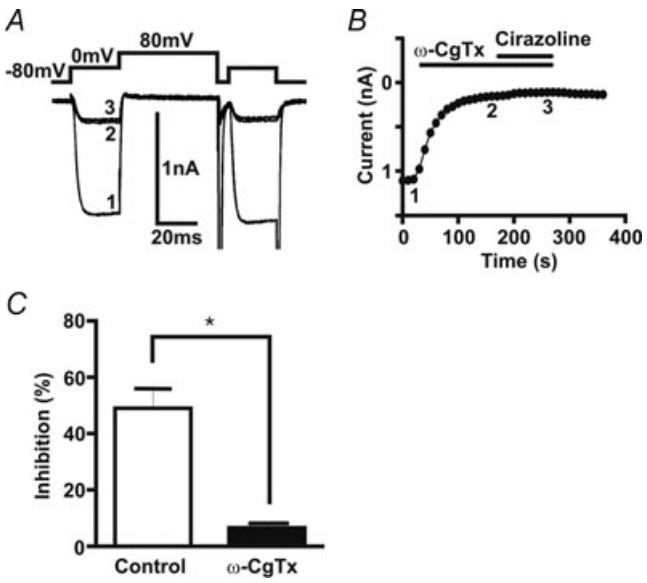

Figure 6. Effects of ω-CgTx on I_{Ca} and cirazoline-induced I_{Ca} inhibition
A, representative traces of the effects of ω-CgTx (1 μM) on I_{Ca} and cirazoline (30 μM)-induced I_{Ca} inhibition in rat SCG neurons. *B*, time course of the effect of ω-CgTx on cirazoline-induced I_{Ca} inhibition. *A* and *B* were obtained using the same cell. Traces 1, 2 and 3 in *A* were recorded at the corresponding times indicated in *B*. *C*, summary of I_{Ca} inhibition by 30 μM cirazoline in the absence and presence 1 μM ω-CgTx. Data are presented as means + S.E.M. $^*P < 0.01$.

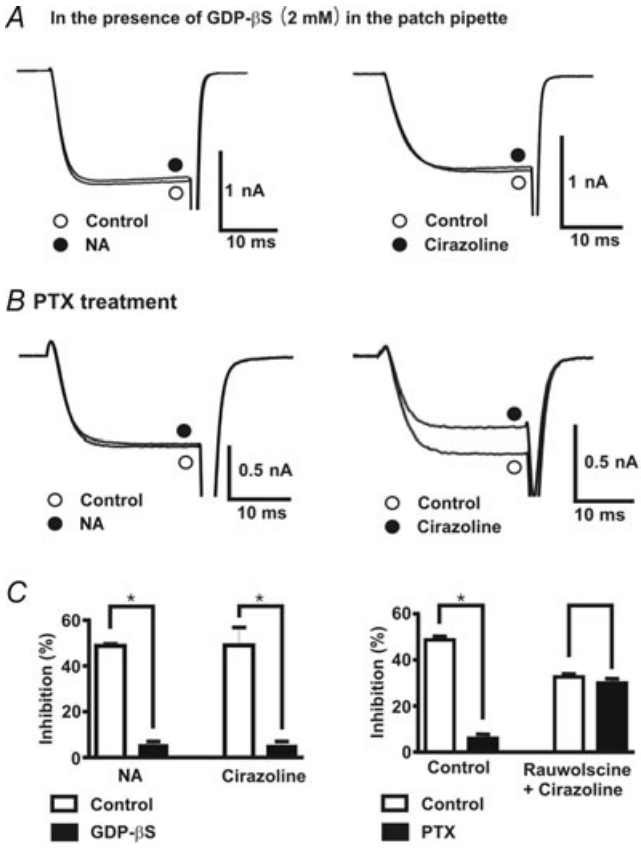

Figure 7. Effects of GDPβS and PTX on cirazoline-induced I_{Ca} inhibition
A, representative I_{Ca} trace for the control conditions (○) *versus* the presence (●) of 1 μM NA (left panel) or 30 μM cirazoline (right panel) after 10 min dialysis with 2 mM GDPβS in the pipette solution. The I_{Ca} was evoked by 25 ms depolarizing step pulses to a test potential of 0 mV from a holding potential of −80 mV. *B*, representative I_{Ca} trace in the control conditions (○) and in the presence (●) of 1 μM NA (left panel) and 30 μM cirazoline (right panel), respectively, after pretreatment with 500 ng ml^{-1} PTX for 15–18 h. *C*, left panel shows a summary of the effects of GDPβS on I_{Ca} inhibition by 30 μM cirazoline in neurons dialysed with control pipette solution (0.3 mM GTP) or with pipette solution containing GDPβS (2 mM, 0 GTP). Bars indicate mean inhibition of I_{Ca} by 30 μM cirazoline. Noradrenaline (1 μM) was also tested, as a positive control. From left to right, $n = 7$, 5, 5 and 7. $^*P < 0.01$. Right panel shows a summary of the effects of PTX on cirazoline-induced I_{Ca} inhibition. Cirazoline was applied in the presence of 3 μM rauwolscine. Bars indicate mean inhibition of I_{Ca} by 30 μM cirazoline in the presence of 3 μM rauwolscine. As positive control, NA (1 μM) was also tested. From left to right, $n = 5$, 8, 8 and 7. $^*P < 0.01$.

(from $47 \pm 2\%$, $n = 5$ to $6 \pm 1.9\%$, $n = 8$; Fig. 7*B* and *C*). However, cirazoline-induced I_{Ca} inhibition was still significant even after PTX pretreatment (Fig. 7*B*). On average, cirazoline-induced I_{Ca} inhibitions in the presence of 3 μM rauwolscine were 34 ± 3 ($n = 8$) and $30 \pm 2\%$ ($n = 7$) before and after PTX pretreatment, respectively (Fig. 7*C*). These results suggest that cirazoline-induced $R_{i\text{-}pre}$ activation modulated $I_{Ca\text{-}N}$ via a PTX-insensitive G protein.

Effects of cirazoline on M-type potassium current (I_M) in SCG neurons

In rat SCG neurons, muscarinic agonist and angiotensin II strongly suppress I_M (Marrion *et al.* 1989; Bernheim *et al.* 1992; Shapiro *et al.* 1994*a*). Modulations of I_M and I_{Ca} by these agonists share a common mechanism in rat SCG neurons (Hille, 1994). We therefore examined the modulation of I_M in SCG neurons cultured overnight. We found that cirazoline suppressed I_M by $35 \pm 7\%$ in the presence of 3 μM rauwolscine ($n = 5$; Fig. 8*A*). Oxotremorine methiodide (Oxo-M) also significantly suppressed the current (Fig. 8*A*). This suppression was in turn significantly prevented by 30 μM rauwolscine pretreatment for 10 min ($13 \pm 2\%$, $n = 4$; Fig. 8*B*).

Effects of cirazoline on repetitive firing of action potentials (APs) in SCG neurons

The role of $I_{Ca\text{-}N}$ in regulating neuronal excitability was determined by measuring firing frequency of action potentials in rat SCG neurons. In current-clamp mode,

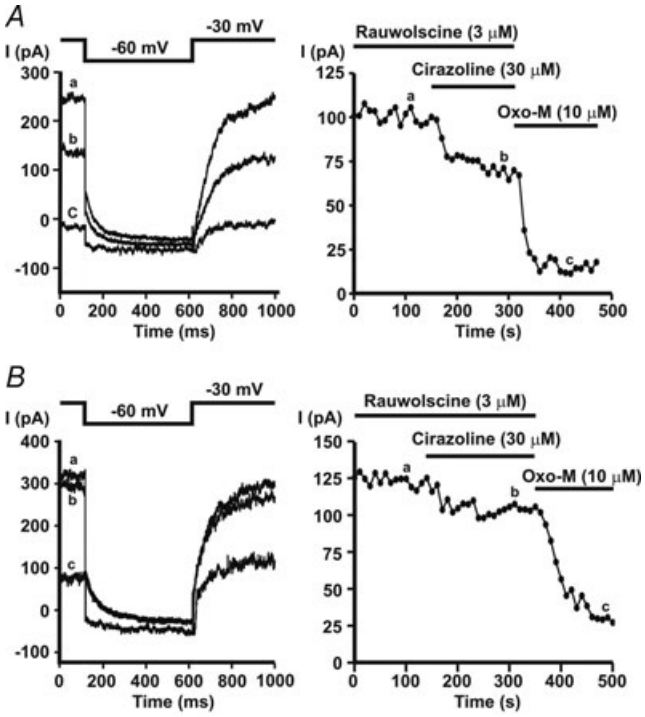

Figure 8. Effects of cirazoline on M-type potassium current (I_M) in SCG neurons
A, left panel shows representative I_M traces before application of agonists (a) and during application of 30 μM cirazoline (b) or 10 μM Oxo-M (c). Current was elicited by the pulse protocol as shown above. Cirazoline (30 μM) inhibited I_M in the presence of 3 μM rauwolscine. *A*, right panel shows time course of I_M amplitudes for the time-dependent relaxations during the step to −60 mV by bath applications of cirazoline or Oxo-M. The amplitude was measured as the difference between the current 10–20 ms after the beginning of the voltage step and the current at the end of the step. *B*, left panel shows representative I_M traces recorded as described in *A* (left panel), except that cirazoline was applied in the presence of 30 μM rauwolscine. Rauwolscine (30 μM) significantly attenuated cirazoline-induced I_M inhibition. *B*, right panel shows time course of I_M amplitudes measured as described in *A* (right panel).

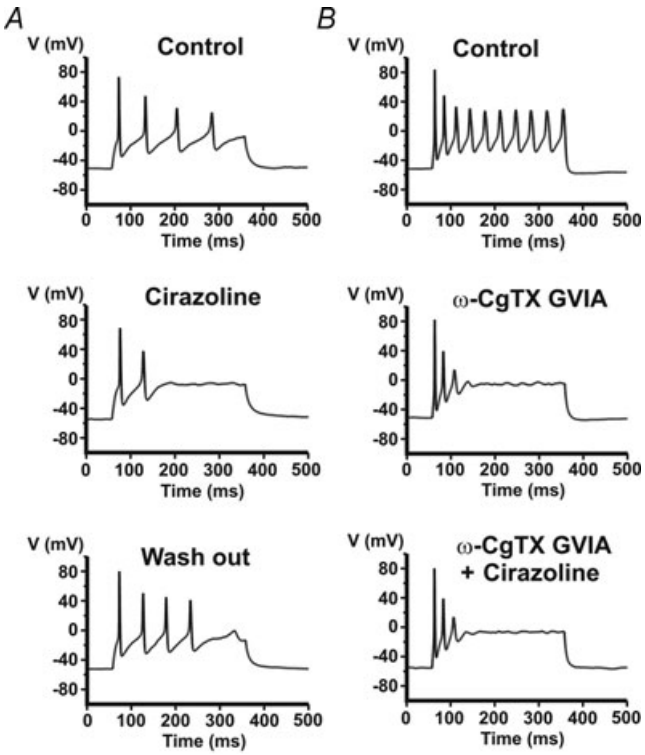

Figure 9. Effects of cirazoline on repetitive firing of action potentials (APs) in SCG neurons
All current-clamp experiments were performed in the presence of rauwolscine (3 μM). *A*, representative traces of repetitive AP firing before (top panel) and during application of cirazoline (30 μM; middle panel) in current-clamp mode. Action potentials were elicited by positive current (100–200 pA) injection for 300 ms via the patch pipette. Cirazoline significantly inhibited repetitive AP firing in a reversible manner (bottom panel). *B*, representative traces of AP firing before (middle panel) and during application of cirazoline (30 μM; bottom panel) in presence of ω-CgTx (1 μM) in current-clamp mode. The ω-CgTx (1 μM) significantly blocked the repetitive AP firing (middle panel). Cirazoline (30 μM) had no effects on repetitive AP firing in the presence of ω-CgTx (bottom panel).

APs were evoked by constant-current injection through the patch pipette. All current-clamp experiments were performed in the presence of rauwolscine (3 μM). As shown in Fig. 9*A* and *B*, injection of positive current (100–200 pA) for 300 ms evoked APs in rat SCG neurons with a frequency of $7.0 \pm 0.7\ s^{-1}$ ($n = 12$). Cirazoline (30 μM) significantly decreased the frequency of AP firing in a partly reversible manner (control, $7.3 \pm 1.7\ s^{-1}$; cirazoline, $5.3 \pm 1.7\ s^{-1}$, $n = 6$, $P < 0.05$; (Fig. 9*A*). In a similar manner, ω-CgTx GVIA (1 μM) significantly blocked the AP firing (control, $9.7 \pm 1.6\ s^{-1}$; ω-CgTx GVIA, $2.1 \pm 0.5\ s^{-1}$, $n = 6$, $P < 0.01$), but cirazoline (30 μM) had no effects on AP firing in the presence of ω-CgTx (ω-CgTx GVIA, $2.1 \pm 0.5\ s^{-1}$; ω-CgTx GVIA + cirazoline, $2.3 \pm 0.6\ s^{-1}$, $n = 6$; Fig. 9*B*).

Discussion

Our results showed that the activation of $R_{i\text{-}pre}$ inhibited $I_{Ca\text{-}N}$ in a voltage- and PTX-independent manner in rat SCG neurons. A non-I_1/non-I_2 imidazoline receptor has been identified as $R_{i\text{-}pre}$ in animal and human hearts and blood vessels (Molderings & Göthert, 1999; Molderings *et al.* 2002). This $R_{i\text{-}pre}$ inhibits NA release from sympathetic nerve endings in cardiovascular tissue and does not belong with classical presynaptic inhibitory receptors, such as $R_{\alpha 2}$, for several reasons, as follows: (1) $R_{i\text{-}pre}$ is activated by both imidazoline and guanidine derivatives; (2) $R_{i\text{-}pre}$ is blocked with low potency by rauwolscine, an $R_{\alpha 2}$ antagonist; and (3) $R_{i\text{-}pre}$ is also blocked with moderate potency by SR141716A and LY320135, CB1 cannabinoid receptor antagonists (Molderings & Gothert, 1998, 1999; Molderings *et al.* 1999, 2002). Unfortunately, a specific agonist or antagonist for $R_{i\text{-}pre}$ has not yet been developed.

Calcium channels play various roles in neurons, but their most crucial function is excitation–secretion coupling. Neurotransmitter release is highly dependent on intracellular Ca^{2+} in a co-operative manner, and a rise in intracellular Ca^{2+} concentration is mediated mostly by Ca^{2+} influx through voltage-operated Ca^{2+} channels. Any substance that modulates Ca^{2+} channel activity can therefore affect synaptic release and significantly alter information transmission. In peripheral sympathetic neurons, N-type Ca^{2+} channels primarily regulate the release of the sympathetic neuroeffector, NA. In addition, various substances, such as NA, somatostatin, substance P and magnesium, which readily modulate N-type Ca^{2+} channels, can regulate NA release from sympathetic nerve termini (Molderings *et al.* 2000; Shimosawa *et al.* 2004). The $R_{i\text{-}pre}$-mediated inhibition of NA release may thus be associated with N-type Ca^{2+} channels in sympathetic nerve terminals. To test this possibility, we determined the effect of cirazoline, a potent putative $R_{i\text{-}pre}$ agonist (Molderings & Göthert, 1999; Molderings *et al.* 2002), on $I_{Ca\text{-}N}$ in rat SCG neurons. We found that cirazoline (30 μM) inhibited I_{Ca} in a reversible and voltage-dependent manner by about 50% (Fig. 1), and that this cirazoline-induced inhibition was nearly completely occluded by ω-CgTx GVIA (Fig. 6), suggesting that cirazoline inhibited mainly $I_{Ca\text{-}N}$ in rat SCG neurons.

Imidazoline derivatives have been reported to bind to R_α as well as to imidazoline receptor (Molderings *et al.* 1999). In fact, cirazoline is known also to be an α_1-adrenoceptor ($R_{\alpha 1}$) agonist and α_2- adrenoceptor ($R_{\alpha 2}$) antagonist, as well as a putative $R_{i\text{-}pre}$ agonist (Cavero *et al.* 1982; Ruffolo & Waddell, 1982; Göthert & Molderings, 1991). To isolate the effects of imidazoline derivatives on only $R_{i\text{-}pre}$ activation, it is necessary to rule out the adrenoceptor-mediated effects. We therefore compared the inhibitory effect of cirazoline on I_{Ca} with that of NA, a non-selective R_α agonist. Noradrenaline (1 μM) inhibited I_{Ca} by 50% in a reversible manner (Fig. 2) and increased the prepulse facilitation that represents voltage-dependent inhibition (Fig. 3). Pretreatment with low-dose rauwolscine (3 μM), which blocks $R_{\alpha 2}$ almost completely, prevented NA-induced I_{Ca} inhibition and prepulse facilitation, suggesting that NA-induced Ca^{2+} inhibition is mainly mediated by $R_{\alpha 2}$ activation, consistent with previous reports (Schofield, 1990; Fig. 3*B*). Cirazoline (30 μM) also inhibited I_{Ca} by about 50% in a reversible and voltage-dependent manner (Fig. 1). This cirazoline-induced I_{Ca} inhibition was partly attenuated to about 30% by 3 μM rauwolscine without noticeable prepulse facilitation (Fig. 5*B*). This result suggested that cirazoline may also behave as a partial $R_{\alpha 2}$ agonist. In fact, Gaiser *et al.* (1999) suggested that cirazoline acts as an α_{2A}-adrenoceptor agonist. Moreover, α_{2A}-adrenoceptor subtypes, as well as other $R_{\alpha 2}$ ($\alpha_{2B,}$ α_{2C}) subtypes, exist in rat SCG neurons (Gold *et al.* 1997), and all three $R_{\alpha 2}$ subtypes serve as autoreceptors in postganglionic sympathetic neurons (Trendelenburg *et al.* 2003). It is therefore possible that cirazoline may inhibit I_{Ca} in part through a α_{2A}-adrenoceptors, but this will have to be determined in future studies.

To investigate the mechanism of $R_{\alpha 2}$-resistant (e.g. in the presence of 3 μM rauwolscine) inhibition of I_{Ca} by cirazoline, we pretreated neurons with 30 μM rauwolscine, which potently blocked the $R_{i\text{-}pre}$ for 10 min. This pretreatment nearly completely prevented cirazoline from inhibiting I_{Ca} (Fig. 5*C*). In addition, pretreatment with 10 μM LY320135, an $R_{i\text{-}pre}$ and a CB1 cannabinoid receptor antagonist, in combination with 3 μM rauwolscine (Molderings & Göthert, 1998, 1999; Molderings *et al.* 1999, 2002) also significantly attenuated $R_{\alpha 2}$-resistant I_{Ca} inhibition by cirazoline (Fig. 5*D*). These results strongly suggest that $R_{\alpha 2}$-resistant I_{Ca} inhibition by cirazoline may mediated by activation of $R_{i\text{-}pre}$. However, several other possibilities must be ruled out to

determine the mechanism of this $R_{\alpha 2}$-resistant inhibition of cirazoline. First, cirazoline may inhibit I_{Ca} via CB1 cannabinoid receptor activation. In fact, rauwolscine at the imidazoline-receptor-blocking concentration (30 μM) blocks the effect of CB1agonists, such as cP55, 940 and anandamide (Molderings et al. 1999). Moreover, LY320135 is a CB1 cannabinoid receptor antagonist. However, this mechanism can be excluded because rat SCG neurons lack endogenous cannabinoid receptors (Mackie & Hille, 1992; Pan *et al.* 1996). The second possible mechanism is that cirazoline-induced I_{Ca} inhibition may be mediated by $R_{\alpha 1}$ activation. However, this is unlikely because: (1) phenylephrine, a selective $R_{\alpha 1}$, produces no significant effect on I_{Ca} in rat SCG neurons (Schofield, 1990); and (2) 3 μM rauwolscine, which antagonizes $R_{\alpha 2}$, nearly completely prevented inhibition of I_{Ca} by NA, a non-selective adrenergic agonist, as previously reported (Schofield, 1990). This suggested that I_{Ca} inhibition by NA may have been mediated not by $R_{\alpha 1}$ activation, but by $R_{\alpha 2}$ activation in rat SCG neurons (Fig. 3). Taken together, these results suggest that activation of $R_{i\text{-}pre}$ may modulate $I_{Ca\text{-}N}$ in a voltage-independent manner in rat SCG neurons.

In most neurons, PTX-sensitive G protein is activated by various neurotransmitters, such as NA, somatostatin and adenosine, and is involved in inhibition of $I_{Ca\text{-}N}$ (Shapiro & Hille, 1993; Hille, 1994). The characteristics of voltage-dependent $I_{Ca\text{-}N}$ inhibition are slowing of the activation kinetics, relief of $I_{Ca\text{-}N}$ inhibition by the conditioning depolarizing pulses (prepulse facilitation), and the absence of diffusible messenger (membrane-delimited manner; Hille, 1994; Elmslie, 2003). However, other neurotransmitters inhibit $I_{Ca\text{-}N}$ in different ways. For example, substance P and pancreatic polypeptide inhibit $I_{Ca\text{-}N}$ in a voltage-independent, PTX-insensitive and membrane-delimited manner (Shapiro & Hille, 1993; Wollmuth *et al.* 1995). In contrast, Oxo-M, a muscarinic agonist, and angiotensin II inhibit $I_{Ca\text{-}N}$ in a voltage-independent, PTX-insensitive and second messenger-utilizing manner (Beech *et al.* 1991, 1992; Bernheim *et al.* 1992; Shapiro *et al.* 1994*a*). The inhibition of $I_{Ca\text{-}N}$ by Oxo-M is initiated by M_1 muscarinic receptors (Bernheim *et al.* 1992; Shapiro *et al.* 1999) and requires $G\alpha_{q/11}$ class G protein (Haley *et al.* 2000; Kammermeier *et al.* 2000) and phospholipase C activation (Suh & Hille, 2002; Ruiz-Durantez *et al.* 2003). Moreover, this inhibition of $I_{Ca\text{-}N}$ by Oxo-M results from depletion of phosphatidylinositol-4,5-bisphosphate (Gamper *et al.* 2004).

Oxotremorine methiodide also suppresses I_M using a very similar signalling mechanism to that of $I_{Ca\text{-}N}$. In fact, this pathway is initiated by activation of M_1 muscarinic receptors (Marrion *et al.* 1989) and PTX-insensitive G proteins (Brown *et al.* 1989; Bernheim *et al.* 1992), and the specific G protein that is primarily involved is $G\alpha_{q/11}$ (Haley *et al.* 1998, 2000). Moreover, this suppression of I_M by Oxo-M is mediated through depletion of phosphatidylinositol-4,5-bisphosphate (Winks *et al.* 2005). Bradykinin also inhibits I_M via $G\alpha_{q/11}$, which is activated by B2 BK receptor (Jones *et al.* 1995). These results suggest that inhibition of I_{Ca} and I_M by M_1 muscarinic agonists share, at least in part, a common pathway (Shapiro *et al.* 1994*b*). In this study, NA-induced I_{Ca} inhibition was almost completely prevented by pretreatment with PTX, consistent with previous data (Shapiro *et al.* 1994*b*; Elmslie, 2003). In contrast, the inhibitory effects of cirazoline were similar to those of Oxo-M and angiotensin II in SCG neurons because: (1) PTX did not affect cirazoline-induced $I_{Ca\text{-}N}$ inhibition significantly in SCG neurons (Fig. 7*B*); (2) in the presence of 3 μM rauwolscine, cirazoline inhibited $I_{Ca\text{-}N}$ in a voltage-independent manner; and (3) cirazoline also inhibited I_M in the presence of 3 μM rauwolscine (Fig. 8). These results suggest that activation of $R_{i\text{-}pre}$ inhibited $I_{Ca\text{-}N}$ mainly in a PTX-independent and voltage-independent pathway.

In autonomic ganglia neurons, repetitive firing of APs needs Ca^{2+} influx through N-type Ca^{2+} channels to maintain the repetitive activity. In fact, Ca^{2+}-free buffer or ω-CgTx GVIA significantly blocked the repetitive action potentials during the prolonged depolarizing stimulus in bronchial ganglion neurons (Myers, 1998). Thus, inhibition of $I_{Ca\text{-}N}$ by neurotransmitter or inflammatory mediators released near a ganglion may attenuate the ability to conduct excitatory stimuli from the preganglionic synapse and, consequently, regulate the peripheral autonomic target organ. In the present experiments, cirazoline (30 μM) significantly decreased the frequency of AP firing in a partly reversible manner (Fig. 9*A*). Likewise, ω-CgTx GVIA (1 μM) significantly blocked the AP firing. This cirazoline-induced inhibition of AP firing was almost completely occluded in the presence of ω-CgTx (Fig. 9*B*). This result suggests that $I_{Ca\text{-}N}$ inhibition induced by activation of $R_{i\text{-}pre}$ may inhibit repetitive AP firing and relay of excitatory stimuli to the sympathetic nerve terminal. In addition, if N-type Ca^{2+} channels are functionally coupled to $R_{i\text{-}pre}$ at synaptic nerve terminals of SCG neurons much as they are at the soma, activation of $R_{i\text{-}pre}$ may depress NA release by reducing intracellular Ca^{2+} concentration via direct blockade of N-type Ca^{2+} channels at peripheral sympathetic nerve terminals, as in the general concept of presynaptic inhibition proposed by Dunlap & Fischbach (1981).

In conclusion, we have demonstrated that activation of $R_{i\text{-}pre}$ inhibited $I_{Ca\text{-}N}$ via voltage- and PTX-independent pathways, and this inhibition attenuated repetitive AP firing in SCG neurons. These results suggest, for the first time, cellular mechanisms for pharmacological effects of $R_{i\text{-}pre}$ activation in the peripheral sympathetic nervous system and provide basic and theoretical information

about developing new agents for the treatment of hypertension.

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