Activation of Alpha-2 Adrenergic Receptors Inhibits Norepinephrine Release by a Pertussis Toxin-Insensitive Pathway Independent of Changes in Cytosolic Calcium in Cultured Rat Sympathetic Neurons

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ABSTRACT
Whole-cell electrophysiological studies suggest that sympathetic nerve alpha-2 adrenergic receptors are coupled to voltage-dependent N-type calcium channels through the G_i family of proteins to inhibit neurotransmitter release. Because most nerve terminals are too small for direct electrophysiological recordings, the aim of this study was to examine the relationship between alpha-2 adrenergic receptor-mediated inhibition of norepinephrine release and the rise in cytosolic calcium in neurites from cultured sympathetic neurons. In cultured rat superior cervical ganglion neurons, the alpha-2 adrenergic receptor agonists, UK-14304 (0.01–10 μM) and oxymetazoline (0.1–10 μM), and the N-type calcium channel blocker, ω-conotoxin GVIA (0.1–10 nM), inhibited the release of tritiated norepinephrine in response to electrical stimulation (1 Hz, 30 pulses, 0.1 ms, 70 V). The inhibitory effect of the alpha-2 adrenergic receptor agonists was not altered by pretreatment with pertussis toxin (200 ng/ml, 18 h), although pertussis toxin blocked the inhibition of forskolin-stimulated cAMP accumulation by UK-14304. In fura-2 loaded cells, electrical stimulation (1 Hz, 30 pulses, 0.1 ms, 70 V) increased cytosolic calcium in sympathetic neuronal processes. Blockade of N-type calcium channels with ω-conotoxin (1 and 10 nM) reduced the rise in cytosolic calcium by 25 ± 3% and 52 ± 6%, respectively, whereas UK-14304 and oxymetazoline did not alter the electrically stimulated rise in cytosolic calcium. These data suggest that blockade of N-type calcium channels with ω-conotoxin GVIA inhibits stimulated norepinephrine release and cytosolic calcium measured with fura-2 at similar concentrations, whereas activation of alpha-2 adrenergic receptor inhibits norepinephrine release by a pathway that is insensitive to pertussis toxin and changes in cytosolic calcium in neurites from cultured rat superior cervical ganglion cells.

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ABBREVIATIONS: cyclic AMP, cAMP; norepinephrine, NE; SCG, superior cervical ganglia; SCGCs, SCG cells; HEPES, N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid; BSS-HEPES, balanced salt solution supplemented with HEPES; EFS, electrical field stimulation; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N’ N’-tetraacetic acid.
tenuates the alpha-2 adrenergic receptor-mediated decrease in cAMP accumulation (Ui, 1988) and vasoconstriction (Boyer et al., 1983), but its effect on norepinephrine release from sympathetic nerves is controversial. In NG108-15 cells, pertussis toxin inhibits alpha-2 adrenergic receptor-mediated reduction in cAMP accumulation through G_{i} and decreases calcium influx through G_{o}. The alpha-2 adrenergic receptor coupled to calcium channels in frog ganglia (Lipscombe et al., 1989), potassium efflux in rat locus ceruleus (Aghajanian and Wang, 1986) and the inhibition of norepinephrine release in brain slices (Allgaier et al., 1985) is sensitive to pertussis toxin, which implicates a member of the G_{i} protein family in these responses. On the other hand, pertussis toxin does not alter alpha-2 adrenergic receptor-mediated inhibition of norepinephrine release in rat vas deferens or atria (Docherty, 1990), mouse atria (Musgrave et al., 1987) or in the pithed rat (Docherty, 1988). The reason for the differences in the ability of pertussis toxin to block the inhibition of transmitter release elicited by alpha-2 adrenergic receptor activation is unclear but may represent differences in the mechanism by which the receptor inhibits neurotransmitter release in various tissues or possible differences in receptor subtypes.

The present study sought to examine the role of changes in cytosolic calcium in mediating the inhibition of norepinephrine release by alpha-2 adrenergic agonists in neurites from cultured rat sympathetic neurons. Because most neuronal terminals are too small for electrophysiological recordings, cultured superior cervical ganglion neurons were loaded with fura-2 and the rise in cytosolic calcium in response to electrical stimulation was determined in neurite processes. Similar electrical stimulation parameters were used for the measurement of norepinephrine release and the rise in cytosolic calcium to correlate these changes with activation of alpha-2 adrenergic receptors and blockade of N-type calcium channels. The data from this study suggest that activation of alpha-2 adrenergic receptors inhibits norepinephrine release by a pertussis toxin-insensitive pathway in cultured rat sympathetic neurons without altering cytosolic calcium measured with fura-2.

**Methods**

**Cell isolation.** Superior cervical ganglia (SCG) were removed from 1- to 2-day-old Sprague-Dawley (Harlan) rat pups as described previously (Schwartz and Malik, 1991). The SCG were placed in L-15 media and cleaned of blood vessels and connective tissue. The SCG were incubated with collagenase D (1 mg/ml) (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 min, washed three times with L-15 media and resuspended in M-199 media containing 10% heat-inactivated fetal calf serum, glutamine (1.0 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), uridine and 5-fluoro-2-deoxyuridine (2 × 10^{-5} M) and nerve growth factor (50 ng/ml). SCG were isolated and incubated with collagenase as described. The ganglia were placed on collagen-coated plastic strips as described above for ganglion cells. The explants were allowed to adhere to the coverslips overnight in a small amount of medium before additional medium was added. Explants were cultured for 7 to 10 days. On the day of the experiment, the neurites were carefully severed from the explants by a scalpel under low-power magnification of an inverted microscope and the explants removed with fine forceps. The release of [H]NE in the remaining neurites was carried out as described above for neuronal cells.

**Cytosolic calcium measurement.** Calcium measurements were performed on multiple small neurites from cultured ganglion cells loaded with the fluorescent calcium indicator fura-2. SCGCs were plated in the center of collagen-coated round glass coverslips (0.11 mm thick, 31 mm diameter, Biophysics Technologies, Inc., Sparks, MD) for 7 to 10 days as described above. Cells were loaded with fura-2 by incubation with 3 μM fura 2-AM for 30 min in culture media at 37°C followed by a 20-min incubation in BSS-HEPES supplemented with 25 mg/l bovine serum albumin at room temperature in the dark. Coverslips containing the loaded cells were fixed on a Biophysics Tissue Chamber, and 1 ml HEPES-BSS containing sulfipyrazone (100 μM, as an anion-exchange inhibitor) was added to the well and the chamber mounted on the stage of a Nikon Diaphot microscope. Buffer temperature was maintained at 37°C by a Therm-Adapt digital thermoregulator (Biophysics Technologies, Inc.,). Loaded cells were visualized with a Nikon Fluor 40 or Fluor100 phase-contrast oil immersion objective. A Photon Technology International (PTI) model RF-M2010 ratio fluorescence system consisting of a single monochromator-based illuminator coupled to a Nikon epifluorescence microscope with OSCAR software was used for all ratio fluorescence. Excitation wavelengths were alternated between 340 nm and 380 nm. Emission intensities were measured at 510 nm. The 340:380 ratios of emitted fluorescence were calculated for each time point, and calcium concentration was estimated according to the following equation: \[
\frac{[Ca^{2+}]_{i}}{K_{d}} = \left(\frac{F_{380 \text{ max}} - F_{380 \text{ min}}}{R_{\text{max}} - R}\right) \times (R - R_{\text{min}}) / (R_{\text{max}} - R)
\] with a K_{d} value for fura-2 of 225 nM (Gryniewicz et al., 1997).
et al., 1985). At the end of the experiment, $R_{\text{max}}$ was determined with 20 $\mu$M ionomycin and $R_{\text{min}}$ with 10 mM EGTA. Two platinum wire electrodes connected to a Grass S48 Stimulator were placed in the well on both sides of the cells for EFS (1 Hz, 10 pulses, 0.1 ms duration, 70 V). Cells were electrically stimulated first in the absence and then in the presence of various pharmacological agents or their vehicle which were added directly to the tissue chamber in 10-$\mu$L bolus doses to obtain the desired final molar concentration. Basal calcium measurements were calculated by averaging intracellular calcium for five successive periods before electrical stimulation. The peak rise in cytosolic calcium was calculated by averaging cytosolic calcium for five successive periods during electrical stimulation. The peak rise in cytosolic calcium above base line in response to electrical stimulation was calculated by subtracting the average basal cytosolic calcium from the average maximum rise in cytosolic calcium in response to electrical stimulation. The peak rise in cytosolic calcium was used for statistical analysis.

Multiple neuronal neurites were monitored with either a Fluor40 or a Fluor100 oil immersion epifluorescent objective. The signal from the selected neurites was optically isolated from surrounding areas by a bilaterally adjustable iris of the photometer with a photon-counting photomultiplier detector. Cytosolic calcium was measured in areas of neurites devoid of cell bodies. Selected fields contained 5 to 20 narrow neurites (approximately $<1 \mu$m in diameter) to allow the cytosolic calcium measurements represent to the total population of neurites.

**cAMP accumulation.** cAMP was measured essentially as described (Schwartz and Malik, 1993b). To determine cellular cAMP accumulation, medium was removed and the cells incubated in BSS-HEPES containing 1 mM 3-isobutyl-1-methylxanthine for 30 min. Agonists or vehicle were added during the first 10-min period and remained in the BSS-HEPES for an additional 10 min in which forskolin was added. The experiment was stopped by placing the cells in ice-cold 50 mM sodium acetate (pH 4.0) and immediately freezing on dry ice.

Samples were stored at $-80^\circ$C until assayed. To process the cells for cAMP accumulation measurements, samples were thawed, cells scraped and the extract boiled in a water bath for 3 min. The extract was centrifuged in an Eppendorf microfuge for 5 min. and the supernatant used to estimate cAMP accumulation. cAMP was detected by standard radioimmunoassay techniques.

**Analysis of data.** Differences in fractional overflow ratios and changes from basal levels for cytosolic calcium and cAMP accumulation were expressed as mean and S.E.M. and compared with the Student’s t test.

**Drugs.** Tissue culture media and serum were purchased from Mediatech, Inc. (Herndon, VA), nerve growth factor from Harlan Bioproducts (Indianapolis, IN) and tissue culture supplements from Sigma Chemical Co. (St. Louis, MO). The following drugs used in this study were purchased: tritiated NE $\cdot$ (lsqb)7-$\text{H}(N)$ (NE specific activity, 10–30 Ci/mmol, New England Nuclear, Boston, MA), Fura-2 AM (Molecular Probes, Eugene, OR), $\alpha$-conotoxin GVIA, Conus geographus (Calbiochem, San Diego, CA), UK-14304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine), Idazoxan hydrochloride ((cis)-2-(1,4-benzodioxan-2-yl)-2-imidazoline hydrochloride) and oxymetazoline hydrochloride (Research Biochemicals International, Natick, MA).

**Results**

**Effect of alpha-2 adrenergic receptor agonists on electrically induced release of tritiated NE.** Cultured superior cervical ganglion cells loaded with $[3H]NE$ were superfused, electrically stimulated (1 Hz, 10 pulses, 0.1 ms, 70 V) and the superfusate collected. The tritium released in response to electrical stimulation has previously been demonstrated to be predominantly intact $[3H]NE$ (Schwartz and Malik, 1993b). In control experiments, the fractional tritium overflow ratio (S2/S1) in response to electrical stimulation was 1.09 $\pm$ 0.09. Addition of the alpha-2 adrenergic receptor agonists, UK-14304 (0.01–10 $\mu$M) or oxymetazoline (0.1–10 $\mu$M) to the superfusion buffer 8 min before S2 had no effect on basal tritium overflow but dose-dependently inhibited the increase in fractional tritium overflow in response to electrical stimulation (Fig. 1). UK-14304 (10 $\mu$M) and oxymetazoline (10 $\mu$M) reduced stimulated norepinephrine release by 58 $\pm$ 4% and 66 $\pm$ 5%, respectively. The alpha-2 adrenergic receptor antagonist, idazoxan (10 $\mu$M) alone had no effect on basal or stimulated fractional tritium release but attenuated the inhibition of stimulated $[3H]NE$ release by UK-14304 and oxymetazoline (Fig. 1, insert).

The fractional tritium overflow ratio (S2/S1) in isolated neurites from explanted ganglia in response to electrical stimulation in control was 0.91 $\pm$ 0.21. UK-14304 (10 $\mu$M) added to the superfusion buffer 8 min before S2 inhibited the fractional tritium overflow ratio (S2/S1) in response to electrical stimulation (S2/S1 = 0.58 $\pm$ 0.22, n = 3).

**Effect of alpha-2 adrenergic receptor agonists on stimulated rise in cytosolic calcium in neurites of ganglion cells.** Cytosolic calcium was measured in neurites of ganglion cells loaded with fura-2. In each experiment, two stimulations were conducted (S1, S2). Basal cytosolic calcium in neurites was $114 \pm 15$ nM. EFS (1 Hz, 10 pulses, 70 V, 0.1-ms duration) caused a rapid increase in cytosolic calcium that returned to base line after the stimulation (Fig. 2). The increase in cytosolic calcium in response to electrical stimulation between different cell populations was variable (range, 50–230 nM). However, the S2/S1 ratio for the rise in cytosolic calcium above base line in response to electrical stimulation was 1.04 $\pm$ 0.05. Figure 2A depicts a tracing from an individual experiment in which the stimulated rise in cytosolic calcium
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To examine the possibility that NE released from the neurons competed with UK-14304 for the alpha-2 adrenergic receptor, and therefore masked the effect of UK-14304 on changes in cytosolic calcium, the effect of yohimbine was examined on the electrically stimulated rise in intracellular calcium. Yohimbine (1 μM) added to the well for 10 min did not alter basal or stimulated rise in cytosolic calcium elicited by electrical stimulation.

**Effect of the N-type calcium channel antagonist, ω-conotoxin GVIA, on NE release and the rise in cytosolic calcium.** To determine the effect of N-type calcium channel blockade on stimulated NE release and the rise in cytosolic calcium, the N-type calcium channel blocker ω-conotoxin GVIA was used. In [3H]NE-loaded neurons, ω-conotoxin GVIA (0.1–10 nM) had no effect on basal tritium overflow but significantly inhibited the fractional tritium overflow elicited by electrical stimulation (fig. 4). ω-Conotoxin GVIA (10 nM) reduced stimulated release of norepinephrine by 72 ± 5%. In neurites from fura-2 loaded cells, ω-conotoxin GVIA (1–10 nM) significantly attenuated the peak rise in cytosolic calcium elicited by electrical stimulation (figs. 2B and 4). ω-Conotoxin (10 nM) inhibited the rise in cytosolic calcium in response to electrical stimulation by 54 ± 4%. ω-Conotoxin GVIA produced a concentration-dependent inhibition of both cytosolic calcium and NE release over the same concentration range (fig. 4).

**Effect of pertussis toxin on cAMP accumulation and neurotransmitter release in response to alpha-2 adrenergic receptor activation.** SCGCs were incubated with pertussis toxin (200 ng/ml, 18 h) or vehicle and cAMP accumulation and NE release measured in separate experiments. Basal and forskolin-stimulated cAMP accumulation was not significantly different between vehicle- and pertussis toxin-treated cells (fig. 5). UK-14304 (10 μM) did not affect basal cAMP accumulation but attenuated the increase in cAMP accumulation produced by forskolin in vehicle-treated cells.

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Fig. 3. Comparison of the effect of UK-14304 on stimulated [3H]NE release and the rise in cytosolic calcium. Data are expressed as a percent of the response in the presence of vehicle. Symbols and lines are the mean and S.E.M. for ω-conotoxin GVIA on stimulated fractional tritium overflow for five experiments repeated in triplicate. Bars and lines represent the mean and S.E.M. for ω-conotoxin GVIA on stimulated rise in cytosolic calcium for 8 to 12 individual coverslips from five different preparations. Basal intracellular calcium: vehicle, 101 ± 11 nM; 1 μM ω-conotoxin GVIA, 125 ± 21; 10 nM ω-conotoxin GVIA, 134 ± 13 nM. Electrical stimulation in the presence of vehicle increased cytosolic calcium to 246 ± 21 nM. An asterisk indicates significantly different from vehicle (P < .05).

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Fig. 4. Comparison of the effect of ω-conotoxin GVIA (1–10 nM) on stimulated [3H]NE release and the rise in cytosolic calcium. Data are expressed as a percent of the response in the presence of vehicle. Symbols and lines are the mean and S.E.M. for ω-conotoxin GVIA on stimulated fractional tritium overflow for five experiments repeated in triplicate. Bars and lines represent the mean and S.E.M. for ω-conotoxin GVIA on stimulated rise in cytosolic calcium for 8 to 12 individual coverslips from five different preparations. Basal intracellular calcium: vehicle, 101 ± 11 nM; 1 μM ω-conotoxin GVIA, 125 ± 21; 10 nM ω-conotoxin GVIA, 134 ± 13 nM. Electrical stimulation in the presence of vehicle increased cytosolic calcium to 246 ± 21 nM. An asterisk indicates significantly different from vehicle (P < .05).
In pertussis toxin-treated cells, UK-14304 had no effect on forskolin-stimulated cAMP accumulation.

Figure 5 also depicts the effect of pertussis toxin on electrically stimulated \(^{3}\text{H}\)NE release. Pertussis toxin treatment did not affect basal fractional tritium overflow (1.66 ± 0.11% per 2.5-min collection and 1.48 ± 0.09% per 2.5 min-collection for vehicle- and pertussis toxin-treated cells, respectively) but significantly enhanced the fractional amount of \(^{3}\text{H}\)NE released in response to EFS (1.25 ± 0.05% per 2.5 min versus 1.65 ± 0.07% per 2.5 min for vehicle- and pertussis toxin-treated cells, respectively, P < .05). The fractional overflow ratio (S2/S1) in response to EFS in the two groups was similar (1.10 ± 0.03 versus 1.08 ± 0.02 for vehicle- and pertussis toxin-treated cells, respectively). The addition of oxymetazoline (10 µM) or UK-14304 (10 µM) to the superfusion buffer 8 min before S2 reduced stimulated \(^{3}\text{H}\)NE release in both vehicle- and pertussis toxin-treated cells.

**Discussion**

The aim of this study was to determine whether activation of \(\alpha_2\)-adrenergic receptors inhibits NE release by reducing cytosolic calcium through a pertussis toxin-sensitive \(G_i\) protein in neurites from cultured rat superior cervical ganglion cell. NE release was measured in superfused cultured sympathetic neurons and isolated neurites of ganglia explants loaded with \(^{3}\text{H}\)NE. Cytosolic calcium was measured in neurites of cultured cells loaded with fura-2. Electrical stimulation was used to elicit increases in NE release and cytosolic calcium. The data from this study suggest that blockade of N-type calcium channels inhibits both NE release and the rise in cytosolic calcium. However, inhibition of NE release by \(\alpha_2\)-adrenergic receptor agonists occurs independent of a reduction in cytosolic calcium concentration as measured with fura-2 and was insensitive to pertussis toxin treatment. These data suggest that at least part of the effect of \(\alpha_2\)-adrenergic receptor activation is mediated by a signaling cascade independent of the \(G_i\) family of proteins and changes in cytosolic calcium in cultured rat sympathetic nerves.

UK-14304 and oxymetazoline inhibited fractional tritium overflow in response to electrical stimulation (1 Hz, 30 pulses, 0.1-ms duration, 70 V). The inhibition of NE release by UK-14304 and oxymetazoline was attenuated by the \(\alpha_2\)-adrenergic receptor antagonists, idazoxan and yohimbine, which suggests that UK-14304 and oxymetazoline activated \(\alpha_2\)-adrenergic receptors. Idazoxan alone had no effect on either the stimulated release of tritium or the rise in cytosolic calcium, which suggests a lack of negative feedback control from released NE at these stimulation parameters. A similar lack of autoinhibition in chick sympathetic neurons has been reported by Boehm et al. (1991).

To determine whether activation of \(\alpha_2\)-adrenergic receptors and/or blockade of N-type calcium channels decreased NE release by reducing the stimulated rise in cytosolic calcium, cells were loaded with fura-2 and cytosolic calcium monitored in neurites during electrical stimulation. The effect of these agents on cytosolic calcium were then related to their effects on NE release. The stimulation parameters for NE release and cytosolic calcium experiments were similar except for the number of impulses used (10 impulses for cytosolic calcium and 30 impulses for NE release). Electrical stimulation (1 Hz, 10 pulses) increased cytosolic calcium \(137 ± 16\) nM above base line. This rise in cytosolic calcium is similar to that reported by Przywara et al. (1991, 1993a, b) for electrical stimulation in neurites of cultured chick sympathetic nerves. In the present study, both UK-14304 and oxymetazoline dose-dependently inhibited fractional tritium overflow with the maximum effect occurring at 1 and 10 µM, respectively. Over the same concentration range, neither UK-14304 nor oxymetazoline reduced the rise in cytosolic calcium in response to electrical stimulation. Other frequencies of stimulation were also used. Elevation of cytosolic calcium in response to 1 pulse and 5 Hz (25 pulses) was not affected by UK-14304 or oxymetazoline (data not shown). Therefore, under conditions in which \(\alpha_2\)-adrenergic receptor activation inhibited electrically stimulated NE release, no measurable change in cytosolic calcium with fura-2 was detected.

One explanation for our inability to detect changes in cytosolic calcium in response to \(\alpha_2\)-adrenergic receptor activation may be related to our experimental design. We used fura-2 to measure cytosolic calcium as an index of calcium influx into neuronal processes. It was assumed that if \(\alpha_2\)-adrenergic receptors are negatively coupled to N-type calcium channels and these channels mediate the influx of calcium into the nerve terminal, then inhibition of these channels would decrease the influx of calcium into the neurites and the cytosolic calcium concentration. The use of fura-2 is a popular approach to assessing cytosolic calcium signaling in many cells including neurons. There are, however, some limitations to the use of this compound in assessing cytosolic calcium changes related to neurotransmitter...
release. Augustine et al. (1992) have reported that the presynaptic calcium concentrations measured with fura-2 may not represent the source of calcium responsible for neurotransmitter release. In that study, when EGTA was injected into squid “giant” presynaptic nerve terminals, neurotransmitter release was not altered, yet the rise in presynaptic calcium detected by fura-2 was blocked, which suggests that the calcium responsible for release may be localized. Therefore, the use of fura-2 to monitor changes in calcium influx responsible for NE release in neurites from sympathetic neurons may not distinguish whether inhibition of these channels accounts for a decrease in NE release. We attempted to circumvent this apparent limitation of fura-2 by examining whether direct blockade of N-type calcium channels with ω-conotoxin GVIA altered the cytosolic calcium concentration measured with fura-2 and whether this change in cytosolic calcium related to a change in NE release. Blockade of N-type calcium channels with ω-conotoxin GVIA reduced both the electrically stimulated rise in cytosolic calcium measure with fura-2 and NE release. The inhibition of both parameters were within the same concentration range. Therefore, in our experiments, blockade of N-type calcium channels and the resultant decrease in calcium influx results in a decrease in cytosolic calcium measured by fura-2. This decrease in fura-2-detected cytosolic calcium also can be correlated with a reduction in NE release. Whether the absolute cytosolic calcium concentration measured with fura-2 after application of ω-conotoxin GVIA is the calcium that triggers NE release is subject to contention; however, there does appear to be a relationship between cytosolic calcium measured with fura-2 and NE release. On the other hand, activation of α2 adrenergic receptors with UK-14304 and oxymetazoline did not alter the stimulated rise in cytosolic calcium despite inhibiting NE release. The inhibition of release produced by the α2 adrenergic receptor agonist was similar to that produced by ω-conotoxin GVIA. This would suggest that if α2 adrenergic receptors were coupled to N-type calcium channels to inhibit the influx of calcium to decrease NE release, then activation of these receptors should have sufficiently decreased the activity of these channels to decrease cytosolic calcium in a manner similar to ω-conotoxin GVIA.

Another explanation for the lack of effect of α2 adrenergic receptor activation on cytosolic calcium could be that calcium measurements were taken in areas that either do not possess N-type calcium channels, do not release NE or do not have α2 adrenergic receptors. The observation that ω-conotoxin GVIA decreased the stimulated rise in cytosolic calcium indicates that these neurites possess N-type calcium channels. But do the neurites themselves possess α2 adrenergic receptors and do these neurites release NE? Przybylarski et al. (1993a) have reported that [3H]NE is preferentially taken up and released by neurites and not cell bodies in explants of cultured sympathetic ganglia. In the present study, a similar approach was taken in that the effect of α2 adrenergic receptor activation on NE release was assessed in isolated neurites from explaned ganglia. In the isolated neurite preparation which was devoid of cell bodies, activation of α2 adrenergic receptors with UK-14304 inhibited the electrically stimulated release of NE to an extent similar to that seen in the intact neuronal population. These data indicate that neurites from sympathetic explants possess α2 adrenergic receptors that are coupled to a signaling cascade that inhibits NE release. In these neurites, however, activation of α2 adrenergic receptors do not reduce the rise in cytosolic calcium.

The pertussis toxin sensitivity of the α2 adrenergic receptor-mediated inhibition of NE release was also examined in this study. Pretreatment of SCGC with pertussis toxin (200 ng/ml, 18 h) did not affect the basal overflow of [3H]NE but significantly enhanced stimulated NE release, which suggests that Gi/Go tonically inhibits NE release in sympathetic nerves. This potentiating effect of pertussis toxin has been demonstrated by others in sympathetic nerves (Hill et al., 1993; Ikeda, 1991) and adrenal chromaffin cells (Ohara-Imaizumi et al., 1992; Sontag et al., 1991). In insulin-secreting HIT-T15 cells, the transient expression of constitutively active mutants of Gi1, Gi2, Gi3 and G12,13 inhibit insulin release, which supports the notion that Gi/Go is inhibitory on secretion (Lang et al., 1995). In the present study, Gi/Go appear to be tonically active, because inactivation with pertussis toxin enhances stimulated NE release. The tonic inhibition of NE release by Gi/Go, however, is not mediated by tonic activation of α2 adrenergic receptors for two reasons. First, α2 adrenergic receptor antagonists did not enhance NE release as would be expected if released NE was activating these receptors. Second, the inhibition of NE release caused by α2 adrenergic receptor activation was not sensitive to pertussis toxin. The mechanism for this tonic inhibition of NE release is presently unknown.

The effect of α2 adrenergic receptor activation on stimulated NE release has been reported to be both pertussis toxin sensitive (Allgaier et al., 1985; Boehm et al., 1992) and insensitive (Docherty, 1988,1990; Hill et al., 1993; Murphy and Majewski, 1989). Along these same lines, cells display differing pertussis toxin sensitivities to α2 adrenergic receptor-mediated inhibition of calcium currents (Boehm et al., 1992; Plummer et al., 1991; Schofield, 1990, 1991; Song et al., 1989, 1991). In the present study, the sympathetic neuronal α2 adrenergic receptor can couple to Gi, as demonstrated by the ability of pertussis toxin to block the inhibitory effect of UK-14304 on forskolin-stimulated cAMP accumulation. The reason for its inability to couple to Gi to decrease NE release is unknown, but several possibilities exist. The α2 adrenergic receptor that couples to Gi to decrease cAMP accumulation may be segregated into a compartment away from release sites such that the population of α2 adrenergic receptors that inhibit adenyl cyclase is different from that which inhibits NE release. The pertussis toxin-sensitive G-protein may be on the cell body, whereas the inhibition of NE release occurs at the nerve terminal. These receptor populations may also be composed of different subtypes that couple with different efficiencies to the various G-proteins. However, which if any of these explanations is correct is unknown at this time.

The mechanism for α2 adrenergic receptor-mediated inhibition of NE release in cultured sympathetic nerves independent of altering cytosolic calcium is unclear. In neuroendocrine cells, α2 adrenergic receptor activation has been demonstrated to directly inhibit the exocytotic process independent of a rise in cytosolic calcium (Gilon et al., 1993; Hsu et al., 1991; Lang et al., 1995; Wollheim and Sharp, 1981). Actin polymerization in bovine adrenal chromaffin cells has been implicated in regulating accessibility of secretory granules to the plasma membrane before exocytosis.
(Trifaro and Vitale, 1993). Treatment of chromaffin cells with phorbol esters causes a disruption of the actin cytoskeleton and potentiates nicotine-stimulated catecholamine release (Vitale et al., 1992). Of particular interest is a recent report that activation of alpha-2 adrenergic receptors results in an increase in F-actin formation in insulin-secreting HIT-T15 cells (Cable et al., 1995). The authors suggest that regulation of F-actin formation could contribute to the mechanisms by which alpha-2 adrenergic receptors inhibit insulin secretion independent of cytosolic calcium concentrations. Whether this mechanism occurs in sympathetic nerves is unknown, but activation of protein kinase C has been shown to enhance NE release as well as block the inhibitory effects of alpha-2 receptor activation in sympathetic nerves (Schwartz and Malik, 1993a).

Whether activation of presynaptic alpha-2 adrenergic receptors inhibits NE release by decreasing the influx of calcium through voltage-sensitive N-type calcium channels remains controversial. With use of fluorescent calcium indicator dyes to measure cytosolic calcium, studies to date have reported complete (Bhave et al., 1990), partial (Dolezal et al., 1995) and no blockade (this study) of the stimulated rise in cytosolic calcium by alpha-2 adrenergic receptors. Bhave et al. (1990) reported that 30 µM NE reduced the electrically stimulated release of NE in cultured rat neurons, but completely blocked the rise in cytosolic calcium in the growth cone region. Whether this blockade of the calcium current was sensitive to alpha-2 adrenergic receptor antagonism and why a complete blockade of the rise in cytosolic calcium still produced an increase in NE release, however, was not discussed. Dolezal et al. (1995) reported that in terminals of chicken sympathetic neurons, in the presence of reduced extracellular calcium (0.13 mM), UK-14304 (10 µM) decreased the release of NE and cytosolic calcium elicited by nicotinic receptor activation. A reduction in extracellular calcium was necessary to detect both an effect of UK-14304 on release and cytosolic calcium because of a significant degree of autoinhibition from endogenously released NE. In our study, autoinhibition due to released NE was negligible because blockade of alpha-2 adrenergic receptors neither enhanced electrically stimulated NE release nor the rise in cytosolic calcium. Additionally, activation of alpha-2 adrenergic receptors decreased stimulated NE release but did not affect cytosolic calcium, whereas omega-conotoxin GVIA inhibited both stimulated NE release and the rise in calcium. It appears that more studies with different techniques as well as different calcium dye indicators are necessary to determine the role of calcium in mediating the inhibition of NE release by presynaptic alpha-2 adrenergic receptors.

In conclusion, the concentration range of UK-14304 and oxytmazolone that reduced fractional tritium overflow in cultured rat superior cervical ganglion cells did not reduce the rise in cytosolic calcium in response to electrical stimulation; whereas omega-conotoxin decreased cytosolic calcium and NE release at similar concentrations. Additionally, although pertussis toxin blocked the inhibitory effect of UK-14304 on forskolin-stimulated cAMP accumulation, it did not block the alpha-2 adrenergic receptor-mediated inhibition of NE release. These data suggest that at least part of the inhibitory effect of alpha-2 adrenergic receptor activation is mediated by a pertussis toxin-insensitive signaling cascade independent of a change in cytosolic calcium in cultured rat sympathetic neurites.

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References


References


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