Multiple Cellular Mechanisms Mediate the Effect of Lobeline on the Release of Norepinephrine

ERNŐ SÁNTHA, BEÁTA SPERLÁGH, TIBOR ZELLES, GABRIELLA ZSILLA, PÉTER T. TÓTH, BALÁZS LENDVAI, MÁRIA BARANYI, and E. SYLVESTER VIZI

Department of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

Accepted for publication April 3, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

The complex effect of lobeline on [3H]norepinephrine ([3H]NE) release was investigated in this study. Lobeline-induced release of [3H]NE from the vas deferens was strictly concentration-dependent. In contrast, electrical stimulation-evoked release was characterized by diverse effects of lobeline depending on the concentration used: at lower concentration (10 μM), it increased the release and at high concentration (100 and 300 μM), the evoked release of [3H]NE was abolished. The effect of lobeline on the basal release was [Ca2+]i-independent, insensitive to mecamylamine, a nicotinic acetylcholine receptor antagonist, and to desipramine, a noradrenaline uptake inhibitor. However, lobeline-induced release was temperature-dependent: at low temperature (12°C), at which the membrane carrier proteins are inhibited, lobeline failed to increase the basal release. Lobeline dose dependently inhibited the uptake of [3H]NE into rat hippocampal synaptic vesicles and purified synaptosomes with IC50 values of 1.19 ± 0.11 and 6.53 ± 1.37 μM, respectively. Lobeline also inhibited Ca2+ influx induced by KCl depolarization in sympathetic neurons measured with the Fura-2 technique. In addition, phenylephrine, an α1-adrenoceptor agonist, contracted the smooth muscle of the vas deferens and enhanced stimulation-evoked contraction. Both effects were inhibited by lobeline. Our results can be best explained as a reversal of the monoamine uptake by lobeline that is facilitated by the increased intracellular NE level after lobeline blocks vesicular uptake. At high concentrations, lobeline acts as a nonselective Ca2+ channel antagonist blocking pre- and postjunctional Ca2+ channels serving as a counterbalance for the multiple transmitter releasing actions.

Although lobeline was originally considered as a pure nicotinic agonist, recent observations suggest that its pharmacological action is more complex than was thought previously. In our earlier study, it was found that the inhibition of carrier-mediated processes possibly underlies the loss of serotonin-releasing effects of lobeline at 7°C in hippocampal slices. Therefore, it has been proposed that lobeline acts by reversal of the monoamine uptake (Lendvai et al., 1996). Teng et al. (1997) observed that lobeline increased dopamine (DA) release via inhibition of vesicular DA uptake, which leads to a depletion of vesicular DA content and to an increase in cytosolic DA level. Furthermore, lobeline was found to be 28-fold more potent to inhibit vesicular [3H]DA uptake than to release [3H]DA from rat striatum (Teng et al., 1998). In contrast to plasma membrane carriers, low temperature does not influence the exocytotic type of transmitter release (for review, see Vizi, 1998). Therefore, using low temperature, the two primary types of transmitter release can be separated.

However, the release process from a nerve terminal is also regulated by several factors, perhaps fluctuation of resting cytoplasmic Ca2+ concentration being most important (Zucker, 1999). Voltage-dependent Ca2+ channel (VDCC) activity regulates transmitter release and significantly contributes to the operation of intracellular Ca2+ stores. The finding that lobeline (10–300 μM) antagonized the high voltage-activated calcium current in a dose-dependent manner in rat sympathetic neurons suggests that lobeline can cause direct inhibition of VDCCs (Toth and Vizi, 1998). None of these effects fit well to the classic view of nicotinic receptor function, which is thought to mediate Na+, K+, and Ca2+ fluxes on activation. Nevertheless, there is evidence indicating that lobeline is a true nicotinic receptor ligand: 1) because it is able to displace [3H]nicotinic binding from central nicotinic receptors with high affinity (Yamada et al., 1985; Lippileno and Fernandes, 1986; Benerjee and Abood, 1989; Broussolle et al., 1989); 2) it has a positive effect on learning and memory (Decker et al., 1993); and 3) it causes tachycardia, hyper-
trichloroacetic acid. To determine radioactivity released from the tissue, we made an attempt to understand further the complex effect of lobeline on transmitter release using different model systems. It was demonstrated that lobeline can induce norepinephrine (NE) release from peripheral tissues using several distinct mechanisms, including the reversal of membrane carrier and the inhibition of vesicular uptake. In addition, these effects are also accompanied by a “brake” mechanism, the inhibition of VDCCs.

**Experimental Procedures**

**Release of Tritiated NE from Guinea Pig Vas Deferens.** The experiments were performed on male guinea pigs (body weight, 200–300 g). The vasa deferentia were excised and incubated for 40 min at 37°C in 1 ml of Krebs’ solution containing 1-[$\text{J}$-$\text{H}$]NE ($10 \mu\text{Ci/ml}, 36.0 \text{Ci/mmol};$ Amersham Pharmacia Biotech, Arlington Heights, IL). Experiments were performed at 37°C in modified Krebs’ solution 118 mM NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 25 NaHCO$_3$, 0.03 Na$_2$EDTA, ascorbic acid, 0.3, and D-glucose 12.5, which was continuously saturated with carbogen gas (95% O$_2$ + 5% CO$_2$). After incubation the vasa were transferred to thermostatted (37°C) organ baths (internal volume, 3 ml). In some experiments, the bath temperature was reduced to 12°C using FRIGOMIX R (B. Braun Biotech International, Allentown, PA) from the sixth fraction (18th min after starting the collection) until the end of the experiment. The preparation was perfused at a rate of 1 ml/min. After 60 min of preperfusion, the outflow was collected in 3-ml (3-min) fractions for an additional 60 min. Tissues were stimulated at 60 V, 8 Hz, 1-ms impulse duration for 1 min (480 pulses), via platinum electrodes using a Grass S88 stimulator during the 3rd and 15th collection periods ($S_3$ and $S_{15}$). Mecamylamine and desipramine (DMI) were added at the 6th fraction to the perfusion fluid. Lobeline was perfused from the 8th collection period until the end of the experiment. At the end of the perfusion period, the tissue was removed from the organ bath and suspended in 500 $\mu$l of 10% trichloracetic acid. To determine radioactivity released from the tissue, aliquots (0.5 ml) of the perfusate samples were assayed, and a 100-$\mu$l aliquot was assayed for tissue radioactivity. Radioactivity was determined in a liquid scintillation counter (Packard 1900; Packard, Meriden, CT). The outflow of tritium was expressed as fractional release (FR), i.e., as the percentage of the amount of radioactivity in the tissue at the time of the release. To calculate the gradient interface was carefully collected, diluted with 0.32 M sucrose, and layered onto 1/2 volume of 1.2 M sucrose and centrifuged at 220,000g ($\omega^2 = 1.6 \times 10^{13}$ rad$^2$/s). The supernatant was layered onto 1/2 volume of 0.8 M sucrose and centrifuged as described above. The pellet containing synaptosomes was used for uptake experiments.

**Measurement of $\text{[H]}$NE Uptake into Hippocampal Synaptosomes.** Synaptosomal pellet was suspended in carbenogen Krebs’ assay buffer containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, 0.3 mM ascorbic acid, and 0.01 mM pargyline (pH 7.4). Aliquots of synaptosomal suspension were preincubated with lobeline (0.001–100 $\mu$M) for 5 min at 37°C in a final volume of 1 ml of assay solution. After preincubation, [$\text{H}$]NE (5 mM) was added to the tubes and incubation was continued for 5 min. Uptake was terminated by adding 3 ml of assay buffer. Samples were filtered through GF/B filters by using a Brandel cell harvester. Filters were washed with Krebs’ solution. Nonspecific uptake was determined by incubation of the samples at 0°C. The protein content of the preparation was measured by the method of Lowry et al. (1951) using CuEDTA.

**Preparation of Synaptosomes from Hippocampus.** Purified synaptosomes were prepared according to the method of Dodd et al. (1981). Hippocampi of male rats (160–180 g) were homogenized in 0.32 M sucrose (1:10 g/ml) by a Teflon pestle and centrifuged at 1000g ($\omega^2 = 1.57 \times 10^{10}$ rad$^2$/s). The supernatant was layered onto 1/2 volume of 1.2 M sucrose and centrifuged at 220,000g ($\omega^2 = 1.6 \times 10^{13}$ rad$^2$/s). The gradient interface was carefully collected, diluted with 0.32 M sucrose, and layered onto 1/2 volume of 0.8 M sucrose and centrifuged as described above. The pellet containing synaptosomes was used for uptake experiments.

**Measurement of Intracellular Calcium.** Intracellular calcium measurements were performed on single sympathetic neuronal cells prepared from the superior cervical ganglion of 1- to 3-day-old Wistar rat pups as described by Toth and Miller (1995). The cells, plated on 22-mm diameter coverslips, were loaded with 3 $\mu$l of flourescein diacetate (Fura-2 AM; Molecular Probes Inc., Eugene, OR) for 60 min in the dark at room temperature. Cells were then rinsed three times and incubated further for 30 min to complete hydrolysis of acetoxymethyl ester groups. Dye loading, de-esterification, and the entire experiment were performed in a HEPES-buffered salt solution, pH 7.4, composed of 154 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES. DE-esterification, the coverslips were mounted on a metal recording chamber (Intracell Ltd., Herts, UK) and placed on the stage of a Nikon Diaphot inverted epifluorescence microscope. A Photon Technology International (South Brunswick, NJ) microfluorometric system was used for the ratio measurements. Fura-2 was excited with light from a 75-W xenon arc lamp, alternately at 340 and 380 nm with a bandwidth of 4 nm through a Nikon Fluor ×100 oil immersion objective. Fluorescence intensity at 510 ± 20 nm was measured by means of a photomultiplier tube. Measurements were limited to a field of view slightly larger than the cells, with a rectangular diaphragm. Background correction was performed before the fluorescence ratio was calculated. Background light levels were determined on a cell-free area. Intracellular calcium levels were expressed as the ratio of
fluorescence intensities at 340 to 380 nm excitations. The metal recording chamber contained 1 ml of buffer, and the cells were superfused at a rate of 3 ml/min. Depolarization-induced calcium influx was produced by changing the perfusion solution for 20 s from low K⁺ (5 mM) to high K⁺ (50 mM) with K⁺ exchanged for Na⁺ reciprocally.

**HPLC Analysis.** The concentrations of [³H]NE and its ³H-metabolites of the guinea pig vas deferens superfusate fluid were determined using a Gilson HPLC system with electrochemical and liquid scintillation detection. For the separation, Nucleosil 3 C-18 analytical column (150 x 4.0 mm) was used. The mobile phase was 50 mM sodium phosphate, 25 mM citric acid, pH 3.6, 0.25 mM EDTA, 0.75 mM octane sulfonic acid sodium salt, and 5% acetonitrile:methanol 0.25 mM concentration, inhibited the uptake of [³H]NE with an IC₅₀ value of 6.53 ± 1.37 μM (Fig. 3). Furthermore, lobeline (100 μM) was able to decrease significantly the uptake of [³H]NE in vesicles with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration. Lobeline inhibited the uptake of [³H]NE with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration. Lobeline inhibited the uptake of [³H]NE with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration. Lobeline inhibited the uptake of [³H]NE with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration. Lobeline inhibited the uptake of [³H]NE with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration. Lobeline inhibited the uptake of [³H]NE with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration. Lobeline inhibited the uptake of [³H]NE with an IC₅₀ value of 3.4 fmol/mg of protein/5 min using 5 x 10⁻⁹ M NE concentration.
Fig. 2. Lobeline reverses the uptake carrier to release [3H]NE. A, time course of the effect of low temperature on the 100-μM lobeline-induced release. Electrical field stimulation (arrowheads) and lobeline induce release of [3H]NE from vas deferens. The large [3H]NE outflow by the application of lobeline at 37°C is completely abolished by lowering the temperature of the perfusate to 12°C. In sharp contrast, the release attributable to electrical stimulation is unaffected. The effect of lobeline on the [3H]NE release is expressed as FR. B, quantification of the effect of various treatment suggests the uptake reversal as basic releasing mechanism. Numbers below the bars represent the used concentrations of lobeline (μM). The ratio of FRR/FRRt, as a measure for transmitter releasing action of lobeline, is not changed by DMI treatment or in Ca2+-free medium, but low temperature (12°C) abolished the [3H]NE release by lobeline in all concentration used. Results represent mean ± S.E. (n = 4 in all groups) (**P < .001 versus lobeline alone).

Discussion

In this study, we have shown various aspects of pre- and postjunctional effects of lobeline to the release of [3H]NE. Although previous studies have suggested that the uptake of NE (Lindmar and Loffelholz, 1972) and carrier-mediated neurotransmitter release (Vizi et al., 1985, 1986) are temperature-dependent, it has just recently been recognized that 12°C is the cut-off point at which exocytotic and carrier-mediated release can be separated (Vizi, 1998; Vizi and Sperlagh, 1999). In agreement with this theory, our experiments showed that electrical stimulation-evoked release of [3H]NE, known to be the result of vesicular exocytosis, remained unaffected at 12°C. In contrast, the direct transmitter-releasing effect of lobeline was low temperature-sensitive. Therefore, it is reasonable to suggest that the action of lobeline involves a carrier-mediated outward transport of [3H]NE by a reversal of the flow of carrier-mediated monoamine uptake.
in the presynaptic membrane. Simple inhibition of the plasma membrane carrier by lobeline can be excluded because low temperature would then further increase the release or, at least, cause no change.

In agreement with the findings of Teng et al. (1997, 1998) on dopaminergic neurons, our data also suggest that lobeline inhibits vesicular catecholamine uptake that most likely results in a cytoplasmic NE accumulation in the nerve terminal. Our results that IC50 values for vesicular and synaptosomal [3H]NE uptake are in the same order of magnitude suggest that the inhibitory effect of lobeline on synaptosomal uptake is primarily attributable to the vesicular inhibition because the synaptosomal preparation includes synaptic vesicles, and the effect of lobeline on uptake must include it. However, the finding that lobeline has a surplus inhibitory effect on NE uptake after TB treatment needs explanation. When the direction of the NE transport has already been changed by lobeline, it is assumed that the transporter is not ready to bind the released transmitters on the extracellular side. This should result in an apparent inhibition of the synaptosomal uptake flow after the vesicles have been depleted by TB. There are drugs such as tyramine (Driessen et al., 1996) and histamine (Boudreau and Vohra, 1991) that can enter the cell using the membrane uptake carrier to evoke NE release. The uptake of tyramine and histamine can be blocked by DMI. The fact, that the monoamine uptake blocker DMI did not influence the effect of lobeline clearly shows that lobeline operates the carrier in a mode that is different from the one sensitive to DMI. As a highly lipophilic compound, lobeline can pass the membrane via passive diffusion. The failure of DMI to inhibit the effect of lobeline not only shows the route used by lobeline to enter the cell, but also that the carrier cannot be blocked by DMI when NE transport is reversed by lobeline. Although cytoplasmic NE is available for the monoaminooxidase enzyme, a significant amount of [3H]NE must have escaped from intracellular metabolism, because not only the metabolites but also NE itself considerably contribute to the released radioactivity in response to lobeline (Fig. 1). Therefore, the cellular-releasing effect of lobeline is based on multiple sites: first, it increases the releasable pool of NE in the cytoplasm; second, lobeline enhances the release from the cytoplasmic transmitter pool by the reversal of the plasma membrane uptake (Fig. 5).

The question arises whether lobeline acted on the nicotinic acetylcholine receptor(s) (nAChRs). It is well known that stimulation of nAChR causes relatively high calcium influx (Patrick et al., 1993; Seguela et al., 1993). The nAChR-induced release of NE from the hippocampus is [Ca2+]o-dependent (Vizi et al., 1995). Lobeline is able to bind to the central nicotinic receptors with very high affinity (Yamada et al., 1985; Lippiello and Fernandes, 1986; Benerjee and Abood, 1988) and in the hippocampus. Here, lobeline inhibits pre- and postjunctional VDCCs. A, effect of lobeline on high K+ depolarization-induced [Ca2+]i increase using single-cell fluorescence measurements of rat sympathetic ganglion cells. Twenty-second perfusions of 50 mM KCl elicited [Ca2+]i transients. Lobeline (300 μM) inhibited the depolarization-induced [Ca2+]i increase (horizontal bar). B, the ratio of releases induced by the second and first electrical stimulations (FRS2/FRS1) increases when lobeline is used in 10 μM, showing a facilitation of VDCCs. FRS2/FRS1 decreases at a higher concentration of lobeline, suggesting an evolving inhibition on prejunctional VDCCs. C, effect of lobeline on contraction of guinea pig vas deferens. Phenylephrine (100 μM) potentiated the stimulation-induced contraction and the induced spontaneous contractions (C/a). When phenylephrine (100 μM) and lobeline (300 μM) were applied together, the effect of phenylephrine on the postjunctional site was blocked (C/b). (*P < .05; ***P < .001 versus control).

Fig. 4. Lobeline inhibits pre- and postjunctional VDCCs. A, effect of lobeline on high K+ depolarization-induced [Ca2+]i, increase using single-cell fluorescence measurements of rat sympathetic ganglion cells. Twenty-second perfusions of 50 mM KCl elicited [Ca2+]i transients. Lobeline (300 μM) inhibited the depolarization-induced [Ca2+]i increase (horizontal bar). B, the ratio of releases induced by the second and first electrical stimulations (FRS2/FRS1) increases when lobeline is used in 10 μM, showing a facilitation of VDCCs. FRS2/FRS1 decreases at a higher concentration of lobeline, suggesting an evolving inhibition on prejunctional VDCCs. C, effect of lobeline on contraction of guinea pig vas deferens. Phenylephrine (100 μM) potentiated the stimulation-induced contraction and the induced spontaneous contractions (C/a). When phenylephrine (100 μM) and lobeline (300 μM) were applied together, the effect of phenylephrine on the postjunctional site was blocked (C/b). (*P < .05; ***P < .001 versus control).
1989; Broussolle et al., 1989). On the other hand, lobeline-induced release was independent on the extracellular Ca$^{2+}$ level in our experiments, and the effect of lobeline was insensitive to mecamylamine, a nicotinic receptor antagonist. Similarly, lobeline-induced outflow has been shown to be mecamylamine-insensitive in the hippocampus (J. P. Kiss, K. Windisch, and E. S. Vizi, unpublished data) and striatal slices (Teng et al., 1997). The participation of the mecamylamine-insensitive $\alpha$7-nAChR in the effect of lobeline also seems unlikely, because lobeline acts as an antagonist on human $\alpha$7-nAChR expressed in Xenopus oocytes (Briggs and McKenna, 1998). In sharp contrast, NE release, induced by other nicotinic agonists such as 1,1-dimethyl-4-phenylpyridinium and nicotine, can be reversed by nicotinic antagonists in the vas deferens (Todorov et al., 1991) and hippocampus (Kiss et al., 1997; Sershen et al., 1997). Therefore, it appears that lobeline does not behave as a classic nicotinic agonist on the NE release in the vas deferens.

Different types of Ca$^{2+}$ channels take part in the releasing process in sympathetic neurons of vas deferens that involves N-, P-, and Q-type VDCCs (Waterman, 1997). In our experiments, high concentrations (100 and 300 $\mu$M) of lobeline inhibited $[^{3}H]$NE release evoked by electrical stimulation. Lobeline inhibited Ca$^{2+}$ influx induced by KCl in cultured sympathetic neurons measured by the Fura-2 technique, in inhibition of synaptosomal and vesicular $[^{3}H]$dopamine uptake. Calcium and sodium currents evoked by action potentials were also inhibited by lobeline. In addition, lobeline increased the high voltage-activated calcium current in a dose-dependent manner using whole-cell patch clamp in rat sympathetic neurons (Toth and Vizi, 1998). The inhibition of these channels by lobeline can stop release-activating mechanisms and appears as an opposing effect of the releasing action of lobeline. One advantage of the vas deferens preparation is the possibility of the simultaneous recording of smooth muscle contractions and the release of $[^{3}H]$NE that shows pre- and postjunctional effects at the same time. Electrical nerve stimulation results in biphasic contraction mediated by the cotransmitter action of ATP and NE, released from sympathetic nerve terminals (Kasakov et al., 1988; Vizi et al., 1992) and acting on postjunctional $\alpha$1-adrenoceptors and $\beta_{2}$x purinoceptors (Burnstock, 1990). Lobeline abolished the smooth muscle contraction evoked by electrical field stimulation consistently with its suggested presynaptic VDCC inhibitory effect. Because muscle contraction by the $\alpha$1-adrenoceptor agonist phenylephrine, which acts on the postjunctional site, can also be prevented by lobeline, it seems reasonable to conclude that lobeline inhibits both pre- and postjunctional VDCCs.

Together, the results of our experiments show that two primary routes converge after the application of lobeline to release NE from the cytoplasm, including a vesicular uptake inhibition and a reversal of the plasma membrane carrier. At a higher concentration range (100–300 $\mu$M), blockade of the pre- and postsynaptic VDCCs contributes to the complex action of lobeline.

References


Send reprint requests to: Dr. E. Sylvester Vizi, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Szegyony u. 43., H-1450 P.O. Box 67, Hungary. E-mail: esvizi@koki.hu