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Norepinephrine Inhibits Calcium Currents and EPSPs via a G-Protein-coupled Mechanism in Olfactory Bulb Neurons

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The most pronounced effect of norepinephrine (NE) in the olfactory bulb is disinhibition of mitral/tufted (M/T) cells. Although it has been previously proposed that the effects of NE are mediated by a direct inhibitory action on granule cells, we have demonstrated that NE could exert it effects through inhibition of excitatory synaptic transmission from M/T cells to granule cells (Trombley and Shepherd, 1992). In order to define further the mechanism underlying NE-mediated inhibition of synaptic transmission, the effects of NE on calcium channel currents were examined using whole-cell recording techniques on bulb neurons in primary culture. NE inhibited high-threshold calcium currents at concentrations that were effective in reducing synaptic transmission. Clonidine, but not isoproterenol, mimicked the effects of NE on calcium currents, suggesting that the effects were mediated through activation of presynaptic α-adrenergic receptors. The effects of NE on calcium currents were irreversible in the presence of internal GTPγS and prevented by preincubation with pertussis toxin, results that are consistent with a G-protein-coupled mechanism. Preincubation with pertussis toxin also prevented the effects of NE on synaptic transmission, suggesting that a similar G-protein couple mechanism mediates both effects. Intracellular dialysis with staurosporin or calcium buffering with EGTA did not prevent the effects of NE, suggesting that neither protein phosphorylation nor elevated intracellular calcium were required. These results suggest that NE may inhibit synaptic transmission in the olfactory bulb by reducing calcium currents via a G-protein-coupled α-adrenergic receptor.

Norepinephrine (NE) can produce a variety of effects in neurons of several brain regions, including changes in membrane potential or voltage-sensitive currents. In the hippocampus and olfactory bulb, however, the most pronounced effect of NE is a reduction in synaptic inhibition (Jahr and Nicoll, 1982; Madison and Nicoll, 1988). In the hippocampus, NE reduces the amplitude of IPSPs and increases the number of population spikes in CA1 pyramidal neurons evoked by orthodromic stimulation (Madison and Nicoll, 1988). The disinhibitory action of NE in the hippocampus is mediated by α-adrenergic receptors (Madison and Nicoll, 1988) and has been recently shown to be presynaptically localized (Doze et al., 1991).

In the mammalian olfactory system, sensory information is transmitted from olfactory receptors neurons to the olfactory cortex via mitral/tufted (M/T) cells in the olfactory bulb. M/T cell activity is modified en route to the cortex by granule (GR) cells, which are inhibitory interneurons that release GABA onto M/T cells at reciprocal dendrodendritic synapses. Antidromic stimulation of M/T cell axons excites GR cells, which in turn generate IPSPs in the M/T cells (Jahr and Nicoll, 1980). NE reduces these IPSPs, an effect that could result in increased M/T cell excitability and enhanced orthodromic transmission from sensory neurons to the olfactory cortex. Trombley and Shepherd (1992) demonstrated that NE reduces excitation of GR cells by inhibiting mitral cell to GR cell EPSPs via activation of presynaptic α-adrenergic receptors. Noradrenergic-mediated disinhibition of mitral cells, therefore, may be a consequence of decreased excitation of GR cells. Although the effects of NE in the hippocampus and the olfactory bulb are similar—that is, NE is disinhibitory, α-adrenergic mediated, and presynaptically localized—details of the underlying mechanism of action have not yet been examined.

It has been demonstrated that NE can inhibit transmitter release in other neuronal systems including substance P from dorsal root ganglia (Rane et al., 1987) and NE from sympathetic neurons (Lipscombe et al., 1989). It has long been known that calcium entry is essential for transmitter release (Katz and Miledi, 1967), and recent evidence suggests that NE inhibition of high-threshold calcium channels is responsible for its effects on transmitter release (Rane et al., 1987; Lipscombe et al., 1989). Using primary cultures of olfactory bulb neurons and whole-cell voltage-clamp techniques, I examined the effects of NE on calcium currents in M/T cells. A reduction of transmitter release via an inhibition of voltage-gated calcium currents could explain the effects of NE on EPSPs reported in Trombley and Shepherd (1992). Here I report that NE, via α-adrenergic receptors, inhibits both voltage-sensitive calcium currents and EPSPs by a pertussis toxin (PTX)-sensitive G-protein-coupled mechanism that does not require protein phosphorylation or elevations in intracellular calcium.

Materials and Methods

Cell cultures. The methods for preparing primary cultures of olfactory bulb neurons have been described in detail (Trombley and Westbrook, 1990; Trombley and Shepherd, 1992). Briefly, olfactory bulbs were dissected from neonatal rat pups, incubated in papain, triturated, and plated at a density of ≈50,000-60,000 cells/cm² on a confluent layer of olfactory bulb astrocytes. The neuronal growth medium contained 95% Minimal Essential Medium (GIBCO) with 5% horse serum, 6 gm/liter

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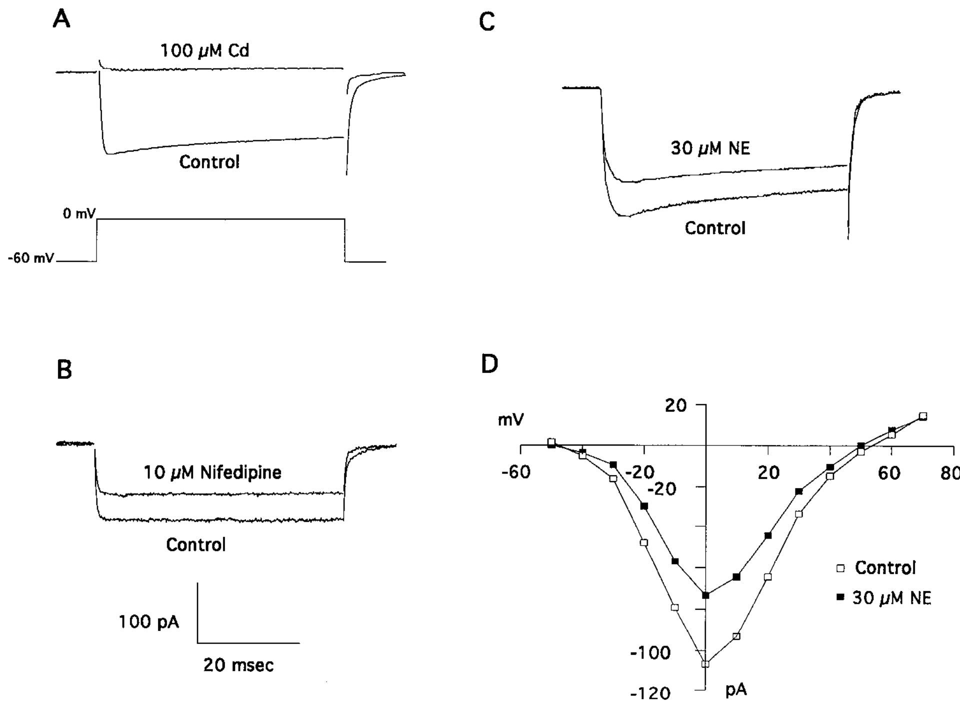

Figure 1. NE reversibly blocks high-threshold calcium currents in M/T cells in short-term culture. Under voltage clamp, a 50 msec depolarizing voltage step to 0 mV from a holding potential of −60 mV evokes a sustained inward Ba current through high-threshold calcium channels. *A*, Flow pipe application of 100 μM Cd reversibly blocked the current. *B*, This inward current was inhibited by 10 μM nifedipine, suggesting that the current is through high-threshold calcium channels. *C*, Flow pipe application of 30 μM NE reversibly reduced the calcium channel current by approximately 30%. *D*, Current–voltage relationship for the effects of 30 μM NE on the Ba current. CsCl patch solution (in mM): 5 ATP, 0.5 GTP, 11 EGTA; extracellular solution (in mM): 10 Ba, 1 Mg. Holding potential, −60 mV.

glucose, and a nutrient supplement (Serum Extender, Collaborative Research, Inc.). Electrophysiological recordings were made after 6–48 hr in culture for experiments on calcium currents and after 7–10 d for experiments on synaptic potentials.

Electrophysiology. Voltage- and current-clamp recordings were performed at room temperature using a discontinuous voltage-clamp amplifier (Axoclamp 2A, Axon Instruments). Switching frequencies of 10–15 kHz were used during voltage-clamp experiments, and the membrane voltage was continuously monitored. The recording chamber was perfused at 1.0–2.0 ml/min with a solution containing (in mM) NaCl, 162.5; KCl, 2.5; $CaCl_2$, 2.0; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10; glucose, 10; $MgCl_2$, 1; and no added glycine. The pH was adjusted to 7.3 with NaOH, and the osmolarity was 325 mOsm. For the experiments examining calcium currents, 10 mM barium replaced external calcium as the charge carrier and tetrodotoxin (TTX; 1 μM) was added to block voltage-sensitive Na currents. Experiments using synaptic pairs followed the protocol described in Trombley and Shepherd (1992). Patch electrodes were pulled from borosilicate glass, fire polished, and filled with solution containing (in mM) $KMeSO_4$ or CsCl, 145; $MgCl_2$, 5; HEPES, 10; Na-ATP, 5; Na-GTP, 0.5; and EGTA, 1.1 or 11; pH 7.2, osmolarity 310 mOsm. Electrodes had resistances of 4–6 MΩ. Most drug stocks were made in water and nifedipine in absolute ethanol (final concentration, 0.01%). Drug stocks were diluted in the recording solution and delivered by a flow pipe perfusion system consisting of an array of 400-μm-i.d. glass barrels fed by gravity from drug reservoirs. The flow pipes were positioned within 250 μm of the neuron using a hydraulic manipulator, and flow was controlled with pinch clamps. Neurons were always bathed with control solution from one barrel except during application of drugs. Drugs applied were staurosporin (Kamiya Biochemical Company), clonidine, GTPγS, tetrodotoxin, nifedipine, pertussis toxin, and norepinephrine (Sigma).

Voltage and current protocols were generated using pCLAMP software (Axon Instruments); EPSPs and membrane currents were digitized at 5–10 kHz and stored for later analysis on an IBM-386 computer. Calcium currents in olfactory bulb neurons were isolated by blocking sodium currents with tetrodotoxin and potassium currents by including Cs in the patch pipette. Under these conditions, calcium currents could be evoked and adequate space clamp was achieved by recording prior to extensive neuritic outgrowth. Unless otherwise indicated, whole-cell recording was restricted to presumptive M/T cells identified by their physiological, morphological, and immunohistochemical criteria (Trombley and Westbrook, 1990). Often the currents were small in 2 mM calcium, so for ease of analysis external calcium was replaced with 10 mM Ba to increase the amplitude of the current. Current records were filtered at 1–3 kHz (eight-pole Bessel filter). The control calcium channel current amplitude was taken as the average of peak current values immediately before and after drug application in order to compensate for current rundown. Current amplitude was measured as the average of a 2 msec data epoch beginning 10 msec after the onset of the voltage jump. A *P*/4 subtraction procedure was used to compensate for leak and capacitative currents. The initial 400 μsec after a voltage jump had uncanceled capacitative transients and was blanked. Statistical results are reported as mean ± SD.

Results

Effects of NE on calcium currents

As shown in Figure 1*A*, a 50 msec depolarizing voltage step to 0 mV from a holding potential of −60 mV evoked a sustained inward current that could be completely blocked by 100 μM Cd. The inward current activated near −40 mV, peaked at 0 mV, and reversed near +55 mV. This current showed little inactivation during the step even when the duration was increased to 150 msec. The sustained inward current was sensitive to nifedipine (10 μM; Fig. 1*B*), which reduced the current by 40 ± 14% ($n = 9$). These characteristics suggest that at least some component of the high-threshold current was from L-type calcium channels. I did not, however, attempt to determine the relative contribution of N- and L-type channels to the high-threshold current, which can vary considerably between neuronal cell types (Regan et al., 1991). Transient or T-type calcium currents were small and rarely observed even from a holding potential of −100 mV, where most T-type channels would be available for activation. I therefore used a holding potential of −60 mV, where the T-current would be inactive (Fox et al., 1987), and further distinctions between different types of calcium channels were not made.

Flow pipe application of 30 μM NE reduced the calcium channel current in 36 out of 42 M/T neurons by 29 ± 10% (Fig. 1*C*). A current–voltage relationship for effects of NE on the calcium channel current is shown in Figure 1*D*. NE was used at 30 μM throughout these experiments since it has previously been shown that this concentration is effective in reducing monosynaptic EPSP amplitudes in cultures of olfactory bulb

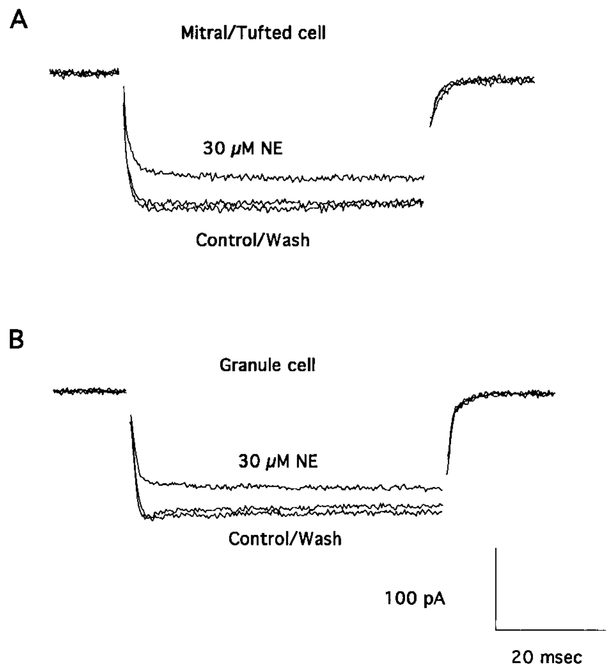

Figure 2. NE had similar effects on both M/T cells and GR cells. Flow pipe application of 30 μM NE reversibly reduced the calcium channel current both in M/T cells (*A*) and in GR cells (*B*). Voltage protocol and electrode were the same as in Figure 1*A–C*.

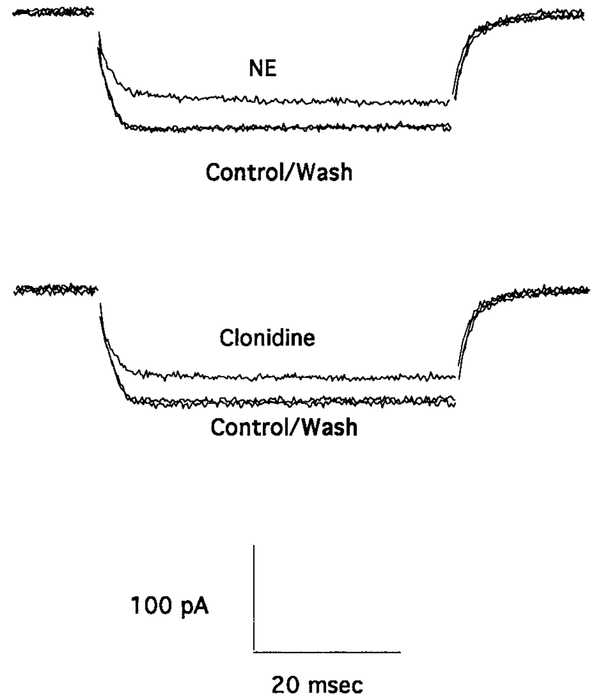

Figure 3. The effects of NE on the Ba current are similar to the effects of the α_2-adrenergic receptor agonist clonidine. Flow pipe application of 30 μM NE reversibly reduced the Ba current by 29% in an M/T cell. This effect was mimicked by 1 μM clonidine, which reduced the current by 26% in the same neuron. Voltage protocol and electrode were the same as in Figure 1*A–C*.

neurons (Trombley and Shepherd, 1992) and other studies have shown similar concentrations to be effective in inhibiting both calcium currents and transmitter release (Holz et al., 1989; Lipscombe et al., 1989). With 4 mM Mg/ATP and 0.5 mM GTP in the pipette, measurable calcium channel currents often could be evoked for up to 30 min after gaining whole-cell access. NE was still effective in reducing the calcium channel current after more than 15 min of whole-cell recording, indicating that intracellular dialysis did not prevent the effects of NE under these recording conditions. NE had similar effects on calcium channel currents in olfactory bulb GR cells, where it inhibited the current by 27 ± 7% in seven out of nine neurons (Fig. 2).

NE can inhibit calcium channel currents in neurons from other regions including sympathetic and sensory ganglia as well as the spinal cord (Dunlap and Fischbach, 1981; Hirning et al., 1988; Bean, 1989; Sah, 1990; Bernheim et al., 1991), an effect mediated through activation of α_2-adrenergic receptors (Lipscombe et al., 1989; Surprenant, 1989). The effect of NE on the calcium channel current in M/T cells was also mimicked by selective α_2-adrenergic receptor activation. The α_2-adrenergic agonist clonidine (500 nM) reduced the calcium channel current by 23 ± 7% in seven out of nine neurons (Fig. 3).

A PTX-sensitive G-protein mediates the effects of NE

A G-protein has been proposed to mediate the effects of NE on both high-threshold calcium currents and transmitter release (Holz et al., 1989; Lipscombe et al., 1989). In order to determine whether NE acts via a G-protein–coupled mechanism in M/T cells, I examined the effects of NE in the presence of a nonhydrolyzable analog of GTP, GTPγS, which generates a permanently active form of the α-subunit (Gilman, 1987). With 100 μM GTPγS in the recording pipette, 30 μM NE reduced the calcium channel current by 21 ± 7% in 7 out of 10 M/T cells. This antagonism, however, was irreversible, and the calcium channel current failed to recover from the effects of NE even after several minutes of perfusion with control solution (Fig. 4*A*).

Because PTX prevents the interaction of receptors with G_i or G_o proteins by ADP ribosylation (Gilman, 1987), I examined the effects of NE on the Ba current in M/T cells after the neurons were incubated in 250 ng/ml PTX for 15–36 hr. NE reduced the Ba current by 26 ± 5% in 9 out of 10 neurons but had no effect on 14 out of 18 PTX-treated neurons from sister cultures (Fig. 4*B*), suggesting that a PTX-sensitive G-protein mediated the effects of NE.

Some G-protein–coupled receptors exert their effects on membrane currents through diffusible second messengers (Trautwein et al., 1986; Dunlap et al., 1987; Rane et al., 1989; Bernheim et al., 1991), while the effects of others are mediated by direct action of the activated α-subunit (Brown and Birmbaumer, 1988; Lipscombe et al., 1989; Toselli et al., 1989). In order to determine whether the action of NE on the Ba current in olfactory M/T cells was via a protein kinase, the effects of NE were examined in the presence of internal staurosporin, a broad-spectrum kinase inhibitor (Rüegg and Burgess, 1989). In cells that had been dialyzed with 1 μM staurosporin, 30 μM NE was still able to depress the calcium channel current reversibly by 25 ± 10% in five out of five M/T neurons (data not shown). Buffering of intracellular calcium to pCa <8 with 11 mM EGTA also did not block the action of NE, suggesting that neither protein phosphorylation nor release of intracellular calcium stores is required for the effects of NE on the calcium channel current.

The effects of NE on EPSPs are also PTX sensitive

Trombley and Shepherd (1992) demonstrated that NE reduced the amplitude of evoked monosynaptic EPSPs in cultures of

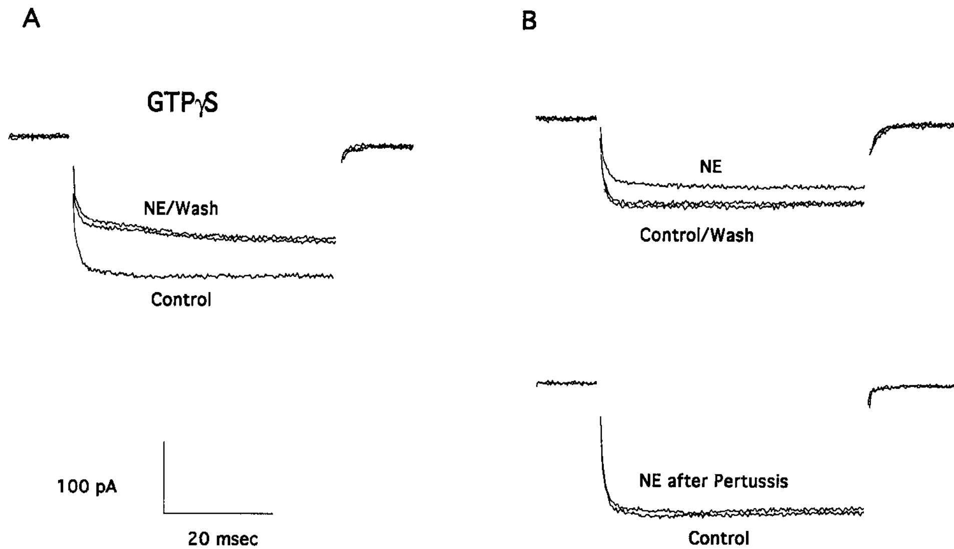

Figure 4. The effects of NE on calcium channel currents are G-protein mediated. *A*, Flow pipe application of 30 μM NE reduced the inward current in an M/T cell dialyzed with 100 μM GTPγS. The current does not recover, however, after return to control. *B*, The effects of NE are blocked by pretreatment with PTX. Calcium channel currents in M/T cells were not inhibited by 30 μM NE following treatment for 15–24 hr with 250 ng/ml PTX. However, NE reduced the inward current by 26 ± 5% (n = 9) in M/T cells from untreated sister cultures. Voltage protocol and electrode were the same as in Figure 1*A–C*.

olfactory bulb neurons by a presynaptic, α-adrenergic receptor mechanism. As in the previous study, monosynaptic EPSPs were evoked in a GR cell by stimulation of an action potential in the M/T neuron using a brief depolarizing current injection. Flow pipe application of 30 μM NE reduced the EPSP amplitude by 47 ± 16% (n = 4; Fig. 5*A*).

In sister cultures that had been pretreated with 250 ng/ml PTX for 15–36 hr, EPSPs could not be inhibited by NE (n = 5; Fig. 5*B*). However, EPSPs could still be blocked in PTX-treated neurons using postsynaptic receptor antagonists. These results suggest that NE antagonizes both calcium channel currents and EPSPs in olfactory bulb neurons by a similar mechanism that involves a PTX-sensitive G-protein.

Discussion

These results demonstrate that NE inhibits Ba currents through high-threshold calcium channels in olfactory bulb neurons. Noradrenergic inhibition of calcium channel currents could modulate excitation–secretion coupling at synaptic contacts between mitral and GR cells and may explain the inhibitory actions of NE on synaptic transmission reported in Trombley and Shepherd (1992). This conclusion is further supported by the evidence that both actions of NE appear to be mediated by a PTX-sensitive G-protein coupled to presynaptic α-adrenergic receptors. A model of noradrenergic modulation of M/T–GR cell synapses is shown in Figure 6.

Pharmacology

Noradrenergic inhibition of voltage-sensitive calcium channels in M/T cells appears to be mediated through activation of α-adrenergic receptors, as the effects of NE were mimicked by the α-adrenergic agonist clonidine. α-Adrenergic receptor–mediated inhibition of calcium currents may underlie the actions of NE on synaptic transmission between mitral and GR cells, since the effect of NE on EPSPs was also mimicked by clonidine but not by the selective β-adrenergic receptor agonist isoproterenol (Trombley and Shepherd, 1992). This conclusion is further supported by the collective results from several laboratories that have demonstrated a role for α-adrenergic receptors in inhibition of calcium currents (e.g., Bernheim et al., 1991), synaptic transmission (e.g., Madison and Nicoll, 1988; Dodt et al., 1991),

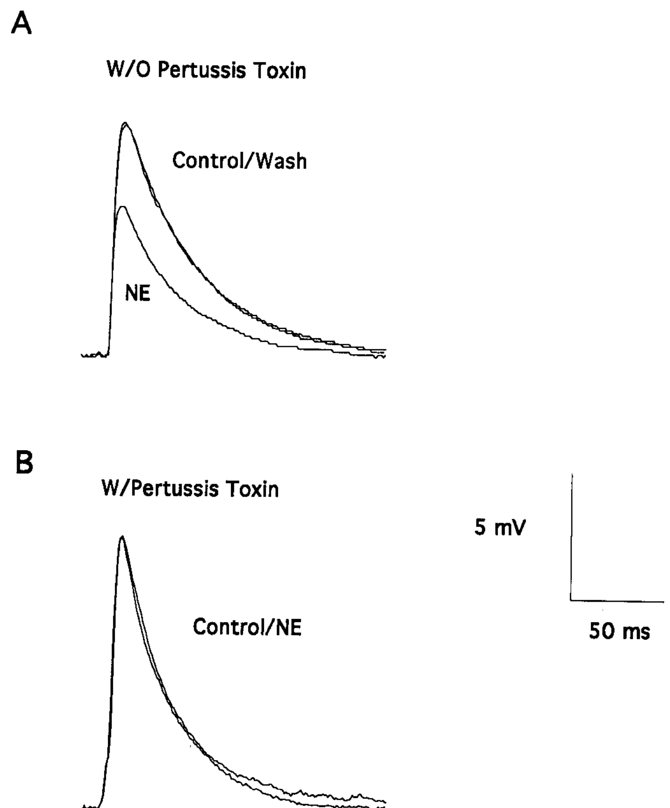

Figure 5. EPSPs evoked from pairs of bulb neurons are not reduced by NE after the neurons have been pretreated with PTX. Monosynaptic EPSPs evoked in GR cells by intracellular stimulation of an M/T cell were reduced by 30 μM NE. In PTX-treated neurons, 30 μM NE failed to reduce the EPSP (n = 5). Electrode solution: $KMeSO_4$, 5 mM ATP, 0.5 mM GTP; extracellular solution: 2 mM Ca, 1 mM Mg.

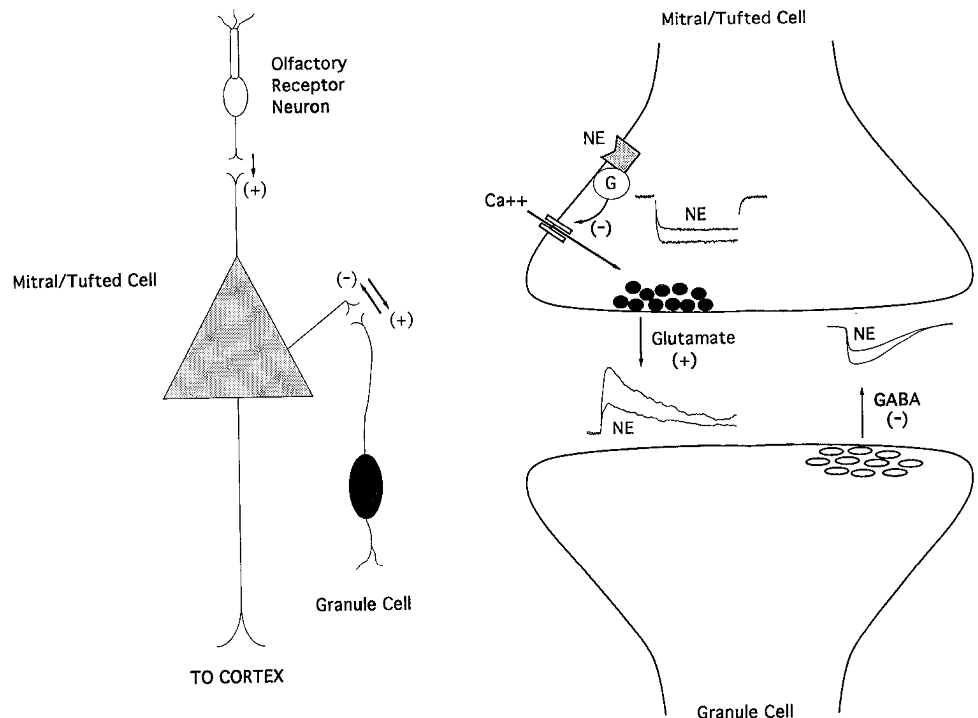

Figure 6. Model for noradrenergic disinhibition of olfactory bulb neurons. NE activates a presynaptic α-adrenergic receptor that inhibits voltage-gated calcium currents via a G-protein–coupled mechanism. Reduced calcium influx results in decreased transmitter release from M/T cells onto GR cells. Reduced excitation of GR cells reduces subsequent GR cell–mediated inhibition of M/T cells. Because NE can also inhibit calcium currents in GR cells, it may also inhibit transmitter release from GR cells, which would further contribute to M/T cell disinhibition.

and transmitter release (e.g., Lipscombe et al., 1989) in other neurons. In several types of neurons, including neurons from both sympathetic ganglia and sensory ganglia, α-adrenergic receptor activation mediates the inhibitory effects of NE on both high-threshold calcium currents and transmitter release (Holz et al., 1989; Lipscombe et al., 1989). In these studies, however, the effects of NE are prevented by α_2-adrenergic antagonists but not mimicked by clonidine. This difference may be due to the variability in the partial agonist actions of clonidine in different types of neurons (Surprenant, 1989).

Direct action of a G-protein mediates the effects of NE

The actions of NE on voltage-sensitive calcium channel currents appear to be mediated through a G-protein since its effects were irreversible after intracellular dialysis with GTPγS and prevented by pretreatment with PTX. Because PTX blocks the actions of both G_i and G_o, the identity of the G-protein mediating the effects of NE cannot be determined from these data. In dorsal root ganglion neurons and NG108 cells, however, G_o appears to couple inhibitory receptors to calcium channels (Hescheler et al., 1987; Ewald et al., 1989; McFadzean et al., 1989). These properties are similar to several transmitter systems, including, for example, glutamate, GABA, and adenosine, which have presynaptic receptors coupled to PTX-sensitive G-proteins. As with NE, activation of either 2-amino-4-phosphonobutyrate, $GABA_B$, or adenosine A1 receptors inhibits voltage-sensitive calcium currents and transmitter release (Holz et al., 1989; Scholz and Miller, 1991; Trombley and Westbrook, 1992). Collectively these results suggest a general mechanism by which activation of presynaptic receptors can modulate synaptic efficacy.

There has been some controversy about the mechanism of action of G-protein–coupled receptors, specifically whether their effects are mediated indirectly via a diffusible second messenger or by direct action of the activated G-protein on the ion channel. In several transmitter systems, including, for example, the cholinergic and the noradrenergic systems, there is evidence to support direct (Lipscombe et al., 1989; Toselli et al., 1989; Bernheim et al., 1991) and second messenger–mediated (Trautwein et al., 1986; Rane et al., 1989; Bernheim et al., 1991) actions on voltage-sensitive calcium channels. Both G_s (Yatani et al., 1987) and G_o (Hescheler et al., 1987; Toselli et al., 1989) have also been implicated in the direct modulation of calcium currents. G-proteins may use both direct and indirect mechanism to exert differential effects. Direct coupling would allow fast, local effects, while second messengers could produce long-lasting or global effects.

Neurotransmitter-mediated calcium channel inhibition may involve activation of protein kinase C (Rane et al., 1989) or release of calcium from intracellular stores (Bernheim et al., 1991; Kramer et al., 1991). The results of the present study suggest that the effects of NE are not mediated through either protein kinase activation or elevated intracellular calcium, however, as intracellular dialysis with staurosporin or EGTA did not prevent its effects. These results suggest that the G-protein may act directly on calcium channels in M/T cells. This conclusion is supported by the recent report of Bernheim et al. (1991) that demonstrated an α-adrenergic–mediated inhibition of calcium currents in rat superior cervical ganglion neurons that was not prevented by intracellular BAPTA or mediated by cAMP, cGMP, or protein kinase C. Although EGTA does not rapidly buffer calcium, transmembrane calcium influx was minimized by using calcium-free solutions in these experiments. NE was also effective on repeated applications during whole-cell dialysis lasting more than 15 min, which should deplete intracellular calcium stores (Murphy and Miller, 1988; Pfaffinger et al., 1988; Kramer et al., 1991).

Calcium current inhibition reduces transmitter release

Trombley and Shepherd (1992) demonstrated that NE could reduce EPSPs by a presynaptic mechanism. The results of the

present study further support the notion that the effects of NE on EPSPs are mediated through inhibition of presynaptic voltage-sensitive calcium channels as both effects of NE are mediated through a PTX-sensitive G-protein. A central role for calcium in regulating transmitter release has been known since Katz and Miledi (1967) demonstrated not only that removal of extracellular calcium inhibits transmitter release but also that transmitter release was dependent on the presence of calcium during depolarization of the presynaptic terminal. Neurotransmitter-mediated inhibition of high-threshold calcium channel currents and a reduction in transmitter release is also not without precedent. In dorsal root ganglion neurons, both GABA and NE can inhibit the high-threshold calcium current (Dunlap and Fischbach, 1981). Application of either GABA or NE also reduces substance P release, an effect mimicked by dihydropyridines, lending further support for a role for high-threshold calcium currents in transmitter release (Perney et al., 1986; Rane et al., 1987). A correlation between inhibition of voltage-sensitive calcium currents and an inhibition of synaptic transmission can also be drawn from the results of Dolphin and Scott (1987) and Harrison et al. (1988). The $GABA_B$ receptor agonist baclofen reduced calcium channel currents in rat sensory neurons (Dolphin and Scott, 1987) and inhibited EPSPs and IPSPs by a presynaptic mechanism in hippocampal neurons (Harrison et al., 1988). In olfactory bulb neurons, a causal relationship between inhibition of presynaptic calcium currents and EPSPs is further supported by the evidence that NE can inhibit both.

The results of these experiments suggest a mechanism where inhibition of voltage-sensitive calcium channels may mediate an inhibitory neuromodulatory mechanism in synaptic pathways that use excitatory amino acids as transmitters. In the olfactory system these pathways include axonal projections to the cortex via the lateral olfactory tract and axon collaterals that terminate within the bulb. In addition to these, the M/T cells make reciprocal excitatory–inhibitory synapses with periglomerular cells in the glomerular layer and with GR cells in the external plexiform layer. Although the focus of these experiments has been on calcium currents in M/T cells, NE appears to have a similar effect on calcium currents in GR cells. The net effect of inhibition of transmitter release from M/T and/or GR cells would be disinhibition of M/T cells. Thus, presynaptic inhibitory mechanisms may play a significant role in early stages of olfactory processing.

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