The N-Type Ca Channel in Frog Sympathetic Neurons and Its Role in α-Adrenergic Modulation of Transmitter Release

SATHAPANA KONGSAMUT,[a] DIANE LIPSCOMBE,[a,b] RICHARD W. TSIEN[a,b,c]

[a] *Department of Cellular and Molecular Physiology*
Yale University School of Medicine
New Haven, Connecticut 06510

[b] *Department of Molecular and Cellular Physiology*
Beckman Center
Stanford University Medical Center
Stanford, California 94305

Free cytosolic calcium ions regulate a variety of different cellular functions. In neurons, for example, the generation of an intracellular calcium transient triggers such diverse events as neurotransmitter release, short- and long-term changes in cell excitability, altered Ca-dependent enzyme activity, and altered gene expression. Given the diverse nature of these Ca-dependent responses, it is significant that several types of voltage-gated Ca channels have been described over the last few years.[1-13] With the general acceptance of the existence of multiple Ca-channel types, attention has turned toward basic questions about the physiological significance of diverse Ca channels and their control by neurotransmitters and hormones.[7,10,13]

This paper focuses on a particular class of voltage-gated Ca channels, referred to as N-type Ca channels.[13-15] In many neuronal preparations, N-type and L-type Ca channels appear to be distinct entities that contribute to high voltage activated Ca current.[8,9,14-18] Here we review information about their properties in frog sympathetic neurons, a particularly favorable preparation for studying N-type channels as distinct from L-type Ca channels.[19] We describe properties of neurotransmitter release from sympathetic neurons and the relative importance of N- and L-type Ca channels in regulating this release. Finally, we describe the selective down-modulation of N-type Ca channels by α-adrenergic agents and its possible role in inhibitory effects of norepinephrine (NE) on its own release.[20]

[c] Address for correspondence: R. W. Tsien, Department of Molecular and Cellular Physiology, Beckman Center, Stanford University Medical Center, Palo Alto, California 94305.

DISTINCTIONS BETWEEN N- AND L-TYPE Ca CHANNELS

In frog sympathetic neurons, N- and L-type Ca channels are most easily identified and studied in single-channel recordings (FIGS. 1 and 2). The virtual lack of T-type Ca-channel activity in these cells,[19] as in rat sympathetic neurons,[21,22] makes the identification and separation of N and L-type Ca channels simpler than in other preparations that express T-type as well as N and L-type channels (e.g., chick sensory neurons[15]). N and L-type Ca channels differ in several respects, including single-channel conductance, voltage-dependence of activation and inactivation, and sensitivity to dihydropyridines (DHPs, see Tsien *et al.*[13] for review). In sympathetic neurons, L-type Ca channels have a unitary conductance of 25-28 pS (110 m*M* Ba, 20°C), are sensitive to modulation by DHPs, and are relatively resistant to inactivation with changes in holding potential. N-type Ca channels have a unitary conductance of 15-16 pS (110 m*M* Ba, 20°C), are insensitive to DHPs, and readily inactivate with depolarized holding potentials.

FIGURES 1 and 2 highlight the striking difference in the voltage-dependence of inactivation of N- and L-type Ca channels. Recordings from a cell-attached patch containing only L-type Ca channels are shown in FIGURE 1. The opening probability of L-type Ca channels evoked by test pulses to +20 mV is not affected by changing the holding potential from −100 mV to −40 mV. In contrast, the opening probability of N-type Ca channels decreases significantly within 10-20 seconds after the holding potential is changed from −80 mV to −40 mV. This can be seen in the two examples of N-type Ca-channel recordings shown in FIGURE 2. The recordings also illustrate that N-type Ca channels can inactivate with widely different time courses following a step depolarization. Panel A shows an example of a patch of N-type Ca channels that inactivate relatively rapidly ($\tau \sim 50$ msec) during a step depolarization to −10 mV. This may be compared with the example in panel B, which shows little or no inactivation of N-type Ca channels during a 320-msec pulse in another patch. The time course of N-channel inactivation is quite variable from patch to patch, ranging between these two extreme examples (see also Aosaki & Kasai[23]). With respect to unitary conductance and lack of sensitivity to dihyropyridines, the properties of N-type Ca channels recorded in different patches and in different regions of the neuron appear indistinguishable.[19,20]

FIGURE 3A shows an ensemble average of N-type Ca-channel current, calculated from 11 cell-attached patches recorded under identical conditions. The average current is composed of a prominently decaying component and a sustained component, reflecting the kinds of unitary activity illustrated in FIGURE 2. FIGURE 3B shows a series of whole-cell currents recorded with 2 m*M* external Ba for comparison with the averaged single-channel currents. The holding potentials are set at more negative values than in the single-channel recordings to allow for differences in external surface charge with 2 m*M* rather than 110 m*M* Ba. The whole-cell current is composed of different components that correspond rather well to the properties of N- and L-type channels found in single-channel recordings (FIGS. 1-3). The sustained current that remains available for activation even at a holding potential of −60 mV is dominated by current through L-type channels, which are less prone to inactivation with changes in holding potential (FIG. 1). The extra whole-cell current recruited at a holding potential of −100 mV is comprised of a prominently decaying and a more sustained current, similar in time course to the averaged currents carried by the inactivating (FIG. 2A and B) and sustained N-type Ca-channel activity (FIG. 2C and D).

The present results suggest that it is incorrect to assume that N-type Ca channels contribute only to the decaying component of whole-cell Ca-channel current, and that

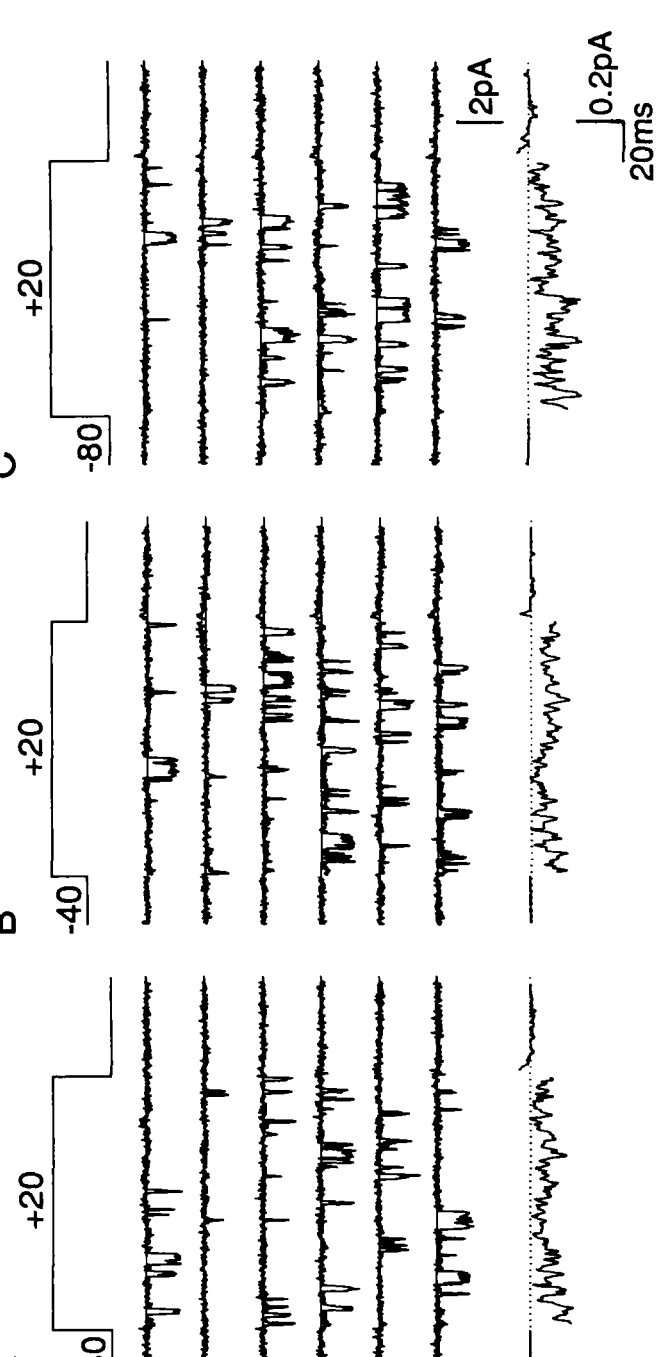

FIGURE 1. L-type Ca-channel activity is not responsive to changes in holding potential. Cell-attached patch recording containing only the L-type Ca channel, identified by its unitary conductance (25 pS between +20 mV and +40 mV), held at −80 mV (**A and C**) and −40 mV (**B**). Depolarizations to potentials more positive than +10 mV were required to activate the channel. Six sequential leak-subtracted current recordings are shown for each stimulation protocol. Average currents, plotted below the individual recordings, were calculated from more than 20 individual sweeps. The dotted line indicates zero current level. Patch pipette contained 110 m*M* Ba as the charge carrier and depolarizing pulses of 130-msec duration were applied every 4 seconds. No DHP was present (see Lipscombe *et al.*[19] for further details).

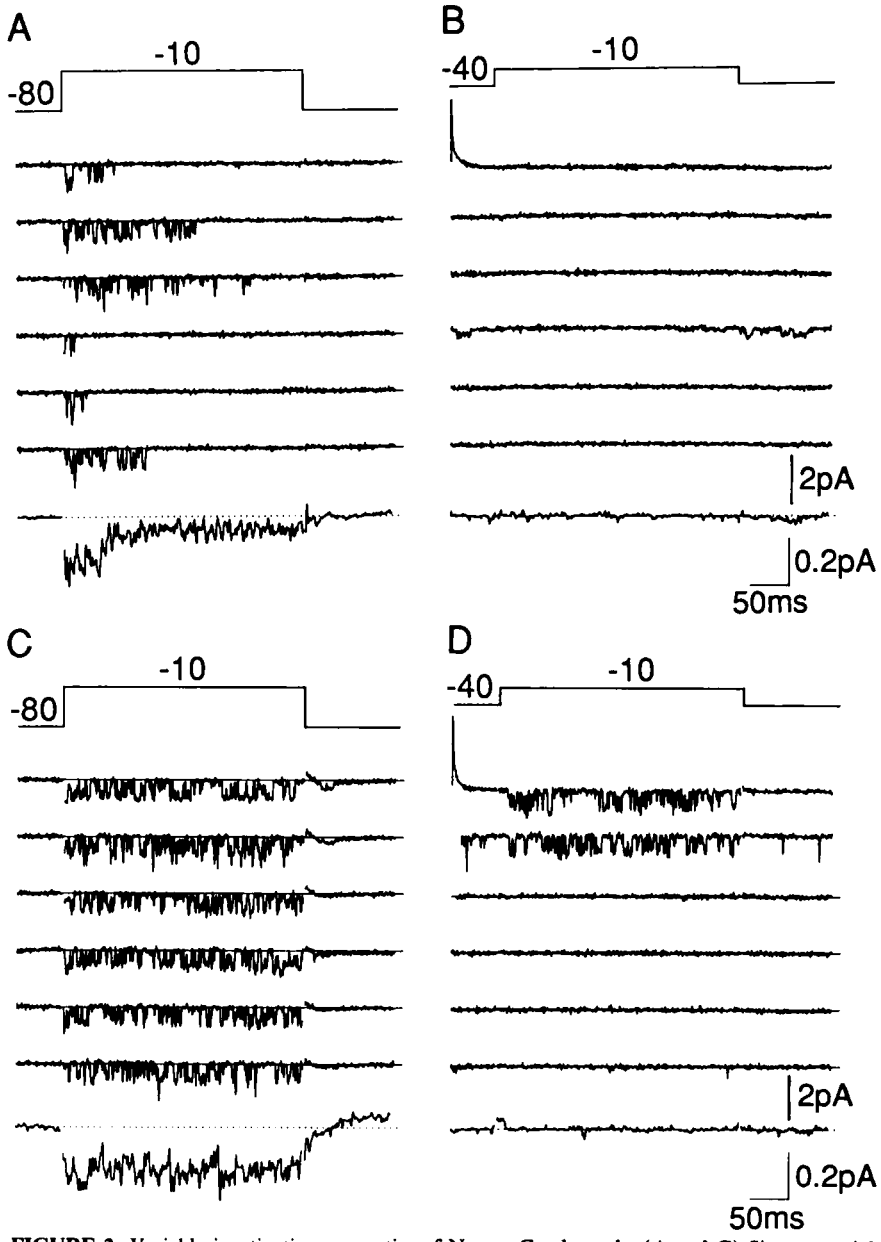

FIGURE 2. Variable inactivation properties of N-type Ca channels. (**A and C**) Six sequential sweeps showing unitary N-channel activity from two different patches evoked by a depolarization to −10 mV from a holding potential of −80 mV. (**B and D**) Six sequential sweeps from the same patches recorded immediately after a sudden change in holding potential from −80 to −40 mV (marked by upward capacity transient in first sweep). Average currents are plotted below each stimulation protocol. Average N-channel current in patch A is transient while average current in C shows little inactivation. Depolarizing pulses of 320-msec duration were applied every 10 seconds. Recording conditions as previously described in FIGURE 1 and Lipscombe *et al.*[20]

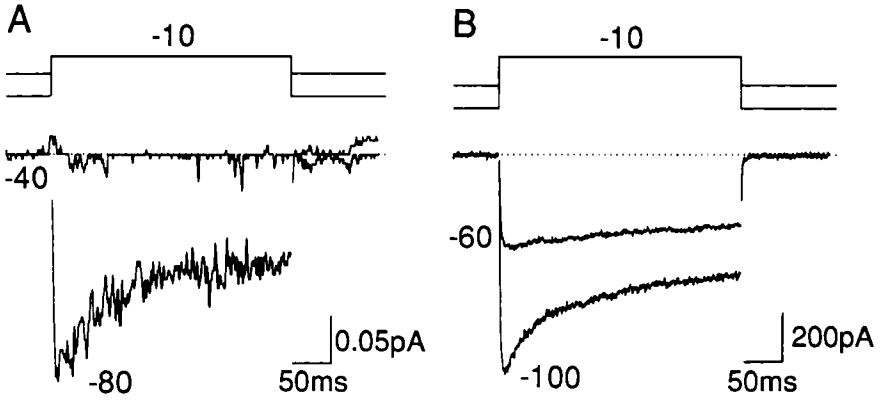

FIGURE 3. Comparison of average N-type Ca-channel activity from cell-attached patch recordings to a whole-cell current recording. (**A**) average of mean currents calculated from 11 cell-attached patches (including the two patches illustrated in FIG. 2) recorded under the same conditions as previously described.[19] Average currents evoked by depolarizing pulses to −10 mV from holding potentials of −80 mV and −40 mV are shown superimposed. In calculating the final average, contributions of individual patches were weighted equally. (**B**) Whole-cell currents recorded with 2 m*M* external Ba evoked by depolarizing pulses to −10 mV from holding potentials of −60 mV and −100 mV.

L-type channels underlie all of the late current. In earlier analysis of results from chick dorsal root ganglion (DRG) neurons, it was assumed as a first approximation that N-type Ca-channel current decayed rapidly and relatively completely.[16] The present data, along with single-channel analysis in rat sympathetic neurons,[22,24] PC12 cells,[24] and chicken or frog DRG neurons,[7,23] suggest that N-type Ca channels may contribute substantially to steady components of macroscopic Ca-channel current.

At present, no perfect method exists for separating N- and L-type Ca current in whole-cell recordings. Nevertheless, there is little doubt of the distinctness of these channels, given the combination of differences in single-channel conductance, kinetics, and pharmacology (see below).

STUDIES OF SYMPATHETIC TRANSMITTER RELEASE

Individual sympathetic varicosities are not readily accessible for direct study with patch-clamp methods. Thus, it has been difficult to study the role of different Ca entry pathways in the release of sympathetic transmitter and their electrophysiological basis in the same preparation. For this purpose, we took the approach of measuring radiolabeled transmitter release from cell bodies of sympathetic neurons. Evidence is available from earlier studies of Koketsu and colleagues[25,26] that cell bodies in sympathetic ganglia can release catecholamines. Miyagawa *et al.*[25] first suggested this possibility on the basis of electrophysiological measurements of synaptic transmission from preganglionic (cholinergic) terminals. Later, Suetake *et al.*[26] examined the catecholamine fluorescence intensity in cell bodies of sympathetic neurons (FIG. 4). Application of a prolonged electrical stimulation in the presence of an inhibitor of

catecholamine synthesis produced a marked decrease in catecholamine content (B) relative to that found in matched ganglia that were left unstimulated (A).

FIGURE 5 illustrates our results using radiotracer methods to study the release of catecholamine from frog sympathetic ganglion cells. Most of the experiments were carried out with whole paravertebral sympathetic ganglia, although similar results have been obtained in experiments with dissociated neurons in culture (S. K. and K. R. Bley, unpublished). Sympathetic ganglia were incubated in [^{3}H]NE, washed, and stimulated with 50 m*M* K^+ to evoke secretion (see FIG. 5 legend for details). These challenges with K^+ allow for direct depolarization of the cells bypassing possible changes due to action potential propagation or duration. The transmitter release is steeply dependent on K^+ (range 30-60 m*M*) and extracellular Ca^{2+} ($EC_{50} \sim 0.5$ m*M*) and is completely inhibited by cadmium ions ($IC_{50} \sim 10$ μM). The effect of 5 μM Cd is illustrated in FIGURE 5A. In addition, ω-toxin from the marine snail *Conus geographus* is a potent inhibitor of secretion. All these observations are as expected for secretion triggered by Ca entry through voltage-gated Ca channels.[20]

In contrast to the pronounced inhibitory effects of cadmium ions and ω-conotoxin, DHP Ca-channel agonists and antagonists have little or no effect on transmitter release. FIGURE 5B and C show representative data obtained with experimental conditions chosen to favor a DHP effect. DHPs are added during a predepolarization period

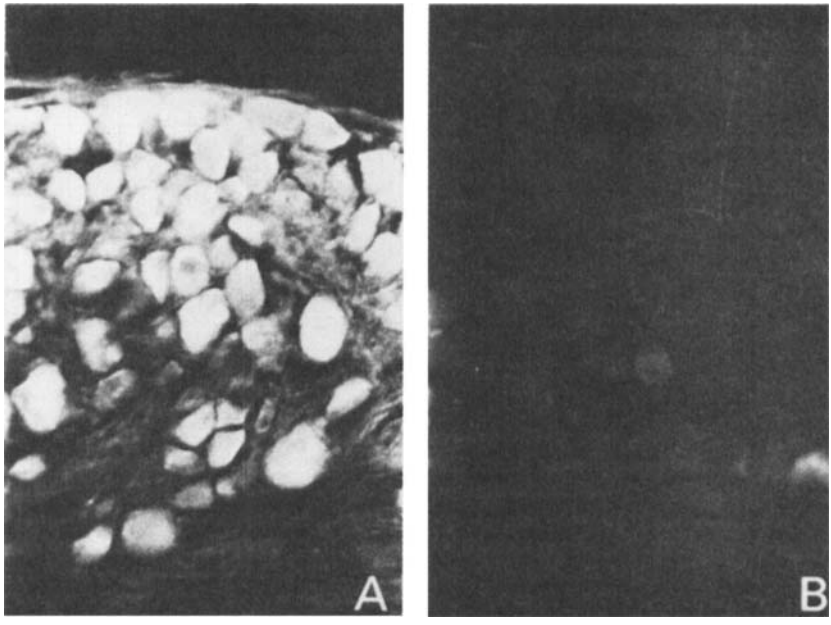

FIGURE 4. Effect of electrical stimulation on catecholamine fluorescence in the cell bodies of sympathetic neurons. Photomicrographs of formaldehyde-induced fluorescence of a pair of bullfrog paravertebral sympathetic ganglia. Ganglia were preincubated in Ringer's solution containing an inhibitor of catecholamine synthesis, α-methyl-*p*-tyrosine (80 μg/ml), for five hours. The specimens were exposed to formaldehyde vapor of 75% relative humidity at 80°C for three hours. (**A**) Unstimulated control ganglion. (**B**) Ganglion stimulated at 30 Hz for 60 min. (From Suetake, Kojima & Koketsu.[26] Used with permission.)

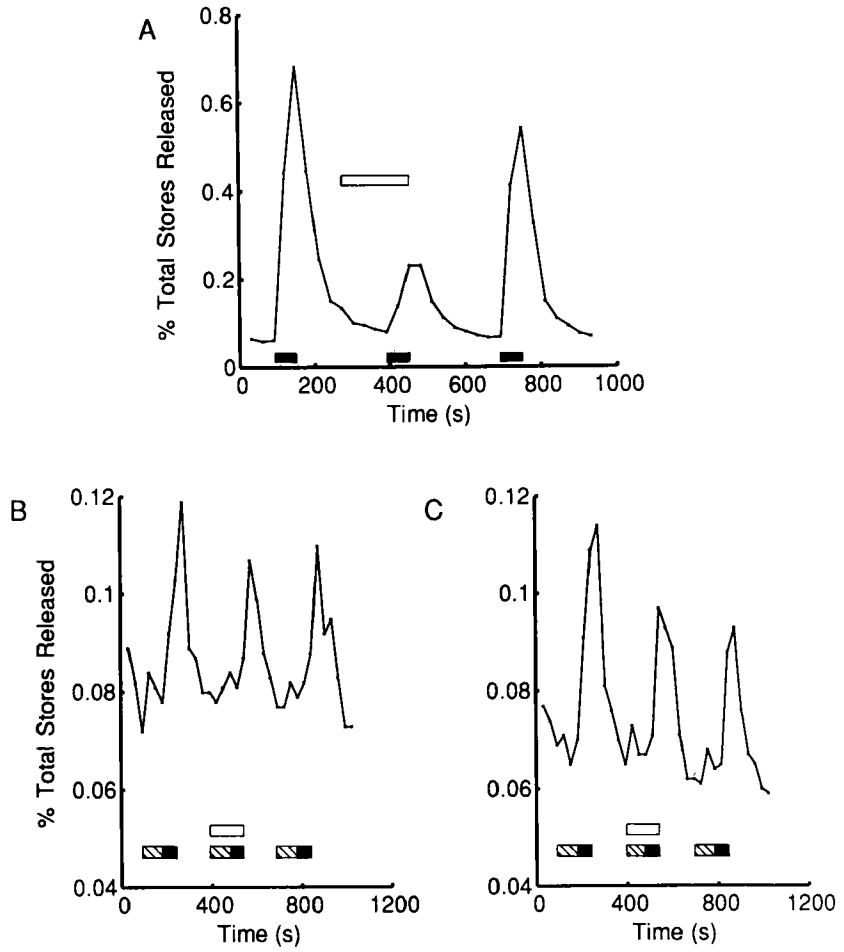

FIGURE 5. Properties of transmitter release from frog sympathetic neurons. (**A**) Effect of 5 μM Cd (open bar) on release evoked by 50 mM K^+ stimulation (solid bars). (**B,C**) Ganglia were superfused with low-Ca^{2+} solutions except where Ca^{2+} additions are indicated. Ganglia were predepolarized with 50 mM K^+ (no added Ca) for 1.5 min before evoking release (hatched bars). Release was evoked by addition of 2 mM Ca for 1 min in the continued presence of 50 mM K^+ (solid bars). Lack of effect of DHPs (open bars), 1 μM BAY K8644 (**B**) and 10 μM nitrendipine (**C**) on Ca^{2+}-evoked release. **Methods:** Sympathetic chains from frogs (*Rana pipiens pipiens*) were removed, incubated in a Ringer's solution (128 mM NaCl, 2 mM KCl, 10 mM HEPES, 10 mM glucose, 1 mM ascorbic acid [antioxidant], 0.1 mM pargyline [monoamine oxidase inhibitor], pH adjusted to 7.3 with NaOH) containing 10 μCi/ml [^{3}H]norepinephrine (NE) for two hours at room temperature. Excess ^{3}H was washed off by perfusing with Ringer's containing 10 μM desipramine (reuptake blocker) and 0 or 2 mM $CaCl_2$ for 35-40 minutes after which time a stable baseline release was achieved; thirty-second fractions were then collected continuously from this point. Results presented are individual representative experiments.

(without added Ca^{2+}) in order to promote DHP binding and to cause inactivation of transient Ca channels, thus favoring any release mediated by the long-lasting DHP-sensitive L-type Ca channels. Despite these measures, DHPs produce little inhibition or stimulation of release. Taken together with available electrophysiological evidence (see above), these observations indicate that release of transmitter from frog sympathetic neurons must be mediated primarily by N-type Ca channels, as has been previously reported by Hirning *et al.*[22] for rat sympathetic neurons.

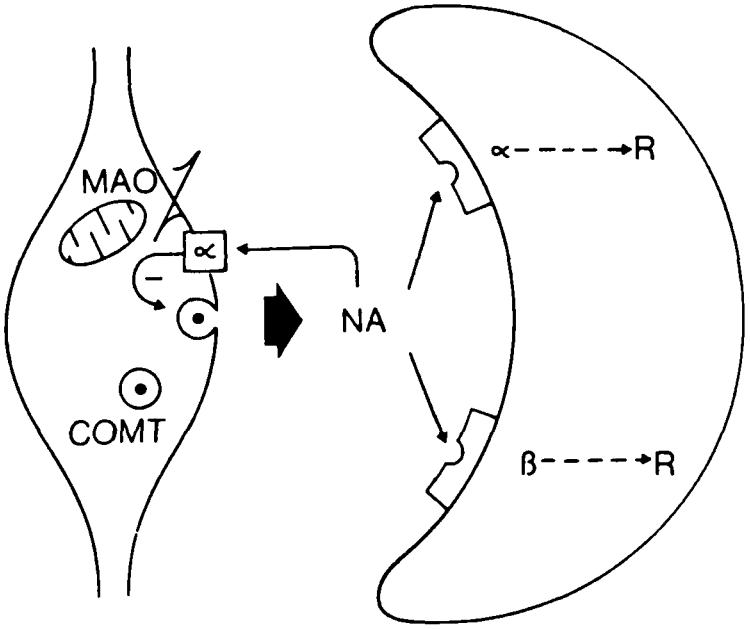

FIGURE 6. Schematic representation of a negative feedback mechanism for norepinephrine (NE) mediated by presynaptic α-adrenoceptors. Once it reaches a threshold concentration in the cleft, NE, released by nerve stimulation, activates presynaptic α-adrenoceptors leading to inhibition of further transmitter release. The presynaptic negative feedback mechanism is present both in tissues where the response (R) of the effector organ is mediated through α- or through β-adrenoceptors. MAO, monoamine oxidase; COMT, catechol-*O*-methyl transferase. (From Langer.[27] Used with permission.)

INHIBITION OF SECRETION BY NE

In the early 1970s, Langer and others proposed the idea of autoinhibition of transmitter release from sympathetic neurons.[27–33] In essence, locally released catecholamines act through α-adrenergic receptors to inhibit subsequent transmitter release through an autoinhibitory feedback loop (FIG. 6). The α-adrenergic response to released or circulating catecholamines appears to be a significant mechanism for

regulating the level and spatial uniformity of transmitter output. This is the earliest and most extensively studied example of autoinhibition of release, but such a mechanism can also be seen in peripheral and central neurons containing other transmitters such as acetylcholine, ATP, dopamine, GABA, histamine, and serotonin.[29,30,32-34]

TABLE 1 and FIGURE 7 illustrate the inhibitory effect of NE on high K^+-evoked release of transmitter from frog sympathetic neurons. Experiments are performed essentially as described and illustrated in FIGURE 5A. The inhibition is reversible and dose dependent, reaching a maximal inhibition of ~50% at 10 to 30 μM NE (TABLE 1). The NE inhibition shows some pharmacological properties expected for an α_2 antagonist (TABLE 1; FIG. 7). Interestingly, no inhibition is seen with application of the α_2 agonist clonidine (up to 100 μM). These pharmacological properties, the lack of clonidine effect included, are characteristic of a distinct subtype of α_2 receptor previously described in sensory neurons[35] and neuroblastoma cells.[36]

Many investigators[28,29,32] have suggested that the mechanism underlying NE autoinhibition might involve altered phosphorylation of Ca channels or of intracellular or membrane-bound proteins such as synapsin I by cyclic AMP- or Ca-dependent protein kinases, as found in other systems.[37] The prevailing hypothesis may be summarized as follows:

$$\text{NE} \rightarrow \alpha_2 \text{ receptor} \rightarrow G_i \rightarrow \downarrow \text{cAMP} \rightarrow \downarrow \text{phosphorylation of Ca channels or synapsin I or other intracellular protein} \rightarrow \downarrow \text{transmitter release}$$

TABLE 1. Effect of Norepinephrine on High K^+-Evoked Release of Transmitter from Frog Sympathetic Neurons

Treatment	[NE]	Percent Inhibition	SE	n
NE only	1	12.4	3.7	4
	3	31.9	3.8	4
	10	49.6	3.7	4
	30	50.0	5.2	7
NE + 10 μM yohimbine	10	17.2	4.3	4
	30	8.2	2.0	3
NE + 1 mM dibutyryl cyclic AMP	10	37.1	3.0	5
	30	42.8	4.8	7
NE + 300 μM H-7	30	47.5	2.4	8
NE with release stimulated by Ca addition in the presence of 10 μM ionomycin	30	12.1	5.2	4

NOTE: Experiments were performed essentially as described in the legend to FIGURE 5. Transmitter release was stimulated with three periods of depolarization by 50 mM K^+ in 2 mM Ca^{2+}: control, NE added and wash (see FIG. 5A; Lipscombe *et al.*[20]) with NE present for two minutes before and during the second depolarization. For each run, a fitted baseline was subtracted and the area under each peak was calculated (A_{con}, A_{NE}, and A_{wash}). Percent inhibition was calculated according to the equation: % inhibition = $100 \cdot [1 - (A_{NE}/(A_{con} + A_{wash})/2)]$. For ionomycin-induced release, basal release was obtained by washing with Ringer containing 2 mM EGTA and 100 nM free calcium; 10 μM ionomycin was added to the perfusion Ringer 10 minutes before collecting fractions; release was evoked by addition of 1 mM free calcium (buffered with 2 mM EGTA) in the continued presence of 10 μM ionomycin to bypass calcium entry through voltage-sensitive Ca channels. SE = standard error; n = number of runs.

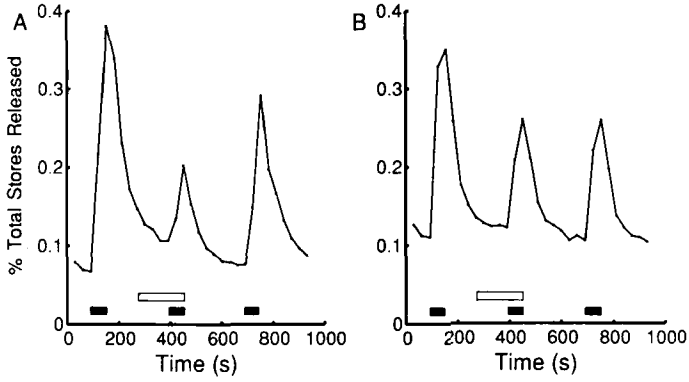

FIGURE 7. Inhibitory effect of NE on K^+-stimulated transmitter release. Experiments were conducted essentially as described in FIGURE 5A. Two representative examples are shown. (**A**) Inhibitory effect of 30 μM NE (open bar), present during the second K^+ challenge (filled bars). (**B**) Inhibitory effect of 30 μM NE (open bar) is antagonized when 10 μM yohimbine is present throughout the run.

This mechanism cannot, however, account for our findings in sympathetic neurons (TABLE 1; Lipscombe *et al.*[20]). The inhibition of NE persists in the presence of a saturating concentration (1 mM) of dibutyryl cyclic AMP, indicating that NE is not acting by reducing cyclic AMP levels (see also Johnston *et al.*[38]). Furthermore, the NE inhibition is not prevented by 300 μM H-7, a nonspecific inhibitor of several protein kinases,[39] including cyclic AMP- and calmodulin-dependent protein kinases and protein kinase C. This suggests that phosphorylation by H-7-sensitive protein kinases is not involved in the mechanism of inhibition (see also Wanke *et al.*[40]). Thus, it appears that NE can act on transmitter release without the involvement of the intracellular messengers that have previously been invoked.

To determine the locus of the inhibitory action, transmitter release was evoked with Ca in the presence of ionomycin to bypass Ca entry through voltage-gated calcium channels. Under these conditions, NE is ineffective in inhibiting transmitter release (TABLE 1 and Lipscombe *et al.*[20]), suggesting that NE does not inhibit processes subsequent to a rise in intracellular Ca. It is likely that NE acts by modulating Ca entry because NE and other catecholamines have been shown to modulate Ca currents or Ca-dependent action potentials in this (see below and Lipscombe *et al.*[20]) and other preparations of sympathetic neurons[21,41,42] in DRG neurons[21,35,43–46] and in neuroblastoma cells.[36]

NE MODULATION OF Ca-CHANNEL CURRENT

In frog sympathetic neurons, NE rapidly and reversibly inhibits whole-cell Ca-channel currents. FIGURE 8A illustrates the effect of NE on whole-cell currents recorded with 10 mM Ca in the external solution. Qualitatively similar results can be obtained with either external Ca (2-10 mM) or Ba (1-2 mM) as the charge carrier. The pharmacology of the NE-mediated inhibition of Ca current is identical to the

inhibitory effects on sympathetic transmitter release. The response to NE is antagonized by phentolamine and by yohimbine, but not by propranolol, a β-antagonist. As in the release studies, clonidine does not mimic the action of NE. This strong correlation between the pharmacological characteristics of the noradrenergic effect on Ca-channel currents and release is consistent with the same receptor mediating both events.[20]

Although the detailed mechanism by which α-adrenoceptor stimulation leads to inhibition of Ca channels is not clear, it is likely that a GTP-binding protein mediates at least part of the response. Thus, (1) GTP (0.3 m*M*) has to be present in the recording pipette in order to obtain multiple responses to NE, (2) substituting GTP with low concentrations of a nonhydrolyzable analogue, GTP-γ-S (0.05 m*M*), largely

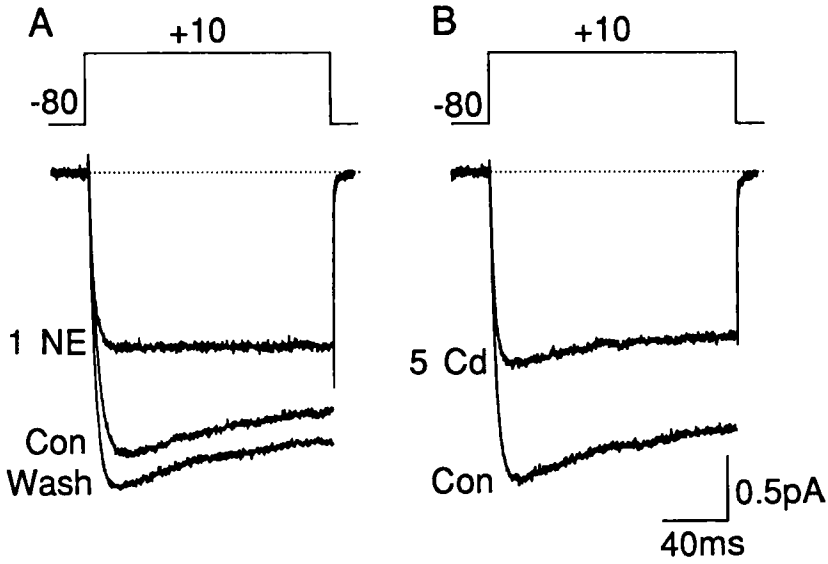

FIGURE 8. Effect of 1 μM NE (**A**) and 5 μM Cd (**B**) on whole-cell, Ca-channel currents recorded from the same cell. Currents were recorded with 10 m*M* external Ca as the charge carrier in solutions (internal and external) that effectively blocked other voltage-gated conductances (see Fox *et al.*[15] for details). Depolarizing pulses lasting 160 msec were applied every 10 sec. Leak-subtracted Ca-channel currents are shown superimposed below the appropriate voltage pulse protocols.

prevents recovery from the inhibitory effect of NE, and (3) high concentrations of GTP-γ-S (0.5 m*M*) mimic the inhibitory effects of NE.[20]

Selective Inhibition of N-Type Ca Channels in Whole-Cell Recordings

The inhibitory effects of NE on peak Ca-channel current are incomplete, usually not exceeding about 50% of control, even at maximally effective NE concentrations (between 10 and 100 μM NE in 2 m*M* external Ba, Lipscombe *et al.*[20]) and even in

combination with intracellular GTP-γ-S. The incompleteness of the NE effect raises the question of whether one or both types of high-voltage-activated Ca channel might be partially inhibited (see also Refs. in Tsien *et al.*[13]).

FIGURE 8 shows the inhibitory effect of 1 μ*M* NE on whole-cell Ca currents (A) and compares it to the effect of 5 μ*M* Cd (B), a nonselective inhibitor of N- and L-type Ca channels. The inhibitory effects of these two blockers on the same cell are quite different. NE eliminates the decaying component and partially inhibits a sustained component of the control Ca current (A). The NE-sensitive current has a time course of inactivation consistent with the kinetic properties of N-type Ca channels described in FIGURE 2. In contrast, Cd inhibits the Ca current without affecting the time course of current decay compared to control, as expected for a nonselective inhibitor of both types of Ca channels. Thus, the lack of an inactivating component in the presence of NE cannot be attributed to a simple reduction in overall Ca influx and less current-dependent or Ca-dependent inactivation (*cf.* Docherty & McFadzean[36]). The most likely explanation is that NE is selectively inhibiting the N-type Ca-channel current. This idea is supported by the dependence of the NE effect on the holding potential (FIG. 9). While NE strongly reduces the current evoked from a negative holding potential of −120 mV (A), it has less effect on the sustained, largely L-type Ca current evoked from a less negative holding potential of −60 mV (B). The holding potential sensitivity of the NE-sensitive current parallels that of N-type Ca-channel currents described in FIGURES 2 and 3.

Modulation of Single N-Type Ca Channels

Unitary recordings of Ca-channel activity provide a means for testing our hypothesis that NE acts selectively on the N-type Ca channel and allows us to analyze the mechanism of inhibition in more detail. While many neurotransmitters have been shown to inhibit whole-cell, high-voltage-activated Ca currents in neurons (see Miller[10] and Tsien *et al.*[13] for references), little is known so far about how inhibition occurs at the level of single channels. One impediment has been rundown of N and L-type channels, which is particularly problematic for studies of inhibitory modulation. This may have discouraged the use of outside-out patches as applied in studies of NE modulation of low voltage-activated T-type Ca channels by Marchetti *et al.*[21]

To avoid problems of Ca channel rundown, we can take the approach of comparing large numbers of cell-attached patch recordings with and without NE in the pipette (external) solution. Because the majority of cell-attached patch recordings contain both N- and L-type Ca channels,[19] we can choose a stimulation protocol that allows them to be studied in isolation (FIG. 10). N-type Ca channels are activated selectively by stepping from a relatively negative holding potential of −80 mV to −10 mV (A). At this test potential, openings of L-type channels are extremely rare in the absence of DHP agonists. Depolarizing the holding potential from −80 mV to −40 mV largely inactivates N-type channels (B), but leaves L-type channels available for opening with stronger test depolarizations to +20 mV (C).

With these voltage protocols, we can distinguish between changes in the activity of N- and L-type Ca channels in membrane patches exposed to NE.[20] A representative example of the effect of 100 μ*M* NE on N- and L-type Ca channels is illustrated in FIGURE 10D-F. NE inhibits the N-type Ca-channel current activated from a holding potential of −80 mV (D) while the activity of L-type Ca channels evoked by de-

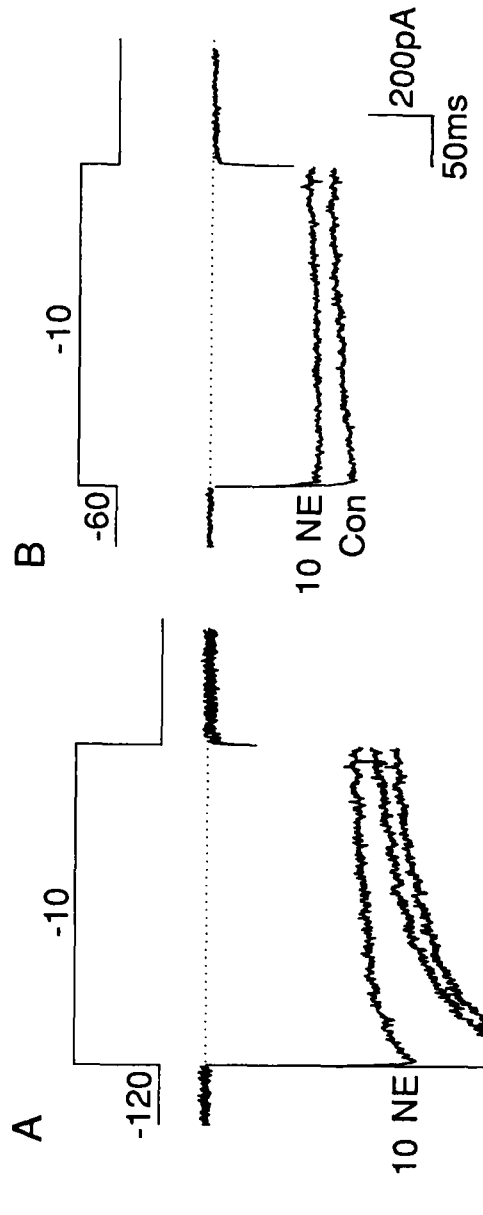

FIGURE 9. Holding potential dependence of NE-sensitive whole-cell Ca current. Whole-cell currents were recorded with 2 m*M* external Ba as the charge carrier. Depolarizing pulses lasting 320 msec were applied every 10 sec. The effect of 10 μM NE on currents evoked by pulses to -10 mV from holding potentials of -120 mV (*A*) and -60 mV (*B*) recorded from the same cell. Leak-subtracted records are shown superimposed below the appropriate voltage pulse protocol.

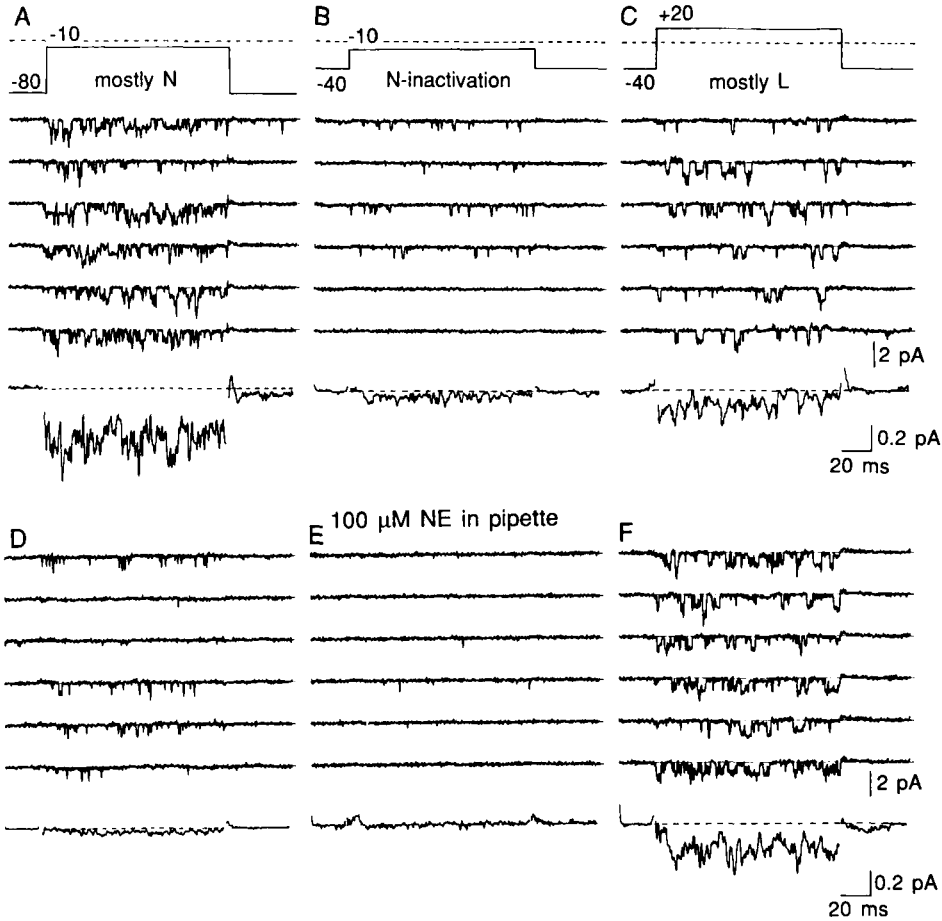

FIGURE 10. Properties of unitary N-type and L-type Ca-channel currents in a control patch and in a patch exposed to 100 μM NE present in the pipette. Recordings from two cell-attached patches with 110 mM Ba in the absence (**A-C**) and presence (**D-F**) of 100 μM NE in the patch pipette. (Exemplars of collected data from a large number of patches; see FIG. 11.) Six sequential current recordings are shown above average currents from at least 20 individual sweeps for each voltage protocol. Depolarizing pulses, 130 msec in duration, were applied every 4 sec. (**A**) N-type Ca-channel activity evoked by voltage pulses to -10 mV from a holding potential of -80 mV. (**B**) N-type Ca-channel activity mostly inactivated by changing the holding potential to -40 mV. (**C**) In the same patch recording stronger depolarizing pulses to $+20$ mV recruits L-type Ca-channel activity from a holding potential of -40 mV. (**D-F**) N-type and L-type Ca-channel activity in a cell-attached patch exposed to 100 μM NE present in the recording pipette. (**D**) Openings of N-type Ca channels are briefer and less frequent in the presence of NE compared to control (**A**). (**F**) The kinetics of L-type Ca channels are not obviously affected by the presence of NE.

polarizing pulses to +20 mV from a holding potential of −40 MV (F) is not significantly different from control patches (C). FIGURE 11A-D shows collected data from 33 control patches and 30 patches exposed to 10-100 μM NE.[20] Currents carried by N-type channels are strongly inhibited at 30 μM (C) and 100 μM (D) NE, though inhibition of N-type channel activity is not complete even at these high concentrations. In contrast, L-type Ca-channel activity is not significantly inhibited at any NE concentration over the range between 10-100 μM.[20]

To test whether signal transduction involves production of a readily diffusible second messenger such as cyclic AMP, cyclic GMP, or diacylglycerol, we can expose

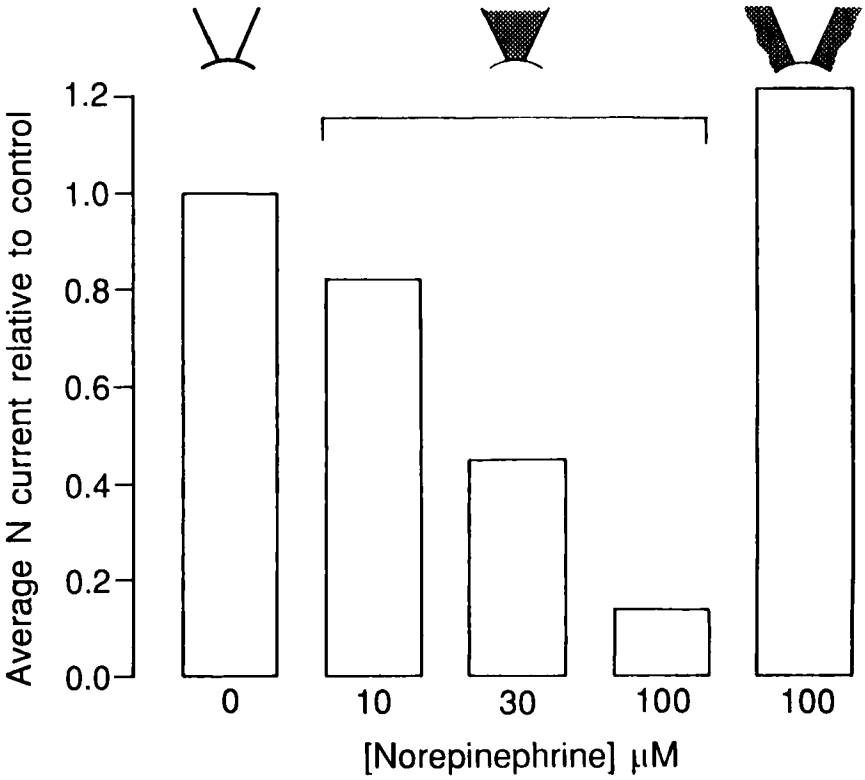

FIGURE 11. NE-mediated inhibition of N-type Ca-channel current is dose-dependent and not mediated by a readily diffusable cytosolic messenger. Averaged N-type Ca-channel currents from groups of cell-attached patches are normalized relative to currents in control patches (1.0) and plotted as bars. N-type Ca-channel activity was evoked by depolarizing pulses to −10 mV from a holding potential of −80 mV. Channel activity was identified as N-type because of its unitary conductance and sensitivity to holding potential as illustrated in FIG. 2. Illustrations at the top of the bar graph indicate control recordings with no drug (left), with 10, 30 and 100 μM NE in the recording pipette (middle), or with 100 μM NE applied to the bulk of the cell outside of the pipette (right). Mean currents from n individual patches were weighted equally to produce the average currents for each experimental condition: 0 NE inside the pipette (n = 22), 10 μM NE (n = 15), 30 μM NE (n = 10), and 100 μM NE (n = 5). The rightmost bar shows the average N-type Ca-channel activity after addition of 100 μM NE to the outside of cells (n = 5), normalized by activity recorded from the same patches before addition of NE.

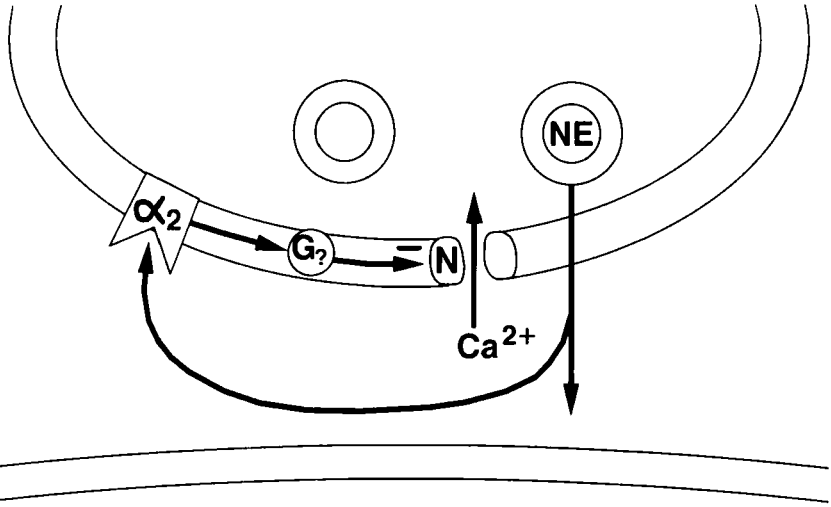

FIGURE 12. Scheme for NE-mediated autoinhibition. Depolarization causes opening of Ca channels leading to Ca entry. The Ca influx through N-type Ca channels is dominant in triggering release of vesicular sympathetic transmitter (NE) by exocytosis. Released or circulating NE can bind to an α_2 receptor to activate a G-protein leading to inhibition of N-channel activity, Ca entry and release. This scheme allows for discrete, localized regulation of Ca influx and release.

the bulk of the cell to NE and look for changes in the activity of N-type channels effectively isolated from the bathing solution by the patch pipette. In five patches, formed with pipettes of ~ 1 μm diameter, the mean current through N-type channels remains unchanged following application of 100 μM NE to the rest of the cell (FIG. 11E and F). In none of the experiments is there a detectable decrease in activity after the drug addition.[20] Thus, the inhibitory action of NE on Ca channels is not likely to be mediated by a second messenger that is free to diffuse from receptors outside the patch to channels within the patch.

Kinetic Basis of Downmodulation

NE inhibits N-type Ca-channel activity by altering its gating kinetics while having no significant effect on the unitary current amplitude (FIG. 10). The mean open time ($<t_o>$) of the N-type Ca channel decreases from 0.87 $\pm$ 0.14 msec in control to 0.38 $\pm$ 0.07 msec or 0.40 $\pm$ 0.09 msec in 30 or 100 μM NE, respectively.[20] The more than twofold abbreviation of N-type channel openings contributes substantially to the more than threefold decrease in average current ($<I>$) seen overall.

Changes in the kinetic steps leading to channel opening are also evident. The latency between the depolarizing step and the first detectable channel opening is roughly twice as large in the presence of 10-100 μM NE (21 msec) relative to the control (12 msec). Detailed kinetic interpretation of this measurement or of other closed time intervals is complicated by uncertainty about the total number of channels

in individual patches. Nevertheless, the results suggest a slowing in rate constants leading to channel opening. Changes in gating kinetics on a time scale slower than the duration of the depolarizing pulses are also suggested by a significant increase in the percentage of sweeps that contain no detectable opening (8% in control, 23% in 30-100 μM NE).

DISCUSSION

In this paper we discuss information about properties of N- and L-type Ca channels, their contribution to whole-cell Ca current, and their relative importance in triggering sympathetic transmitter release. We present evidence that N-type channels are selectively inhibited by NE and describe the mechanism of inhibition at the level of single channels. Down-modulation of N-type channel activity appears to play an important role in α-adrenergic control of transmitter release.

Two Major Types of High-Voltage-Activated Ca Channels

Single-channel recordings from frog sympathetic neurons provide very clear distinctions between N-type and L-type Ca channels. As we have illustrated, these channels differ markedly in their single-channel conductance, voltage dependence, and kinetics.[13,19] Thus, it is possible with appropriate voltage protocols to study each of these channel types in isolation even if both are present in the same patch (e.g. FIG. 10). The rate of inactivation of the N-type Ca channel may vary widely during depolarizing pulses that activate the channel. Although this variability is evident in earlier published records from sympathetic neurons,[19,22] it is particularly striking in the series of cell-attached patch recordings reported here. Variability of the rate of inactivation has also been documented for cardiac L-type Ca channels by Cavalie *et al.*[47] Other groups have described rapidly decaying or slowly decaying N-type channel behavior in rat sympathetic neurons and PC12 cells[24] and chick and frog DRG neurons.[7,23] Regardless of their rate of inactivation during depolarizing pulses, N-type Ca channels differ consistently from L-type channels in their steady-state inactivation with prolonged depolarizations (FIG. 1-3).

All of this analysis suggests that it is precarious and possibly incorrect to split whole-cell current into different decaying exponential components and to assign the components to particular types of Ca channels. Rapidly or slowly inactivating N-type channel activity could also contribute to tail currents with similar deactivation kinetics following short or long depolarizing pulses.[24,48]

Selective Inhibition of N-Type Channels

Stimulation of α-receptors by NE provides a selective means of inhibiting N-type but not L-type Ca-channel activity. The sustained and decaying forms of N-type Ca-channel activity are both strongly, although not completely, inhibited. The absence

of an α-adrenergic effect on L-type Ca channels was demonstrated with both whole-cell recordings (FIG. 9) and single-channel recordings from many cell-attached patches (FIGS. 10 and 11). Our results provide an explanation for the incompleteness of NE inhibition of Ca current in earlier voltage-clamp experiments.[36,41,44,45]

On the bases of whole-cell Ca-channel recordings, it seems likely that NE shares a common inhibitory mechanism of action with several neuroeffectors such as acetylcholine, LHRH, GABA, adenosine, and substance P (e.g., Refs. 10, 11, 17, 40, 49). Evidence for this hypothesis is based on (1) the similarity in the voltage and time-dependence of the transmitter-sensitive currents, (2) mutual occlusion of NE and LHRH effects in frog sympathetic neurons (K. R. Bley, unpublished observations), and (3) effects of internal GTP-γ-S, acting at low concentrations to render the inhibitory effects of the Ca current effectively irreversible[20] and at high concentrations to directly mimic neurotransmitter effects.[17,50,51]

Apart from involvement of G-proteins, the mechanism of noradrenergic modulation of Ca channels in sympathetic neurons appears to be fundamentally different from that in sensory neurons. Dunlap and colleagues[43,52,53] have demonstrated that PKC activation is required for NE-mediated downmodulation of Ca currents in chick DRG neurons. In contrast, the effect of NE on sympathetic neurons is not mimicked by agents known to stimulate protein kinase C, such as phorbol diacetate or phorbol dibutyrate (0.1-1 μM). In fact, acute application of phorbol esters consistently increases the activity of both N- and L-type Ca channels recorded in cell-attached patches by increasing their probability of opening.[54] Likewise, in earlier experiments in rat sympathetic neurons, Wanke *et al.*[40] found that ACh modulation via muscarinic receptors was not blocked by H-7 or mimicked by activators of PKC. Diversity in the signaling mechanisms linking transmitter-receptor activation and Ca-channel inhibition in different cell types have been highlighted by Wanke and Ferroni.[55] They showed, in parallel studies, that PKC activation did not mediate the effects of ACh in rat sympathetic neurons, while PKC stimulation is an important link in the signaling pathway in chick sensory neurons.[52,53]

Mechanism of NE Inhibition at the Single-Channel Level

Previously published studies of downmodulation of neuronal high-voltage-activated Ca currents by neurotransmitters have relied on whole-cell recordings. The experiments described here provide new information about the downmodulation at the single-channel level. NE produces a pronounced change in the rapid gating kinetics, which includes a marked abbreviation of N-type Ca-channel openings and an increase in the latency-to-first opening. The results clearly indicate that the mechanism of NE inhibition is not a simple elimination of channels or a mere prolongation of steps leading up to channel opening.

The signaling mechanism that links the α-receptor to the N-type Ca channel involves a GTP-binding protein. Further experiments are needed to determine whether the G-protein directly interacts with the N-type Ca channel or whether an intermediary molecule is involved. Our results indicate that the coupling between the α-adrenergic receptor and the N-type Ca channel does not involve the production of a readily diffusible second messenger (FIG. 11).

Mechanisms Controlling Transmitter Release

We have measured K^+-evoked transmitter release from cell bodies of frog sympathetic neurons. This system offers the possibility of directly measuring transmitter release and Ca currents in essentially the same preparation, an advantage over most preparations containing synaptic terminals. We have used this model system to determine which type(s) of voltage-gated Ca channel dominates transmitter release and which channel is the target of α-adrenergic mediated inhibition.

Effects of DHPs

Our findings suggest that the N-type Ca channel dominates transmitter release from frog sympathetic neurons, similar to previous findings in cultured rat superior cervical ganglion neurons.[22] [^{3}H]NE release is not affected by DHPs, agents known to act selectively on L-type channels. Insensitivity to DHPs is found even when the drug is applied during a predepolarization in the absence of external Ca; such conditions have been shown to favor DHP binding.[56,57] In contrast, Cd and ω-conotoxin, agents known to block N-type Ca channels, are effective inhibitors of K^+-evoked transmitter release.

Effect of α-Adrenergic Stimulation

Additional support for the importance of N-type Ca channels in regulating release comes from experiments studying effects of α-adrenergic stimulation with exogenous NE. NE inhibition of N-type Ca-channel activity is accompanied by a consistent and sizeable reduction of K^+-evoked transmitter release. Inhibition of Ca current and transmitter release are closely correlated in several respects. In both cases (1) the IC_{50} for NE inhibition is ~ 3 μM with 1-2 mM external Ca or Ba[20]; (2) NE block is incomplete, even at maximally effective concentrations; (3) the pharmacology is that of a subtype of α_2-like receptor that is blocked by phentolamine and yohimbine and is not stimulated by clonidine (similar to α_2-like receptors in other neuronal preparations[35,36]). Because α-stimulation fails to significantly reduce transmitter release evoked by ionomycin-induced Ca entry, we conclude that inhibition of Ca entry, particularly through N-type channels, is responsible for the reduction in K^+-evoked transmitter release.

Thus, experiments with DHPs and α-adrenergic stimulation provide complementary approaches to identifying the Ca entry system(s) that dominate transmitter release. On one hand, selective enhancement or reduction of Ca influx via L-type Ca channels by DHPs has little or no effect on transmitter release; on the other hand, selective reduction of Ca influx via N-type Ca channels by α-adrenergic agents strongly decreases release.

Because L-type channels have been shown to coexist with N-type channels on the cell body of sympathetic neurons,[19] these results reinforce the idea that L-type channels are somehow at a disadvantage in triggering catecholamine release. The reasons for this are not obvious because DHP-sensitive L-type Ca channels make a substantial

contribution to the macroscopic whole-cell current and to Ca transients detected with fura-2.[22] Global measurements of intracellular calcium transients and calcium-channel currents do not necessarily predict which Ca delivery systems are important for evoking transmitter release. In this respect it is notable that caffeine induces sizeable intracellular Ca transients[58] but is completely ineffective in evoking sympathetic transmitter release (SK, unpublished).

We do not know yet why Ca entry through N-type Ca channels is so effective in producing transmitter release. One possibility is that the transmitter release mechanism has fast on and off kinetics and a low affinity for Ca so that it fails to respond to global Ca transients in the micromolar range. Such release mechanisms might be anchored near the mouths of N-type channels where the local concentration of Ca ions might transiently achieve much higher levels. Similar ideas have been invoked to explain the speed of transmitter release at presynaptic terminals.[59–61]

Mechanism(s) of Autoinhibition

Our results support the following mechanism for noradrenergic inhibition of K^+-evoked transmitter release:

NE → α_2-like receptor → $G_?$ → ↓N-type Ca channel activity → ↓release

However, it remains possible that the physiological effect of NE on transmitter released by action potentials involves other mechanisms. For example, one hypothesis might be as follows:

NE → α_2 receptor → G_i → ↓cAMP → ↓phosphorylation → ↑K channel activity → ↓release

This scenario is based on evidence in other neurons where K conductances are increased by stimulation of a clonidine-sensitive α_2-receptor (e.g. Williams *et al.*[62]) and inhibited by increases in cyclic AMP.[63] A mechanism based on potassium-channel modulation would modify release through changes in action potential duration. This is an electrical effect that would spread along axonal varicosities over distances of the order of the electrical space constant. In contrast, α-adrenergic modulation of N-type Ca channels might provide short-range feedback control: signaling from receptor to channel and from Ca entry to transmitter release would be highly localized.

CONCLUSIONS

Frog sympathetic neurons allow a relatively direct comparison between electrophysiological measurements of Ca-channel properties and radiotracer measurements of transmitter release. Selective α-adrenergic inhibition of N-type Ca-channel activity is seen in both whole-cell and single-channel recordings, and provides an attractive explanation for autoinhibition of sympathetic transmitter release, especially because transmitter release is thought to be triggered selectively by Ca influx through N-type Ca channels. The downmodulation of N-type Ca channels is mediated by a G-protein

and is dominated by changes in rapid gating kinetics on a millisecond time scale. Overall, our experiments support the idea that N- and L-type channels are distinct entities, differing in their importance for catecholamine release and their responsiveness to neurotransmitters.

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