α-Adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating

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IN sympathetic neurons, catecholamines interact with prejunctional α-adrenergic receptors to reduce delivery of transmitter to postjunctional target organs[1–4]. This autoinhibitory feedback is a general phenomenon seen in diverse neurons containing a variety of transmitters[2–4]. The underlying mechanisms of α-adrenergic inhibition are not clear, although decreases in cyclic AMP and cAMP-mediated phosphorylation have been implicated[1–4] (compare ref. 5). We have studied depolarization-induced catecholamine release and calcium-channel currents in frog sympathetic neurons. Here we show that α-adrenergic inhibition of transmitter release can be explained by inhibition of Ca^{2+}-channel currents and not by modulation of intracellular proteins. Noradrenaline strongly reduces the activity of N-type Ca^{2+} channels, the dominant calcium entry pathway triggering sympathetic transmitter release[6], whereas L-type Ca^{2+} channels are not significantly inhibited. The down-modulation of N-type channels involves changes in rapid gating kinetics but not in unitary flux. This is the first detailed description of inhibition of a high-voltage activated neuronal Ca^{2+} channel at the single-channel level. The coupling between α-adrenergic receptors and N-type channels involves a G protein, but not a readily diffusible cytoplasmic messenger or protein kinase C, and may be well suited for rapid and spatially localized feedback-control of transmitter release.

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Figure 1 shows the inhibitory effects of noradrenaline (NA) on transmitter release in isolated frog sympathetic ganglia[7], to facilitate comparisons with measurements of ionic currents in cell bodies (Fig. 2). Noradrenaline strongly and reversibly reduced transmitter outflow evoked by exposure to K^+ (Fig. 1*a*). The inhibition reached a maximum of ~50% at 10–100 μM NA (Fig. 1*b*). Inhibition was unaffected by the β-adrenergic blocker, propranolol, but was prevented by phentolamine (10 μM), a general α-adrenergic antagonist, or by yohimbine (10 μM), an α_2-adrenergic antagonist (Fig. 1*b*). Application of the α_2-adrenergic agonist clonidine (at concentrations ≤100 μM) had no effect. These pharmacological properties are characteristic of a distinct subtype of α_2-adrenergic receptor described in other neurons[8,9]. It has been suggested[1–4] that NA autoinhibition might involve altered phosphorylation of Ca^{2+} channels or of intracellular proteins, such as synapsin I (ref. 10), by cyclic AMP- or Ca^{2+}-dependent protein kinases. We found, however, that the inhibition by 30 μM NA remained unchanged in 1 mM dibutyryl cAMP, or in H-7 (a protein kinase blocker) at a concentration (300 μM) that inhibits several protein kinases[11] (Fig. 1*b*). Transmitter release induced by adding Ca^{2+} in the presence of the Ca^{2+} ionophore ionomycin, bypassing calcium entry through voltage-gated Ca^{2+} channels, was not affected by NA (Fig. 1*b*; ref. 12). This indicates that noradrenergic inhibition does not depend on mechanisms subsequent to a rise in cytosolic Ca^{2+} but is likely to involve modulation of Ca^{2+} entry.

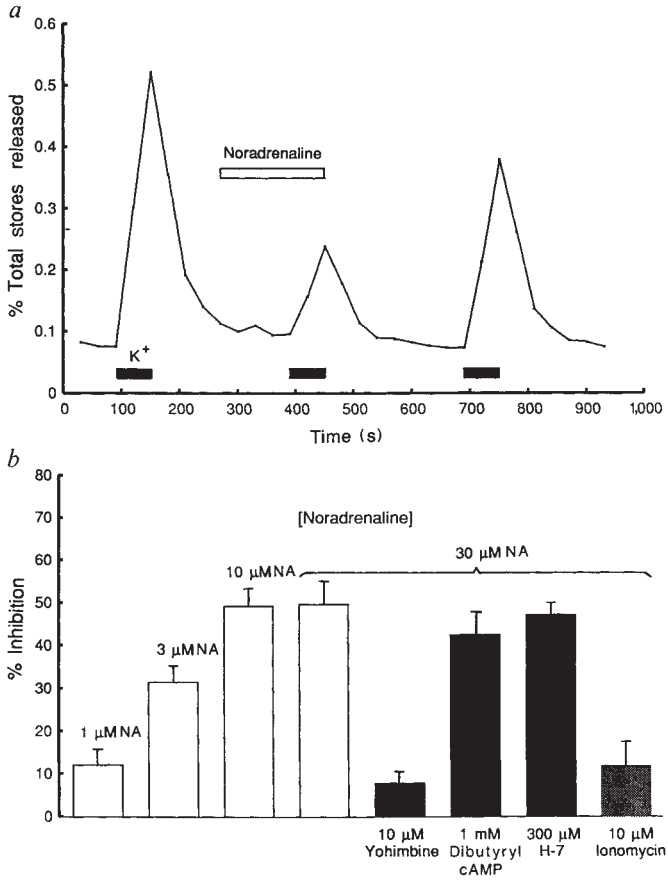

FIG. 1 *a*, Effect of 30 μM NA (open bar) on radiolabelled transmitter release evoked by exposure to 50 mM K^+ (solid bars). *b*, Characteristics of NA effect. Bars represent means ± s.e.m. of percentage of NA-induced inhibition in 3–8 experiments. Open bars, dose-dependence of NA-induced inhibition; solid bars, effect of 30 μM NA in the presence of the indicated agents. Dibutyryl cAMP or H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) did not significantly affect NA inhibition ($P > 0.05$). Grey bar, NA did not significantly affect transmitter release evoked by Ca^{2+} in ionomycin ($P > 0.05$). METHODS. Sympathetic chains (3–4 ganglia) from frogs (*Rana pipiens pipiens*) were incubated for 2 h at 22 °C in Ringer's solution containing (mM): NaCl (128), KCl (2), glucose (10), HEPES (10) (*p*H 7.3 with NaOH); also added were ascorbic acid (antioxidant), (1.0 mm) pargyline (monoamine oxidase inhibitor) (0.1 mm), and [^{3}H]-NA (10 μCi ml^{-1} ≈ 0.2 μM) (New England Nuclear). After [^{3}H]-NA loading, the ganglia were enclosed in a chamber and perfused at 1.6 ml min^{-1} with Ringer's solution containing 10 μM desipramine (reuptake blocker), 10 μM propranolol and 2 mM $CaCl_2$. Once a stable baseline of ^{3}H-release was achieved (30–40 min), 30-s fractions were collected continuously. Sympathetic transmitter release (probably a mixture of noradrenaline and adrenaline[36], from neuronal somata[7,27]) was induced by perfusing ganglia with Ringer's solution containing 50 mM K^+ to directly depolarize the cells, bypassing possible changes in action-potential propagation or duration. Release showed dependence on K^+ (30–80 mM) and extracellular calcium (effector concentration for half-maximum response ≈0.5 mM), and complete inhibition by cadmium (50% inhibitory concentration ≈10 μM) as expected for exocytosis triggered by voltage-gated Ca^{2+} influx. The amount of release was calculated as the area (*A*) under each peak after baseline subtraction. The release in the presence of NA (A_{NA}) was expressed as a percentage of the average of release evoked before (A_{con}) and after washing out NA (A_{wash}). Thus, percentage inhibition = $[1-(2A_{NA}/A_{con}+A_{wash})]\times 100$. For ionomycin-induced release, 10 μM ionomycin was added to the perfusion solution (external free calcium concentration buffered at 0.1 μM with 2 mM EGTA) 10 min before collecting fractions. Release was evoked by exposure to 1 mM free calcium. Peak ionomycin-induced release (0.2–0.4%) was similar to that evoked by 50 mM K^+.

Noradenaline inhibits Ca^{2+} entry, as measured by whole-cell Ca^{2+} currents from isolated frog sympathetic neurons (Fig. 2), as it does in other neurons[13–19]. In parallel with the release studies, the inhibition of Ca^{2+} current by NA was antagonized by yohimbine (Fig. 2*b*) and phentolamine, but not by propranolol. Likewise, clonidine (at concentrations up to 100 μM) had no effect. The inhibition was maximal with 10 μM NA (Fig. 2*c*; refs 9, 15). Replacement of GTP in the recording-pipette solution with a non-hydrolysable analogue, GTP-γ-S (0.05 mM), largely prevented recovery from the NA-induced inhibition (Fig. 2*d*); internal GTP-γ-S (0.5 mm) alone mimicked the inhibitory effect of NA (results not shown). Both results suggest involvement of G proteins, as in other neuronal systems[16,20–22].

Sympathetic neurons have two types of high-voltage activated (HVA) Ca^{2+} channels, N-type and L-type, which differ in their voltage and time dependence of inactivation and in their single-channel conductance[6,23–25]. The NA-sensitive current showed partial, but not complete, decay over hundreds of milliseconds[6,25–27] and a strong dependence on holding potential (Fig. 2*e–g*), all consistent with inhibition of N-type Ca^{2+} channels.

Unitary current recordings provide the most direct approach to identifying the NA-sensitive Ca^{2+} channel and characterizing the modulatory effect. Although many neurotransmitters inhibit HVA Ca^{2+} currents[8,9,14–22,24,26], little is known about the mechanism of inhibition at the single-channel level (see ref. 28). Figure 3*a–c* shows N-type and L-type Ca^{2+} channels studied in isolation in the same patch-recording using appropriate voltage protocols[6,23,24]. The N-type channel current illustrated in Fig. 3*a* is sustained during the depolarizing pulse, but in other recordings it often shows a more pronounced time-dependent inactivation[6,24–27]. In all cases, however, the N-type channel current inactivates following prolonged (10–20 s) depolarizations of the holding potential[24,27]. To assess the effect of NA, a series of cell-attached patch recordings were obtained with or without NA (10–100 μM) in the recording pipette. The average N-type Ca^{2+} channel currents were reduced from 0.24 ± 0.09 pA ($n = 22$) to 0.09 ± 0.05 pA ($n = 10$) in 30 μM NA, and to 0.03 ± 0.02 pA ($n = 5$) in 100 μM NA (Fig. 3*f–h*). The L-type Ca^{2+} current was not significantly changed ($P > 0.05$), although there was some hint of inhibition at 100 μM NA. Noradrenaline did not significantly change unitary current amplitude at any concentration tested (legend to Fig. 4). By contrast, there was a marked change in rapid gating kinetics. The mean open time decreased from 0.87 ± 0.14 ms (control; $n = 23$) to 0.38 ± 0.07 ms in 30 μM NA ($n = 7$), or 0.40 ± 0.09 ms in 100 μM NA ($n = 5$). This more than two-fold abbreviation of N-type channel openings contributes substantially to the large decrease in average current seen overall (Fig. 3*f–h*). Changes in gating kinetics on a time scale slower than the pulse duration are suggested by an increase in the percentage of blank sweeps from 8% in the control to 24% in 30–100 μM NA.

The coupling between the α-adrenoceptor and N-type channel inhibition does not involve a readily diffusible second messenger, as application of 100 μM NA to the bath had no effect on N-type channels under the patch pipette (Fig. 3*i, j*; see also ref. 17). In particular, the messenger is unlikely to be cAMP, because dibutyryl cAMP had no effect on N-type channels, whereas it did increase the activity of L-type channels. Protein kinase C is also unlikely to be involved, because phorbol esters increase N-type and L-type Ca^{2+}-channel activity in these cells[29].

Our results demonstrate that NA acts through α-adrenoceptors to selectively inhibit the activity of N-type Ca^{2+} channels, and link this inhibition to attenuation of sympathetic transmitter release. Noradrenaline reduces the probability of channel opening by accelerating the rate of channel closing, and possibly slowing the kinetics of opening, without changing unitary channel flux. Because the mechanism of inhibitory modulation of unitary HVA neuronal Ca^{2+}-channel currents has not previously

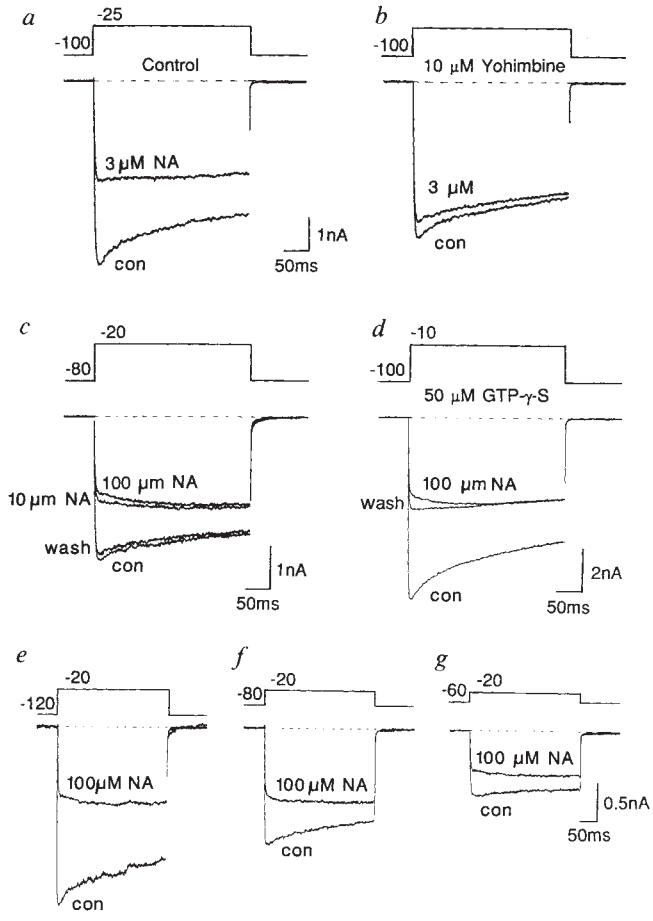

FIG. 2 Noradrenaline inhibition of whole-cell Ca^{2+}-channel currents in sympathetic neurons. *a, b*, Effect of 3 μM NA in the absence and presence of 10 μM yohimbine. Yohimbine block was reversible (data not shown). *c*, Incomplete inhibition of Ca^{2+}-channel currents at maximally effective NA concentrations. Four records were taken: before (con) and after exposure to 10 μM NA, ~30 s after NA removal (wash), and after exposure to 100 μM NA; 10 μM and 100 μM NA were equally effective. Note reversibility of NA effect with standard internal solution (300 μM GTP). *d*, With 50 μM GTP-γ-S instead of internal GTP, the inhibitory effects of NA were irreversible over 9 min of washing in drug-free solution. *e–g*, Recordings of Ca^{2+}-channel currents evoked by step depolarizations to −20 mV from different holding potentials in the absence and presence of NA (100 μM). Same cell as in *c*, 2 mM external Ba^{2+}. The dependence of the NA effect on holding potential is consistent with inhibition of N-type Ca^{2+}-channel current. Under these recording conditions, L-type channels contribute a relatively sustained current, whereas N-type channels show a greater but not complete degree of inactivation with depolarization[6,25–27]. In other experiments with 2–10 mM external Ca^{2+}, NA had little or no effect on sustained currents (presumably L-type channel current) evoked by depolarization from −40 or −30 mV (ref. 37).

METHODS. Neurons were isolated from sympathetic ganglia of adult frogs by a combination of enzymatic and mechanical dissociation (see, for example, ref. 24). Currents carried by voltage-gated Ca^{2+} channels were recorded with the whole-cell patch-clamp method. To minimize Ca^{2+}-dependent inactivation and to block residual outward K^+ currents, Ba^{2+} was usually used as the permeant divalent cation but similar results have been obtained with 2–10 mM Ca^{2+}. The standard internal (pipette) solution contained (mM): CsCl (100), EGTA (10), Na-ATP (2), MgCl (5), GTP (0.3), HEPES (40) (*p*H 7.2 with CsOH). Recording pipettes had resistances <1 MΩ. The external bathing solution contained (mM): tetraethylammonium (TEA) (130), CsCl (5), Glucose (10), $BaCl_2$ (2), tetrodotoxin (TTX) (1 μM), propranolol (10 μM) and HEPES (5, *p*H 7.4 with TEA-OH). Cells were continually superfused at 1–2 ml min^{-1} with NA-free or NA-containing solutions. A computer (PDP 11/23) controlled the command voltage, and digitized and stored the filtered current signals (−3 dB at 1 kHz). Step depolarizations lasting 320 ms were applied every 10–12 s. All current records were leak-subtracted and are shown together with the voltage protocol. All experiments were carried out at room temperature (~22 °C).

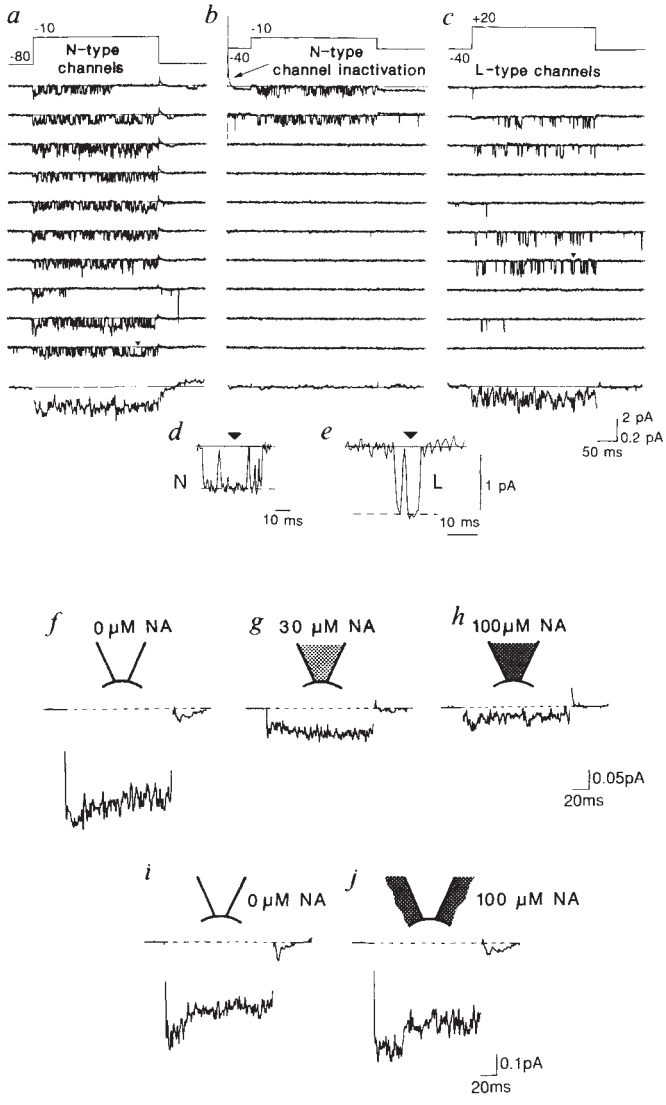

FIG. 3 *a–e*, Separation of unitary N-type and L-type Ca^{2+}-channel currents in the same cell-attached patch. *a*, Sequential recordings of N-type Ca^{2+}-channel currents evoked every 10 s by 320 ms pulses from −80 mV to −10 mV. *b*, Sequential current recordings showing N-type Ca^{2+} channels inactivating within 10–20 seconds following a displacement of the holding potential from −80 to −40 mV (arrow marks corresponding capacitive transient). *c*, Sequential recordings of L-type Ca^{2+}-channel currents evoked by pulses from −40 mV to +20 mV. Average currents, each calculated from ~30 sweeps, are shown below individual current records. *d*, *e* Openings of N- and L-type Ca^{2+} channels (arrow heads in *a*, *c*) enlarged to show clearly resolved and different unitary amplitudes. As the unitary L-type currents at +20 mV (*e*) are larger in amplitude than unitary N-type currents at −10 mV (*d*), despite the smaller driving force for Ba^{2+} entry, they must represent different Ba^{2+} conductances (N-type, 15–16 pS; L-type, 26–28 pS; refs 24, 27). *f–h*, Mean currents from separate groups of cell-attached patches, showing inhibition of N-type Ca^{2+}-channel current evoked by 130 ms pulses from −80 mV to −10 mV. Channel activity was identified as N-type by unitary conductance and sensitivity to holding potential as in *a*, *b*. Averages weighted individual patches equally. *f*, Control ($n=22$ patches); *g*, *h*, 30 μM NA ($n=10$) or 100 μM NA ($n=5$) in the patch pipette. Noradenaline (10 μM) reduced N-type channel currents only slightly; a difference in NA-sensitivity between recordings with 110 mM external Ba^{2+} (*f–h*) and 2 mM Ba^{2+} (Fig. 2) might be expected from alterations in surface potential and local catecholamine concentration. *i*, *j*, Evidence against involvement of a readily diffusible messenger. In five cell-attached patches (pipettes ~1 μm in diameter), the mean current through N-type channels remained unchanged following application of 100 μM NA to the rest of the cell. In none of the experiments was there a detectable decrease in activity after the drug addition.

METHODS. Unitary currents recorded in a series of cell-attached patches with and without NA present in the patch pipette. Attempts at perfusing the pipette with drug while continuously monitoring channel activity were hampered by instability of the recordings, and outside-out patches gave inconsistent results. Recording pipettes had resistances of 5–10 MΩ and contained (mM): $BaCl_2$ (110), HEPES (10), (*p*H adjusted to 7.4 with TEA-OH) and TTX (1 μM). The external bathing solution, used to set the membrane potential to zero, contained (mM): K-aspartate (140), Glucose (10), HEPES (5), EGTA (10) (*p*H adjusted to 7.4 with KOH). A computer (PDP 11/23) controlled the command voltage; and digitized and stored the filtered currents (−3 dB at 1 kHz). Individual current recordings were leak-subtracted before determination of average currents.

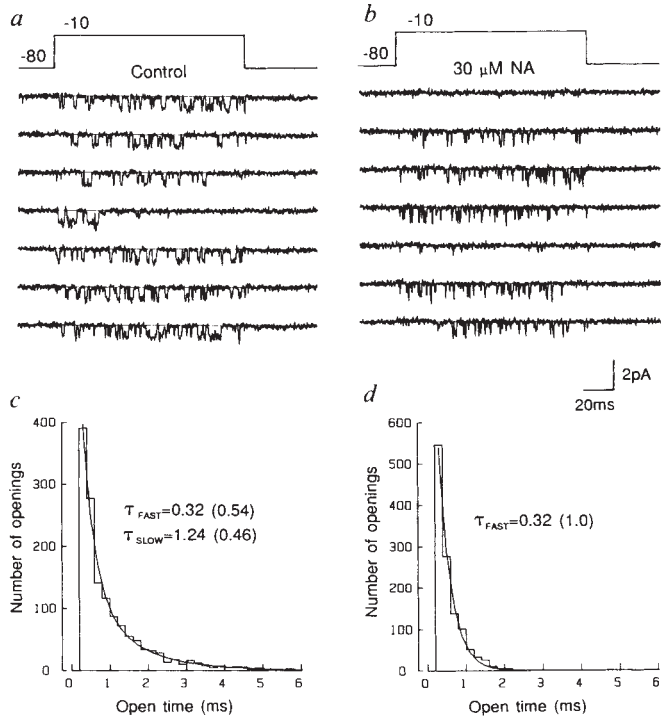

FIG. 4 Effect of NA on the gating of unitary N-type Ca^{2+} channels. *a*, *b*, Sequential sweeps of N-type Ca^{2+} channel currents recorded in two cell-attached patches, in the absence of NA (*a*) and with 30 μM NA present in the pipette solution. (*b*). Noradenaline altered gating kinetics, but did not change unitary current size: control, 0.92 ± 0.02 pA ($n=22$); 30 μM NA, 0.95 ± 0.05 pA ($n=8$); 100 μM NA, 1.0 ± 0.02 pA ($n=5$). *c*, *d*, Histograms of open time durations measured from the patches illustrated in *a*, *b*. Opening and closing events were detected as crossings of a threshold set halfway between open and closed levels. The distribution of open times in the absence of NA (*c*) was fitted by least-squares with the sum of two exponential components of the form $B \exp(-t/\tau)$ with time constants and relative area $[B \cdot \tau / \Sigma(B \cdot \tau)]$ as indicated. In the presence of 30 μM NA, the slow component was essentially absent (*d*), consistent with the diminished appearance of long openings in the current records (*b*). In other experiments, single channel analysis was performed on records with little or no overlap of unitary currents, like those illustrated in *a*, *b*. The slow component was too small to measure in 6 our of 12 patches with 30–100 μM NA. In the other 6 patches, a slow component remained detectable, but it decayed more rapidly and was smaller in amplitude and relative area.

been reported, it will be interesting to see if this pattern of modulation of rapid gating kinetics holds true for other neurotransmitters and other cells[24,26]. Changes in the kinetics of opening and closing might be consistent with allowed voltage-dependence of gating reported by Bean[19].

The pharmacological properties of NA inhibition of whole-cell N-type channel current and transmitter release are very similar. This points to a functional relationship between these phenomena and reinforces earlier evidence that dihydropyridine-insensitive N-type Ca^{2+} channels are the main Ca^{2+} entry mechanism controlling transmitter release from sympathetic neurons[6]. Interestingly, the triggering of transmitter release may be dominated by L-type channels in certain other neuronal systems[30–32]. Stimulation of α-adrenergic receptors is coupled to inhibition of N-type Ca^{2+}-channels and reduction of transmitter release by means of a G protein but not by a readily diffusible second messenger such as cAMP (as in current hypotheses[2–4]), nor by protein kinase C (as in sensory neurons[33]). A relatively direct coupling mechanism would be appropriate for rapid feedback control of transmitter release. The feedback may also be localized, because (1) the concentration of NA falls off steeply with increasing distance from the release sites, (2) the α-adrenergic modulation of N-type Ca^{2+} channels works only at short range, and (3) the attenuation of Ca^{2+} entry may strongly affect only nearby release sites. Our results do not exclude additional effects of NA on potassium channels[34,35], possibly mediated by clonidine-sensitive α_2-receptors[34] and lowered cAMP[35], that would result in less localized decreases in transmitter release through reduction of action-potential duration and global Ca^{2+} entry. □

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A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning

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VOLTAGE-activated potassium channels play an important part in the control of excitability in nerve and muscle. Different K^+ channels are involved in establishing the resting potential, determining the duration of action potentials, modulation of transmitter release, and in rhythmic firing patterns and delayed excitation[1]. Using *in vitro* transcripts made from a directional complementary DNA library we have isolated, by expression cloning in *Xenopus* oocytes, a novel K^+-channel gene (*drk1*). Functionally, *drk1* encodes channels that are K^+ selective and belong to the delayed rectifier class of channels, rather than the A-type class encoded by the *Shaker* gene of *Drosophila*. The channels show sigmoidal voltage-dependent activation and do not inactivate within 500 ms. Structurally, *drk1* encodes an amino-acid sequence which is more closely related to the *Drosophila Shab* gene than to the *Shaker* gene.

Several cDNA clones encoding K^+ channels have been isolated from *Drosophila*[2–8]. Microinjection of *Xenopus* oocytes, with RNA transcribed *in vitro* from some of the clones produces voltage-dependent, fast transient outward K^+ currents, characteristic of A-type channels[9–11]. The different cDNA clones that have been isolated contain an identical core region, yet differ in the regions encoding the amino and carboxyl termini of the proteins. Using *Shaker* sequence information, two mammalian K^+-channel cDNA clones encoding the same gene were recently isolated by cross-hybridization[12,13]. Expression of rat cDNAs in *Xenopus* oocytes yielded K^+ currents with properties of delayed rectifier channels[14] and possibly A-type channels[15]. We designed a sequence-independent approach to isolate cDNA clones encoding channel and receptor genes expressed in the brain. Size-fractionated rat-brain messenger RNA, enriched for mRNA between 3.3 and 4.2 kilobases (kb), was used to generate a directional cDNA library in the transcription-competent vector λZAP[16]. Pools of 100,000 recombinant phages (independent cloning events) were amplified and used to prepare DNA templates for RNA synthesis. Following microinjection of *in vitro* synthesized RNA, *Xenopus* oocytes were tested for outward currents (I_K) produced by depolarizing voltage steps. Transcripts from pools of 100,000 recombinants yielded small I_K-like currents. One pool was divided into smaller 'cocktails', each containing 10,000 recombinants. Three of seven such cocktails elicited sustained outward currents of several hundred nA in amplitude upon depolarization of the oocyte membrane. The currents activated relatively slowly (>100 ms for full activation) and did not inactivate during the test pulse (500 ms). One cocktail was chosen and, by reducing the pool size to 1,000, then 100 and finally 12 recombinants, we eventually isolated a single 'positive' clone (*drk1*) with a 3.4-kb insert.

The cDNA clone we isolated encoded a K^+ channel with the properties of a delayed rectifier[1]. The channels opened at test potentials more positive than −20 mV and showed sigmoidal voltage-dependent activation (Fig. 1*a*, *b*). The time to half-maximal activation ranged from 20 to 100 ms. Injection of as little as 20 pg *in vitro*-synthesized transcripts produced I_K-current amplitudes of up to 1 μA at +40 mV. At higher current