An R-Type Ca^{2+} Current in Neurohypophysial Terminals Preferentially Regulates Oxytocin Secretion

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Multiple types of voltage-dependent Ca^{2+} channels are involved in the regulation of neurotransmitter release (Tsien et al., 1991; Dunlap et al., 1995). In the nerve terminals of the neurohypophysis, the roles of L-, N-, and P/Q-type Ca^{2+} channels in neuropeptide release have been identified previously (Wang et al., 1997a). Although the L- and N-type Ca^{2+} currents play equivalent roles in both vasopressin and oxytocin release, the P/Q-type Ca^{2+} current only regulates vasopressin release. An oxytocin-release and Ca^{2+} current component is resistant to the L-, N-, and P/Q-type Ca^{2+} channel blockers but is inhibited by Ni^{2+}. A new polypeptide toxin, SNX-482, which is a specific α_{1E}-type Ca^{2+} channel blocker (Newcomb et al., 1998), was used to characterize the biophysical properties of this resistant Ca^{2+} current component and its role in neuropeptide release. This resistant component was dose dependently inhibited by SNX-482, with an IC_{50} of 4.1 nM. Furthermore, SNX-482 did not affect the other Ca^{2+} current types in these CNS terminals. Like the N- and P/Q-type Ca^{2+} currents, this SNX-482-sensitive transient Ca^{2+} current is high-threshold activated and shows moderate steady-state inactivation. At the same concentrations, SNX-482 blocked the component of oxytocin, but not of vasopressin, release that was resistant to the other channel blockers, indicating a preferential role for this type of Ca^{2+} current in oxytocin release from neurohypophysial terminals. Our results suggest that an α_{1E} or "R"-type Ca^{2+} channel exists in oxytocinergic nerve terminals and, thus, functions in controlling only oxytocin release from the rat neurohypophysis.

Key words: class E (α_{1E}) Ca^{2+} channel; secretion; SNX-482; vasopressin; posterior pituitary; oxytocin

Voltage-dependent channels are responsible for the Ca^{2+} that enters nerve terminals and elicits vesicular release of neurotransmitters (Augustine et al., 1987). Neurotransmitter release in the CNS is regulated by multiple types of Ca^{2+} channels (Dunlap et al., 1995). A number of studies have defined several electrophysiologically distinct Ca^{2+} channels on neuronal cell bodies: L-, N-, T-, and P-types (Fox et al., 1987; Bean, 1989; Tsien et al., 1991; Llinas et al., 1992). Other classes of channels, such as the Q- and R-types, have been revealed by molecular cloning (Snutch and Reiner, 1992; Ellinor et al., 1993; Sather et al., 1993; Perez-Reyes et al., 1998) and the use of Ca^{2+} antagonists (Olivera et al., 1984; Hillyard et al., 1992; Ramachandran et al., 1993; Newcomb et al., 1998). The specific role at CNS terminals of these different types of Ca^{2+} channels, however, is still unclear.

The N-type channel seems to be involved in classical neurotransmission (Hirning et al., 1988), whereas the L-type is known to regulate the secretion of certain peptides (Cazalis et al., 1987; Dunlap et al., 1995). The class E (α_{1E}) and G (α_{1G}) Ca^{2+} channels have been localized recently to the CNS (Westenbroek et al., 1995; Perez-Reyes et al., 1998). However, the phenotype of the expressed α_{1E} channel is controversial (Snutch and Reiner, 1992; Randall and Tsien, 1995), and the biophysical properties of the class E current in CNS terminals remain to be determined.

To determine any role for class E channels in CNS secretion (see Wu et al., 1998, 1999), we studied the nerve terminals of the rat neurohypophysis. This is a population of relatively homogeneous peptidergic nerve endings that allows comparative study by a number of different techniques. This has been a very useful model system for characterization of nerve terminal Ca^{2+} channels (Lemos and Nowycky, 1989; Wang et al., 1992, 1997a; Wang and Lemos, 1994; Fisher and Bourque, 1995) and of mechanisms underlying depolarization–secretion coupling (Cazalis et al., 1987; Lim et al., 1990; Lindau et al., 1992; Wang et al., 1993b, 1997a; Branchaw et al., 1998). We have shown previously that "L"- and "N"-type Ca^{2+} channels exist in nerve terminals of the neurohypophysis (Lemos and Nowycky, 1989; Wang et al., 1992) and that they control both vasopressin (AVP) and oxytocin (OT) release, except for a significant resistant component (Cazalis et al., 1987; Dayanithi et al., 1988; Wang et al., 1993b). More recently we have shown that a "Q"-type Ca^{2+} current component also exists in approximately one-half of these CNS terminals (Wang et al., 1997a). Moreover, when blockers of the Q-type Ca^{2+} current were added to the terminals, the resistant component of AVP release was essentially abolished. In contrast, a similar resistant component of OT release was unchanged by the same concentrations of the Q-type channel blockers.

Most recently we have shown that purified native SNX-482, a specific α_{1E} channel blocker, could inhibit the neurohypophysial Ca^{2+} current (Newcomb et al., 1998). This led us to examine,

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using a combination of pharmacological and biophysical techniques, whether class E or R-type Ca^{2+} channels might also exist on these CNS terminals and functionally contribute to neurosecretion.

MATERIALS AND METHODS

Electrophysiological recordings. As we have described previously (Wang et al., 1997a), after sedation by CO_2 the rats were killed by decapitation using a guillotine. The neurohypophysis was then excised, following previous protocols, and homogenized in a solution containing (in mM): sucrose, 270; HEPES-Tris, 10; and K-EGTA, 0.01, pH 7.25 (Cazalis et al., 1987). All chemicals were obtained from Sigma (St. Louis, MO). The isolated neurohypophysial nerve terminals could be identified under an inverted microscope (Nordmann et al., 1987). Normal Locke's solution [containing (in mM), NaCl, 145; KCl, 5; $CaCl_2$, 2.2; $MgCl_2$, 1; Na-HEPES, 10; and glucose, 15, pH 7.35] was then used to perfuse the terminals. Before patch-clamp recordings, the terminals (usually 5–8 μm in diameter) were perfused with the 5 mM Ba^{2+} (replacing $CaCl_2$) Locke's solution, which also contained 1 μM TTX with 0.02% BSA. To obtain perforated-patch (Rae et al., 1991) recordings in the "whole-terminal" configuration (Hamill et al., 1981), freshly made amphotericin B (240–300 μg/ml) was mixed with the pipette solution that contained (in mM): Cs-glutamate, 135; HEPES, 10; glucose, 5; $CaCl_2$, 2; $MgCl_2$, 1; and TEA, 20, pH 7.25.

As reported previously (Wang et al., 1997a), the perforated-patch recording configuration enables us to overcome problems with the run-down of Ca^{2+} currents that complicated former studies (Lemos and Nowycky, 1989; Wang et al., 1992; Wang and Lemos, 1994; Fisher and Bourque, 1995; Branchaw et al., 1998). Only terminals with perforated-patch access resistances of <10 MΩ were chosen for further recordings. The Ba^{2+} current (I_{Ba}), which was activated by depolarizing from −80 to +10 mV and demonstrated both transient and long-lasting components (see, e.g., Fig. 1*A*), could be maintained for >1 hr without appreciable rundown. The I_{Ba} was filtered at 3 kHz and sampled at 10 kHz. pClamp (Axon Instruments, Burlingame, CA) was used for acquisition and analysis of data.

Peptide release. Rat neurohypophyses (see Electrophysiological recordings) were homogenized as described previously (Cazalis et al., 1987). The homogenate was centrifuged at 2400 × *g* for 6 min. The resulting pellet contains highly purified nerve terminals. The nerve endings were loaded onto filters (0.45 μm Acro disk; Gelman Sciences, Ann Arbor, MI) and perfused at 37°C with normal Locke's solution. Four minute fractions of perfusate were collected, and the evoked release was triggered by an 8-min-duration pulse of a depolarizing concentration (50 mM) of K^+. The results are given as AVP or OT release per fraction using specific radioimmunoassays (Wang et al., 1997a). The medium before and after the depolarizing period contained (in mM): NaCl, 40; $KHCO_3$, 5; *N*-methyl-D-glucamine (NMG)-Cl, 100; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; and Tris-HEPES, 10, with 0.02% BSA, pH 7.25. Depolarization medium contained 50 mM K^+, in which the NMG was reduced to maintain the osmolarity (300–310 mOsm).

Polypeptide toxins. The polypeptide toxins used in this study were synthetic versions prepared by Neurex Pharmaceutical Corporation (Ramachandran et al., 1993). These were termed SNX-482, the synthetic version of a novel 41 amino acid peptide isolated from the venom of the West African tarantula *Hysterocrates gigas* (Newcomb et al., 1998), SNX-111, the synthetic version of ω-conopeptide MVIIA (Olivera et al., 1994), SNX-194, the methionine-12 to norleucin-12 derivative of SNX-111, and SNX-230, the synthetic version of MVIIC (Hillyard et al., 1992). The synthetic version of ω-AgaIVA (Mintz et al., 1992) was purchased from Peptides International (Louisville, KY) or synthesized as described by Gaur et al., (1994). In the text we refer to the synthetic peptides by their original names or by the Neurex terms.

Data analysis. All results are given as means ± SEM, and the statistical significance of differences in groups was analyzed using SigmaStat (Jandel Scientific, San Rafael, CA) with Tukey's *t* tests.

RESULTS

Ca^{2+} channel currents

In the isolated neurohypophysial terminals, the peak I_{Ba}, which was activated by depolarizing from −80 to +10 mV, demonstrates both transient and long-lasting components (Fig. 1*A*). As we have reported previously (Wang et al., 1997a), the use of the dihydropyridine (DHP) Ca^{2+} channel antagonist nicardipine (2.5 μM) selectively inhibits the long-lasting (L-) component of the Ba^{2+} currents (Fig. 1). Subsequent addition of the N-type Ca^{2+} channel blocker MVIIA (3000 nM) led to rapid inhibition of a large portion of the isolated transient component (and, to a lesser extent, the long-lasting component) of the Ba^{2+} current. This concentration has been shown previously to block the N-type component maximally (Wang et al., 1997a).

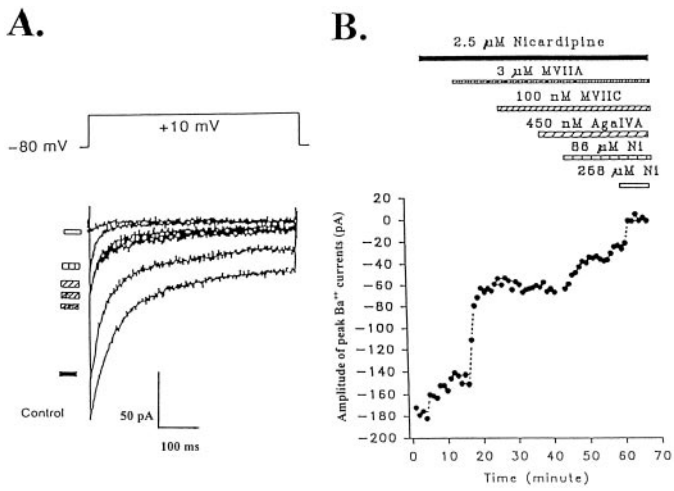

Figure 1. A toxin-resistant Ba^{2+} current in neurohypophysial terminals is blocked by Ni^{2+}. Subtypes of the macroscopic Ba^{2+} current (I_{Ba}) in nerve terminals can be pharmacologically dissected by applying different Ca^{2+} channel blockers. *A,* In an isolated rat neurohypophysial terminal, the I_{Ba} was elicited by depolarizations (see *template above*) and first recorded under control conditions (5 mM Ba^{2+} Locke's solution) and then after subsequent applications (shown by *horizontal bars* in *B*) of the L-type blocker nicardipine (2.5 μM), the N-type Ca^{2+} channel blocker MVIIA (3 μM), and the P/Q-type blockers MVIIC (100 nM) and AgaIVA (450 nM). There was a resistant Ba^{2+} current component that could only be dose dependently (86–258 μM) inhibited by Ni^{2+}. *B,* The corresponding time–response plot of the peak values of the macroscopic I_{Ba} is shown.

In ~50% of the neurohypophysial terminals investigated, subsequent addition of low (36 nM) concentrations of MVIIC inhibited this remaining component, and higher (150 nM) concentrations almost completely abolished it (Wang et al., 1997a). In another group of terminals (~46%), however, the non-L- and -N-types of Ca^{2+} currents could not be blocked by the P/Q-type Ca^{2+} channel blockers MVIIC or AgaIVA (Fig. 1). This resistant part of the transient Ca^{2+} current appeared to be analogous to an R-type Ca^{2+} channel current (Randall and Tsien, 1995).

Pharmacology of resistant Ca^{2+} channel currents

To test whether this resistant component of the Ba^{2+} current could indeed be classified as an R-type Ca^{2+} channel current, Ni^{2+}, a T- and R-type Ca^{2+} channel blocker, was applied to this terminal. Low concentrations (86μM) of Ni^{2+} inhibited the resistant current (Fig. 1). Because of the low selectivity of Ni^{2+} between Ca^{2+} channels, however, the identity of the resistant component of the Ca^{2+} current in the nerve terminal was still unclear.

A newly discovered polypeptide toxin, SNX-482, was found to be a specific blocker of the class E (α_{1E}) Ca^{2+} channel (Newcomb et al., 1998). This toxin made it possible for us to identify the Ni^{2+}-sensitive type of Ca^{2+} current and to probe its function in neurohypophysial nerve terminals (Wang et al., 1997b; Dayanithi et al., 1999).

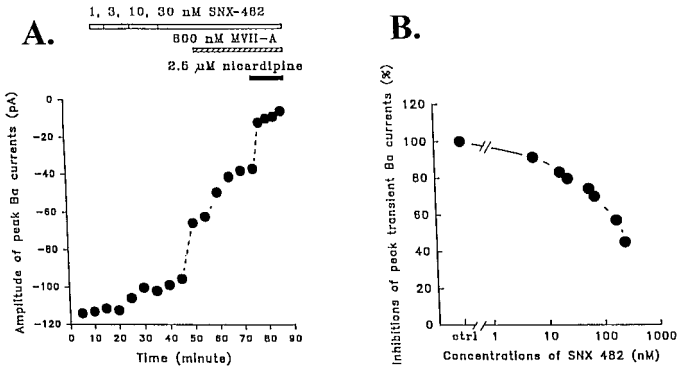

Figure 2. SNX-482 inhibits only the transient I_{Ba} in nerve terminals. Dose-dependent inhibition by SNX-482 of the total macroscopic I_{Ba} of neurohypophysial terminals is shown. *A,* A representative time–response plot (see, e.g., Fig. 1*B*) of the effect of 1–30 nM SNX-482 on the total macroscopic I_{Ba} current. Note that the remaining current in the nerve terminal was sensitive to MVIIA and nicardipine. *B,* Dose–response curve for the effect of SNX-482 on the undifferentiated transient macroscopic I_{Ba} ($n = 3$). The *solid line* was obtained from fitting with the equation $I = I_{max}\{1 - [x/(IC_{50} + x)]\}$, where I is the current amplitude at a given voltage, I_{max} is the maximum current, and x is the blocker's concentration. *ctrl*, Control.

First, the effects of SNX-482 on the long-lasting and transient components of the Ba^{2+} current of the neurohypophysial terminals were examined (Fig. 2*A*). The isolated, transient component of the Ba^{2+} current usually includes an N-type and either a P/Q-type or a resistant component of Ca^{2+} channel currents (Wang et al., 1997a). The IC_{50} for the undifferentiated transient I_{Ba}, obtained from the equation $I = I_{max}\{1 - [x/(IC_{50} + x)]\}$, is 226 nM (Fig. 2*B*). This is similar to that for SNX-482 to inhibit the cloned α_{1B} (N-type) Ca^{2+} channel current but much higher than that (IC_{50} = 10 nM) to block the heterologously expressed α_{1E}-type currents (Newcomb et al., 1998). The toxin does not affect the DHP-sensitive or L-type current in these terminals. These results indicate that, at high concentrations, SNX-482 could block some combination of N-, P/Q-, and/or class E-type Ca^{2+} channels in the terminals.

Application of a combination of DHP, MVIIA, and MVIIC or of high concentrations of MVIIA/SNX-194 and MVIIC/AgaIVA allowed us to obtain isolated "resistant" Ba^{2+} or Ca^{2+} currents (Fig. 3*A*). SNX-482, in a dose-dependent manner (in a total of seven terminals), inhibited the isolated resistant currents (Fig. 3*A,B*) with an IC_{50} of 4.1 nM (Fig. 3*C*), similar to that found for the α_{1E} Ca^{2+} currents expressed in human embryonic kidney (HEK) cells (Newcomb et al., 1998). The inhibition by SNX-482 of the resistant-type Ba^{2+} current is reversible (Fig. 3*D*). Furthermore, both SNX-482 and Ni^{2+} inhibited the same previously resistant component of the Ba^{2+} current (Fig. 3*D*).

Any sensitivity of P/Q-type currents in the nerve terminals to SNX-482 was then examined. As shown in Figure 4*A*, in the presence of the L-type blocker nicardipine and the N-type blocker MVIIA, the remaining Ba^{2+} component was not affected by SNX-482, although it was inhibited by the P/Q-type blocker AgaIVA. This confirmed that SNX-482 is not a P/Q-type or class A channel blocker (Newcomb et al., 1998). The inhibition of the resistant Ba^{2+} current component, in ~46% of the neurohypophysial terminals investigated, by both Ni^{2+} and SNX-482 lead us to conclude that this channel current most closely resembles that of the α_{1E} Ca^{2+} channel subunit expressed in HEK cells (Newcomb et al., 1998).

Interestingly, in ~5% of the terminals investigated ($n = 21$), in addition to the L- and N-type Ca^{2+} channel currents, there appears to exist both P/Q- and SNX-482-sensitive-type Ba^{2+}

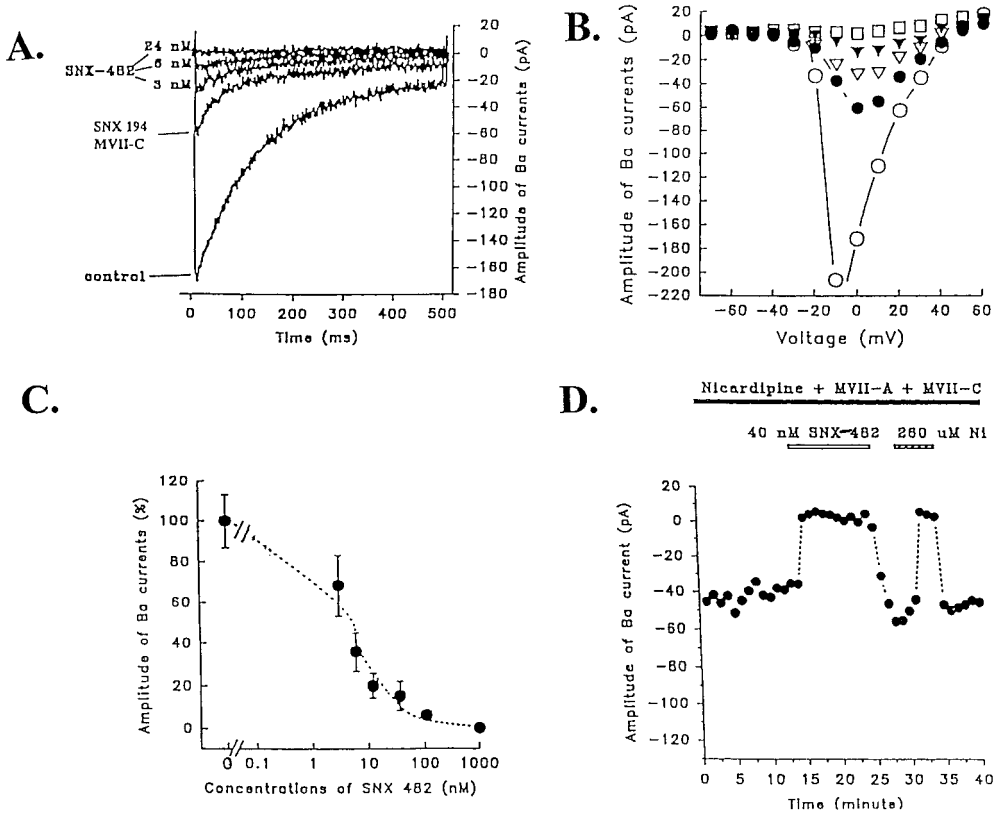

Figure 3. SNX-482 blocks the previously resistant neurohypophysial I_{Ba}. The I_{Ba} was elicited by depolarizations to 0 mV. *A,* Representative *traces* of resistant macroscopic I_{Ba} inhibited by SNX-482 in a dose-dependent manner after an application of high concentrations of SNX-194 (3 μM) and MVIIC (2 μM) to block the other components. *B,* The *I–V* relation of the macroscopic I_{Ba} under control conditions (○) and in the presence of SNX-194 and MVIIC (●) and 3 nM (▽), 6 nM (▼), and 24 nM (□) SNX-482. *C,* The dose–response curve of the effects of SNX-482 on the isolated resistant I_{Ba} in neurohypophysial terminals. The *dotted line* fit was obtained from the equation $I = I_{max}\{1 - [x/(IC_{50} + x)]\}$. *D,* Reversibility of the effects of SNX-482 and Ni^{2+} on the isolated resistant (to nicardipine + MVIIA + MVIIC) I_{Ba}. Ni^{2+} and SNX-482 appear to inhibit the same component of the current.

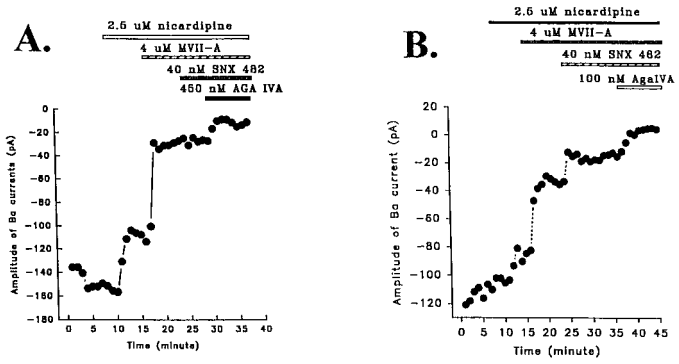

Figure 4. SNX-482 does not block the neurohypophysial P/Q-type Ca^{2+} current. *A*, A representative time–response curve of the peak values of the macroscopic I_{Ba} of an isolated nerve terminal insensitive to SNX-482 is shown. The I_{Ba} was recorded under control conditions and after subsequent applications of the L-type Ca^{2+} channel blocker nicardipine (2.5 μM) and the N-type Ca^{2+} channel blocker MVIIA (4 μM). SNX-482 (40 nM) did not affect the remaining Ba^{2+} current, which was subsequently blocked by the P/Q-type blocker AgaIVA (450 nM). *B,* By the use of the same protocol described in *A*, SNX-482 and AgaIVA each had a partial effect on the non-L and non-N-type Ba^{2+} current in a different nerve terminal. Therefore, some (~5%) neurohypophysial terminals have both P/Q- and R-type currents.

currents. Figure 4*B* is an example of this, showing that the non-L- and non-N-type Ba^{2+} currents were partially sensitive to both SNX-482 and AgaIVA.

Biophysical properties

Biophysical characterization of the resistant component of the neurohypophysial terminal I_{Ba} also favors a class E or R-type Ca^{2+} channel classification. This component of the current is a transient, high-voltage-activated Ba^{2+} current with an inactivation rate constant of 21 ± 3 msec (n = 7) during a step to 0 mV (see Fig. 3*A*). Figure 5*A* illustrates the activation ($V_{1/2}$ = −14.2 mV) and steady-state inactivation ($V_{1/2}$ = −58.8 mV) of the SNX-482-sensitive component of the neurohypophysial terminal Ba^{2+} current. The inactivating rate constant and activation and steady-state inactivation curves (Fig. 5*A*) of this neurohypophysial Ca^{2+} current component are most consistent with those of the R-type Ca^{2+} channel in granule cells (Randall and Tsien, 1995). Nevertheless, the other transient Ca^{2+} current components (N- and P/Q-type) appear to have biophysical properties similar to those of this "R"-type Ca^{2+} component (Wang et al., 1997a).

The relative permeabilities for Ca^{2+} versus Ba^{2+} between the total channel currents and the isolated SNX-482-sensitive or R-type current were compared, as shown in Figure 5*B*. Ba^{2+} currents were significantly larger than the corresponding Ca^{2+} currents for both the total and the isolated components. The inactivation rate constant, however, differed. The total current showed slower inactivation with Ba^{2+} as compared with Ca^{2+} as the charge carrier, whereas the R-type currents showed no difference in their inactivation with either Ba^{2+} or Ca^{2+}.

Peptide release

In a previous report (Wang et al., 1997a), we found that a significant portion of OT release could not be inhibited even by simultaneous applications of L-, N-, and P/Q-type Ca^{2+} channel blockers. To determine whether the class E or R-type Ca^{2+} channel could play a role in this secretion, we measured both OT and AVP release in the same samples collected from perfused

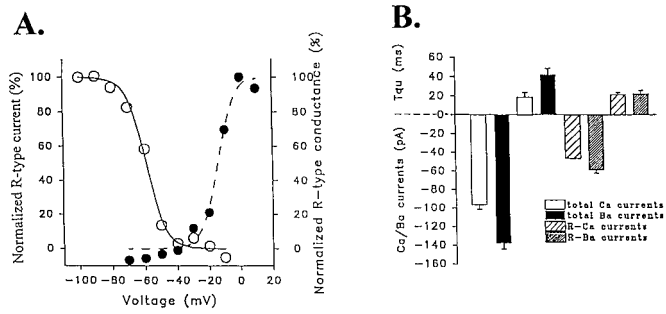

Figure 5. Voltage dependence and kinetics of Ba^{2+} and Ca^{2+} currents in neurohypophysial terminals. *A,* Activation (●) and steady-state inactivation (○) curves for the pharmacologically isolated (see Fig. 1) R-type macroscopic Ba^{2+} currents are illustrated (n = 3). The peak Ba^{2+} currents elicited were normalized to the maximal currents (for the steady-state inactivation curve) or conductances (for the activation curve) and plotted versus the holding potentials or the step potentials, respectively. Data for activation and steady-state inactivation were fit using appropriate forms of the Boltzmann equation. For activation, $C = C_{max}\{1 + \exp[(V_{1/2} - V_s)/k]\}^{-1}$, and for steady-state inactivation, $I = I_{max}\{1 + \exp[(V_s - V_{1/2})/k]\}^{-1}$, where C is the conductance at a given voltage, C_{max} is the maximum conductance, I is the current amplitude at a given voltage, I_{max} is the maximum current, V_s is the voltage step, $V_{1/2}$ is the midpoint potential, and k is the slope parameter. The fitting of the two curves for the isolated resistant (R)-type I_{Ba} gives $V_{1/2}$ values of −14.1 mV (k = 5.0) and −58.8 mV (k = 5.7) for activation and inactivation, respectively. *B,* Comparison of amplitudes and inactivation rates of the total versus R-type Ca^{2+} and Ba^{2+} currents is shown. *Bottom histograms*, The 5 mM Ba^{2+} total currents (n = 5) and the isolated R-type Ba^{2+} currents (n = 4) were larger than the 5 mM Ca^{2+} total currents (n = 5) and isolated R-type Ca^{2+} currents (n = 3), respectively ($p < 0.01$). *Top histograms*, The inactivation time constant (τ) was obtained from the same groups of terminals (as above). The 5 mM Ba^{2+} total currents had larger τ values, or slower inactivation, than did the 5 mM Ca^{2+} current group ($p < 0.01$). The R-type Ba^{2+} currents, however, had τ values similar to those of the R-type Ca^{2+} currents ($p > 0.05$).

populations of nerve terminals (Fig. 6). Capitalizing on the same pharmacological protocol used to isolate the R-type component electrophysiologically (see Fig. 3*A*), we revealed a similar resistant component (42.3%) of Ca^{2+}-dependent OT release (Fig. 6*B*). In these experiments, high K^+ alone induced OT release of 4258 ± 306 pg (n = 4), and both nicardipine and MVIIA, given in combination, reduced (by 57.7 ± 3.8%) high-K^+-stimulated release to 1812 ± 376 pg. SNX-482 (20 nM) completely blocked the remaining stimulated OT release (to basal level, 159 ± 33 pg). In contrast, a similar resistant component (38.4%) of stimulated AVP release (406 ± 30 pg) was essentially unchanged (458 ± 63 pg; n = 4) by the same concentration of this R-type channel blocker (Fig. 6*A*). As shown previously (Wang et al., 1997a), this resistant component of AVP release was blocked (to basal level, 60 ± 3 pg) by the P/Q-type blocker MVIIC. These results were the same even if the order of drugs was reversed or scrambled (data not shown). Furthermore, stimulated release was stable during prolonged applications of each of the Ca^{2+} channel blockers, indicating that steady-state effects had been established.

We have also performed a set of experiments to compare quantitatively the SNX- 482 block of OT release with the IC_{50} of this toxin on R-type calcium channels. As described in the Figure 6 legend, the nerve terminals were challenged with 50 mM K^+ either in the absence of any channel blocker (control) or in the presence of both 2.5 μM nicardipine (L-type channel blocker) and 1 μM MVIIA (N-type channel blocker) and then subsequently with varying concentrations of SNX-482 (1, 5, 10, 20, 50, or 100 nM). In this batch of experiments, K^+ alone evoked 3678 ± 139

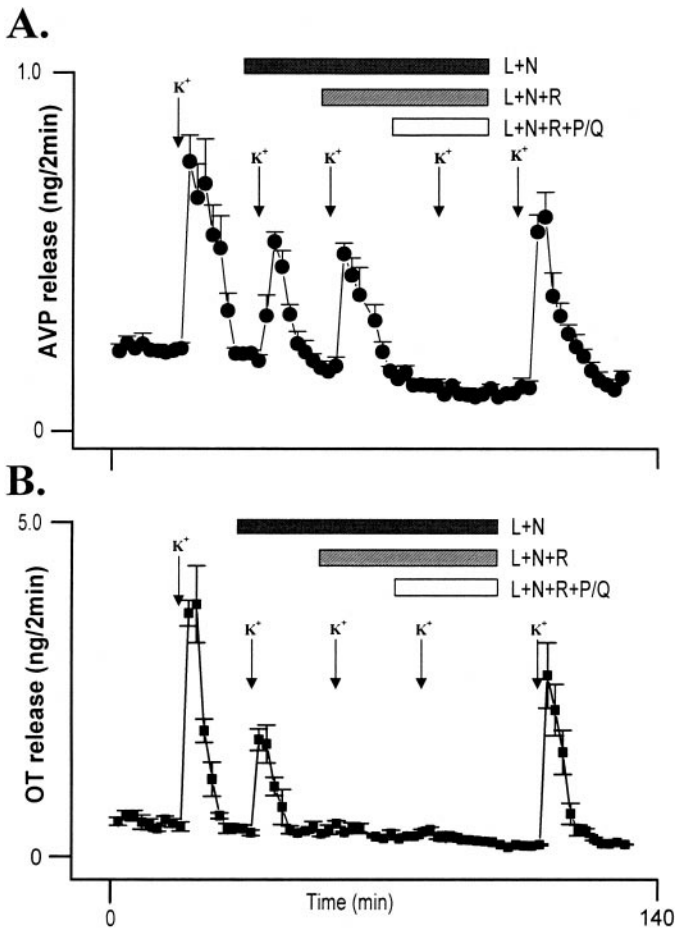

Figure 6. Effects of the Ca^{2+} channel blocker SNX-482 on AVP versus OT release from nerve terminals. *A,* AVP release was repeatedly stimulated (see *arrows*) with 50 mM K^+. The isolated neurohypophysial terminals were challenged with elevated K^+ either in the absence (*first arrow*) of any channel blockers or in the presence of 2.5 μM nicardipine (L-type Ca^{2+} channel blocker) and 1 μM MVIIA (N-type Ca^{2+} channel blocker) and then subsequently with the addition of 20 nM SNX-482 (R-type Ca^{2+} channel blocker) and finally plus 300 nM MVIIC (P/Q-type Ca^{2+} channel blocker). All these drugs were present for at least 20 min before, during, and after the K^+ stimulation (see *differently shaded horizontal bars above*). The inhibitions were mostly reversible, as indicated by control K^+ stimulation after washout (*last arrow*). All data points represent the mean of four to six experiments. *B,* OT release was repeatedly stimulated with 50 mM K^+. The nerve terminals were challenged with elevated K^+ either in the absence (*first arrow*) of any channel blockers or in the presence of 2.5 μM nicardipine (L) and 1 μM MVIIA (N), then 20 nM SNX-482 (R), and finally 300 nM MVIIC (P/Q) (see *horizontal bars above*). This inhibition was also mostly reversible (*last arrow*). Note that the same perfusate samples were assayed for both OT and AVP.

pg ($n = 3$). L- and N-type channel blockers reduced this OT release by 59.7% (to 2194 ± 100 pg). Further addition of 1, 5, 10, 20, 50, or 100 nM SNX-482 suppressed the resistant OT release by 8% (to 2018 ± 30 pg), 32.6% (to 1478 ± 54 pg), 61.4% (to 846 ± 40 pg), 93.4% (to 145 ± 7 pg), 95.6% (to 96 ± 6 pg), and 97.6% (to 52 ± 6 pg), respectively. The IC_{50} calculated from the equation $r = R_{max}\{1 - [x/(IC_{50} + x)]\}$ for the SNX-482 block of OT release is 6.8 nM, which is comparable with the IC_{50} for the toxin on R-type calcium currents. Finally, the effects of SNX-482 on OT versus AVP release were significantly ($p < .001$) different, thus revealing the importance of the class E or R-type component in only OT release.

DISCUSSION

The isolated neurohypophysial terminals are uniquely useful for studying the pharmacological, biophysical, and functional properties of Ca^{2+} channels at the site of secretion, and they have revealed a surprising pharmacological and functional complexity in the CNS presynaptic Ca^{2+} channel family.

Four different components of Ca^{2+}-dependent neuropeptide release

The regulation of neurotransmission in the mammalian CNS has been characterized by the involvement of multiple types of voltage-dependent Ca^{2+} channels, each of which might play a specific role in the regulation of neurotransmission. In the mammalian neurohypophysial system, the L- and N-type Ca^{2+} channels play an equivalent role in both AVP and OT release. This is quite different from the role of Ca^{2+} channels in classical neurotransmission, in which the N- and P/Q-type, instead of the L-type, Ca^{2+} channel current are dominant in controlling neurotransmitter release (Hirning et al., 1988; Wheeler et al., 1994; Dunlap et al., 1995). Furthermore, the P/Q-type Ca^{2+} channel has turned out to be critical for AVP release from neurohypophysial nerve terminals (Wang et al., 1997a). Finally, the present results have demonstrated that an SNX-482-sensitive Ca^{2+} current is responsible for an important part of OT release.

Identity of the resistant Ca^{2+} channel in nerve terminals

We have now shown that the SNX-482-sensitive Ca^{2+} current has similar biophysical properties to that of the class E channel. The phenotype of the expressed class E channel (Zhang et al., 1993) can resemble that of native currents described as either R- (Randall and Tsien, 1995) or T-type (Snutch and Reiner, 1992). The T-type Ca^{2+} channel in the CNS is a low-voltage-activated channel that is affected by Ni^{2+} (Tsien et al., 1991; Fisher and Bourque, 1996). The terminal SNX-482-sensitive Ca^{2+} current, although also blocked by Ni^{2+}, is moderately high voltage activated and more permeable to Ba^{2+} than to Ca^{2+} (Fig. 5*B*). Thus, in terms of its biophysical properties, this channel appears to be different from the T-type Ca^{2+} channel.

A correspondence between cloned expressed class E calcium channels and various currents described as resistant, or R-type, is suggested by similar electrophysiological properties and resistance to selective antagonists of N, P/Q, and L-type calcium channels (Newcomb et al., 1998). In the absence of a selective antagonist of the class E calcium channel, however, the correspondence between calcium channel classes defined by cDNA sequencing and electrophysiology has been unclear, and the role of class E calcium channels in physiology has not been studied previously (but see Wu et al., 1998, 1999). Our study capitalizes on the recent discovery of a selective class E antagonist from tarantula venom, SNX-482, and it has allowed us to analyze directly the identity, function, and pharmacology of the resistant-type calcium channels in CNS terminals.

The initial studies of the *in vitro* actions of native SNX-482 have revealed unanticipated diversity in the response of native R-type currents to the peptide (Newcomb et al., 1998). Nevertheless, because low nanomolar concentrations of SNX-482 have no effects on other calcium channel subtypes (see Figs. 2, 4) (Newcomb et al., 1998), the potent block of the neurohypophysial R-type current demonstrates that the resistant current isolated pharmacologically is not simply residual current flowing through incompletely blocked N-, P/Q-, and L-type calcium channels. Thus, the

variability of the response of native R-type currents almost certainly indicates pharmacological heterogeneity of the distinct entities, perhaps splice variants, which are responsible for these currents.

These variants could also explain the fact that class E mRNA has, so far, not been detected in the hypothalamic magnocellular somata that project to the neurohypophysis (Gainer and Chin, 1998). In contrast, preliminary evidence (G. Dayanithi, unpublished results), using antibodies raised against class E channels, has localized these channels to isolated neurohypophysial terminals.

The R-type Ca^{2+} channel controls OT, but not AVP, release

Our data suggest that in one group of terminals, there is a Ni^{2+}- and SNX-482-sensitive Ca^{2+} channel able to regulate OT release preferentially, whereas in another group of terminals the P/Q-type Ca^{2+} channel plays a converse role in AVP release. We demonstrate here that the α_{1E} class or R-type Ca^{2+} channel exists on these neurohypophysial terminals, where it participates in the control of neuropeptide secretion. These results lead to the hypothesis that the R-type channels are preferentially localized on OT peptide-containing nerve terminals and thus do not affect Ca^{2+} currents in vasopressinergic terminals. Interestingly, some (5%) terminals appear to have both types of channels (Fig. 4*B*), comparable with the observed percentage of terminals containing both OT and AVP (Wang et al., 1997b). In any case, the data clearly show that the R-type component plays an important role in OT, but not in AVP, secretion from these CNS terminals.

In conclusion, we have demonstrated that an R-type Ca^{2+} channel exists in at least one type of CNS terminal and is important in depolarization–secretion coupling. This lends support to the idea that R-type channels may play a specific role in synaptic transmission in other CNS synapses (Newcomb et al., 1998; Wu et al., 1998, 1999). The data presented here clarify the specific identities and functional importance of the Ca^{2+} channels actually located at nerve terminals and point out that the R- and P/Q-channels, at least, may be heterogeneously localized for different functions.

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