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

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Multiple types of voltage-dependent Ca\textsuperscript{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\textsuperscript{2+} channels in neuropeptide release have been identified previously (Wang et al., 1997a). Although the L- and N-type Ca\textsuperscript{2+} currents play equivalent roles in both vasopressin and oxytocin release, the P/Q-type Ca\textsuperscript{2+} current only regulates vasopressin release. An oxytocin-release and Ca\textsuperscript{2+} current component is resistant to the L-, N-, and P/Q-type Ca\textsuperscript{2+} channel blockers but is inhibited by Ni\textsuperscript{2+}. A new polypeptide toxin, SNX-482, which is a specific \(\alpha_{1E}\)-type Ca\textsuperscript{2+} channel blocker (Newcomb et al., 1998), was used to characterize the biophysical properties of this resistant Ca\textsuperscript{2+} current component and its role in neuropeptide release. This resistant component was dose dependently inhibited by SNX-482, with an IC\textsubscript{50} of 4.1 nM. Furthermore, SNX-482 did not affect the other Ca\textsuperscript{2+} current types in these CNS terminals. Like the N- and P/Q-type Ca\textsuperscript{2+} currents, this SNX-482-sensitive transient Ca\textsuperscript{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\textsuperscript{2+} current in oxytocin release from neurohypophysial terminals. Our results suggest that an \(\alpha_{1E}\) or “R”-type Ca\textsuperscript{2+} channel exists in oxytocinergic nerve terminals and, thus, functions in controlling only oxytocin release from the rat neurohypophysis.

Key words: class E \((\alpha_{1E})\) Ca\textsuperscript{2+} channel; secretion; SNX-482; vasopressin; posterior pituitary; oxytocin
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 Cl\(^{-}\)) Locke’s solution, which also contained 1 μM TTX with 0.02% BSA. To obtain perforated-patch recording configuration enables us to overcome problems with the rundown of Ca\(^{2+}\) currents that complicated former studies (Lemos and Noyce, 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. 1A), 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-o-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 mM).

*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 norleucine-12 derivative of SNX-111, and SNX-230, the synthetic version of MVIC (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\(_{Na}\), which was activated by depolarizing from −80 to +10 mV, demonstrates both transient and long-lasting components (Fig. 1A). 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 (3 μM), and the P/Q-type blockers MVIC (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.

**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 (86(μM) 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 Ba\(^{2+}\) 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\(_{4}\)) 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).
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. 2A). 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 component of the Ba\(^{2+}\) current (Fig. 2B) was sensitive to MVIIA and nicardipine. 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\(^{2+}\) channel currents.

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. 3A). SNX-482, in a dose-dependent manner (in a total of seven terminals), inhibited the isolated resistant currents (Fig. 3A,B) with an IC\(_{50}\) of 4.1 nM (Fig. 3C), similar to that found for the \(\alpha_{1E}\) 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. 3D).

Any sensitivity of P/Q-type currents in the nerve terminals to SNX-482 was then examined. As shown in Figure 4, in the presence of the L-type blocker nicardipine and the N-type channel blocker AgaIVA, 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 led us to conclude that this channel current most closely resembles that of the \(\alpha_{1E}\) 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\(^{2+}\) channel currents.

The IC\(_{50}\) was obtained from fitting with the equation \(I = I_{\text{max}} \left[ 1 - \frac{x}{(IC_{50} + x)} \right]\), where \(I\) is the current amplitude at a given voltage, \(I_{\text{max}}\) is the maximum current, and \(x\) is the blocker’s concentration. **ctrl**, Control.

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

![Figure 2](image-0.png)

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

![Figure 3](image-1.png)
currents. Figure 4B 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 $I_{\text{Ba}}$ 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. 3d). Figure 5a 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. 5a) 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 5b. 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 populations of nerve terminals (Fig. 6). Capitalizing on the same pharmacological protocol used to isolate the R-type component electrophysiologically (see Fig. 3a), we revealed a similar resistant component (42.3%) of Ca$^{2+}$-dependent OT release (Fig. 6b). 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. 6a). 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 MVIIIC. 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
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. 5B). 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 cer-
tainly 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, unpub-
ilished results), using antibodies raised against class E channels,
has localized these channels to isolated neurohypophyseal
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 \(\alpha_{\text{ME}}\) class or R-type Ca\(^{2+}\) channel
exists on these neurohypophysial terminals, where it participates
in the control of neupeptide 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. 4B),
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 sup-
port 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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