Pharmacological Discrimination of N-Type from L-Type Calcium Current and Its Selective Modulation by Transmitters

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GABA and norepinephrine inhibit high voltage-activated calcium current in chick sensory neurons. Using specific pharmacological tools, we have dissected this current into two components: the major one is ω -conotoxin sensitive and dihydropyridine resistant (N-type) while the minor one is dihydropyridine sensitive and ω -conotoxin resistant (L-type). The ability to selectively eliminate these two components has allowed us to determine whether the transmitters target the same or different channel types. Both GABA and norepinephrine modulate the N-type component as evidenced by their lack of effect on (1) ω -conotoxin-resistant current and (2) pure L-type tail current, prolonged by a dihydropyridine calcium channel agonist. This simple pharmacological profile will allow future tests of the significance of the two channel types in regulating sensory neuron functions.

In the past decade, research on voltage-dependent calcium channels has demonstrated multiple channel types, differing from one another biophysically, biochemically, and pharmacologically (Swandulla et al., 1991). Considering the wide variety of cellular functions controlled by the voltage-dependent influx of calcium ions across plasma membranes, it is possible that calcium channel types with unique functional properties have evolved as a means of controlling particular cellular functions—one type for altering firing threshold, for example, and another for triggering muscle contraction. In addition to playing different functional roles, calcium channel subtypes are likely to serve as differential targets for the action of both exogenous signals (such as hormones or neurotransmitters) and endogenous signals (such as GTP-binding proteins, cyclic nucleotides, and kinases).

Studying the roles of the different calcium channel subtypes and their modulators in cellular function has been experimentally problematic due, in large part, to the absence of specific pharmacological tools that can selectively block one or another channel type. Antagonists of high voltage-activated (HVA) calcium channels have been particularly wanting. Original reports of multiple HVA calcium channel subtypes suggested that two types (L and N) could be discriminated in dorsal root ganglion neurons on the basis of their inactivation kinetics as well as

their pharmacology (Nowicky et al., 1985a; Fox et al., 1987a,b). Subsequent investigations, however, suggest (1) that macroscopic L- and N-type calcium currents in many cells cannot be well separated kinetically (Hirning et al., 1988; Aosaki and Kasai, 1989; Plummer et al., 1989; Carbone et al., 1990); (2) that dihydropyridines (DHPs), although selective for L-type channels, exhibit voltage-dependent properties that limit their usefulness for studies of multicomponent HVA calcium currents (Sanguinetti and Kass, 1984; Sanguinetti et al., 1986; Rane et al., 1987); and (3) that subtypes of DHP-resistant current can be discriminated (Aosaki and Kasai, 1989; Plummer et al., 1989; Regan et al., 1991).

ω -Conotoxin GVIA (ω -CgTx), a peptide toxin from the snail *Conus geographus* (Olivera et al., 1985), is likely to be a potent, highly selective blocker of one type of HVA current (N-type). Although early experiments, based on kinetic separation of L- and N-type current, concluded that both types in chick dorsal root ganglion neurons were blocked irreversibly by the toxin (McCleskey et al., 1987; Fox et al., 1987a), recent studies of chick dorsal root ganglion neurons (Aosaki and Kasai, 1989), rat superior cervical ganglion (SCG) neurons and pheochromocytoma (PC12) cells (Plummer et al., 1989) indicate that L-type current is not a target for irreversible toxin action.

In the work reported here, we have taken advantage of the two selective pharmacological agents, DHPs and ω -CgTx, to characterize the HVA calcium currents present in embryonic chick dorsal root ganglion neurons *in vitro*. Such characterization has allowed us to determine whether two transmitters [norepinephrine (NE) and GABA] that modulate the HVA current in these cells target the same or different channel types. Considering that both HVA calcium current and neurotransmitters play significant roles in the regulation of neurosecretion from sensory (Holz et al., 1988, 1989) and sympathetic (Perney et al., 1986; Hirning et al., 1988) neurons, results of this study are important for understanding the mechanisms involved in synaptic transmission between sensory neurons and their targets in the spinal cord.

Materials and Methods

Culture. Dorsal root ganglia were dissected from 11–12-d-old chicken embryos and grown in dissociated cell culture using previously described methods (Holz et al., 1988). The neurons were studied between days 0 and 6 *in vitro*.

Electrical recording. Standard tight-seal, whole-cell recording techniques were employed to study HVA calcium current from the cell bodies of the neurons (Hamill et al., 1981). A List EPC-7 patch-clamp amplifier was used for these recordings. Capacitive transients were routinely cancelled using the EPC-7's cancellation circuitry, and series resistance (typically 3.5 M Ω) was compensated to about 70%. Current traces were digitized at 10 kHz, stored, and analyzed on an Atari Mega

Received Aug. 1, 1991; revised Oct. 2, 1991; accepted Oct. 18, 1991.

We thank Drs. W. Gruner and T. J. Turner for critical comments on the manuscript. The work was supported by PHS Grant NS-16483 and The McKnight Foundation.

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4 ST computer using data acquisition and analysis software from Instrutech Corporation (Elmont, NY). Linear components of capacitive and leak current were subtracted from all traces before analysis using a standard P/4 protocol starting from a holding potential of -100 mV. For experiments in which the time course of tail current decay was studied, records were digitized at 10 kHz and were not filtered further (see Figs. 3*A,B*; 5*A,B*). In all other experiments, displayed records were filtered to 1 kHz. Unless otherwise specified, during an experiment, voltage steps were applied to the test cell every 10 sec. External solutions contained (in mM) NaCl, 150 ; CaCl₂, 1 ; MgCl₂, 0.8 ; tetraethylammonium chloride (TEA), 10 ; HEPES, 25 ; and glucose, 5 . Tetrodotoxin (0.3 μ M) was added to all solutions. In certain indicated experiments, 40 mM TEA and 100 μ M 4-aminopyridine were also added to the external solution (replacing NaCl) to suppress a small amount of contaminating outward current. Patch pipettes were made from Fisherbrand glass microhematocrit capillary tubes (wall thickness, 1.1 – 1.2 mm) and filled with a solution containing (in mM) CsCl, 150 ; HEPES, 10 ; 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetra-acetic acid (BAPTA), 5 ; and Mg-ATP, 5 . Pipette resistances prior to forming high-resistance seals ranged from 1.6 to 2.3 M Ω .

During the first 24 hr, the cells extended only short processes and excellent spatial control of voltage could be achieved. With time in culture, however, a slow component to the tail current could be observed in some cells, which likely represents a slow charging of the process membrane. Tail current experiments reported were performed on cells in which this slow component was minimal.

Solutions/chemicals. (+)-(S)-202-791 was a generous gift of Dr. Robert Hof, SANDOZ AG Preclinical Research (Basel, Switzerland). Nimodipine was a generous gift of Dr. Scriabine, Miles Pharmaceuticals. These dihydropyridines were dissolved in 95% ethanol and stored as 10 mM stock solutions. For experiments, they were diluted to 1 – 5 μ M as indicated in extracellular recording solution. ω -CgTx GVIA was obtained from Peninsula Laboratories, stored as a stock solution of 1 mM in distilled water, and diluted to 9 μ M in external recording solution on the day of the experiment. Similarly, 10 mM stock solutions of GABA, bicuculline methiodide, and NE were prepared in distilled water and diluted to their final experimental concentrations of 100 μ M (GABA and bicuculline) and 10 – 50 μ M (NE), in external recording solution. Drugs and transmitters were applied using puffer pipettes with tip diameters approximately 3 – 5 μ m as previously described (Rane and Dunlap, 1990).

Results

ω -CgTx selectively blocks a DHP-resistant calcium current

In our studies, we found few cells that exhibited low voltage-activated calcium current when measured from a holding potential of -80 mV. Those cells that did were excluded from our study so as to simplify the measurement of the peak of the HVA current. The efficacy of ω -CgTx on the HVA current in dorsal root ganglion neurons *in vitro* varied with age of the cultured neurons. Acutely dissociated cells (or cells tested within 1 d of plating) exhibited virtually no ω -CgTx-resistant current (i.e., 9 μ M toxin blocked 100% of the HVA calcium current) (Fig. 1*A*). As first described by Aosaki and Kasai (1989), these pure ω -CgTx-sensitive currents activated following step depolarizations to potentials more positive than -30 mV, reached a maximum at 0 mV, and reversed near 55 mV (Fig. 1*C*). Such HVA current inactivated as a function of the holding potential. In 80% of the cells studied within 1 d of plating, half-maximal inactivation occurred at -53 mV ($n = 15$; Fig. 1*B*); in these cells, a 70% reduction of the peak current (measured at 0 mV) was produced when the holding potential was changed from -80 to -40 mV. The other 20% of the cells studied exhibited a less pronounced dependence on holding potential. In these, only a 30% reduction in peak current was produced following depolarization of the holding potential from -80 to -40 mV. An example of such a holding potential-resistant current (that is ω -CgTx sensitive) is shown in Figure 2 for a cell that had been 5 d in culture.

The inactivation rate of the ω -CgTx-sensitive current during

a test pulse was also variable and dependent upon the holding potential from which an activating test pulse was given. During a 150 msec depolarization to 0 mV from a holding potential of -60 mV, ω -CgTx-sensitive current decayed steadily and incompletely to an average $50 \pm 7\%$ (mean $\pm$ SD; $n = 12$) of its peak value (Fig. 1*A*, trace 1). The extent of decay in this series of experiments ranged between 40% and 80% . In general, the currents that displayed the more prominent inactivation during a test pulse were also more sensitive to inactivation with depolarized holding potentials. When the neurons were held at -40 mV, the peak current during the test pulse was not only reduced, as discussed, but the ω -CgTx-sensitive current decayed approximately five times more slowly ($72 \pm 9\%$ of peak by 150 msec; $n = 12$) than currents evoked by depolarizations from -60 mV holding potentials. Observed on this limited time scale, ω -CgTx-sensitive calcium current elicited from a -40 mV holding potential often appeared to be noninactivating (Fig. 1*A*, trace 2). Interestingly, voltage steps from -60 mV to $+80$ mV elicited outward currents that could be blocked by ω -CgTx and presumably represent cesium ions moving out of the cell through ω -CgTx-sensitive calcium channels (Fenwick et al., 1982; Lee and Tsien, 1982). This outward current did not inactivate at all over the course of a 150 msec voltage step ($n = 5$; Fig. 1*D*, trace 2), suggesting that the inactivation of ω -CgTx-sensitive inward current during a test pulse may represent a calcium-dependent rather than a voltage-dependent process. Thus, variability in the rate of this process may be due to some variability in the efficacy of intracellular calcium buffering.

Over time *in vitro*, the proportion of ω -CgTx-resistant current increased. For neurons between 3 and 6 d in culture, 60 sec applications of the toxin blocked $86 \pm 9\%$ ($n = 44$) of the HVA calcium current activated by step depolarizations to 0 or $+10$ mV from a holding potential of -60 mV (Fig. 2). The percentage inhibition produced by ω -CgTx was largely independent of holding potentials between -80 and -40 mV as shown in Figure 2*C*.

At all times studied, the portion of HVA calcium current inhibited by ω -CgTx was larger than that previously attributed to any one dorsal root ganglion neuron calcium current type when defined by whole-cell current inactivation kinetics or holding potential dependence (Nowycky et al., 1985a; Fox et al., 1987a,b). Based on a classification of HVA currents in which the long-lasting (L) and inactivating (N) components represent distinct current types, our results with ω -CgTx would suggest that both N- and L-type currents are blocked by the peptide, as McCleskey et al. (1987) reported.

In order to directly test whether ω -CgTx blocks L current in chick dorsal root ganglion neurons, we employed the DHP (+)-(S)-202-791. Like Bay K 8644, this pure calcium channel agonist shifts the voltage dependence of activation of L-type calcium channels and prolongs their apparent mean channel open time (Hess et al., 1984; Nowycky et al., 1985b; Plummer et al., 1989). This increase in mean channel open time results in a prolongation of macroscopic tail current deactivation kinetics (Plummer et al., 1989; Regan et al., 1991). We have investigated the ability of ω -CgTx to reduce this slow, pure L-type tail current. Figure 3 shows an example of one such experiment performed on a cell with a larger-than-average ω -CgTx-resistant component of calcium current. Prior to the application of (+)-(S)-202-791 (2 μ M), the time course for tail current deactivation was relatively fast, decaying to 37% of its peak in approximately 600 μ sec (Fig. 3*A*, trace 1). Application of the DHP agonist

Figure 1. Properties of ω -CgTx-sensitive calcium current measured in chick dorsal root ganglion neurons prior to 1 d *in vitro*. *A*, Whole-cell calcium currents elicited with 150 msec voltage steps from holding potentials of -60 mV (trace 1) or -40 mV (trace 2) to a test potential of 0 mV. Trace 3 shows current measured after the application of $9 \mu\text{M}$ ω -CgTx (holding potential, -80 mV). *B*, Steady-state inactivation curve for ω -CgTx-sensitive current. Data points represent the average normalized peak currents measured from five cells. The data are fit with a Boltzmann relation, $I/I_{\text{max}} = 1/(1 + \exp((V - V_{1/2})/r))$ (solid line) with $V_{1/2} = -53$ mV and $r = 10$. *C*, Current-voltage relationship for normalized peak ω -CgTx-sensitive calcium current. Data points represent the average of four measurements. *D*, Inward and outward current flowing through ω -CgTx-sensitive channels. Currents were elicited with 150 msec voltage steps from -60 mV to either 0 mV (trace 1) or 80 mV (trace 2). Any residual currents measured in the presence of ω -CgTx were subtracted from the control currents to yield ω -CgTx-sensitive current. Traces in *A* and *B* were recorded from cells bathed in saline containing 50 mM TEA and 100 μM 4-aminopyridine.

resulted in the appearance of a slow component to the tail current with an average ($n = 12$) time constant of 4 msec (Fig. 3*B*, trace 2). At times later than ~ 3 msec (when the fast component has decayed to near zero; Fig. 3*B*, arrow), the tail current represents a pure L-type current. Upon application of ω -CgTx to this cell, the test pulse current was reduced 45% while the (+)-(S)-202-791-sensitive component of tail current was virtually unaffected by the toxin (Fig. 3*B*, trace 3). Similar results have been described in rat SCG (Plummer et al., 1989) and dorsal root ganglion (Regan et al., 1991) neurons.

The apparent difference between traces 2 and 3 in Figure 3*B* at early times results from changes in the rapidly deactivating, ω -CgTx-sensitive component of tail current. Currents were fit beginning 3.0 msec after repolarization in order to eliminate interference by changes in this rapidly deactivating current. The extrapolated amplitudes of these fits were used as an index of L-type calcium current inhibition by ω -CgTx. In four cells selected for study because they showed relatively large toxin-resistant currents, we found ω -CgTx blocked an average of $50.5 \pm 7.3\%$ of the test pulse current elicited with a voltage pulse

from -60 mV to 10 mV. In contrast, the inhibitory effect on the (+)-(S)-202-791-prolonged tail component was $0.35 \pm 9.7\%$. Thus, ω -CgTx appears to be selective for a DHP-resistant current.

ω -CgTx-resistant current is highly sensitive to DHPs

Unlike Aosaki and Kasai (1989) and Plummer et al. (1989), we did not observe, over a 10 min period, any component of HVA current that recovered from ω -CgTx block ($n = 10$; Fig. 3*C*). We were thus able to study ω -CgTx-resistant current in isolation after a single application of the toxin. Applying (+)-(S)-202-791 to cells that had previously been treated with ω -CgTx greatly enhanced ($172 \pm 81\%$; $n = 7$) the residual current recorded following test pulses to -20 mV (Fig. 4*A*, traces 1 and 2). This enhanced current was unaffected by the reapplication of ω -CgTx (Fig. 4*A*, trace 3). Furthermore, when elicited from a holding potential of -40 mV, the ω -CgTx-resistant current was blocked completely by the DHP antagonist nimodipine ($5 \mu\text{M}$; $n = 9$; Fig. 4*B*, trace 4). On the basis of these results, we conclude that there are two types of pharmacologically distinct HVA calcium

Figure 2. ω -CgTx sensitivity of calcium current recorded from chick dorsal root ganglion neurons after 3–6 d *in vitro*. Whole-cell calcium currents were measured from holding potentials of -60 mV (*A*) and -40 mV (*B*) before and after the application of $9 \mu\text{M}$ ω -CgTx. *C*, Histogram illustrating the average amplitudes ($n = 6$) of calcium currents in control (dark shading) and ω -CgTx-treated (light shading) cells. Currents were evoked by depolarizing pulses to 10 mV from holding potentials of -40 , -60 , and -80 mV.

current in chick dorsal root ganglion neurons: L-type, which is sensitive to DHPs and resistant to ω -CgTx, and N-type, which is irreversibly blocked by ω -CgTx and resistant to DHPs. We define here N-type current solely on the basis of these pharmacological criteria and by doing so do not mean to attribute to this current component any specific kinetic characteristics. In contrast to other studies on peripheral and central neurons (Aosaki and Kasai, 1989; Plummer et al., 1989; Regan et al., 1991), no component of HVA current in any of the dorsal root ganglion neurons tested was found to be resistant to both ω -CgTx and DHPs.

Neurotransmitters modulate the ω -CgTx-sensitive current

Superfusion of dorsal root ganglion neurons with $100 \mu\text{M}$ GABA substantially inhibits whole-cell calcium current. This GABA-induced inhibition is characterized by a slowing of the activation kinetics (Fig. 5C, trace 2; Grassi and Lux, 1989). On average, calcium current was inhibited $37 \pm 5\%$ ($n = 5$) when measured 15 msec after the start of a depolarizing test pulse to 0 mV and $21 \pm 5\%$ when measured at 150 msec (Fig. 5C). The ability of GABA to inhibit a larger percentage of calcium current than that carried by ω -CgTx-resistant channels ($\sim 14\%$) strongly suggests that GABA inhibits, at least in part, N-type channels. It is possible, however, that GABA also modulates L-type current.

In order to investigate this, we examined the ability of GABA to inhibit the (+)-(S)-202-791-prolonged component of calcium tail current observed upon repolarization to -60 mV. Figure 5A shows the results of one such experiment. Calcium current recorded following a 10 msec depolarization to 10 mV was inhibited 35% by $100 \mu\text{M}$ GABA; however, GABA had no significant inhibitory effect ($<1\%$) on the slowly decaying component of tail current produced by superfusion of this cell with (+)-(S)-202-791 (Fig. 5A, trace 3). Figure 5B compares the (+)-(S)-202-791-sensitive component of calcium tail current before and after application of GABA. This tail current component was fit with a single exponential in the manner described for the experiment of Figure 3 (see above). Any change in L-type current magnitude was gauged by the difference between the amplitudes of the current fits before and after GABA treatment. In seven experiments of this type, GABA inhibited the test pulse current an average of $30 \pm 7\%$ but produced no significant reduction in the DHP-sensitive tail current ($6 \pm 6\%$).

To control for the possibility that (+)-(S)-202-791 protects L-type channels from being modulated by GABA, we examined the ability of the transmitter to inhibit whole-cell calcium current before and after block of N-type current with ω -CgTx, in the absence of the DHP agonist. Studying cells that showed large GABA-induced current inhibitions prior to ω -CgTx application,

Figure 3. ω -CgTx does not block L-type calcium current in chick dorsal root ganglion neurons. *A*, Whole-cell calcium currents measured during a 20 msec voltage step from -60 mV to 10 mV and after repolarization to -60 mV under the following conditions: control (trace 1), during application of 2 μ M (+)-(S)-202-791 (trace 2), and during coapplication of both (+)-(S)-202-791 and 9 μ M ω -CgTx (trace 3). *B*, Tail currents from the experiment in *A* viewed on an expanded time scale. Numbered traces correspond to the same conditions as in *A*. Traces 2 and 3 are fit at points later than 3 msec after repolarization (arrow) to an exponential function with a time constant of 3.9 msec and an initial amplitude of -2140 pA (smooth curve). *C*, Time courses for the effects of (+)-(S)-202-791 and ω -CgTx on the amplitude of test pulse current measured at the end of a 20 msec voltage step (open circles) and on the tail current amplitude measured 3 msec after repolarization (solid circles). Data in *C* are from a different cell.

Figure 4. ω -CgTx-resistant calcium current is sensitive to DHPs. *A*, Whole-cell calcium current measured after an initial application of 9 μ M ω -CgTx (trace 1), during subsequent application of (+)-(S)-202-791 (trace 2), and during reapplication of ω -CgTx in the presence of (+)-(S)-202-791 (trace 3). *B*, Whole-cell calcium currents measured following 150 msec voltage steps to 0 mV under the following conditions: from a holding potential of -60 mV (trace 1), from a holding potential of -60 mV after 9 μ M ω -CgTx was applied to the cell (trace 2), from a holding potential of -40 mV after ω -CgTx application (trace 3), and from a holding potential of -40 mV after ω -CgTx application and then subsequent application of 5 μ M nimodipine (trace 4). In *B*, the solution bathing the cell contained 50 mM TEA and 100 μ M 4-aminopyridine.

Figure 5. GABA inhibits an ω -CgTx-sensitive but not a DHP-sensitive calcium current in chick dorsal root ganglion neurons. *A*, Whole-cell calcium currents measured during a 10 msec voltage step from -60 mV to 10 mV and after repolarization to -60 mV under the following conditions: control (trace 1), during application of 2μ M (+)-(S)-202-791 (trace 2), and during coapplication of both (+)-(S)-202-791 and 100μ M GABA plus 100μ M bicuculline, to block GABA_A-induced increases in chloride permeability (trace 3). *B*, Tail currents from the experiment in *A* viewed on an expanded time scale. Numbered traces correspond to the same conditions as in *A*. Traces 2 and 3 are fit at points later than 3 msec after repolarization (arrow) to an exponential function (smooth line) with a time constant of 6.9 msec and an initial amplitude of 940 pA. *C*, Whole-cell calcium currents measured during 50 msec voltage steps to 0 mV from -60 mV under the following conditions: control (trace 1), during superfusion of the test cell with 100μ M GABA (trace 2), after recovery from GABA application (trace 3), after application of 9μ M ω -CgTx (trace 4), and during subsequent application of GABA (trace 5). All solutions in *C* contained 100μ M bicuculline, 50 mM TEA, and 100μ M 4-aminopyridine.

we found no significant inhibition of ω -CgTx-resistant calcium current by the transmitter (Fig. 5C). In five such experiments, GABA inhibited HVA calcium current by $37 \pm 5\%$ (measured at 15 msec) before ω -CgTx application, while no effect was observed on ω -CgTx-resistant current ($<2\%$ inhibition). In all cases, the amplitude of GABA-sensitive calcium current was substantially larger than the ω -CgTx-resistant current in these cells. It is therefore clear from the above experiments that GABA inhibits an ω -CgTx-sensitive, DHP-resistant component of calcium current, defined as N-type.

We have also examined the ability of NE to inhibit dorsal root ganglion neuron whole-cell calcium current before and after the test cell was treated with 9μ M ω -CgTx (Fig. 6). As with GABA, a saturating dose of NE (10 – 50μ M) inhibited a large percentage of calcium current ($22\% \pm 7\%$; $n = 12$; Fig. 6, traces 1 and 2) before ω -CgTx treatment, but had no effect on the residual inward current after ω -CgTx treatment ($<1\%$; Fig. 6, traces 4 and 5). Thus, like GABA, NE appears to inhibit exclusively N-type current.

Differences in NE- and GABA-induced inhibition of calcium current

Inhibition of HVA calcium current by GABA is characterized by an invariant and prominent slowing of the activation kinetics (Fig. 5C). This effect is clearly seen even with short-duration (10 msec) depolarizations and is mimicked by the GABA_B receptor agonist baclofen (data not shown). Upon depolarization to 0 mV, currents taken before GABA application reach a peak within 20 msec, while after application of 100μ M GABA calcium currents often do not reach a peak by the end of a 50 msec voltage step. In contrast, the inhibition produced by NE is most often not accompanied by such a kinetic change (Fig. 6). In these cells, the NE-resistant and NE-sensitive components of the current are kinetically similar (Dunlap and Fischbach, 1981; Forscher and Oxford, 1985). A small percentage of dorsal root ganglion neurons, however, do exhibit an effect of NE on the activation kinetics that differs from the slowing produced by GABA. In these cells, the initial activation rate remains little

Figure 6. NE also inhibits an ω -CgTx-sensitive calcium current in chick dorsal root ganglion neurons. Whole-cell calcium currents measured during 50 msec voltage steps to 0 mV from -60 mV under the following conditions: control (trace 1), during superfusion of the test cell with $10 \mu\text{M}$ NE (trace 2), after recovery from NE application (trace 3), after application of $9 \mu\text{M}$ ω -CgTx (trace 4), and during subsequent application of NE (trace 5). All solutions contained 50 mM TEA and $100 \mu\text{M}$ 4-aminopyridine.

affected by the transmitter but an additional, slower component to the activation can be observed with long (> 100 msec) pulses. The significance of such differences in transmitter action is unclear at present.

Discussion

Pharmacological separation of calcium current subtypes

Using the calcium channel blocker ω -CgTx and two DHPs—(+)-(*S*)-202-791, which enhances L-type Ca channel activity, and nimodipine, which inhibits these same channels—we have discerned only two pharmacologically separable components of HVA calcium current present in dorsal root ganglion neurons of embryonic chick. The ω -CgTx-sensitive component makes up the vast majority of the HVA current, confirming results of others for chick dorsal root ganglion neurons (Kasai et al., 1987; Aosaki and Kasai, 1989) and rat SCG neurons and PC12 cells (Plummer et al., 1989). Our studies, however, do not demonstrate the reversible component to the ω -CgTx-sensitive HVA current reported by these investigators. Additionally, we have found that the component of calcium current in chick dorsal root ganglion neurons that is insensitive to ω -CgTx is completely blocked by nimodipine, indicating an L-type current. In contrast to other tissues, no HVA calcium current that was resistant to both DHPs and ω -CgTx was observed. Such a resistant component makes up $> 90\%$ of the HVA current in cerebellar Purkinje neurons, 50% in hippocampal CA1 neurons (Regan et al., 1991), 40% in PC12 cells, and 10% in SCG neurons (Plummer et al., 1989).

We report that ω -CgTx is unable to inhibit the slow component of tail current produced by (+)-(*S*)-202-791 modulation of L-type calcium current, and on this basis have concluded that ω -CgTx does not inhibit L-type channels. While our experiments do not rule out the possibility that there is a component of DHP-sensitive current that is protected from the blocking effects

of ω -CgTx by (+)-(*S*)-202-791, we feel, for the following reasons, that if such a current exists, it must be a minor component of the total calcium current. (1) In the absence of (+)-(*S*)-202-791, ω -CgTx often does not inhibit all calcium current, but rather leaves behind a current that is exquisitely sensitive to DHPs. (2) In the great majority of cells, ω -CgTx blocks most of the calcium current even in the presence of (+)-(*S*)-202-791. Thus, our investigations have led us to a simple pharmacological profile of calcium current types in chick dorsal root ganglion neurons. N-type current, which is irreversibly blocked by ω -CgTx, and L-type, which is unaffected by ω -CgTx but sensitive to DHPs.

Kinetic separation of calcium current subtypes

Original reports of L- and N-type calcium currents in chick dorsal root ganglion neurons suggested that the two current components could be resolved by their differential inactivation kinetics and sensitivity to holding potential (Nowycky et al., 1985a; Fox et al., 1987a). As a result, conclusions of many previous studies that have relied on these kinetic distinctions to determine the pharmacological profiles and physiological significance of L- and N-type calcium channels are likely to be incorrect. As a case in point, McCleskey et al. (1987) and Fox et al. (1987a) interpreted the ω -CgTx block of long-lasting current in dorsal root ganglion neurons as indicating a block of L-type current. Similarly, the fact that NE and GABA inhibited a long-lasting component of HVA calcium current (that remained available at -40 mV holding potentials) prompted us to conclude that the transmitters also targeted L-type channels (Ranc et al., 1987; Holz et al., 1989). However, steady-state inactivation studies reported here for the HVA current indicate that a significant fraction of the ω -CgTx-sensitive (non-L-type) component in dorsal root ganglion neurons remains at such depolarized holding potentials. Furthermore, both the ω -CgTx-sensitive and the DHP-sensitive current components inactivate only slightly during a 150 msec pulse (when depolarized holding potentials are used) and thus exhibit similar long-lasting wave forms. Since the pharmacological agents employed in the present study (ω -CgTx and DHPs) can differentiate these kinetically similar current components, they allow us now to argue that the N-type channel is the more likely target and that L-type calcium channels are not modulated by the two transmitters.

Differences in transmitter-mediated modulation of HVA calcium channels

Although NE and GABA both appear to target the same current type, certain characteristics of the modulation differ for the two transmitters. Inhibition by GABA of HVA current in peripheral neurons is characterized by a very prominent slowing of the activation kinetics (Fig. 5; Grassi and Lux, 1989). Such effects on peripheral neurons are common to many other transmitters and peptides as well, including dopamine (Marchetti et al., 1986), somatostatin (Ikeda and Schofield, 1989), ACh (Wanke et al., 1987), adenosine (Kasai and Aosaki, 1989), and leutinizing hormone-releasing hormone (Elmslie et al., 1990). Inhibition of peripheral neuron HVA calcium current by transmitters such as NE, neuropeptide Y, and bradykinin, on the other hand, is most often not associated with such kinetic alterations (Dunlap and Fischbach, 1981; Forscher and Oxford, 1985; Bean, 1989; Ewald et al., 1989). Similar discrepancies have been observed on central neurons as well (Toselli et al., 1989; Sah, 1990). At present, it is unclear how such differences in transmitter action

are brought about. Differing kinetics of activation between GABA- and NE-modulated currents in embryonic chick dorsal root ganglion neurons are unlikely to be due to different subtypes of ω -CgTx-sensitive calcium channels: Swandulla and Armstrong (1988) demonstrated in acutely dissociated neurons that the deactivation kinetics of HVA calcium current are well described by a single exponential function. They argue that freshly dissociated neurons contain a single type of HVA calcium current.

All transmitters reported to inhibit HVA calcium current are thought to require the activation of GTP-binding proteins (Tsien et al., 1988; Rane and Dunlap, 1990). Experiments in which GTP- γ -S has been used to activate G-proteins in the absence of transmitter (Dolphin et al., 1988; Marchetti and Robello, 1989; Elmslie et al., 1990) demonstrate a prominent inhibition of calcium current and slowing of the activation kinetics, mimicking the action of several transmitters. In contrast to GTP- γ -S, which promotes the indiscriminate activation of all available G-proteins (Gilman, 1987), the transmitters are likely to exhibit some selectivity in their G-protein interactions (Hescheler et al., 1987; Ewald et al., 1989). It is possible that the slowing of HVA calcium current activation kinetics is associated with some, but not all, G-proteins, and that differences in transmitter effects on current kinetics reflect selectivity of receptor-G-protein interactions.

Implications for the regulation of neurotransmitter release

The presence of only two HVA current components blocked by selective and potent antagonists makes chick dorsal root ganglion neurons a powerful experimental system for testing the role of calcium channel subtypes in the regulation of cellular function. This new information also allows us to review past results in a new light. Holz et al. (1988) demonstrated that the electrically stimulated release of substance P from cultured dorsal root ganglion neurons was eliminated by ω -CgTx, suggesting that N-type channels play a prominent role in triggering exocytosis. Significantly, the electrically evoked release was relatively resistant to blockade by the DHP antagonist nifedipine but was enhanced 45% by the DHP agonist Bay K 8644. This indicates that the L-type calcium channel might only play a significant role in triggering peptide release under conditions in which its calcium permeability is potentiated (Miller, 1987). In contrast, substance P release evoked by chronic depolarization of the neurons with elevated potassium is much more sensitive to DHPs (blocked 58% by nifedipine and enhanced 115% by Bay K 8644). Whether this enhanced sensitivity results from the voltage-dependent action of DHPs (Sanguinetti and Kass, 1984; Sanguinetti et al., 1986; Rane et al., 1987) or differential inactivation of N-type over L-type channels (Jones and Jacobs, 1990) remains unclear. Knowledge of the distinct pharmacological identities of these channel types, however, will now enable a determination of their relative contributions to release of peptide and classical transmitters from these neurons.

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