Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture

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ABSTRACT Opioid receptors are differentially coupled to ion channels. μ- and δ-opioid receptors are coupled to calcium- and/or voltage-dependent potassium channels and κ-opioid receptors are coupled to voltage-dependent calcium channels. Using the single-electrode voltage-clamp technique, we investigated the effect of the κ-opioid receptor agonist dynorphin A on somatic calcium currents of mouse dorsal root ganglion (DRG) neurons in culture. Three different calcium currents were recorded: a small transient current activated positive to −60 mV; a large, inactivating current activated positive to −50 mV; and a moderate, slowly inactivating current activated positive to −40 mV. The first was less sensitive to cadmium block than the others. These calcium currents were similar to those described in other cells, which have been designated T, N, and L calcium currents, respectively. The opioid peptide dynorphin A reduced calcium current by selectively reducing the large inactivating (N) calcium current. Naloxone, an opioid receptor antagonist, reversed this action of dynorphin A. N calcium current is the predominant calcium current in DRG neurons. If N calcium channels are present in primary afferent terminals, and if they are coupled to κ-opioid receptors as in the soma, these results suggest a mechanism by which dynorphin A inhibits calcium influx and neurotransmitter release.

Recent studies have established that multiple opioid receptors including μ-, δ-, and κ-opioid receptors are present in the nervous system (1–3) and that they are differentially coupled to ion channels (5–10). μ- and δ-opioid receptors have been shown to be coupled to potassium channels (5–8, 11, 12). Opioid ligands selective for μ-opioid receptors hyperpolarized myenteric (13, 14) and locus ceruleus (5, 11) neurons and reduced the duration of calcium-dependent action potentials of dorsal root ganglion (DRG) (15–17) and locus ceruleus (12) neurons. Opioid agonists selective for δ-opioid receptors hyperpolarized coecal submucosal neurons (18) and reduced the duration of calcium-dependent action potentials of mouse DRG neurons (16–18). All of these actions of μ- and δ-opioids were due to enhancement of potassium current. Dynorphin A is an endogenously occurring opioid peptide (19, 20) that is a κ-opioid receptor agonist (21–24). Dynorphin A reduced calcium-dependent action potential duration of DRG (9, 10, 25, 26) and myenteric (27) neurons. The dynorphin A effect was due to a reduction of a voltage-dependent calcium current (26, 28).

The coexistence of two different calcium currents has been demonstrated in various tissues (e.g., refs. 29–34) as well as in neurons (35–37). Recently, a third type of calcium current was described in chick DRG neurons (38). The three calcium currents had different activation and inactivation voltage ranges, and the calcium channels had different conductances and kinetics. The currents were differentially affected by the calcium channel blocker cadmium, the agonist dihydroxyindine Bay K 8644, and the antagonist dihydropyridines (38–40). The calcium currents were designated T, N, and L by Nowycky et al. (38).

These observations raise the possibility that the κ-opioid receptor could be linked to one or more calcium channel types. In the present study we recorded three different somatic calcium currents in mouse DRG neurons and assessed the effect of dynorphin A on these T, N, and L calcium currents.

MATERIALS AND METHODS

Cell Culture. DRG neurons were grown in cell culture as described (41). Spinal cords, with DRG attached, were dissected from 12- to 14-day fetal mice and mechanically dissociated. The resulting suspension was plated onto 35-mm collagen-coated dishes at various densities, usually about one-eighth cord per dish. Initial medium contained 5% horse serum and 5% Nu-Serum (Collaborative Research, Waltham, MA) in minimum essential medium (MEM) (Eagle’s; GIBCO). Nerve growth factor (NGF) was added at a final concentration of 10 ng/ml. After 4–5 days in culture, 5′-fluoro-2′-deoxyuridine and uridine were added to the cultures to suppress the growth of nonneuronal cells. Subsequent medium changes were at 3- to 4-day intervals; the media had the same composition as above, including NGF at a concentration of 10 ng/ml to promote growth and maturation of DRG neurons. Cultures were used for experiments at 4–12 weeks after plating.

Intracellular Recording and Single-Electrode Voltage Clamp. For voltage-clamp experiments, recording medium was modified to suppress sodium and potassium currents, thereby virtually isolating calcium currents. Media (pH 7.3–7.4) contained (in mM): choline chloride, 67.0 or 142.0; Tris base, 13.0; KCl, 5.3, or CsCl, 5.3; CaCl2, 2.0; MgCl2, 0.8; glucose, 5.6; tetraethylammonium ion, 25 or 100. As a rule, when Cs+-substituted medium was used, the concentration of tetraethylammonium ion was 25 mM (choline chloride = 142.0); otherwise, it was 100 mM (choline chloride = 67.0).

For all experiments, cultures were placed on the heated (35°C) stage of an inverted phase-contrast microscope. Medium was covered with mineral oil to prevent evaporation. Impalements under visual observation were made with micropipettes filled with 3 M CsCl to further suppress potassium currents. A modified bridge circuit allowed simultaneous current passage and voltage recording with single micropipettes (20–30 ΜΩ). A single microelectrode voltage-clamp amplifier (Axoclamp-2, Axon Instruments, Burlington, CA) was used that switched between voltage sampling and current injection at 6–7 kHz with a 70–30% duty cycle. Head stage outputs were continuously monitored on a separate oscilloscope to ensure that voltage settling occurred prior to the voltage sample and hold measurement.

Voltage step commands were generated using the program p-Clamp (Axon Instruments) on a microcomputer (IBM-XT).

Abbreviations: DRG, dorsal root ganglion; NGF, nerve growth factor; Vh, holding potential(s).
Current traces were digitized, stored, and analyzed using this program as well. Voltage commands were 100 or 300 msec in duration and were sampled at approximately 5.0 and 1.6 kHz, respectively (512 points per sweep). Leak currents were determined by two different methods. Hyperpolarizing voltage commands of magnitudes equal to the depolarizing commands used to evoke the inward currents yielded currents that were digitally added to the inward currents to give calcium currents ("leak-subtracted"). Alternatively, leak currents were recorded during equivalent depolarizing commands after block of calcium currents by cadmium; these currents were digitally subtracted from the relevant inward currents ("cadmium leak-subtracted").

Recordings were made from neurons 15–30 μm in diameter. Estimates of neuronal input resistance were made from hyperpolarizing and depolarizing voltage steps in the absence or presence of cadmium. Initial resistances at −70 to −90 mV ranged from 14 to 50 MΩ and inward calcium currents ranged from 5 to 15 nA. Although the recording medium allowed recording of inward currents virtually free of sodium and potassium currents, cell viability was limited. In general, cells were not used unless resting membrane potentials were at least −45 mV. Barium could be substituted for calcium, but this tended to obscure T calcium currents and enhance L calcium currents (unpublished results).

**RESULTS**

**Three Calcium Currents Were Recorded from Mouse DRG Neurons.** Initial experiments were directed at isolating separate components of the inward currents recorded from mouse DRG neurons (Fig. 1). Because these currents were similar to those described in chick DRG neurons, we have adopted the terminology of Nowycky et al. (38). T calcium current (Fig. 1A) was a small transient inward current requiring a V₃ more negative than −80 mV to remove inactivation. It was activated positive to −60 mV. When evoked from V₃ greater than −80 mV by commands to −50 to −40 mV, T calcium current was 0.5–1.0 nA. L calcium current (Fig. 1B) was a moderate, very slowly inactivating current. It was activated positive to −40 mV and was maximal at 0 to +10 mV. When evoked from V₃ between −50 and −40 mV by commands to −20 to 0 mV, L calcium current was 1.0–3.0 nA. N calcium current (Fig. 1C) was a moderately rapidly inactivating current (100–800 msec). V₃ negative to −70 mV were necessary to remove inactivation. It was activated positive to −50 mV and was maximally activated at about −20 mV; the inactivation range was −80 to −30 mV. When evoked from V₃ greater than −80 mV by commands to −20 to 0 mV, both N and L calcium currents were evoked (Fig. 1C). The N calcium current, however, was larger than the L calcium current, ranging from 5 to 15 nA. In 20% of neurons, T and N calcium current activation ranges also overlapped, so that T calcium current could not be isolated unequivocally. N and L calcium currents, however, could be recorded in all neurons studied. They were quite sensitive to blockade by cadmium ions (≥20 μM), whereas T calcium current was partially resistant to concentrations ≤100 μM (Fig. 1D). The differential activation and inactivation voltage ranges, inactivation rates, and sensitivity to cadmium suggested three separate components to the calcium current.

In practice, T calcium current was elicited from a V₃ of −90 mV with depolarizing steps of +30 to +40 mV for 100 msec. Combined N and L calcium currents were evoked from a V₃ of −80 mV with steps of +40 to +60 mV. At a V₃ of −80 mV, a partially inactivated T calcium current was often evoked as well but usually contributed <10–15% of the peak current. If the voltage command was prolonged to 300 msec, N calcium current was largely inactivated by the end of the command in most neurons, allowing an estimate of L calcium current as the residual inward current (Fig. 1C). This was usually more reliable than isolating L calcium current (V₃ = −50 to −40 mV, with steps of +40 to +20 mV) since at these V₃, L calcium current was partially activated and, therefore, slowly inactivating.

**Dynorphin A Selectively Reduced N Calcium Current.** Recordings were obtained from 255 DRG neurons. In 61 neurons (24%), inward currents were reversibly reduced by dynorphin A. This proportion was similar to that of previous studies (9, 10, 26, 28) and may reflect differences in expression of κ-opioid receptors among DRG neurons. Of these 61 neurons, 37 yielded stable recordings for analysis. The remaining neurons were eliminated because of unstable or
variable leak current, making comparisons between trials difficult.

Fig. 2 illustrates the effect of dynorphin A (1 μM) on calcium currents. T and L calcium currents were unaffected by dynorphin A (Fig. 2 A and B). Peak N and L calcium currents, however, were reduced 5–36% by dynorphin A in those neurons analyzed using digitized leak-subtracted current traces (Fig. 2C). The mean reduction was 19% ± 2.3% (± SEM, n = 14). As expected, if only N calcium current was affected by dynorphin A, the early (N and L calcium currents) but not the late (primarily L calcium current) calcium current was reduced (Fig. 2C). This effect was completely reversible.

Although the results shown suggest that dynorphin A reduced N calcium current, an enhancement of a residual early transient outward potassium current was possible. Fig. 2D shows the lack of effect of dynorphin A on the current elicited by a depolarizing step in the presence of cadmium. The trace shown was generated by subtracting the current produced by a depolarizing step in the presence of cadmium from that produced in the presence of cadmium and dynorphin A. No outward current was evoked. Cadmium prevented activation of calcium-dependent potassium current and thus did not eliminate the possibility that dynorphin A was activating this current. However, this issue was addressed in a previous study (ref. 28; see Discussion).

Calcium current–voltage plots were obtained from DRG neurons prior to and following dynorphin A application. Neurons were held at −90 mV and 100- to 300-msec commands were applied. Negative commands to −120 mV and positive commands to 0 mV were applied in 10-mV steps. Current–voltage relationships were plotted for peak current (measured at 5–15 msec) and for late current (measured at the end of the voltage-clamp steps—i.e., 100 or 300 msec). The late current–voltage relationships were plotted for neurons in which the large transient current (N calcium current) appeared to be fully inactivated. Dynorphin A did not affect currents evoked with negative commands or with positive commands up to −50 mV. The positive commands included the voltages activating T calcium current. Positive to −50 mV, T/N/L calcium currents were evoked, and dynorphin A reduced the magnitude of the peak inward current at all commands above −50 mV (Fig. 3). Dynorphin A did not affect the late currents measured 100–300 msec after the onset of the voltage command, either with hyperpolarizing and depolarizing small commands or with large depolarizing commands after inactivation of the large transient (N) calcium current (Fig. 4).

The Effect of Dynorphin A on N Calcium Current Was Reversed by Naloxone. In three of three cells tested, naloxone (1 μM) blocked the reduction in peak calcium current produced by dynorphin A (1 μM). Dynorphin A reduced the early calcium current when applied by pressure ejection to a neuron held at −80 mV (Fig. 5A). After the current returned to control amplitude (not shown), naloxone (1 μM) was applied by diffusion from a large-tipped micropipette for 30 sec. Reapplication of dynorphin A then had no effect (Fig. 5B).

**DISCUSSION**

The present experiments show that mouse DRG neurons in primary cell culture had three separate calcium currents, similar to those designated T, N, and L calcium currents in chick DRG neurons (38). In addition, we have shown that dynorphin A acted primarily, if not solely, on the N calcium current. This effect was reversed by naloxone. The calcium current–voltage relationship for the N calcium current was not altered by dynorphin A application (Fig. 4).

**Fig. 2.** Dynorphin A selectively reduced N calcium current. The currents shown in A–D were recorded from different neurons. In each case, dynorphin A reduced peak inward current. Specific voltage-clamp protocols were then used to isolate the separate calcium currents (see Fig. 1). A control trace (trace 1) and traces obtained after pressure ejection of 1 μM dynorphin A for 2–4 sec (trace 2) and after recovery (trace 3) are shown. Currents were evoked every 15–30 sec. Digitized leak-subtracted currents are shown. (A) T calcium current was unaffected by dynorphin A. Vh was −92 mV; the 100-msec depolarizing step was to −52 mV. (B) L calcium current was unaffected by dynorphin A. Vh was −45 mV; the 100-msec depolarizing step was to −5 mV. (C) The early phase of the T/N/L current was selectively reduced by dynorphin A, suggesting an action on the N current alone (see text). Vh was −65 mV; the 100-msec voltage command was to −15 mV. (D) Dynorphin A affected calcium current only. Recordings were from a neuron that had a response to dynorphin A similar to that shown in C. A micropipette containing 100 μM cadmium (Cd) was brought near the neuron, and the cadmium was applied by pressure ejection for 5 sec and then by diffusion. A depolarizing voltage command yielded an outward current (leak). Dynorphin A (DYN) was then applied by pressure ejection for 4 sec, and the voltage step was repeated. This yielded another outward current; from this, the current obtained after the initial cadmium application was digitally subtracted. The resulting trace is shown. Dynorphin A produced no change in the current in the presence of cadmium. Vh was −95 mV; the 100-msec voltage command was to −25 mV.

**Fig. 3.** Peak current–voltage relationship of the dynorphin A block of calcium current. Cadmium leak-subtracted data are shown. To differentiate between N and L effects, the current traces were measured at peak calcium current (7 msec) and at the late calcium current (Fig. 4). Dynorphin A application reduced the peak current, without a change in the voltage dependence of the peak current. Vh was −90 mV; 100-msec voltage steps were to the indicated voltages.
Dynorphin A may decrease the number of N calcium channel openings. The major effect of dynorphin A was on the peak inward current and not on the rate of inactivation, suggesting against enhancement of inactivation. Most experiments were conducted at a VH at which N calcium channels were not inactivated. Therefore, it is unlikely, though possible, that dynorphin was acting on the inactive channel to slow its return to the active state. To test this, we applied dynorphin A to cells at Vh at which N channels were either noninactivated or ~50% inactivated. In preliminary experiments, dynorphin A produced similar reductions in calcium current evoked from each potential (also, compare Figs. 2 and 5). A more definitive answer will require further study.

Dynorphin A (1 μM) produced a 5–36% reduction in the peak calcium current, which was probably due to a reduction in the N calcium current. This concentration produced a maximal reduction of the duration of calcium-dependent action potentials in DRG neurons (10). Though the percentage reduction of N calcium current would be somewhat greater (i.e., if L and T calcium current components were subtracted from the peak current), it is clear that a large proportion of N calcium current was still activated in the presence of dynorphin A. This suggests that only a proportion of N calcium channels may be available for coupling to κ-opioid receptors.

The mechanism by which dynorphin A reduced N calcium current is not known at present. It is possible that the κ-opioid receptor is directly coupled to N calcium channels by way of a GTP binding protein or is indirectly coupled by way of an intracellular second messenger, or both. Modulation of calcium channels by cyclic AMP or A kinase has been reported (43-45) as has an enhancement of calcium current by phorbol esters (presumably by way of protein kinase C) in Aplysia (46, 47). Previous work has shown a reduction of DRG neuron calcium currents by phorbol esters (48-50) that seems to be mediated largely through N channels (unpublished observations). The effect of dynorphin A on N calcium current may or may not be mediated through protein kinase C. These data suggest, however, that dynorphin A and protein kinase C may both have independent actions on specific calcium currents in sensory neurons.

Opioid receptors have been localized on both dorsal horn cells and terminals of primary afferents (51–53). These receptors have also been found on DRG neuron somata in vitro and in vivo (16–18, 54). Dynorphin A, acting at κ-opioid receptors, reduced calcium currents and appeared to do so by acting on the N calcium channel. Of particular interest in this regard are recent data demonstrating a role of N calcium channels in neurotransmitter release. Block of L calcium channels by dihydropyridines was not sufficient to inhibit depolarization-induced release of neurotransmitter in sympathetic ganglion cells (34, 55). Block of both N and L calcium channels with a calcium channel-specific toxin, ω-toxin from Conus geographus, did, however, prevent neurotransmitter release (55). In this way, the reduction of the predominant (N) calcium current in DRG neurons by dynorphin A could reduce neurotransmitter release. A presynaptic action of dynorphin A on neurotransmitter release has been reported in the locus ceruleus in vitro (56). In this way, dynorphin may modulate release of neurotransmitter from primary afferents onto spinal cord dorsal horn neurons.

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