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DYNORPHIN A DECREASES VOLTAGE-DEPENDENT CALCIUM CONDUCTANCE OF MOUSE DORSAL ROOT GANGLION NEURONES

BY ROBERT L. MACDONALD AND MARY ANN WERZ

From the Department of Neurology, University of Michigan, Ann Arbor, MI 48104, U.S.A.

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SUMMARY

1. The actions of the opioid peptides dynorphin A and (Leu)enkephalin were assessed on calcium-dependent action potentials and inward calcium currents recorded from somata of mouse dorsal root ganglion (d.r.g.) neurones grown in primary dissociated cell culture. Dynorphin A and (Leu)enkephalin decreased the duration of somatic calcium-dependent action potentials in a portion of d.r.g. neurones impaled with potassium acetate-filled micropipettes. When substantial potassium conductance was blocked by intracellular injection of caesium acetate, d.r.g. neurones continued to respond to dynorphin A but responses to (Leu)enkephalin were abolished.

2. In voltage-clamp experiments, dynorphin A but not (Leu)enkephalin reduced the magnitude of inward calcium currents. Dynorphin A responses were blocked by the opiate antagonist naloxone. The dynorphin A effect was due to reduction of voltage-dependent calcium conductance since dynorphin A reduced depolarization-evoked inward currents but did not alter membrane conductance following blockade of calcium channels by cadmium, and because dynorphin A reduced the instantaneous current–voltage slope (chord conductance) during step commands that produced maximal activation of voltage-dependent calcium conductance.

3. Dynorphin A binds with high affinity to κ-opioid receptors. (Leu)enkephalin, which has affinity for both μ- and δ-receptors but not for κ-opioid receptors, was without effect on calcium conductance. Therefore, we suggest that κ-receptors are coupled to voltage-dependent calcium-channels and that binding of dynorphin A produces a decrease of calcium current.

INTRODUCTION

Multiple opioid receptors have been suggested to be independently distributed within the nervous system. The most carefully studied receptors have been μ-, δ- and κ-opioid receptors. Evidence for these receptor types includes: (1) different rank order potencies for displacement of radio-labelled opioid ligands (Chang, Hazum & Cuatrecasas, 1980; Kosterlitz, Paterson & Robson, 1981), (2) differences in receptor localization in brain regions (Chang, Cooper, Hazum & Cuatrecasas, 1979; Goodman, Snyder, Kuhar & Young, 1980; Goodman & Snyder, 1982), (3) selective protection

of opioid receptors against alkylation (Robson & Kosterlitz, 1979; Chavkin & Goldstein, 1981; James & Goldstein, 1984), (4) development of selective receptor agonists and antagonists (Lahti, Von Voigtlander & Barsuhn, 1982; Mosberg, Hurst, Hruby, Gee, Yamamura, Galligan & Burks, 1983; Cotton, Giles, Miller, Shaw & Timms, 1984), and (5) differences in behavioural effects (Wood, Rackham & Richard, 1981; Schmauss & Yaksh, 1984). At present, however, little is known regarding the coupling of opioid receptors to specific ion channels. It is likely that μ-receptors (Pepper & Henderson, 1980; Williams, Egan & North, 1982; Werz & Macdonald, 1983*a*,*b*; Yoshimura & North, 1983) and δ-receptors (Werz & Macdonald, 1982*a*,*b*, 1983*a*,*b*) are coupled to potassium channels and that binding of opioid ligands to these receptors results in an enhancement of potassium conductance. Furthermore, because of this increase of potassium conductance, ligands with affinity for μ- and δ-receptors have been reported to decrease the duration of somatic calcium-dependent action potentials of locus coeruleus neurones (North & Williams, 1983) and dorsal root ganglion (d.r.g.) neurones (Werz & Macdonald, 1983*a*,*b*) by hastening membrane repolarization. In contrast, dynorphin A, an opioid peptide with high affinity at κ-receptors, decreased the duration of d.r.g. neurone calcium-dependent action potentials that were associated with decreased action potential after-hyperpolarization and persisted after intracellular injection of caesium, a potassium channel blocker (Macdonald & Werz, 1983; Werz & Macdonald, 1984*a*,*b*, 1985). Recently this finding has been confirmed in myenteric neurones (Cherubini & North, 1985). Thus, we suggested that dynorphin A might act on d.r.g. neurones at a distinct opioid receptor, probably the κ-receptor, that is coupled to voltage-dependent calcium channels. In the present investigation we directly assessed dynorphin A actions on inward calcium currents using the single-electrode voltage-clamp technique. A preliminary report of this work has appeared in abstract form (Macdonald & Werz, 1984).

METHODS

Cell culture

Neuronal cultures were prepared, as previously described (Ransom, Christian, Bullock & Nelson, 1977), by dissecting spinal cords with attached dorsal root ganglia from 12–14-day-old fetal mice, then mechanically dissociating the tissue, and plating the resultant cell suspension on 35 mm collagen-coated plates at a density of $\frac{1}{8}$ or $\frac{1}{4}$ spinal cord per plate. Initial culture medium consisted of 80% Eagle's minimum essential medium, 10% horse serum, and 10% fetal calf serum. Approximately 4 days after initial plating, 5′-fluoro-2′-deoxyuridine and uridine were added to the cultures to inhibit the division of non-neuronal background cells, after which the growth medium was modified to 90% Eagle's minimum essential media and 10% horse serum. All culture media contained nerve growth factor at a concentration of 5 ng/ml to promote survival and growth of d.r.g. neurones. 2–12-week-old cultures were used in electrophysiology experiments.

Intracellular recording and single micro-electrode voltage clamp

For intracellular recording of somatic calcium-dependent action potentials, neurones were placed in a Tris-buffered balanced saline (pH 7·3, 320 mosmol) that contained (mM): NaCl, 135·0; KCl, 5·3; $MgCl_2$, 0·8; $CaCl_2$, 5·0; Tris-HCl, 13; glucose, 5·6; and tetraethylammonium (TEA), 5·0. The calcium concentration was elevated to enhance the magnitude of calcium-dependent action potentials and currents. For electrophysiological experiments, cultures were placed on the heated stage (35 °C) of an inverted phase contrast microscope allowing neuronal impalement under visual observation. Intracellular recordings were made with high resistance (20–50 MΩ) micropipettes

filled with 4 M-potassium acetate or 4 M-caesium chloride. A modified bridge circuit allowed simultaneous current passage and voltage recording using single micropipettes.

For voltage-clamp experiments, the potassium channel blocker caesium was substituted for potassium in the bathing medium. Prior to recording in a caesium-containing (5·3 mM), potassium-free medium, cultures were bathed in the recording medium for 15 min with three changes to reduce internal potassium concentration. In addition, the sodium channel blocker, tetrodotoxin (TTX) was added (5–10 μM). Neurones were voltage clamped using a single micro-electrode voltage-clamp pre-amplifier (Axoclamp 2) that switched between voltage sampling and current injection modes at 6 kHz with a 70–30 duty cycle. Micropipettes used for voltage-clamp recordings were filled with 3 M-caesium chloride and had resistances of 15–25 MΩ.

Drug application by pressure ejection

(Leu)enkephalin (Peninsula) and dynorphin A (Peninsula) were dissolved in acetic acid (1 mM) and frozen in aliquots. Peptides were diluted in the recording medium containing 0·1 % bovine serum albumin and administered to neurones by application of pressure (0·5–1·5 lbf/in^2 with 1 lbf/in^2 = 6·7 kPA) to micropipettes (tip diameters 2–5 μm) that contained the diluted solution. Micropipette tips were broken under direct visual observation, approximately 400 × magnification, to be of similar tip diameters. Micropipettes containing opioid peptides were positioned to within 5 μm of d.r.g. neurones during pressure application and then were removed from the position near the cell. Using this paradigm, opioid peptide-free medium was without effect as long as the osmolarity of the medium bathing the culture was the same as that contained in the pressure ejection micropipette. The development of osmolarity differences was prevented by placing a thin coat of mineral oil over the medium bathing the culture to retard evaporation. The opioid antagonist, naloxone (Endo Laboratories), and the calcium channel blocker, cadmium ion, were applied by diffusion from separate micropipettes with tip diameters of 10–15 μm which were positioned 10–15 μm from d.r.g. neurones. The solution in the cadmium-containing diffusion micropipettes was recording medium with cadmium chloride added (100–200 μM).

Statistical analysis

Results are expressed as means ± S.E. of mean.

RESULTS

Action potentials and action potential after-hyperpolarizations have calcium-dependent components

Mouse d.r.g. neurone action potentials were previously shown to have two components: these were an initial sodium-dependent component that was only partially sensitive to block by tetrodotoxin as well as a slower calcium-dependent component (Heyer & Macdonald, 1982). Action potentials had durations of about 2 ms and had a convex inflexion on the repolarizing phase. Local application of the calcium channel blocker, cadmium ion at 100 μM, abolished the convex inflexion. Addition of 5 mM-TEA to the recording medium enhanced action potential duration to 5–25 ms (Fig. 1*A*1, *B*1), and cadmium reversed this prolongation. However, substantial potassium conductance remained in the presence of 5 mM-TEA as indicated by large after-hyperpolarizations and resting membrane potentials of −50 to −70 mV. Further blockade of potassium conductance was obtained by intracellular injection of caesium. Initially, upon impalement with caesium acetate-filled micropipettes, action potentials did not differ from those recorded with potassium acetate-filled micropipettes. Over 5–15 min, membrane potential declined to 0 to −20 mV and input resistance increased. Thereafter, the membrane potential was held at about −60 mV by injection of current. Action potentials could then be evoked with

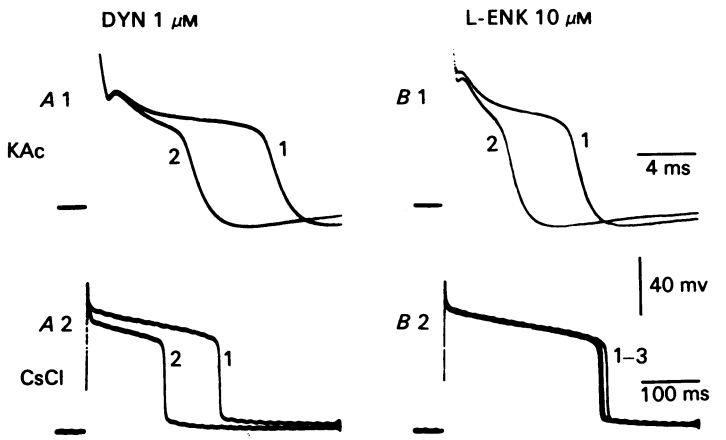

Fig. 1. Dynorphin A (DYN) responses persisted while (Leu)enkephalin (L-ENK) responses were abolished by intracellular caesium injection. Comparison of responses to the opioid peptides during recording with potassium acetate-filled micropipettes (*A*1, *B*1) and during subsequent re-impalement with caesium chloride-filled micropipettes (*A*2, *B*2). Intracellular injection of caesium did not attenuate neuronal responses to dynorphin A (*A*2) but abolished the response to (Leu)enkephalin (*B*2). All traces are from the same neurone. Action potentials were evoked every 15 s. In all traces, action potential (1) was evoked prior to and action potentials (2, 3) after a 1 s application of opioid peptide.

durations of 100 ms to 2 s which were without after-hyperpolarizations (Fig. 1 *A*2, *B*2). Cadmium abolished the broad plateau of these action potentials.

Dynorphin A decreased action potential duration following intracellular caesium acetate injection

As previously reported (Werz & Macdonald, 1983*a*, 1985), during recording with potassium acetate-filled micropipettes, dynorphin A (10 nM–1 M) (Fig. 1 *A*1) and (Leu)enkephalin (50 nM–5 M) (Fig. 1 *B*1) decreased calcium-dependent action potential duration of a subpopulation of neurones. Both dynorphin A and (Leu)enkephalin decreased the duration of somatic calcium-dependent action potentials without affecting resting membrane potential or conductance. To assess opioid effects on resting membrane conductance, depolarizing stimuli to evoke action potentials were discontinued and small, constant, hyperpolarizing current pulses were applied. Neither dynorphin A ($n = 3$) nor (Leu)enkephalin ($n = 4$) affected resting membrane conductance as indicated by the absence of effect by the opioids on the amplitude of voltage responses to the constant current injections. Dynorphin A responses differed from (Leu)enkephalin responses in that dynorphin A reponses persisted following intracellular caesium injection (Fig. 1 *A*2, *B*2).

Dynorphin A decreased inward currents

For voltage clamp, neurones were bathed in potassium-free medium containing caesium and were impaled with caesium chloride-filled micropipettes. Neurones were held at potentials of about −60 mV and voltage step commands of 75 ms duration and varying amplitude were applied. Currents were linearly related to voltage

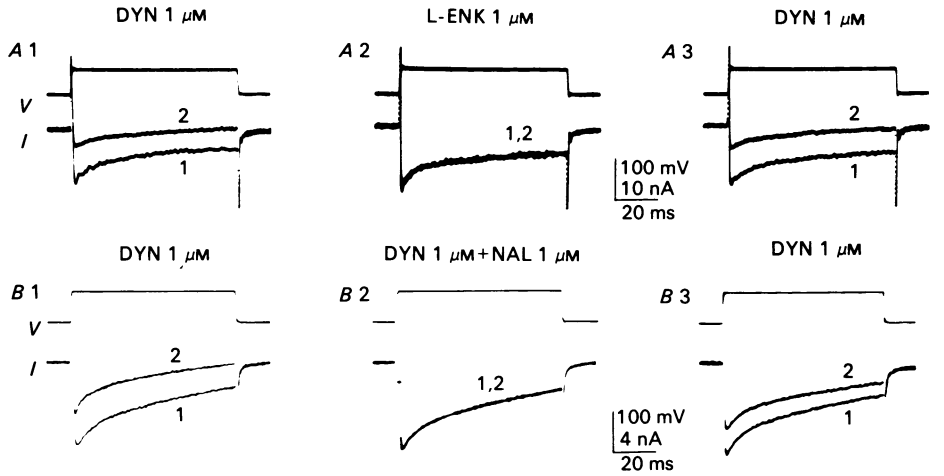

Fig. 2. Dynorphin A (DYN) decreased calcium-dependent inward currents. For voltage-clamp experiments, neurones were bathed in potassium-free medium containing caesium and were impaled with caesium chloride-filled micropipettes. Step depolarizations from a holding potential of −60 mV evoked currents that were net inward over the 75 ms duration of the command. *A*, dynorphin A decreased the magnitude of the inward current (*A*1). (Leu)enkephalin (L-ENK) was without effect (*A*2) even though the neurone continued to respond to dynorphin (*A*3). *B*, a d.r.g. neurone that responded to dynorphin A with a reduction of inward current when the depolarization-evoked inward current had returned to base line, a micropipette with tip diameter of 20 μm and containing 1 μM-naloxone (NAL) was positioned to within 15 μm of the neurone. After naloxone was permitted to diffuse from the micropipette for 1 min, application of dynorphin did not affect the amplitude of the inward current (*B*2). Following removal of the 1 μM-naloxone-containing micropipette from its position near the cell, dynorphin A application again decreased the amplitude of the inward current (*B*3). *V*, voltage; *I*, current.

commands for hyperpolarizing and for small (less than 20 mV) depolarizing commands. Step depolarizations to membrane potentials between −40 and +25 mV produced net inward current that peaked at 3–4 ms, and partially decayed over 75 ms. In the presence of cadmium (200 μM) the depolarization-induced inward currents were blocked. Pressure application of dynorphin A at 1 μM reversibly decreased the magnitude of depolarization-induced inward currents in a sub-population of neurones (thirteen of thirty-eight) (Fig. 2*A*1, *A*3). In contrast, (Leu)enkephalin at 1–10 M did not affect inward current amplitude in any of eleven neurones, including five neurones that did respond to dynorphin A (Fig. 2*A*2).

Naloxone antagonism of dynorphin A effects was assessed. First, a control response to application of dynorphin A was obtained (Fig. 2*B*1). Then a 10–15 μm diameter micropipette containing 1 μM-naloxone was positioned near the neurone and the antagonist was allowed to diffuse into the medium surrounding the neurone for 1 min before re-application of opioid peptide. Responses of neurones to dynorphin A were blocked by 1 μM-naloxone (Fig. 2*B*2). Finally, the naloxone-containing micropipette was removed from its position near the d.r.g. neurone, and dynorphin A was re-applied and again decreased inward current (Fig. 2*B*3). Naloxone reversibly antagonized dynorphin A reduction of inward currents in five of five neurones.

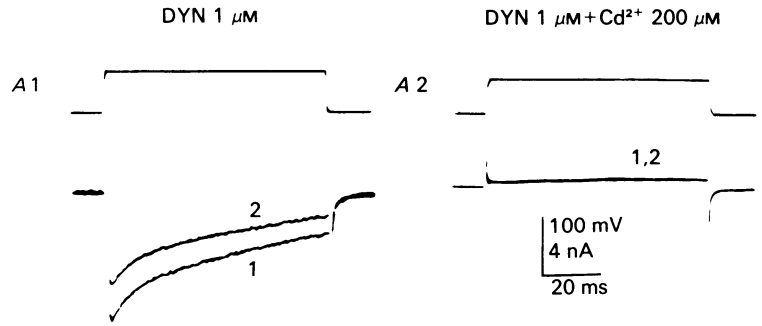

Fig. 3. Dynorphin A did not affect leak conductance. Dynorphin A decreased inward current when potassium conductances were largely blocked by caesium (*A*1). Cadmium (200 μM) abolished the inward current. Dynorphin A did not affect leak currents when the inward current was blocked by 200 μM-cadmium (*A*2).

Dynorphin A decreased calcium conductance

Dynorphin A decreases of inward current could be mediated by an enhancement of outward potassium (or caesium) current or by a decrease of inward calcium current. To distinguish between these alternatives, a neurone was determined to respond to dynorphin A with a reduction of inward current (Fig. 3*A*1). Cadmium was applied then to block the calcium-dependent component, and dynorphin A was re-applied. In the presence of cadmium the remaining outward current was unaffected by dynorphin A (three of three neurones) (Fig. 3*A*2), suggesting that dynorphin A did not affect a voltage-dependent outward current, but rather, that it acted on a calcium-dependent inward current.

The effect of dynorphin A was assessed on the current–voltage relation over the voltage range of −120 mV to +30 mV obtained with command steps from a holding potential of −60 mV (Figs. 4 and 5). With hyperpolarizing and small (< 20 mV) depolarizing command steps, dynorphin A did not affect the current–voltage relation. With larger depolarizing commands, dynorphin A decreased the inward current throughout the voltage range examined (Figs. 4 and 5). We determined dynorphin A effects on calcium-dependent currents directly by subtracting the current–voltage relation obtained in the presence of cadmium from that obtained in the absence of cadmium. In the presence of cadmium, the N-shaped current–voltage relation became linear with step depolarizations to membrane potentials negative of about 0 mV. With larger step depolarizations, the current–voltage relationship became non-linear. When the current–voltage relation in the presence of cadmium was subtracted from the base-line current–voltage relation, calcium-dependent current remained (Fig. 6). Calcium current was activated at a membrane potential of about −30 mV and was fully activated at about −10 mV. The extrapolated reversal potential of calcium-dependent current was about +30 mV. Dynorphin A did not alter the potential for the onset of the inward current, the potential at which the inward current was maximal, or the extrapolated reversal potential of the inward current (five of five neurones) (Fig. 6).

Cadmium blocks inward calcium current but also prevents activation of calcium-

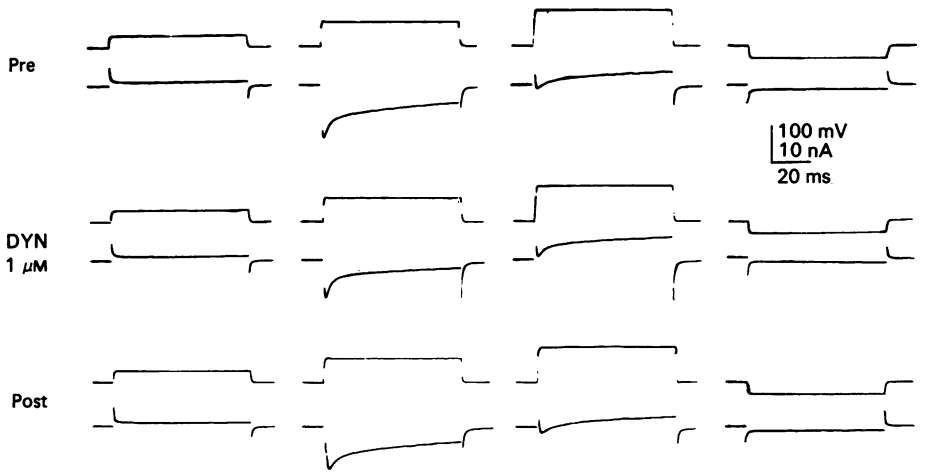

Fig. 4. Dynorphin A reduced calcium currents. Current–voltage relation of a d.r.g. neurone during command steps of +35, +75, +115 and −35 mV from a holding potential of −65 mV prior to (pre), during (DYN), and after (post) dynorphin A application are illustrated. Dynorphin A did not affect the current–voltage relation obtained with hyperpolarizing step commands but did reduce the inward current evoked by depolarizing commands.

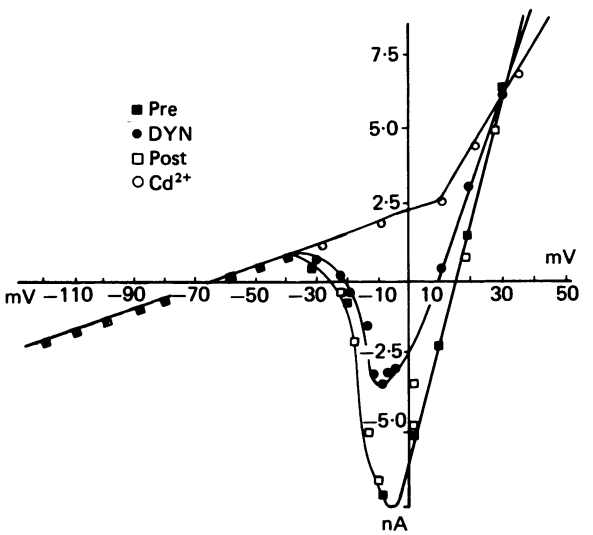

Fig. 5. Dynorphin A reduced the amplitude of inward currents. The current–voltage relation obtained from the neurone illustrated in Fig. 4 is shown. Cadmium (Cd^{2+}) abolished the depolarization-induced inward current, producing a linear current–voltage relation to potentials of about 0 mV but a concave upward relation with larger step depolarizations.

dependent outward currents. Thus, the absence of a dynorphin A effect on cadmium-insensitive current does not exclude an augmentation of calcium-dependent potassium or caesium current. To determine whether dynorphin A decreased voltage-dependent calcium conductance or augmented calcium-dependent potassium (or caesium) conductance, depolarizing step commands were applied from a holding

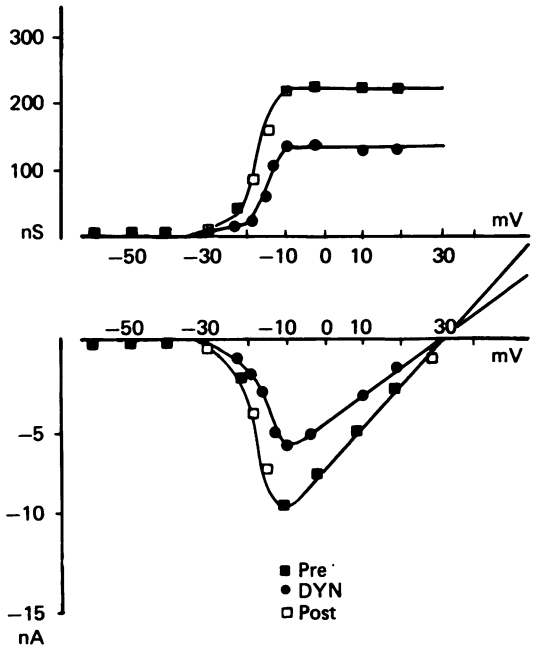

Fig. 6. Dynorphin A decreased inward currents but did not affect their voltage dependence or extrapolated reversal potential. Lower Figure illustrates the current–voltage relation shown in Fig. 5 but after leak current, the current–voltage relation obtained in the presence of cadmium, was subtracted. Upper Figure is the conductance–voltage relation following leak subtraction and is derived from lower Figure.

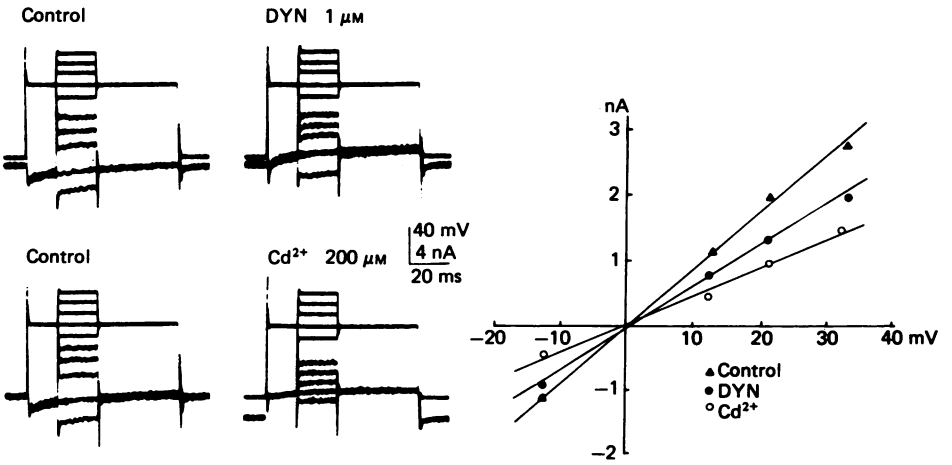

Fig. 7. Dynorphin A reduced chord conductance at step commands that fully activated calcium conductance. From a holding potential of −60 mV, depolarizing step commands were applied to fully activate voltage-dependent calcium conductance. During the large depolarizing commands, small hyperpolarizing and depolarizing voltage steps (±10 mV) were applied. Dynorphin A (DYN) and cadmium (Cd^{2+}) application resulted in less current being required to attain a given voltage during step commands that fully activated calcium conductance.

potential of −60 mV that were sufficient in magnitude to activate fully voltage-dependent calcium conductance. During the large depolarizing command, small hyperpolarizing and depolarizing voltage steps (±10 mV) were applied. Dynorphin A application resulted in less current being required to attain a given voltage during step commands that fully activated calcium conductance (six of six) (Fig. 7). Thus, the reduction of inward currents by dynorphin A was associated with a decrease in membrane chord conductance consistent with an action to decrease calcium conductance rather than to augment a potassium (or caesium) conductance. At step commands that fully activated calcium conductance, dynorphin A at 1 μM reduced chord conductance by 30±3·8% (mean±S.E. of mean, five neurones) (Fig. 7). The effect of dynorphin A on calcium conductance was also assessed directly on two neurones. Using the paradigm described, neuronal calcium conductance was estimated by subtracting leak conductance, the conductance remaining in the presence of 200 μM-cadmium, from total conductance. In the two neurones, dynorphin A reduced membrane conductance by 34 and 36% and calcium conductance by 65 and 64% respectively.

DISCUSSION

We observed that dynorphin A decreased calcium entry at d.r.g. neurone somata in the absence of an effect on either resting membrane potential or conductance. The reduction in calcium entry could be mediated by a direct decrease of voltage-dependent calcium conductance or by an enhancement of a voltage- and/or calcium-dependent potassium conductance.

We suggest that dynorphin A decreases voltage-dependent calcium conductance. First, dynorphin A decreases in duration of calcium-dependent action potentials persisted when substantial potassium conductance was blocked following intracellular caesium injection. Secondly, dynorphin A reduced depolarization evoked calcium-dependent inward currents but did not alter membrane conductance following blockade of calcium channels by cadmium. Thirdly, dynorphin A reduced the instantaneous current–voltage slope (chord conductance) during step commands that produced maximal activation of voltage-dependent calcium conductance. Reduction of calcium current could occur by several mechanisms. Binding of dynorphin A to opioid receptors could: (1) reduce the absolute number or mean open time of calcium channels activated either directly or by an intracellular mediator, (2) hasten calcium channel inactivation, (3) alter the voltage dependence of calcium channel gating, (4) decrease the calcium equilibrium potential and thus the driving force for calcium ions. Our data suggest that dynorphin A does not alter the voltage dependence of calcium channel activation or the calcium reversal potential. With regard to calcium channel activation, while our data are not adequate to provide a definitive answer to the question, several points can be made. First, peak inward current was reduced and there was no effect on the rate of decay of the inward current and no obvious change in rate of activation. While the single-electrode voltage-clamp technique has limitations, a substantial slowing in activation rate could probably have been detected. Thus, it is likely that dynorphin A reduced calcium current by decreasing the absolute number of calcium channels or their mean open time. The reduction in

effective calcium channel number could occur by a direct interaction of opioid receptor and calcium channel or by modulation of intracellular second messengers. A role for cyclic nucleotides in the modulation of voltage-dependent calcium channels has been suggested in heart muscle (Cachelin, de Peyer, Kokubun & Reuter, 1983), *Helix* neurones (Chad & Eckert, 1984), and dorsal root ganglion neurones (Kostyuk, Veselovsky & Fedulova, 1981). Alternatively, calcium conductance may be modulated by calcium/phosphatidyl serine/diacylglycerol-dependent protein kinase (Berridge & Irvine, 1984; DeRiemer, Strong, Albert, Greengard & Kaczmarek, 1985). Further investigation is required to determine the nature of opioid–receptor coupling to the calcium channel.

The opioid-mediated direct reduction of calcium conductance appears specific for dynorphin A and opioids with affinity for κ-opioid receptors. Opioids lacking affinity for κ-opioid receptors such as (Leu)enkephalin and (N-MePhe^3, D-Pro^4)morphiceptin appear to act by enhancing potassium conductance (Werz & Macdonald, 1982*b*, 1983*b*). Morphine, enkephalin, and enkephalin analogues have previously been shown to enhance potassium conductance of neurones in myenteric plexus (North & Tonini, 1977; Morita & North 1982), locus coeruleus (Pepper & Henderson, 1980; Williams *et al.* 1982), and substantia gelatinosa (Yoshimura & North, 1983). Additionally, morphine has been reported to enhance calcium-dependent after-hyperpolarizations of myenteric plexus neurones (Tokimasa, Morita & North, 1981). The effects of opioids on potassium conductance are mediated by receptors with high affinity for naloxone, consistent with μ-receptors. Effects of opioids on potassium conductance have also been shown to decrease calcium entry by hastening repolarization of calcium action potentials (North & Williams, 1983; Werz & Macdonald, 1983*b*). Thus, opioid receptors may be coupled to different ion channels, calcium or potassium, but may have a similar net effect on calcium entry.

Opioid receptors have been localized in spinal cord both on primary afferent terminals as well as on intrinsic elements of the dorsal horn. Opioid receptor localization on primary afferents is suggested by the 50 % reduction in opiate receptor binding observed in the substantia gelatinosa following dorsal rhizotomy. (Lamotte, Pert & Snyder, 1976; Fields, Emson, Leigh, Gilbert & Iversen, 1980). Additionally, opioid receptors are present on neurites of dorsal root ganglion explants (Hiller, Simon, Crain & Peterson, 1978). At present it is thought that opioids mediate analgesic actions at spinal levels at least in part by depressing neurotransmitter release from primary afferents. Opioid receptors have also been found on the somata of a portion of dorsal root ganglion neurones grown in primary dissociated culture (Mudge, Leeman & Fischbach, 1979; Werz & Macdonald, 1982*a*,*b*) and on a portion of adult d.r.g. neurones *in vivo* (Ninkovic, Hunt & Gleave, 1982) and opioids decrease calcium entry at the somatic membrane of d.r.g. neurone (Werz & Macdonald, 1982*a*,*b*). Interestingly, a similar effect at the d.r.g. neurone terminal membrane would result in a decrease of neurotransmitter release (Macdonald & Nelson, 1978). We have observed that only a small portion of d.r.g. neurones respond to opioids. Furthermore, we have observed a heterogeneous response of d.r.g. neurones to μ-, δ- and κ-receptor ligands (Werz & Macdonald 1982*b*, 1984). The heterogenous distribution of opioid receptors as well as the absence of opioid receptors on the majority of d.r.g. neurones is suggestive of distinct, highly specific roles for these

receptor types in the processing of information in the spinal cord, consistent with observations that μ-, δ-, and κ-receptors differentially modulate nociceptive information in spinal cord (Wood *et al.* 1981; Upton, Sewell & Spencer, 1982; Schmauss & Yaksh, 1984).

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