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ENKEPHALIN REDUCES CALCIUM ACTION POTENTIALS IN ROHON-BEARD NEURONS *IN VIVO*[1]

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Abstract

Rohon-Beard neurons of the *Xenopus* spinal cord develop somatic Ca^{++}-dependent action potentials at early embryonic stages. Because many Ca^{++}-dependent events are blocked or reduced by enkephalin in other neuronal systems, we tested the effect of met-enkephalin on the Ca^{++} spikes of Rohon-Beard cells. Our results indicate that met-enkephalin, which has no effect on the resting membrane potential or conductance in these cells, reversibly and specifically shortens their Ca^{++} action potentials, probably by decreasing the Ca^{++} conductance. Enkephalin has a similar effect on *Xenopus* dorsal root ganglion cells *in vivo*. Examination of Rohon-Beard cells at various stages of development shows that enkephalin shortens Ca^{++} spikes from the earliest times that the spikes appear (~21 hr after fertilization) to the latest times they are detectable (7 to 9 days). This very early response suggests either that enkephalin binds directly to the Ca^{++} channels or that an "enkephalin receptor" is among the earliest neuronal phenotypes to appear in these cells.

Since their discovery, the opiate peptides have been implicated in an increasing variety of physiological roles. These various effects seem to be mediated through a similar array of physiological mechanisms, of which the most prominent is a reduction or block of events dependent on a voltage-sensitive Ca^{++} influx. Such an action has been demonstrated in regions of both the central and peripheral nervous systems (Jessell and Iversen, 1977; MacDonald and Nelson, 1978; Konishi et al., 1979; Mudge et al., 1979; Lightman et al., 1982). These actions are generally interpreted as indicating a role of opiates, particularly enkephalin, in regulating transmitter release.

Calcium-dependent action potentials are a feature of several populations of developing excitable cells (Kano, 1975; Baccaglini and Spitzer, 1977; Baccaglini, 1978; Strichartz et al., 1980). In addition, mature neurons in some cases possess a Ca^{++} component in largely Na^{+}-dependent spikes (Koketsu and Nishi, 1969; Akaike et al., 1978; Yoshida et al., 1978). Mudge and co-workers (Mudge et al., 1979; Fischbach et al., 1981) found that the Ca^{++} component of spikes in cultured chick dorsal root ganglion cells is reduced by met-enkephalin applied to the soma, and they suggested that enkephalin may regulate the *in vivo* release of substance P by a similar action on Ca^{++} channels at the terminal. However, in developing cells having action potentials wholly or predominantly dependent on Ca^{++}, effects of enkephalin on voltage-sensitive Ca^{++} channels could be significant without regard to the control of transmitter release.

The Rohon-Beard neurons of the *Xenopus* tadpole spinal cord develop long duration Ca^{++} action potentials very early during development (at the stage of neural tube closure), which later are transformed to brief, predominantly Na^{+}-dependent events (Baccaglini and Spitzer, 1977). Because these neurons are accessible to study *in vivo* prior to their developing electrical or chemical excitability, and they go through a stage of Ca^{++}-dependent impulses, we examined the effect of enkephalin on the Rohon-Beard cell action potentials. We found that met-enkephalin reduces or blocks the Ca^{++} component of Rohon-Beard neuron action potentials, without affecting the resting membrane voltage or conductance. Furthermore, this effect is present from the earliest times that the Ca^{++} channels are physiologically detectable and remains at least until the time of onset of cell death in the population.

Materials and Methods

Xenopus laevis embryos were obtained by conventional breeding procedures and staged according to the method of Nieuwkoop and Faber (1956). Preparations were dissected and mounted in chambers as described previously (Baccaglini and Spitzer, 1977; Bixby and

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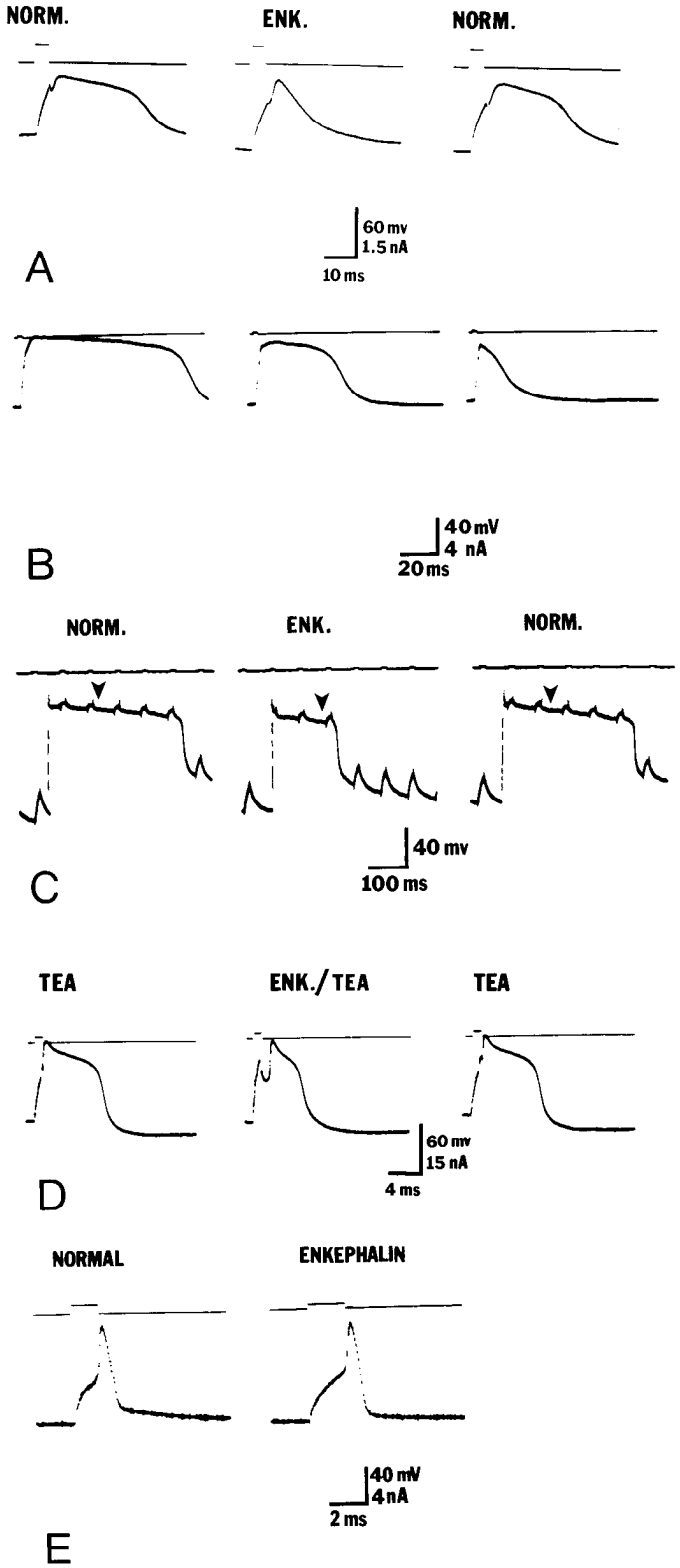

Figure 1. Puffer application of enkephalin shortens the Ca^{++} component of Rohon-Beard cell action potentials. Voltage records, *lower traces*; injected currents, *upper traces*. *A*, 20 μM enkephalin shortens the Ca^{++} plateau of the Na^{++}/Ca^{++} action potential at stage 26. The response returns when the puffer is turned off. *B*, 10 μM enkephalin reduces the amplitude and duration of the Ca^{++} action potential at stage 22/23. *Center trace*, immediately following onset of application; *right trace*, after several seconds of application. *C*, 20 μM enkephalin decreases membrane conductance during the Ca^{++} action potential at stage 23. During enkephalin application (*center traces*), constant hyperpolarizing current pulses yield 20 to 30% *larger* voltage changes at comparable times after action potential initiation (*arrowheads*), indicating a *decrease* in conductance. The action potential amplitude, duration, and conductance return to normal when the enkephalin puff is turned off (*right-hand traces*). *D*, 1 μM enkephalin shortens the Ca^{++} plateau of the Na^{+}/Ca^{++} action potential elicited in the presence of TEA^{+} at stage 46. The effect disappears when the puffer is turned off. *E*, 20 μM enkephalin has no apparent effect on the Na^{+} action potential at stage 46.

Spitzer, 1982). Rohon-Beard neurons and dorsal root ganglion neurons were visualized with Nomarski interference contrast optics at × 500 magnification.

Cells were impaled with glass microelectrodes filled with 3 M potassium acetate, having resistances of 100 to 160 megohms. Currents and voltages were measured with a cathode follower amplifier having current injection capability and were displayed on an oscilloscope. Action potentials were elicited with short (1 to 2 msec) depolarizing current pulses of 0.5 to 3 × 10^{-9} A. Data were accepted only from cells having resting potentials (rps) more negative than −40 mV, and most cells had rps between −50 and −90 mV. Cells with small rps were often held at −60 to −70 mV with steady hyperpolarizing current.

We perfused the preparations continuously with amphibian saline, containing (in mM): Na^{+}, 125; Cl^{-}, 148; Ca^{++}, 10; K^{+}, 3; HEPES buffer, 5 (pH 7.4). For animals younger than stage 30, 10 mM $MgCl_2$ was added to this saline to reduce muscle contraction. For older animals (stages 46 to 49), 20 mM tetraethylammonium chloride (TEA^{+}) was often added to the normal saline to reduce outward K^{+} current.

Met-enkephalin and other peptides (Sigma) were pressure ejected from blunt-tipped puffer electrodes as described previously (Bixby and Spitzer, 1982). These electrodes contained the peptides in the same saline as that bathing the preparation, and pressures of 1 to 15 p.s.i. were used with tip sizes ranging from 3 to 9 μm. In some experiments naloxone was present in the perfusion solution.

Results

We initially tested Rohon-Beard neurons for a response to met-enkephalin at stage 26 (1 day after fertilization), the time at which they become sensitive to GABA and glycine. No change in resting membrane voltage or conductance was observed when concentrations of enkephalin up to 30 μm were applied to the cells. However, the action potentials of these neurons, which depend on an influx of Na^{+} and Ca^{++}, were consistently shortened with enkephalin concentrations greater than or equal to 5 μM; the duration and amplitude of the Ca^{++}-dependent component was reduced (Fig. 1*A*; Table I). The extent of this reduction varied from cell to cell; Figure 1*A* illustrates one of the larger effects. Enkephalin can shorten the action potential at this stage in concentrations as low as 1 μM (five of eight cells), but not at 0.1 μM (zero of four cells).

The action potentials remained shortened for the entire period of enkephalin application (up to 1 min), with no apparent desensitization; durations returned to nor-

TABLE I

Stage dependence of enkephalin[a] effect on Rohon-Beard neurons

Stage	Action Potential Shortened	Action Potential Lengthened	No Effect
	no. of cells		
20	2		
21/22	8		
23/24	6		1
26	14	3[b]	1
41/42	2		
45/46	20	—	1
Total	52	3	3

[a] Enkephalin concentration ≥ 5 μM.

[b] All of these cells were in one preparation.

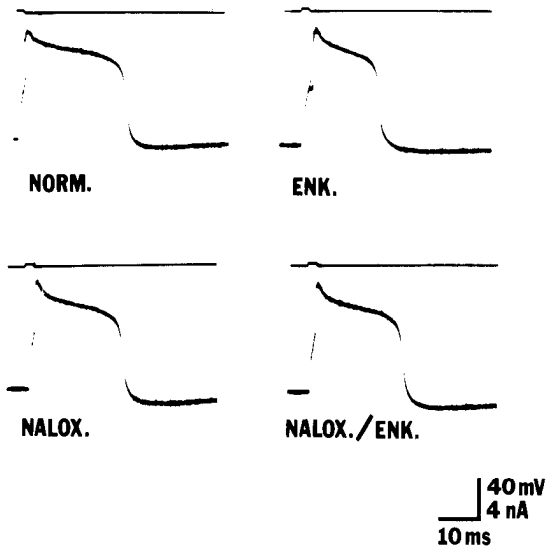

Figure 2. Five micromolar enkephalin shortens the Ca^{++} component of the action potential elicited in the presence of TEA^+ at stage 41 (*upper traces*). One micromolar naloxone prevents this effect (*lower traces*).

mal upon cessation of application. The latencies from onset of application to onset of full effect, and from end of application to the return of normal action potential duration, varied from undetectable to several seconds, but they seemed to be concentration dependent. The shortening of action potentials by enkephalin was a specific effect at all stages observed, in the sense that it was always abolished by low concentrations (1 to 10 μM) of naloxone ($n = 8$; Fig. 2).

Of the 18 cells tested at stage 26, three exhibited an abnormal response, in which the action potential was *longer* in the presence of 10 μM enkephalin. These cells were all from the same animal, in which other cells gave the usual response, and such an effect was never seen in any of the cells examined at other stages ($n = 34$). We have no explanation for this result.

Rohon-Beard neurons become capable of generating impulses around stage 20, and we tested enkephalin on the Ca^{++} action potentials of these early differentiating cells. Enkephalin (5 to 10 μM) markedly reduces the duration and peak amplitude of the early spike (Fig. 1*B*; Table I), and in some cases can prevent the generation of the impulse. Thus the response of the action potential to enkephalin is present in these neurons as soon as the Ca^{++} channels become detectable by our techniques. The opiate reduces the conductance increase at the peak of these impulses (Fig. 1*C*; $n = 3$), suggesting that it acts by decreasing the inward current.

Although the Ca^{++} component of the Rohon-Beard cell action potential is gradually reduced during the first week or so of development, Ca^{++} channels can be revealed at stage 46 (the time onset of cell death; Lamborghini, 1981). The cells now generate predominantly Na^+-dependent impulses in normal saline, but addition of TEA^+ decreases delayed rectification, and the Ca^{++} component is revealed as a plateau on the action potential (see Baccaglini and Spitzer, 1977). We took advantage of this procedure to determine whether Ca^{++} currents of mature Rohon-Beard cells can be reduced by enkephalin. The characteristic Ca^{++}-dependent plateau present in TEA^+ could be reliably shortened by enkephalin (1 to 10 μM; Fig. 1*D*). Enkephalin was effective in concentrations as low as 0.1 μM (two of five cells tested), a concentration ineffective on younger neurons. The explanation for the lower effective concentration with stage 46 neurons could be a lower density of Ca^{++} channels.

The action of met-enkephalin on Rohon-Beard neurons seems to be limited to the effect on Ca^{++}-dependent spikes. No change in the height or duration of stage 46 Na^+-dependent action potentials was seen when enkephalin (20 μM) was applied for 10 to 30 sec ($n = 7$; Fig. 1*E*). Enkephalin was also without consistent effect on the iontophoretic response to GABA in stage 46 cells ($n = 5$) and on the voltage-dependent uncoupling present in the embryonic neurons at earlier stages ($n = 4$) (see Bixby and Spitzer, 1982; Spitzer, 1982).

Other opiate peptides besides met-enkephalin can reduce the impulse duration of Rohon-Beard cells. Leu-enkephalin is nearly as potent as met-enkephalin, working consistently at 10 μM and sometimes at 2 μM (two of four cells). Dynorphin and β-endorphin are also effective at 10 μM, but 1 μM dynorphin elicited a barely detectable shortening, and 1 μM β-endorphin was ineffective ($n = 4$). In contrast, oxytocin and substance P, two nonopiate neuroactive peptides, did not influence the height or duration of Rohon-Beard cell Ca^{++} spikes at a concentration of 10 μM ($n = 3$, substance P; $n = 7$, oxytocin).

No effect on Rohon-Beard cell spike duration (stage 26) was observed when norepinephrine ($n = 7$), dopamine ($n = 5$), or 5-hydroxytryptamine ($n = 3$) were perfused in the bathing saline at a concentration of 100 μM. In cultured chick dorsal root ganglion neurons (where enkephalin reduces the Ca^{++} component of the spike), as well as in rat sympathetic cells, norepinephrine reduces the duration of Ca^{++}-dependent impulses (Dunlap and Fischbach, 1978; Horn and McAfee, 1980).

Xenopus dorsal root ganglion neurons also exhibit action potentials with Ca^{++} components (Baccaglini, 1978). We found that 20 μM met-enkephalin reversibly shortened the duration of dorsal root ganglion neuron action potentials *in vivo* (Fig. 3) ($n = 3$).

Discussion

The shortening of Ca^{++}-dependent action potentials reported here for spinal neurons *in vivo* is like that seen

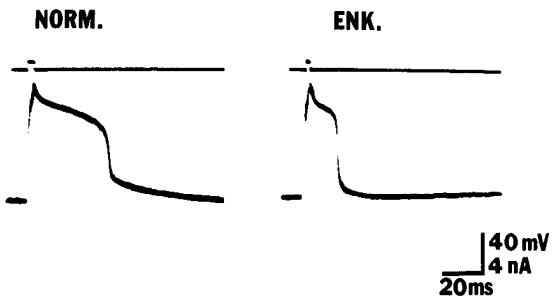

Figure 3. Twenty micromolar enkephalin reduces the Ca^{++} component of the action potential of a dorsal root ganglion neuron in TEA^+ saline at stage 49.

in cultured chick dorsal root ganglion neurons, where the effect has been shown to be on Ca^{++} channels (Fischbach et al., 1981). We believe that enkephalin is reducing Ca^{++} current in the Rohon-Beard neurons as well, rather than increasing K^+ current (Williams et al., 1982), for the following reasons. First, there is no effect on the height or duration of the Na^+ action potential present in the older neurons. Second, the shortening is seen in high concentrations of TEA^+, which suggests that voltage-sensitive K^+ channels are unlikely to be involved. Third, the principally Ca^{++}-dependent action potential of the embryonic neurons can be appreciably reduced in height or even blocked by enkephalin, lessening the possibility of the effect being mediated through K^+ channels which open with some delay upon depolarization (see, however, MacDermott and Weight, 1982). Finally, direct measurements of the membrane conductance during these action potentials suggest that it is decreased by enkephalin.

The opiate peptides have been shown to be involved in a diverse set of physiological effects, including changes in resting membrane potential and conductance, and in the postsynaptic response to neurotransmitters (e.g., Barker et al., 1978). A consistent finding has been, though, that opiates reduce or block responses involving voltage-sensitive Ca^{++} channels, an effect seen both *in vitro* and in several systems *in vivo*, including the present result in *Xenopus* dorsal root ganglion neurons. It seems that the action of enkephalin on Rohon-Beard cells is restricted to this latter category.

There are various possible explanations for the observed variability in the magnitude of the enkephalin effect. There may be biological variation in the number or types of receptors. Alternatively, difficulties in delivering known concentrations to the cell surface could have arisen, due to differences in diffusion barriers or in puffer characteristics.

The Rohon-Beard cell response to enkephalin is present at least as early as stage 20, approximately 21 hr after fertilization. It is, therefore, among the earliest signs of physiological differentiation detected in these embryonic neurons to date. By contrast, the other known chemosensitivities of the cells, to GABA and glycine, appear first around stage 25 (Bixby and Spitzer, 1982). We have commented before on the apparent tendency for the simultaneous appearance of sensitivity to different neurotransmitters, which has been seen for GABA and glycine in Rohon-Beard neurons and for GABA and acetylcholine in the dorsal unpaired median neurons of the grasshopper (Goodman and Spitzer, 1979; Bixby and Spitzer, 1982). The early appearance of enkephalin sensitivity in Rohon-Beard neurons is an exception to this tendency. However, it may be that the binding site for enkephalin in Rohon-Beard neurons is a transmitter receptor only in the most general sense. That is, our results would be explained if enkephalin exerted its action by binding directly to the voltage-dependent Ca^{++} channel. If enkephalin had the ability to block Ca^{++} channels in this way, one might expect that it would do so in a wide variety of neurons, as seems to be the case. We have recently shown, for example, that enkephalin reduces quantal content at the frog neuromuscular junction, apparently by blocking Ca^{++} influx (Bixby and Spitzer, 1983). It should be noted, however, that we have no direct evidence supporting this mechanism of action of enkephalin.

The physiological role of the Rohon-Beard cell sensitivity to enkephalin is unknown. Preliminary immunocytochemical results suggest that enkephalin is present in the dorsal spinal cord during development (J. E. Lamborghini and H. J. Karten, personal communication). It is possible, as has been suggested for other neural systems, that the reduction in Ca^{++} current is related to the regulation of neurotransmitter release, presumably involving Ca^{++} channels at the axonal terminals. However, it may also be that the function of enkephalin is in modulating the somatic Ca^{++} action potentials which are transiently expressed in Rohon-Beard cells. It is unlikely that the shift in ionic dependence of the inward current of the action potential is the result of suppression of the Ca^{++} component by endogenous opiate peptides, because it also occurs in neurons in dissociated cell culture (Spitzer and Lamborghini, 1976). The physiological significance of these long duration Ca^{++} action potentials is still obscure.

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