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Voltage-dependent Effects of Opioid Peptides on Hippocampal CA3 Pyramidal Neurons *in vitro*

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Opioid peptides, and especially the dynorphins, have been localized to several circuits in the CA3 hippocampal region, yet electrophysiological studies often find mixed effects of opiates on the excitability of CA3 neurons. Reasoning that these mixed effects might involve voltage-dependent actions, we tested the effect of several opiates on CA3 pyramidal neurons using single-electrode voltage-clamp recording in a slice preparation of rat hippocampus. In most CA3 neurons, the voltage-dependent K^+ current known as the M-current (I_M) was uniquely sensitive to the opioid peptides, with the direction of response dependent upon the opiate type and concentration. Thus, an opiate selective for κ receptors, U-50,488H, significantly augmented I_M. The κ-selective agonists dynorphin A and dynorphin B, which exist in mossy fiber afferents to CA3 pyramidal neurons, also markedly augmented I_M at low concentrations (20–100 nM). By contrast, dynorphin A at higher concentrations (1–1.5 μM) often reduced I_M. Similarly, several opiates [e.g., D-Ala^2,D-Leu^5-enkephalin: (DADL), [D-Pen^{2,5}]-enkephalin (DPDPE)] known to act on the δ receptor subtypes reduced the M-current, with partial reversal of this effect by naloxone. Neither the selective μ-receptor agonist [D-Ala^2, NMe-Phe^4, Gly-ol]-enkephalin (DAMGO) nor the nonopioid fragment of dynorphin, des-Tyr-dynorphin, consistently altered I_M. These opiate effects on I_M were accompanied by changes in conductance and holding current consistent with their respective effects on I_M. Dynorphin A did not measurably affect the Q-current, a conductance known to contribute to inward rectification in hippocampal pyramidal neurons. The opiate effects on I_M were not altered by pretreatment with Cs^+ (which blocks I_Q) or Ca^{2+} channel blockers. The opposing effects of the dynorphins (both A and B) and DADL on I_M were antagonized by naloxone (1–3 μM), and the dynorphin-induced augmentations of I_M were usually reversed by the κ receptor antagonist norbinaltorphimine. These results suggest that the opiates can have opposing effects on the same voltage-dependent K^+ channel type (the M channel) in the rat CA3 pyramidal neuron, with the direction of the response depending on which receptor subtype is activated. These data not only help explain the mixed effects of opiates seen in other studies, but also suggest a potential postsynaptic function for the endogenous opiates contained in the CA3 mossy fibers.

[Key words: opiates, electrophysiology, hippocampal slice, M-current, K^+ conductance, κ receptors, dynorphin, enkephalin]

The function of the hippocampal opioid peptides has been under considerable study since their discovery in the mid-1970s. Scattered cells and fibers immunohistochemically labeled for enkephalins were first described in both CA1 and CA3 subfields (see Bloom, 1983, for review). Later studies found a dense opioid projection from dentate granule cells to the CA3 region, via the mossy fiber pathway (Gall et al., 1981). Subsequent biochemical and cytochemical findings suggested that this pathway contained mainly dynorphin peptides as well as some proenkephalin-derived fragments (Chavkin et al., 1983b, 1985b; McGinty et al., 1983). Stimulation of the hippocampal CA3 region releases several dynorphins and enkephalins (Chavkin et al., 1983a; Terrian et al., 1988), as well as the excitatory amino acid glutamate (Terrian et al., 1988).

In the last decade, physiological studies began to delineate the function of hippocampal opioid peptides. Most studies have focused on CA1, where pyramidal neurons do not appear to be directly affected by the opioid peptides, although the inhibitory synaptic mechanisms in these cells are curtailed (Zieglgänsberger et al., 1979; Nicoll et al., 1980; Siggins and Zieglgänsberger, 1981). Thus, the most likely function for enkephalins in CA1 is disinhibition, exerted by dampening the activity or activation of inhibitory interneurons (Zieglgänsberger et al., 1979; Nicoll et al., 1980; Siggins and Zieglgänsberger, 1981; Siggins and Gruol, 1986). A direct study of enkephalin effects on such interneurons supports this disinhibition hypothesis (Madison and Nicoll, 1988). A similar disinhibitory mechanism may be exerted by enkephalins in CA3 and dentate gyrus (Gruol et al., 1983; Caudle et al., 1990). In all hippocampal regions, this effect appears to derive from activation of either the μ or δ subtype of opioid receptor (Chavkin et al., 1985b; Siggins et al., 1986; Wimpey et al., 1989; Caudle and Chavkin, 1990; Lupica et al., 1992; Watson and Lanthorn, 1993).

CA3 neurons would appear to be ideal subjects for testing the physiological role of the dynorphins, because of the profuse dentate–CA3 dynorphin projection via mossy fibers. However, electrophysiological findings to date have been somewhat ambiguous. In extracellular and intracellular studies both excitatory and inhibitory effects have been reported (Henriksen et al., 1982;

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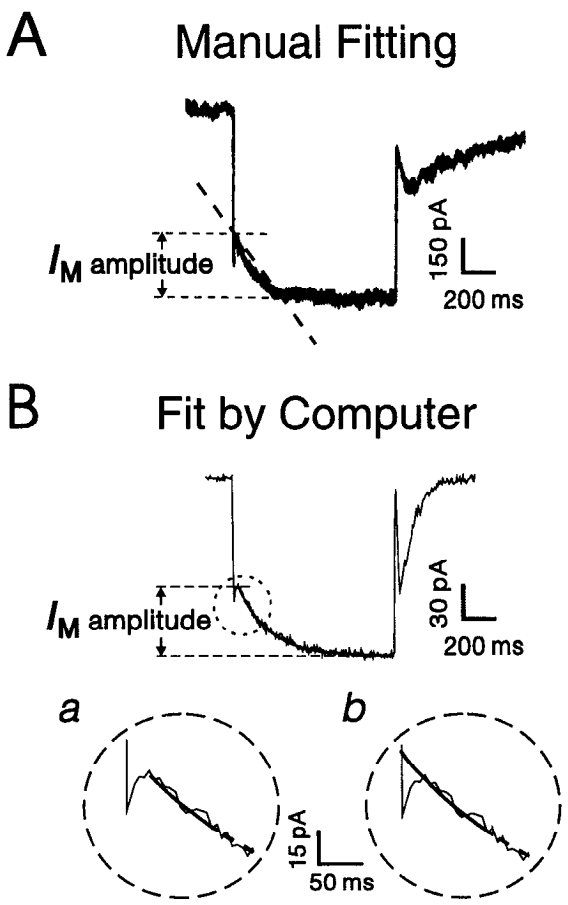

Figure 1. Methods used to quantitate the amplitude of the inward M-current relaxation in CA3 pyramidal neurons. Examples show membrane currents of pyramidal neurons clamped at −47 mV (*A*) or −43 mV (*B*) and submitted to a 15 mV (*A*) or 5 mV (*B*) hyperpolarizing step. The slow inward (downward) relaxation reflects the reduction of the noninactivating time- and voltage-dependent M-current (I_M), carried by the outward flow of K^+. *A*, Estimation of I_M by manual fitting of a straight line to a polygraph record of an I_M relaxation evoked by a single hyperpolarizing command. The lower end of the line was usually started at some point (not necessarily the steady state) about halfway through the trajectory, to avoid effects of the complexities caused by the confounding outward relaxation (see Results). *B*, I_M quantification by computerized exponential curve-fitting methods (CLAMPFIT software). The I_M relaxation amplitude is estimated from the peak of the relaxation (instantaneous current) to the end of the voltage step where a steady baseline current is reached (steady state current). Here, we often estimated the I_M relaxation amplitude by two methods (see Materials and Methods): *a* shows fitting to peak (with magnification of the current trace shown in the *dashed circle* in *B*); *b*, fitting with extrapolation back to command onset. The shape of the peak and extrapolated plots of the I_M versus voltage are the same with both computerized methods, except that I_M amplitudes are about 20% larger when extrapolating the fit to command onset. Differences in the thickness of the current traces in *A* versus *B* are due to the use of an unaveraged polygraph tracing in *A*; the traces in *B* were obtained from averaged (five sweeps), digitized data imaged in and printed from the HPGL format (modified from Fig. 2 of Schweitzer et al., 1993).

Gruol et al., 1983; Iwama et al., 1986). In intracellular studies *in vitro*, dynorphin superfusion evoked either depolarizations or hyperpolarizations in different CA3 pyramidal neurons (Gruol et al., 1983). By contrast, enkephalins had little or no effect on membrane potential in either CA1 or CA3 pyramidal neurons.

Several heuristic models have been proposed to account for the mixed effects of dynorphin in CA3 (Siggins and Gruol, 1986; Siggins et al., 1986). One explanation is that different CA3 pyramidal neurons have different opioid receptor subtypes, perhaps selectively activated by different dynorphin concentrations or dynorphin fragments that produce qualitatively different responses. Indeed, it is known that dynorphin is a preferential κ agonist at low concentrations (Chavkin et al., 1982), whereas the other opioid peptides and high dynorphin concentrations can act at either μ or δ receptors. Furthermore, there now are reports of at least two subtypes of the κ-binding sites, κ_1 and κ_2, with differential species and regional distributions (Nock et al., 1988; Zukin et al., 1988; Traynor, 1989; Wagner et al., 1992; but see Nock et al., 1990). Interestingly, κ_1 receptors appear to predominate in guinea pig, whereas κ_2 receptors predominate in rat brain (Zukin et al., 1988). Studies of the guinea pig dentate gyrus support a role for the κ_1 receptor in reducing excitatory synaptic transmission (Wagner et al., 1992), and a recent report suggests that dynorphin may be involved in presynaptic modulation of long-term potentiation (LTP) in the guinea pig mossy fiber–CA3 pathway (Weisskopf et al., 1993).

Another possibility is that dynorphin actions are voltage dependent, producing different effects at different membrane potentials. Indeed, voltage-dependent effects of μ receptor agonists have been seen in a small percentage of acutely isolated CA1 neurons (Wimpey and Chavkin, 1991). A preliminary current-clamp study (Pacheco et al., 1983) in fact found evidence for such voltage-dependent dynorphin effects in CA3 pyramidal neurons. Other peptides like somatostatin can alter voltage-dependent conductances such as the M-current (I_M; Brown, 1988; Jacquin et al., 1988; Moore et al., 1988a). I_M is a noninactivating K^+ conductance seen at slightly depolarized membrane potentials. Voltage dependency is best studied with voltage-clamp methods, where peptide actions on ionic currents can be examined directly. Therefore, we have applied these methods to CA3 pyramidal neurons in the rat hippocampal slice, and verify that dynorphin and other opioid peptides exert concentration- and voltage-dependent actions.

Preliminary reports of early studies on this subject have been reported elsewhere (Moore et al., 1988b; Siggins, 1990).

Materials and Methods

Slice preparation. We used standard intracellular recording techniques in the rat hippocampal slice, prepared as described previously (Moore et al., 1988a, 1990; Schweitzer et al., 1993). In brief, we cut transverse hippocampal slices (taken from male Sprague–Dawley rats of 100–170 gm body weight) of 350–400 μm thickness on a brain McIllwain slicer and incubated them in gassed (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl, 130; KCl, 3.5; NaH_2PO_4, 1.25; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10. Other ions and agents were added to this ACSF medium as desired. After a short incubation period in an interface configuration, the slices were completely submerged and continuously superfused with warm (30–32°C) ACSF at a constant rate within the range of 2.0–4.0 ml/min. We used sharp glass micropipettes filled with KCl (3 M; tip resistances of 50–80 MΩ) or K-acetate (3 M; tip resistances of 80–95 MΩ) to penetrate CA3 pyramidal neurons. Methods of superfusion, voltage-clamp recording, cell identification, drug administration, and data analysis were as described previously (Moore et al., 1988a, 1990; Schweitzer et al., 1993).

Electrophysiology. Current- and voltage-clamp studies were performed with either an Axon Instruments Axoclamp 2A or a Dagan preamplifier. In voltage-clamp mode, tetrodotoxin (0.5–1 μM) was added to block synaptic transmission and Na^+-dependent action potentials. Using discontinuous single-electrode voltage clamp, the switching frequency between current injection and voltage sampling was 3–4 kHz. Electrode "settling time" and input capacitance neutralization at the headstage were monitored continuously on an oscilloscope (Finkel and

Redman, 1985). Current and voltage records were filtered at 0.3 kHz and stored on polygraph paper, DC tape recordings, and oscillograph film, and in later studies also were acquired by D/A sampling and acquisition software (pCLAMP, Axon Instruments). For I_M studies, we most often applied −5, −10, −15, −20, and −25 mV voltage commands in sequence; with D/A sampling via computer, we used two to five sweeps at each of these voltage commands for subsequent averaging. The various problems (e.g., space clamping) associated with voltage clamping of neurons with extended processes are discussed elsewhere (Halliwell and Adams, 1982; Johnston and Brown, 1983; Finkel and Redman, 1985). These problems may be less acute when dealing with relative changes following drug application (see, e.g., Madison et al., 1987). Notwithstanding, we repeated some studies with opiates in the presence of various pharmacological agents, to minimize the contribution of large non-K^+ conductances (e.g., Na^+ and Ca^{2+}) in remote dendrites.

M-current analysis. In hippocampal CA1 pyramidal neurons, I_M is best seen with holding potentials of −40 to −45 mV, hyperpolarizing command steps of 5–25 mV, and durations of 700–1000 msec (Halliwell and Adams, 1982; Schweitzer et al., 1993), when it appears as a slow inward current "relaxation" following the instantaneous (ohmic) inward current drop (Fig. 1). Similar M-current relaxations have been described for CA3 neurons (Gustafsson et al., 1982; Zbicz and Weight, 1983; Charpak et al., 1990). Our CA3 voltage-clamp studies were done at 30–32°C to slow down I_M kinetics. These temperatures do not appear to alter agonist actions on I_M: in a previous study, somatostatin superfusion at 35–37°C elicited the same augmenting effect on I_M as at lower temperatures (Moore et al., 1988a).

I_M amplitude was measured either by manual fitting of curves to the relaxation, or by software (CLAMPFIT, Axon Instruments) that fitted exponential curves (one exponent; r values = 0.90–0.99 in the presence of Ca^{2+} channel blockers; see below) to the I_M relaxation (Fig. 1). To quantify the magnitude of I_M, we calculated the difference between the instantaneous peak current at command onset and either (1) the most inward but stable point along the trajectory (Fig. 1*A*) for cases showing "contamination" by an outward relaxation (see Results) or (2) the steady state current just before command offset (Fig. 1*B*). Tail (off-command) currents were not analyzed because of contamination with other currents (e.g., the fast I_A or I_T), especially with larger voltage commands. Usually, a current (capacitative) artifact of 5–20 msec duration was present at command onset (see also Halliwell and Adams, 1982). Therefore, for the computerized method of estimation of the instantaneous portion of I_M with hyperpolarizing commands, the current relaxation was fitted via two methods: (1) curve fitting to the peak of the initial (quasi-instantaneous) current (Fig. 1*Ba*) that usually fell within 5–20 msec of step onset after complete settling of the capacity transient, or (2) fitting through the peak with extrapolation to command onset (Fig. 1*Bb*), based on the finite duration of the initial capacitative artifact and the exponential (monotonic) nature of I_M relaxation. These methods gave essentially equivalent results with respect to relative effects of drugs, although the extrapolated I_M amplitude was obviously bigger (10–25%; average of 20%) than amplitude at peak. However, to be conservative we used measures of the peak (method 1) for statistical analyses of opiate action (see also Schweitzer et al., 1993).

Q-current. The Q-current (I_Q) is an inward or anomalous rectifier current likely carried by Na^+ and K^+ that is seen in CA1 hippocampal pyramidal neurons (Halliwell and Adams, 1982); this current also appears to be present in CA3 neurons (see Results). Like I_M, it produces a slow inward current relaxation with hyperpolarizing voltage steps, although only from hyperpolarized holding potentials (more negative than −65 mV). The amplitude of the relaxation increases as a function of the command hyperpolarization. I_Q and other forms of anomalous rectification were pharmacologically distinguished from I_M using extracellular cesium (2 mM) as a blocker of I_Q, or the muscarinic agonist carbachol (20–40 μM) to block I_M.

Drug and ion administration. Drugs, peptides, and ion channel blockers were added from a concentrated stock solution to the ACSF in known concentrations immediately before administration to the slice chamber. The usual opiate-testing protocol was as follows: recording of currents for 10–15 min during superfusion of ACSF alone ("control"), followed by switching to ACSF with drug and repeating these current measures after 5–20 min of drug, then followed by switching again to ACSF alone for 30–35 min with subsequent current measures ("washout"). The cell was depolarized to about −40 to −45 mV for I_M analysis at each of these three periods, but was held near resting potential between these periods to avoid the instabilities that occasionally develop with prolonged depolarization (see Halliwell and Adams, 1982).

We obtained dynorphin A (1–17) from Drs. N. Ling, J. Rivier, and W. Vale at the Salk Institute, and from Peninsula Laboratories. Dynorphin B, des-Tyr-dynorphin, the selective μ receptor agonist [D-Ala^2, NMe-Phe^4, Gly-ol]-enkephalin (DAMGO), and the selective δ receptor agonist [D-Pen^{2,5}]-enkephalin (DPDPE) were all purchased from Peninsula Laboratories, and the κ-selective agonist U-50,488H was a gift from Upjohn Co. D-Ala^2,D-Leu^5-enkephalin (DADL), an opioid peptide relatively unselective for receptor subtypes but having strong μ and δ action, was obtained from Dr. N. Ling and Peninsula Laboratories. We purchased nifedipine and the opiate antagonist naloxone from Sigma, the κ_1-specific antagonist norbinaltorphimine (NBNI) from Research Biochemicals International, and tetrodotoxin (TTX) from Calbiochem.

The putative I_M recorded in many CA3 pyramidal neurons displayed some properties seemingly different from those in hippocampal CA1 neurons (see Results and Discussion), including the apparent superposition of an opposing outward relaxation when using KCl pipettes. Therefore, in many experiments we added ion channel blockers (e.g., 50–200 μM $CdCl_2$, 10 μM nifedipine, 2 mM CsCl, or 1 mM $BaCl_2$) or the muscarinic agonist carbachol (20–40 μM) the ACSF to help characterize the currents altered by the opiate peptides.

Quantification and statistics. All opiate-evoked changes in holding currents are given as mean ± SEM pA. To simplify reporting of effects on input conductance and I_M relaxations, these values are given as mean ± SEM of the percentage change from control. To determine the significance of the effects of the opiates on I_M, we averaged the I_M relaxation amplitudes for control, drug (opiate), and washout conditions over all hyperpolarizing steps (at roughly equivalent holding currents; mean = −41.2 mV) and submitted these results to ANOVA of repeated measures, followed by post hoc analysis by Newman-Keuls. p values less than 0.05 were considered to be significant.

Results

Neuronal sample and membrane properties

We used voltage recording during cell penetration and studied a total of 91 CA3 pyramidal neurons. These neurons had an average resting membrane potential (RMP) of −69 ± 0.7 mV (mean ± SEM; n = 86) and a mean (current-evoked) action potential amplitude of 103 ± 1.1 mV (n = 65). Stable recordings could be maintained for up to 4 hr, suggesting a relative lack of injury by the electrode penetration.

We studied the M-current using single-electrode voltage clamp at holding potentials of −32 to −54 mV (mean = −41.2 ± 1.1 mV). Without drugs such as Cd^{2+} or nifedipine in the bath (see below), the mean holding current reached at these depolarized potentials was +678 pA (n = 29). The I_M inward relaxation was voltage dependent with a maximum amplitude (averaged over five different hyperpolarizing steps) of 78–145 pA (mean = 123 pA) in control conditions (see Figs. 2, 3), using commands of −5 to −25 mV. Several lines of evidence suggest, as we reported previously for CA1 pyramidal neurons (Moore et al., 1988a), that the inward relaxations represent I_M: (1) the relaxations are suppressed (leaving only a "rectangular" ohmic current response) by the muscarinic agonist carbachol (20–40 μM; n = 7; Fig. 2*A*) or by 1 mM Ba^{2+} (Fig. 2*A,B*; n = 3; see below); and (2) the magnitude, kinetics, and voltage dependence of the relaxations appear similar to those of the M-current reported previously for CA1 pyramidal neurons (Fig. 2*A*; Halliwell and Adams, 1982; Moore et al., 1988a).

However, in 55% of the CA3 cells studied, hyperpolarizing commands of 15–25 mV (from depolarized holding potentials) elicited a complex current trajectory with a slow underlying *outward* relaxation (Fig. 2*B*; see also Gustafsson et al., 1982; Gähwiler and Brown, 1987) that ran counter to the M-current *inward* relaxation (especially with stronger hyperpolarizing commands) and thus interfered with measurement of the latter.

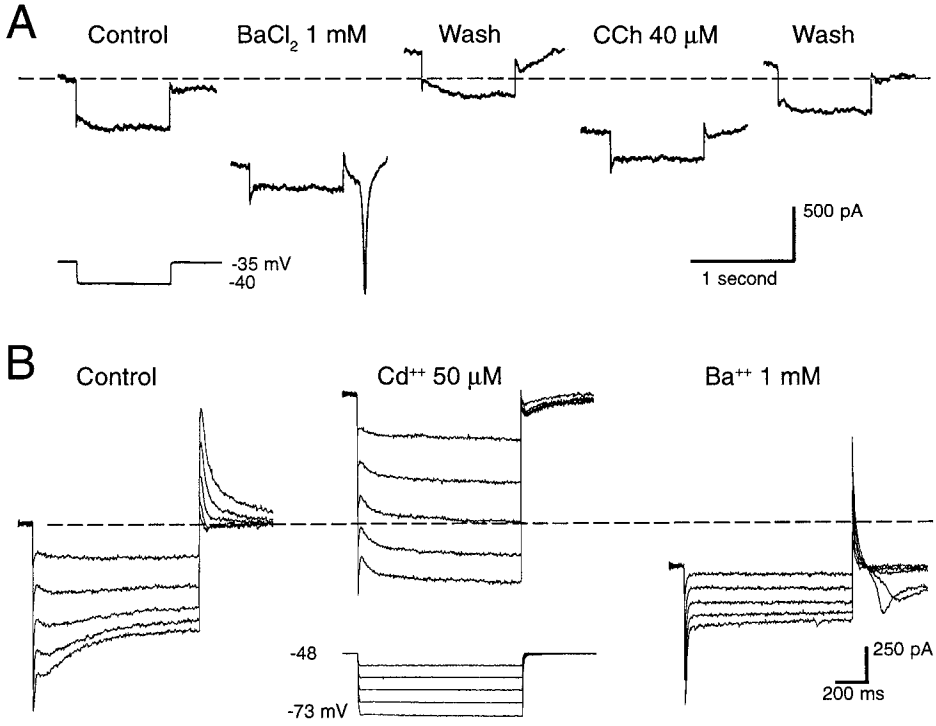

Figure 2. Characterization of the M-current in rat CA3 pyramidal neurons. *A,* Polygraph current records of individual I_M relaxations (in response to 5 mV hyperpolarizing commands; voltage protocol at *lower left*) before (*Control*) and after superfusion of $BaCl_2$ (12 min) and carbachol (*CCh*; 6 min). Both agents flatten the I_M relaxation, associated with a large inward holding current and a conductance decrease (shown by the decrease in the ohmic current step occurring at command onset). All effects return to control levels or even overshoot after washout of the two agents (34 min and 17 min, respectively). RMP of this cell was −68 mV. *B,* Another CA3 neuron. Computer-averaged (two sweeps) current responses to five different hyperpolarizing commands (voltage protocol at *lower middle*). The I_M relaxation clearly seen in the control condition with a 5 mV command is obscured by a slowly developing outward relaxation with larger commands; likewise, the I_M tail current on command offset is obliterated by a large transient outward current (see Zbicz and Weight, 1985). However, superfusion of Cd^{2+} (50 μM) in the ACSF (for 28 min) eliminates the outward relaxation and part of the outward tail current, leaving an obvious inward I_M relaxation. Superfusion of Ba^{2+} 1 mM fully blocks the inward relaxation, supporting the idea that it arises from a slow closing of M channels during the hyperpolarization. RMP of this cell was −62 mV. In both *A* and *B* during Ba^{2+} superfusion, note the abrupt inward currents at command offset, likely to represent Ba^{2+} spikes. *Dashed lines* in this and subsequent figures of current records represent control holding current.

Therefore, in later experiments we attempted to minimize this outward relaxation to verify better the validity of our I_M measures. Superfusion of 2 mM CsCl to block inward-rectifying conductances such as I_Q had no effect on the outward relaxation or on the I_M relaxation, although the I_Q relaxation was completely blocked (n = 17; data not shown). However, low concentrations (50–100 μM) of the general Ca^{2+} channel blocker Cd^{2+} (Fig. 2*B*) or 10 μM nifedipine (an antagonist of high-threshold Ca^{2+} or L-channels) usually reduced or obliterated the *outward* relaxation, leaving behind an inward current relaxation equivalent in form to the M-current seen in sympathetic ganglion neurons and CA1 pyramidal neurons (Brown and Adams, 1980; Halliwell and Adams, 1982; Moore et al., 1988a, Schweitzer et al., 1993). Cd^{2+} and especially nifedipine superfusion also resulted in tail currents more representative of I_M relaxations in CA1 pyramidal neurons, with the ohmic step at command offset smaller than at onset (see Fig. 1). Therefore, in later studies of the dynorphins we continually superfused either Cd^{2+} or, in most experiments, nifedipine onto all CA3 neurons displaying a marked outward relaxation.

Opioid effects

We tested six types of opiates: (1) U-50,488H, an opiate selective for κ opiate receptor subtypes; (2) dynorphins A and B, opioid peptides also relatively selective for κ receptors at low concentrations, but less selective at higher concentrations; (3) DADL, a broadly effective opioid peptide capable of activating several receptor subtypes but especially μ and δ; (4) DPDPE, a δ-selective opioid; (5) DAMGO, a μ-selective opioid peptide; and (6) des-Tyr-dynorphin, a fragment of dynorphin that does not bind to κ receptors. For any given CA3 neuron, the effects of a given peptide were usually measured across four electrophysiological measures: (1) holding current, (2) input conductance at depolarized potentials, (3) the M-current, and (4) the Q-current. The following results are organized according to these four measures; in each section we discuss the effects of the κ-selective agonists (low dynorphin concentrations and U-50,488H) first, followed by the effects of the opioids probably acting on other, non-κ opiate receptors (DADL, DAMGO, DPDPE, des-Tyr-dynorphin, and high concentrations of dynorphin A).

Holding current. Superfusion of low concentrations of dynorphin A (20–100 nM) either had no effect on the holding current at resting potential (n = 2) or caused a small outward (hyperpolarizing) current in four cells (see Table 1). Dynorphin B (100 or 1000 nM) also had little effect on holding current at holding potentials near rest. However, these low dynorphin concentrations evoked a pronounced outward holding current at depolarized potentials around −40 mV (Fig. 3), in keeping with a voltage-dependent action. Likewise, the κ agonist U-50,488H (2–10 μM) produced little holding current in most neurons at

Table 1. Effect of several opioid peptides on the holding current (mean pA ± SEM) in CA3 pyramidal neurons held at two different membrane potentials

Opioid tested	Holding near RMP	Holding near −40 mV	"*n*" cells
Dynorphin A (low conc.)[a]	45 ± 44 pA	245 ± 202 pA	6:6
Dynorphin B	8 ± 14	226 ± 33	6:6
U-50,488H[a]	−18 ± 16	190 ± 73	10:10
DADL[a]	9 ± 44	−217 ± 90	12:9
Dynorphin A (high conc.)[a]	−32 ± 27	−142 ± 72	10:9
Des-Tyr-dynorphin	−28 ± 19	−16 ± 37	4:4

Negative numbers represent inward currents. Low conc. = concentrations of 20–100 nM; high conc. = 1–1.5 μM. "*n*" cells refer to number of cells measured at RMP: near −40 mV.

[a] Data only from early studies without Cd^{2+} or nifedipine (which could artifactually influence holding currents) in the superfusate.

resting potential, but evoked large outward currents up to 600 pA at depolarized potentials (Table 1).

By contrast, superfusion of DADL (1–10 μM, usually 2 μM) elicited either weak inward or outward holding currents when CA3 neurons were held at or near resting potential, but at depolarized potentials DADL evoked an inward current (in eight of nine cells; Table 1). Similar effects were observed with high concentrations of dynorphin A (1–1.5 μM): small outward or inward currents at resting potential, but a clear inward current at depolarized membrane potentials (in seven of nine cells: Fig. 3*A*; Table 1). Des-Tyr-dynorphin (1 μM) had little effect on holding currents at either holding potential.

Input conductance at depolarized potentials. Because the opiates elicited clear or reproducible effects on holding currents at depolarized potentials but not at normal resting potentials, we estimated the average conductances only at depolarized holding potentials, using the inward steady state current (a control mean of 353 ± 96 pA for all opiate-treated cells) evoked by the 15 mV hyperpolarizing command of the I_M protocol (see Materials and Methods). Low dynorphin A concentrations increased mean input conductance by 18 ± 6% (n = 7), with washout of the effect. Low dynorphin B concentrations increased conductance by 21 ± 7% (n = 4), with complete recovery on washout. U-50,488H also increased mean conductance at depolarized potentials by 18 ± 3% (n = 8), again with washout of the effect. By contrast, DADL caused an average 5 ± 7% decrease in conductance (n = 8), with recovery on washout, and DPDPE decreased conductance by 16 ± 3% (n = 6). High concentrations

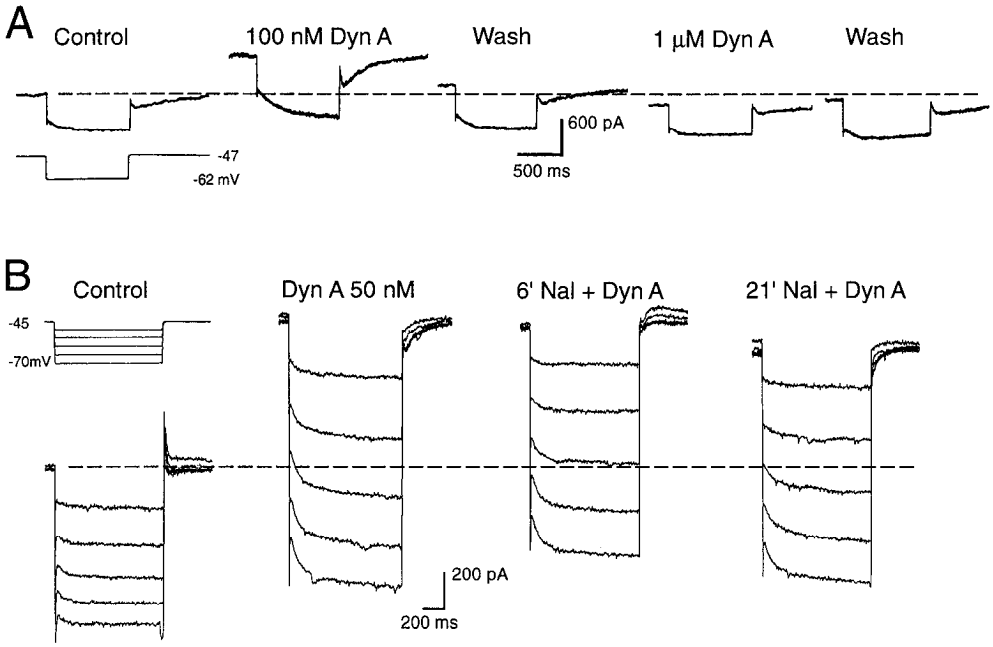

Figure 3. Dynorphin A (*Dyn A*) superfusion alters holding current, input conductance, and the M-current in CA3 pyramidal neurons. *A*, Polygraph record of current responses to a single −15 mV command (voltage protocol at *lower left*); no Ca^{2+} channel blockers were present. A low concentration of dynorphin A (superfused for 7 min) dramatically increases the I_M relaxation, in association with an outward holding current and a conductance increase, whereas later superfusion of a higher dynorphin A concentration (for 6.5 min) decreases I_M, causes a slight inward holding current, and reduces conductance. All these dynorphin effects are at least partially reversed by washout (8 and 12 min, respectively), although, as with DADL (see Fig. 8), the effects of 1 μM dynorphin A are more difficult to reverse than those of 100 μM dynorphin A. RMP of this cell was −68 mV. *B*, Another cell: computer-averaged current responses to several voltage commands (voltage protocol at *upper left*), all recorded in the presence of Cd^{2+} (50 μM). Dynorphin A (50 nM) dramatically increases the I_M relaxation and conductance, and appears to cause a large outward holding current. These effects are at least partially reversed by concomitant superfusion of 3 μM naloxone (*Nal*; 6 and 21 min). However, the sustained large outward holding current (and possibly part of the persistent increase in conductance) may be an artifact of prolonged Cd^{2+} superfusion (see Results). RMP of this cell was −67 mV.

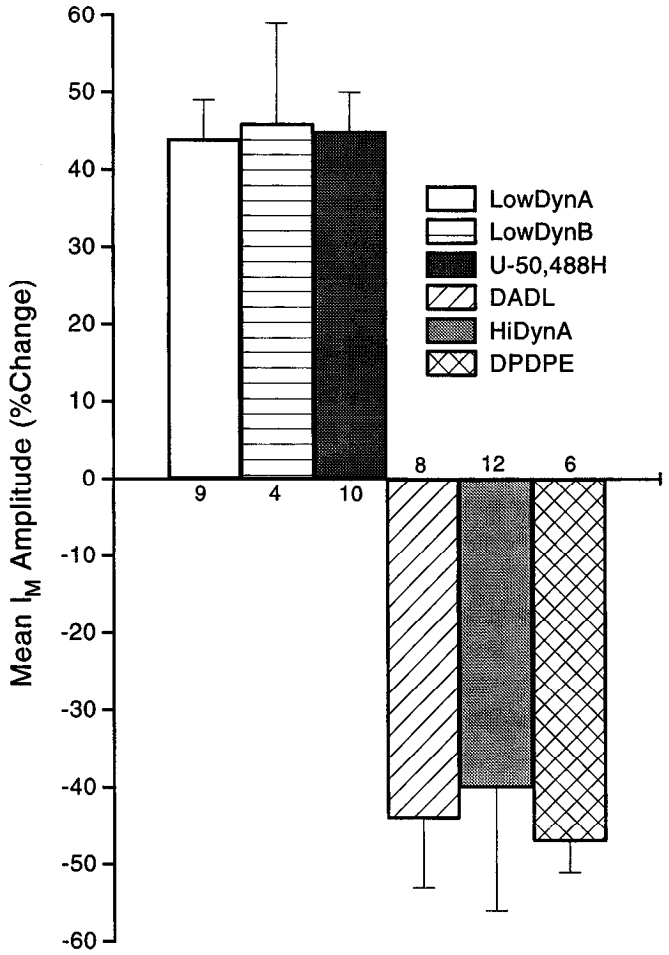

Figure 4. Summary of opioid effects on I_M amplitude, expressed as the mean percentage change from control (±SEM) when averaged over all the hyperpolarizing steps for all cells. Only the effects of those opioids that caused a significant effect by ANOVA (see text) are shown. Note that the κ-selective agonists U-50,488H or low concentrations of dynorphin A or B (*LowDynA, LowDynB*) all increase the mean M-current amplitude (*upward-going bars*) by about 45%. In contrast, the non-κ-selective agonists DADL and DPDPE, and high concentrations of dynorphin A (*HiDynA*) all decrease the mean I_M amplitude by 40–47% (*negative-going bars*). The *numbers associated with the zero line* indicate "*n*" cells. Error bars represent SEM.

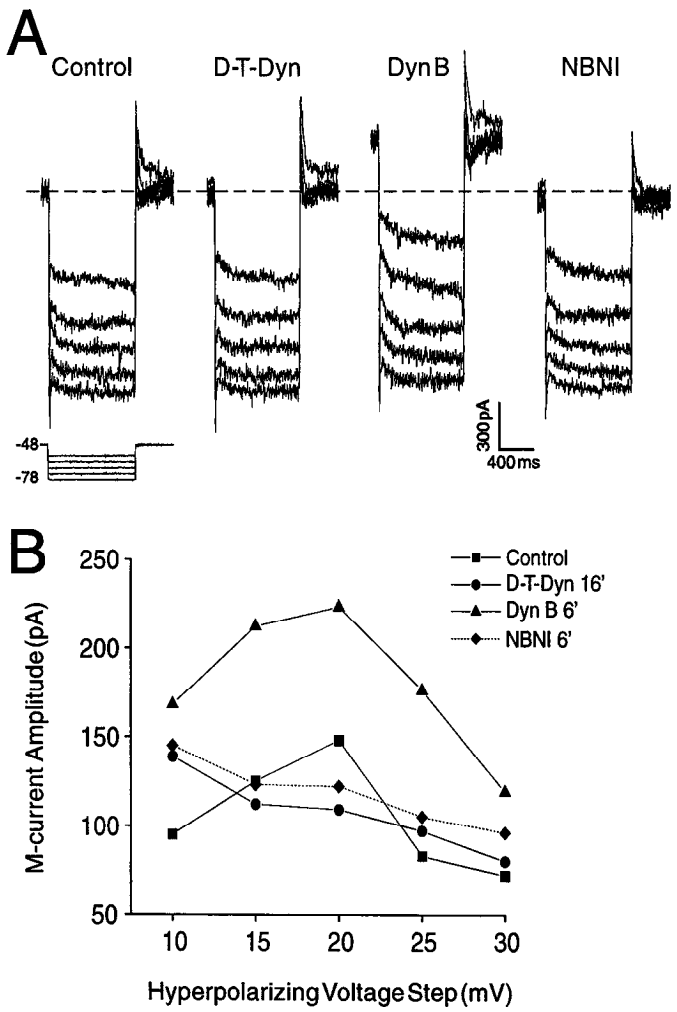

Figure 5. Dynorphin B (*DynB*), but not des-Tyr-dynorphin (*D-T-Dyn*), augments the M-current with reversal of the effect by (*NBNI*). *A*, Computer-averaged current responses to five voltage commands (protocol at *lower left*; holding potential = −48 mV), all taken in the presence of nifedipine (10 μM). Superfusion of des-Tyr-dynorphin (1 μM, 16 min) has little or no effect on the holding current, input conductance, or the M-current relaxations. By contrast, superfusion of 100 nM dynorphin B for 6 min causes an outward holding current and a conductance increase and dramatically increases M-current relaxations. Superfusion of 1 μM NBNI with DynB (6 min) resulted in the return of all measures back to control levels. *B*, Chart of I_M relaxation amplitudes taken from the same records as in *A* (from single exponential fits for each relaxation) plotted against command voltage. Note the large increase in I_M evoked by dynorphin B and the complete reversal of the effect by NBNI (1 μM). RMP of this cell was −73 mV.

of dynorphin A (10 cells) and des-Tyr-dynorphin (four cells) had no reversible, net average effect on conductance.

M-current. The effects of the opiates on the M-current relaxation is in keeping with their voltage-dependent effects on the holding currents and input conductances at depolarized potentials. Thus, all of the κ agonists enhanced the mean M-current relaxation amplitudes by an average of 40–50%. Figure 4 shows the effects of all opioids that elicited a significant effect on the I_M amplitude, expressed as the percentage change (±SEM; compared to control) averaged over all the hyperpolarizing steps. When submitted to statistical analysis by ANOVA of repeated measures (post hoc by Newman-Keuls) over all command steps, low concentrations of dynorphin A (20–100 nM) significantly [$F(2,102) = 7.272$; $p = 0.0011$] augmented I_M relaxations (Figs. 3*A,B*; 4) in 9 of 13 CA3 neurons showing recovery on washout. Low dynorphin B (100 nM) elicited significant [$F(2,57) = 9.943$; $p = 0.0002$] augmentations of I_M in four of four cells (Figs. 4, 5). Likewise, superfusion of U-50,488H (2–10 μM) significantly [10 of 10 cells; $F(2,117) = 15.055$; $p = 0.0001$] increased I_M relaxations, with a significant washout of the effect. Figure 6 illustrates the augmenting effects of low dynorphin A concentrations and U-50,488H on the I_M relaxation averaged for all hyperpolarizing steps across all tested cells showing reversal of the effect with naloxone or on washout. The I_M-augmenting effects of low dynorphin A concentrations were at least partially reversed by the concomitant superfusion of 1–5 μM (usually 1–2 μM) naloxone (four of five cells; Figs. 3, 6). The κ antagonist NBNI completely and significantly reversed dynorphin B augmentations of I_M (Fig. 5; $n = 4$); that is, M-currents during the antagonists were not significantly different from control levels, in all four cells tested. Naloxone 1–5 μM did not reverse aug-

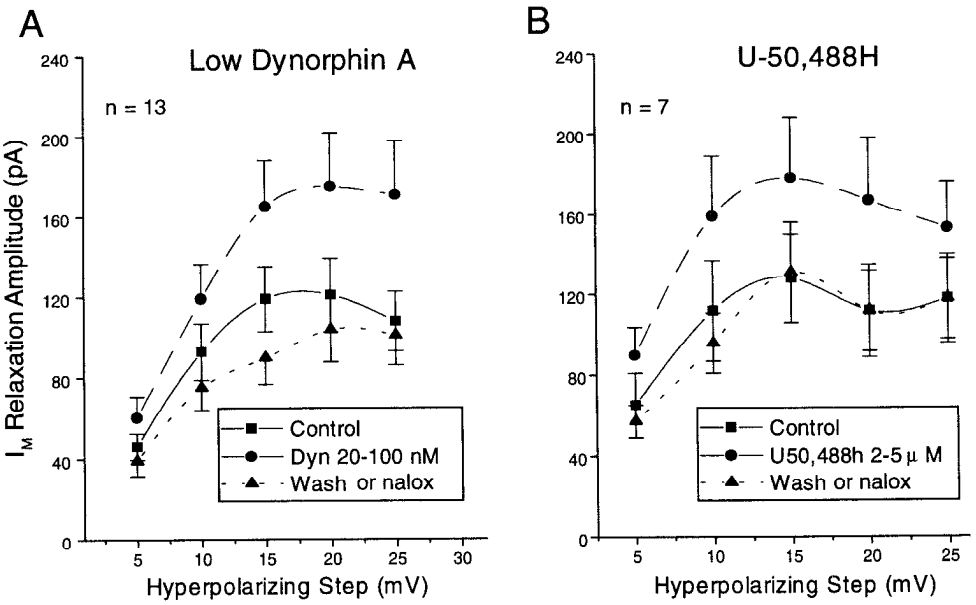

Figure 6. Augmenting effects of κ-selective agonists on M-current relaxations in response to five hyperpolarizing voltage steps (5, 10, 15, 20, and 25 mV) averaged from all cells showing at least a partial recovery on washout or with naloxone. *A*, Effect of low concentrations of dynorphin A (20–100 nM) on I_M relaxations averaged over 13 neurons showing recovery of effect with washout or naloxone treatment. There was a significant increase in I_M relaxations with a significant recovery (see Results). *B*, M-current data averaged from seven CA3 neurons held at around −40 mV and treated with U-50,488H (2–5 μM). Here again, the κ agonist significantly (by ANOVA and Newman-Keuls; see Results) increased M-currents in these cells, with significant recovery to control levels after washout or naloxone (*nalox*). Error bars represent SEM.

mentation of I_M by 5 μM U-50,488H in two cells but reversed this effect in another CA3 cell. In keeping with opiate receptor involvement in the dynorphin actions, the nonopiate des-Tyr-dynorphin had no significant effect on I_M [$F(1,38) = 0.248$; $p = 0.62$] even in the same cells showing pronounced I_M augmentation by dynorphin B (Fig. 5).

In those cells displaying the contaminating outward relaxation and therefore treated with Cd^{2+} (50–100 μM) or nifedipine (10 μM) to block Ca^{2+} channels, the I_M-augmenting effects of low dynorphin concentrations were particularly obvious ($n = 11$). However, prolonged superfusion of Cd^{2+} was often complicated by a slowly developing outward current and conductance increase that sometimes obscured complete washout or naloxone reversal of the dynorphin effects (Fig. 3), accounting for the apparent resistance to naloxone reversal in some cells. This problem did not occur with the use of nifedipine (Figs. 5, 7).

By contrast to the effects of κ-selective agonists, superfusion of less selective or δ-selective agonists depressed the mean M-current amplitudes by 40–47% on average (Figs. 4, 8). Thus, 1–10 μM DADL significantly [$F(2,102) = 7.999$; $p = 0.0006$] reduced the I_M relaxations (even in cells showing I_M augmentations by a κ agonist: Fig. 8), with significant reversal on washout with ACSF alone. Naloxone (1–3 μM) at least partially reversed this I_M-reducing effect of DADL in two of five cells. High dynorphin A concentrations (1–1.5 μM; nine cells tested in the presence of Cd^{2+} or nifedipine) reduced the relaxations in 7 of 12 neurons (Figs. 3*A*, 4), but also clearly augmented them in four cells (Fig. 7), and had no effect in one cell. When the data from all cells tested with high dynorphin A concentrations were averaged, there was a significant decrease [$F(2,57) = 6.816$; $p = 0.0022$] in I_M (Fig. 4), although without a significant group washout of the effect (however, several individual cells did show clear washout: see Fig. 3*A*). Naloxone (1–3 μM) antagonized both the I_M-decreasing (two cells) and the I_M-augmenting (four of four cells) effects of high dynorphin A concentrations, as well as the small (19 ± 11%) I_M-augmenting effects of high dynorphin B concentrations (two of two cells). Similarly, the δ receptor–specific agonist DPDPE (0.5–2 μM) significantly [$F(2,66) = 13.375$; $p = 0.0001$, $n = 6$] decreased the average I_M, again without a significant group recovery on washout (although several individual cells showed a clear reversal: Fig. 8*B*). By contrast, there was no clear effect on I_M of the μ receptor–specific agonist DAMGO (0.2–1 μM; six cells).

Q-current. To determine if these voltage-dependent effects of the opiates on membrane currents derived from specific actions on some non-I_M membrane conductance, we tested the effects of several opiates on the Q-current seen in CA3 pyramidal neurons. In five cells, 1 μM dynorphin A had no effect on the Q-current relaxation seen with hyperpolarizing commands delivered from holding potentials around −70 mV (data not shown). In one CA3 neuron, DADL (10 μM) slightly increased the I_Q relaxation. In two other neurons U-50,488H had no effect or decreased I_Q but without recovery on prolonged washout. Furthermore, the effects of the opiates on the M-current relaxations described above were not altered by pretreatment of the CA3 neurons with 2 mM Cs^+ (15 cells), which totally flattened the I_Q relaxations seen at more hyperpolarized holding potentials.

Discussion

Voltage-dependent opioid effects. As noted in the introductory remarks, there is an abundance of opioid-containing fibers innervating rat hippocampal CA3 neurons, and under appropriate stimulus conditions these fibers appear to release a variety of prodynorphin- and proenkephalin-derived peptides. The results of our voltage-clamp investigations (1) suggest that these opioid peptides have direct, postsynaptic voltage-dependent actions on CA3 pyramidal neurons; (2) suggest that these actions depend upon the concentration of the peptide and the opiate receptor subtype(s) present on the target neurons; and (3) provide possible explanations for the rather covert and previously confusing role played by the major class of naturally occurring opioids in CA3, the prodynorphin-derived peptides previously shown to have κ receptor–mediated effects. Our studies show that U-50,488H and low concentrations of dynorphin A and B, likely to be most specific for κ receptors, cause an increased conductance and a pronounced outward (hyperpolarizing) holding current when the CA3 pyramidal cell is depolarized from resting potential. Higher (and probably less receptor-selective) concentrations of dynorphin A, as well as opioids likely to activate δ receptors, usually exert actions opposite to those of the κ ago-

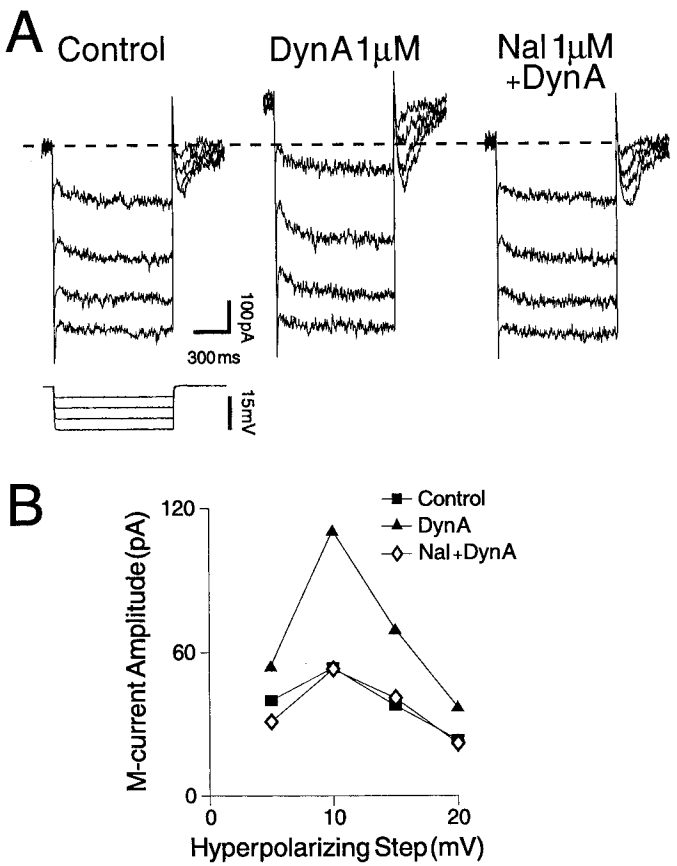

Figure 7. High concentrations of dynorphin A can also increase I_M relaxations in some CA3 neurons, with naloxone reversal. *A*, Computer-averaged (two sweeps) current responses to four voltage commands (protocol at *lower left*; holding potential = −43 mV), all taken in the presence of the Ca^{2+} channel blocker nifedipine (10 μM). Note the tail (offset) current typical of M-channel opening. Superfusion of 1 μM dynorphin A (*DynA*; 10 min) increases the I_M relaxations and conductance and causes an outward holding current. Concomitant superfusion of naloxone (*Nal*; 1 μM) for 19 min completely reverses all dynorphin effects. *B*, Chart of I_M relaxation amplitudes taken from the same records as in *A*, obtained by fitting single exponential curves to each relaxation, and plotted against voltage command. Note that naloxone completely reverses the I_M augmentation by dynorphin. RMP of this cell was −66 mV.

nists: inward (depolarizing) currents associated with a conductance decrease, especially at depolarized membrane potentials. We attribute at least a portion of these opposing effects on holding currents to actions on the M-current, with κ receptor activation increasing it and δ receptor activation decreasing it. Indeed, there are considerable data showing that several peptides can regulate the M-current (Brown, 1988), and somatostatin, like dynorphin A and B, can increase it in neurons (Jacquin et al., 1988; Moore et al., 1988a). The recently reported sequence homologies between δ opiate and somatostatin receptors (Evans et al., 1992) may be pertinent to these M-current data.

However, there is at least one confound to be considered in this interpretation. M-current recording in CA3 is not as straightforward as in CA1, because of apparent "contamination" by other currents when using standard I_M voltage-clamp protocols. Thus, the outward (non-I_M) relaxation described here (and shown in the figures of several earlier publications; e.g., Gustafsson et al., 1982; Gähwiler and Brown, 1987) often has a trajectory that runs counter to the usual inward I_M relaxation. Blocking this outward relaxation with Cd^{2+} (see also Charpak et al., 1990) or nifedipine helped verify the opioid effects on the M-current to our satisfaction. Although the nature of the outward relaxation is unknown, the fact that it is blocked with Ca^{2+} channel blockers and is less often seen when using K-acetate–containing pipettes (data not shown) suggests involvement of a Ca^{2+}-dependent Cl^- conductance. The use of Cd^{2+} together with TTX, by greatly reducing Na^+ and Ca^{2+} conductances, also helps reduce the problem of space-clamp artifacts due to large conductances in remote dendrites, and minimizes the likelihood that a Ca^{2+}-dependent K^+ conductance contributes to the opioid effects.

From these data we conclude that opioids can regulate the M-current in CA3 pyramidal neurons. Because of the voltage dependence of I_M, these opioid effects will be more pronounced at depolarized membrane potentials. It is possible that the opioids, like somatostatin in CA1 (Schweitzer et al., 1993), influence other ionic conductances in CA3 neurons. One candidate is the inward (or anomalous) rectifier current seen in pyramidal neurons; this K^+ conductance is augmented by enkephalins in several peripheral and central neuron types (North et al., 1987; Tatsumi et al., 1990), including a sample of CA1 cells likely to be interneurons (Wimpey and Chavkin, 1991). However, our studies with Cs^+ do not support opiate alteration of the inwardly rectifying I_Q. Ca^{2+} conductances might also be altered. Opiates, including dynorphin A (Gross et al., 1990), can alter Ca^{2+} currents in several neuron types (Gross and MacDonald, 1987; Crain and Shen, 1990; Surprenant et al., 1990; Regan et al., 1991; Seward et al., 1991). Such a Ca^{2+} channel effect is interesting in the light of recent studies suggesting a role for intracellular Ca^{2+} in the regulation of I_M in sympathetic ganglia neurons (Beech et al., 1991; Kirkwood et al., 1991; Marrion et al., 1991). However, our data showing the persistence of opiate effects on I_M in the presence of Ca^{2+} channel blockers do not support a primary role for Ca^{2+} currents in this I_M effect in hippocampal CA3 neurons.

Mixed or opposing opioid actions in hippocampus. The opposing opioid effects on I_M could explain the mixed excitatory and inhibitory effects of opiates, including dynorphin and other κ agonists, seen in both extracellular and intracellular current-clamp studies of CA3 neurons (Henriksen et al., 1982; Walker et al., 1982b; Gruol et al., 1983; Moises and Walker, 1985; Siggins et al., 1986). The mixed effects may be more often encountered when using dynorphin application from pipettes, where drug concentrations are not known or not easy to control. In addition, the specificity of dynorphin and κ agonists for opiate receptors has been brought into question because of the often reported inability to block their effects by naloxone (Walker et al., 1982a; Moises and Walker, 1985; Alzheimer and ten Bruggencate, 1990). However, it is not yet certain whether this is due to a nonopiate action of the κ agonists or to a higher resistance of the κ receptors (compared to μ or δ receptors) to low concentrations of naloxone in the presence of possibly high concentrations of the agonist.

The involvement of opiate receptors. In the present studies, naloxone at reasonably low concentrations (1–3 μM) most often antagonized the I_M-augmenting effects of low dynorphin A, suggesting involvement of an opiate receptor. Likewise, superfusion of the κ_1-specific antagonist NBNI (100–2000 nM) partially or completely reversed the dynorphin A– and B–induced augmentation of I_M in most cells. Although some cells showed partial

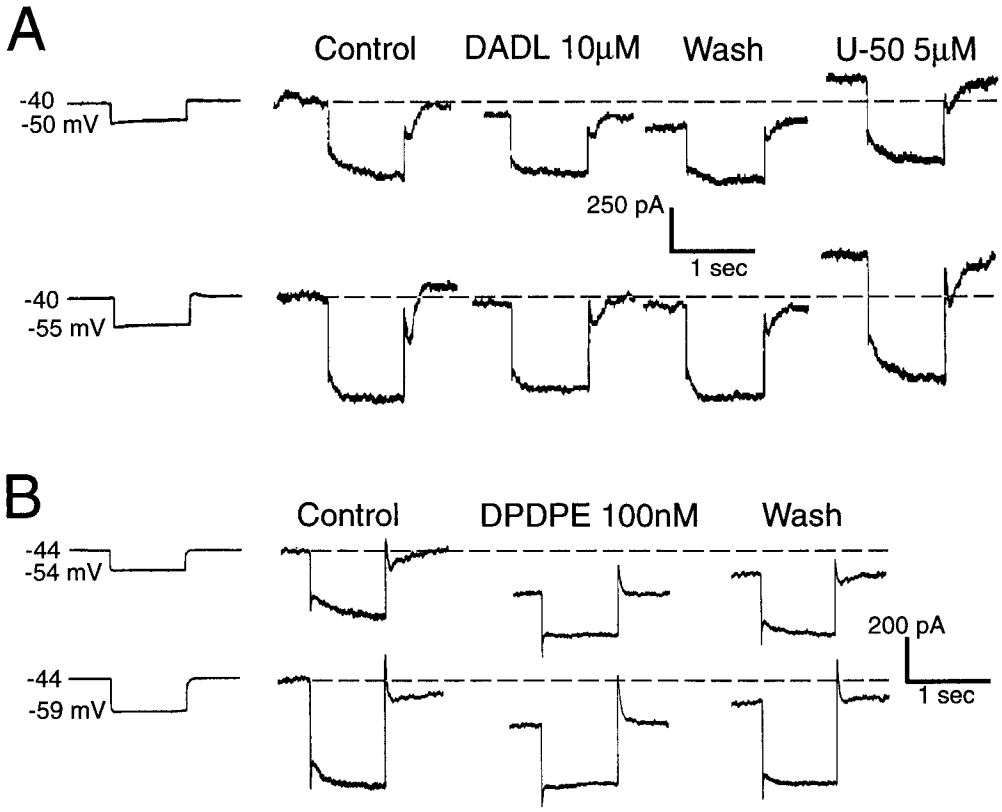

Figure 8. Opiates can reciprocally modulate the M-current in CA3 pyramidal neurons; those selective for δ receptors reduce I_M. *A,* Polygraph record of voltage-clamp recording of selected membrane currents at a −40 mV holding potential, with voltage steps (protocols at *left*) of −10 mV (*upper traces*) and −15 mV (*lower traces*), taken during superfusion of control ACSF and ACSF with added opiates. DADL (10 μM, 8 min) reduces the M-current relaxation and input conductance (note the decrease in the size of the ohmic step at command onset), in association with a small inward holding current. Also note the failure to obtain complete recovery of these measures (especially the inward holding current) with washout (*Wash*) of DADL. By contrast, subsequent superfusion of the κ agonist U-50,488H (*U-50*; 5 μM; 9 min) dramatically augments the relaxation and the conductance and causes a pronounced outward baseline current in the same cell. *Gaps between records* represent periods of 8–10 min when the membrane potential was returned to rest. RMP in this cell was −75 mV. *B,* Polygraph recordings from another CA3 cell, held at −44 mV (voltage protocols at *left*), showing that the δ-selective agonist DPDPE (superfused for 16 min) causes an inward holding current, a conductance decrease, and a flattening of the M-current, with partial recovery to control levels with 20 min of washout of the opiate. RMP of this cell was −72 mV.

resistance to naloxone or NBNI reversal, most of these resistant neurons had been concomitantly superfused with Cd^{2+}, which appears to prevent total reversal by superimposing a nonspecific (nonopioid) outward conductance. Also, with dynorphin A effects, NBNI may be less effective than naloxone, suggesting that some other κ subtype could be involved. The report that κ_2 receptors predominate in rat brain (Zukin et al., 1988; but see Nock et al., 1990) is consistent with this conclusion. Moreover, the lack of effect on I_M of des-Tyr-dynorphin, the dynorphin fragment that does not bind to opiate receptors (see Walker et al., 1982a), further supports the involvement of some type of κ receptor in the I_M augmentation by dynorphins A and B. The receptor(s) involved in the I_M-*reducing* effects usually evoked by the less receptor-selective agonist DADL or high dynorphin concentrations is less clear, although mimicry of this effect by the δ agonist DPDPE and the lack of effect of the μ agonist DAMGO suggest a role for δ receptors. Naloxone effectively antagonized the I_M-reducing opiate effects, again pointing to the involvement of an opiate receptor.

Functional implications. As for the function of endogenous opioids in CA3 hippocampus, regulation of the M-current should play an important role in events that involve prolonged depolarizations, such as the bursting activity often seen in CA3 pyramidal neurons. Dynorphin-induced augmentation of I_M would be predicted to counter prolonged depolarizations and reduce spike bursts (Adams et al., 1982; Halliwell and Adams, 1982). Indeed, depending on the activation kinetics of I_M (a property difficult to measure in these neurons), it is possible that the reduced action potential amplitudes seen with dynorphin (Pacheco et al., 1983) and the κ agonists U-50,488H and U-69,593 (Alzheimer and ten Bruggencate, 1990) derive from M-current augmentation, via indirect shortening (rather than direct reduction) of Na^+ currents.

Recent evidence suggests that opiates play some as yet undefined role in epileptiform activity. The epileptigenic action of μ agonists in CA1 and CA3 neurons may arise from inactivation of inhibitory interneurons in hippocampus (Zieglgänsberger et al., 1979; Nicoll et al., 1980; Siggins and Zieglgänsberger, 1981; Siggins et al., 1986). By contrast, results of several studies point to a dampening effect of dynorphins or mossy fiber activation on epileptiform activity (Tortella and Holaday, 1986; Jones, 1991), in keeping with an inhibitory action via I_M augmentation. The differential alterations of enkephalin and dynorphin levels and metabolism in hippocampus following evoked seizure activity (Gall, 1988; Hong et al., 1988) also suggest a role for hippocampal opioids in seizure-induced behaviors.

Possible role of opioids in LTP and memory. The role of the hippocampus in memory processing is well known, as is the

utility of the phenomenon of LTP as a cellular model of memory and learning. It has also become clear that LTP in the mossy fiber–CA3 pathway is quite different from that in the Schaffer collateral–CA1 pathway (for details, see Harris and Cotman, 1986; Ishihara et al., 1990; Jaffe and Johnston, 1990; Zalutsky and Nicoll, 1990). Interestingly, both somatostatin (Matsuoka et al., 1991) and opiates (Martinez et al., 1990) can enhance LTP or synaptic transmission in the rat mossy fiber–CA3 system, whereas naloxone is usually reported to prevent LTP induction here (Martin, 1983; Derrick et al., 1991; Williams and Johnston, 1992).

However, a recent report suggests that dynorphin may reduce LTP, in a naloxone-reversible fashion, in the guinea pig mossy fiber–CA3 system, at least in part by a presynaptic action (Weisskopf et al., 1992, 1993). A similar finding has recently been described for the perforant path–dentate granule cell pathway (Wagner et al., 1993). Unfortunately, the mechanism(s) underlying LTP in the mossy fiber–CA3 system (e.g., whether it arises pre- or postsynaptically) is still under some debate (Jaffe and Johnston, 1990; Zalutsky and Nicoll, 1990). Nonetheless, it would appear from our data and that of Weisskopf et al. (1992, 1993) that dynorphin in CA3 hippocampus can act at both pre- and postsynaptic sites. As the presynaptic effects were seen in CA3 of guinea pig, which displays mostly κ_1 receptors, it is possible that species differences account for the contrasting sites of dynorphin action. It seems pertinent that these and other researchers studying guinea pig hippocampus have failed to find dynorphin effects on CA3 pyramidal neuronal excitability (Caudle and Chavkin, 1990; Weisskopf et al., 1992).

Postsynaptically, there are hints of processes (e.g., several types of ionic conductances) in the rat that might regulate mossy fiber–CA3 LTP. Dynorphins or enkephalins released from mossy fibers (Gall et al., 1981; Chavkin et al., 1983a, 1985b; McGinty et al., 1983; Terrian et al., 1988) could act on distinct κ or δ receptors to either up- or downregulate this form of synaptic plasticity, perhaps via bimodal modulation of I_M, or via the pronounced low- and high-threshold Ca^{2+} currents seen in CA3 pyramidal neurons (Gähwiler and Brown, 1987). Based on their potent hippocampal actions, it is reasonable to suggest that systemic opiates also could alter memory function. This notion is strengthened by recent data showing naloxone-sensitive impairment of spatial memory by dynorphin microinjection into the hippocampus (McDaniel et al., 1990). Further studies will be required to determine whether the M-current is involved in these memory-related phenomena.

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