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POSSIBLE MECHANISMS OF ENKEPHALIN ACTION ON HIPPOCAMPAL CA1 PYRAMIDAL NEURONS[1]

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Abstract

(*1*) Intracellular and extracellular recordings were made from CA1 pyramidal neurons in an *in vitro* rat hippocampal slice preparation, while [D-Ala^2, D-Leu^5]enkephalin (DADL) was applied by perfusion at a known concentration (1 to 5 × 10^{-7} M), in a small droplet, or by iontophoresis into the cellular and dendritic layers of the slice. The effects of DADL on synaptic potentials and membrane properties were studied in an effort to determine the mechanisms underlying its epileptogenic action in the hippocampus.

(*2*) DADL increased the size and often the duration of excitatory postsynaptic potentials (EPSPs) generated on either the apical or basal dendrites; this resulted in an increased discharge probability for a constant orthodromic stimulus. Extracellular field potential recordings showed a larger population spike for a given size field EPSP. These effects of DADL could be reversed substantially by perfusion with naloxone (1 to 5 × 10^{-7} M) and appeared qualitatively different from the epileptiform actions of penicillin.

(*3*) DADL did not appear to increase the intrinsic excitability of the soma membrane, since membrane potential, input resistance, spike threshold, and antidromic field potentials all were unchanged. In addition, the shape of the membrane charging curve during hyperpolarizing current injection was not changed noticeably by DADL.

(*4*) At the concentrations tested, DADL did not attenuate recurrent inhibition in the CA1 region, as evaluated by comparing in the absence and presence of DADL: (*a*) antidromically evoked recurrent inhibitory postsynaptic potentials (IPSPs) and their dependence on membrane potential, (*b*) the reduction of a synaptically driven population spike by a prior antidromic volley, (*c*) iontophoretic GABA (γ-aminobutyric acid) responses. Similarly, IPSPs evoked by orthodromic stimulation appeared either unaffected or occasionally enhanced by DADL.

(*5*) By iontophoretic mapping, it was shown that the DADL-sensitive sites are limited to stratum oriens and stratum pyramidale. Local application of DADL into stratum radiatum was relatively ineffective in enhancing the efficacy of synapses located in this region.

(*6*) The dendritic input-output relationship between the presynaptic fiber volley and the field EPSP was not changed by DADL. This finding and the results of the iontophoretic mapping experiments suggest that increased excitatory transmitter release was not involved.

(*7*) The data are consistent with the proposal that DADL selectively attenuates a dendritic IPSP which is virtually invisible to the soma, although the possibility cannot be ruled out that DADL may, in addition, act to enhance the responsiveness of pyramidal dendritic membrane to excitatory synaptic activation.

The functional roles of opioid peptides in the central nervous system are of considerable neurobiological interest. Intraventricular injection of subanalgesic doses of opioid peptides in rats can produce electrographic and behavioral evidence of limbic seizure activity (Urca et al., 1977; Frenk et al., 1978a, b; Henriksen et al., 1978), which appears at least partly confined to the hippocampal formation (Henriksen et al., 1979). Similar expressions of interictal seizure activity in electrically kindled rats in some cases can be attenuated by naloxone (Frenk et al., 1979). These results raise the possibility that one function of endogenous opioid systems may involve the elaboration of certain epileptiform phenomena in limbic areas, although the precise nature of such a role has yet to be defined. The hippocampus is a structure well suited for investigating cellular mechanisms of drug action. Our present knowledge of its synaptic organization together

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with the recently reported distribution of opioid receptors (Goodman et al., 1980; Herkenham and Pert, 1980) and enkephalin-containing fiber systems (Sar et al., 1978; Gall et al., 1981) provide a basis for defining opioid mechanisms in terms of identified synapses and neurons.

The unexpected finding that iontophoretic application of opioid peptides elevates the firing rate of rat hippocampal pyramidal neurons (Nicoll et al., 1977) triggered a search for the cellular mechanisms of opioid action in this brain region. Based on extracellular *in vivo* studies, Zieglgänsberger et al. (1978) proposed that the observed rise in pyramidal cell spike frequency was a result of inhibition of the tonic activity of basket cell interneurons. Later studies in hippocampal slices and cultures were conflicting; several reports supported the original suggestion of recurrent disinhibition (Dunwiddie et al., 1980; Lee et al., 1980; Gähwiler, 1980; Nicoll et al., 1980) although two investigations reported no decrease in the intensity of synaptic inhibition when hippocampal slices were exposed to opioid peptides (Haas and Ryall, 1981; Lynch et al., 1981).

In the present investigation, intracellular and extracellular recordings have been made from identified hippocampal pyramidal neurons in an *in vitro* slice preparation. It will be shown that [D-Ala^2, D-Leu^5]enkephalin (DADL) can increase the size of orthodromically evoked excitatory postsynaptic potentials (EPSPs) without measurably reducing inhibitory postsynaptic potentials (IPSPs) recorded at the soma, including recurrent IPSPs. We are left with the possibilities that DADL enhances the responsiveness of pyramidal dendritic membrane to excitatory synaptic activation or that an unusual and novel form of dendritic inhibition is blocked selectively by DADL. A preliminary report of the results was presented to the Society for Neuroscience (Dingledine, 1980).

Materials and Methods

Rats (125 to 200 gm) were anesthetized with ether; the hippocampus was dissected out of each hemisphere and placed into chilled artificial cerebrospinal fluid (ACSF). A Sorvall tissue chopper was used to cut nearly transverse slices which then were transferred to the recording chamber. Slices were maintained at 33 to 36°C and superfused at a rate of 0.3 to 1 ml/min with ACSF of the following composition (in millimolar concentrations): NaCl, 130; KCl, 3.5; NaH_2PO_4, 1.25; $NaHCO_3$, 24; $CaCl_2$, 1.5 (or, in initial studies, 2.0); $MgSO_4$, 1.5; glucose, 10; bubbled with 95% O_2, 5% CO_2 to a pH of 7.3 to 7.4. A humidified stream of warm O_2/CO_2 was directed over the upper surface of the slices. The procedures for preparing and maintaining slices and the recording chamber design are described in more detail elsewhere (Dingledine et al., 1980).

Extracellular recordings of field potentials were made with glass micropipettes (10 to 40 megohms, filled with 3 M NaCl) positioned in the various cellular and dendritic layers of the slices as required. Intracellular recordings were made with glass micropipettes pulled on a Brown-Flaming type puller (David Kopf Instruments) and filled with 3 M potassium acetate. Electrode resistance, evaluated with square current pulses, was 100 to 200 megohms. In some cases, electrodes were beveled to a final resistance of 70 to 150 megohms (Ogden et al., 1978); these electrodes were quieter and had superior current passing capability although they appeared to offer no distinct advantage in terms of ease or healthiness of penetration.

Satisfactory intracellular recordings were obtained from 34 CA1 pyramidal neurons (nomenclature of Lorente de Nó, 1934). Although the resting properties of each cell varied somewhat over the course of an experiment, the action potential in different cells ranged from 87 to 120 mV (mean, 104 ± 2 mV SEM), input resistance ranged from 9 to 65 megohms (mean, 36 ± 2 megohms), and membrane potential ranged from −62 to −89 mV (mean, −72 ± 1 mV). Except in a few cases, recordings from individual cells were maintained for 1 to 7 hr, with the quality of the penetration usually improving gradually over the initial part of this period as a result of the electrode sealing into the membrane.

The pyramidal cell population was stimulated orthodromically with constant current negative square wave pulses (10 to 200 μA, 0.1 msec) by sharpened tungsten electrodes positioned visually among the afferent fibers of the stratum (st.) radiatum and/or the st. oriens. For antidromic activation, a similar stimulating electrode was placed in the alveus on the subicular side of the recording site. For all experiments in which recurrent inhibition was studied, a razor blade chip was used to make a cut through st. oriens and st. pyramidale at a position between the antidromic stimulating electrode and the recording site. This lesion resulted in a purer antidromic volley by reducing the possibility that current spread from the alveus electrode would activate afferent fibers running in the st. oriens (Dingledine and Langmoen, 1980). A test of the effectiveness of the lesion in each slice was made by delivering a high intensity (100 μA) tetanizing stimulus (10 Hz) through the alveus electrode; only slices in which this stimulus train evoked a clean antidromic population spike, with no evidence of a facilitating orthodromic population spike, were used further. This arrangement was deemed necessary to study the properties of recurrent inhibitory potentials in isolation, since, without such a lesion, antidromically evoked recurrent IPSPs usually were contaminated with EPSPs. To eliminate the complications of spike afterpotentials when studying recurrent IPSPs, the antidromic stimulus intensity was set below threshold for the impaled cell.

[D-Ala^2, D-Leu^5]Enkephalin (Peninsula Laboratories), sodium benzyl penicillin (Sigma), normorphine sulfamate (gift of Dr. E. L. May, National Institutes of Health), and morphine sulfate (Mallinckrodt) were applied by perfusion. The recording chamber had a dead space of approximately 1.5 ml. With a flow rate of 0.5 to 1 ml/min, drug effects reached a plateau within 12 to 20 min of changing solutions. In some experiments, single barrel iontophoretic pipettes were used to deliver DADL (1 mM in 100 mM NaCl or sodium acetate, pH 5.5), sodium benzyl penicillin (0.5 M, pH 7.3), sodium glutamate (1 M, pH 7.5, Sigma), and GABA (γ-aminobutyric acid, 1 M, pH 4.5, Sigma) into various regions of the slice, or DADL solution (10^{-5} M) was applied to the surface of the slice in a small (<1 nl) droplet. Iontophoretic electrodes had resistances of 30 to 150 megohms; backing currents ranged

from 0 to 5 nA and ejection currents ranged from 5 to 180 nA. All iontophoretic pipettes were positioned independently by separate micromanipulators.

Recorded signals were led through negative capacitance DC-coupled electrometers, displayed on an oscilloscope, and stored on magnetic tape. During intracellular recordings, a chart recorder was used to monitor membrane potential. The electrometer used for intracellular recording had a bridge configuration that allowed current pulses to be passed across the cell membrane to change the membrane potential and monitor input resistance. Whenever this was done, the bridge balance was routinely checked and adjusted if necessary (Engel et al., 1972). In most experiments, the recorded signals were averaged (Nicolet model 1072) before photography; measurements were made by hand from the film or chart.

Results

Characteristics of the enkephalin effect. DADL, when washed into the bath (1 to 5 × 10^{-7} M), applied iontophoretically, or in a droplet into st. pyramidale or st. oriens, enhanced the response of CA1 pyramidal neurons to excitatory synaptic activation. This effect, which occurred regardless of whether the activated synapses were located on the apical or basal dendrites, was manifested in intracellular recordings by an increase in the size and often the duration of evoked EPSPs (Fig. 1). DADL increased the size of evoked EPSPs in 24 of the 27 neurons tested. The magnitude of this effect varied and was difficult to estimate since a previously subthreshold EPSP in the presence of DADL often would trigger a spike. With suprathreshold stimulation, EPSPs that normally triggered only one spike could, in the presence of DADL, elicit two or occasionally three spikes (Fig. 2). In extracellular recordings of orthodromic population spikes, DADL increased the amplitude of the primary response and, at higher stimulus intensities, evoked two, or rarely three, population spikes (Fig. 3), in confirmation of previous reports (Martinez et al., 1979; Dunwiddie et al., 1980; Lynch et al., 1981; Haas and Ryall, 1981). These effects of DADL were undiminished for at least 30 min in bath applications and could be reversed by washing or by adding naloxone (1 to 5 × 10^{-7} M) to the perfusion fluid. A similar augmentation of the response to orthodromic stimulation could be seen in slices perfused with normorphine or morphine, although the required concentration was higher than for the enkephalin derivative used here. The threshold concentration for these alkaloid opiates was around 10^{-6} M, while the threshold concentration of DADL was around 10^{-8} M.

The effect of DADL on the orthodromic response of pyramidal cells was milder and appeared to be qualitatively different from that of the well studied epileptogenic agent, penicillin. Intense burst responses (i.e., more than five population spikes) to moderate stimulation intensities and large intracellular "depolarizing shifts," which

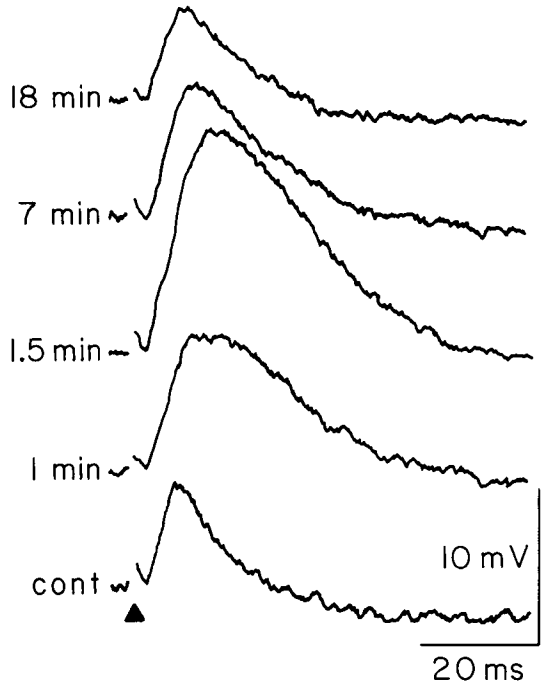

Figure 1. Effect of DADL on evoked EPSP. Each *trace* is the average of four responses to st. radiatum stimuli in control (*cont*) and at various times after application of a droplet (<1 nl) of 10^{-5} M DADL to the surface of the slice. The *arrowhead* marks the stimulus artifact in the *lowest trace*. Membrane potential, −70 mV throughout; action potential, 110 mV; input resistance, 15 megohms before and after DADL application.

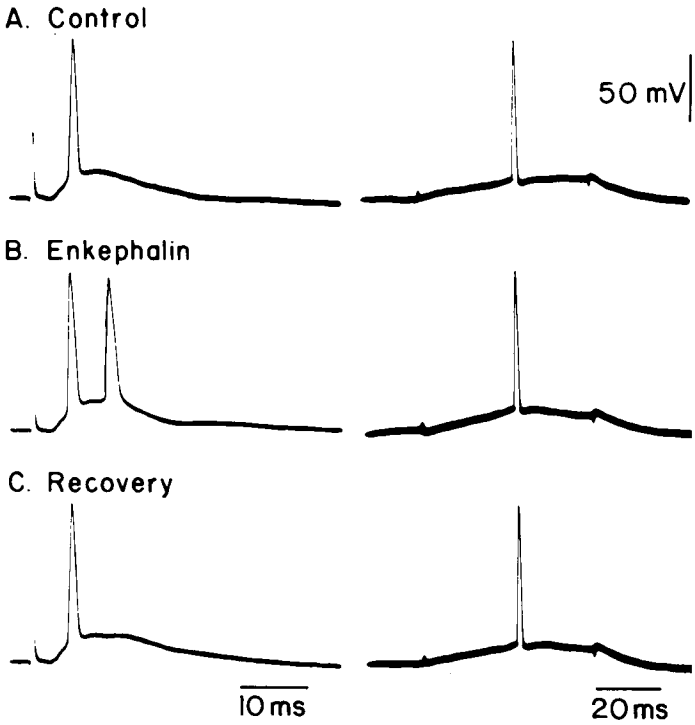

Figure 2. Effect of DADL on synaptic and direct activation of a CA1 pyramidal neuron. This cell was activated synaptically (*left column*) by a stimulating electrode in st. radiatum and directly (*right column*) by a 0.4-nA depolarizing current pulse delivered through the recording electrode. The synaptic stimulus and current pulse were repeated alternately at 0.5 Hz by an automatic sequencer. *A*, Control responses. *B*, Responses 1 min after an iontophoretic application (50 nA, 20 sec) of DADL into st. oriens. Synaptic but not direct activation is enhanced by DADL. *C*, Recovery 4 min later. Membrane potential, −72 mV; input resistance, 32 megohms.

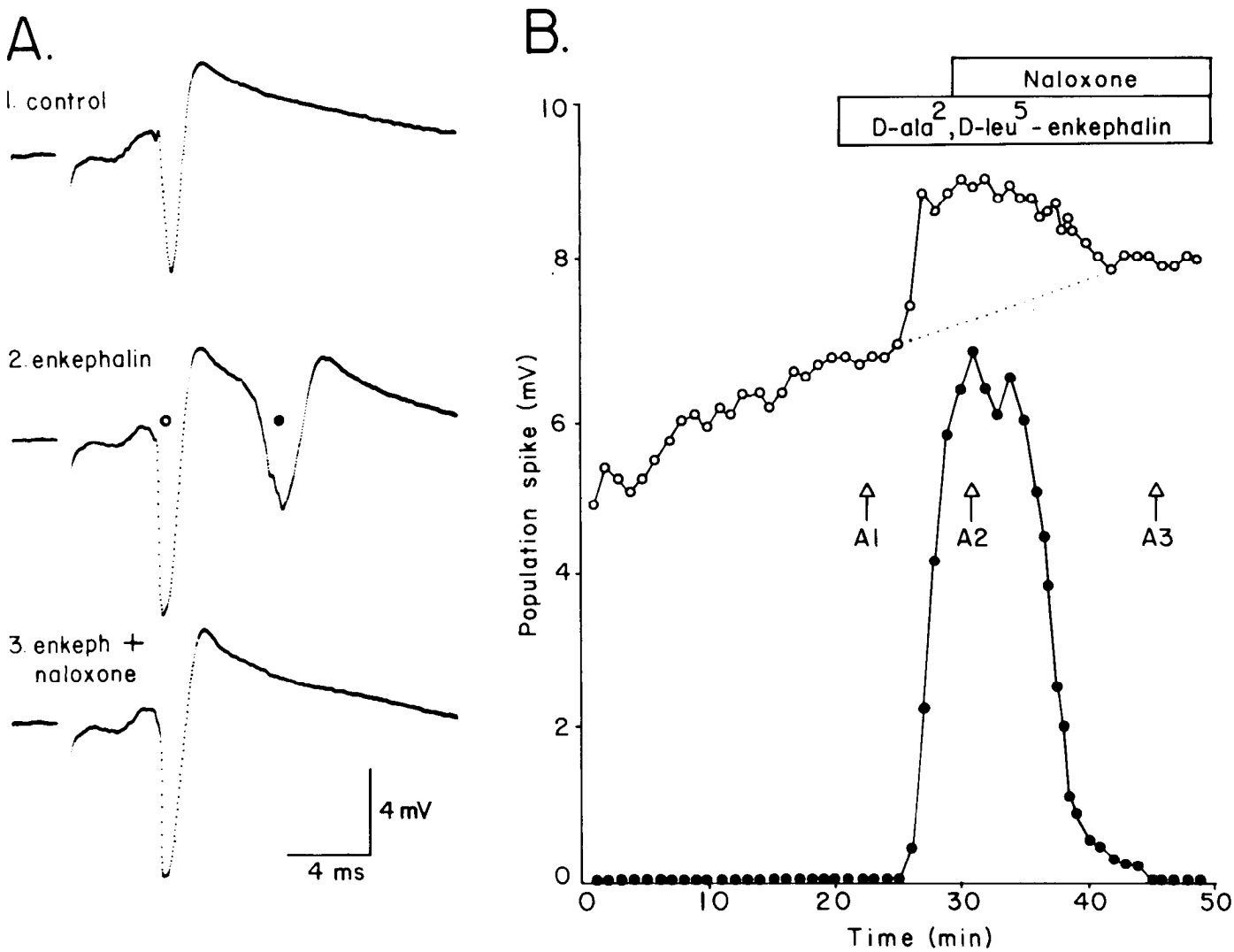

Figure 3. Somatic field potentials in the CA1 region. An extracellular micropipette located in st. pyramidale recorded population spike responses evoked by 1 Hz stimulation in st. radiatum. Each sample recorded in *A* is the digital average of eight consecutive responses. *A1*, Control; *A2*, 11 min after perfusing the slice with 5×10^{-7} M DADL; *A3*, 15 min after changing to a perfusion fluid containing DADL plus 5×10^{-7} M naloxone. *B*, Time course of the opioid effect. The size of the first (○) and second (●) evoked population spikes are plotted versus time over the experiment. Drugs are present as indicated by the *boxes*. Note that naloxone completely reversed the effect of DADL. The times chosen for the sample records in *A* are shown by *arrows* in *B*.

TABLE I

Effect of enkephalin on membrane properties of pyramidal cells

The data were gathered from 18 pyramidal neurons that were exposed to [D-Ala^2, D-Leu^5]enkephalin, by either bath application (1 to 5×10^{-7} M) or iontophoresis, one or more times. The values reported are the mean ± SEM, with the number of DADL applications given in parentheses. In the columns marked "Hyperpolarizing Charging Curve," the values reported are the times required to reach 10%, 30%, and 63% of the maximum voltage deflection produced by a weak hyperpolarizing transmembrane current pulse. For each DADL application, measurements for enkephalin were taken at a time when the orthodromic EPSP was clearly potentiated, and measurements for recovery or enkephalin plus naloxone were taken at a time when the EPSP had returned to normal. No value in those rows is significantly different from 0. Not all cells were held long enough for a satisfactory EPSP recovery; therefore, the number of measured values for recovery or enkephalin plus naloxone is less than the number of DADL applications.

	Membrane Potential	Input Resistance	Spike Threshold	Hyperpolarizing Charging Curve		
				10% Maximum	30% Maximum	63% Maximum
	mV	*megohms*	*mV*		*msec*	
Control	−76 ± 1	30 ± 3	12.5 ± 1	2.4 ± 0.5	6.1 ± 1.1	15.4 ± 1.9
Absolute change						
Enkephalin	0.3 ± 0.6 (26)	−0.2 ± 0.5 (18)	0.2 ± 0.2 (19)	−0.2 ± 0.2 (7)	−0.7 ± 0.5 (7)	−1.0 ± 0.5 (7)
Recovery or enkephalin + naloxone	0.7 ± 0.5 (19)	−0.8 ± 0.9 (15)	0.1 ± 0.3 (13)	0.1 ± 0.7 (6)	−0.1 ± 0.9 (6)	1.1 ± 1.9 (6)

are characteristic of the penicillin-treated slice (Schwartzkroin and Prince, 1977), usually were not seen in the presence of even high concentrations (3×10^{-6} M) of DADL. Additionally, even relatively mild enhancement of the orthodromic response by penicillin is accompanied by a dramatic blockade of GABA-mediated recurrent inhibition (Fig. 7; see also Wong and Prince, 1979; Dingledine and Gjerstad, 1979, 1980; Schwartzkroin and Prince, 1980), while, as shown below, DADL did not attenuate classical recurrent IPSPs.

The enhanced orthodromic responses produced by DADL did not appear to be due to a general increase in

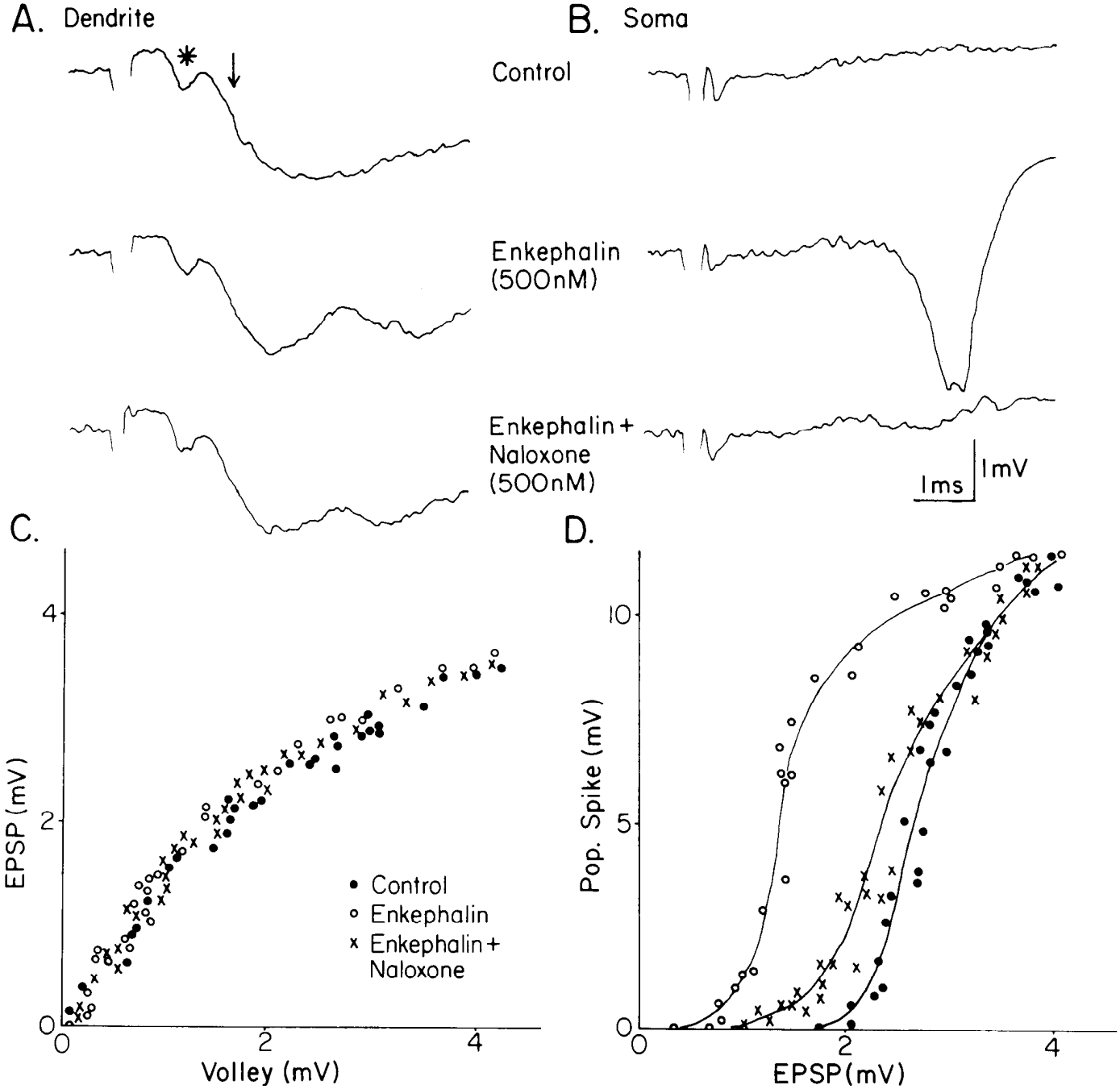

Figure 4. Dendritic and somatic field potentials in the CA1 region. A stimulating cathode in st. radiatum was used to activate apical dendritic synapses. An extracellular micropipette situated "on-beam" in the apical dendritic field of the pyramidal cells recorded the triphasic afferent fiber volley (*asterisk* in *A*) and the following slow negativity (field EPSP) taken to reflect the magnitude of the postsynaptic current. A second micropipette placed in the cell layer recorded population spikes (*B*). By varying the stimulus current, input-output curves could be constructed that relate the size of EPSP to the afferent volley (*C*) and the population spike to the EPSP (*D*). An *arrow* in *A* shows the time at which all EPSPs were measured in this experiment. The three pairs of averaged sample responses in *parts A* and *B* were matched for a similar size fiber volley. In a naloxone-reversible action, DADL increased the probability of pyramidal cell discharge for a given postsynaptic current density (*D*) without changing the synaptic transfer relationship (*C*).

the excitability of the pyramidal soma membrane, since membrane potential, input resistance, and spike threshold (e.g., Fig. 2) were unaltered (Table I). Antidromic field potentials also were unaffected by DADL. An attempt was made to examine the effect of DADL on the shape of the charging curve during the injection of a hyperpolarizing pulse, since, if the dendritic membrane resistance were increased selectively to a large degree by DADL, one might expect the initial part of the charging curve to be slower (Rall, 1960). However, no consistent effect of DADL on the charging time constants could be detected (Table I).

Effects on dendritic field potentials. One possible explanation for the potentiated EPSP in the presence of DADL might be an increased excitatory transmitter release. This possibility was explored by iontophoretic mapping experiments (described below) and by dendritic field potential recordings. Extracellular recording electrodes were placed in the cell layer and "on-beam" in the apical dendritic layer at the level of activated synapses. The former electrode was used to record population spikes (Fig. 4*B*); the dendritic electrode recorded the presynaptic afferent fiber volley (marked with an *asterisk* in Fig. 4*A*) and the field EPSP, a slow negativity whose initial slope is proportional to local inward currents underlying postsynaptic depolarization (Andersen et al., 1978). Input-output curves were constructed by varying the stimulus intensity and plotting, first, the field EPSP (measured at a fixed latency shown by the *arrow* in Fig. 4*A*) as a function of the peak-to-peak amplitude of the fiber volley (Fig. 4*C*) and, second, the population spike as a function of the field EPSP (Fig. 4*D*). As demonstrated in Figure 4, bath application of 5×10^{-7} M DADL shifted the EPSP-to-spike curve to the left but had no discernible effect on the volley-to-EPSP relationship; subsequent addition of 5×10^{-7} M naloxone to the DADL-containing perfusion fluid resulted in a substantial reversal of this effect. Thus, the large increase in population spike caused by DADL can be accounted for entirely by a higher firing probability at a given excitatory postsynaptic current. By this test, it appears that DADL does not increase excitatory transmitter release onto the apical dendrites of pyramidal cells. Similar results were obtained in 10 experiments. This finding supports several earlier studies (Martinez et al., 1979; Robinson and Deadwyler, 1980; Dunwiddie et al., 1980; Corrigall and Linseman, 1980; Lynch et al., 1981; but see Haas and Ryall, 1981) but has the added advantage that the fiber volley itself was used as a direct measure of afferent input strength rather than the less direct measure provided by the stimulus current (or voltage). It should be noted that the dendritic input-output transfer as plotted in Figure 4*C* is sensitive to treatments that alter transmitter release; a change in the extracellular

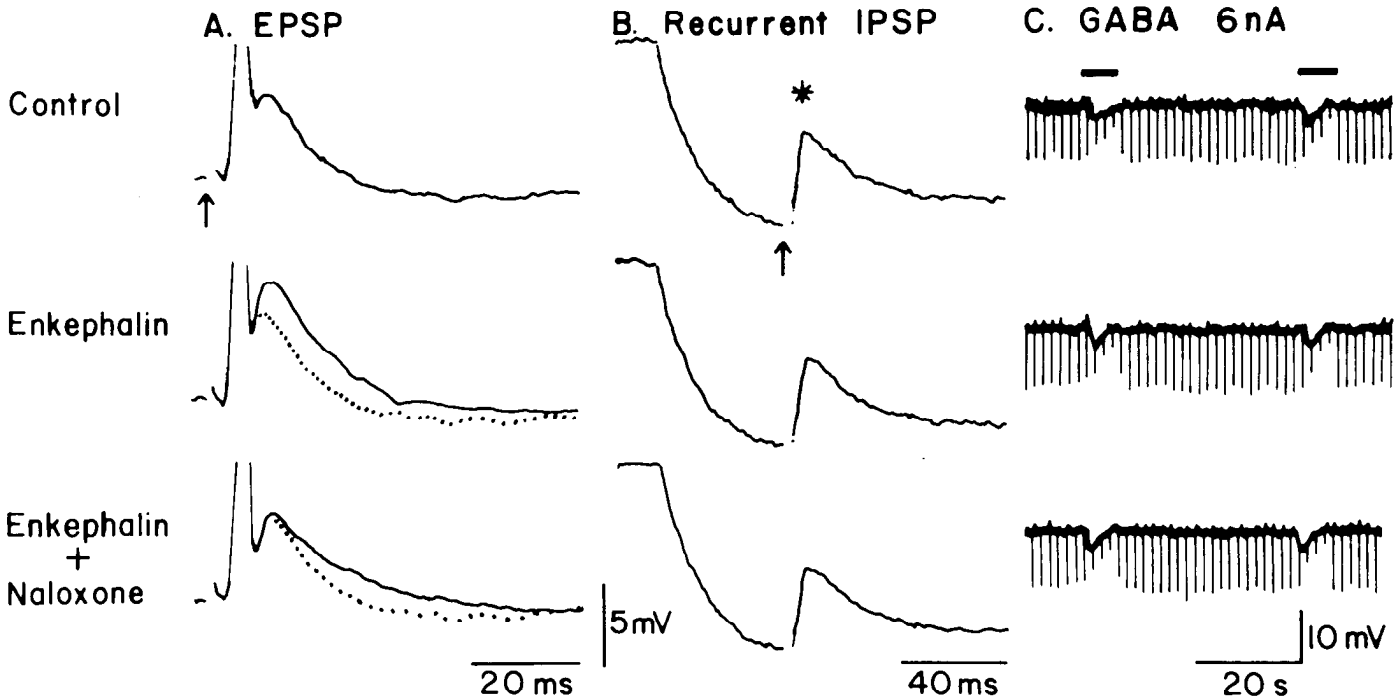

Figure 5. Excitatory and GABA-mediated inhibitory responses in a CA1 pyramidal neuron. This cell was presented alternately with an orthodromic stimulus, which evoked an EPSP and spike (*A*), and an antidromic stimulus, which evoked a recurrent IPSP (*asterisk* in *B*). In addition, GABA was applied iontophoretically into st. pyramidale at regular intervals throughout the experiment (*C*). The recurrent IPSP in *B* was evoked at the base of a current-induced hyperpolarization of sufficient magnitude (−0.25 nA) to reverse the sign of the IPSP. *Top row*, Control responses; *middle row*, responses after perfusing the slice for 16 min with 5×10^{-7} M DADL; *bottom row*, responses 19 min after adding 5×10^{-7} M naloxone to the DADL-containing perfusion fluid. Each *trace* in *A* and *B* is the digital average of 32 evoked responses; stimulus artifacts are marked with an *arrow* in the *top row*. The *dotted lines* in *A* show the control response for comparison. In *C*, the sharp, *downward deflections* are the responses to hyperpolarizing current pulses passed across the membrane to monitor its resistance (same pulses shown in *B*). DADL prolongs the orthodromic EPSP but does not decrease the size of the recurrent IPSP or attenuate the inhibitory effects of GABA. Membrane potential, −62 mV; spike, 107 mV; input resistance, 40 megohms.

Ca^{2+} concentration from the resting level by as little as 0.1 mM results in an approximately 15% change in the initial slope of this plot (Dingledine and Somjen, 1981).

Effects on synaptic inhibition. The epileptogenic mechanism of penicillin in the hippocampal slice is now considered to include a reduction in GABA-mediated recurrent and feed-forward synaptic inhibition (Wong and Prince, 1979; Dingledine and Gjerstad, 1979, 1980; Schwartzkroin and Prince, 1980), which allows unchecked EPSPs to trigger dendritic currents that underlie burst responses. The possible effect of DADL on recurrent inhibition was examined in two series of experiments.

Since the response to orthodromic stimulation consists of a mixed EPSP/IPSP, it is difficult to determine whether an increase in the overall response (e.g., Fig. 1) results from a removal of IPSPs or a potentiation of EPSPs. This problem was partially overcome by developing a microlesioning technique that allowed relatively pure antidromically evoked recurrent IPSPs to be studied in the absence of EPSP contamination (see "Materials and Methods"). In the first set of experiments, intracellular recordings were made from antidromically identified pyramidal neurons. Orthodromic and antidromic stimuli were presented alternately to the cell and the effects of DADL on the recurrent IPSP were examined at a time when the orthodromic response was clearly enhanced. The results of such an experiment are shown in Figure 5. In this cell, the apparent reversal potential for the recurrent IPSP (−65 mV) was near the resting potential (−62 mV); therefore, to allow an adequate assessment of the changes in the size of the IPSP, it was triggered at the base of a hyperpolarization produced by a −0.25-nA current pulse. At this level of membrane potential, the IPSP was depolarizing (*asterisk* in Fig. 5*B*). Since there is good evidence that GABA is the transmitter for recurrent inhibition in the hippocampus (Curtis et al., 1970; Andersen et al., 1980), the postsynaptic effects of an antidromic stimulus were mimicked by regular, periodic, iontophoretic applications of GABA into the cell layer (Fig. 5*C*). Bath application of 5×10^{-7} M DADL increased the orthodromic response (Fig. 5*A*), and this effect was partially reversed after naloxone (5×10^{-7} M) was added to the perfusion fluid. Each *trace* in Figure 5, *A* and *B*, is the average of 32 trials, with the control response shown (*dotted line*) for comparison in the *lower traces* of *part A*. At a time when a clear potentiation by DADL of the orthodromic response had been demonstrated, there was no discernible change in either the recurrent IPSP (Fig. 5*B*) or the hyperpolarization and conductance increase produced by iontophoretic application of GABA (Fig. 5*C*). By changing the

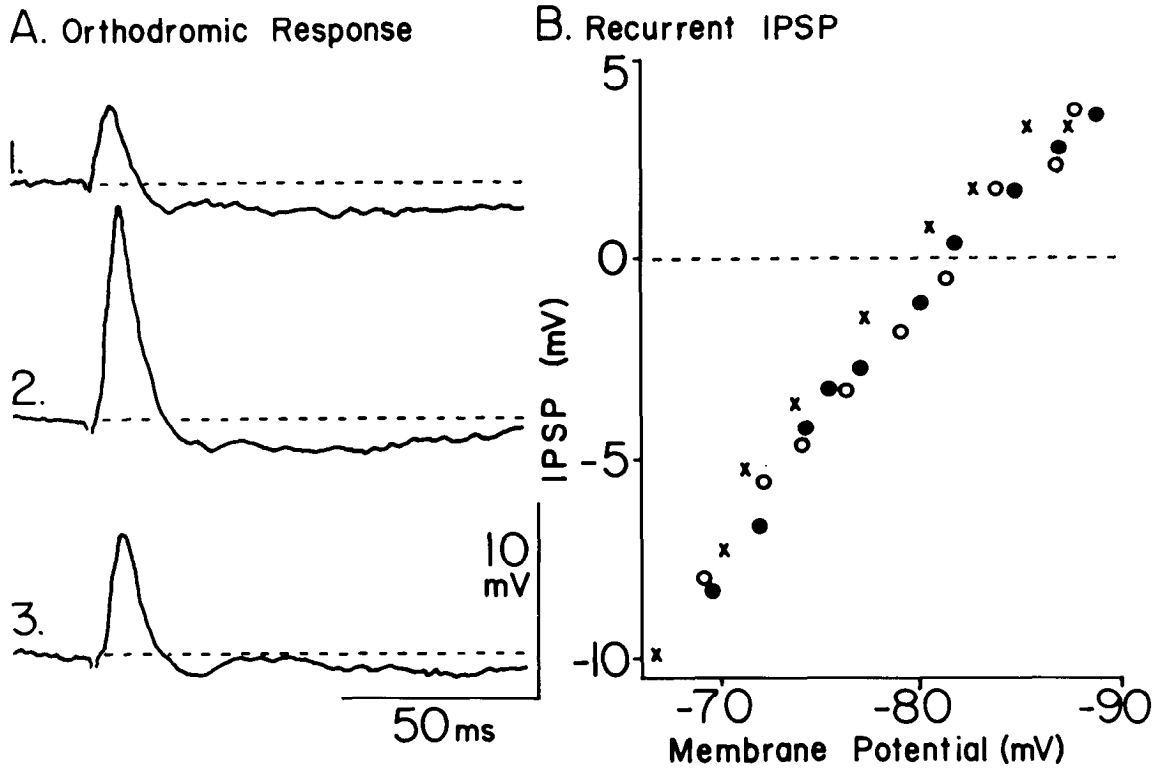

Figure 6. Excitatory and recurrent inhibitory postsynaptic potentials. This CA1 pyramidal cell was presented alternately with an orthodromic stimulus to st. radiatum, which evoked an EPSP followed by an IPSP, and an antidromic stimulus, which evoked a recurrent IPSP. The recurrent IPSP was triggered at the plateau of 150-msec current pulses of different magnitude in order to measure the IPSP at different membrane potentials. *A1*, Control orthodromic response; *A2*, 17 min after perfusing with 3×10^{-7} M DADL; *A3*, 20 min after adding 5×10^{-7} M naloxone to the DADL-containing perfusion fluid. *B*, IPSP as a function of membrane potential in control (●), DADL (○), and DADL plus naloxone (×). DADL potentiated the EPSP without reducing either orthodromic or recurrent IPSPs. The *dashed line* in *A* shows the resting potential. Membrane potential, −76 mV; input resistance, 27 megohms; spike, 95 mV.

magnitude of the injected current pulse, it was possible to examine the dependence of the IPSP on membrane potential in the presence and absence of DADL. When this was done for the cell illustrated in Figure 5, no effect of DADL was noted either on the apparent reversal potential of the IPSP or on the slope of the IPSP-membrane potential plot. An example of such a plot is shown in another cell in Figure 6, where, in a naloxone-reversible fashion, bath application of 3×10^{-7} M DADL greatly potentiated the EPSP but had no effect on the pure

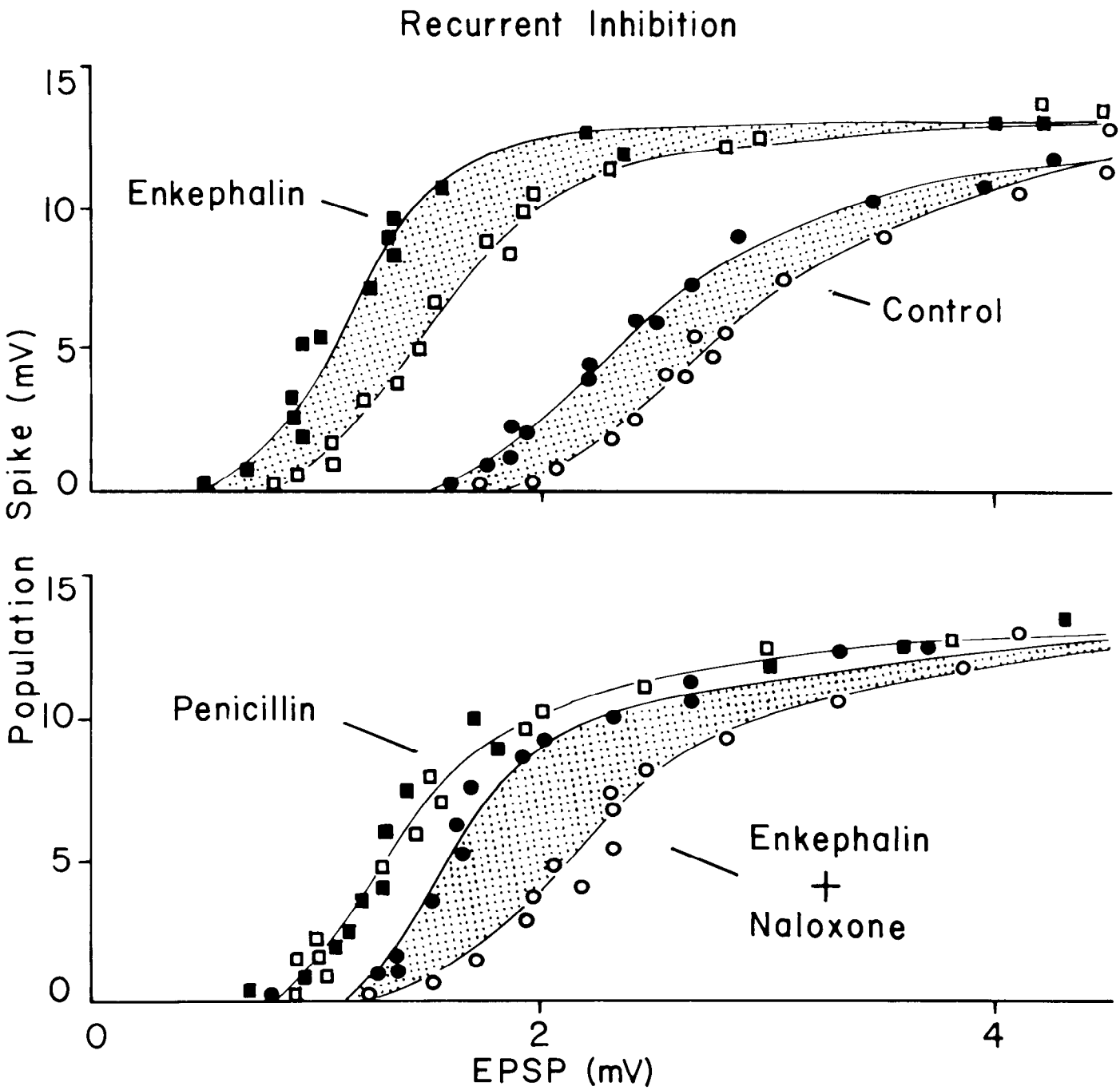

Figure 7. Recurrent inhibition judged by field potentials. Input-output curves of the population spike as a function of (st. radiatum) field EPSP were plotted as in Figure 4 either without (■, ●) or with (□, ○) a conditioning antidromic stimulus delivered 25 msec prior to the orthodromic stimulus. The antidromic volley activated a recurrent inhibitory circuit that caused a small shift to the *right* of the illustrated input-output curve. The amount of this shift (*shaded area*) is taken as a measure of the intensity of recurrent inhibition. The *top panel* shows that the shift to the *left* of the input-output curve that reflects the excitatory effect of DADL is not accompanied by a reduction of recurrent inhibition. ●, ○, Control values; ■, □, values obtained after perfusion with 5×10^{-7} M DADL. The *bottom panel* shows partial recovery of the excitatory effect of DADL after perfusion with 5×10^{-7} M naloxone (pair of curves labeled *enkephalin + naloxone*) and the expected obliteration of recurrent inhibition caused by subsequent perfusion with a known GABA antagonist, penicillin (0.4 mM). This low concentration of penicillin produced only mild enhancement of the orthodromic response. In none of the above conditions did the conditioning antidromic volley affect the synaptic transfer curve (field EPSP as a function of fiber volley).

recurrent IPSP. In this series, the effect of DADL on recurrent IPSPs was tested in a total of nine cells; in only one instance did the recurrent IPSP appear to be reduced in size by DADL, but, in this case, no recovery was seen.

In the second series of tests, a paired antidromic-orthodromic stimulus paradigm was used to assess the strength of recurrent inhibition. Field potentials were recorded from both cellular and dendritic layers of the slice and input-output curves were constructed as in Figure 4*D*. Recurrent inhibition was monitored as follows: by activating the recurrent inhibitory loop with a prior antidromic volley, the whole curve of EPSP versus population spike was shifted to the right; the amount by which the curve was shifted (*shaded areas* in Fig. 7) was considered a measure of the intensity of recurrent inhibition. It was reasoned that, if DADL increased the orthodromic response by reducing recurrent inhibition, then the shift to the left of the EPSP-spike curve that is seen in the presence of DADL should be associated with a reduction in the *shaded area*. However, this was not the case. As shown in Figure 7, recurrent inhibition by this test was not reduced when 5×10^{-7} M DADL was washed into the bath, even though a dramatic potentiation of the orthodromic response itself occurred. After reversing the DADL effect by perfusing with 5×10^{-7} M naloxone (*lower panel*), the GABA antagonist, penicillin (0.4 mM), was washed into the bath. Although this concentration of penicillin produced a milder effect on the orthodromic response than did DADL, recurrent inhibition in the presence of penicillin was obliterated as expected. This experiment was repeated three times with similar results, although, in one case, DADL appeared to produce a naloxone-reversible enhancement of recurrent inhibition.

It seemed possible that, although antidromic, presumably somatic inhibition was not reduced by DADL, some form of synaptic inhibition elicited exclusively by orthodromic stimulation might be attentuated. However, when orthodromically evoked IPSPs were prominent enough to be recorded easily by a somatically placed electrode (e.g., Fig. 6*A*), DADL either had no effect or occasionally even appeared to potentiate the hyperpolarizing phase of the orthodromic response. However, the early part of the IPSP invariably was contaminated by the EPSP, and therefore, no firm conclusion could be reached on this point.

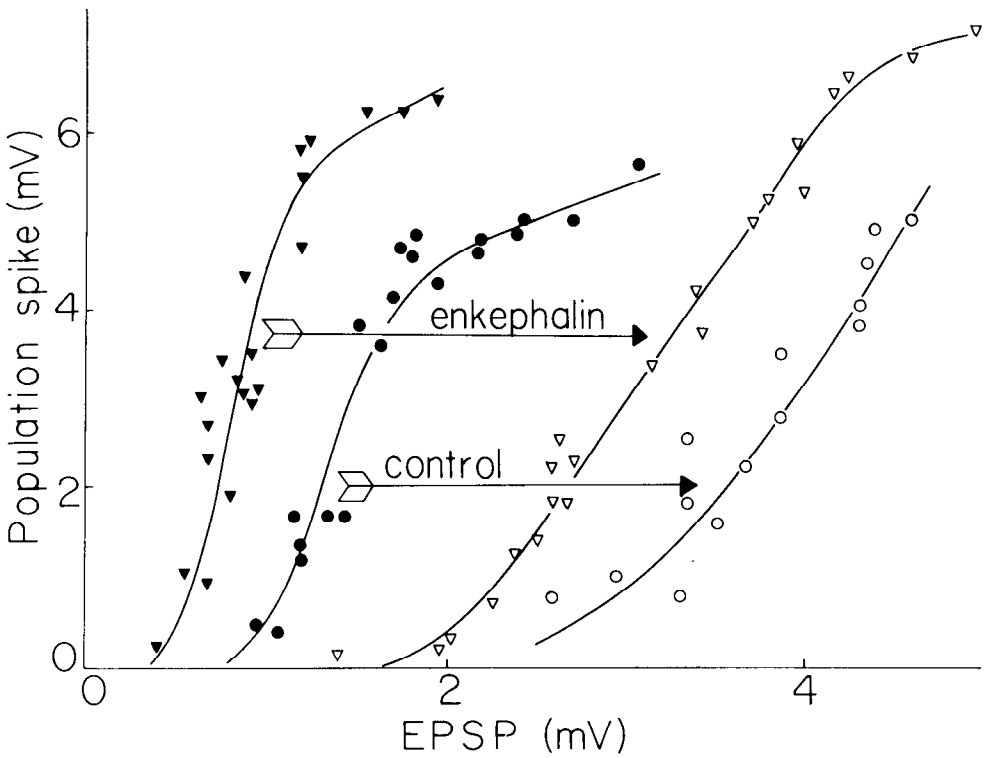

Figure 8. Feed-forward inhibition judged by field potentials. As in Figure 7, the field EPSP and population spike responses to 0.1 Hz st. radiatum stimuli were plotted as input-output curves. However, in this experiment, orthodromic responses were obtained either without (▼, ●) or with (▽, ○) a preceding *orthodromic* stimulus delivered also into the st. radiatum but by a separate stimulating electrode. The conditioning orthodromic stimulus produced a very powerful inhibition of the response to the test stimulus, as evidenced by a large shift to the *right* in the control input-output curve (normal and conditioned response curves connected by *arrow* labeled *control*). ●, ○, Control response curves; ▼, ▽, responses obtained after perfusion with 5×10^{-7} M DADL. The shift to the *left* in the unconditioned response curves (▼, ●) caused by DADL was not associated with a significant reduction of feed-forward inhibition, as the lateral distance between curves in *control* and *enkephalin* is nearly the same. The delay between conditioning and test orthodromic stimuli was 40 msec.

Another form of inhibition of orthodromic population spikes can be demonstrated by preceding a st. radiatum stimulus with a second orthodromic stimulus, which is delivered through a separate stimulating electrode placed some distance away but in the same dendritic layer. The anatomical substrate of this type of inhibition is unknown, although it appears to be postsynaptic inhibition onto the pyramidal cell since the conditioning stimulus need not reduce the field EPSP of the test stimulus. This form of synaptic inhibition, which will be termed feedforward inhibition, appears distinct from classical recurrent inhibition since it seems much more powerful (compare the separation between the control curves of Fig. 8 with that of Fig. 7) and fatigues more easily. Indeed, feedforward inhibition usually could not be demonstrated with stimulation at 1 Hz and required a stimulus fre-

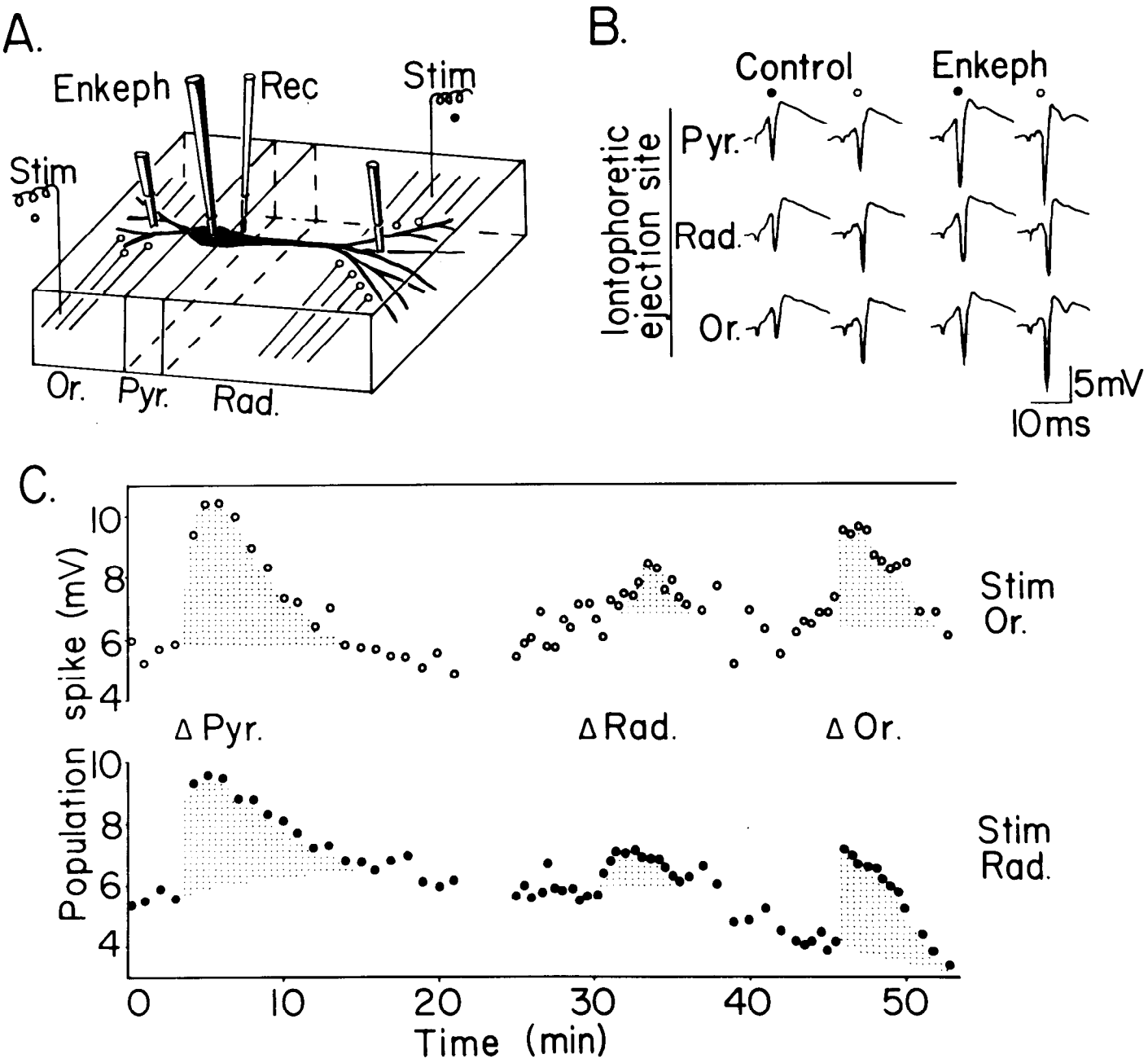

Figure 9. Iontophoretic mapping of DADL-sensitive sites. *A*, Experimental arrangement. Population spikes were recorded (*Rec*) and averaged from the cell layer (*Pyr*) in response to orthodromic stimuli (*Stim*) delivered alternately into st. radiatum (*Rad*) or st. oriens (*Or*). An iontophoretic pipette (*Enkeph*) ejected a standard dose of DADL (60 nA, 10 sec) into each of the three layers as shown, while the responses to the two stimulating lines were monitored continuously. *B*, Sample records, showing control responses evoked by stimulating in st. radiatum (*left column*) and st. oriens and responses at the time of peak DADL effect, for each of the three iontophoresis sites. *C*, Time course and intensity of DADL effect at the three iontophoresis sites. *Triangles* mark the time and site of DADL iontophoresis. The *upper panel* shows responses to st. oriens stimulation and the *bottom panel* shows st. radiatum responses. The approximate duration and magnitude of the drug effects are indicated by *stippling*. For both stimulating lines, DADL was most effective when injected into st. pyramidale or st. oriens.

quency as low as 0.05 to 0.1 Hz to be maximally effective; in contrast, recurrent inhibition was maximal at stimulus frequencies less than approximately 0.5 Hz. As shown in Figure 8, by this test, a marked effect on the orthodromic response caused by bath application of DADL at 5×10^{-7} M did not appear to be associated with a reduction in the intensity of feed-forward inhibition when tested at a 40-msec delay between the paired stimuli.

Iontophoretic mapping of enkephalin-sensitive sites. Bath application achieves a known and uniform drug concentration but gives no information concerning the spatial distribution of drug-sensitive sites. This informa-

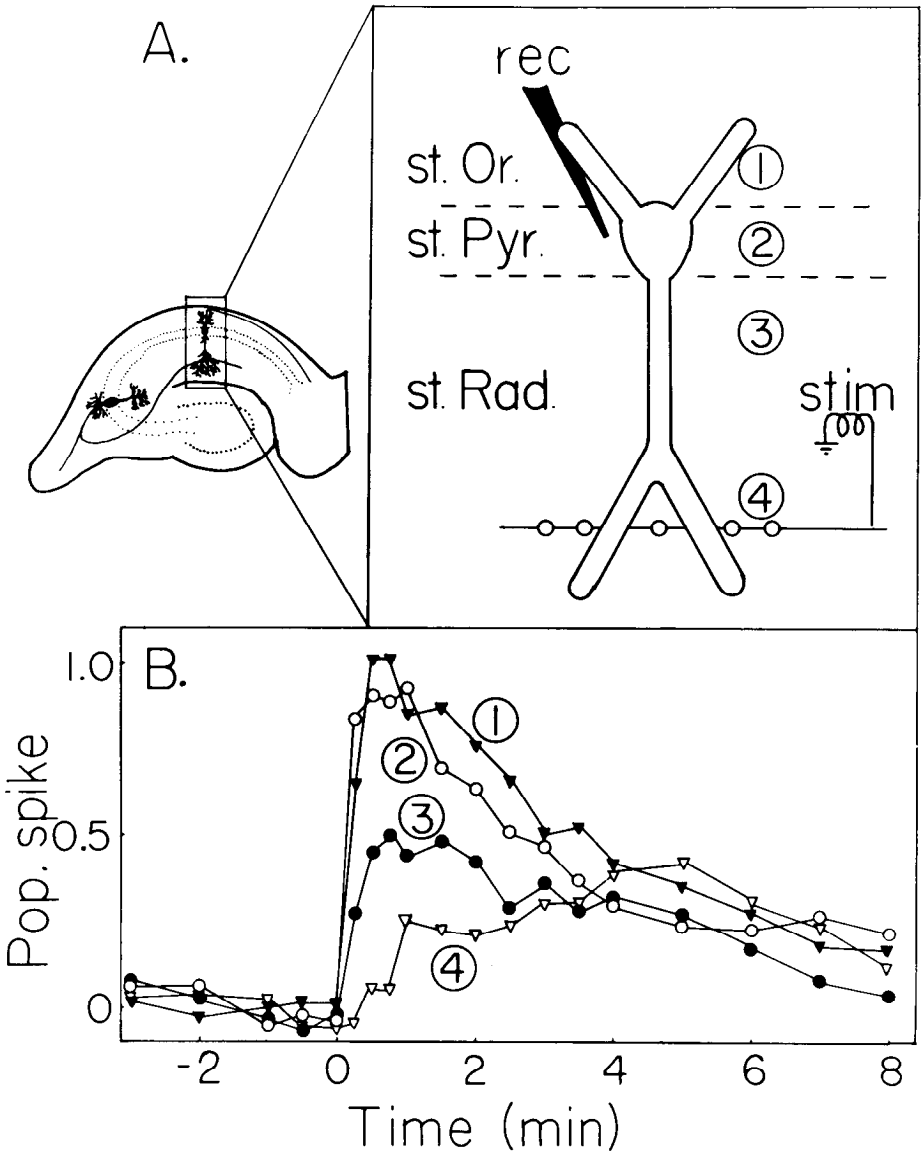

Figure 10. Time course of DADL effect related to iontophoresis site. *A*, Experimental arrangement. Constant current stimulation through a microcathode placed in the distal quarter of st. radiatum (*st. Rad., stim*) evoked population spikes recorded by a micropipette in st. pyramidale (*st. Pyr., rec*). An iontophoretic micropipette was used to deliver standard applications of DADL into the slice at sites marked *1* to *4*. DADL applications were made 15 to 20 min apart in the order *2-4-1-3*. At standard intervals, four responses were averaged and the measured values were normalized with respect to the maximum response increase produced by iontophoresis of DADL into st. pyramidale (*site 2*). *st. Or.*, st. oriens. *B*, The mean of the normalized population spike responses from seven slices are plotted over time for each of the four iontophoresis sites. DADL (usually 100 nA for 4 to 8 sec) was applied at time 0. Application of DADL directly into the zone of activated synapses (*site 4*) invariably produced a small, slowly developing response when compared with drug application into st. oriens or st. pyramidale (*sites 1* and *2*). Iontophoresis of DADL into the proximal third of st. radiatum (*site 3*) elicited an intermediate effect.

tion can be obtained by applying the drug iontophoretically to various parts of the cell. Such laminar mapping studies have been successful in demonstrating spatially separate effects of GABA (Andersen et al., 1980) and acetylcholine (Valentino and Dingledine, 1981) in the hippocampal slice. A similar approach was used here to determine the location of DADL-sensitive sites in the CA1 region.

For the experiment illustrated in Figure 9, stimuli were presented alternately through two stimulating electrodes, one (●) activating fibers in the st. radiatum that synapse on apical dendrites and the other (○) activating fibers in the st. oriens that synapse on basal dendrites. An electrode (*Rec*) placed in the pyramidal cell layer recorded population spikes evoked by the two stimulating lines. For both inputs, the effect of DADL was evaluated by applying a standard dose (60 nA for 10 sec) into each of three layers as diagramed in Figure 9*A*. The effect of DADL was much greater on both inputs when delivered into st. oriens or st. pyramidale than into st. radiatum; this result was not critically dependent on the depth of the iontophoretic pipette in the slice. For each drug application, the best response is shown, together with control records, in the averaged population spikes shown in Figure 9*B*. The left two columns (*Enkeph*) show the maximum population spike reached following each application of DADL. The time course of the two responses to iontophoretic DADL administration is shown in Figure 9*C*. The effect of DADL applied into st. radiatum was smaller and more sluggish than either of the other application sites, even when the response to activation of synapses located within st. radiatum itself was tested.

The above finding suggested the possibility that the observed responses to apical dendritic application of DADL may have been due to diffusion of the drug into the cell layer. This possibility was tested in a series of seven experiments, in which population spikes were recorded in response to st. radiatum volleys as a standard iontophoretic dose of DADL (usually 100 nA for 4 or 8 sec) was ejected into each of four sites along the dendritic axis (numbered *1* to *4* in Fig. 10*A*). In each experiment, the population spike responses were normalized as a percentage of the maximum increase in response seen following the application of DADL into st. pyramidale (*site 2*); the normalized population spike amplitude then was averaged over the seven experiments and plotted against time (Fig. 10*B*). Application of DADL into st. oriens and st. pyramidale yielded virtually identical effects on the radiatum volley responses (cf., *curves 1* and *2* in Fig. 10*B*). On the other hand, ejection of DADL into st. radiatum (*site 4*) caused only a small, very slow response. The data are consistent with the idea that receptors mediating the excitatory effect of DADL are limited to st. pyramidale and st. oriens. In contrast, a similar mapping experiment with penicillin revealed that both st. oriens and st. radiatum were equally sensitive throughout their whole extent (not shown).

Even the most rapid responses to iontophoretic or droplet application of DADL required approximately 30 to 60 sec to achieve maximal effect (Fig. 10*B*), which is 100 to 1000 times slower than the fastest effects seen after iontophoretic applications of glutamate and GABA. The relatively prolonged time course of DADL action is also noteworthy. In contrast to the several second duration of action of even large iontophoretic doses of penicillin, a 4- to 8-sec application of DADL enhanced synaptic activation of pyramidal cells for 10 min or more.

Discussion

The principal finding of this study is that [D-Ala^2, D-Leu^5]enkephalin, acting via a naloxone-reversible action in st. oriens and/or st. pyramidale, can increase the size and duration of EPSPs of dendritic origin without altering soma resting membrane properties, recurrent somatic IPSPs, or dendritic field potentials taken to reflect excitatory postsynaptic currents. The opioid effect appears qualitatively different from the epileptiform action of penicillin both in its lack of effect on recurrent inhibition and by the virtual absence of paroxysmal depolarizing shifts in DADL-treated neurons. Assuming the presence of tonic synaptic input to pyramidal neurons *in vivo*, the observed potentiation of EPSPs may provide an explanation for the mild electrographic seizure activity seen following the intraventricular injection of opioid peptides (Urca et al., 1977; Frenk et al., 1978a, b; Henriksen et al., 1978; Elazar et al., 1979) and the previously reported "excitatory" effect of these peptides on hippocampal neurons *in vivo* (Nicoll et al., 1977).

The lack of effect of DADL on membrane potential and input resistance is in accord with studies of other opioid peptides (Gähwiler, 1980; Nicoll et al., 1980; Haas and Ryall, 1981), and in addition, it was shown here that the voltage threshold for initiating action potentials is unchanged by DADL. However, the data presented here do not support the recent suggestion of *recurrent* disinhibition as the mechanism of action of opioid peptides (Zieglgänsberger et al., 1979; see also Dunwiddie et al., 1980; Lee et al., 1980; Nicoll et al., 1980; Gähwiler, 1980) or morphine (Corrigall and Linseman, 1980; Robinson and Deadwyler, 1980) in the hippocampus. It should be noted that others also have failed to confirm the original hypothesis of recurrent disinhibition (Haas and Ryall, 1981; Lynch et al., 1981). While much of the experimental support for disinhibition relies on indirect evidence for which alternative explanations may be found (see discussion in Haas and Ryall, 1981), two reports appear to be of critical importance for this hypothesis: the observation of a reduction in the evoked burst responses of a population of non-pyramidal neurons by a Met-enkephalin derivative (Lee et al., 1980) and the report that, in the presence of 10^{-4} M pentobarbital, orthodromic and antidromic stimulation evoke enkephalin-sensitive long lasting inhibitory potentials in CA1 pyramids (Nicoll et al., 1980). While the experimental findings appear to be solid, the interpretation that the primary epileptiform action of enkephalins is to reduce synaptic inhibition should be viewed within the framework of several assumptions. Lee et al. (1980) proposed that their population of "non-pyramidal" cells were indeed basket cells or other inhibitory interneurons, although the presumed inhibitory nature of these cells was not demonstrated. Alger and Nicoll (1979) have presented evidence that the enkephalin-sensitive inhibitory potentials recorded in pentobarbital represent IPSPs. However, the extent to which

reduced inhibition of the sort illustrated by Nicoll et al. (1980) contributes to the epileptiform actions of enkephalins is uncertain, as it is clear that an augmentation of excitatory responses can be produced in the absence of detectable effects on synaptic inhibition (Figs. 5 to 8). The apparent discrepancy between the results of Nicoll et al. (1980) and those presented here may reflect differences in concentrations and/or enkephalin derivatives used in the two studies.

Haas and Ryall (1981) suggested that FK33-824, a Met-enkephalin derivative, facilitates excitatory transmitter release, but this is unlikely to underlie the epileptiform action of DADL, as dendritic field potentials indicative of excitatory postsynaptic currents were unchanged (Fig. 4; see also Corrigall and Linseman, 1980; Dunwiddie et al., 1980; Robinson and Deadwyler, 1980; Lynch et al., 1981), and the site of action within the CA1 region of DADL appears to be several hundred micrometers distant from the affected synapses (Figs. 9 and 10).

The observations reported here indicate that the effect of DADL is to enhance the electrical coupling between an apparently normal EPSP current generator and a normal soma spike trigger zone. One can place constraints on two remaining possible mechanisms for the epileptiform action of DADL. First, although blockade of the classical type of recurrent, somatic inhibition does not appear to be involved in the augmentation by DADL of orthodromic responses, it is conceivable that a form of postsynaptic *dendritic* inhibition is attenuated selectively by opioid peptides. Nicoll et al. (1980) point out that, in pentobarbital, the exaggerated orthodromic IPSPs appear to be considerably more sensitive to enkephalin than are antidromic IPSPs. More recently, we have provided evidence for two forms of orthodromic inhibition that appear to be blocked by DADL in the absence of pentobarbital (Dingledine, 1981). If dendritic disinhibition is involved, a novel type of inhibitory interneuron must be postulated for the hippocampal CA1 region. To comply with published data, the putative enkephalin-sensitive inhibitory interneuron should have its cell body located in st. pyramidale or st. oriens but should make synapses exclusively or predominantly on electrotonically distant dendrites of pyramidal cells. The non-pyramidal interneuron described by Schwartzkroin and Mathers (1978) meets some of the requirements for such an interneuron, as it is apparently not a basket cell and is found in st. pyramidale where DADL receptors have been demonstrated (Figs. 9 and 10; Goodman et al., 1980) and where a population of enkephalin-sensitive non-pyramidal cells has been described (Lee et al., 1980). Although the weight of the published evidence favors this hypothesis, it will be necessary to examine the effect of enkephalins on a physiologically identified inhibitory interneuron directly (Knowles and Schwartzkroin, 1981).

An alternative to the disinhibition hypothesis is the concept that DADL may modulate *excitatory* synaptic transmission onto hippocampal dendrites by a postsynaptic action that would entail some amplification mechanism (Lynch et al., 1981). This amplification mechanism could involve a coupled calcium/potassium system thought to regulate dendritic electroresponsiveness in these neurons (Wong and Prince, 1978; Traub and Llinas, 1978; Schwartzkroin and Wyler, 1979; Hotson and Prince, 1980), a decrease in dendritic spine stem resistance (Rall, 1978), or perhaps some other electrical event triggered only by activation of the excitatory synaptic receptor. However, no data directly support this proposed mechanism, and two pieces of information would appear to complicate it. First, the site of action of DADL (st. pyramidale/oriens) is several hundred micrometers away from the affected excitatory synapses in st. radiatum (Figs. 9 and 10) so that a propagating intracellular messenger would seem to be required to mediate the opioid effect on the EPSP. Second, DADL has not been found to facilitate calcium spikes evoked in tetrodotoxin (Dingledine, 1981) nor to potentiate the excitatory actions of dendritically applied *dl*-homocysteic acid (Haas and Ryall, 1981) or *N*-methyl aspartate (Dingledine, 1981). Admittedly, these negative findings are not a crucial argument against the hypothesis, as it is not entirely clear to what extent an iontophoretic application of these compounds mimics the postsynaptic action of the natural transmitter.

Although the selective δ receptor agonist, DADL, was used in this study, it is likely that the effects demonstrated were mediated via interaction with μ type opiate receptors. The selectivity of DADL for δ receptors is not great (Chang and Cuatrecasas, 1979; Chang et al., 1979) so that the concentrations of 1 to 5 $\times$ 10^{-7} M used here would be expected to occupy a significant proportion of μ receptors. The observed location of DADL-sensitive sites in st. pyramidale and st. oriens (Figs. 9 and 10) matches the distribution of μ receptors shown by autoradiography (Goodman et al., 1980; Herkenham and Pert, 1980). Finally, the effects of morphiceptin, a β-casein fragment that shows great selectivity for the μ receptor (Chang et al., 1981), are essentially identical to those of DADL in the slice preparation (R. Dingledine, unpublished observations).

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