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Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration

(peptides/opiates/calcium)

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ABSTRACT **Sensory neurons grown in dispersed cell culture in the absence of non-neuronal cell types contain immunoreactive substance P that is chemically similar to synthetic substance P. When depolarized in high-K^+ media (30–120 mM), the neurons release this peptide by a Ca^{2+}-dependent mechanism. An enkephalin analogue, [D-Ala^2]enkephalin amide, at 10 μM inhibits the K^+-evoked release of substance P. At the same or lower concentrations, [D-Ala^2]enkephalin amide and enkephalin decrease the duration of the Ca^{2+} action potential evoked and recorded in dorsal root ganglion cell bodies without affecting the resting membrane potential or resting membrane conductance. This modulation of voltage-sensitive channels may account for the inhibition of substance P release.**

The peptide substance P is thought to be involved in some way in synaptic transmission between primary sensory fibers and neurons within the spinal cord (1–3). Its apparent selective localization in small-diameter neurons of sensory ganglia and in fine unmyelinated processes that terminate in the dorsal horn of the spinal cord have focused attention on the role of this peptide in pain pathways (4–6). Iontophoresis of substance P into Rexed laminae I, IV, or V of the spinal cord increases the firing rate of cells that can also be excited by noxious stimuli (7, 8).

The peptide enkephalin is associated anatomically with substance P in many areas of the nervous system, particularly those related to pain and analgesia (5). For example, antibodies directed against enkephalin label nerve cell bodies in lamina II (substantia gelantinosa) and nerve processes in the regions that contain substance P (5, 9). Iontophoresis of enkephalin into the dorsal horn depresses the firing of cells in lamina IV and V. When applied in the substantia gelatinosa, enkephalin selectively depresses firing of cells (in laminae IV and V) that respond to noxious stimuli (10).

Opiates inhibit the release of neurotransmitters in the peripheral nervous system (11–13), so it is quite possible that enkephalin depresses transmission in the spinal cord by an action on primary afferent nerve terminals. The number of [^{3}H]naloxone binding sites in spinal cord dorsal horn decreases after dorsal rhizotomy (14), providing some evidence for presynaptic opiate receptors. Macdonald and Nelson (15) have recently shown by quantal analysis that etorphine can decrease transmitter release at sensory neuron–spinal cord neuron synapses in cell culture. The transmitter in this case is unknown. Jessell and Iversen (16) demonstrated that [D-Ala^2]enkephalin amide (DAEA) can inhibit potassium-evoked release of substance P from the rat trigeminal nerve nucleus. Considering the experiments mentioned above, it is likely that DAEA acted directly on sensory neuron nerve terminals within this nucleus. However, the site and mode of DAEA action were not determined.

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We have investigated these questions, using sensory neurons in dispersed cell culture grown in the absence of both target spinal cord neurons and ganglionic, non-neuronal "supporting" cells. The cultured sensory neurons contain immunoreactive substance P, and this peptide can be released when the neurons are depolarized in a high-potassium medium.[§] This paper reports an inhibitory effect of DAEA on the release of substance P from isolated sensory neurons. An effect of DAEA on the Ca^{2+} component of the sensory neuron action potential that suggests a mechanism for inhibition of transmitter release is also described.

MATERIALS AND METHODS

Cell Culture. Dorsal root ganglia (DRGs) were dissected from thoracic and lumbar segments of 9 to 10-day chicken embryos, incubated for 15 min at 37°C in 0.01% collagenase (Sigma), and then mechanically dissociated into single cells by trituration through a pasteur pipette. The cells were plated on collagen-coated tissue culture dishes (Falcon). Usually neurons from 5 ganglia were added to each 35-mm dish in 1.5 ml of medium or neurons from 15 ganglia were placed in each 60-mm dish in 3.0 ml of medium. The growth medium was Eagle's minimum essential medium (GIBCO) supplemented with 2 mM glutamine, penicillin at 50 μg/ml, streptomycin at 50 units/ml, 10% (vol/vol) heat-inactivated horse serum (GIBCO), 5% (vol/vol) chicken embryo extract, methocel (Dow) at 4 mg/ml, and nerve growth factor at 1 μg/ml. The 7S form of nerve growth factor was prepared from mouse salivary glands (17). In order to kill dividing ganglionic non-neuronal cells, 5 μM cytosine arabinonucleoside (cytosine arabinoside) (18) was added for the first 3 days of culture.

Release Experiments. Cultures were studied 12–18 days after plating. The cells were washed free of growth medium and placed in a solution containing (in mM): Na^+, 130; K^+, 6; Ca^{2+}, 1.8; Mg^{2+}, 0.8; Cl^-, 123; SO_4^{2-}, 0.8; HPO_4^{2-}, 1.2; Hepes, 25; and also glucose at 1 mg/ml and 0.2% bovine serum albumin. The pH of this solution is 7.4. High-K^+ solutions were prepared by substituting KCl for NaCl isosmotically. In each experiment the neurons were incubated for two successive 5-min periods in control K^+ (6 mM) followed by a solution containing high K^+ (30–120 mM). At the end of each 5-min incubation the bathing solution was removed and a fresh solution was added to the dishes. The cells were extracted with 2 M acetic acid; both extract and incubation solutions were lyophilized. To determine if substance P was degraded by the cells, 50 fmol of synthetic substance P was added to some cultures; recovery was greater than 90%.

Abbreviations: DAEA, [D-Ala^2]enkephalin amide; DRG, dorsal root ganglion.

§ Mudge, A. W., Fischbach, G. D. & Leeman, S. E. (1977) in *Seventh Annual Meeting of the Society for Neuroscience, Abstracts*, Abstr. 1306, p. 410.

In the inhibition experiments, groups of three or four cultures were depolarized in the presence or absence of drugs. DAEA was added to both the low-K^+ and high-K^+ solutions so that neurons in this group were exposed to the drug for two 5-min periods in low K^+ preceding the period in high K^+. There was no detectable degradation of peptides added to the incubation solution; however, DAEA rather than enkephalin (Peninsula Labs, San Carlos, CA) was used because it exhibited less cross-reactivity in the substance P radioimmunoassay. When naloxone was used, the neurons were also preincubated with the antagonist. Solutions of DAEA were made fresh and the concentration was checked by amino acid analysis. A group of sibling cultures that were not exposed to the drug served as a control.

Radioimmunoassay. After lyophilization, samples were resuspended in buffer and assayed for substance P by using antiserum R6P (19). Antibodies in this serum recognize the carboxy terminus of the undecapeptide and show 50% cross-reactivity with the carboxy-terminal octapeptide (residues 4–11). The assay is sensitive to 1.5 fmol; a 12% displacement of radioactive substance P bound by the antibody was arbitrarily taken as the limit of detection of the assay.

Chromatography. Sephadex G-25-80 (Sigma) columns (1 × 20 cm) were developed with 0.5 M pyridine/acetic acid, pH 5.5, containing 0.2% bovine serum albumin; 0.5-ml fractions were collected. Acetic acid (2 M) extracts of the cultures and of pooled samples of released material were lyophilized and applied to the column in running buffer. Immunoreactive material from the above columns (cell extracts and released material) was then subjected to cation-exchange chromatography in 0.5-ml columns of sulfopropyl-Sephadex SP-C25-120 (Sigma). Samples were applied in 5 ml of 0.3 M pyridine/acetic acid, pH 5.5, and eluted by steps of 0.5 M pyridine/acetic acid (5 ml) and 1.0 M pyridine/acetic acid (10 ml) at pH 5.5. Samples were lyophilized before substance P was assayed.

Electrophysiology. Intracellular microelectric recordings were performed on the stage of an inverted phase-contrast microscope as previously described (20). The cells were bathed in the same control solution used during release experiments except that the Ca^{2+} concentration was raised to 5.4 mM to accentuate the Ca^{2+} component of the action potential. The entire stage was warmed to 30–32°C. Drugs made up in recording medium were applied by pressure ejection [1- to 2-sec pulse at 1–2 pounds/in^2 (7–14 kPa)] from a 3- to 5-μm-tip micropipette located about 50 μm from the penetrated neuron.

Control experiments in which the effect on membrane potential of various concentrations of ejected K^+ was determined indicated that target cells were bathed in the same concentration of drug present in the loaded pipette (21). A small (3–5 mV) hyperpolarization, coincident with the pressure pulse, was observed on occasion. This is an artifact: the same response was observed with the recording electrode outside of the cell. Effects of ejected DAEA or enkephalin on evoked spikes were sought after the pulse when the resting potential had returned to the prepulse level.

RESULTS

DRG neuron culture

Fig. 1 shows a typical cluster of round DRG neurons 16 days after plating. This culture was treated with cytosine arabinonucleoside. Although there was a dense network of nerve processes, no satellite cells or other non-neuronal cells were evident along the neurites or on the cell bodies. Few, if any, fibroblastlike cells were found even after staining with methylene blue. Without background cells, the processes were not firmly adherent, and, in the absence of Methocel, the entire nerve network sometimes detached.

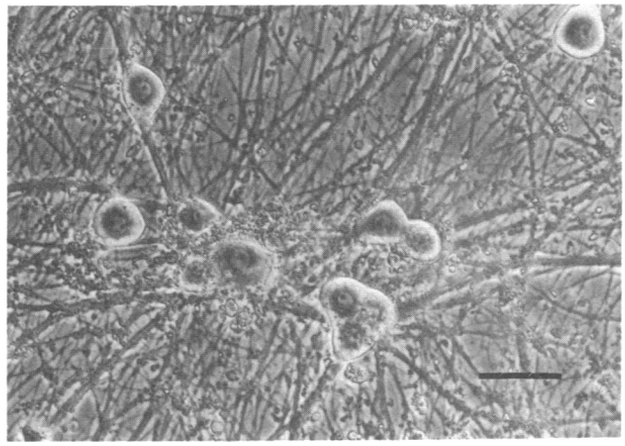

FIG. 1. Phase contrast micrograph of isolated chicken DRG neurons after 16 days in culture. The cultures were grown in 5 μM cytosine arabinonucleoside for the first 3 days. No non-neuronal cells are present. Bar = 50 μm.

A suspension of cells freshly dissociated from 15 ganglia contained about 60 fmol of immunoreactive substance P. The amount of substance P increased with time in culture: 2 weeks after plating, dishes seeded with cells from 15 ganglia contained between 2 and 3 pmol of immunoreactive material.

The material present in the cultures showed a dose response in the radioimmunoassay parallel to that of synthetic substance P, indicating immunological similarity with the carboxy terminus of this peptide. The immunoreactive material was further characterized by gel-exclusion and cation-exchange chromatography. A single peak that was indistinguishable from synthetic substance P was obtained in both procedures (Fig. 2, Table 1); recoveries were better than 90%. The carboxy-terminal octapeptide (residues 4–11) was clearly separated from the undecapeptide under these conditions.

Release of substance P

The histogram in Fig. 3A shows the amount of substance P present in the medium at the end of successive 5-min incubations in control and in high-K^+ solutions. The amount released is expressed as a percent of the total peptide present at each time point, assuming no net synthesis or degradation. There was little substance P (<0.25%) released into control (6 mM K^+) medium, but when the neurons were depolarized in medium containing 120 mM potassium, about 4% of the substance P present was released during the next 5 min. Similar amounts of substance

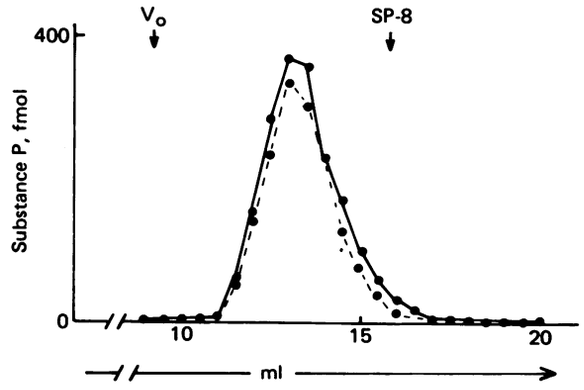

FIG. 2. Sephadex G-25 chromatography of synthetic substance P (●- - -●) and an acetic acid (2 M) extract of cultured sensory neurons (●—●). V_o indicates the void volume and SP-8 indicates the elution volume of the carboxy-terminal octapeptide (residues 4–11).

Table 1. Sulfopropyl-Sephadex chromatography using step elution with pyridine/acetic acid, pH 5.5

Material	% recovery Pyridine/acetic acid 0.3 M	0.5 M	0.1 M	Total
Substance P	<2	<2	91	91
Cell extract	<2	<2	92	92
Released material	<2	<2	98	98
Octapeptide	87	7	<2	94

Substance P:
$^{2+}$Arg-Pro-$^{+}$Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.
Octapeptide: $^{+}$Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.

P were released when the high-K^+ pulse was repeated a second and third time. There was no difference in the amount released with 60 or 120 mM K^+ as stimulus. However, there was a steep K^+-release relationship between 30 and 60 mM K^+.

The release of substance P does not simply reflect a nonspecific loss of soluble cytoplasmic molecules. Cultures in which the neurons had been preloaded with 2-deoxy[^{14}C]glucose showed no increase over baseline efflux of radioactivity in high-K^+ (120 mM) media.

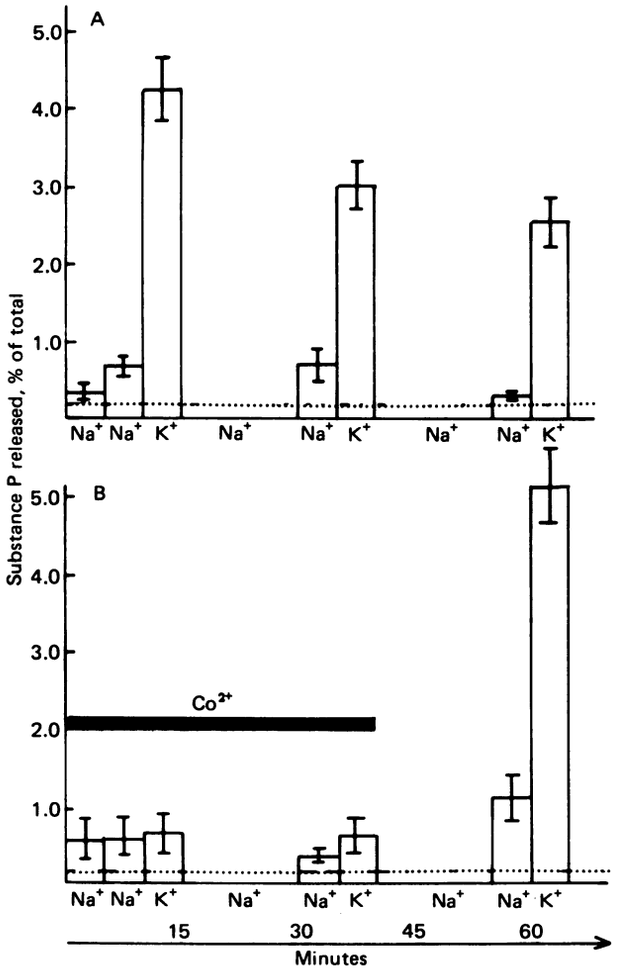

FIG. 3. (*A*) Potassium-evoked release of immunoreactive substance P. Cultures were incubated for successive 5-min periods either in control solutions (Na^+), or in 120 mM K^+ solutions (K^+). The amount of peptide present in the solution at the end of each period is expressed as percent of the total peptide present in the cells. The dotted line indicates the limit of detection of the assay. Results are the means of nine cultures from three platings; lines indicate SEM. (*B*) Same as *A*, except that 5 mM Co^{2+} was added to all solutions for the first 40 min of incubation. The Ca^{2+} concentration was lowered to 0.2 mM and Mg^{2+} was omitted when Co^{2+} was present. Results are means for eight cultures from two platings.

Calcium influx into nerve terminals is required for the release of neurotransmitters (see ref. 22 for review). Cobalt blocks inward Ca^{2+} currents in cultured sensory neurons (20) and other cells (23), and it blocks acetylcholine release at the neuromuscular junction (24). As shown in Fig. 3*B*, the addition of cobalt prevented the K^+-stimulated release of substance P. The block was rapidly reversible: after the cobalt was removed the neurons released the usual amount of substance P on depolarization. We have studied the calcium dependence of substance P release in detail; the amount released (in response to 60 mM K^+) increases 5-fold as Ca^{2+} is raised from 0.2 to 5.4 mM.

Otsuka and Konishi (1) suggested that a carboxy-terminal fragment of substance P may be the biologically active molecule, so a pool of released material was also chromatographed. The released material was indistinguishable from synthetic substance P in two systems (Fig, 2, Table 1); therefore these cultured sensory neurons release a molecule whose size and charge are similar to those of the undecapeptide.

Inhibition of substance P release by DAEA

The effect of DAEA was tested in cultures from eight different platings. Release was evoked with 30–40 mM K^+. In six of the eight experiments, 10 μM DAEA significantly inhibited the K^+-evoked release of substance P. The mean release as percentage of control in each of these experiments was 34%, 48%, 57%, 66%, 67%, 72%, 94%, and 96%. We cannot yet explain the variability from plating to plating, but preliminary data indicate that the relative concentrations of K^+ and Ca^{2+} are important in determining the extent of DAEA inhibition. Fig. 4 shows the most pronounced effect; DAEA release = 34% of control. As illustrated in this figure, 50 μM naloxone completely prevented the DAEA inhibition.

Effect of DAEA on the DRG action potential

Action potentials recorded in perikarya of cultured chicken DRG neurons are relatively prolonged, and the repolarization phase is marked by a distinct plateau (20). DAEA applied at 10 μM by pressure ejection from a nearby extracellular pipette decreased the duration of the plateau (Fig. 5*A*). The spike duration was usually decreased immediately after the 1- to 2-sec ejection pulse, and it returned to control gradually over the next 1–2 min. In one series of experiments performed between 1 and

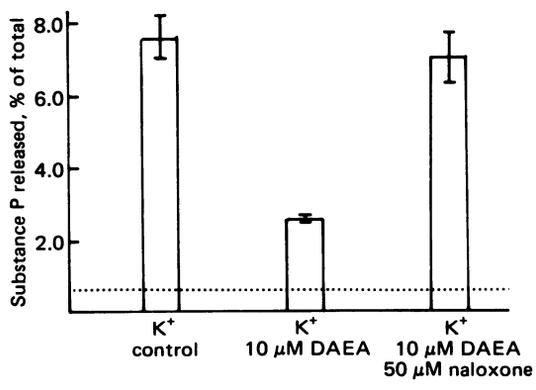

FIG. 4. Inhibition of substance P release by DAEA. Sibling cultures were depolarized by 30 mM K^+ for 5 min. The second group was depolarized in the presence of 10 μM DAEA and the third group was depolarized in the presence of 10 μM DAEA plus 50 μM naloxone. The dotted line indicates the limit of detection of the assay. Means and SEMs of triplicate cultures are given.

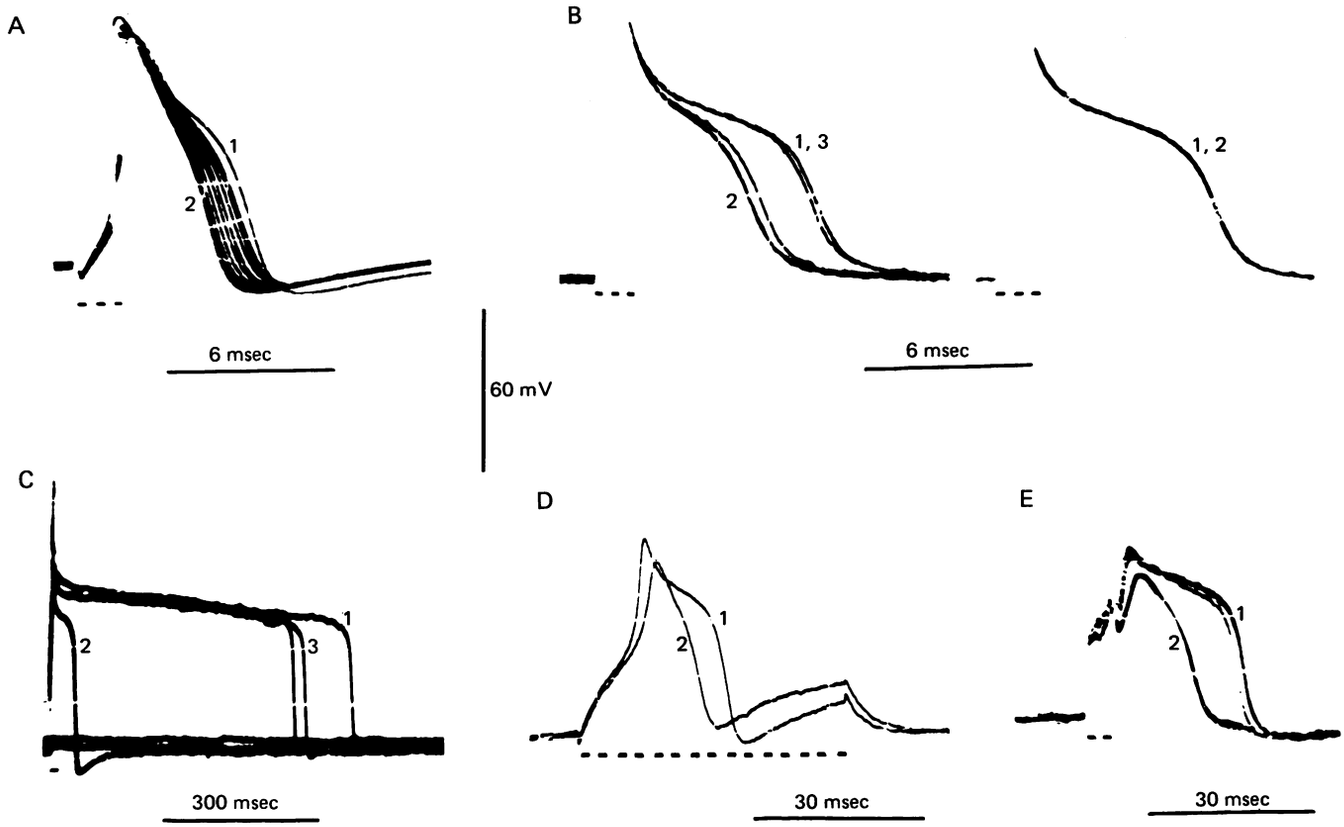

FIG. 5. Effect of DAEA and enkephalin on DRG soma action potentials. In all cases (*A–E*), 1 denotes the control spike; 2 denotes the spike after application of drug; and 3, when shown, denotes the return to control. (*A*) Superimposed oscilloscope traces showing the effect of 10 μM DAEA in control medium. Intervening traces were evoked at 0.1 per sec and indicate the rate of return to control. (*B*) Trace on left shows effect of 0.1 μM enkephalin. Superimposed traces on right, recorded in the same cell, show that 1 μM naloxone blocked the response to enkephalin. Naloxone and enkephalin were delivered simultaneously from a second extracellular pipette. (*C*) Effect of 10 μM DAEA on a prolonged action potential recorded in the presence of 5 mM Ba^{2+}/1.8 mM Ca^{2+}. (*D, E*) Effect of 10 μM DAEA on spikes evoked in the presence of 0.1 μM tetrodotoxin. Ba^{2+} (5 mM) was present in *E*, but not in *D*. Note the decreased rate of rise and amplitude. The broken line beneath each trace indicates the duration of the stimulating current pulse (delivered through the intracellular electrode).

3 weeks after plating, DAEA at 10 μM produced an effect similar to that shown in Fig. 5*A* in 35 of 49 cells tested. Complete dose–response curves were not defined, but it is noteworthy that the effect of 1 μM DAEA was not as marked and only a few cells responded to 0.1 μM. Enkephalin produced the same effect. It was at least as potent as DAEA: most (21 of 26) cells responded to 1 or 0.1 μM. In every case tested, the effect of DAEA or enkephalin was blocked by naloxone at a concentration of 1 μM (Fig. 5*B*); lower concentrations of naloxone were not tested.

Neither DAEA nor enkephalin produced a change in resting membrane potential or membrane conductance. The latter was tested with small pulses of inward current that produced 5- to 20-mV electrotonic potentials.

The early phase of the DRG soma spike is dominated by a relatively large inward Na^+ current, whereas the plateau is due in large part to an inward Ca^{2+} current (20). Thus a decrease in spike duration implies a decrease in Ca^{2+} entry. It is not clear, however, if DAEA affects Ca^{2+} channels directly; an increase in outward K^+ current might produce the same result. However, it is unlikely that a delayed, voltage-sensitive K^+ channel is involved. DAEA reduced the duration of prolonged spikes recorded in the presence of Ba^{2+} (Fig. 5*C*), an ion that carries current through Ca^{2+} channels and also blocks delayed voltage-sensitive K^+ channels (25). Moreover, the effect of DAEA was evident early in the spike. When the fast Na^+ component of the spike was blocked with 0.1 μM tetrodotoxin, DAEA produced a clear decrease in the rate of rise and the amplitude of the spike (Fig. 5 *D* and *E*). Thus we favor a direct effect on inward Ca^{2+} current. However, it is still possible that an early, perhaps Ca^{2+}-dependent, K^+ current (26) is present in these sensory neurons. Further experiments are required.

The effect of DAEA was not common to all peptides. Bradykinin, neurotensin, thyrotropin-releasing factor, and substance P (10 μM) did not alter the spike. Somatostatin, another peptide present in sensory neurons (4, §) did decrease the spike duration.

DISCUSSION

Embryonic chicken sensory neurons in dissociated cell culture contain immunoreactive substance P and they develop a voltage-sensitive, Ca^{2+}-dependent release mechanism for substance P in the absence of target spinal cord neurons. Our finding that DAEA can inhibit the stimulated release of substance P from cultured sensory neurons confirms and extends Jessell and Iversen's report (16) that DAEA reduces the release of substance P from the trigeminal nucleus. Because no other cell types are present in cytosine arabinonucleoside-treated DRG cultures, we conclude that DAEA must act directly on sensory neurons.

The concentrations of DAEA required to inhibit release from both cultured sensory neurons (this study) and the trigeminal nerve nucleus (16) were in the range 1–10 μM. These concentrations are 100- to 1000-fold higher than concentrations that effectively inhibit neurally evoked contractions in the gut (27). The unphysiological stimulus used in our experiments (K^+

depolarization) may account for the higher dose required; alternatively, different receptors may be involved (27).

DAEA (and enkephalin) decreased the duration and magnitude of the Ca^{2+} spike evoked and recorded in DRG perikarya. A similar effect at sites of substance P release may account for the observed inhibition. The notion that cell body Ca^{2+} spikes might serve as indicators of Ca^{2+} influx across inaccessible synaptic membranes is not new (28). Recent studies of habituated sensory neuron–motoneuron synapses in the abdominal ganglion of *Aplysia* support the applicability of this model (29). Serotonin "dishabituates" by increasing transmitter output from the sensory nerve terminals; it also prolongs the Ca^{2+} spike recorded in the sensory neuron cell body. In addition we have found that γ-aminobutyric acid, norepinephrine, and serotonin—three neurotransmitters present in the spinal cord of higher vertebrates—decrease the DRG spike duration (30). Each of these compounds also inhibits the release of substance P (unpublished results).

Our focus on a possible presynaptic mechanism of opiate action does not, of course, exclude other, perhaps direct, postsynaptic actions (cf. refs. 31 and 32).

It should be emphasized that DAEA (and other effective drugs) altered the DRG spike at concentrations that did not produce changes in resting membrane conductance. Whatever the precise mechanism for DAEA modulation of voltage-sensitive ion channels, it does not involve a simple shunting of the membrane resistance.

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