Two distinct modulatory effects on calcium channels in adult rat sensory neurons

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ABSTRACT D-ala^2-D-leu^5-enkephalin (100 to 1000 nM) reduces HVA Ca^{2+} currents of approximately 60% in 92% of the adult rat sensory neurons tested. In 80% of the cells sensitive to enkephalin, the reduction in Ca^{2+} current amplitude was associated with a prolongation of the current activation that was relieved by means of conditioning pulses in a potential range only about 10 mV positive to the current activation range in control conditions. The time course of the current activation was fitted to a single exponential in control, (τ = 2.23 msec ± 0.14 n = 38) and double exponential with enkephalin, (τ_1 = 2.18 msec ± 0.25 and τ_2 = 9.6 msec ± 1, test pulse to −10 mV, 22°C). A strong conditioning depolarizing prepulse speeded up the activation time course, completely eliminating the slow, voltage-sensitive exponential component, but it was only partially effective in restoring the current amplitude to control values. The voltage-independent inhibitory component that was not relieved could be recovered only by washing out enkephalin. In the remaining 20% of the cells affected, enkephalin decreased Ca^{2+} current amplitude without prolongation of Ca^{2+} channel activation. In these cases the conditioning voltage pulse was not effective in relieving the inhibition that persisted also at strong positive test potentials, on the outward currents. The voltage-dependent inhibition occurred slowly after enkephalin superfusion ($\tau \simeq 12$ sec), whereas the voltage-independent one developed about ten times more rapidly. Dopamine (100 μM) could also induce both voltage-dependent and independent modulations. In some sensory neurons the two different effects were separately induced by the two substances. GTP-γ-S (100 μM) intracellularly perfused mimicked both the modulatory effects. The two modulations may have different functions in processing nociceptive inputs.

INTRODUCTION

The modulation of high voltage-activated (HVA) calcium channels by neurotransmitters is frequently referred to as *time and voltage-dependent* since the inhibitory effect is relieved at the most positive membrane potentials during the test pulse (1–5). On the other hand, calcium current suppression by certain substances at some locations in the nervous system is reported as *voltage-independent* (6–8). Previous observations from our laboratory indicated two different modulatory effects in adult rat sensory neurons. A voltage-independent modulation was observed on HVA, dihydropyridine and/or ω-conotoxin-insensitive calcium currents, by muscarinic $GABA_B$ or dopamine receptor activation, and a voltage-dependent prolongation of current activation was induced by means of dopamine or GTP-γ-S intracellularly perfused (9–11). On this basis, we have investigated the inhibition induced on HVA Ca^{2+} currents by enkephalin, a potent analgesic reported to mediate presynaptic inhibition on nociceptive primary afferents (12, 13) and already known to inhibit calcium channels and transmitter release (14–20). These inhibitory effects were compared to those produced by GTP-γ-S and dopamine.

METHODS

Cell preparation

Dorsal root ganglia were dissected from Wistar adult rats (175–200 gr, 6–7 weeks old) (21). Animals in ether anesthesia were decapitated and their dorsal columns were excised. After dorsal laminectomy the spinal cord was removed and ganglia were collected in cool Ca^{2+}-free Tyrode solution. The ganglia were then minced with fine iridectomy scissors and treated enzymatically in a Ca^{2+}-free Tyrode solution containing 0.5% trypsin (type XII-S) and 0.75% collagenase (type I-S) for 35 min at 37°C. The fragments of ganglia were then gently triturated using Pasteur pipettes with reduced tip diameter. After centrifugation, the cells were plated on collagen-poly-D-lysine-coated Petri-dishes. The cultures were fed with MEM Eagle supplemented with 10% fetal calf serum, 10% horse serum, 2 mM L-glutamine, 30 mM glucose, 10 mM HEPES, 27 mM $NaHCO_3$, 0.1 IU/l penicillin, 0.1 mg/l streptomycin and 0.15 mg/l amphotericin B. Collagenase (type I-S), trypsin (type XII-S), glucose, HEPES free acid, $NaHCO_3$ and poly-D-lysine were all supplied by SIGMA (St. Louis, MO-USA). L-glutamine, penicillin, streptomycin, amphotericin were all supplied by FLOW (Irvine, UK). Heat-inactivated horse and fetal calf sera were supplied by GIBCO (Paisley, UK). Cultures were incubated at 37°C with carbon dioxide adjusted to give a pH of 7.4.

Patch-clamp recordings

Recordings were carried out a few hours after plating up to 36 hours at 22°C from cells of 15–30 μm in diameter, using the whole-cell patch-clamp technique (22). Cells were voltage-clamped using patch pipettes of 2–3 MΩ resistance, with an NPI SecIL switching amplifier (Tamm, Germany), sampling rate 15–30 Khz, interfaced with an IBM-compatible OLIVETTI M290 (Ivrea, Italy) computer. Stimulation, acquisition and off-line data analysis were done using pClamp programs AXON INSTRUMENTS (Foster City, CA-USA) and data were digitized at sampling times of 100 μsec using a 12-bit A/D Tecmar Lab Master board. Unless noted otherwise, capacitive transients and leakage currents were compensated by subtracting residual Cd^{2+}-insensitive currents recorded after adding 500 μM Cd^{2+} to the external medium. A multiple least-squares regression algorithm was used for curve-fitting. The results obtained were based only on cells showing adequate voltage-clamping in which current modulation was always, at least partially, followed by recovery after washing. Cells with low voltage activated (LVA) calcium currents were not used.

Solutions

External solution contained (in mM): 5 $CaCl_2$, 105 choline-Cl, 20 TEA, 1 $MgCl_2$, 10 HEPES free acid and 10 glucose (pH to 7.4 with

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CsOH). Choline replaced sodium in order to avoid tetrodotoxin-insensitive sodium currents. Internal solution contained (in mM): 110 CsCl, 30 TEA-Cl, 0.8 $MgCl_2$, 10 EGTA free acid, 10 HEPES free acid, 8 glucose (pH 7.2 using CsOH). Where indicated, this solution was supplemented with 100 μM GTP-γ-S (guanosin-5'-O-(3-thiotriphosphate)). Ascorbic acid (1 mg/ml of bathing solution) was added to the external solutions when dopamine (3-hydroxytiramine-HCl 50–100 μM) was used. D-ala^2-D-leu^5-enkephalin was dissolved in distilled water and then added to the external medium. All the drugs were supplied by SIGMA (St. Louis, MO-USA).

RESULTS

Enkephalin inhibited HVA calcium currents

The opioid D-ala^2-D-leu^5-enkephalin at various concentrations caused an amplitude decrease of HVA Ca^{2+} currents in 92% of the adult rat sensory neurons tested (100 nM −60% of peak amplitude ± 5.4 SEM, n = 5; 400 nM −59% ± 2.47 n = 33; 1000 nM −65% ± 4.7 n = 10). Naloxone, an opioid-receptor antagonist, at the concentration of 1 μM fully antagonized the effect of 100 nM enkephalin (n = 5) (Fig. 1).

Prolongation of Ca^{2+} current activation

In about 80% of the cells affected by enkephalin the current inhibition was particularly marked at the beginning of the pulse, with an increase in time to peak (from 17 msec ± 1.3 in control, up to 108 msec; mean increment 38 msec ± 4.9 n = 39). In these cases the current amplitude decrease was accompanied by a prolongation of Ca^{2+} channel activation. The best fit of the current rising phase was calculated. Neglecting the very early phase of activation (23), the activation time course of the Ca^{2+} currents in control could be well fitted with a single-exponential curve:

$$I_{(t)} = A \exp^{-t/\tau} \quad (1)$$

where τ was 2.23 msec ± 0.14 n = 38, whereas in the modulated currents, the prolongated current activation was well fitted with a two-exponential curve:

$$I_{(t)} = A_1 \exp^{-t/\tau_1} + A_2 \exp^{-t/\tau_2} \quad (2)$$

where τ_1 was 2.18 msec ± 0.25 and τ_2 was 9.6 msec ± 1 n = 38, (test pulses to −10 mV). The relative amplitudes A_1 and A_2 of the fast and slow components were 42% ± 3.5 and 58% ± 3.5 n = 38 respectively. The relative amplitudes did not change significantly, varying the agonist concentration from 100 to 1,000 nM. Fig. 2 shows the voltage-dependence of the time constant (τ) in control and of the two time constants (τ_1 and τ_2) during modulation with enkephalin. All the time constants and especially τ_2 decreased at increasing potentials.

The influence of voltage on current modulation was tested using a double pulse protocol (Fig. 1 *A, upper trace*). This protocol was adopted in order to allow the resting HVA Ca^{2+} channels to recover from the drug-induced inhibition during the first conditioning depolarizing pulse and then to deactivate during the brief repolarization. The conditioned currents obtained by the following test pulse were compared with the unconditioned currents elicited by an analogous test pulse. A conditioning pulse positive to +30 mV was effective in completely restoring the activation time course to a single exponential curve (τ = 2.57 msec ± 0.24 n = 38) as observed in control, eliminating the slow component induced by the drug and reducing the time to peak to control values. However, the conditioning pulse even at higher poten-

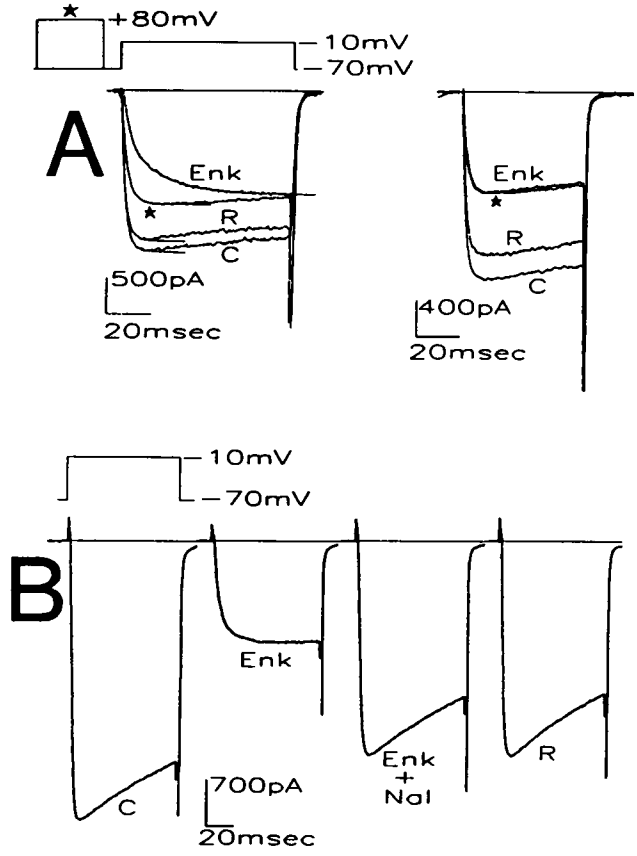

FIGURE 1 Enkephalin inhibited HVA calcium currents. In all diagrams, trace *C* refers to control and *R* to recovery. *A (left)*: the modulatory effects induced by enkephalin (*Enk*, 400 nM) could be partially relieved by a 30 msec conditioning pulse to +80 mV (*asterisk*). The current amplitude was almost completely restored to control levels only after washing out the drug (*R*). The best fitting was calculated for the rising phase to peak of all the traces (*thin lines*) using the equations reported in the text. Control trace could be best fitted with a single exponential curve (τ = 2.36 msec), while enkephalin induced a second slow exponential component in the rising phase (*Enk;* τ_1 = 2.3 msec τ_2 = 10 msec). The conditioning pulse (*asterisk*) was effective in completely restoring the activation time course to a simple exponential curve as in control (τ = 2.37 msec) and the same occurred in washing. A different cell is shown on the right, subjected to a similar experimental protocol as in *A*, the conditioning pulse failed to relieve the inhibitory effect of enkephalin. *R:* recovery. *B:* naloxone (Nal 1 μM) antagonized the inhibitory effects induced by 100 nM enkephalin.

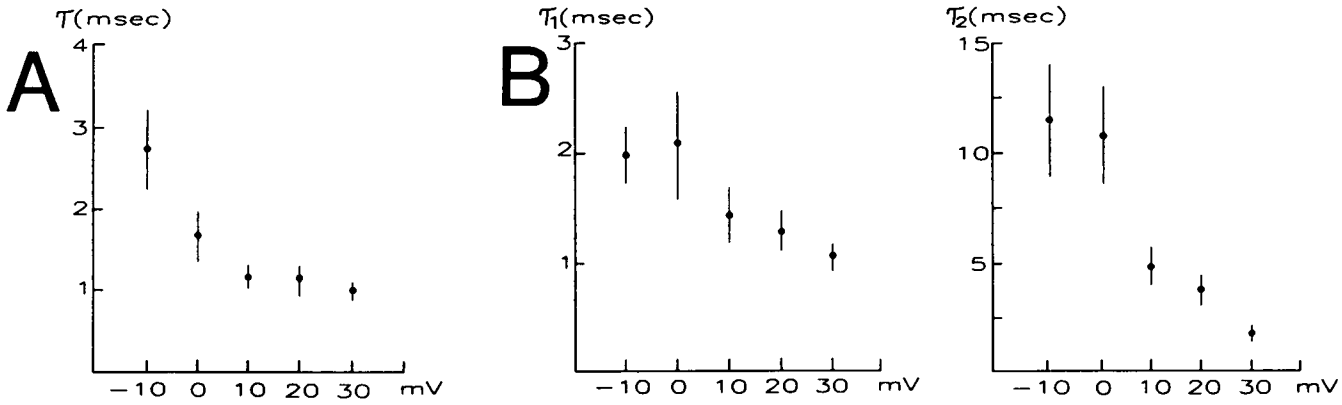

FIGURE 2 Analysis of the activation time course of the Ca^{2+} currents in control and with 400 nM enkephalin. Currents were elicited by means of depolarizing steps from a holding potential of −70 mV to the potentials indicated on the abscissae. The rising phase of the control currents were well fitted with a single exponential (Eq. 1 in the text) *A:* time constant τ of the rising phase as a function of the test potential. Enkephalin induced a prolongation of current activation that was well fitted with a double exponential curve (Eq. 2 in the text). *B:* the two diagrams show the two time constants τ_1 and τ_2 versus test potential. Each point represents the mean value from 7 cells ± SEM.

tials was only partially effective in restoring Ca^{2+} current amplitude. The current amplitude was almost completely restored only after washing (Fig. 1 *A*).

Enkephalin scaled down Ca^{2+} currents

In about 20% of the cells affected by enkephalin, HVA Ca^{2+} currents were scaled down without a significant prolongation of current activation (time to peak in control: 13.6 msec ± 1.4; with enkephalin 14.7 msec ± 2 n = 11, test pulse at −10 mV). In these cases the effect was present also at the highest test potentials and the conditioning pulse completely failed to relieve the drug induced inhibition (Fig. 1 *A, right*). Both the effects were observed on the Ca^{2+} currents evoked from a range of holding potentials between −100 and −50 mV.

Time- and voltage-dependence of the modulatory effects

Time-dependence of the modulatory effects of enkephalin was evaluated, measuring current amplitudes both at 10 msec and at 99 msec from the beginning of pulses at scaled potentials ranging from −60 to +90 mV, from a holding potential of −70 mV. Current–voltage relationships indicate that enkephalin induced a Ca^{2+} current decrease clearly evident at 10 msec and only slightly relieved during time at 99 msec (Fig. 3 *A, B,* and *C*). Fig. 3 *E* shows the time-dependent current recovery during the pulse, between 10 and 99 msec, versus pulse voltage. Recovery occurred around the voltage range in which the voltage-sensitive inhibition was relieved. It is evident from the *I–V* curves in Fig. 3 *B* and *C* that an inhibitory component persists even at positive potentials on the outward currents, both at 10 and 99 msec.

The voltage-dependent inhibition was studied as a function of the conditioning pulse voltages. Currents evoked by a test pulse to a fixed voltage were conditioned by 30 msec prepulses from a holding potential of −70 mV to scaled potentials ranging from −50 to +120 mV. The relief of the enkephalin-induced voltage-dependent inhibition occurred in a range of conditioning potentials about 10 mV more positive than the voltage range of the activation-curve in control unconditioned currents (Fig. 4 *A* and *B*).

After the relief by means of a suitable conditioning pulse, the voltage-sensitive inhibitory component induced by enkephalin reverted, reducing the Ca^{2+} current and slowing down the activation kinetics. A protocol of two stimuli with a changing duration of repolarization between conditioning and test pulse was adopted in order to evaluate the time course of this reversion. The relative amplitude of the voltage-sensitive slow exponential component, estimated by the fitting of the activation phase of the currents with Eq. 2, increased as a function of the duration of repolarization, with a time-constant of about 100 msec (Fig. 5).

The relief of the enkephalin induced voltage-dependent inhibition was studied as a function of the duration of a +50 mV conditioning pulse. The time constant of the relief was about 12 msec (Fig. 6).

The two modulatory effects developed through different limiting steps

We observed in almost all the cells in which the two inhibitory effects of enkephalin were present that the inhibitions developed with different time courses in onset and offset. In order to evaluate this aspect, paired unconditioned and conditioned test pulses were imposed at different times from the beginning of drug superfusion, as indicated in Fig. 7. A first amplitude decrease occurred in less than 2 seconds. However, the effect on the

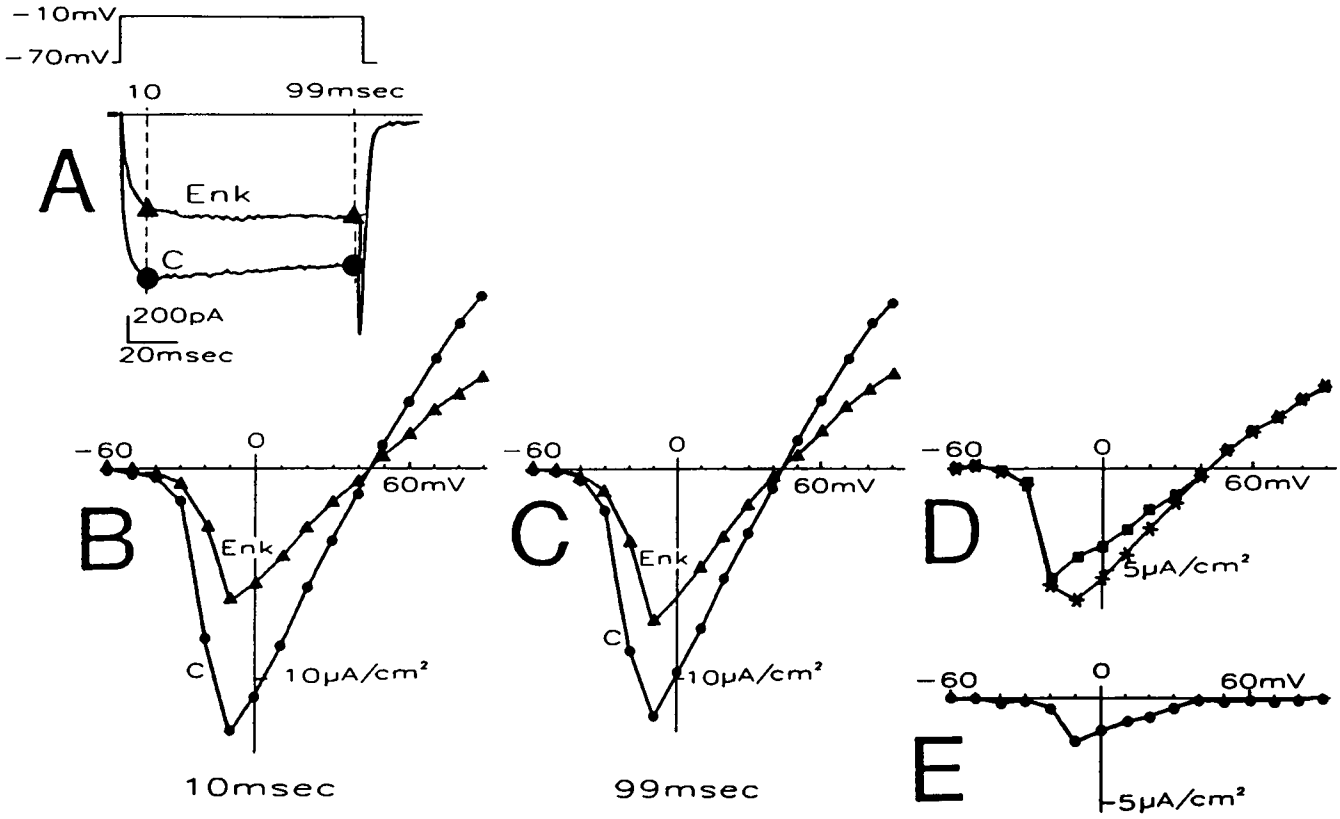

FIGURE 3 Time- and voltage-dependence of enkephalin induced modulatory effects. Ca^{2+} currents were evoked by 100 msec pulses from a holding potential of −70 mV to voltage values ranging from −60 to +90 mV. The current amplitude was measured at two different times along the current curves in control and inhibited with enkephalin (400 nM) (*A*). The Current-voltage relationship indicate that enkephalin induced a decrease in calcium current both at 10 msec (*B*) and at 99 msec from the beginning of the depolarizing pulse (*C*). Panel *D* shows the differences between the currents in control and those inhibited by the drug at 10 msec (*asterisks*) and 99 msec (*squares*), respectively. There is a stronger inhibition, at 10 msec in the range between −20 mV and +30 mV shown in trace *E*, that is the difference between the two curves in *D* and this represents the time-dependent recovery between 10 msec after the beginning of the pulse and 99 msec. Note that although the inhibition persisted at the most positive potentials (*B* and *C*), there was not relief of inhibition during the time between 10 and 99 msec at the potentials positive to +30 mV (*E*). Each point represents average current density from 4 experiments.

activation kinetics and, therefore, the larger amplitude decrease at the beginning of the pulse became fully evident only after tens of seconds ($\tau_{onset} \simeq 12$ sec), and reverted more slowly than the effect on the current amplitude during washing (voltage-dependent effect: $\tau_{offset} \simeq 28$ sec; voltage-independent effect: $\tau_{offset} \simeq 7$ sec). When the prolongation of current activation took place, the conditioning pulse became more effective in partially relieving the ongoing inhibition, especially at the beginning of the pulse, speeding the activation kinetics up to control value.

Dopamine (100 μM) caused a peak amplitude decrease in HVA Ca^{2+} currents (−42% ± 3.5 n = 41) in 76% of the cells tested. Modulatory effects were either voltage-dependent or independent, although dopamine was more effective than enkephalin in inducing a voltage-independent inhibition without prolongation of current activation, frequently speeding up the inactivation kinetics. The effects were fully reversed during washing.

In 5 neurons out of 16, in which HVA Ca^{2+} currents were sensitive to both dopamine and enkephalin, the two different inhibitory effects were separately induced by dopamine and enkephalin. Dopamine scaled down the Ca^{2+} currents in a voltage-independent manner, whereas enkephalin could also induce the voltage-dependent inhibition of Ca^{2+} current (Fig. 8). The two drugs could be tested many times on the same cell without a qualitative change in the effects. The voltage-independent modulation induced by dopamine was also rapid in onset and offset.

GTP-γ-S mimicked the modulatory effects

After a few minutes in whole-cell configuration, intracellular GTP-γ-S (100 μM) induced a Ca^{2+} current inhibition with prolongation of current activation. The activation time course changed from single exponential in control (τ = 2.26 ± 0.25 n = 18, test potential = −10 mV) to double exponential when the effect of GTP-γ-S was stabilized (τ_1 = 1.8 msec ± 0.3; τ_2 = 7.3 msec ± 0.6 n = 18). A 30 msec conditioning pulse positive to +30 mV was effective in accelerating the current activation, completely restoring the activation time course to a single

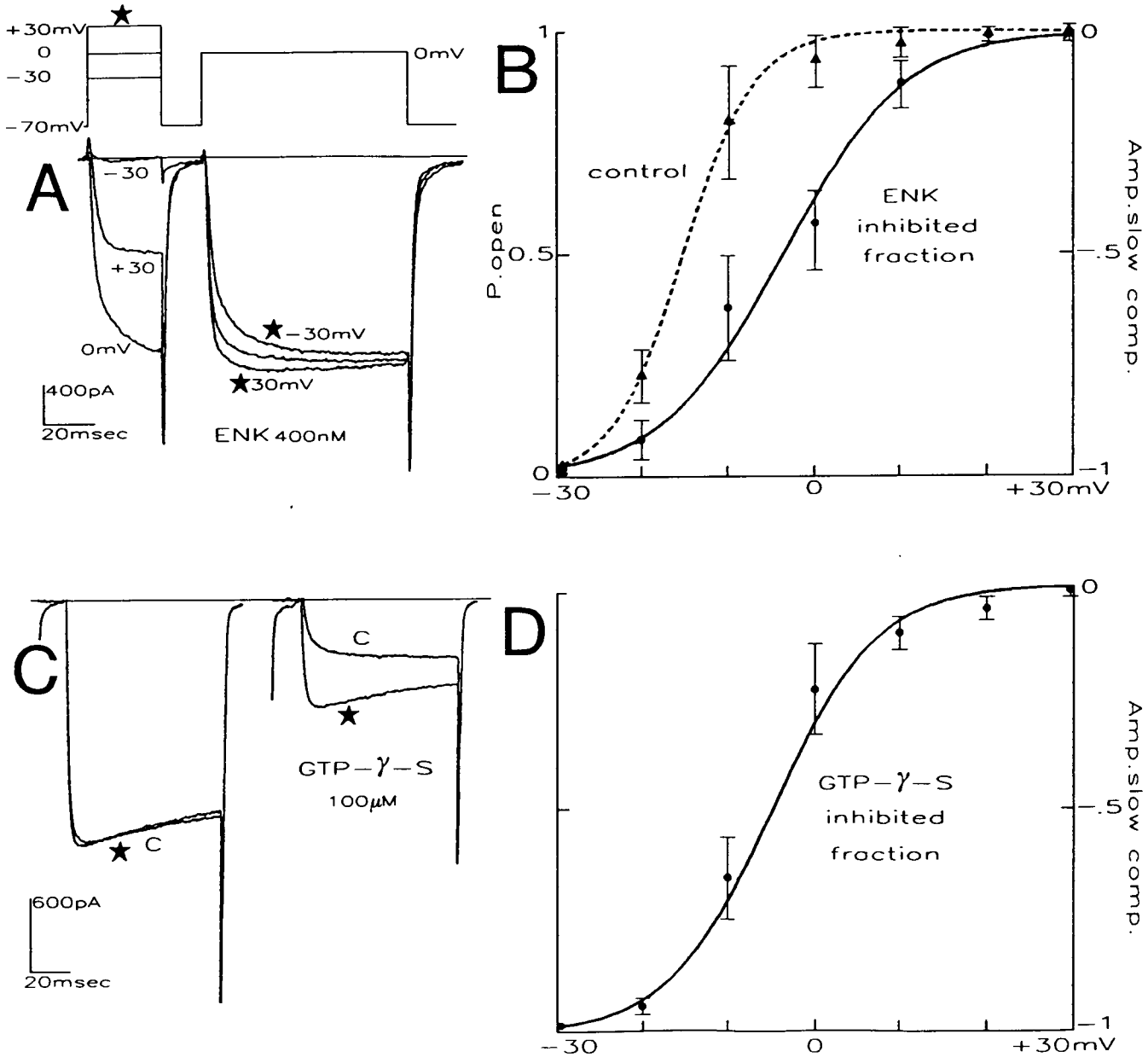

FIGURE 4 Voltage-dependent inhibitory component studied as a function of the conditioning pulse voltage. A double pulse protocol with a conditioning pulse at increasing potentials was adopted to induce a gradual relief of the slow exponential component of the current rising phase elicited by a test pulse to 0 mV as shown in *A*. The amplitude of the voltage-sensitive slow exponential component (Amp. slow comp.) was estimated with a double exponential fitting of the current activation (Eq. 2 in the text). The relative values ($A_2/A_1 + A_2$) were normalized and plotted in diagram *B* (*dots* ± SEM $n = 5$), as a function of the conditioning potential. The current activation curve obtained in control conditions (*triangles*) was plotted on the same diagram. The two series of values were fitted with Boltzmann curves: $1/[1 + \exp^{-(V-V_{0.5})/k}]$ where $V_{0.5}$, the midpoint, is −15.2 mV for activation curve in control and −3.7 mV for the "disinhibition curve"; and k is the slope factor: 4 mV in control and 7 mV for the disinhibition curve. *C*: A 30 msec conditioning pulse to +30 mV partially relieved the inhibition of HVA calcium currents elicited by an 80 msec test pulse to −10 mV, in a GTP-γ-S intracellularly perfused cell (*asterisk*). Left: the currents recorded at the beginning of the recording. Right: 5 min later, when the effect of intracellular GTP-γ-S had stabilized. The conditioning pulse speeded up the activation time course, partially restoring the current amplitude. (Artifacts were partially truncated). *D*: the relative amplitude of the slow exponential component induced by GTP-γ-S was plotted versus the conditioning potential, as indicated above for enkephalin. The midpoint of the Boltzmann curve was −5 mV and the slope factor was 6 mV. Note that the voltage-dependent inhibition induced by GTP-γ-S was relieved at about the same voltage range as that induced by enkephalin.

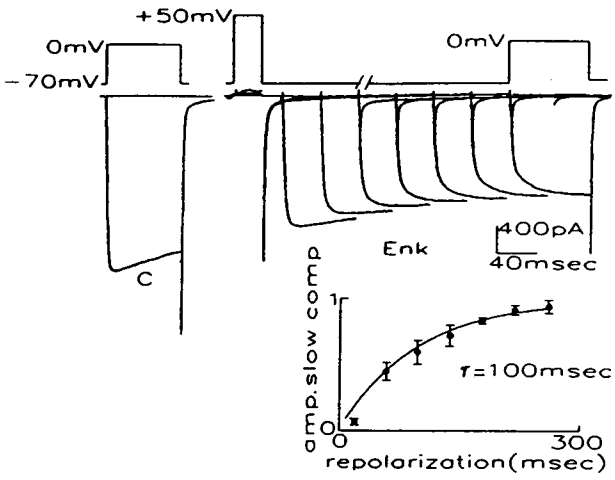

FIGURE 5 Reversion time course of the voltage-sensitive inhibition induced by 400 nM enkephalin after it was relieved by a conditioning pulse. *C*: control current. On the right, seven superimposed current traces recorded during enkephalin superfusion (*Enk*) with a stimulation protocol consisting of two pulses with decreasing time of repolarization between conditioning and test pulse (*traces on top*). The current curves were fitted with a double exponential (Eq. 2 in the text) and the relative amplitude of the slow exponential component ($A_2/A_1 + A_2$) of 3 different cells was normalized and plotted in the diagram below versus the duration of repolarization. The points ± SEM were fitted with an exponential curve with time constant of 100 msec.

fast exponential curve (τ = 2 msec ± 0.3 n = 18) as observed in control, but was only partially effective in restoring Ca^{2+} current amplitude. (Fig. 4 *C*). The diagram in Fig. 4 *D* shows the relative amplitude ($A_2/A_1 + A_2$) of the slow exponential component normalized and plotted as a function of the conditioning potential. The relief of the GTP-γ-S-induced voltage-dependent inhibition occurred in a range of conditioning potentials about 10 mV more positive than the voltage range of the activation-curve in control unconditioned currents, as observed for enkephalin (Fig. 4 *B*).

With both enkephalin and dopamine the voltage-independent inhibition was found to be less sensitive to intracellular dialysis than the voltage-dependent prolongation of current activation.

DISCUSSION

Two types of inhibition

The major finding of the present study is that enkephalin could induce two types of inhibition on HVA Ca^{2+} channels. These data confirm a first observation about two electrophysiologically distinct modulations, on calcium currents in adult rat sensory neurons (9, 10).

The two modulations were characterized according to *a*) voltage and time-dependence, *b*) the different action on the channel, *c*) the onset and offset rates of the effects, and *d*) the sensitivity to the intracellular dialysis.

The prolongation of the current activation accounted for the transient inhibition at the beginning of the pulse. It can be explained if a Ca^{2+} channel model is assumed, in which the opiate receptor activation induces a fraction of the Ca^{2+} channels to switch to a second closed state with slower, opening kinetics, as previously proposed (2–4, 24–27).

The prolongation of the current activation was voltage and time-dependent. Increasing depolarizations speeded the current rising phase up to control values, decreasing the amplitude of the slow exponential component that is characteristic of this prolongation. On the other hand, the current amplitude recovery was not obtained, even at high potentials, and the time did not contribute indefinitely to the relief of the current inhibition. As can be observed in Fig. 3 *C*, an inhibitory component persisted even at potentials positive to +40 mV and at the end of the pulse. Moreover, at those potentials, the time between 10 and 99 msec during the pulse did not contribute to the recovery (Fig. 3 *E*). (This can simply be explained if we consider that the slow exponential component of the rising phase at those potentials had a short time constant ($\tau_2 \simeq 2$ msec at +30 mV) and at 10 msec from the beginning of the pulse it was already at its maximum, i.e., all the channels with slower opening kinetics were activated). Therefore enkephalin could induce also a voltage and time-independent scaledown of the currents.

As a consequence of the model adopted, the amplitude of the slow exponential component of the current activation should reflect the amount of channels in the closed-and-inhibited state at the beginning of the test pulse.

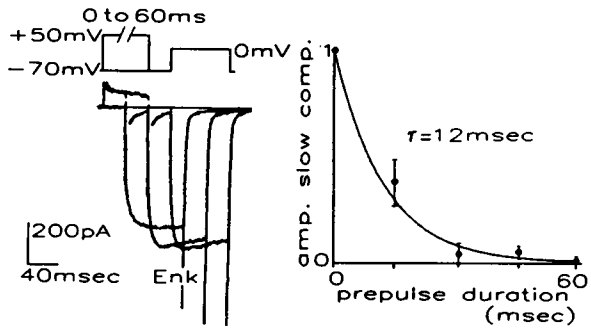

FIGURE 6 Relief of the voltage-sensitive inhibition induced by 400 nM enkephalin as a function of the conditioning pulse duration. A double pulse protocol was used, consisting of conditioning pulses to +50 mV that lasted from 0 to 60 msec in 15 msec intervals, followed by a test pulse to 0 mV as indicated. On the left, three superimposed current traces conditioned with prepulses of increasing duration (0, 30, and 60 msec respectively). The current curves were fitted with a double exponential (Eq. 2 in the text) and the relative amplitude of the slow exponential component ($A_2/A_1 + A_2$) of 4 different cells was normalized and plotted in the diagram versus the prepulse duration. The points ± SEM were fitted with an exponential curve with time constant of 12 msec.

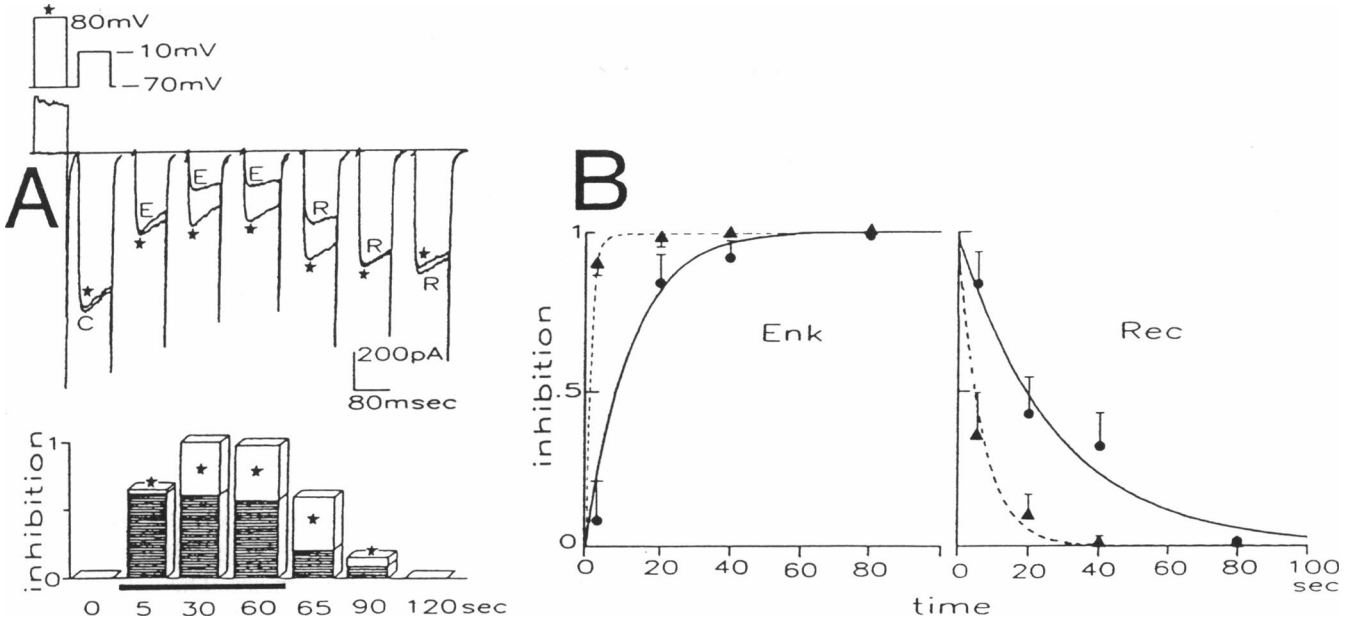

FIGURE 7 Onset and offset time course of the modulatory effects of enkephalin. *A:* the upper part shows paired unconditioned and conditioned Ca^{2+} currents elicited by means of a double pulse protocol as indicated above, recorded at different times: first on the left with control solution (*C*); from second to fourth with enkephalin (*E*) and then during recovery (*R*). The conditioning pulse only partially relieved the inhibited currents (*asterisks*). The stacked bar histogram shows the voltage-dependent inhibitory component, measured as current amplitude relieved by the prepulse (*empty bars*) and the voltage independent one (*filled bars*) expressed in normalized values as a function of time from the beginning of superfusion with enkephalin (*horizontal black bar under the abscissa*). Note that the voltage-dependent effect occurred slowly after enkephalin superfusion and reverted slowly during washing, whereas the voltage-independent one developed more rapidly. The diagrams in *B* show the onset (*Enk*) and offset (*Rec*) of the two modulations. Dots and triangles indicate the voltage-dependent and -independent effects, respectively, each point representing the mean value ± SEM from 5 experiments. Values were fitted with exponential curves. Continuous lines represent voltage-dependent effects (τ_{onset} = 12 sec; τ_{offset} = 28 sec). Dashed lines represent voltage-independent effects (τ_{onset} = 1.3 sec; τ_{offset} = 7 sec).

A double pulse protocol with a conditioning depolarizing pulse at scaled potentials (Fig. 4) allowed the reluctant channels to open during the first pulse and then to deactivate during the brief repolarization, relieving the inhibition. After the relief, the inhibition reverted quite slowly. This made it possible to estimate the amount of Ca^{2+} channels which had remained in the inhibited state after the conditioning pulse as a function of the conditioning potential, estimating the relative amplitude of the slow exponential component of the current rising phase elicited by the following test pulse. The curve was shifted by ~10–15 mV towards positive potentials compared to the activation curve in control conditions.

An inhibitory action on a transient current component through a distinct channel subtype cannot be completely ruled out (28–30). However it seems, unlikely. The effects were present on the Ca^{2+} currents evoked from a wide range of holding potentials. Moreover a positive conditioning pulse should inactivate a transient current component instead of relieving it. On the other hand, it seems unlikely that the time and voltage-independent scale down of Ca^{2+} currents may develop through a distinct channel subtype, since previous results indicate a voltage-independent scale down, on dihydropyridine insensitive and/or ω-conotoxin insensitive HVA Ca^{2+} currents, in the same neurons, exerted either by muscarinic or $GABA_B$ receptor activation (10), or by dopamine (Formenti, A., E. Arrigoni, and M. Mancia, unpublished observations).

In a recent paper, Beech et al. (1992) showed that transmitter inhibition of HVA Ca^{2+} channels in rat sympathetic neurons could occur with both a fast and a slow time course of action. Moreover they distinguished two fast effects insensitive to intracellular BAPTA: a voltage-dependent and a voltage-independent one, and they characterized the slow effect as voltage-independent and calcium-dependent since high intracellular calcium buffer concentration abolished it (32, 33). Interestingly, we observed that the two inhibitory effects of enkephalin displayed different onset and offset time courses. Whereas the fast, voltage and BABTA insensitive modulation observed by Beech et al. (1992) is similar to that induced by muscarinic and $GABA_B$ (10), dopamine (11) and enkephalin receptor activation in sensory neurons, the slow effect that we report here is different since it was voltage-dependent and occurred despite the presence of 10 mM EGTA in the intracellular solution even in the situation when using barium as charge carrier (Formenti, A., E. Arrigoni, and M. Mancia, unpublished observations).

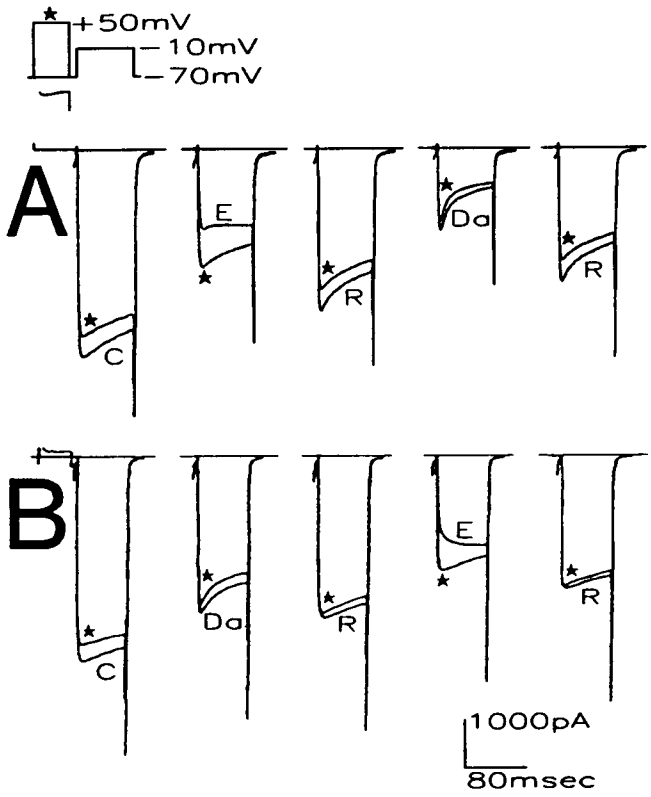

FIGURE 8 Voltage-dependent and independent inhibitory effects of enkephalin (*Enk*, 400 nM) and dopamine (Da, 100 μM), respectively, on HVA Ca^{2+} currents, evoked by coupled unconditioned and conditioned test pulses at the potentials indicated on the above trace, in two different sensory neurons (*A* and *B*). Whereas a conditioning pulse (*asterisk*) was partially effective in relieving the inhibitory effect induced by enkephalin, it was totally uneffective on the currents inhibited by dopamine.

Further evidence as to the existence of two electrophysiologically distinct mechanisms of modulation on HVA calcium channels comes from the observation that in, some neurons, the two different inhibitory effects were separately induced by dopamine and enkephalin in the same cell.

GTP-γ-S intracellularly perfused could induce a Ca^{2+} channel current inhibition with prolongation of current activation. As observed in enkephalin superfused cells, also with intracellular GTP-γ-S, current inhibition was only partially relieved by a strong conditioning depolarizing pulse (although in our experiments we cannot exclude a certain run-down of the currents). On the other hand, the voltage-dependent prolongation of Ca^{2+} current activation induced by either enkephalin or GTP-γ-S presents similar features, and it is relieved at the same potentials. Therefore, there are points of similarity between the action of GTP-γ-S and that of enkephalin. The possibility that Ca-channel modulation may develop through two distinct pathways mediated by G-proteins is supported by recent findings which suggest that more than one type of G-protein is involved in receptor-channel coupling (34).

This suggests, but does not prove, that both types of modulation may be mediated by G-proteins (35).

Two modulations electrophysiologically distinct: a physiological consideration

Strong evidence suggests that enkephalin mediates presynaptic inhibition on nociceptive primary afferents at spinal level (12, 13). The differences in voltage-dependence of the two inhibitory effects may be of physiological relevance in nociceptive sensory transmission. Since the voltage-dependent effect could be readily relieved with some msec depolarization in the voltage range of the action potential overshot (Fig. 6) and since it takes about 100 msec to revert (Fig. 5), then it might act as a filter, blocking low spike frequencies. The voltage-independent inhibition, on the other hand, reduces calcium entry and transmitter release at all frequencies. Therefore voltage-relieved inhibition may provide a threshold mechanism for qualitative discrimination of nociceptive inputs, whereas voltage-independent inhibition could more properly act in noxious inhibitory control (13, 36).

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