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Block of calcium channels by enkephalin and somatostatin in neuroblastoma–glioma hybrid NG108-15 cells

(opiate receptors/neuropeptides/patch clamp)

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ABSTRACT **Leucine-enkephalin, methionine-enkephalin, and morphine caused a reversible block of Ca^{2+} channel currents in neuroblastoma–glioma hybrid cells (NG108-15). The long-lasting (type 2) component of the Ca^{2+} channel current was blocked by leucine-enkephalin, while the transient (type 1) component was not affected. The enkephalin-induced blocking action was antagonized by naloxone and appears to be mediated by δ-opiate receptors. Two different aspects of the blocking effect were detected, a resting block and a recovery from block during prolonged depolarizing pulses. Recovery from block was more complete, and its time course was more rapid, with depolarization to more positive potentials. The dose dependence of the type 2 channel block at rest indicated a one-to-one binding stoichiometry, with an apparent dissociation constant of 8.8 nM. Somatostatin exerted a similar selective blocking action on the type 2 Ca^{2+} channel. The time- and voltage-dependent block of type 2 Ca^{2+} channels may provide a mechanism underlying the enkephalinergic presynaptic inhibition of transmitter release and the somatostatin block of pituitary growth hormone release.**

Since the discovery of methionine-enkephalin and leucine-enkephalin (Leu-EK) by Hughes *et al.* (1), the physiological roles of the endogenous opioid peptides have been of pivotal interest in neuroscience. Convincing evidence has been obtained that enkephalin (EK) inhibits transmitter release from presynaptic nerve terminals in central and peripheral nervous systems (2–8). However, the mechanism underlying this inhibition still remains unclear. Since Ca^{2+} is known to be essential for exocytosis, it is possible that EK inhibits transmitter release by blocking voltage-gated Ca^{2+} influx at the nerve terminals. In support of this notion, there have been reports that opioid peptides reduce the duration and amplitude of Ca^{2+} action potential in dorsal root ganglion neurons (5, 9, 10). Similarly, somatostatin is known to inhibit release of pituitary growth hormone and pancreatic endocrine hormones (11, 12). Since secretory cells such as pituitary cells have voltage-gated Ca^{2+} channels as well as Na^+ channels (13), it is possible that this peptide exerts its inhibitory effect through voltage-gated Ca^{2+} channels.

Diversity of Ca^{2+} channels in excitable membranes is well documented (13). Studies with various excitable cells using patch clamp techniques (11–28) have confirmed the notion proposed by Llinás and Yarom (29) and Fishman and Spector (30) that there is more than one type of Ca^{2+} channel in a cell: one type of Ca^{2+} channel can be activated by small depolarizations and inactivated (referred to as type 1 in this paper), whereas the other type can be activated only by large depolarizations and shows little or no inactivation (referred to as type 2). Cells capable of secretion, such as pituitary cells and chromaffin cells, have been found to generate long-lasting Ca^{2+} currents (16, 31, 32), suggesting that this type of Ca^{2+} channel is involved in the release of transmitters or hormones.

The properties of the inactivating and noninactivating types of Ca^{2+} channels in neuroblastoma N1E-115 cells (20, 26, 27, 30, 33) and neuroblastoma–glioma hybrid NG108-15 cells (30) have been characterized electrophysiologically and pharmacologically. Both N1E-115 and NG108-15 cells have specific opiate receptors (34–36). NG108-15 cells contain only δ subtype of opiate receptors (35), and their properties have been biochemically well characterized (34, 37, 38). NG108-15 cells have specific binding sites for somatostatin (39). Thus, NG108-15 cells provide an excellent opportunity to examine the effects of EK and somatostatin on the two types of Ca^{2+} channels. We report here that EK selectively blocks the noninactivating type of Ca^{2+} channels in a manner antagonized by naloxone. Somatostatin has also been found to block the noninactivating Ca^{2+} channels selectively. Similar results were obtained with N1E-115 cells (40).

METHODS

Neuroblastoma–glioma hybrid cells (NG108-15) were cultured for 5–10 days in the presence of 1 mM dibutyryladenosine 3′,5′-cyclic monophosphate to facilitate development of Ca^{2+} channels and chemical sensitivity (41). Ionic currents through Ca^{2+} channels were recorded from the cells using the whole-cell variation of the patch clamp technique (42). To improve space-clamp conditions, cells with processes exceeding the cell diameter were not used. Compensation for the series resistance was carried out by adding a part of the output voltage of the membrane current recording to the command pulse. Unless otherwise stated, leakage currents were subtracted from the membrane currents assuming a linear current–voltage relationship. Further details of electrical recordings were described (21). The external and internal (or pipette-filling) solutions were designed to separate Ca^{2+} channel currents from other potential-dependent or Ca^{2+}-activated currents (38). Ba^{2+} (50 mM) was used as a charge carrier through Ca^{2+} channels. The external solution contained 50 mM $BaCl_2$, 30 mM NaCl, 5 mM CsCl, 25 mM glucose, 25 mM tetraethylammonium chloride, 0.5 μM tetrodotoxin, and 5 mM Hepes with pH adjusted to 7.3–7.4 with CsOH (calculated total concentration = 302.5 mM). The internal solution contained 130 mM cesium glutamate, 2.5 mM $MgCl_2$, 5 mM glucose, and 10 mM Pipes with pH adjusted to 7.0–7.1 with NaOH (calculated total concentration = 297.5 mM). The experiments were performed at room temperature (21–23°C).

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Abbreviations: EK, enkephalin; Leu-EK, leucine-EK.

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RESULTS

Ca^{2+} Channel Currents. Inward Ba^{2+} currents recorded from neuroblastoma–glioma hybrid cells (NG108-15) are shown in Fig. 1*A* (control). As reported (20, 21, 26, 27, 30), step depolarizations from a holding potential of −80 mV to potentials more positive than −50 mV induced transient (type 1) inward currents and further depolarizations to potentials more positive than −20 mV induced additional long-lasting (type 2) inward currents. The current–voltage (*I–V*) relationships at the peak and at the end of a 135-msec pulse (steady-state) are illustrated in Fig. 1 *B* and *C*, respectively (control). Peak *I–V* relationship in control shows a hump around −20 mV and a maximum around +10 mV (Fig. 1*B*). The current around −20 mV represents the maximal amplitude of the type 1 component, whereas the current around +10 mV represents a combination of both type 1 and type 2 components (20, 21, 27). The steady-state currents shown in Fig. 1*C* attain a maximum around +10 mV. Since type 1 currents inactivate completely by the end of a 135-msec depolarizing pulse to the potentials more positive than −20 mV, the current at +10 mV in Fig. 1*C* represents the maximal amplitude of the type 2 current (20, 21, 27). Both types of inward currents were abolished by 0.5 mM Cd^{2+} or La^{3+}. The amplitude of type 2 current was increased by 30% in the presence of 1 mM 8-bromoadenosine 3′,5′-cyclic monophosphate without alteration of type 1 current (data not shown). These electrophysiological and pharmacological properties of the two types of Ca^{2+} channels of NG108-15 cells were similar to those observed with neuroblastoma N1E-115 cells (20, 21, 26, 27).

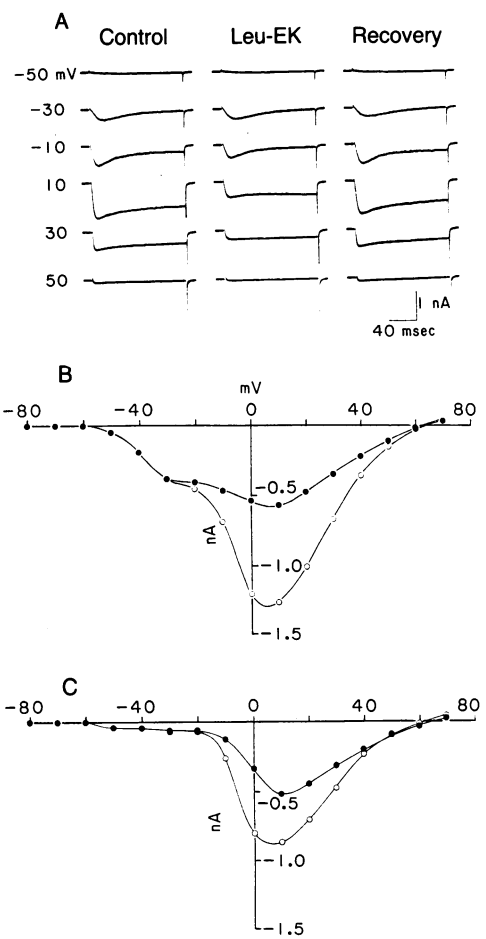

FIG. 1. Reversible block of Ca^{2+} channel currents by 10 nM Leu-EK (*A*) and the current-voltage relationships (*B* and *C*). (*A*) Membrane current records made during 135-msec step depolarizing pulses to various potentials indicated from a holding potential of −80 mV. The linear leak conductance was 0.45 nS. (*B*) Peak current–voltage relationship for the sample records shown in *A*. (*C*) Steady-state current–voltage relationship for the sample records in *A*. The current amplitude was measured at the end of a 135-msec depolarizing pulse. In *B* and *C*, leakage currents were subtracted. Open and filled circles represent current amplitudes in the absence and presence of Leu-EK, respectively.

EK Block. Leu-EK at concentrations of 0.5–500 nM and methionine-EK at concentrations of 50–1000 nM caused a reversible decrease in the amplitude of Ba^{2+} currents. In the presence of 10 nM Leu-EK, the amplitudes of Ba^{2+} currents of both transient and long-lasting components were reversibly reduced (Fig. 1*A*). However, the transient component observed alone at potentials more negative than −20 mV was not altered by Leu-EK (Fig. 1*A*). The absence of the effect is more clearly seen in the *I–V* relationships, in which an EK-induced decrease in the amplitude either at the peak (Fig. 1*B*) or at the steady state (Fig. 1*C*) is apparent only at potentials more positive than −20 mV. There were no obvious reductions in the steady-state currents for potentials more positive than +40 mV (Fig. 1*C*). The results suggest that the type 1 component is not affected by EK. The holding current at −80 mV was not affected by Leu-EK.

[D-Ala^2,D-Leu^5]EK, a δ-opiate receptor agonist, showed a similar effect at concentrations of 50–1000 nM. In one experiment at 200 nM, it reduced the amplitude of type 2 current by 93% when measured at −10 mV. However, the type 1 component was unaffected. By contrast, morphine, a μ-opiate receptor agonist, at 1 μM caused only an 18 ± 2% (mean ± SEM, n = 4) decrease in the amplitude of type 2 current, and a 21 ± 1% decrease in the amplitude of type 1 current.

Naloxone Antagonism. To examine whether the action of EK on Ca^{2+} channels is mediated by activation of opiate receptors, naloxone, a specific opiate receptor antagonist, was used. Leu-EK-induced block in NG108-15 cells was rather resistant to naloxone. Complete antagonism was not observed with naloxone even at a concentration 10 times higher than that of Leu-EK. As shown in Fig. 2, however, 1000 nM naloxone completely antagonized the inhibitory effect of 25 nM Leu-EK. Naloxone alone did not affect the Ba^{2+} current. This suggests that enkephalin acts on specific opiate receptors. The low sensitivity of the receptors to naloxone is characteristic of δ-opiate receptors (35).

Absence of EK Effect on Type 1 Ca^{2+} Channels and K^{2+} Channels. The experiments shown in Fig. 1 suggest that EK has no action on type 1 Ca^{2+} channel. To confirm this point, higher concentration (250 nM) of Leu-EK was applied to cells that showed only the transient component of Ba^{2+} current. As shown in Fig. 3 *A* and *B*, type 1 currents were not affected by Leu-EK in a wide range of the membrane potential from −50 mV to +70 mV.

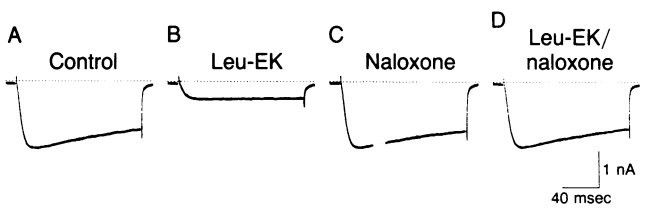

FIG. 2. Naloxone antagonism of Leu-EK-induced block of Ca^{2+} channels. Ca^{2+} channel currents were evoked by a depolarization from a holding potential of −80 mV to +10 mV. (*B*) Record was taken 3 min after exposure to 25 nM Leu-EK. Then, Leu-EK was washed out with a saline solution containing 1 μM naloxone. (*C*) Record was taken 5 min after naloxone treatment. (*D*) Record was taken 3 min after reapplication of 25 nM Leu-EK in the presence of naloxone. Dotted lines represent zero current level.

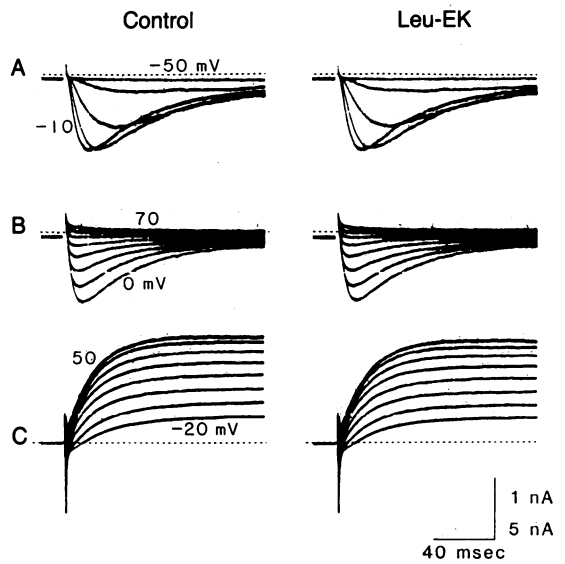

FIG. 3. Effects of Leu-EK on currents through type 1 Ca^{2+} channels, Na^{2+} channels, and K^+ channels. Current records on the *Left* and on the *Right* were obtained before and 3–4 min after exposure to 250 nM Leu-EK, respectively. (*A* and *B*) Families of type 1 Ca^{2+} channel currents in a cell as induced by depolarizing step pulses from a holding potential of −80 mV. (*A*) Steps to levels between −50 and −10 mV in 10 mV increments. (*B*) Steps to levels between 0 and +70 mV in 10 mV increments. The linear leak conductance was 0.38 nS. (*C*) A family of inward Na^{2+} channel currents and outward K^+ channel currents induced by depolarizing pulses from a holding potential of −80 mV to levels between −20 and 50 mV in 10 mV increments. The linear leak conductance was 0.56 nS. Unlike the experiments to measure Ca^{2+} channel current, the cell was bathed in a Ca^{2+}-deficient physiological saline containing no tetrodotoxin or tetraethylammonium. External Ba^{2+} and tetraethylammonium were replaced with Na^+, and Cs^+ was replaced with K^+. Internal Cs^+ was also replaced with K^+. Dotted lines represent zero current level in *A*, *B*, and *C*. Upper current calibration applies to *A* and *B*, and lower current calibration to *C*.

To exclude the possibility that EK causes an increase in the outward current resulting in an apparent decrease in the amplitude of the inward Ba^{2+} current, outward K^+ currents were recorded from cells bathed in a Ca^{2+}-deficient physiological saline solution in which Na^+ was substituted for Ba^{2+} and tetraethylammonium, and K^+ for Cs^+. The suction pipette contained an internal solution in which K^+ was substituted for Cs^+. As shown in Fig. 3*C*, Leu-EK at concentrations of 25–500 nM did not increase the outward current, but rather decreased it slightly. This outward current appears to be carried through voltage-dependent K^+ channels (30). Even in the presence of 1.8 mM Ca^{2+} in the physiological saline solution, Leu-EK did not affect the outward current (data not shown). Neither holding nor leakage current was changed. The transient inward current preceding the outward current was blocked by 0.5 μM tetrodotoxin (data not shown). This Na^+ channel current remained unchanged after exposure to Leu-EK (Fig. 3*C*). These results suggest that enkephalin blocks type 2 Ca^{2+} channels selectively. We cannot completely exclude the possibility that Leu-EK increases a Ca^{2+}-activated K^+ current that is known to be evoked in this preparation (30). However, this is rather unlikely because no effect of Leu-EK is seen in the absence of Ca^{2+} in the external solution.

Block of Type 2 Ca^{2+} Channel. To see the direct effect of Leu-EK on type 2 Ca^{2+} channel current, the type 1 channel was completely inactivated by applying a 10-sec conditioning pulse to −30 mV (20, 27, 40). Leu-EK at 25 nM caused a 73 ± 3% (n = 7) decrease in the amplitude of type 2 current evoked by a step pulse to −10 mV without a marked change in its time course (Fig. 4*A*). However, the current traces obtained with further depolarizations showed a slow increase in amplitude following the initial fast rise (Fig. 4*A*). The second slow phase seemed to be accelerated by increasing the depolarization. The amplitude of the current at the end of a 135-msec pulse approached the control value as the membrane was depolarized to potentials more positive than +30 mV suggesting that the EK-induced block was removed during a maintained depolarization. The tail current upon repolarization was not significantly affected by Leu-EK (Fig. 4*A*).

To examine the time course of recovery from EK block during depolarization, the current amplitude in the presence of 25 nM Leu-EK [$I_{\text{Leu-EK}(t)}$] is plotted in an amount relative to the control amplitude [$I_{\text{control}(t)}$] (Fig. 4*B*). The relative current amplitudes after the onset of the pulses to various potentials maintained a constant value at 0.27–0.28 initially, indicating that the initial reduction was due to the "resting" block during the conditioning pulse to −30 mV. The initial block lasted for a certain period (delay time, t^*) and was followed by an exponential recovery. The delay time was shortened and the time course of recovery was accelerated with an increase in depolarization. The values of t^* were estimated to be 56 ± 8, 19 ± 2, 5.4 ± 0.2, and 2.2 ± 1.0 msec (mean ± SEM, n = 4) for depolarizations to 0, 10, 20, and 30 mV, respectively. The time constant of the recovery decreased with increasing the depolarization; i.e., 131 ± 7, 43 ± 3, and 14 ± 1 msec (mean ± SEM, n = 4) for 20, 30, and 40 mV, respectively.

Whereas the time course of the relative current amplitude for 40 mV showed full recovery by the end of a 135-msec pulse, traces at less-positive potentials did not reach steady-state levels (Fig. 4*B*). The relative current amplitude at the end of a prolonged pulse of 400 msec showed no recovery at −10 mV, whereas it showed full recovery at +30 mV (recovery not illustrated). The potential at which the half recovery was observed at the end of a 400-msec pulse was around +10 mV.

The results indicate that EK blocks type 2 Ca^{2+} channels in a voltage-dependent manner. The slow increase in current amplitude during a depolarizing step in the presence of EK may represent a relaxation process from a resting block to an unblocked state during depolarization.

Dose–Response Relationship. The dose–response relationship was obtained by measuring the values of the resting block of type 2 Ca^{2+} channel current by Leu-EK (Fig. 5). Leu-EK at 0.5 nM caused a noticeable reduction of the current. The data could be fitted by a one-to-one stoichiometric binding curve. The apparent dissociation constant was calculated to be 8.8 nM.

Somatostatin Block. Somatostatin (25–1000 nM) was also found to block the type 2 Ca^{2+} channel current (Fig. 6). The block by somatostatin was similar to that by enkephalin. With a 135-msec depolarizing pulse to 0 mV from a 10-sec conditioning pulse to −30 mV, somatostatin at 50 nM caused a 70 ± 3% (n = 3) decrease in type 2 current amplitude without marked change in its time course. The recovery from the resting block started 17 msec after the beginning of the pulse to +10 mV. With large depolarizations, the relaxation became faster, and the delay in its onset was shortened. The value of the resting block was not voltage dependent. Somatostatin at 50–500 nM did not affect type 1 Ca^{2+} channel current. The outward currents such as shown in Fig. 3*C* were not augmented by somatostatin (25–200 nM) but rather were reduced slightly.

DISCUSSION

The major finding from the present study with neuroblastoma–glioma hybrid cells (NG108-15) is that Leu-EK reversibly

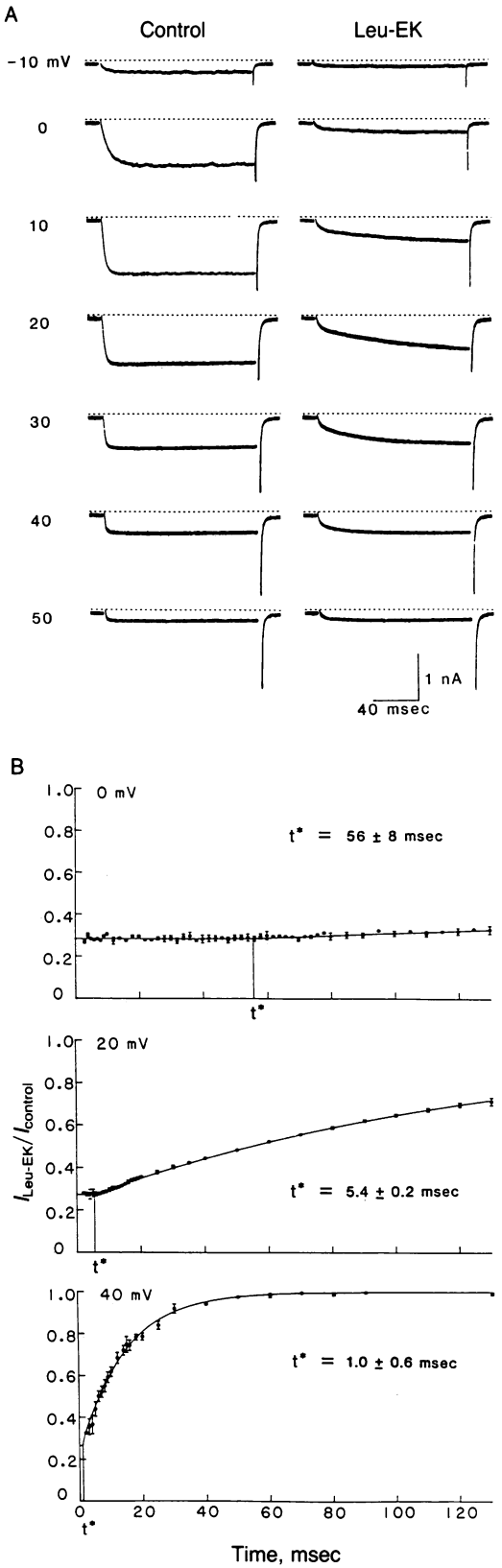

FIG. 4. Effect of Leu-EK on type 2 Ca^{2+} channel currents (*A*) and the time courses of type 2 Ca^{2+} channel currents in the presence of 25 nM Leu-EK (*B*). (*A*) Currents were evoked by step depolarizing pulses as indicated at left following a 10-sec conditioning pulse to −30 mV. Current records on the *Left* and on the *Right* were obtained before and 3–5 min after bath application of 25 nM Leu-EK, respectively. Dotted lines represent zero current level. (*B*) Ratios of the current amplitudes in the presence of Leu-EK to that in control ($I_{Leu\text{-}EK}/I_{control}$) are plotted as a function of time. A depolarizing step pulse was applied following a 10-sec prepulse to −30 mV. The potential during each step depolarizing pulse is indicated at the upper left corner of each graph. The data were pooled from four experiments (filled circles and vertical bars, mean ± SEM). t^* indicates the delay of onset of recovery.

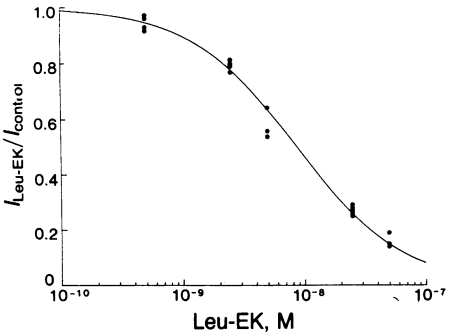

FIG. 5. Dose dependence of resting block of type 2 Ca^{2+} channels by Leu-EK. The currents were evoked by a step pulse to 0 mV following a 10-sec prepulse to −30 mV before and 3–4 min after application of Leu-EK at various concentrations. The ratios of current at the beginning of the pulse in Leu-EK to that in control are plotted ($I_{Leu\text{-}EK}/I_{control}$). The curve was drawn according to a one-to-one stoichiometric binding with an apparent dissociation constant of 8.8 nM.

blocks the type 2 Ca^{2+} channel without affecting the type 1 Ca^{2+} channel. In the presence of Leu-EK, the type 2 Ca^{2+} channel current is restored slowly during a depolarizing step in a manner dependent upon the membrane potential and time. Recovery is more complete and its time course faster with depolarization to more positive potentials. Somatostatin exerts a similar selective blocking action on the type 2 channel. Thus EK and somatostatin block type 2 Ca^{2+} channels in a voltage-dependent manner.

The slow recovery of type 2 Ca^{2+} channel current during a depolarizing step in the EK-treated membrane may represent a time- and voltage-dependent unblocking. Alternatively, it may represent a slowing of the channel activation process. If the latter were the case, however, only the activation process would be altered, because the deactivation process as observed from the time course of tail current upon repolarization was not affected by Leu-EK.

The block of type 2 Ca^{2+} channels by EK is antagonized by naloxone. However, the potency of naloxone is low; 1 μM naloxone is required to completely antagonize the action of 25 nM Leu-EK. In addition, the potency of morphine in blocking the type 2 Ca^{2+} channel is considerably lower than that of EK. These observations suggest that the EK block in the NG108-15 cells is mediated by activation of δ-opiate receptors. In dorsal root ganglion cells, κ-opiate receptors are suggested to mediate the dynorphin block of Ca^{2+} channels (10).

There is evidence to indicate that noninactivating Ca^{2+} channels are involved in excitation–secretion coupling (43). We have suggested that type 2 Ca^{2+} channels in neuroblastoma cells are very similar to those present in nerve terminals (21). Therefore, the EK block of type 2 Ca^{2+} channels may explain the mechanism underlying the presynaptic inhibition of transmitter release observed in endogenous enkephalinergic pathway (6) or caused by EK applied exogenously (2–5). EK has also been reported to modulate synaptic transmission by altering the postsynaptic sensitivity to transmitters or by hyperpolarizing the membrane through activation of K^+ channels (44, 45).

It should be noted that the EK and somatostatin block is maximal at potentials near the threshold for Ca^{2+} action potentials. This would effectively prevent Ca^{2+} spikes from

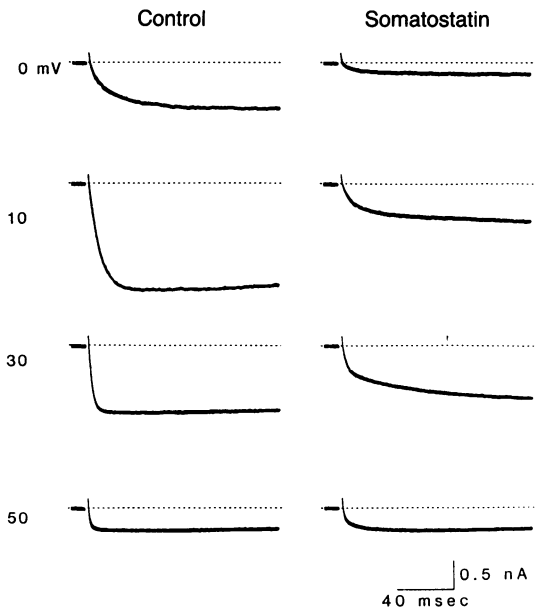

FIG. 6. Effect of somatostatin on type 2 Ca^{2+} currents. The currents were evoked by step depolarizing pulses following a 10-sec prepulse to −30 mV. Current records on the *Left* and *Right* were obtained before and 4–5 min after exposure to 50 nM somatostatin, respectively. Dotted lines represent zero current level.

being evoked regeneratively. The secretory cells so far examined also contain the noninactivating type of Ca^{2+} channels (16, 31, 32, 43). Since this type of Ca^{2+} channel is likely to be involved in hormone release, somatostatin inhibition of release of various hormones such as pituitary growth hormone, pancreatic insulin, and glucagon (12) may be due to its blocking action on the Ca^{2+} channels in secretory cells in a manner similar to that observed in the present study.

A histochemical study has shown that the distribution of opiate receptors is not confined to presynaptic nerve terminals and that membranes of nonsynaptic regions also contain opiate receptors in caudate–putamen (46). Noninactivating Ca^{2+} channels are also distributed in somal and dendritic membranes (15, 19, 21, 29). Therefore, EK may play a role in modulation of the intracellular concentration of Ca^{2+} in nonsynaptic as well as synaptic regions of a neuron.

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