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Thyrotropin-releasing-hormone (TRH) and its physiological metabolite TRH-OH inhibit Na^+ channel activity in mammalian septal neurons

(septum)

J. LÓPEZ-BARNEO, A. CASTELLANO, AND J. TOLEDO-ARAL

Departamento de Fisiología y Biofísica, Facultad de Medicina, Avenida Sánchez Pizjuán, 4, 41009 Sevilla, Spain

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ABSTRACT **The interaction of thyrotropin-releasing hormone (TRH) and its physiological metabolite TRH-OH with Na^+ channels was studied in enzymatically dissociated guinea pig septal neurons by using the whole-cell variant of the patch-clamp technique. In about 60% of the cells tested, the neuropeptides at concentrations between 0.01 and 2.5 μM produced a dose-dependent reversible attenuation of Na^+ currents. With 2 μM TRH-OH, peak Na^+ current amplitude was reduced by 20–50% (27 ± 8%, mean ± SD; n = 16), whereas at the same concentration TRH was approximately half as effective as TRH-OH. In the presence of the tripeptides, the voltage-dependent parameters of the Na^+ current were unaltered. TRH-induced reduction of Na^+ current amplitude was transient and recovered almost completely during maintained exposure to the peptides. In addition, the response to either TRH-OH or TRH decreased with repeated treatment. Our results demonstrate that neuronal Na^+ channels can be modulated by naturally occurring neuropeptides.**

It is well established that voltage-gated neuronal Ca^{2+} and K^+ channels can be modulated by transmitters and neuropeptides (for reviews, see refs. 1 and 2), but it is generally believed that Na^+ channels are unaffected by physiological metabolites and ligands. A common view is that Ca^{2+} and K^+ channels are directly implicated in the subtle mechanisms underlying neuronal integration and responses to changes in the cell environment, whereas Na^+ channels are specialized in the generation of the fast transmembrane current required for electrical signaling and thus are less susceptible to modulation (see, however, ref. 3). In this report we show unmistakable evidence indicating that thyrotropin-releasing hormone (TRH; pyroGlu-His-Pro-NH_2) and, with more potency, its physiological derivative TRH-OH (pyroGlu-His-Pro-OH) reversibly inhibit Na^+ channel activity in freshly dissociated guinea pig septal neurons. It has been claimed that TRH, besides its role in pituitary control, has numerous functions in the mammalian central nervous system (4, 5). The hormone and its metabolites are broadly distributed in the brain, and the septal region is particularly rich in TRH-like immunoreactivity and contains saturable TRH receptors (5–7). Extrahypothalamic TRH is very unstable and after being released by nerve terminals, is rapidly converted into new products. Therefore, TRH is considered to be a "prohormone" or "precursor" of peptides with their own biological activity (8, 9). TRH-OH, produced after cleavage of the C-terminal amide group by a specific proline endopeptidase, is one of the most stable derivatives of TRH and is found at high levels in numerous brain regions (8, 9). It was known that TRH has several neuropharmacological effects and that it can modify the electrical activity of brain cells (10–12), but the modulatory action of Na^+ channels of both the hormone and the metabolite was totally unexpected. Thus, our findings discover a novel aspect in the physiology of Na^+ channels and in addition provide significant clues regarding the action at the cellular level of brain TRH and its biotransformation products.

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METHODS

Septal cells were obtained from slices 300–400 μm thick of the guinea pig brain. The procedure followed for slicing was the same as described (13). For cell dissociation, six slices of the septal nucleus, isolated from the rest of the brain with fine scissors, were placed in a vial containing 3 ml of Earle solution (Flow Laboratories) with 0.2 mg of trypsin type IX and 0.05 mg of DNase type II (Sigma) per ml. This solution was continuously bubbled with 95% O_2/5% CO_2 and incubated for 20 min at 37°C. The tissue was washed twice with solution containing 1 mg of albumin per ml to remove the enzymes. Cells were mechanically dispersed with fire-polished Pasteur pipettes of various sizes, centrifuged, and resuspended in minimal Essential Medium (Flow Laboratories) supplemented with 1% glutamine, 10% (vol/vol) fetal bovine serum, and antibiotics. Cells were plated on fragments of glass coverslips treated with poly(L-lysine) and maintained in a CO_2 incubator until use (2–10 hr after plating). At this stage the cells had an almost spherical shape and no appreciable processes under the light microscope.

During the experiments, a coverslip was transferred to a chamber of 0.2 ml with continuous flow of solution that could be changed in ≈15 s. Ionic currents were recorded in the "whole-cell" configuration of the patch-clamp technique (14). The general procedures and the setup used were the same as described (15). We used a patch-clamp amplifier built by following the design by Sigworth (16) with the modifications described by Matteson and Armstrong (17). A fast and uniform control of the membrane potential was favored by the use of low-resistance electrodes (0.8–2 MΩ), a feedback resistance of only 100 MΩ in the current-to-voltage converter, and electronic compensation of series resistance. The amplifier was interfaced to an IBM-PC/AT computer, which was used for generation of command voltage pulses and for acquisition, display, and analysis of the data (18). Linear ionic and capacity currents were subtracted by using the P/2 procedure (19). The composition of the recording solutions is indicated in the figure legends. High-purity peptides were obtained from Sigma, dissolved in distilled water, and kept at −20°C. Fresh aliquots of neuropeptides were used in each experiment. Experiments were done at room temperature (20–25°C).

Abbreviations: TRH, thyrotropin-releasing hormone; TTX, tetrodotoxin.

RESULTS

TRH-OH and TRH Reversibly Reduce Na^+ Channel Activity. Freshly dissociated septal neurons subjected to voltage clamp can generate voltage-dependent Na^+, Ca^{2+}, and K^+ currents. The characteristics of these currents are described elsewhere (20). Fig. 1 summarizes the effect of TRH-OH on the Na^+ and K^+ currents in a neuron dialyzed with high K^+ and bathed in the standard 130 mM Na^+ solution. Inward currents with a time course typical of Na^+ currents could be recorded during voltage steps to −20 (Fig. 1*A*) or 0 (Fig. 1*B*) mV (control traces). With a larger depolarization (Fig. 1*C*), the inward current was followed by a slower outward K^+ current. Exposure of the cell to a test external solution of the same ionic composition but with 2.5 μM TRH-OH added produced a decrease in the amplitude of the Na^+ current by about 50%, leaving the outward current unaltered (TRH-OH trace). The effect of the tripeptide was almost perfectly reversible as illustrated by the recovery traces. Small differences seen between control and recovery currents in some experiments are likely because of a 5- to 10-mV shift toward negative potentials in the current–voltage curve of Na^+ channels in dialyzed cells (21). Peak inward and outward current amplitude as a function of the pulse membrane potential is plotted in Fig. 1*D* before and during the application of TRH-OH. Thus, in septal neurons TRH-OH can attenuate Na^+ current without affecting the K^+ current. Similar qualitative effects were observed with TRH. In all TRH-sensitive neurons (n = 71), the inhibition of the Na^+ current appeared in 20–30 s after introduction of either TRH-OH or TRH, and the recovery was complete in about 60 s after switching to the control solution. Ca^{2+} channels, although not studied in detail, seemed to be unresponsive to the tripeptides. There are, however, septal cells where the K^+ currents were also modified by both TRH and TRH-OH (20). The present report focuses on the modulation of Na^+ channels.

The interaction of the neuropeptides with Na^+ channels was further studied in neurons where the internal solution (inside the pipette and the cell) contained Cs^+ instead of K^+ to block K^+ channels. Fig. 2*A* shows recordings of isolated TRH-OH Na^+ currents during depolarizations to 0 mV. In this experiment 2 μM TRH-OH diminished the peak current amplitude by about 30%. After TRH-OH wash-out, the addition of 1 μM tetrodotoxin (TTX) to the same cell abolished the inward current, which indicates that in this respect Na^+ channels exposed to the tripeptide behaved normally. Inhibition of Na^+ current by the peptides is dose dependent and, in Fig. 2 *B* and *C*, the effect of lower concentrations of TRH-OH (0.1 μM in *B* and 0.01 μM in *C*) is shown for comparison. Na^+ current amplitude was reduced with complete reversibility by 20% and 10%, respectively. We have tested so far the effect of TRH, TRH-OH, or both (at concentrations between 0.01 and 2.5 μM) in more than 100 septal neurons, and in about 60% of them, a dose-dependent attenuation of the Na^+ current between 10% and 50% was observed. At a concentration of 2 μM, TRH-OH decreased current amplitude by 27 ± 8% (mean ± SD; n = 16). With 0.1 and 0.01 μM TRH-OH, the average reduction in the current

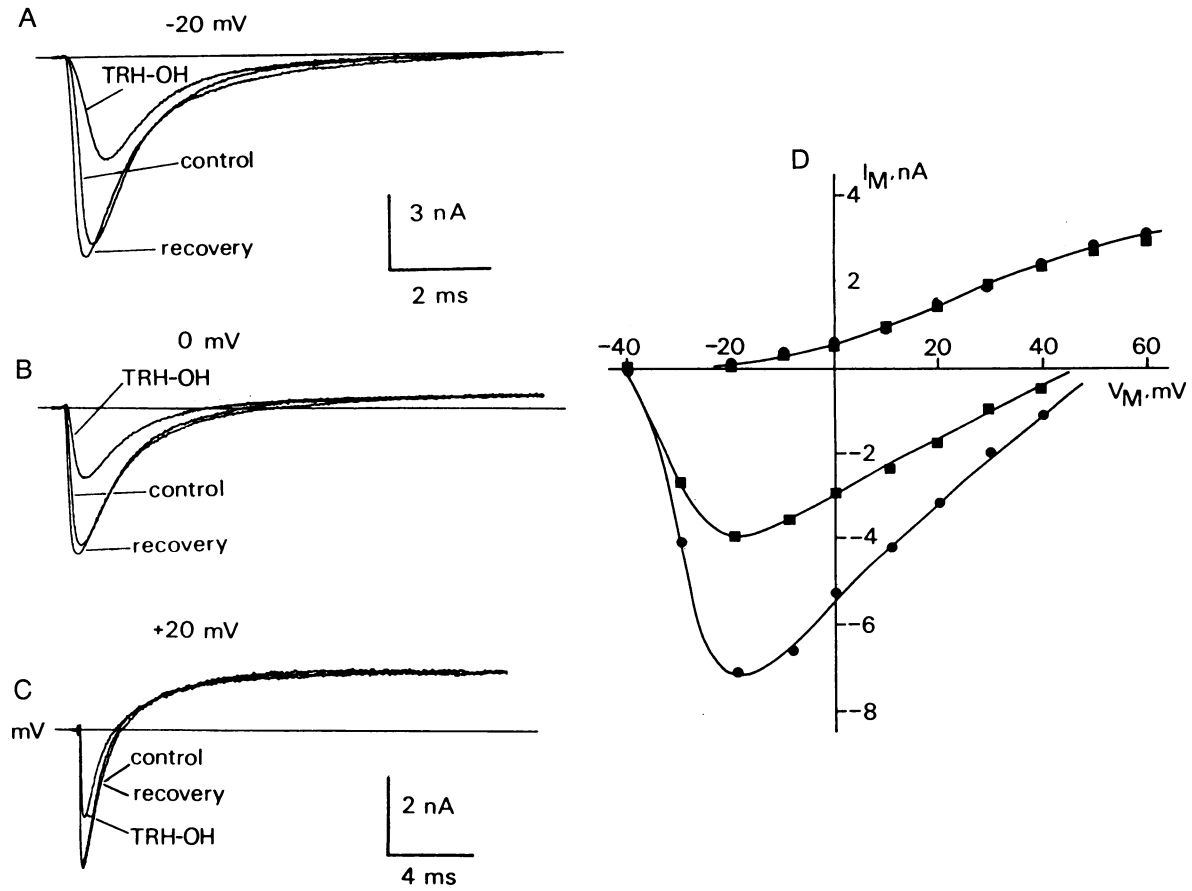

FIG. 1. Effect of TRH-OH on the Na^+ and K^+ currents of septal neurons. (*A–C*) Currents recorded during 9-ms (*A* and *B*) and 20-ms (*C*) voltage steps to the indicated membrane potentials from a holding potential of −80 mV. Recordings were obtained with (TRH-OH trace) and without (control and recovery traces) 2.5 μM TRH-OH added to the external solution. (*D*) Current–voltage relations of peak outward and inward currents measured in the absence (●) and presence (■) of TRH-OH. The external solution was 130 mM NaCl/2.7 mM KCl/10 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes, pH 7.4. The internal solution was 80 mM potassium glutamate/20 mM KF/30 mM KCl/2 mM $MgCl_2$/3 mM Mg-ATP/10 mM EGTA/10 mM Hepes, pH 7.3.

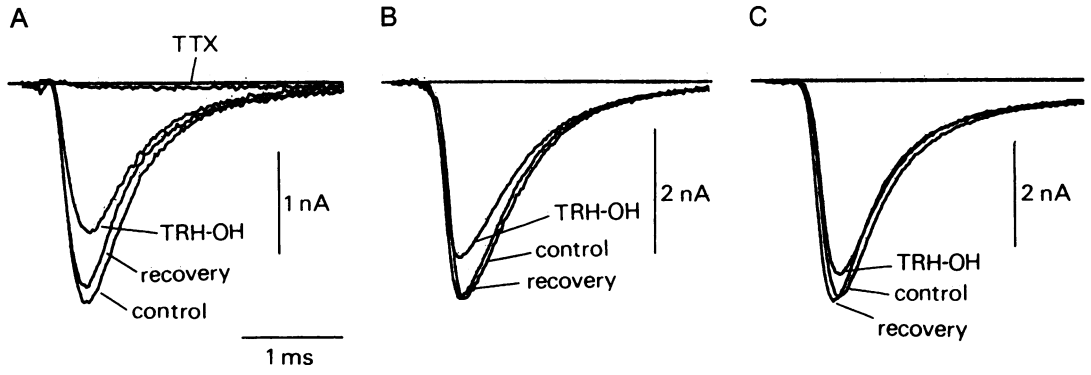

FIG. 2. Dose-dependent inhibition of isolated Na^+ currents by TRH-OH. Recordings were obtained in three different cells before (control trace), during (TRH-OH trace), and after (recovery trace) exposure to 2 μM (*A*), 0.1 μM (*B*), and 0.01 μM (*C*) TRH-OH. In *A*, after wash-out of TRH-OH, the current was completely blocked by 1 μM TTX. Note that in *C* there is a slow Ca^{2+} component in the inward current that is not affected by the tripeptide. The holding potential was −80 mV, and the test potentials were 0 mV (*A*), −20 mV (*B*), and −10 mV (*C*). Pulse duration was 7 ms. The external solution was 130 mM NaCl/2.7 mM KCl/2.5 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes. The internal solution was 70 mM CsCl/20 mM CsF/40 mM NaCl/4 mM Mg-ATP/5 mM Hepes/5 mM EGTA.

amplitude was 16% (n = 5) and 9% (n = 10) respectively. At the same concentrations, TRH was almost half as effective as TRH-OH.

These results indicate that TTX-sensitive Na^+ currents with normal activation and inactivation kinetics are inhibited by TRH-OH and, less effectively, by TRH. However, the effect is specific to some cells, which suggests that the tripeptides do not directly block the channels but that a different mechanism, perhaps involving a receptor and some intracellular mediator, is required.

TRH-OH Does Not Alter the Voltage-Dependent Parameters of Na^+ Currents. Although Fig. 1*D* already shows that the Na^+ current–voltage relation was not displaced by TRH-OH, we further examined the possibility that this peptide could shift the voltage-dependent parameters of Na^+ currents. This is also of importance because it has been recently shown that

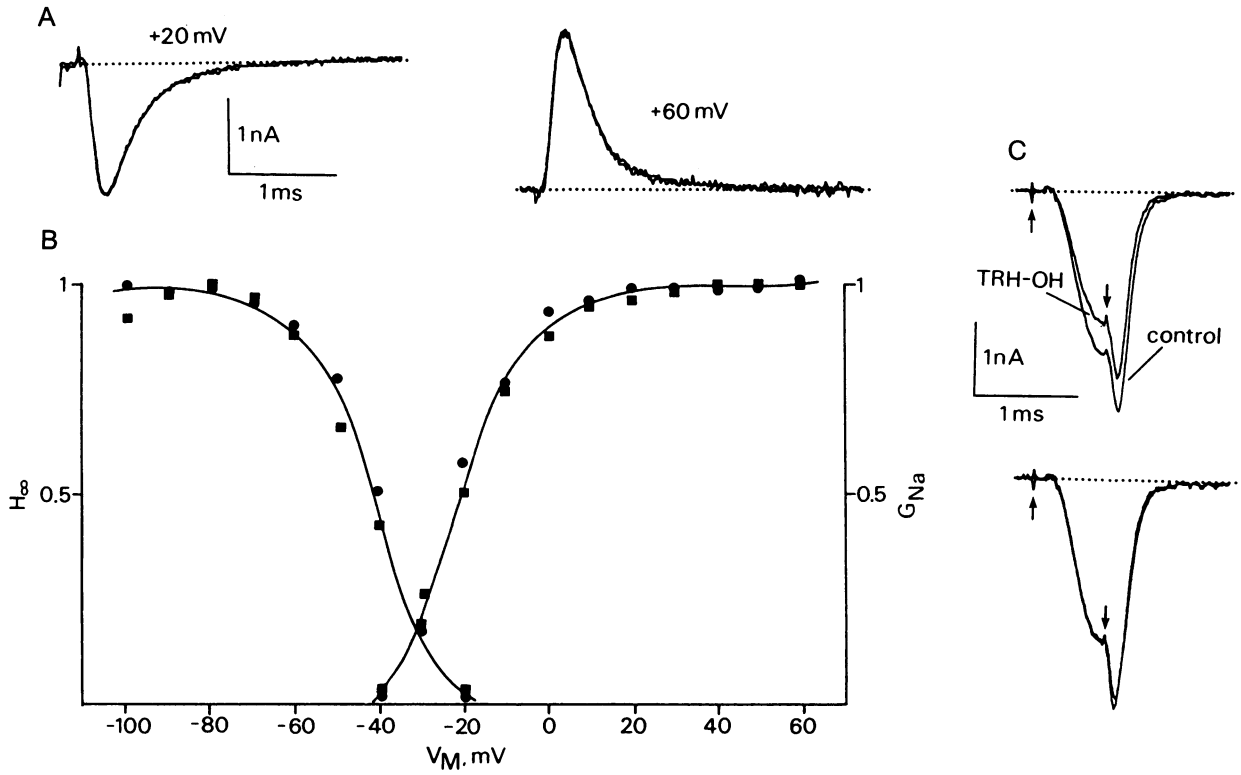

FIG. 3. Effect of TRH-OH on the voltage-dependent parameters of Na^+ currents. (*A*) Superimposed sweeps of Na^+ currents elicited by depolarization to the indicated membrane potentials with and without 2 μM TRH-OH added to the external solution. Current sweeps recorded during exposure to the peptide are scaled by a factor of 1.3. (*B*) Voltage dependence of steady-state inactivation (H_∞) and of the peak Na^+ conductance (G_{Na}) in the control solution (●) and in the presence of 2 μM TRH-OH (■). The continuous lines were fitted by eye. H_∞ was determined by applying 50-ms depolarizing prepulses of variable amplitude (abscissa) followed by a pulse to 0 mV. The normalized peak current amplitude during the pulse is represented on the ordinate. Normalized G_{Na} (ordinate) was calculated by the formula $G_{Na} = I_{max}/(V_M - E_{Na})$. I_{max} is the peak current elicited by steps to different membrane potentials (V_M, abscissa). E_{Na} was +32 mV. Solutions for *A* and *B* are as in Fig. 2. (*C Upper*) Na^+ currents recorded during 700-μs steps to 0 mV from −80 mV in the control solution and during application of 2 μM TRH-OH. Step duration is indicated by the arrows. (*C Lower*) Superposition of the two traces after scaling the TRH-OH sweep by a factor of 1.24. The external solution was 80 mM Na/70 mM Trizma, pH 7.4/2.7 mM KCl/2.5 mM $CaCl_2$/1 mM $MgCl_2$. The internal solution was 100 mM CsCl/30 mM CsF/10 mM Hepes/5 mM EGTA/4 mM Mg-ATP.

isoproterenol (although only partially reversible) shifts steady-state inactivation of cardiac Na^+ channels toward negative voltages (3). Fig. 3 summarizes the effect of TRH-OH on activation, inactivation, and closing of septal Na^+ channels. In Fig. 3*A*, inward and outward Na^+ currents recorded at the indicated membrane potentials with and without external addition of TRH-OH are shown. The traces in TRH-OH are scaled by a factor of 1.3 to match the amplitude of the control current. The two superimposed current sweeps, recorded with a time interval of ≈80 s, are undistinguishable, demonstrating that although TRH-OH reduced current amplitude by about 30%, activation and inactivation time courses were unchanged. In this experiment we used an internal solution with 40 mM Na^+ to obtain a well-defined reversal potential (E_{Na}); before and during application of TRH-OH, E_{Na} was +32 mV. Fig. 3*B* shows the voltage-dependence of steady-state inactivation (H ∞) and of the peak Na^+ conductance (G_{Na}) with values measured in the absence and presence of TRH-OH. The two sets of normalized values are superimposable, indicating that these parameters were also unaltered by TRH-OH. The effect of the peptide on Na^+ deactivation is shown in Fig. 3*C*. The top traces are Na^+ currents elicited by a brief voltage step to 0 mV, indicated by the arrows, before and during exposure to TRH-OH. Repolarization occurs when the pulse current is maximal and is followed by tail currents that reflect the progressive closure of the channels open during the pulse. At the bottom it is clearly illustrated that scaling the TRH-OH sweep by a factor of 1.24 perfectly overlaps the control current.

These results indicate that TRH-OH does not shift the voltage-dependence of Na^+ channels and that those that remain functional during exposure to the neuropeptide behave normally. Thus, the reduction of the Na^+ current amplitude is most likely due to a decrease of either single-channel conductance or the open probability of the channels.

Maintained Exposure to TRH Produces a Transient Attenuation of Na^+ Current Amplitude. Although in most experiments cells were bathed by the test solution (with TRH or TRH-OH) for only 30–60 s, we studied in a few neurons the effect of a more permanent exposure to the tripeptides. Fig. 4*A* is a plot of the normalized peak Na^+ current amplitude (ordinate) versus the time after obtaining the whole-cell configuration. The cell was repeatedly exposed to TRH-OH, and switching to a new solution is indicated by vertical arrows. The four sweeps drawn in Fig. 4*B* are indicated by their respective number. TRH-OH produced in 30–40 s a maximal decrease of Na^+ current amplitude of 23% (sweep 2). However, this effect was transient, and in the course of the following 2 min, current amplitude increased to almost 90% of the initial value. Current amplitude completely recovered after reintroduction of the control solution (compare sweeps 1 and 3). A second exposure to the peptide produced a maximal decrease in the current amplitude of only 10%, which was also transient. Similar qualitative results were seen either with TRH or TRH-OH at concentrations as low as 0.1 μM in all neurons studied with this experimental protocol (n = 9). These observations reveal that the effect of the tripeptides on Na^+ channels is transient and that there is a loss of responsiveness during repeated exposure to TRH or TRH-OH.

DISCUSSION

In this report there are two major findings: (*i*) Na^+ channels can be modulated by ligands normally occurring in the mammalian brain, and (*ii*) extrahypothalamic TRH and its physiological derivative TRH-OH modify membrane ionic permeability in central nervous system neurons with com-

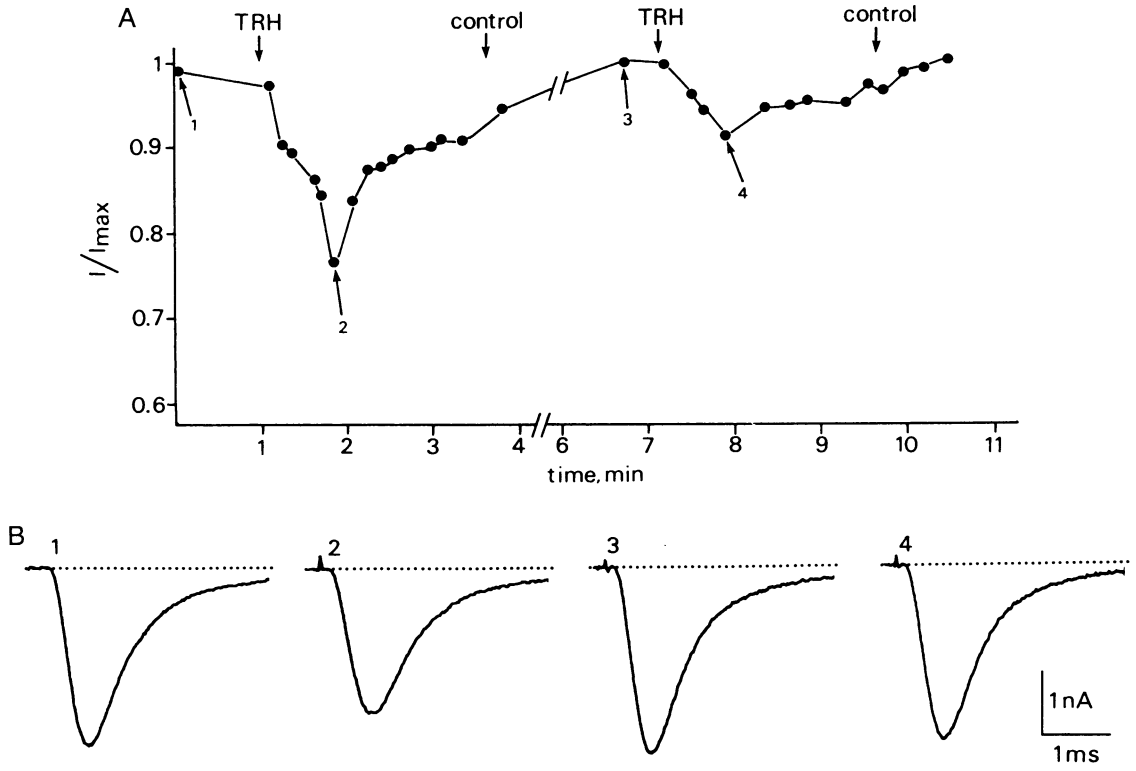

FIG. 4. Effect of maintained and repeated exposure to 2.5 μM TRH-OH. (*A*) Normalized peak Na^+ current amplitude (ordinate) recorded during 7-ms steps to 0 mV from −80 mV at different times in min after the onset of the whole-cell recording mode. Measurements are represented by dots, and the vertical arrows indicate the switching to a new solution. In *B* there are four selected current sweeps identified by their respective number in *A*. Solutions were as in Fig. 3*C*.

plete reversibility. There are previous data indicating that in the μM range TRH can produce enhancement or depression of neuronal excitability (11, 22–24). TRH-induced depolarization is compatible with inhibition of voltage-dependent K^+ channels, an effect demonstrated in clonal adenohypophyseal cells (25) and also observed by us in preliminary experiments (20). On the contrary, the depression of neuronal excitability could be explained by the attenuation of the Na^+ current reported here. Inhibition of Na^+ channel activity is expected to produce an increase in the firing threshold and a decrease in the rate of rise of action potentials. On physiological grounds it can be speculated that these effects would favor discrimination of large synaptic inputs and a more maintained Ca^{2+} entry during longer-lasting action potentials.

The effect of TRH and TRH-OH was selective to some cells, and they did not seem to directly block the channels. This implies that the target neurons must specifically express, with the appropriate distribution, receptors for the tripeptides and the deamidating enzyme that synthetizes TRH-OH. The concentrations of TRH and TRH-OH that attenuate Na^+ currents are within the range of the dissociation constant of low- and high-affinity receptors for TRH-like peptides described in neuronal bodies of the septum and other parts of the brain (7, 8). The regional distribution of proline endopeptidase is not well studied, but high enzymatic activity is found in the soluble fraction of homogenated cerebral tissue (8, 9). Interestingly, the effect of TRH and TRH-OH was transient, and their potency decreased with repeated treatment. In pituitary cells subjected to voltage clamp it is known that TRH induces a transient release of Ca^{2+} from internal stores (26) with a time course similar to the recovery of Na^+ current amplitude reported here during maintained exposure to the neuropeptides. TRH-induced Ca^{2+} mobilization also decreases with repetitive exposure to the stimulus, presumably because intracellular mediators are being exhausted (26). Inhibition of Na^+ channels by TRH may also involve an intracellular mediator that could induce phosphorylation of the channel protein. Several phosphorylation sites, missing in the electroplax Na^+ channel, are known to exist in the α subunit of neuronal Na^+ channels (27). In conclusion, TRH and its biologically active metabolite TRH-OH regulate Na^+ channels in septal neurons; the effect of the peptides is selective and reversible. This phenomenon, which confers a new perspective upon the physiology of Na^+ channels, may be also caused by other neuropeptides and may occur in other central neurons as well. The molecular mechanism responsible for modulation is still unknown and could be addressed in future experimental work.

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