The Journal of Neuroscience, July 1995, 15(7): 4982–4991

Three Phases of TRH-Induced Facilitation of Exocytosis by Single Lactotrophs

Alla F. Fomina[a] and Edwin S. Levitan

Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Membrane capacitance measurements were used to study neuropeptide modulation of exocytosis by perforated patch clamped rat lactotrophs. We report that depolarizing voltage-clamp pulses evoke exocytosis that is steeply dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels. Furthermore, we find that the neuropeptide TRH (thyrotropin-releasing hormone) acts in three phases to promote exocytosis. First, TRH transiently (within ~0.5 min) triggers depolarization- and extracellular Ca^{2+}-independent exocytosis. Second, within 3 min of application, TRH facilitates depolarization-evoked exocytosis while inhibiting voltage-gated Ca^{2+} current. Finally, after 8 min, TRH further enhances depolarization-evoked exocytosis by increasing high-voltage-activated (HVA) Ca^{2+} channel current. Activation of protein kinase C (PKC) with a phorbol ester also stimulates depolarization-evoked exocytosis by increasing Ca^{2+} current. Therefore, PKC can only account for the last effect of TRH. Thus, a single neuromodulator may employ several temporally distinct mechanisms to stimulate peptide secretion.

[Key words: exocytosis, calcium channel, neuropeptide, prolactin, protein kinase C, pituitary]

Many neuromodulators regulate peptide secretion by neurons and endocrine cells. Because the effects of secreted peptides are typically slow, it has not been possible to study neuromodulation of peptidergic transmission in the nervous system with quantal analysis. Rather, secretion of neuropeptides and peptide hormones has usually been assayed biochemically, with the use of the reverse hemolytic plaque assay, or has been inferred from slow synaptic responses or other bioassays. The limited time resolution of these methods, however, has hindered direct linkage of neuromodulator effects on production of second messengers, membrane potential and Ca^{2+} channel activity to changes in peptide secretion. Recently, recordings of membrane capacitance changes have allowed rapid and high resolution measurements of exocytotic activity from a variety of peptidergic cells including melanotrophs (Thomas et al., 1990; Okano et al., 1993), pancreatic β cells (Ammala et al., 1993a,b), gonadotrophs (Tse et al., 1993), neurohypophysis terminals (Lim et al., 1990), and lactotrophs (Sikdar et al., 1989, 1990; Zorec et al., 1991). Those studies have examined the influence of intracellular Ca^{2+}, G-proteins, and cAMP on exocytotic activity. In most cases, conventional whole-cell recording was used which, by dialyzing the cytoplasm, can alter normal control of exocytosis (Augustine and Neher, 1992; Ammala et al., 1993b). Furthermore, few experiments have examined the effects of neuromodulators on exocytosis. Therefore, the full complexity of control of peptide secretion by neuromodulators may have not been revealed by previous studies.

We chose to study the action of the neuropeptide thyrotropin-releasing hormone (TRH) on metabolically intact peptidergic cells. TRH and its receptor are widely distributed throughout the CNS (Faglia and Persani, 1991; Wu et al., 1992; Satoh et al., 1993). This neuropeptide affects behavior, augments long-term potentiation, and stimulates catecholamine secretion in the brain (Faglia and Persani, 1991; Ishihara et al., 1991, 1992). In addition, TRH secreted by hypothalamic neurons promotes secretion of the peptide hormone prolactin by pituitary lactotrophs, which in turn affects the reproductive and immune systems. Work with clonal pituitary cells suggests that TRH-induced prolactin secretion occurs in two phases. First, TRH initially evokes a transient burst of secretion that is associated with release of intracellular Ca^{2+}. Then TRH evokes a second sustained phase of secretion that requires Ca^{2+} influx through voltage-gated Ca^{2+} channels and is associated with increased action potential activity and activation of protein kinase C (PKC) (Mason et al., 1988, 1989; Gershengorn, 1989; Iijima et al., 1990). PKC activators also stimulate prolactin secretion from normal lactotrophs (Negro-Vilar and Lapetina, 1985) via an unknown mechanism. However, in normal pituitary cells, Ca^{2+} influx through voltage-gated Ca^{2+} channels plays a major role in both phases of TRH-induced prolactin secretion (Sato et al., 1992). To further explore the role of voltage-gated Ca^{2+} channels in TRH-induced secretion, we have used the perforated patch technique, which leaves cells metabolically intact, to examine the action of TRH on lactotroph voltage-gated Ca^{2+} currents and exocytotic activity.

Here we report that exocytosis measured as an increase in membrane capacitance is steeply dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels. Furthermore, we show that TRH acts in three phases to promote exocytosis by voltage-clamped rat lactotrophs. Within the first minute of application, the neuropeptide transiently evokes exocytosis that is independent of Ca^{2+} influx and membrane depolarization. During the first few minutes, TRH also facilitates depolarization-evoked exocytosis while inhibiting Ca^{2+} influx through voltage-gated Ca^{2+} channels. Finally, after ~8 min, TRH further stimulates

Received Dec. 16, 1994; revised Feb. 8, 1995; accepted Feb. 10, 1995.

We thank Drs. J. P. Horn and W. C. DeGroat for reading an earlier version of the manuscript. Antisera were provided by the National Hormone and Pituitary Program (University of Maryland School of Medicine) and NIDDK. This research was supported by a Klingenstein Fellowship in the Neurosciences, an American Heart Association (PA affiliate) Grant-in-aid, and NIH Grant NS29804 to E.S.L.

Correspondence should be addressed to Edwin S. Levitan at the above address.

[a]Permanent address: Bogomoletz Institute of Physiology, Kiev, Ukraine.

depolarization-evoked exocytosis by increasing voltage-gated Ca^{2+} channel activity. Only this latter effect appears to be mediated by activation of protein kinase C. Thus, a single neuromodulator can act via several temporally distinct mechanisms to promote peptide secretion.

Materials and Methods

Cell preparation and identification. Anterior pituitary glands were dissected from retired breeder female Sprague–Dawley rats. Cells were dissociated by incubations at 37°C first in a Mg-Ca-free buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 15 mM glucose, pH 7.4) containing 10 mg/ml bovine serum albumin (BSA, fraction V, Sigma) with 1 mg/ml trypsin (type III, Sigma) for 30 min, and then in Mg-Ca-supplemented buffer (the same buffer above supplemented with 1 mM $MgSO_4$ and 1.8 mM $CaCl_2$) with 1 mg/ml Viokase (Pancreatin, Sigma) for 90 min. Cells were then triturated using Pasteur pipettes. The cell suspension harvested from one rat was centrifuged through 4 ml layer of Mg-Ca-containing buffer supplemented with 200 mg/ml BSA at 500 × *g* for 5 min. The pellet was resuspended in Ham's F-10 culture medium supplemented with 2.5% fetal bovine serum and 12.5% horse serum (GIBCO) to 1–2 × 10^4 cells/ml; 2 ml of the cell suspension were placed into 35 mm plastic dishes with fire-flamed glass gridded coverslip (Eppendorf) on the bottom and cultured for up to 7 d in a 5% CO_2 incubator at 37°C. By immunofluorescence (see below), 50–80% cells isolated by this method were identified as immunoreactive for prolactin.

Immunocytochemical labeling. For identification of the PRL-containing cells we followed a procedure described previously (St. John et al., 1986). Briefly, for immunofluorescent surface labeling of living cells, the culture media was removed and washed once with phosphate buffer (PBS, pH 7.4) and then kept for 1 hr in buffer solution B (140 mM NaCl, 5 mM KCl, 20 mM NaHEPES, 4 mg/ml BSA, pH 7.4) with diluted (1:1000) primary anti-rat prolactin antibody (anti-RPRL-IC-4, rabbit, NIDDK-NIH) at room temperature. The cells were rinsed three times with buffer solution A, then incubated at room temperature in fluorescein-conjugated secondary antibody (anti-rabbit IgG FITC-conjugate, Sigma) used at final concentration 1:200 for 60 min. After this incubation, cells were rinsed and the coverslip was transferred into experimental chamber on an Olympus IMT-2 fluorescent microscope.

To assay intracellular hormone content after experiments, the recording solution was replaced with fixative [4% (w/v) paraformaldehyde in PBS, pH 7.4] and incubated for 30 min at 4°C. After fixation, cells were rinsed with PBS, then permeabilized by treatment with Triton X-100 (0.25% in buffer solution B) for 5 min and rinsed again. Labeling was performed with 1:1000 dilutions of primary antibodies against rat PRL and growth hormone (NIDDK-anti-rGH-IC-1, #AFP411S, monkey, NIDDK-NIH). Primary antibodies were applied for 1 hr at 4°C. Cells were rinsed three times with buffer solution and then incubated for 60 min in fluorescein anti-monkey IgG FITC-conjugate (Sigma) and then rhodamine-conjugated anti-rabbit IgG TRITC-conjugate (Sigma) secondary antibodies used at final dilutions of 1:1000. Cells were rinsed with buffer solution before observation.

Electrical recording. Cells were voltage clamped in whole-cell amphotericin-perforated patch recording mode (Rae et al., 1991) using List EPC-9 patch-clamp amplifier with ATARI computer and E9Screen (3.0) software (HEKA electronic) to control the amplifier and acquire data. Bath solutions were (1) for Ca^{2+} current measurements, 60 mM NaCl, 90 mM NaOH, 20 mM tetraethylammonium hydroxide (TEA·OH), 5 mM $Ca(OH)_2$, 1 mM $MgCl_2$, 10 mM NaHEPES, 10 mM glucose, 1 μM tetrodotoxin [pH was adjusted to 7.4 with methanesulfonic acid (Fluka)] (in some experiments [Ca^{2+}] was varied between 2 and 10 mM); (2) for Ba^{2+} current measurements, 60 mM NaCl, 90 mM NaOH, 20 mM TEA·OH, 5 mM $Ba(OH)_2$, 1 mM $MgCl_2$, 10 mM NaHEPES, 10 mM glucose, 1 μM tetrodotoxin (pH adjusted to 7.4 with methanesulfonic acid); or (3) 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM KOH, 10 mM NaHEPES, 10 mM glucose (pH adjusted to 7.4 with methanesulfonic acid). Pipette solutions were (1) for Ca^{2+} and Ba^{2+} current measurements, 55 mM CsCl, 75 mM Cs-aspartate, 5 mM $MgCl_2$, 10 NaHEPES, pH 7.4.; or (2) 55 mM KCl, 75 mM K_2SO_4, 5 mM $MgCl_2$, 10 mM NaHEPES, pH 7.4. Osmolarity of bath solutions was 310–320 mOsm and internal solutions 290–300 mOsm. A stock solution of amphotericin B (4 mg/50 μl DMSO) was prepared before each experiment; 4 μl of stock solution was dissolved by sonication in pipette solution and stored in the dark for no longer then 1 hr. Resistance of pipettes filled with these solutions ranged between 2.8–4.0 MΩ. Access resistance (R_a) stabilized at 21.1 ± 0.6 MΩ (N = 94) after 5–10 min of perforation and did not changed significantly during experiment.

Membrane capacitance was measured with the time-domain technique (Lindau and Neher, 1988) using the EPC-9 capacitance tracking feature. Capacitance measurements were obtained at ~1 Hz. Capacitance, current, voltage, and R_a data were recorded on PCM videocassette recorders and then displayed with a four-channel digital chart recorder (WR7700, Western Graphtec, Inc.). Membrane currents were acquired and stored on hard disk along with *p*/4 leak subtraction records. To ensure that capacitance responses were consistent (i.e., to prevent frequency dependent facilitation or depression), cells were stimulated once every 1–3 min. Except when indicated otherwise, experiments were done at 32°C. All measurements are given ±SEM.

Results

Depolarization-evoked Ca^{2+} influx triggers rapid exocytosis and slow endocytosis

Exocytotic secretion was monitored as a change in membrane capacitance (ΔC_m) in single voltage clamped rat lactotrophs. The average cell membrane capacitance at the beginning of experiments was 6.6 ± 0.3 pF (mean ± SEM, N = 94). However, capacitance could be increased with voltage steps: 50–1000 msec depolarizations from −70 mV to +10 mV evoked increases in membrane capacitance that were complete within the time resolution of our measurements (i.e., within 1 sec) (Fig. 1). These capacitance increases were not correlated with changes in access conductance or holding current. Furthermore, the capacitance responses were very temperature dependent: at room temperature, depolarizations less than 500 msec never evoked capacitance changes (n = 55), while at 32°C, C_m increased more than 50 fF in response to 100 msec depolarization in 28 of 39 cells. Hence, depolarization to +10 mV triggers rapid exocytosis in a temperature dependent manner.

The exocytosis evoked by depolarization to +10 mV was associated with Ca^{2+} influx through the voltage-gated Ca^{2+} channels (Fig. 1*A*). In contrast, depolarizing pulses to +60 mV elicited outward current and were not followed by capacitance changes. The dependence of depolarization-induced exocytosis on Ca^{2+} entry was further confirmed by demonstrating that the exocytotic response to depolarization was proportional to the bath Ca^{2+} concentration. Elevating the extracellular Ca^{2+} concentration increased voltage-gated Ca^{2+} entry and exocytosis (Fig. 1*B*) (N = 3), while replacing bath Ca^{2+} with Co^{2+} completely and reversibly abolished depolarization-evoked exocytosis (N = 5) (Fig. 2). Thus, Ca^{2+} influx through voltage-gated Ca^{2+} channels can trigger rapid exocytosis by single lactotrophs.

Capacitance recordings can also be used to monitor endocytosis. We found that after voltage clamping the membrane potential to −70 mV with the perforated patch-clamp method, membrane capacitance slowly declined ($\Delta C_m/\Delta t$ = −55.9 ± 4.7 fF/min, N = 22). Under our standard conditions, depolarizations that triggered exocytosis did not significantly affect the rate of the gradual decrease in membrane capacitance (−50.4 ± 3.9 fF/min, N = 41). Likewise, basal endocytosis did not change in response to depolarizations in the presence of Co^{2+} (Fig. 2). However, when voltage-gated Ca^{2+} current was increased by elevating bath Ca^{2+} to concentrations >5 mM, endocytosis became more rapid (Fig. 1*B*); for example, raising bath [Ca^{2+}] from 2 mM to 6 mM increased the rate of postdepolarization endocytosis from −62.7 ± 19.1 fF/min to −133 ± 41.6 fF/min (N = 3). Even after stimulation, endocytosis was far slower than depolarization-evoked exocytosis. Thus, the detected changes in the rate of endocytosis did not significantly affect our measurements

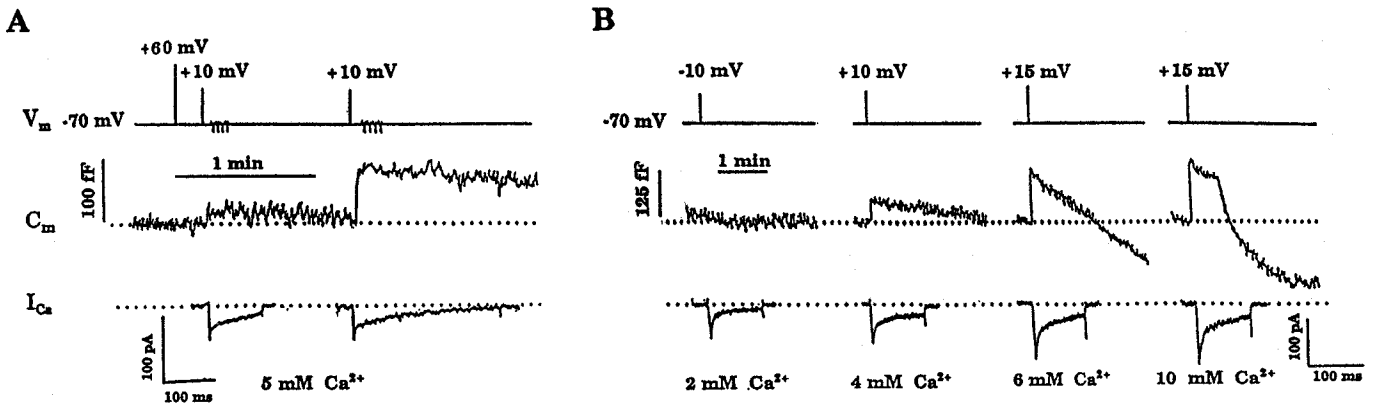

Figure 1. Voltage-gated Ca^{2+} currents and exocytotic activity measured from single lactotrophs. Ca^{2+} current (I_{Ca}), capacitance (C_m), and voltage (V_m) are shown. *A*, Depolarization from −70 mV to +60 mV for 250 msec induced outward current (not shown) which was not associated with capacitance changes. Depolarization to +10 mV for 100 msec and 250 msec induced inward Ca^{2+} currents and C_m increases. Hyperpolarizing pulses for leak subtraction did not affect C_m. Initial cellular capacitance and access resistance were 5.23 pF and 16.7 MΩ, respectively. *B*, Elevating bath [Ca^{2+}] from 2 to 10 mM increased voltage-gated Ca^{2+} current and depolarization-evoked exocytosis and endocytosis. Because of the changes in bath [Ca^{2+}], different voltage steps were used to evoked maximal Ca^{2+} current. *p*/4 leak subtraction pulses are not shown in voltage trace. Recording solutions: bath (1), pipette (1).

of rapid exocytotic responses. Interestingly, further enhancement of the rate of endocytosis sometimes occurred after a delay (Fig. 1*B*, response in 10 mM Ca^{2+}). Hence, large increases in Ca^{2+} influx can trigger events that accelerate basal endocytosis.

Exocytosis is steeply dependent on voltage-gated Ca^{2+} influx

To examine the relationship between Ca^{2+} influx and exocytosis, we first varied the duration of the depolarizing pulse to +10 mV. In all cells tested ($N = 20$), the amplitude of the capacitance changes increased with the duration of the depolarizing pulse (e.g., Fig. 1*A*). Figure 3*A* shows the relationship between the exocytotic response and Ca^{2+} influx (measured as integrated Ca^{2+} current (I_{Ca}). Because the magnitude of the exocytotic response and Ca^{2+} current density varied from cell to cell and from culture to culture, data from each cell were normalized to value of ΔC_m (ΔC_{m100}) and integrated Ca^{2+} current (I_{Ca100}) evoked by a 100 msec depolarization to +10 mV ($\Delta C_{m100} = 68.5 \pm 10.4$ fF, $N = 15$). The dependence of exocytosis on Ca^{2+} influx was fitted with

$$\Delta C_m/\Delta C_{m100} = A(I_{Ca}/I_{Ca100})^B, \quad (1)$$

with $A = 1.02$ and $B = 2.65$ being the best values computed with a nonlinear regression least square fit (smooth curve). To independently verify this relationship, we fixed the duration of depolarizations and varied Ca^{2+} influx by changing the concentration of Ca^{2+} in the extracellular solution between 2 and 10 mM (e.g., Fig. 1*B*). With this protocol, a computer fit of Equation 1 gave a value of $B = 2.91$ (Fig. 3*B*). Hence, exocytosis by lactotrophs is steeply dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels.

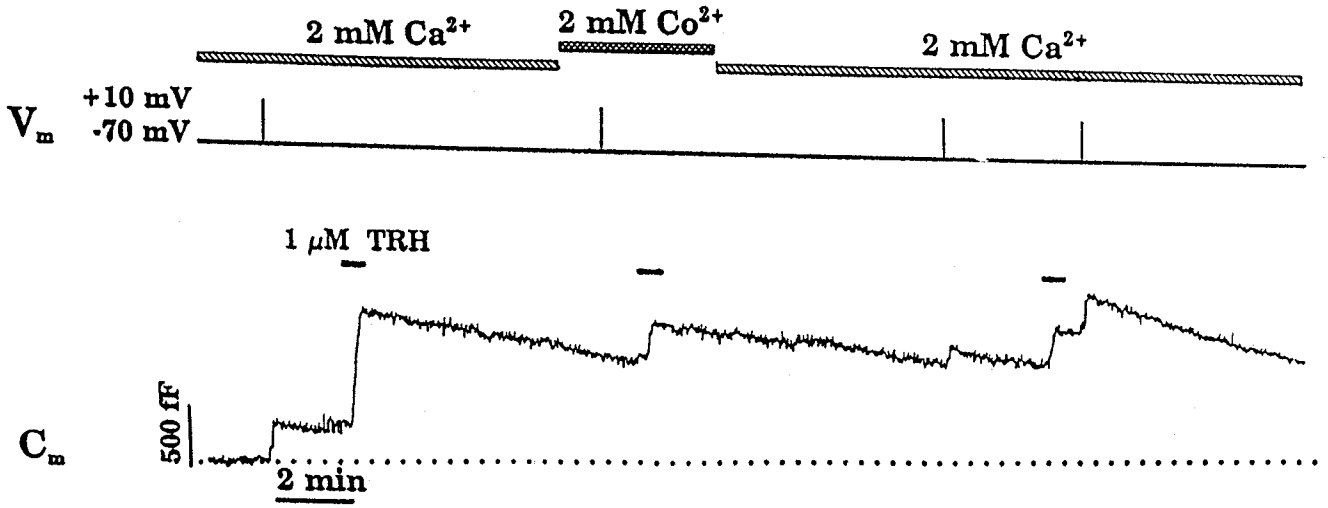

Figure 2. Depolarization-induced and TRH-induced exocytosis differ in their dependence on extracellular Ca^{2+}. Each vertical bar in the voltage trace indicates a train of three 500 msec depolarizations to +10 mV with 500 msec interpulse repolarizations. Capacitance changes were triggered by trains of depolarizations in Ca^{2+} containing solution. Equimolar substitution of external Ca^{2+} with Co^{2+} completely and reversibly abolished the exocytotic response to depolarization. In contrast, application of 1 μM TRH for 30 sec induced exocytosis at −70 mV that was not blocked by substitution of Ca^{2+} with Co^{2+}. The decrease in the amplitude of the second TRH response is due to desensitization. This experiment was performed at 22°C. Initial membrane capacitance was 8.0 pF. Recording solutions: bath (3), pipette (2).

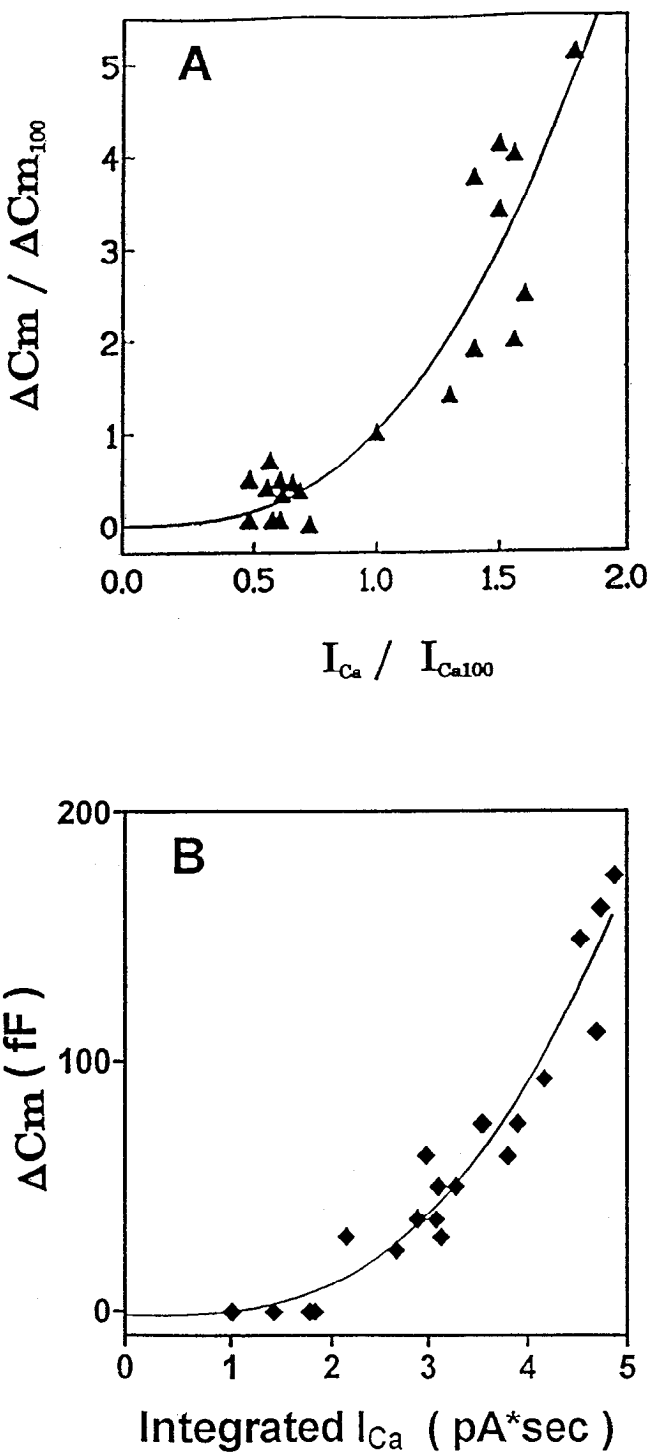

Figure 3. Dependence of ΔC_m on Ca^{2+} influx. *A*, Ca^{2+} influx was measured as integrated Ca^{2+} current (I_{Ca}). Changes in C_m in response to depolarizations to +10 mV for 50, 100, and 250 msec were measured. Data from each cell (N = 16) were normalized to value of ΔC_m (ΔC_{m100}) and integrated Ca current (I_{ca100}) evoked by a 100 msec depolarization. The dependence of exocytosis on Ca influx was fitted with Equation 1, with A = 1.02 and B = 2.65 being the best values computed with a nonlinear regression least square fit (smooth curve). *B*, Ca^{2+} influx was varied in a single lactotroph by changing the concentration of extracellular Ca^{2+}. 2, 4, 6, 8, and 10 mM were used. Depolarizing voltages were adjusted to evoke maximal Ca^{2+} current. Best fit with Equation 1 was obtained with B = 2.91. Same cell as Figure 1*B*.

TRH transiently evokes depolarization- and Ca^{2+}-independent exocytosis

Application of 1 μM TRH triggered exocytosis at −70 mV, a voltage where voltage-gated Ca^{2+} channels are closed (Fig. 2). Like exocytosis evoked by depolarization, the probability of TRH-induced exocytosis at the hyperpolarized membrane potential was temperature dependent: TRH-induced exocytosis at −70 mV was seen in 10 of 36 cells at room temperature, but in 15 of 27 cells at 32°C. The exocytotic response to TRH at −70 mV was not sustained: after 10–15 sec delay, TRH induced a 214 ± 34 fF increase in Cm (N = 22) that was complete in the next 16.7 ± 1.8 sec in 18 cells and in the next 56.2 ± 7.4 sec in the other four cells examined. In the continued presence of TRH, exocytosis was followed by endocytosis (−73.4 ± 7.5 fF/min, N = 31) that was ~40% faster than before application of the neuropeptide ($p < 0.05$). Our demonstration of Ca^{2+} dependent stimulation of endocytosis (see above) suggests that this could be caused by the known elevation of cytoplasmic Ca^{2+} produced by TRH (Malgaroli et al., 1987; Gershengorn, 1989). The exocytotic response obtained with a second exposure to TRH was always smaller than with the initial exposure. Thus, this effect of TRH partially desensitized. However, the ability to evoke multiple responses allowed us to test if TRH-evoked exocytosis required extracellular Ca^{2+}. As can be seen in Figure 2, TRH-induced exocytosis at −70 mV, unlike depolarization-evoked exocytosis, was not blocked by substitution of bath Ca^{2+} with Co^{2+}. Therefore, at least some of the depolarization-independent exocytosis triggered by TRH occurs in the absence of extracellular Ca^{2+} entry through Ca^{2+} channels.

TRH facilitates Ca^{2+} influx-induced exocytosis

To investigate the possible involvement of modulation of voltage-gated Ca^{2+} channels in TRH-induced secretion, we studied simultaneous changes in depolarization-dependent exocytosis and Ca^{2+} currents during bath application of TRH. In seven of nine investigated cells TRH acted within 30 sec to inhibit the voltage-gated Ca^{2+} current. During the next 4–5 min, the current gradually recovered to the control level. For example, in Figure 4, Ca^{2+} influx was reduced by 43% after 2 min of TRH application (compare traces 1 and 2, Fig. 4*C*). Based on Equation 1 one would expect that depolarization-evoked exocytosis should have been reduced dramatically (i.e., by 75%). However, depolarization-evoked exocytosis did not change substantially at this time point. Moreover, in eight of nine cells where voltage-gated Ca^{2+} influx was reduced or remained the same, depolarization-evoked exocytosis was enhanced (Fig. 5). This figure also illustrates that the increase in depolarization-evoked exocytosis did not necessarily have to follow large depolarization-independent exocytosis or dramatic inhibition of voltage-gated Ca^{2+} current. Quantitation from a series of experiments reveals that TRH application reduced average Ca^{2+} influx by ~20% within first 30 sec (Fig. 6*A*) leading to a prediction that exocytosis should have been reduced to ~55% of the control (Fig. 6*B*, solid bar). Yet, average depolarization-evoked exocytosis was *enhanced* to ~150% of control (Fig. 6*B*, open bar). A plot of the relationship between Ca^{2+} influx and depolarization-evoked exocytosis reveals that the secretory responses to depolarization-evoked reduced Ca^{2+} influx in the first 3 min of TRH exposure were greater than predicted by Equation 1 in nearly all cases (Fig. 6*C*, open circles). This indicates that during this period induction of exocytosis by Ca^{2+} influx was enhanced by the neuropeptide.

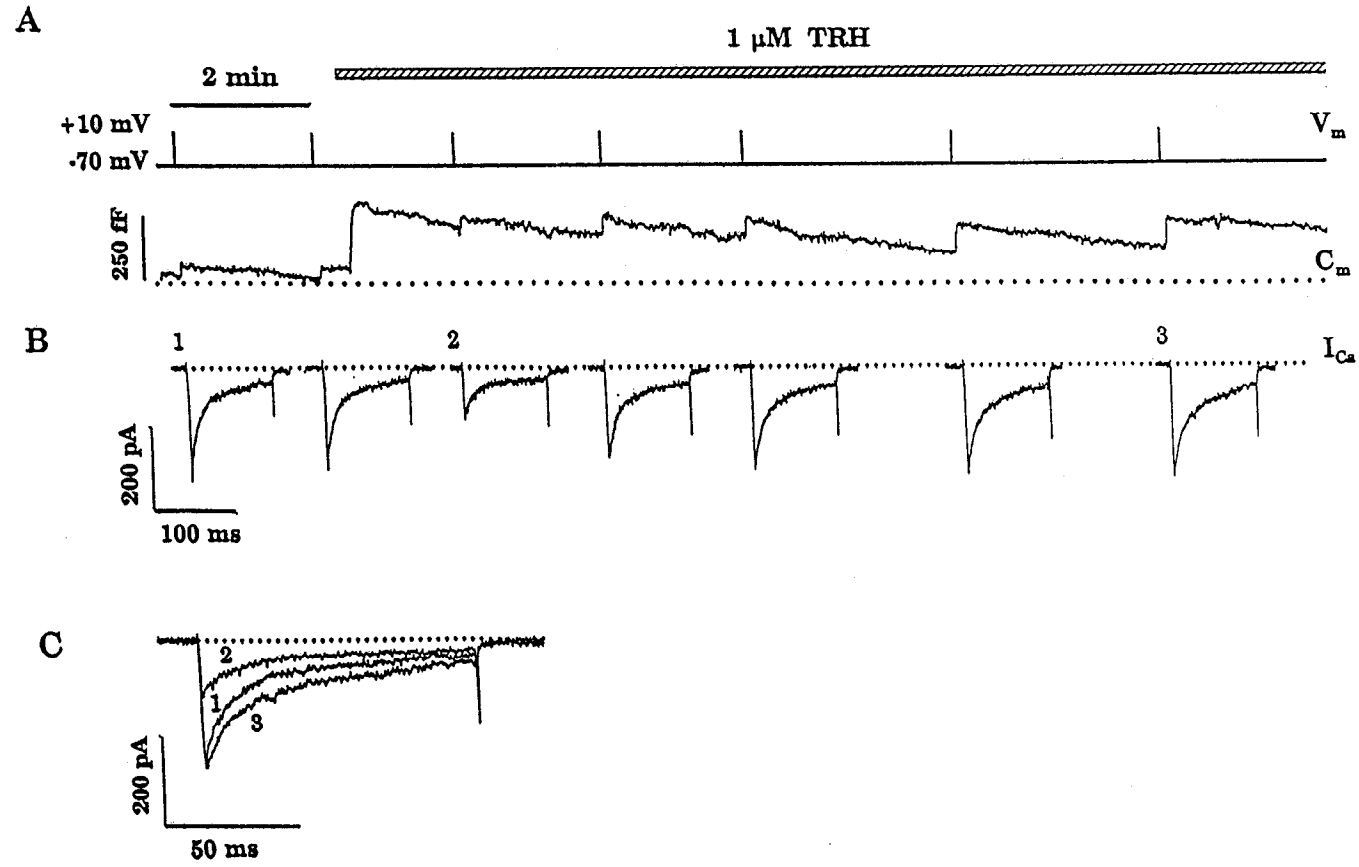

Figure 4. TRH affects exocytosis and Ca^{2+} currents. Vertical bars indicate single 100 ms depolarizations to +10 mV (*p*/4 leak subtraction pulses are not shown in voltage trace). *A*, C_m changes in response to 100 msec pulses to +10 mV before and after application of 1 μM TRH. *B*, Corresponding Ca^{2+} current traces before and after TRH application (leak subtracted). *C*, Superimposed Ca^{2+} current traces before (*1*), 1.5 min (*2*), and 12 min (*3*) after TRH application. Initial membrane capacitance was 7.9 pF. Recording solutions: bath (1), pipette (1).

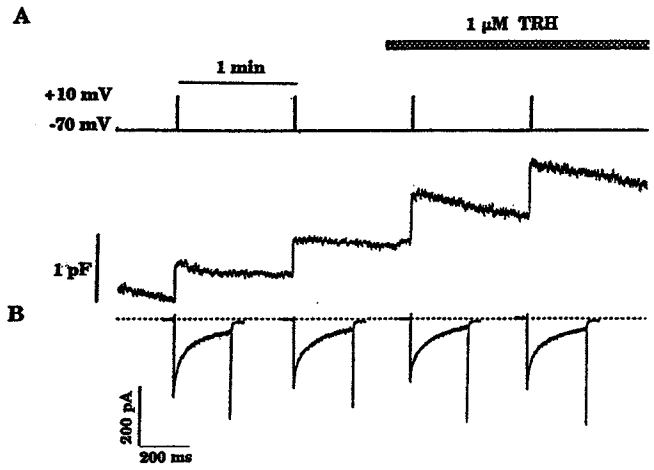

Figure 5. TRH can increase depolarization-induced exocytosis without increasing Ca^{2+} currents within the first minutes of application. Vertical bars indicate single 250 msec depolarizations to +10 mV (*p*/4 leak subtraction pulses are not shown in voltage trace). *A*, Exocytotic responses are shown; 1 μM TRH induced a small exocytotic response at −70 mV, but significantly potentiated depolarization-evoked exocytosis. *B*, Ca^{2+} currents were not increased by TRH application. Initial membrane capacitance was 8.8 pF. Recording solutions: bath (1), pipette (1).

At later times TRH increases voltage-gated Ca^{2+} channel activity to further promote exocytosis

Following the transient decrease and recovery of voltage-gated Ca^{2+} current, an increase in total voltage-gated Ca^{2+} current was observed in the continued presence of TRH (Fig. 4*B*, traces 2 and 3). Typically, the increase in the peak current was modest when compared to control, but the apparent inactivation of current was slowed. The combination of these two effects produced an increase in total Ca^{2+} influx to ~130% of control after 10–13 min of TRH treatment (Fig. 6*A*).

The dependence of exocytosis on Ca^{2+} influx in untreated cells (Eq. 1) predicts that the increase in voltage-gated Ca^{2+} influx should increase exocytosis. If TRH along with increasing of Ca^{2+} influx also simultaneously increased the efficiency of coupling Ca^{2+} influx to exocytosis (as was seen with shorter exposures to the neuropeptide), then the depolarization-evoked increases in Cm seen after 4 min of TRH application should be significantly larger than predicted by Equation 1. However, as can be seen in Figure 6, *B* and *C*, the dependence of the exocytosis on Ca^{2+} influx was well predicted by Equation 1 after 4 min of TRH application. This finding suggests that the enhanced coupling of Ca^{2+} influx to exocytosis is marked for the first 3

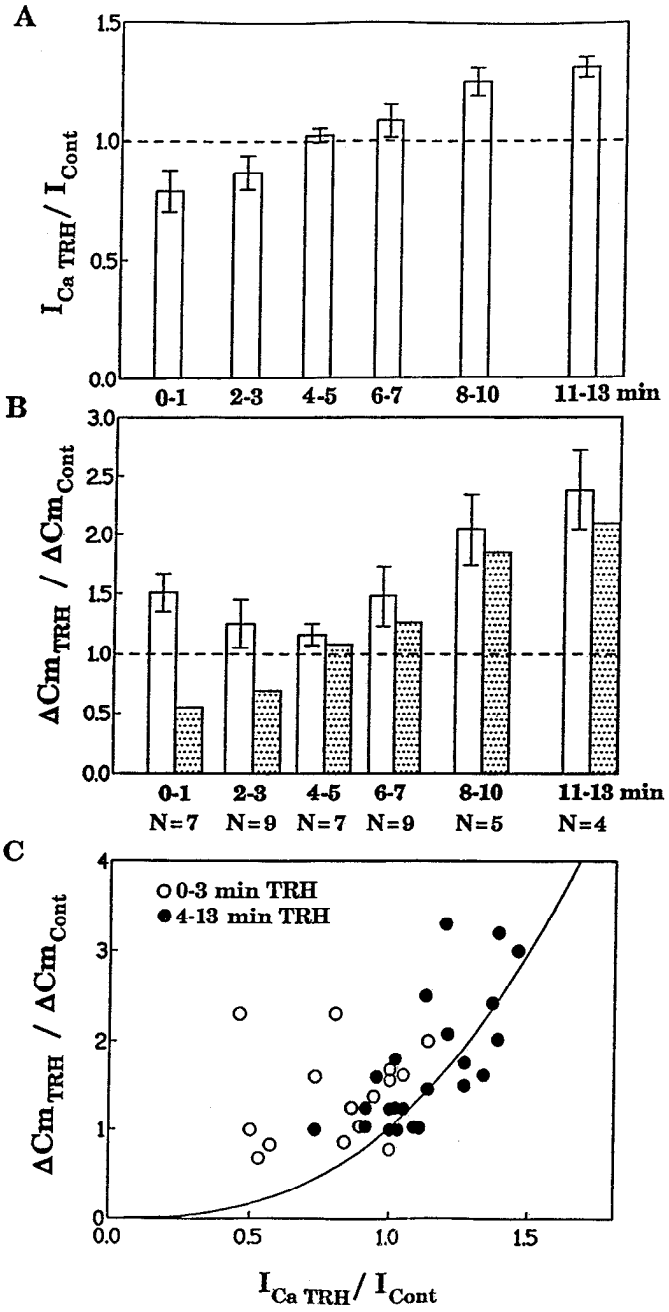

Figure 6. Changes in depolarization-evoked Ca^{2+} influx and exocytosis during continuous application of 1 μM TRH. *A*, Time course of the changes in depolarization-evoked Ca^{2+} influx (presented as integrated current normalized to control). Note that TRH has a biphasic effect on depolarization-evoked Ca^{2+} influx. *B*, Exocytotic responses were measured and normalized to responses before TRH application (*open bars*) and predicted from Equation 1 (*hatched bars*). Note that while Equation 1 predicts that TRH should have initially decreased depolarization-evoked exocytosis, exocytotic responses increased. At later times, exocytotic responses are similar to predictions of Equation 1. *C*, Scatter plot showing the effect of TRH on the dependence of exocytosis (ΔC_m) on Ca^{2+} influx. Each point represents an individual depolarization-evoked exocytotic response after superfusion with TRH. Values are normalized to responses from the same cell before application of the neuropeptide. The smooth curve is identical to the curve shown in Figure 3*A*. *Open circles* show responses within the first 3 min of TRH application. Note only one of these points is below the curve. *Solid circles* show responses evoked after TRH had been applied for 4–14 min. Note that these points appear to be distributed along the standard curve.

min of TRH application. Then, as that effect subsides, depolarization-evoked exocytosis is promoted by increasing voltage-gated Ca^{2+} channel activity.

TRH modulates high-voltage-activated Ca^{2+} channels

HVA (high-voltage-activated) and LVA (low-voltage-activated) Ca^{2+} channel currents in lactotrophs can be most reliably separated with the use of Ba^{2+} as a charge carrier (DeRiemer and Sakmann, 1986; Lingle et al., 1986; Cobbett et al., 1987; Lewis et al., 1988). Therefore, we examined TRH action on voltage-gated Ba^{2+} currents. Superfusion with 1 μM TRH first acted within 10 sec to inhibit maximal voltage-gated Ba^{2+} current elicited from holding potential −70 mV by ~20% on average (Fig. 7*A*). The current amplitude then gradually increased so that it reached the initial level after 4 min of superfusion with TRH, and a level of 20% higher than the initial control value (i.e., before TRH application) after 10 min of neuropeptide application. TRH inhibition and stimulation of channel activity did not alter current kinetics (Fig. 7*B*), which showed little inactivation, suggesting that the neuropeptide does not affect the voltage-dependent activation and inactivation of the channels. Most importantly, these experiments demonstrate that the sequential opposing effects of TRH on voltage-gated Ca^{2+} channels occur regardless of whether Ca^{2+} or Ba^{2+} is used as the charge carrier.

Ba^{2+} currents were then evoked from a holding potential of −40 mV to isolate HVA currents and subtracted from the total current evoked from −70 mV to estimate the amplitude of the LVA currents. As can be seen in Figure 7*C*, prolonged TRH application increased the magnitude of the HVA current without altering LVA currents. No change in HVA current kinetics was produced by TRH (data not shown) suggesting that the apparent kinetic changes in the Ca^{2+} currents described above (Fig. 4) were probably due to the selective increase in the HVA component of the total current. In separate experiments where the holding potential was maintained at −40 mV, we found that HVA current was transiently inhibited and then later stimulated by TRH (data not shown). Thus, TRH modulates HVA Ca^{2+} channel activity.

Activation of protein kinase C can account for the third effect of TRH

To test if PKC could mediate one or more of the three TRH actions, we superfused patch clamped lactotrophs with TPA (12-*O*-tetradecanoylphorbol 13-acetate), a phorbol ester activator of PKC, or its inactive stereoisomer 4α-TPA. We found that neither TPA nor 4α-TPA affected exocytosis in cells voltage clamped at −70 mV (N = 32). However, 100–500 nM TPA acted over a period of 3–4 min to increase depolarization-evoked exocytosis in 17 of 28 cells (Fig. 8*A*). In contrast, equivalent doses of 4α-TPA did not produce a significant effect (N = 6). Simultaneous measurements of voltage-gated Ca^{2+} currents and exocytosis indicated that 100 nM TPA facilitated depolarization-evoked exocytosis to 220 ± 38% of control while increasing Ca^{2+} influx to 151 ± 27% of control (N = 4) (Fig. 8*B*). Since exocytotic responses were not greater than predicted by Equation 1, the efficiency of coupling Ca^{2+} influx to exocytosis was not increased. Rather, PKC facilitated exocytosis by stimulating voltage-gated Ca^{2+} channel activity. This finding indicates that activation of PKC alone does not mimic the first two TRH effects. Instead, activation of PKC appears to be sufficient to produce the third phase of TRH action.

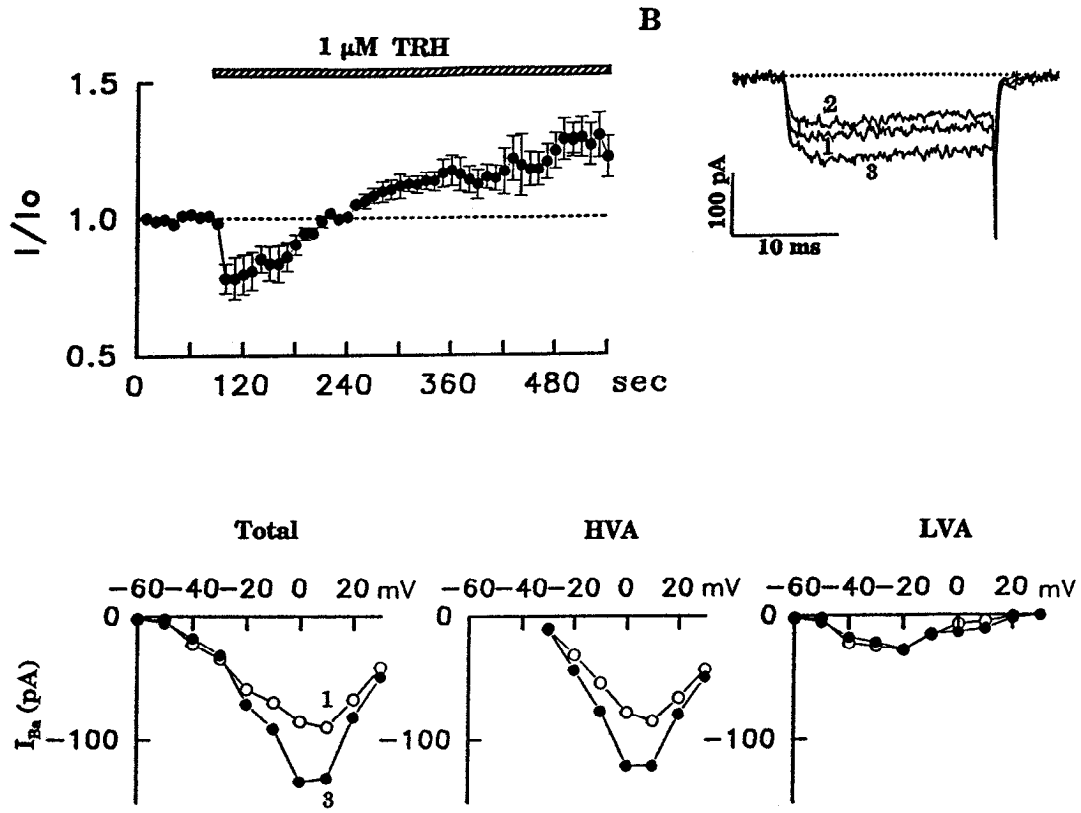

Figure 7. TRH modulates high-voltage-activated (*HVA*) Ca^{2+} channels. *A*, Time course of modulation of peak voltage-gated Ba^{2+} current by 1 μM TRH. Currents were evoked by depolarizations from −70 mV to +10 mV. Each point is average from six cells. *B*, Representative current traces showing I_{Ba} before (*1*), 10 sec after (*2*), and 8 min after (*3*) superfusion with TRH. *C*, Representative current–voltage relationships for total I_{Ba} (*left*), high-voltage-activated (*HVA*) current (*middle*), and low-voltage-activated (*LVA*) currents (*right*) before (*open circles*) and 8 min after (*solid circles*) superfusion with TRH. HVA currents were recorded from holding potential −40 mV. LVA currents were obtained by subtracting HVA current traces from total current traces. Data is from same cell shown in *B*, Recording solutions: bath (2), pipette (1).

Discussion

Exocytosis by a peptidergic cell is steeply dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels

It was known that brief depolarizations (i.e., <250 msec) trigger exocytosis, presumably reflecting peptide secretion, by neurohypophysis terminals (Lim et al., 1990), intermediate lobe melanotrophs (Thomas et al., 1990), and pancreatic β cells (Ammala et al., 1993a,b). However, previous experiments performed with conventional whole cell recording demonstrated that it was very difficult to trigger exocytosis with voltage-clamp depolarization of bovine lactotrophs (Mason et al., 1989) and rat gonadotrophs (Tse et al., 1993). Those latter results suggested that action potentials might play a minor role in evoking peptide secretion by anterior pituitary cells. However, we found that 50–250 msec voltage-clamp depolarizations could effectively trigger exocytosis from rat lactotrophs. Since it is known that exocytotic responses to depolarization wash out with whole cell recording (Augustine and Neher, 1992; Ammala et al., 1993b), it is possible that the use of the perforated patch-clamp configuration and performing experiments at 32°C were essential for detecting depolarization-evoked exocytosis. Therefore, under physiological conditions Ca^{2+} influx through voltage-gated Ca^{2+} channels may effectively trigger peptide secretion from some anterior pituitary cells.

Our experiments revealed that increasing Ca^{2+} influx through voltage-gated Ca^{2+} channels by lengthening the duration of depolarization or by increasing bath Ca^{2+} dramatically increased exocytosis: exocytotic responses are proportional to Ca^{2+} influx raised to the third power. This finding is reminiscent of the previously described cooperative relationship between the rate of exocytosis and cytoplasmic Ca^{2+} concentration in chromaffin cells (Augustine and Neher, 1992) and melanotrophs (Thomas et al., 1990). Likewise, in the squid giant synapse, transmission is cooperatively dependent on Ca^{2+} influx with voltage clamp depolarizations >5 msec in duration (Augustine et al., 1985). Perforated patch-clamp recordings reveal that lactotroph action potential duration is long (>10 msec) and can vary in response to modulators (data not shown). The steep dependence of exocytosis on Ca^{2+} influx suggests that even subtle changes in action potential activity might affect prolactin secretion. This may be a general feature of regulation of peptide secretion by neuroendocrine cells (Bondy et al., 1986; Gainer et al., 1987).

Three phases of TRH-induced facilitation of exocytosis

Our finding that TRH produces three temporally distinct effects to promote exocytosis is unprecedented. Previous work with pituitary tumor cells had suggested that TRH induces two phases of prolactin secretion: a transient burst associated with intracellular Ca^{2+} release and a plateau phase that requires extracellular Ca^{2+} influx (Gershengorn, 1989). Electrophysiological studies had suggested that the plateau phase is due to increased action potential activity (Mason et al., 1988, 1989; Iijima et al., 1990). It is likely that the transient depolarization- and extracellular calcium-independent exocytosis triggered by TRH (e.g., Figs. 2,

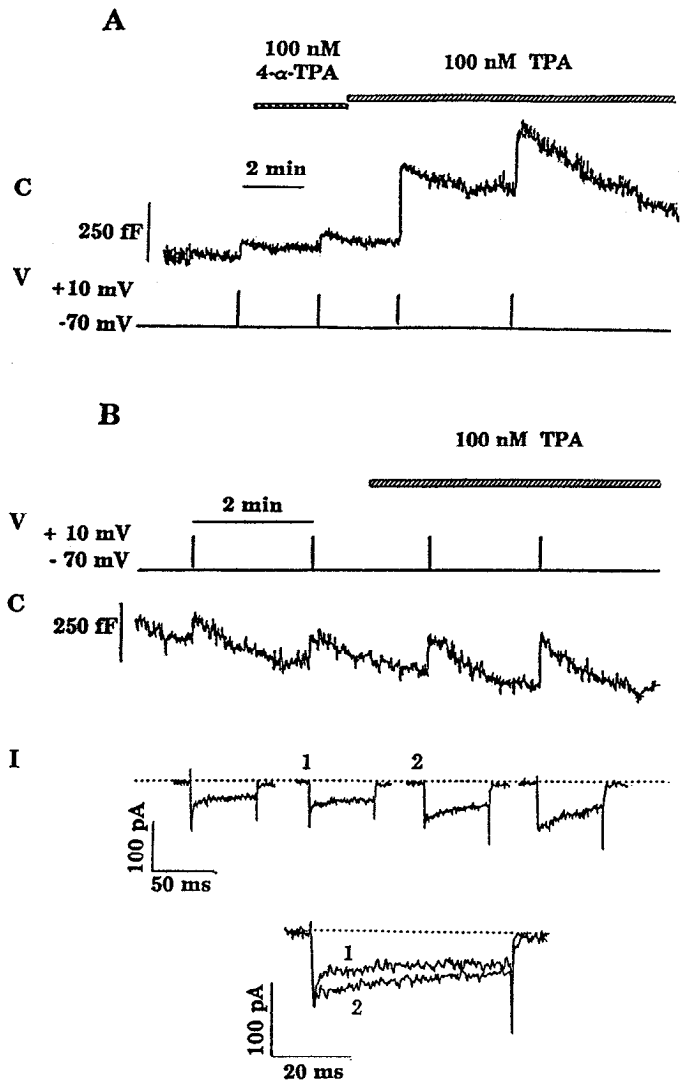

Figure 8. Activation of protein kinase C increases depolarization-evoked exocytosis and voltage-gated Ca^{2+} current. *A*, Facilitation of exocytotic responses by 100 nM TPA, a PKC activator. 4-α-TPA does not produce this effect. Recording solutions: bath (3), pipette (2). *B*, 100 nM TPA increases depolarization-evoked exocytosis and voltage-gated Ca^{2+} current. *p*/4 leak subtraction pulses are not shown in voltage trace. Recording solutions: bath (1), pipette (1).

4) is evoked by the brief (i.e., <1 min) release of intracellular Ca^{2+} seen in normal rat lactotrophs at 32°C (Malgaroli et al., 1987). However, if the plateau phase was only caused by increased action potential firing, then we would not have expected to detect TRH modulation of exocytosis evoked by voltage-clamp depolarizations. Thus, our finding that TRH facilitates depolarization-evoked exocytosis was not anticipated from previous work with the neuropeptide.

Interestingly, two phases of facilitation of depolarization-evoked exocytosis were induced by the neuropeptide. During the first few minutes, TRH enhanced exocytosis while inhibiting Ca^{2+} influx through voltage-gated Ca^{2+} channels. This early action might account for the contribution of voltage-gated Ca^{2+} channels to the initial 2–5 min burst of TRH-induced prolactin secretion by normal lactotrophs described previously (Sato et al., 1992). At later times, exocytosis was augmented by increasing voltage-gated Ca^{2+} channel current. This effect could participate in producing the plateau phase of secretion.

It is established that TRH stimulates PKC and that activation of this enzyme increases prolactin secretion (Negro-Vilar and Lapetina, 1985; Gershengorn, 1989). Therefore, we tested whether the activation of PKC alone could account for any of the three facilitatory effects of the neuropeptide. Work with pituitary tumor cells suggested that PKC sensitizes the secretory apparatus to increase basal and Ca^{2+} influx-dependent prolactin secretion (Ronning and Martin, 1986; Haymes and Hinkle, 1993), but does not stimulate Ca^{2+} channels (Kramer et al., 1991). However, in normal lactotrophs phorbol ester activation of PKC increased exocytotic responses to depolarization by stimulating voltage-gated Ca^{2+} channels. Furthermore, PKC did not induce exocytosis at a hyperpolarized membrane potential. Thus, PKC may act via different mechanisms to stimulate secretion by normal and clonal pituitary cells. In addition, our work provides direct evidence that PKC modulation of Ca^{2+} channels can facilitate secretion. Finally, it is apparent that PKC activation can only account for the third phase of TRH action in normal lactotrophs.

The limited action of PKC suggests that the second phase of facilitation of depolarization-evoked exocytosis requires a different signal transduction mechanism (possibly in conjunction with PKC). There are several messengers that might facilitate depolarization-evoked exocytosis without stimulating voltage-gated Ca^{2+} channels. TRH activates novel G protein and tyrosine kinase pathways (Bauer et al., 1994; Brady et al., 1994; Ohmichi et al., 1994). In addition, release of intracellular Ca^{2+} may be involved: an increase in baseline $[Ca^{2+}]_i$ could sum with Ca^{2+} influx through voltage-gated channels to trigger exocytosis. However, this latter hypothesis is called into question by the fact that Ca^{2+} release in rat lactotrophs at 32°C is too brief (~1 min; Malgaroli et al., 1987) to account for the second phase of TRH action. A plausible alternative is that transient Ca^{2+} release produces a sustained increase in the coupling efficacy of between Ca^{2+} influx and exocytosis (von Ruden and Neher, 1993). Testing this mechanism is difficult because blockers of TRH-induced intracellular Ca^{2+} release affect basal Ca^{2+} concentration, Ca^{2+} channel activity, and prolactin secretion (Kramer et al., 1991; Sato et al., 1992). Thus, extensive studies with intracellular Ca^{2+} measurements and a variety of pharmacological agents will be required to fully analyze all of the possible mechanisms underlying the second phase of TRH action. Nevertheless, our studies suggest that the ability of this neuromodulator to activate multiple second messenger systems provides the basis for producing temporally distinct effects on exocytosis.

Modulation of voltage-gated Ca^{2+} channel currents and exocytotic secretion

This report constitutes the first demonstration that TRH regulates lactotroph voltage-gated Ca^{2+} channels and exocytotic activity. There are few other preparations where modulation of Ca^{2+} channels and exocytosis have been followed simultaneously (Ammala et al., 1993a; Artalejo et al., 1994). In the absence of direct measurements, most investigators have assumed that secretion is proportional to Ca^{2+} influx. Our results indicate that this is not always the case. During the first few minutes of TRH application, depolarization-evoked exocytosis is enhanced despite the fact that voltage-gated Ca^{2+} channels are inhibited by the neuropeptide. Thus, Ca^{2+} current measurements alone do not predict exocytosis because induction of exocytosis by Ca^{2+} in-

flux can be augmented by the neuropeptide. In contrast, after 8 min of TRH exposure, voltage-gated Ca^{2+} current is increased. Since the relationship between Ca^{2+} influx and exocytosis is not altered during this period, we conclude that the modulation of these channels is sufficient to explain the observed increase in depolarization-evoked exocytosis. Thus, the ability to predict the impact of modulating Ca^{2+} influx on peptide secretion may depend on the duration of neuropeptide exposure.

Rat lactotrophs express two types of voltage-gated Ca^{2+} channels: high-voltage-activated (HVA) and low-voltage-activated (LVA) channels (DeRiemer and Sakmann, 1986; Lingle et al., 1986; Cobbett et al., 1987; Lewis et al., 1988). We found that TRH produces a biphasic effect on lactotroph HVA Ca^{2+} current. However, LVA current was not increased by the neuropeptide. It is likely that most of the LVA current is inactivated with physiological action potential activity (Cobbett et al., 1987). Hence, modulating HVA channels may be an efficient mechanism for altering Ca^{2+} influx into lactotrophs.

TRH modulation of peptide secretion and synaptic transmission

TRH stimulates secretion of prolactin and TSH by anterior pituitary cells and catecholamines by some CNS neurons (Faglia and Persani, 1991). In addition, TRH augments long-term potentiation in the hippocampus (Ishihara et al., 1991, 1992). Since the same TRH receptor is expressed in the brain and in the anterior pituitary (Wu et al., 1992; Satoh et al., 1993), we suggest that TRH might employ similar mechanisms to facilitate secretion by neurons and lactotrophs. It is possible that each phase of TRH action may differentially depend on the dose and the duration of exposure to the neuropeptide. Furthermore, each facilitating mechanism might be differentially affected by other transmitters. This may be a crucial feature in the central nervous system and in the anterior pituitary where secretion sites can be simultaneously exposed to a variety of interacting neurotransmitters. Therefore, the use of temporally distinct regulatory mechanisms might facilitate intricate control of secretion by neuromodulators.

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