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Thyrotropin-releasing hormone induces opposite effects on Ca^{2+} channel currents in pituitary cells by two pathways

(Ca^{2+} release/guanine nucleotide-binding regulatory proteins/pertussis toxin/GH_3 cells)

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ABSTRACT **Thyrotropin-releasing hormone (TRH) stimulates pituitary secretion by steps involving a cytosolic Ca^{2+} rise. We examined various pathways of Ca^{2+} elevation in pituitary GH_3 cells. By using the patch clamp technique in the whole-cell configuration and Ba^{2+} as divalent charge carrier through Ca^{2+} channels, TRH (1 μM) reversibly reduced the current by about 55%. This hormonal effect was prevented by infusing guanine 5′-[β-thio]diphosphate (GDP[βS]) intracellularly but not by pretreating the cell with pertussis toxin (PT). Since PT-insensitive guanine nucleotide-binding regulatory (G) proteins are known to mediate a hormone-stimulated inositol trisphosphate-mediated Ca^{2+} release from intracellular stores, we assume that the inhibitory effect of TRH on Ba^{2+} currents through Ca^{2+} channels is caused by the increased intracellular Ca^{2+}. To prevent a Ca^{2+}-release-dependent inhibition of Ca^{2+} channels, we preincubated GH_3 cells in a medium free of divalent charge carriers and measured the Na^+ current through Ca^{2+} channels. When fura-2 was used as indicator for the cytosolic Ca^{2+}, TRH induced a release from intracellular stores only once and had no effect on the intracellular Ca^{2+} concentration during further applications. In line with this observation, TRH initially reduced the Na^+ current through Ca^{2+} channels but stimulated it during subsequent applications. The stimulation was sensitive to GDP[βS] and was abolished by pretreatment with PT, suggesting that the stimulatory action of TRH is mediated by a G protein different from the one that functionally couples the receptor to phosphatidylinositol 4,5-bisphosphate hydrolysis. In conclusion, the present data suggest that TRH increases the intracellular Ca^{2+} concentration by two interacting pathways, that release from intracellular stores causes a secondary blockage of Ca^{2+} channels, and that, especially with empty intracellular Ca^{2+} stores, Ca^{2+} channels are stimulated by a PT-sensitive G protein.**

Pituitary-hormone-releasing factors such as thyrotropin-releasing hormone (TRH), luteinizing-hormone-releasing hormone (LHRH), and angiotensin II (ATII) increase the cytosolic Ca^{2+} in anterior pituitary cells in two successive phases. They first induce an inositol trisphosphate-dependent Ca^{2+} release (1–6) and then stimulate extracellular Ca^{2+} entry, which is sustained in the presence of the releasing factor (7, 8). The nature of this retarded and prolonged Ca^{2+} entry remains unclear (9–12). A biphasic TRH action was also seen in membrane potential recordings. The membrane potential hyperpolarized within the first phase (about 2 min); action potentials induced thereafter occurred at a higher rate than the basal activity (13, 14). The hyperpolarization was attributed to an activation of Ca^{2+}-dependent K^+ channels, which may be related to the intracellular Ca^{2+} mobilization (11, 15), and the increased firing pattern was thought to be associated with an inhibition of inwardly rectifying K^+ channels (16). However, the TRH-induced inhibition of voltage-dependent Ca^{2+} channels previously reported (17, 18) was puzzling in light of the overall stimulation of secretion (6, 8, 19, 20).

To prevent the hormone-induced Ca^{2+} release in the present approach, we created ionic conditions that did not allow a refilling of the internal stores by removal of divalent cations from the external solution. Under these conditions, the current through Ca^{2+} channels occurs as Na^+ current (21, 22). Our data demonstrate, in pituitary GH_3 cells, that (*i*) TRH releasing Ca^{2+} from internal stores may induce a Ca^{2+}-dependent Ca^{2+}-channel-current (I_{Ca}) inhibition and that (*ii*) TRH directly stimulates Ca^{2+} channels when the internal Ca^{2+} stores are emptied. The latter effect may be a mechanism for refilling the Ca^{2+} stores. The opposite TRH-induced effects on cytoplasmic Ca^{2+} could be differentiated by their sensitivity to pertussis toxin (PT).

MATERIALS AND METHODS

Cell Culture. Rat pituitary GH_3 cells (passage 24–40) were purchased from the American Type Culture Collection and cultured as described (23).

Electrophysiology. Whole-cell membrane currents were measured according to the method of Hamill *et al.* (24) (for some details, see ref. 23). Patch pipettes had an average resistance of 1–5 MΩ (open diameter, 1–3 μm). Recording of currents was usually started 5 min after disruption of the membrane patch for intracellular dialysis with the pipette solution. The "run-down" of the I_{Ca} (see refs. 25 and 26) could be roughly approximated as a linear process. It was faster with Ba^{2+} as charge carrier (50% decline within 5–8 min) than with Na^+ (50% decline within 7–15 min).

The membrane capacity was measured as a current response to a fast voltage-ramp pulse (slope, 10 V/s) and amounted to 13.9 ± 4.1 pF (n = 26). The unspecific ohmic conductance amounted to about 0.6 ± 3.1 nS (n = 12) with solution E2 (see below) and 1.4 ± 2.9 nS (n = 14) with solution E3. Only those experiments were analyzed in which the unspecific conductance remained unchanged.

Measurement of Intracellular Free Ca^{2+}. Measurements of intracellular free Ca^{2+} in single GH_3 cells were carried out as described (27). Maximal fluorescence was determined by adding 40 μM ionomycin (using a 0.01 M stock solution in dimethyl sulfoxide). Minimal fluorescence was obtained by adding 0.25 M EGTA (pH 7.8). The ratio of the two signals

Abbreviations: TRH, thyrotropin-releasing hormone; I_{Ca}, Ca^{2+} channel current; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PT, pertussis toxin; ωCT, ω-conotoxin; LHRH, luteinizing-hormone-releasing hormone; ATII, angiotensin II; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; *IV*, current–voltage.

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was used to calculate the intracellular free Ca^{2+} concentration as described by Grynkiewicz et al. (28).

Solutions and Materials. Divalent cation fluxes through Ca^{2+} channels were measured as Ca^{2+} and Ba^{2+} currents using modified Tyrode's solutions E1 and E2, respectively. E1 contained 140 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 10 mM glucose, and 10 mM Hepes·NaOH (pH 7.4 at 37°C). E2 contained 125 mM NaCl, 10.8 mM $BaCl_2$, 1 mM $MgCl_2$, 5.4 mM CsCl, 10 mM glucose, and 10 mM Hepes·NaOH (pH 7.4 at 37°C). Monovalent cation fluxes through Ca^{2+} channels were recorded in the nominally Ca^{2+}-free solution E3 containing 140 mM NaCl, 5.4 mM CsCl, 10 mM glucose, 1 mM EGTA, and 10 mM Hepes·NaOH (pH 7.4 at 37°C). The patch pipettes were filled with a solution containing 120 mM CsCl, 3 mM $MgCl_2$, 5 mM Mg-ATP, 10 mM EGTA, and 10 mM Hepes, cesium salt (pH 7.4 at 37°C). If indicated, the pipette solution contained 5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) instead of EGTA. TRH was obtained from Sigma (Deisenhofen, F.R.G.). ω-Conotoxin (ωCT) was purchased from Bissendorf (Hannover, F.R.G.). Nimodipine was from Bayer (Leverkusen, F.R.G.). (+)-Isradipine was a gift of Sandoz Pharmaceutical (Basel). PT was kindly provided by M. Yajima (Kyoto, Japan).

Statistics. All values are expressed as the mean ± SEM. Differences between means were tested for statistical significance by the Wilcoxon rank sum test. $P < 0.05$ was considered significant.

RESULTS

Effect of TRH on Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$). The way in which Ca^{2+} withdrawal from the extracellular solution (change from solution E1 to solution E3) influences the TRH effect on $[Ca^{2+}]_i$ was first tested in experiments using the fura-2 method. As in previous reports (5, 10), superfusion of GH_3 cells with 1 μM TRH led to a biphasic increase in $[Ca^{2+}]_i$ in the presence of Tyrode's solution (E1). A fast transient $[Ca^{2+}]_i$ spike (duration, about 30 s) was followed by a sustained phase during which $[Ca^{2+}]_i$ fluctuated around a mean amplitude above the basal $[Ca^{2+}]_i$ but below the peak level. Resting Ca^{2+} levels were in the range of 150–180 nM, rising to >1 μM initially and fluctuating between 300 and 400 nM during the sustained phase. Washing out TRH reduced $[Ca^{2+}]_i$ to its basal level within about 60 s. When TRH was applied for a second time, the spike phase was shorter, which is in line with a reduced loading of the intracellular Ca^{2+} stores. LHRH (1 μM) and ATII (1 μM) were less potent than TRH in triggering the fast transient $[Ca^{2+}]_i$ spike (approximately 20% and 10%, respectively, of the TRH-induced $[Ca^{2+}]_i$ spike) but were similarly effective in inducing the second sustained phase.

If GH_3 cells were incubated in Ca^{2+}-free saline (E3), 1 μM TRH induced a transient $[Ca^{2+}]_i$ spike similar in size to that obtained with Tyrode's solution, but the sustained phase was completely absent; within about 2 min, $[Ca^{2+}]_i$ returned to its basal level despite the presence of TRH. A second TRH application had no effect on $[Ca^{2+}]_i$, suggesting that a single TRH application is sufficient to empty internal Ca^{2+} stores completely. This was confirmed by subsequent superfusion of 1 mM caffeine, which induced a Ca^{2+} release with Tyrode's solution but was unable to increase $[Ca^{2+}]_i$ with solution E3. The TRH-induced Ca^{2+} release was also absent when GH_3 cells were incubated in E3 for more than 60 min prior to application of TRH (see also ref. 29).

Ca^{2+} Channel Currents Carried by Ba^{2+} and Na^+. The whole-cell inward current through voltage-dependent Ca^{2+} channels was characterized using Ba^{2+} as divalent charge carrier in modified Tyrode's solution (E2). K^+ currents were minimized by Cs^+ in the internal and external medium. As shown (23), GH_3 cells exhibited a slowly inactivating Ba^{2+} current when pulsed to a test potential of 0 mV (Fig. 1*B*, trace Con). A fast inactivating current was also observed in about 30% of all cells tested, as described (ref. 23, see also refs. 30 and 31). To estimate the current–voltage (*IV*) relation, the test potential was linearly varied from −100 to 100 mV. The recorded current (Fig. 1*A*, trace Con) was U-shaped and exhibited a maximum at about −10 mV. The current amplitudes corresponded to those measured during pulses to the respective potentials. The apparent threshold occurred at −40 mV and the reversal potential at 50 mV. The latter may be underestimated because of a possible Cs^+ flux through Ca^{2+} channels at positive potentials (32).

With the external solution E3, which was nominally Ca^{2+}-free, the current through Ca^{2+} channels was mainly carried by Na^+ (see refs. 21 and 22). The holding potential of −80 mV has been shown to fully inactivate Na^+-carried currents through low-threshold (T-type) Ca^{2+} channels (31). The currents were about 3 times the amplitude of those recorded in E2 and showed more pronounced single channel noise (Fig. 1 *D* and *E*) (21). Compared to the Ba^{2+} condition, the *IV* relation for the Na^+ current through Ca^{2+} channels was markedly shifted to more negative potentials, as may be expected from the effects of a changing surface potential (33). The apparent threshold and reversal potential occurred at about −70 and 25 mV, respectively, and the maximal current amplitude was measured at about −40 mV.

To demonstrate that the currents measured under both conditions, E2 and E3, were mediated by voltage-dependent Ca^{2+} channels, we used the dihydropyridines (+)-isradipine (1 μM) and nimodipine (200 nM, see below), known to inhibit Ca^{2+} channels, and Bay K 8644 (1 μM), known to stimulate Ca^{2+} channels. ωCT (10 μM) was tested for its ability to block N-type Ca^{2+} channels (34). I_{Ca} measured with E2 or E3 was nearly completely blocked by (+)-isradipine [reduction from

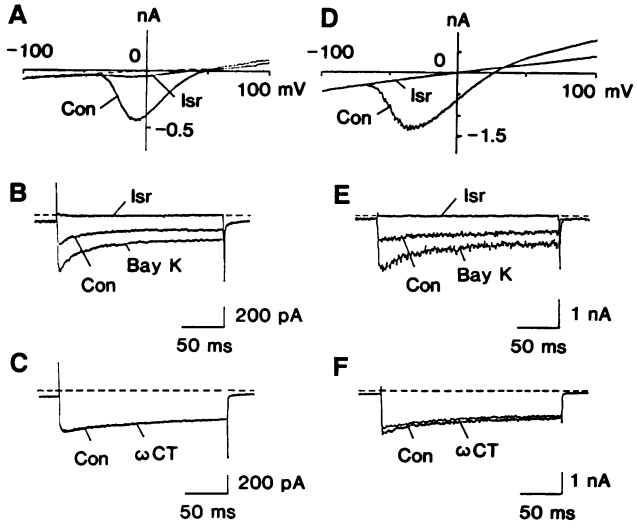

FIG. 1. Characterization of I_{Ca} carried by Ba^{2+} (*A–C*) or by Na^+ (*D–F*) in GH_3 cells. Cells were held at −80 mV and linear voltage-ramp pulses at 0.67 V/s from −100 mV to +100 mV (*A* and *D*) or depolarizing step pulses to 0 mV (*B* and *C*) and to −50 mV (*E* and *F*) were applied. The stimulation frequency was 0.3 Hz. Currents were recorded in the absence (traces Con) and presence of (+)-isradipine (1 μM, traces Isr), Bay K 8644 (1 μM, traces Bay K), or ωCT (10 μM). In *B* and *E*, Bay K 8644 was added first and washed out before application of (+)-isradipine. The effects of Bay K 8644 and (+)-isradipine were completely reversible upon wash-out (data not shown). The dotted lines in the *IV* relationships represent the assumed leak conductances, visually extrapolated from negative voltages through the zero voltage level; in the current traces they indicate the zero current level.

-311 ± 79 to -27 ± 36 pA ($n = 7$) with solution E2 and from -1459 ± 838 to -96 ± 214 pA ($n = 5$) with solution E3; see Fig. 1]. Bay K 8644 stimulated the Ba^{2+} current by $62 \pm 26\%$ ($n = 8$) and the Na^+ current by $87 \pm 27\%$ ($n = 6$). Under both conditions, Bay K 8644 accelerated the time course of inactivation (see ref. 35). All (+)-isradipine and Bay K 8644 effects were fully reversed by washing out the drugs. In contrast to other cells of endocrine or neuronal origin (for review, see ref. 34), GH_3 cells showed no reduction of I_{Ca} after application of ωCT (1–2 min, $n = 6$).

Effect of TRH on I_{Ca}. In a first series of experiments, we tested the TRH effect on I_{Ca} in GH_3 cells superfused with E2 (i.e., under conditions allowing the filling of Ca^{2+} stores with divalent cations). In the representative time course shown in Fig. 2*B*, TRH (1 μM) was applied in three successive phases separated by 45-s agonist-free intervals. The strongest inhibition of the Ba^{2+}-carried I_{Ca} was seen during the first application; the current was reduced from about -430 to -150 pA. In 10 cells, we determined the mean reduction to $55 \pm 20\%$. The TRH-induced inhibition could be fully reversed by washing out the agonist. Further TRH applications produced smaller reductions; the second and third TRH superfusion suppressed I_{Ca} by $19 \pm 9\%$ ($n = 8$) and by $15 \pm 8\%$ ($n = 3$), respectively. Prolonged intervals between the TRH applications led to a more pronounced recovery of its inhibitory action, suggesting the involvement of a Ca^{2+} release from internal stores that must be refilled. At 10 nM TRH, the reduction of I_{Ca} amounted to $34 \pm 10\%$ and $18 \pm 7\%$ during the first and second application, respectively ($n = 3$). TRH shifted the maximum of the *IV* relation obtained during ramp pulses, by about 10 mV toward more negative potentials (see Fig. 4*A*).

We next focused on the TRH (1 μM) action on I_{Ca} under a condition where the internal stores could not be refilled [i.e., with solution E3 (Fig. 3)]. As expected from the fura-2 measurements and in line with the assumption that an internal Ca^{2+} release can induce a Ca^{2+} channel inhibition, the first application of TRH strongly reduced the Na^+ current through Ca^{2+} channels (see Fig. 3, traces a–c). The mean reduction amounted to $60 \pm 20\%$ ($n = 5$) and was very similar to that observed with solution E2.

In contrast, a stimulatory action on I_{Ca} became evident when TRH was superfused for a second time (Fig. 3, traces

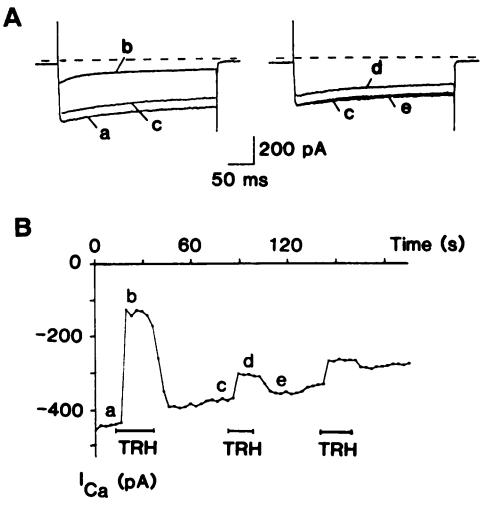

FIG. 2. Effect of 1 μM TRH on Ba^{2+}-carried I_{Ca} in GH_3 cells under external solution E2. (*A*) Superimposed current traces (a–e) defined in the time course of *B*. Currents were measured as maximal inward currents during test pulses from -80 mV to 0 mV (stimulatory frequency, 0.3 Hz). Recording of currents was started after 5 min of intracellular infusion of pipette solution containing 10 mM EGTA. The presence of hormone is indicated by horizontal bars.

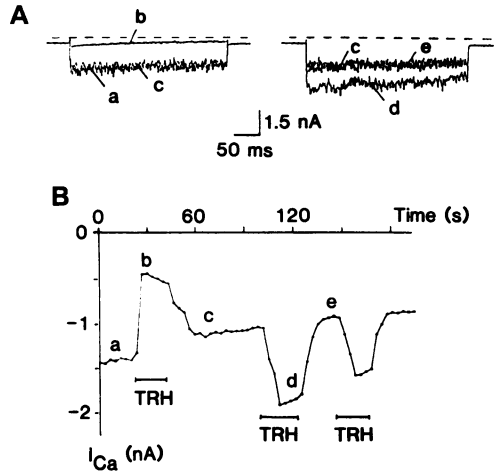

FIG. 3. Effect of 1 μM TRH on Na^+-carried I_{Ca} in GH_3 cells under external Ca^{2+}-depleted solution E3. (*A*) Superimposed current traces (a–e) defined in the time course of *B*. The cell was incubated in solution E3 for 5 min and intracellularly dialyzed with pipette solution containing 10 mM EGTA for 8 min prior to the experiment. Currents were measured as maximal inward currents during test pulses from -80 mV to -50 mV (stimulatory frequency, 0.3 Hz). The presence of hormones is indicated by horizontal bars.

c–e). The current was augmented by $43 \pm 23\%$ on the average ($n = 5$). On further applications (up to four times), the stimulation was reproducible and of similar quantity. The stimulatory effect was accompanied by a shift of the threshold potential and of the potential, where the maximum occurred, by about 5 mV and 10 mV, respectively, in the hyperpolarizing direction. The reversal potential was unchanged (Figs. 4*A* and 5*D*). To further characterize the current stimulated by TRH, we used 200 nM nimodipine, which reportedly fails to affect the Na^+ current through low-threshold (T-type) Ca^{2+} channels in pituitary GH_3 cells (31). Nimodipine reversibly inhibited the current measured by the voltage-ramp pulse protocol to $12 \pm 4\%$ ($n = 4$) of the control. This nimodipine-insensitive current was unaffected by 1 μM TRH ($13 \pm 4\%$ of the control, $n = 4$), suggesting an involvement of dihydropyridine-sensitive Ca^{2+} channels in TRH-induced current stimulation.

If GH_3 cells were incubated for 50–70 min in solution E3 prior to the experiment, the stimulation occurred on the first TRH application (Fig. 4*A*). Intracellular application of BAPTA (5 mM; infusion time, 5–8 min), which is supposed to chelate Ca^{2+} more efficiently than EGTA (36), inverted the inhibitory TRH effect to a stimulatory TRH effect even with solution E2. On first application of TRH, the stimulation was preceded by a slight transient inhibition of I_{Ca}. The transient inhibition was absent on further TRH applications. TRH (1 μM) increased the Ba^{2+}-carried current by $25 \pm 24\%$ on average ($n = 9$; Fig. 4*B*).

LHRH (1 μM) and ATII (1 μM) also induced stimulation of I_{Ca} by $26 \pm 12\%$ ($n = 5$) and $23 \pm 11\%$ ($n = 5$), respectively, with solution E3 (Figs. 4*C* and 5*D*).

Involvement of Guanine Nucleotide-Binding Regulatory (G) Proteins in the Hormonal Effects. Like other receptors for releasing hormones, the TRH receptor interacts with its effectors through G proteins (37–39). Therefore, we tested whether the TRH effects on Ca^{2+} channels were sensitive to guanine 5′-[β-thio]diphosphate (GDP[βS]), the GDP analog that stabilizes all G proteins in their inactive form, and to PT, which by ADP-ribosylation of G-protein α subunits prevents coupling of activated receptors to G proteins of the G_i and the G_o G-protein families (37). Intracellular infusion of GDP[βS] (100 μM, for about 8 min) by the patch pipette completely abolished both the inhibitory (with solution E2, Fig. 5*B*) and

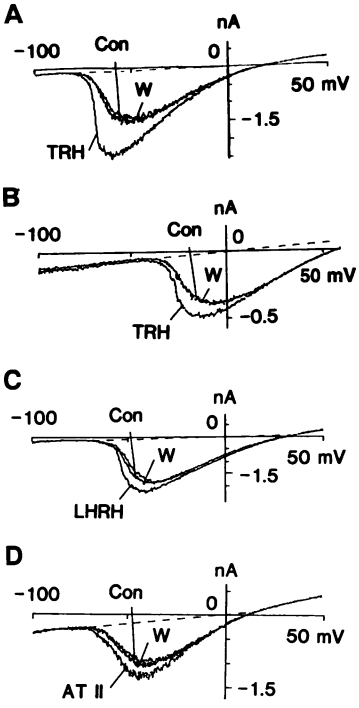

FIG. 4. Effects of TRH, LHRH, and ATII on I_{Ca} in GH_3 cells. (*A*) Effect of the first TRH application on the Na^+-carried I_{Ca} in a GH_3 cell incubated in solution E3 for 50 min prior to the experiment. (*B*) Effect of TRH on the Ba^{2+}-carried I_{Ca} in a GH_3 cell intracellularly dialyzed (5–9 min) with the Ca^{2+}-chelator BAPTA (5 mM) instead of EGTA. (*C* and *D*) Effects of LHRH and ATII on Na^+-carried I_{Ca} in GH_3 cells prestimulated with TRH. TRH (1 μM) was applied and washed out before addition of LHRH or ATII. Traces: Con, control *IV* relationship before application of the hormone; TRH, LHRH, and ATII, *IV* relationships recorded in the presence of 1 μM TRH, 1 μM LHRH, and 1 μM ATII, respectively; W, *IV* relationship recorded after removing the hormone from the bath.

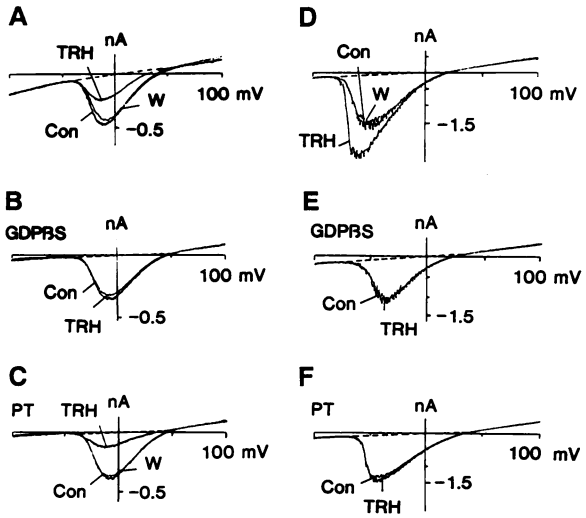

FIG. 5. Effects of GDP[βS] and PT on the TRH-induced hormonal modulation of I_{Ca} in GH_3 cells. (*A–C*) Effect of the first TRH application on *IV* relationships of Ba^{2+}-carried I_{Ca} of cells incubated in E2 and intracellularly dialyzed with pipette solution containing 10 mM EGTA for 5–7 min. (*D–F*) Effect of the second application of TRH on Na^+-carried I_{Ca} of cells incubated in E3 and intracellularly dialyzed with pipette solution containing 10 mM EGTA for 5–8 min. (*B* and *E*) Currents were recorded after 5–10 min of intracellular infusion with 100 μM GDP[βS]. (*C* and *F*) Cells were pretreated with PT (25 ng/ml) for 24 hr. Traces: Con, control *IV* relationship; TRH, *IV* relationship recorded in the presence of 1 μM TRH; W, *IV* relationship recorded after removal of TRH from the bath. Intracellular GDP[βS] application or PT pretreatment had no effect on the control currents carried by Ba^{2+} or Na^+.

the stimulatory (with solution E3, second application, Fig. 5*E*) effects of TRH [$-9 \pm 8\%$ ($n = 3$) and $+5 \pm 9\%$ ($n = 10$), respectively]. In contrast, although PT pretreatment of GH_3 cells did not abolish the TRH-induced current inhibition [with solution E2 and E3, TRH reduced the currents by $37 \pm 12\%$ ($n = 5$) and $41 \pm 15\%$ ($n = 10$) respectively; see Fig. 5*C*], it fully blocked the stimulation of the Na^+ current through Ca^{2+} channels by TRH (solution E3, $n = 6$; see Fig. 5*F*).

DISCUSSION

The present findings demonstrate that, in pituitary cells, hormone-releasing factors, including TRH, LHRH, and ATII, modulate intracellular free Ca^{2+} through multiple mechanisms. If the Ca^{2+} stores are filled, hormones induce a PT-insensitive inositol trisphosphate-mediated Ca^{2+} release (see refs. 11, 40, and 41); if they are empty, the releasing factors stimulate voltage-gated Ca^{2+} channels in a PT-sensitive manner (see also ref. 24).

The Ca^{2+} release was found to coincide with a Ca^{2+} current inhibition (see also refs. 17 and 18). A Ca^{2+}-mediated inhibition of voltage-dependent Ca^{2+} channels has previously been suggested. Injection of Ca^{2+} into *Helix aspera* neurons (42), Ca^{2+} dialysis of isolated ventricular cardiocytes (43), and photorelease of Ca^{2+} in dorsal root ganglion neurons (44) reversibly reduce the amplitude of Ca^{2+} currents. Furthermore, agents like caffeine and capsaicin, which liberate Ca^{2+} from internal stores (45, 46), can also decrease Ca^{2+} currents (ref. 46; J.H., unpublished observation). Our data add the possibility that hormones releasing endogenous Ca^{2+} from internal stores by an inositol trisphosphate-dependent mechanism (10, 19, 39) may also induce a Ca^{2+}-dependent inhibition of Ca^{2+} channels (see also refs. 17 and 18).

The nature of this Ca^{2+}-mediated process, which apparently is partially activated by Ba^{2+} and other divalent cations (46), remains unclear. Since photolytically released Ca^{2+} induces a Ca^{2+} current inhibition in the millisecond range, a binding site closely associated with the inner mouth of the Ca^{2+} channel has been suggested (44). The present results clearly demonstrate that cell dialysis with 10 mM EGTA was insufficient to suppress the Ca^{2+}-mediated inhibition of the Ca^{2+} current. Furthermore, the stimulatory TRH effect became evident on intracellular application of BAPTA. These results are in line with findings of Marty and Neher (36) indicating that, in chromaffin cells, EGTA-buffered internal solutions were unable to suppress Ca^{2+}-dependent K^+ currents in contrast to BAPTA buffers. This difference is most likely due to BAPTA having the faster association and dissociation rates than EGTA (37). Thus, Ca^{2+} may interact rapidly with its intracellular binding site(s) responsible for the Ca^{2+}-mediated inhibition of Ca^{2+} channels. It can be speculated that (*i*) Ca^{2+} stores and Ca^{2+} channels are in close proximity (47–49) or that (*ii*) the released Ca^{2+} may activate a Ca^{2+}/calmodulin-dependent protein kinase (50) that phosphorylates the Ca^{2+} channel.

Furthermore, the present experiments show that Ca^{2+} released during the first TRH administration is not able to refill the internal stores. Under these conditions, we observed a stimulation of voltage-dependent dihydropyridine-sensitive Ca^{2+} channels that may imply a refilling mechanism for internal Ca^{2+} stores. Such a Ca^{2+} channel stimulation was found to be induced in adrenocortical cells by ATII (51, 52) and in GH_3 cells by LHRH (23). The ability of these factors to enhance Ca^{2+} currents carried by Ba^{2+} in EGTA-dialyzed cells points to a less marked stimulation of the phosphatidylinositol 4,5-bisphosphate hydrolysis by LHRH and ATII than by TRH. Indeed, we found that LHRH and ATII

induced lower Ca^{2+} spikes than TRH. In analogy to the present data, the stimulatory effect of LHRH on the Ba^{2+}-carried I_{Ca} was PT-sensitive but did not depend on the intracellular cAMP level (23).

It has to be assumed that TRH receptors couple to more than one G protein. A PT-sensitive G protein, presumably of the G_i family (see ref. 52), may mediate the stimulation of voltage-gated Ca^{2+} channels, whereas a PT-insensitive G protein may couple to the phospholipase C. This diversity is also in line with the biochemical finding that TRH stimulates PT-sensitive and PT-insensitive high-affinity GTPases in membranes of GH_3 cells (53). Further experiments should be performed to specify the PT-sensitive and -insensitive G proteins mediating Ca^{2+} entry and Ca^{2+} release, respectively.

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1. Schlegel, W., Roduit, C. & Zahnd, G. R. (1984) *FEBS Lett.* **168,** 54–59.
2. Rebecchi, M. J. & Gershengorn, M. C. (1983) *Biochem. J.* **216,** 287–294.
3. Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306,** 67–68.
4. Gershengorn, M. C., Geras, E., Purrello, V. S. & Rebecchi, M. J. (1984) *J. Biol. Chem.* **259,** 10675–10681.
5. Winiger, B. P. & Schlegel, W. (1988) *Biochem. J.* **255,** 161–167.
6. Stoijkovic, S. S., Iida, T., Virmani, M. A., Izumi, S.-I., Rojas, E. & Catt, K. J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8855–8859.
7. Tan, K. N. & Tashjian, J. A. H. (1981) *J. Biol. Chem.* **256,** 8994–9002.
8. Schlegel, W., Winiger, B. P., Mollard, P., Vacher, P., Wuarin, F., Zahnd, G. R., Wollheim, C. B. & Dufy, B. (1987) *Nature (London)* **329,** 719–721.
9. Davidson, J. S., Wakefield, I. K., King, J. A., Mulligan, G. P. & Millar, R. P. (1988) *Mol. Endocrinol.* **2,** 382–390.
10. Kuan, S. I., Login, I. S., Judd, A. S. & MacLeod, R. M. (1990) *Endocrinology* **127,** 1841–1848.
11. Mollard, P., Vacher, P., Dufy, B., Winiger, B. P. & Schlegel, W. (1988) *J. Biol. Chem.* **263,** 19570–19576.
12. Dubinsky, J. M. & Oxford, G. S. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4282–4286.
13. Ingram, C. D., Bicknell, R. J. & Mason, W. T. (1986) *Endocrinology* **119,** 2508–2518.
14. Sartor, P., Dufy-Barbe, L., Corcuff, J. B., Taupignon, A. & Dufy, B. (1990) *Am. J. Physiol.* **258,** E311–E319.
15. Ritchie, A. (1987) *J. Physiol. (London)* **385,** 611–625.
16. Bauer, C., Meyerhof, W. & Schwarz, R. (1990) *J. Physiol.* **429,** 169–189.
17. Yamashita, N., Takuwa, Y. & Ogata, E. (1985) *J. Membr. Biol.* **87,** 241–247.
18. Levitan, E. S. & Kramer, R. H. (1990) *Nature (London)* **348,** 545–547.
19. Tan, K. N. & Tashjian, A. H., Jr. (1984) *J. Biol. Chem.* **259,** 427–434.
20. Naor, Z., Capponi, A. M., Rossier, M., Ayalon, D. & Limor, R. (1988) *Mol. Endocrinol.* **2,** 512–520.
21. Hess, P., Lansman, J. B. & Tsien, R. (1986) *J. Gen. Physiol.* **88,** 293–319.
22. Lux, H. D., Carbone, E. & Zucker, H. (1990) *J. Physiol. (London)* **430,** 159–188.
23. Rosenthal, W., Hescheler, J., Hinsch, K.-D., Spicher, K., Trautwein, W. & Schultz, G. (1988) *EMBO J.* **7,** 1627–1633.
24. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
25. Belles, B., Malecot, C. O., Hescheler, J. & Trautwein, W. (1988) *Pflügers Arch.* **411,** 353–360.
26. Fenwick, E. M., Marty, A. & Neher, E. (1982) *J. Physiol. (London)* **331,** 599–635.
27. Korbmacher, C., Helbig, H., Haller, H., Erickson-Lamy, K. A. & Wiederholt, M. (1989) *Biochem. Biophys. Res. Commun.* **164,** 1031–1039.
28. Grynkiewicz, G., Poenie, M. & Tsien (1985) *J. Biol. Chem.* **260,** 3440–3450.
29. Brostrom, C. O. & Brostrom, M. (1990) *Annu. Rev. Physiol.* **52,** 577–590.
30. Kalman, D., O'Lague, P., Erxleben, C. & Armstrong, D. L. (1988) *J. Gen. Physiol.* **92,** 531–548.
31. Suarez-Kurtz, G., Katz, G. M. & Reuben, J. P. (1987) *Pflügers Arch.* **410,** 345–347.
32. Bean, B. P. (1989) *Nature (London)* **340,** 153–156.
33. Kostyuk, P. G., Mironov, S. L. & Shuba, Y. M. (1983) *J. Membr. Biol.* **76,** 83–93.
34. Bean, B. P. (1989) *Annu. Rev. Physiol.* **51,** 367–368.
35. Hess, P., Lansman, J. B. & Tsien, R. W. (1984) *Nature (London)* **311,** 538–544.
36. Marty, A. & Neher, E. (1985) *J. Physiol. (London)* **367,** 117–141.
37. Birnbaumer, L., Abramowitz, J. & Brown, A. M. (1990) *Biochim. Biophys. Acta* **1031,** 163–224.
38. Wojcikiewicz, R. J. H., Kent, P. A. & Fain, J. N. (1986) *Biochem. Biophys. Res. Commun.* **138,** 1383–1389.
39. Aub, D. L., Frey, E. A., Sekura, R. D. & Cote, T. E. (1986) *J. Biol. Chem.* **261,** 9333–9340.
40. Schlegel, W., Wuarin, F., Zbaren, C., Wollheim, C. B. & Zahnd, G. R. (1985) *FEBS Lett.* **189,** 27–32.
41. Martin, T. F. J., Bajjalieh, S. M., Lucas, D. O. & Kowalchyk, J. A. (1986) *J. Biol. Chem.* **261,** 10041–10049.
42. Plant, T. D., Standen, N. B. & Ward, T. A. (1983) *J. Physiol. (London)* **334,** 189–212.
43. Kokubun, S. & Irisawa, H. (1984) *Jpn. J. Physiol.* **34,** 599–611.
44. Morad, M., Davies, N. W., Kaplan, J. H. & Lux, H. D. (1988) *Science* **241,** 842–844.
45. Rousseau, E. & Meissner, G. (1989) *Am. J. Physiol.* **256,** H328–H333.
46. Bleakman, D., Brorson, J. R. & Miller, R. J. (1990) *Br. J. Pharmacol.* **101,** 423–431.
47. Charbonneau, M. & Grey, R. D. (1984) *J. Biol. Chem.* **102,** 90–97.
48. Muallem, S., Fimmel, C. J., Pandol, S. J. & Sachs, G. (1986) *J. Biol. Chem.* **261,** 2660–2667.
49. Putney, J. W. (1986) *Cell Calcium* **7,** 1–12.
50. Sakakibara, M., Alkon, D. L., DeLorenzo, R., Goldenring, J. R., Neary, J. T. & Heldman, E. (1986) *Biophys. J.* **50,** 319–327.
51. Cohen, C. J., McCarthy, R. T., Barrett, P. Q. & Rasmussen, H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2412–2416.
52. Hescheler, J., Rosenthal, W., Hinsch, K. D., Wulfern, M., Trautwein, W. & Schultz, G. (1988) *EMBO J.* **7,** 619–624.
53. Offermanns, S., Schultz, G. & Rosenthal, W. (1989) *Eur. J. Biochem.* **180,** 283–287.