Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6265–6269, July 1993
Cell Biology

G_{i2} and protein kinase C are required for thyrotropin-releasing hormone-induced stimulation of voltage-dependent Ca^{2+} channels in rat pituitary GH_3 cells

(cytoplasmic calcium/G proteins/pertussis toxin/signal transduction/antisense oligonucleotides)

MAIK GOLLASCH*†, CHRISTIANE KLEUSS‡, JÜRGEN HESCHELER*, BURGHARDT WITTIG‡, AND GÜNTER SCHULTZ*§

Institute für *Pharmakologie und ‡Molekularbiologie und Biochemie, Freie Universität Berlin, D-1000 Berlin 33, Federal Republic of Germany; and †Institut für Physiologie, Humboldt-Universität, D-1040 Berlin, Federal Republic of Germany

Communicated by Norman Davidson, March 22, 1993 (received for review January 20, 1993)

ABSTRACT **In rat pituitary GH_3 cells, thyrotropin-releasing hormone (TRH) and other secretion-stimulating hormones trigger an increase in the cytosolic Ca^{2+} concentration by two mechanisms. Ca^{2+} is released from intracellular stores in response to inositol 1,4,5-trisphosphate and can enter the cell through voltage-dependent L-type Ca^{2+} channels. Stimulation of these channels is sensitive to pertussis toxin, indicating that a pertussis toxin-sensitive heterotrimeric guanine nucleotide-binding regulatory protein (G protein) is involved in functional coupling of the receptor to the Ca^{2+} channel. We identified the G protein involved in the stimulatory effect of TRH on the Ca^{2+} channel by type-selective suppression of G-protein synthesis. Antisense oligonucleotides were microinjected into GH_3 cell nuclei, and 48 h after injection the TRH effect was tested. Whereas antisense oligonucleotides hybridizing to the mRNA of G_o or G_{i1} α-subunit sequences did not affect stimulation by TRH, oligonucleotides suppressing the expression of the G_{i2} α subunit abolished this effect, and oligonucleotides directed against the mRNA of the G_{i3} α subunit had less effect. The requirement of a concurrent inositol phospholipid degradation and subsequent protein kinase C (PKC) activation for the TRH effect on Ca^{2+}-channel activity was demonstrated by inhibitory effects of antisense oligonucleotides directed against $G_q/G_{11}/G_z$ α-subunit sequences and treatment of GH_3 cells with PKC inhibitors, respectively. Our results suggest that TRH elevates the cytosolic Ca^{2+} concentration in GH_3 cells transiently via Ca^{2+} release from internal stores, followed by a phase of sustained Ca^{2+} influx through voltage-dependent Ca^{2+} channels stimulated by the concerted action of G_{i2} (and G_{i3}) plus PKC.**

Thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), angiotensin II (A-II), and vasopressin are hormones that stimulate hormone secretion in pituitary and other endocrine cells by elevating the cytosolic Ca^{2+} concentration (1–3). In addition to stimulation of inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from internal stores, which induces a transient increase in cytosolic Ca^{2+} (4, 5), these hormones stimulate voltage-dependent Ca^{2+} channels in the plasma membrane (6–12), thereby inducing sustained Ca^{2+} entry from the extracellular space into the cells (1, 2, 13, 14).

The two transduction pathways involve different heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). G proteins insensitive to the main exotoxin of *Bordetella pertussis*, pertussis toxin (PT), probably belonging to the G_q family (G_q, G_{11}) (15), functionally couple the receptors to phospholipase C-β, which catalyzes phosphatidylinositol 4,5-bisphosphate hydrolysis. The immediate products of this reaction, IP_3 and 1,2-diacylglycerol, act as the second messengers at the beginning of a bifurcating signal transduction pathway to induce (*i*) Ca^{2+} mobilization from intracellular storage pools and (*ii*) activation of protein kinase C (PKC) (16). Although some authors (17, 18) have suggested the involvement of PKC for regulation of sustained Ca^{2+} entry in the presence of release-inducing factors, the importance of hormonally activated PKC in the regulation of Ca^{2+} channels remains unclear, since activation of PKC by phorbol esters inhibited Ca^{2+} currents in rat pituitary GH_3 cells (19) and other endocrine and neuroendocrine cells (20, 21). Moreover, stimulation of voltage-dependent Ca^{2+} channels by TRH, LHRH, and A-II involves G proteins sensitive to PT (7, 8, 10).

Membranes of pituitary and other endocrine cells contain several PT-sensitive G proteins that belong to the G_i and G_o families. G_{o1}, G_{o2}, G_{i2}, and G_{i3} have been detected in GH_3 cells (8, 22, 23). Although activated forms of G_i and G_o were identified by affinity labeling with azidoanilido-[α-^{32}P]GTP after stimulation with agonists inducing Ca^{2+}-channel stimulation and inhibition, respectively (ref. 22; and S. Offermanns, personal communication), the specific G proteins mediating hormonal Ca^{2+}-channel stimulation are not known.

Here we report on the identification of the G proteins involved in the TRH-induced stimulation of Ca^{2+} channels in GH_3 cells. We used intranuclear injection of antisense oligonucleotides specifically hybridizing with mRNAs of G_i and G_o α-subunit sequences to suppress the expression of these proteins (24, 25). We show that G_{i2} is the main PT-sensitive G protein involved in hormonal Ca^{2+}-channel stimulation and that this pathway requires concurrent PKC activation mediated by PT-insensitive G proteins, as revealed by the use of corresponding antisense oligonucleotides and PKC inhibitors.

MATERIALS AND METHODS

Rat pituitary GH_3 cells were obtained from the American Type Culture Collection and cultured as described (10). Two

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: G protein, heterotrimeric guanine nucleotide-binding regulatory protein; TRH, thyrotropin-releasing hormone; LHRH, luteinizing hormone-releasing hormone; A-II, angiotensin II; PT, pertussis toxin (main exotoxin of *Bordetella pertussis*); IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; G_i and G_s, inhibitory and stimulatory G proteins for adenylyl cyclases; G_o, G protein mediating inhibition of Ca^{2+} channels; G_q and G_{11}, G proteins mediating stimulation of phospholipase C-β.

§To whom reprint requests should be addressed at: Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69-73, D-1000 Berlin 33, F.R.G.

days prior to microinjection, cells (passages 30–48) were seeded at a density of about 10^3 cells per mm^2 on glass slides imprinted with numbered squares for convenient localization of the cells intranuclearly injected with antisense oligonucleotides. The sequences of the oligonucleotides used were determined by sequence comparison and multiple alignment with the MACMOLLY TETRA software (Soft Gene, Berlin). Oligonucleotides were synthesized in a DNA synthesizer (Milligen model 8600) or purchased (TIB Molbiol, Berlin). Injections of oligonucleotides (5 μM solution in water) were performed with an automated injection system (24). The calculated injected volume was 10 to 20 fl, which contained 10,000–20,000 full-length oligonucleotides. After incubation in culture medium for 48 h, glass slides with injected cells were transferred to a perfusion chamber (volume of 200 μl, perfusion rate of 5 ml/min) for electrophysiological determinations.

Whole-cell membrane currents were measured according to the method of Hamill *et al.* (26). The extracellular solution in the perfusion chamber 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). After obtaining the whole-cell configuration, the external solution was changed to a nominally Ca^{2+}-free solution 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). Since apparent TRH effects on Ca^{2+} channels strongly depend on intracellular Ca^{2+}, a nominally Ca^{2+}-free solution was used, which does not allow refilling of internal Ca^{2+} stores depleted by first exposure of cells to the hormone (10, 13). Use of this solution makes it possible to study the multiple mechanisms by which TRH modulates voltage-dependent Ca^{2+} channels (10). Under these conditions, currents through Ca^{2+} channels occurred as monovalent ion fluxes. The patch pipettes (average resistance of 1–1.5 MΩ) were filled with a solution containing 120 mM CsCl, 3 mM $MgCl_2$, 5 mM MgATP, 10 mM EGTA, and 10 mM Hepes/CsOH (pH 7.4 at 37°C). For estimation of current–voltage relations of Na^+ currents carried through high-threshold, dihydropyridine-sensitive (L-type) Ca^{2+} channels, cells were held at −80 mV, and every 3 s the potential was linearly altered from −100 to +100 mV at 0.67 V/s. The holding potential of −80 mV was sufficient to fully inactivate currents through low-threshold (T-type) Ca^{2+} channels. The cells exhibited a U-shaped Ca^{2+} channel current with a maximum of 1.77 ± 0.55 nA (mean ± SEM; n = 36) at about −40 mV. The apparent threshold occurred at −70 mV, and the reversal potential was at 25 mV. The current amplitudes corresponded to those measured during step pulses to the respective potentials.

Peak Ca^{2+} channel currents were determined as maximal inward currents in reference to the assumed leak conductance. The assumed leak conductance (1.8 ± 2.4 nS, mean ± SEM; n = 56) was extrapolated from measured currents at test potentials of −80 mV and −100 mV and subtracted from the measured current–voltage relation. Only those experiments in which the unspecific conductance remained unchanged were analyzed. Recording of currents was usually started 5 min after disruption of the membrane patch for intracellular dialysis with the pipette solution. The membrane capacity amounted to 15.8 ± 5.1 pF (n = 31). Basal Ca^{2+}-channel currents and membrane capacities were not affected by microinjection of any oligonucleotide used.

TRH, H-7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], and staurosporine were obtained from Sigma. Calphostin C was purchased from Calbiochem.

RESULTS

TRH modulates the cytoplasmic free Ca^{2+} through multiple mechanisms. If Ca^{2+} stores were filled, TRH (1 μM) induced an IP_3-mediated, PT-insensitive Ca^{2+} release; if they were empty, the hormone stimulated voltage-dependent Ca^{2+} channels in a PT-sensitive manner (10). During the first TRH application to cells incubated in Ca^{2+}-containing medium or in nominally Ca^{2+}-free external solution, the Ca^{2+} release caused an inhibition of the Ca^{2+} current, presumably due to Ca^{2+}-dependent Ca^{2+} channel inhibition. In cells incubated in the nominally Ca^{2+}-free external solution, the Ca^{2+} release during the first TRH administration is not sufficient for refilling of the internal stores (10, 13); during further applications of TRH, we were able to observe the PT-sensitive stimulation of dihydropyridine-sensitive (L-type) Ca^{2+} channels (Fig. 1*A*).

Membranes of pituitary GH_3 cells contain several PT-sensitive G proteins; α subunits of G_{i2}, G_{i3}, G_{o1}, and G_{o2} have been detected (22, 23). To determine the PT-sensitive G-protein type(s) involved in the current stimulation, we injected short antisense oligonucleotides, complementary to specific regions of RNAs encoding α subunits, to suppress selectively the synthesis of individual G-protein α subunits. After intranuclear injection of the antisense oligonucleotide i_{com}, which is complementary to a translated region of all mRNAs encoding G_i α subunits, stimulation of peak Ca^{2+} channel currents by TRH was abolished in a time-dependent manner

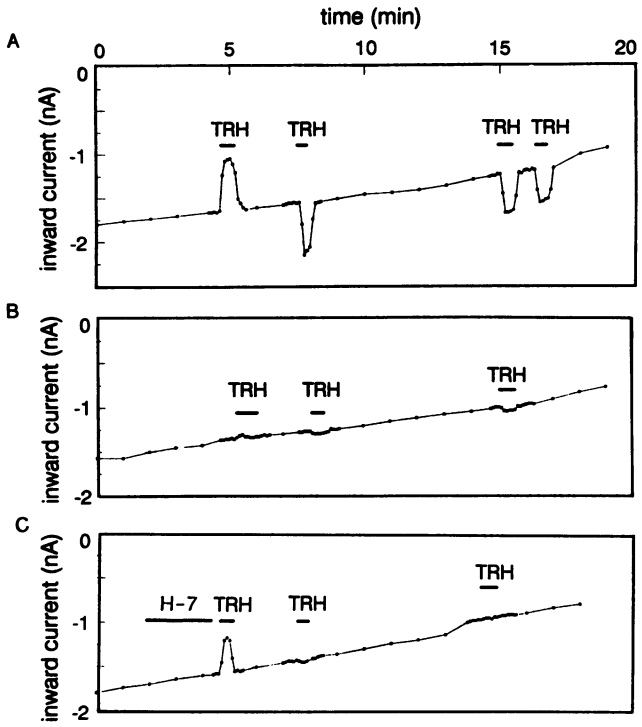

FIG. 1. Effect of TRH on Na^+-carried Ca^{2+}-channel currents in pituitary GH_3 cells. Time courses of Ca^{2+}-channel inward currents evoked by repetitive (0.3 Hz) linear voltage-ramp pulses at 0.67 V/s from −100 mV to +100 mV are shown. Peak currents were determined as maximal inward currents during the voltage pulses. (*A*) Noninjected GH_3 cell. (*B*) Cell injected with the antisense oligonucleotides tq, t11, and tz directed against mRNAs encoding α subunits of G_q, G_{11}, and G_z, respectively. (*C*) Cell pretreated with H-7 (10 μM for 3 min). Recording of currents was started 4–6 min after intracellular infusion of the pipette solution. The presence of TRH (1 μM) and H-7 (10 μM) is indicated by horizontal bars. tq (CAACCTCTC-GAACCAATTGTG) corresponds to nt 310–330 of the identical strand of the $G\alpha_q$ gene sequence (27) and is able to hybridize with the mRNA of $G\alpha_q$. t11 (GGAGTGCATTGGCCTTGTTCTGCT-CATAC) corresponds to nt 287–315 of the identical strand of the $G\alpha_{11}$ gene sequence (27) and is able to hybridize with the mRNA of $G\alpha_{11}$. tz (TCAGCGGGGCCCGTCAGCGCAAAGAGC) corresponds to nt 317–343 of the identical strand of the $G\alpha_z$ gene sequence (28) and is able to hybridize with the mRNA of $G\alpha_z$.

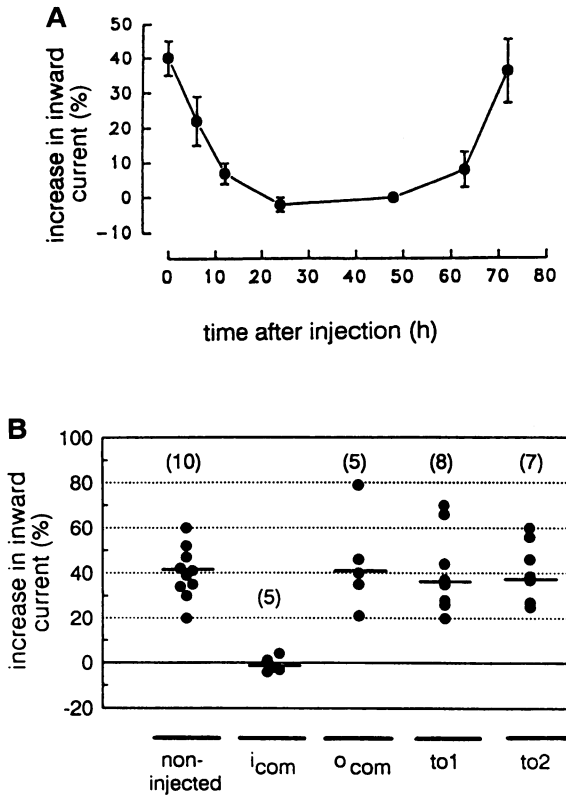

FIG. 2. Ca^{2+}-current stimulation by TRH in GH_3 cells injected with antisense oligonucleotides directed against mRNAs encoding α subunits of G proteins. Cells were prestimulated by short-term extracellular application of TRH (1 μM) in Ca^{2+}-free solution to eliminate inhibitory hormonal effects on currents depending on TRH-induced intracellular Ca^{2+} mobilization (compare with Fig. 1*A*). (*A*) Time course of current stimulation by TRH (1 μM) in GH_3 cells injected with the antisense oligonucleotide i_{com} at time point zero. The increase in current is shown as a percent of the peak current observed in the absence of the receptor agonist. Mean values ± SEM are shown. The number of cells tested at 0, 6, 12, 24, 48, 62, and 72 h was 10, 8, 3, 4, 5, 11, and 12, respectively. (*B*) Current stimulation by TRH (1 μM) in GH_3 cells 48 h after injection with antisense oligonucleotides. The lines represent the medians; the numbers enclosed in parentheses indicate the number of recorded cells. The injected oligonucleotides are indicated on the abscissa. The recording conditions were as described in the legend to Fig. 1. i_{com} (ARGTTSYKGTCGATCAT) corresponds to nt 51–67 of the identical strand of all known $G\alpha_i$ gene sequences (29). i_{com} is able to hybridize with the mRNAs of all known isoforms of $G\alpha_i$. Abbreviations for degenerate base positions are R (G or A), Y (T or C), K (G or T), and S (G or C). o_{com} (TGCGACTCACCACGTCACACAC) corresponds to nt 318–339 of the identical strand of the $G\alpha_o$ gene sequence (29). o_{com} is able to hybridize with the mRNAs of all known variants of $G\alpha_o$. to1 (AGGCAGCTGCATCTTCATAGGTGTT) corresponds to nt 907–930 of the identical strand of the $G\alpha_{o1}$ gene sequence (30). It is able to hybridize with the described $G\alpha_{o1}$ mRNA. to2 (GAGCCACAGCTTCTGTGAAGGCACT) corresponds to nt 907–930 of the identical strand of the $G\alpha_{o2}$ gene sequence (30). It is able to hybridize with the described $G\alpha_{o2}$ mRNA.

(Fig. 2*A*). Between 24 and 48 h, the peptide hormone was without effect (current increase by −2% ± 4%, n = 5, 48 h after injection of i_{com}), but after 72 h the response of Ca^{2+} channel currents to TRH was restored. As previously demonstrated in GH_3 cells, i_{com} was ineffective in suppressing Ca^{2+}-channel inhibitions via muscarinic and somatostatin receptors, which are mediated by G_{o1} and G_{o2}, respectively (24). Complete prevention of TRH-induced current stimulation by i_{com} was also seen using only 1/10th of the concentration of oligonucleotides routinely used in the present and previous studies (24, 25).

In contrast to i_{com}, injection of the oligonucleotide o_{com}, which is complementary to a translated region of all mRNAs encoding G_o α subunits, was ineffective in preventing TRH-induced Ca^{2+}-channel stimulation 48 h after injection (Fig. 2*B*). In line with this finding, injection of oligonucleotides that selectively target regions on the $G\alpha_{o1}$ (oligonucleotide to1) and $G\alpha_{o2}$ (oligonucleotide to2) mRNAs encoding the G_{o1} and G_{o2} α subunits, respectively, did not prevent the hormonal current stimulation. In GH_3 cells, oligonucleotides to1 and to2 have been demonstrated to abolish G_{o1}- and G_{o2}-mediated Ca^{2+}-channel inhibition through muscarinic and somatostatin receptors, respectively (24).

Our results suggest involvement of proteins of the G_i family in the stimulation of Ca^{2+} channels by TRH. To determine which G-protein subtype is involved, we injected oligonucleotides that are reverse complementary oriented to specific regions of mRNAs specifically encoding one subtype of G_i α subunit. An antisense oligonucleotide hybridizing with a translated region (oligonucleotide ti1) or a nontranslated region (oligonucleotide 5′i1) of the $G\alpha_{i1}$ mRNA did not affect the Ca^{2+}-channel stimulation by TRH (Fig. 3). This is in line with previous data (23) demonstrating that, in contrast to pituitary tissue, $G\alpha_{i1}$ mRNA is not detectable in the pituitary-derived GH_3 cell lines. Injection of the antisense oligonucleotide ti2, which is complementary to a translated region of $G\alpha_{i2}$, largely reduced the TRH-dependent Ca^{2+}-channel stimulation, suggesting involvement of G_{i2} in TRH-induced cur-

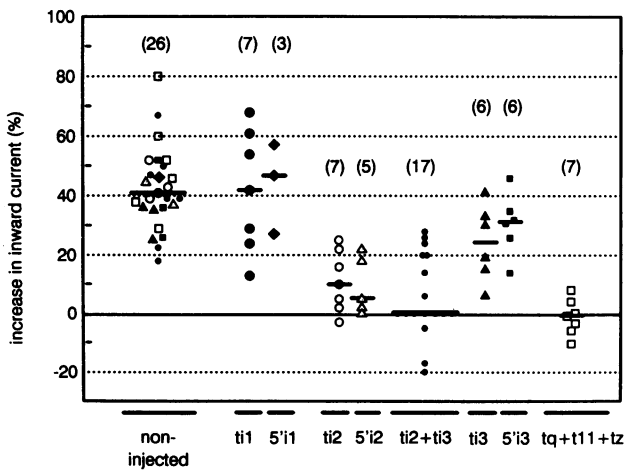

FIG. 3. Ca^{2+}-current stimulation by TRH in GH_3 cells injected with antisense oligonucleotides directed against mRNAs encoding α subunits of G proteins. The percent increase in peak Ca^{2+}-channel current by TRH (1 μM) in noninjected cells and in cells injected with the oligonucleotides indicated on the abscissa is shown. The lines represent the medians; the numbers enclosed in parentheses indicate the number of recorded cells. Identical symbols indicate identical groups of observations. The recording conditions were as described in the legend to Fig. 1. ti1 (CAGCAAGCACGAAGAGTTGGC) corresponds to nt 316–336 of the identical strand of the $G\alpha_{i1}$ gene sequence (29). It is able to hybridize with the described $G\alpha_{i1}$ mRNA. 5′i1 (GGTGGCCGAGCGTCGCCGCG) corresponds to nt −20 to −1 of the identical strand of the $G\alpha_{i1}$ gene sequence (29). It is able to hybridize with the described $G\alpha_{i1}$ mRNA. ti2 (CGGCAGCACAGGACAGTGCGAACAGC) corresponds to nt 317–342 of the identical strand of the $G\alpha_{i2}$ gene sequence (29). It is able to hybridize with the described $G\alpha_{i2}$ mRNA. 5′i2 (CCTGCCGTCCGCCGGCCCGG) corresponds to nt −20 to −1 of the identical strand of the $G\alpha_{i2}$ gene sequence (29). It is able to hybridize with the described $G\alpha_{i2}$ mRNA. ti3 (CAGCACTGCCAGCTAAAACAA) corresponds to nt 322–342 of the identical strand of the $G\alpha_{i3}$ gene sequence (29). It is able to hybridize with the described $G\alpha_{i3}$ mRNA. 5′i3 (GACGGCGGCCGGAGAGGGGA) corresponds to nt −20 to −1 of the identical strand of the $G\alpha_{i3}$ gene sequence (29). It is able to hybridize with the described $G\alpha_{i3}$ mRNA. The sequences of tq, t11, and tz are given in the legend to Fig. 1.

rent stimulation. The likely involvement of G_{i2} was confirmed by microinjecting the antisense oligonucleotide 5'i2, which is complementary to a 5' nontranslated region of the $G\alpha_{i2}$ mRNA and induced similar effects on the TRH effect as did ti2 (see Fig. 3). Intranuclear injection of antisense oligonucleotides that target the $G\alpha_{i3}$ mRNA (i.e., ti3 and 5'i3, which are complementary to translated and 5' nontranslated regions of the $G\alpha_{i3}$ mRNA, respectively) only slightly affected TRH stimulation. ti2 together with ti3 completely prevented the stimulatory effect of TRH on Ca^{2+}-channel currents. This suggests that G_{i2} and G_{i3} synergistically transduce the stimulatory hormonal signal to the channel.

The present data show that stimulation of pituitary, dihydropyridine-sensitive Ca^{2+} channels by TRH involves the widely distributed G_i subtype G_{i2}. G_{i2} is assumed to be involved in the receptor-induced inhibition of adenylyl cyclase (31). Release-inducing hormones, however, cause no or insignificant inhibition of adenylyl cyclase (32, 33). Instead, these secretion-stimulating hormones, via PT-insensitive G proteins of the G_q family (G_q, G_{11}; ref. 15), activate phospholipase C-β1 and cause hydrolysis of phosphatidylinositol 4,5-biphosphate to IP_3 and diacylglycerol with subsequent PKC activation. To determine whether TRH-induced Ca^{2+}-channel stimulation requiring G_{i2} activation is primed by simultaneous PKC activation, we studied the possible interference of antisense oligonucleotides against the α subunits of the PT-insensitive G proteins G_q, G_{11}, and G_z and of various inhibitors of PKC.

Simultaneous intranuclear injection of antisense oligonucleotides tq, t11, and tz, which are complementary to individual and translated regions of the $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_z$ mRNAs, respectively, suppressed both the IP_3-mediated current inhibition during the first TRH application (see Fig. 1*B*) and the stimulatory TRH action on the channels during further applications of the peptide hormone (see Fig. 1*B* and Fig. 2). This observation indicates that hormone-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol with subsequent PKC activation is required to allow G_{i2}-mediated Ca^{2+}-channel stimulation. It is likely that PKC represents the active component of the priming inositol phospholipid response and that G_{i2} or a Ca^{2+}-channel component may serve as a target for PKC action, allowing stimulatory G_i–Ca^{2+}-channel interaction. The assumption that PKC plays an essential role in Ca^{2+}-channel stimulation is supported by the finding that H-7 and staurosporine, specific inhibitors of PKC at the catalytic domain (34), completely suppressed the TRH-induced Ca^{2+} channel stimulation. When H-7 (10 μM) was extracellularly applied, the current inhibition by TRH persisted; in contrast, the subsequent enhancement observed during further application of the hormone was blocked (4% ± 2% current increase; n = 11) (see Fig. 1*C*). In the presence of staurosporine intracellularly infused at 10 μM via the patch pipette for 5 min, the current increase induced by TRH was 7% ± 5% (n = 5). Similar results were obtained with calphostin C, a specific inhibitor of PKC at the regulatory domain (34). In the presence of calphostin C intracellularly infused at 1 μM via the patch pipette for 5 min, the current was increased by 3% ± 2% (n = 5).

DISCUSSION

Our experiments show that G_i proteins are involved in TRH receptor-mediated stimulation of pituitary Ca^{2+} channels. The identification of proteins of the G_i family, mainly G_{i2}, in stimulatory effects on Ca^{2+} channels extends the general concept of multiple G-protein-mediated regulations of Ca^{2+} channels.

The first observed G-protein effect in the regulation of Ca^{2+} channels was that of G_s. In cardiac cells, G_s- and adenylyl cyclase-stimulating hormones increase dihydropyridine-sensitive (L-type) Ca^{2+} currents by a dual pathway; Ca^{2+}-channel activity is indirectly enhanced by phosphorylation of channel components by the catalytic subunit of cyclic AMP-dependent protein kinase and, more directly, by $G\alpha_s$–Ca^{2+}-channel interaction (35).

The essential role of G proteins of the G_i family in hormonal Ca^{2+}-channel stimulation was previously hypothesized, based on findings in adrenal cortical Y1 cells (7), where the stimulatory effect of A-II was PT-sensitive and the membranes contained PT-sensitive G proteins of the G_i type but no G_o. PT-sensitive Ca^{2+}-channel stimulation was also observed in GH_3 cells by TRH, LHRH, and A-II (8, 10) and in pheochromocytoma PC-12 cells by platelet-activating factor (M.G., unpublished data). Whereas G_{i2} occurs ubiquitously, additional G_{i3} expression was detected in membranes of GH_3 cells (23), and G_{i1} is expressed in PC-12 cells (36).

Identification of the involved G_i subtype became possible by intranuclear injection of antisense oligonucleotides to suppress the expression of individual G-protein subunits in

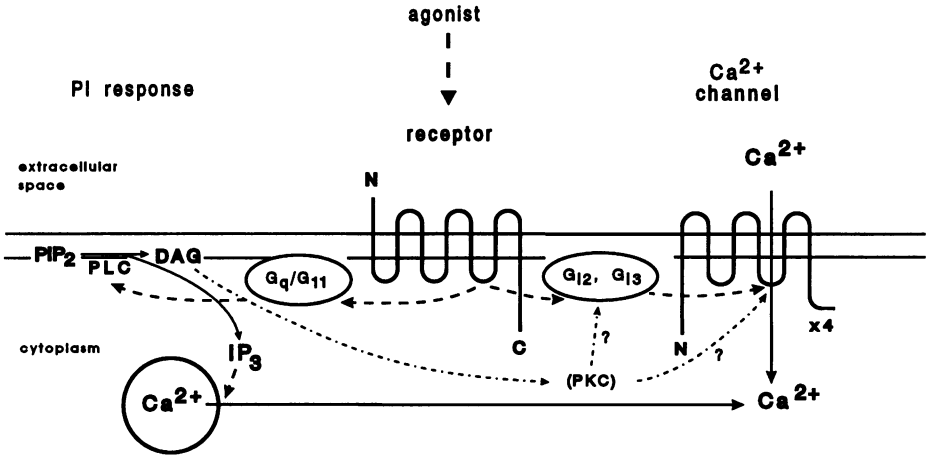

FIG. 4. Signal-transduction pathways involved in hormonal Ca^{2+}-channel stimulation. From the data presented, two pathways emerge to result in confluent regulation of the L-type Ca^{2+} channel in secretory cells. Whereas a PT-sensitive pathway appears to involve G_{i2}, PKC stimulation resulting from G_q/G_{11}-mediated activation of phospholipase C-β is likely to represent an additional permissive signal priming the Ca^{2+} channel for G_{i2} modulation. Whether PKC acts on G_{i2} or a Ca^{2+}-channel component and whether TRH acts through one or separate receptor subtypes remain to be determined. ×4 indicates four hexahelical repeats in the structure of the pore-forming α_1 subunit of the calcium channel. PI, inositol phospholipid; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol.

pituitary GH_3 cells (24, 25). This approach allowed us to show that stimulation of dihydropyridine-sensitive Ca^{2+} channels by TRH is mainly mediated by the widely distributed G_{i2} with a minor contribution of G_{i3}. G_{i2} is assumed to be involved in hormonal inhibition (e.g., by α_2-adrenergic agonists) of adenylyl cyclase (31). On the other hand, G_{i1} has been reported to couple somatostatin receptors of pituitary AtT-20 cells to adenylyl cyclase (37). Although G_{i3} can also be activated by α_2-adrenergic agonists, G_{i3} apparently does not contribute to inhibitions of adenylyl cyclase (38); $G\alpha_{i3}$ has been shown to activate a Na^+ channel in the epithelial cell line A6 (39) and to stimulate K^+ channels, including inwardly rectifying channels in atrial and pituitary cells as well as ATP-dependent K^+ channels in ventricular cells (40).

Secretion-inducing hormones cause no or insignificant inhibition of adenylyl cyclase (32, 33). Although 8-bromo-cAMP has been shown to stimulate L-type Ca^{2+} channels in chromaffin cells (41), release-inducing hormones stimulate Ca^{2+} channels independently of intracellular cyclic AMP levels (8). In addition to stimulation of Ca^{2+} channels in pituitary and other endocrine cells (6–12), TRH, LHRH, A-II, vasopressin, and platelet-activating factor induce a inositol phospholipid response with subsequent PKC activation (4, 18, 42, 43). Our data demonstrate that the TRH-induced Ca^{2+}-channel stimulation was abolished by antisense oligonucleotides preventing the expression of G proteins of the G_q family, which are presumably involved in the TRH-induced stimulation of phospholipase C-β. The involvement of PKC is additionally supported by the block of TRH-induced current stimulation by H-7, staurosporine, and calphostin C, which are relatively specific inhibitors of PKC. Staurosporine has also been reported to inhibit A-II-induced Ca^{2+}-channel current stimulation in adrenal bovine cells (44). Taken together, these results suggest an essential involvement of a $G_{q/11}$-mediated PKC effect on the TRH-induced stimulatory pathway through G_{i2} on the Ca^{2+} channel (Fig. 4). Whether TRH activates G_{i2} and $G_{q/11}$ via the same or different receptors has not been determined. A PKC-dependent step modifying G_{i2} appears to be possible as G_{i2} has been reported to be a target for phosphorylation by PKC (45). Whereas this possible PKC-induced modification of G_{i2} may interfere with the inhibitory effect of G_{i2} on adenylyl cyclase, the altered G_{i2} protein may exert a stimulatory effect on Ca^{2+} channels. On the other hand, a PKC-mediated modification of a Ca^{2+}-channel component may be necessary for G_{i2}–Ca^{2+}-channel interaction. Further studies are necessary to specify the PKC isoform involved in the hormone-induced channel stimulation and its exact role in G_{i2} or Ca^{2+}-channel phosphorylation.

We thank M. Bigalke, I. Reinsch, and W. Stamm for expert technical assistance and D. Lewinsohn for computer programming. This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

1. Albert, P. R. & Tashjian, A. H., Jr. (1984) *J. Biol. Chem.* **259**, 15350–15363.
2. Kuan, S. I., Login, I. S., Judd, A. S. & MacLeod, R. M. (1990) *Endocrinology* **127**, 1841–1848.
3. Stojilkovic, S. S., Iida, T., Virmani, M. A., Izumi, S.-I., Rojas, E. & Catt, K. J. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8855–8859.
4. Imai, A. & Gershengorn, M. C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8540–8544.
5. Mollard, P., Dufy, B., Vacher, P., Barker, J. L. & Schlegel, W. (1990) *Biochem. J.* **268**, 345–352.
6. Cohen, C. J., McCarthy, R. T., Barrett, P. Q. & Rasmussen, H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2412–2416.
7. Hescheler, J., Rosenthal, W., Hinsch, K. D., Wulfern, M., Trautwein, W. & Schultz, G. (1988) *EMBO J.* **7**, 619–624.
8. Rosenthal, W., Hescheler, J., Hinsch, K.-D., Spicher, K., Trautwein, W. & Schultz, G. (1988) *EMBO J.* **7**, 1627–1633.
9. Mollard, P., Vacher, P., Rogawski, M. A. & Dufy, B. (1988) *FASEB J.* **2**, 2907–2912.
10. Gollasch, M., Haller, H., Schultz, G. & Hescheler, J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10262–10266.
11. Thorn, P. & Petersen, O. H. (1991) *J. Membr. Biol.* **124**, 63–71.
12. Suzuki, N., Takagi, H., Yoshioka, T., Tanakadate, A. & Kano, M. (1992) *Biochem. Biophys. Res. Commun.* **187**, 529–536.
13. Shorte, S. L. & Schofield, J. G. (1991) *Mol. Cell. Endocrinol.* **79**, 167–176.
14. Törnquist, K. (1991) *Biochem. Biophys. Res. Commun.* **180**, 860–866.
15. Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H. & Ross, E. M. (1992) *J. Biol. Chem.* **267**, 8081–8088.
16. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341**, 197–205.
17. Dufy, B., Jaken, S. & Barker, J. L. (1987) *Endocrinology* **121**, 793–802.
18. Stojilkovic, S. S., Torsello, A., Iida, T., Rojas, E. & Catt, K. J. (1992) *J. Steroid Biochem.* **41**, 453–467.
19. Marchetti, C. & Brown, A. M. (1988) *Am. J. Physiol.* **254**, C206–C210.
20. DiVirgilio, G., Pozzan, T., Wollheim, C., Vicenti, L. M. & Meldolesi, J. (1986) *J. Biol. Chem.* **261**, 32–35.
21. Lewis, D. L. & Weight, L. L. (1988) *Neuroendocrinol.* **47**, 169–175.
22. Offermanns, S., Gollasch, M., Hescheler, J., Spicher, K., Schmidt, A., Schultz, G. & Rosenthal, W. (1991) *Mol. Endocrinol.* **5**, 995–1002.
23. Paulssen, E. J., Paulssen, R. H., Haugen, T., Gautvik, K. M. & Gordeladze, J. O. (1991) *Mol. Cell. Endocrinol.* **76**, 45–53.
24. Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G. & Wittig, B. (1991) *Nature (London)* **353**, 43–48.
25. Kleuss, C., Scherübl, H., Hescheler, J., Schultz, G. & Wittig, B. (1992) *Nature (London)* **358**, 424–426.
26. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391**, 85–100.
27. Strathmann, M. & Simon, M. I. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9113–9117.
28. Fong, H. K. W., Yoshimoto, K. K., Eversole-Cire, P. & Simon, M. I. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3066–3070.
29. Jones, D. T. & Reed, R. R. (1987) *J. Biol. Chem.* **262**, 14241–14249.
30. Hsu, W. H., Rudolph, U., Bertrand, P., Olate, J., Nelson, C., Moss, L. G., Boyd, A. E., Codina, J. & Birnbaumer, L. (1990) *J. Biol. Chem.* **265**, 11220–11226.
31. Simmonds, W. F., Goldsmith, P. K., Codina, J., Unson, C. G. & Spiegel, A. M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7809–7813.
32. Gordeladze, J. O., Sletholt, K., Thorn, N. A. & Gautvik, K. M. (1988) *Eur. J. Biochem.* **177**, 665–672.
33. Offermanns, S., Schultz, G. & Rosenthal, W. (1989) *Eur. J. Biochem.* **180**, 283–287.
34. Tamaoki, T. (1991) *Methods Enzymol.* **201**, 340–347.
35. Trautwein, W. & Hescheler, J. (1990) *Annu. Rev. Physiol.* **52**, 257–274.
36. Gollasch, M., Hescheler, J., Spicher, K., Klinz, F.-J., Schultz, G. & Rosenthal, W. (1991) *Am. J. Physiol.* **260**, C1282–C1289.
37. Tallent, M. & Reisine, T. (1992) *Mol. Pharmacol.* **41**, 452–455.
38. McClue, S. J., Selzer, E., Freissmuth, M. & Milligan, G. (1992) *Biochem. J.* **284**, 565–568.
39. Cantiello, H. F., Patenaude, C. R. & Ausiello, D. A. (1989) *J. Biol. Chem.* **264**, 20867–20870.
40. Birnbaumer, L., Abramowitz, J. & Brown, A. M. (1990) *Biochem. Biophys. Acta* **1031**, 163–224.
41. Doupnik, C. A. & Pun, R. Y. K. (1992) *Pflügers Arch.* **420**, 61–71.
42. Kiley, S. C., Parker, P. J., Fabbro, D. & Jaken, S. (1991) *J. Biol. Chem.* **266**, 23761–23768.
43. Yue, T., Stadel, J. M., Sarau, H. M., Friedman, E., Gu, J. L., Powers, D. A., Gleason, M. M., Feuerstein, G. & Wang, H. Y. (1992) *Mol. Pharmacol.* **41**, 281–289.
44. Doupnik, C. A. & Pun, R. Y. K. (1992) *Biophys. J.* **61**, A392.
45. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **151**, 431–437.