Recent evidence demonstrates that the α subunits of some heterotrimeric GTP-binding proteins (G proteins) are subject to modification by protein kinase C (PKC). For the family of G proteins that activate the phospholipase C/inositol trisphosphate/calcium/PKC pathway, such modification could result in G protein autoregulation. To examine the potential regulation of members of the Gαq family by PKC phosphorylation, we expressed the thyrotropin-releasing hormone (TRH) receptor in combination with Gαq, Gα11, Gα14, Gα15, or Gα16 in Xenopus oocytes and examined the regulation of signaling by PKC activators and inhibitors. For Gα16 and Gα12, the two family members of hematopoietic lineage, PKC activators reduce both the magnitude and the time course of TRH-mediated responses; PKC inhibitors have the opposite effect. The PKC-mediated effects are evident in measurements of GTPase activity, suggesting that the regulation is occurring early in the signaling pathway. In vivo phosphorylation experiments demonstrate that Gα16 is a substrate for PKC modification. By comparison, Gαq is not phosphorylated by PKC in vivo, and oocytes expressing Gαq are not functionally modulated by PKC. Repeated TRH stimulation of oocytes expressing Gα16 mimics the effects of PKC activators, and this functional regulation is correlated with an increase in Gα16 phosphorylation. A mutant Gα16 with four consensus PKC phosphorylation sites removed is not phosphorylated in vivo, and TRH responses mediated through the mutant are not regulated by PKC. These results demonstrate that signaling involving hematopoietic G proteins is subject to PKC-mediated autoregulation, at least in part, by phosphorylation of the G protein α subunit.

Heterotrimeric guanine nucleotide-binding proteins (G proteins)1 transduce signals from cell-surface receptors for hormones, neurotransmitters, and growth factors to intracellular second messengers and ion channels (1–5). One subset of G proteins (the Gαq family) is responsible for coupling receptor-mediated signals to activation of PLCγ, in turn, PLC catalyzes the hydrolysis of phosphotidyl-4,5-bisphosphate to produce IP3 and diacylglycerol. IP3 causes the release of Ca2+ from intracellular stores, and diacylglycerol activates PKC (6–14). Five members comprise the Gαq family: Gαq, Gα11, Gα14, Gα15, and Gα16 (2). All members are refractory for modification by pertussis toxin, although they differ with respect to their potency for activation of PLCγ and their tissue distribution. For example, the human clone Gα16 and its murine homolog Gα13 are specifically expressed in cells of hematopoietic lineage (15, 16).

The signals mediated through G proteins are subject to regulation by a myriad of factors that can exert their effects throughout the signaling pathway from surface seven-helix receptors (17) to intracellular IP3 receptors (18–20). Some of these factors directly modify G proteins and alter G protein function. Pertussis toxin and cholera toxin associate with certain G protein α subunits to maintain them in an inactive state or render them constitutively active, respectively (21, 22). Posttranslational events, such as myristoylation and palmitoylation, also influence G protein action (23). Recently, a family of proteins has been identified that act as GTPase-activating proteins of heterotrimeric G protein α subunits. Isoforms of these regulators of G protein signaling (RGS proteins) show specificity toward particular G protein α subunits and exert their effects by competing with downstream effectors for activated Ga subunit binding (24, 25). Tyrosine kinases phosphorylate Gαq and the inhibition of tyrosine phosphorylation prevents accumulation of IP3 (26). Protein kinase C directly phosphorylates members of other G protein families: Gαi, Gαo, and Gα12 (27–32). Phosphorylation by PKC has been shown to regulate signaling through Gαi and Gα12 by preventing the association of the Go subunit with GBγ subunits (31, 32).

The evidence that signaling mediated through several different G proteins can be regulated by PKC phosphorylation of Ga subunits is particularly intriguing for members of the Gαq family, because such effects of PKC could represent a form of G protein autoregulation. Although Gαq does not appear to be phosphorylated by PKC (32), the action of PKC on other members of the Gαq family is not known. Previously, we have characterized the signaling properties of members of the Gαq family expressed in Xenopus oocytes (20, 33), exploiting the fact that the oocyte has an endogenous Cl− channel that is normally activated by the PLC/IP3/Ca2+ pathway and that can be used as a sensitive tool for the electrophysiological characterization of G protein activity (33, 34). In the present report, we used this system to evaluate the regulation of G proteins of the Gαq family by PKC phosphorylation. We find that signaling mediated by Gα15 and Gα16 is functionally regulated by PKC and that this change in function is correlated with the level of phosphorylation of the Ga subunit. These results suggest an important role for PKC in the regulation of G protein signaling in hematopoietic cells.
EXPERIMENTAL PROCEDURES

**In Vitro Synthesis of RNA**—In vitro transcription of sense RNA was performed as described previously (20). Recombinant plasmids containing Gα cDNA inserts were linearized by digestion with appropriate restriction enzymes. The transcription of linearized templates was performed in 7.6 mM Tris-Cl, pH 7.6, 6 mM MgCl₂, 0.6 mM NaCl, and 10 mM dithiothreitol containing 0.5 mM each ATP, CTP and UTP, 0.1 mM GTP, unlabeled GTP, 5-17-methyl-GTP, and 150 units of the appropriate polymerase in a total volume of 250 μl. The reaction mixture was incubated for 150 min at 37 °C. The DNA template was subsequently removed by treatment with 5 units of RNase-free DNase I for 15 min at 37 °C. Free nucleotides were removed using a Sephadel G-50 column. The mRNA was phenol/chloroform-extracted and recovered by ethanol precipitation. The mRNA was dissolved in RNase-free water at the corresponding concentration (see legends), divided into aliquots, and stored at −80 °C until used. A mutant Gα16 was created in which four consensus phosphorylation sites (Ser⁴, Thr⁶, Ser⁵³, and Ser³³⁶) were mutated to Ala (pALTER 1, Promega).

**Oocyte Expression and Electrophysiology**—These procedures are described in detail elsewhere (35). Briefly, oocytes were defolliculated and maintained at 18 °C in incubation medium containing ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.4), 1.8 mM CaCl₂, 50 μg/ml gentamycin, and 5% horse serum. Whole cell currents were measured at room temperature using an Axon Instruments GeneClamp in a standard two-microelectrode voltage-clamp configuration. Current was measured on-line by chart recorder and digitized using Axoscope software. Electrodes were filled with 3 M KCl and had a resistance of 1–3 MΩ. During an experiment, the oocytes were clamped at −80 mV and superfused continuously in ND96 medium; all drugs were applied in this solution. For some drugs, Me₂SO or ethanol was used as a solvent; final concentrations of these chemicals were always less than 0.1%. To minimize the contribution of the oocyte’s endogenous Gα, to the measurements, Gα, activates the PLC pathway in oocytes; Ref. 36), all oocytes were treated for 24 h prior to recording with 5 μg/ml pertussis toxin; this inhibits Gα, signaling but has no effect on signaling through the pertussis toxin-insensitive Gα, family (33).

**GTP Measurements**—GTPase measurements were performed on cell lysates prepared as described (37). Oocytes were homogenized in ice-cold 10 mM Tris-Cl, pH 8.0, 0.32 M sucrose, 1 mM MgSO₄, and the protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (200 μM). The homogenate was pelleted twice at 1000 × g to remove cell debris, and the supernatant fraction was centrifuged at 100,000 × g for 30 min. GTPase measurements were made as described (38) and performed for 15 min at room temperature in the presence of 100 μM of membrane protein resuspended in 50 mM Tris-Cl and protease inhibitors. Protein content was determined by the method of Bradford.

**In Vivo Phosphorylation and Immunodetection**—Immunodetection and phosphorylation experiments were performed using antibodies made against peptides specific to Gα₁₆ (LARYLDEI) and Gα₁₄ (LN-LKEYNL). Detection of Gα protein was by Western blot of oocyte membranes (39). Expressing oocytes were homogenized in 0.32 M sucrose in TE buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA) with protease inhibitors. Crude membrane pellets were obtained following 100,000 × g centrifugation and resuspended in SDS sample buffer. Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis gels, transferred onto polyvinylidene difluoride membranes (Pierce), immunoblotted with antibody (1:800 dilution of primary; 1:1000 dilution of horseradish peroxidase-conjugated secondary), and visualized using ECL reagents (Amersham Pharmacia Biotech). In vivo phosphorylation was performed essentially as described (32). Oocytes expressing Gα₁₆ subunits were injected with [³²P] (0.5 μCi/ml) 3 h prior to antibody immunoprecipitation. For immunoprecipitation, the homogenization, centrifugation, and resuspension steps were performed in RIPA buffer (20 mM NaHEPES, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% sodium deoxycholate, 0.5% SDS, 5 mM NaF, and 10 mM mercaptoethanol, subsequently centrifuged with protein G-agarose and 0.8 mg of Gα antibody overnight at 4 °C. The precipitate was centrifuged, resuspended, run on a 6% acrylamide gel, and exposed to film. Where applicable, data were analyzed by t test or one-way analysis of variance.

RESULTS

To examine the role of PKC in the functional regulation of signaling mediated by the Gα₁₆ family of heterotrimeric G proteins, the TRH receptor was expressed in combination with various G protein α subunits in Xenopus oocytes, and the resulting TRH-mediated signal was measured (Fig. 1). The oocyte provides an excellent system for functional analysis of signals mediated by the Gα, family because it contains an endogenous Gα₂⁻activated Cl⁻ channel that acts as a sensitive sensor for G protein/PLC/IP₃/Gα₂⁻mediated changes that occur following receptor stimulation. We have demonstrated previously that the ligand-induced TRH receptor couples to endogenous Gα, in oocytes (33). Furthermore, we have shown that co-expression of the TRH receptor with different members of the Gα, family (Gα₁₁, Gα₁₄, Gα₁₅, and Gα₁₆) results in increases in TRH-induced Cl⁻ currents; these results indicate functional coupling of exogenously expressed Gα subunits to the TRH receptor in oocytes (20, 33).

Fig. 1, A and B, shows results from individual oocytes recorded first during control conditions and then 20 min after injection of the PKC-activating phorbol ester PMA. Under control conditions, application of 100 nM TRH to oocytes expressing the TRH receptor and either Gα₁₁ (Fig. 1A) or Gα₁₅ (Fig. 1B) resulted in characteristic inward Cl⁻ currents. Following PMA treatment, oocytes expressing Gα₁₆ showed a reduction in both the peak amplitude and the time course of these TRH-mediated Cl⁻ currents (Fig. 1A); oocytes expressing Gα₁₄ were unaffected by PMA treatment (Fig. 1B).

Summary data for oocytes expressing TRH receptor and each of the PLC-activating G proteins are shown in Fig. 1, C and D. PMA significantly reduced peak TRH-mediated Cl⁻ currents in oocytes expressing Gα₁₁ (p < 0.05) and Gα₁₅ (p < 0.05); PMA had no effect on oocytes expressing Gα₁₄ and Gα₁₃ (Fig. 1C). In addition to examining the effect of peak responses, we also investigated the effect of PMA on the response time course. Because the magnitude of the peak response could potentially influence the time course of the response, the post-PMA peak response data from Fig. 1C were examined; only peak Cl⁻ currents that were of comparable size were included in the time course analyses. For oocytes showing comparable peak TRH-mediated Cl⁻ currents following PMA treatment, only oocytes expressing Gα₁₆ or Gα₁₅ showed PMA-mediated decreases in response time course (Fig. 1D). These results suggest that PKC regulates the cascade induced by TRH receptor activation only in the presence of Gα₁₆ or Gα₁₅ subunits.

To elucidate further the PKC-dependent modulation of Gα₁₆ signaling, TRH-induced currents were measured in the presence of PMA, staurosporine, or the inactive phorbol ester 4αPDD (Fig. 2). Fig. 2A shows data from a single oocyte recorded first during control conditions and then 20 min after injection of PMA. These results, from a separate oocyte batch, reiterate the PMA effect shown in Fig. 1; TRH-mediated Cl⁻ currents in oocytes injected with PMA exhibit a reduction in both the peak amplitude and the time course of the response. Treatment with staurosporine, a PKC inhibitor, had the opposite effect; both the peak amplitude and the response time course of TRH-mediated currents were increased (Fig. 2B). To rule out the possibility that the reduction in Gα₁₆-mediated signaling seen with PMA treatment was due to desensitization of the TRH receptor (because of repeated applications of TRH), a subset of Gα₁₆-expressing oocytes was recorded first during control conditions and then 20 min following injection of water. Neither peak currents nor the time course of the response were altered in these oocytes (Fig. 2C).

Data summarizing the modulation of the magnitude of the peak response are shown in Fig. 2D. PMA injection caused a 56% reduction (p < 0.01) of peak Cl⁻ currents in oocytes expressing TRH receptors and Gα₁₆, staurosporine caused a 29% increase (p < 0.01). Neither injection of vehicle solution nor 4αPDD altered the TRH-mediated currents, which further supports the role of PKC in mediating this effect. Concentration-
Protein Kinase C Regulation of Ga\text{16}

FIG. 1. PMA-induced changes in the magnitude and kinetics of TRH-induced Cl\textsuperscript{−} currents are specific to Ga\text{16}-injected and Ga\text{q}-injected oocytes. A, oocytes were injected with 0.2 ng of TRH receptor cRNA and 0.005 ng of Ga\text{q} cRNA 48 h prior to measurement. TRH-induced Cl\textsuperscript{−} currents in an individual oocyte were measured before (control) and 20 min after (PMA) injection of 1 \mu M (approximate final concentration) PMA. The solid bars above each trace represent the application of 100 nM TRH. B, same as in A except that oocytes were injected with 0.2 ng of TRH receptor cRNA and 0.5 ng of Ga\text{16} cRNA 48 h prior to measurement. C, changes in the magnitude of peak Cl\textsuperscript{−} currents of treated oocytes expressing TRH receptor and the G protein \( \alpha \) subunits of the Ga\text{16} family. The amount of cRNA injected was as follows: 0.2 ng of TRH receptor; 0.005 ng of Ga\text{15} and Ga\text{16}; 0.5 ng of Ga\text{16}, Ga\text{15}, and Ga\text{14}. The values in parentheses above the bars denote the number of oocytes recorded first in control conditions and then 20 min after PMA injection. A value of 100% represents no change in responses between the two applications. D, changes in the time course of responses of oocytes treated as in C. Data are plotted as the time for the peak response to decay to one-half its maximal value. To control for changes in time course due to differences in peak Cl\textsuperscript{−} currents, oocytes were selected post hoc such that peak Cl\textsuperscript{−} currents for each group were within 10% of each other. The values in parentheses above the bars denote the number of oocytes recorded in each condition. A baseline TRH-mediated peak responses were obtained for oocytes in all conditions. Next, oocytes were injected with PMA, staurosporine, or vehicle. Twenty minutes later, oocytes were repeatedly challenged with 30-s applications of TRH at 4-min intervals. Repeated TRH stimulation of oocytes expressing Ga\text{16} resulted in a time-dependent decrease in the peak response that approached the levels obtained in oocytes treated with PMA. Oocytes treated with PMA showed the characteristic reduction in peak currents that were insensitive to further reduction following repeated TRH stimulation. Oocytes treated with staurosporine showed the characteristic increase in peak currents but failed to show a decrease in peak response following repeated TRH application. Oocytes injected with Ga\text{q}, which is insensitive to PKC regulation, did not show a decrease in peak Cl\textsuperscript{−} currents during repeated TRH stimulation. The evidence that repetitive stimulation caused a reduction in the TRH-induced response (i) only in the presence of Ga\text{16}; (ii) similar to that seen with PMA treatment, and (iii) under conditions in which receptor homologous desensitization was negligible suggests the possibility that the Ga\text{16} cascade can be feedback-regulated.

To rule out further that desensitization of the receptor was contributing to the reduction in Ga\text{16}-mediated signaling during repetitive stimulation, we performed heterologous desensitization experiments using repetitive stimulation of multiple receptors that couple to Ga\text{16} in oocytes (20). Experiments were performed similar to those discussed in Fig. 3A using oocytes...
expressing Go16 and both TRH and 5HT2c receptors. These data are shown in Fig. 3B. Once again, base-line TRH-mediated peak responses were obtained for oocytes in all conditions. Next, oocytes were injected with PMA or vehicle. Twenty minutes later, oocytes were re-tested with TRH and then repeatedly stimulated with 30-s applications of 5HT at 4-min intervals. After three applications of 5HT, the oocytes were re-tested with TRH. Oocytes expressing Go16 showed decreases in TRH-mediated Cl⁻ currents following repeated 5HT stimulation that were comparable to the decreases obtained in PMA-treated oocytes. Oocytes expressing Gαq did not show stimulation-mediated decreases in Cl⁻ currents. Thus, repeated Go16 stimulation, even through different receptors, is capable of down-regulating the Go16-mediated signal. These data not only reinforce the possibility of Go16-mediated autoregulation but suggest that this signaling cascade can be regulated by any pathway that activates PKC.

Assuming that all of the components of the PLC/IP₃/Ca²⁺ pathway are the same following activation by different members of the Gαq family, the data showing that only some members are modulated by PKC suggest that the action of PKC occurs at the level of G protein activation. To support this hypothesis further, two additional experiments were performed. First, to confirm that the regulation by PKC was not occurring at the level of the IP₃ receptor or at the level of the Ca²⁺-activated Cl⁻ channel, TRH-mediated G protein activation was bypassed by direct injection of IP₃ into the oocyte. The results of this experiment are shown in Fig. 4. Fig. 4A shows the responses of an oocyte-expressing TRH receptor and Go16 before and after treatment with PMA. One minute after the PMA measurement, the oocyte was injected with IP₃. The PMA-induced decrease in peak response following TRH stimulation was reversed by IP₃ (Fig. 4B, p < 0.05), suggesting that components of the signaling cascade downstream of IP₃ are not

Fig. 2. Activators and inhibitors of PKC alter the magnitude and kinetics of TRH-induced Cl⁻ currents in oocytes expressing Go16. Oocytes were injected with 0.2 ng of TRH receptor cRNA and 0.005 ng of Go16 cRNA 48 h prior to measurement. A, TRH-induced Cl⁻ currents in an individual oocyte measured before (control) and 20 min after (PMA) injection of 1 μM (approximate final concentration) PMA. The solid bars above each trace represent the application of 100 nM TRH. B, same as in A except currents were recorded before (control) and 20 min after (staurosporine) injection of 100 nM (approximate final concentration) staurosporine. C, same as in A except Cl⁻ currents were measured before (control) and after (water) injection of 50 nl of water. D, changes in the magnitude of the peak response of oocytes treated with PMA, the inactive phorbol ester 4α-PDD, staurosporine, or vehicle (50 nl) compared with the response obtained 20 min prior to drug injection. Drug concentrations listed below the abscissa are in μM. The values in parentheses above the bars denote the number of oocytes recorded first in control conditions and then 20 min after drug injection. Data are plotted relative to control values. E, concentration-response curves for the effects of PMA (filled circles) and staurosporine (open circles) on the magnitude of peak Cl⁻ currents. Data are plotted relative to the response elicited in untreated oocytes. Solid lines are exponential fits to the data. Each data point represents measurements from 4 to 6 oocytes. F, changes in the time course of responses of oocytes treated as in D. Data are plotted as the time for the response to decay to one-half its maximal value. To control for changes in time course due to differences in peak responses, oocytes were selected post hoc such that peak responses across all conditions were within 10% of each other. The values in parentheses above the bars denote the number of oocytes recorded in each condition.
action of PMA occurs at a step in the cascade that can affect GTP hydrolysis, either at the level of the G protein or at the level of G protein/receptor coupling.

In theory, a reduction in GTPase activity due to PMA treatment could result either from a decrease in intrinsic GTPase activity (either directly or through a change in GTPase-activating protein function) or from a decrease in the number of activated G protein molecules (e.g., through an inhibition of G protein/receptor coupling). In the former case, a decrease in GTPase activity would be correlated with an increase in Cl⁻ channel activation; in the latter case, TRH-mediated Cl⁻ channel activation would be decreased. Since oocytes expressing Ga16 show a decrease in both GTPase activity and Cl⁻ currents, the data are consistent with a model in which PMA interferes with receptor-mediated activation of the G protein. To address this hypothesis further, we repeated the GTPase measurements in the presence of increasing TRH concentrations (Fig. 5B), and we compared these results to peak TRH-mediated Cl⁻ currents (Fig. 5C). Treatment of oocytes with both PMA and staurosporine resulted in a change in the maximal amount of GTPase activity that could be elicited; this effect was mirrored by functional changes as assessed by Cl⁻ current activation. These data suggest that PKC activators and inhibitors act to alter the fraction of activable Ga16 molecules.

If the fraction of activable Ga16 molecules is reduced, perhaps by interfering with the coupling of the TRH receptor to Ga16, then the time course to peak Cl⁻ channel activation should be altered. Changes in the latency to peak activation following TRH application in oocytes treated with either PMA or staurosporine are shown in Fig. 5D. PMA treatment resulted in a 3-fold slowing in response latency; staurosporine treatment produced a small decrease in the latency. The much smaller change in latency in the presence of staurosporine may reflect a smaller amount of endogenous PKC phosphorylation in control oocytes in that oocyte batch.

Since the above data were consistent with the hypothesis that PKC is acting on the G protein, experiments were performed to determine if Ga16 is phosphorylated by PKC and if the level of phosphorylation correlated with the functional effects of PKC activation. These results are shown in Fig. 6. We first examined the effect of PMA treatment on oocytes expressing either Ga16 or Gaq. For this experiment, oocytes were preincubated with labeled phosphate in the absence or presence of PMA, homogenized, and immunoprecipitated using subtype-specific antibodies. The specificity of the antibodies was confirmed in a Western blot analysis of oocyte membranes expressing either Ga16 or Gaq (Fig. 6A). Autoradiographs of extracts immunoprecipitated with the Ga16 antibody revealed a band only in oocytes expressing Ga16 and treated with PMA (Fig. 6B). This band was absent or near absent in oocytes not treated with PMA or in oocytes expressing Gaq and treated with PMA. These results indicate that phosphorylation of Ga16 is specific and observed upon activation of PKC.

To determine if phosphorylation of Gaq is correlated with receptor activation, oocytes expressing TRH receptor and Ga16 were preincubated with labeled phosphate, unstimulated or repetitively stimulated with various applications of TRH, homogenized, and immunoprecipitated. Repetitive TRH stimulation resulted in an increase in the state of Gaq phosphorylation (Fig. 6C). The amount of Ga16 phosphorylation following repetitive TRH application was correlated with the reduction in peak TRH-mediated Cl⁻ currents as assessed by voltage clamp (Fig. 6D). These results demonstrate that the phosphorylation of Ga16 is influenced by receptor stimulation and that the degree of Ga16 phosphorylation correlates with the amount of receptor-mediated activation. The evidence that both repet-

![Fig. 3. Repeated TRH-induced stimulation of Ga16-mediated Cl⁻ currents mimics the effects of PMA. A, oocytes (five per condition) were injected with 0.2 ng of TRH receptor cRNA and either 0.5 ng of Gaq or 0.005 ng of Ga16 cRNA 48 h prior to measurement. Control peak Cl⁻ currents were recorded at time 0 following application of 100 nM TRH. The measured oocyte was then water-injected or injected with 1 μM PMA or 100 nM staurosporine. Twenty minutes after the first measurement, the oocyte was then stimulated with a 30-s application of 100 nM TRH in 4-min intervals. Peak Cl⁻ currents were recorded and plotted as a percentage of the initial response. B, oocytes (six per condition) were injected with 0.2 ng of TRH receptor cRNA, 1 ng of 5HT2c cRNA, and either 0.5 ng of Gaq or 0.005 ng of Ga16 cRNA 48 h prior to measurement. TRH-induced currents were measured as in A except that 30-s applications of 10 nM 5HT were substituted at the 24-, 28-, and 32-min time points.](image-url)
positive stimulation of the receptor and PMA treatment induce \( G_{a_{16}} \) phosphorylation supports the hypothesis that \( G_{a_{16}} \) can be regulated by feedback phosphorylation.

If phosphorylation of \( G_{a_{16}} \) by PKC is responsible for the functional regulation of TRH-mediated responses, then oocytes expressing a mutant \( G_{a_{16}} \) protein that is not phosphorylated by PKC should not mediate modulation of TRH-induced responses. To test this hypothesis, we simultaneously mutated four sites on \( G_{a_{16}} \) (Ser\(^4\), Thr\(^6\), Ser\(^53\), and Ser\(^336\)) that are consensus sequences for PKC phosphorylation which are not present in \( G_{a_{q}} \). We expressed this mutant along with TRH receptors in oocytes. The results of this experiment are shown in Fig. 7. Unlike wild-type \( G_{a_{16}} \), the mutant \( G_{a_{16}} \) protein was not phosphorylated \textit{in vivo} by PMA (Fig. 7A). In addition, TRH stimulation of oocytes expressing the mutant \( G_{a_{16}} \) resulted in inward Cl\(^-\) currents that appeared comparable to wild-type \( G_{a_{16}} \); however, these currents could not be regulated by PMA (Fig. 7B). In the same batch of oocytes, whereas oocytes expressing wild-type \( G_{a_{16}} \) showed the characteristic regulation of peak Cl\(^-\) currents in the presence of PMA and staurosporine, oocytes expressing the mutant \( G_{a_{16}} \) were not regulated (Fig. 7C). The response time course in the mutant-expressing oocytes was also not regulated (Fig. 7D). These data strongly support the hypothesis that PKC phosphorylation of \( G_{a_{16}} \) is responsible for the functional regulation of TRH-mediated signaling.
G proteins serve as both the upstream initiators and the downstream targets of a variety of intracellular second messengers. An important aspect of these cascades must be their ability to prevent uncontrolled cell stimulation. Such regulation of G protein signaling could occur through second messenger pathways that are part of an autoregulatory feedback loop or that are initiated by cross-talk between second messenger systems. Several types of kinases are known to modify different proteins of the G protein cascade. These include G protein-coupled receptor kinases (40, 41), tyrosine kinases (26), and protein kinase-mediated signals have been shown to be modified. For instance, PKC can have functional effects by acting on various seven-helix receptors and on downstream signaling components. Furthermore, PKC may not be the only mechanism for regulatory transduction pathway and (ii) a mechanism for regulatory cross-talk by any transduction pathway that activates PKC.

We believe that the phosphorylation of \( \text{Go}_{16} \) by PKC is responsible for the functional regulation of TRH-mediated signaling. In fact, we show that a mutant \( \text{Go}_{16} \) protein that is not phosphorylated by PKC does not mediate modulation of TRH-induced responses, suggesting that PKC phosphorylation of \( \text{Go}_{16} \) is responsible for the functional regulation of TRH-mediated signaling. Furthermore, the evidence that signaling mediated by \( \text{Go}_{16} \), a protein that is highly related to \( \text{Go}_{15} \) and which activates a comparable signaling pathway in oocytes, is not affected by PKC supports the conclusion that the PKC effect on \( \text{Go}_{16} \) is direct. We cannot rule out that some other protein in the oocyte may be responsible for some of the PKC-mediated effects in the \( \text{Go}_{16} \) pathway, and the likelihood that the functional effects of PKC are solely due to its action on \( \text{Go}_{16} \) is remote given the multiple mechanisms through which G protein-mediated signals have been shown to be modified. For instance, PKC can have functional effects by acting on various seven-helix receptors and on downstream signaling components. Furthermore, PKC may not be the only mechanism acting to control \( \text{Go}_{16} \) activity. We and others (19, 20) have demonstrated that chronic activation of the PLC pathway by
Protein Kinase C Regulation of \( \text{G}_{\alpha_{16}} \)

The high expression of wild-type or GTPase-deficient \( \text{G}_{\alpha_{16}} \) induces signal adaptation through down-regulation of IP\(_{3}\) receptors. Thus, \( \text{G}_{\alpha_{16}} \)-mediated signaling cascades initiated by different receptors in hematopoietic cell types is subject to regulatory control involving multiple mechanisms, including PKC auto-regulation and chronic desensitization of IP\(_{3}\) receptors.

We are presently determining the site(s) necessary for this effect and the mechanism(s) by which such biochemical modification causes functional inhibition of \( \text{G}_{\alpha_{16}} \)-signaling. For \( \text{G}_{\alpha_{16}} \) and \( \text{G}_{\alpha_{12}} \), PKC phosphorylation occurs in the amino-terminal \( \beta_{y} \) binding region of the \( \text{G}_{\alpha} \) subunit and inhibits signaling by preventing trimer association (31, 32). Two serine residues and one threonine residue are present in this region of \( \text{G}_{\alpha_{16}} \) and \( \text{G}_{\alpha_{12}} \) that could serve as the sites of PKC phosphorylation. The \( \text{G}_{\alpha_{12}} \) and \( \text{G}_{\alpha_{12}} \) studies also suggest that the active phosphorylation sites are surrounded by arginine residues (31, 32). One arginine-rich serine site is present in \( \text{G}_{\alpha_{16}} \) and \( \text{G}_{\alpha_{12}} \); it is found near the carboxyl terminus in a putative receptor-binding region. Our GTPase data suggest that PKC phosphorylation of \( \text{G}_{\alpha_{16}} \) reduces the number of activable G protein molecules; a mechanism in which phosphorylation of a site interferes with receptor/G protein coupling is consistent with these data.

Our data reveal that the only members of the \( \text{G}_{\alpha} \) family that are functionally regulated by PKC phosphorylation are the \( \text{G}_{\alpha_{16}} \) and \( \text{G}_{\alpha_{12}} \) subunits. These two G proteins have a number of interesting features. They can be activated by a wide variety of functionally different receptors (43); the potential for these G proteins to couple to a broad spectrum of receptors makes them a good target for feedback regulation in order to prevent aberrant signaling. Regulation of the expression and activity of \( \text{G}_{\alpha_{16}} \) may be critical for controlling cellular proliferation rates.

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