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GTP-Binding Protein $G_{\alpha z}$: Its Down-regulation by Dexamethasone and Its Credentials as a Mediator of Antigen-Induced Responses in RBL-2H3 Cells

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SUMMARY

We have investigated the possible role of guanine nucleotide-binding proteins in the process of antigen-induced exocytosis in a cultured rat mast cell line, RBL-2H3 cells. The mRNAs for the α subunits of the guanine nucleotide-binding proteins $G_{\alpha s}$ (short and long forms), $G_{\alpha i\text{-}2}$, $G_{\alpha i\text{-}3}$, and $G_{\alpha z}$ were detected by hybridization with G_α-specific oligonucleotide probes. The corresponding proteins were identified in membranes of RBL-2H3 cells on the basis of size, immunoreactivity with specific antibodies, and their ability to serve as substrates for ADP-ribosylation by cholera toxin or pertussis toxin. Treatment of cells with as little as 10^{-9} to 10^{-7} M dexamethasone markedly decreased the amount of $G_{\alpha z}$ mRNA and membrane $G_{\alpha z}$, as well as the responsiveness of the cells to antigen stimulation. In the same cells, the exposure to dexamethasone caused an increase in the amounts of certain other G_α subunits, particularly $G_{\alpha i\text{-}3}$, and in the responsiveness of the cells to an adenosine analog, *N*(ethylcarboxamido)-adenosine. Because of the apparent decrease in $G_{\alpha z}$ mRNA and protein in dexamethasone-treated cells and the fact that neither cholera toxin nor pertussis toxin inhibits the stimulatory signals to antigen [*J. Biol. Chem.* **265**:745–753 (1990)], we suggest that $G_{\alpha z}$ is a potential candidate for regulating the early signals in antigen-stimulated RBL-2H3 cells.

The cultured rat RBL-2H3 cell is widely used as an experimental model for the study of antigen-induced signals for exocytosis in mast cells. The physiological trigger for the stimulation of these cells is the aggregation of membrane receptors for IgE by multivalent binding of antigen to receptor-bound IgE (1). Although the mechanism of the communication between the receptor and effector systems is still unclear, much is known about the kinetics of receptor aggregation and the intervening signals that are thought to promote exocytosis in these cells (2). The same array of intervening events, namely the hydrolysis of inositol phospholipids (3) and phosphatidylcholine,[2] the mobilization of intracellular and extracellular Ca^{2+} (3), and the activation of protein kinase C,[2] are induced by analogs of adenosine through an unusual type of receptor (3). The stimulatory effects of the adenosine analogs, however, are too transient to promote exocytosis, but they can markedly synergize the antigen-induced signals for exocytosis.

Although the responses to antigen and the adenosine analog NECA are qualitatively similar and are suppressed to the same extent by GDPβS (4),[3] the coupling mechanisms are quite distinct. All responses to antigen, for example, are suppressed and all those to NECA are enhanced when RBL-2H3 cells are exposed for several hours to nanomolar concentrations of dexamethasone. These effects are probably exerted at the level of the receptor and coupling proteins rather than the effector systems (5). In addition, RBL-2H3 cells that have been treated with either cholera toxin or pertussis toxin no longer respond to NECA but do so to antigen (3). Indeed, the mobilization of Ca^{2+}, and as a consequence exocytosis, is enhanced in response to antigen in cholera toxin-treated cells by a mechanism that is independent of the activation of adenylyl cyclase (3, 6, 7). If the responses to NECA and antigen are mediated by G proteins, then clearly different G proteins must be recruited by these two stimulants in RBL-2H3 cells.

As reported here, by use of 1) specific oligonucleotide probes to detect mRNA of the α subunits (G_α) of known G proteins

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[2] H. M. S. Gonzaga, H. Ali, and M. A. Beaven, unpublished observations.
[3] H. Ali, unpublished observations.

ABBREVIATIONS: NECA, 5′-(*N*-ethylcarboxamido)-adenosine; G protein, guanine nucleotide-binding protein; G_s and G_i, stimulatory and inhibitory G proteins that can couple to adenylyl cyclase; G_o, a G protein that is abundant in brain; G_t, the predominant G protein in retina; G_z, a recently described G protein of unknown function; $G_{\alpha i\text{-}1}$, an example to indicate the designation and subtype of the α subunits of these G proteins; DNP, dinitrophenol; DNP-BSA, one molecule of bovine serum albumin conjugated with 25 molecules of dinitrophenol; SDS, sodium dodecyl sulfate; GDPβS, guanosine 5′-(2-*O*-thio)diphosphate; $[Ca^{2+}]_i$, concentration of free cytosolic Ca^{2+}; kb, kilobases; MOPS, 3[*N*-morpholino]propanesulfonic acid.

by Northern analysis, 2) antibodies to detect G_α subunits in cell extracts by Western immunoblotting techniques, and 3) cholera and pertussis toxins as catalysts of ADP-ribosylation of G_α subunits, we have screened the RBL-2H3 cell for known G proteins. In addition, analysis of the expression of these proteins, before and after exposure to dexamethasone, has revealed that the recently described toxin-insensitive G protein $G_{\alpha z}$ (8–10) is a potential candidate for coupling of the IgE receptor to the effector systems that mediate exocytosis.

Experimental Procedures

Materials. DNP-specific IgE and the antigen DNP-BSA were gifts from Dr. Henry Metzger (National institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health). The cDNA probe for hybridization with γ-actin was kindly supplied by Dr. Peter Gunning. The random-primed DNA labeling kit and oligo(dT)-cellulose were purchased from Boehringer Mannheim; [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]dATP (6000 Ci/mmol) from Dupont/New England Nuclear; terminal deoxynucleotidyltransferase and RNA standards from Gibco BRL, Gaithersburg, MD; bacterial toxins from List Biochemicals; Ficoll (type 400) and dextran sulfate from Pharmacia; salmon sperm DNA from Lofstrand Laboratories; MOPS, formamide, guanidinium isothiocyanate, and sodium lauroyl isothiocyanate from Fluka Chemie AG; and cesium chloride from Schwartz/Mannheim Biotechnologies. Other materials were of the best available quality from major suppliers.

The oligonucleotide probe for mRNA of $G_{\alpha i\text{-}2}$ was synthesized by Pharmacia. All other oligonucleotide probes were synthesized on an Applied Biosystems model 380B DNA synthesizer. Preparations of antibodies against the G_α subunits were obtained from Dupont/New England Nuclear (designated AS/7, EC/2, GC/2, and RM/1); antibodies 2921 and 8645 were kindly supplied by Dr. David Manning (University of Pennsylvania, Philadelphia, PA). A $G_{\alpha\,common}$ antibody, which was prepared by Dr. Jane L. Halpern (LCM, National Hearth, Lung, and Blood Institute) (11), was used as an additional reagent to confirm location and apparent molecular size of the G_α subunits. The specificities of these antibodies are described in the text.

Experiments with cultured cells. Techniques for cell culture, the sensitization of cells with DNP-specific IgE to give 100% occupancy of receptors, and the labeling of cells with [^{3}H]inositol and [^{14}C]5-hydroxytryptamine were based on published descriptions (3–5). For the experiments with dexamethasone, RBL-2H3 cells were incubated with the indicated concentration of dexamethasone for 14 hr, in minimal essential medium with Earle's salts and 15% fetal calf serum, either in suspension (25×10^6 cells/200 ml) for the assay of proteins or in monolayer culture (150-cm^2 flasks) for the isolation of poly(A)$^+$ RNA. Cells were then harvested from these cultures by centrifugation ($250 \times g$, 5 min) or by treatment with trypsin (0.05% trypsin in 0.02% EGTA; GIBCO) for 5 min for the isolation of protein or poly(A)$^+$ RNA (see below). The responsiveness of the cells to antigen or NECA was assessed by plating cells in 24-well cluster plates (2×10^5 cells/0.4 ml of medium/well) and incubating them for an additional 2 hr with 4 μCi/ml [^{3}H]inositol, 1 μCi/ml [^{14}C]5-hydroxytryptamine, and 0.5 μg/ml DNP-specific IgE (in addition to the indicated concentration of dexamethasone). Release of radiolabeled inositol phosphates and 5-hydroxytryptamine was then determined in triplicate cultures exactly as described (3–5). Values cited were the mean ± standard error of the average values from individual experiments, as noted in the text.

Isolation and hybridization of poly(A)$^+$ RNA. Total RNA was isolated from RBL-2H3 cells by the guanidinium/cesium chloride method (12); from this, the poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography, as described by Davis *et al.* (13). The average yield of poly(A)$^+$ RNA was 0.31 ± 0.03 μg/10^6 cells.

Poly(A)$^+$ RNA (10 μg) was fractionated by electrophoresis in 1% agarose/2.2 M formaldehyde gels, in a buffered solution (pH 7.0) of 40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA, transferred to nitrocellulose membranes, and cross-linked to the membranes by UV irradiation. The poly(A)$^+$ RNA was hybridized with synthetic oligonucleotide probes of 18 to 48 bases. The oligonucleotide probe for $G_{\alpha o}$ was complementary to the 3′ untranslated region of the mRNA. Other probes were complementary to a hypervariable region of the G_α proteins. Specific probes for the long and short species of $G_{\alpha s}$ were constructed around a sequence that is unique to $G_{\alpha s\text{-}1}$ (Fig. 1). Probes were labeled with [α-^{32}P]dATP by use of terminal deoxynucleotidyltransferase (18). Hybridizations and washes were performed as described (19), except that formamide was included in the hybridization buffer when the 48-base oligomers were used.

Immunoblotting techniques. Cells (25×10^6) were collected by centrifugation ($250 \times g$, 5 min), washed twice in phosphate-buffered saline, and then resuspended in 0.6 ml of sucrose-Tris buffer of the following composition: 250 μM sucrose, 20 mM Tris-HCl, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM NaN_3, 2 mM dithiothreitol, 1 M benzamidine, with lima and soy bean trypsin inhibitors (each 2 μg/ml). In two initial experiments, pepstatin and leupeptin were used instead of the trypsin inhibitors; this did not alter the quality of the final immunoblots. Suspended cells were disrupted by two 20-sec periods of sonication (maximum setting; Kontes microultrasonic cell disrupter) and then fractionated into cytosolic and membrane fractions by centrifugation ($105{,}000 \times g$, 60 min). The membrane fraction was washed once with 1 ml of the sucrose-Tris buffer and then suspended in 0.1–0.5 ml of the same medium. Protein in membrane and cytosol fractions were assayed with a protein assay kit (Bio-Rad).

Samples of cytosolic and membrane fractions (100 μg of protein), in addition to extracts of rat brain cortex membranes (50 μg of protein), were fractionated by electrophoresis in 10% SDS-polyacrylamide gels, as described by Goldsmith *et al.* (20). The proteins were transferred to nitrocellulose filters. Immunoactive proteins were detected exactly as described (21), using antibodies diluted as follows: 8645 and 2921, 1/100; AS/7, 1/250; $G_{\alpha common}$ 1/2000; all others, 1/1000. The second antibody, biotinylated goat anti-rabbit IgG (BRL), was used at a 1/5000 dilution. The bands were quantified by scanning densitometry (LKS) Ultrascan XL laser densitometer), and the data were calculated in arbitrary units.

ADP-ribosylation of cell membranes and cytosolic proteins. Cells were collected by centrifugation ($250 \times g$, 8 min), washed twice in phosphate-buffered saline, and suspended (25×10^6 cells/ml) in the sucrose-Tris buffer before disruption by sonication and separation into cytosol and membrane fractions by centrifugation ($100{,}000 \times g$, 40 min, 4°). The membrane fraction was washed twice with 100 volumes of the sucrose-Tris buffer. ADP-ribosylation of cell proteins was performed essentially as described by Burns *et al.* (22). Membrane or cytosol fractions (100 μg of protein) were incubated in 100 μl of 20 mM potassium phosphate, pH 7.5, 3.75 mM glycine, 0.05 mM ATP, 20 mM thymidine, 2 mM $MgCl_2$, 15 mM dithiothreitol, 20 μCi/ml [^{32}P]NAD, with 0.5 μg/ml pertussis toxin, or 5 μg/ml cholera toxin, as indicated. After incubation for 60 min at 30°, the reaction was stopped by addition of 750 μl of 10% trichloroacetic acid. Precipitated proteins were collected by centrifugation ($2500 \times g$, 30 min, 4°), dissolved in 1% SDS, and separated by SDS-polyacrylamide gel electrophoresis (in 12% polyacrylamide gels). Radiolabeled proteins were detected by autoradiography.

Results

Expression of mRNA for G_α subunits in RBL-2H3 cells. Hybridization of poly(A)$^+$ RNA from three batches of unstimulated RBL-2H3 cells with specific oligonucleotide probes (Fig. 1), under conditions of high stringency, indicated the presence of mRNA for the short and long species of $G_{\alpha s}$ (1.85 kb), $G_{\alpha i\text{-}2}$ (2.35 kb), $G_{\alpha i\text{-}3}$ (3.5 kb), and $G_{\alpha z}$ (3.0–3.2 kb). The sizes of the mRNAs for $G_{\alpha s}$, $G_{\alpha i\text{-}2}$, $G_{\alpha i\text{-}3}$, and $G_{\alpha z}$ were consistent with previous reports (8, 9, 14–17), except that one additional mRNA for $G_{\alpha z}$ at 2.3 kb was detected with the probe

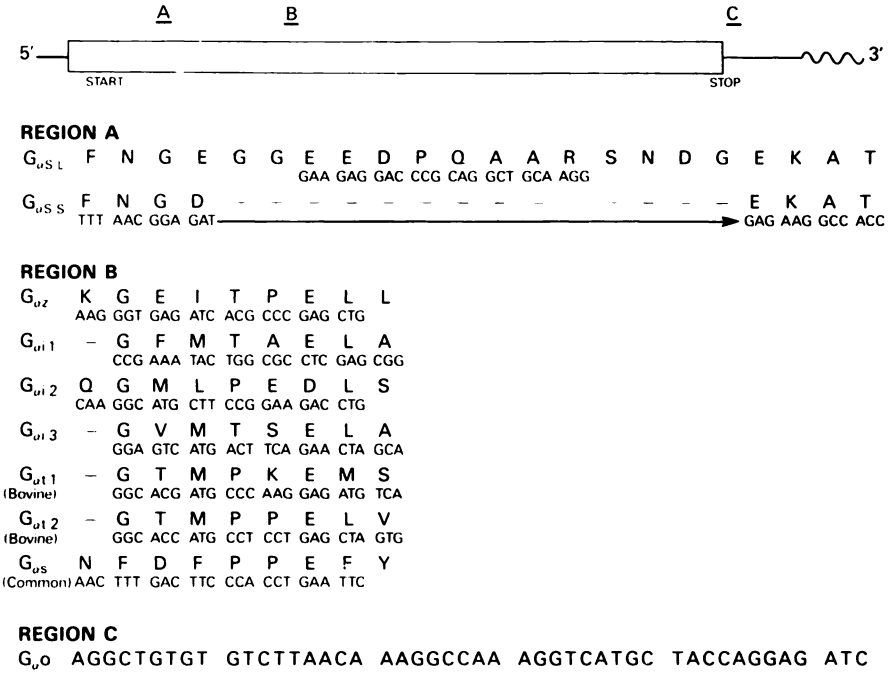

Fig. 1. Sequence of G_α-specific oligonucleotide probes. *Upper*, a schematic representation (not to scale) of a consensus G_α cDNA. *Open box*, the coding regions; *line*, the 3′ untranslated region. *Bars above the scheme*, three regions (A, B, and C) selected for the preparation of the complementary oligonucleotide probes. *Lower*, probes selected from each of these regions. The deduced amino acid (indicated by the single-letter abbreviations) sequences, along with the actual nucleotide sequence of the cDNA probe, are shown. For optimal alignment of sequences, gaps (—) were introduced. Alignment was based on published sequences for the rat G_α subunits (8, 9, 14, 15), except for $G_{\alpha t}$, which was based on the sequence for bovine $G_{\alpha t}$ (16, 17).

for $G_{\alpha z}$. No mRNAs for $G_{\alpha i\text{-}1}$, $G_{\alpha t\text{-}1}$, $G_{\alpha o}$, and $G_{\alpha t\text{-}2}$ were detected (Fig. 2).[4]

Effect of dexamethasone on the amounts of mRNA for G_α subunits. The same mRNAs were observed in dexamethasone-treated cells (0.1 μM dexamethasone) as in untreated cells, but the amount of mRNA for $G_{\alpha z}$ was markedly decreased in relation to the amounts of mRNAs for $G_{\alpha s}$ (long and short forms), $G_{\alpha i\text{-}2}$, and $G_{\alpha i\text{-}3}$, which appeared to be unaffected by the treatment with dexamethasone (Fig. 3). Identical results were obtained in two additional experiments. The decrease in mRNA for $G_{\alpha z}$, as measured by densitometric analysis, was 62 ± 3% in all three experiments. This decrease was associated with a decreased release of inositol phosphates (by 67 ± 3%) and secretion (by 63 ± 7%) in response to antigen.

[4] Hybridization with the $G_{\alpha o}$ probe yielded a faint band (4.3 kb) after 14 days of exposure to the photographic film in one early experiment. The presence of trace amounts of mRNA for $G_{\alpha o}$ was possible but of doubtful significance.

Effects of dexamethasone treatment on levels of membrane G_α subunits. To investigate the effects of dexamethasone on the amounts of the various G_α subunits, cells were incubated with increasing concentrations of dexamethasone, and the membrane G_α subunits were identified by their immunoreactivity with G_α-specific antibodies (Table 1; Fig. 4*A*). A 40-kDa membrane protein that reacted with antibody EC/2, consistent with the presence of $G_{\alpha i\text{-}3}$, increased in amount, by up to 80%, with increasing concentrations of dexamethasone. A 39.5-kDa membrane protein or proteins that reacted with antibody AS/7 ($G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$, or $G_{\alpha t}$) did not significantly change in amount in response to dexamethasone. Two membrane proteins, 43 and 45 kDa in size, reacted with antibody RM/1 ($G_{\alpha s\text{-}s}$ and $G_{\alpha s\text{-}l}$); the levels of the 43-kDa protein increased slightly in cells incubated with high concentrations of dexamethasone. A 40.8-kDa protein reacted with antibody 2921 ($G_{\alpha z}$), and the amount of this protein decreased with increasing

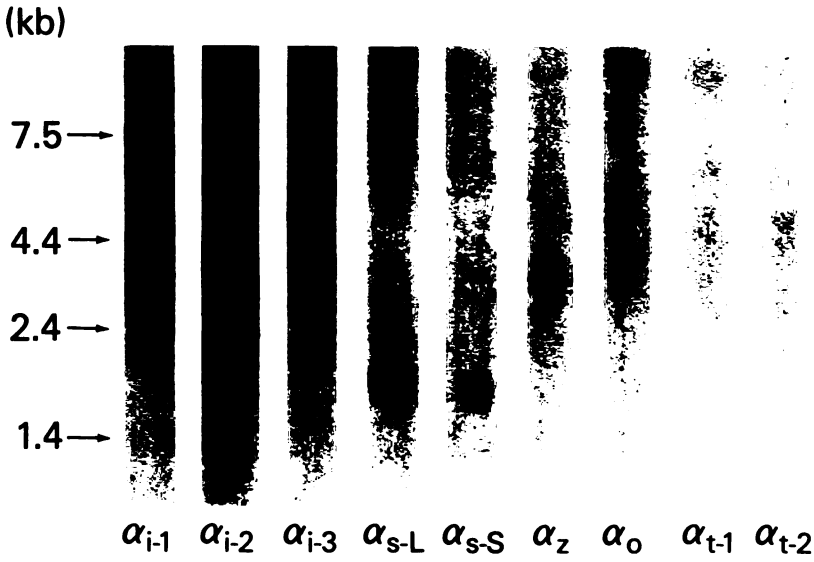

Fig. 2. Hybridization of poly(A)$^+$ RNA from unstimulated monolayer cultures of RBL-2H3 cells with G_α-specific oligonucleotide probes. Poly(A)$^+$ RNA (10 μg) was hybridized with the indicated G_α-specific oligonucleotide probes (Fig. 1), as described in Experimental Procedures. Similar results were obtained in two additional experiments. The blots for $G_{\alpha i\text{-}2}$, $G_{\alpha i\text{-}3}$, $G_{\alpha s\text{-}s}$, $G_{\alpha s\text{-}1}$, and $G_{\alpha z}$ were exposed for 24–48 hr and blots for $G_{\alpha t\text{-}1}$, $G_{\alpha o}$, $G_{\alpha t1}$, and $G_{\alpha t2}$ for >10 days. The positions of the RNA standards are shown on the *left*.

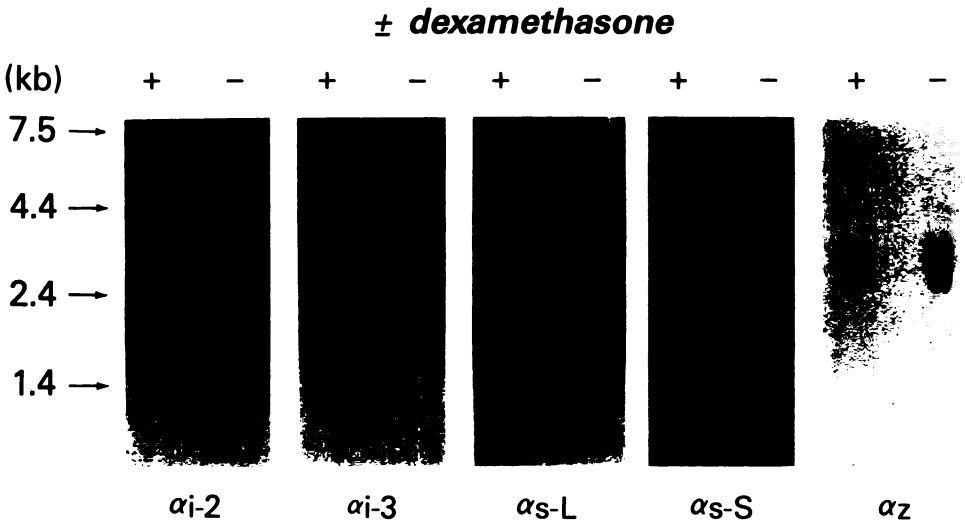

Fig. 3. Hybridization of poly(A)$^+$ RNA from monolayer cultures of control and dexamethasone-treated RBL-2H3 cells with G_α-specific oligonucleotide probes. Cultures were incubated for 14 hr without (−) or with (+) 0.1 μM dexamethasone. Blots were prepared from poly(A)$^+$ RNA (10 μg) and hybridized with the indicated oligonucleotide probes. Positions of the RNA standards are shown on the left. The effect of dexamethasone treatment on the responses to antigen in this experiment is indicated in the text.

TABLE 1

Identification of membrane G_α subunits in RBL-2H3 cells

+ or −, presence or absence of mRNA or protein for the indicated α subunit. Where identification is uncertain, all possible candidates are indicated in parentheses. Arrows, significant increase or decrease in amounts of these substances in dexamethasone-treated cells.

G_α subunit	Presence of mRNA	Protein recognized by antibody[a]						Substrate for[b]		Size of subunit[c]
		AS/7	EC/2	2921	RM/1	GC/2	8645	CTx	PTx	
										kDa
$G_{\alpha i-1}$	−	(+)					(+)		(+)	
$G_{\alpha i-2}$	+	(+)					(+)		(+)	39
$G_{\alpha i-3}$	+		+↑				(+)		(+)	39.5–40
$G_{\alpha z}$	+↓			+↓			(+)			40.8
$G_{\alpha s-S}$	+				+			+		43
$G_{\alpha 2-1}$	+				+			+		45
$G_{\alpha o}$	−					−				
$G_{\alpha i-2}$ and $G_{\alpha t-2}$	−	(+)	(+)							

[a] The α subunits recognized by the antibodies are as follows: AS/7, $G_{\alpha t-1}$, $G_{\alpha t-2}$, $G_{\alpha i-1}$, and $G_{\alpha i-2}$; EC/2, $G_{\alpha t-1}$, $G_{\alpha t-2}$, and $G_{\alpha i-3}$; 2921, $G_{\alpha z}$; RM/1, $G_{\alpha s-S}$ and $G_{\alpha s-L}$; GC/2, $G_{\alpha o}$; 8645, $G_{\alpha i}$ (all forms) and $G_{\alpha z}$.

[b] Proteins ADP-ribosylated by treatment of cells with cholera toxin (CTx) or pertussis toxin (PTx).

[c] As determined by electrophoretic migration of protein (detected by immunoblotting) or of the ADP-ribosylated protein.

concentrations of the glucocorticoid. Although some of these antibodies cross-reacted with additional membrane proteins in both RBL-2H3 cells and rat brain cortex, the correspondence of the 40–50-kDa bands in both preparations provided reasonable certainty of the identity of $G_{\alpha i-3}$, $G_{\alpha s-L}$, and $G_{\alpha z}$ (Fig. 4A). Moreover, the detection of two bands of ADP-ribosylated proteins in cholera toxin-treated membranes and a smaller ADP-ribosylated protein in pertussis toxin-treated membranes was consistent with the existence of at least two forms of $G_{\alpha s}$ and one or more forms of $G_{\alpha i}$ (Fig. 4B). The studies revealed another 40-kDa protein that was ADP-ribosylated by treatment of cytosolic extracts with either pertussis toxin or cholera toxin (data not shown). The significance of this protein was unclear, because we failed to detect it in membrane fractions (Fig. 4B). There have been reports of a 39- to 41-kDa membrane protein that served as a substrate for both toxins in other types of cells (23–29), and the protein is being investigated as a potential candidate for mediating the effects of NECA, which were blocked by both toxins (3).

The apparent decrease in the amount of $G_{\alpha z}$ (Fig. 4A) was observed consistently, with as little as 10^{-9} M dexamethasone. In three series of experiments, the release of [^{3}H]inositol phosphates in response to an optimal concentration of antigen (20 ng/ml DNP-BSA) was reduced by 17%, 42%, and 60% in cells exposed to, respectively, 10^{-9}, 10^{-8}, and 10^{-7} M dexamethasone. A correlation was observed between the decreases in the amounts of $G_{\alpha z}$ and in release of the [^{3}H]inositol phosphates (Fig. 5). In contrast, there was no decrease in amounts of other G_α subunits. If anything, there was an increase in the amount of $G_{\alpha i-3}$ (range of values 20–80%) and possibly $G_{\alpha s-s}$ (0–50%) after treatment of cells with 10^{-7} M dexamethasone. As in previous studies (5), the same cells showed enhanced responses to 100 μM NECA, with increased hydrolysis of inositol phospholipids (Table 2) and an increase in secretion from <5% to 22 ± 4%. Therefore, both $G_{\alpha i-3}$ and the aforementioned soluble 40-kDa protein were candidates for mediating responses via the adenosine receptor(s) in RBL-2H3 cells.

Discussion

The series of events in antigen-stimulated RBL-2H3 cells is similar to those observed in many other types of cells whose functional responses are dependent on Ca^{2+}, namely, mobilization of Ca^{2+} ions and the activation of various phospholipases, including phospholipases A_2 (30, 31), C (2), and D (32). Secondary reactions include translocation of protein kinase C to the membrane (33) and a substantial protein kinase C-catalyzed phosphorylation of the light and heavy chains of myosin (34). These reactions are thought to promote functional responses such as the exocytosis of secretory granules (1), the release of arachidonic acid (31), and, in mast cells, induction of synthesis of various cytokines (35, 36). All of these effects are dependent on influx of Ca^{2+} and a rise in $[Ca^{2+}]_i$, although molecular details of the communication between the aggregated IgE receptor and the various effector systems are undefined.

The G proteins, by analogy with the known role of these proteins in coupling receptors with adenylyl cyclase (37), are thought to serve a similar role in the activation of phospholipase C (38) and possibly other phospholipases (39). The only evidence, however, for the involvement of G protein(s) in antigen-mediated responses is the ability of GDPβS to suppress antigen-induced hydrolysis of the inositol phospholipids, exocytosis (4,

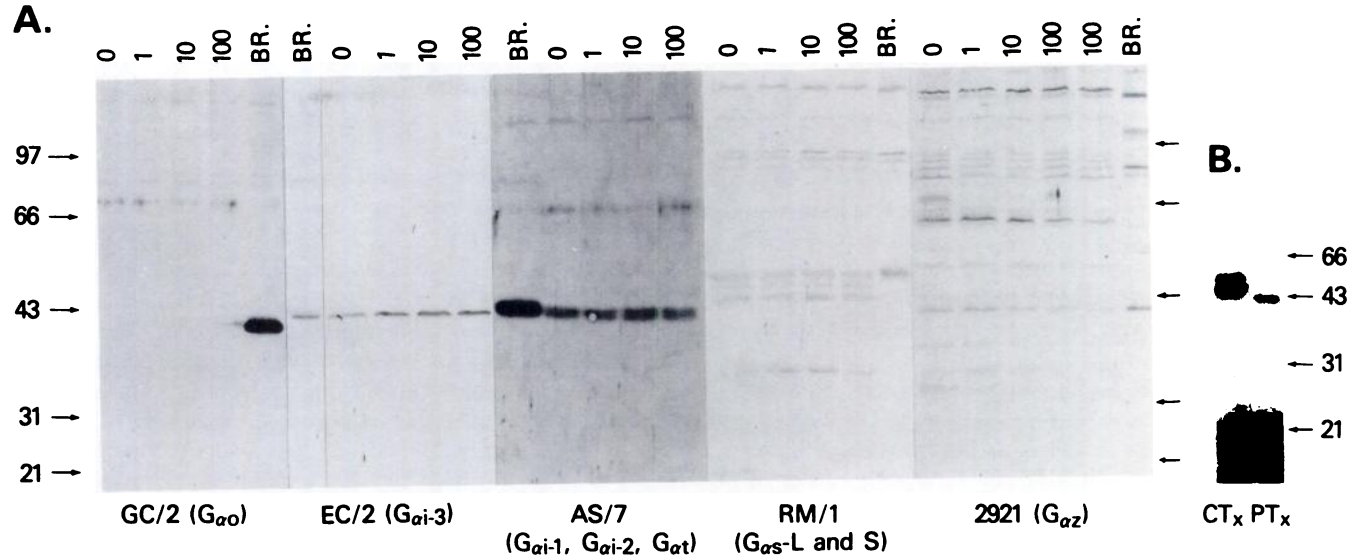

Fig. 4. Effects of dexamethasone on the levels of the G_α subunits in the membrane fraction. A, RBL-2H3 cells were incubated with dexamethasone for 14 hr. *Numbers above each lane*, concentration of dexamethasone (nM). Solubilized membrane preparations were subjected to electrophoresis and immunoblotting with the indicated antibody. The assumed specificity of the antibodies is noted in parentheses. *Lanes BR*, extracts of rat brain cortical membranes. Sizes (kDa) of standard proteins are indicated on the *left*. Representative immunoblots from separate experiments in which each antibody was tested at least twice are shown. The actual changes in the amounts of the protein and cellular responses to antigen for these and other experiments are summarized in the text and Fig. 5. B, RBL-2H3 cell membrane proteins [^{32}P]ADP-ribosylated by incubation with [^{32}P] NAD and cholera toxin (*CTx*) or pertussis toxin (*PTx*). Sizes (kDa) of standard proteins are indicated on the *right*.

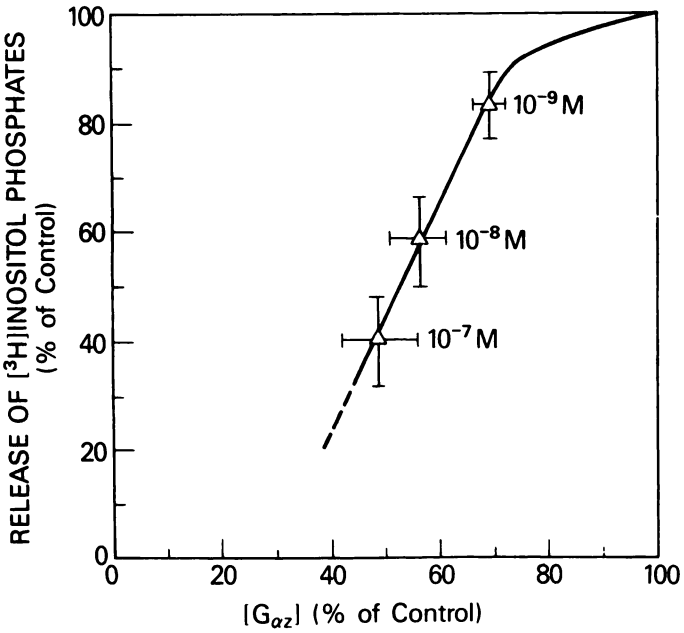

Fig. 5. Correlation between membrane $G_{\alpha z}$ protein and antigen-induced hydrolysis of inositol phospholipids in dexamethasone-treated cells. The decrease in the amount of membrane $G_{\alpha z}$ is plotted as a function of the decrease in phosphoinositide response to antigen (DNP-BSA, 20 ng/ml). Suspension cultures of RBI-2H3 cells were exposed to 0 (control), 10^{-9}, 10^{-8}, or 10^{-7} M dexamethasone for 14 hr before determination of the content of $G_{\alpha z}$ and the response to antigen. The values (mean ± standard error) from three such experiments with the indicated concentrations of dexamethasone are shown. The calculations of the amounts of $G_{\alpha z}$ were based on arbitrary densitometric units. The release of inositol phosphates in the control antigen-stimulated cultures was 36–44% (range of values for all experiments). The correlation coefficient (*r*) between decrease in $G_{\alpha z}$ and responsiveness to antigen was 0.49 for individual data points and 0.997 for the mean values, with a slope of 2.1 ± 0.2 and an intersect with the *x*-axis of about 25%.

TABLE 2

Alteration in expression of $G_{\alpha z}$ and $G_{\alpha i\text{-}3}$ and the responses to antigen and NECA in RBL-2H3 cells treated with dexamethasone

Values (mean ± standard error of three separate experiments) indicate percentage of decrease (↓) or increase (↑) in the amount of α subunit, as determined by immunoblot, after overnight (14-hr exposure of cells to dexamethasone and the generation (percentage of total [^{3}H]inositol phospholipids) of [^{3}H]inositol phosphates in response to stimulation wtih antigen (20 ng/ml DNP-BSA) or 100 μM NECA in both control (no treatment) and dexamethasone-treated cells.

Treatment	Change in expression of		Release of inositol phosphates with	
	$G_{\alpha z}$	$G_{\alpha i\text{-}3}$	DNP-BSA	NECA
	%		%	
None			40 ± 2	2 ± 0.5
Dexamethasone (10^{-7} M)	52 ± 6↓	47 ± 7↑	16 ± 4	7 ± 1

40), and release of arachidonic acid[3] in permeabilized cells and the enhancement of antigen-induced influx of Ca^{2+} in cholera toxin-treated cells (3, 6). Even so, relatively high (millimolar) concentrations of GDPβS are needed to suppress these responses (6). Also, we and others[3,5] have been unable to demonstrate that GTP augments antigen-induced hydrolysis of the inositol phospholipids. These caveats do not apply to the stimulation of mast cells with compound 48/80 (30, 41) or of RBL-2H3 cells with NECA (3), because the responses to these two agents are effectively abolished by incubation of cells with pertussis toxin and, in the case of NECA stimulation, by incubation with cholera toxin as well. It is probable, therefore, that pertussis toxin-sensitive G proteins are required for the signal transduction processes of these two stimulants.

The present studies strengthen the arguments for the possible involvement of a G protein, specifically G_z, in the mediation of responses to antigen. Firstly, the responses to antigen in toxin-treated cells (3) are consistent with the fact that $G_{\alpha z}$ is not a substrate for cholera toxin- or pertussis toxin-catalyzed

[5] S. Dreskin and H. Metzger, personal communication.

ADP-ribosylation (8–10). Secondly, the decrease in the amounts of mRNA for $G_{\alpha z}$ and of the $G_{\alpha z}$ subunit in dexamethasone-treated cells correlates with the attenuation of the phosphoinositide and secretory responses of these cells to antigen. This is in contrast to the increase in the amounts of $G_{\alpha i\text{-}3}$, and possibly $G_{\alpha s\text{-}s}$, and the increased sensitivity of the cells to NECA (5). Finally, the activation of phospholipase C and increase in $[Ca^{2+}]_i$ in antigen-stimulated cells are exquisitely sensitive to the inhibitory effects of activators of protein kinase C, such as phorbol myristate (42), and it is known that a $G_{\alpha z}$-like protein is selectively phosphorylated in platelets stimulated with phorbol myristate acetate or thrombin (43).

The cDNA that encodes $G_{\alpha z}$ (alternatively referred to as $G_{\alpha x}$) was recently characterized (8, 9). Although the deduced sequence of amino acids indicates a high degree of homology between $G_{\alpha z}$ and the $G_{\alpha i}$ subunits, the molecules differ in two critical regions. $G_{\alpha z}$ lacks the cysteine, located four residues from the carboxyl terminus, that is the site of ADP-ribosylation by pertussis toxin in $G_{\alpha o}$, $G_{\alpha t}$, and the $G_{\alpha i}$ species. In addition, $G_{\alpha z}$ contains three amino acids that differ from the other G_α subunits in the region believed to be responsible for GTPase activity (GXXXXGK) (44). This latter difference is thought to account for the extraordinarily low rates of guanine nucleotide exchange and intrinsic GTPase activity of recombinant $G_{\alpha z}$ from *Escherichia coli* (10). Consequently, activation of $G_{\alpha z}$ would be expected to cause a long lasting signal unless, as speculated, GTPase-activating proteins, such as those recently described for *ras* and *ras*-related proteins, enhance the GTPase activity of $G_{\alpha z}$ (10).

If $G_{\alpha z}$ is indeed the transducer for signals from the IgE receptor, then additional enhancement of this signaling pathway is necessary to account for several earlier observations. For example, the observed $t_{1/2}$ for hydrolysis of the recombinant $G_{\alpha z}$-bound GTP is 10–12 min, compared with seconds for other G_α subunits (10). This rate of hydrolysis, and presumably of deactivation of $G_{\alpha z}$, is too slow to account for the immediate abrogation of all stimulatory responses to antigen when antigen is displaced from the receptor-bound IgE with excess monovalent hapten (1, 33). There is also evidence that a Ca^{2+}-independent tyrosine phosphorylation of several proteins (45), which include the phospholipase C γ isozyme,[6] is an early event in antigen-stimulated RBL-2H3 cells. These phosphorylations, however, are not attenuated in cells treated with dexamethasone.[7] Moreover, the tyrosine kinase inhibitor genistein (46, 47) blocks all responses to low concentrations of antigen, but the blockade is largely reversed by increasing the concentration of antigen.[3] This result could indicate that a tyrosine kinase-catalyzed phosphorylation synergizes the transduction of the primary signal across the membrane at low levels of stimulation. More definitive information on the roles of $G_{\alpha z}$ and tyrosine phosphorylation must await further studies of the phosphorylation of the phospholipase C γ isozyme (48) in RBL-2H3 cells and of the effects of overexpression of the $G_{\alpha z}$ gene in transfected RBL-2H3 cells.

The identification of $G_{\alpha i\text{-}2}$, $G_{\alpha s\text{-}s}$, $G_{\alpha s\text{-}L}$, and $G_{\alpha z}$ in RBL-2H3 cells was based on their immunoreactivity to antibodies, the presence of mRNA, and their ability to serve as substrates for toxin-catalyzed ADP-ribosylation (Table 1). The studies with toxins revealed additional soluble proteins, which remain uncharacterized but which resemble those previously described in cytosol of rat mast cells (30) and membranes of various cells (23–29).

In conclusion, incubation of RBL-2H3 cells with physiologic concentrations of dexamethasone results in the alteration of expression of the various G_α subunits, primarily a decrease in $G_{\alpha z}$ and an increase in $G_{\alpha i\text{-}3}$. Therefore, one mechanism by which glucocorticoids, such as dexamethasone, might induce selective changes in signal transduction systems is by up-regulating or down-regulating the expression of G proteins or receptors. Our data are consistent with previous proposals that increased expression of $G_{\alpha s}$ (49, 50) and β-adrenergic receptors (51, 52) in different types of dexamethasone-treated cells accounts for the enhanced activation of adenylyl cyclase by β agonists in these cells. Although treatment with dexamethasone results in decreases in the expression of several proteins (e.g., Fig. 4), the decrease in $G_{\alpha z}$ was unique with respect to the G_α subunits and, therefore, its characteristics fit the criteria for a G protein subunit that can couple to the IgE receptor. The identity of the G protein that couples to the adenosine receptor is presently unclear, but $G_{\alpha i\text{-}3}$ and a soluble protein that serves as a substrate for ADP-ribosylation by either pertussis toxin or cholera toxin are potential candidates.

[6] D. J. Park, H. K. Min and S. G. Rhee, submitted for publication.
[7] R. A. Baumgartner, C. L. Jelsema, and M. A. Beaven, unpublished data.

References

1. Metzger, H., G. Alcaraz, R. Hohman, J. P. Kinet, V. Pribluda, and R. Quarto. The receptor with high affinity for immunoglobulin E. *Annu. Rev. Immunol.* **4**:419–470 (1986).
2. Beaven, M. A., and J. R. Cunha-Melo. Membrane phosphoinositide-activated signals in mast cells and basophils. *Prog. Allergy* **42**:123–184 (1988).
3. Ali, H., J. R. Cunha-Melo, W. F. Saul, and M. A. Beaven. The activation of phospholipase C via adenosine receptors provides synergistic signals for secretion in antigen stimulated RBL-2H3 cells: evidence for a novel adenosine receptor. *J. Biol. Chem.* **265**:745–753 (1990).
4. Ali, H., D. M. Collado-Escobar, and M. A. Beaven. The rise in concentration of free Ca^{2+} and pH provide sequential, synergistic signals for secretion in antigen-stimulated rat basophilic leukemia (RBL-2H3) cells. *J. Immunol.* **143**:2626–2633 (1989).
5. Collado-Escobar, D., H. Ali, and M. A. Beaven. On the mechanism of action of dexamethasone in a rat mast cell line (RBL-2H3 cells): evidence for altered coupling of receptors and G-proteins. *J. Immunol.* **144**:3449–3457 (1990).
6. Narasimhan, V., D. Holowka, C. Fewtrell, and B. Baird. Cholera toxin increases the rate of antigen-stimulated calcium influx in rat basophilic leukemia cells. *J. Biol. Chem.* **263**:19626–19632 (1988).
7. McCloskey, M. A. Cholera toxin potentiates IgE-coupled inositol phospholipid hydrolysis and mediator secretion by RBL-2H3 cells. *Proc. Natl. Acad. Sci. USA* **85**:7260–7264 (1988).
8. Fong, H. K., K. K. Yoshimoto, P. Eversole-Cire, and M. I. Simon. Identification of a GTP-binding protein α subunit that lacks an apparent ADP-ribosylation site for pertussis toxin. *Proc. Natl. Acad. Sci. USA* **85**:3066–3070 (1988).
9. Matsuoka, M., H. Itoh, T. Kozasa, and Y. Kaziro. Sequence analysis of cDNA and genomic DNA for a putative pertussis toxin-insensitive guanine nucleotide-binding regulatory protein α subunit. *Proc. Natl. Acad. Sci. USA* **85**:5384–5388 (1988).
10. Casey, P. J., H. K. W. Fong, M. I. Simon, and A. G. Gilman. G_z a guanine nucleotide-binding protein with unique biochemical properties. *J. Biol. Chem.* **265**:2383–2390 (1990).
11. Noda, M., B. C. Kunz, S. C. Tsai, R. Adamik, J. J. Murtagh, H. C. Chen, J. L. Halpern, and J. Moss. Isolation and immunological properties of adenosine kinase. *Prep. Biochem.* **19**:351–361 (1989).
12. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
13. Davis, G. D., M. D. Dibner, and J. F. Batte. *Basic Methods in Molecular Biology*. Elsevier Science Publishing Co., New York (1986).
14. Itoh, H., T. Kozasa, S. Nagata, S. Nakamura, T. Katada, M. Ui, S. Iwai, E. Ohtsuka, H. Kawasaki, K. Suuki, and Y. Kaziro. Molecular cloning and sequence determination of cDNAs for α subunits of the guanine nucleotide-binding proteins G_s, G_i and G_o from rat brain. *Proc. Natl. Acad. Sci. USA* **83**:3776–3780 (1986).
15. Itoh, H., R. Toyama, T. Kozasa, T. Tsukamoto, M. Matsuoka, and Y. Kaziro. Presence of three distinct molecular species of G_i protein α subunit: structure

of rat cDNAs and human genomic DNAs. *J. Biol. Chem.* **263**:6656–6664 (1988).
16. Tanabe, T., T. Nukada, Y. Nishikawa, K. Sugimoto, H. Suzuki, H. Takahashi, M. Noda, T. Haga, A. Ichiyama, K. Kangawa, N. Minamino, H. Matsuo, and S. Numa. Primary structure of the α-subunit of transducin and its relationship to *ras* proteins. *Nature* (*Lond.*) **315**:242–245 (1985).
17. Lochrie, M. A., J. B. Hurley, and M. I. Simon. Sequence of the *alpha* subunit of photoreceptor G protein: homologies between transducin, *ras*, and elongation factors. *Science* (*Washington D. C.*) **228**:96–99 (1985).
18. Eschenfeldt, W. H., R. S. Puskas, and S. L. Berger. Homopolymeric tailing. *Methods Enzymol.* **152**:337–342 (1987).
19. Tsuchiya, M., S. R. Price, M. S. Nightingale, J. Moss, and M. Vaughan. Tissue and species distribution of mRNA encoding two ADP-ribosylation factors, 20-kDa guanine nucleotide binding proteins. *Biochemistry* **28**:9668–9673 (1989).
20. Goldsmith, P., K. Rossiter, A. Carter, W. Simonds, C. G. Unson, R. Vinitsky, and A. M. Spiegel. Identification of the GTP-binding protein encoded by G_{is} complementary DNA. *J. Biol. Chem.* **263**:6476–6479 (1988).
21. Kenna, J. G., H. Satoh, D. D. Christ, and L. R. Pohl. Metabolic basis for a drug hypersensitivity: antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane. *J. Pharmacol. Exp. Ther.* **245**:1103–1109 (1988).
22. Burns, D. L., E. L. Hewlett, J. Moss, and M. Vaughan. Pertussis toxin inhibits enkephalin stimulation of GTPase of NG108-15 cells. *J. Biol. Chem.* **258**:1435–1438 (1983).
23. Owens, J. R., L. T. Frame, M. Ui, and D. M. Cooper. Cholera toxin ADP-ribosylates the islet-activating protein substrate in adipocyte membranes and alters its function. *J. Biol. Chem.* **260**:15946–15952 (1985).
24. Aksamit, R. R., P. S. Backlund, Jr., and G. L. Cantoni. Cholera toxin inhibits chemotaxis by a cAMP-independent mechanism. *Proc. Natl. Acad. Sci. USA* **82**:7475–7479 (1985).
25. Verghese, M., R. J. Uhing, and R. Snyderman. A pertussis/cholera toxin-sensitive N protein may mediate chemoattractant receptor signal transduction. *Biochem. Biophys. Res. Commun.* **138**:887–894 (1986).
26. Gierschik, P., and K. H. Jakobs. Receptor-mediated ADP-ribosylation of a phospholipase C-stimulating G protein. *FEBS Lett.* **224**:219–223 (1987).
27. Milligan, G., and F. R. McKenzie. Opioid peptides promote cholera-toxin-catalysed ADP-ribosylation of the inhibitory guanine-nucleotide-binding protein (G_i) in membranes of neuroblastoma × glioma hybrid cells. *Biochem. J.* **252**:369–373 (1988).
28. Milligan, G. Foetal calf serum enhances cholera toxin-catalysed ADP-ribosylation of the pertussis toxin-sensitive guanine nucleotide binding protein, G_i, in rat glioma C6BU1 cells. *Cell. Signalling* 1:65–74 (1989).
29. Iiri, T., M. Tohkin, N. Morishima, Y. Ohoka, M. Ui, and T. Katada. Chemotactic peptide receptor-supported ADP-ribosylation of a pertussis toxin substrate GTP-binding protein by cholera toxin in neutrophil-type HL-60 cells. *J. Biol. Chem.* **264**:21394–21400 (1989).
30. Nakamura, T., and M. Ui. Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release, and histamine secretion in mast cells by islet-activating protein, pertussis toxin: a possible involvement of the toxin-specific substrate in the Ca^{2+}-mobilizing receptor-mediated biosignaling system. *J. Biol. Chem.* **260**:3584–3593 (1985).
31. Garcia-Gil, M., and R. P. Siraganian. Phospholipase A_2 stimulation during cell secretion in rat basophilic leukemia cells. *J. Immunol.* **136**:259–263 (1986).
32. Gruchalla, R. S., T. T. Dinh, and D. A. Kennerly. An indirect pathway of receptor-mediated 1,2-diacylglycerol formation in mast cells. I. IgE receptor-mediated activation of phospholipase D. *J. Immunol.* **144**:2334–2342 (1990).
33. White, K. N., and H. Metzger. Translocation of protein kinase C in rat basophilic leukemic cells induced by phorbol ester or by aggregation of IgE receptors. *J. Immunol.* **141**:942–947 (1988).
34. Ludowyke, R. I., I. Peleg, M. A. Beaven, and R. S. Adelstein. Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells. *J. Biol. Chem.* **264**:12492–12501 (1989).
35. Plaut, M., J. H. Pierce, C. J. Watson, J. Hanley-Hyde, R. P. Nordan, and W. E. Paul. Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature* (*Lond.*) **339**:64–67 (1989).
36. Wodnar-Filipowicz, A., and C. Moroni. Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions. *Proc. Natl. Acad. Sci. USA* **87**:777–781 (1990).
37. Gilman, A. G. G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56:615–649 (1987).
38. Gomperts, B. D., M. M. Barrowman, and S. Cockcroft. Dual role for guanine nucleotides in stimulus-secretion coupling. *Fed. Proc.* **45**:2156–2161 (1986).
39. Exton, J. H. Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265**:1–4 (1990).
40. Saito, H., K. Ishizaka, and T. Ishizaka. Effect of nonhydrolyzable guanosine phosphate on IgE-mediated activation of phospholipase C and histamine release from rodent mast cells. *J. Immunol.* **143**:250–258 (1989).
41. Saito, H., F. Okajima, T. F. Molski, R. I. Sha'Afi, M. Ui, and T. Ishizaka. Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. *J. Immunol.* **138**:3927–3934 (1987).
42. Beaven, M. A., D. F. Guthrie, J. P. Moore, G. A. Smith, T. R. Hesketh, and J. C. Metcalfe. Synergistic signals in the mechanism of antigen-induced exocytosis in 2H3 cells: evidence for an unidentified signal required for histamine release. *J. Cell Biol.* **105**:1129–1136 (1987).
43. Carlson, K. E., L. F. Brass, and D. R. Manning. Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than G_i in human platelets. *J. Biol. Chem.* **264**:13298–13305 (1989).
44. Price, S. R., A. Barber, and J. Moss. Structure-function relationships of guanine nucleotide-binding proteins, in *ADP-Ribosylating Toxins and G Proteins. Insights into Signal Transduction* (J. Moss and M. Vaughan, eds.). American Society of Microbiology, Washington D. C., 397–424 (1990).
45. Benhamou, M., J. S. Gutkind, K. C. Robbins, and R. P. Siraganian. Tyrosine phosphorylation coupled to IgE receptor-mediated signal transduction and histamine release. *Proc. Natl. Acad. Sci. USA* **87**:5327–5330 (1990).
46. Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S.-I. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262**:5592–5595 (1987).
47. Mustelin, T., K. M. Coggeshall, N. Isakov, and A. Altman. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* (*Washington D. C.*) **247**:1584–1587 (1990).
48. Nishibe, S., M. I. Wahl, P. B. Wedegaertner, J. J. Kim, S. G. Rhee, and G. Carpenter. Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor, and their cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* **87**:424–428 (1990).
49. Chang, F.-H., and H. R. Bourne. Dexamethasone increases adenylyl cyclase activity and expression of the α-subunit of G_s in GH_3 cells. *Endocrinology* **121**;1711–1715 (1987).
50. Rodan, S. B., and G. A. Rodan. Dexamethasone effects on β-adrenergic receptors and adenylate cyclase regulatory proteins G_s and G_i in ROS 17/2.8 cells. *Endocrinology* **118**:2510–2518 (1986).
51. Collins, S., M. G. Caron, and R. J. Lefkowitz. β_2-Adrenergic receptors in hamster smooth muscle cells are transcriptionally regulated by glucocorticoids. *J. Biol. Chem.* **263**:9067–9070 (1988).
52. Hadcock, J. R., and C. C. Malbon. Regulation of β-adrenergic receptors by "permissive" hormones: glucocorticoids increase steady-state levels of receptor mRNA. *Proc. Natl. Acad. Sci. USA* **85**:8415–8419 (1988).

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