Regulation of Constitutive Exocytic Transport by Membrane Receptors

A BIOCHEMICAL AND MORPHOMETRIC STUDY*

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Biochemical and morphometric approaches were combined to examine whether constitutive secretory transport might be controlled by plasma membrane receptors, as this possibility would have significant physiological implications. Indeed, IgE receptor stimulation in rat basophilic leukemia cells potently increased the rate of transport of soluble pulse-labeled $^{35}$S-sulfated glycosaminoglycans from distal Golgi compartments to the cell surface. This effect was largely protein kinase C (PKC)-dependent. Direct activation of PKC also stimulated constitutive transport of glycosaminoglycans, as indicated by the use of agonistic and antagonistic PKC ligands. PKC ligands also had potent, but different, effects on the exocytic transport from distal Golgi compartments to the plasma membrane of a membrane-bound protein (vesicular stomatititis virus glycoprotein), which was slightly stimulated by activators and profoundly suppressed by inhibitors of PKC. Morphological analysis showed impressive changes of the organelles of the secretory pathway in response to IgE receptor stimulation and to direct PKC activation (enhanced number of buds and vesicles originating from the endoplasmic reticulum and Golgi and increase in surface and volume of Golgi compartments), suggestive of an overall activation of exocytic movements. These results show that rapid and large changes in constitutive transport fluxes and in the morphology of the exocytic apparatus can be induced by membrane receptors (as well as by direct PKC stimulation).

Constitutive membrane transport is fundamental to a number of cellular functions including growth and differentiation, secretion of proteins such as immunoglobulins, proteoglycans, serum, matrix, and milk proteins, as well as generation, homeostasis, and turnover of cellular organelles. Such essential functions are likely to be precisely controlled.

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† To whom correspondence should be addressed: Laboratory of Molecular Neurobiology, Consorzio Mario Negri Sud, Laboratory of Molecular Neurobiology and the Physiopathology of Secretion Unit, 66030 S. Maria Imbaro (Chieti), Italy. Tel.: 39-872-570354; Fax: 39-872-578240.

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¶ Present address: Depts. of Cell and Molecular Biology, The Scripps Research Inst., La Jolla, CA 92037.

** Present address: Laboratorio di Biologia Cellulare e dello Sviluppo, Università di Pisa, Via Carducci 13, 56100 Ghezzano (PI) Italy.

Certain basic steps in membrane and protein transport such as vesicle formation, docking, and fusion have been studied extensively over the last decade, and a fundamental set of molecular mechanisms executing these events has been elucidated (1). Superimposed on this core machinery, regulatory mechanisms must exist to fine tune membrane traffic, maintain organelle homeostasis, and mediate adaptive responses of transport to variations in extracellular conditions. In spite of their potentially great physiologic importance, however, they have received relatively little attention.

Evidence has nevertheless accumulated showing that regulatory molecules play a role in constitutive membrane traffic. Heterotrimeric GTP-binding proteins have been implicated in the secretion of proteoglycans (2), apical secretion in polarized cells (3), endosome-endosome fusion (4), transcytosis (5, 6), in the association of coatomer with Golgi membranes (7), vesicle formation from the distal Golgi (8), and ER to Golgi transport (9). Protein phosphorylation has been shown to be involved in the regulation of traffic; ER to Golgi transport is suppressed by protein phosphatase inhibitors (10). Vesicle formation from the trans Golgi/trans Golgi network (TG/TGN) is dependent on protein phosphorylation (11); hyperphosphorylation of a yet unidentified protein(s) leads to a block of homologous endosome fusion in vitro (12); the mitotic kinase Cdc2 has also been proposed to be responsible for the endosome fusion block at mitosis (13).

On the basis of these considerations, we have recently investigated the possibility that signal transduction pathways might control constitutive membrane traffic and reported that protein kinase C (PKC) and PKC-coupled receptors modulate the association of ADP-ribosylation factor (ARF) to the Golgi apparatus (14). ARF binding to Golgi membranes is a key molecular step in vesicular traffic (1). This finding has helped bring into focus the link between constitutive traffic and signal transduction and further stimulated interest in the question as to whether segments of the membrane trafficking pathways may be regulated by second messengers (15) and by which mechanisms. Most recently, a number of reports have produced evi-
dence that, indeed, diverse transport pathways can be affected by bacterial toxins or drugs modifying the level of second messengers (16–21).

To assess the physiological significance of these regulatory phenomena, we have studied the effects of receptor stimulation on constitutive membrane traffic. Biochemical analysis indicates that in RBL cells, the IgE receptor can control membrane and solute exocytic fluxes from the TGN to the cell surface mostly via PKC. Morphological analysis extends this observation, revealing changes of the secretory apparatus (enhanced number of buds and vesicles originating from both the ER and the Golgi complex, and increase in surface and volume of Golgi compartments), suggestive of an overall acceleration of exocytic transport. The results suggest that signal transduction systems can play a crucial role in the mechanisms controlling size and transport activity of the exocytic organelles.

EXPERIMENTAL PROCEDURES

Materials—Ro31-8282 was a gift from Roche Research Centre (Welwyn Garden City, UK). Brefeldin A (BFA), phorbol 12-myristate 13-acetate (PMA), streptavidine-agarose, mouse anti-dinitrophenol (DNP) IgE, horseradish peroxidase type II, p-nitrophenyl β-D-xylopyranoside (xyloside), and adenosine 3′-phosphate 5′-phosphosulfate were purchased from Sigma; NHS-LC-biotin was from Pierce (Rockford IL). Calphostin C, DNP-bovine serum albumin (BSA) conjugate (DNP-BSA), and A23187 were from Calbiochem (San Diego, CA). 12-Deoxyphorbol 13-phenacylacetate (DPP), 12-deoxyphorbol 13-phenylacetate-20-acetoxy (DPPA), and thymeleatoxin were purchased from LC Laboratories (Woburn, MA). Cell culture media and sera were from Life Technologies, Inc. and Seramed (Berlin, Germany). TranS−S (a mixture of 35S-labeled methionine and cysteine) was purchased from ICN (Irvine, CA). [3H]Hydroxytriptamine (serotonin) binoxalate was from New England Nuclear (Boston, MA). Various reagents used for the experiments were from Sigma Chemical Co., St. Louis, MO, or obtained from various sources. 

Fig. 1. Cell-free formation of secretory vesicle from the TGN in PC12 cells. A postnuclear supernatant from PC12 cells prelabeled with [35S]sulfate, was incubated at 37 °C to allow vesicle formation in vitro. After the reaction, the suspension was centrifuged on a continuous sucrose gradient to separate small vesicles (upper fractions) from larger membranes (lower fractions). Fractions were analyzed by SDS-PAGE and radioactivity was measured in an scintillation counter using Beckman Ultima Gold XR.

Analysis of Regulated Release of Serotonin from RBL Cells—The release of serotonin from secretory granules in RBL cells was measured as follows. Cells at confluence in 24-well plates were labeled with 1 μCi/ml [3H]serotonin for 12 h. The control sample was incubated for the same time at 0°C. The distribution of labeled proteoglycans between upper and lower fractions (from fraction 1 to 12) is shown for a sample incubated for 30 min at 37 °C and a control sample incubated for the same time at 0°C. The distribution of sialyltransferase activity across the gradient is also shown for samples incubated at 0°C (filled symbols) and at 37°C (empty symbols). The ratio between radioactivity in the upper versus lower fractions is taken as an index of vesicle formation from the TGN (see Table III).
vitro, as measured by the shift of labeled proteoglycans from the lower into the upper fractions, as compared with the 0 °C control (26), occurred rather efficiently in basal conditions; it was independent of the presence of Ca
+2 and reached a plateau at 30–60 min (60 min not shown). The shift of labeled material from lower to upper fractions was very likely due to active vesicle formation rather than damage (fragmen-
tation). In intact Golgi membranes, since the profile of the resident trans Golgi marker sialyltransferase was not significantly changed by the different treatments (Fig. 1). The small amount of labeled material present in upper fractions in the 0 °C sample is most probably due to some degree of TGN fragmentation and/or to the formation of transport vesicles in vivo prior to homogenization.

VSV (m) was a gift from Dr. J. Rothman. The VSV glycoprotein (VSV-G) transport by RBL cells was platted at confluence in 12-well plates and infected the following day at 31 °C with the ts045 mutant (28) of VSV in MEM containing 10% FCS and 5 μM actino-
mycin D. 1 h later, the medium was replaced with MEM, and the incubation continued for an additional 2 h. To label proteins, cells were starved 10 min at the nonpermissive temperature of 40 °C in DMEM lacking methionine and cysteine, pulse-labeled with [35S]methionine (50–60 μCi/ml) for 10 min at 40 °C, and then shifted to 19.5 °C for 2 h (to block proteins in the TGN) in complete MEM supplemented with 2 μM methionine. The cells were then incubated at 37 °C with or without agonists for various periods. A control was performed by incubating cells at 0 °C. Cell surface proteins were biotinylated according to Refs. 29 and 30, after washing in phosphate-buffered saline (PBS) and one washing in PBS containing 10 μM CaCl
2 and 5 mM MgCl
2. Cells were biotinylated at 37 °C for 20 min, quenched in 10× PBS containing 50 mM NH4Cl, and extensively washed with PBS-Ca/Mg. Biotinylated cells were lyzed for 60 min in lysis buffer (150 mM NaCl, 20 mM Tris pH 9, 5 mM EDTA, 1% Triton X-100, 0.2% BSA). Material from two wells was collected and centrifuged 10 min at 14,000 × g, and aliquots of the supernatant were precipitated with trichloroacetic acid or incubated overnight at 4 °C with 30 μl of a streptavidin-agarose bead suspension. The beads were centrifuged at 14,000 × g for 30 s and washed extensively with PBS containing 1% Triton X-100. Bound material was eluted from the beads with 150 mM NaCl, 20 mM Tris pH 9, 5 mM EDTA, 1% Triton X-100, and 0.2% BSA. Material from two wells was eluted from the bead suspension, and eluted material was analyzed by 8% acrylamide SDS-PAGE. Finally, clusters were defined as 50–80-nm round structures with a slightly denser appearance, vesicular (round) over tubular or oval profiles, we used the approxima-
tion that the endosomal compartment by horseradish peroxidase staining showed that endosomes represent a negligible fraction of the mem-
branes in the Golgi exclusion zone (Fig. 2B). Buds were defined as spherical-cylindrical elevations (diameter 60–100 nm) protruding from the surface of the ER (Fig. 2C) or of the Golgi complex (Fig. 2D) by at least 60% of their diameter, showing a direct connection with the donor membrane (ER or Golgi complex) and containing an enlargement of the endosomal compartment. The mean diameter of the round vesicles was 50–90 nm, and the average number of vesicles per cell section was 7. These structures were distinguishable from clathrin coats (Fig. 2D, inset). Vesicular profiles were defined as 50–80-nm round structures with a slightly denser content than that of Golgi cisternae. Given the high prevalence of vesicular (round) over tubular or oval profiles, we used the approxima-
tion that all round profiles represent vesicles, in order to calculate their number, surface, and volume. The tubular-vesicular clusters (TVC) derived from the ER (putative transport intermediates, see “Results”) were defined as distinct aggregates of at least three vesicles adjacent to the ER or at least two vesicles associated with an ER bud (Fig. 2C and inset). The average number of vesicular profiles in thin sections of such clusters was six.

To assess the volume and surface density (Nv and Sv, respectively) of the above defined structures, five random meshes on a grid, each containing at least 30 cell profiles from the same ultratrace, were used for each treatment and time point, photographed at 3000×, and photographically enlarged 14,000 times (to analyze whole cells), 31,120 times (for the ER), or 66,700 times (for the Golgi complex and TVC). The mean area (Sv) and perimeter (Pv) of buds, vesicles, and TVC in cells was estimated by the formula: 

\[ \text{Sv} = \frac{1}{2} \text{Pv} \cdot \text{Nv} \]

where Nv is the number of profiles of these structures per cell sectional area and C is a constant related to their shape (33). Since these structures are rather spherical, we used a C coefficient equal to 1. The correction of bias due to section thickness and section compression was done according to Ref. 33. The correction factor for bud and vesicle number density was 0.41, as in Fig. 4 in Ref. 33. The correction factors for Golgi volume and surface were estimated as 0.6 and 0.64, respectively, based on Figs. 5 and 6 in Ref. 33 and on the reasonable assumption that two-thirds of the Golgi complex was com-
posed of the endosome with an average length of 1 μm and one-third of the tubules of 70 nm in diameter. The absolute values of surface (Sv), volume (Vv), and number (Nv) of the structures were calculated by the formulae:

\[ Vv = \frac{1}{2} \text{Pv} \cdot \text{Nv} \]

\[ Sv = \frac{1}{2} \text{Pv} \cdot \text{Nv} \]

\[ Nv = \frac{1}{2} \text{Pv} \cdot \text{Nv} \]

Cytochemistry—For horseradish peroxidase labeling of endosomal compartments, cells were incubated with 15 mg/ml horseradish pero-
idase at 37 °C for 2 h. Cells were then stained and embedded in Epon 812, and ultrathin sections were stained with uranyl acetate and lead citrate and photographed with a Zeiss 109 transmission electron microscope.

Transmission Electron Microscopy—Cells were grown on Thermanox coverslips (Nunc Inc., Illinois) to 80% confluence, fixed with 2.5% glutaraldehyde in 10 mM PBS for 12 h, postfixed in 1% buffered OsO4 for 1 h, and embedded in Epon as described above and sectioned. The average of these two values (780 μm3) was taken as a reliable estimate of RBL cell volume and was used to calculate all other morphometric parameters (see below and Table 1).

The Golgi apparatus was defined as a complex of cisternae organized in stacks and tubular structures in the Golgi exclusion zone (Fig. 2A). In these areas, there may be two non-Golgi tubular structures: endosomes and transitional elements of the ER. The latter were excluded based on continuity with rough ER, presence of ribosomes on a contour, character-
estic floccular content, and more abrupt membrane outline. Tubular endosomes were excluded based on their thinner diameter (40–50 in-
stead of 60–90 nm for Golgi-associated tubules), very uniform width, and worm-like appearance; moreover, experiments designed to visual-
ize the endosome with or without confinement by horseradish peroxidase staining showed that endosomes represent a negligible fraction of the mem-
branes in the Golgi exclusion zone (Fig. 2B). Buds were defined as spherical-cylindrical elevations (diameter 60–100 nm) protruding from the surface of the ER (Fig. 2C) or of the Golgi complex (Fig. 2D) by at least 60% of their diameter, showing a direct connection with the donor membrane (ER or Golgi complex) and containing an enlargement of the endosomal compartment. The mean diameter of the round vesicles was 50–90 nm, and the average number of vesicles per cell section was 7. These structures were distinguishable from clathrin coats (Fig. 2D, inset). Vesicular profiles were defined as 50–80-nm round structures with a slightly denser content than that of Golgi cisternae. Given the high prevalence of vesicular (round) over tubular or oval profiles, we used the approxima-
tion that all round profiles represent vesicles, in order to calculate their number, surface, and volume. The tubular-vesicular clusters (TVC) derived from the ER (putative transport intermediates, see “Results”) were defined as distinct aggregates of at least three vesicles adjacent to the ER or at least two vesicles associated with an ER bud (Fig. 2C and inset). The average number of vesicular profiles in thin sections of such clusters was six.

To assess the volume and surface density (Nv and Sv, respectively) of the above defined structures, five random meshes on a grid, each containing at least 30 cell profiles from the same ultratrace, were used for each treatment and time point, photographed at 3000×, and photographically enlarged 14,000 times (to analyze whole cells), 31,120 times (for the ER), or 66,700 times (for the Golgi complex and TVC). The mean area (Sv) and perimeter (Pv) of buds, vesicles, and TVC in cells was estimated by the formulae:

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idase at 37 °C for 2 h. Cells were then stained and embedded in Epon 812, and ultrathin sections were stained with uranyl acetate and lead citrate and photographed with a Zeiss 109 transmission electron microscope.
RESULTS

We have studied the effects of membrane receptor stimulation on constitutive transport of soluble and membrane markers from TG/TGN to plasma membrane and on the morphology of the exocytic apparatus. Most experiments were carried out in RBL cells, where we had previously described the modulatory role of membrane receptors and PKC on ARF binding (14); other cell lines were employed to confirm and extend the role of membrane receptors and PKC on ARF binding (see "Experimental Procedures"). The horseradish peroxidase-labeled endosomes (arrows) were clearly distinct from the Golgi structure (arrowheads), and no horseradish peroxidase was detected in the Golgi area. C, ER-derived buds (arrows) and tubular-vesicular clusters (arrowheads and inset, asterisk) are situated near the ER (small arrows; see definitions in "Experimental Procedures"). D, tangential section of the Golgi cisternae (G) and buds therefrom (arrows). Clathrin-coated vesicles (arrowhead and inset) are distinguishable from non-clathrin-coated buds and vesicular profiles. Bars, 0.1 \( \mu \)m.

Fig. 2. Ultrastructure of the components of the secretory pathway. A, a Golgi apparatus with membrane stacks (arrows), tubules, vesicles, and vacuoles (arrowheads). B, horseradish peroxidase labeling of early endosomes treated with 1 \( \mu \)M PMA for 15 min and processed for cytochemical visualization of horseradish peroxidase (see "Experimental Procedures"). The horseradish peroxidase-labeled endosomes (arrows) were clearly distinct from the Golgi structure (arrowheads), and no horseradish peroxidase was detected in the Golgi area. C, ER-derived buds (arrows) and tubular-vesicular clusters (arrowheads and inset, asterisk) are situated near the ER (small arrows; see definitions in "Experimental Procedures"). D, tangential section of the Golgi cisternae (G) and buds therefrom (arrows). Clathrin-coated vesicles (arrowhead and inset) are distinguishable from non-clathrin-coated buds and vesicular profiles. Bars, 0.1 \( \mu \)m.

Receptor and PKC Regulation of Constitutive Traffic

After long chases. In mastocyte-type cells (among which are the RBL cells) a considerable amount of proteoglycans is stored in regulated secretory granules (Ref. 25 and references therein), suggesting that retained GAG might also be associated with the granule compartment. Fig. 3D shows how, after 6 h of chase, the vast majority (more than 70%) of the GAG remaining associated with RBL cells were released by stimuli typically effective on regulated secretory pools, indicating that it represents granule content.

Of note, proteoglycans (the high \( M_w \) sulfated material in Fig. 3A) appeared to behave similarly (although not identically) to GAG both in time course of secretion and responsiveness to stimulants (Fig. 3C) (experiments using various stimulants are described below). As GAG measurement involving SDS-PAGE and imaging is expensive and time consuming, we measured the release of sulfated material by cetylpyridinium chloride precipitation (a simpler technique that collectively detects GAG and proteoglycans, hereafter indicated as "glycans"), as an index of constitutive release. As expected, a good numerical correspondence was found between the two methods (for total glycans and GAG; Fig. 4). The cetylpyridinium chloride method was thus routinely used, and the SDS-PAGE/InstantImager technique was only employed to separately quantitate GAG and proteoglycans in selected experiments.

IgE Receptor Activation Stimulates Constitutive Glycan Release in RBL Cells Largely via PKC—The IgE receptor is the main activator of RBL cells. To study the effects of membrane receptor activation on constitutive release, RBL cells primed with anti-DNP monoclonal IgEs were preincubated with xyloside and pulse-labeled for 5 min with \( ^{35}S \) sulfate to label glycans in the TG/TGN compartment. Cells were then treated with DNP-BSA in the absence of extracellular calcium, a condition known to completely abolish regulated secretion in these cells (Ref. 36 and this report). This increased the release of glycans by about 3–4-fold, but, as expected, it did not stimulate the regulated secretion of serotonin (Table II and Fig. 4A). The effect of the antigen on glycan secretion was completely inhib-
ited by 10 μg/ml BFA (Fig. 4A). BFA did not affect the antigen-induced stimulation of serotonin release via the regulated pathway (when this was effected in the presence of extracellular calcium; Table II). The IgE receptor-coupled signal transduction machinery is complex and includes the phospholipase C-PKC cascade (37). In order to assess whether the receptor modulation of constitutive membrane transport was mediated by PKC, two specific PKC inhibitors (with two different binding sites on PKC, see below) were used: Ro 31-8220 and calphostin C. Fig. 4B shows how the stimulation of glycan release through IgE activation is largely, albeit not completely, inhibited by both inhibitors, indicating that PKC is involved in IgE receptor-activated glycan release. Both inhibitors were used at “specific” concentrations at which they had no effect on the stimulation of release induced by agents acting through protein kinase A or tyrosine kinases, in addition they did not appear to be toxic at the practiced concentrations. Moreover, an activator of PKC, the synthetic DAG analog PMA, mimicked the action of the IgE receptor. Fig. 4C shows the effects of 100 nm PMA added during the chase period. When cells were incubated at 19.5 °C for 1.5 h after the pulse to block labeled glycans in the distal Golgi, IgE receptor stimulation and direct stimulation of PKC both increased constitutive release (not shown), similar to the stimulation without the temperature block indicating direct acceleration of exit from the TGN/TGN. Of note, the effect of PMA on GAG release was comparable with that on total glycan release evaluated by cetylpyridinium chloride method. Values represent means ± S.D. Experiments were performed at least 3 times with similar results.

While the above clearly suggests the involvement of PKC in

2 R. Buccione, S. Bannykh, I. Santone, M. Baldassarre, F. Facchiano, Y. Bozzi, G. Di Tullio, A. Mironov, A. Luini, and M. A. De Matteis, unpublished observations.
For constitutive glycan release, cells preincubated in the presence of xylitol and then pulse labeled with [35S]sulfate for 5 min were chased for 15 min in the presence or absence of 100 nM PMA with or without 10 μM Ro 31-8220. Table II lists the effects of activatory and inhibitory agents on constitutive and regulated release in RBL cells. For regulated glycan release, cells preincubated in the presence of xylitol and then pulse labeled with [35S]sulfate for 5 min were chased for 15 min in the presence or absence of 100 nM PMA with or without 10 μM Ro 31-8220. Table II lists the effects of activatory and inhibitory agents on constitutive and regulated release in RBL cells.

### Table II

| Treatment | Release (% of total ± SD) |
|-----------|--------------------------|
|           | Ca2+-containing medium | Ca2+-deprived medium |
| Glycans   | Serotonin                | Glycans   | Serotonin                |
| Control   | 17.2 ± 1.1               | 11.6 ± 0.1 | 18.6 ± 0.3 | 11.5 ± 0.6              |
| PMA       | 50.7 ± 3.1               | 11.8 ± 0.5 | 43.4 ± 3.1 | 11.4 ± 0.8              |
| BFA       | 8.0 ± 1.9                | 11.6 ± 0.1 | 8.4 ± 1.0  | 11.6 ± 0.1              |
| BFA/PMA   | 13.2 ± 2.2               | 11.4 ± 0.3 | 13.4 ± 1.8 | 11.5 ± 0.2              |
| DNP - BSA | 58.3 ± 3.4               | 63.3 ± 0.1 | 43.6 ± 2.9 | 10.5 ± 0.2              |
| DNP - BSA/BFA | 14.7 ± 0.1   | 69.7 ± 1.1 | 9.7 ± 0.2  |

The inactive phorbol ester A-PDD had no effect (not shown). The specific PKC inhibitor calphostin C, which acts directly on the phorbol binding site of PKC (41), markedly reduced the stimulatory effect of PKC (Fig. 5B). Ro 31-8220, a highly specific PKC inhibitor acting at the ATP binding site of PKC (42), completely abolished the stimulatory effect of PMA (Fig. 5B). H7 and staurosporine, less selective compounds of the latter class, had similar effects (not shown). In addition, PMA was no longer able to stimulate glycan release after down-regulation of PKC with 1 μM calphostin C, 3 μM Ro 31-8220, or, after 6 h of treatment, with 1 μM PMA which down-regulates PKC isoforms α and β (71, 72). Release was expressed as above. Values represent means ± S.D. Experiments were performed at least 3 times with similar results.

The regulation of constitutive exocytosis, it has been recently shown that a domain homologous to the phorbol ester binding region of PKC is present in other proteins (38, 39), raising the possibility that these proteins might be responsible for the stimulatory effects of PMA. A series of experiments was thus carried out to determine if the effect of PMA was due to PKC activation. Indeed, several other phorbol esters among which DPP, DPPA, and thymeleatoxin had a stimulatory effect on activation. Several other phorbol esters among which carried out to determine if the effect of PMA was due to PKC stimulatory effects of PMA on glycan release, being slightly effective only at the highest concentration (1 μM). The inactive phorbol A-PDD had no effect (not shown). The specific PKC inhibitor calphostin C, which acts directly on the phorbol binding site of PKC (41), markedly reduced the stimulatory effect of PKC (Fig. 5B). Ro 31-8220, a highly specific PKC inhibitor acting at the ATP binding site of PKC (42), completely abolished the stimulatory effect of PMA (Fig. 5B). H7 and staurosporine, less selective compounds of the latter class, had similar effects (not shown). In addition, PMA was no longer able to stimulate glycan release after down-regulation of PKC with 1 μM calphostin C, 3 μM Ro 31-8220, or, after 6 h of treatment, with 1 μM PMA which down-regulates PKC isoforms α and β (71, 72). Release was expressed as above. Values represent means ± S.D. Experiments were performed at least 3 times with similar results.

As noted above, RBL cells also feature regulated exocytosis and a considerable fraction of proteoglycans, and GAG (this report) accumulates in secretory granules. PKC alone does not stimulate release of secretory granules and RBL cells (Table I and Beaven et al. (36)), and, in addition, regulated secretion of serotonin was strictly dependent on external calcium, whereas PKC-stimulated glycan release was not. Finally, BFA did not inhibit serotonin release in clear contrast with its ability to inhibit glycan release. Thus, the pattern of responsiveness of the regulated secretory route in RBL cells to a variety of stimulators and inhibitors is completely different from that of the constitutive pathway. To further verify the separatedness of the two pathways, we followed the time-course of entry of GAG into the regulated compartment. After chase of increasing lengths, RBL cells were treated with PMA or DNP-BSA in the absence of extracellular calcium (to stimulate constitutive release) or a combination of calcium ionophore and PMA or DNP-BSA in the presence of external calcium (to induce regulated exocytosis, which is absolutely calcium-dependent in these cells; Ref. 36 and this report). When release was measured immediately after labeling, PMA (or DNP-BSA in the absence of calcium) potently stimulated it. After 1 h of chase in the absence of stimuli, PMA moderately stimulated GAG release, whereas PKA/A23187 induced the release of about 60% of the total GAG content (not shown); the same was observed with αIgE stimulation in the absence or the presence of external calcium, respectively. After 6 h of chase (Fig. 3D), PMA alone or αIgE stimulation in the absence of external calcium (not shown) had no effect, whereas the vast majority of GAG content was released by PMA/A23187 or αIgE stimulation in the presence of external calcium (not shown); the response to the latter stimuli precisely paralleled that seen with serotonin. This is taken to indicate that GAG had moved into a constitutively releasable compartment, functionally colocalized with the serotonin-containing granule compartment. Thus, the kinetic data are in line with the other above results, indicating that in RBL cells, constitutive and regulated GAG secretion can be readily distinguished and that the former is selectively stimulated by PKC or by IgE receptor activation in the absence of calcium.

Effect of PKC Activators and Inhibitors on Transport of the VSV-G Protein from the TGN to the Plasma Membrane in RBL Cells—To determine whether the transport of a membrane-bound protein was also susceptible to regulation by PKC, we used the VSV-G (28), a widely accepted marker of membrane protein transport. Treatment with 100 nM PMA reproducibly increased the amount of VSV-G transported to the cell mem-
brane after an incubation at 19.5°C to block material in the TGN, but the effect was quite small (up to about 30% over the control level). By contrast, the PKC inhibitor Ro 31-8220 profoundly and rapidly inhibited the basal rate of transport of VSV-G to the cell surface (Fig. 6). Therefore, like glycan transport, VSV-G traffic out of the TGN appears to be regulated by PKC; however, the transport of the two markers and their regulation are different in their requirement for PKC activity, which appears to be crucial for stimulated but not basal constitutive glycan release, whereas the opposite seems to be true for VSV-G transport.

Effects of PMA on Secretory Vesicle Formation from the TG/TGN in Vitro—The effect of PKC activation was examined on the formation of vesicles from the TG/TGN in a cell-free system. RBL cells proved unsuitable for fractionation techniques aimed at producing a functioning TG/TGN. Many other cell types, including the pheocromocytoma-derived cell line PC12, polarized epithelial Madin-Darby canine kidney cells and the promielocytic cell line HL-60, displayed a significant, albeit variable, stimulation of constitutive exocytosis in response to PMA (Fig. 7A). PC12 cells were thus used since they have been thoroughly characterized for use in assays of vesicle production in vitro from the TGN (26). In detail, in these cells, after an initial delay of 2–3 min, the stimulatory effect of PMA peaked (up to 200% of basal) between 5 and 15 min and declined thereafter (Fig. 7B). PMA affected the rate, but not the total amount, of glycan released at plateau. BFA was an extremely effective inhibitor of both basal and PMA-stimulated release (Fig. 7B). SDS-PAGE/InstantImager analysis showed that the great majority (80–90%) of released glycans was represented by low molecular weight species (not shown). Biotinylated cell surface proteins were precipitated with streptavidin-agarose and quantified with the InstantImager after SDS-PAGE. Top panel shows the autoradiogram of a time-course of VSV-G transport to the cell surface. Bottom panel shows InstantImager quantification.

The effect of PKC activation was examined on the formation of vesicles from the TG/TGN. Cells were then chased at 37°C in the presence or absence of 100 nM PMA or 5 μM Ro 31-8220 (Ro). Biotinylated cell surface proteins were precipitated with streptavidin-agarose and quantified with the InstantImager after SDS-PAGE. Top panel shows the autoradiogram of a time-course of VSV-G transport to the cell surface. Bottom panel shows InstantImager quantification.

To measure vesicle production in vitro, PC12 cells were homogenized after a short pulse with [35S]sulfate to produce a postnuclear supernatant containing, among other membranes, the Golgi complex. This postnuclear supernatant was incubated at 37°C for various lengths of time in the presence or absence of 100 nM PMA or 10 μM Ro 31-8220 and then fractionated by velocity gradient centrifugation to separate smaller secretory vesicles from larger TGN membranes between upper and lower fractions at 0°C and 37°C, according to Ref. 26 (see Fig. 1). The addition of PMA stimulated the transfer of labeled proteoglycans from lower (Golgi) into upper (vesicular) fractions (Table III). The inclusion of Ro 31-8220 during the incubation inhibited vesicle formation (not shown). A similar increase in vesicle formation by PKC, albeit only in the presence of okadaic acid, was recently reported (11). These results indicate that PMA can increase vesicle formation and therefore that at least part of the acceleration of glycan release in vivo by PMA is due to a direct effect on export from the TGN/TGN.

Effects of Plasma Membrane Receptor Stimulation and Direct PKC Activation on the Morphology of the Exocytic System—A series of morphometric analyses were carried out in RBL cells to examine whether relevant features of the secretory organelles might be quantitatively altered by IgE receptor activation and PMA stimulation. Under control conditions, the ER surface exhibited several coated buds that were often associated with TVC (see “Experimental Procedures”; possibly representing elements of the intermediate compartment) (45) whose main components were vesicular profiles of 60–65 nm in diameter. 11% of these buds were situated on the external surface of the nuclear envelope (Fig. 2C, inset). The Golgi complex also exhibited several coated buds (Fig. 2D) and was formed by 3.6 ± 0.1 stack profiles per cell; the number of cisternae per stack was 4.9 ± 0.2.

![Fig. 6. Regulation of VSV-G transport from the TGN to the cell surface in RBL cells.](image)

![Fig. 7. Stimulation of glycan release by PMA in PC12 cells and other cell lines.](image)
When cells were treated with PMA, the number of buds on the ER increased by 90% within 2 min (Fig. 8A), while the distribution of the buds and the shape and size of the ER did not change appreciably. The number of ER-derived vesicular profiles and TVC rose to a somewhat lesser extent (Fig. 8A), while the number of vesicular profiles per TVC did not change. The number of total vesicular profiles was also significantly increased (Fig. 8D). Only less than half of this effect could be ascribed to ER vesicles; we did not attempt to determine the nature of the non-ER vesicular profiles, but they are likely to be mostly endocytic and Golgi-derived. A similar effect of PMA on cytoplasmic vesicles has been observed previously in human basophils (46). Concomitantly (within 2 min), the volume and surface of the Golgi apparatus increased by 40 and 55%, respectively (Fig. 8B), and the number of Golgi buds increased to a similar extent (Fig. 8C). The rims of Golgi profiles appeared to have become larger, and the number of Golgi profiles per cell appeared to be slightly increased. The latter effect, however, might be due to increased probability of section through larger stacks rather than to an actual increase in stack profile number. Endocytosis in RBL cells can be stimulated by PMA (47); thus, to examine whether the changes attributed to the Golgi complex might in part be due to endocytic structures located in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, which was thus treated with PMA and 100 ng/ml DNP-BSA for different times and then processed for morphometry as described under "Experimental Procedures." The number of vesicles (A), ER buds (B), and the Golgi surface (C) rapidly increased reaching a plateau in about 20 min.

When cells were treated with PMA, the number of buds on the ER increased by 90% within 2 min (Fig. 8A), while the distribution of the buds and the shape and size of the ER did not change appreciably. The number of ER-derived vesicular profiles and TVC rose to a somewhat lesser extent (Fig. 8A), while the number of vesicular profiles per TVC did not change. The number of total vesicular profiles was also significantly increased (Fig. 8D). Only less than half of this effect could be ascribed to ER vesicles; we did not attempt to determine the nature of the non-ER vesicular profiles, but they are likely to be mostly endocytic and Golgi-derived. A similar effect of PMA on cytoplasmic vesicles has been observed previously in human basophils (46). Concomitantly (within 2 min), the volume and surface of the Golgi apparatus increased by 40 and 55%, respectively (Fig. 8B), and the number of Golgi buds increased to a similar extent (Fig. 8C). The rims of Golgi profiles appeared to have become larger, and the number of Golgi profiles per cell appeared to be slightly increased. The latter effect, however, might be due to increased probability of section through larger stacks rather than to an actual increase in stack profile number. Endocytosis in RBL cells can be stimulated by PMA (47); thus, to examine whether the changes attributed to the Golgi complex might in part be due to endocytic structures located in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, cells were incubated with a high concentration of horseradish peroxidase (15 mg/ml) for 2 h to allow extensive labeling of the endosomal system. Several structures, mostly located in the cell periphery and identifiable as endosomes, became strongly horseradish peroxidase-labeled. In contrast, no horseradish peroxidase was detectable in the Golgi area, which was thus treated with PMA and 100 ng/ml DNP-BSA for different times and then processed for morphometry as described under "Experimental Procedures." The number of vesicles (A), ER buds (B), and the Golgi surface (C) rapidly increased reaching a plateau in about 20 min.

**DISCUSSION**

The Stimulation of Constitutive Transport by Plasma Membrane Receptors—This paper provides biochemical and morphometric evidence that the activation of the IgE receptor stimulates the constitutive exocytic pathway in RBL cells. PKC appears to be a key component in the transduction pathway mediating this response. This represents, to our knowledge, the first demonstration that a membrane receptor can activate exocytic membrane transport and concurrently induce rapid and profound morphological changes consistent with the functional activation.

The mechanism by which signals generated at the plasma membrane by the IgE receptor translocate to the transport apparatus is probably linked to the fact that IgE receptor activation stimulates the activity of soluble tyrosine kinases (48), which can, in turn, activate cytosolic phospholipase C γ1 (49); PKC could thus be stimulated directly on the target organelle through the translocation of activated phospholipase C γ1 and the consequent local production of DAG, the natural activator of the kinase. Very recently, we have found that other
receptors such as growth factor receptors, can stimulate constitutive secretion in a variety of cell lines, and that they can exert this effect even through PKC-independent mechanisms.\textsuperscript{2} Thus, different signaling pathways appear to control constitutive traffic. To understand the physiological significance of these responses, it will be important to determine which types of receptors activate specific stations of the transport route and which second messengers are involved. It is likely that receptor-induced modulation of intracellular protein transport and sorting is involved in a variety of physiological phenomena. For instance, the stimulation of the membrane transport apparatus in mast cells via the IgE receptor might be required to prepare the complex recovery process that these cells must undergo after degranulation.

Analysis of the Role of PKC in the TGN to Plasma Membrane Transport—The observations that, in RBL cells, the activation of IgE receptors generates DAG (49), and that PMA mimics the stimulatory effect of IgE receptor on constitutive transport from the TGN to the plasma membrane obviously suggests that PKC is involved in receptor-induced activation of this traffic pathway. Moreover, we show that PKC activators stimulate constitutive exocytosis in a variety of cell types, indicating a remarkable generality of this phenomenon. It has recently been reported, however, that several proteins containing a domain homologous to the PMA/DAG binding region of PKC exist and may respond to PMA (38, 39, 50), suggesting the possibility that the IgE receptor- and PMA-induced regulation of constitutive traffic might be mediated by a protein(s) other than PKC. Moreover, a protein of this family has recently been implicated in maintaining the basal rate of ER to Golgi traffic (20). We provide several lines of evidence indicating that the IgE receptor and PMA effects on TGN to plasma membrane traffic are mediated by PKC including that the effects of PMA and IgE receptors on transport were (a) mimicked only by phorbol esters active on PKC, (b) prevented by long PMA treatments inducing PKC down-regulation, and (c) suppressed not only by calphostin C, an inhibitor at the PMA/DAG binding site, but also by specific inhibitors acting at the catalytic site of the kinase. Therefore, both non-PKC PMA/DAG-binding protein(s) (20) and proteins of the PKC family (this study) seem to be able to exert regulatory effects on exocytic transport, a possibility that will have to be kept in account in future studies.

A relevant question concerning the role of PKC in constitutive release is whether the kinase can act directly on the TGN to stimulate export toward the plasma membrane. In fact, in addition to the ER to Golgi traffic segment (20), endocytosis (19, 51) and apical endosome-plasma membrane recycling in polarized cells (16) have been reported to be stimulated by PMA. It is thus possible that the stimulation of TGN to plasma membrane transport might be due to enhanced membrane flow in other districts of the cell rather than a direct activation of export from the TGN. For instance, the stimulation of endocytosis and ER to Golgi transport could increase the input of surface and volume into the TGN, thereby stimulating a compensatory output from it. However, the demonstration, in PC12 cells, that PMA accelerates the formation of proteoglycan-containing vesicles from cell-free TGN preparations (where such indirect effects are very unlikely to occur) indicates that at least part of the effect of PMA on glycan release is due to a direct action on the TGN. It should be noted, at the same time, that this experiment does not exclude the possibility that other (indirect) effects of PMA may occur in vivo, especially because the effect observed in cell-free vesicle formation experiments is smaller than the overall acceleration of glycan release seen in intact cells (it is also possible, however, that this discrepancy may simply be due to a lower efficiency of the in vitro reaction). Further support for a direct action of PMA on the TGN comes from a different set of experiments in intact RBL cells, showing that there is no detectable input from the endocytic pathway into the TGN (see Fig. 2) and that, after a prolonged temperature block at 19.5°C, IgE receptor and PKC activation strongly accelerate exit from the TGN. In conclusion, the collective data indicate that the stimulatory action of PMA on TGN to plasma membrane transport is, at least in part, due to a direct activation of membrane export from the TGN.

Another question concerns the differential roles of PKC in the transport of fluid-phase and membrane markers, since the transport of GAG (a fluid-phase marker) was potently accelerated by PKC activators, whereas the transport of VSV-G (a membrane protein) was rapidly and markedly inhibited by PKC inhibitors (compare Figs. 4 and 6). A difficulty in interpreting these results is that the appearance of membrane markers on the plasma membrane depends on the balance between exocytic and endocytic activities, whereas the release of soluble molecules is mainly due to exocytic transport. Nevertheless, it seems to us that the most economical explanation of the results may be based on recent reports that soluble and membrane proteins are transported to the plasma membrane in distinct vesicular carriers (43, 44). Another alternative explanation is based on the fact that, in RBL cells, a large amount of soluble glycan and VSV-G are different transport mechanisms for GAG and VSV-G, they might be differentially regulated by PKC.

Mechanisms of Activation and Targets of the PKC Isoform(s) Involved in Regulating Membrane Traffic—As mentioned above, a likely mechanism through which IgE receptors exert their control on membrane traffic is the induction of increased DAG levels in the TGN (as well as presumably in other stations of the traffic pathways), and the consequent local activation of a PKC isoform(s). PMA could mimic the receptor-induced effects by directly activating PKC at the same intracellular sites. In connection with this, it is interesting to note that DAG can be formed not only in response to receptor stimulation but also (at least in the ER and the Golgi complex) during lipid metabolic reactions (53); in addition, several heterotrimeric G proteins that activate DAG-forming enzymes such as phospholipases C in “canonical” transduction pathways are located on intracellular organelles including the ER and the Golgi complex (2, 54). It is thus conceivable that DAG may not only be a direct mediator of secretory responses to receptor activation but also that DAG levels (and, consequently, PKC activity) in secretory organelles (controlled either by lipid metabolic pathways or by “local” G proteins; see Ref. 55 and discussion below) may play a significant role in determining basal traffic rates (53, 56).

What is the target of PKC in the traffic machinery? A good candidate is the mechanism of ARF activation and/or binding to Golgi membranes, since this is a key event in vesicle formation already known to be modulated by PKC (14). The involvement of ARF in promoting the assembly of different kinds of coat onto Golgi membranes, is indicated both by direct evidence and by the use of BFA that blocks the activation of ARF (7, 57, 58). With regard to the TGN/BFA, BFA is known to block constitutive transport from the TGN to the plasma membrane...
(59) (this report); moreover, ARF has also been found to be associated with proteoglycan-carrying post-TGN vesicles in rat hepatocytes (42). Other targets of PKC in the traffic machinery cannot be excluded. For instance, the phospholipase D-stimulating activity of ARF (60, 61) has been recently shown to be potentiated by PMA in human neutrophils (62).

The identity and the localization of the PKC isoform(s) that controls membrane traffic is presently unclear. PKC is part of a family of several isozymes with different sensitivities to modulators and substrate specificities (63, 64). The α, β, and ε isoforms have previously been hypothesized to be involved in the regulation of membrane traffic (14, 16, 19, 65). A series of experiments in COS 7 cells, which normally express these isoforms and respond to PMA with an increase in glycan secretion have, however, revealed that these kinases are not involved in regulating membrane traffic (at least in these cells). In fact, when α- and β-PKC were overexpressed together with the secretory mutant of human alkaline phosphatase (66), neither the basal secretion rate of the secretory mutant of human alkaline phosphatase nor the potency of PMA in eliciting the secretory response appeared to change, as one would have expected if the overexpressed isoforms had been involved in the PMA stimulation of membrane traffic (67, 68). Interestingly when the ε isoform was overexpressed, the response to PMA was completely lost in accordance with a recent report (65).

More work using the co-transfection approach employed in this study will be necessary to identify the relevant PKC isoform(s).

Significance of the Morphological and Functional Changes in the Exocytic Pathway Induced by IgE Receptor and PKC Stimulation—The morphometric data, combined with the activatory effect of IgE receptors and PMA on secretory traffic shown in this study, together with the recent finding that PMA strongly (4-fold) stimulates ER to Golgi transport (20), suggest a picture consistent with a regulatory role of PKC (and other PMA-binding proteins) on the entire secretory pathway. The simplest interpretation of the observed increase in the number of ER buds and ER-derived vesicles accompanied by a transient increase in surface and volume of the Golgi complex (Fig. 8) is that PMA enhances the probability of budding and vesicle formation from the ER, thereby driving an accelerated flow of membrane into the Golgi (20) and causing the consequent rapid increase of the organelle’s size. Moreover, the increase in number of Golgi buds and total vesicular profiles is consistent with the acceleration in exocytic export from the TG/TGN induced by PKC stimulation. These conclusions are in agreement with a comprehensive study of the morphological effects of PMA on transport organelles.

If one makes the reasonable assumption that the increase in Golgi size (60% in 2 min) is mostly due to enhanced membrane input from the ER, one obtains that the observed PMA-induced Golgi increase requires an acceleration in net membrane transport from the ER to the Golgi corresponding to at least 5% of the ER surface/min. Clearly this effect, if continued, would rapidly lead to gross imbalances in the sizes of the secretory organelles. Similar considerations can be made based on functional (GAG release) data, with respect to the TG/TGN. The half-time of GAG release from the cell lines here used ranges from 15 to 30 min; these estimations, assuming that GAG are homogenous markers of the TG/TGN luminal space, would also reflect the half-time of turnover of this organelle. If so, a 100% increase in export rate from the TG/TGN such as that described here would, again, lead to substantial changes in the size of the organelle within a short time if homeostatic mechanisms were not in place.

Thus, this study introduces a novel appreciation of the rapidity and extent of the variations in membrane traffic rates that may occur under physiological conditions and supports the notion that there must be ways in which traffic movements in different organelles are precisely matched to preserve the “homeostasis” of cell membranes. While this idea has occasionally surfaced in the literature, the mechanisms presiding over such fine regulation have not been investigated. One possibility that may now be suggested is that DAG or other second messengers (see recent data implicating CAMP in the regulation of transcytosis and endocytosis) (17, 18, 19) may be involved not only in mediating the variations in traffic rates induced by a membrane receptor, as shown here, but also in restoring, when necessary, “normal” conditions in the secretory pathway. It is possible, for instance, that individual organelles may possess sensors for excessive changes in their size and shape that would generate messengers to re-establish “correct” functioning status. The heterotrimeric G proteins located on secretory organelles might play a role in these intracellular signaling systems functioning in and between organelles (55). Interestingly, an example of interorganellar signaling (albeit apparently not involving G proteins) has been recently provided by the finding that events in the lumen of the ER can be sensed and signaled via a protein kinase to the nucleus, to control the transcription of ER chaperon proteins (69, 70). A variety of transduction signals may be used as one of the mechanisms functioning to maintain the homeostasis of intracellular traffic pathways and to mediate their adaptive responses to receptor stimuli.

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Regulation of Constitutive Exocytic Transport by Membrane Receptors: A BIOCHEMICAL AND MORPHOMETRIC STUDY
Roberto Buccione, Sergei Bannykh, Ivana Santone, Massimiliano Baldassarre, Francesco Facchiano, Yuri Bozzi, Giuseppe Di Tullio, Alexander Mironov, Alberto Luini and Maria Antonietta De Matteis

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