Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6965–6969, October 1987
Biochemistry

Intestinal brush border membranes contain regulatory subunits of adenylyl cyclase

(cholera toxin/ADP ribosylation/enterocytes/reconstitution of adenylyl cyclase/adenylate cyclase)

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Communicated by Severo Ochoa, May 18, 1987

ABSTRACT **Cholera toxin alters intestinal function by stimulation of adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing) or adenylate cyclase, EC 4.6.1.1]. The mechanism of this activation is unknown and particularly puzzling because adenylyl cyclase is confined to the basal lateral membrane of enterocytes, whereas it is the brush border membrane that binds the toxin and contains proteins that undergo cholera toxin-catalyzed ADP ribosylation. It is shown that cholate extracts from cholera toxin-treated brush border membranes can efficiently reconstitute adenylyl cyclase activity in the guanine nucleotide-binding regulatory component (G_s)-deficient cyc$^-$ variant of the S49 mouse lymphoma cell line (cyc$^-$ cells lack the α subunit of G_s needed to activate the catalytic subunit of adenylyl cyclase). Moreover, NaF (in the presence of Al^{3+}) and guanyl-5′-yl imidodiphosphate mediate strong activation of cyc$^-$ adenylyl cyclase provided the cholate extracts of brush border membranes are also present. Therefore, it appears that brush border membranes contain high levels of regulatory subunits of adenylyl cyclase in the absence of catalytic subunits. This represents a previously unrecognized feature of this transduction system that presumably plays an important role in the derangement of intestinal cell function by cholera toxin.**

Over the last few years, it has been firmly established that activation of adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing) or adenylate cyclase, EC 4.6.1.1] by cholera toxin is mediated by the ADP-ribosylation of the guanine-nucleotide-binding regulatory component (G_s) of the enzyme (1, 2). This modification results in the permanent activation of the adenylyl cyclase (1–3). However, most of the studies have been performed with cellular types and systems where the toxin was allowed to interact directly with the cyclase (3, 4). The enterocytes, which are the intestinal cells naturally affected by the toxin in the watery diarrhea characteristic of clinical cholera (4), present an additional and interesting problem. In these cells, the toxin binds to the brush border membrane (5, 6), while adenylyl cyclase appears to be located in the basal lateral membrane (7–10). This implies that upon binding of cholera toxin to the apical face of the enterocyte, the ADP-ribosylating A_1 fragment of the toxin (11) would have to travel through the intestinal cell to reach the adenylyl cyclase in the contraluminal side. To our knowledge, however, the internalization of the toxin has not been demonstrated to date. A second possibility is that regulatory components of adenylyl cyclase are ADP-ribosylated in the brush border membrane and that such modified components move by a still-undefined mechanism to interact with the catalytic subunits in the basal lateral membrane. We have shown that cholera toxin catalyzes the ADP-ribosylation of brush border membrane proteins in spite of the absence of adenylyl cyclase in this membrane (12, 13). By reconstituting adenylyl cyclase activity in G_s-deficient cyc$^-$ S49 lymphoma cell membranes (refs. 14 and 15; cyc$^-$ cells lack the α subunit of G_s needed to activate the catalytic subunit of adenylyl cyclase), we demonstrate here that the proteins ADP-ribosylated by cholera toxin in intestinal brush border membranes are indeed regulatory components of adenylyl cyclase. We also show that basal lateral membranes contain regulatory subunits that are not affected by treatment of the membranes with the toxin. The presence of G_s subunits in brush border membranes of enterocytes is discussed in relationship to the mechanism of activation of intestinal adenylyl cyclase by cholera toxin.

MATERIALS AND METHODS

Membrane Preparation and Assay of Marker Enzymes. Crude plasma membranes, brush border membranes, and basal lateral membranes were prepared from rabbit small intestine as described (12, 16). Protein, sucrase, Na^+/K^+-ATPase and adenylyl cyclase activities were determined by established procedures (12, 16).

Activation of Cholera Toxin and Detection of ADP-Ribosylated Membrane Proteins in Polyacrylamide Gels. Cholera toxin (2 mg/ml) was dissolved in 20 mM Tris·HCl, pH 7.4/80 mM NaCl/0.4 mM EDTA/50% (vol/vol) glycerol. The toxin was activated prior to the ADP ribosylation reaction by incubation for 10 min at 37°C after diluting appropriately in an activation buffer [10 mM Hepes/NaOH (pH 6.8) containing 0.13 M NaCl, 0.5% sodium dodecyl sulfate, 4 mM dithiothreitol, and 2% (wt/vol) bovine serum albumin]. Crude plasma membranes, brush border membranes, and basal lateral membranes were incubated with activated cholera toxin or the same amount of activation buffer in 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)/NaOH (pH 6.8) containing 5 mM ATP, 50 μM GTP, 5 μM [^{32}P]NAD (about 20 Ci/mmol; 1 Ci = 37 GBq), 2 mM EDTA, 10 mM thymidine, 10 mM isoniazid, 5 mM $MgCl_2$, and an ATP-regenerating system in a final volume of 0.1 ml. The reaction was carried out at 25°C for 30 min and stopped by dilution with 1 ml of ice-cold 10 mM Hepes/NaOH (pH 6.8) containing 0.13 M NaCl. The mixture was centrifuged at 29,000 × *g* for 20 min, and the pellet containing the ADP-ribosylated membranes was washed twice with stopping buffer. After dissociation, the reaction products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 12% polyacrylamide gels (17). The gels were stained for protein

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Abbreviations: p[NH]ppG, guanyl-5′-yl imidodiphosphate; VIP, vasoactive intestinal peptide; Mops, 3-(*N*-morpholino)propanesulfonic acid; G_s, guanine nucleotide-binding regulatory component; cyc$^-$, adenylyl cyclase-inactive phenotype (cells that lack the 45-kDa α subunit of G_s needed to activate the catalytic subunit of adenylyl cyclase).

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and dried, after which autoradiographies were taken by exposure of Kodak X-Omat film to the dried gels.

Reconstitution of Adenylyl Cyclase Activity in cyc⁻ S49 Plasma Membranes by Extracts of Untreated and Cholera Toxin-Treated Intestinal Membranes. Membranes (5–8 mg of protein per ml) were treated for 30 min at 25°C with the indicated concentrations of activated cholera toxin in 15 mM Mops/NaOH (pH 7.0) containing 3 mM NAD, 70 mM NaCl, 6 mM thymidine, 3 mM ATP, and 30 μM GTP in a final volume of 0.2 ml. The reaction was stopped by 1:6 dilution with 10 mM Hepes/NaOH (pH 8.0) at 4°C, and the samples were centrifuged at 29,000 × *g* for 20 min. The pellets were designated as cholera toxin-treated membranes. Cholate extracts were prepared from untreated and cholera toxin-treated membranes after resuspending them in an extraction mixture so that the final concentrations were: 15 mM Hepes/NaOH (pH 8.0), 20 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1% sodium cholate, and about 10 mg of membrane protein per ml. The extraction was maintained for 60 min at 4°C with continuous stirring and terminated by centrifugation at 29,000 × *g* for 60 min. To the supernatants, EDTA and ethylene glycol were added to 11 mM and 30% (wt/vol), respectively. These "cholate extracts" contained about 2 mg of protein per ml and 0.5% sodium cholate. For the reconstitution reaction, cholate extracts were diluted 1:9 with 10 mM Tris·HCl (pH 8.0) containing 1 mg of bovine serum albumin per ml, 1 mM EDTA, and 20 mM 2-mercaptoethanol. To 10 μl of the diluted samples, 10 μl of cyc⁻ S49 plasma membranes (1 mg of protein per ml) in 10 mM Tris·HCl, pH 8.0/1 mM EDTA was added. The mixture was preincubated for 15 min at 0°C. After this period 30 μl of reaction mixture was added, and adenylyl cyclase activity was measured in the presence or absence of different activators as indicated. The reaction was maintained for 40 min at 30°C. [^{32}P]cAMP was purified by the double-column procedure of Salomon *et al.* (18).

Materials. All chemicals were purchased from Sigma unless otherwise indicated. [^{3}H]cAMP, [α-^{32}P]ATP, and [*adenylyl*-^{32}P]NAD were from New England Nuclear. Cholera toxin was from Schwarz/Mann. Cholate and forskolin were from Calbiochem. S49 cyc⁻ plasma membranes were a generous gift from J. Codina (Baylor College of Medicine, Houston).

RESULTS

We have studied the mechanism of activation of intestinal adenylyl cyclase using crude and purified plasma membranes from both the luminal and the serosal side of rabbit intestinal epithelium. The membranes were prepared from rabbit mucosal scrapings (see above), and their purity was assessed by comparing the specific activities of the marker enzymes sucrase (for brush border) and Na^+/K^+-ATPase (for basal lateral). The brush border membranes used throughout this study showed a specific activity for sucrase that was 20- to 25-fold higher than that in the homogenate (Table 1). The specific activity of Na^+/K^+-ATPase in these membranes was the same as in the homogenate. Basal lateral membranes showed a specific activity for Na^+/K^+-ATPase that was 15- to 20-fold higher than that in the homogenate. In agreement with previous observations (7, 8, 12), the specific activity of stimulated adenylyl cyclase was 20–50% lower in brush border than in crude plasma membranes. We used forskolin, guanyl-5′-yl imidodiphosphate (p[NH]ppG), or cholera toxin to activate the enzyme. When adenylyl cyclase was determined in basal lateral membranes with either forskolin or p[NH]ppG as activators, the specific activity of basal lateral membranes was 2- to 4-fold higher than that of crude plasma membranes. However, the specific activity with cholera toxin as activator was similar to that of crude plasma membranes (Table 1; see also ref. 12).

Table 1. Enzymatic activity of marker enzymes and adenylyl cyclase in homogenates and plasma membrane fractions of rabbit intestinal epithelium

Enzyme activity	Homogenate	Membranes		
		Crude	Brush border	Basal lateral
Sucrase	87	168	2017	40
Na^+/K^+-ATPase	6	12	6	113
Adenylyl cyclase stimulation				
+GTP (10 μM)	ND	18	6	25
+forskolin (0.1 mM)	ND	80	38	190
+p[NH]ppG (10 μM)	ND	116	29	421
+cholera toxin (50 μg/ml)	ND	97	22	101

Membranes were prepared as described. Sucrase and Na^+/K^+-ATPase activities are expressed in nmol/min per mg of protein. Adenylyl cyclase activity is expressed in pmol/min per mg of protein. In the series activated with forskolin or cholera toxin, 10 μM GTP was also present during the reaction. Results are from a representative experiment. ND, not determined.

In striking contrast with the relatively low activity of adenylyl cyclase in brush border membranes, proteins therein showed a high level of cholera toxin-catalyzed ADP-ribosylation (Fig. 1). The molecular weight (M_r, 45,000) of the major substrate corresponds to that of the α subunit of the G_s regulatory component known to be modified in other systems (19, 20). We also have shown (13) that brush border membranes bind [^{3}H]GTP with a K_d of 1.8×10^{-7} M, although we have not been able to observe a cholera toxin-inhibitable GTPase, probably because of the high nonspecific phosphatase activity present in these membranes.

To test whether the proteins modified by cholera toxin correspond to regulatory components of adenylyl cyclase, we reconstituted the enzyme using cholate extracts of intestinal membranes and plasma membranes of the cyc⁻ clone of the S49 mouse lymphoma cell line (14, 21). Fig. 2 shows that, indeed, the cholera toxin-modified proteins in intestinal brush border membranes correspond to G_s components of adenylyl cyclase because cholate extracts of cholera toxin-treated membranes can reconstitute adenylyl cyclase activity in cyc⁻ membranes. Extracts of crude plasma membranes and brush border membranes pretreated with cholera toxin showed a high reconstitution activity. The presence of G_s regulatory components in brush border membranes is further supported by the high activation of adenylyl cyclase obtained with p[NH]ppG or NaF in cyc⁻ plasma membranes reconstituted with cholate extracts of brush border membranes (Table 2).

The low enrichment of cholera toxin activatable adenylyl cyclase in basal lateral membranes is in good agreement with the low capacity of cholera toxin to catalyze ADP-ribosylation of basal lateral membrane proteins (Fig. 1) and with the low reconstitution activity found in cholate extracts of cholera toxin-treated basal lateral membranes. Taken together, the ADP-ribosylation and the reconstitution experiment data suggest that the number of G_s components accessible to cholera toxin in brush border membranes is at least 5-fold higher than in basal lateral membranes. However, this should not be interpreted as a lack of regulatory components of adenylyl cyclase in basal lateral membranes because p[NH]-ppG (Table 1) as well as NaF or vasoactive intestinal peptide (VIP) (12) showed the capacity to activate adenylyl cyclase in these membranes. Rather, it may be due to a reduced accessibility to the toxin of G_s components or to the presence of an activity that may deplete any of the cofactors required for the activation. The reduced accessibility to cholera toxin of the G_s components present in basolateral membranes is

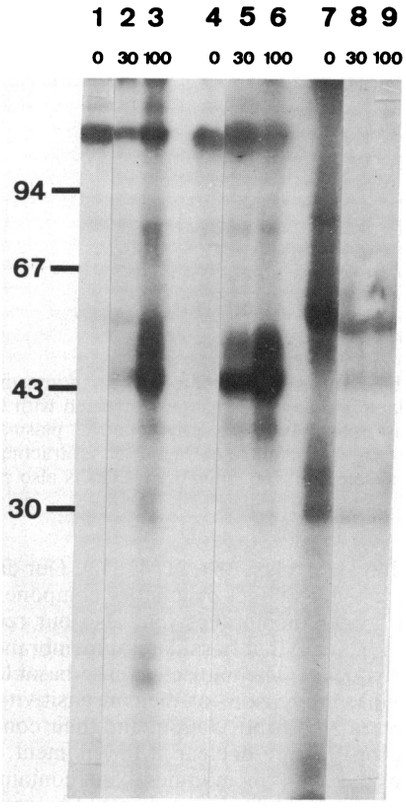

FIG. 1. Cholera toxin-dependent ADP-ribosylation of intestinal epithelial membrane proteins. Autoradiographic profiles of ^{32}P-labeled proteins of crude plasma membranes (lanes 1–3), brush border membranes (lanes 4–6), and basal lateral membranes (lanes 7–9) after incubation with [^{32}P]NAD in the absence of cholera toxin (lanes 1, 4, and 7) or in the presence of preactivated cholera toxin at 30 μg/ml (lanes 2, 5, and 8) or 100 μg/ml (lanes 3, 6, and 9). ADP-ribosylation of membranes was performed as indicated. The whole sample (180 μg of membrane protein from crude plasma membranes, 100 μg of brush border membranes, and 160 μg of basal lateral membranes) was loaded onto the gel, except in lane 2 in which half of the sample was loaded onto the gel. Migration of molecular mass standards expressed in kDa is indicated.

also substantiated by the data presented in Table 2. Thus, whereas extracts obtained from toxin-treated basal lateral membranes generally showed a reconstitution activity similar to that of crude plasma membrane extracts, the reconstitution was better when the cyclase was activated with NaF or p[NH]ppG.

The different capacity of cholera toxin and NaF to activate regulatory components in basal lateral membranes is consistent with the fact that NaF stimulates adenylyl cyclase in mucosal scrapings even after prior treatment of the animals with crude cholera toxin (22, 23). Analogous results can be obtained by treatment of intestinal crude plasma membranes with activated cholera toxin and subsequent activation with NaF (P.D. and P.S.L., unpublished data). These results are unlike those obtained with erythrocyte membranes (1) in which pretreatment with cholera toxin abolished the ability of NaF to activate adenylyl cyclase.

It is unknown at present whether the large population of G_s components present in brush border membranes can be coupled to any membrane receptor. Enterocyte receptors (24) appear to be exclusively located in the basal lateral membranes because they are receptors for modulators of intestinal secretion, which either act from the serosal side after being released from nerve endings or reach the epithelium from the blood stream. We have measured the ability of VIP to activate cyc$^-$ adenylyl cyclase reconstituted with cholate extracts from the three types of membranes. However, even in cyc$^-$ plasma membranes reconstituted with extracts from basal lateral membranes, the reconstituted activity was comparable to the basal GTP-dependent activity (Table 2), indicating that the VIP receptors were either not incorporated into the cyc$^-$ plasma membrane or incorporated without functional coupling to G_s components.

DISCUSSION

It is currently considered that upon binding of cholera toxin to cell membranes, the A_1 protomer is inserted in the membrane (25) and catalyzes the ADP-ribosylation of the G_s component of adenylyl cyclase (1, 2). This modification maintains the enzyme in a permanently activated state (1–3). In intestinal tissue, the toxin binds to the luminal side of enterocytes (5, 6), but adenylyl cyclase is exclusively located in the basal lateral membrane (7–10). This implies that activation of intestinal adenylyl cyclase should take place by one of the following mechanisms: (*i*) cholera toxin or more likely the active A_1 protomer moves from the brush border to the basal lateral membrane in order to ADP-ribosylate G_s subunits, or (*ii*) the regulatory subunits are modified by the toxin in the luminal side, and they are subsequently transferred to the contraluminal side to interact with the catalytic subunits. No data are available indicating any movement of the whole or part of the toxin to the basal lateral membrane of intestinal cells. On the other hand, brush border membranes contain components that incorporate ^{32}P after treatment with [^{32}P]NAD and cholera toxin (Fig. 1; see also refs. 12 and 13). Moreover, basal lateral membranes containing the catalytic subunit of the cyclase (Table 1; see also ref. 12) do not contain proteins that become labeled under the same conditions in which proteins within the brush border membranes are ADP-ribosylated by the toxin (Fig. 1).

One possible explanation for the presence of ADP-ribosylatable brush border membrane proteins may be that these proteins do not correspond to guanine nucleotide-binding proteins directly related to adenylyl cyclase activation. They might be components of a different transduction mechanism of the enterocyte. Recent evidence indicates that there is a family of regulatory proteins that participate as transduction elements in cellular processes independent of adenylyl cyclase, such as activation of phospholipase C (26, 27), coupling of muscarinic receptors to potassium channels (28, 29), exocytosis (30), or photoreception (31). Some of these regulatory proteins are modified by cholera toxin and some others by pertussis toxin. It is also known that cholera toxin catalyzes the ADP-ribosylation of more than one protein in the same membrane (Fig. 1; see also refs. 12 and 32), and it also has been shown that (*i*) sometimes cholera and pertussis toxins both modify the same regulatory component (33–35) and (*ii*) stimulation of inositolphospholipid degradation by thyrotropin in GH_3 cells affects a guanine nucleotide-binding component that is modified by neither cholera toxin nor pertussis toxin (36–38). Although all these data suggest caution before ruling out that ADP-ribosylation of some brush border proteins is also related to changes in membrane constituents other than adenylyl cyclase components, the presence of regulatory subunits of the enzyme in the brush border is supported by the following facts: (*i*) the molecular masses of the major components ADP-ribosylated by cholera toxin correspond to those of the cyclase regulatory components found in other systems (Fig. 1; see also refs. 4, 19, and 39), (*ii*) the ADP-ribosylation of brush border proteins appears to be similar in many respects to well-characterized ADP-ribosylation of other G_s subunits (12, 40), and (*iii*) cholate extracts of cholera toxin-treated brush border membranes can efficiently reconstitute adenylyl cyclase activity

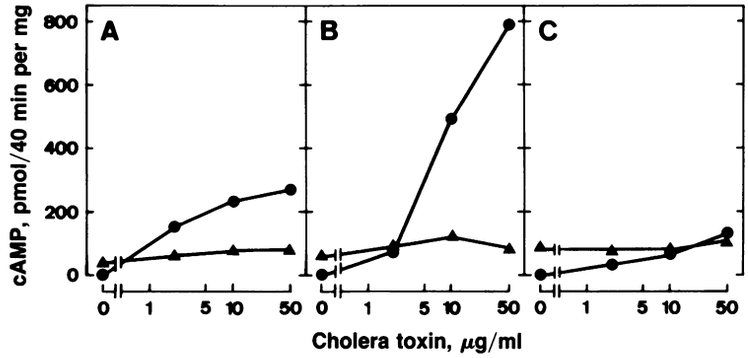

FIG. 2. Reconstitution of adenylyl cyclase activity in cyc⁻ S49 plasma membranes by extracts of cholera toxin-treated intestinal epithelial membranes. Crude plasma membranes (*A*), brush border membranes (*B*), and basal lateral membranes (*C*) were treated with the indicated concentrations of activated cholera toxin, extracted with cholate, and used to reconstitute adenylyl cyclase activity in cyc⁻ plasma membranes as indicated. Results are expressed as reconstituted adenylyl cyclase activity obtained per mg of cholate extract after subtracting the activity of cholate extracts and cyc⁻ membranes (●). The activity of cholate extracts in the absence of cyc⁻ membranes (▲) is also presented for comparison. Each value is the mean of triplicate determinations.

in plasma membranes of the cyc⁻ S49 lymphoma cell line (Fig. 2 and Table 2).

The quantitation of G_s regulatory components in brush border membranes appears quite difficult. The reconstitution value obtained by treating the membranes with 50 μg of cholera toxin per ml is near saturation (notice the logarithmic scale in the abscissa of Fig. 2). However, in our hands (Fig. 2 and Table 2) as well as in other hands (14), reconstitution of cyc⁻ adenylyl cyclase shows a wide variability from experiment to experiment. This makes rather doubtful any conclusion on the number of regulatory components present in the solubilized fraction being tested. For this same reason, it is important to use data from the same experiment when comparing solubilized fractions from the various types of membranes used in this study.

The use of cyc⁻ plasma membranes and their reconstitution by addition of detergent extracts containing regulatory components of adenylyl cyclase has been of great significance in the development of the current model of hormone-sensitive adenylyl cyclase (20, 21, 41, 42). Our data not only prove the existence of such regulatory components in intestinal brush border membranes, but also our reconstitution experiments indicate that basal lateral membranes also contain G_s components. The nature of these basal lateral membrane proteins, the reason for their insensitivity to cholera toxin-catalyzed ADP-ribosylation, and their connection to a given receptor type are unclear at the moment.

Table 2. Reconstitution of adenylyl cyclase activity in cyc⁻ plasma membranes by detergent extracts of membranes from rabbit small intestinal epithelium

Adenylyl cyclase stimulation	Adenylyl cyclase activity in donor membranes, pmol/40 min per mg of protein in the extract		
	Crude	Brush border	Basal lateral
GTP (10 μM)	NR	35	NR
VIP (10 μM)	NR	NR	NR
p[NH]ppG (10 μM)	336	5696	1464
F^- (10 mM) Al^{3+} (0.1 mM)	1288	4139	1675
Cholera toxin (50 μg/ml)	239	2068	430

Membranes from rabbit small intestine were obtained, treated with 50 μg of cholera toxin per ml when indicated, and cholate extracts were prepared as described. The cholate extracts were mixed with cyc⁻ plasma membranes, and adenylyl cyclase was determined in the presence of the indicated concentrations of activators. For the series stimulated with NaF, VIP, or cholera toxin, 10 μM GTP was present during the reaction. Results are expressed as reconstituted adenylyl cyclase activity per 40 min, from which was subtracted the activity of the cholate extract in the absence of cyc⁻ membranes plus the endogenous cyc⁻ adenylyl cyclase activity. The intestinal membranes used in this experiment were those obtained in the preparation described in Table 1. Each value represents the mean of triplicate determinations. NR, not detectable reconstituted activity (i.e., the actual activity measured was equal to or slightly lower than the value obtained by adding the endogenous cyc⁻ activity plus the activity of the cholate extract).

Since basal lateral membranes also contain regulatory components of adenylyl cyclase, it could be argued that the components detected in the brush border originate from a contamination of basal lateral membrane proteins redistributed all across the cell membrane of the enterocyte after disruption of the tight junctions during the purification procedure. This appears improbable because: (*i*) there is no redistribution of marker enzymes corresponding to one or the other side of the cell (Table 1), and (*ii*) cholate extracts of brush border membranes reconstitute adenylyl cyclase activity in cyc⁻ S49 plasma membranes much more efficiently than do extracts of basal lateral membranes (Table 2). It would be hard to visualize a way in which regulatory components of adenylyl cyclase not only move from the contraluminal membrane but also concentrate in brush borders during isolation of the membranes.

The presence in the intestinal brush border membrane of a large population of free G_s regulatory components opens new possibilities for understanding the mechanism by which cholera toxin activates adenylyl cyclase in this tissue. It is conceivable that after interaction of cholera toxin with the brush border membrane, the A_1 protomer dissociates from the holotoxin within the membrane bilayer, therein catalyzing the ADP-ribosylation of G_s components. Then, the dissociated α subunit of the modified G_s component would reach in a yet-undefined way the catalytic subunit in the basal lateral membrane. It is interesting in this regard that G_s regulatory components have been found in the cytosolic fraction of various tissues (43), and specially interesting is a recent report demonstrating the release of α subunits of G_s from rat liver plasma membranes as a consequence of the action of cholera toxin (44). Whether this or another mechanism allows intestinal brush border G_s to reach adenylyl cyclase in the basal lateral membrane remains to be determined.

We are grateful to Professor S. Gascón for his continuous support; to Dr. J. Codina, Baylor College of Medicine, Houston, for the generous gift of S49 cyc⁻ membranes; and to Dr. S. B. Shears for critical reading of the manuscript. This work was supported by grants

from the Fondo de Investigaciones Sanitarias (85/1132) and the Comisión Asesora de Investigación Científica y Técnica (PB85-0399). G.V. was the recipient of a fellowship from the Fondo de Investigaciones Sanitarias (F.I.S.).

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