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Regulation of Intestinal Guanylate Cyclase by the Heat-Stable Enterotoxin of *Escherichia coli* (STa) and Protein Kinase C

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The heat-stable enterotoxin of *Escherichia coli* (STa) stimulates membrane-bound guanylate cyclase in intestinal epithelium and induces fluid and ion secretion. Using the T84 human colon carcinoma cell line as a model, we observed that phorbol esters markedly enhanced STa-stimulated cyclic GMP accumulation in T84 cells (C. S. Weikel, C. L. Spann, C. P. Chambers, J. K. Crane, J. Linden, and E. L. Hewlett, Infect. Immun. 58:1402–1407, 1990). In this study we document that the phorbol ester treatment increases ^{125}I-STa-binding sites as well as membrane-bound guanylate cyclase activity in T84 cells and provide evidence that both effects are mediated by phosphorylation. Guanylate cyclase activity was increased approximately 50% in membranes prepared from intact T84 cells treated with phorbol-12,13-dibutyrate (β-PDB) and after treatment of homogenates with β-PDB in a manner dependent on ATP, $MgCl_2$, and cytosol. Similarly, treatment of membranes with purified bovine brain protein kinase C in the presence of appropriate cofactors and β-PDB resulted in an increase in STa-stimulated guanylate cyclase activity of about 70%. Likewise, the number of ^{125}I-STa-binding sites was increased by about 25 to 40% in membranes prepared from intact cells or homogenates treated with β-PDB; no effect on binding affinity (K_d = 0.15 nM) was noted. These experiments suggest that protein kinase C may phosphorylate the STa receptor-guanylate cyclase or a closely related protein and increase guanylate cyclase activity. The stimulatory effects of protein kinase C on STa-sensitive guanylate cyclase are opposite in direction to the profound inhibitory effects of the kinase on atrial natriuretic peptide-stimulated guanylate cyclase, demonstrating differential regulation by protein kinases within the guanylate cyclase-receptor family.

The heat-stable enterotoxin of *Escherichia coli* (STa) is an 18- or 19-amino-acid peptide toxin that causes diarrhea by binding to and stimulating intestinal membrane-bound guanylate cyclase (1, 19, 25, 43, 45, 46). The T84 human colon carcinoma cell line possesses toxin-sensitive guanylate cyclase and toxin-stimulated vectorial ion secretion (16, 27, 37, 45) and has served as an in vitro model of enterotoxin action. Previously we reported that β-phorbol dibutyrate (β-PDB) doubled the cyclic GMP (cGMP) accumulation observed in response to maximal concentrations of the toxin STa (52) and that carbachol, which stimulates protein kinase C (PKC) in this cell line (10), also potentiates STa-stimulated cGMP accumulation (11). Moreover, both carbachol and histamine produce a marked, synergistic ion-secretory response when added in combination with STa in T84 monolayers (37). These findings were unexpected since phorbol esters, acting through PKC, abolish the ability of atrial natriuretic peptide (ANP) to stimulate membrane-bound guanylate cyclase in various tissues (30, 34, 38). Several possible mechanisms by which the phorbols and other agonists might enhance STa-stimulated cGMP production were considered, including increased recruitment of receptors from intracellular pools to the plasma membrane, synthesis of new receptors, and phosphorylation-mediated changes in the catalytic domain of the receptor-guanylate cyclase molecule. In this study we investigated the mechanism of this phorbol ester effect on STa action in intact and broken cells. We found that β-PDB treatment of intact cells caused a rapid increase in the number of binding sites for ^{125}I-STa and in guanylate cyclase activity in T84 membranes and that these effects on guanylate cyclase and toxin binding could also be observed in vitro in homogenates and in membranes treated with purified bovine brain PKC.

MATERIALS AND METHODS

Cell culture. T84 cells were obtained from the American Type Culture Collection, Rockville, Md., and were at passage 50 to 52 on receipt; the cells were maintained for an additional 23 passages by previously described methods (12, 52). Cells were grown for at least 5 to 7 days after exposure to a dissociating agent (trypsin-EDTA) before harvest for use in experiments.

Cellular fractions. Cells were harvested by scraping in ice-cold phosphate-buffered saline containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride and then pelleted at 2,000 × *g* for 5 min at 4°C. The cells were lysed by being placed on ice for 10 min in hypotonic buffer consisting of 50 mM Tris (pH 7.4), 2.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mg of leupeptin per liter and then homogenized in a Polytron homogenizer at 80% power for 8 s. The crude homogenate contained no intact cells or nuclei as assessed by phase-

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contrast microscopy. The crude homogenate was centrifuged at 24,000 × g for 45 min and resuspended in 50 mM Tris (pH 7.4)–2 mM $MgCl_2$–1 mM EDTA to yield a membrane fraction which was sometimes frozen at −70°C until use in guanylate cyclase assays and binding experiments.

Guanylate cyclase assay and cGMP measurement. Guanylate cyclase assays were performed as previously described (12), and the reaction was stopped by addition of 0.9 ml of 0.1 M HCl. cGMP was measured by automated radioimmunoassay after acetylation with a 3.5:1 mixture of triethylamine and acetic anhydride as previously described (6, 11, 12, 52). Manual radioimmunoassay was performed in some experiments by incubating 50 μl of ^{125}I-labeled succinyl cGMP tyrosine methyl ester, 50 μl of anti-cGMP antibody diluted 1:8,000, and 50 μl of sample or standard for 4 h at room temperature. Radioactive tracer was precipitated by the addition of 50 μl of rabbit anti-goat Immunobeads (Bio-Rad, Richmond, Calif.) followed by an 18-h incubation at 4°C. Protein was measured by the modified Lowry method as described previously (12).

Iodination and purification of ^{125}I-STa. Purified *E. coli* STa, which migrated as a single sharp peak on high-pressure liquid chromatography, was obtained from Donald C. Robertson, University of Kansas. STa (20 μg) was allowed to react with 1 mCi of Na^{125}I in 250 to 300 μl of 0.2 M sodium phosphate buffer (pH 7.4) at 22°C for 5 min in a tube previously coated with 2 μg of Iodogen Reagent (Pierce Chemical Co., Rockford, Ill.) as specified by the manufacturer. Enzymobeads (Bio-Rad) were also used in some experiments but gave a lower yield of iodinated product.

The product of the iodination reaction was subjected to high-pressure liquid chromatography on a Phenomenex C18 column exactly as described by Thompson et al. (47) to separate the desired biologically active ligand, monoiodinated ^{125}I-4Tyr-STa, from ^{125}I-18Tyr-STa. Later it was learned (40a) that a 30 to 55% methanol gradient over 60 min could be used instead of an acetonitrile gradient to elute the iodinated toxin, and this was used in later purifications for convenience. ^{125}I-4Tyr-STa was identified by its position of elution (the later of the two major iodinated peaks) and by its superiority over ^{125}I-18Tyr-STa in binding to T84 membranes. The specific activity of the freshly labeled toxin was ca. 2,000 Ci/mmol. ^{125}I-4Tyr-STa was used in all binding experiments presented in this paper and is henceforth referred to as ^{125}I-STa.

^{125}I-STa binding. Conditions for ^{125}I-STa binding were initially based on those used by Guarino et al. for labeled STa in T84 cell membranes (21). Binding was carried out in 50 mM sodium acetate (pH 5.8)–0.1 g of bovine serum albumin per liter (binding buffer) with or without 100 mM NaCl and 4 mM $MgCl_2$; 40 to 80 μg of membrane protein was used per tube in a total volume of 200 μl. In other experiments, 50 mM Tris (pH 7.4) was substituted for sodium acetate. Binding was initiated by the addition of ^{125}I-STa and continued at 30°C for 90 min, a time determined to give steady-state binding, and was terminated by addition of 2.5 ml of ice-cold binding buffer and immediate filtration on Whatman GF/C 24-mm glass fiber filters on a vacuum manifold. Each filter was then washed twice more with 2.5 ml of ice-cold binding buffer, and ^{125}I counts were determined. Nonspecific binding was determined in the presence of 1 μM unlabeled STa. Nonspecific binding increased linearly with the concentration of ^{125}I-STa and increased from 7.5% of total binding at 0.1 nM ^{125}I-STa to 25% at 2 nM ^{125}I-STa at pH 5.8.

To assess the effect of pH on binding and guanylate cyclase, a series of buffers was prepared as follows (final concentrations): 50 mM sodium acetate, titrated to pH 4.0 and 5.8; with acetic acid, and 50 mM sodium phosphate at pH 6.8 and 7.8.

Phorbol esters. β-PDB and the inactive isomer α-PDB were obtained from LC Services Corp., Woburn, Mass., and were dissolved at 10 mM in ethanol and stored at −20°C for up to 3 months; this stock was diluted to 0.1 mM just before use and added to cells or homogenate to yield a final concentration of 1 μM in 0.01% ethanol. α- and β-PDB were used because previous studies showed that β-PDB was more active than phorbol myristate acetate and other phorbol esters in intestine in two mammalian species (17, 51).

Treatment of homogenates and membranes. Phorbol ester effects in homogenates were tested by preincubating homogenates for 5 min at 37°C in the presence of 5 mM $MgCl_2$ and 1 mM ATP or other adenine nucleotides and with or without $CaCl_2$ added to 0.1 mM above the EGTA concentration. Effects of purified rat brain PKC on T84 membranes were tested by preincubating membranes with 40 to 60 μl of purified PKC at 30°C for 5 min in the presence of 6 mM $MgCl_2$, 0.1 mM $CaCl_2$ (in absence of chelator), 1 mM ATP or other nucleotide, and phorbol ester and then returning the membranes to ice.

Purification of PKC. Purified bovine brain PKC was prepared in the laboratory of J. Sando by the method of Walker et al. (49) through the hydroxylapatite column step, in which fractions II (a mixture of α and β isozymes) and III (predominantly isozyme) were frozen at −70°C, shipped on dry ice, and used for these experiments. To confirm enzyme activity after storage and transport, we assayed this material for PKC activity by phosphorylation of histone as described previously (49). In this assay, 2 × 10^6 cpm of [^{32}P]ATP, 40 μM unlabeled ATP, 100 μM $CaCl_2$, 40 μg of phosphatidylserine per ml, 1.6 μg of diolein per ml, 0.6 mg of histone V-S per ml, and PKC fractions were incubated in a total volume of 75 μl at 30°C for 3 min; 60-μl aliquots were withdrawn and spotted onto phosphocellulose filters and rinsed as described previously (49). Purified PKC (50 μl) catalyzed the incorporation of 10,000 to 18,000 cpm of ^{32}P dry (Cerenkov) counts into histone in this assay.

Materials. Purified *E. coli* STa, obtained from Donald C. Robertson, was prepared by the method of Dreyfus et al. (18). Na^{125}I was obtained from Amersham, and other reagents were from the sources described previously (12). The PKC pseudosubstrate inhibitor, PKC(19–36), was purchased from Peninsula, Belmont, Calif. Anti-cGMP antibody was prepared by immunizing goats with succinyl cGMP conjugated to bovine serum albumin (Elmira Biologicals, Iowa City, Iowa).

Data analysis. Results are presented as means ± standard deviations unless otherwise stated. Binding isotherms were transformed by the method of Scatchard (41); the binding parameters (total binding and dissociation constant, K_d) were calculated by a nonlinear least-squares curve-fitting program (Kaleidagraph; Synergy Software, Reading, Pa). For displacement of ^{125}I-STa by unlabeled STa, the 50% inhibitory concentrations (IC_{50}s) were obtained graphically and the inhibitory constant, K_i, was calculated by the method of Cheng and Prusoff (7).

RESULTS

Conditions for STa binding and guanylate cyclase activation. The goals of this investigation were to determine whether PKC activators increased guanylate cyclase activity

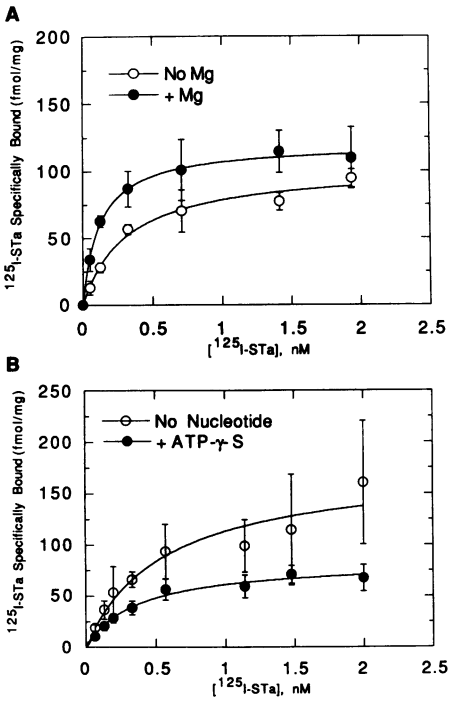

FIG. 1. Effect of $MgCl_2$ and ATP-γ-S on ^{125}I-STa binding in T84 cell membranes. (A) Saturation-binding curves were generated in the presence and absence of 4 mM $MgCl_2$ at pH 7.4, without NaCl, as described in Materials and Methods. Similar results were obtained when binding was performed at pH 5.8. This experiment is typical of two experiments at pH 7.4, both done in triplicate. (B) Membranes frozen in Tris–1 mM $MgCl_2$ were resuspended in binding buffer at pH 7.4 (50 mM Tris, 0.1% bovine serum albumin) without NaCl or $MgCl_2$, yielding a final $MgCl_2$ concentration of 0.1 mM, and binding was measured in the absence and presence of 0.6 mM ATP-γ-S. This experiment is typical of three performed with ATP-γ-S.

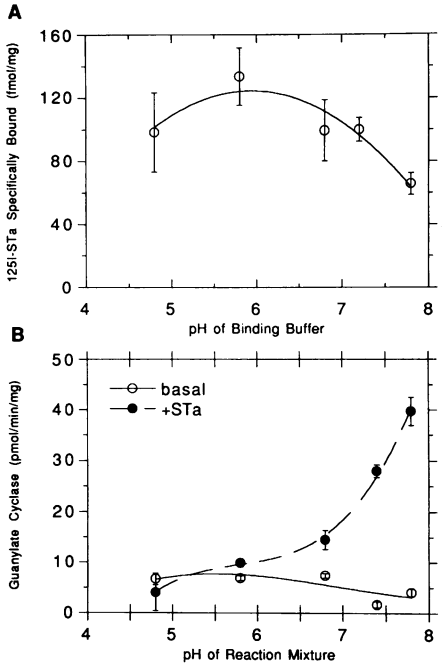

FIG. 2. Effect of pH on ^{125}I-STa binding and guanylate cyclase activity in T84 membranes. A series of buffers were prepared as described in Materials and Methods and used for both the toxin-binding and guanylate cyclase assays. (A) Effect of pH on ^{125}I-STa binding. The concentration of radiolabeled tracer was 0.21 nM, and the predicted receptor occupancy was 58%. (B) Effect of pH on basal and toxin-stimulated guanylate cyclase activity.

or ^{125}I-STa binding in T84 cells. Preliminary binding experiments with ^{125}I-4Tyr-STa yielded a single class of binding sites with a K_d of 0.1 to 0.2 nM, which was almost 50-fold lower than that reported previously by Guarino et al. for this ligand in T84 cell membranes (21) and somewhat lower than that reported for toxin binding to rat intestinal membranes (26, 48). Since our binding conditions differed from those of Guarino et al. with respect to the presence of $MgCl_2$ and the concentration of NaCl, we sought to determine whether these variables accounted for the higher affinity of binding we observed. Figure 1A demonstrates the effect of 4 mM $MgCl_2$ on ^{125}I-STa binding in a saturation-binding curve at pH 7.4. In two such experiments, $MgCl_2$ increased the number of toxin-binding sites by 21% ± 7% whereas the mean K_d values, calculated by a nonlinear least-squares curve-fitting program, were not significantly altered (0.21 nM in the absence and 0.11 nM in the presence of $MgCl_2$). In other experiments at pH 7.4, 100 mM NaCl increased the number of apparent binding sites by 48% ± 23% (n = 5 experiments) but again had no effect on binding affinity. The effects of $MgCl_2$ and NaCl were not independent; that is, the binding observed in the simultaneous presence of 100 mM NaCl and 4 mM $MgCl_2$ at pH 7.4 was not increased over that seen with magnesium alone (data not shown).

Since ATP and its analog adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S) enhance STa-stimulated guanylate cyclase activity (20), we tested the effect of ATP-γ-S on ^{125}I-STa binding. Figure 1B shows that ATP-γ-S decreased the number of apparent STa-binding sites by about 50% without affecting the binding affinity. ATP (1 mM) produced a similar but slightly smaller reduction in the number of apparent sites (mean 37% decrease in the number of sites in five separate experiments). The presence of 4 mM $MgCl_2$ in the binding assay did not diminish the inhibitory effect of ATP and ATP-γ-S on binding (data not shown). Larose et al. (35) have recently reported that ATP decreases the number of adrenal ANP receptors of the R_1 receptor (guanylate cyclase-coupled) subtype in membranes and in purified receptor preparations.

Guarino et al. noted that ^{125}I-STa binding to intact T84 colon cells increased as the pH was lowered from neutrality to pH 4.5 and chose pH 5.8 for their binding studies in membranes as well (21). Therefore we investigated whether a difference in pH accounted for the increase in binding affinity in our results. Figure 2A shows the effect of pH on ^{125}I-STa binding by using a single, moderately high concentration of ligand. ^{125}I-STa binding increased 1.5- to 2-fold as the pH was lowered from 7.8 to 5.8 but then decreased again at pH 4.8. Figure 2B demonstrates that the ability of STa to stimulate guanylate cyclase activity declined markedly below neutrality and was abolished at pH 5 and below. These findings suggest that although the extracellular toxin-binding domain remains functional at lowered pH, this is not the case for toxin-activated guanylate cyclase activity in a broken-cell preparation. Although neither the pH nor the magnesium or sodium concentration explained the higher binding affinity we observed than that seen by Guarino et al. (21), the K_d values we obtained were virtually identical to those recently

TABLE 1. Summary of experiments demonstrating an enhancement in guanylate cyclase activity in membranes prepared from intact T84 cells treated with phorbol esters

Expt	Mean guanylate cyclase activity (pmol/min/mg of protein)[a] ± SD											
	Basal				Manganese stimulated				STa stimulated			
	α-PDB	β-PDB	Fold stimulation (β/α)	P	α-PDB	β-PDB	Fold stimulation (β/α)	P	α-PDB	β-PDB	Fold stimulation (β/α)	P
A. Longer treatment (45–60 min, 9 expts)	7.9 ± 4.1	11.9 ± 6.9	1.6	0.04	135 ± 89	162 ± 105	1.2	0.02	35.3 ± 11	53.4 ± 22	1.5	0.002
B. Shorter treatment (10–15 min, 5 expts)[b]	13.4 ± 1.8	15.7 ± 1.8	1.2	0.03	312 ± 48	384 ± 45	1.2	0.02	40.4 ± 5.3	52.0 ± 3.5	1.3	0.003

[a] The T84 cells used in row A and row B were from separate batches, so that guanylate cyclase activities between these groups of experiments are not comparable. Fold stimulation (β/α) was calculated for each experiment individually and the mean is shown.
[b] Except for the Mn^{2+}-stimulated condition, for which $n = 2$.

measured by de Sauvage et al. in T84 cells by using a truncated STa analog (15).

Effect of phorbol ester treatment on guanylate cyclase activity. A previous report from our laboratories showed that the addition of β-PDB to T84 cells caused a 1.6- and 2.4-fold enhancement in STa-stimulated cGMP accumulation at 1 and 2 h, respectively. Because the enhancement in cGMP accumulation in intact cells was seen at 1 h, initial experiments seeking an effect of phorbol esters on guanylate cyclase or toxin binding were performed with membranes made from cells treated with β-PDB for 1 h. Table 1, row A, shows that membranes from cells treated with β-PDB for 45 to 60 min manifested increased basal, manganese-stimulated, and STa-stimulated guanylate cyclase activities compared with those of the control (α-PDB) membranes. The magnitude of the increase was 50 to 70% for basal and STa-stimulated activity and 20% for Mn^{2+}-stimulated activity. In five subsequent experiments with membranes made from cells treated with β-PDB for only 10 or 15 min (Table 1, row B), the increase in guanylate cyclase activity under all three conditions was still observed, although the magnitude of the increase was somewhat less than after the longer treatment. These results indicated that the β-PDB treatment of cells caused a rapid and stable alteration in guanylate cyclase activity and that this alteration was observed under all assay conditions and not just with STa stimulation. Furthermore, the change in guanylate cyclase activity was observed after only a 10-min exposure to β-PDB, even though the maximum effect of the phorbol ester on cGMP accumulation in intact cells was at 1 and 2 h. Experiments with intact cells have indeed shown that β-PDB-induced enhancement of cGMP accumulation can be seen as early as 5 min after addition of β-PDB and STa (52). Since the guanylate cyclase assay is linear for at least 10 min, the phorbol ester effect acutely is to increase the enzyme V_{max}, although an additional effect leading to enhanced stability of the enzyme cannot be ruled out.

Previously we reported that β-PDB had no effect on cGMP levels in the absence of STa in these cells (52), but resting cGMP levels are low in this cell line and previous measurements were made with a rapid, automated radioimmunoassay technique which is less sensitive for low levels of cGMP. Since an increase in basal guanylate cyclase activity was detected in membranes prepared from β-PDB-treated cells, we reexamined the effect of β-PDB alone on cGMP levels in intact T84 cells. We repeated our measurements of basal cGMP using larger cell wells (to increase the cGMP signal) and a more sensitive, 18-h radioimmunoassay and were able to detect an effect of β-PDB on cGMP levels in whole cells treated for 45 min: ethanol vehicle control, 5.4 ± 0.16 pmol/mg; 1 μM α-PDB, 8.8 ± 3.6 pmol/mg; and 1 μM β-PDB, 14.4 ± 2.8 pmol/mg ($P < 0.02$ compared with the α-PDB control). Thus, β-PDB did elevate basal cGMP levels in intact cells, as predicted from the guanylate cyclase results in Table 1.

Effect of β-PDB on STa binding. To determine whether phorbol ester treatment caused any change in the toxin binding, we measured the binding of ^{125}I-STa in T84 membranes made from control and β-PDB-treated cells. Binding was performed at pH 5.8 in these experiments in the presence of $MgCl_2$ to optimize the binding. Figure 3 depicts the results of a typical experiment, in which membranes from β-PDB-treated cells showed an approximate 30% increase in the number of ^{125}I-STa-binding sites. In four similar experiments, β-PDB treatment of cells for 1 h increased the ^{125}I-STa-binding sites from 217 ± 36 to 272 ± 45 fmol/mg

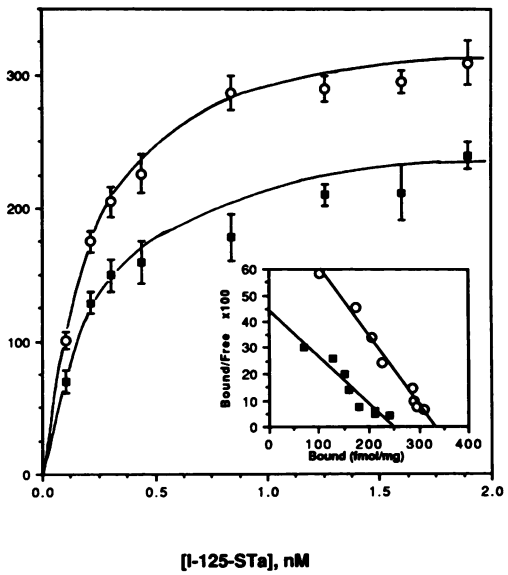

FIG. 3. Effect of phorbol ester treatment on binding of ^{125}I-STa to T84 membranes. Intact T84 cells were treated for 1 h with either the active phorbol ester β-PDB (○) or the inactive α-PDB (■). Membranes were prepared from these cells, and binding of ^{125}I-STa was measured at pH 5.8 with 0.1% bovine serum albumin, 4 mM $MgCl_2$, and 100 mM NaCl as described in Materials and Methods. Inset: Scatchard plot of the data showing a single class of binding sites.

(25% increase; $P = 0.01$ by the paired t test) without affecting the K_d. A 17% increase in binding sites ($P < 0.05$) was also observed in membranes prepared from cells treated with β-PDB for 10 min. The STa binding in membranes from phorbol ester-treated and control cells was also investigated in experiments in which a fixed concentration of ^{125}I-STa (0.3 to 0.37 nM) was displaced by increasing amounts of unlabeled toxin. Although β-PDB-treated membranes bound 20 to 30% more ^{125}I-STa, no differences in IC_{50}s were noted for displacement of ^{125}I-STa by unlabeled toxin from β-PDB with respect to control membranes (IC_{50}s were 1.5 to 2.0 nM in three experiments). Since IC_{50}s vary depending on the concentration of labeled ligand used, K_i values were calculated by the method of Cheng and Prusoff (7), using the K_d value for ^{125}I-STa of 0.15 nM. In three experiments the K_i for native STa was 0.49 ± 0.03 nM for α-PDB membranes and 0.54 ± 0.05 nM for β-PDB membranes. Thus, ^{125}I-STa may have a slightly higher apparent affinity for T84 membranes (K_d, 0.1 to 0.2 nM) than does the native toxin.

Binding of ^{125}I-STa to intact T84 cells also increased 20% ± 8% after β-PDB treatment in two experiments (data not shown; $P = 0.03$ by the paired t test).

Although the K_d value observed for ^{125}I-STa binding in T84 cells was lower than those previously reported by some workers in this field (9, 21), it lies between the K_ds for the high-affinity and low-affinity binding sites recently reported by Hugues et al. for rat intestinal membranes (26). We observed only a single apparent binding site, but high-affinity sites constituting less than 10% of binding could have been missed by the methods we used. One puzzling feature of STa action which other investigators have long noted and which we also observed is the discrepancy between the K_d for binding and the 50% effective concentration (EC_{50}) for activation of guanylate cyclase (30 to 50 nM). Our results do not provide a ready explanation for this phenomenon.

Effect of PKC activators on STa binding and guanylate cyclase in broken-cell preparations. Next we sought to determine whether a phorbol ester effect on guanylate cyclase or toxin binding could be observed in broken-cell preparations. In these experiments, crude, fresh T84 homogenates were incubated for 5 min at 37°C in the presence or absence of cofactors (ATP, $MgCl_2$, $CaCl_2$, and phorbol ester) necessary for activation of endogenous PKC and then guanylate cyclase activity was assayed. Exogenous phospholipid was not added since cell membrane lipid was present in the homogenates. Figure 4 shows that preincubating crude homogenates with ATP with or without calcium increased the STa-stimulated guanylate cyclase activity. Preincubation with ATP had a more variable effect on basal guanylate cyclase activity, probably owing to the small amount of ATP carried over to the guanylate cyclase assay (~0.2 mM), since direct addition of ATP to homogenates and membranes resulted in small increases in basal guanylate cyclase activity, consistent with the recent report of Gazzano et al. (20). Addition of $CaCl_2$ to a concentration 0.1 mM above the chelator concentration caused an increase in basal and STa-stimulated guanylate cyclase activity (Fig. 4, compare the first and fifth sets of conditions). In the presence of ATP, treatment of homogenates with β-PDB resulted in an additional increase in STa-stimulated guanylate cyclase activity (Fig. 4, fourth and eighth sets of conditions). The enhancement of activity by β-PDB was seen in homogenates both with and without calcium, but the β-PDB effect was most prominent in the absence of calcium (i.e., there was a 1.8-fold enhancement in activity by β-PDB over vehicle in the absence of calcium compared with a 1.2-fold enhancement in the presence of calcium). Although the prototypic PKC isozymes (α, β, and γ) are calcium dependent, their calcium requirement is decreased in the presence of phorbol esters. The more recently cloned isozymes are calcium independent (PKC-δ [36] and PKC-ε [42]) or are hypothesized to be calcium independent (PKC-η and PKC-ζ) on the basis of deduced sequences (40). The results in Fig. 4 demonstrated that the phorbol ester effect on guanylate cyclase could be reproduced in a broken-cell system.

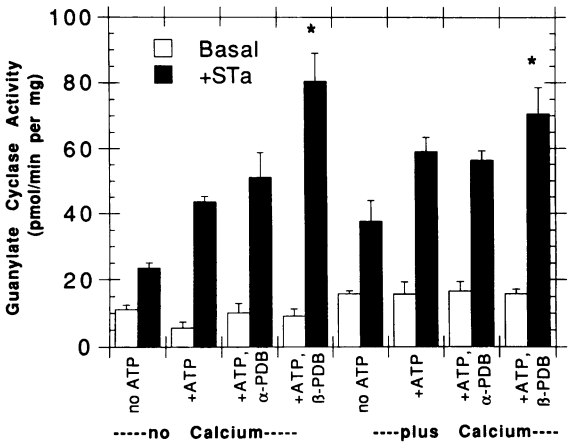

FIG. 4. Effect of ATP, calcium, and phorbol esters on guanylate cyclase activity in T84 cell homogenates. Homogenates containing 1.25 mM EGTA and 0.25 mM EDTA plus 4 mM $MgCl_2$ were prepared as described in Materials and Methods. For the preincubation, some homogenates also contained 1 mM ATP (bars marked +ATP) or 1.6 mM $CaCl_2$ (bars marked plus Calcium) or both and 1 μM phorbol esters or 0.01% ethanol vehicle. The homogenates were preincubated at 37°C for 5 min and then returned to ice; the homogenates were diluted fivefold, and the guanylate cyclase activity was measured in triplicate in the presence (+STa, black bars) or absence (basal, open bars) of 1 μM STa. *, statistically significant difference compared with the α-PDB control for each calcium condition ($P < 0.05$). The results shown are typical of results from three experiments.

Next we examined the effect of phorbol esters on ^{125}I-STa binding after treatment of homogenates in the presence of ATP and $MgCl_2$; since ^{125}I-STa binding could not be measured directly in homogenates because of high nonspecific binding, we prepared membranes from the treated and control homogenates by centrifugation and used them for the binding assay. Treatment of homogenates with β-PDB for 5 min resulted in a 40% increase in the specific binding of toxin, which was due solely to an increase in the number of toxin-binding sites (Fig. 5A, binding at pH 5.8). This effect was not observed if ATP was omitted, if 5'-adenylyl-β-γ-imidodiphosphate (AppNHp) was substituted for ATP, or if the preincubation was shortened to 2 min (Fig. 5B). The phorbol ester-induced increase in binding sites was also observed if binding was performed at pH 7.4 (mean 33% increase in sites in four experiments).

Kurose et al. (33) showed that the ability of ANP to stimulate membrane-bound guanylate cyclase was enhanced markedly by ATP and that the nonphosphorylating analog AppNHp was partially able to substitute for ATP in that regard. Table 2 summarizes the effect of ATP, AppNHp, and phorbol esters on guanylate cyclase activity in T84 homogenates. Both ATP and AppNHp increased STa-stimulated guanylate cyclase activity, and in this regard AppNHp was as efficacious as ATP. The further enhancement in activity

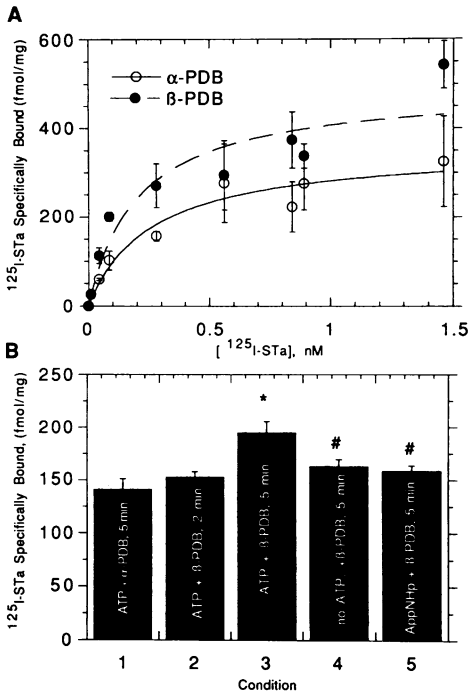

FIG. 5. Effect of preincubating homogenates with phorbol esters on ^{125}I-STa binding. Homogenates were treated with 1 mM ATP, 4 mM $MgCl_2$, and the β-PDB or inactive α-PDB for 5 min at 37°C and then centrifuged to prepare membranes as described in Materials and Methods. (A) Saturation-binding curves for membranes prepared from homogenates treated with α-PDB and β-PDB were generated at pH 5.8. (B) Effect of adenine nucleotides and duration of the preincubation. Binding was performed by using 0.48 nM ^{125}I-STa at pH 5.8. *, statistically significant compared with the α-PDB control ($P < 0.01$); #, significantly less than the β-PDB condition in column 3 ($P < 0.05$). Conditions 4 and 5 were not significantly increased compared with condition 1.

with β-PDB, however, was observed only in the presence of ATP and not with the nonphosphorylating analog. In addition, no effect of β-PDB was observed if washed membranes were substituted for homogenates in this procedure (see Fig. 6A below). This requirement for ATP and for cytosol is consistent with an action of phorbol esters through PKC-mediated phosphorylation of substrates. Despite this, homogenate experiments occasionally showed an effect of β-PDB on guanylate cyclase if no adenine nucleotide was present or if ADP was the nucleotide used in the preincubation step. This β-PDB-mediated effect was most probably due to regeneration of ATP during the guanylate cyclase assay from the 1 mM GTP and the nucleotide-regenerating system present in that assay, since it was blunted when the regenerating system was omitted and was abolished by the addition of the PKC inhibitor peptide, PKC(19–36), after the preincubation step (24).

TABLE 2. Effect of preincubation with adenine nucleotides and phorbol esters on guanylate cyclase activity in T84 cell homogenates

Condition	Adenine nucleotide[a]	Phorbol ester[a]	Mean guanylate cyclase activity ± SD (pmol/min/mg of protein)	
			No STa	+STa
1	None	None	11.1 ± 1.3	23.4 ± 1.7
2	ATP	Vehicle	5.6 ± 1.8	43.7 ± 1.6[b]
3	ATP	α-PDB	10.1 ± 2.8	51.2 ± 7.6[c]
4	ATP	β-PDB	9.1 ± 2.2	80.5 ± 8.5[d]
5	AppNHp	Vehicle	7.1 ± 1.0	38.0 ± 3.3[b]
6	AppNHp	β-PDB	7.6 ± 1.8	43.1 ± 1.8[c]

[a] Adenine nucleotides, when present, were at 1 mM and phorbol ester was at 1 μM.
[b] Significantly different compared with condition 1 (+STa), $P < 0.01$.
[c] Not significantly different compared with the value directly above it.
[d] Significantly different compared with conditions 2, 3, and 6 (+STa), $P < 0.01$.

To determine whether the phorbol ester effects observed in intact cells and homogenates were indeed due to a direct PKC-mediated phosphorylation, we tested the effect of adding purified bovine brain PKC directly to membranes. As shown in Fig. 6, phorbol ester treatment in the presence of appropriate cofactors but in the absence of exogenous PKC did not cause any increase in guanylate cyclase activity in these membranes, presumably owing to the absence of the kinase from the membranes which were prepared in the presence of EGTA. Addition of the bovine brain kinase in the presence of β-PDB caused a 68% increase in STa-stimulated guanylate cyclase activity and a twofold increase in basal activity (Fig. 6A). The effect on basal guanylate cyclase activity was inconsistent, being observed and statistically significant in only four of nine experiments; this variability is probably due to the low basal guanylate cyclase activities in these preparations, resulting in raw cGMP levels near the limit of detection of the immunoassay (~1 to 2 pmol/ml).

As in the experiments with T84 cell homogenates, the effect of PKC plus β-PDB on guanylate cyclase activity in membranes was dependent on ATP. The ATP analogs ATP-γ-S, AppNHp, and ADP-β-S did not permit the PKC-mediated increase in activity (Fig. 6B and C). When adenine nucleotides were omitted, or when ADP was substituted for ATP (Fig. 6B), stimulation by PKC plus β-PDB was still observed in some experiments. This was again believed to be due to regeneration of ATP from GTP during the guanylate cyclase assay, since these stimulations were abolished when 1 μM PKC(19–36) inhibitor peptide was added immediately after the 5-min preincubation step (Fig. 6C, in which conditions 6 through 9 received the inhibitor). The effect of PKC plus β-PDB on toxin-stimulated guanylate cyclase was also completely abolished by the addition during the preincubation step of 1 μM PKC inhibitor peptide (Fig. 6C, columns 2 and 3), 10 μM staurosporine, or 25 μM kinase inhibitor H7 (data for the last two inhibitors not shown).

We next examined whether treatment of membranes with PKC plus β-PDB increased ^{125}I-STa binding. In contrast to the results with guanylate cyclase, no increase in ^{125}I-STa binding was observed, even in membrane preparations which exhibited the expected increase in guanylate cyclase activity. Adding cytosol back to the membranes during the preincubation with PKC did not restore the increase in binding observed in the homogenate experiments (data not shown). Although technical problems could explain these results, these findings tentatively suggest that the phorbol ester-mediated increase in toxin binding seen in intact cells and homogenates may not be a simple conformational change due to phosphorylation of receptor within the membrane. Possible explanations include phorbol-mediated translocation of receptors from microsomes to plasmalemma, uncoupling of receptors from cytoskeletal anchors, protection of receptors from proteolytic degradation during

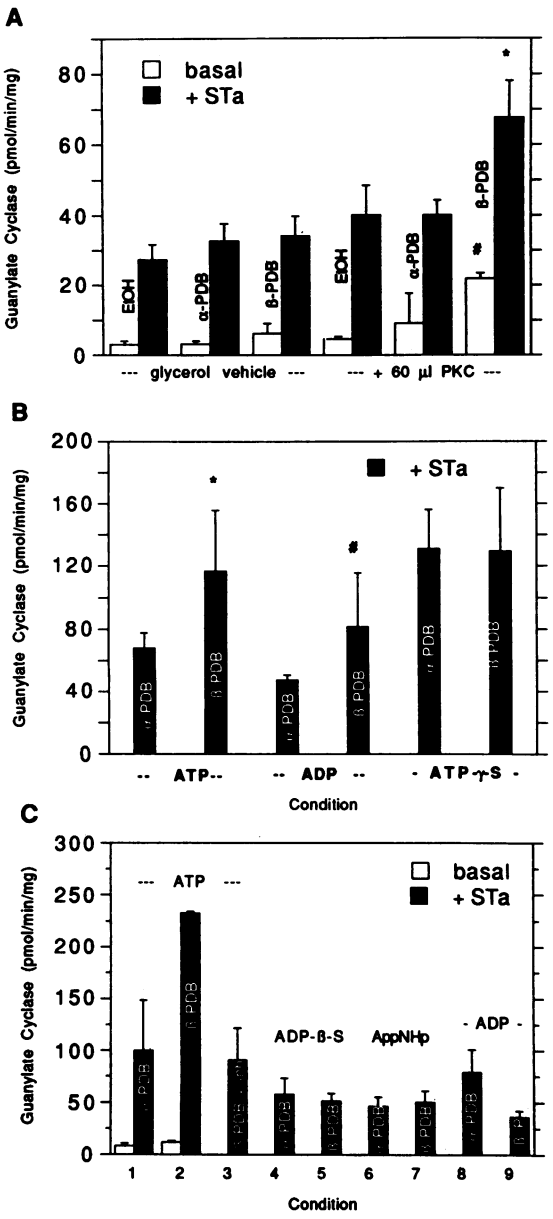

FIG. 6. Effect of purified PKC on guanylate cyclase activity in T84 membranes. Washed membranes (100 µg) were treated with 50 µl (panel B) or 60 µl (panels A and C) of purified PKC together with 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM ATP or other adenine nucleotide at 30°C for 5 min in a total volume of 200 µl. (A) Dependence on added PKC. *, significant increase ($P = 0.01$); #, not significant ($P = 0.17$). (B) Dependence on ATP. *, $P = 0.04$; #, $P = 0.08$. (C) Effect of inhibitor peptide and adenine nucleotides. In condition 3, 1 µM PKC inhibitor peptide, PKC(19–36) was added prior to the 5-min preincubation. In conditions 6 through 9, the PKC inhibitor peptide (1 µM) was added immediately after the preincubation step, before the guanylate cyclase assay. Inh, PKC inhibitor.

preparation, or the release of bound inhibitors which partially block or mask toxin-binding sites. Since little or nothing is known about the intracellular trafficking or recycling of the STa receptor or other guanylate cyclases, all of these possibilities remain speculative.

DISCUSSION

Weikel et al. previously showed that phorbol esters markedly potentiate STa-stimulated cGMP accumulation in intact T84 cells with a peak effect at 1 to 2 h (52). Crane et al. demonstrated that carbachol mimics phorbol esters in this regard (11). Levine et al. recently showed a more dramatic synergism at the level of chloride ion secretion in T84 cell monolayers, when they found that carbachol and histamine, both of which mobilize intracellular calcium and activate PKC, caused a secretory response when added with STa that is two- to fivefold higher than expected from mere additivity (37). In the present study we investigated possible mechanisms for the phorbol ester effect and have provided evidence for a PKC-mediated phosphorylation which results in an increase in guanylate cyclase activity. An increase in the number of STa-binding sites is observed when intact cells or homogenates are treated with phorbol esters but not when membranes are treated with purified PKC, suggesting that a more complex mechanism is involved in the binding increase.

Previous examples of PKC-mediated effects on membrane-bound guanylate cyclases, primarily ANP-stimulated guanylate cyclase, have been uniformly inhibitory. For example, phorbol esters or PKC strongly inhibit or totally abolish ANP-stimulated cGMP production in a variety of tissues including adrenal cortex, vascular smooth muscle, and kidney (4, 22, 30, 38). In the case of the adrenal cortex, the rat ANP receptor-guanylate cyclase has been purified and is phosphorylated in vitro by partially purified PKC (44). PKC mediates increases in cGMP levels in pinealocytes, but this appears to be an effect on soluble guanylate cyclase rather than on the membrane-bound enzyme (23). Indeed, Zwiller et al. reported that rat brain PKC can phosphorylate and activate soluble guanylate cyclase in vitro (53).

The ability of ATP and ATP analogs to increase STa-stimulated and ANP-stimulated guanylate cyclase activity has been noted by other investigators. Similarly, we observed an ATP- and ATP-γ-S-mediated decrease in the number of STa-binding sites, as has been observed for ANP receptors. The site of action of ATP in STa-stimulated guanylate cyclase has not been investigated, but for ANP-sensitive guanylate cyclase, ATP is believed to act allosterically on the membrane-proximal "kinase-like" cytoplasmic domain (8). In the T84 cell system described here, ATP has dual effects on guanylate cyclase. First, ATP and its analogs ATP-γ-S and AppNHp enhance guanylate cyclase activity and decrease the number of STa-binding sites, which, by analogy with ANP receptors, are probably direct allosteric effects on the receptor enzyme. Second, ATP alone (not the nonphosphorylating analogs) serves as a substrate for PKC in homogenates and experiments involving the purified kinase, resulting in an additional increase in guanylate cyclase activity.

Membrane-bound guanylate cyclase belongs to a larger family of receptor-enzymes including the epidermal growth factor receptor, the insulin receptor, and other tyrosine kinase receptors. PKC phosphorylates these receptors on specific residues, resulting in a decreased receptor number owing to increased internalization, attenuated signal-generating (including tyrosine kinase) activity, and, in some cases, diminished affinity of binding for ligand (2, 14, 29, 39). The intestinal STa receptor-guanylate cyclase interaction with PKC differs from the tyrosine kinase receptor model in almost every aspect. PKC or its activators enhance toxin-stimulated cGMP production in intact and broken cells and

phorbol esters increase the number of toxin-binding sites in intact cells and homogenates without altering the binding affinity.

The intestinal STa receptor-guanylate cyclase has been cloned from rat intestinal, human ileal, and T84 cell cDNA (43, 45). Inspection of the deduced amino acid sequence reveals that all three possess sequences, such as Arg-546–Gly–Ser–Leu–Arg, which conform to the consensus phosphorylation site for PKC (2, 31) and which differ from the ANP receptor-guanylate cyclases at that position (Arg-Gly-Ser-Leu-Gln). Thus, the primary structure may provide a clue to the mechanisms by which closely related receptors might be differentially regulated. As a caveat, it should be noted that PKC, like other kinases, has a relatively loose substrate specificity and that a consensus sequence does not prove phosphorylation at that site.

Our results are consistent with three potential explanations: the first is direct PKC-mediated phosphorylation of the STa receptor-guanylate cyclase polypeptide itself. Second, PKC may activate another kinase or kinases, resulting in an alteration (presumably phosphorylation) of the STa receptor-guanylate cyclase. Third, PKC may phosphorylate an accessory protein which modifies receptor binding. In vitro phosphorylation experiments with T84 homogenates demonstrated that β-PDB increased incorporation of ^{32}P from [γ-^{32}P]ATP into at least five membrane proteins with molecular masses of 102, 78, 67, 62, and 51 kDa (data not shown). The 78-kDa band may be the same as an 80-kDa band which we (unpublished data) and others (28, 32) have observed to be labeled by cross-linking of ^{125}I-STa with disuccinimidyl suberate and believed to represent a portion of the STa receptor. Definitive identification of the STa receptor-guanylate cyclase as a substrate for PKC will require development of anti-receptor antibody or improved purification techniques for isolating the receptor-enzyme.

The phorbol ester and PKC-mediated effects on guanylate cyclase reported here are relatively modest. Reasons for this are unclear, but the receptor may be partially phosphorylated prior to phorbol ester or PKC treatment. The sea urchin sperm guanylate cyclase, for example, is heavily phosphorylated in its basal state (5, 50), although this has not been demonstrated for mammalian intestinal guanylate cyclases. Nevertheless, the fact that the effects of PKC are opposite in direction to those reported for ANP-sensitive guanylate cyclase and tyrosine kinases shows that the intestinal STa receptor-guanylate cyclase has a distinct mode of regulation. For many receptors and signaling pathways in various cell types, PKC serves to dampen or attenuate cellular responses to agonists, whereas in intestinal cells PKC augments the cellular cGMP response and biological effects. Since PKC can be activated by a variety of neurohumoral mediators of intestinal secretion (acetylcholine [10] and perhaps histamine and serotonin) and even other intestinal pathogens (3), this biochemical difference may have far-reaching physiological and pathophysiological consequences. In addition, we would predict that the actions of a recently identified endogenous peptide ligand for the STa receptor, termed guanylin (13), will be enhanced by PKC activators as well.

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