Nucleotide regulation of heat-stable enterotoxin receptor binding and of guanylate cyclase activation

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Certain nucleotides were found to regulate the binding of the *Escherichia coli* heat-stable enterotoxin (STa) to its receptor in pig intestinal brush border membranes. ATP and adenine nucleotide analogues inhibited ¹²⁵I-STa binding, while guanine nucleotide analogues stimulated binding, with maximal effects at 0.5–1.0 mM. The strongest inhibitors were adenosine 5'-[β -imido]triphosphate (App[NH]p) (36%) and adenosine 5'-[β -thio]diphosphate (ADP[S]) (41%). Inhibition did not require Mg²⁺, and was blocked by *p*-chloromercuribenzenesulphonate (PCMBS). Stimulation of binding required Mg²⁺, was not prevented by PCMBS and was maximal with GDP[S] (41%). While App[NH]p and MgGDP[S] appeared to be acting at different sites, they also interfered with each other. These nucleotides exerted only inhibitory effects on STa-stimulated guanylate cyclase activity, in contrast with the stimulatory effects of adenine nucleotides on atrial natriuretic peptide (ANP)-stimulated guanylate cyclase. Inhibition by low concentrations of MgApp[NH]p and MgATP was weaker above 0.1 mM, while MgGDP[S] and magnesium guanosine 5'-[γ -thio]triphosphate (MgGTP[S]) inhibited in a single phase. Inhibition by MgApp[NH]p, at all concentrations, was competitive with the substrate (MgGTP), as was that by MgGDP[S] and MgGTP[S]. Whereas membrane guanylate cyclases usually show positively co-operative kinetics with respect to the substrate, STa-stimulated activity exhibited Michaelis–Menten kinetics with respect to MgGTP. This changed to positive co-operativity when Lubrol PX was the activator, or when the substrate was MnGTP. These results suggest the presence of both a regulatory and a catalytic nucleotide-binding site, which do not interact co-operatively with STa activation.

INTRODUCTION

The heat-stable enterotoxin, STa, is secreted by pathogenic strains of *Escherichia coli*, and produces secretory diarrhoea in man and animals (Greenberg & Guerrant, 1981). There are two *E. coli* STas, with great structural similarity, one containing 18 and the other 19 amino acids (Chan & Giannella, 1981; Aimoto *et al.*, 1982). Our work is with the first, which is also called STp since it originated from a porcine strain of enterotoxigenic *E. coli*. There is an STa receptor located in the brush border of the intestinal epithelium, which is also the location of the larger part of the enterocyte guanylate cyclase (de Jonge, 1975a). STa activates this membranous guanylate cyclase, but not other soluble or particulate guanylate cyclases from placental mammals (Field *et al.*, 1978; Guerrant *et al.*, 1980; Rao *et al.*, 1980; Dreyfus *et al.*, 1984). Rao & Field (1984) and de Jonge (1984) have reviewed the evidence supporting this activation as the single initial event resulting in intestinal ion secretion.

In our preliminary attempts to better characterize STa activation (ElDeib *et al.*, 1986), we were aided by the discovery that mucosa from porcine jejunum contained Mg²⁺-dependent guanylate cyclase that could be stimulated 30–50-fold by STa, as compared with 5–10-fold in rats (Field *et al.*, 1978; Guerrant *et al.*, 1980; Rao *et al.*, 1980). The maximal STa-stimulated activity of purified brush border membrane vesicles was from 2–3 orders of magnitude greater than the response in rodent intestinal mucosa (Field *et al.*, 1978; Guerrant *et al.*, 1980; Rao *et al.*, 1980; Dreyfus *et al.*, 1984). The only other particulate mammalian guanylate cyclase of comparable activity is that in retinal rod outer segments (Bensinger *et al.*, 1974). The porcine system

was therefore capable of providing relatively unequivocal results. From studies on the effects on enzyme activity of dimethyl sulphoxide, haemin and aromatic disulphides, we postulated the existence of a tonic inhibitor of activity, and concluded that STa activation may involve a de-repression (ElDeib *et al.*, 1986, 1987, 1991).

The demonstration that ATP enhanced stimulation of guanylate cyclase by atrial natriuretic peptide (ANP) in membranes from various rat tissues (Kurose *et al.*, 1987) caused us to perform similar studies with the porcine brush border enzyme. However, the results we obtained were opposite to the effects observed with the ANP-stimulated system, i.e. adenine nucleotides and their derivatives only inhibited STa-stimulated activity. There were similarities between the two in that ATP inhibited ¹²⁵I-STa binding to brush border membranes, just as ¹²⁵I-ANP binding to its receptor was also inhibited (DeLean, 1986; Cole *et al.*, 1989). When we extended our studies by the use of certain guanine nucleotide analogues, we were able to demonstrate stimulatory effects on ¹²⁵I-STa binding, although their effects on STa-stimulated guanylate cyclase were always inhibitory. These results suggest that STa-stimulated guanylate cyclase may be a distinct form of membrane guanylate cyclase (Garbers, 1989). A portion of these results have appeared in abstract form (Katwa *et al.*, 1990).

MATERIALS AND METHODS

Brush border membrane vesicles

Male pigs (4 weeks old) were anaesthetized with CO₂ and exsanguinated. The entire jejunum was removed, everted and the

Abbreviations used: ANP, atrial natriuretic peptide; DTT, dithiothreitol; Gpp[NH]p, guanosine 5'-[β -imido]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; ADP[S], adenosine 5'-[β -thio]diphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; App[NH]p, adenosine 5'-[β -imido]triphosphate; App[CH₂]p, adenosine 5'-[β -methylene]triphosphate; PCMBS, *p*-chloromercuribenzenesulphonate; cGMP, cyclic GMP.

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segments were washed in cold 1.8% NaCl in a cold room. We have described previously the procedure used for the isolation of brush border membranes, their storage in liquid N₂, and their washing before use (Katwa *et al.*, 1991). Protein was determined by a bicinchoninic acid method (Kaushal & Barnes, 1986), using BSA as standard.

Adrenal gland membranes

The adrenals were removed from a pig at the same time as the jejunum, and homogenized with a Willems Polytron equipped with a PT 20ST generator in the same homogenization medium as used for intestinal cells (Katwa *et al.*, 1991). The homogenate was centrifuged at 1085 *g* for 10 min, and the resulting supernatant suspension was centrifuged at 171 000 *g* for 50 min. This pellet was washed twice with the same medium, and finally stored in liquid N₂ (Katwa *et al.*, 1991). The membranes were washed with ice-cold water before use.

Guanylate cyclase determination

The reaction mixture contained 50 mM-Tris (pH 7.6 with HCl), 0.6 mM-[α -³²P]GTP (approx. 1 μ Ci), 3.6 mM-MgCl₂ (or 0.9 mM-MnCl₂), 10 mM-NaN₃, 10 mM-phosphocreatine, 10 units of creatine kinase, 0.2 mM-EGTA, approx. 5 μ g of membrane protein and, when present, 15 ng of STa (1.014×10^{-7} M) or 0.2% Lubrol PX, in a final volume of 75 μ l. When the substrate was varied, it was always as the Mg²⁺ or Mn²⁺ chelate (1:1), and the same was true when other nucleotides were added to the reaction. Excess metal was maintained at 3 mM in the case of Mg²⁺ and 0.3 mM in the case of Mn²⁺ (Mg²⁺-dependent activity is assumed unless otherwise noted). Reactions were initiated by adding cold enzyme to the reaction mixture, prewarmed to 30 °C, and were terminated (after 5 or 10 min) by adding 0.15 ml of 1 M-HClO₄. The cyclic [³²P]GMP formed was purified by sequential chromatography on Dowex 50 and alumina columns (White & Karr, 1978) and counted for radioactivity. Determinations were in triplicate and are presented as means $\pm$ S.D. The Figures have bars representing the S.D., except where this was smaller than the symbol, usually a representative experiment is presented. Kinetic analysis was performed with the computer program Enzfitter, 1987, by R. J. Leatherbarrow, published by Elsevier Science Publishers BV, Amsterdam.

¹²⁵I-STa binding

The procedures for radioiodination of STa and for purification of ¹²⁵I-[Tyr⁴]STa have been described (Thompson *et al.*, 1985). Binding was performed in polypropylene tubes, in 0.1 ml of a mixture consisting of 50 mM-Tris/HCl, pH 7.6, 0.1 mM-EDTA, 0.1% bacitracin, 10 mM-NaN₃, 9 mM-MgCl₂ and approx. 160 pM-¹²⁵I-STa. Non-specific (non-saturable) binding was determined in the presence of 0.3 μ M unlabelled STa. Incubation was always at 30 °C for 30 min in the studies on nucleotide regulation, and for 120 min when steady-state binding was desired. Separation of membrane-bound from free ¹²⁵I-STa, and determination of the bound radioactivity, was as described (Katwa *et al.*, 1991). Determinations of both total binding and non-specific binding were in triplicate, and the specific binding was calculated from the difference. Presented are the means $\pm$ S.D., usually from a representative experiment. Binding data were evaluated by computer-assisted non-linear regression analysis (GraphPAD, Version 2.0, 1987, by H. J. Motulsky, ISI Software, Institute for Scientific Information, Philadelphia, PA, U.S.A.).

Pretreatment of membranes with *p*-chloromercuribenzenesulphonate (PCMBS)

Washed brush border membranes were resuspended in 50 mM-Tris/HCl, pH 7.6, containing the appropriate concentration of

PCMBS. The control membranes were resuspended in buffer alone. The suspensions were placed in ice for 30 min, after which they were diluted 6–10-fold with Tris/HCl buffer and centrifuged at 171 360 *g* for 20 min. The resulting pellets were washed twice with Tris/HCl buffer, and finally resuspended in water. When it was desired to reverse the effects of PCMBS treatment by reduction, the treatment period in ice was terminated by the addition of dithiothreitol (DTT) to a final concentration of 3 mM. After a further 10 min in ice, the suspension was diluted with Tris/HCl, and the membranes were washed by centrifugation as before.

ADP-ribosylation catalysed by pertussis toxin

Pertussis toxin was activated by incubating in 25 mM-Tris/HCl containing 25 mM-DTT and 5 mM-EDTA at 30 °C for 30 min, after which the solution was placed in ice. Aliquots (80 μ l) of this solution, containing 4 μ g of pertussis toxin, were added to reaction mixtures containing 2.5 mM-NAD⁺, 20 mM-thymidine, 2.5 mM-ATP, 2 mM-GTP, 40 mM-Tris/HCl, pH 7.6, and approx. 240 μ g of membrane protein in a final volume of 0.4 ml. One control omitted pertussis toxin, and another control omitted both NAD⁺ and pertussis toxin. The reactions were incubated with shaking at 30 °C for 20 min, after which 40 μ l of 25 mM-NAD⁺ was added to the reactions containing NAD⁺ (the control received water), and the incubation was continued for another 20 min. Each tube was diluted with 7 ml of ice-cold 5 mM-Hepes, pH 7.6, centrifuged as before, and the pellets resuspended in the same buffer.

Materials

STa was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., as were undesignated biochemicals. Purified Lubrol PX was from Pierce, Rockford, IL, U.S.A., and DTT was from Research Organics, Cleveland, OH, U.S.A. GTP was purchased from Pharmacia, Piscataway, NJ, U.S.A., and the other nucleotides were from either Sigma or Boehringer Mannheim, Indianapolis, IN, U.S.A. ANP was the rat synthetic 28-amino-acid peptide (Sigma A 2288). Na¹²⁵I (carrier-free, 350–600 mCi/ml) was from Amersham Corporation, Arlington Heights, IL, U.S.A., and [α -³²P]GTP (> 600 Ci/mM) was from Du Pont-NEN, Boston, MA, U.S.A.

RESULTS

Nucleotide regulation of ¹²⁵I-STa binding

Since brush border membranes contain high nucleoside triphosphatase and nucleoside triphosphate pyrophosphorylase activities (ElDeib *et al.*, 1986), we had to optimize the conditions in order to obtain reproducible effects of nucleotides on binding. We found maximal effects using a 30 min incubation period at 30 °C, in the presence of 10 mM-azide to inhibit nucleotide hydrolysis (ElDeib *et al.*, 1986). Although the absolute response of each membrane preparation to a certain nucleotide varied, the relative responses of different preparations to the different nucleotides were similar. Table 1 contains the results from a single experiment, where nearly all of the nucleotides studies were used, as a way of summarizing these comparative effects. The most obvious generalization one can make is that adenine nucleotides inhibited binding while guanine nucleotides were stimulatory. Furthermore, the inhibition of binding by the adenine nucleotides was not dependent on the presence of Mg²⁺, while the stimulatory effect of the guanine nucleotides was Mg²⁺-dependent. Finally, the dinucleotides were of equal or greater potency compared with the trinucleotides. However, there were discrepancies that may have been due to rapid breakdown of a

Table 1. Nucleotide regulation of ^{125}I -STa binding

Binding to brush border membranes was determined in the absence and the presence of 9 mM-MgCl₂. Control binding (without nucleotide) was 93.8 fmol/mg of protein without MgCl₂ and 148.9 fmol/mg of protein in its presence. All nucleotides were at 1 mM. Mean values are given; S.D. values were less than 5% of the mean. Minus values indicate inhibition of binding.

Nucleotide present	Change in ^{125}I -STa binding (%)	
	- MgCl ₂	+ MgCl ₂
ATP	-22.1	-29.9
App[NH]p	-32.8	-36.3
App[CH ₂]p	-25.5	-23.7
ATP[S]	-34.0	-18.8
ADP	-32.4	-28.0
ADP[S]	-40.6	-27.7
AMP	-2.7	-7.0
Cyclic AMP	-20.0	-22.3
Adenosine	+0.4	+1.3
GTP	+4.4	-4.3
Gpp[NH]p	-7.8	+9.8
GTP[S]	-8.5	+29.9
GDP	0.0	+4.2
GDP[S]	-6.0	+41.2
GMP	+0.2	-5.5
cGMP	+1.2	-4.6
Guanosine	-3.2	-1.5

Fig. 1. Concentration-dependent effects of some adenine and guanine nucleotide analogues on ^{125}I -STa binding

○, App[NH]p; ●, ADP[S]; ▲, GTP[S]; △, GDP[S].

particular compound. For example, GDP was relatively ineffective in stimulating binding, while guanosine 5-[β -thio]diphosphate (GDP[S]) was the most effective compound. Presumably this was due to rapid hydrolysis of GDP by nucleoside diphosphatase (EC 3.6.1.6) (Plaut, 1963) or by ATP diphosphohydrolase (EC 3.6.1.5) (Knowles *et al.*, 1983), to both of which GDP[S] is resistant (Eckstein *et al.*, 1979). In contrast, ADP was an effective inhibitor of binding in both the presence and the absence of Mg²⁺, perhaps because of the strong inhibition of its hydrolysis by the presence of 10 mM-NaN₃ (Knowles *et al.*, 1983).

As previously reported (Kuno *et al.*, 1986b), low concentrations

Fig. 2. Competitive inhibition by unlabelled STa of the binding of ^{125}I -STa to membranes, in the absence (○) and in the presence (●) of 1 mM-App[NH]p

Incubation was for 2 h in the absence of MgCl₂. Control is ^{125}I -STa bound in the absence of unlabelled STa.

Fig. 3. Effect of membrane treatment with PCMBs on App[NH]p inhibition of ^{125}I -STa binding

The membranes were treated with the indicated concentrations of PCMBs, after which half the suspension was reduced with 3 mM-DTT (△, ▲) as described in the Materials and methods section. ^{125}I -STa binding was determined in the absence (○, △) and the presence (●, ▲) of 0.5 mM-App[NH]p.

of Mg²⁺ increased ^{125}I -STa binding, and in our system did not increase non-specific binding. In experiments to determine the optimum Mg²⁺ concentration with which to study effects of nucleotides on binding, we varied [Mg²⁺] in the presence of 1 mM-ATP, App[NH]p, App[CH₂]p or GDP[S] (results not shown). While binding progressively increased with higher [Mg²⁺], plateauing near 24 mM, the largest percentage change in binding induced by the presence of a nucleotide occurred at lower [Mg²⁺] (3–9 mM). We chose 9 mM as a compromise between the two effects. The effect of 9 mM-Mg²⁺ on specific binding was evaluated in a competition binding experiment with unlabelled STa (results not shown), using a 2 h incubation. The EC₅₀ in the presence of Mg²⁺ was 2.1 nM, compared with 3.6 nM in its absence. There was little change in B_{max} , which was 5.9 and 5.6 pmol/mg of protein with and without Mg²⁺ respectively. The effect of Mg²⁺ was therefore to increase the affinity of the receptor for ^{125}I -STa, without a change in the receptor number.

Fig. 1 compares the concentration-dependent effects on binding of four of the most effective nucleotides. Since Mg²⁺ had to be present to demonstrate guanine nucleotide stimulation,

Table 2. Effect of PCMBS treatment on the regulation of ^{125}I -STa binding by App[NH]p and MgCl_2

Brush border membranes were treated with 1 mM-PCMBS in 50 mM-Tris/HCl, pH 7.6, or with Tris/HCl alone, as described in the Materials and methods section.

Present in binding mixture		^{125}I STa bound (fmol/mg of protein)	
9 mM- MgCl_2	1 mM-App[NH]p	Control membranes	PCMBS-treated membranes
—	—	122.3 $\pm$ 2.3	132.7 $\pm$ 2.2
—	+	81.2 $\pm$ 0.8	137.2 $\pm$ 5.8
+	—	194.0 $\pm$ 3.0	253.5 $\pm$ 7.0
+	+	129.4 $\pm$ 1.2	246.1 $\pm$ 4.6

Table 3. Effect of PCMBS treatment on the regulation of ^{125}I -STa binding by App[NH]p and GDP[S]

Brush border membranes were treated with 0.5 mM-PCMBS in 50 mM-Tris/HCl, pH 7.6, or with Tris/HCl alone, as described in the Materials and methods section. The nucleotides were each present at 1 mM. This experiment was performed three times, with the same membrane preparation; each determination was in triplicate. The value shown represents the mean $\pm$ S.D. of the nine determinations; a minus value indicates inhibition.

Membrane treatment	Nucleotide added	^{125}I -STa bound (fmol/mg of protein)	Change in binding (%)
Control	None	183.5 $\pm$ 3.3	—
	App[NH]p	120.7 $\pm$ 5.8	—33.9 $\pm$ 3.1
	GDP[S]	215.0 $\pm$ 4.4	+17.8 $\pm$ 2.4
	App[NH]p + GDP[S]	170.2 $\pm$ 5.3	—6.7 $\pm$ 2.9
With PCMBS	None	225.0 $\pm$ 11.1	+22.6 $\pm$ 6.1
	App[NH]p	218.6 $\pm$ 5.9	+19.1 $\pm$ 3.2
	GDP[S]	250.1 $\pm$ 6.2	+36.3 $\pm$ 3.4
	App[NH]p + GDP[S]	230.4 $\pm$ 10.3	+25.5 $\pm$ 5.6

ADP[S] was less potent than App[NH]p (compare with Table 1). The EC_{50} for App[NH]p was 70 μM , and for the other nucleotides it was approx. 120 μM . We chose App[NH]p as the inhibitory nucleotide in most of our studies, and GDP[S] as the stimulatory nucleotide.

Fig. 2 shows results from one of several competition binding experiments, in which App[NH]p induced a shift of the STa competition curve to the right. The EC_{50} increased from 1.8 nM in the absence of App[NH]p to 2.7 nM in its presence. Conversely, App[NH]p induced a decrease in the B_{max} from 3.5 to 2.5 pmol/mg of protein. Thus App[NH]p appeared to decrease both the affinity of the receptor for ^{125}I -STa and the number of receptors. The experiment was performed in the absence of Mg^{2+} ; in its presence, App[NH]p induced similar but less marked effects. Because of the relative instability of GDP[S] in long incubations containing Mg^{2+} , competition binding experiments in the presence of this nucleotide were limited to 30 min rather than the 2 h required to achieve a steady state. Under these conditions, GDP[S] induced a small shift to the left, decreasing

Fig. 4. Concentration-dependent effects of App[NH]p on Mg^{2+} -dependent (a) and Mn^{2+} -dependent (b) guanylate cyclase activity

Activity was determined in the presence of no activator (○), STa (●) or Lubrol PX (▲), and the indicated concentration of either MgApp[NH]p (a) or MnApp[NH]p (b).

Fig. 5. Comparison of the inhibitory effects of adenine and guanine nucleotides on STa-stimulated guanylate cyclase activity

In (a), activity was determined in the presence of the indicated concentrations of MgATP (○), MgApp[NH]p (△), MgATP[S] (□), MgGTP[S] (●) and MgGDP[S] (▲). (b) is a Dixon plot of the data in (a). In (b), the left y axis refers to the open symbols and right y axis to the filled symbols.

Fig. 6. Lineweaver-Burk plots showing effects of substrate concentration on STA-stimulated (a, b, c) and Lubrol PX-stimulated (d) guanylate cyclase activity

(a) Activity determined in the presence of 0 μM - (○), 50 μM - (●), 200 μM - (□) and 400 μM - (■) App [NH]p. (b) activity determined in the presence of 0 μM - (○), 50 μM - (●), 100 μM - (□) and 200 μM - (■) GDP[S]. (c) Activity determined in the presence of 0 μM - (○), 100 μM - (●), 200 μM - (□) and 400 μM - (■) GTP[S]. (d) Activity determined in the presence of 0 μM - (○), 10 μM - (●), 50 μM - (□) and 200 μM - (■) App[NH]p.

the EC_{50} from 1.9 to 1.4 nM, with no change in B_{max} . (results not shown).

Effects of thiol-reactive reagents on ^{125}I -STa binding and regulation by nucleotide

Fig. 3 shows that pretreating membranes with increasing concentrations of PCMBs resulted in a concentration-dependent lifting of App[NH]p inhibition. The effects of PCMBs were reversed by reduction with DTT (Fig. 3). The results in Table 2 demonstrate that PCMBs was a useful tool in elucidating the regulation of ^{125}I -STa binding. The data show that PCMBs treatment prevented inhibition by App[NH]p in both the presence and the absence of Mg^{2+} . Furthermore, App[NH]p exerted the same percentage inhibition of control membrane binding in the absence of Mg^{2+} (33.6%) as in its presence (33.3%). Thus App[NH]p inhibited binding independently of Mg^{2+} .

Table 2 also shows that PCMBs treatment stimulated ^{125}I -STa binding; however, this stimulation was largely Mg^{2+} -dependent. This is demonstrated by the fact that Mg^{2+} , in the absence of App[NH]p, stimulated binding by 58.6% before PCMBs treatment and by 91.0% afterwards. Since PCMBs only stimulated binding by 8.5% in the absence of Mg^{2+} (and App[NH]p), we conclude that its primary effect was to enhance the stimulatory effect of Mg^{2+} . The results in Table 3 show that, while PCMBs treatment prevented App[NH]p inhibition of ^{125}I -STa binding, it did not prevent stimulation by GDP[S], although this stimulation (11.2%) was decreased from the 17.8% stimulation induced by

GDP[S] with control membranes. These results also show that, while App[NH]p and GDP[S] appear to have largely separate effects on binding, there may be some interaction of the nucleotides at their effective sites. With control membranes, the simultaneous presence of both nucleotides did not result in the expected difference (−16.1%) between the stimulatory (+17.8%) and inhibitory (−33.9%) effects, but only a value of −6.7%. This suggests competition between the nucleotides at the effective sites. Also, with PCMBs-treated membranes, where the inhibitory effect of App[NH]p was eliminated, GDP[S] increased binding by 11.2%, as previously mentioned, while in the presence of both nucleotides binding was increased by only 2.9%. We conclude that the sites operative in regulating ^{125}I -STa binding are not absolutely specific, at least at the nucleotide concentrations used here.

Nucleotide regulation of guanylate cyclase activity

Fig. 4 depicts concentration-dependent inhibition of guanylate cyclase by App[NH]p, showing Mg^{2+} -dependent activity in Fig. 4(a) and Mn^{2+} -dependent activity in Fig. 4(b). In Fig. 4(a), STA-stimulated activity was inhibited in two phases: a strong inhibition by low concentrations of Mg App[NH]p which changed to a less sensitive response near 0.1 mM. In contrast, when activity was stimulated by Lubrol PX, the first phase was much weaker and the second phase nearly absent, although the two curves were parallel above 0.6 mM. There was no first-phase inhibition of STA-stimulated Mn^{2+} -dependent activity by

Table 4. Comparison of adenine nucleotide regulation of guanylate cyclase activation by STa and ANP

Adrenal membranes were prepared as described in the Materials and methods section, and 16 g of membrane protein was used in the standard reaction mixture (see the Materials and methods section), except for the substitution of 1 μ M-ANP for STa. The activity of brush border membranes was determined at the same time. Nucleotides (as Mg^{2+} chelates) were present at 1 mM.

Membranes	Activator	Nucleotide added	Activity (pmol of cGMP/min per mg of protein)
Brush border	None	None	48.2 $\pm$ 2.5
	None	ATP	9.9 $\pm$ 0.9
	None	App[NH]p	18.7 $\pm$ 8.1
	None	ATP[S]	7.7 $\pm$ 0.9
	STa	None	3966.7 $\pm$ 20.1
	STa	ATP	2664.3 $\pm$ 82.6
	STa	App[NH]p	2142.3 $\pm$ 76.0
	STa	ATP[S]	1554.0 $\pm$ 21.1
Adrenal	None	None	14.3 $\pm$ 1.1
	None	ATP	28.1 $\pm$ 0.2
	None	App[NH]p	22.5 $\pm$ 0.2
	None	ATP[S]	31.7 $\pm$ 0.7
	ANP	None	18.6 $\pm$ 1.4
	ANP	ATP	73.2 $\pm$ 1.1
	ANP	App[NH]p	47.0 $\pm$ 0.7
	ANP	ATP[S]	75.7 $\pm$ 4.2

MnApp[NH]p (Fig. 4b), although there was a small first-phase inhibition of both basal and Lubrol-stimulated activity.

Fig. 5(a) compares the concentration-dependent inhibition of STa-stimulated guanylate cyclase by MgATP, MgApp[NH]p, MgATP[S], MgGTP[S] and MgGDP[S]. (The effects on basal activity are not shown, since with these co-ordinates all of the curves approximate to the *x*-axis.) All adenine nucleotides gave two-phase inhibition curves, while the guanine nucleotides produced a single-phase response. This is obvious when the data from this experiment were presented in a Dixon plot (Fig. 5b). We investigated the type of inhibition of STa-stimulated activity by MgApp[NH]p, MgGDP[S] and MgGTP[S] (Figs. 6a, 6b and 6c). When the initial reaction velocities were determined with various levels of MgGTP, the reciprocal plots were linear, with intersections on the ordinate. When these data were fitted to Hill plots, parallel lines were observed with Hill coefficients of 1.0. The diagrams shown in Fig. 6 are examples of multiple experiments performed with each of these nucleotides: MgApp[NH]p, *n* = 13; MgGTP[S], *n* = 6; MgGDP[S], *n* = 5. Similar results were obtained with MgATP (not shown, *n* = 2). The data indicated that the enzyme obeyed classical Michaelis–Menten kinetics with respect to MgGTP, and suggested competitive inhibition by all of the nucleotides. The mean uninhibited apparent K_m was 0.242 $\pm$ 0.120 mM.

Since the results obtained with adenine nucleotides (Fig. 5) differed from stimulatory effects previously observed with ANP-stimulated guanylate cyclase (Kurose *et al.*, 1987), we performed positive control experiments with an ANP-stimulated guanylate cyclase in pig adrenal membranes (Trembley *et al.*, 1985). Table 4 shows results from one such experiment, in which the effects of ATP, App[NH]p and ATP[S] on STa-stimulated activity in brush border membranes and on ANP-stimulated activity in adrenal membranes were directly compared under the same assay conditions. The results show that these nucleotides induced a 2.5–4.1-fold increase in ANP-stimulated activity at the same time as they consistently inhibited STa-stimulated activity.

Since particulate guanylate cyclases typically show positive co-operativity with respect to the substrate (Goldberg & Haddox, 1977), rather than the Michaelis–Menten kinetics we found (Figs. 6a, 6b and 6c), we performed a few experiments using different assay conditions. Fig. 6(d) shows reciprocal plots with Lubrol PX rather than STa as activator, again using MgApp[NH]p as inhibitor. The reciprocal plots were not linear, and the average of the Hill coefficients was 1.35. When we used MnGTP as substrate, we confirmed previous observations on intestinal brush border membranes, in which positive co-operativity with respect to substrate was demonstrated with Mn^{2+} -dependent activity and no other activators (de Jonge, 1975b). We found this also to be true in the presence of STa or Lubrol PX.

Effects of ADP-ribosylation catalysed by pertussis toxin

We used conditions that we found to be effective in labelling pertussis toxin substrates in brush border membranes with [α - 32 P]ADP-ribose, using [α - 32 P]NAD⁺ as donor (Katwa *et al.*, 1991). However, in these experiments we used a high concentration of unlabelled NAD⁺, and replenished it half way through the incubation. These membranes were then used for 125 I-STa binding, and there was little or no effect of presumed ADP-ribosylation on binding, on inhibition of binding by 1 mM-App[NH]p or on stimulation of binding by 1 mM-GDP[S]. Likewise, when these pretreated membranes were assayed for guanylate cyclase activity, there was no effect on basal or STa-stimulated activity, nor on inhibition of stimulated activity by 0.1 mM-MgApp[NH]p and 0.1 mM-MgGDP[S]. Both 125 I-STa binding and cyclase activity were determined after three separate ADP-ribosylations. The lack of effect of pertussis toxin-catalysed ADP-ribosylation on STa stimulation of guanylate cyclase was previously reported (Crane *et al.*, 1989). Our results suggest that a pertussis toxin substrate is not involved in nucleotide regulation of 125 I-STa binding or cyclase activity.

DISCUSSION

There is previous evidence that mammalian particulate guanylate cyclase contains an allosteric ATP-binding site (Schulz *et al.*, 1989), and ATP has been shown to regulate both the binding of 125 I-ANP to the ANP receptor/guanylate cyclase and the stimulation of activity. In bovine adrenal zona glomerulosa membranes (DeLean, 1986), ATP induced a concentration-dependent inhibition of 125 I-ANP binding, with an IC_{50} of 50 μ M and a maximum decrease of 30–50 % at 1 mM. While no effect of ATP was shown on the binding of 125 I-ANP to rat liver plasma membranes (Kurose & Ui, 1985), ATP did stimulate Mg^{2+} -dependent but not Mn^{2+} -dependent guanylate cyclase activity in these and other rat tissue membranes. ANP-stimulated activity was more markedly increased than was basal activity. Since non-hydrolysable ATP analogues also stimulated activity, as did ADP and ADP[S], it was concluded that phosphorylation by ATP was not involved, but that an allosteric ATP-binding site facilitated ANP-induced activation. ATP inhibited 125 I-ANP binding to rat glomerular membranes, but ATP and non-hydrolysable analogues only inhibited both basal and ANP-stimulated guanylate cyclase (Cole *et al.*, 1989). However, since this activity was determined in the presence of both Mg^{2+} and Mn^{2+} , the Mn^{2+} may have been responsible for the lack of stimulation by ATP (Gerzer *et al.*, 1981). Finally, App[NH]p, ATP and ATP[S], with increasing potency, activated Mg^{2+} -dependent basal activity and potentiated ANP activation of guanylate cyclase in rat lung membranes (Chang *et al.*, 1990). Since activation by an adenine nucleotide and ANP together was greater than the sum of the individual activations, it was suggested

that ATP was physiologically required for ANP activation. These workers did not perform studies on ^{125}I -ANP binding.

Both the sea-urchin-sperm and the ANP-receptor guanylate cyclases have protein kinase domains in the intracellular portion of the transmembrane protein, just inside the plasma membrane, which are suggested to contain the ATP-binding sites (Garbers, 1989; Schulz *et al.*, 1989). Chinkers & Garbers (1989) showed that this domain functions as a regulatory element in the ANP receptor/cyclase. When the kinase-like domain was removed by deletion mutagenesis, the resulting ANP receptor retained guanylate cyclase activity, but this activity was independent of ANP, and its stimulation by ANP was markedly decreased. This group also showed that one of the ANP receptor/cyclases, GC-A, could be expressed in insect cells using a recombinant baculovirus, and the expressed protein retained its ability to bind ^{125}I -ANP and to function as an ANP-activated guanylate cyclase (Chinkers *et al.*, 1991). Optimal ANP activation of GC-A was dependent upon the presence of millimolar concentrations of ATP or non-hydrolysable analogues, which were suggested to bind to the protein kinase-like domain. Finally, Shultz *et al.* (1990) described a new plasma membrane form of guanylate cyclase which was encoded by a clone (GC-C) derived from a rat small intestine cDNA library. Expression of the protein in COS-7 cells resulted in high guanylate cyclase activity, binding of ^{125}I -STa and STa-stimulated accumulation of cGMP in the transfected cells. GC-C encoded a protein containing an extracellular amino acid sequence different from that of previously cloned guanylate cyclases; however, the protein contained the intracellular protein kinase-like domain and the cyclase catalytic domain. These workers concluded that this guanylate cyclase serves as a cell surface receptor for STa, and therefore resembles other members of this enzyme/receptor family (Garbers, 1989). The cDNA for the human receptor for STa has also been cloned and expressed, with similar results (de Sauvage *et al.*, 1991).

ADP[S] was the most effective of the adenine nucleotides as an inhibitor of ^{125}I -STa binding in the absence of Mg^{2+} (Table 1). This suggested that inhibition might be mediated by a purinergic receptor, since ADP[S] was a particularly potent stimulator of phospholipase C in turkey erythrocytes (Boyer *et al.*, 1989), and since enterocytes have been shown to have an ATP receptor (Korman *et al.*, 1982), and a colonic epithelial cell has an adenosine receptor on both apical and basolateral aspects of cell monolayers (Barrett *et al.*, 1990). The erythrocyte receptor was suggested to be a P_{2y} -purinergic receptor subtype, since the order of potency of a series of ATP and ADP analogues was: 2-methylthioadenosine 5'-triphosphate > ADP[S] > ATP[S] > ATP > App[NH]p ~ ADP (Burnstock & Kennedy, 1985). However, we did not find a similar order of potency with respect to inhibition of ^{125}I -STa binding, since 2-methylthioadenosine 5'-triphosphate exerted a very weak concentration-dependent inhibition of ^{125}I -STa binding when compared with App[NH]p (results not shown). Furthermore, the inhibition by cyclic AMP (Table 1), whether or not Mg^{2+} was present, did not resemble other purinergic receptors (Gordon, 1986). Since AMP and adenosine had little or no effect, it does not appear that the effect of cyclic AMP resulted from a breakdown product. Our results showed some similarity with recent studies on the purinergic inhibition of ANP-induced cyclic GMP (cGMP) accumulation in FRTL-5 thyroid cells (Okajima & Kondo, 1990). These workers suggested that two mechanisms were involved in the action of the different agonists: (1) stimulation of cGMP hydrolysis via a phospholipase C/ C^{2+} -phosphodiesterase system, and (2) inhibition of cGMP generation, probably by a pertussis toxin-sensitive G-protein-mediated inactivation of guanylate cyclase. However, we found no effect on inhibition of STa-stimulated guanylate cyclase by 0.1 mM-MgApp[NH]p if the EGTA con-

centration was increased from 0.2 to 2.2 mM, or by the inclusion of 5 mM-cGMP or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at 0.1 or 1.0 mM (results not shown). We also showed that pertussis toxin-catalysed ADP-ribosylation did not affect nucleotide regulation of ^{125}I -STa binding and STa-stimulated guanylate cyclase. Thus neither of the above mechanisms appear to be involved in this regulation.

Our results on ^{125}I -STa binding resemble some of those obtained with ^{125}I -ANP and its receptor (DeLean, 1986; Cole *et al.*, 1989), in that adenine nucleotides and their analogues were inhibitory in the same concentration range; however, these nucleotides did not stimulate brush border membrane guanylate cyclase activity or potentiated STa activation (Figs. 4, 5 and 6; Table 4). A model for the effects we have observed might have ^{125}I -STa binding, as stimulated by Mg^{2+} and inhibited by an App[NH]p-binding domain, occurring either in a separate protein or in the protein kinase-like domain in the receptor/cyclase. The inhibition by the App[NH]p-binding domain was enhanced on binding App[NH]p, and removed by reaction with PCMBs. This suggests that a free thiol is present at the App[NH]p-binding site. Since stimulation of binding by Mg^{2+} was independent of App[NH]p, and stimulation by PCMBs was largely Mg^{2+} -dependent (Table 2), PCMBs appeared to stimulate binding by removing the tonic inhibition exerted by the App[NH]p-binding domain.

The stimulation of ^{125}I -STa binding by certain guanine nucleotide analogues in the presence of Mg^{2+} (Table 1, Fig. 1), which apparently resulted from an increased affinity of the STa receptor (Fig. 2), was opposite to other known effects of guanine nucleotides. Their addition usually decreases the apparent affinity of G-protein-mediated receptors for agonists, presumably by dissociating G-proteins from receptors (Birnbauer *et al.*, 1985). A possible role in STa activation of guanylate cyclase, for one of the G-proteins that can be ADP-ribosylated by pertussis toxin, has been disproved (Crane *et al.*, 1989). However, there is a similarity between the STa receptor and G-protein-mediated receptors, in that GDP analogues were more potent than GTP analogues (Birnbauer *et al.*, 1985). Weikel *et al.* (1990) showed that a protein kinase C activator, phorbol dibutyrate, acted synergistically with STa to elevate cGMP in intact T84 cells. They proposed 'cross-talk' between the two second messenger systems. It is therefore quite possible that a G-protein does modify STa binding to its receptor. de Jonge *et al.* (1989) have reported the enrichment of several G-proteins in the apical membranes of enterocytes in spite of the apparent lack of G-protein-coupled hormone receptors on this aspect of the cells.

It has been a common observation with particulate guanylate cyclases that they display positively co-operative kinetic patterns with respect to substrate, usually MnGTP, while the cytosolic enzyme exhibited classical Michaelis-Menten kinetics (Kimura & Murad, 1974; Garbers *et al.*, 1974, 1975; Chrisman *et al.*, 1975). Hill coefficients were in the range 1.40–1.74, and two or more interactive substrate sites were proposed (Kimura & Murad, 1974; Garbers *et al.*, 1974, 1975; Chrisman *et al.*, 1975). Both the ANP-stimulated particulate guanylate cyclase from rat lung (Kuno *et al.*, 1986a) and the sea-urchin-sperm guanylate cyclase (Garbers, 1976) shifted to Michaelis-Menten kinetics on purification. Similar positive co-operative kinetics with MnGTP were previously found with rat intestinal brush border membranes (de Jonge, 1975b). Although we also found positive co-operativity with pig intestinal membranes when MnGTP was the substrate, or with MgGTP as substrate when Lubrol PX was the activator (Fig. 6d), we found Michaelis-Menten kinetics with respect to MgGTP when STa was the activator (Figs. 6a, 6b and 6c). Since Mn^{2+} is an apparent activator of the brush border enzyme (Fig. 4), positive co-operativity in brush border mem-

branes appears to depend on the activator. This is different from the ANP-stimulated enzyme, where Hill coefficients of about 2.0 were found with both Mn^{2+} -dependent and Mg^{2+} -dependent activity (Waldman *et al.*, 1989). Because of the extremely low Mg^{2+} -dependent activity of unstimulated brush border guanylate cyclase, we did not attempt kinetic studies. We therefore did not know if the basal activity also exhibits Michaelis–Menten kinetics, or if STa activation is associated with a change in kinetic behaviour.

The biphasic inhibition of STa-stimulated guanylate cyclase by MgApp[NH]p (Fig. 4a) suggests two inhibitory sites, one of high affinity (K_i 25–30 μM) and one of low affinity (K_i 0.4–0.5 mM). The K_i for the low-affinity site is in the same range previously suggested for the catalytic site (Goldberg & Haddox, 1977), and this assumption is supported by the inhibition in the same concentration range of both basal and Lubrol-stimulated Mn^{2+} -dependent activity (Fig. 4b). Since MgApp[NH]p appears to be a purely competitive inhibitor at both high and low concentrations (Fig. 6a), we have to assume that the substrate, MgGTP, can also bind to the high-affinity site. Since varying the substrate concentration resulted in regular Michaelis–Menten kinetics, this suggests either a single catalytic site or two independent sites. If there were two independent sites, MgApp[NH]p might bind to the first site with greater affinity than to the second site, which could result in two-phase inhibition. An alternative model assumes a single catalytic site, and that the high-affinity site is a regulatory and not a catalytic site. By analogy with previous studies on particulate guanylate cyclases (Kimura & Murad, 1974; Garbers *et al.*, 1974, 1975; Chrisman *et al.*, 1975), we assume that the high-affinity site is a regulatory site.

Our model proposes that under conditions of STa activation, the substrate binds first to the regulatory site, and that while this binding is required for the subsequent binding of substrate to the catalytic site, it does not affect the affinity of the catalytic site. The binding of low concentrations of MgApp[NH]p, MgATP, MgATP[S], MgGDP[S] or MgGTP[S] to the regulatory site inhibits activity, which could occur if this interfered with a required binding of substrate to this site. The difference between the adenine nucleotides and the guanine nucleotides would depend upon their inhibition at the catalytic site. MgGTP[S] and MgGDP[S] inhibited activity in a single phase (Fig. 5) and were purely competitive inhibitors (Figs. 6b and 6c), with K_i values of 25–30 μM and 50–100 μM respectively. Since this is much lower than the K_m for the substrate (240 μM), and is in the same range as the first K_i for App[NH]p (25–30 μM), only a single inhibitory phase would be seen. In contrast, the K_i for App[NH]p at the catalytic site is 0.4–0.5 mM, which results in two distinct inhibitory phases. A corollary of the hypothesis is that, in the presence of a different activator (Lubrol PX or Mn^{2+}), there is interaction between the binding sites so that substrate binding to the regulatory site increases the binding affinity of the catalytic site (Fig. 6d) (Kimura & Murad, 1974; Garbers *et al.*, 1974, 1975; Chrisman *et al.*, 1975).

It is attractive to assume that the App[NH]p-binding domain inhibiting ^{125}I -STa binding is the same as the high-affinity binding site inhibiting STa-stimulated guanylate cyclase. However, there is an apparent discrepancy between the relative insensitivity of the receptor to regulation by nucleotides as compared with their effects on enzyme activity (compare Fig. 1 and Fig. 5). While it might be argued that there occurred a more extensive breakdown of the nucleotide during the 30 min incubation in the binding assay as compared with the 5–10 min incubation in the cyclase assay in the presence of GTP, this would imply that the effects of the nucleotides on ^{125}I -STa binding should be more pronounced using shorter incubations. This was not the case, since we found that the percentage change in binding induced by App[NH]p,

GDP[S] or GTP[S] was not greatly different between 10 and 30 min of incubation (results not shown). The concentration of STa used in the cyclase assays (0.1 μM) is well beyond the linear portion of the dose-response curve and approaches a maximal effect. It has to be lowered to 4 nM to halve the activity. Therefore, activity would be relatively unresponsive to inhibitors of STa binding unless relatively large changes were made, and the inhibition of activity that was measured at low inhibitor concentrations probably does not reflect effects on the STa receptor, but only on the enzyme. This argument has a weakness in that there was a 1000-fold difference in ligand concentrations used in the two determinations (the ^{125}I -STa concentration was approx. 0.09 nM). The apparent discrepancy in sensitivity may therefore arise from the different conditions. However, competition binding experiments (Fig. 2) showed that App[NH]p decreased the B_{max} by 29% (see the Results section), which is approximately the same maximum inhibition as occurred in first phase inhibition of cyclase activity (Figs. 4 and 5).

Adenine nucleotide inhibition of ^{125}I -ANP binding also occurred at millimolar concentrations (DeLean, 1986; Cole *et al.*, 1989). Also, optimal ANP activation of GC-A, the expressed ANP receptor/cyclase, was dependent upon the presence of millimolar concentrations of ATP, which was suggested to bind to the protein kinase-like domain (Chinkers *et al.*, 1991). It should be noted, however, that the protein kinase-like domain in the STa receptor/cyclase, GC-C, was only 39% identical with GC-A, and 35% identical with the other ANP receptor/cyclase, GC-B (Schulz *et al.*, 1990). This difference may be the basis for the difference in response to adenine nucleotides between STa receptor/cyclase and ANP/receptor cyclase (Table 4). Our results are in contrast with the recent report on adenine nucleotide activation of basal and STa-stimulated rat intestinal guanylate cyclase (Gazzano *et al.*, 1991). These workers demonstrated an approx. 2.5-fold increase in STa stimulation by 1 mM-ATP[S], which was the most effective of the adenine nucleotides. There may be a species-dependent difference in GC-C between rat and pig, since from comparison of the predicted amino acid sequences of the human and rat STa receptors, de Sauvage *et al.* (1991) showed that the intracellular domain was more highly conserved (91% identity) than the extracellular domain (71% identity). In contrast, the entire predicted sequences of GC-A (92%) and GC-B (98%) are highly conserved between rat and human. The authors therefore suggested that a putative endogenous ligand for the STa receptors may be species-specific.

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REFERENCES

- Aimoto, S., Toshifumi, T., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y. & Miwatani, T. (1982) *Eur. J. Biochem.* **129**, 257–263
- Barrett, K. E., Huott, P. A., Shah, S. S., Dharmathaphorn, K. & Wasserman, S. I. (1990) *Am. J. Physiol.* **256**, C197–C203
- Bensinger, R. E., Fletcher, R. T. & Chader, G. J. (1974) *Science* **183**, 86–87
- Birnbaumer, L., Codina, J., Mattera, R., Cerione, R. A., Hildebrandt, J. D., Sunyer, T., Rojas, F. J., Caron, M. J., Lefkowitz, R. J. & Iyengar, R. (1985) *Recent Prog. Hormone Res.* **41**, 41–99
- Boyer, J. L., Downes, C. P. & Harden, T. K. (1989) *J. Biol. Chem.* **264**, 884–890
- Burnstock, G. & Kennedy, C. (1985) *Gen. Pharmacol.* **16**, 433–440
- Chan, S.-K. & Giannella, R. G. (1981) *J. Biol. Chem.* **256**, 7744–7746
- Chang, C.-H., Kohse, K. P., Chang, B., Hirata, M., Jiang, B., Douglas, J. E. & Murad, F. (1990) *Biochim. Biophys. Acta* **1052**, 159–169
- Chinkers, M. & Garbers, D. L. (1989) *Science* **245**, 1392–1394
- Chinkers, M., Singh, S. & Garbers, D. L. (1991) *J. Biol. Chem.* **266**, 4088–4093
- Chrisman, T. D., Garbers, D. L., Parks, M. A. & Hardman, J. G. (1975) *J. Biol. Chem.* **250**, 374–381

- Cole, F. E., Rondon, I., Iwata, T., Hardee, E. & Frohlich, E. D. (1989) *Life Sci.* **45**, 477–484
- Crane, J. K., Hewlett, E. L. & Weikel, C. S. (1989) *Infect. Immun.* **57**, 1186–1191
- de Jonge, H. R. (1975a) *FEBS Lett.* **53**, 237–242
- de Jonge, H. R. (1975b) *FEBS Lett.* **55**, 143–152
- de Jonge, H. R. (1984) *Biochem. Soc. Trans.* **12**, 180–184
- de Jonge, H. R., van den Berghe, N., Tilly, B. C., Kansen, M. & Bijman, J. (1989) *Biochem. Soc. Trans.* **17**, 816–818
- DeLean, A. (1986) *Life Sci.* **39**, 1109–1116
- de Sauvage, F. J., Camerato, T. R. & Goeddel, D. V. (1991) *J. Biol. Chem.* **266**, 17912–17918
- Dreyfus, L. A., Jaso-Friedmann, L. & Roberston, D. C. (1984) *Infect. Immun.* **44**, 493–501
- Eckstein, F., Cassel, D., Levkovitz, H., Lowe, M. & Selinger, Z. (1979) *J. Biol. Chem.* **254**, 9829–9834
- ElDeib, M. M. R., Parker, C. D., Veum, T. L., Zinn, G. M. & White, A. A. (1986) *Arch. Biochem. Biophys.* **245**, 51–65
- ElDeib, M. M. R., Parker, C. D. & White, A. A. (1987) *Biochim. Biophys. Acta* **928**, 83–91
- ElDeib, M. M. R., Parker, C. D. & White, A. A. (1991) *Biochem. J.* **275**, 29–34
- Field, M., Graff, L. H., Jr., Laird, W. J. & Smith, P. L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2800–2804
- Garbers, D. L. (1976) *J. Biol. Chem.* **251**, 4071–4077
- Garbers, D. L. (1989) *J. Biol. Chem.* **264**, 9103–9106
- Garbers, D. L., Hardman, J. G. & Rudolph, F. B. (1974) *Biochemistry* **13**, 4166–4171
- Garbers, D. L., Dyer, E. L. & Hardman, J. G. (1975) *J. Biol. Chem.* **250**, 382–387
- Gazzano, H., Wu, H. I. & Waldman, S. A. (1991) *Infect. Immun.* **59**, 1552–1557
- Gerzer, R., Hofmann, F. & Schultz, G. (1981) *Eur. J. Biochem.* **116**, 479–486
- Goldberg, N. D. & Haddox, M. K. (1977) *Annu. Rev. Biochem.* **46**, 823–896
- Gordon, J. L. (1986) *Biochem. J.* **233**, 309–319
- Greenberg, R. N. & Guerrant, R. L. (1981) *Pharmacol. Ther.* **13**, 507–531
- Guerrant, R. L., Hughes, J. M., Chang, B., Robertson, D. C. & Murad, F. (1980) *J. Infect. Dis.* **142**, 220–228
- Katwa, L. C., Parker, C. D. & White, A. A. (1990) *FASEB J.* **4**, A458
- Katwa, L. C., Parker, C. D., Dybing, J. K. & White, A. A. (1991) *Arch. Biochem. Biophys.* **290**, 397–406
- Kaushal, V. & Barnes, L. D. (1986) *Anal. Biochem.* **157**, 291–294
- Kimura, H. & Murad, F. (1974) *J. Biol. Chem.* **249**, 6910–6916
- Knowles, A. F., Isler, R. E. & Reece, J. F. (1983) *Biochim. Biophys. Acta* **731**, 88–96
- Korman, L. Y., Lemp, G. F., Jackson, M. J. & Gardner, J. D. (1982) *Biochim. Biophys. Acta* **721**, 47–54
- Kuno, T., Andresen, J. W., Kamisaki, Y., Waldman, S. A., Chang, L. Y., Saheki, S., Leitman, D. C., Nakane, M. & Murad, F. (1986a) *J. Biol. Chem.* **261**, 5817–5823
- Kuno, T., Kamisaki, Y., Waldman, S. A., Garipey, J., Schoolnik, G. & Murad, F. (1986b) *J. Biol. Chem.* **261**, 1470–1476
- Kurose, H. & Ui, M. (1985) *Arch. Biochem. Biophys.* **238**, 424–434
- Kurose, H., Inagami, T. & Ui, M. (1987) *FEBS Lett.* **219**, 375–379
- Okajima, F. & Kondo, Y. (1990) *J. Biol. Chem.* **265**, 21741–21748
- Plaut, G. W. E. (1963) *Methods Enzymol.* **6**, 231–236
- Rao, M. C. & Field, M. (1984) *Biochem. Soc. Trans.* **12**, 177–180
- Rao, M. C., Guandalini, S., Smith, P. L. & Field, M. (1980) *Biochim. Biophys. Acta* **632**, 35–46
- Schulz, S., Chinkers, M. & Garbers, D. L. (1989) *FASEB J.* **3**, 2026–2035
- Schulz, S., Green, C. K., Yuen, P. S. T. & Garbers, D. L. (1990) *Cell* **63**, 941–948
- Thompson, M. R., Luttrell, M., Overmann, G. & Giannella, R. A. (1985) *Anal. Biochem.* **148**, 26–36
- Trembley, J., Gerzer, R., Pang, S. C., Cantin, M., Genest, J. & Hamet, P. (1985) *FEBS Lett.* **194**, 210–214
- Waldman, S. A., Rapoport, R. M., Fiscus, R. R., Leitman, D. C., Chang, L. Y. & Murad, F. (1989) *Biochim. Biophys. Acta* **999**, 157–162
- Weikel, C. S., Spann, C. L., Chambers, C. P., Crane, J. K., Linden, J. & Hewlett, E. K. (1990) *Infect. Immun.* **58**, 1402–1407
- White, A. A. & Karr, D. B. (1978) *Anal. Biochem.* **85**, 451–460

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