Activation of intestinal brush border guanylate cyclase by aromatic disulphide compounds

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Guanylate cyclase in pig intestinal brush border membranes was stimulated by certain aromatic disulphides. The most effective were 6-thioguanine disulphide [(TGS)₂], 6-mercaptapurine disulphide, 6,6'-dithiodinicotinic acid, 5,5'-dithiobis-(2-nitrobenzoic acid) and 5-carboxy-2-thiouracil disulphide. (TGS)₂ stimulated activity 15-fold when present at 0.1 mM. The optimum concentration for each disulphide was different, and higher concentrations were inhibitory. There was no activation by alkyl disulphides or by *N*-ethylmaleimide. Activation by 50 µM-(TGS)₂ was partially reversed by later addition of 0.1 mM-dithiothreitol, whereas activation by the *Escherichia coli* heat-stable enterotoxin STa was relatively unaffected. Pretreatment of the membranes with (TGS)₂ produced a concentration-dependent inhibition of STa-stimulated activity, while stimulating basal activity, until the activities were equal at 50 µM. Activity was [Mg²⁺]-dependent, the optimal [Mg²⁺] progressively increasing as the enzyme was stimulated by (TGS)₂, STa and Lubrol PX respectively. However, (TGS)₂ pretreatment prevented the shift to higher [Mg²⁺] optima induced by STa or Lubrol alone. Substitution of Mn²⁺ for Mg²⁺ in the reaction elevated basal activity and eliminated by activation (TGS)₂. (TGS)₂ only inhibited Mn²⁺-dependent activity (both basal and stimulated). The affinity of ¹²⁵I-STa for its receptor was slightly increased by (TGS)₂. We propose that (TGS)₂ undergoes thiol–disulphide exchange with at least three different protein thiols of decreasing reactivity. The first is associated with Mg²⁺-dependent activation, the second is associated with a tonic inhibition of activity and the third is associated with the catalytic activity, although probably not at the active site.

INTRODUCTION

The heat-stable enterotoxin STa, which is secreted by pathogenic strains of *Escherichia coli*, produces secretory diarrhoea in man and animals. There is an STa receptor located in the brush border of the intestinal epithelium, which is also the location of the larger part of mature enterocyte guanylate cyclase (De Jonge, 1975; Frantz *et al.*, 1984). Field *et al.* (1978) and later Rao *et al.* (1980) and Guerrant *et al.* (1980) showed that STa could activate this brush border membrane guanylate cyclase, but not other soluble or particulate guanylate cyclases. Both De Jonge (1984) and Rao & Field (1984) have reviewed the evidence supporting this activation as the single initial event resulting in intestinal ion secretion. The mechanism of activation is unknown; however, both we (ElDeib *et al.*, 1986a) and Kuno *et al.* (1986) have presented evidence suggesting that the STa receptor and cyclase activity are on separate proteins.

STa is an 18- or 19-amino-acid peptide containing three intramolecular disulphide bonds (Staples *et al.* 1980), the reduction of which destroys biological activity. We found that whereas free STa is rapidly inactivated by thiol compounds, it is more resistant when bound to its receptor (ElDeib *et al.*, 1986b). However, certain of these thiol compounds were able to reverse STa activation of guanylate cyclase when they were added after binding to the receptor had occurred, and these compounds decreased biological activity in a ligated intestinal loop bioassay. When screening a large series of compounds for their ability to reverse guanylate cyclase activation, we found that certain aromatic thiol compounds could themselves induce enzyme activation. This was traced to their oxidation in the reaction mixture to disulphides, followed by activation by the disulphides. Because of the magnitude of this activation, e.g. [15-fold for 6-thioguanine disulphide] [(TGS)₂], we at first thought that the

aromatic disulphides might be mimicking STa; however, the present results demonstrate a separate mechanism of activation. We propose that these compounds undergo a disulphide–thiol exchange with a thiol group on a protein that is part of the guanylate cyclase complex. Although De Jonge (1984) and Dreyfus *et al.* (1984) have suggested that STa itself might undergo such an exchange reaction with its receptor, the demonstration of the reversibility of its binding (Cohen *et al.*, 1987) makes such a reaction unlikely. Although the two modes of activation are different, there are similarities in the activated states when compared with activation induced by a non-ionic detergent.

MATERIALS AND METHODS

Brush border membrane vesicles

Castrated male pigs (4 weeks old) were anaesthetized with CO₂ and exsanguinated. The entire jejunum was removed and everted, and the segments were washed in cold 1.8 % NaCl in a cold room (4 °C). We have described the procedure used for the isolation of brush border membranes (ElDeib *et al.*, 1986a, 1987), which is a modification of the method of Gerke & Weber (1983). The solutions used for enterocyte isolation (solution A), homogenization and differential centrifugation (solution I), were all supplemented with 0.2 mM-phenylmethanesulphonyl fluoride, added from a freshly prepared 1000-fold concentrate in dimethyl sulphoxide (Me₂SO). In addition, solution I also contained leupeptin (1 µg/ml), pepstatin A (2.5 µg/ml) and aprotinin (5 µg/ml), which were also added from a 1000-fold concentrate in Me₂SO (stored frozen). The final pellets were suspended in 20 % (w/v) glycerol and stored in liquid N₂ in small portions. Before use the membranes were thawed in ice, diluted approx. 50-fold with ice-cold water and then sedimented at 171 360 g for 20 min. They were then resuspended in ice-cold water and briefly

Abbreviations used: Me₂SO, dimethyl sulphoxide; (TGS)₂, 6-thioguanine disulphide; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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sonicated to a uniform suspension. Protein was determined by a bicinchoninic acid method (Kaushal & Barnes, 1986), using bovine plasma albumin as standard.

Guanylate cyclase determination

The reaction mixture contained 50 mM-Tris/HCl, pH 7.6, 1.2 mM-[α - 32 P]GTP (approx. 1 μ Ci), 3 mM-MgCl₂, 10 mM-NaN₃, 15 mM-phosphocreatine, 10 units of creatine kinase, 0.2 mM-EGTA, approx. 5 μ g of membrane protein and, when present, STa or Lubrol PX, in a final volume of 75 μ l. (TGS)₂ and other water-insoluble disulphides were added to the mixture in 5 μ l of Me₂SO. Reactions were initiated by adding cold enzyme to the reaction mixture, prewarmed to 30 °C, and were terminated (usually after 10 min) by adding 0.15 ml of 1 M-HClO₄. The cyclic [32 P]GMP formed was purified by sequential chromatography on Dowex 50 and alumina columns (White & Karr, 1978) and counted for radioactivity. Determinations were in triplicate or in quadruplicate, 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.

Pretreatment of membranes with (TGS)₂

Membranes were incubated at 30 °C for 10 min in a complete guanylate cyclase reaction mixture (in order to stabilize activity), but the GTP was not radioactive. The mixture also contained (TGS)₂, which was added dissolved in Me₂SO. The final concentration of Me₂SO was always 6.6% (v/v), and this was also present in the control. The incubation was terminated by placing the tubes in ice, after which 240 μ l was removed from each tube and centrifuged in the A-100/30 rotor of a refrigerated Beckman Airfuge. The samples were centrifuged at approx. 167000 g at 2 °C for 5 min. After removal of the supernatant solutions the pellets were resuspended in 200 μ l of 6.6% Me₂SO containing 1 mM-EGTA (adjusted to pH 7.6 with NaOH) and the centrifugation was repeated. After one more resuspension and centrifugation, this time in 1 mM-EGTA alone, the membranes were resuspended in the same solution and used for guanylate cyclase and protein determinations.

125 I-STa binding studies

The procedures of Thompson *et al.* (1985) were used for radioiodination of STa and for purification by reverse-phase chromatography of the 125 I-STa monoiodinated in the tyrosine-4 position. We have assumed a specific radioactivity of 1200 Ci/mmol (Thompson *et al.*, 1985) in our calculations. Competition binding studies were performed in a final volume of 0.1 ml containing 50 mM-Tris (pH 7.6 with HCl), 0.15 M-NaCl, 0.1% bacitracin, 0.2 mM-EDTA, 153 pM- 125 I-STa, 7–9 μ g of membrane protein and increasing concentrations of unlabelled STa. Polypropylene tubes were used and the mixtures were incubated at 30 °C for 2 h, at which time steady-state binding was achieved. The tubes were then placed in ice, and immediately after the addition of 3 ml of ice-cold phosphate-buffered saline (20 mM-KH₂PO₄/154 mM-NaCl, pH 7.6 with NaOH) to a tube, the tube contents were poured on to a Whatman GF/B filter, under vacuum, which had been pretreated with 0.3% polyethyleneimine. The filter was immediately washed for four times with 3 ml of cold phosphate-buffered saline. The radioactivity on the filters was determined in a γ -radiation counter. Determinations were in triplicate, and presented are competition profiles from one of three similar experiments. Although treatment of the filters with polyethyleneimine was originally devised to allow separation of solubilized receptor from free ligand (Bruns *et al.*, 1983), it also enhances recovery of the brush border

membrane- 125 I-STa complex (Guarino *et al.*, 1987). In our hands this amounted to a 3-fold increase in specific binding.

Materials

STa was purified from the culture medium of *Escherichia coli* strain 431, as previously described (ElDeib *et al.*, 1987). This was the 18-amino-acid peptide (STp or ST1a). The same STa was also obtained from Sigma, and was used for the binding studies. Sigma was also the source for 6,6'-dithiodinicotinic acid and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and for the thiols used for disulphide synthesis, as well as other unspecified biochemicals. (TGS)₂, 6-mercaptapurine disulphide and 5-carboxy-2-thiouracil disulphide were synthesized from the corresponding thiol compounds by oxidation with I₂ (Doerr *et al.*, 1961) and purified by recrystallization. Purified Lubrol PX was obtained from Pierce, and dithiothreitol (DTT) was from Research Organics (Cleveland, OH, U.S.A.).

RESULTS

Assay of guanylate cyclase in the presence of aromatic disulphides

Our early observations were that 6-mercaptapurine and 6-thioguanine produced concentration-dependent stimulation of activity. Activity peaked at 7.5–10.0 mM, and amounted to 3.8-fold for the first thiol and 6.8-fold for the second. Higher concentrations of these and related compounds were inhibitory. Because of the high concentrations required for the maximum effect of these thiols, we hypothesized that the true activators were oxidation products generated during the enzyme assay. Since disulphides of these compounds were easily prepared and purified (Doerr *et al.*, 1961), this was readily confirmed. The optimum concentration of the disulphides of these two thiols was approx. 0.1 mM (Fig. 1). In order to be able to screen other disulphides for stimulatory activity under comparable conditions, these were also incorporated into the guanylate cyclase reaction mixture. Fig. 1 shows results obtained with those disulphides that were most effective, from a large series that were synthesized or purchased. Since the concentration-activity curves were biphasic, the activity at each concentration must represent the relative rates of both activation and inactivation. Activity stimulated by (TGS)₂ appeared to be relatively poorly inhibited by higher concentrations (Fig. 1); however, this is probably due to its poor solubility at those concentrations. (TGS)₂ (and 6-mercaptapurine disulphide) was added to the reactions dissolved in Me₂SO, and after dilution (to 6.66% Me₂SO), a

Fig. 1. Activation of guanylate cyclase by various aromatic disulphides

Shown is the increase in activity induced by the indicated concentrations of (TGS)₂ (●), 6-mercaptapurine disulphide (■), 6,6'-dithiodinicotinic acid (△), DTNB (○) and 5-carboxy-2-thiouracil disulphide (□).

Table 1. Reversal of (TGS)₂-induced activation by the subsequent addition of DTT

Reactions were initiated with enzyme, and after a 5 min incubation 15 μ l of either water or 0.5 mM-DTT was added, and the reactions were continued for an additional 25 min. Determinations were in quadruplicate. * $P = 0.0272$, ** $P < 0.0001$, compared with control.

Activator	Cyclic GMP formed (nmol/30 min per mg of protein)	
	Water added at 5 min	0.1 mM-DTT added at 5 min
None (control)	1.47 $\pm$ 0.12	1.48 $\pm$ 0.10
+ Sta (5 ng)	65.77 $\pm$ 1.53	62.93* $\pm$ 1.22
+ 50 μ M-(TGS) ₂	16.35 $\pm$ 0.50	11.61** $\pm$ 0.23
+ STA + (TGS) ₂	35.42 $\pm$ 0.46	38.06** $\pm$ 0.34

Fig. 2. Effect of the order of addition of STA and (TGS)₂ on the time course of the reaction

Reactions were initiated by the addition of enzyme to tubes containing 5 ng of STA (○), 50 μ M-(TGS)₂ (□) or both together (▲), and terminated at the indicated times. At the 5 min reaction time, half of the tubes with a single activator had the other activator (or vehicle) added, and the reactions were continued. Specifically, tubes containing STA had 5 μ l of Me₂SO added containing either nothing (○) or 0.75 mM-(TGS)₂ (●), while to the tubes containing (TGS)₂ was added 5 μ l of water containing either nothing (□) or 5 ng of STA (■).

portion was probably re-precipitated when the (TGS)₂ concentration exceeded 0.25 mM.

The only common structural feature for the effective compounds was that they were all aromatic disulphides. The alkyl disulphides we tried (GSSG, cystamine and 2-hydroxyethyl disulphide) had only inhibitory effects, if any, when tested over the same concentration range. The same was true of two other types of thiol-reactive compounds, alloxan and *N*-ethylmaleimide.

Two previously known activators of intestinal mucosa-guanylate cyclase are STA and non-ionic detergents (ElDeib *et al.*, 1986a). Activation by the first is receptor-mediated (Gianella *et al.*, 1983), whereas activation by the second group of compounds, although described for other guanylate cyclases for many years (White, 1975), is still not understood. It appeared improbable that activation by aromatic disulphides was receptor-mediated, since the required concentrations of disulphides was too high and the structures of the active compounds were too different. A direct chemical reaction was more probable, involving a disulphide-thiol exchange reaction, resulting in a mixed disulphide with one or more thiols on a component(s) of the guanylate cyclase complex.

Characteristics of the exchange reaction with (TGS)₂

If a disulphide-thiol exchange reaction occurred, it should be reversed by the addition of excess thiol. The results presented in Table 1 and Fig. 2 are complementary, and will be considered together. Fig. 2 shows that the reaction rate in the presence of either STA or 50 μ M-(TGS)₂ was essentially linear for the first 10 min, indicating that activation by either was complete in a negligible time. We therefore assumed that a change in the reaction rate of a (TGS)₂-stimulated reaction, induced by the addition of DTT at 5 min would have to result from the action of the DTT on membrane-bound (TGS)₂. The results in Table 1 demonstrate a 29% decrease in (TGS)₂-stimulated activity after a 25 min reaction. This supports our proposal of mixed disulphide formation. We made a similar assumption as to the effect of DTT on STA-stimulated activity, since STA is known to dissociate relatively slowly from its receptor (Guarino *et al.*, 1987). In the experiment presented in Table 1 we used only 0.1 mM-DTT, since we had previously shown that at this concentration it was relatively ineffective in reducing receptor-bound STA (ElDeib *et al.*, 1986b). The results confirmed the earlier work, showing only a 4.3% decrease in STA-stimulated activity and also demonstrating a difference in the rates of reduction of receptor-bound STA and of the putative mixed disulphide(s).

When 50 μ M-(TGS)₂ was added to a STA-stimulated reaction, we observed a time-dependent decrease in the reaction rate (Fig. 2). The reverse addition of STA to a (TGS)₂-stimulated reaction resulted in a small increase in rate; however, this could be prevented either by increasing the length of incubation with (TGS)₂ before adding STA (see Fig. 3) or by increasing the (TGS)₂ concentration (results not shown). Fig. 2 also shows that a reaction initiated in the presence of both (TGS)₂ and STA resulted in activity that was intermediate between those obtained with the two used individually. These results demonstrated that concentrations of (TGS)₂ that could stimulate basal activity were also capable of inhibiting STA-stimulated activity. The extent of inhibition increased as the order of addition of (TGS)₂ was changed from after the STA to simultaneous with STA to, finally, preceding the STA. This suggests that the inhibitory site(s) was partially protected after STA had bound to its receptor.

Returning now to Table 1, we note that the addition of 0.1 mM-DTT to a reaction initiated with both 50 μ M-(TGS)₂ and STA resulted in a 7.5% increase in activity. If we assume that (TGS)₂ forms mixed disulphides with protein thiols mediating both stimulatory and inhibitory effects, we must conclude that both effects will be reversed by reduction of the mixed disulphides. However, since the stimulation by STA is four times that induced by (TGS)₂, the net effect seen in this experiment was stimulatory, i.e. a reversal of the inhibition of STA activation.

Pretreatment of brush border membranes with (TGS)₂

In order to study the activation mechanism further we altered the experimental design. Membranes were incubated for 10 min at 30 °C, with increasing concentrations of (TGS)₂, after which they were washed and assayed for activity. The results in Fig. 3 demonstrate that activation by STA was very strongly inhibited by pretreatment of the membranes with 20 μ M-(TGS)₂, and this inhibition was unchanged as the (TGS)₂ concentration was increased to 50 μ M. However, stimulation of basal activity increased over the same concentration range, reaching a maximum at 40–50 μ M. The sensitive response of STA-stimulated activity to 20 μ M-(TGS)₂ supported our earlier proposal that (TGS)₂ reacted with a protein thiol that was important to the activation pathway of STA, which we will term SH-1. However, this cannot be the same protein thiol as that involved in the activation by (TGS)₂, which required higher concentrations for

Fig. 3. Effect of preincubating membranes with increasing concentration of $(TGS)_2$ on activity

The preincubation conditions and washing procedure are described in the Materials and methods section. Activity was then determined with no addition (○), with 5 ng of STa (●) or with 0.2% Lubrol PX (■). This experiment was performed three times with the same enzyme preparation, and shown are the resulting means $\pm$ S.D. for all determinations under the same conditions.

Fig. 4. Effect of preincubating membranes with increasing concentrations of $(TGS)_2$ on activity determined in the presence of Mn^{2+}

All conditions were as for Fig. 3, except for the substitution of 3 mM- $MnCl_2$ for $MgCl_2$ in the guanylate cyclase determinations.

full activity (SH-2). Activity stimulated by Lubrol PX was also inhibited by 20 μM -($TGS)_2$, although not as markedly as was STa-stimulated activity. However, Lubrol-stimulated activity slowly increased again between 20 and 50 μM -($TGS)_2$, which we interpret as reflecting reaction with SH-2. In the light of these two stimulatory responses to increasing $(TGS)_2$ concentrations, it is difficult to account for the apparently flat response of STa-stimulated activity to $(TGS)_2$ between 20 and 50 μM , unless this resulted from two opposing effects: an inhibitory effect and a stimulatory effect. If this was the case, the concealed inhibition would have to be a much less sensitive response to $(TGS)_2$ than the two previous effects and must reflect reaction with a third protein thiol (SH-3).

Fig. 4 shows results from an experiment similar to that shown in Fig. 3, with the same membrane preparation, but with activity determined in the presence of 3 mM- $MnCl_2$ rather than $MgCl_2$. Mn^{2+} greatly elevated basal activity without a concomitant increase in STa-stimulated activity. Pretreatment of the membranes with $(TGS)_2$ progressively diminished and finally erased the small increase in activity (40%) induced by STa. There was no obvious stimulation of basal activity, although the small but not statistically significant increase in basal activity at 30 μM ($TGS)_2$ may represent such an effect. In the presence of Mn^{2+} , Lubrol PX induced higher activity than did STa; however, the inhibitory response to $(TGS)_2$ was similar for both activators.

Fig. 5. Effect of Mg^{2+} concentration on enzyme activity before and after $(TGS)_2$ pretreatment

Both (a) and (b) show activities stimulated with 5 ng of STa (□), 0.2% Lubrol PX (△) or with no addition (○, ●), at the indicated $MgCl_2$ concentrations. In (a) (●) and (b) (●, △, □) the membranes had been pretreated with 50 μM -($TGS)_2$.

These results resemble those obtained with Mg^{2+} in that relatively high concentrations of $(TGS)_2$ (30–40 μM), were required to block STa activation completely. However, the unique sensitivity of STa-stimulated activity to $(TGS)_2$ concentrations between 10 and 20 μM , as compared with the inhibition of Lubrol-stimulated activity, was eliminated by substituting Mn^{2+} for Mg^{2+} (compare Figs. 3 and 4).

Although inhibition of Mn^{2+} -dependent activity by $(TGS)_2$ was not as obviously biphasic as was its effect on Mg^{2+} -dependent activity, the largest increase in inhibition occurred in both cases between 10 and 20 μM -($TGS)_2$. This was true for basal activity as well as for activity stimulated by STa or Lubrol PX (Fig. 4). Since Mn^{2+} was obviously responsible for the elevated basal activity, and must also be considered to be an enzyme activator (Gerzer *et al.*, 1981), these results are consistent with the proposal that the most reactive thiol (SH-1) becomes exposed to a differing extent with any type of enzyme activation. The progressive inhibition of Mn^{2+} -dependent activity by $(TGS)_2$ concentrations higher than 20 μM is quite different from the results obtained with Mg^{2+} (Figs. 3 and 4). Since the $(TGS)_2$ inhibition curves for both STa- and Lubrol-stimulated activities were parallel, we assume a common site of action. This may represent reaction with a thiol associated with the catalytic activity, and we will assume this to be SH-3 (see above). However, this thiol is unlikely to be at the active site, since the inhibition curves do not converge.

Because the results in Figs. 3 and 4 indicated that Mg^{2+} was required to demonstrate activation by $(TGS)_2$, we investigated the effects of varying the Mg^{2+} concentration (Fig. 5). The responses of the enzyme to $[Mg^{2+}]$, in the presence of different activators are shown in Fig. 5(a). STa-stimulated activity was maximal at between 2 and 6 mM- Mg^{2+} , whereas stimulation by Lubrol PX resulted in a more gradual response to $[Mg^{2+}]$, reaching a maximum between 9 and 12 mM. In contrast, activity induced by pretreatment with 50 μM -($TGS)_2$ was maximal in the relatively narrow range 1.5–2.0 mM- Mg^{2+} . With guanylate

Fig. 6. Competitive inhibition by unlabelled STa of the binding of ^{125}I -STa to brush border membranes pretreated with $50\ \mu\text{M}$ -(TGS) $_2$ (●) or with vehicle (○).

cyclase, as well as with other enzymes utilizing nucleotides, the substrate is assumed to be the metal-nucleotide complex (here at a concentration of $1.2\ \text{mM}$), and since activity initially increased with excess Mg^{2+} the excess metal must serve as an activator (Cleland, 1970). Since Lubrol-stimulated activity approached STa-stimulated activity at high Mg^{2+} concentrations (Fig. 5a), this suggests that activation was driven by excess Mg^{2+} and that both STa and Lubrol PX had permissive roles. Activity stimulated by (TGS) $_2$ initially increased with increasing $[\text{Mg}^{2+}]$ at a rate similar to that for STa-stimulated membranes. However, at about $2\ \text{mM}$ - Mg^{2+} it began to decrease, as if prematurely entering the inhibitory phase of the Mg^{2+} optimum curve, which for STa-stimulated activity was above $6\ \text{mM}$ - Mg^{2+} (Fig. 5a). Fig. 5(b) shows the effects on the Mg^{2+} optimum curves when membranes pretreated with $50\ \mu\text{M}$ -(TGS) $_2$ were assayed in the presence of STa or Lubrol but with no other activator. The differences in the activity response to $[\text{Mg}^{2+}]$ were eliminated. The implication is that (TGS) $_2$ reacted with a thiol group required for $[\text{Mg}^{2+}]$ were eliminated. The implication is that (TGS) $_2$ reacted with a thiol group required for Mg^{2+} -dependent activation. We conclude, therefore, that (TGS) $_2$ inhibited activation by STa, by Lubrol and by (TGS) $_2$ itself, and that this inhibition was at a site common to all three, which involved the role of excess Mg^{2+} in activation. We tentatively propose this to be the least reactive thiol (SH-3).

Since we had no direct evidence as to whether (TGS) $_2$ treatment was affecting the binding of STa to its receptor, we performed three competition binding studies with a fixed concentration of ^{125}I -STa and increasing concentrations of unlabelled STa. Fig. 6 shows the competition profiles from one of these experiments. Using computer-assisted non-linear regression analysis (Motulsky, 1987), the EC_{50} (concn. causing half-maximal stimulation) for STa was calculated to be $1.37\ \text{nM}$ after (TGS) $_2$ treatment, as compared with $1.64\ \text{nM}$ for the control. The B_{max} was essentially unchanged, being $5.31\ \text{pmol/mg}$ of protein for (TGS) $_2$ -treated and $5.42\ \text{pmol/mg}$ for the control. Similar results were obtained in the other experiments. Since (TGS) $_2$ treatment always increased the binding of ^{125}I -STa (10% in the experiment depicted in Fig. 5), we concluded that this resulted from an increase in the affinity of the receptor for ^{125}I -STa rather than from a change in the number of receptors. Increased ^{125}I -STa binding also resulted from treatment of membranes with the thiol reagents *N*-ethylmaleimide and *p*-chloromercuribenzenesulphonic acid (results not shown).

DISCUSSION

The present work suggests that (TGS) $_2$ reacts with at least three different protein thiol groups in the guanylate cyclase complex. The most reactive thiol (SH-1) appears to be on a protein mediating Mg^{2+} -dependent activation by either STa or Lubrol (Fig. 3). Although STa-stimulated activity is more sensitive to (TGS) $_2$ inhibition than is Lubrol-stimulated activity, the lack of inhibition of STa binding (Fig. 6) suggests that the reactive thiol is not on the receptor. The next most reactive thiol (SH-2) is associated with activation by aromatic disulphides (Fig. 3). The least reactive thiol (SH-3) appears to be associated with the catalytic activity. This apparent lack of reactivity may be deceptive, since the substrate (MgGTP) was always present during incubations with disulphides. Others have demonstrated substrate protection of thiols at the active site of guanylate cyclase (Ignarro *et al.*, 1981), and our preliminary work suggested that maximal activation by aromatic disulphides was obtained in the presence of substrate. We should add, however, that particulate guanylate cyclase is in general less sensitive to inhibition by thiol reagents than is the soluble form (Goldberg & Haddox, 1976).

Thiol-disulphide exchange reactions involving a thiol, RSH , and a symmetrical disulphide, XSSX , occur as follows:

The unsymmetrical mixed disulphides found with protein thiols are stable intermediate products (RSSX), but with non-protein thiols they are rapidly reduced with a second thiolate anion (Jocelyn, 1987). A large contribution to thiol-disulphide exchange equilibrium constants is attributed to the relative values of the pK_a of the reducing thiol, and of the thiol derived from the disulphide. pK_a values for aryl thiols are typically 5–6, whereas for alkyl thiols the values are 8–10 (Houk *et al.*, 1987). It is this difference in pK_a that provides the driving force for the ability of DTNB to completely oxidize aliphatic thiols (Houk *et al.*, 1987). We assume that it is this increased reactivity of aromatic disulphides that is responsible for their ability to activate guanylate cyclase whereas alkyl disulphides cannot.

Ziegler (1985) has reviewed much of the literature on the regulation of enzymes by thiol-disulphide exchange. He used the term *S*-thiolation to denote the reaction of protein thiols with low- M_r disulphides to form protein-mixed disulphides, and listed a considerable number of enzymes that are thereby inactivated. In contrast, there are few examples of enzymes that are activated by *S*-thiolation. One group that may be relevant to our work is the vertebrate collagenases, which are present in latent forms that can be activated by limited proteolysis or by disulphides (Macartney & Tschesche, 1980). Latent collagenase from human polymorphonuclear leukocytes was shown to be activated by GSSG, cystine or peptides containing disulphides, e.g. insulin. Activation proceeded by a disulphide-thiol exchange by which the active enzyme ($M_r = 65\,500$ – $67\,000$) is released from the latent enzyme ($M_r = 91\,000$ – $94\,000$), which is a mixed disulphide of an inhibitor ($M_r = 20\,000$ – $25\,000$) and the collagenase. The reactions are freely reversible and changes in the GSH/GSSG ratio cycle the enzyme between active and inactive forms.

There have been some earlier reports on the effects of thiol-reactive reagents on intestinal brush border guanylate cyclase. The first was the observation that alloxan slightly inhibited Mn^{2+} -dependent basal activity (De Jonge, 1975). Later it was shown that activation by STa was prevented by 6,6'-dithiodinicotinic acid and Cd^{2+} (De Jonge *et al.*, 1986). The latter report foreshadowed the present work, since these workers

showed that 40 μM - Cd^{2+} also induced a 4-fold increase in basal activity, and the authors indicated that Cu^{2+} , Zn^{2+} and Pb^{2+} would also be stimulatory. Activation by 6,6'-dithiodinicotinic acid was not observed, probably because it was used at 0.1 mM, which we found to be suboptimal (Fig. 1). It is likely that the metal ions were catalysing the oxidation of protein thiols to disulphides (Friedman, 1973), and that those thiols are the same as those reacting with aromatic disulphides. Supporting the possibility of an oxidation is the activation by haemin, but not by haem, of guanylate cyclase activity in several cell membrane preparations (Waldman *et al.*, 1984), including intestinal brush border membranes (De Jonge *et al.*, 1986; ElDeib *et al.*, 1987).

The actual mechanism by which aromatic disulphides stimulate brush border guanylate cyclase will not be definitively understood until the components of the system are purified. $(\text{TGS})_2$ and related compounds can obviously react with several different protein thiols, and thus are not specific activators. However, the activated state induced by $(\text{TGS})_2$ certainly bears some resemblance to that induced by the physiological agonist STa. As a working hypothesis, we are attracted to the concept that S-thiolation by $(\text{TGS})_2$ is inactivating a protein exerting a tonic inhibition on the catalytic moiety. In this sense the system would resemble the collagenase system previously discussed (Macartney & Tschesche, 1980), although we have no direct evidence to either support or disprove a covalent bonding of inhibitor to enzyme. Our hypothesis is in accordance with a model recently proposed for the activation of certain plasma membrane guanylate cyclase by the atrial natriuretic peptide (Chinkers & Garbers, 1989). This enzyme contains the receptor for the activating peptide projecting to the cell surface, and the intracellular portion of the protein contains at least two domains with sequence similarity with other proteins. One domain possesses catalytic activity and the other domain has sequence similarity to protein kinase catalytic domains. The protein-kinase-like domain was shown to function as a regulatory element, and its deletion elevated basal guanylate cyclase activity. This elevated activity was not further stimulated by atrial natriuretic peptide, and the authors propose that the binding of the peptide to the receptor initiates a conformational change in the protein-kinase-like domain that results in de-repression of the catalytic activity. Although the available evidence points to the STa receptor as residing on a separate protein from the intestinal brush border guanylate cyclase (ElDeib *et al.*, 1986a; Kuno *et al.*, 1986), a similar de-repression could occur. It remains to be determined if both $(\text{TGS})_2$ and STa activate guanylate cyclase by de-repression. In a preliminary study on crude particulate fractions prepared from rat heart, kidney and lung we were unable to demonstrate guanylate cyclase activation by 0.1 mM $(\text{TGS})_2$. Such activation may therefore be limited to the intestinal mucosa enzyme.

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REFERENCES

- Bruns, R. F., Lawson-Wendling, K. & Pugsley, T. A. (1983) *Anal. Biochem.* **132**, 74–81.
- Chinkers, M. & Garbers, D. L. (1989) *Science* **245**, 1392–1394.
- Cleland, W. W. (1970) *Enzymes* 3rd Ed. **2**, 1–65.
- Cohen, M. B., Thompson, M. R., Overmann, G. J. & Gianella, R. A. (1987) *Infect. Immun.* **55**, 329–334.
- De Jonge, H. R. (1975) *FEBS Lett.* **55**, 143–152.
- De Jonge, H. R. (1984) *Biochem. Soc. Trans.* **12**, 177–180.
- De Jonge, H. R., Bot, A. G. M. & Vaandrager, A. B. (1986) *Zentralbl. Bakteriol. Hyg. Suppl.* **15**, 335–340.
- Doerr, I. L., Wempfen, I., Clarke, D. A. & Fox, J. J. (1961) *J. Org. Chem.* **26**, 3401–3409.
- Dreyfus, L. A., Jaso-Friedmann, L. & Robertson, D. C. (1984) *Infect. Immun.* **44**, 493–501.
- ElDeib, M. M. R., Parker, C. D., Veum, T. L., Zinn, G. M. & White, A. A. (1986a) *Arch. Biochem. Biophys.* **245**, 51–65.
- ElDeib, M. M. R., Dove, C. R., Parker, C. D., Veum, T. L., Zinn, G. M. & White, A. A. (1986b) *Infect. Immun.* **51**, 24–30.
- ElDeib, M. M. R., Parker, C. D. & White, A. A. (1987) *Biochim. Biophys. Acta.* **928**, 83–91.
- Field, M., Graff, L. H., Jr., Laird, W. J. & Smith, P. L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2800–2804.
- Frantz, J. C., Jaso-Friedman, L., & Robertson, D. C. (1984) *Infect. Immun.* **43**, 622–630.
- Friedman, M. (1973) *The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins*, pp. 34–42, Pergamon Press, New York.
- Gerke, V. & Weber, K. (1983) *Eur. J. Cell. Biol.* **31**, 249–255.
- Gerzer, R., Hofmann, F. & Schultz, G. (1981) *Eur. J. Biochem.* **116**, 479–486.
- Gianella, R. A., Luttrell, M. & Thompson, M. (1983) *Am. J. Physiol.* **245**, G492–G498.
- Goldberg, N. D. & Haddox, M. K. (1976) *Annu. Rev. Biochem.* **46**, 823–896.
- Guarino, A., Cohen, M. B., Overmann, G., Thompson, M. R. & Gianella, R. A. (1987) *Dig. Dis. Sci.* **32**, 1017–1026.
- Guerrant, R. L., Hughes, J. M., Chang, B., Robertson, D. C. & Murad, F. (1980) *J. Infect. Dis.* **142**, 220–228.
- Houk, J., Singh, R. & Whitesides, M. (1987) *Methods Enzymol.* **143**, 129–140.
- Ignarro, L. J., Kadowitz, P. J. & Baricos, W. H. (1981) *Arch. Biochem. Biophys.* **208**, 75–86.
- Jocelyn, P. C. (1987) *Methods Enzymol.* **143**, 44–67.
- Kaushal, V. & Barnes, L. D. (1986) *Anal. Biochem.* **157**, 291–294.
- Kuno, T., Kamisaki, Y., Waldman, S. A., Garipey, J., Schoolnik, G. & Murad, F. (1986) *J. Biol. Chem.* **261**, 1470–1476.
- Macartney, H. W. & Tschesche, H. (1980) *FEBS Lett.* **119**, 327–332.
- Motulsky, H. J. (1987) *GraphPAD, Version 2.0*, ISI Software, Institute for Scientific Information, Philadelphia.
- 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.
- Staples, S. J., Asher, S. E. & Giannella, R. A. (1980) *J. Biol. Chem.* **255**, 4716–4721.
- Thompson, M. R., Luttrell, M., Overmann, G. & Gianella, R. A. (1985) *Anal. Biochem.* **148**, 26–36.
- Waldman, S. A., Sinacore, M. S., Lweicki, J. A., Chang, L. Y. & Murad, F. (1984) *J. Biol. Chem.* **259**, 4038–4042.
- White, A. A. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 353–373.
- White, A. A. & Karr, D. B. (1978) *Anal. Biochem.* **85**, 451–460.
- Ziegler, D. M. (1985) *Annu. Rev. Biochem.* **54**, 305–329.