Effect of antipsychotic drugs on the molecular action of cholera toxin in rabbit intestinal epithelial cells

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Antipsychotic drugs of known antidiarrhoeal and anticalmodulin activity inhibited the cholera-toxin-catalysed ADP-ribosylation of proteins of M_r 37000, 40000 and 45000 (thought to be regulatory components of the adenylate cyclase complex) that was previously shown to occur in plasma membranes from rabbit intestinal epithelial cells [(1989) Biochim. Biophys. Acta 1014, 289–297]. There was no obvious correlation between the different activities of the drugs. The drugs also inhibited adenylate cyclase activity, but in this case the inhibition correlated well with the known IC_{50} values of the drugs for anticalmodulin activity and with their antidiarrhoeal activities.

Antipsychotic drug; Cholera toxin; Adenylate cyclase; ADP-ribosylation

1. INTRODUCTION

Cholera toxin triggers the hypersecretion of fluids into the lumen of the small intestine, leading to the characteristic watery diarrhoea of cholera. This massive water loss is almost certainly caused by the permanent activation of the adenylate cyclase catalytic subunit. The A1 peptide of the toxin transfers the ADP-ribose moiety of NAD^+ to the α subunit of G_s (the regulatory, stimulatory subunit of adenylate cyclase). $G_{s\alpha}$ interacts with the catalytic subunit of adenylate cyclase, resulting in the permanent activation of the enzyme and, hence, in an increase in the production of cyclic AMP in the cell [1,2]. This increase in the cyclic AMP content is presumably responsible, through protein kinases [3], for the stimulation of chloride secretion and the inhibition of sodium chloride absorption that give rise to the loss of water.

The concentration of calcium is also thought to be involved in the control of ion secretion [4–6], and the calcium-binding protein calmodulin has been suggested to be important in mediating these events [7,8]. The binding of calcium-activated calmodulin activates a number of calcium-dependent enzymes including adenylate cyclase [9] and protein kinases [10,11]. A class of antipsychotic drugs known as the phenothiazines has been found to inhibit the cholera-toxin-induced diarrhoea by mechanisms which are not as yet fully understood. However, these phenothiazines bind to calmodulin in a calcium-dependent manner with both high affinity and specificity, and so this has been proposed as a mechanism of their action [12–15]. In fact, a correlation between the high affinity for calmodulin and the antisecretory activity of many drugs has been shown [16–19].

In this paper, we have investigated the effects of six drugs, with differing anticalmodulin and antidiarrhoeal activities, on the activation of adenylate cyclase and the ADP-ribosylation of membrane proteins. The anticalmodulin activity of a drug is its ability to inhibit the calmodulin-induced activation of phosphodiesterase in vitro [15]. The IC_{50} values for this inhibition of 17, 28, 40, 110 and 340 μM for trifluoperazine, triflupromazine, chlorpromazine, promazine and promethazine (all phenothiazines), respectively, and of 100 μM for amitriptyline (a dibenzazepine) [15,19,20], correlate well with their abilities to inhibit the diarrhoea induced by cholera toxin and other agents, such as prostaglandins and vasoactive intestinal peptide [7,18,19,21,22]. Trifluoperazine, chlorpromazine and triflupromazine have a greater antidiarrhoeal activity than amitriptyline, promazine and promethazine.

2. MATERIALS AND METHODS

2.1. Materials
[α-^{32}P]ATP was bought from Amersham International and [adenylate-^{32}P]NAD$^+$ from New England Nuclear. Cholera toxin and the six drugs were from Sigma Chemical Co.

2.2. Preparation of membranes
A mixture of brush border and basal-lateral membranes (mixed plasma membranes) was prepared from one male white New Zealand rabbit as described previously by Longbottom and van Heyningen [23].

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2.3. *Preparation of drug solutions*

The drugs were freshly prepared for each experiment, as a 5 mM stock solution in double-distilled water, because they are very light-sensitive and oxidize on prolonged exposure to air, acquiring a blue or pink colour.

2.4. *ADP-ribosylation assay*

Membrane proteins (50 μg) were ADP-ribosylated with 20 μg/ml activated cholera toxin and 5 μM [adenylate-^{32}P]NAD$^+$ (25–35 Ci/mmol) as in Longbottom and van Heyningen [23]. For investigating drug effects, solutions of freshly prepared drugs were added to the
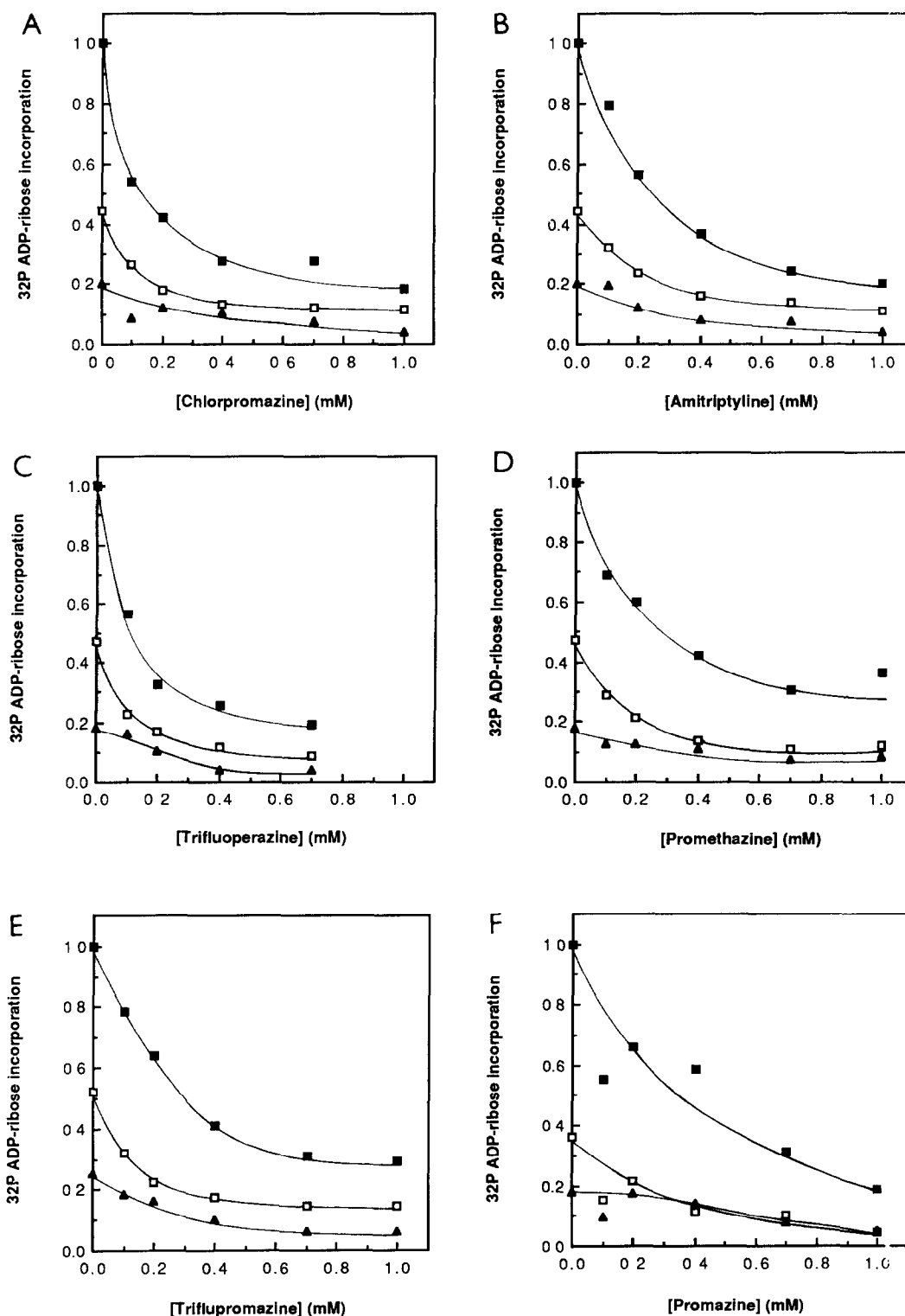

Fig. 1. Effect of antidiarrhoeal drugs on the ADP-ribosylation of mixed plasma membrane proteins: 45 kDa ($\square$), 40 kDa ($\blacksquare$) and 37 kDa ($\blacktriangle$). A value of 1 for the incorporation of [^{32}P]ADP-ribose into proteins corresponds to an integrated value of 30000 arbitrary density units, obtained from the densitometric analysis of the autoradiographs.

ADP-ribosylation assay reaction mixture giving final concentrations of 0.1, 0.2, 0.4, 0.7 and 1.0 mM. Membranes were solubilized and analyzed by polyacrylamide gel electrophoresis in SDS on a 10% gel as in [23]. The incorporation of ^{32}P into membrane proteins was estimated by densitometric analysis after autoradiography of dried gels. The absorbance signals corresponding to radiolabelled proteins were converted to arbitrary units from the printout of integrated values.

2.5. Adenylate cyclase assay

Adenylate cyclase was assayed by determining the amount of cyclic [^{32}P]AMP produced from [α-^{32}P]ATP [23]. For investigating drug effects, the freshly prepared drugs were added to the adenylate cyclase assay reaction mixture in [23] to give final concentrations of 0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mM.

3. RESULTS AND DISCUSSION

The action of the toxin is complicated, but is thought to be ultimately due to its ADP-ribosylation of the regulatory proteins of the adenylate cyclase complex, and the consequent increase in the concentration of cyclic AMP in the intestinal cells. An involvement of prostaglandins has also been suggested [24]. Our previous work using rabbit intestinal epithelial cells showed that the toxin causes ADP-ribosylation of three proteins of 45, 40 and 37 kDa in the brush border membrane [23]. The identities of these proteins have not been determined, although the results indicated that the 45 and 40 kDa proteins were probably two forms of $G_{s\alpha}$.

The effects of the drugs on the cholera-toxin-induced ADP-ribosylation of the 45, 40 and 37 kDa proteins in the mixed plasma (brush border and basal-lateral) membrane fraction are shown in Fig. 1. Purified brush border and basal-lateral membranes were also investigated but the results were similar to those for the mixed fraction and so are not shown in this paper: the patterns of inhibition were the same, but the values for the incorporation of [^{32}P]ADP-ribose into the proteins in the absence of drug were different because of cross-contamination of the membrane preparations, as discussed in [23]. No concentrations of trifluoperazine above 0.7 mM are shown in Fig. 1C because the drug was at least partly insoluble above this concentration. However, since inhibition had reached a maximum by about 0.5 mM, this has not affected the result in any way.

Although there is so much inhibition of ADP-ribosylation, there does not appear to be any correlation of this inhibition with the known antidiarrhoeal and anticalmodulin activities of the drugs. An exception to this is trifluoperazine, which has both the greatest anticalmodulin and antisecretory activity of all the drugs, as well as the greatest inhibitory effect on the ADP-ribosylation of the 40 kDa protein in particular. The fact that they do all cause a clear inhibition of ADP-ribosylation does suggest some unknown mechanism that might involve both calmodulin and ADP-ribosyltransferase activities. Calmodulin has been

found to have an action in so many different systems that it must be possible that it is also involved here.

Although the inhibition of ADP-ribosylation by the phenothiazines was unexpected, there was good reason to believe that they would inhibit adenylate cyclase activity, since the drugs are known to bind calmodulin [15] and calmodulin is known to be involved in the activation of adenylate cyclase [9]. Fig. 2 shows that such inhibition was observed, and illustrates the effects of the drugs on adenylate cyclase activity in the mixed plasma membrane fraction. Again purified brush border and basal-lateral membranes were investigated, but gave the same pattern of inhibition as the mixed membrane fraction and so are not shown. Trifluoperazine appeared to come out of solution at concentrations greater than about 1.5 mM (higher than in the ADP-ribosylation experiments probably because of a difference in the ionic strengths), but, as before, inhibition by this drug had reached a maximum at about 1.0 mM (Fig. 2).

The order of the drugs for producing the greatest inhibition of cyclase activity (trifluoperazine > chlorpromazine, triflupromazine > amitriptyline > promazine > promethazine, see Fig. 2) correlates well with the known IC$_{50}$ values for the inhibition of calmodulin activity [15], as well as with the known antidiarrhoeal activities of the drugs [19,20; trifluoperazine, chlorpromazine and triflupromazine have greater antidiarrhoeal activities than the other drugs.

Therefore, the results of this study show that there is a relationship between the binding of the drugs to calmodulin, with their effect on the movement of ions
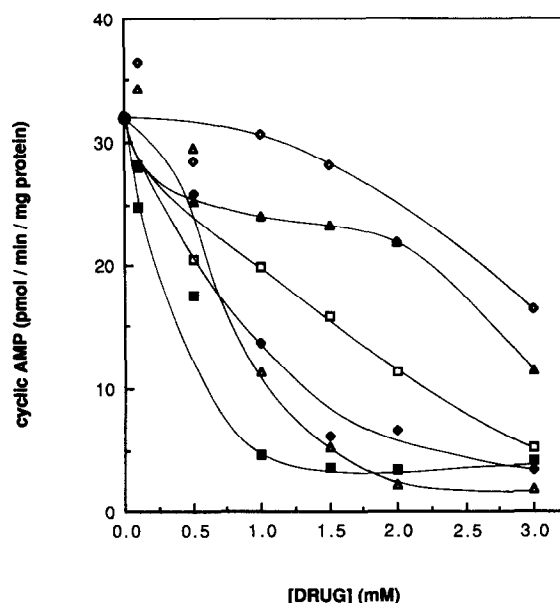

Fig. 2. Effect of antidiarrhoeal drugs on the activation of adenylate cyclase by cholera toxin. The drugs used were chlorpromazine (△), amitriptyline (□), trifluoperazine (■), promethazine (◇), triflupromazine (♦) and promazine (▲).

across the brush border membrane and with their effect on adenylate cyclase activity. Unfortunately, no similar relationship could be found when investigating the effects of the drugs on ADP-ribosylation, although the drugs do cause inhibition.

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