Effects of chlorpromazine, berberine and verapamil on *Escherichia coli* heat-labile enterotoxin-induced intestinal hypersecretion in rabbit ileal loops

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Summary. The effects of chlorpromazine (CPZ), berberine and verapamil on intestinal hypersecretion in the rabbit ileal loop model by the heat-labile enterotoxin (LT) of *Escherichia coli* were studied in relation to their ability to inhibit the stimulation of intestinal adenylate cyclase (AC) by LT. CPZ 5 mg by the intraluminal (i.l.) route and 4 mg/kg by the intramuscular (i.m.) route significantly reduced LT-induced intestinal hypersecretion. Berberine (10 mg) exerted an inhibitory effect, but only after i.l. administration, whereas verapamil did not exert any significant inhibitory effect when administered either i.l. (2.5 mg) or i.m. (4 mg/kg). At concentrations of $(0.17-1.34) \times 10^{-3}$ M CPZ, the anti-secretory effect of CPZ correlated with its inhibitory effect on rabbit LT-stimulated intestinal AC. Inhibition of cAMP synthesis was probably not involved in the mechanism of action of the two other substances. These results indicate that CPZ and phenothiazines in general are efficient drugs for reducing LT-induced intestinal hypersecretion and could represent a model for synthesis of new anti-secretory drugs with no tranquiliser side effects.

Introduction

Many natural and synthetic drugs have been reported to inhibit intestinal hypersecretion induced by cholera toxin or *Escherichia coli* heat-labile enterotoxin (LT) in rabbit or mouse ileal loops (Holmgren *et al.*, 1978; Smith and Field, 1980; Larsen, 1982) and in piglets (Lönnroth *et al.*, 1979). More recently, progress has been made in the development of anti-secretory drugs as therapy in human experimental infectious diarrhoeas (Rabani *et al.* 1982, 1987).

Anti-secretory drugs are classified into three groups according to their mode of action (Donowitz *et al.*, 1986). The anti-secretory effect can result from stimulation of fluid absorption, inhibition of secretion, or a combination of both. The mechanisms by which these drugs act upon enterotoxin-induced intestinal hypersecretion at a molecular level are not well established. Water and electrolyte (Na^+ , Cl^-) transport across intestinal mucosa, mediated by intracellular agents such as cyclic

AMP (cAMP), cyclic GMP (cGMP), or ionised calcium (via calmodulin), is amplified when the concentrations of these agents are dramatically increased by enterotoxins (cholera toxin, *E. coli* heat-stable toxin, *E. coli* LT) during infectious diarrhoeas (Ilundain and Naftalin, 1979; Donowitz, 1983). Drugs that prevent cAMP synthesis through adenylate cyclase inhibition, cGMP synthesis through guanylate cyclase inhibition, calcium release from intracellular stores, calcium entry across the mucosa or calmodulin activation by Ca^{2+} can be considered as potential antidiarrhoeal drugs and are able to reduce loss of water in diarrhoea.

On the basis of Donowitz's classification the following drugs were chosen: (1) chlorpromazine (CPZ), a drug that stimulates absorption and inhibits secretion, being a Ca^{2+} -calmodulin antagonist and adenylate cyclase inhibitor; (2) verapamil, a calcium channel blocker that stimulates intestinal absorption; and (3) berberine, which inhibits secretion. This study investigated the effects of CPZ, verapamil and berberine on *E. coli* LT-induced intestinal hyper-secretion in the rabbit ileal loop, in relation to their ability to inhibit stimulation of intestinal adenylate cyclase by LT.

Materials and methods

Enterotoxigenic E. coli strain

E. coli strain EWD₂₉₉ was kindly supplied by Dr S. Falkow (Stanford University, USA). This strain, an *E. coli* K12 transformed strain, contains multiple copies of the Ent plasmid from the porcine strain P₃₀₇, which codes for LT production.

Preparation of enterotoxigenic periplasmic proteins

E. coli was cultured in casamino acids-yeast extract medium (CAYE II) for 18 h at 37°C with continuous aeration and stirring. Bacterial cells were then collected and treated with 10 mM EDTA, and lysozyme 100 µg/ml according to the procedure of Grasser-Regallet *et al.* (1986) to obtain periplasmic proteins with large amounts of LT. This preparation was used for biological and enzymic assays.

Ligated jejunal loop assay

Jejunal ligated loops were prepared in rabbits, weighing 2.5 kg, after a 24-h fast, according to the method of Pierce and Wallace (1972). LT was injected into loops at doses of 0.8–25 µg of protein; 25 µg produced a virtually maximal response. CPZ (Largactil®, Specia) 5 mg, berberine sulphate (Sigma) 10 mg, verapamil (Isoptine®, Knoll) 2.5 mg, were injected as appropriate into each loop immediately after LT challenge.

For intramuscular (i.m.) administration, CPZ, berberine and verapamil were injected in the rabbit leg at a dose of 4 mg/kg.

When drugs were given intraluminally (i.l.) and i.m., 4 rabbits and 4–6 rabbits respectively were used per drug. Rabbits were killed 18 h later by a lethal dose of pentobarbital and the intestines were removed.

The index of fluid accumulation (IFA) was calculated as the ratio of the accumulated fluid weight to the intestinal loop dry weight as previously described (Scheftel *et al.*, 1980). To reduce the bias of varying rabbit susceptibility to LT, each IFA index per dose was expressed as a percentage of the IFA_{max} obtained with a 25-µg LT dose.

Statistical methods

When drugs were injected i.l., five control loops containing increasing LT doses were compared, respectively, with five loops containing the same LT doses in the presence of the drug. Thus, each rabbit could be considered as its own control. Statistical analysis was by Student's paired *t* test.

When drugs were injected i.m., IFA mean values for each treated group were compared to the IFA mean value of the untreated control group (*n* = 4) by Student's *t* test. Results were expressed as mean ± SEM.

Intestinal cells homogenate preparation

Intestinal epithelial cells were isolated from rabbit mucosa by a modification of the method of Stern (1966). Briefly, immediately after a lethal pentobarbital intravenous injection, the jejunum was removed and flushed free of contents with ice-cold 0.9% NaCl. Citrate solution (0.027 M sodium citrate) was used to loosen the cells, which were collected and further treated with 1.5 mM EDTA in phosphate-buffered 0.9% NaCl solution (PBS); only epithelial cells were isolated. Then cells were centrifuged at 9000 *g* for 5 min and washed several times with PBS. After being washed, the cells were homogenised for 3 min in 50 ml of triethanolamine buffer (10 mM, pH 7.6) containing 250 mM sucrose. The resulting homogenate was used for adenylate cyclase assay.

Adenylate cyclase assay

The adenylate cyclase level of the homogenate was assayed as follows: in a final volume of 400 µl, the incubation medium contained 20 mM Tris HCl, pH 7.5, bovine serum albumin 1 mg/ml, 0.1 mM GTP, 2.5 mM MgCl₂, 5 mM DTE, 0.2 mM 3-isobutyl-1-methylxanthine, 20 mM creatine phosphate, creatine kinase 1 mg/ml, 0.5 mM EGTA and homogenate (about 800 µg of protein). Incubation was started by adding 0.5 mM ATP. LT and drugs were added to the medium at appropriate concentrations. Incubation was for 10 min, 20 min, 30 min and 45 min at 37°C with stirring. The enzymic reaction was stopped by boiling at 100°C for 3 min. cAMP levels were determined by the radio-competition assay of Gilman (1970) with the cAMP binding protein (Amersham International, Aylesbury).

Protein assays

Proteins were measured by the method of Bradford (1976), with bovine serum albumin as standard.

Results

Figs. 1a, 1b and 1c show histograms of the effects on net fluid accumulation induced by LT, when drugs were injected by the i.l. route. CPZ 5 mg (fig. 1a) reduced significantly the effects of all but the highest dose of LT, although the evidence for a reduction by the 3.1-µg dose was weak (*p* < 0.1). Berberine 10 mg (fig. 1b) significantly reduced fluid accumulation by all but the two lowest doses of LT, but the significance of the reduction by the 3.1-µg and 6.2-µg doses (*p* < 0.1) was weak. CPZ and berberine reduced fluid accumulation by 30–60% depending on the LT dose. However, compared with CPZ, berberine significantly reduced the maximal effects of LT as a non-competitive inhibitor. The effects of i.m. injections of these drugs on LT-induced hypersecretion are shown in the table.

Table. Index of fluid accumulation (IFA) obtained with increasing doses of LT in rabbits treated with chlorpromazine, berberine, verapamil injected i.m. at 4 mg/kg of weight

Treatment	Number of experiments	IFA (mean of 4-6 experiments $\pm$ SEM) at LT dose				
		1.6 μ g	3.1 μ g	6.2 μ g	12.5 μ g	25 μ g
Control	4	0.22 $\pm$ 0.19	2.9 $\pm$ 1.5	16.6 $\pm$ 0.7	18.6 $\pm$ 1.7	25.8 $\pm$ 1.7
Chlorpromazine	6	<0.1	<0.1	<0.1	4.7 $\pm$ 3.0 ($p < 0.001$)	10.4 $\pm$ 3.4 ($p < 0.01$)
Berberine	4	1.5 $\pm$ 0.9	12.2 $\pm$ 4.9	22.4 $\pm$ 2.4	24.3 $\pm$ 2.3	31.6 $\pm$ 4.5
Verapamil	4	1.1 $\pm$ 0.4	9.5 $\pm$ 3.0	15.7 $\pm$ 3.0	27.2 $\pm$ 1.6	32.5 $\pm$ 5.8

Fig. 1. Effect of (a) chlorpromazine 5 mg, (b) berberine 10 mg, (c) verapamil 2.5 mg injected i.l. on LT-enterotoxin-induced rabbit intestinal hypersecretion. The index of fluid accumulation (IFA) was calculated as ratio of accumulated fluid weight to weight of dry loop and then expressed as a fraction of the maximum IFA. □—response to LT in control loops; ▨—response to LT in presence of drug; bar represents 1 SEM. * $p < 0.05$; ** $p < 0.01$; NS = not significant.

In these assays, only CPZ reversed completely the effect of low LT doses (1.6, 3.1, 6.2 μ g) and reduced significantly the hypersecretion at the higher doses of 12.5 and 25 μ g ($p < 0.001$ and $p < 0.01$ respectively). In these assays, berberine and verapamil did not antagonise the hypersecretion caused by LT.

In-vitro assays were performed to measure the effects of the drugs on the stimulation of membrane-bound intestinal adenylate cyclase (AC) by LT. Stimulation of AC activity, by increasing doses of LT, is shown in fig. 2. Stimulation of AC activity was maximal with an LT dose of 19 μ g of protein/ml for 45 min. This dose, similar to that producing maximal hypersecretion *in vivo*, was used for following assays in the presence of inhibitors.

The effects of the drugs on LT-stimulated AC activity are shown in fig. 3. Berberine, 4.5×10^{-4} M, inhibited LT-stimulated AC activity weakly (about 25%). Verapamil, 3.5×10^{-4} M, reduced LT-stimulated AC activity by 65% after

Fig. 2. Stimulation of intestinal adenylate cyclase activity by increasing doses of LT enterotoxin during incubation time of 45 min.

Fig. 3. Effects of 5×10^{-4} M chlorpromazine ($\triangle$ — $\triangle$), 4.5×10^{-4} M berberine ($\blacksquare$ — $\blacksquare$), 3.5×10^{-4} M verapamil ($\circ$ — $\circ$) on LT-induced stimulation of intestinal AC activity as function of time of incubation: $\triangle$ — $\triangle$ = AC basal activity; $\bullet$ — $\bullet$ = LT-stimulated AC activity.

incubation for 45 min and was a more potent inhibitor, CPZ, 5×10^{-4} M, abolished entirely LT-stimulated AC activity and also depressed strongly AC basal activity. Fig. 4 shows the dose-response curve of CPZ inhibition on LT-stimulated AC activity. The inhibition curve has a sigmoid shape between 1.7×10^{-4} and 13.4×10^{-4} M CPZ. Nearly

Fig. 4. Inhibition of AC activity stimulated by LT-enterotoxin by increasing doses of chlorpromazine during incubation time of 45 min.

maximal inhibition of AC activity occurred at a CPZ concentration of 1.34×10^{-3} M.

Discussion

Of the three substances tested in this study, CPZ was the most effective inhibitor of LT-induced intestinal hypersecretion in the rabbit. In contrast to berberine and verapamil, CPZ significantly reduced fluid intestinal secretion, when given i.l. or i.m.

The inhibitory effect of CPZ, given by the i.l. route, was in the range 30–60% whereas CPZ injected i.m. (4 mg/kg of body weight) reversed fluid secretion completely at lower LT doses and decreased it significantly at higher doses. The i.m. CPZ administration seemed to be the most efficient. Moreover, Holmgren *et al.* (1978) have also reported that in mice parenteral CPZ at a dose of 4 μ g/g body weight completely inhibited the intestinal hypersecretion induced by cholera toxin.

Berberine caused a similar degree of inhibition when tested by the i.l. route. However, this drug was inefficient when given i.m. This contrasts with the findings of Sack and Froehlich (1982) who observed that i.l. and i.m. berberine injections (10 mg and 20 mg/kg respectively) inhibited intestinal hypersecretion in the rabbit when induced by LT and cholera toxin. Verapamil, 2.5 mg, like berberine, was entirely ineffective when administered i.m. and less effective than CPZ and berberine when given i.l. These results are in agreement with those of Zinner *et al.* (1986), who found that verapamil was ineffective in reducing intestinal transport stimulated by cholera toxin.

This anti-secretory effect of CPZ appears to be related to its inhibition of LT-stimulated intestinal AC activity. Indeed CPZ, 5×10^{-4} M, markedly inhibited AC activity and also depressed basal enzyme activity. CPZ inhibits AC activity stimulated by hormones in various tissues (Wolff and Jones, 1970) or by secretagogues such as cholera toxin (Lönnroth *et al.*, 1977). However, CPZ action on intestinal fluid secretion probably involves other mechanisms of action (Jennische and Lönnroth, 1982). The reversal of LT-induced hypersecretion through inhibition of cAMP synthesis is clear, but other mechanisms are possible, such as the inhibition of Ca^{2+} calmodulin-activated AC (unpublished data) which may be responsible for a reduction in secretion, or the membrane stabilising effect of CPZ due to its hydrophobicity that alters small bowel fluid absorption and secretion (Rabani *et al.*, 1982).

The anti-secretory effect of berberine, when

injected by the i.l. route does not seem to be mediated via cAMP, since its inhibition of AC activity is weak. Sack and Froelich (1982) did not observe any decrease in cAMP levels in rabbit enterocytes after cholera toxin stimulation, when berberine was injected i.l. The mechanism of action of berberine is unknown. Berberine may act at a biochemical step after adenylate cyclase activation (Tai *et al.*, 1981).

Verapamil, which is a drug that stimulates absorption, does not affect LT-induced hypersecretion after i.l. or i.m. injection. This might suggest that extracellular Ca^{++} does not play a critical role in LT hypersecretion in contrast to hypersecretion induced by *E. coli* heat-stable toxin (Abbey and Knoop, 1979) or serotonin (Zinner *et al.*, 1986). Verapamil does not significantly alter the effects of secretagogues that act by increasing cAMP (Donowitz, 1983); nevertheless we noted an inhibitory

effect of verapamil on LT-stimulated AC that cannot be explained.

These results indicate that the most effective drug for preventing water and electrolyte losses during enterotoxigenic *E. coli* diarrhoea is CPZ, a drug that markedly inhibits LT-stimulated AC activity and also antagonises calmodulin-stimulated adenylate cyclase.

As many authors have noted, CPZ, and phenothiazines in general, represent a pharmacological group with anti-secretory properties, but with undesirable tranquiliser side effects. Modifications of the chemical structure of compounds belonging to this group may yield drugs with anti-secretory effects for the prevention of infectious diarrhoea but without these side effects.

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REFERENCES

- Abbey D M, Knoop F C 1979 Effect of chlorpromazine on the secretory activity of *Escherichia coli* heat-stable enterotoxin. *Infection and Immunity* **26**: 1000–1003.
- Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.
- Donowitz M, Asarkof N 1982 Calcium dependence of basal electrolyte transport in rabbit ileum. *American Journal of Physiology* **243**: G28–G35.
- Donowitz M 1983 Ca^{++} in the control of active intestinal Na and Cl transport: involvement in neurohumoral action. *American Journal of Physiology* **245**: G165–G177.
- Donowitz M, Wicks J, Sharp G W G 1986 Drug therapy for diarrheal diseases: a look ahead. *Review of Infectious Diseases* **8** Suppl 2: S188–S201.
- Gilman A G 1970 A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proceedings of the National Academy of Sciences of the USA* **67**: 305–312.
- Grasser-Regallet F, Scheffelt J M, Monteil H 1986 Isolation of a heat-labile enterotoxin produced by a human strain of *Escherichia coli* by wheat-germ agglutinin affinity chromatography. *FEMS Microbiology Letters* **35**: 239–243.
- Holmgren J, Lange S, Lönnroth I 1978 Reversal of cyclic AMP-mediated intestinal secretion in mice by chlorpromazine. *Gastroenterology* **75**: 1103–1108.
- Ilundain A, Naftalin R J 1979 Role of Ca^{2+} dependent regulator protein in intestinal secretion. *Nature* **279**: 446–448.
- Jennische E, Lönnroth I 1982 Effects of chlorpromazine on fluid transport across the intestinal mucosa of the rat. *Acta Pharmacologica et Toxicologica* **50**: 305–309.
- Larsen J J 1982 A study on inhibition of cholera toxin-induced intestinal hypersecretion by neuroleptics. *Acta Pharmacologica et Toxicologica* **50**: 294–299.
- Lönnroth L, Holmgren J, Lange S 1977 Chlorpromazine inhibits cholera toxin-induced intestinal hypersecretion. *Medical Biology (Helsinki)* **55**: 126–129.
- Lönnroth I, Andren B, Lange S, Martinsson K, Holmgren J 1979 Chlorpromazine reverses diarrhea in piglets caused by enterotoxigenic *Escherichia coli*. *Infection and Immunity* **24**: 900–905.
- Pierce N F, Wallace C K 1972 Stimulation of jejunal secretion by a crude *Escherichia coli* enterotoxin. *Gastroenterology* **63**: 439–448.
- Rabbani G H, Greenough W B, Holmgren J, Kirkwood B 1982 Controlled trial of chlorpromazine as antisecretory agent in patients with cholera hydrated intravenously. *British Medical Journal* **284**: 1361–1364.
- Rabbani G H, Buttler T, Knight J, Sanyal S C, Alam K 1987 Randomized controlled trial of berberine sulfate therapy for diarrhea due to enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. *Journal of Infectious Diseases* **155**: 979–984.
- Sack R B, Froelich J L 1982 Berberine inhibits intestinal secretory response of *Vibrio cholerae* and *Escherichia coli* enterotoxins. *Infection and Immunity* **35**: 471–475.
- Scheffelt J C, Martin C, Bober C, Monteil H 1980 Isolation of an enterotoxic factor elaborated by human enteropathogenic *Escherichia coli*. *FEMS Microbiology Letters* **9**: 125–130.
- Smith P L, Field M 1980 In vitro antisecretory effects of trifluoperazine and other neuroleptics in rabbit and human small intestine. *Gastroenterology* **78**: 1545–1553.
- Stern B K 1966 Some biochemical properties of suspensions of intestinal epithelial cells. *Gastroenterology* **51**: 855–867.
- Tai Y H, Feser J F, Marnane W G, Desjeux J F 1981 Antisecretory effects of berberine in rat ileum. *American Journal of Physiology* **241**: G253–G258.
- Wolff J, Jones A B 1970 Inhibition of hormone-sensitive adenyl cyclase by phenothiazines. *Proceedings of the National Academy of Sciences of the USA* **65**: 454–459.
- Zinner M J, McFadden D, Sherlock D, Jaffe B M 1986 Verapamil reversal of serotonin-induced jejunal secretion of water and electrolytes in awake dogs. *Gastroenterology* **90**: 515–519.