THE INFLUENCE OF DRUGS ON THE RESPONSE OF A CELL CULTURE PREPARATION TO BACTERIAL TOXINS

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SUMMARY. The influence was studied of lanthanum chloride, chlorpromazine hydrochloride, indomethacin and sodium cromoglycate on the morphological changes induced in Vero cells by the action of the cholera toxin, the thermolabile enterotoxin (LT) and the Vero cell cytotoxin (VT) of *Escherichia coli*, the enterotoxin of *Clostridium perfringens*, and the cytotoxin of *Clostridium difficile*. These drugs were able to inhibit the effects produced by *C. difficile* cytotoxin but not by the other toxins examined.

INTRODUCTION

Recent studies of the pharmacological interruption of the secretory effects of cholera toxin and of the heat-labile (LT) and heat-stable (ST) enterotoxins of *Escherichia coli* have shown that drugs such as chlorpromazine, lanthanum chloride, indomethacin and sodium cromoglycate (cromolyn) inhibit the effects induced by these toxins *in vivo*.

Investigation of the mechanism of cholera toxin has led to a better understanding of the process of active secretion in the intestine. Cyclic nucleotides are important intracellular mediators of the action of intestinal secretagogues, such as cholera toxin, the ST and LT enterotoxins of *E. coli*, Vasoactive Intestinal Peptide, prostaglandins, and bile acids (Rask-Madsen and Bukhave, 1981; Turnberg, 1981). Cholera toxin, the ST and LT enterotoxins of *E. coli* and certain prostaglandins have been shown to stimulate intestinal fluid secretion *in vivo* and to activate intestinal mucosal adenylate or guanylate cyclase (Kimberg *et al.*, 1971; Evans *et al.*, 1972; Finkelstein, 1973; Hughes *et al.*, 1978).

Other recent evidence indicates that calcium and the intracellular calcium-binding protein calmodulin play a crucial role in stimulating intestinal secretion. This conclusion was based primarily on the observation that the calcium ionophore A23187 induces intestinal secretion (Bolton and Field, 1977; Frizzell, 1977) and that trifluoroperazine, an inhibitor of the formation of the calcium-calmodulin complex, inhibits this effect. Furthermore, trifluoroperazine inhibits the changes in electrolyte

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transport caused by those secretagogues that act through cyclic nucleotides and this suggests that calcium is involved in cAMP- and cGMP-induced intestinal secretion (Ilundain and Naftalin, 1979; Smith and Field, 1980).

As several phases of toxin-stimulated secretion can now be distinguished, the present study seeks to exploit drugs that inhibit different stages in the secretory process to elucidate mechanisms of toxin action. Most of the studies of the influence of drugs on the action of toxins have been performed with animal models. In the present study, we have examined the influence of drugs on cell-toxin interaction with special reference to the action of enterotoxins. Vero cells were used in these studies because they are sensitive to a wide range of toxins including cholera toxin, the LT enterotoxin and Vero cell cytotoxin (VT) of *E. coli*, *Clostridium difficile* cytotoxin and *C. perfringens* enterotoxin.

MATERIALS AND METHODS

Drugs. Lanthanum chloride ($\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$; Sigma L4131), chlorpromazine hydrochloride (CRP; Sigma), indomethacin (IND; donated by Merck, Sharp and Dohme Research Laboratories) and sodium cromoglycate (CRM; donated by Fisons PLC, Pharmaceutical Divisions) were used. Seven two-fold serial dilutions of the drugs were prepared in 50mm TRIS-HCl, pH 7.0 (TRIS-hydroxymethyl methylamine, BDH Chemicals Ltd, Poole). Initial concentrations of CRM, CRP and IND were 44 $\mu\text{g/ml}$ and that of LaCl_3 462 $\mu\text{g/ml}$; 25- μl volumes of each of the drugs in each of the dilutions were added to the wells of microtitration plates (Falcon, Microtest II, Becton Dickinson and Co., Cockeysville, MD, USA) containing 25 μl each of the 10 two-fold serial dilutions of the toxin under test. Drugs were tested singly and in combination.

Toxin preparations. *E. coli* strains H296 and H19 were used for the production of LT and VT toxins respectively, as described by Giugliano, Mann and Drasar (1982). A cytotoxin-producing strain of *C. difficile* (HA) donated by Dr P. Borriello, Clinical Research Centre, Harrow Road, London, was used for the production of cytotoxic culture filtrates as described by Larson *et al.* (1978). Purified *C. perfringens* type A enterotoxin (5 mg/ml) was provided by Dr R. Skjelkvale (Norwegian Food Research Institute, Oslo, Norway). A purified preparation of cholera toxin obtained from Sigma was used at an initial dilution of 0.5 $\mu\text{g/ml}$.

Tissue culture assay. The influence of the drugs on the action of the bacterial toxins was assayed with cultures of African Green monkey kidney (Vero) cells. The procedures for maintenance and toxin assay were described by Giugliano *et al.* (1982). Briefly, the mixtures of the drugs and bacterial toxin dilutions were added to freshly seeded cells in microtitration plates and incubated at 37°C. Cells were examined microscopically after incubation for 18–20, 48 and 72 h at 37°C. Any observed effect on cell growth and morphology was scored in arbitrary units: 0, 1, 2, 3 and 4 corresponded respectively to 0, 25, 50, 75 and >90% cells with abnormal appearances. The titre of the toxin was taken as the highest dilution that gave a 50% end-point, calculated, if necessary, by interpolation.

RESULTS

The effects of lanthanum chloride (LaCl_3), chlorpromazine (CRP), indomethacin (IND) and sodium cromoglycate (CRM) on the response of Vero cells to the toxins were studied in cell-culture preparations.

None of the drugs showed any influence on the activity of the filtrates of LT-producing or VT-producing *E. coli*, purified enterotoxin of *C. perfringens* or cholera enterotoxin. However, the drugs inhibited the effect produced on the cells by filtrates of *C. difficile*. Figs 1 and 2 show the percentage of cells affected in the presence of several concentrations of CRP and LaCl_3 and diluted filtrates of a cytotoxicogenic strain of *C. difficile*. Both drugs inhibited the *C. difficile* cytotoxin, and the effect was

FIG. 1.—Effect of culture filtrates of *C. difficile* on Vero cells in the presence of chlorpromazine.

FIG. 2.—Effect of culture filtrates of *C. difficile* on Vero cells in the presence of lanthanum chloride.

FIG. 3.—Effect of culture filtrates of *C. difficile* on Vero cells in the presence of graded amounts of lanthanum chloride and chlorpromazine 2.7 $\mu\text{g/ml}$.

FIG. 4.—Effect of culture filtrates of *C. difficile* on Vero cells in the presence of graded amounts of chlorpromazine and lanthanum chloride 14 $\mu\text{g/ml}$.

dose-related. Indomethacin and CRM also inhibited the effect of *C. difficile* filtrates, but the degree of inhibition was the same within the limits of the concentration of each of these drugs. There was some evidence that the inhibition of the effects of toxin was potentiated by combining the drugs. Thus, more effective protection was observed when CPR and LaCl_3 were used together (Figs 3 and 4). It was not possible to test combinations of CRM and IND, or CRM and LaCl_3 , because precipitation of the mixtures occurred.

DISCUSSION

The effects of drugs on intestinal secretion may be explained by reference to their influence on the metabolism of cyclic nucleotides or of calcium and to interactions with prostaglandins (fig. 5). The action of the drugs used in this study has not been

FIG. 5.—Outline of the secretory system stimulated by cholera toxin and the LT and ST enterotoxins of *E. coli*. The points of action of chlorpromazine (CRP), lanthanum chloride (LaCl_3), indomethacin (IND) and sodium cromoglycate (CRM) are marked. The possible sites of action of *C. difficile* toxins are indicated (*). Key: Reactions →, Interactions ⇨, Blocked reactions † and interactions ‡.

completely elucidated, but it is known that indomethacin (IND) acts as an inhibitor of cyclo-oxygenase and, therefore, of the synthesis of prostaglandins. IND has been shown to inhibit the secretion induced by cholera, and by the LT and ST enterotoxins of *E. coli*, but it does not affect the levels of cAMP or the toxin-induced activation of adenylate cyclase (Kimberg *et al.*, 1971; Wald *et al.*, 1977; Madsen and Knoop, 1978; Greenberg *et al.*, 1980), although it does inhibit the activation of guanylate cyclase induced by ST (Greenberg *et al.*, 1980).

Chlorpromazine (CRP) has a variety of properties, among them the binding of calmodulin (Wolff and Brostrom, 1979) and inhibition of secretion induced by cholera enterotoxin, the LT and ST enterotoxins of *E. coli*, and cGMP and cAMP analogues (Holmgren, Lange and Lonnroth, 1978; Abbey and Knoop, 1979; Greenberg *et al.*, 1980). Furthermore, Holmgren *et al.* (1978) have shown that CRP reduces cholera toxin-mediated activation of adenylate cyclase and the secretion induced by cAMP analogues. Greenberg *et al.* (1980) have shown that this drug reduces ST-mediated activation of guanylate cyclase. Thus, CRP can influence both the production of cyclic nucleotides and their effects on membranes as mediated by the calcium-binding protein, calmodulin.

Lanthanum chloride (LaCl_3) has been shown to act as a calcium antagonist (Weiss, 1974) and to inhibit secretion caused by cholera toxin and *E. coli* LT and ST toxins. It seems to act in the reaction sequence beyond the formation of the cyclic nucleotides. The accumulation of cAMP and the activation of guanylate cyclase by cholera and ST toxins respectively were not reduced by LaCl_3 . Furthermore, it has been shown to inhibit secretion caused by cGMP analogues (Leitch and Amer, 1975; Greenberg, Murad and Guerrant, 1982). Sodium cromoglycate (CRM) inhibits calcium ion uptake and ST-induced secretion caused by cGMP analogues (Thomas and Knoop, 1982).

The sites of action of the drugs used in this study on mechanisms of secretion stimulated by cholera toxin and the ST and LT toxins of *E. coli* are shown in outline in fig. 5. For clarity the secretory mechanisms are divided into three phases: (i) the calcium-calmodulin system; (ii) prostaglandins; (iii) guanylate and adenylate cyclase. That none of the drugs used in this study affected the responses of the cells to cholera toxin or to the LT enterotoxin of *E. coli* suggests that the accumulation of cAMP necessary to induce morphological changes in cells was not impaired by the drugs. These results support the findings of Kimberg *et al.* (1974) and Leitch and Amer (1975) who showed that LaCl_3 and IND did not affect the levels of cAMP induced by cholera and LT toxins on intestinal mucosa. Furthermore, Holmgren *et al.* (1978) have shown that CRP inhibits the secretion of intestinal fluid mediated by dibutyryl cAMP. This indicates that the primary action of CRP may be at a stage subsequent to the formation of cAMP in the secretory pathway and that the inhibition of toxin-dependent stimulation of adenylate cyclase is unimportant in Vero cells. Because LaCl_3 , CRP, CRM and IND can inhibit calcium-dependent cellular events and cholera and *E. coli* enterotoxin-induced intestinal secretion, it has been assumed that calcium has an important role in the action of these toxins. The lack of influence of these drugs on the induction of morphological changes in cells in culture may indicate that calcium metabolism is not involved in the typical morphological changes of the cells in culture produced in response to cholera and LT enterotoxins.

The VT cytotoxin of *E. coli* does not stimulate adenylate cyclase, and cAMP is

probably not involved (Konowalchuck *et al.*, 1978). The results of the present study do not provide further clues as to the mechanism of its action.

The mechanism of action of *C. perfringens* enterotoxin is largely unknown. However, it has been shown that its mode of action is different from that of cholera enterotoxin and the LT enterotoxin of *E. coli*. Levels of cAMP are not affected but glucose absorption by the intestinal mucosa is inhibited (McDonel, 1974 and 1979). Furthermore, the results of work by McDonel and Duncan (1977) on the effect of *C. perfringens* enterotoxin on mitochondrial respiration suggested that alterations in electrolyte transport could be caused by a reduction in the energy supply available to the cell. Because none of the drugs used in this study inhibited the effect of *C. perfringens* enterotoxin on cells, it is likely that neither cyclic nucleotides nor calcium is involved in its action. This possibility was considered by McDonel and Duncan (1977). Alternatively, the action of the drugs in the reaction sequence may occur only after the main metabolic event responsible for the morphological changes observed on the cells. This seems to be the case with cholera and LT enterotoxins.

The mechanism of action of *C. difficile* on the intestinal mucosa is not well understood but at least two toxins, the cytotoxin and the enterotoxin, are involved. The enterotoxin is only slightly cytotoxic. *C. difficile* also produces several other extracellular products. The filtrates used in the present study contained various products and the effects observed may result from their combined action.

The effect of *C. difficile* toxins on cells in culture was inhibited by all of the drugs used, and the combination of LaCl_3 and CRP produced a greater inhibition of the toxin-induced effect than either of the drugs alone. These results indicate that calcium-dependent cellular events were involved in the induction of morphological changes in the cells. Enhancement of inhibition by combining LaCl_3 and CRP has been reported for cGMP-mediated ST enterotoxin-induced secretion in infant mice. Whilst the mechanism of action of *C. difficile* toxin remains unclear, the studies of Hughes *et al.* (1983) on *C. difficile* toxin-induced secretion in rabbit ileum *in vitro* provided evidence that cyclic nucleotides are not involved but that calcium may play an important role in the activity of toxin. Thus, the *C. difficile* toxins may influence the activation of the calcium-calmodulin complex or act at some subsequent point of the pathway leading to the final cellular response. The response would in any case be dependent on the presence of activated calcium-calmodulin complex, and the inhibition of the effects of *C. difficile* toxin by cromoglycate indicates that calcium influx plays a significant role in the cell response to the toxin (fig. 5).

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