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INHIBITION OF ARACHIDONIC ACID RELEASE AS THE MECHANISM BY WHICH GLUCOCORTICOIDS INHIBIT ENDOTOXIN-INDUCED DIARRHOEA

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1 Dexamethasone blocked endotoxin-induced diarrhoea in mice, but not that induced by arachidonic acid or prostaglandin E_2.

2 Indomethacin blocked endotoxin and arachidonic acid-induced diarrhoea, but not that induced by prostaglandin E_2.

3 Codeine blocked all three forms of diarrhoea.

4 The above data, when considered in relation to literature reports that endotoxin induces prostaglandin synthesis, suggest that dexamethasone blocks diarrhoea by preventing the release of arachidonic acid, the substrate for prostaglandin biosynthesis.

5 The activities of indomethacin and dexamethasone in castor oil diarrhoea support the above conclusion and their inactivity in 5-hydroxytryptophan-induced diarrhoea confirms the absence of 'codeine-like' direct effects on the gut.

6 Other glucocorticoids (hydrocortisone, prednisolone) were also able to block endotoxin diarrhoea, but oestradiol, testosterone and progesterone did not.

7 The inhibitory action of dexamethasone on endotoxin diarrhoea could not be blocked by the protein synthesis inhibitor, cycloheximide, nor by the glucocorticoid receptor antagonist, progesterone. Thus, involvement of glucocorticoid receptor-mediated gene activation could not be demonstrated.

Introduction

Recent evidence suggests that anti-inflammatory glucocorticoids block the release of arachidonic acid from phospholipid pools following a variety of stimuli (McMillan, Brinkerhoff & Harris, 1978; Flower & Blackwell, 1979; Gryglewski, 1979; Carnuccio, Di-Rosa & Persico, 1980; Hirata, Schiffman, Venkatasubramanian, Salomon & Axelrod, 1980). Since free arachidonic acid can be metabolized to a range of potent biologically active products (prostaglandins, thromboxanes, hydroxylated fatty acids: Flower, 1978a; slow-reacting substance: Murphy, Hammarstrom & Samuelsson, 1979), this action of glucocorticoids could well explain, at least in part, their potent anti-inflammatory and anti-allergic properties.

The data supporting this hypothesis are largely from *in vitro* studies, using either isolated tissues, organs, or cultured cells. The present paper describes data obtained *in vivo* which provide strong, albeit indirect, support for the *in vitro* data. However, it was not possible in these studies to demonstrate involvement of the classical mechanism of steroid action, i.e. steroid-receptor interaction followed by synthesis of an effector protein (King & Mainwaring, 1974), although the possibility was not definitely excluded.

Methods

Female CD1 mice were obtained from Charles River Labs (Wilmington, Mass.) and weighed 25–30 g at the time of use. The animals were randomly allocated to the different treatment groups, dosed with the drugs under investigation, and after a suitable time interval, treated with a diarrhoea-inducing agent. Immediately after administration of the diarrhoea-inducing agent, the animals were put into individual wire-bottomed cages placed over clean paper. The treated animals were allocated to the individual cages in random order and at the appropriate time, diarrhoea was scored by an observer who was unaware of the allocation of treatments.

The scoring system used was as follows: 0 = no faeces or normal faeces; 1 = swollen, moist faeces; 2 = wet shapeless stool. There was very good agreement between the scores assigned by different observers. The intermediate score of 1 was found to be necessary to avoid arbitrary classification of some faeces into either extreme category. Statistical analysis was by the method of Kruskal-Wallis rank sums as modified by Dunn (Hollander & Wolfe, 1973) using t instead of z for group sizes of less than 10.

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Table 1 Effect of diarrhoea-inducing agents and inhibitors in the mouse

	Mean diarrhoea score				
Dose (mg/kg)	*Prostaglandin E_2*	*Arachidonic acid*	*Endotoxin*	*Castor oil*	*5-Hydroxy-tryptophan*
Codeine phosphate (orally)					
0	2.0 (6)‡	1.5 (10)	1.8 (13)	2.0 (10)	2.0 (10)
100	0.7* (7)	0.3* (10)	0.3* (12)	0.6* (10)	0.1* (10)
Indomethacin (orally)					
0	2.0 (6)	2.0 (5)	1.6 (9)	2.0 (10)	2.0 (10)
0.3	—	0.8 (5)	0.2* (5)	—	—
1.0	—	0.2* (5)	0.2* (5)	0 * (10)	—
3.0	—	0 * (5)	0 * (5)	—	—
10.0	1.7† (7)	—	—	—	1.9 (10)
Dexamethasone sodium phosphate (subcutaneously)					
0	2.0 (6)	1.9 (10)	1.6 (14)	2.0 (10)	2.0 (10)
1	—	1.5 (10)	0.2* (13)	—	—
3	—	1.4 (10)	0.2* (13)	—	—
10	2.0† (7)	1.6 (10)	0 * (14)	0.5* (10)	2.0 (10)

All compounds were administered 1 h before the diarrhoea-inducing agent, with the exception of those marked (†) which were administered 2 h before.

‡ Figures in parentheses indicate the number of animals per group.

* $P < 0.05$ corresponding control group.

The various diarrhoea-inducing agents were: (a) prostaglandin E_2 (PGE_2, Sigma, Chemical Co. St. Louis, M.O., U.S.A.), 0.4 ml of a 1 mg/ml solution of PGE_2 in ethanol was added to 0.72 ml of 0.1% w/v sodium bicarbonate and diluted to 20 ml with 0.9% w/v sodium chloride solution (saline) immediately before injection, intraperitoneally, at 10 ml/kg (200 μg/kg); (b) arachidonic acid (Sigma Chemical Co.), 0.3 ml of a 10 mg/ml solution of arachidonic acid in ethanol was added to 0.15 ml of 16.4 mg/ml sodium carbonate and diluted to 10 ml with saline immediately before injection intraperitoneally at 10 ml/kg (3 mg/kg); (c) endotoxin from *Salmonella enteritidis* (Sigma Chemical Co.) was dissolved in saline at 0.04 mg/ml and injected intravenously at 10 ml/kg (0.4 mg/kg); (d) castor oil U.S.P., 0.3 ml per mouse orally; (e) 5-hydroxytrytophan (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was dissolved in saline at 5 mg/ml and injected intraperitoneally at 10 ml/kg (50 mg/kg).

Drugs

Dexamethasone, dexamethasone disodium phosphate, and indomethacin were gifts from Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Codeine phosphate was purchased from S.B. Penick and Co., New York, N.Y., U.S.A. All other compounds were purchased from Sigma Chemical Co., St. Louis, M.O., U.S.A.

The drugs were given in a volume of 10 ml/kg in water (orally) or saline (subcutaneously), except for the water insoluble steroids. These were prepared in olive oil which was used at 5 ml/kg when injected subcutaneously. Indomethacin was given as a suspension with one drop of Tween 80 added per 10 ml to facilitate dispersion; the particle size was reduced by homogenization with a power-driven teflon in glass homogenizer.

Results

Induction of diarrhoea

Preliminary studies established that 200 μg/kg PGE_2 (i.p.), 3 mg/kg arachidonic acid (i.p.), and 0.4 mg/kg endotoxin (i.v.) gave reasonably consistent diarrhoea without excessive systemic toxicity. Although intraperitoneal administration of endotoxin was effective, even at high doses, this route was never as effective as the intravenous one. Skarnes & Harper (1972) also used this route and dose of endotoxin to induce diarrhoea in mice. The onset of diarrhoea was more rapid with PGE_2 and arachidonic acid than with endotoxin, but all three agents had produced their maximal incidence and severity of diarrhoea by 1.5 h after injection.

Fifty mg/kg 5-hydroxytryptophan (i.p.) produced severe diarrhoea with 100% incidence within 15 min, as previously reported by Wooley (1958) and Garattini & Valzelli (1965).

Castor oil diarrhoea was slower in onset, 100% incidence only occurring after 2 h following 0.3 ml/kg. Similar results were obtained by Niemegeers, Lenaerts & Awouters (1976).

Table 2 The effects of various steroids on diarrhoea induced by endotoxin

Drug‡	*Dose*	*Mean score*
Dexamethasone	10	0.4 (8)†*
Hydrocortisone	100	0.1 (8)*
Prednisolone	100	0.0 (8)*
Testosterone	100	1.8 (8)
Progesterone	100	2.0 (8)
Oestradiol	100	1.4 (8)
Control	—	1.7 (15)

‡ Drugs were given orally in olive oil vehicle 2 h before intravenous injection of endotoxin (0.4 mg/kg). † Numbers in parentheses indicate number of mice per group.
* $P < 0.05$ compared with controls.

Effects of codeine phosphate, indomethacin, and dexamethasone sodium phosphate

These three compounds were used to characterize all five models of diarrhoea (Table 1). The diarrhoea produced by all five agents was inhibited by codeine phosphate at 100 mg/kg orally, given 1 h or 2 h before the diarrhoea-inducing agent. Indomethacin was effective at 1 mg/kg, or less, against endotoxin, arachidonic acid and castor oil diarrhoea, but was ineffective against PGE_2 or 5-hydroxytryptophan (5-HTP). Dexamethasone sodium phosphate was effective only against endotoxin and castor oil diarrhoea (note: 10 mg/kg dexamethasone sodium phosphate is equivalent to 7.6 mg/kg of dexamethasone as the free alcohol).

Effects of various steroids on endotoxin-induced diarrhoea

Endotoxin-induced diarrhoea was selected for further study and it was shown that, of the six steroids examined, only the glucocorticoids (dexamethasone, prednisolone, hydrocortisone) proved effective (Table 2). Oestradiol, testosterone and progesterone were ineffective despite the very high doses used. In this experiment, in order to standardize formulations, all the steroids were administered as the free alcohol in olive oil vehicle.

Effect of the time of administration of dexamethasone on its ability to block endotoxin-induced diarrhoea

The influence of time of administration on the ability of dexamethasone disodium phosphate to block endotoxin diarrhoea is shown in Table 3. A single dose of 10 or 3 mg/kg (s.c.) given 72, 48 or 24 h before endotoxin was ineffective. However, when dosed 4 or 2 h before, or at the same time as endotoxin, a marked inhibitory effect was obtained.

The role of protein synthesis in the inhibitory action of dexamethasone against endotoxin-induced diarrhoea

The role of protein synthesis in the inhibitory effect of dexamethasone against endotoxin was studied

Table 3 The effect of time of administration on the action of dexamethasone sodium phosphate in endotoxin-induced diarrhoea

Time of administration (h)	*Mean diarrhoea score* 10 mg/kg s.c.	3 mg/kg s.c.
−72	1.4 (8)†	1.9 (8)
−48	1.1 (8)	1.4 (7)
−24	1.1 (8)	1.6 (8)
−4	0.4 (8)*	0.1 (8)*
−2	0.1 (8)*	0.7 (9)
0	0.4 (8)*	0.1 (8)*
Controls	1.6 (31)	

Mice were given either 10 mg/kg or 3 mg/kg dexamethasone sodium phosphate (s.c.), or the vehicle (controls), at various times before challenge with 0.4 mg/kg endotoxin (i.v.). 0 = time of endotoxin administration. † Figures in parentheses indicate the number of mice per group.
* $P < 0.05$ compared with controls.

using the protein synthesis inhibitor, cycloheximide. The dosage of cycloheximide chosen (6 mg/kg s.c., 1 h before and again immediately before endotoxin injection) was that shown by Tsurufuji, Sugio & Takemasa (1979) and Tsurufuji, Sugio, Takemasa & Yoshizawa (1980) to block the anti-inflammatory effect of dexamethasone. This dosage schedule did not block the anti-diarrhoeal activity of dexamethasone given subcutaneously at 10 or 3 mg/kg (data not shown). Cycloheximide itself did not induce diarrhoea over the period of observation.

The effect of progesterone on the ability of dexamethasone to block endotoxin-induced diarrhoea

Progesterone (200 mg/kg s.c.) given 2 h before endotoxin, did not antagonize the ability of dexamethasone (3 mg/kg s.c.) 1 h before endotoxin, to inhibit diarrhoea (data not shown). The dose of progesterone used was the same as that found by Tsurufuji *et al.* (1979; 1980) to block the anti-inflammatory effect of dexamethasone.

Discussion

Codeine is known to bind to opiate receptors (Pert & Synder, 1973) thereby decreasing gut motility (Cox & Weinstock, 1966). Thus, its antidiarrhoeal action is independent of the nature of the diarrhoeal stimulus, as illustrated in Table 1. Codeine, in effect, acts as a positive control in our studies, demonstrating that all five models of diarrhoea employed are sensitive to a standard antidiarrhoeal agent.

Although high concentrations of indomethacin have many biochemical and pharmacological activities, low concentrations are relatively specific in blocking the conversion of arachidonic acid to prostaglandins and related compounds (Flower, 1974). Thus, the activity of indomethacin in endotoxin, castor oil and arachidonic acid-induced diarrhoea suggests that prostaglandin biosynthesis from arachidonic acid may be involved in the production of diarrhoea by these agents. Prostaglandins themselves have strong stimulatory effects on the gut, as indicated by the ability of PGE_2 to induce diarrhoea (Table 1). Indomethacin was inactive against diarrhoea induced by PGE_2, indicating that indomethacin does not have a direct codeine-like effect on the gut. Furthermore, 5-HTP-induced diarrhoea, which has been shown to be mediated by the conversion of the 5-HTP to 5-hydroxytryptamine (Wooley, 1958; Garattini & Valzelli, 1965), was also insensitive to indomethacin.

Endotoxin has been shown in many different systems to cause the liberation of arachidonic acid (Conde, Garcia-Barreno & Suarez, 1980) and prostaglandins (Skarnes & Harper, 1972; Bult, Rampart, Van Hove & Herman, 1979; Bult, Beetens & Herman, 1980). Skarnes & Harper (1972) demonstrated increased levels of prostaglandins and inhibition of diarrhoea by indomethacin in a model involving the same dose and type of endotoxin as used in the present study. Since the substrate for the synthesis of the relevant prostaglandin is arachidonic acid, and the main source of arachidonic acid in mammalian tissues is the phospholipid esters, endotoxin diarrhoea must involve activation of the mechanism which releases this arachidonic acid prior to its metabolism to the diarrhoea-inducing prostaglandins.

The activity of indomethacin against castor oil diarrhoea has been confirmed by several other workers (Awouters, Niemegeers, Lenaerts & Janssen, 1978; Strub & Muller, 1979) but there does not appear to be direct biochemical evidence of the involvement of prostaglandins in this model. However, a wide range of prostaglandin biosynthesis inhibitors are active against castor oil diarrhoea and their potency is closely correlated to their potency in inhibiting prostaglandin synthesis (Awouters *et al.*, 1978; Strub & Muller, 1979). Therefore, castor oil diarrhoea probably also involves the release of arachidonic acid from phospholipid and its subsequent metabolism to prostaglandins.

Arachidonic acid-induced diarrhoea has been used as an *in vivo* screen for compounds with the ability to inhibit prostaglandin biosynthesis and the activity of indomethacin in this model has been described previously (Baruth & Randall, 1974; Yellin, Buck, Sperow & Reavey-Cantwell, 1976). There seems to be little reason to doubt that diarrhoea in this model is mediated by metabolism of the exogenous arachidonic acid to prostaglandins since it has been reported that most tissues promptly metabolize free arachidonic acid to prostaglandins, the regulation of prostaglandin biosynthesis normally being achieved by regulation of substrate availability (Flower, 1978a,b).

Dexamethasone does not have a direct 'codeine-like' effect on gut activity, as indicated by its lack of activity against PGE_2 and 5-HTP-induced diarrhoea. Nor does it have the ability to block the conversion of arachidonic acid to prostaglandins as indicated by its lack of activity against arachidonic acid-induced diarrhoea. Numerous *in vitro* studies have also failed to demonstrate an inhibitory effect of glucocorticoids on prostaglandin synthesis at pharmacological or physiological concentrations (Shen, 1979). However, dexamethasone did block both endotoxin-induced diarrhoea, as also shown by Ferluga, Kaplun & Allison (1979), and castor oil-induced diarrhoea, the two models mediated by prostaglandins. The data therefore suggest that dexamethasone is able to block the synthesis of prostaglandins by a mechanism other than a direct effect on prostaglandin synthetase and

the obvious mechanism is reduction in the availability of the substrate, arachidonic acid.

One possible way in which dexamethasone could reduce the availability of arachidonic acid is by blocking the interaction of the diarrhoea-inducing agent with its target or receptor. However, since castor oil and endotoxin are two very different stimuli, it seems unlikely that dexamethasone would have the ability to interfere at the stimulus/receptor level of both of them. Furthermore, glucocorticoids were found to have no effect on the binding of endotoxin to cultured macrophages (D.L. Peavey and C.L. Brandon, personal communication). A more likely site of action is a possible common pathway after receptor activation. Thus, the data suggest that the antidiarrhoeal action of dexamethasone is due to inhibition of, or inhibition of the activation of, the phospholipase(s) that liberate arachidonic acid from membrane phospholipids. Such a mechanism has been suggested by several *in vitro* studies (Flower, 1978b; Gryoglewski, 1979; Flower & Blackwell, 1979; Carnuccio *et al.*, 1980; Hirata *et al.*, 1980; McMillan *et al.*, 1980) but not previously by *in vivo* studies.

The doses of the protein synthesis inhibitor, cycloheximide, used in the present study proved capable in other workers' hands of blocking some of the anti-inflammatory properties of dexamethasone (Tsurufuji *et al.*, 1979; 1980). This observation is consistent with the known mechanism of action of steroids in many systems, i.e. induction of the synthesis of an effector protein molecule (King & Mainwaring, 1974). The failure of cycloheximide, in the present studies, to block the antidiarrhoeal action of dexamethasone suggests that: (a) a protein effector molecule is not involved; (b) the effector molecule is stored and it is its release that is triggered by dexamethasone; or (c) the dose of dexamethasone is so high that anything less than 100% inhibition of protein synthesis, which probably cannot be achieved, will still enable sufficient synthesis of the effector molecule to take place. Unfortunately, perhaps because of the quantal nature of the test, it was not possible to use lower doses of dexamethasone. Although 10 and 3 mg/kg subcutaneously were consistently active, the effect of 1 mg/kg was variable. Thus, it was not possible to reproduce the conditions of Tsurufuji *et al.* (1979; 1980). Since a glucocorticoid-induced protein with the ability to inhibit phospholipase A_2 has been identified and partially characterized (Flower & Blackwell, 1979; Carnuccio *et al.*, 1980; Hirata *et al.*, 1980; Blackwell, Carnuccio, DiRosa, Flower, Parente & Persico, 1980), the first of these possibilities seems to be excluded. There is evidence that this protein is stored (Blackwell *et al.*, 1980) so the second possibility remains open, although it is not possible to exclude the third.

In support of the data obtained here, Brune & Wagner (1979) were unable to block the ability of dexamethasone to inhibit prostaglandin synthesis in cultured macrophages by adding cycloheximide to the medium.

No pretreatment interval was required to demonstrate the antidiarrhoeal action of dexamethasone. This is a marked contrast to the widely reported latency of action of steroids in anti-inflammatory tests *in vivo* which is attributed to the time required to synthesize adequate amounts of effector protein (Church & Miller, 1978; Tsurufuji *et al.*, 1979; 1980). The absence of detectable latency in the present model may be due to the delayed onset of the response which could mask any latency in the action of the steroid.

Of the steroids tested, the ability to block endotoxin-induced diarrhoea was confined to glucocorticoids, suggesting the involvement of the classical glucocorticoid receptor. However, progesterone, a competitive antagonist at the glucocorticoid receptor *in vitro* (Munck & Wira, 1971; Rousseau, Baxter & Tomkins, 1972; King & Mainwaring, 1974) and *in vivo* (Tsurufuji *et al.*, 1979; 1980), did not antagonize the antidiarrhoeal action of dexamethasone. Due to the requirement for relatively high doses of dexamethasone in this test, it is possible that the dose of progesterone used was insufficient to displace effectively dexamethasone from its receptor although it does at least raise the possibility that the glucocorticoid receptor is not involved in this pharmacological effect of dexamethasone.

In conclusion, it appears that *in vivo* glucocorticoids can block the release of arachidonic acid which is initiated by a variety of different stimuli. The absence of latency and the failure to inhibit the response with progesterone and cycloheximide does suggest that gene activation via the glucocorticoid receptor is not involved in the phenomenon. However, these results should be regarded with some reservation because of the high doses of dexamethasone used. Lower doses of dexamethasone did not give reliable anti-diarrhoeal activity with the present assay. The use of a more sensitive system, for example the 'enteropooling' assay (Robert, Nezamis, Lancaster, Hanchar & Klepper, 1976) and lower doses of dexamethasone are required to confirm these findings.

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