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THE INVOLVEMENT OF CALCIUM IN THE INTESTINAL RESPONSE TO SECRETAGOGUES IN THE RAT

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SUMMARY

1. The involvement of Ca^{2+} in the regulation of intestinal secretion was investigated in stripped sheets of rat mid-intestine.

2. Removal of serosal Ca^{2+} together with the addition of EGTA at concentrations of 0·5 and 1 mM inhibited the rise in short-circuit current (s.c.c.) induced by both acetylcholine and theophylline, a similar degree of inhibition being observed with both secretagogues.

3. Ca^{2+}-free serosal fluid with 0·5 mM-EGTA added reduced significantly the rises in s.c.c. induced by A23187, acetylcholine, 5-hydroxytryptamine, theophylline, dibutyryl cyclic AMP and prostaglandin E_2, but not the increased s.c.c. associated with glucose absorption. The Ca^{2+} channel blocker verapamil produced similar results.

4. The calmodulin antagonist trifluoperazine inhibited secretagogue action while its sulphoxide derivative was without effect at the same concentration.

5. The intracellular Ca^{2+} antagonist TMB-8 reduced the increased s.c.c. observed with acetylcholine and dibutyryl cyclic AMP.

6. The net Cl^- secretion, but not the decreased mucosal-to-serosal Na^+ flux, induced by acetylcholine was abolished in Ca^{2+}-free conditions. There was no consistent effect on the reduction in the residual ion flux caused by acetylcholine.

7. Absence of Ca^{2+} converted the stimulation of Cl^- secretion induced by dibutyryl cyclic AMP observed under control conditions to an enhancement of net Na^+ and Cl^- absorption.

8. It is concluded that intestinal secretagogues, whether they act through cyclic AMP or not, require both internal and external sources of Ca^{2+} if they are to produce their full effects. Moreover, it appears that the nature of the response to dibutyryl cyclic AMP depends on the prevailing Ca^{2+} concentration.

INTRODUCTION

Many agents are capable of stimulating intestinal secretion and these can be divided into two groups: those whose effects are mediated via a rise in mucosal cyclic AMP production, e.g. prostaglandins and vasoactive intestinal polypeptide (VIP), and those whose actions do not involve any change in cyclic nucleotide levels, e.g. acetylcholine and 5-hydroxytryptamine (Bolton & Field, 1977). Ca^{2+} plays a central

role in the actions of both types of secretagogue, combining with calmodulin to stimulate the secretory process (Ilundain & Naftalin, 1979) and it has been suggested that the source of the Ca^{2+} is different for the two groups of secretagogues (Bolton & Field, 1977). Cyclic AMP is said to utilize intracellular stores of Ca^{2+}, while agents whose actions do not involve this nucleotide increase Ca^{2+} entry from the extracellular fluid. Evidence for this view has come from work carried out in rabbit ileum (Bolton & Field, 1977; Donowitz, Asarkof & Pike, 1980) and rabbit (Frizzell, 1977) and rat (Zimmerman, Dobbins & Binder, 1983) colon, but preliminary experiments in rat small intestine suggested that internal and external sources of Ca^{2+} were required for the actions of both types of secretagogue (Hardcastle, Hardcastle & Noble, 1983*a*). The present study was designed to investigate further the involvement of Ca^{2+} in the regulation of intestinal secretion.

METHODS

Experiments were carried out on male albino rats weighing between 230 and 260 g. These were obtained from the Sheffield Field Laboratories and were allowed free access to food (Diet 86, Oxoid, London) and water. They were anaesthetized with sodium pentobarbitone (60 mg/kg I.P.).

Measurement of intestinal electrical activity in vitro

The potential difference (p.d.), short-circuit current (s.c.c.) and resistance (R) were measured *in vitro* using paired sheets of rat mid-intestine from which the muscle layers had been removed. These were clamped between two Perspex chambers and incubated at 37 °C in Krebs bicarbonate saline (Krebs & Henseleit, 1932), gassed with 95% O_2/5% CO_2. The serosal solution contained 10 mM-glucose and the mucosal solution 10 mM-mannitol. The p.d. was measured by salt bridge electrodes connected via calomel half-cells to a Vibron electrometer (Electronic Instruments Ltd., Model 33B-2). Current was applied across the tissue using Ag/AgCl electrodes which made contact with mucosal and serosal solutions via wide-bore salt bridges. When short-circuiting the tissue, a correction was made for the resistance of the medium as described by Field, Fromm & McColl (1971). Tissue resistance was calculated from p.d. and s.c.c. measurements using Ohm's law. Preliminary experiments showed that there was no significant difference ($P > 0.05$) in the electrical characteristics of the two sheets and in their responses to secretagogues.

In experiments where Ca^{2+}-free conditions were required, Ca^{2+} was omitted from the serosal solution and varying concentrations of EGTA were added to remove interstitial Ca^{2+}. The Ca^{2+} concentration was reduced on the serosal side of the tissue only for two reasons: (a) lowering mucosal as well as serosal calcium levels has been shown to lead to a sharp fall in tissue resistance, indicating disruption of the structural integrity of the mucosa (Bolton & Field, 1977) and (b) the increased intestinal Ca^{2+} content associated with secretagogue action is due to a rise in Ca^{2+} entry across the serosal surface of the tissue (Donowitz, Fogel, Battisti & Asarkof, 1982).

A change in s.c.c. was taken as the difference between the maximum value obtained in the presence of a secretagogue and the value immediately before its addition. After setting up, tissues were left for 10 min before readings began. In the case of the experiments with TMB-8 this period was extended to 20 min. Readings were taken for 5 min before the addition of a secretagogue. In control sheets the mean p.d. was 2.6 ± 0.1 (87) mV and the mean s.c.c. was 70.0 ± 3.4 (87) $\mu A/cm^2$ at the end of this 5 min period.

Measurement of Na^+ and Cl^- fluxes in vitro

Na^+ and Cl^- fluxes were measured simultaneously using the stripped intestinal sheet preparation described above. The method for determining and calculating the unidirectional fluxes has been described by Corbett, Isaacs, Riley & Turnberg (1977). 1·5 μCi ^{22}Na and 2·5 μCi ^{36}Cl were added to either the mucosal or serosal solution and two 10 min incubations began 20 min after the addition of the isotopes, by which time steady-state fluxes had been achieved. The first period acted as a control while during the second period acetylcholine or dibutyryl cyclic AMP was present at a concentration of 1 mM. Both mucosal and serosal reservoirs were sampled at the beginning and end

of each incubation period, the volume removed being replaced with unlabelled buffer. Samples were added to 5 ml Bray's scintillation fluid (Bray, 1960) in inserts and counted for ^{22}Na in an automatic well-type γ counter (Packard Auto-Gamma 500), together with a ^{22}Na standard. After counting, the inserts were placed in scintillation vials and counted in a liquid scintillation counter (LKB, 1215 Rackbeta). The ^{22}Na γ counts, multiplied by a factor for the relative efficiency of the two counters for ^{22}Na, were then subtracted from the total β counts to give the ^{36}Cl counts.

The net flux was calculated as the difference between the two unidirectional fluxes measured in paired sheets of mid-intestine taken from adjacent regions. Their resistances did not differ by more than 15%. The residual ion flux was taken as the difference between the mean s.c.c. and the sum of net Na^+ and Cl^- fluxes.

Expression of results

Results are expressed as mean values $\pm$ 1 S.E. of the mean with the number of observations in parentheses. Significance has been assessed using Student's *t* test, paired or unpaired as appropriate.

Chemicals

Mannitol was obtained from May & Baker Ltd., Dagenham; glucose from BDH Chemicals Ltd., Poole; acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, theophylline, dibutyryl (D6) cyclic AMP and ethylene glycol-bis-(β-amino-ethyl ether)*N,N'*-tetra-acetic acid (EGTA) from the Sigma Chemical Co., St. Louis, MO 643168, U.S.A.; atropine sulphate from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Prostaglandin E_2 (PGE_2) was supplied by Upjohn Co., Kalamazoo, MI, U.S.A. A23187 was obtained from Boehringer Mannheim GmbH, West Germany. TMB-8(8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride was generously supplied by Dr P. W. O'Connell, Upjohn Co., Kalamazoo, MI, U.S.A. and DL-verapamil by Abbott Laboratories Ltd., Queenborough, Kent. Trifluoperazine and trifluoperazine sulphoxide were gifts from Smith, Kline & French Laboratories Ltd., Welwyn Garden City, Herts. Radioactive tracers were supplied by Amersham International PLC, Amersham, Bucks.

RESULTS

Effect of Ca^{2+} lack on the electrical responses to acetylcholine and theophylline

Removal of serosal Ca^{2+} had no significant effect on the rise in s.c.c. induced by acetylcholine when 0·2 mM-EGTA was present in the Ca^{2+}-free solution (Fig. 1). With 0·5 mM-EGTA however, the response to acetylcholine was reduced by 44%, while with 1 mM-EGTA it was decreased by 71%. A very similar pattern of results was obtained with theophylline: no inhibition with 0·2 mM-EGTA, 40% inhibition with 0·5 mM-EGTA and 74% inhibition with 1 mM-EGTA (Fig. 1). These changes could not be correlated with a fall in tissue resistance when this was calculated from the first p.d. and s.c.c. readings. Control tissues had a resistance of 42·1 $\pm$ 1·4 (87) Ω cm^2, while in Ca^{2+}-free conditions tissue resistance was 36·7 $\pm$ 2·1 (21) Ω cm^2 with 0·2 mM-EGTA, 38·3 $\pm$ 3·7 (13) Ω cm^2 with 0·5 mM-EGTA and 41·0 $\pm$ 3·6 (13) Ω cm^2 with 1 mM-EGTA.

Effect of Ca^{2+}-free serosal fluid on the electrical responses to intestinal secretagogues

The effects of a number of intestinal secretagogues were tested under normal conditions and in Ca^{2+}-free serosal fluid which contained 0·5 mM-EGTA. In all cases an inhibition of the response was observed in the Ca^{2+}-free conditions (Fig. 2), regardless of whether cyclic AMP mediated the response (theophylline, dibutyryl cyclic AMP, PGE_2) or not (A23187, acetylcholine, 5-hydroxytryptamine). The rise in s.c.c. associated with the absorption of glucose however, was not affected by the lack of Ca^{2+}, suggesting that these conditions had not caused a general deterioration of the tissue.

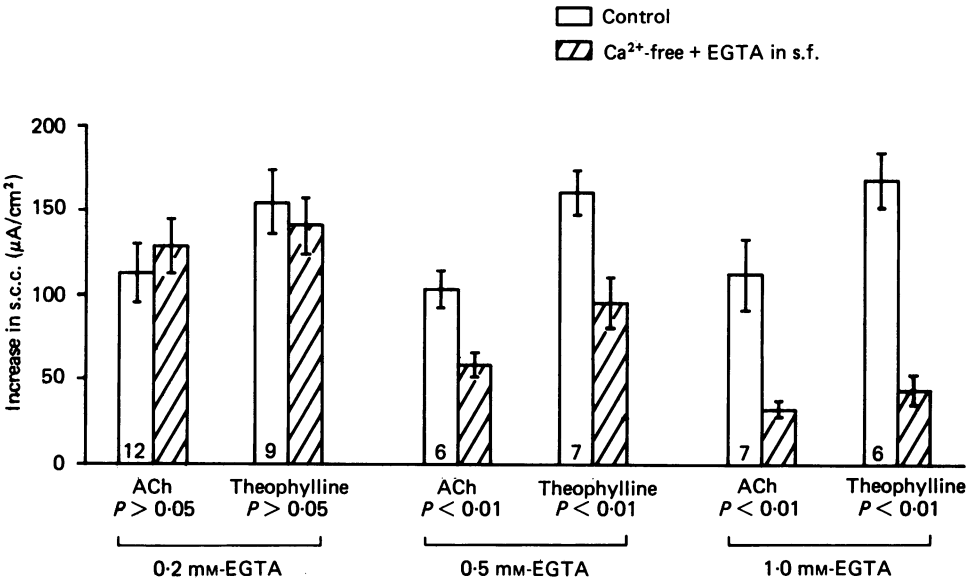

Fig. 1. Increases in short-circuit current (s.c.c.) across stripped sheets of rat mid-intestine induced by acetylcholine (ACh, 1 mM in serosal fluid (s.f.)) and theophylline (10 mM in mucosal fluid) in control conditions (open bars) and when Ca^{2+} was omitted from the serosal solution with EGTA added at the concentrations shown (hatched bars). Experiments were carried out on paired sheets of intestine using the number of pairs indicated and significance was assessed using a paired t test.

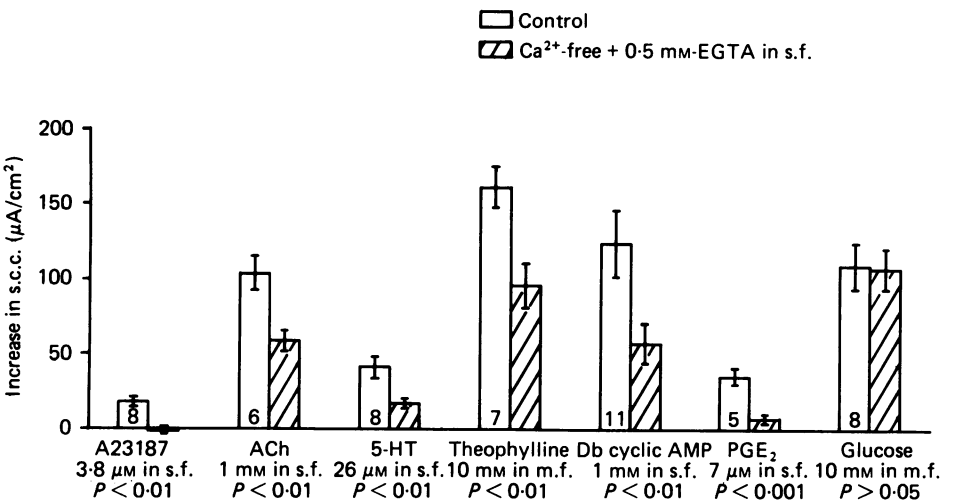

Fig. 2. Increases in s.c.c. across stripped sheets of rat mid-intestine in response to secretagogues and glucose under control conditions (open bars) and in the absence of serosal Ca^{2+} with 0·5 mM-EGTA added (hatched bars). Experiments were carried out on the numbers of tissue pairs indicated and a paired t test was used to assess significance. In this and subsequent Figures s.f. = serosal fluid and m.f. = mucosal fluid.

It was possible that the lack of serosal Ca^{2+} had led to a depletion of intracellular stores of this ion. This seems unlikely however, since the response to $BaCl_2$ (5 mM in the serosal fluid), which acts primarily by releasing Ca^{2+} from intracellular stores (Hardcastle, Hardcastle & Noble, 1983*b*), is not reduced by the Ca^{2+}-free conditions ($37{\cdot}5 \pm 7{\cdot}5$ (9) $\mu A/cm^2$ in control conditions and $51{\cdot}1 \pm 9{\cdot}4$ (9) $\mu A/cm^2$ in Ca^{2+}-free serosal fluid with 0·5 mM-EGTA, $P > 0{\cdot}05$).

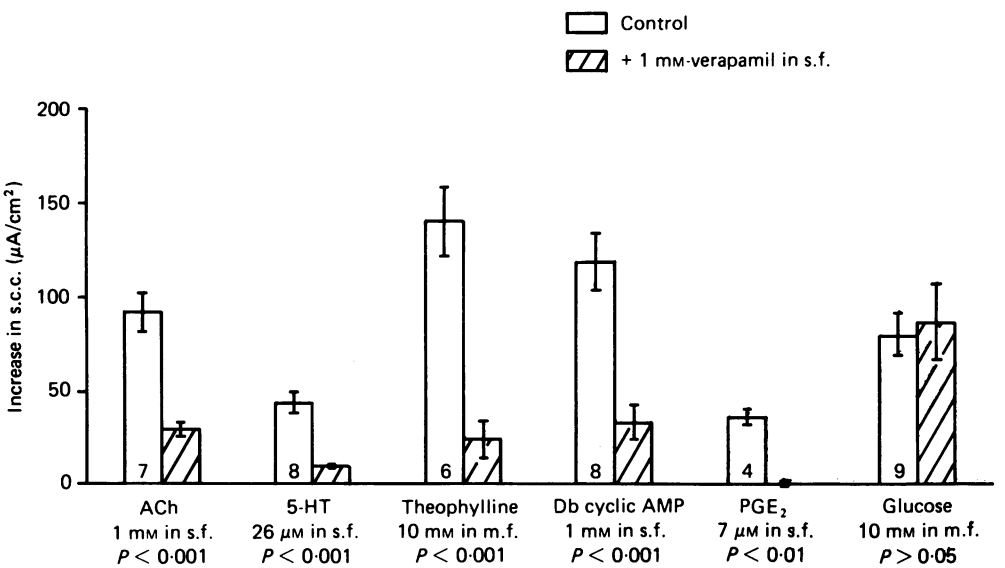

Fig. 3. Increases in s.c.c. across stripped sheets of rat mid-intestine in response to secretagogues and glucose in the absence (open bars) and presence (hatched bars) of verapamil (1 mM in serosal fluid). Experiments were carried out on the numbers of tissue pairs indicated and a paired *t* test was used to assess significance.

Effect of verapamil on the electrical responses to intestinal secretagogues

The addition of the Ca^{2+} channel blocker verapamil (1 mM) to the serosal side of the intestine produced a similar pattern of results to those obtained in the absence of serosal Ca^{2+} (Fig. 3). Once again the increased s.c.c. induced by glucose was unaffected by the experimental conditions.

Effect of trifluoperazine on the electrical responses to intestinal secretagogues

The effect of the calmodulin antagonist trifluoperazine was tested at a concentration of 0·4 mM in the serosal solution. This concentration is in the same range (0·1–0·5 mM) as those employed by Ilundain & Naftalin (1979) and Smith & Field (1980). Trifluoperazine reduced significantly the electrical responses to all the secretagogues tested (Fig. 4). The phenothiazines are also known to affect membrane behaviour and, at high doses, cause cell lysis (Seeman, 1972). These actions do not appear to be primarily responsible for the trifluoperazine-induced inhibition of secretagogue action since trifluoperazine had no significant effect ($P > 0{\cdot}05$) on the basal s.c.c., nor did it reduce the response to glucose (Fig. 4).

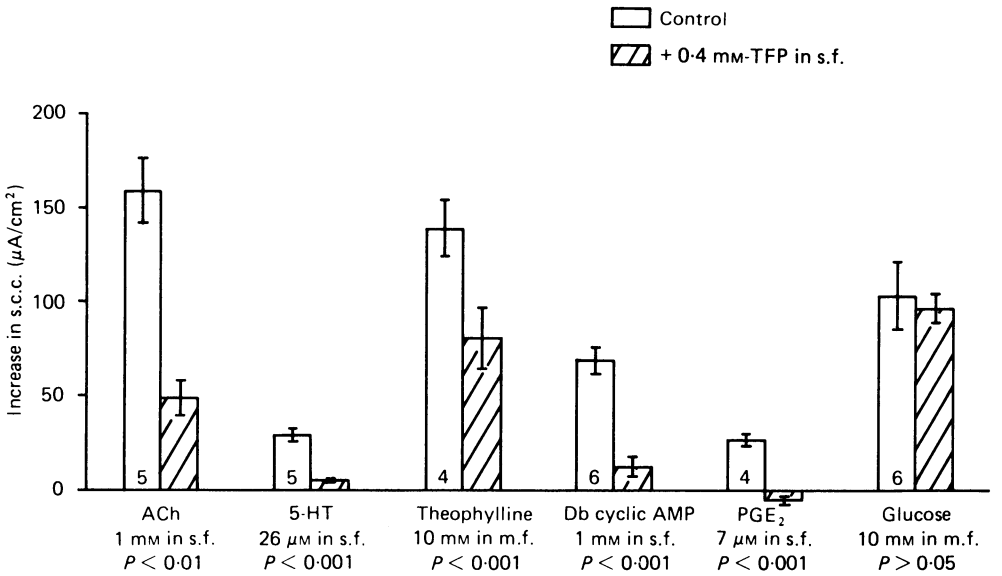

Fig. 4. Increases in s.c.c. across stripped sheets of rat mid-intestine in response to secretagogues and glucose in the absence (open bars) and presence (hatched bars) of trifluoperazine (TFP, 0·4 mM in serosal fluid). Experiments were carried out on the numbers of tissue pairs indicated and a paired t test was used to assess significance.

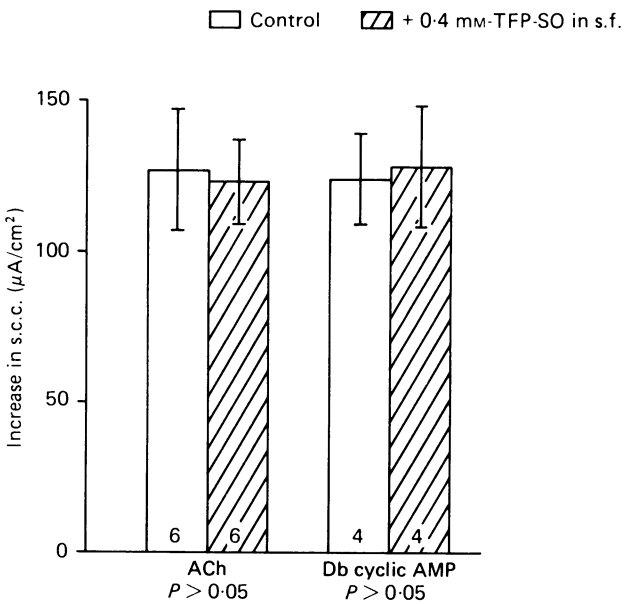

Fig. 5. Increases in s.c.c. across stripped sheets of rat mid-intestine induced by acetylcholine (ACh, 1 mM in serosal fluid) and dibutyryl cyclic AMP (Db cyclic AMP, 1 mM in serosal fluid) under control conditions (open bars) and in the presence of 0·4 mM-trifluoperazine sulphoxide (TFP-SO) in the serosal fluid (hatched bars). Experiments were carried out on the numbers of tissue pairs indicated and a paired t test was used to assess significance.

Trifluoperazine sulphoxide, which is much less effective as an inhibitor of calmodulin (Weiss, Prozialeck, Cimino, Barnette & Wallace, 1980), was also investigated. At a concentration of 0·4 mM in the serosal fluid trifluoperazine sulphoxide failed to reduce the rises in s.c.c. induced by acetylcholine and dibutyryl cyclic AMP (Fig. 5). It was not however, without effect on the tissue at this concentration since it completely abolished the secretory response to 5 mM-$BaCl_2$ (increase

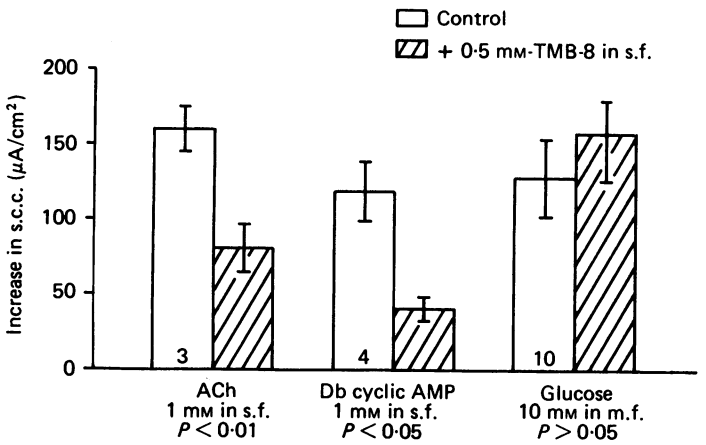

Fig. 6. Increases in s.c.c. across stripped sheets of rat mid-intestine induced by acetylcholine (ACh, 1 mM in serosal fluid), dibutyryl cyclic AMP (Db cyclic AMP, 1 mM in serosal fluid) and glucose (10 mM in mucosal fluid) under control conditions (open bars) and in the presence of 0·5 mM-8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) in the serosal fluid (hatched bars). Experiments were carried out on the numbers of tissue pairs indicated and a paired *t* test was used to assess significance.

in s.c.c. = $21·1 \pm 5·9$ (5) $\mu A/cm^2$ under control conditions, $-3·1 \pm 1·4$ (5) $\mu A/cm^2$ with trifluoperazine sulphoxide, $P < 0·01$). This inhibition cannot be ascribed to a direct action on the secretory process since normal responses to acetylcholine and dibutyryl cyclic AMP were obtained (Fig. 5). It may result from an effect of trifluoperazine sulphoxide on the membrane, preventing the entry of Ba^{2+} into the enterocyte. This suggests that the membrane actions of the phenothiazines cannot explain their inhibition of secretion as seen in Fig. 4 and this is therefore more likely to be a consequence of their ability to interfere with the actions of calmodulin.

Effect of TMB-8 on the electrical responses to intestinal secretagogues

TMB-8 is described as an intracellular Ca^{2+} antagonist that prevents the release of this ion from intracellular stores (Malagodi & Chiou, 1974). At a concentration of 0·5 mM in the serosal fluid it reduced significantly the responses to acetylcholine and dibutyryl cyclic AMP, without affecting the rise in s.c.c. induced by glucose (Fig. 6).

Effect of acetylcholine and dibutyryl cyclic AMP on Na^+ and Cl^- fluxes in control and Ca^{2+}-free conditions

In the absence of secretagogue the intestinal sheet exhibited a net absorption of Na^+ ions and a net secretion of Cl^- ions. This spontaneous secretion of Cl^- is in

agreement with earlier work in rat small intestine (Munck, 1970, 1972; Tai & Decker, 1980; Hardcastle, Hardcastle, Read & Redfern, 1981). In addition there was a negative residual ion flux, which could be due either to anion absorption or cation secretion (Tables 1 and 2). Since mid-intestine was used in these experiments it is probable that the residual ion flux represents HCO_3^- absorption (Schultz, Frizzell & Nellans, 1974). The net Na^+ and Cl^- fluxes and residual ion flux were not significantly altered by lack of serosal Ca^{2+} together with the addition of either 0·5 or 1 mM-EGTA ($P > 0·05$ in all cases) but there were small increases in the unidirectional fluxes of both ions which were significant with 1 mM-EGTA ($P < 0·05$). These may reflect an increased tissue permeability with a consequent rise in paracellular flow in the Ca^{2+}-free conditions. In addition, the s.c.c. decreased significantly ($P < 0·001$ with 1 mM-EGTA) on removal of serosal Ca^{2+}.

Acetylcholine

Under control conditions acetylcholine caused a marked stimulation of s.c.c. ($P < 0·001$) and this was accompanied by significant changes in active ion transport (Table 1). Net Na^+ absorption was reduced as a consequence of a decrease in the mucosal-to-serosal flux of this ion. Net Cl^- secretion was enhanced as a result of an increased serosal-to-mucosal Cl^- movement. In addition, the residual ion flux, which was consistent with anion absorption or cation secretion, was reduced significantly.

In Ca^{2+}-free serosal conditions with 0·5 mM-EGTA present, acetylcholine still produced a substantial increase in s.c.c. although this was significantly smaller ($P < 0·05$) than that obtained in control conditions. This secretagogue however, failed to elicit significant alterations in any of the other indices measured, with the exception of the mucosal-to-serosal Na^+ flux which was still reduced.

There was no significant difference between the effects of acetylcholine on ion fluxes in control and Ca^{2+}-free conditions with 0·5 mM-EGTA when an unpaired t test was used. Thus further experiments were carried out using the higher EGTA concentration of 1 mM. Under these conditions acetylcholine still reduced significantly the mucosal-to-serosal Na^+ flux and residual ion flux. It also increased the s.c.c. There was however, no stimulation of net Cl^- secretion and the unidirectional Cl^- fluxes were similarly unaffected. When compared with the results obtained under control conditions it was found that acetylcholine induced a significantly smaller increase in s.c.c. ($P < 0·001$), net Cl^- secretion ($P < 0·05$) and serosal-to-mucosal Cl^- flux ($P < 0·05$).

Thus removal of serosal Ca^{2+} has reduced the electrical response to acetylcholine and abolished its ability to stimulate Cl^- secretion.

Dibutyryl cyclic AMP

Under control conditions dibutyryl cyclic AMP (Table 2) increased the s.c.c. and this was entirely accounted for by a stimulation of net Cl^- secretion due to a rise in the serosal-to-mucosal movement of this ion. There were no significant changes in mucosal-to-serosal Cl^- movement, Na^+ movement or the residual ion flux.

When Ca^{2+} was omitted from the serosal solution and 0·5 mM-EGTA added, dibutyryl cyclic AMP still increased the s.c.c. although this response was significantly smaller than that observed under control conditions ($P < 0·01$). However, there was

TABLE 1. Effect of 1 mM-acetylcholine (ACh) on Na^+ and Cl^- fluxes and mean short-circuit current (s.c.c.) across stripped sheets of rat mid-intestine in control conditions and when Ca^{2+} was omitted from the serosal fluid (Ca^{2+}-free) with the addition of 0·5 or 1 mM-EGTA. The mucosal-to-serosal flux (J_{ms}) and the serosal-to-mucosal flux (J_{sm}) were measured in adjacent segments of tissue and the net flux (J_{net}) determined as the difference between the two unidirectional fluxes. The residual ion flux (J_R) was calculated as the difference between the s.c.c. and the sum of the net Na^+ and Cl^- fluxes. Each value represents the mean ± 1 S.E. of mean in μmol/cm^2.h, with the number of observations in parentheses. The significance of secretagogue action was assessed using a paired t test

	Na^+ fluxes			Cl^- fluxes				
	J_{ms}	J_{sm}	J_{net}	J_{ms}	J_{sm}	J_{net}	S.c.c.	J_R
Control (8)	25·22 ± 1·19	21·24 ± 1·38	3·98 ± 1·57	10·88 ± 0·90	15·03 ± 1·10	−4·15 ± 0·75	1·34 ± 0·16	−6·79 ± 1·45
+ ACh	22·49 ± 1·16	22·46 ± 1·70	0·03 ± 2·23	11·22 ± 0·52	18·82 ± 1·44	−7·60 ± 1·08	5·89 ± 0·28	−1·74 ± 1·51
	$P < 0·01$	n.s.	$P < 0·05$	n.s.	$P < 0·01$	$P < 0·05$	$P < 0·001$	$P < 0·01$
Ca^{2+}-free + 0·5 mM-EGTA (8)	29·05 ± 2·09	25·44 ± 2·36	3·61 ± 1·43	12·51 ± 1·07	13·84 ± 2·04	−1·33 ± 1·94	1·26 ± 0·41	−3·68 ± 1·80
+ ACh	26·43 ± 1·59	23·81 ± 2·20	2·62 ± 2·17	12·34 ± 1·30	15·44 ± 2·13	−3·10 ± 2·36	4·54 ± 0·55	−1·17 ± 2·74
	$P < 0·01$	n.s.	n.s.	n.s.	n.s.	n.s.	$P < 0·001$	n.s.
Ca^{2+}-free + 1 mM-EGTA (8)	28·84 ± 1·89	24·58 ± 0·85	4·26 ± 1·25	14·71 ± 1·36	18·14 ± 1·54	−3·43 ± 1·20	0·69 ± 0·12	−7·00 ± 1·20
+ ACh	24·03 ± 1·08	22·87 ± 1·12	1·16 ± 1·35	17·03 ± 1·13	17·61 ± 1·08	−0·58 ± 1·87	1·73 ± 0·18	−0·01 ± 1·25
	$P < 0·01$	n.s.	n.s.	n.s.	n.s.	n.s.	$P < 0·001$	$P < 0·01$

TABLE 2. Effect of 1 mM-dibutyryl cyclic AMP (Db cyclic AMP) on Na^+ and Cl^- fluxes and mean short-circuit current (s.c.c.) across stripped sheets of rat mid-intestine in control conditions and when Ca^{2+} was omitted from the serosal fluid (Ca^{2+}-free) with the addition of 0·5 or 1 mM-EGTA. The mucosal-to-serosal flux (J_{ms}) and the serosal-to-mucosal flux (J_{sm}) were measured in adjacent segments of tissue and the net flux (J_{net}) determined as the difference between the two unidirectional fluxes. The residual ion flux (J_R) was calculated as the difference between the s.c.c. and the sum of the net Na^+ and Cl^- fluxes. Each value represents the mean ± 1 S.E. of mean in μmol/cm^2.h, with the number of observations in parentheses. The significance of secretagogue action was assessed using a paired t test

	Na^+ fluxes			Cl^- fluxes				
	J_{ms}	J_{sm}	J_{net}	J_{ms}	J_{sm}	J_{net}	S.c.c.	J_R
Control (8)	29·00 ± 0·92	23·62 ± 1·68	5·38 ± 1·38	10·24 ± 0·31	13·36 ± 0·89	−3·12 ± 0·78	1·43 ± 0·14	−7·07 ± 1·06
+ Db cyclic AMP	30·30 ± 1·08	25·39 ± 1·61	4·91 ± 1·10	11·01 ± 0·37	17·40 ± 1·00	−6·39 ± 1·05	3·27 ± 0·19	−8·03 ± 1·17
	n.s.	n.s.	n.s.	n.s.	$P < 0·01$	$P < 0·01$	$P < 0·001$	n.s.
Ca^{2+}-free + 0·5 mM-EGTA (7)	31·35 ± 1·43	26·48 ± 1·84	4·87 ± 1·76	12·60 ± 0·92	13·35 ± 0·75	−0·75 ± 0·91	1·15 ± 0·21	−4·47 ± 1·03
+ Db cyclic AMP	35·83 ± 0·94	29·86 ± 1·27	5·97 ± 1·03	14·74 ± 1·40	17·93 ± 1·44	−3·19 ± 2·03	2·16 ± 0·42	−7·00 ± 1·38
	$P < 0·01$	n.s.	n.s.	n.s.	$P < 0·01$	n.s.	$P < 0·01$	n.s.
Ca^{2+}-free + 1 mM-EGTA (9)	32·71 ± 2·92	31·18 ± 1·70	1·53 ± 1·87	15·43 ± 1·02	17·88 ± 1·12	−2·45 ± 1·07	0·71 ± 0·10	−3·27 ± 1·67
+ Db cyclic AMP	38·90 ± 2·67	31·65 ± 2·13	7·25 ± 1·42	20·17 ± 1·15	19·51 ± 1·61	0·66 ± 1·30	0·84 ± 0·11	−5·75 ± 1·61
	$P < 0·01$	n.s.	$P < 0·01$	$P < 0·001$	n.s.	$P < 0·01$	$P < 0·01$	n.s.

no significant increase in net Cl^- secretion although it was still possible to detect a rise in the serosal-to-mucosal flux of this ion. Net Na^+ movement and the serosal-to-mucosal Na^+ flux did not change but there was a significant increase in mucosal-to-serosal Na^+ movement. The residual ion flux did not alter significantly. Comparing these fluxes with those obtained under control conditions there was a significant increase in the mucosal-to-serosal Na^+ flux which was not obtained under control conditions ($P < 0.05$), but no significant differences ($P > 0.05$) in any of the other fluxes.

With the EGTA concentration in the Ca^{2+}-free serosal solution increased to 1 mM, dibutyryl cyclic AMP produced changes which differed markedly from those obtained under control conditions. Instead of a stimulation of net Cl^- secretion dibutyryl cyclic AMP caused a significant inhibition. This resulted from an increase in the mucosal to serosal Cl^- flux with no significant change in the oppositely directed flux. In addition, dibutyryl cyclic AMP significantly enhanced net Na^+ absorption due to an increased mucosal-to-serosal Na^+ movement with no significant alteration of the serosal-to-mucosal flux. The rise in s.c.c. was reduced to 7 % of its control value but was still significantly different from zero. Once again the residual ion flux did not change. The effects of dibutyryl cyclic AMP on the mucosal-to-serosal Na^+ flux ($P < 0.01$), net Na^+ flux ($P < 0.01$), mucosal-to-serosal Cl^- flux ($P < 0.001$), serosal-to-mucosal Cl^- flux ($P < 0.05$), net Cl^- flux ($P < 0.001$) and s.c.c. ($P < 0.001$) all differed significantly from those obtained under control conditions.

It therefore appears that while dibutyryl cyclic AMP stimulates Cl^- secretion under control conditions, in the absence of serosal Ca^{2+} it stimulates Na^+ and Cl^- absorption.

DISCUSSION

Ca^{2+} is important for stimulus–secretion coupling in many epithelial tissues (Berridge, 1979; Case, Hardcastle & Hardcastle, 1983). It appears to act by combining with the Ca^{2+}-dependent protein calmodulin to form an active complex which then triggers the secretory process. Calmodulin seems to be similarly involved in the activation of secretion in the small intestine, since calmodulin antagonists block the secretory effects of a number of different agents (Ilundain & Naftalin, 1979; Smith & Field, 1980). Bolton & Field (1977) were the first to suggest that the source of the Ca^{2+} that combined with calmodulin depended on whether a secretagogue acted via cyclic AMP or not. They showed that lack of serosal Ca^{2+} reduced the secretory response to A23187, carbachol and 5-hydroxytryptamine, but not to theophylline, PGE_1 and VIP in rabbit ileum, but their study was limited by the fact that the secretory effect was monitored only as a change in p.d. and ion flux measurements were not performed. Subsequently Donowitz *et al.* (1980), also using rabbit ileum, found that the absence of serosal Ca^{2+} or addition of the Ca^{2+} channel blocker verapamil inhibited both electrical and ion transport changes induced by 5-hydroxytryptamine, but not those caused by theophylline. In addition, it has been shown that carbachol and 5-hydroxytryptamine increase Ca^{2+} influx into and the Ca^{2+} content of rabbit ileal mucosa (Donowitz *et al.* 1982). It was therefore suggested that secretagogues whose actions did not involve cyclic AMP, e.g. 5-hydroxytryptamine and cholinergic agonists, increased the entry of Ca^{2+} into the cell through

specific Ca^{2+} channels while cyclic AMP-mediated secretagogues, e.g. theophylline, prostaglandins and VIP, released Ca^{2+} from intracellular stores. In both cases the resultant rise in cytosolic Ca^{2+} levels leads to an increased Ca–calmodulin formation and a stimulation of secretion. The present study however, suggests that in rat mid-intestine at least, the situation is not so simple.

Initial experiments showed that in the presence of 0·5 or 1 mM-EGTA Ca^{2+}-free serosal conditions reduced the rise in s.c.c. induced by both acetylcholine and theophylline, with a remarkable similarity between the degree of inhibition observed with these two secretagogues (Fig. 1). Further experiments with a range of secretagogues demonstrated that in all cases lack of serosal Ca^{2+} with 0·5 mM-EGTA reduced the electrical response, regardless of whether a secretagogue acted via cyclic AMP or not (Fig. 2). This inhibition was not associated with an over-all loss of cell function since the rise in s.c.c. associated with glucose absorption was unaffected (Fig. 2). Similar results were obtained with verapamil (Fig. 3). These data suggested that in rat mid-intestine all secretagogues depended, at least in part, on external Ca^{2+}. However, the experimental conditions employed may have depleted intracellular calcium stores, thus accounting for the reduction in the response to cyclic AMP-mediated secretagogues. This seems unlikely since these Ca^{2+}-free conditions do not inhibit the secretory response to $BaCl_2$, an agent that appears to act solely by releasing intracellular Ca^{2+} (Hardcastle *et al.* 1983*b*).

Removal of serosal Ca^{2+} did not completely abolish secretagogue action, with the exception of the Ca^{2+} ionophore A23187, whose effects were eliminated (Fig. 2). This raises the possibility that not all the Ca^{2+} involved in linking stimulus with secretion originates extracellularly. Further support for this view comes from experiments with TMB-8. This agent is an intracellular Ca^{2+} antagonist which is said to prevent the release of Ca^{2+} from intracellular stores in smooth muscle (Malagodi & Chiou, 1974), platelets (Charo, Feinman & Detwiler, 1976), macrophages (Trotter & Quintana, 1981), neutrophils (Smith & Iden, 1979) and adrenocortical cells (Garcia, Laychock & Rubin, 1982). In rat small intestine TMB-8 inhibited the responses to both acetylcholine and dibutyryl cyclic AMP (Fig. 6). This indicates that some intracellular Ca^{2+} may be involved in stimulus–secretion coupling not only for cyclic AMP-mediated secretagogues but also for agents whose actions do not involve this nucleotide, a view first suggested by Bolton & Field (1977).

It therefore appears that both internal and external sources of Ca^{2+} are involved in the responses to secretagogues in the rat small intestine. That the Ca^{2+} entering the cytoplasm combines with calmodulin to stimulate secretion is supported by the finding that secretagogue action was, in all cases, inhibited by trifluoperazine (Fig. 4). This seems to be related to the ability of trifluoperazine to antagonize calmodulin rather than to its non-specific effects.

The conclusions drawn so far are all based on the s.c.c. increases observed in response to secretagogue challenge. To identify the ionic basis of these electrical changes Na^+ and Cl^- fluxes were measured in the absence and presence of secretagogues under control and Ca^{2+}-free conditions. Lack of serosal Ca^{2+} increased the unidirectional fluxes of both Na^+ and Cl^-. Since serosal-to-mucosal Na^+ movement takes place virtually entirely via the paracellular pathway (Munck & Schultz, 1974) the increase observed in this flux in Ca^{2+}-free conditions (Tables 1 and 2) implies an

increase in paracellular flow. However, this increase was insufficient to alter significantly the net fluxes of Na^+ and Cl^-, nor did it prevent a stimulation of net Na^+ absorption being observed in the presence of dibutyryl cyclic AMP (Table 2). Thus it seems unlikely that the increased paracellular permeability caused by lack of Ca^{2+} could have masked the secretory actions of acetylcholine and dibutyryl cyclic AMP. Donowitz & Asarkof (1982), using rabbit ileum, found that decreased Ca^{2+} stimulated the net absorption of Na^+ and Cl^-, even though the paracellular permeability was enhanced, as indicated by large increases in unidirectional fluxes and tissue conductance. The fact that Ca^{2+} was reduced in both mucosal and serosal solutions in these experiments may explain the difference between their effects on net Na^+ and Cl^- fluxes and those reported here.

With normal levels of Ca^{2+} the rise in s.c.c. induced by acetylcholine was due to a stimulation in Cl^- secretion and a reduction in the residual ion flux which is consistent with an inhibition of HCO_3^- absorption. There was also a decrease in Na^+ absorption (Table 1). These changes are similar to those obtained with pilocarpine in rat jejunum (Hubel, 1976). When Ca^{2+} was removed from the serosal fluid and 0·5 mM-EGTA added, acetylcholine failed to alter significantly any flux except mucosal-to-serosal Na^+ movement which was again reduced (Table 1). It was not possible however, to demonstrate a statistically significant difference between the effects of acetylcholine on ion fluxes under these conditions and those obtained at normal Ca^{2+} levels, although the acetylcholine-induced rise in s.c.c. was significantly reduced in the Ca^{2+}-free conditions. This illustrates the greater sensitivity of the electrical technique. Increasing the EGTA concentration to 1 mM revealed significant differences between the effects of acetylcholine on net Cl^- secretion as well as the s.c.c. under control and Ca^{2+}-free conditions (Table 1). In contrast, removal of Ca^{2+} failed to reduce the inhibition of the mucosal-to-serosal Na^+ flux and was without consistent effects on the residual ion flux. Thus the inhibition of Na^+ and HCO_3^- absorption caused by acetylcholine does not appear to be Ca^{2+} sensitive. This inability of Ca^{2+}-free conditions to affect the anti-absorptive actions of acetylcholine was not observed with 5-hydroxytryptamine (Donowitz *et al.* 1980) and electrical field stimulation (Hubel & Callanan, 1980) in rabbit ileum, nor with bethanechol and theophylline in rat colon (Zimmerman *et al.* 1983).

In the case of dibutyryl cyclic AMP a rather different picture emerged. Under control conditions the rise in s.c.c. induced by this nucleotide was due solely to an increase in Cl^- secretion (Table 2). Thus in rat mid-intestine there was no evidence for the inhibition of NaCl absorption observed with cyclic AMP in rabbit ileum (Field, 1971). Once again the electrical technique proved to be more sensitive in detecting changes brought about by lack of Ca^{2+}, since with 0·5 mM-EGTA the rise in s.c.c. was the only dibutyryl cyclic AMP-induced change to be decreased significantly. With the EGTA concentration increased to 1 mM, dibutyryl cyclic AMP no longer induced a net secretion of Cl^- ions. Indeed, under these Ca^{2+}-free conditions the action of dibutyryl cyclic AMP was totally reversed, resulting in a stimulation of both net Na^+ and net Cl^- absorption. This raises the intriguing possibility that the intestinal response to this agent depends on the prevailing Ca^{2+} concentration, inducing a net secretory state under control conditions yet a net absorptive state in Ca^{2+}-free

conditions. Further studies are required to investigate the mechanisms involved in these absorptive actions of dibutyryl cyclic AMP in the absence of Ca^{2+}.

In other transporting epithelia (toad urinary bladder, frog skin, renal proximal tubule) cytosolic Ca^{2+} levels are thought to regulate Na^+ transport, with an increase in Ca^{2+} ion activity inhibiting Na^+ transport (Taylor & Windhager, 1979). It is unlikely that the increase in net Na^+ absorption induced by dibutyryl cyclic AMP in Ca^{2+}-free conditions (Ca^{2+}-free serosal fluid containing 1 mM-EGTA) results from a lowered cytosolic Ca^{2+} concentration since basal net Na^+ absorption was not significantly different from that observed with normal Ca^{2+} concentration (Table 2). A similar situation was observed in rat colon where basal net Na^+ absorption was not affected by varying the serosal Ca^{2+} concentration from 0 to 3 mM (Zimmerman *et al.* 1983). All these studies are hampered by the fact that cytosolic Ca^{2+} activity in the transporting cells has not been measured directly, although changes in intracellular Ca^{2+} levels have been inferred from the alterations in $^{45}Ca^{2+}$ efflux that occur in response to secretagogue challenge (Donowitz, 1983). The apparent lack of effect of the Ca^{2+}-free conditions used in this study on basal ion transport may result from an inability to alter the minor part of the free Ca^{2+} ions which is active. Direct determinations of active Ca^{2+} in the cytosol of transporting epithelial cells are necessary.

The present study confirms the fundamental importance of Ca^{2+} not only in the regulation of intestinal secretion but also in the control of absorption. In rat small intestine, both intra- and extracellular sources of Ca^{2+} contribute to the control of the secretory process.

REFERENCES

BERRIDGE, M. J. (1979). Relationship between calcium and the cyclic nucleotides in ion secretion. In *Mechanisms of Intestinal Secretion*, ed. BINDER, H. J., pp. 65–81. New York: Alan R. Liss Inc.

BOLTON, J. E. & FIELD, M. (1977). Ca ionophore-stimulated secretion in rabbit ileal mucosa: relation to actions of cyclic 3′,5′-AMP and carbamylcholine. *Journal of Membrane Biology* **35**, 159–173.

BRAY, G. A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analytical Biochemistry* **1**, 279–285.

CASE, R. M., HARDCASTLE, J. & HARDCASTLE, P. T. (1983). Calcium and secretory processes. *Proceedings of 3rd BSG. SK & F International Workshop on Intestinal Secretion*, ed. TURNBERG, L. A., pp. 24–28. Welwyn Garden City: Smith, Kline & French Laboratories Ltd.

CHARO, I. F., FEINMAN, R. D. & DETWILER, T. C. (1976). Inhibition of platelet secretion by an antagonist of intracellular calcium. *Biochemical and Biophysical Research Communications* **72**, 1462–1467.

CORBETT, C. L., ISAACS, P. E. T., RILEY, A. K. & TURNBERG, L. A. (1977). Human intestinal ion transport *in vitro*. *Gut* **18**, 136–140.

DONOWITZ, M. (1983). Ca^{2+} in the control of active intestinal Na and Cl transport: involvement in neurohumoral action. *American Journal of Physiology* **245**, G165–177.

DONOWITZ, M. & ASARKOF, N. (1982). Calcium dependence of basal electrolyte transport in rabbit ileum. *American Journal of Physiology* **243**, G28–35.

DONOWITZ, M., ASARKOF, N. & PIKE, G. (1980). Calcium dependence of serotonin-induced changes in rabbit ileal electrolyte transport. *Journal of Clinical Investigation* **66**, 341–352.

DONOWITZ, M., FOGEL, R., BATTISTI, L. & ASARKOF, N. (1982). The neurohumoral secretagogues carbachol, substance P and neurotensin increase Ca^{++} influx and calcium content in rabbit ileum. *Life Sciences* **31**, 1929–1937.

FIELD, M. (1971). Ion transport in rabbit ileal mucosa. II. Effects of cyclic 3',5'-AMP. *American Journal of Physiology* **221**, 992–997.

FIELD, M., FROMM, D. & MCCOLL, I. (1971). Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. *American Journal of Physiology* **220**, 1388–1396.

FRIZZELL, R. A. (1977). Active chloride secretion by rabbit colon: calcium-dependent stimulation by ionophore A23187. *Journal of Membrane Biology* **35**, 175–187.

GARCIA, R., LAYCHOCK, S. G. & RUBIN, R. P. (1982). Inhibition of dibutyryl cyclic AMP induced steroidogenesis in rat adrenocortical cells by the putative calcium antagonist TMB-8. *Journal of Steroid Biochemistry* **16**, 317–322.

HARDCASTLE, J., HARDCASTLE, P. T. & NOBLE, J. M. (1983*a*). The role of calcium in intestinal secretion in the rat *in vitro*. *Journal of Physiology* **338**, 51–52*P*.

HARDCASTLE, J., HARDCASTLE, P. T. & NOBLE, J. M. (1983*b*). The effect of barium chloride on intestinal secretion in the rat. *Journal of Physiology* **344**, 69–80.

HARDCASTLE, J., HARDCASTLE, P. T., READ, N. W. & REDFERN, J. S. (1981). The action of loperamide in inhibiting prostaglandin-induced intestinal secretion in the rat. *British Journal of Pharmacology* **74**, 563–569.

HUBEL, K. A. (1976). Intestinal ion transport: effect of norepinephrine, pilocarpine and atropine. *American Journal of Physiology* **231**, 252–257.

HUBEL, K. A. & CALLANAN, D. (1980). Effects of Ca^{2+} on ileal transport and electrically induced secretion. *American Journal of Physiology* **239**, G18–22.

ILUNDAIN, A. & NAFTALIN, R. J. (1979). Role of Ca^{2+}-dependent regulator protein in intestinal secretion. *Nature* **279**, 446–448.

KREBS, H. A. & HENSELEIT, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* **210**, 33–66.

MALAGODI, M. H. & CHIOU, C. Y. (1974). Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studies in smooth muscle. *European Journal of Pharmacology* **27**, 25–33.

MUNCK, B. G. (1970). Interactions between lysine, Na^+ and Cl^- transport in rat jejunum. *Biochimica et biophysica acta* **203**, 424–433.

MUNCK, B. G. (1972). Effects of sugar and amino acid transport on transepithelial fluxes of sodium and chloride of short circuited rat jejunum. *Journal of Physiology* **223**, 699–717.

MUNCK, B. G. & SCHULTZ, S. G. (1974). Properties of the passive conductance pathway across *in vitro* rat jejunum. *Journal of Membrane Biology* **16**, 163–174.

SCHULTZ, S. G., FRIZZELL, R. A. & NELLANS, H. N. (1974). Ion transport by mammalian small intestine. *Annual Review of Physiology* **36**, 51–91.

SEEMAN, P. (1972). The membrane actions of anaesthetics and tranquilizers. *Pharmacological Reviews* **24**, 583–655.

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.

SMITH, R. J. & IDEN, S. S. (1979). Phorbol myristate acetate-induced release of granule enzymes from human neutrophils: inhibition by the calcium antagonist 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride. *Biochemical and Biophysical Research Communications* **91**, 263–271.

TAI, Y.-H. & DECKER, R. A. (1980). Mechanisms of electrolyte transport in rat ileum. *American Journal of Physiology* **238**, G208–212.

TAYLOR, A. & WINDHAGER, E. E. (1979). Possible role of cytosolic calcium and Na–Ca exchange in regulation of transepithelial sodium transport. *American Journal of Physiology* **236**, F505–512.

TROTTER, J. A. & QUINTANA, R. L. (1981). Inhibition of macrophage spreading by antagonists of cellular calcium. *FEBS Letters* **129**, 29–32.

WEISS, B., PROZIALECK, W., CIMINO, M., BARNETTE, M. S. & WALLACE, T. L. (1980). Pharmacological regulation of calmodulin. *Annals of the New York Academy of Sciences* **356**, 319–345.

ZIMMERMAN, T. W., DOBBINS, J. W. & BINDER, H. J. (1983). Role of calcium in the regulation of colonic secretion in the rat. *American Journal of Physiology* **244**, G552–560.