The effect of drugs acting on cholinceptors and mucosal chloride on luminal bicarbonate transport by rat caecum under *in vitro* conditions

¹Paul Canfield & Tarik Abdul-Ghaffar

Department of Physiology & Biophysics, St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG

- 1 The transport of HCO_3^- (Jsm) from a HCO_3^- -buffered serosal to an unbuffered mucosal saline solution has been studied in rat caecum *in vitro*.
- 2 Carbachol, bethanechol and acetylcholine (ACh) caused a concentration-dependent fall in Jsm with similar maximum effects. 1,1-Dimethyl-4-phenyl-piperazinium iodide (DMPP) also inhibited Jsm but the effect was less than with the other drugs. Maximum cholinceptor inhibition was less than that obtained with anoxia.
- 3 Responses were blocked by atropine (10^{-5} M) but hexamethonium (2×10^{-4} M) significantly altered the response only to DMPP.
- 4 Physostigmine (10^{-5} M) shifted the ACh response curve to the left but physostigmine itself caused inhibition of Jsm which was blocked by atropine.
- 5 Substitution of mucosal Cl^- by NO_3^- reduced Jsm to a similar extent to maximum cholinceptor effect and abolished responses to bethanechol. Anoxia further reduced Jsm in the presence of NO_3^- .
- 6 Mucosal SITS and DIDS (1 mM) reduced Jsm but this was less than the maximum inhibition seen with drugs acting on cholinceptors or mucosal Cl^- removal. Serosal DIDS caused a similar inhibition.
- 7 We conclude that cholinceptor agonists inhibit but do not abolish luminal bicarbonate transport by an action on muscarinic receptors.

Keywords: Bicarbonate transport; cholinceptor agonists and antagonists; caecum *in vitro*; mucosal chloride substitution; anion transport inhibitors

Introduction

The rat caecum is a major site for absorption of water, salt and short chain fatty acids (Umesaki *et al.*, 1979; Edmonds & Thompson, 1980). Under *in vitro* conditions, it exhibits an alkaline flux from a bicarbonate-buffered serosal bathing solution to an unbuffered mucosal solution (Jsm) and this is sustained for several hours. Replacement of the bicarbonate/ CO_2 buffer by HEPES/ O_2 abolishes the flux whilst anoxia reduces it by about two thirds (Canfield, 1991). There have been several studies characterizing ion transport in rabbit caecum (Clauss *et al.*, 1985; 1989; Hatch, 1987; Hatch & Freel, 1988) but not in rat and none of the studies has looked at the effects of physiological mediators on ion transport in caecal tissue. This paper describes the effects of drugs acting on cholinceptors on luminal bicarbonate secretion in the rat caecum *in vitro*. Some of these results have been presented to the Physiological Society (Abdul-Ghaffar & Canfield, 1990).

Methods

Tissue preparation

Normally fed, male Wistar rats (250–350 g) were killed by cervical dislocation. The abdomen was opened by a mid-line incision and the caecum exteriorised, dissected free of mesentery and removed. It was opened and the contents washed away with cold, isotonic saline. Portions of the tissue were tied over the end of a plastic tube (cross sectional area 1.13 cm^2) with the mucosa facing the tube lumen. Six pieces of tissue were set up in each experiment from 2 animals. This is essentially the same apparatus previously described for use with rat stomach (Canfield & Price, 1981). The lumen of the tube contained 5 ml of a solution of composition (in mM): NaCl 136, KCl 5,

MgSO_4 1.2, CaCl_2 2.4 and glucose 11.7, gassed with 100% O_2 . The tube was suspended in a bath containing 30 ml of a similar solution in which 26 mM NaHCO_3 replaced an equivalent amount of NaCl. This was gassed with 95% O_2 : 5% CO_2 (pH 7.4) and maintained at 36°C .

Measurement of alkalisation

The unbuffered solution was replaced every 15 min throughout the experiment for determination of HCO_3^- by back-titration with 5 mM HCl using an autotitrator system (ABU 80, Radiometer, Copenhagen). During titration at room temperature the solution was continuously gassed with 95% O_2 : 5% CO_2 . The titration end point was determined as that pH obtained when a sample of unbuffered saline taken directly from the stock reservoir was similarly gassed and this was redetermined several times during an experiment. This method of estimating alkalisation was validated by adding known amounts of NaHCO_3 to 5 ml of unbuffered saline equivalent in amount to that produced by the tissues. A linear regression of 'recovered' bicarbonate by titration against added bicarbonate gave a correlation coefficient of 0.996 ($n = 24$) with a slope of 1.03. The advantage of titrating in this way is that titrations were sufficiently rapid to perform six in 15 min with one autotitrator, in contrast to the need to bubble with N_2 for 20 min when titrating to an alkaline end point as reported by Dietz & Field (1973) or the need for one autotitrator per tissue for the method of continuous pH-stat titration (Flemstrom, 1977; Sullivan & Smith, 1986).

Drugs

The following were used; acetylcholine chloride, hexamethonium chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), physostigmine, bethanechol (carbamyl β -methyl choline chloride), carbachol (carbamylcholine chloride), atropine sulphate, tetrodotoxin (TTX), 4-acetamido-4'-isothiocyanatostilbene-2-2'-disulphonic acid (SITS), and

¹ Author for correspondence.

4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS). All drugs were obtained from Sigma, Poole, Dorset, and were made up on the day of the experiment and added to the serosal side unless otherwise indicated.

Expression of results

Measurements of alkaline flux from serosa to mucosa are designated as J_{sm} . Tissues were allowed to recover for 60 min before flux measurements began and the unbuffered solution was changed every 15 min during this period. Statistical comparisons were made between the mean steady basal value of the 15 min period immediately preceding the experimental procedure and the final 15 min flux value at the end of the procedure period. Results are expressed as mean $\pm$ s.e.mean as $\mu\text{mol cm}^{-2} \text{ h}^{-1}$ of base. Statistical analysis was performed with either a paired or unpaired Student's *t* test as appropriate and *P* values less than 0.05 were taken as significant. The number of pieces of tissue used in each study is indicated by *n*.

Ion substitution

In some experiments Cl^- was excluded from the unbuffered saline in contact with the mucosa, being replaced with nitrate (NaNO_3 , $\text{Ca(NO}_3)_2$). The other ions were unchanged. When solution changes were made the tissue was washed 3 times at 5 min intervals with the new mucosal solution before flux measurements recommenced and the titration end point with the new solution was determined.

Results

Cholinoceptor agonists

Acetylcholine (ACh), carbachol and bethanechol all inhibited J_{sm} and the maximum effect was not significantly different between the drugs. Figure 1 shows the decrease of J_{sm} from the preceding level of flux plotted against drug concentration for these drugs plus the effect of DMPP which was also inhibitory but had a significantly lower maximum effect. Maximum values were; ACh = 2.4 ± 0.5 ($n = 6$ at $5 \times 10^{-4} \text{ M}$), bethanechol = 2.7 ± 0.4 ($n = 11$ at $2.5 \times 10^{-4} \text{ M}$), carbachol = 2.6 ± 0.3 ($n = 6$ at 10^{-5} M) and DMPP

Figure 1 Reduction in rate of bicarbonate secretion from preceding basal value in each tissue ($\mu\text{mol cm}^{-2} \text{ h}^{-1}$) against log drug concentration. In (a) carbachol (●), bethanechol (○) and 1,1-dimethyl-4-phenyl piperazinium (DMPP) (△). In (b) acetylcholine alone (●) and in the presence of physostigmine 10^{-5} M (○). All values are mean with s.e.mean (vertical bars) and number of tissues in parentheses.

Figure 2 The effect of a maximum concentration of carbachol with subsequent application of anoxia on basal bicarbonate secretion (J_{sm} $\mu\text{mol cm}^{-2} \text{ h}^{-1}$). Values are mean with s.e.mean (vertical bars) and $n = 4$.

$1.2 \pm 0.2 \mu\text{mol cm}^{-2} \text{ h}^{-1}$ ($n = 6$ at $5 \times 10^{-4} \text{ M}$). The maximum inhibition obtained was significantly less than that seen following 30 min of anoxia in the same tissues as shown in Figure 2 for bethanechol.

Cholinoceptor antagonists

Atropine (10^{-5} M) inhibited the response to all four agonists (Table 1). Hexamethonium ($2 \times 10^{-4} \text{ M}$) had no significant effect on responses to carbachol and acetylcholine but the response to DMPP was reduced to a value not significantly different from zero.

Effect of physostigmine

One possible explanation for the small response to DMPP is that it results from the release of endogenous ACh which is then rapidly broken down by the tissue, effectively truncating the response. The use of the choline esterase inhibitor, physostigmine (10^{-5} M) should allow testing of this hypothesis. We first tested physostigmine with exogenous ACh (Figure 1b) where it shifted the concentration-response curve to the left indicating that the tissue did indeed break down ACh. However, a complication was found in these experiments in

Table 1 Effect of hexamethonium (C6) and atropine (Atr) on the inhibitory action of cholinoceptor agonists on bicarbonate secretion

Agonist	Control	C6	Atr	n
Carbachol	$2.1 \pm 0.4^*$	$2.2 \pm 0.5^*$	0.5 ± 0.4	8
Bethanechol	$1.9 \pm 0.4^*$	not done	0.2 ± 0.1	6
ACh	$1.4 \pm 0.2^*$	$1.2 \pm 0.2^*$	0.2 ± 0.2	6
DMPP	$0.5 \pm 0.1^*$	0.1 ± 0.3	0.2 ± 0.4	6

Values are mean fall in rate of bicarbonate secretion from preceding basal values (in $\mu\text{mol cm}^{-2} \text{ h}^{-1}$) $\pm$ s.e.mean. Tissues were either controls or were pre-incubated for 60 min with hexamethonium ($2 \times 10^{-4} \text{ M}$) or atropine (10^{-5} M). They were then exposed for a further 60 min to one of the following; carbachol 10^{-5} M , bethanechol 10^{-5} M , acetylcholine 10^{-4} M or the nicotinic agonist 1,1-dimethyl-4-phenyl piperazinium (DMPP) 10^{-4} M .

* indicates a significant ($P < 0.05$) fall in rate of secretion. All responses were blocked by atropine but only DMPP was affected by hexamethonium.

Table 2 Effect of physostigmine and atropine on basal bicarbonate secretion

Treatment	Jsm ($\mu\text{mol cm}^{-2} \text{h}^{-1}$)	
	Experiment A	Experiment B
Initial rate	4.6 ± 0.3	4.3 ± 0.2
+ Physostigmine	$4.0 \pm 0.2^*$	
+ Physostigmine + atropine	4.5 ± 0.3	4.3 ± 0.4

Values are mean rate of bicarbonate secretion (Jsm) $\pm$ s.e.mean, $n = 6$ in both experiments. * indicates a significant difference from the initial value ($P < 0.05$) by paired t test. In experiment A, physostigmine (10^{-5} M) was added for 60 min after a steady initial value was obtained and then atropine (10^{-5} M) was also added for a further 60 min. In experiment B only atropine was added for 60 min. Atropine alone had no effect on basal Jsm but did reverse the effect of physostigmine.

that physostigmine alone resulted in an inhibition of Jsm. Overall, physostigmine (10^{-5} M) resulted in a mean decrease of $0.67 \mu\text{mol cm}^{-2} \text{h}^{-1}$ ($n = 65$, 95% confidence limits 0.45 – $0.88 \mu\text{mol cm}^{-2} \text{h}^{-1}$). The effect of physostigmine was blocked by atropine indicating an action via muscarinic receptors and presumably due to endogenous ACh. Atropine had no effect on basal Jsm in the absence of physostigmine (Table 2). TTX (10^{-6} M) did not block the effect of physostigmine (Table 3)

Table 3 Effect of tetrodotoxin (TTX) on basal bicarbonate secretion and response to physostigmine

Experiment A	
Initial rate	3.9 ± 0.5
+ TTX	3.8 ± 0.6
+ TTX + physostigmine	$3.6 \pm 0.6^*$
Experiment B	
Initial	4.6 ± 0.4
+ Physostigmine	$3.9 \pm 0.3^*$
+ Physostigmine + TTX	$4.0 \pm 0.3^*$

Values are mean rate of bicarbonate secretion (Jsm in $\mu\text{mol cm}^{-2} \text{h}^{-1}$) $\pm$ s.e.mean and $n = 6$ for both experiments. * indicates a significant difference ($P < 0.05$) from initial value by paired t test. In experiment A, tetrodotoxin (TTX 10^{-6} M) was added for 45 min when an initial steady rate had been reached then physostigmine (10^{-5} M) was also added for a further 45 min. In experiment B the drugs were added in the reverse order. TTX alone did not alter basal Jsm nor did it prevent the effect of physostigmine

Figure 3 The effect of substituting mucosal chloride with nitrate and subsequent application of anoxia on basal bicarbonate secretion (Jsm $\mu\text{mol cm}^{-2} \text{h}^{-1}$). Values are mean with s.e.mean (vertical bars) and $n = 5$. **Figure 4** Comparison of the effect on basal bicarbonate secretion (Jsm $\mu\text{mol cm}^{-2} \text{h}^{-1}$) shown by open columns, of 3 treatments (A, B or C), shown by stippled columns and the effect of subsequent application of anoxia (hatched columns) on the treatment response. (A) Maximum concentration of carbachol ($5 \times 10^{-5} \text{ M}$); (B) mucosal Cl^- substituted with NO_3^- and (C) maximum concentration of bethanechol ($5 \times 10^{-4} \text{ M}$). Values are mean with s.e.mean (vertical bars) and in (A) $n = 6$; in (B) $n = 5$ and in (C) $n = 4$. There were no significant differences between the effects on Jsm of the initial treatments nor between the values following subsequent application of anoxia.

suggesting that release of endogenous ACh did not result from spontaneous activity in the intrinsic nervous system of the tissue. These results mean that concentration-response curves in the presence of physostigmine would have to be constructed against an uncontrolled background of ACh-induced inhibition and experiments with physostigmine were discontinued.

Replacement of mucosal chloride

Drugs acting on cholinceptors are well established as stimulants of electrogenic chloride secretion and inhibitors of neutral NaCl uptake in the intestine (Donowitz & Welsh, 1987). Thus, they may inhibit bicarbonate secretion in the caecum by inhibiting a $\text{HCO}_3^-/\text{Cl}^-$ exchange mechanism. We therefore, examined the effect of removing Cl^- from the unbuffered solution in contact with the mucosa, on basal Jsm and the response to bethanechol. The results are shown in

Table 4 Effect of 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) and anoxia on rate of bicarbonate secretion (Jsm)

Treatment	Jsm ($\mu\text{mol cm}^{-2} \text{h}^{-1}$)			
	Initial	+ Drug	Fall Jsm	Anoxia
Mucosal DIDS 1 mM ($n = 15$)	4.7 ± 0.3	3.4 ± 0.2	$1.3 \pm 0.2^*$	1.4 ± 0.1
Mucosal SITS 1 mM ($n = 6$)	4.7 ± 0.6	4.1 ± 0.4	$0.6 \pm 0.2^*$	1.7 ± 0.1
Serosal DIDS 1 mM ($n = 6$)	4.3 ± 0.5	2.6 ± 0.3	$1.7 \pm 0.3^*$	1.1 ± 0.2

The drug (DIDS or SITS) was added when the initial values were steady. The drug value was after 60 min exposure. The anoxia value followed 45 min treatment in the presence of the drug used. The fall in Jsm column was calculated from the difference between initial and drug value for each tissue. The values are mean $\pm$ s.e.mean and all three drug treatments resulted in a significant ($*P < 0.05$) fall in Jsm. There was no significant difference between results with mucosal and serosal DIDS by unpaired t test.

Figure 3. Replacement with nitrate resulted in a decrease of Jsm which became steady after 30 min and was further reduced by anoxia. The results are quantitatively the same as the results obtained with maximal inhibition by bethanechol or carbachol with subsequent application of anoxia and are summarised in Figure 4 for comparison. Bethanechol (2.5×10^{-4} M) was without effect in NO_3^- -substituted mucosal saline.

Action of anion transport inhibitors

To test further the relationship between Cl^- and HCO_3^- , we examined the effect of two putative anion exchange inhibitors; DIDS or SITS (1 mM) were applied to the mucosal side for 60 min and Jsm compared with the pre-drug value. Results are shown in Table 4. Both inhibitors caused a significant reduction in Jsm but this was less than the maximum inhibition obtained with cholinergic agonists or with mucosal Cl^- replacement. Application of 1 mM DIDS to the serosal side also inhibited Jsm and the effect was not significantly different from that obtained with the mucosal drug.

Discussion

Cholinergic agonists

The cholinergic agonists clearly have an inhibitory effect on the luminal bicarbonate flux (Jsm) in this preparation. The reversal of the effect by atropine and the very weak action of hexamethonium indicate that inhibition is via muscarinic receptors. Zimmerman & Binder (1982) have reported that rat colonic enterocytes have muscarinic receptors so that inhibition may be a direct effect on the mucosal cells but our results do not allow us to distinguish between this and an indirect effect mediated by the cholinergic-mediated release of another, unidentified agent from elsewhere in the tissue. The maximum level of inhibition obtained with bethanechol and carbachol was significantly less than that seen in the presence of anoxia showing that part of the oxygen-dependent flux was resistant to cholinergic drugs. Although the nicotinic agonist DMPP inhibited Jsm and the effect was prevented by hexamethonium, the maximum response was small. This may have been the result of a rapid breakdown of acetylcholine released postganglionically but attempts to test this with physostigmine were frustrated by the inhibitory effect of physostigmine itself which is discussed below.

Effect of physostigmine

Physostigmine alone caused a variable amount of inhibition of Jsm which could be prevented by atropine. The most likely explanation for this is that there is a spontaneous release of acetylcholine in the tissue and that this is normally broken down so rapidly that it does not effect the measured Jsm (atropine alone did not alter Jsm). In the presence of physostigmine, this acetylcholine would persist and inhibit Jsm; the variability of the effect between tissues reflecting differing rates of release of endogenous acetylcholine. The inability of TTX to prevent this action of physostigmine suggests that the acetylcholine release did not result from spontaneous activity in the intact intrinsic nerve system of the preparation. Similar results were reported by Cooke (1984) on the effect of physostigmine on short circuit current in guinea-pig ileum. Although physostigmine did result in a leftward shift of the acetylcholine concentration-response curve, this cannot be directly compared with the curve for acetylcholine alone because the concentration of endogenously released acetylcholine at the receptors cannot be determined. A possible alternative explanation for the weak inhibitory effect of DMPP is that non-selective stimulation of the ganglia results in activation of both an inhibitory and stimulatory pathway, each capable of separately influencing Jsm. Harris *et al.* (1989)

have reported that distension of rat distal colon, both *in vivo* and *in vitro*, stimulated bicarbonate secretion via an intrinsic neural reflex but have not identified the mediator. The possible role of the intrinsic nervous system in regulating caecal bicarbonate secretion should be investigated further.

Ion substitution and anion transport inhibitors

Substitution of mucosal Cl^- with NO_3^- reduced Jsm by the same amount as maximal cholinergic inhibition (in the presence of mucosal Cl^-) and also abolished the effect of cholinergic drugs on Jsm. In neither situation was the oxygen-dependent part of Jsm completely inhibited. The inhibitory effect of mucosal Cl^- removal conflicts with the previously reported lack of effect of bilateral Cl^- removal in this preparation (Canfield, 1991) but it is likely that the solution changing in the earlier study was not adequate as the washes were made in rapid succession rather than at 5 min intervals. Interpretation of the results of Cl^- substitution is difficult without further studies. The removal of Cl^- from the mucosal solution will alter ionic concentration gradients across the tissue and its replacement with a less permeant ion is likely to hyperpolarize the luminal surface which would oppose the exit of anions (Feldman *et al.*, 1988). There is also the possibility that the substituting ion may be able to replace Cl^- on the exchanger to some extent and allow it to operate at a reduced rate of transport. Additionally, Cl^- may diffuse passively from the serosal to the mucosal side and thus become available to an exchanger. Such an effect would be limited by the replacement of the mucosal saline every 15 min but might allow a sufficient concentration of Cl^- to permit a reduced rate of operation.

In our experiments, mucosal SITS or DIDS significantly reduced Jsm but this reduction was much smaller than that produced by maximum cholinergic inhibition or replacement of mucosal Cl^- . As serosal DIDS was equally effective, it seems unlikely that this inhibition was the result of a specific action on an apical $\text{HCO}_3^-/\text{Cl}^-$ exchanger. In the earlier study (Canfield, 1991) where lower concentrations were used, only serosal DIDS caused a significant fall in Jsm. Smith *et al.* (1986) have previously drawn attention to non-specific effects of disulphonic stilbenes in rabbit colon. As there are also reports of stilbene-resistant anion exchangers in rabbit kidney (Schuster, 1985), turtle bladder (Kohn *et al.*, 1990) and rat distal colon (Feldman & Stephenson, 1990) epithelia, it would seem that these drugs are of limited general value for investigation of epithelial anion exchange mechanisms; a conclusion reached by Smith *et al.* (1986) in respect of rabbit proximal colon.

The quantitative similarity between the results with drugs acting on cholinergic receptors and mucosal Cl^- substitution may be a coincidence. Alternatively they could be explained if the oxygen-dependent part of Jsm resulted from the operation of at least two transport mechanisms; one which utilised an apical $\text{HCO}_3^-/\text{Cl}^-$ exchange mechanism and was totally inhibited by either cholinergic agonists or Cl^- removal and the other which was Cl^- -independent and unaffected by drugs acting on cholinergic receptors. Previous studies of *in vitro* bicarbonate transport in mammalian large intestine have reported Cl^- -independent secretion in rabbit proximal colon (Sullivan & Smith, 1986), total dependence on mucosal Cl^- in rat distal colon (Feldman *et al.*, 1990) and an electrogenic secretion in rabbit caecum (Hatch & Freel, 1988) and rat distal colon (Harris *et al.*, 1989). The proposed explanation for the present findings requires further investigation of the dependence of Jsm on mucosal Cl^- concentration, transmucosal p.d. and measurement of the relationship between HCO_3^- secretion and Cl^- uptake by tissues under various conditions. If these substantiate this explanation, then mucosal Cl^- substitution may offer the possibility of studying the regulation of these mechanisms separately. Two separately controlled mechanisms for luminal bicarbonate secretion have been proposed previously for amphibian duodenum (Flemstrom *et al.*, 1982).

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