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Reduction in Jejunal Fluid Absorption *In vivo* through Distension and Cholinergic Stimulation Not Attributable to Enterocyte Secretion

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Jejunal fluid absorption *in vivo* was reduced by distension and by hydrostatic pressure and further declined on adding *E. coli* STa enterotoxin but no net fluid secretion was detected. Luminal atropine reduced pressure mediated reductions in fluid absorption to normal values but intravenous hexamethonium was without effect. A neural component to inhibition of absorption by pressure (though not stretch) may be mediated by axon reflexes within cholinergic neurons. Perfusion of cholinergic compounds also reduced net fluid absorption but did not cause secretion. In order to show that these actions were not secretory processes stimulated by cholinergic compounds that offset normal rates of absorption, these compounds were tested for their ability to cause net secretion in loops that were perfused with solutions in which choline substituted for sodium ion. In addition, these perfusates additionally contained *E. coli* STa enterotoxin or EIPA (ethyl-isopropyl-amiloride) to minimize absorption. In these circumstances, where it might be expected to do so if it

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were acting through a secretory rather than an absorptive mechanism, carbachol did not cause net fluid secretion. Cholinergic stimulation and pressure induced distension are thought to reduce net fluid absorption through inducing secretion but are found only to reduce fluid absorption. In conclusion, distension and cholinergic stimulation of the small intestine are two further circumstances in which fluid secretion is assumed to explain their action on fluid movement, as required by the enterocyte secretion model of secretion but, which like STa enterotoxin, instead are only able to reduce fluid absorption. This casts further doubt on the widespread validity of the enterocyte secretion model for fluid appearance in the lumen in diarrhoeal diseases.

Keywords: Intestine; secretion; diarrhea; intestinal stretch; E. coli STa; enterotoxin; enterocyte.

1. INTRODUCTION

For several decades, fluid secretion into the intestinal lumen, mediated by the enterocytes of the small intestinal villi [1], has been assumed to be the mechanism underlying many forms of diarrhoeal disease. Distension [2-4], exposure to *E. coli* STa enterotoxin and cholinergic agents [5] are all thought to cause net secretion but it is evident now that STa is not a secretory toxin when fluid movement is determined by luminal volume recovery [6]. Net secretion is only claimed to occur when non volumetric methods, such as short-circuit current or potential difference measurements [7], are chosen as proxies for mass transport of fluid *in vitro* or when *in vivo* volumetric methods are used that conflate movement of fluid from the vasculature to the interstitial space with the unproven further step of movement from the interstitial space into the lumen [8].

Given the fact that STa enterotoxin is not secretory but is able to achieve almost complete cessation of fluid absorption, it can be used to test circumstances currently believed to act by stimulating fluid secretion. In a poorly absorbing loop, substances deemed to cause secretion should be able to cause further measurable net fluid secretion into the lumen and it should be unnecessary to deduce the occurrence of it from the ambiguous finding of reductions in net fluid absorption. For this reason, intestinal distension and exposure to cholinergic agents was investigated in the presence of STa enterotoxin to determine whether net secretion *in vivo* could be found. Both distension and exposure to cholinergic agents did not cause net secretion but instead showed no further reduction in fluid absorption beyond that already caused by STa enterotoxin.

In addition, conclusions about the deemed neural control of these processes, when investigated by this method, were found to diverge markedly from those drawn when using *in vivo* methods based on measurements that inadvertently also include the weight contributed by the interstitial space [5,9]. Pressure mediated intestinal distension and exposure to cholinergic agents were found to be further circumstances that are able to prevent fluid absorption but unable to extend beyond that point and cause the net secretion required by the enterocyte secretion paradigm.

2. METHODS

2.1 *In vivo* Perfusion Procedures

All experiments complied with UK (1986) animal research legislation. Absorption of fluid *in vivo* from perfused jejunal loops was measured by a recirculation procedure [10], described in detail elsewhere [6,11], using a fluid recovery method [12]. Adult Sprague-Dawley female rats were anaesthetised (70 mg/Kg i. p.) with sodium pentobarbitone (Sagatal). Isolated loops about 25 cm in length were perfused in the distal direction at 1.8 ml/ min by a peristaltic pump (Crouzet 82344, UK) from a 30 ml reservoir maintained at 37^0C. The output cannula was 4 mm, as wide as possible (Fig. 1d) to maintain intraluminal pressure at atmospheric pressure. After the perfusion period, fluid was pumped in to the fluid reservoir, the loop was excised so that remaining fluid in the loop could be retrieved, assisted by flushing with air. Volume was measured to the nearest 0.1ml, this representing 0.4% of the initial 25 ml perfusate volume that consisted of 100 mM sodium bicarbonate and 54 mM sodium chloride, unless otherwise specified. Fluid absorption, the difference between the initial added and final recovered volume, is expressed as microlitres per cm length of intestine per hour, positive values denoting absorption. In some experiments intended to show that the prepared loops could be made to be secretory, experiments were done using the same protocol as had been used to demonstrate

secretion after *Clostridium difficile* exposure [13].

2.2 Stretch and Pressure Distension in the Perfused Loop

Prior to perfusion of a loop open at both ends, a 30 cm long, 1mm diameter, a blunt steel rod was introduced through the lumen in order to pull a ligature thread along its length. The distending device was a 1mm diameter length of polythene tubing folded back on itself and tied in order to give a fifteen cm figure of eight length of distending tubing (Fig. 1e). The introduced ligature thread was tied to the distending coil which was pulled gently into the loop. Once the coil was in place, the pulling ligature was cut and removed. The distended loop was re-introduced into the abdominal cavity with the ends cannulated in the usual way (Fig. 1c). Loops distended by hydrostatic pressure (Fig. 1b) had their outflow tube connected to a second reservoir, raised to 30 cm above the intestine. To maintain constant pressure, inflow was through the base of this reservoir with outflow through an exit port, 5 mm above the base. Any surge in fluid leaving the reservoir by syphonage could not therefore reduce the pressure head. Tissues for histological examination were stained with haematoxylin and eosin.

2.3 Administration of Chemicals and Enterotoxins

Hexamethonium was injected as a bolus dose of 10 mg/Kg into the jugular vein at hourly intervals, making three injections in total. Intravenous atropine was given as a bolus dose of 0.5 mg/Kg at the start of experiment and then at hourly intervals. Intraluminal atropine was perfused at a concentration of 100 μM. The perfusate concentration of STa was 80 ng/ml.

2.4 Source of Chemicals

E.coli STa and other chemicals were purchased from Sigma Chemical Co (Poole, Dorset, UK). Sagatal (sodium pentobarbitone) was obtained from Rhone Merieux Ltd (Harrow, London, UK).

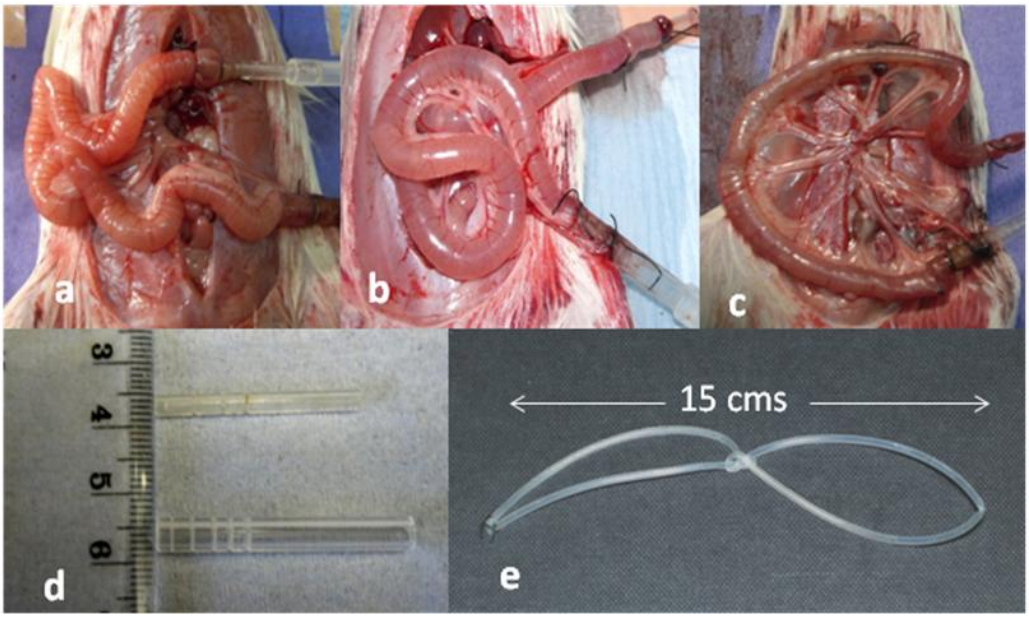

Fig. 1. a) insertion of perfusion cannulae into a loop with the loop undergoing normal perfusion. The upper entry cannula has an internal diameter of 2 mm, the lower exit cannula has an internal diameter of 4 mm (see section d of figure). b) A cannulated loop undergoing hydrostatic pressure distension of 30 cm during perfusion. c) a cannulated loop undergoing stretch after insertion of a polythene insert and undergoing perfusion. d) entry and exit cannulae showing sizes and machined grooves for tie on of ligature threads. e) polyethylene tubing made into a 15 cm long coil that distended the loop on insertion.

2.5 Statistical Analysis of Data

Results are given as the mean and standard error of the mean, followed by the number of experiments, equal to the number of animals in parenthesis. Implementation of all statistical analysis was done using BMDP [14]. Comparison of means was by paired and unpaired "Student's" 't'-values with Dunnett's [15] correction for multiple comparisons, if required.

3. RESULTS

3.1 A Physiological Assessment of the Fluid Recovery Protocol for Measuring Intestinal Fluid Transport

Measuring fluid transport by luminal fluid recovery avoids the alternative method of volume marking using putatively unabsorbable solutes. These solutes are now known to introduce gross errors because of substantial absorption [16] that gives false positive indications of secretion [17], both in short duration experiments and when low absorption rates occur. Three hours perfusion duration was chosen to maximize the chances of detecting small but non-zero rates of fluid secretion. A critique of the fluid recovery method is that the *E. coli* STa induced intestinal secretion that assumedly should occur cannot be shown because of the lengthy perfusion period and other accessory conditions, including perfusate content, poor maintenance of blood pressure, differing responses between male and female rats, paradoxical responses to anaesthesia or simply just an unspecifiable inability to respond to secretory stimuli for unknown reasons. Since all prior observations on the failure to secrete fluid after STa have arisen to date only when the fluid recovery technique was used, with an implied technique mediated failure to show secretion rather than an absence *per se* of secretion, it was necessary to consider maintenance of blood pressure and heart rate over the duration of these experiments.

Using the recovered volume preparation, initial heart rates of 394 ± 22 (5) beats per minute were found that did not vary throughout perfusion and ended at 379 ± 4.6 (5) beats per minute. Mean arterial blood pressure of 132 ± 9.0 (5) mmHg at the onset, remained steady and ended at 125 ± 4.0 (5) mmHg. Falls in pressure were inducible by i.v. perfusion with 2.8 mg/Kg/hr cromakalim, from 108 ± 11.7 (5) to 62.4 ± 7.0 (5) mmHg. Hence, while barbiturate anaesthetised animals did not suffer from deleterious blood pressure changes that might have prejudiced the detection of secretion, falls in blood pressure were detectible when deliberately induced. Sex of the animals was also not relevant to maintenance of mean arterial blood pressure using this protocol, since initial and final blood pressure were similar in both cases.

Intravenous infusion of glucose-containing saline is sometimes advocated for maintenance of adequate mean arterial blood pressure. In the absence of intravenous hydration, blood pressure was well maintained using the present protocol. Perfusate bicarbonate anion concentrations resembling those that arise in the lumen during pancreatic secretion, promote fluid absorption [18]. Intestinal fluid intake of 5 - 6 mls over three hours already approximates substantial hourly rates of i.v. fluid infusion. Hence, superimposed i.v. infusion would predispose to fluid secretion by expansion of plasma volume. Glucose in the intestinal perfusate by stimulating fluid absorption would obscure any STa mediated secretion taking place. The experiments would therefore be on intestine subjected to bacterial enterotoxin but also simultaneously to a form of oral rehydration solutions as occurs with most, if not all, *in vitro* and some *in vivo* experiments.

In assessing fluid transport, measurements are now rarely done directly by fluid recovery but more frequently estimated indirectly by electrical measurements, having assumed that these faithfully reflect alterations in fluid movement. A further particular difficulty in assessing secretion and its inhibition by pharmaceutical means is used of an ostensibly reliable preparation that conflates interstitial as well as luminal volume change by aggregating them both into a single weight measurement. The preparation, devised originally to estimate capillary filtration coefficient by loop weight change [9, Fig. 3; 19, Fig. 2], was thereafter often used to measure luminal fluid secretion.

To be in conformity with the enterocyte secretion hypothesis, the interpretation to be deduced from weight increase is that increased enterocyte secretion into the lumen has occurred. However, given the method of measurement, it is not proven that fluid has entered the lumen and by extension, that the driving force for fluid movement is provided by the enterocytes. In contrast, luminal volume recovery as used in the present paper unambiguously measures whether net fluid secretion has occurred or not, since

change in luminal volume occurs free from distortions arising from altered interstitial volume.

3.2 The Effect of Distension on Jejunal Fluid Absorption

3.2.1 Distension by stretch through coil insertion and by hydrostatic pressure

The unstretched intestine absorbed fluid at a rate of 105.0 ± 11.3 (7) ul/cm/hr. In the stretched loop (Fig. 2 & Table 1), there was a significantly lower ($p<0.001$) rate of 40.0 ± 5.0 (10) ul/cm/hr absorption, that was also higher ($p< 0.001$) than zero net absorption. When STa was added to mechanically distended loops, fluid absorption was 14 ± 13 (6) ul/cm/hr, not different from zero absorption, and highly significantly less ($p<0.001$) than control rates of absorption, although not significantly below rates of absorption in the presence of distension alone.

When the loop was distended by hydrostatic pressure, the rate of absorption was 54.4 ± 7.9 (9) ul/cm/hr, significantly ($p<0.01$) less than unstretched intestine (Fig. 2) but no different (Table.1) from the rate of absorption in the intraluminally stretched loops. On addition of STa to pressure distended loops, the rate of absorption fell significantly ($p<0.05$) further to 19 ± 7 (7) ul/cm/hr, less than the pressure treated loops without STa and also less than normal loops ($p<0.01$) but the rate was still significantly ($p< 0.02$) absorptive and not secretory.

3.2.2 Effect of i.v. hexamethonium and 100 µm luminal atropine on fluid absorption after stretch or pressure distension

Hexamethonium (i.v. 10 mg/Kg) had no effect (Table 2) on the reduced fluid absorption from the jejunal loops after coil distension or after raised hydrostatic pressure. In separate

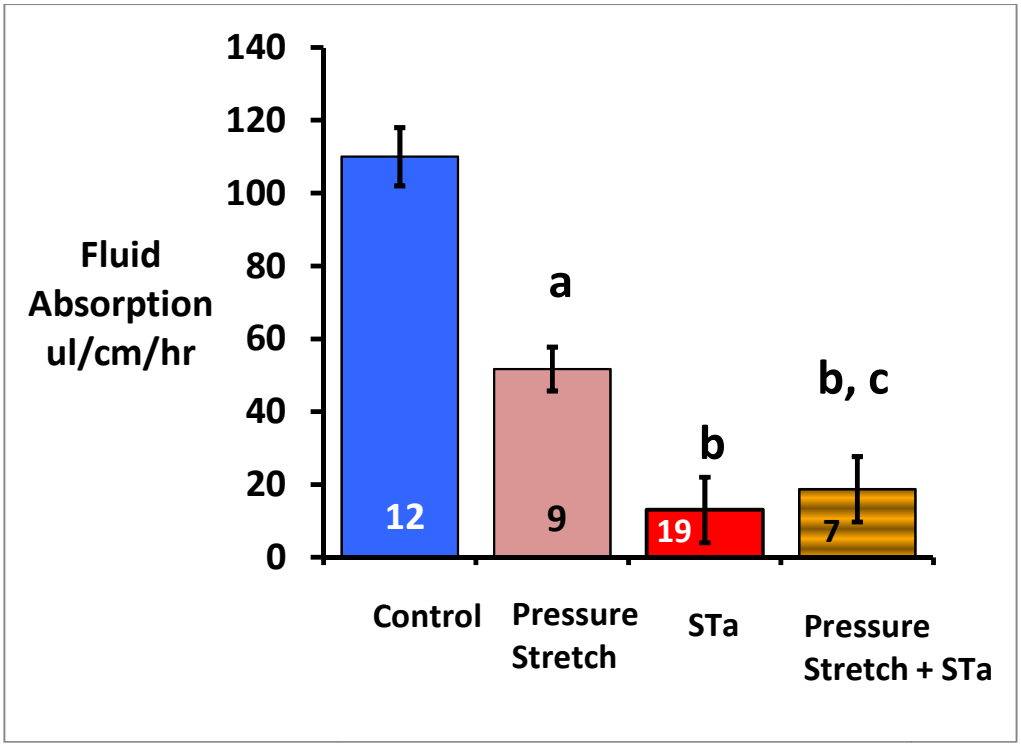

Fig. 2. The effect of intraluminal distension caused by 30 cm hydrostatic pressure on fluid absorption from the proximal jejunum *in vivo* of the anaesthetised rat, with and without co-perfusion of 80 ng/ml *E. coli* STa enterotoxin

Results are expressed as the mean and standard error of the mean with the number of experiments (equal to the number of animals) in parenthesis. Statistical significance was calculated by "Student's" 't'-test, a = $p<0.01$, b = $p<0.001$, comparison with control value, c = $p<0.05$ for comparison with value for pressure stretch.

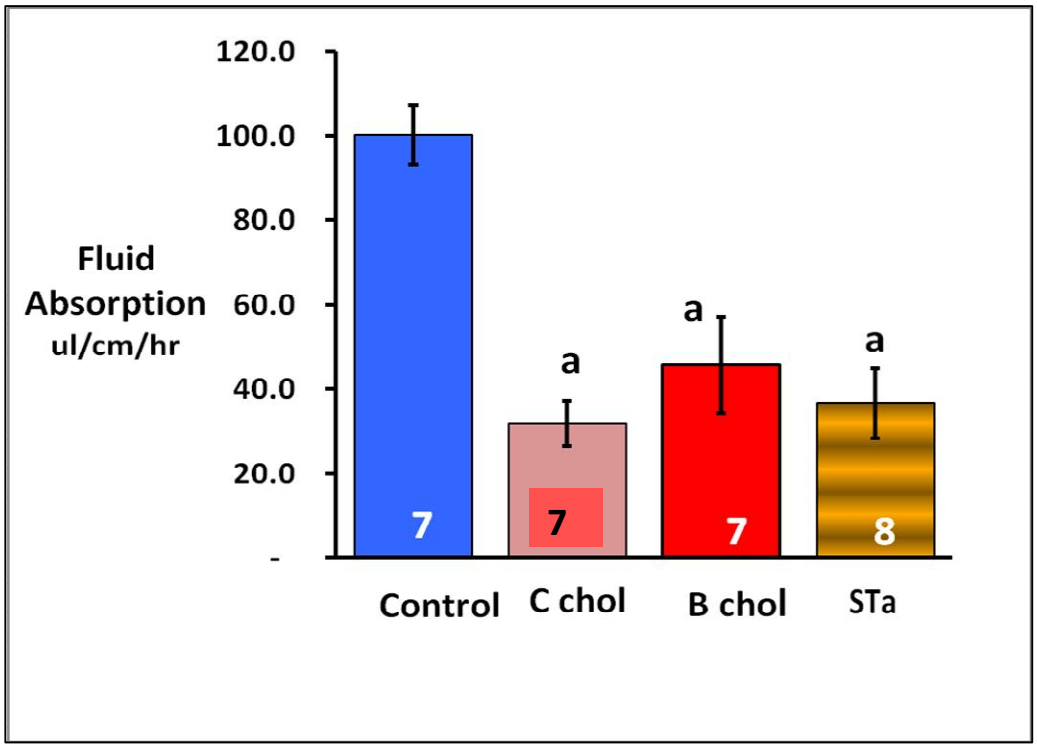

Fig. 3. The effect of 100 uM luminal carbachol (C), luminal bethanechol (B) and 80 ng/ml *E. coli* STa on fluid absorption from the proximal jejunum *in vivo* of the anaesthetised rat perfused with bicarbonate saline. Results are expressed as the mean and standard error of the mean with the number of experiments (equal to the number of animals) in parenthesis

Statistical significance, a = p< 0.001 for comparison with control values and a = not significant for differences between carbachol, bethanechol and STa mean values.

experiments, this dose reduced mean arterial blood pressure, showing that the lack of effect of hexamethonium on fluid absorption was not because the dose or route precluded any effects from happening. In the loops distended by coil, the fluid absorption rate of 40.0 ± 5.0 (10) ul/cm/hr was significantly ($p<0.001$) below the undistended control value of 105.0 ± 11.3 (12) ul/cm/hr (Table 1) and luminal atropine did not reverse this (Table 2). In contrast, atropine completely restored (Table 2) the reduction ($p<0.001$) caused by hydrostatic pressure from 54.4 ± 7.7 (9) to 102.8 ± 14.6 (5) ul/cm/hr. Luminal perfusion of atropine alone while restoring fluid absorption had no effect when administered alone in control loops.

3.3 Lack of Effect of i.v. Hexamethonium and Luminal Atropine on STa Inhibited Fluid Absorption

Since atropine but not hexamethonium restored fluid absorption in the jejunum that had been stretched by hydrostatic pressure, these substances were also investigated in loops that had been perfused with STa to inhibit fluid absorption. If STa had an additional secretory action, net fluid secretion should be detected rather than a reduction in absorption to rates close to zero absorption. Perfusion of STa expectedly reduced net fluid absorption. Luminal 100 uM atropine in loops perfused with STa did not restore net fluid absorption since this was 32.3 ± 8.1 (5) ul/cm/hr after atropine (Table 2) but 33.0 ± 8.5 (5) ul/cm/hr without atropine. The mean of 33.0 ± 8.5 (5) ul/cm/hr is taken from a subset of littermates derived from a larger mean of 16.1 ± 2.7 (19) experiments with STa alone but even comparison with this mean did not show any significant action of atropine. Similarly, i.v. administration of hexamethonium also failed to restore net fluid absorption inhibited by perfusion with STa since this was 15.5 ± 2.4 (5) ul/cm/hr after hexamethonium. The anti-cholinergic effect mediated by atropine did not restore the reduced fluid absorption after STa treatment as it did after pressure distension.

3.4 Lack of Effect of Luminal 0.1 mM Carbachol on STa and on EIPA Inhibited Fluid Absorption from Choline Perfusate

When either 100uM carbachol or bethanechol was perfused luminally in sodium ion containing perfusate, both substances significantly (P<0.01) reduced net fluid absorption rates (Fig. 3) from 98.8 ± 8.8 (7) ul/cm/hr. to 31.9 ± 5.4 (7) ul/cm/hr in the presence of carbachol and 47.5 ± 5.1 (7) ul/cm/hr in the presence of bethanechol i.e. close to values of 35.8 ± 9.2 (8) ul/cm/hr, obtained on STa perfusion. The reduction in net fluid absorption might have been caused by fluid secretion offsetting normal fluid absorption or simply from a reduction in fluid absorption. In these experiments, salivary secretion that was normally quiescent became copious. In a subset of experiments, the salivary flow was assessed as 1.1 ± 0.07 (5) ul/g body weight/hour versus 0.0 ± 0.0 (6) without perfusion of cholinergic agents.

Previous experiments [6] had demonstrated that when choline substituted for sodium ion, fluid absorption reversed to modest rates of fluid secretion and that STa increased this but when ethyl-isopropyl-amiloride preceded perfusion with STa, no increase in fluid secretion was noted. The increase in secretion detected when STa was perfused in sodium ion-free buffers was therefore still dependent on affecting a residual sodium ion uptake process incompletely inhibited by luminal absence of sodium ion i.e. notionally completely free sodium ion perfusates. STa was not therefore further inhibitory to fluid movement if all sodium ion absorption mechanisms were inhibited. This type of experiment was repeated using STa and 1.0 mM luminal carbachol.

The normal intestine when perfused with choline substituting for sodium ion did not absorb fluid but instead showed modest secretion at a rate of -10.0 ± 2.2 (10) ul/cm/hr. In the STa treated loop (Fig. 4), there was a significantly higher (p<0.001) rate of -27.7 ± 3.6 (7) ul/cm/hr secretion, which in the presence of STa and

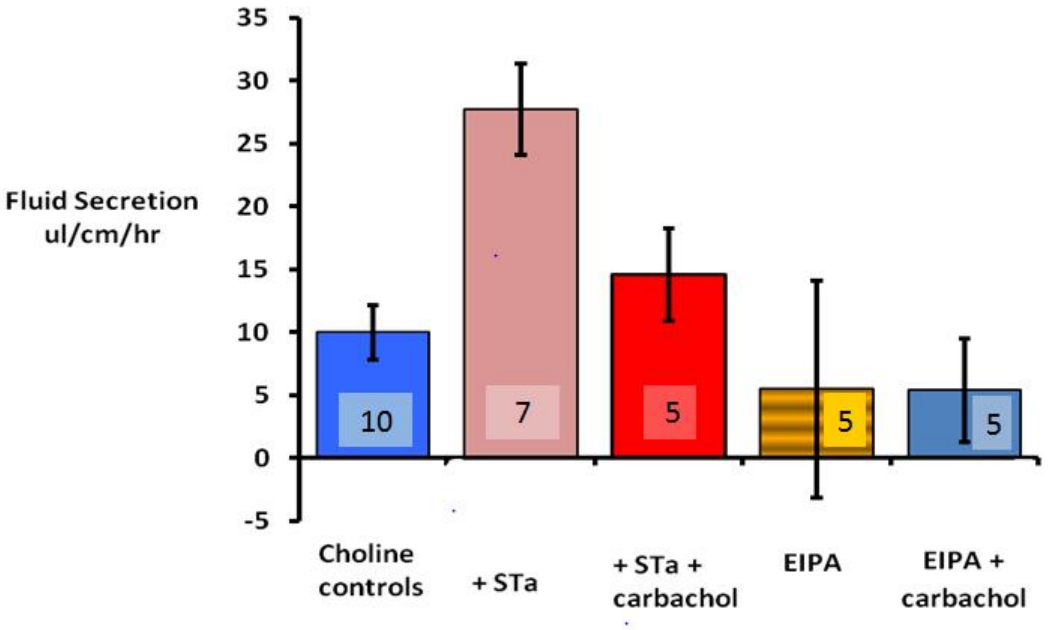

Fig. 4. The effect of luminal carbachol on fluid absorption from the proximal jejunum *in vivo* of the anaesthetised rat perfused by sodium-free choline substituted saline additionally containing 80 ng/ml *E. coli* STa or 0.1 mM EIPA (ethyl-isopropyl-amiloride) to minimise fluid absorption.

Results are expressed as the mean and standard error of the mean The number of experiments (equal to the number of animals) was n = 10 for the choline perfused control loops, n=7 for the choline plus STa perfused loops, n=5 for the choline plus STa plus 100uM carbachol loops, n=5 for the choline plus EIPA perfused loops and n =7 for the choline plus EIPA plus carbachol perfused loops

Table 1. The effect of mechanical stretch and pressure mediated stretch on jejunal fluid absorption in the presence and absence of luminal *E. coli* STa toxin, using the recovered volume technique in perfused loops *in vivo* in anaesthetised (Sagatal) rats.

Fluid absorption	No addition	STa addition
	ul/cm/hr	
Control loops	105.0 ± 11.3 (12)	16.1 ± 2.7 (19) ††
Stretch distension	40.0 ± 5.0 (10) **	13.8 ± 13.4 (6)
Pressure distension	54.4 ± 7.9 (9)	18.7 ± 6.9 (7)††

*Statistical significance by paired t-test, * = p < 0.02; **= p< 0.01 for stretch comparisons. † = p < 0.05, †† = p< 0.001 for +/- STa comparisons. Positive values indicate absorption.*

Table 2. The effect of i.v. hexamethonium (10 mg/kg) and 100 uM luminal atropine on mechanically stretched and pressure mediated stretched loops and STa treated loops on jejunal fluid absorption using the recovered volume technique in perfused loops *in vivo* in anaesthetised (Sagatal) rats.

Fluid absorption	No drug treatment	hexamethonium	atropine
	ul/cm/hr		
Stretch Distension	40.0 ± 5.0 (10)	36.5 ± 9.6 (5)	55.2 ± 5.9 (5)
Pressure Distension	54.4 ± 7.7 (9)	55.6 ± 6.0 (6)	102.8 ± 14.6 (5)**
E. coli STa treatment	16.1 ± 2.7 (19)†	15.5 ± 2.4 (5)†	32.3 ± 8.1 (5)

*Statistical significance by paired t-test, * = p < 0.02; **= p< 0.01 for drug comparisons. † = p < 0.05, †† = p< 0.001 for within drug differences STa comparisons. Positive values indicate absorption.*

carbachol was -14.6 ± 3.7 (5) ul/cm/hr and did not differ from values for choline perfusion alone. Similarly, when EIPA was used as the agent for blocking sodium ion uptake, fluid secretion was -5.5 ± 8.6 (5) ul/cm/hr, not different from zero absorption. When luminal carbachol was additionally perfused, there was no increase in secretion since the net rate was -5.4 ± 4.6 (5) ul/cm/hr, not different from perfusion in the absence of carbachol and not different from zero net absorption. The absence of sodium ion and addition of substances to inhibit sodium ion uptake further showed that carbachol did not elicit any fluid secretory event. Not shown in Fig. 4 is that 1.0 mM carbachol added to isosmotic sodium bicarbonate (154 mM) perfusate reduces fluid absorption significantly (p<0.001) from 100.2 ± 7.0 (10) ul/cm/hr to 31.9 ± 5.4 (7) ul/cm/hr. Hence, it is clear that carbachol reduces net fluid absorption but it only becomes clear that this is not through any secretory process when the possibility of fluid absorption is prevented by the combination of sodium ion free perfusates plus additional inhibitors.

4. DISCUSSION

An important aspect of investigating diarrhoeal diseases has been to determine the means by which fluid enters the small intestine. Enterotoxin mediated secretory diarrhoea is widely held to be achieved through causing the epithelial cells (enterocytes) of the villus to secrete abnormally large volumes of fluid [1,20,21]. Since the inception of this concept, various methods have been used in an attempt to measure enterocyte mediated secretion, including *in vitro* electrical measurements, *in vitro* radioactive tracer transport measurements or *in vivo* methods that are flawed [5] in that fluid could originate from the vasculature through vasodilatation and by-pass the enterocyte. This has led to the situation where the proxy methods of detecting secretion consistently demonstrate the features associated with exposure to enterotoxins, yet the enterotoxin need not cause net fluid secretion. A particularly clear expression of this dichotomy is the undoubted elevation in short-circuit current when small intestinal tissue is exposed to heat stable *E. coli* STa [7], whereas no net secretion is found *in vivo* in a perfused loop [6].

In order to determine whether net fluid secretion has occurred, irrespective of whether it is of enterocyte origin or not, the chosen method should unequivocally measure mass transport of fluid as mass or volume and not as is commonly the case, by electrical measurements. This is

only achievable by eliminating the possibilities for estimation error and introducing no assumptions about what is being measured. These strict criteria rule out use of 'unabsorbable' markers and then calculating volume change because the known absorption of markers violates Fick's principle [16] and will falsely indicate secretion. Collection of fluid at the end of the experiment is therefore the required technique, since increased luminal content can only be attributed to secretion. In contrast, any method that measures total loop weight *in situ* necessarily measures luminal content plus interstitial and vascular fluid as well [5]. Weight changes cannot unequivocally be attributed to secretion into the lumen because interstitial fluid weight can increase, without any fluid entering the lumen. The use of the recirculation technique provides a means of clearly establishing whether net secretion of fluid has occurred irrespective of whether it is of vascular origin or generated by the enterocytes. The failure to detect widely expected secretion in some circumstances can be shown not to be a question of failure of this technique or inadequate maintenance of blood pressure under anaesthesia since secretion is detected in this preparation when a loop is exposed to LT, *Clostridium difficile* toxin A or adverse osmotic pressure gradients.

Using this method, the effect of stretch was re-examined. Mechanical stretch has long been associated with intestinal secretion [22] particularly in denervated loop preparations. The effects of this and of the neurally active agents hexamethonium and atropine on this manoeuvre and on the action of *E.coli* heat stable (STa) enterotoxin were examined to investigate whether there were circumstances other than STa challenge that were deemed to cause secretion but did not in fact do so. Both mechanical and pressure induced stretch reduced the net fluid absorption although not quite to the extent that exposure to STa did. Hexamethonium treatment was unable to restore fluid absorption, whereas atropine completely restored pressure mediated reductions but could not restore stretch and *E. coli* mediated reductions. In no case was net fluid secretion detected. The present fluid recirculation technique of detecting a lack of net secretion can be enhanced by co-perfusion of STa enterotoxin that reduces fluid absorption to very low rates despite itself not actually causing secretion. The investigation of potentially secretory circumstances can be better examined with co-perfusion because any secretion in the almost complete absence of absorption can be expected to manifest itself as genuine rather than assumed increased luminal volume. Mechanical stretch caused some loss of villus cells when examined histologically but pressure stretched tissue did not show this feature. For this reason, investigation into mechanical stretch was discontinued as it seemed to indicate mechanical damage only and was not susceptible to improvement on atropine co-perfusion.

Pressure stretch in combination with STa did not lead to net secretion indicating the mode of action of pressure stretch on fluid absorption was not based on fluid secretion but on inhibition of an absorptive mechanism that was common to STa action. It is likely therefore that only fluid absorption is altered by pressure distension, through a cholinergic neural mechanism, not involving ganglionic transmission.

For this reason, the effect of two cholinergic agents on fluid absorption was investigated. Both luminal bethanechol and luminal carbachol reduced fluid absorption to rates that were comparable to tissue exposed to STa enterotoxin. When tested, luminal carbachol also induced copious salivary secretion which showed that the carbachol was absorbed and was able to cause secretion in tissues that are known to be secretory. Although both cholinergic agents reduced absorption, fluid secretion cannot be inferred on these grounds alone.

To investigate this further, loops were perfused with low sodium ion perfusates additionally containing STa or isopropyl-amiloride to prevent fluid absorption while cholinergic agonists were also administered. Under these circumstances, low sodium ion perfusates together with STa and also with EIPA reduced absorption to modest net secretion. Substitution of choline for sodium ion in perfusates is known to cause some minor secretion because of enhanced vasodilatation [23]. In a state of modest fluid secretion and with sodium ion dependent fluid absorption mechanisms inhibited either by EIPA or STa, carbachol inclusion in the perfusate did not further enhance the rate of fluid secretion. It is clear that although cholinergic agonists prevent fluid absorption, it is unlikely that they provoke fluid secretion. If that were the case, then in the *in vivo* loop of intestine that was not absorbing fluid, net fluid secretion should have been detected but carbachol did not induce this.

The present findings are that cholinergic innervation and pressure induced stretch of the tissue prevent fluid absorption but do not cause fluid secretion. These are two more circumstances in which fluid absorption is inhibited and can lead to diarrhoeal disease without the intervention of any putative enterocyte secretion mechanism or through vasodilatation. These findings seem to be at variance with and in some cases completely opposite to many reports in the literature proposing an action on secretion mechanisms. The empirical findings that have led to these mutually exclusive conclusions require detailed consideration, since assumedly secretory phenomena are demonstrable when electrical methods or weighed loop preparations are used but do not arise when secretion is assessed by luminal volume recovery.

First, the weighed loop preparation, that was the subject of recent discussion [24,25], from any schematic of the technique can be seen to additionally weigh fluid in the vascular and interstitial volume and add this to the total; this will misattribute expansion of the interstitial space after vasodilatation to the further, unproven step of movement of fluid into the lumen. This may explain many discordant findings between the Gothenberg loop preparation and the re-circulated or single pass perfused but unweighed loop used in the present study, where luminal content is assessed and not total loop weight. Using the weighed loop technique, fluid secretion is claimed on exposure *in vivo* to live Salmonella bacteria [26], to cholera toxin [27] and to *E.coli* STa [5]. In all cases, hexamethonium can apparently inhibit the rate of secretion whilst atropine cannot. In contrast, using recovered luminal volume to assess secretion, *Salmonella typhimurium* infection had little effect on net jejunal fluid absorption [28] while the weighed loop preparation apparently shows [26] copious rates of secretion. Similarly, perfused loop preparations, with rates of net fluid absorption similar to the *Salmonella* study, showed [6] that STa prevents absorption but does not exhibit the apparent copious secretion that characterises the weighed loop preparation [5]. Neither atropine nor hexamethonium affects the secretion caused by *Clostridium difficile* toxin A in the perfused loop [29]. Similarly, these compounds could not restore to normal values the inhibited fluid absorption on perfusion with *E. coli* heat stable STa enterotoxin, as presented here. In contrast, using the weighed loop protocol, hexamethonium apparently inhibited the secretory actions of *C. difficile* toxin A and the anti-absorptive actions of STa [5, 30]. A reasonable explanation for these discrepant findings is that the Gothenberg weighed loop preparation measures both luminal and vascular routes of fluid entry into the interstitial space. As a consequence, many pharmacological interventions that are apparently anti-secretory are likely only to have caused vasoconstriction or prevent vasodilatation leading to the erroneous conclusion that fluid secretion into the lumen had been altered.

Second, while generally but erroneously accepted that measurement of voltage and short-circuit current measures enterocyte mediated fluid secretion through chloride exit across the brush border, STa is found to elevate the short circuit current [7] but does not cause secretion [6]. A likelihood is that the very reliable current increase that does occur on STa exposure is an associated phenomenon dependent on reduced interstitial volume but does not represent secretion. Any vascular event that leads to interstitial volume filling would reduce short-circuit current but would additionally indicate wrongly that vascularly active pharmacological agents are associated with control of enterocyte secretion.

Sensitivity to atropine but not to hexamethonium arises often when electrical measurements substitute for volumetric measurements. Muscarinic agonists carbachol and acetylcholine increase potential difference [31-33] as does pilocarpine but while net fluid absorption is reduced to zero, it does not become secretory [34] in the perfused jejunal loop. Cholinergic agonists can cause modest net fluid secretion in a non-absorbing loop but only if the mean arterial blood pressure is much lower than normal and with the intestinal villus capillary pressure higher than usual resulting from vasodilatation [35], greater than normal capillary pressure and fluid flows into the lumen. The sensitivity of electrical changes to atropine indicates cholinergic receptors on the enterocyte, with acetylcholine inhibiting enterocyte mediated absorption but is inconsistent with secretion mechanisms residing within the epithelial cell since net secretion after cholinergic stimulation was not detected *in vivo* using the recovered volume protocol.

The effect of pressure stretch on fluid absorption presented here using volume recovery contrasts strongly, differing on almost all points with reported findings when electrical measurements

are made [4] to assess secretion. The increase in PD after cholera toxin treatment (assumed to reflect greater secretion) is reduced by hexamethonium but not by atropine, paralleling what is found when the weighed tissue preparation is used [5]. In stretched control loops [4] unexposed to CT, atropine does nothing and hexamethonium reduces the stretch induced pd. This is the opposite of what was found in the present studies in which hexamethonium did not restore fluid absorption in the stretched loop but atropine did. These findings are reconcilable with the findings presented here derived from luminal volume measurements, by acknowledging that the weighed tissue preparation responds to fluid entering the interstitial space from the vasculature, as do transmucosal electrical measurements.

5. CONCLUSION

In conclusion, local distension caused by pressure, like STa action on enterocytes, is also a circumstance that inhibits fluid absorption but does not cause fluid secretion. It is likely that distension provokes a local neural response that results in localised acetylcholine release that is inhibitable by atropine. These observations were made to distinguish between circumstances and enterotoxins that cause intestinal fluid secretion from those that simply reduce fluid absorption. This is a necessary examination of the enterocyte secretion model because pressure distension did not cause the net fluid secretion often claimed on the basis of electrical measurements and the weighed loop preparation. It is probable that these measurements may be linked only by underlying disruption of fluid absorption but not necessarily by direct causality. In addition, pharmacological interventions that apparently reduce secretion as measured by these methods may simply result from reductions in total weight of the tissue as vasodilatation is prevented (if this is caused by any particular toxin) or simply offset (if the toxin has no effect on the vasculature). The present experiments, showing that pressure distension is not secretory, help to challenge the enterocyte fluid secretion paradigm and draw attention to the fact that intestinal secretion may be better understood as a consequence of changes to mucosal permeability because of inflammatory changes [28] or of changes in vascular tone coupled with altered paracellular permeability [36] rather than as a consequence of biochemical occurrences within the enterocytes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the authors.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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