Role of the enteric nervous system in the maintained hypersecretion induced by enterotoxin STa in the nutritionally deprived intestine

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Abstract

Electrogenic (Cl^-) secretion was measured as the short circuit current (Isc, $\mu\text{A}/\text{cm}^2$) across muscle-stripped sheets of jejunum and ileum incubated in vitro after removal from fed rats, rats starved for three days, and chronically undernourished rats (50% of fed control intake for 21 days). Concentration and Isc response curves for serially-added mucosal *Escherichia coli* STa enterotoxin showed that the rats which had undergone dietary deprivation had a larger secretory Isc maximum but the ED_{50} values were unchanged compared with fed animals. In fed intestine the action of STa was transient, with an Isc peak and subsequent decay to the baseline over 60 minutes but in the undernourished intestine the response consisted of a significantly greater peak than that of the fed state (jejunum=94%; ileum=168%) and the Isc was maintained at or near the peak for at least 60 minutes. The starved intestine had a less well developed maintenance of its enhanced peak Isc. Serosal tetrodotoxin ($1 \mu\text{M}$) had no effect on the initial peak Isc values but caused a decay of the maintained Isc down to the basal or fed levels in the starved and, especially, in the undernourished intestines. Thus, dietary deprivation, especially chronic undernutrition, enhances the maximum electrogenic secretion due to STa and creates a new neural path in the submucosal plexus that, when activated by STa, maintains its enhanced secretory action. Its putative role in exacerbating secretory diarrhoea in malnourished human subjects could be an important component underlying the known relation between malnourishment and the increased severity of diarrhoea.

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secretion is also greatly prolonged compared with that in the fed or starved intestine.⁹ In this study we present evidence that this prolongation of the hypersecretory response to *E coli* STa in chronically undernourished jejunum and ileum is driven by a tetrodotoxin-sensitive neural component in the submucosal plexus of the enteric nervous system. This neural pathway seems less well developed in prolonging the hypersecretory response of the starved small intestine to the toxin. Preliminary accounts of the work have been communicated to the Physiological Society and the European Intestinal Transport Group.^{10 11} Interestingly, other studies have shown that starvation also induces a greatly enhanced, neurally-driven, basal secretory tone in the mouse colon.¹² The induction of neural mechanisms by dietary deprivation highlights the plasticity of the enteric nervous system and is an example of the influence of the level of food intake on enteric neural pathways and their control of small intestinal secretory activity.

Methods

ANIMALS AND DIETARY STATUS

Male albino rats of the Sheffield Wistar strain (weight range 230-260 g) were housed in individual plastic cages with raised wire mesh bottoms to minimise coprophagy. The controls were allowed free access to their food (Diet CRM, Labsure, London). They consumed approximately 24 g of the diet per day. Another group of rats was fed 50% (12 g per day) of the diet for 21 days (chronically undernourished group). A further group of rats was starved for 72 hours. All the rats were allowed free access to their drinking water. The temperature ($23 (1)^\circ\text{C}$), humidity (72%), and the 12 hour light-dark cycle (lights on from 6 00 to 18 00) were all controlled.

IN VITRO PREPARATION

Rats from the various groups were anaesthetised with intraperitoneal sodium pentobarbitone (Sagatal, May and Baker; 60 mg/kg body weight). On achieving surgical anaesthesia, a mid-line abdominal incision was made and approximately 5 cm of jejunum, some 15 cm caudal to the ligament of Treitz, and 5 cm of ileum, approximately 20 cm proximal to the ileo-caecal junction, were removed. These were washed through with prewarmed 0.9% NaCl and then stripped of their external muscle. The segments were cut

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When rats are starved for more than 24 hours, or are acutely or chronically undernourished, their small and large intestines hypersecrete in relation to a variety of neurotransmitters and secretagogues.¹⁻⁶ This hypersecretion in the starved and undernourished intestine mimics features of the human diarrhoea of starvation and malnutrition.⁶⁻⁸ The response of the jejunum and ileum from chronically undernourished rats to the bacterial enterotoxin *Escherichia coli* STa in vitro is not only that of an enhanced maximal secretion but the duration of maximal

Figure 1: Log concentration and short circuit current (I_{sc}) response curves for *Escherichia coli* STa (A) in jejunum from fed control (open circles, $n=9$), three-day starved (solid circles, $n=10$), and chronically undernourished rats (open squares, $n=10$) and (B) in ileum from fed control (open circles, $n=10$), three-day starved (solid circles, $n=10$) and chronically undernourished rats (open squares, $n=8$). Results are given as mean (SEM).

open along their mesenteric border and mounted as flat sheets between two identical chambers in an Ussing apparatus as described by Przyborski and Levin.¹³ The exposed tissue area was approximately 2 cm². The sheets were incubated at 37°C in bicarbonate saline¹⁴ containing 10 mM serosal glucose and 10 mM mucosal mannitol gassed continuously with 95% O₂, 5% CO₂. All preparations were mounted and allowed to stabilise for approximately 10 minutes before any recordings of short circuit current (I_{sc}) were taken.

Electrogenic ion transport was monitored continuously as the I_{sc} (μA/cm²) using an automatic voltage clamp (DVC 1000, WPI Inc) linked through a Maclab 4 to an Apple Macintosh computer. The potential difference across the intestine was sensed by two M KCl in agar bridges in close approximation to the tissue while the applied current was delivered by two carbon electrodes fixed at the ends of the chambers.¹³

Two indices were used to assess the electrogenic secretory responses to STa, (i) the maximum change in the I_{sc} ($\Delta I_{sc_{max}}$) obtained by subtracting the basal I_{sc} from the maximum value and (ii) the change in I_{sc} after 60 minutes incubation ($\Delta I_{sc_{60}}$) obtained by subtracting the basal I_{sc} from the I_{sc} at 60 minutes after the addition of the toxin.

To assess the viability of the intestinal preparations, 28 mM glucose was occasionally added to the mucosal fluid and the resultant increase in I_{sc} created by the hexose active

transfer current (cotransport of the hexose with Na⁺ ions) was monitored. Whenever tetrodotoxin (TTX, 1 μM) was used to block neural transmission in the intestinal sheets it was added to the serosal solution and allowed to act for 10 minutes before STa was added.

CHEMICALS

All chemicals were purchased from Sigma Chemical Company Ltd, Poole, England.

GRAPHIC DATA PRESENTATION AND STATISTICAL ANALYSIS

The time course data (response-duration curves) for the changes in I_{sc} after addition of the various agents are plotted as changes in the I_{sc} from the last five minutes of the basal state, which is taken as the zero I_{sc} baseline. Increases in the I_{sc} are plotted as positive and decreases as negative compared with this initial zero baseline. Results are shown as the mean (SEM) with the number of animals used in brackets. It should be noted that the I_{sc} data plotted in the time course data graphs (Figs 2–4) are from individual experiments whose maximum values occur at different times after the addition of STa, thus the mean maximum I_{sc} obtained from each of these experiments given in the tables or text will not always correspond exactly with the maximum values shown in the graphical data.

Statistical analysis used the unpaired Student's t test with significance taken at the 5% level. When multiple comparisons were made, the Kruskal-Wallis analysis of variance was used followed by Conover's multiple t test to identify specific differences.¹⁵

Results

CONCENTRATION-RESPONSE CURVES TO STa IN FED AND UNDERNOURISHED INTESTINES

Stripped jejunum and ileum from fed, starved, and undernourished rats were stimulated *in vitro* with cumulative additions of STa to the mucosal solution over the range 5 to 100 ng/ml. The responses, calculated as the $\Delta I_{sc_{max}}$ and plotted against the log of the STa dose, showed typical sigmoidal concentration-response relationships. The maximum responses to STa in both the jejunum and ileum were observed at 50 ng/ml and were not significantly smaller compared with the responses at 100 ng/ml (Fig 1 (A) and (B)). In the undernourished jejunum, the ΔI_{sc} induced were significantly greater than the fed values at all doses of STa (Fig 1 (A)) but in the ileum, only those doses above 25 ng/ml were significantly greater than the fed values. The maximum increase in $I_{sc_{max}}$ in the undernourished compared with the fed jejunum was 104% greater ($p<0.001$), and in the ileum it was 124% greater ($p<0.001$). With the starved jejunum only the doses above 25 ng/ml were significantly different from the fed values (50 ng/ml, $p<0.02$; 100 ng/ml, $p<0.01$), but in the starved ileum all doses were significantly greater than the fed

(5 ng/ml, $p<0.01$; 10 ng/ml, $p<0.05$; 25 ng/ml, $p<0.01$; 50 ng/ml, $p<0.01$; 100 ng/ml, $p<0.001$). In the starved jejunum, the $\Delta I_{sc_{max}}$ was 113% greater ($p<0.001$) and in the ileum it was 176% greater ($p<0.001$) than the respective fed values. The ED_{50} values (the concentration of STa required to produce a half

maximal response) were 30 (3), 27 (5), and 28 (6) ng/ml respectively in the fed, starved, and undernourished jejunum and 28 (2), 27 (4), and 25 (5) ng/ml in the fed, starved, and undernourished ileum respectively. The values indicated that there were no differences between the ED_{50} in the different parts of the intestine

Figure 2: Response-duration curves for the short circuit current (I_{sc}) induced by mucosal *Escherichia coli* STa (30 ng/ml) in the presence (open squares) and absence (solid circles) of serosal tetrodotoxin: (A) (1×10^{-6} M) in the jejunum of fed control ($n=8$) and (B) of chronically undernourished rats ($n=6$). Results are given as the mean (SEM).

Figure 3: Response-duration curves for the short circuit current (I_{sc}) induced by mucosal *Escherichia coli* STa (30 ng/ml) in the presence (open squares) and absence (solid circles) of serosal tetrodotoxin (1×10^{-6} M) (A) in the ileum of (A) fed control ($n=8$) and (B) of chronically undernourished rats ($n=6$). Results are given as the mean (SEM).

Figure 4: Response-duration curves for the short circuit current (Isc) induced by mucosal Escherichia coli STa (30 ng/ml) in the presence (open squares) and absence (solid circles) of serosal tetrodotoxin (1×10⁻⁶ M) (A) in the jejunum (n=6) and (B) ileum (n=6) of three day starved rats. Results are given as the mean (SEM).

or in the different nutritional states. On the basis of these data, subsequent studies used 30 ng/ml STa as the standard enterotoxin mucosal challenge.

TIME COURSE OF THE ACTION OF STa IN THE PRESENCE AND ABSENCE OF TTX IN FED, STARVED, AND UNDERNOURISHED INTESTINE The maintained duration of action of STa on the enhanced Isc in the undernourished jejunum (Fig 2 (A)) and ileum (Fig 3 (A)) is obvious when compared with the smaller peaks and decay of the Isc in the fed jejunum and ileum (Figs 2 (B) and 3 (B) respectively). The starved intestine (Fig 4 (A) and (B)) shows less dramatic evidence of a prolongation of STa's action but the Isc values are significantly

greater after 60 minutes of STa than those of the fed (Table I) intestine especially in the case of the jejunum. Statistical analysis of the Isc values induced by the STa 60 minutes after its addition are shown in Table I.

To assess whether a neural component played a part in the prolongation of action of STa in the starved and undernourished intestine, TTX was added to the serosal bathing fluid and allowed to act before the addition of mucosal STa. The small decrease in the basal Isc after the addition of the TTX was not significant in the fed, starved, or undernourished intestines, indicating that very little neural tone drives their secretory activity in the basal condition. The addition of TTX did not significantly affect the maximum Isc induced by the STa in the fed intestine (Table I). Similarly, in both the starved and undernourished jejuna and ilea the presence of TTX did not significantly affect the enhanced Isc maximum induced by STa when compared with values in the absence of TTX. Compared with the fed ΔIsc_{max} in the starved jejuna and ilea, the increases in the absence of TTX were 97% (p<0.01) and 116% (p<0.02) respectively while values in the presence of TTX was 121% (p<0.01) and 222% (p<0.001) respectively. Similarly, in the undernourished jejuna and ilea the values were 94% (p<0.001) and 168% (p<0.001) in the absence of TTX and 56% (p<0.02) and 271% (p<0.001) respectively in the presence of TTX (Table I).

The effect of the added TTX was assessed in the various intestines by measuring the induced STa Isc after 60 minutes (Isc₆₀). In

TABLE I The maximum short circuit current (ΔIsc_{max}) and the Isc at 60 minutes (Isc₆₀) after the addition of mucosal Escherichia coli STa (30 ng/ml) in the presence (+ TTX) and absence (- TTX) of serosal tetrodotoxin (1×10⁻⁶ M) in the jejunum and ileum of fed control rats, chronically undernourished rats, and rats starved for three days. Results are given as the mean (SEM) with the number of rats used in brackets. Superscript letters facilitate selected statistical comparisons by Conover's multiple t test after Kruskal-Wallis analysis of variance (ANOVA)

Tissue/group	ΔIsc _{max} (μA/cm ²)		ΔIsc ₆₀ (μA/cm ²)	
	- TTX	+ TTX	- TTX	+ TTX
Jejunum:				
Fed	21.3 (2.9) (8) ^a	19.4 (4.3) (8) ^b	7.0 (2.3) (8) ^c	3.5 (1.2) (8) ^d
Chronic	41.4 (5.4) (6) ^e	30.3 (4.2) (6) ^f	35.5 (4.2) (6) ^g	3.6 (1.2) (6) ^h
Starved	42.0 (2.3) (6) ^a	36.2 (2.9) (6) ^r	13.7 (1.4) (6) ^s	3.5 (1.2) (6) ^t
Ileum:				
Fed	14.1 (1.8) (8) ⁱ	9.6 (1.5) (8) ^j	2.4 (0.8) (8) ^k	1.1 (0.6) (8) ^l
Chronic	37.8 (5.0) (6) ^m	35.6 (1.5) (6) ⁿ	27.8 (3.1) (6) ^o	2.2 (1.7) (6) ^p
Starved	30.5 (3.7) (6) ^u	30.9 (3.8) (6) ^v	14.2 (3.8) (6) ^w	5.5 (1.3) (6) ^x

a v c=p<0.001, a v e=p<0.01, a v q=p<0.001, b v r=p<0.02, c v g=p<0.001, c v s=p<0.01, i v m=p<0.001, i v u=p<0.05, j v n=p<0.001, j v v=p<0.001, l v x=p<0.01, s v t=p<0.001.

TABLE II *Transfer increase in short circuit current ($\Delta I_{sc_{max}}$) generated by 28 mM mucosal glucose in the presence (+TTX) and absence (–TTX) of serosal tetrodotoxin (1×10^{-6} M) in the jejunum and ileum of fed control rats, chronically undernourished rats, and rats starved for three days. Results are given as the mean (SEM) with the number of rats used in brackets. Superscript letters facilitate selected statistical comparisons by Conover's multiple t test after Kruskal-Wallis analysis of variance (ANOVA)*

Tissue/group	$\Delta I_{sc_{max}}$ ($\mu A/cm^2$)	
	–TTX	+TTX
Jejunum:		
Fed	91.3 (8.2) (8) ^a	63.0 (5.4) (8) ^b
Chronic	172.0 (20.8) (6) ^c	160.5 (10.1) (6) ^d
Starved	140.2 (14.2) (6) ⁱ	105.0 (12.8) (6) ^j
Ileum:		
Fed	108.2 (16.1) (8) ^e	71.2 (6.5) (8) ^f
Chronic	162.2 (18.0) (6) ^g	200.5 (10.6) (6) ^h
Starved	137.0 (11.6) (6) ^k	144.4 (15.8) (6) ^l

a v b=p<0.02, a v c=p<0.002, a v i=p<0.01, b v d=p<0.001, b v j=p<0.01, e v j=p<0.05, f v h=p<0.001, f v l=p<0.02.

the fed jejunum and ileum, the presence or absence of TTX had no significant effect on the $I_{sc_{60}}$ (Table I). In the case of the starved intestine, TTX significantly lowered, three- to fourfold, the values of the $I_{sc_{60}}$ both in the jejunum and in the ileum. The effect was much greater in the undernourished jejunum and ileum, however, where the decrease of the $I_{sc_{60}}$ in the presence of TTX was approximately 10 fold the value in its absence. In the presence of TTX, none of the jejunal $I_{sc_{60}}$ values were significantly different from one another.

To show that the reduction of the $I_{sc_{60}}$ by TTX in the starved and undernourished intestines was not due to deterioration of the preparations, 28 mM glucose were added to the mucosal bathing solutions after the 60 minutes and the increase in the I_{sc} brought about by the coupled active transfer of Na^+ ions with the hexose was measured. The values for these hexose transfer currents are shown in Table II. Apart from the unaccountable decrease in the fed jejunum, there were no significant differences in the I_{sc} values generated in any of the intestines in the absence or presence of TTX. The I_{sc} values for the starved and undernourished intestines, however, were all significantly greater than the fed values, whether TTX was present or absent. This clearly shows that the decrease in secretory I_{sc} caused by the TTX was not due to a general deterioration of the intestines. The enhanced electrogenic transfer of glucose is a well known feature of starved or malnourished intestine.^{3 4}

Discussion

The results show that *E coli* STa elicits an electrogenic secretory concentration-dose response in the jejunum and ileum from fed, starved, and chronically undernourished rats, and the maximum values for the starved and undernourished intestines were significantly greater than those induced in the fed intestine. In both the jejunum and ileum, this was obtained at STa concentrations of 50 ng/ml with a similar ED₅₀. This suggests that starvation and chronic undernutrition are unlikely to affect the affinity of the receptors to the enterotoxin.

It is also unlikely that the increased maximum response is due to an increase in the number of STa receptors as other workers¹⁶ have not found an increase in the binding sites for STa on the undernourished enterocyte.

The time course profiles for the STa I_{sc} response in the fed intestines were very different to those in the undernourished intestines – they were transient in the former and maintained in the latter. In the case of the starved intestine, there was some evidence of a maintained STa induced $I_{sc_{max}}$ compared with the fed values, but it was less obvious than that in the undernourished gut. In the presence of the neurotoxin TTX, none of the initial $I_{sc_{max}}$ induced by STa in any of the intestines was affected, but prolongation of the I_{sc} response in the undernourished ileum and jejunum was reduced back to the initial basal level after 60 minutes. The starved ileum and jejunum also showed an increase in the speed of decay of the I_{sc} in the presence of TTX (more so in the jejunum) but the effect was very much smaller than that observed in the undernourished intestine. The action of TTX in inhibiting the maintenance of the I_{sc} activated by STa clearly indicates that there is a neural mechanism operating in the starved, and especially the undernourished, intestines that maintains the duration of action of the STa.

The intestinal preparations have their outer smooth muscle coat stripped away, which also removes the myenteric plexus but leaves intact the submucosal and mucosal plexus of the enteric nervous system.¹⁷ Thus the neural path that is activated or uncovered by 21 days of chronic undernourishment and partially induced by the three day starvation must reside in the submucosal-mucosal plexus. While previous studies in the fed rat indicated that both hexamethonium and TTX partially depressed the action of STa on inducing jejunal fluid secretion *in vivo*¹⁸ this neural mechanism clearly does not influence the electrogenic secretion measured in our experiments in the fed stripped intestine *in vitro* as TTX has no effect on either the maximal I_{sc} generated by STa or on its duration. Recent studies with fed small intestine incubated *in vitro* in the unstripped, intact condition have shown that TTX will partially inhibit the I_{sc} generated by mucosal STa even though it has no effect in the stripped intestine, implicating involvement of the myenteric plexus. Moreover, this myenteric reflex pathway is inhibited by capsaicin and by L-NAME and is thus dependent on C afferent fibres and nitric oxide (Rolfe and Levin¹⁹). We are presently examining the action of STa in invoking electrogenic secretion *in vitro* via the myenteric plexus reflex in the unstripped intestine from chronic undernourished rats.

Our results clearly show that nutritional deprivation can induce or uncover a neural path in the enteric nervous system that activates intestinal secretion. Such a mechanism enhances the duration of action of the STa enterotoxin which will create greater secretion in undernourished subjects. As secretory diarrhoea caused by enterotoxigenic *E coli* is the cause of a large proportion of childhood

diarrhoea²⁰ and it is well known that reduced nutrition gives rise to an increased frequency and duration of diarrhoea,^{21,22} the discovered neural reflex path may well be a component of the diarrhoea via the enhanced action of STa. We have shown in previous studies that dietary deprived intestine not only responds to STa with an enhanced electrogenic secretion but also with an increased fluid secretion.^{6,9,23}

Other investigations in undernourished rats *in vivo*¹⁶ superficially seem to have confirmed the original finding of Young *et al*⁹ that STa has a greater and prolonged action on intestinal secretion in this condition. These workers, however, could not find any change in the receptor density, avidity of binding of STa, or in its activation of guanylyl cyclase in the undernourished intestine. Moreover, they did not observe any significant differences in fluid secretion by the undernourished compared with the fed intestine until the enterotoxin had had approximately two hours of contact with the ligated jejunal loop. They proposed that the longer duration of action on intestinal secretion was due to a reduced enzymatic breakdown of the enterotoxin by the enterocytes. While such a mechanism may play a role in affecting the duration of STa's action in undernourished intestine it cannot account for the greater intestinal secretion previously reported by Young *et al*⁹ and the results of the present study. Our results show the rapidity with which the enhanced electrogenic Isc is generated by STa and its maintained duration by the induced neural reflex. Both mechanisms are major, if not primary, factors in the exacerbation of the enterotoxin's secretory action in nutritional deprivation. Clearly, any reduced breakdown of STa in the undernourished intestine will allow STa to act on these mechanisms for an even longer duration than in the fed state. It is important to note that in our stripped intestine preparation the submucosal-mucosal plexus has no part to play in the enhancement of the Isc induced by STa in either the undernourished or starved intestine compared with the fed. The latter observation confirms previous studies in the starved jejunum and ileum.^{3,4} The cellular mechanism(s) underlying this hypersecretion has not yet been evaluated but it is likely to involve processes after the direct action of STa on the enterocyte via its binding to its brush border receptor guanylate cyclase.²⁴ Obvious factors such as changes in the production and catabolism of cyclic guanosine monophosphate are under investigation. Detailed studies examining the neural characteristics of the STa activated submucosal mechanism induced by the undernutrition^{11,25,26} are to be published in a further paper elsewhere. At present, we have no experimentally obtained information about possible mechanisms involved in the dietary induction or uncovering of the neural path(s) prolonging the STa induced secretory activity and we feel it would be premature to speculate about them. Our findings, however, suggest that there is an exciting new field of study on the inter-relationships of dietary intake and enteric nervous system neural connections.

Finally, the recent discovery of guanylin, the endogenous ligand for the guanylate cyclase receptor²⁷ may have implications for the control of secretory activity of the intestine during nutritional deprivation. The ligand has homology with STa and is present in the ileum and colon. If guanylin is released from the cells it too could activate ileal secretion in the same manner as STa causing possible hypersecretion of enhanced duration. Such a mechanism might underlie previously inexplicable secretory states of the intestine.

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