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**Research Article**

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# The Effects of Electrical Field Stimulation and Tetrodotoxin on Ion Transport by the Isolated Rabbit Ileum

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ABSTRACT To determine whether intramural nerves affect intestinal ion transport, we studied the effect of electrical field stimulation (EFS) on the movement of ions across isolated rabbit ileum. EFS increased the transmural electrical potential difference and the short circuit current (Isc), caused Cl secretion, and reduced conductance, but did not alter fluxes of Na or the residual current ($J^R_{net}$). The neurotoxin, tetrodotoxin, prevented all the changes caused by EFS but did not prevent the increase in Isc caused by theophylline (5 mM), carbachol (10 $\mu$M), or glucose (10 mM), or the reduction in Isc caused by norepinephrine (10 $\mu$M), implying that tetrodotoxin prevented responses to EFS by affecting electrically excitable cells rather than epithelial cells. Tetrodotoxin also enhanced the mucosa to serosa fluxes of Na and Cl, reduced the potential difference and Isc, and increased conductance. The site of tetrodotoxin action is uncertain because it may affect the release of at least four neurotransmitters and the release of peptides from endocrine cells. The Isc response to EFS was not affected by atropine (10 $\mu$M), physostigmine (10 $\mu$M), or by hemicholinium (1 $\mu$M). The mechanism by which EFS causes Cl secretion remains to be determined.

## INTRODUCTION

How important are the intestinal nerves in controlling the rates of ionic absorption and secretion in the intestine? The classic study on the influence of extrinsic nerves by Wright et al. (1), demonstrated in cats that stimulating the vagal fibers causes secretion from the duodenum and that transecting the vagus has little effect. However, segments of small intestine secrete when the sympathetic nerve supply is interrupted, and the entire small intestine secretes if the celiac ganglia are removed. The secretion seems to be caused by acetylcholine because it is prevented by atropine and enhanced by physostigmine (1). Other studies with cholinergic and adrenergic transmitters in vivo and in vitro have added evidence that in the small intestine, cholinergic stimuli cause secretion and adrenergic stimuli enhance absorption (2–6).

Although the ways in which the extrinsic nerves affect epithelial cell function have been studied in some detail, very little is known of the comparable effects of the intrinsic nerves. Smooth muscle physiologists have studied the effect of the intrinsic nerves on smooth muscle function by depolarizing the nerves with an electrical field (7, 8). We have used the same method to determine, in the rabbit ileum, the effect of the intrinsic nerves on the transport of Na and Cl, the short circuit current (Isc),[1] the transmural electrical potential difference (PD), and the conductance.

## METHODS

*Procedure with rabbits.* New Zealand White male rabbits, weighing 2–3 kg, were fasted overnight and killed by a blow to the neck. 15–20 cm of distal ileum was removed just proximal to the attachment of the mesoappendix. The lumen was flushed with saline, and the segment was opened longitudinally along its mesenteric attachment to make a sheet. This was kept in an oxygenated balanced salt solution (Ringer's) at room temperature until portions of it were mounted in the flux chamber. The solution was bubbled with 94% $O_2$–6% $CO_2$ and had the following composition (millimolar): NaCl, 113; KCl, 4.5; $MgSO_4$, 1.0; $Na_2HPO_4$, 1.8; $NaH_2PO_4$, 0.2; $NaHCO_3$, 25; $CaCl_2$, 1.25; mannitol, 10. A 3–4-cm square of ileum was excised and impaled serosa side up on the mounting needles of the half-chamber. In early studies, efforts were made to remove only the superficial serosal membrane overlying the longitudinal muscle, and histologic studies confirmed that it was possible to do so. In recent studies, it is apparent that layers of longitudinal muscle of varying thicknesses are also removed, occasionally exposing the under-

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[1] *Abbreviations and nomenclature used in this paper*: EFS, electrical field stimulation; Isc, short circuit current; J, flux, m, mucosa; PD, potential difference; R, residual; s, serosa.

lying myenteric plexus. Examination of numerous sections demonstrated that the myenteric plexus remains even when the longitudinal muscle is completely removed. This tissue was then clamped in the flux chamber leaving either 1.77 or 1.00 $cm^2$ of surface available for transport. The mucosal surface of the tissue was bathed with 10–12 ml of Ringer's solution, and the solution that bathed the serosal surface was identical except that it contained glucose in place of mannitol. The bath fluid was kept at 37°C with water jackets as described by Schultz and Zalusky (9). The fluid was circulated and oxygenated with a gas lift using 94% $O_2$–6% $CO_2$ which was hydrated before it entered the bath fluid.

*Electrical measurements.* Transmural electrical PD and the Isc were monitored with an automatic voltage clamp which compensated for the fluid resistance between the bridges used to sense the PD and reduced the transmural PD to zero (University of Iowa Bioengineering Laboratory). Junctional potentials measured in the absence of tissue did not exceed 0.5 mV. Measurements of tissue resistance and conductance were calculated with open circuit values of PD and the Isc measured immediately before opening the circuit.

*Radioisotope fluxes.* The unidirectional fluxes of $^{22}Na$ and $^{36}Cl$ were studied using paired tissues taken from the same ileal segment. Tissues were paired if their conductances did not differ by >25% or their PD's by >1 mV. 10 min after mounting, isotopes were added to the "hot" side of each chamber and 20 min elapsed before beginning the first study period. At that time, if the criteria for pairing were not met, the tissues were discarded and a new study begun.

The radioactivity of $^{22}Na$ and $^{36}Cl$ were determined and fluxes calculated using the methods described by Field et al. (10). The Isc and PD were recorded every 2 min. The measurement of Isc and PD during periods of electrical field stimulation (EFS) were made 15 s after the cessation of the stimulus. The Isc was then interrupted for 2 s to allow for measurement of the PD. Hence, over the 20-min study period, Isc was interrupted for a 20-s total period.

The significance of difference among means was determined with an analysis of variance and Tukey test.

*EFS.* Electrical current was passed through the tissue parallel to the plane of the muscularis propria. Three pins on either side of the flux chamber cavity served as electrodes that were attached to the output of a Grass S-88 stimulator through two stimulus isolation units (Grass Instrument Co., Quincy, Mass.) (Fig. 1). The stimulus wave form was rectangular and bipolar with a total duration of 510 μs. The end of the 250-μs positive wave was separated from the start of the negative wave by a 10-μs interval. These stimuli were applied with a frequency of 10 Hz in trains of 600 ms duration, at one train a second. The symmetry of the wave form was monitored with an oscilloscope. Although the nominal output voltage is known (100 V), it has not been possible to estimate the density of the current flowing through the tissue under the conditions of the study because the resistance of bath fluid on either side is in parallel with the tissue resistance.

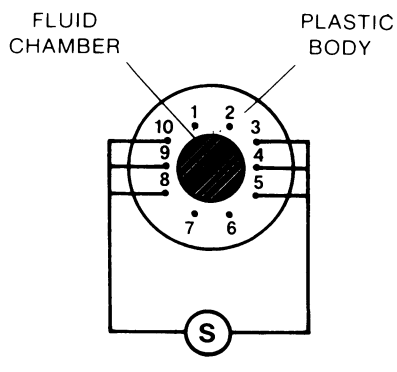


FIGURE 1 Method of EFS. This is the end view of the flux chamber half on which the sheet of intestine is impaled on stainless steel pins numbered 1–10. Pins 1, 2, 6, and 7 are electrically neutral. Pins 8–10 and 3–5 are connected to the output of a Grass S-88 neural stimulator (S).

*Effect of stimulus characteristics on Isc response.* The initial studies to determine if EFS affected Isc or PD showed an apparent maximal change in Isc could be attained with a bipolar square wave 510 μs in duration passed at a frequency of 10 Hz in trains 0.600 s in duration at one train per second. When we found that EFS evoked changes in ion transport, we examined in greater detail the effect of stimulus characteristics on the change in Isc from base-line values (ΔIsc). We used a latin square design in which two of three variables (frequency, wave duration, or train duration) were held constant. In all studies, we passed one train per second for 30 s of each minute. The following frequencies were studied in six tissues: 1, 2, 5, 10, 20, and 30 Hz (wave duration, 510 μs; train duration, 0.600 s). The effect of bipolar wave duration was studied in four tissues with durations of 1010, 810, 510, and 210 μs (10 Hz, train duration of 0.600 s). We studied five tissues with train durations of 0.800, 0.600, 0.400, 0.200, and 0.100 s (10 Hz, 510 μs wave duration). EFS was applied for 30 s followed by a 30-s rest period. A second 30-s period of EFS then followed. We measured the maximal change in Isc caused by EFS during the first period relative to the Isc that immediately preceded the period of EFS. A rest period of 5 min was allowed to elapse between the test periods with EFS.

*Ion fluxes and electrical characteristics of rabbit ileum.* To determine whether the rabbit ileal preparation would be suitable for study, we measured the ion fluxes and electrical characteristics of 19 tissue pairs for five successive 20-min periods. Fluid was sampled before the first period and at 20-min intervals for 100 min.

*Effects of EFS on ion fluxes and electrical measurements.* To determine the effects of EFS on rabbit ileum, we compared ion fluxes and electrical measurements made during the three successive 20-min periods in which the first and third periods were control periods and EFS was applied during the second period.

*Effects of tetrodotoxin on the responses to EFS.* To assess the effect of the neurotoxin, tetrodotoxin, on the response of the ileum to EFS, tetrodotoxin (0.1 μM) was added after the control (first) period and a 20-min period of equilibration was allowed to elapse before beginning the 20-min period of study. The effect of tetrodotoxin on the response to EFS was studied in the next period, which was then followed by a second control period.

*Effects of tetrodotoxin on the responses to epinephrine, glucose, theophylline, and carbachol.* To help localize the site of action of tetrodotoxin, we studied the effects of epinephrine (10 μM), glucose (10 mM), theophylline (5 mM), and carbachol (10 μM) in the manner described in Results.

*Effects of atropine, physostigmine, and hemicholinium on the Isc responses to EFS.* If the Isc response to EFS is mediated by acetylcholine, the increment in Isc should be reduced by atropine and may be increased by the cholinesterase inhibitor, physostigmine. The Isc responses to EFS before and 20 min after atropine were compared. Atropine was present in the bath fluid in concentrations increasing from 10 mM to 10 mM. A similar design was used to determine the effect of physostigmine (10 μM) ($n = 4$).

Hemicholinium-3 interferes with the synthesis of acetylcholine and causes responses mediated by cholinergic fibers

TABLE I

*Ion Flux Measurements during Five Successive 20-min Study Periods*

| | Study period | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| $J^{Cl}_{sm}$ | 6.1±0.5 | 6.1±0.4 | 6.1±0.5 | 6.3±0.4 | 6.4±0.5 |
| $J^{Cl}_{ms}$ | 8.3±0.5 | 8.4±0.6 | 8.2±0.5 | 9.2±0.4 | 8.7±0.4 |
| $J^{Cl}_{net}$ | 2.2±0.6 | 2.4±0.5 | 2.1±0.4 | 2.9±0.5 | 2.3±0.5 |
| $J^{Na}_{sm}$ | 10.6±0.3 | 10.4±0.4 | 10.8±0.4 | 10.7±0.3 | 10.5±0.6 |
| $J^{Na}_{ms}$ | 11.2±0.4 | 11.9±0.3 | 11.9±0.4 | 11.6±0.4 | 12.1±0.4 |
| $J^{Na}_{net}$ | 0.6±0.4 | 1.5±0.3 | 1.1±0.5 | 0.9±0.3 | 1.6±0.4 |
| $J^{R}_{net}$ | 2.7±0.7 | 1.7±0.6 | 2.0±0.7 | 2.9±0.5 | 1.6±0.6 |
| Isc | 1.1±0.2 | 1.1±0.2 | 1.0±0.2 | 0.9±0.2 | 0.9±0.2 |
| PD | 1.3±0.2 | 1.2±0.2 | 1.1±0.2 | 1.0±0.2 | 1.0±0.2 |
| Conductance | 24.1±0.6 | 24.2±0.6 | 24.8±0.7 | 25.1±0.8 | 24.8±0.8 |

Ionic fluxes and Isc are expressed as $\mu$eq cm$^{-2}$/h. PD is the transmural electrical potential difference whose polarity is that of the serosal solution. Conductance expressed as mmho cm$^{-2}$. Within any one category, there were no significant differences ($P > 0.05$) among the five periods ($n = 19$).

to diminish with time when cholinergic nerves are electrically stimulated. We stimulated the ileal preparation continuously (5 Hz, 0.600 s train; one train per second) to determine how long it took for the Isc to fall to one-half of its original value ($t_{1/2}$). Control tissues ($n = 14$) were compared with those treated with hemicholinium-3 (1 $\mu$M) ($n = 14$).

## RESULTS

*Flux studies and electrical characteristics of the ileal preparation.* Sodium and chloride were absorbed at a stable rate during all five periods. The residual current was in the expected range, but its variance was generally larger than that noted for the ion fluxes. The Isc in this study was generally lower than we noted in the other control periods studied (Table I), gradually decreasing from 1.1 to 0.9 $\mu$eq·cm$^{-2}$/h. Similarly, the PD tended to be low and decreased from 1.3 to 1.0 mV (serosal polarity) over the five study periods. Conductance, on the other hand, was in the range measured in most studies and varied very little.

*Effect of stimulus characteristics on Isc response.* The characteristic changes in Isc caused by EFS are shown in a sample tracing in Fig. 2. During the 20-min control period, the Isc diminished slightly from 50 to 45 $\mu$A cm$^{-2}$. Within 30 s of the onset of EFS, the Isc increased to 98 $\mu$A cm$^{-2}$. When EFS was stopped for 30 s, the Isc decreased and it increased again when EFS was resumed: this accounts for the sawtooth pattern of the tracing. Over the 20-min period of EFS, the mean Isc diminished from 96 to 75 $\mu$A cm$^{-2}$. When EFS was stopped, the Isc fell rapidly and returned to base-line values in ≅5 min. It appears that the gradual reduction in base-line Isc seen during the first control period continued throughout the period of EFS since the base-line Isc after EFS was less than that of the preceding control period.

If the mucosa was stripped from the ileum and only

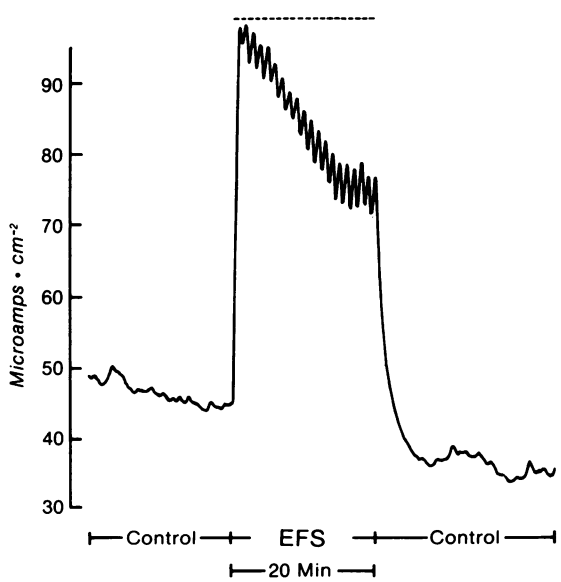


FIGURE 2 Effect of EFS on Isc. Isc rises abruptly with the onset of stimulation for the first 30-s of each min for 20 min. It diminishes with time, then returns to base-line values within ≅5 min after stimulation stops.

the muscularis was mounted in the flux chamber, EFS did not induce an Isc ($n$ = 6).

Only changes in frequency affected the magnitude of ΔIsc (Table II). The increase in Isc was greatest at frequencies of 5 and 10 Hz and diminished progressively at frequencies above and below. Varying wave duration from 210 to 1,010 μs and train duration from 100 to 800 μs had no effect on the Isc response.

*Effect of EFS on ionic fluxes and electrical measurements.* In the control period, Na was absorbed, and there was no net movement of Cl or $J^{R}_{net}$. During the period of EFS, there was no change in $J^{Cl}_{ms}$, but an increase in $J^{Cl}_{sm}$ caused chloride secretion (Table III). There were no significant changes, however, in the unidirectional or net fluxes of Na. In the control period that followed EFS, net Cl movement, as in the first control period, did not differ from zero.

EFS caused the mean Isc to increase from 2.5 μeq·cm$^{-2}$/h in the control period to 3.4. In the control period that followed, Isc diminished to 2.1 μeq·cm$^{-2}$/h. In these studies, PD was not recorded at fixed intervals, so trends in conductance could not be calculated. This information was obtained in later studies, however, and the changes in conductance and PD are shown in Fig. 3. During the initial control period, conductance increased slightly. EFS then reduced conductance ($P < 0.05$) from 24.4 to 21.3 mmho·cm$^{-2}$ during the 20-min of stimulation, but the values then steadily increased in the following control period. The PD, which was in the range of 1.7 mV in the control period increased to ≅4 mV during EFS, then returned to values similar to those of the prior control period.

*Effect of tetrodotoxin on the responses to EFS.* To determine whether the changes associated with EFS were caused by depolarization of intramural nerves (11), we studied the effect of tetrodotoxin on epithelial ion transport and on the response of the epithelium to EFS. Tetrodotoxin, at a concentration of 0.1 nM began to inhibit the rise in Isc caused by EFS, and at a concentration of 0.1 μM, the response was abolished (Table IV).

Tetrodotoxin, 0.1 μM, prevented the changes in Cl transport caused by EFS. In Table V, the $J^{Cl}_{net}$ measured in the presence of tetrodotoxin alone was 2.7 μeq cm$^{-2}$/h; when the tissue was stimulated in the presence of tetrodotoxin (tetrodotoxin plus EFS), $J^{Cl}_{net}$ was 2.8.

Tetrodotoxin had an additional potent effect on transport, however; it reduced the Isc when added to the bath fluid. This effect of tetrodotoxin was studied in

TABLE II
*Effect of EFS Frequency on the Increase in Isc*

| Frequency | ΔIsc (μA cm$^{-2}$) | |
|---|---|---|
| | Mean | SE |
| *Hz* | | |
| 30 | 14.3 | 8.1 |
| 20 | 28.8 | 7.4 |
| 10 | 50.2 | 8.1 |
| 5 | 56.3 | 8.7 |
| 2 | 42.8 | 4.2 |
| 1 | 25.7 | 3.0 |

The maximal increase in ΔIsc in response to EFS at the frequency shown. (Wave duration 510 μs; train duration 0.600 s [$n$ = 6].)

TABLE III
*The Effect of EFS on Ionic Transport and Isc in Rabbit Ileum*

| | Control 1 | EFS | Control 2 |
|---|---|---|---|
| $J^{Cl}_{sm}$ | 6.4±0.3 | 7.8±0.5* | 6.9±0.5 |
| $J^{Cl}_{ms}$ | 6.4±0.4 | 6.5±0.4 | 7.6±0.7 |
| $J^{Cl}_{net}$ | 0.0±0.3 | −1.3±0.4‡ | 0.7±0.6 |
| $J^{Na}_{sm}$ | 11.7±0.4 | 12.2±0.5 | 11.7±0.4 |
| $J^{Na}_{ms}$ | 13.4±0.5 | 12.7±0.7 | 13.6±0.7 |
| $J^{Na}_{net}$ | 1.7±0.5 | 0.5±0.5 | 1.9±0.7 |
| $J^{R}_{net}$ | 0.9±0.7 | 1.4±0.8 | 1.0±1.0 |
| Isc | 2.5±0.4 | 3.4±0.4‡ | 2.1±0.3 |

Ionic fluxes and Isc are expressed as μeq cm$^{-2}$/h.
* Denotes a significant difference between the value during EFS and that in control 1 ($n$ = 15).
‡ Denotes a significant difference ($P < 0.05$) between the value obtained during EFS and values in control 1 and control 2.

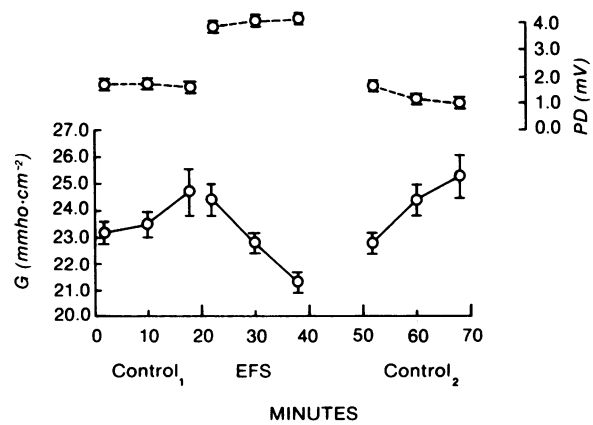


FIGURE 3 Effect of EFS on conductance (G) and transmural electrical PD. The rabbit ileum was electrically stimulated for 20 min after the control period of 20 min. G decreased with time, and PD increased and remained constant. In the second control period (from 50 to 70 min), PD returned to base-line values and G steadily increased ($n$ = 23).

TABLE IV
*Tetrodotoxin Inhibition of Isc Response to EFS*

| Column no. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Tetrodotoxin concentration | 1 pM | 10 pM | 0.1 nM | 1 nM | 10 nM | 0.1 μM |
| Inhibition, % | 7±6 | −4±9 | 37±13 | 52±16 | 41±12 | 100±5 |
| Significant difference from column no. | 6,4 | 5,4 | 6,2 | 6,2,1 | 6,2 | 5,4,3,2,1 |

The effect of increasing concentrations of tetrodotoxin on the response of the Isc to EFS expressed as percent inhibition calculated as follows: percentage of inhibition equals $(1 - \Delta Isc_{TTX}/\Delta Isc_C \times 100$. $\Delta Isc_C$ and $\Delta Isc_{TTX}$ are the changes caused by EFS before and 20 min after the addition of tetrodotoxin to the bathing solutions. $n = 4$ at each concentration.
$P < 0.05$.

four tissues at each of six concentrations ranging from 1 pM to 0.1 μM (Table VI). 20 min after the addition of the tetrodotoxin when the Isc had reached a new steady state, the change in Isc was measured. At the two lowest concentrations, 1 pM and 0.1 μM, Isc increased slightly, an effect that often occurred in the absence of tetrodotoxin. The reduction in Isc began at 0.1 nM and when the concentration was 0.1 μM the Isc decreased by 116 μA $cm^{-2}$. The sequential changes in electrical measurements when tetrodotoxin 0.1 μM was added to the bath fluid are shown in Table VIII. The PD fell from 3.9 V to 0.4 mV and that reduction persisted despite EFS. Because it reflects the changes in PD, the Isc diminished from 3.8 to 0.2 μeq $cm^{-2}$/h, and actually reversed polarity in 5 of 13 studies. The trend continued even during the following period of EFS when the polarity was reversed in 7 of 13 studies. The effect of tetrodotoxin was to reduce the mean Isc to zero. To find out whether the tissue was dead, glucose 10 mM was added to both the mucosal and serosal bath fluids before the final period. In the fourth column, PD increased from 0.1 to 2.4 mV and Isc increased from −0.1 to 2.9 μeq $cm^{-2}$/h, proving that the tissue could respond to glucose stimulation of ion transport. Tetrodotoxin increased the conductance of the tissue from a control value of 27.2 to 34 mmho $cm^{-2}$ and the trend continued during the period of EFS. Glucose caused no further increase.

*Effects of tetrodotoxin on the responses to theophylline and to carbachol.* To determine whether the epithelial cells treated with tetrodotoxin were able to respond to other stimuli that cause Cl secretion and that increase the Isc, we measured the Isc and PD in 18 tissues during a 20-min control period (Table VII). Tetrodotoxin was then added to the bath fluid in one-half of the group ($n = 9$), and the other one-half served as control. After 20 min, theophylline ethylenediamine (aminophylline) 5 mM was added to the bath fluid of both groups and the maximal increase in Isc and PD was measured. The effect of carbachol (10 μM) was studied in an identical manner. There were no significant differences between controls and tetrodotoxin-treated tissues when theophylline was added. The Isc increased by 58 μA $cm^{-2}$ in controls and by 72 μA $cm^{-2}$ in tetrodotoxin-treated tissues. Similarly, the Isc and PD of tissues treated with tetrodotoxin did not respond differently to carbachol. In controls, Isc increased by 51 μA $cm^{-2}$, whereas the increment in tetrodotoxin-treated tissues was 60 μA $cm^{-2}$.

*Effects of atropine, physostigmine, and hemicholinium on the Isc responses to EFS.* Atropine began to reduce the Isc response to EFS at a concentration of 0.1 mM, and the inhibition was complete at 10 mM. It had no effect at 10 μM (Table IX).

Physostigmine, 10 μM, had no effect on the Isc response, which increased by 32±5 and by 30±5 μA $cm^{-2}$, respectively, before and after the agent was added.

Hemicholinium, 1 μM, did not significantly reduce the time that it took for Isc to fall one half its original value with continuous EFS. The $t_{1/2}$ for the control group was 17.1±6.0 min, and for the hemicholinium group 9.9±2.0 min ($P > 0.05$).

TABLE V
*Effect of Tetrodotoxin (0.1 μM) on ion Transport before and during EFS*

| | Control | Tetrodotoxin | Tetrodotoxin + EFS |
|---|---|---|---|
| $J^{Cl}_{sm}$ | 5.2±0.2 | 4.4±0.2* | 5.1±0.3 |
| $J^{Cl}_{ms}$ | 5.1±0.2 | 7.1±0.4* | 7.9±0.4* |
| $J^{Cl}_{net}$ | −0.1±0.2 | 2.7±0.4* | 2.8±0.5* |
| $J^{Na}_{sm}$ | 12.1±0.2 | 12.5±0.4 | 12.7±0.4 |
| $J^{Na}_{ms}$ | 12.5±0.5 | 14.9±0.4* | 15.9±0.4* |
| $J^{Na}_{net}$ | 0.5±0.5 | 2.4±0.6* | 3.2±0.6* |
| $J^{R}$ | 2.6±0.5 | 0.4±0.6* | −0.8±0.6 |

Ionic fluxes and Isc are expressed as μeq $cm^{-2}$/h.
* Differs significantly ($P < 0.05$) from control. $n = 23$.

TABLE VI
*The Effect of Increasing Concentrations of Tetrodotoxin on Isc*

| Column no. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Tetrodotoxin concentration | 1 pM | 10 pM | 0.1 nM | 1 nM | 10 nM | 0.1 μM |
| $\Delta\mu A\ cm^{-2}$ | −12±6 | −10±3 | 0±3 | 27±4 | 85±22 | 116±12 |
| Significant difference from column no. | 6,5,4,3 | 6,5,4,3 | 6,5,2,1 | 2,1 | 3,2,1 | 3,2,1 |

The column number is noted on the first line to facilitate statistical comparisons. On the second line is the concentration of tetrodotoxin in the bath fluid expressed in moles. On the third line is the absolute change in Isc ($\mu A\ cm^{-2}$). $n = 4$ at each concentration. On the fourth line are the numbers of columns containing values with which the percentage of change differs significantly ($P < 0.05$).

## DISCUSSION

*Flux studies and electrical characteristics of full-thickness rabbit ileum.* The rabbit ileum proved to be a stable preparation for study (Table I). With the exception of the first period, Na was always absorbed and at the low rate expected when glucose is absent and the concentration of $HCO_3$ in the bathing fluids is ≅25 mM (10). There was considerable variation in $J^{Cl}_{net}$ among groups of rabbits: In Table I, Cl was absorbed, whereas during the control periods in Table III and V, $J^{Cl}_{net}$ did not differ significantly from zero. In Table V it is clear that the tissue had the ability to absorb Cl actively, since, after the addition of tetrodotoxin, $J^{Cl}_{net}$ increased from −0.1±0.2 to 2.7±0.4 μeq $cm^{-2}$/h. Thus, the absence of Cl transport or the presence of a small Isc does not denote that the transporting epithelium is moribund. These results imply that there are factors in the tissue that are altering the Cl transport of otherwise healthy epithelial cells.

*Effect of electrical stimulus characteristics on Isc response.* Frequency was the only stimulus characteristic that influenced the change in Isc (Table II). In the range of frequencies from 1 to 30 Hz, the response was maximal at 5–10 Hz. In the studies of Gershon (8), 10 Hz was also the frequency that most enhanced contraction of the rabbit jejunum and that caused smooth muscle relaxation when the contractile response was prevented by the muscarinic antagonist, hyoscine. Tetrodotoxin prevented both responses, implying that a stimulus of 10 Hz caused the liberation of at least two neurotransmitters: one cholinergic muscarinic agonist that caused contraction, and one or more compounds that caused relaxation (8).

*Effect of EFS on ionic fluxes and electrical characteristics.* The major change caused by EFS was an increase in $J^{Cl}_{sm}$ that was sufficient to cause Cl secretion (Table III). There were no significant changes in either Na absorption or $J^{R}_{net}$, but the variance of both measurements was large. The Cl secretion of 1.3 μeq $cm^{-2}$/h could account for the entire increase in Isc of 0.9 μeq $cm^{-2}$/h, but changes in $J^{Na}_{net}$ and $J^{R}_{net}$ may have contributed to the difference in the increments. Total conductance decreased linearly with time (Fig. 3), and the reduction is similar in magnitude to that caused by theophylline or cyclic AMP in other studies (12). In the control period that followed EFS, conductance steadily increased, mean PD decreased, and Cl was absorbed.

TABLE VII
*Effect of Tetrodotoxin on the Increases in Isc and PD Caused by Theophylline and Carbachol*

| | Theophylline | | | | Carbachol | | | |
|---|---|---|---|---|---|---|---|---|
| | ΔIsc | | ΔPD | | ΔIsc | | ΔPD | |
| | Control | Tetrodotoxin | Control | Tetrodotoxin | Control | Tetrodotoxin | Control | Tetrodotoxin |
| Mean | 58 | 72 | 3.3 | 3.8 | 51 | 60 | 2.7 | 2.8 |
| SE | 8 | 11 | 0.3 | 0.4 | 6 | 8 | 0.3 | 0.3 |

Increases in Isc and PD caused by the addition of either theophylline (5 mM) or carbachol (10 μM) to bath fluid of control tissues and tissues to which tetrodotoxin was added to bath fluid 20 min before theophylline or carbachol. Nine tissues were studied under each condition (total $n = 36$). The responses of tissues treated with tetrodotoxin did not differ from controls.

TABLE VIII

*Effects of Tetrodotoxin (0.1 μM) on Electrical Characteristics of Ion Transport before and during EFS and after Addition of Glucose to Mucosal Solution (10 mM)*

| Column no. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | Control | Tetrodotoxin | EFS tetrodotoxin | Glucose tetrodotoxin |
| Isc | 3.8±0.3 | $0.2\pm0.3^{1,4}$ | $-0.1\pm0.2^{1,4}$ | $2.9\pm0.3^{1,2,3}$ |
| PD | 3.9±0.4 | $0.4\pm0.2^{1,4}$ | $0.1\pm0.2^{1,4}$ | $2.4\pm0.2^{1,2,3}$ |
| Conductance | 27.2±0.8 | $34\pm1.0^{1,3,4}$ | $37.3\pm1.0^{1,2}$ | $37.1\pm1.2^{1,2}$ |

Mean (±SE) changes in the electrical characteristics of transport caused by tetrodotoxin. TTX (0.1 μM) was present in the bathing solutions in columns 2, 3, and 4; in column 3, the ileum was electrically stimulated (EFS), and in column 4, glucose 10 mM was present in serosal and mucosal solutions. $n = 9$ for PD and Conductance in column 4. $n = 13$ for all other data. The superscripts are the numbers of the columns containing values with which the given value differs significantly ($P < 0.05$), e.g., $0.2\pm0.3^{1,4}$ differs significantly from values in columns 1 and 4.

It is of interest that $J_{ms}^{Cl}$ was higher in the second control period than in either of the preceding periods. Although $J_{ms}^{Cl}$ of the second control period did not differ significantly from that in the EFS period in this study, it was significantly greater in three later studies (not reported here), and in two of these, $J_{ms}^{Cl}$ of the second control period was also significantly higher than that in the first control period. There were no comparable changes in $J_{ms}^{Na}$. Thus, for reasons that are not apparent, $J_{ms}^{Cl}$ is enhanced after the cessation of EFS. With that exception, the changes induced by EFS are reversible. We have not determined whether $J_{ms}^{Cl}$ would have returned to its previous control value given sufficient time.

Were these changes in ion movement caused by an asymmetrical electrical field that favored the movement of anions into the lumen? No, because if asymmetry caused the secretion of Cl during EFS when tetrodotoxin was absent, it should have done the same when tetrodotoxin was present.

Because the increase in Isc does not appear to have been electrical artifact, EFS must have acted on some tissue that was excitable electrically. It is unlikely that EFS directly affected the transporting epithelial cells because their membranes are not considered excitable and because they have a low intracellular electrical PD unlike that of excitable tissues like nerve or muscle.

It is also unlikely that smooth muscle contraction induced by EFS caused the increase in Isc or the Cl secretion, because the increase in Isc did not occur in the absence of the mucosa, but did occur when the muscle was stripped from the preparation and only the mucosa was mounted. Because the smooth muscle serves only as a diffusion barrier that is presumably nonselective, muscular contraction, if it occurred, might have enhanced ion movement, but it should not have selectively increased $J_{sm}^{Cl}$, particularly because the paracellular shunt pathway is more permeable to Na than Cl (13).

*Effects of tetrodotoxin on ion transport and the response to EFS.* To determine whether EFS affected the epithelial cells directly or whether it acted indirectly through nerves, we used the neurotoxin, tetrodotoxin. In studies of the innervation of smooth muscle from a variety of organs and animals, Gershon (8) found that the action of tetrodotoxin (0.1 μM) was limited to the nervous tissue of innervated smooth muscle preparations, and that no actions of tetrodotoxin were found which could not be explained by a block of nervous activity. The effect of tetrodotoxin on intestinal ion transport has not been studied previously, however.

In the period preceding EFS, tetrodotoxin increased the absorption of both Na and Cl chiefly by increasing the rate of $J_{ms}^{Na}$ and $J_{ms}^{Cl}$. With the exception of a small reduction in $J_{sm}^{Cl}$ during the tetrodotoxin treatment period, $J_{sm}^{Cl}$ and $J_{sm}^{Na}$ were not affected.

It seems fairly certain that the blockade of the response to EFS was caused by the well-documented action of tetrodotoxin on intramural nerves (8) or perhaps on paracrine cells, which, in tissue culture are electrically-excitable (14). Although tetrodotoxin might have prevented the EFS response by acting directly on chloride secretory mechanisms of the epithelial cell, this seems unlikely because the cells had the capacity to respond to two other Cl secretagogues when tetrodotoxin was present in the bathing fluid. Theophylline increased the Isc by 72 μA $cm^{-2}$ and carbachol by 60 μA $cm^{-2}$, increments that were as great or greater than those seen in tissues not exposed to tetrodotoxin (Table VII).

The mechanism by which tetrodotoxin increased the absorption of Na and Cl can be surmised with less certainty. Absorption was enhanced because the mucosal to serosal fluxes of Na and Cl increased, and not because the fluxes in the opposite direction diminished. If tetrodotoxin acts on the epithelial cell membrane, its action is contrary to that which might be

TABLE IX

*Atropine Inhibition of Isc Response to EFS*

| Atropine concentration | 10 μM | 0.1 mM | 1 mM | 10 mM |
|---|---|---|---|---|
| Inhibition, % | 9±7 | 21±9 | 85±6 | 100±0 |
| $n$ | 8 | 8 | 4 | 4 |

The effects of increasing concentration of atropine on the response of the Isc to EFS expressed as percentage of inhibition (mean±SE). The percentage of inhibition equals $(1 - \Delta Isc_{TTX}/\Delta Isc_C) \times 100$.

expected from its effect on the nerve cell membrane, for in the epithelial cell, it enhances Na movement, whereas in the nerve cell membrane, Na movement is inhibited (11). The effect of tetrodotoxin is like that of the alpha adrenergic agonists, norepinephrine and epinephrine (3) and of aspirin (15) whose sites of action are also unknown. Each of these agents increases the absorption of Na and Cl, and reduces $J^{R}_{net}$ and Isc. If tetrodotoxin acts on the epithelial cells, does it interact with the pathways of Na absorption that are affected by epinephrine or by glucose? In the case of epinephrine, further study is required to answer the question. The available evidence suggests, however, that if tetrodotoxin affects glucose-dependent Na transport, the influence is minimal, for when glucose (10 mM) was added to the mucosal solution, there was a significant increment in Isc from −0.1 to 2.9 $\mu$eq cm$^{-2}$/h (Table VII). The responses to glucose, theophylline, and carbachol support the view that tetrodotoxin does not act directly on the epithelial cell, although there remains the possibility that tetrodotoxin might act on an epithelial ion transport pathway that is not affected by these agents.

Could all the effects of tetrodotoxin on ion transport be mediated by its action on the nerve cell membrane? Possibly, but there is some evidence to the contrary. The substance liberated by EFS causes Cl secretion by increasing $J^{Cl}_{sm}$. It follows that if the liberation of the substance is prevented by tetrodotoxin, Cl absorption should be enhanced because of a reduction in $J^{Cl}_{sm}$. However, tetrodotoxin causes Cl absorption, not because of a reduction in $J^{Cl}_{sm}$, but because of an increase in $J^{Cl}_{ms}$. Another disparity is that tetrodotoxin increases Na absorption whereas the substance liberated by EFS does not alter Na transport significantly, implying that if tetrodotoxin affects only neural tissue, it must ultimately prevent the release of a substance that normally inhibits Na absorption. These considerations suggest that the actions of tetrodotoxin are too complex to be explained by its blocking the release of a single substance.

If a single substance does cause all of the observed changes, it is not acetylcholine. The threshold concentration of atropine required to inhibit the EFS response was 0.1 mM. At such high concentration, atropine exerts nonspecific effects in addition to its inhibition of the muscarinic effects of acetylcholine (16). The inability of atropine to prevent the increase in Isc at a concentration of 10 $\mu$M is evidence that acetylcholine is not the chemical transmitter. This view is also supported by the studies showing that the Isc response is neither augmented by physostigmine nor attenuated by hemicholinium.

Given the nature of the stimulus, it would be surprising if not more than one chemical transmitter were released. The responses we measure may be caused by the interaction of several substances because the intestine contains fibers that are cholinergic, adrenergic, nonadrenergic inhibitory, and noncholinergic excitatory (17). The potential sites of action of tetrodotoxin are also increased by the presence in the small intestine of 12 endocrine cell types (18), for tetrodotoxin may affect not only nerve fibers but paracrine cells, because depolarizing currents cause action potentials similar to those of sympathetic neurons in a wide variety of cells capable of secreting amines or polypeptides (APUD cells) (14). The effects of EFS may be mediated by several interdependent systems, for there are ample precedents for interactions among nerves, endocrine cells, and epithelial cells (19). In contrast to the innervation of skeletal muscle with its final common path and single mode of response, that of the small intestine appears to have uncommon final pathways and multiple responses. The mapping of those pathways should provide an uncommon challenge.

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