EFFECT OF ETHACRYNIC ACID ON GUINEA PIG ILEUM

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Abstract—The effect of ethacrynic acid on the motor function of guinea pig ileum was studied in vitro. Ethacrynic acid produced dose-related (5-160 μg/ml) contractions in this tissue. Morphine, tetrodotoxin and sodium-free medium prevented the contractions while hexamethonium, diphenhydramine, methysergide or indomethacin did not. Atropine in a high concentration (0.1 μg/ml) only inhibited the contractions. Ethacrynic acid inhibited the contraction of ileum induced by electrical stimulation of intramural nerves. This was not prevented by pretreatment with reserpine. Repeated exposure to ethacrynic acid developed tachyphylaxis in contractile response. Inhibition of electrically elicited contraction of guinea pig ileum also diminished with repeated treatment. Ethacrynic acid (80-160 μg/ml) inhibited the peristaltic reflex of the guinea pig ileum. It is concluded that the excitatory effect of ethacrynic acid is most probably mediated by the release of neurotransmitter, however, the mechanism of the inhibitory effect remains to be elucidated.

Ethacrynic acid is a potent diuretic agent and is clinically prescribed in conditions related to edema (1). The drug has positive inotropic and chronotropic effects (2) and depletes the heart muscle of norepinephrine (3, 4). Ethacrynic acid contracts the guinea pig vas deferens presumably by liberating endogenous catecholamine (5) and potentiates the response of the rabbit aortic strips to norepinephrine (6). It has also been reported that ethacrynic acid has a vasodilatory effect (7) without involvement of alpha- or beta-adrenergic, cholinergic or histaminic receptor mechanisms (8). Clinical reports indicate that gastrointestinal symptoms may develop, particularly with long term administration and range from mild epigastric distress to vomiting and profuse diarrhoea (9). To our knowledge, pharmacological studies on intestinal motor function as related to this drug have not been reported. The present work was an attempt to determine the effect of ethacrynic acid on isolated guinea pig ileum. Some of our findings were presented to 6th International Congress of Pharmacology (10).

MATERIALS AND METHODS

Segments of guinea pig ileum were obtained from exsanguinated animals and tissues were placed in Tyrode solution and carefully flushed free of food residue. Segments of 2-3 cm in length, were mounted in an organ bath containing Tyrode solution and which was bubbled with air and maintained at 37°C. The adjacent segments were used for comparison. The composition of Tyrode solution was as follows (mM): NaCl 136.8, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.1, NaH₂PO₄ 0.4, NaHCO₃ 11.9 and glucose 5.5. In experiments to determine the
effect of Na-free solution on ethacrynic acid-induced contractions, modified McEwen’s solution with the following composition was used in control experiments (mM): NaCl 129.9, KCl 5.64, CaCl₂ 2.16, Tris-buffer 26.0 (pH 7.4, adjusted with HCl), glucose 11.1 and sucrose 13.6. Sodium-free solution was prepared by replacement of NaCl with sucrose. Osmotic pressure of the two solutions was checked with an osmometer (Osmette, Precision Systems).

The intestine was stimulated electrically by means of electrodes arranged coaxially (11). The stimuli (0.5 ms, 0.1 Hz, supramaximal voltage) were applied through a Grass model S88 stimulator.

To study the effect of ethacrynic acid on the peristaltic activity of the guinea pig ileum, the method of Trendelenburg (12) was used. The peristaltic reflex was elicited by raising the Tyrode reservoir to a constant height (10–30 mm H₂O in different experiments) for 30 sec at 3 min intervals.

Recordings of isotonic longitudinal muscle contractions were obtained by means of a frontal writing lever with 10-fold magnification on a smoked kymograph drum. The preparations were allowed to equilibrate for about one hour before commencing the experiments. The constant tension applied to the tissue was 0.5 grams. In Trendelenburg preparations, simultaneous recordings were made of the tension of the longitudinal muscle and of the intra-intestinal pressure by means of Grass FT03 microdisplacement transducer and Statham pressure transducer, respectively. The latter was connected through a T tube to the reservoir and intra-luminal fluid. Air space in the reservoir was about 500 ml.

The following drugs were used: ethacrynic acid (Merck, Sharp & Dohme), tetrodotoxin (Sankyo), atropine sulfate (Sigma), hexamethonium bromide (Koch-Light), indomethacin (Merck, Sharp & Dohme), morphine HCl (E. Merck), acetylcholine HCl (E. Merck), DMPP (1,1-dimethyl-4-phenylpiperazinium, Fluka AG), methysergide (Sandoz), diphenhydramine HCl (Benadryl, Park Davis), reserpine (Serpasil, CIBA-Geigy) and nicotine sulfate (Sigma). Doses of drugs are expressed in terms of salts.

RESULTS

Increasing doses of ethacrynic acid (5–160 μg/ml) produced dose related contractions in guinea pig ileum (Fig. 1). In the study of dose response relationships, only the response to the first dose of ethacrynic acid was considered in each preparation. The magnitude of response was compared with the maximum contraction produced by acetylcholine. Repeated administration of large doses of ethacrynic acid clearly demonstrated a diminishing effect upon each successive addition of the same dose (Fig. 2). The interval between doses of ethacrynic acid ranged from 10 to 20 min and the drug was added when the tonus of the preparation had returned to the control level.

The guinea pig ileum was electrically stimulated as described in the Methods section. Introduction of ethacrynic acid to the bathing medium temporarily contracted the intestine (Fig. 3). Later the amplitude of electrically evoked contractions was reduced or abolished, depending on the concentration of ethacrynic acid (Figs. 3 & 4). The response to stimulation gradually recovered even in the presence of the drug, though the amplitude of con-
FIG. 1. Effect of ethacrynic acid (EA) on guinea pig ileum. A single dose was tested on each preparation. The responses are given as a percentage of the maximum contraction produced by acetylcholine. • • control, ■ ■ in the presence of 0.1 \( \mu \text{g}\ \text{ml} \) of atropine, • • in the presence of 1 \( \mu \text{g}\ \text{ml} \) of morphine. Each dose was tested on at least 6 preparations. Points and vertical bars represent means with s.e. of mean.

FIG. 2. Effect of repeated doses of ethacrynic acid (EA) on guinea pig ileum. A constant dose was tested 4-6 times on each preparation (contact time 3 min, interval between doses 10-20 min). A subsequent dose was added when, after washout, the tonus of the intestine had returned to the initial level. With concentrations of 40 and 80 \( \mu \text{g}\ \text{ml} \) of ethacrynic acid, the response to the 3rd and following doses showed tachyphylaxis. A dose of 20 \( \mu \text{g}\ \text{ml} \) showed no tachyphylactic effect. Each dose was tested on at least 5 preparations. Points and vertical bars represent means with s.e. of mean.

FIG. 3. Effect of repeated doses of ethacrynic acid (●, 80 \( \mu \text{g}\ \text{ml} \)) and acetylcholine (●, 100 \( \mu \text{g}\ \text{ml} \)) on the guinea pig ileum. The ileum was transmurally stimulated by single electric pulses of 0.5 ms duration and supramaximal voltage every 10 sec. At ▲ the duration of electric pulses was increased to 5 ms. Then electric stimulation of the intestine was discontinued and acetylcholine was added to the bath while ethacrynic acid was also present. After washout of acetylcholine and ethacrynic acid the kymograph was stopped until the amplitude of contractions induced by electric pulses was constant. w—washout Calibration, vertical: 20 mm of kymograph recording; horizontal: time 5 min.
tractions was not restored to pretreatment level. Washing the tissue in the first few minutes facilitated the restoration of contractions. A 10-fold increase of the duration of pulses (from 0.5 to 5 ms) augmented slightly the amplitude of contractions which had already been reduced by ethacrynic acid (Fig. 3). Repeated exposure of the electrically stimulated guinea pig ileum to ethacrynic acid resulted in the development of tachyphylaxis to both

![Graph](image)

**Fig. 4.** Inhibitory effect of ethacrynic acid (EA, 10–80 µg/ml) on the amplitude of electrically induced contractions of guinea pig ileum. Only one dose was tested on each preparation. Bars indicate the mean percentage inhibition (±SEM) of the amplitude of contractions. Each concentration was tested on at least 5 preparations.

![Graph](image)

**Fig. 5.** Inhibitory effect of repeated doses of ethacrynic acid (EA, 80 µg/ml) on the amplitude of electrically induced contractions of guinea pig ileum. A subsequent dose was added when, after washout, the amplitude of contractions was constant. Bars indicate the mean percentage inhibition (±SEM) of the amplitude of contractions. Numbers in the bars indicate the number of experiments. The percent inhibition observed with the first dose of ethacrynic acid gradually decreased in subsequent exposures.
contractile and inhibitory phases of the effect of ethacrynic acid (Figs. 3 & 5). The intestine was contracted by acetylcholine before and after addition of the ethacrynic acid to the medium. Figure 3 shows that while the response of the tissue to electrical stimulation was reduced, the height of contraction induced by a maximal dose of acetylcholine remained much the same.

The effect of ethacrynic acid (5-160 μg/ml, each dose on at least 8 preparations) was studied on the Trendelenburg preparation of the guinea pig ileum. 160 μg/ml of ethacrynic acid inhibited the peristaltic reflex (Fig. 6). With 40 and 80 μg/ml, the inhibition was not constant, and appeared 4-15 min after exposure to the drug. The reflex was restored after washing. Lower doses of ethacrynic acid (1-10 μg/ml) did not change the threshold for peristaltic reflex.

When the guinea pig intestine was incubated with morphine (0.1 or 1 μg/ml) for 15 min there was no response of the ileum to 40 and 80 μg/ml of ethacrynic acid (Fig. 1).

In a series of 6 experiments, in Na-free McEwen's solution, DMPP (2 μg/ml), nicotine (0.1 μg/ml) or ethacrynic acid (80 μg/ml) produced no contraction of the tissue, while with acetylcholine (0.01 μg/ml) contraction occurred (Fig. 7A). When the medium was changed to modified McEwen's solution with a normal sodium concentration, the intestinal tissue contracted more than 15 min. Testing of the drugs was carried out only when the tonus of the tissue had returned to its normal value. In this state, ethacrynic acid as well as nicotine and DMPP were effective (Fig. 7B).

Since myenteric neurons are comparably resistant to tetrodotoxin (13), a concentration of 1 μg/ml of tetrodotoxin was used. Ethacrynic acid (80 μg/ml), nicotine (5 μg/ml) and acetylcholine (100 μg/ml) were separately applied to the bath before and 20 min after the

![Fig. 6. Effect of ethacrynic acid on the peristaltic reflex of isolated guinea pig ileum preparation. Upper record, intra-intestinal pressure; lower record, longitudinal muscle contraction. Reflex elicited by raising intra-luminal pressure to 2 cm H₂O for 30 sec. A, before and B, after introduction of 160 μg/ml of ethacrynic acid. C, shows normal recording after washout. Calibration, vertical: 2 cm H₂O intra-luminal pressure, 1 gram increase in longitudinal muscle tension; horizontal: time 30 sec.](image)
introduction of tetrodotoxin. This concentration of tetrodotoxin decreased the amplitude of contractions induced by acetylcholine by 23.6 ± 8.3% (mean ± S.E.). The magnitude of contractions induced by ethacrynic acid or nicotine was calculated as a percent of contractions induced by a supramaximal concentration of acetylcholine (100 ng/ml) under the same conditions (presence or absence of tetrodotoxin). As shown in Table 1, tetrodotoxin reduced the contractions induced by ethacrynic acid or nicotine.

Hexamethonium (30 ng/ml), diphenhydramine (0.1 ng/ml) and methysergide (0.2 ng/ml) did not alter the contractile response of the intestine to ethacrynic acid. Incubation of the

<table>
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<th>Drug</th>
<th>Control</th>
<th>After 1 μg/ml of TTX</th>
<th>% change</th>
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<tr>
<td>EA (80 ng/ml)</td>
<td>36.5 ± 6.7 (7)*</td>
<td>9.5 ± 5.0 (5)**</td>
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<tr>
<td>Nicotine (5 μg/ml)</td>
<td>50.3 ± 4.9 (9)</td>
<td>7.1 ± 1.7 (7)**</td>
<td>−86</td>
</tr>
</tbody>
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* Numbers in parenthesis indicate the number of experiments.

** Significantly different from the control (p < 0.01).
Intestine with indomethacin (10 μg/ml) for 1 hr also did not change significantly the contractile response of the intestine to ethacrynic acid.

Effects of atropine on the contractions elicited by ethacrynic acid were also studied. Only one dose of ethacrynic acid was tested on each preparation. The magnitude of response was calculated as a percent of contraction induced by a supramaximal dose of acetylcholine. Atropine (0.01 μg/ml, contact time 10 min) abolished the contractions induced by single electrical stimuli but did not alter significantly the dose response curve of ethacrynic acid. In a concentration of 0.1 μg/ml, however, atropine changed the dose response curve in an unusual way. The response to doses of 40 μg/ml or less shifted slightly to the right in a competitive fashion, whereas responses to 80 and 160 μg/ml of ethacrynic acid were inhibited more than the response to 40 μg/ml and the dose response curve became bell-shaped (Fig. 1). The maximum contraction of the tissue elicited by acetylcholine was not depressed by 0.1 μg/ml of atropine.

Pretreatment of guinea pigs with reserpine (5 mg/kg, 24 hr before experiment) or incubation of the tissue with indomethacin (10 μg/ml, for 1 hr) did not prevent the inhibitory effect of ethacrynic acid on the electrically evoked contractions of the ileum.

The effect of 40 μg/ml of ethacrynic acid on the response of guinea pig ileum to acetylcholine was studied in the tissue that had developed tachyphylaxis to ethacrynic acid. The maximum response to acetylcholine did not change significantly. ED50 of individual experiments was calculated by the method of least squares. Mean negative log ED50 of acetylcholine was 6.52±0.05 in the absence and 6.26±0.05 in the presence of ethacrynic acid (p<0.01, dose ratio 1.8).

**DISCUSSION**

Ethacrynic acid contracted intestinal smooth muscle of guinea pig in a dose dependent manner. Tachyphylaxis was produced by repeated treatment indicating the possibility of an indirect effect through the release of neurotransmitter substances. Morphine depresses the output of neurotransmitter substance in certain tissues (14). The prevention of ethacrynic acid-induced contractions of guinea pig ileum by pretreatment with morphine indicates that the release of a transmitter probably is responsible for the observed effect of ethacrynic acid. The results of experiments carried out in a Na-free medium are in agreement with this conclusion and indicate that, whatever the mechanism, sodium ions are required for the action of ethacrynic acid.

Although different mammalian smooth muscles are quite resistant to tetrodotoxin, this compound has been shown to interfere selectively with sodium conductance in most nerves. In the present study, tetrodotoxin (1 μg/ml) significantly inhibited the contractions of the guinea pig ileum induced by ethacrynic acid (80 μg/ml) or nicotine (5 μg/ml). This is in agreement with the view that ethacrynic acid acts primarily on neuronal elements of the intestine. Absence of complete prevention of ethacrynic acid- or nicotine-induced contractions may be due to existence of myenteric neurons resistant to tetrodotoxin (13). In the light of our findings with Na-free bathing media, with morphine, and with tetro-
dotoxin, a direct contractile effect of ethacrynic acid on the smooth muscle can be excluded. Cold storage of rabbit colon for 14 days also abolishes the effect of ethacrynic acid in this tissue without preventing the response to acetylcholine (unpublished observation).

As ethacrynic acid does not have pronounced cholinergic receptor blocking activity and maximum response to acetylcholine is not significantly depressed by ethacrynic acid, the inhibition of both phases of peristaltic reflex and electrically induced contractions of guinea pig ileum seem to be mediated through an effect on neuronal elements. Development of tolerance to the inhibitory effect of ethacrynic acid on electrically induced contractions in guinea pig ileum is similar to the tolerance reported for morphine in this tissue (15 & 16) and is in agreement with the above suggestion.

Na⁺,K⁺-ATPase inhibition and ionic changes induced by ethacrynic acid (10⁻³ M) have been observed in kidney cortex and uterus of the rat (17 & 18). Compared with the rat preparation, the guinea pig Na⁺,K⁺-ATPase is two orders of magnitude more sensitive to ethacrynic acid (17 & 19). Therefore, it is possible that concentrations of ethacrynic acid used in the present experiments induced ionic changes in neuronal elements of intestine and released the transmitter(s). The other possibility is that ethacrynic acid, being a moderate SH-inhibitor (19), alters the membrane excitability (20) and the movement of Ca⁺⁺ ion in the nerve terminals (21) which in turn leads to transmitter release.

The contractions induced by ethacrynic acid were not prevented by pretreatment with hexamethonium, diphenhydramine or methysergide indicating that nicotinic, histamine H₁, or 5-hydroxytryptamine receptors are not involved. The inability of indomethacin to inhibit the contractions excludes prostaglandins as possible mediators of the contractile effect of ethacrynic acid on the intestine.

Atropine (0.01 μg/ml) abolished the response of the small intestine to electrical stimulation, but did not affect significantly the contractile response to ethacrynic acid, a finding which may indicate that release of substances other than acetylcholine may be involved. Since contractions induced by ethacrynic acid are completely prevented by morphine, the question is raised whether morphine inhibits the release of substances other than acetylcholine. At a concentration of 0.1 μg/ml, atropine is not a specific antagonist of acetylcholine. Changes in the pattern of the dose response curve of ethacrynic acid in the presence of 0.1 μg/ml of atropine shows that, whatever the mediator may be, the response of the guinea pig intestine to higher doses of ethacrynic acid is the sum of two opposite components, namely excitatory and inhibitory effects. Due to the existence of inhibitory effects only at higher concentrations of ethacrynic acid, the response to higher doses of ethacrynic acid was less than the response to lower doses when the contractile component was inhibited by atropine.

The inability of reserpine and indomethacin pretreatment to affect ethacrynic acid-induced inhibition of electrically elicited contractions excludes the involvement of endogenous norepinephrine and prostaglandins as the responsible mediators. Since the contractile effect of acetylcholine is not altered by the presence of ethacrynic acid, a direct inhibitory effect of ethacrynic acid on the smooth muscle can also be excluded.
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