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TACHYPHYLAXIS TO ETHACRYNIC ACID IN THE ISOLATED ATRIUM OF GUINEA-PIG AND ITS RELATION TO NORADRENALINE STORES

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1 The isolated electrically-paced atrium of the guinea-pig developed a dose-dependent increase in the force of contraction in response to ethacrynic acid (12–100 μg/ml) which was blocked by pretreatment of the animals with reserpine but was unaffected by desipramine or colchicine added to the bathing medium.

2 There was a rapidly developing tachyphylaxis to repeated doses of ethacrynic acid which was not reversed by rest or incubation of the tissue with noradrenaline.

3 There was no cross tachyphylaxis between ethacrynic acid and tyramine, amphetamine or nicotine.

4 Ethacrynic acid (200 μg/ml) decreased the noradrenaline content of the atria by 32%.

5 It is concluded that ethacrynic acid exerts its effects indirectly through the release of endogenous noradrenaline and that the mechanism of release seems to be different from that of other known indirect sympathomimetic drugs.

Introduction

The potent diuretic agent, ethacrynic acid (Beyer, Baer, Michaelson & Russo, 1965), administered chronically to rats, depletes the heart muscle of catecholamines (Torsti, Vapaatalo & Neuvonen, 1968; Neuvonen, Vapaatalo & Torsti, 1969). In previous reports (Pousti, Zarrindast, Sadeghi & Khoyi, 1973; Khoyi, Pousti & Zarrindast, 1974) we have shown that ethacrynic acid increases the force and rate of contraction of spontaneously beating isolated atria and contracts the vas deferens of the guinea-pig. The cardiac effects are blocked by propranolol or reserpine pretreatment whilst the effects on the vas deferens are prevented by phentolamine or reserpine pretreatment and are potentiated by cocaine and low concentrations of desipramine. The spontaneously beating atrium preparation presents problems, however, in that the change in the rate of contraction often prevents a correct assessment of the effect of a drug on the force of contraction.

The present work is a further investigation of the mode of action of ethacrynic acid. Changes in contractile force have been studied on the isolated electrically-paced atria under different conditions. The results suggest that ethacrynic acid acts by release of the adrenergic transmitter; but the mechanism is different from that of other known indirect sympathomimetic drugs.

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Methods

Guinea-pigs of either sex, weighing between 280 and 450 g were used. The method of preparation and recording from the isolated atrium was as described previously (Pousti *et al.*, 1973), except that left atria were used to study alterations in the amplitude of contractions and right or whole atria were used to study the changes in the rate of contractions. The isolated atria were suspended in a bath containing 20 ml of oxygenated Ringer Locke solution, pH 7.4, (37°C) of the following composition (mM): NaCl 158.8, KCl 5.6, $CaCl_2$ 2.16, $NaHCO_3$ 5.9 and glucose 5.5. Rectangular pulses of 3 ms duration at 1.5 times the threshold voltage were applied from a Grass 44 stimulator through the electrodes to which the left atrium was tied, whilst the indifferent electrode was in the bath fluid. A frequency of stimulation of 1 Hz was used in order to minimize the possible stimulation of sympathetic fibres (Levy, 1971). The change

in amplitude of contraction was expressed as a percentage of that before the drugs were added. The tissues were left to equilibrate in the bath for 30 min and then exposed to the different drugs for (in min) ethacrynic acid 3, tyramine 3, amphetamine 3, cocaine 5, colchicine 15, desipramine 15, nicotine 2 and nialamide 60. Ethacrynic acid was dissolved in 5% sodium carbonate solution immediately before use.

When the noradrenaline content of the tissues was being measured, whole atria were equilibrated for 30 min in Ringer Locke solution at 37°C, exposed to ethacrynic acid (200 μg/ml) for 30 min and then returned to solution containing no drug for a further 5 minutes. Controls were left in the bath for 65 minutes. The tissues were dried on filter paper, transferred to liquid nitrogen, weighed and their noradrenaline content determined by the enzymatic method of Iversen & Jarrot (1970) using as the chromotographic solvent butanol:methanol:1 N formic acid, 60:20:20. In some experiments the standard fluorometric trihydroxyindole procedure described by Blakeley, Dearnaley & Harrison (1970) was also used and the results with the two methods were found not to differ.

The following drugs were used: (−)-noradrenaline bitartrate (Winthrop), tyramine hydrochloride (Sigma), ethacrynic acid (Merck, Sharp & Dohme), cocaine hydrochloride (May & Baker), desipramine hydrochloride (Ciba Geigy), nialamide (Sigma), (+)-amphetamine sulphate (Sigma), reserpine (Serpasil ampoules, Ciba Geigy), colchicine (Sigma) and nicotine dihydrogen tartrate (BDH).

The significance of the difference between means was evaluated by Student's t test.

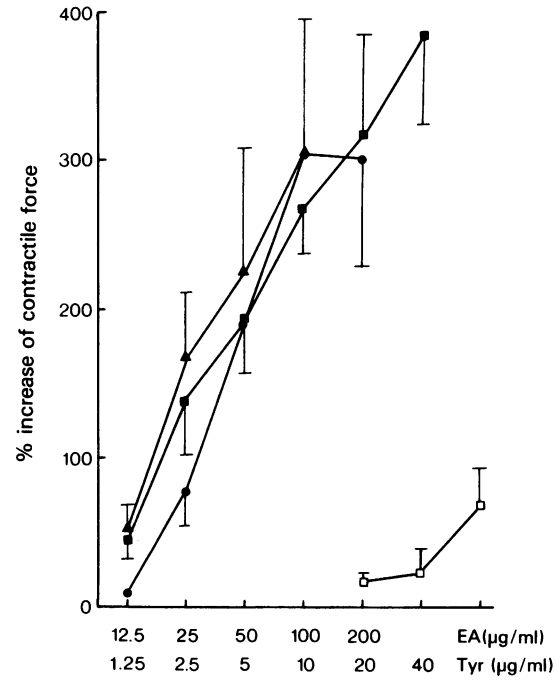

Figure 1 The effect of ethacrynic acid and tyramine on isolated electrically-paced left guinea-pig atria. Abscissa scale: dose of ethacrynic acid (EA) and tyramine (Tyr); ordinate scale: % increase in contractile force. Only one dose of ethacrynic acid was used in each preparation. (●) Ethacrynic acid, (▲) ethacrynic acid in presence of 1.2 μg/ml of desipramine, (■) tyramine, (□) tyramine in presence of desipramine. Each point is mean of at least 4 experiments; vertical lines show s.e. means.

Results

In isolated electrically-paced atria of guinea-pigs, ethacrynic acid (12.5 to 100 μg/ml) produced a dose-dependent increase in the force of contraction (Figure 1). In each preparation, only one dose of ethacrynic acid was tested. To study the degree of involvement of endogenous noradrenaline in the response to ethacrynic acid, guinea-pigs were pretreated with reserpine, 5 mg/kg by intraperitoneal injection 24 h before the experiment. This pretreatment decreased the inotropic effect of ethacrynic acid (200 μg/ml) to 33.1 ± 10.6% (6 experiments, cf. Figure 1). The inotropic effect of tyramine (5 μg/ml) in reserpine-treated atria was reduced to 7.7 ± 1.8% (5 experiments).

On repeated exposure of a tissue to an indirect sympathomimetic drug, tachyphylaxis develops. To find out whether atria develop tachyphylaxis to ethacrynic acid they were exposed repeatedly, at 10 min intervals, to 200 μg/ml of ethacrynic acid, for 3 minutes. The first dose of ethacrynic acid increased the rate of contraction of whole or right atria by 18% but subsequent treatments led to a rapidly developing tachyphylaxis with a complete abolition of the response by the fourth exposure (Figure 2a). In 11 out of 25 atria a negative chronotropic response (3 to 61%) was observed with the third or fourth dose of ethacrynic acid. The inotropic effect of ethacrynic acid, measured in electrically-paced left atria, exhibited less tachyphylaxis and complete abolition of the response did not occur until the sixth or seventh exposure (Figure 2b). The subsequent incubation of the atria with noradrenaline bitartrate, 0.1 μg/ml, for 30 min or a rest period of 2 h with several washings failed to restore the response of the atria to ethacrynic acid.

It was of interest to see whether cross tachyphylaxis developed between ethacrynic acid and other known indirect sympathomimetic drugs. Dose-response curves were prepared for tyramine in 4 left atria before and after the development of complete tachyphylaxis to ethacrynic acid and, as can be seen in

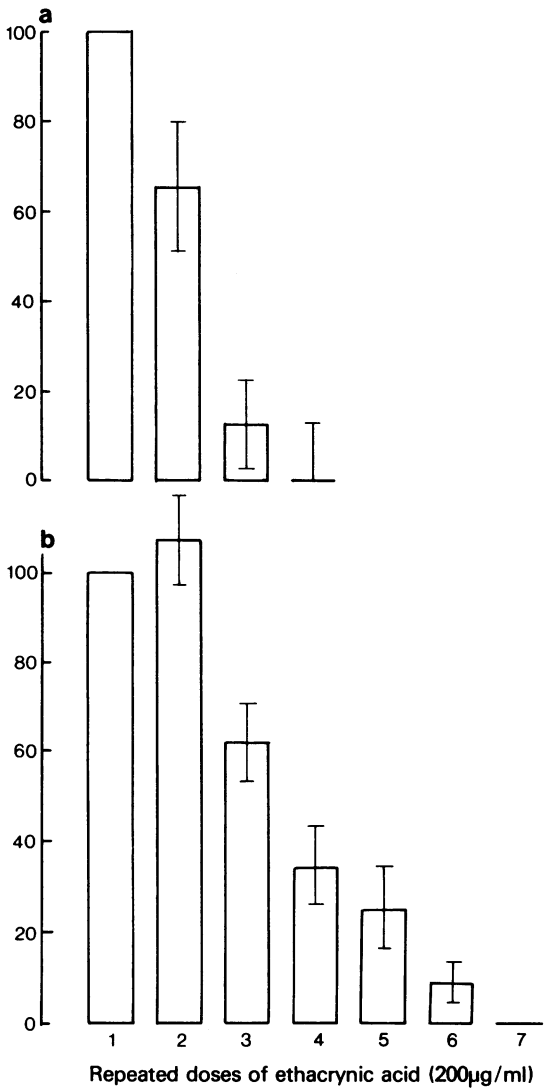

Figure 2 (a) Effect of repeated treatments with ethacrynic acid (200 μg/ml) on the rate of spontaneously beating right or whole atria. The increase observed with the first exposure (18 ± 2.3%) was taken as 100%. (b) Effect of ethacrynic acid (200 μg/ml) on the force of contraction of electrically-paced left atria. The increase observed with the first exposure taken as 100%. Each column is the mean of at least 9 experiments; vertical lines show s.e. mean.

Figure 3, the magnitude of the inotropic response to tyramine did not change significantly after the development of tachyphylaxis to ethacrynic acid. It has been reported (Day, 1967) that there is no cross tachyphylaxis between tyramine and amphetamine and we therefore examined the interaction between amphetamine and ethacrynic acid treatment. Amphetamine sulphate, 20 μg/ml, was tested in 4 non-treated atria and in 4 atria which had been repeatedly exposed to ethacrynic acid until complete tachyphylaxis had developed (Figure 4). There was no significant difference between the two groups ($P > 0.10$). The mechanism of the release of noradrenaline by nicotine-like indirect sympathomimetics is similar to the effect of electrical stimulation and different from tyramine-like indirect sympathomimetics (Smith, 1973; Chubb, De Potter & De Schaepdryver, 1972). The possibility that

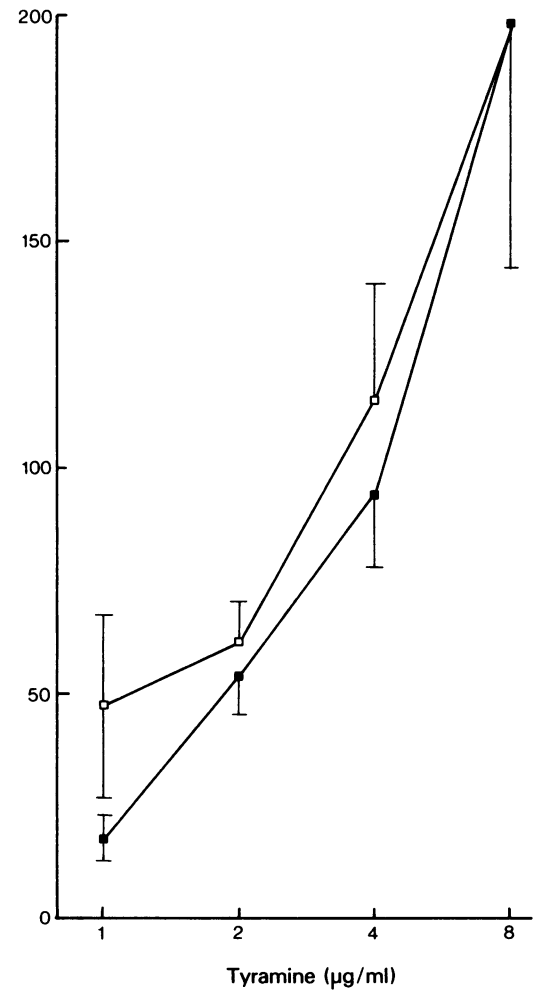

Figure 3 Dose-response curves for tyramine before (■) and after (□) development of tachyphylaxis to ethacrynic acid (200 μg/ml). Each point is mean of 4 experiments. Vertical lines show s.e. means. Abscissa scale: dose of tyramine, ordinate scale: % increase of contractile force of isolated left atria.

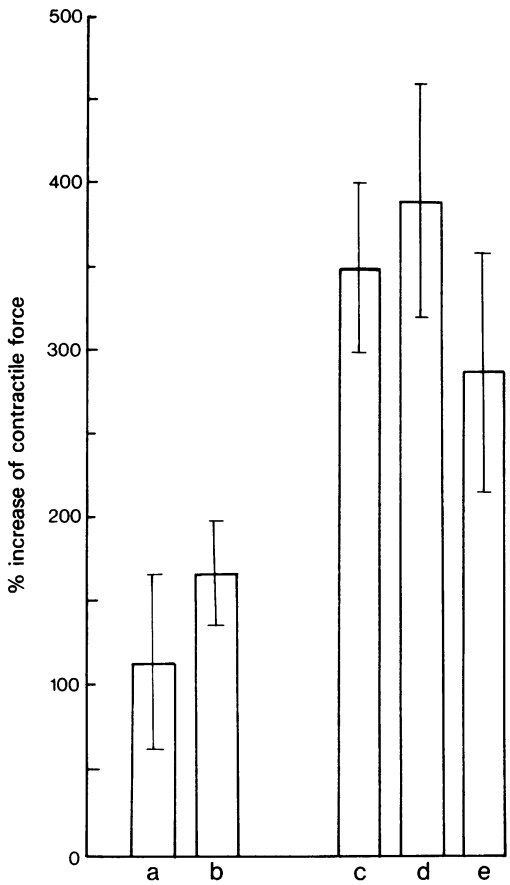

Figure 4 Effect of amphetamine (20 μg/ml, a and b) and nicotine (9.7 μg/ml, c, d and e) on the contractile force of isolated left atria. (a) Amphetamine in 4 control atria; (b) amphetamine in another 4 atria after development of tachyphylaxis to ethacrynic acid; (c) first dose of nicotine (12 atria); (d) second dose of nicotine after a 45 min interval (6 atria) and (e) second dose of nicotine in 6 atria after development of tachyphylaxis to ethacrynic acid. Ordinate scale: % increase of contractile force.

there might be cross tachyphylaxis between nicotine and ethacrynic acid was therefore assessed in 12 atria. Atria were first exposed to nicotine dihydrogen tartrate (30 μg/ml, 9.7 μg base/ml) and then 6 of them acting as controls were washed repeatedly over 45 min and then exposed to a second dose of nicotine. The other 6 atria were treated repeatedly with ethacrynic acid, 200 μg/ml, until complete tachyphylaxis to the drug had developed and were then exposed to a second dose of nicotine. The results, also shown in Figure 4, indicate a 26% decrease in the mean inotropic response to nicotine after treatment with ethacrynic acid but the result was not significantly different from the controls.

Exposure of atria to tyramine, 5 μg/ml, for six 3 min periods was not enough to develop complete tachyphylaxis, that is an absence of a further decrease in contractile force (cf. Lee, Weiner & Trendelenburg, 1967). Similarly, exposure to tyramine 1 mg/ml for 30 min, followed by repeated washings, failed to inhibit the response completely (3 experiments). It has been reported that pretreatment of a tissue with an inhibitor of monoamine oxidase decreases the tachyphylactic dose of tyramine (Maengwyn-Davis, Cown,

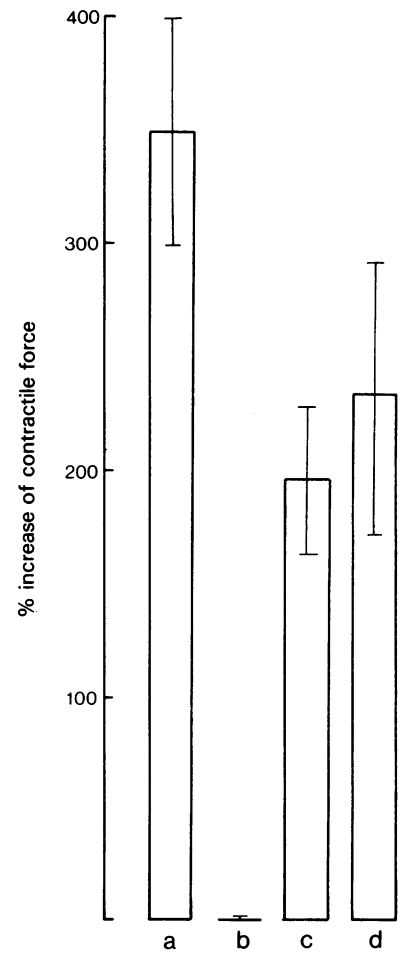

Figure 5 Effect of nicotine (9.7 μg/ml, a and b) and ethacrynic acid (50 μg/ml, c and d) on contractile force of left atria; (a) (12 atria) and (c) (6 atria) responses of control atria; (b) (5 atria) and (d) (4 atria) responses to nicotine and ethacrynic acid in presence of colchicine (400 μg/ml), respectively. Columns indicate mean % increase of contractile force; vertical lines show s.e. mean.

Koppany & Lei, 1966). Left atria were, therefore, incubated with nialamide (120 μg/ml) for 60 min and then repeatedly washed over 30 min before being repeatedly exposed to tyramine (5 μg/ml). Tachyphylaxis developed after only 2 to 3 exposures. The atria were then exposed to ethacrynic acid (200 μg/ml) and the force of contraction increased by 181 ± 67% ($n = 5$), not significantly different from that observed in atria treated with nialamide only (231 ± 38%, $n = 5$, $P > 0.05$).

If ethacrynic acid releases endogenous noradrenaline by a mechanism similar to that of tyramine it should be possible to block its effects by pretreatment of the tissue with desipramine. Dose-response curves of atria to tyramine or ethacrynic acid were obtained in the presence and absence of desipramine (1.2 μg/ml, Figure 1). Only a single dose of ethacrynic acid was tested in each preparation. Desipramine almost completely inhibited the response to tyramine but had no effect upon the response to ethacrynic acid.

The release of noradrenaline by nicotine-like sympathomimetics implies the normal function of microtubules in the axoplasm. It has been shown that 10^{-3} M colchicine interferes with the release of noradrenaline by nicotine but not by tyramine (Sorimachi, Oesch & Thoenen, 1973) presumably by disintegration of microtubules. If ethacrynic acid releases noradrenaline by a mechanism similar to that of nicotine, that is by exocytosis, pretreatment of the tissue with colchicine should prevent its effects. The responses to nicotine (9.7 μg/ml) and ethacrynic acid (50 μg/ml) were studied in left atria before and 15 min after the addition of colchicine (400 μg/ml, Figure 5). Colchicine completely blocked the effects of nicotine whilst having no effect upon the response to ethacrynic acid.

Changes in the tissue noradrenaline content were also measured as described in Methods. The concentration of noradrenaline (μg/g wet tissue) in atria incubated with 200 μg/ml of ethacrynic acid was 3.7 ± 0.21 ($n = 8$) as compared with 5.43 ± 0.37 ($n = 10$) in the control group ($P < 0.005$).

Discussion

The increase in the force of contraction of the isolated electrically-paced left atrium in response to ethacrynic acid and its inhibition by pretreatment of the animals with reserpine confirms previous work with spontaneously beating atria (Pousti *et al.*, 1973) and suggests that the effect of ethacrynic acid is probably mediated through the release of endogenous noradrenaline. The development of tachyphylaxis towards ethacrynic acid seems to be in accord with this view. Also consistent with this, is the observation that reserpine-treated atria, which would be expected to have lost more than 95% of their noradrenaline content (Crout, Muskus & Trendelenburg, 1962) developed a correspondingly smaller positive inotropic effect (11% of the non-reserpine-treated atria) in response to ethacrynic acid. A possible difficulty with this explanation is that atria which have developed complete tachyphylaxis to ethacrynic acid have lost only 32% of their noradrenaline content. It is known, however, that hearts which have developed tachyphylaxis to tyramine show no significant depletion of their noradrenaline content (Axelrod, Gordon, Hertting, Kopin & Potter, 1962; Lee *et al.*, 1967). Therefore it appears that there need be no direct correlation between the tissue noradrenaline content and development of tachyphylaxis to an indirect sympathomimetic such as ethacrynic acid. Damage to vesicular membrane or the microtubules does not seem to be the cause of tachyphylaxis since ethacrynic acid did not abolish the response of atria to nicotine which releases noradrenaline by exocytosis.

The absence of cross tachyphylaxis between tyramine and ethacrynic acid confirms our previous report on guinea-pig vas deferens (Khoyi *et al.*, 1974) and suggests that ethacrynic acid may have a different mechanism of action from that of tyramine. The absence of cross tachyphylaxis between ethacrynic acid and amphetamine and ethacrynic acid and nicotine can be interpreted in the same way.

It is well known that desipramine inhibits the indirect sympathomimetic effect of tyramine (Farrant, 1963; Furchgott, Kirpekar, Rieker & Schwab, 1963) by competing with tyramine for a common amine pump (Trendelenburg, 1961; Farmer & Petch, 1963). In the present study, desipramine failed to inhibit the effects of ethacrynic acid. This is in agreement with our previous work in the guinea-pig vas deferens (Khoyi *et al.*, 1974) and again suggests that ethacrynic acid and tyramine act by different mechanisms. Both ethacrynic acid and ouabain are inhibitors of cardiac Na^+, K^+-ATPase (Gibson & Harris, 1970). Ouabain causes a marked increase in the rate of [^{3}H]-noradrenaline efflux, an effect which is prevented by desipramine and which prompted Paton (1973) to suggest the involvement of a carrier-mediated transport of noradrenaline. The failure of desipramine to affect the response of the atria to ethacrynic acid suggests that ethacrynic acid does not act by accelerating the carrier-mediated transport of noradrenaline.

Colchicine, which disintegrates the microtubules (Borisy & Taylor, 1967), interferes with the release of noradrenaline by nicotine but not by tyramine (Sorimachi *et al.*, 1973). The lack of effect of colchicine on the response to ethacrynic acid suggests that the latter does not depend for its action on intact microtubules.

In summary, therefore, the present work suggests that ethacrynic acid acts through the release of the adrenergic transmitter by a mechanism which differs

from that of tyramine- or nicotine-like sympathomimetic drugs.

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