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Steroid potentiation of responses to sympathomimetic amines in aortic strips

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1. Responses to catecholamines (adrenaline, noradrenaline, nordefrine) were enhanced by 17β-oestradiol, progesterone and desoxycorticosterone in untreated and reserpine pretreated aortic strips. Responses to tyramine, believed mediated via endogenous catecholamines, were enhanced only in untreated strips.

2. Responses to sympathomimetic amines lacking the catechol nucleus (phenylephrine, synephrine, methoxamine) were potentiated inconsistently by the steroids and reserpine pretreatment reduced markedly the frequency of potentiated responses.

3. Known inhibitors of catechol-O-methyl transferase (tropolone, U-0521, pyrogallol) potentiated responses to catecholamines and abolished the enhancing effects of the steroids—when the steroids were given first, there was no further increase in response to catecholamines on adding inhibitors of catechol-O-methyl transferase.

4. Experiments with the oil-immersion technique, to eliminate diffusion of drug from the tissue, indicated that 17β-oestradiol, progesterone and desoxycorticosterone decreased the rate at which aortic strips inactivated adrenaline by O-methylation.

5. It is concluded that 17β-oestradiol, progesterone and desoxycorticosterone potentiate responses to catecholamines in aortic strips by inhibiting a major mechanism for their inactivation.

Hydrocortisone potentiates responses to adrenaline and noradrenaline in aortic strips by inhibiting their inactivation by catechol-O-methyl transferase (Kalsner, 1969). It was of interest to determine whether other steroid hormones also enhance responses to catecholamines and decrease their rate of inactivation by O-methylation. In the present study the effects of 17β-oestradiol, progesterone and desoxycorticosterone on responses to a variety of sympathomimetic amines were investigated.

Methods

Rabbit aortic strips, prepared according to Furchgott (1960), were suspended under 2 g tension in 15 ml. chambers containing Krebs-Henseleit (Krebs) solution

at 37° C. Disodium EDTA was added to the Krebs reservoir to give a final concentration of 0·01 g/l. Responses were recorded isotonically, with 6·8-fold magnification, on a slowly-moving kymograph (usually 1·8 mm/min). Strips were allowed to equilibrate for 90 min before drug testing.

Concentrations of l-noradrenaline and l-adrenaline bitartrates, nordefrine (Cobefrine), l-phenylephrine, methoxamine and tyramine hydrochlorides and l-synephrine are expressed as the base. Cocaine hydrochloride and iproniazid phosphate are expressed as the salts. All drug concentrations are presented as final concentrations in the muscle chambers.

17β-Oestradiol, progesterone and desoxycorticosterone were used as the free alcohols and dissolved in ethanol, or usually propylene glycol, to give stock concentrations of 10 mg/ml. The volume usually added to the muscle chambers was 0·015 ml. In preliminary experiments this volume of ethanol or propylene glycol, alone, had no effect on the basal tone of aortic strips or on their responses to sympathomimetic amines.

Reserpine powder was dissolved in 10% ascorbic acid and solutions prepared every few days. Rabbits were injected intramuscularly with 2–5 mg/kg, usually 18–24 hr before death.

The mineral oil used in the oil-immersion experiments was kept at 37° C in flasks in a water bath and constantly bubbled with 95% oxygen–5% carbon dioxide. A flow of the gas mixture through the muscle chambers was maintained when they were filled with either Krebs solution or oil. After a given response had reached a stable plateau value the aqueous medium was drained from the muscle chamber and it was rapidly refilled with mineral oil, without an intervening wash of the tissue. This trapped a fixed quantity of drug in the tissue. The rate of relaxation of aortic strips in oil can be equated with intrinsic inactivation of drug which had caused the response. Evidence for the absence of any pharmacological action of the oil itself, the lack of accumulation of toxic metabolites and the adequate oxygenation of the tissue during oil immersion has been previously presented (Kalsner & Nickerson, 1968a).

Monoamine oxidase (MAO) was inhibited by iproniazid (1 or 2×10^{-4} g/ml.) (Zeller & Barsky, 1952 ; Furchgott, 1955). Catechol-O-methyl transferase (COMT) was inhibited with pyrogallol (3×10^{-5} g/ml.), tropolone ($1–3 \times 10^{-5}$ g/ml.) or U-0521 (3′-4′-dihydroxy-2-methyl propiophenone) (1×10^{-5} g/ml.) (Belleau & Burba, 1961 ; Mavrides, Missala & D'Iorio, 1963 ; Giles & Miller, 1967). These concentrations produced maximal effects. Evidence for the specificity and completeness of the procedures used to inhibit mechanisms of amines inactivation in aortic strips exposed to oil has been previously presented (Kalsner & Nickerson, 1968a, b ; 1969a). Mean values are shown with standard errors and were compared by Student's *t* test. Differences with *P* values of 0·05 or less were considered significant.

Results

Effects of steroids on responses to adrenaline

The procedure used to assess the effects of steroid hormones on responses to adrenaline and other sympathomimetic amines is illustrated in Fig. 1. Contractions to an agonist were produced on the steep portion of the dose-response curve and after a stable level of response was reached the steroid under investigation was added

to the muscle chamber. A change in size of contraction due to the steroid was readily detectable. The steroids alone, in the concentrations used, had no effect on the basal tone of aortic strips (Fig. 1d).

Responses to adrenaline were enhanced by 1×10^{-6} g/ml. of 17β-oestradiol, progesterone or desoxycorticosterone. Maximal enhancement was produced by 17β-oestradiol (3×10^{-6} to 1×10^{-5} g/ml.), progesterone (1×10^{-6} g/ml.) and desoxycorticosterone (1×10^{-5} g/ml.) (Fig. 1 and Table 1). The potentiation of responses to adrenaline (3×10^{-9} g/ml.) was equivalent to raising the bath concentration of adrenaline to 8·4, 6·2 and $8{\cdot}2 \times 10^{-9}$ g/ml., respectively. Neither pretreatment of rabbits with reserpine nor the presence of cocaine (1×10^{-5} g/ml.) interfered with the action of the steroids.

The enhancing effect of desoxycorticosterone but not that of 17β-oestradiol or progesterone was rapidly reversed after washout of the muscle chambers (Fig. 1). The responses of strips recontracted by adrenaline alone 60 min after washout of adrenaline plus 17β-oestradiol or progesterone were significantly greater than initial responses and no clear increment due to re-addition of the steroids was observed. However, the residual effects of the steroids appeared relatively slight if 120 min elapsed between tests. In the experiments described in this paper, aortic strips were discarded routinely after a single exposure to 17β-oestradiol or progesterone.

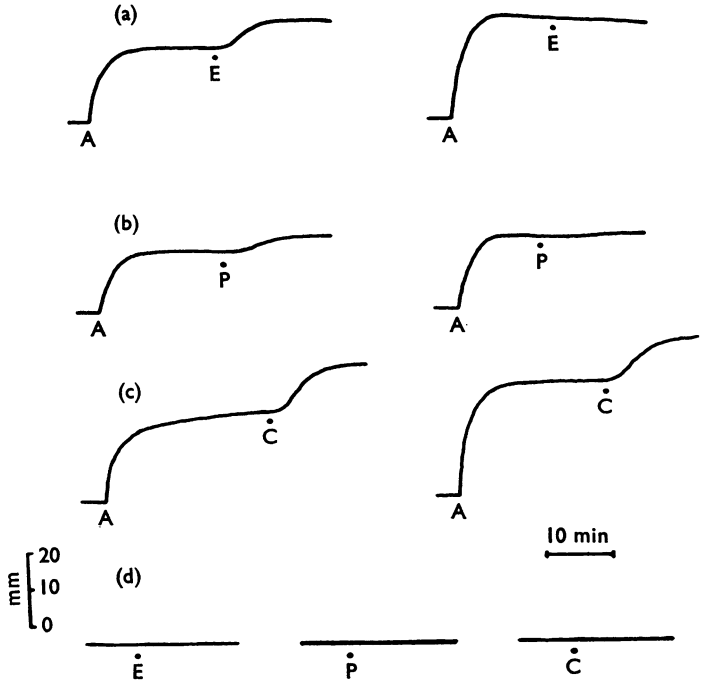

FIG. 1. Effects of steroid hormones on basal tone of aortic strips and responses to adrenaline (A) (3×10^{-9} g/ml.). a,b,c, in each pair, trace on the left shows the initial effect of 17β-oestradiol (E) (1×10^{-5} g/ml.), progesterone (P) (1×10^{-5} g/ml.) or desoxycorticosterone (C) (1×10^{-5} g/ml.), respectively; right, the effects of the steroids on the same strips recontracted by (A) after a 60 min interval. d, Effect of the steroids on the quiescent tone of aortic strips.

TABLE 1. *Effects of steroid hormones on responses to catecholamines*

Agonist (g/ml.)	Steroid: 17β-Oestradiol (1×10^{-5} g/ml.) Agonist contraction amplitude (mm)	17β-Oestradiol (1×10^{-5} g/ml.) Steroid increment (mm)	Progesterone (1×10^{-5} g/ml.) Agonist contraction amplitude (mm)	Progesterone (1×10^{-5} g/ml.) Steroid increment (mm)	Desoxycorticosterone (1×10^{-5} g/ml.) Agonist contraction amplitude (mm)	Desoxycorticosterone (1×10^{-5} g/ml.) Steroid increment (mm)
Adrenaline						
3×10^{-9}	(23) 18·9±1·0	9·7±0·7	(15) 18·1±0·9	6·8±0·9	(8) 21·7±3·2	9·6±1·8
1×10^{-8}	(12) 28·6±2·5	8·2±0·8	(11) 32·0±2·1	8·0±1·1	(12) 32·0±1·9	7·4±0·6
Noradrenaline						
3×10^{-9}	(6) 20·2±5·3	2·3±0·9	(6) 24·1±1·5	3·3±0·6	(7) 20·4±1·8	2·4±0·8
1×10^{-8}	(8) 29·1±2·6	4·3±1·2	(6) 30·5±2·5	3·5±0·8	(8) 32·2±2·7	2·3±0·8
Nordefrine						
3×10^{-8}	(5) 12·8±1·9	7·8±0·6	(5) 17·1±4·6	6·4±1·2	(8) 9·5±1·9	6·9±1·6
Tyramine*						
3×10^{-6}	(4) 13·9±2·5	3·0±0·7	(4) 7·8±2·3	3·9±0·9	(5) 12·6±2·2	5·2±0·6

* Responses to tyramine are believed to be mediated via the release of endogenous catecholamines.
The steroids were added to the muscle chambers after responses to agonists had reached plateau values. Figures in parentheses to the left of contraction amplitudes indicate number of strips.

Effects of steroids on responses to other catecholamines

Responses to noradrenaline (3×10^{-9} g/ml.) were potentiated much less than those to adrenaline (3×10^{-9} g/ml.) by 17β-oestradiol, progesterone and desoxycorticosterone; equivalent to raising the bath concentration of noradrenaline to 4·0, 4·8 and $4{\cdot}2 \times 10^{-9}$ g/ml., respectively. The steroids also enhanced responses to nordefrine and, provided that there had been no reserpine pretreatment, to tyramine, an agent believed to act through the release of endogenous catecholamines. The above findings are summarized in Table 1.

Effects of steroids on responses to amines lacking the catechol nucleus

Responses to phenylephrine, methoxamine, synephrine and those of reserpine-pretreated strips to tyramine were not clearly enhanced on any occasion by desoxycorticosterone (1×10^{-5} g/ml.). Typical traces from several experiments are shown in Fig. 2 and the results are tabulated in Table 2. 17β-Oestradiol (1×10^{-5} g/ml.) and progesterone (1×10^{-5} g/ml.) enhanced inconsistently the responses to phenylephrine, synephrine and methoxamine (Table 2). Reserpine-pretreatment appeared to reduce the frequency of potentiated responses (Table 2).

Effects of COMT inhibitors on steroid potentiation of catecholamine responses

Aortic strips were exposed to the known COMT inhibitors tropolone ($1\text{–}3 \times 10^{-5}$ g/ml.), pyrogallol (3×10^{-5} g/ml.) or U-0521 (1×10^{-5} g/ml.) followed by adrenaline (3×10^{-9} or 1×10^{-8} g/ml.) and treatment with a steroid hormone. The known

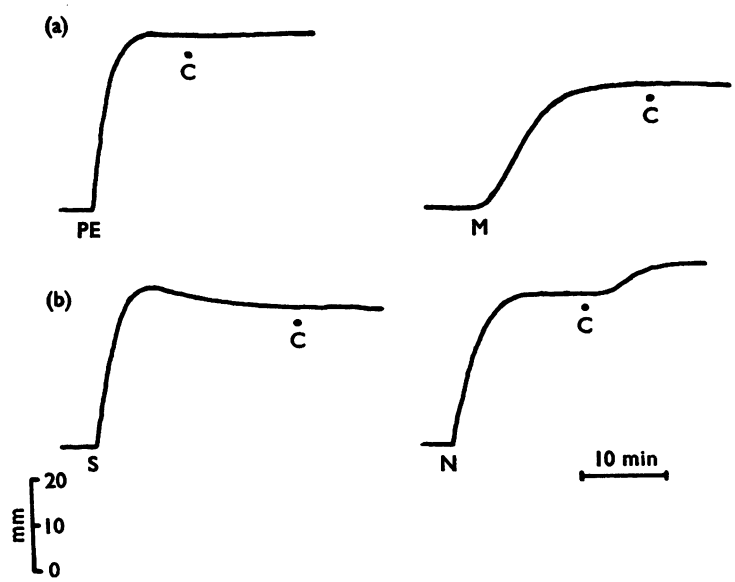

FIG. 2. Effects of desoxycorticosterone (C) (1×10^{-5} g/ml.) on responses to sympathomimetic amines. a, Strips contracted by phenylephrine (PE) (1×10^{-8} g/ml.) or methoxamine (M) (1×10^{-7} g/ml.) and exposed to desoxycorticosterone (C). b, Strips contracted by synephrine (S) (1×10^{-6} g/ml.) or nordefrine (N) (3×10^{-8} g/ml.) and exposed to (C).

TABLE 2. *Effects of steroid hormones on responses to sympathomimetic amines lacking the catechol nucleus*

		Steroid								
		17β-Oestradiol (1×10^{-5} g/ml.)			Progesterone (1×10^{-5} g/ml.)			Desoxycorticosterone (1×10^{-5} g/ml.)		
Agonist (g/ml.)	Pretreatment	Agonist contraction amplitude (mm)	Steroid increment (mm)	No. resp. potentiated	Agonist contraction amplitude (mm)	Steroid increment (mm)	No. resp. potentiated	Agonist contraction amplitude (mm)	Steroid increment (mm)	
Phenylephrine										
2×10^{-8}	None	(5) 28·9±3·4	0	0	(6) 31·1±4·1	0	0	(9) 35·2±1·2	0	
6×10^{-8}	None	(8) 38·4±4·2	0·9±0·2	4	(5) 31·9±2·7	0·8±0·6	2	(4) 39·6±2·2	0	
	Reserp.	(5) 48·7±1·5	0	0	(4) 41·6±6·4	0	0	—	—	
Synephrine										
1×10^{-6}	None	(9) 30·0±2·7	2·2±0·5	8	(8) 30·6±3·3	1·6±0·6	6	(8) 23·6±2·5	0	
	Reserp.	(13) 32·3±2·4	0·8±0·3	8	(8) 29·7±1·7	0	0	—	—	
Methoxamine										
5×10^{-8}	Reserp.	(4) 19·6±3·1	0	0	(4) 20·9±4·5	0	0	—	—	
1×10^{-7}	None	(7) 23·9±3·2	1·4±0·6	4	(8) 20·4±3·1	1·3±0·6	4	(7) 25·6±2·2	0	
	Reserp.	(7) 29·4±3·4	0	0	(8) 25·3±5·1	0·4±0·3	3	—	—	
Tyramine										
3×10^{-5}	Reserp.	(3) 15·8±3·6	0	0	(4) 15·4±1·5	0	0	(4) 19·4±1·1	0	

The steroids were added to the muscle chambers after responses to agonists had reached plateau values. Figures in parentheses to the left of contraction amplitudes indicate number of strips.

COMT inhibitors abolished the enhancing effects of 17β-oestradiol (1×10^{-5} g/ml.) progesterone (1×10^{-5} g/ml.) and desoxycorticosterone (1×10^{-5} g/ml.) in a total of seventeen, thirteen and eight strips, respectively. In similarly designed experiments, known COMT inhibitors reduced or usually abolished the enhancing effect of the steroids on responses to noradrenaline and nordefrine and on responses to tyramine (in the absence of reserpine). In several strips the steroids now produced a slight depression of response amplitude. Typical records from some of these experiments are shown in Fig. 3.

Additional experiments were done in which the steroid hormones and U-0521 (1×10^{-5} g/ml.) or tropolone (1×10^{-5} g/ml.) were added to the chambers containing strips already contracted by a catecholamine. The addition of a known COMT inhibitor did not potentiate further the responses of strips contracted by adrenaline, noradrenaline, nordefrine or tyramine and exposed to either 17β-oestradiol (1×10^{-5} g/ml.), progesterone (1×10^{-5} g/ml.) or desoxycorticosterone (1×10^{-5} g/ml.). Similarly, the steroids did not enhance the responses of strips contracted by a

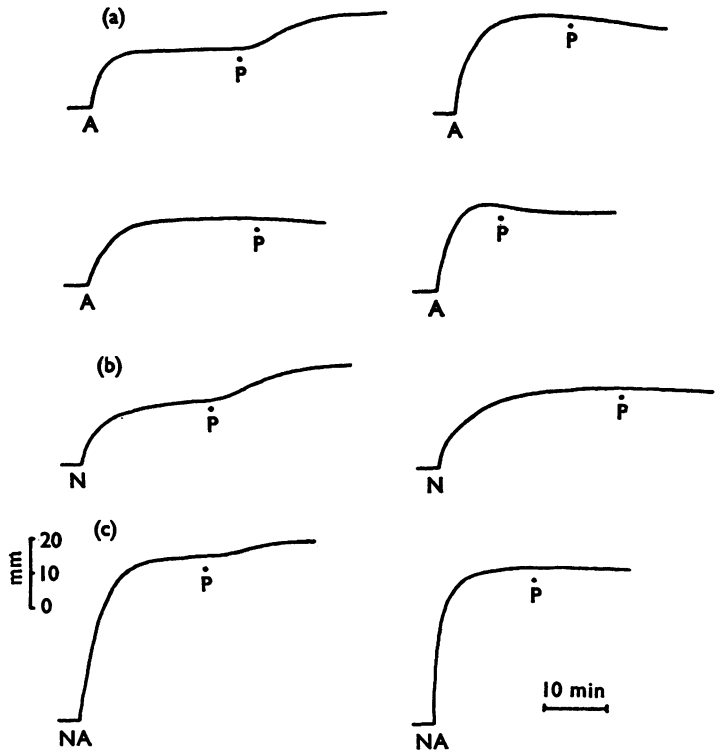

FIG. 3. Effects of COMT inhibitors on responses to progesterone (P) (1×10^{-5} g/ml.) of aortic strips contracted by catecholamines. a, Top left, effect of (P) on untreated aortic strip contracted by adrenaline (A) (3×10^{-9} g/ml.); to right, effect of (P) on response to (A) in the presence of tropolone (1×10^{-5} g/ml.). Lower left and right, aortic strips contracted by (A) and exposed to (P) in the presence of U-0521 (1×10^{-5} g/ml.) or pyrogallol (1×10^{-5} g/ml.), respectively. b, Effect of (P) on response to nordefrine (N) (3×10^{-8} g/ml.) in the absence (left) and in the presence (right) of U-0521 (1×10^{-5} g/ml.). c, Effect of (P) on response to noradrenaline (NA) (3×10^{-9} g/ml.) in the absence (left) and in the presence (right) of U-0521 (1×10^{-5} g/ml.).

catecholamine and exposed to a known COMT inhibitor. Typical traces from several of these experiments are shown in Fig. 4.

Relaxation of adrenaline (1×10^{-8} g/ml.) and phenylephrine (3×10^{-8} g/ml.) contracted aortic strips in oil

Aortic strips were contracted by adrenaline (1×10^{-8} g/ml.) and exposed to oil. After return to Krebs solution and recovery of basal tone, the strips were recontracted by the same concentration of adrenaline and exposed to either 17β-oestradiol (1×10^{-5} g/ml.), progesterone (1×10^{-5} g/ml.) or desoxycorticosterone (1×10^{-5} g/ml.) about 10–20 min before oil immersion. The steroids increased the time for 50% relaxation to 5·9, 1·7, and 3·0 times that of the controls, respectively. The results are tabulated in Table 3a.

The effect of the steroid hormones on the inactivation of adrenaline was also studied in aortic strips in which MAO was inhibited with iproniazid and the uptake and storage processes with cocaine. Strips were treated with iproniazid, contracted

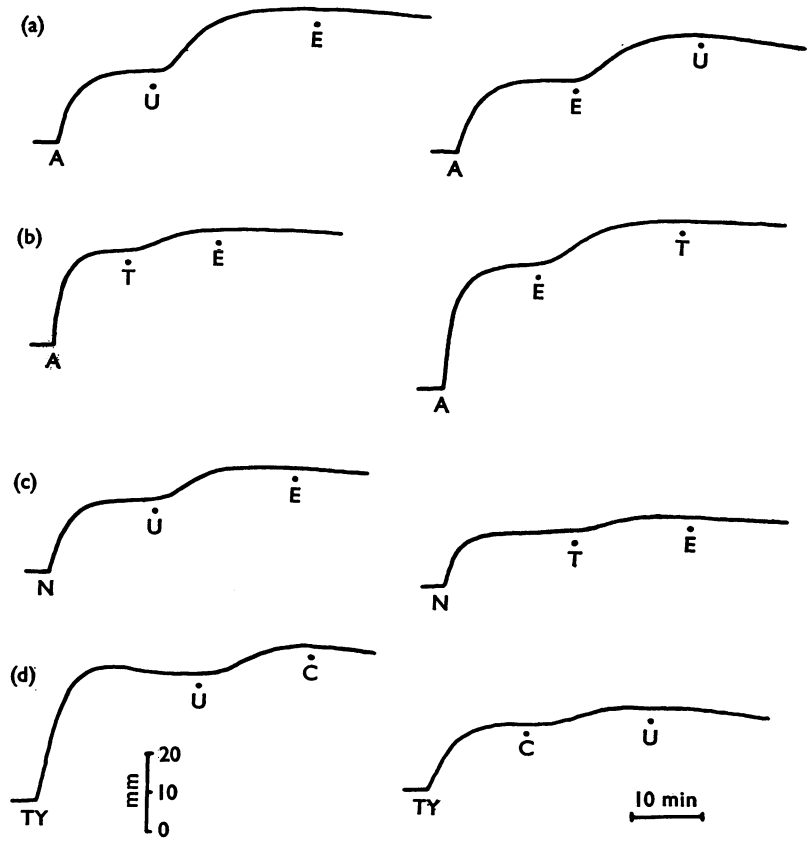

FIG. 4. Interactions of COMT inhibitors and steroid hormones on responses to catecholamines. a, Aortic strips contracted by adrenaline (A) (3×10^{-9} g/ml.) and exposed to 17β-oestradiol (E) (1×10^{-5} g/ml.) and U-0521 (U) (1×10^{-5} g/ml.). b, Aortic strips contracted by (A) and exposed to (E) and tropolone (T) (1×10^{-5} g/ml.). c, Aortic strips contracted by nordefrine (N) (3×10^{-8} g/ml.) and exposed to (U) or (T) followed by (E). d, Aortic strips contracted by tyramine (TY) (3×10^{-6} g/ml.) and exposed to desoxycorticosterone (C) (1×10^{-5} g/ml.) and (U).

by adrenaline (1×10^{-8} g/ml.) for 10 to 20 min and exposed to cocaine (1×10^{-5} g/ml.) for an additional 10 min, and then immersed in oil. After return to Krebs solution and recovery of basal tone the strips were again contracted by adrenaline (the effect of iproniazid is irreversible), re-exposed to cocaine and in addition treated with either 17β-oestradiol (1×10^{-5} g/ml.), progesterone (1×10^{-5} g/ml.) or desoxycorticosterone (1×10^{-5} g/ml.) about 10–20 min before oil immersion. The steroids greatly reduced the residual rate of relaxation (Table 3b). Traces from a typical experiment with 17β-oestradiol are shown in Fig. 5a.

TABLE 3. *Relaxation of adrenaline (1×10^{-8} g/ml.) contracted aortic strips in oil*

a. Treatment	No. of strips	Time to relax 50% (min)	Multiple of control time
Untreated	26	3·3±0·2	
17β-Oestradiol (1×10^{-5} g/ml.)	11	19·3±1·4	5·9
Progesterone (1×10^{-5} g/ml.)	8	5·6±0·5	1·7
Desoxycorticosterone (1×10^{-5} g/ml.)	7	9·7±0·6	3·0

b. Treatment	No. of strips	Time to relax 50% (min)	Multiple of control time
Iproniazid plus cocaine (1×10^{-5} g/ml.)	22	3·8±0·5 *1·4±0·1	
Iproniazid plus cocaine (1×10^{-5} g/ml.) plus 17β-oestradiol (1×10^{-5} g/ml.)	7	*16·4±3·0	11·7
Iproniazid plus cocaine (1×10^{-5} g/ml.) plus progesterone (1×10^{-5} g/ml.)	8	9·5±1·5	2·5
Iproniazid plus cocaine (1×10^{-5} g/ml.) plus desoxycorticosterone (1×10^{-5} g/ml.)	7	21·6±2·5	5·6

Asterisk indicates time to relax 20 rather than 50%.

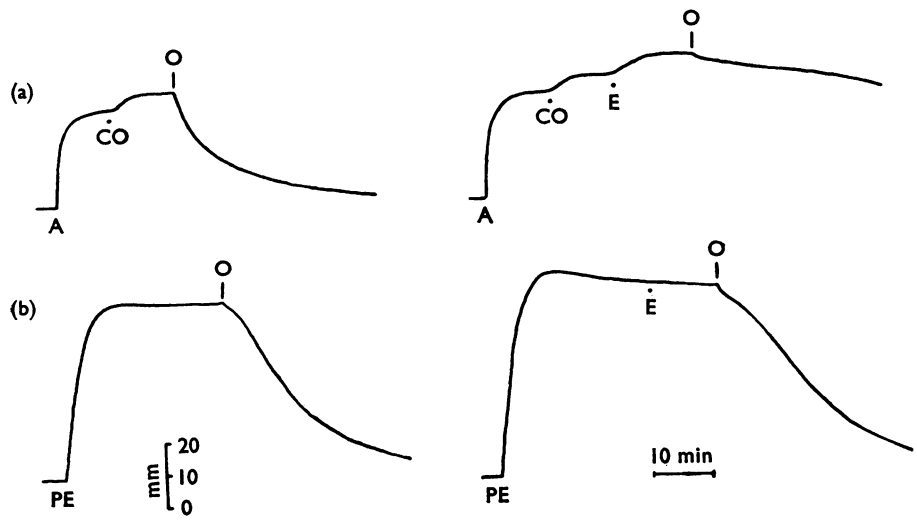

FIG. 5. Relaxation in oil of aortic strips previously contracted by adrenaline (A) (1×10^{-8} g/ml.) and phenylephrine (PE) (3×10^{-8} g/ml.). a, Strip contracted twice by (A): left, strip pretreated with iproniazid and exposed to cocaine (CO) (1×10^{-5} g/ml.) before oil immersion; right, strip re-exposed to (CO) (effect of iproniazid is irreversible) and treated with 17β-oestradiol (E) (1×10^{-5} g/ml.) before oil immersion. b, Strip contracted twice by (PE): left, control relaxation; right, strip exposed to (E) before oil immersion.

The effect of 17β-oestradiol (1×10^{-5} g/ml.) was also studied on the rate of relaxation in oil of eleven aortic strips contracted twice by phenylephrine (3×10^{-8} g/ml.), a sympathomimetic amine which is not a substrate for COMT. Strips relaxed 50% in 10·1 min before and 11·5 min after treatment with the steroid. This difference was significant at the 5% level of probability by the one-tailed t test for paired data. The traces from a typical experiment are shown in Fig. 5b.

Discussion

It was previously reported that hydrocortisone potentiates responses to catecholamines but not to other sympathomimetic amines in aortic strips by inhibiting COMT (Kalsner, 1969). The enhancement of responses to catecholamines by 17β-oestradiol, progesterone and desoxycorticosterone which was observed in the present experiments appears also to be due to inhibition of O-methylation. Bohr, Brodie & Cheu (1958) had previously observed that desoxycorticosterone enhanced responses to adrenaline in aortic strips, but they attributed this to an effect on transmembrane ion gradients.

COMT is the major mechanism for the inactivation of adrenaline and noradrenaline in aortic strips, with MAO an effective alternate mechanism for the inactivation of noradrenaline (Kalsner & Nickerson, 1969a). Known COMT inhibitors potentiate responses to adrenaline much more than those to noradrenaline (Kalsner, 1969). In the present experiments it was observed that responses to the former were enhanced much more than those to the latter amine by each of the steroids investigated. Known COMT inhibitors abolished the enhancing effect of the steroids and vice versa. In a variety of experiments no difference was detectable between the enhancing effect of the steroids on responses to adrenaline, noradrenaline, nordefrine and tyramine and those of known COMT inhibitors (pyrogallol, tropolone, U-0521).

Experiments with the oil immersion technique demonstrated that each of the steroids decreased the rate at which aortic strips inactivated adrenaline (1×10^{-8} g/ml.). The steroids also slowed the relaxation of adrenaline contracted strips after inhibition of MAO and uptake and storage processes. It was previously reported that exposure of strips treated with iproniazid plus cocaine to the COMT inhibitor, tropolone, reduced their residual capacity to inactivate adrenaline by 92% (Kalsner & Nickerson, 1969a). This finding demonstrated that no other independent mechanism of consequence operates to inactivate adrenaline in aortic strips. Exposure of strips treated with iproniazid plus cocaine to 17β-oestradiol (1×10^{-5} g/ml.), progesterone (1×10^{-5} g/ml.) or desoxycorticosterone (1×10^{-5} g/ml.) reduced their residual inactivation capacity by 92%, 60% and 82%, respectively.

17β-Oestradiol (1×10^{-5} g/ml.) increased by a factor of 5·9 the time needed for 50% relaxation of untreated strips contracted by adrenaline (1×10^{-8} g/ml.). A maximally effective concentration of the COMT inhibitor, tropolone, was previously reported to increase the relaxation time of adrenaline (1×10^{-8} g/ml.) contracted strips to 2·9 times that of controls (Kalsner & Nickerson, 1969a). These shifts represent the elimination of 83% and 65% of the capacity of the tissues to inactivate adrenaline, respectively. Maximal potentiation of responses to adrenaline was usually produced by 17β-oestradiol (3×10^{-6} g/ml.). It is possible that 17β-oestradiol (1×10^{-5} g/ml.) exerts an additional, but slight, effect to impair inactivation of sympathomimetic amines by MAO and/or uptake and storage processes.

Evidence supporting such a possibility was the finding that 17β-oestradiol (1×10^{-5} g/ml.) slightly, but significantly, slowed the relaxation of strips contracted by phenylephrine, an amine which is not a substrate for COMT.

It has been reported that there are cyclic variations in the adrenaline and noradrenaline content of the plasma and uterus of various species (Rudzik & Miller, 1962; Wurtman, Chu & Axelrod, 1963; Wurtman, Axelrod & Potter, 1964; Green & Miller, 1965; Cha, Lee, Rudzik & Miller, 1965). Wurtman *et al.* (1964) found that the amount of adrenaline taken up from the circulation by the rat uterus was increased after the administration of 17β-oestradiol. The endogenous content of adrenaline but not of noradrenaline of the heart, spleen and uterus of rats is also elevated by 17β-oestradiol (Spratto & Miller, 1968). This steroid is reported to be without effect on phenylethanolamine-N-methyl transferase or the subcellular distribution of catecholamines in the uterus (Spratto & Miller, 1968).

It is possible that inhibition of COMT by 17β-oestradiol plays some role in the reported cyclic elevations of tissue and plasma adrenaline. It is known that more adrenaline than noradrenaline is O-methylated following the injection of tritiated amine (Axelrod, Weil-Malherbe & Tomchick, 1959; Whitby, Axelrod & Weil-Malherbe, 1961). Also, inhibition of COMT interferes with the inactivation of a low concentration of adrenaline much more than with that of noradrenaline in aortic strips (Kalsner & Nickerson, 1969a). This appears to be due, at lease in part, to the effectiveness of MAO as an alternate pathway of noradrenaline inactivation.

It is not known whether the ability of a number of steroid hormones to inhibit COMT is limited to vascular tissue. In addition, it is possible that the steroids do not directly inhibit the enzyme but decrease access of substrate to the enzyme by impairing movement across certain biological membranes. Such a mechanism of interference with amine metabolism has previously been invoked to explain the effects of the β-haloalkylamines on noradrenaline metabolism (Eisenfeld, Krakoff, Iversen & Axelrod, 1967; Kalsner & Nickerson, 1969b).

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