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Mechanism of potentiation of contractor responses to catecholamines by methylxanthines in aortic strips

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Summary

1. Caffeine and theophylline increased the amplitude of contractor esponses of untreated and reserpine pretreated rabbit aortic strips to catecholamines (adrenaline, α-methylnoradrenaline, noradrenaline). Responses to amines without both the 3- and 4-OH groups in the benzene ring (methoxamine, phenylephrine, Synephrine) were not increased by theophylline and only those to Synephrine were slightly enhanced by caffeine.

2. Compounds which inhibit catechol-O-methyltransferase (pyrogallol, tropolone, U-0521) potentiated responses to catecholamines and abolished the enhancing effect of theophylline and caffeine. Also, the potentiation produced by inhibitors of O-methylation was significantly reduced in the presence of the methylxanthines.

3. Experiments done with the aid of the technique of oil immersion, to eliminate the diffusion of drug from the tissue into the bathing medium, showed that theophylline and caffeine decreased the rate of inactivation of adrenaline by O-methylation.

4. These findings indicate that methylxanthines potentiate the contractor responses to catecholamines in aortic strips by inhibiting their extraneuronal inactivation.

Introduction

Caffeine and theophylline potentiate the contractor response of smooth muscle to adrenaline and noradrenaline, and attempts have been made to relate this effect to one of the known actions of the methylxanthines. Bartelstone, Nasmyth & Telford (1967) raised the possibility that sympathomimetic amine-induced contractions are mediated by cyclic AMP. They suggested that, in their experiments on rat aorta and rabbit uterus, theophylline enhanced responses to noradrenaline by inhibiting cyclic AMP phosphodiesterase. This enzyme degrades cyclic AMP and is inhibited by the methylxanthines (Butcher & Sutherland, 1962). In contrast, Bohr (1965) proposed that caffeine increased responses to adrenaline in rabbit aortic strips by weakening calcium bonds and increasing membrane excitability, an action which it appears to exert in skeletal muscle.

Recent work has indicated that the action of noradrenaline and adrenaline in arterial smooth muscle is terminated by their penetration of effector cell membranes and distribution in cell water (Kalsner & Nickerson, 1969a, b). This is followed by the more definitive processes of enzymatic inactivation, particularly by catechol-O-

methyltransferase. It has also been reported that several compounds, of apparently diverse chemical structure, impair the extraneuronal metabolism of catecholamines (Kalsner & Nickerson, 1969b, c; Kalsner, 1969a, b). In this investigation, the possibility was explored that the enhancement of contractor responses to noradrenaline and adrenaline by the methylxanthines is related to effects on catecholamine metabolism.

Methods

Strips of rabbit aorta were prepared and suspended under 2 g of tension in muscle chambers of 15 ml capacity (Kalsner & Nickerson, 1968a). The strips were immersed in Krebs-Henseleit solution (NaCl, 115·3 mM; KCl, 4·6 mM; $CaCl_2$, 2·3 mM; $MgSO_4$, 1·1 mM, $NaHCO_3$, 22·1 mM, KH_2PO_4, 1·1 mM and glucose, 7·8 mM) to which disodium EDTA was added to give a final concentration of 10 μg/ml. The Krebs solution was maintained at 37°C and constantly bubbled with 95% O_2 and 5% CO_2. Isotonic contractions were recorded, after a 90 min equilibration period, by means of frontal-writing levers on a slowly moving kymograph drum (1·8 mm/min) with a lever magnification of 6·8-fold.

The procedure for the oil immersion of aortic strips has been described (Kalsner & Nickerson, 1968a). Liquid paraffin (U.S.P. 40 St $\times$ 10^{-2}*) was kept at 37° C in flasks and constantly bubbled with 95% O_2 and 5% CO_2. Aortic strips were immersed in oil by draining the muscle chambers free of Krebs solution and refilling them with oil, after responses to agonists had plateaued in the aqueous medium. The purpose of oil immersion is to eliminate diffusion of drug from the tissue into the bathing medium. Evidence for the adequate oxygenation of the tissue during exposure to oil, for the lack of accumulation of toxic metabolites and the absence of any detectable pharmacological action of the oil itself has been presented. Termination of action (relaxation) in oil is a direct measure of the rate at which tissue mechanisms inactivate the fraction of agonist involved in the response (Kalsner & Nickerson, 1968a).

Concentrations of (−)-noradrenaline and (−)-adrenaline bitartrates, (±)-α-methylnoradrenaline hydrochloride (Cobefrin), (−)-phenylephrine and methoxamine hydrochlorides and (−)-Synephrine (1-(4-hydroxyphenyl)-2-methylaminoethanol), caffeine, theobromine and theophylline are expressed as base weights. Cocaine hydrochloride and iproniazid phosphate are referred to as salt weights. Reserpine was dissolved in 10% ascorbic acid solution; rabbits were injected intramuscularly with 1 mg/kg body weight about 18–24 h before death.

Catechol-O-methyltransferase (COMT) was inhibited with maximally effective concentrations of tropolone (10 μg/ml), pyrogallol (3–10 μg/ml) or U-0521 (3′-4′-dihydroxy-2-methyl propiophenone) (10 μg/ml) (Belleau & Burba, 1961 ; Mavrides, Missala & D'Iorio, 1963; Giles & Miller, 1967; Kalsner, 1969a). Monoamine oxidase (MAO) was inhibited by iproniazid (100 μg/ml) (Zeller & Barsky, 1952; Furchgott, 1955 ; Kalsner & Nickerson, 1968b). Strips were exposed to iproniazid for 30 min followed by an additional 30 min period with frequent washes before drug testing. Evidence for the specificity and completeness of the procedures used to inhibit mechanisms of amine inactivation in aortic strips has been presented (Kalsner & Nickerson, 1968a, b ; 1969a). Mean values are shown with their standard errors. Differences with P values of 0·05 or less were considered significant.

* (1 St=10^{-4} m^2 s^{-1})

Results

Effects of theophylline, caffeine and theobromine on adrenaline responses

Aortic strips were contracted by a low concentration of adrenaline (3 ng/ml) and, when responses had reached steady plateau values, exposed to cumulatively increasing concentrations of theophylline (1, 3, 10, 30, 100 μg/ml). The amplitude of response was increased progressively by concentrations of theophylline between 1 and 10 μg/ml. Increasing the concentration to 30 μg/ml usually produced no additional increment or had an inhibitory effect, and 100 μg/ml consistently depressed the amplitude of the response. As shown in Table 1, responses to adrenaline (3 ng/ml) were increased by a mean of 3·8 mm by theophylline (10 μg/ml). This response would have been obtained by a concentration of adrenaline in the bathing medium of 4·2 ng/ml.

Caffeine (10 μg/ml) also enhanced the responses to adrenaline (3 ng/ml). This effect was slightly but significantly greater than that of theophylline at the same concentration and was equivalent to an increase of the adrenaline concentration to 4·7 ng/ml. Raising the concentration of caffeine to 30 μg/ml gave even greater potentiation but a still higher concentration (100 μg/ml) produced no further increment. In contrast, theobromine (10 μg/ml) did not potentiate and 30 μg/ml was only feebly active in enhancing responses to adrenaline (a mean of 0·9 ± 0·3 mm in five strips). Because of its weak activity theobromine was not used in the experiments on the mechanism of the enhancing effect of the methylxanthines. The results of some of the experiments with theophylline and caffeine are given in Table 1 and typical kymograph traces are shown in Fig. 1a.

The effects of the methylxanthines on responses to adrenaline appeared to wear off rapidly after washout of the muscle chambers, since contractions produced by the agonist at 60 min intervals were reproducibly potentiated. Pretreatment of rabbits with reserpine to deplete endogenous catecholamines did not decrease the potentiating effect of theophylline or caffeine (Table 1). Neither theophylline nor caffeine had a direct contractor effect on the aortic strips (Fig. 1a).

TABLE 1. *Effects of methylxanthines on responses to sympathomimetic amines*

Agonist (ng/ml)	No. of strips	Contraction (mm)	Increment (mm) due to theophylline (10 μg/ml)	No. of strips	Contraction (mm)	Increment (mm) due to caffeine (30 μg/ml)
Adrenaline (3)	20	23·3 ± 1·1	3·8 ± 0·2	7	25·3 ± 1·2	*5·6 ± 0·6
	†5	26·6 ± 2·8	4·6 ± 0·5	16	20·7 ± 1·3	9·7 ± 0·9
				†3	29·5 ± 1·9	8·2 ± 1·2
Noradrenaline (3)	6	26·6 ± 3·0	1·2 ± 0·4	5	26·3 ± 2·9	4·5 ± 0·6
α-Methyl-noradrenaline (30)	5	21·6 ± 3·2	2·8 ± 0·4	4	16·4 ± 3·3	6·4 ± 1·1
Phenylephrine (10)	6	29·4 ± 4·8	0	4	22·6 ± 2·5	0
Synephrine (500)	6	25·2 ± 4·0	0	7	26·5 ± 3·2	2·2 ± 0·6
Methoxamine (50)	4	9·6 ± 1·2	0	3	16·5 ± 1·8	0

The methylxanthines were added to the muscle chambers after responses had reached plateau values. * Caffeine concentration (10 μg/ml). † Values obtained from aortic strips of rabbits pretreated with reserpine.

Effects of methylxanthines on responses to other sympathomimetic amines

For these experiments, the concentrations of theophylline and caffeine used were those which were most effective in potentiating responses to adrenaline, namely, 10 and 30 μg/ml, respectively. Theophylline and caffeine enhanced the responses to a low concentration of noradrenaline (3 ng/ml), but to a much lesser extent than they did those to adrenaline (Table 1). Responses to α-methylnoradrenaline were also enhanced.

Responses of moderate amplitude to sympathomimetic amines without both the 3- and 4-OH groups in the benzene ring (phenylephrine, Synephrine, methoxamine) were not increased by theophylline (10 μg/ml) (Table 1). Instead, a slight depressant effect was often observed, probably due to the known relaxant effect on smooth muscle. This was not seen in resting aortic strips because they lack tone. Typical traces obtained from several of these experiments are shown in Fig. 1b. Caffeine (30 μg/ml) either did not affect or slightly decreased the amplitude of responses to phenylephrine and methoxamine. However, it exerted a slight enhancing effect on the responses to Synephrine (Table 1).

Mechanism of methylxanthine potentiation

Theophylline potentiated the responses only to those amines containing the catechol nucleus, and so did caffeine with the exception of its effect on responses to Synephrine. Moreover, the responses to adrenaline were increased more than

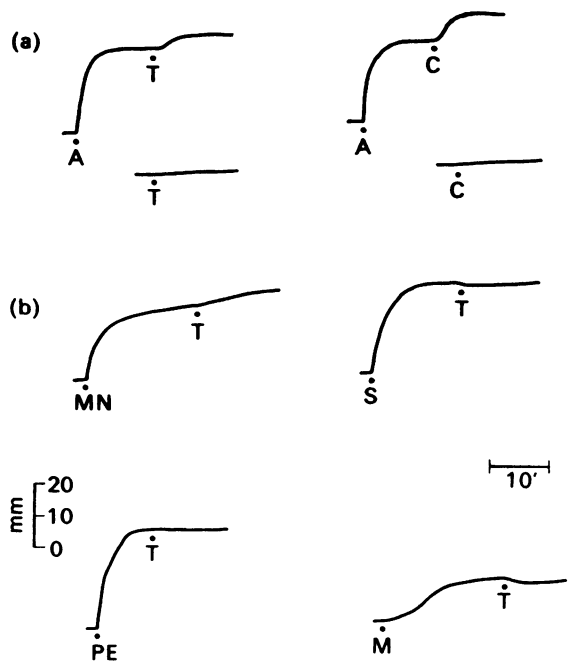

FIG. 1. Effects of theophylline (T) (10 μg/ml) and caffeine (C) (30 μg/ml) on responses to sympathomimetic amines and on basal tone of aortic strips. (a), Strips contracted by adrenaline (A) (3 ng/ml) and exposed to (T) or (C). Lower traces show the lack of effect of (T) and (C) on the tone of uncontracted strips. (b), Strips contracted by α-methylnoradrenaline (MN) (30 ng/ml), Synephrine (S) (500 ng/ml), phenylephrine (PE) (10 ng/ml) and methoxamine (M) 50 ng/ml) and exposed to (T).

those to α-methylnoradrenaline; the responses to noradrenaline were affected least by the methylxanthines. This is the same order of potentiation as was previously observed when inhibitors of COMT were studied (Kalsner, 1969a, b). The relationship between the effects of methylxanthines and inhibition of O-methylation was explored in experiments in which the enzyme was inhibited by U-0521, pyrogallol or tropolone.

Aortic strips were contracted by adrenaline (3 ng/ml) and, after responses had plateaued after about 10 min, exposed to U-0521 (10 μg/ml) or tropolone (10 μg/ml) or pyrogallol (3–10 μg/ml) for approximately 10 min followed by theophylline (10 μg/ml) or caffeine (30 μg/ml) for an additional 10 minutes. The inhibitors of O-methylation potentiated responses to adrenaline and completely blocked the effects of theophylline and caffeine in twenty-three and fourteen tests, respectively. Blockade of potentiation by the methylxanthines was also complete if aortic strips were first treated with an inhibitor of COMT for 10 min and then, without washout of the muscle chambers, contracted by adrenaline and exposed to theophylline or caffeine. The inhibitors also blocked the potentiating effects of the methylxanthines on responses to α-methylnoradrenaline and noradrenaline. Typical records are presented in Fig. 2.

Other experiments were done in which strips, contracted by adrenaline, were first exposed to theophylline or caffeine for about 10 min and then to an inhibitor of O-methylation for an additional 10 minutes. The methylxanthines considerably reduced the potentiation produced by the inhibitors of COMT (Table 2).

In contrast, cocaine (10 μg/ml) exerted its typical enhancing effect in the presence of theophylline or caffeine. Also, potentiation by the methylxanthines was not

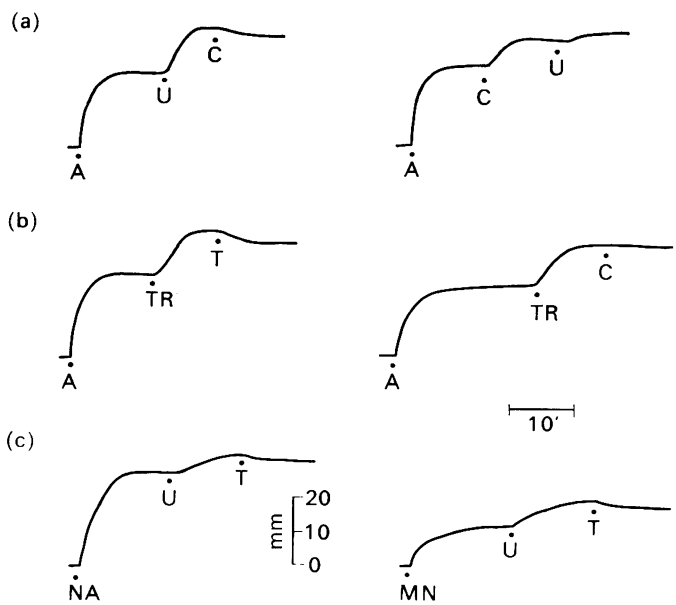

FIG. 2. Interactions of COMT inhibitors and methylxanthines on responses to sympathomimetic amines. (a), Strip contracted twice by adrenaline (A) (3 ng/ml) and exposed to U-0521 (U) (10 μg/ml) and caffeine (C) (30 μg/ml). A 60 min interval with frequent washes of the muscle chamber elapsed between tests. (b), Strips contracted by (A) and exposed to tropolone (TR) (10 μg/ml) followed by theophylline (T) (10 μg/ml) or (C). (c), Strips contracted by noradrenaline (NA) (3 ng/ml) or α-methylnoradrenaline (MN) (30 ng/ml) and followed by (U) and (T).

materially reduced in the presence of cocaine (Fig. 3a). The slight potentiation of the responses to Synephrine by caffeine was not obtained in aortic strips in which MAO was inhibited by iproniazid. However, iproniazid did not modify the potentiating effect of caffeine on the responses to adrenaline (Fig. 3b). The mean value of 8·7 ± 0·8 mm (three strips) did not differ significantly from that obtained in strips without iproniazid (Table 1).

Relaxation in oil

The data presented so far strongly suggest that caffeine and theophylline enhance contractor responses to catecholamines in aortic strips by inhibiting their extraneuronal inactivation by COMT. To provide more definitive evidence, experiments were performed with the aid of the technique of oil immersion.

TABLE 2. *Interactions of methylxanthines and COMT inhibitors on responses to adrenaline*

No. of strips	Contraction (mm) to adrenaline (3 ng/ml)	Methylxanthine	Increment (mm) due to methylxanthine	COMT inhibitor	Additional contraction (mm) due to COMT inhibitor
13	23·1±2·0			U-0521	14·4±1·2
*4	23·4±2·3			U-0521	15·9±0·9
10	22·8±1·7			Tropolone	18·0±1·7
8	24·6±2·6			Pyrogallol	18·1±1·0
6	19·8±2·0	Caffeine	9·3±1·3	U-0521	5·3±1·4
5	20·8±2·4	Caffeine	10·0±1·6	Tropolone	6·2±1·0
4	18·8±0·5	Caffeine	9·0±0·3	Pyrogallol	6·9±0·9
6	23·3±2·2	Theophylline	4·4±2·0	U-0521	8·5±1·3
*5	28·3±1·7	Theophylline	5·4±0·8	U-0521	7·4±1·0
8	25·7±2·0	Theophylline	3·9±0·5	Tropolone	10·3±2·7
3	22·5±1·0	Theophylline	4·2±0·3	Pyrogallol	11·8±2·1

Strips were contracted by adrenaline for about 10 min and then either caffeine (30 μg/ml) or theophylline (10 μg/ml) were added for approximately 10 min followed by a COMT inhibitor for an additional 10 minutes. In control experiments a COMT inhibitor was added without prior exposure to a methylxanthine. The concentrations of U-0521 and tropolone were 10 μg/ml and that of pyrogallol 3–10 μg/ml. * Values obtained from aortic strips of rabbits pretreated with reserpine.

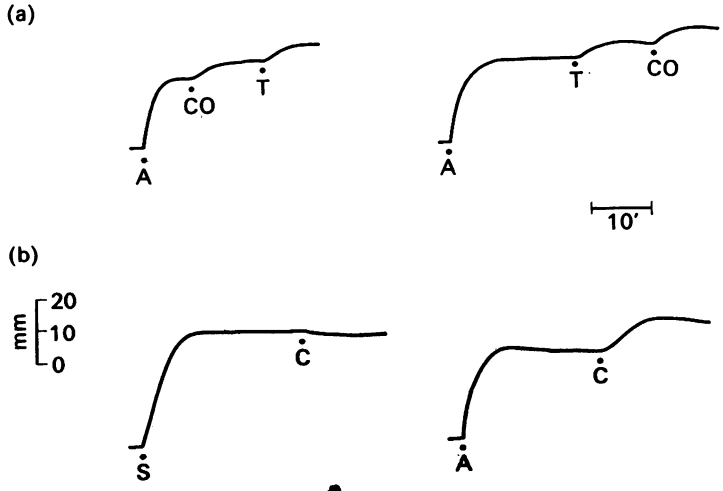

FIG. 3. Effects of iproniazid and cocaine (10 μg/ml) on potentiation by methylxanthines. (a), Strips contracted by adrenaline (A) (3 ng/ml) and exposed to cocaine (CO) and theophylline (T) (10 μg/ml). (b), Strip pretreated with iproniazid, contracted by Synephrine (S) (500 ng/ml) or, 60 min later, by (A) and then exposed to caffeine (C) (30 μg/ml).

Aortic strips, contracted by adrenaline (100 ng/ml) and immersed in oil about 20 min later, relaxed by 25% in 2·4 ±0·3 minutes. Inhibition of COMT with U-0521 (10 μg/ml) slowed relaxation to 23·2±2·1 min (Table 3). Combined treatment of strips with iproniazid and cocaine (10 μg/ml) to inhibit MAO as well as uptake and storage mechanisms produced only a slight slowing of relaxation which was statistically not significant, but additional treatment with an inhibitor of COMT virtually eliminated relaxation (Table 3 and Fig. 4). Such strips relaxed by a mean of only 3·9% in 30 minutes. These findings on the predominant role of

TABLE 3. *Relaxation in oil of aortic strips contracted by adrenaline* (100 *ng/ml*)

Treatment	No. of strips	Time to relax by 50%
Untreated	17	5·3±0·7
	17	*2·4±0·3
Theophylline	4	9·4±0·9
Caffeine	8	8·2±1·0
Theobromine	5	6·0±1·9
U-0521	4	*23·2±2·1
Iproniazid	16	5·7±0·6
Iproniazid plus theophylline	11	9·3±0·7
Iproniazid plus caffeine	9	8·5±1·3
Iproniazid plus cocaine	7	6·8±0·6
Iproniazid plus cocaine plus theophylline	5	16·2±2·4
Iproniazid plus cocaine plus caffeine	4	14·2±2·5
Iproniazid plus cocaine plus U-0521	4	>30·0

Aortic strips were contracted by adrenaline for about 20 min and then immersed in oil for 30 minutes. Methylxanthine, cocaine or U-0521 was added to the muscle chambers at least 5 and usually 10 min before oil immersion. When two of these compounds were used together they were added in random sequence at least 5 min apart. Treatment with iproniazid was as described in **Methods.** The concentrations of other drugs were: theophylline, 30 or 60 μg/ml, caffeine or theobromine, 30 μg/ml; cocaine or U-0521, 10 μg/ml. * Indicated time is to 25% relaxation.

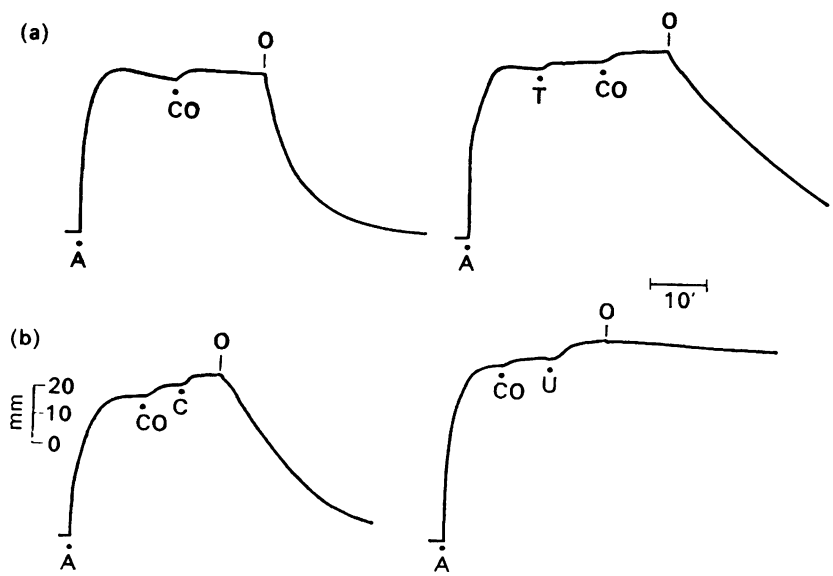

FIG. 4. Relaxation in oil of aortic strips contracted by adrenaline (A) (100 ng/ml). All strips had been pretreated with iproniazid before exposure to adrenaline. (a), Left, strip exposed to cocaine (CO) (10 μg/ml) before oil immersion (O); right, strip exposed to theophylline (T) (60 μg/ml) and (CO), followed by oil immersion. (b), Left, strip exposed to (CO) and caffeine (C) (30 μg/ml) and then immersed in oil; right, strip exposed to (CO) and U-0521 (U) (10 μg/ml) before oil immersion.

O-methylation in the inactivation of adrenaline are similar to those reported previously (Kalsner & Nickerson, 1969a; Kalsner, 1969a).

Aortic strips contracted by adrenaline (100 ng/ml) and exposed to either theophylline (30–60 μg/ml) or caffeine (30 μg/ml), added approximately 10 min before oil immersion, relaxed significantly slower than did controls (Table 3). The time to relax by 50% was increased to 1·8 and 1·5 times that of control values. In contrast, theobromine (30 μg/ml) had no significant effect on relaxation rates.

Treatment of strips with iproniazid alone did not reduce and the combination of iproniazid and cocaine significantly increased the effect of theophylline and caffeine on the relaxation in oil of strips contracted by adrenaline (Table 3). Typical traces obtained from several of the above experiments are shown in Fig. 4.

Discussion

Caffeine and theophylline enhance the contractor response of vascular as well as other smooth muscle to adrenaline and noradrenaline but the mechanism involved has so far been obscure. The results obtained in this investigation indicate that in rabbit aortic strips the methylxanthines enhance the responses to sympathomimetic amines by inhibition of their extraneuronal inactivation. This is predominantly due to a decreased rate of O-methylation.

Potentiation by theophylline was limited to compounds having a catechol structure. Caffeine showed an essentially similar profile of potentiation with the exception that it also slightly increased responses to Synephrine. Theobromine was only feebly active as a potentiating agent. Inhibitors of O-methylation such as tropolone, U-0521 and pyrogallol completely blocked this potentiation by the methylxanthines. Also, the potentiation of the responses to catecholamines by such inhibitors was much reduced in the presence of the methylxanthines. Neither cocaine, which enhances responses to sympathomimetic amines in aortic strips by a different unrelated mechanism (Kalsner & Nickerson, 1969c), nor inhibition of MAO by iproniazid, reduced the potentiating effects of caffeine or theophylline.

COMT is the major mechanism terminating the action of adrenaline and noradrenaline in aortic strips (Kalsner & Nickerson, 1969a). I have found that inhibition of COMT by U-0521 markedly slowed the relaxation in oil of aortic strips contracted by adrenaline. The 9·7-fold lengthening of the relaxation time indicated that O-methylation accounted for about 90% of the capacity of this tissue to inactivate adrenaline. The earlier observation that the residual capacity of strips to inactivate adrenaline after pretreatment with an inhibitor of monoamine oxidase plus cocaine is virtually eliminated by inhibition of COMT (Kalsner & Nickerson, 1969a), has been confirmed in these experiments and serves to demonstrate that, in aortic strips, no unknown independent mechanism inactivates adrenaline to a significant extent.

Theophylline and caffeine, but not theobromine, significantly slowed the relaxation in oil of strips contracted by adrenaline. The increase in relaxation time indicates that theophylline and caffeine reduce the capacity of strips to inactivate adrenaline by 44% and 33% respectively. Inhibition of MAO by iproniazid does not diminish this effect. On the other hand, after pretreatment with iproniazid and cocaine to eliminate alternate mechanisms of inactivation, theophylline and caffeine reduced

the residual capacity of strips to inactivate adrenaline by 58% and 52%, which reflects a marked decrease in the rate of O-methylation.

The finding that responses to Synephrine are slightly enhanced by caffeine is explicable by an inhibitory effect on the rate of deamination since caffeine did not increase the responses to Synephrine after inhibition of MAO with iproniazid. MAO does not make a significant contribution to the initial inactivation of low concentrations of adrenaline or noradrenaline (Kalsner & Nickerson, 1969a) and therefore no part of the potentiation of these amines by the methylxanthines could be attributed to decreased deamination.

Bohr (1965) invoked a membrane effect of caffeine on calcium bonds and Bartelstone *et al.* (1967) the inhibitory effect of the methylxanthines on cyclic AMP phosphodiesterase to account for potentiation. However, the data presented above indicate that there is no need to introduce either hypothesis to explain the effects of these compounds.

Recent work suggests that an increase in cyclic AMP is more probably associated with β-adrenoceptor rather than α-adrenoceptor activation in a variety of tissues (Turtle & Kipnis, 1967; Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970; Goldman & Hadley, 1970), although the nature and extent of this relationship in smooth and cardiac muscle is unsettled (Levy & Wilkenfeld, 1968; Klaus, Krebs & Seitz, 1970; Schonhofer, Skidmore, Forn & Fleisch, 1971). The methylxanthines enhance the excitatory responses of heart muscle to catecholamines mediated by β-adrenoceptors as well as their relaxant effects in smooth muscle. It is unlikely that in these instances inhibition of amine inactivation could account for a major component of the potentiation although it probably plays some role in the total effect achieved.

A question which cannot be answered by my experiments is whether the methylxanthines directly inhibit a main enzyme involved in the inactivation of catecholamines, for example COMT, or, as has been suggested previously to explain potentiation by the haloalkylamines (Kalsner & Nickerson, 1969b), decrease the access of the amines to intracellular sites of enzyme activity by inhibition of passage across effector cell membranes. However, the finding that the deamination of Synephrine, which has no catechol structure, was also impaired by caffeine is in favour of the latter possibility. Since haloalkylamines, hydrocortisone, 17 β-oestradiol and several other steroid hormones inhibit the extraneuronal inactivation of catecholamines (Kalsner & Nickerson, 1969b; Kalsner, 1969a, b), a number of apparently diverse forms of potentiation may have their basis in such inhibition. Alterations in the level of response to sympathomimetic amines, produced by various agents and conditions, may be linked to alterations in their extraneuronal metabolism more often than is currently assumed.

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