Br. J. Pharmac. (1975), 53, 267-277

ROLE OF EXTRANEURONAL MECHANISMS IN THE TERMINATION OF CONTRACTILE RESPONSES TO AMINES IN VASCULAR TISSUE

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1 The role of the uptake and release of agonist from extraneuronal sites in the termination of responses of rabbit aortic strips to amines was studied.

2 Strips were contracted with adrenaline or noradrenaline and after a response plateau was reached, the muscle chambers were washed free of agonist and the relaxation in Krebs solution recorded. After inhibition of catechol-*O*-methyl-transferase, monoamine oxidase and neuronal uptake the relaxation rate was greatly prolonged. Evidence is provided that this very slow relaxation resulted from the accumulation of intact amine at extraneuronal sites during exposure to the agonist and its subsequent release past receptors due to a reversal of the concentration gradient after washout.

3 Pretreatment with the haloalkylamine, GD-131 (*N*-cyclohexylmethyl-*N*-ethyl-β-chloroethylamine), an inhibitor of extraneuronal uptake, returned the slow relaxation rate after enzyme inhibition towards that of control strips. By blocking the extraneuronal transport of amines their accumulation at intracellular loci after enzyme inhibition was prevented.

4 The effects of GD-131 and 17β-oestradiol on the relaxation rate of untreated strips contracted by adrenaline and noradrenaline confirmed that extraneuronal uptake to sites of enzymatic activity is the major mechanism terminating their action.

5 Inactivation of extraneuronal transport sites by GD-131 was prevented by protecting them with 17β-oestradiol or normetanephrine during exposure to the haloalkylamine, pointing to a common site of action of these agents on a specific carrier system for amines.

6 Evidence is presented that the relaxation from contractions induced by histamine and 5-hydroxytryptamine also involves extraneuronal accumulation and release, probably by an uptake process which is identical to the one for catecholamines.

Introduction

An analysis of sensitization of response magnitudes and of relaxation rates of aortic strips after oil immersion has led to the conclusion that the major process terminating the action of noradrenaline and adrenaline in vascular tissue is uptake and distribution in effector cells (Kalsner, 1966; Kalsner & Nickerson, 1969a). It was stated in the latter publication that, although distribution in cell water is the immediate event which terminates the action of these catecholamines, 'the capacity of this process appears to be limited, and its contribution to the body economy is dependent on the associated enzymic processes for definitive inactivation of the mediator'.

The rate of relaxation of aortic strips after washout of a stimulant drug from the muscle chambers has been shown to be a valid and sensitive index of the declining concentration of agonist at the pertinent tissue receptors (Kalsner & Nickerson, 1968a, b). The dissociation of diffusion of agonist out of the tissue from intrinsic inactivation is achieved by replacing the aqueous bathing medium with mineral oil after a steady-state contraction in response to the agonist under study has been established. Under these conditions, relaxation is a direct measure of termination of action by intrinsic mechanisms. In contrast, relaxation in Krebs solution permits movement of agonist out of the tissue into the external bathing medium to function along with intrinsic mechanisms of disposition in terminating action.

The present experiments are based on an analysis of relaxation rates of aortic strips in Krebs solution in order to assess the involvement of uptake and release from extraneuronal sites in terminating responses to moderate concentrations of catecholamines and to define more clearly the nature of these sites and their possible function in terminating contractions to other biogenic amines.

Methods

Helically cut strips of rabbit thoracic aorta about 2.5 x 23 mm were prepared for isotonic recording as described previously (Kalsner & Nickerson, 1968a). All experiments were done at 37° C and the strips were kept under a tension of 2 grams. The strips were allowed to equilibrate in the muscle chambers for at least 90 min before drug testing. The muscle chambers were of 15 ml working volume and contained a modified Krebs-Henseleit solution (mM: NaCl, 115.3; KCl, 4.6; $CaCl_2$, 2.3; $MgSO_4$, 1.1; $NaHCO_3$, 22.1; KH_2PO_4, 1.1; and glucose, 7.8) with disodium edetate added to give a final concentration of 0.03 mM.

All solutions were made with double-distilled water which had been passed through a cation-exchange column. Final concentrations of drugs in the muscle chambers are expressed in terms of molarity and as g/ml (w/v). Concentrations of (−)-noradrenaline and (−)-adrenaline bitartrates, methoxamine hydrochloride, metaraminol bitartrate, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, U-0521 (3,4,dihydroxy-2-methyl propiophenone) and iproniazid were calculated as the free base and cocaine hydrochloride, amodiaquin hydrochloride (Camoquin) and angiotensin amide as the salt.

GD-131 (*N*-cyclohexylmethyl-*N*-ethyl-β-chloroethylamine) was prepared on the day of use in a concentration of 1 mg/ml in propylene glycol to which a few drops of 0.01 N HCl was added. All solutions of sympathomimetic amines, 5-hydroxytryptamine and histamine contained 0.01 N HCl and were prepared fresh daily. Reserpine was dissolved in 10% ascorbic acid and rabbits were injected intramuscularly with 1 mg/kg about 18 h before death. This procedure has been shown to block completely the responses to tyramine ($7.3 \times 10^{-7} - 2.2 \times 10^{-5}$ M; 0.1 − 3.0 μg/ml) a potent releaser of endogenous catecholamines in aortic strips (Kalsner, 1970b).

Aortic strips were exposed to a stimulant drug for a sufficient period, usually 20 min, to allow the contractions to reach a stable plateau before washout. Washout of an agonist drug from the muscle chambers involved three initial changes of the bath fluid, followed by one change every 5 min until relaxation was complete. The chambers were washed by overflow, to minimize any mechanical artifact during the washout procedure. The percentage relaxation at any time was calculated from the ratio of the residual amplitude of contraction to that immediately before washing the stimulant drug from the chamber. To compare the rates of relaxation the time required for each strip to relax 50% was measured and the mean time calculated for each treatment. Details of this procedure were given previously (Kalsner & Nickerson, 1968a, b). Mean values are reported with their standard errors. Differences between means were compared by Student's *t* test and those with *P* values of 0.05 or less were considered significant.

Several treatment conditions were employed to investigate mechanisms for the inactivation of agonists. In a majority of experiments strips cut from the same aorta were utilized for all treatment groups with a given agonist to reduce variability.

(A) *Control*

The agonist was added to the muscle chambers at the required concentration and 20 min later they were washed as indicated above and the relaxations were recorded. In the case of histamine, exposure to the agonist was usually for a total of 10 min prior to washout.

(B) *Cocaine*

Strips were exposed to cocaine (2.9×10^{-5} M; 10 μg/ml) 10 min before contraction with the agonist to inhibit neuronal uptake (Kalsner & Nickerson, 1969b) and about 20 min later the chambers were washed with Krebs solution containing cocaine (2.9×10^{-5} M).

(C) *Iproniazid*

Strips were exposed to iproniazid (5.6×10^{-4} M; 100 μg/ml) for 30 min, the drug was washed out and an additional 30 min, with frequent washes, was allowed to elapse before the addition of the agonist. The strips were then treated in the same way as controls. This procedure was shown previously to produce essentially complete inhibition of monoamine oxidase and diamine oxidase but to produce no other alteration in the responsiveness of the tissue or in the intrinsic disposition of amines (Kalsner & Nickerson, 1968a, b; Kalsner, 1970a).

(D) *Iproniazid plus U-0521 or amodiaquin*

Strips were exposed to iproniazid as in C and then to either U-0521 (5.3×10^{-5} M; 10 μg/ml) or to amodiaquin (6.5×10^{-6} M; 3 μg/ml) for 10 min and without washout, contracted by the agonist as in A. The muscle chambers were washed with Krebs solution containing U-0521 (5.3×10^{-5} M) or amodiaquin (6.5×10^{-6} M). The specificity of action and completeness of the procedures used to

inhibit catechol-*O*-methyltransferase and imidazole-*N*-methyltransferase with U-0521 and amodiaquin, respectively, have been described previously (Kalsner, 1969a, b; Kalsner, 1970a, b).

(E) *Iproniazid plus U-0521 plus cocaine*

Strips were pretreated with iproniazid as in C and then exposed to U-0521 (5.3×10^{-5} M) and to cocaine (2.9×10^{-5} M) followed 10 min later, without washout of the muscle chambers, by the agonist as in A. The chambers were washed with Krebs solution containing both U-0521 and cocaine.

(F) *GD-131*

Strips were exposed to GD-131 (1.3×10^{-5} M; 3 μg/ml) for 15 min followed by a minimum period of 15 min with frequent washes of the muscle chambers and then treated in the same way as the controls. This haloalkylamine compound has been shown to block persistently the uptake of catecholamines in vascular effector cells and to do so at a concentration well below that which blocks the α-receptors (Kalsner & Nickerson, 1969a).

(G) *17β-oestradiol*

Strips were exposed to 17β-oestradiol (3.7 or 11×10^{-5} M; 10 or 30 μg/ml) for 15 min and without washout of the muscle chambers contracted by the agonist for 20 min and treated as in A. This steroid and several others have been reported to block the extraneuronal inactivation of catecholamines in vascular tissue (Kalsner, 1969a, b), a finding which has been confirmed by others for cardiac tissue and established as due to blockade of uptake (Salt, 1972).

(H) *Iproniazid plus GD-131 (or 17β-oestradiol) plus U-0521 plus cocaine*

Strips were pretreated with iproniazid as in C and exposed to GD-131 as in F (or 17β-oestradiol as in G) and then treated with U-0521 plus cocaine as in E. The chambers were washed with Krebs solution containing U-0521 plus cocaine.

(I) *Iproniazid plus GD-131*

Strips were pretreated with iproniazid as in C and with GD-131 as in F and then treated in the same way as group A.

(J) *Iproniazid plus amodiaquin plus GD-131*

Strips were pretreated with iproniazid as in C and with GD-131 as in F and then exposed to amodiaquin (6.5×10^{-6} M) followed 10 min later, without washout of the muscle chambers, by the agonist as in A. The chambers were washed with Krebs solution containing amodiaquin (6.5×10^{-6} M).

(K) *Protection series*

After treatment with iproniazid as in C, strips were exposed to normetanephrine (5.5 or 16×10^{-5} M; 10 or 30 μg/ml) or 17β-oestradiol (3.7×10^{-5} M) for 10 min and without washout to GD-131 (1.3×10^{-5} M) for an additional 15 minutes. The drugs were washed out of the muscle chambers and 120 min later, after exposure to U-0521 and to cocaine as in E, they were contracted by adrenaline as in A. Control strips were treated identically except for the omission of normetanephrine or 17β-oestradiol and/or GD-131.

Results

Relaxation of adrenaline- and noradrenaline-contracted strips in Krebs solution

Aortic strips contracted by a low concentration of adrenaline (1.6×10^{-7} M; 30 ng/ml) reached a mean response amplitude of 36.7 ± 1.4 mm, about half maximal, and relaxed 50% in a mean of 1.4 min after washout of the agonist from the muscle chambers. This process of relaxation towards the pre-contraction baseline reflects the rapidly declining concentration of agonist in the vicinity of receptors due to efficient intrinsic processes of inactivation and also removal by diffusion into the surrounding bathing medium (Kalsner & Nickerson, 1968a, b) (Figure 1a, Table 1). As shown in Table 1, inhibition of neuronal uptake alone, with cocaine, only had a slight effect on the relaxation rate of strips contracted by adrenaline whereas inhibition of extraneuronal uptake with GD-131 produced a material slowing of relaxation, reflecting the importance of this latter process in termination of action, even in the presence of a competing gradient of diffusion of the agonist into the external bathing medium (Table 1, Figure 1).

After direct inhibition of catechol-*O*-methyltransferase and monoamine oxidase with U-0521 and iproniazid the relaxation time of adrenaline-contracted strips was prolonged strikingly to a mean of 20.3 min (Table 1, Figure 1). Additional pretreatment of strips with cocaine had no significant effect on this slow relaxation. The finding that block of neuronal uptake did not speed significantly the relaxation of strips in which

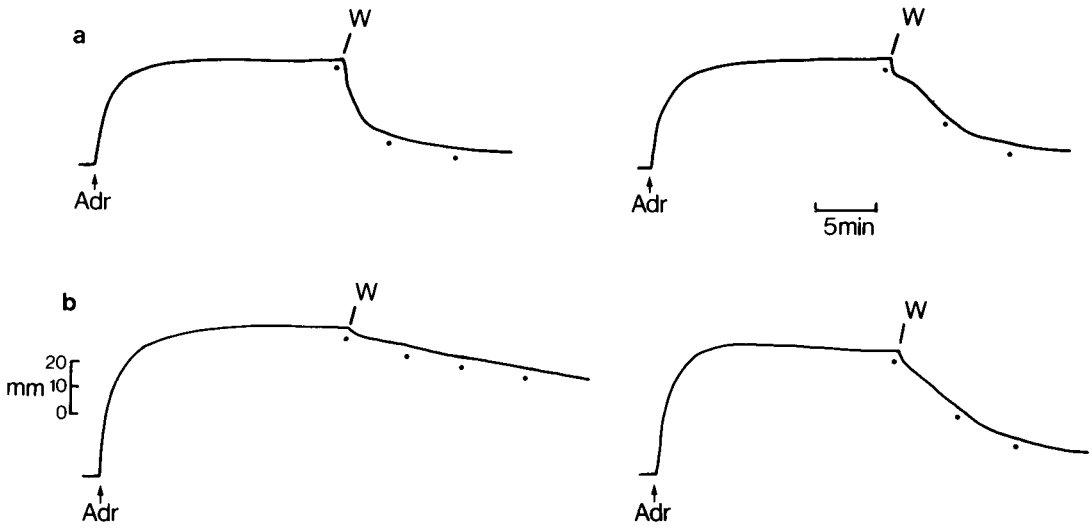


Figure 1 Relaxation in Krebs solution of aortic strips contracted by adrenaline (Adr) (1.6×10^{-7} M). (a) Left, control contraction in response to adrenaline and relaxation in Krebs; right, strip from the same aorta pretreated with GD-131 before contracted by adrenaline; (b) left, strip pretreated with iproniazid and exposed to U-0521 and cocaine before contracted by adrenaline; right, strip from the same aorta pretreated with iproniazid and GD-131 and exposed to U-0521 and cocaine before contracted by adrenaline. Dots indicate washout of the muscle chambers every five minutes. Initial washout is also indicated by (W).

Table 1 Relaxation in Krebs solution of aortic strips contracted by adrenaline and noradrenaline

Agonist	*Treatment*	*No. of strips*	*Time to relax 50%*
Adrenaline 1.6×10^{-7} M	Untreated	18	1.4 ± 0.1
		6	1.9 ± 0.2‡
	Cocaine	12	2.3 ± 0.3*
	Iproniazid plus U-0521	4	20.3 ± 0.6*
	Iproniazid plus U-0521 plus cocaine	23	18.8 ± 1.0*
		7	21.7 ± 1.3*‡
	GD-131	11	4.0 ± 0.3*
		4	6.0 ± 0.7*‡
	GD-131 plus cocaine	4	6.0 ± 0.6†
	17β-Oestradiol	8	4.7 ± 0.9*
	Iproniazid plus GD-131 plus U-0521 plus cocaine	16	4.6 ± 0.3†
		3	7.2 ± 0.3†‡
	Iproniazid plus 17β-oestradiol plus U-0521 plus cocaine	12	5.9 ± 0.7†
Noradrenaline $(1.8 \times 10^{-7}$ M)	Untreated	8	2.4 ± 0.4
	Cocaine	6	3.4 ± 0.2
	Iproniazid plus U-0521	4	15.8 ± 2.7*
	Iproniazid plus U-0521 plus cocaine	10	17.8 ± 2.7*
	GD-131	8	4.0 ± 0.3*
	Iproniazid plus GD-131 plus U-0521 plus cocaine	6	4.8 ± 0.9†

* Significantly different from untreated group.
† Significantly different from corresponding group without GD-131 or 17β-oestradiol treatment.
‡ Values from reserpine-pretreated preparations. In these cases, the indicated statistical comparisons are with other reserpine pretreated groups.

enzymic inactivation was inhibited (Table 1) served to exclude the possibility that uptake and subsequent release from neuronal structures acts as a mechanism prolonging relaxation after enzyme inhibition. Therefore, the likelihood that the slow relaxation of aortic strips after direct inhibition of *O*-methylation and deamination is the consequence of an accumulation and subsequent release of adrenaline from extraneuronal sites onto receptors was investigated with the aid of GD-131.

After inhibition of intrinsic pathways of inactivation, strips exposed to GD-131 and subsequently contracted by adrenaline relaxed much faster than their counterparts without GD-131 pretreatment (Figure 1b, Table 1). In fact, the mean relaxation times of strips pretreated with the haloalkylamine in the presence or absence of functional intrinsic pathways of disposition did not differ significantly, as would be anticipated if extraneuronal uptake to intracellular loci were blocked in both conditions. If penetration to sites of enzymatic inactivation is blocked then the functional status of the enzymes beyond the barrier is irrelevant.

GD-131 in the concentration and time of exposure used here does not block materially uptake into neuronal structures (Kalsner & Nickerson, 1969a) and this is demonstrated in the present experiments by the finding that the combination of cocaine and GD-131 had an approximately additive effect on the relaxation rate (Table 1). It was also noted in experiments with GD-131 that the relaxation curve of strips contracted by adrenaline 30 min or 3 h after a 15 min exposure to the antagonist did not differ significantly, pointing to the formation of a stable chemical bond between the haloalkylamine and some tissue component, as had been suggested previously (Kalsner & Nickerson, 1969a).

Responses to adrenaline were significantly increased by reserpine treatment, reaching a mean of 59.0 ± 6.0 mm in six control strips, and this may account for the slight but consistent decrease in the absolute relaxation rates in these groups as shown in Table 1 (Kalsner & Nickerson, 1969c). However, the results obtained, and the relative effects of the inhibitors, did not differ materially from those recorded in untreated preparations, making it highly improbable that endogenous catecholamine stores are, in any way, involved in the observed relaxation rates (Table 1). In other experiments, strips were contracted with noradrenaline (1.8×10^{-7} M; 30 ng/ml) and the relaxations recorded. The mean amplitude of contraction of control strips was 31.9 ± 3.0 mm. Although there were some differences in the absolute relaxation rates, the effects of inhibitors of intrinsic mechanisms of disposition and of GD-131 were similar to those observed in experiments with adrenaline (Table 1). The specificity of the effects of GD-131 on relaxation rates is indicated by the finding that the relaxation from contractions in response to angiotensin (9.7×10^{-9} M; 10 ng/ml) was not modified by treatment with the haloalkylamine (Table 2, Figure 2b).

Further evidence that the effects of GD-131 are related to blockade of extraneuronal uptake was obtained in experiments with the inhibitor 17β-oestradiol. The relaxation rate of untreated strips contracted by adrenaline (1.6×10^{-7} M) was significantly slowed by treatment of strips with 17β-oestradiol but after inhibition of intrinsic mechanisms of disposition the relaxation time was much reduced by the steroid (Table 1). These are effects similar to those of GD-131, and again are explicable on the basis of inhibition of extraneuronal uptake sites.

Other sympathomimetic amines

Methoxamine is not a substrate for monoamine oxidase or catechol-*O*-methyltransferase and is not taken up into nerve endings in vascular tissue (Kalsner & Nickerson, 1968a). For this reason any extraneuronal uptake of this amine should lead to its accumulation intact and subsequent release past receptors after washout. Blockade of uptake would therefore speed relaxation. The effect of inhibition of extraneuronal uptake on the rate of relaxation from contractions elicited by this agonist was explored. For these experiments a concentration of at least 2.4×10^{-6} M (0.5 μg/ml) of methoxamine was required in order to obtain a sufficient amplitude of response for the study of relaxation rates. The mean contraction amplitudes to 2.4×10^{-6} M and 4.7×10^{-6} M were 34.3 ± 0.9 and 40.5 ± 2.4 mm. As shown in Table 2 the relaxation rates of strips contracted by methoxamine with and without pretreatment with GD-131 did not differ significantly. These experiments were duplicated over a range of methoxamine concentrations in reserpine-pretreated preparations where the results showed a slight but significant effect of GD-131 in hastening relaxation (Table 2).

Metaraminol is not metabolized by catechol-*O*-methyltransferase or monoamine oxidase but it is accumulated by sympathetic nerve terminals in cardiac tissue (Iversen, 1965). Aortic strips were contracted with metaraminol (6×10^{-6} M; 1 μg/ml) with or without prior exposure to GD-131 or to cocaine (2.9×10^{-5} M) as described in the methods section, and 20 min later the muscle chambers were washed and relaxation rates recorded. The mean contraction amplitude of

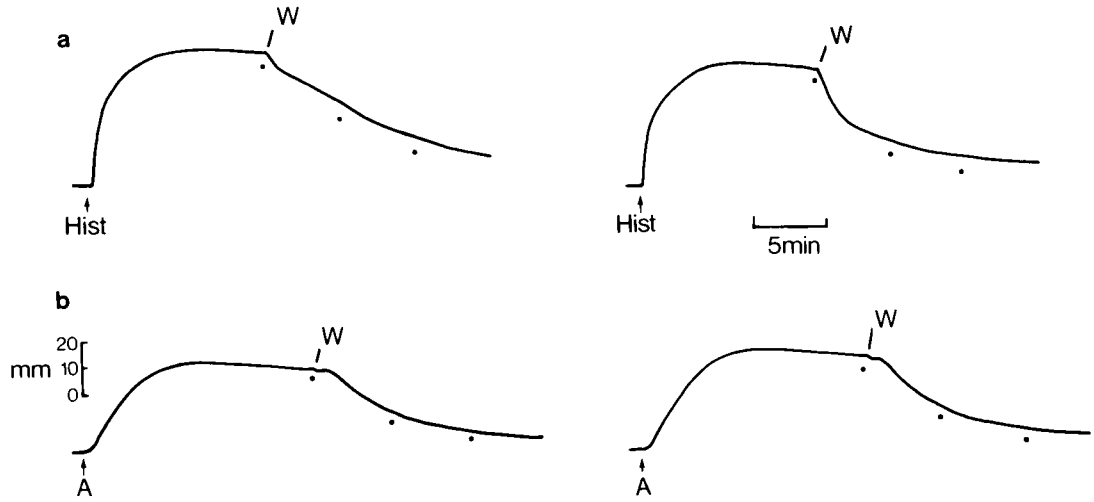


Figure 2 Relaxation in Krebs solution of aortic strips contracted by histamine (Hist) (5.4×10^{-5} M) and angiotensin (A) (9.7×10^{-9}M). (a) Left, control contraction in response to histamine and relaxation in Krebs; right, strip from the same aorta pretreated with GD-131 before contracted by histamine; (b) left, control contraction in response to angiotensin and relaxation in Krebs; right, strip from the same aorta pretreated with GD-131 before contracted by angiotensin. Initial washout of the chambers is indicated by (W) and dots indicate washes every five minutes.

control strips was 37.4 ± 1.8 mm. Cocaine alone had no clear effect on the rate of relaxation, but the rate was increased significantly by GD-131. The combination of the two agents had no greater effect than did GD-131 alone. It appears that the accumulation of metaraminol in sympathetic nerve endings in vascular tissue is not a significant factor affecting relaxation, but that some slight extraneuronal accumulation and release is involved.

Other biogenic amines

The possibility that agonists acting on receptors other than those for sympathomimetic amines also

Table 2 Relaxation in Krebs solution of aortic strips contracted by sympathomimetic amines and angiotensin

Agonist	*Treatment*	*No. of strips*	*Time to relax 50%*
Methoxamine (2.4×10^{-6} M)	Untreated	4	5.4 ± 0.5
	GD-131	4	5.1 ± 0.3
Methoxamine (4.7×10^{-6} M)	Untreated	12	8.9 ± 0.8
		8	8.8 ± 0.7†
	GD-131	12	8.2 ± 0.5
		8	6.7 ± 0.5*†
Methoxamine (4.7×10^{-5} M)	Untreated	5	18.0 ± 0.6†
	GD-131	5	13.6 ± 0.8*†
Metaraminol (6×10^{-6} M)	Untreated	15	5.3 ± 0.6
	Cocaine	18	4.9 ± 0.4
	GD-131	16	3.7 ± 0.3*
	GD-131 plus cocaine	13	4.3 ± 0.2*
Angiotensin (9.7×10^{-9} M)	Untreated	4	5.2 ± 0.5
	GD-131	4	5.2 ± 0.6

* Significantly different from untreated group.
† Values from reserpine-pretreated preparations.

make use of the extraneuronal uptake process to reach intracellular loci and that this affects relaxation was examined. Strips were contracted with histamine (2.7 or 5.4×10^{-5} M; 3 or 6 μg/ml) with and without prior exposure to GD-131 and the relaxation rates recorded after washout of the chambers. The mean contraction amplitudes at the two concentrations did not differ significantly; they were 46.0 ± 5.0 and 42.0 ± 2.4 mm, respectively. GD-131 increased the relaxation rate at both concentrations of histamine, although the effect at the lower concentration (2.7×10^{-5} M) was not statistically significant, probably because of the small number of values (Table 3, Figure 2a). Other strips were contracted with histamine, with and without haloalklylamine pretreatment, after inhibition of imidazole-*N*-methyltransferase and diamine oxidase. It was reported previously that these enzymes are chiefly responsible for inactivating histamine in vascular tissue (Kalsner, 1970a). Inhibition of *N*-methylation and deamination with amodiaquin and iproniazid slowed slightly, but not significantly, the rate of relaxation but after the additional inhibition of extraneuronal uptake with GD-131, the rate of relaxation was significantly increased and comparable with that of control strips treated with the haloalkylamine (Table 3). 17β-Oestradiol had an effect on relaxation after enzyme inhibition similar to that of GD-131. The rate of relaxation after contractions induced by 5-hydroxytryptamine (5.7×10^{-6} M; 1 μg/ml), which reached a mean amplitude of 30.3 ± 2.5 mm, was also significantly increased by GD-131 in control and reserpine-treated strips after inhibition of intracellular inactivation by monoamine oxidase (Table 3).

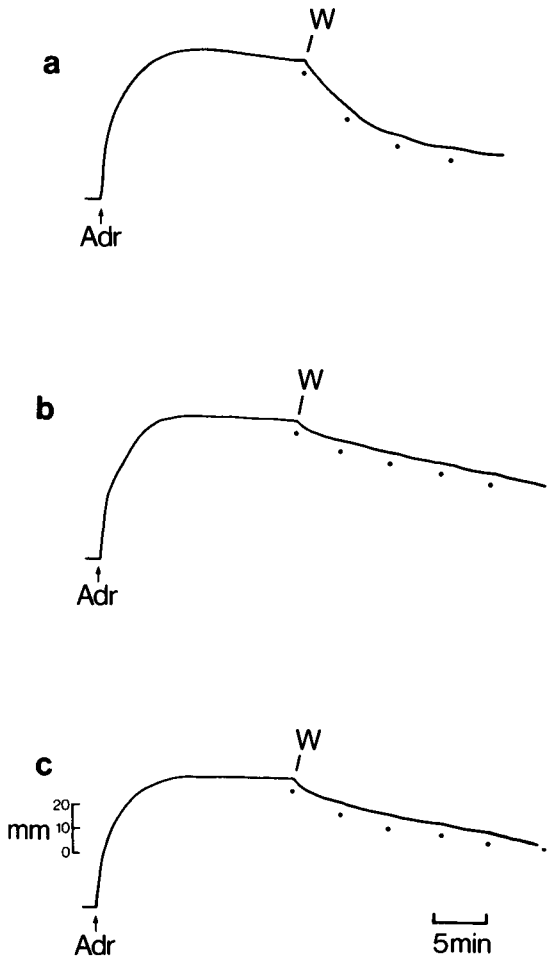
Protection of extraneuronal uptake sites against inactivation by GD-131

Attempts were made to protect the extraneuronal transport sites for sympathomimetic amines against inactivation by GD-131. Aortic strips were exposed to GD-131 in the presence and absence of normetanephrine (5.5 or 16×10^{-5} M; 10 or 30 μg/ml), a compound reported to have high affinity for the extraneuronal uptake sites of cardiac tissue (Iversen, 1965). After inhibition of enzymatic inactivation and neuronal uptake the strips were contracted with adrenaline (1.6×10^{-7} M) and the relaxation rates recorded. Strips exposed to GD-131 in the presence of the higher concentration of normetanephrine (1.6×10^{-4} M) relaxed at a rate comparable with that of strips with no GD-131 treatment, indicating virtually complete protection of the transport sites by the amine (Table 4, Figure 3). The lower concentration of normetanephrine

Table 3 Relaxation in Krebs solution of aortic strips contracted by histamine and 5-hydroxytryptamine (5-HT)

Agonist	*Treatment*	*No. of strips*	*Time to relax 50%*
Histamine (2.7×10^{-5} M)	Untreated	3	5.0 ± 0.4
	Iproniazid plus amodiaquin	12	7.7 ± 1.1
	GD-131	2	1.5 ± 0.3
	Iproniazid plus GD-131 plus amodiaquin	7	3.5 ± 0.4†
	Iproniazid plus 17β-oestradiol plus amodiaquin	3	1.7 ± 0.1†
Histamine (5.4×10^{-5} M)	Untreated	5	5.8 ± 0.4
	Iproniazid plus amodiaquin	5	6.6 ± 0.5
	GD-131	4	2.8 ± 0.4*
	Iproniazid plus GD-131 plus amodiaquin	5	3.1 ± 0.3†
5-HT (5.7×10^{-6} M)	Untreated	10	8.9 ± 0.5
		9	9.2 ± 0.7‡
	Iproniazid	11	15.7 ± 1.1*
		16	17.4 ± 1.6*‡
	GD-131	10	9.4 ± 0.3
		8	8.7 ± 0.4‡
	Iproniazid plus GD-131	12	11.8 ± 0.8†
		16	10.8 ± 0.5†‡

* Significantly different from untreated group.
† Significantly different from corresponding group without GD-131 or 17β-oestradiol treatment.
‡ Values from reserpine-treated preparations.



produced an intermediate level of protection which did not quite reach statistical significance ($P < 0.1 > 0.05$). In matching control strips, exposure to normetanephrine (5.5 or 16 x 10^{-5} M) alone had no significant effect on the relaxation curve of strips subsequently contracted by adrenaline, after inhibition of intrinsic mechanisms of disposition (Table 4). In other experiments, strips were exposed to GD-131 in the presence of 17β-oestradiol (3.7 x 10^{-5} M) as described in the methods section, and after inhibition of intrinsic pathways of inactivation, they were contracted by adrenaline and the relaxation curves recorded. The effectiveness of 17β-oestradiol in protecting against inactivation of extraneuronal uptake sites by GD-131 is demonstrated in Table 4 and Figure 3. In control strips treated identically with 17β-oestradiol, except for exposure to GD-131, the relaxation rate was increased slightly, but not significantly, compared to strips without 17β-oestradiol treatment, undoubtedly due to a

Figure 3 Protection against effects of GD-131 on relaxation of adrenaline (Adr) (1.6 x 10^{-7} M)-contracted strips. (a) Relaxation in Krebs solution of strip pretreated with iproniazid and GD-131 and subsequently exposed to U-0521 and cocaine and contracted by adrenaline; (b) strip treated as in (a) except that exposure to GD-131 was in the presence of normetanephrine (1.6 x 10^{-4} M); (c) strip treated as in (a) except exposure to GD-131 was in the presence of 17β-oestradiol (3.7 x 10^{-5} M). All strips were taken from the same aorta. Details of treatment given in text. Initial washout of the chambers is indicated by (W) and dots indicate washes every five minutes.

Table 4 Protection against effects of GD-131 on relaxation of adrenaline-contracted strips

Treatment	*Additional treatment*	*No. of strips*	*Time to relax 50%*
Iproniazid plus U-0521 plus cocaine	–	7	21.3 ± 2.0
Iproniazid plus U-0521 plus cocaine	GD-131	6	6.6 ± 1.1*
Iproniazid plus U-0521 plus cocaine	GD-131 in presence of NM (5.5 x 10^{-5} M)	3	10.7 ± 1.4*
Iproniazid plus U-0521 plus cocaine	GD-131 in presence of NM (1.6 x 10^{-4} M)	5	25.2 ± 1.0†
Iproniazid plus U-0521 plus cocaine	NM (5.5 x 10^{-5} or 1.6 x 10^{-4} M)	4	21.7 ± 0.4†
Iproniazid plus U-0521 plus cocaine	GD-131 in presence of 17β-oestradiol	6	18.9 ± 2.5†
Iproniazid plus U-0521 plus cocaine	17β-Oestradiol	3	16.8 ± 1.2†

Strips were exposed to GD-131 in the presence or absence of 17β-oestradiol or normetanephrine (NM). The drugs were washed out of the chambers and 120 min later, after inhibition of intrinsic pathways of disposition, the strips were contracted by adrenaline (1.6 x 10^{-7} M). Details of exposure to inhibitors and contraction by adrenaline are given in text.

* Significantly different from control group without additional treatment.

† Significantly different from group which received GD-131 alone as additional treatment.

residual effect of the steroid as an inhibitor of extraneuronal uptake, even 120 min after washout of the muscle chambers. The kymograph traces of a typical protection experiment are shown in Figure 3.

Discussion

The usually rapid relaxation in Krebs of aortic strips contracted by low to moderate concentrations of noradrenaline and adrenaline is prolonged strikingly after inhibition of enzymatic pathways of inactivation. It appears that amine which normally is transported into non-nervous structures and metabolized, is retained intact and accumulated at intracellular loci after enzyme inhibition, and when the agonist is cleared from the bathing medium by washout of the muscle chambers an outwardly directed concentration gradient is established. This movement of intact agonist past the receptors is responsible for the very slow decline of the response.

This interpretation was supported by the finding that the haloalkylamine, GD-131, returned the relaxation rates of treated strips towards control values. By blocking the inward transport of amines and their accumulation at intracellular loci after enzyme inhibition their consequent release past receptors, after washout of the muscle chambers, was prevented. The outward transport of amines also may be blocked by GD-131 but it is probably not relevant to the present discussion since, in all cases, treatment with the inhibitor preceded exposure to the amines.

The uptake and release of amine from neuronal structures could not account for the prolongation of the response to adrenaline and noradrenaline and the effects of GD-131. These experiments were performed in the presence of a concentration of cocaine (2.9×10^{-5} M) that blocks the neuronal membrane transport system virtually completely (Iversen, 1965). In addition, blockade of the nerve membrane transport system alone had only a slight effect on relaxation rates, whereas inhibition of monoamine oxidase and catechol-*O*-methyltransferase produced an effect comparable with that of combined inhibition of all three intrinsic processes of inactivation.

The prolongation by GD-131 of the relaxation time of untreated aortic strips contracted by adrenaline (1.6×10^{-7} M) or noradrenaline (1.8×10^{-7} M) is also explicable on the basis of a single known action of this haloalkylamine on amine transport across extraneuronal membranes. When enzyme systems are functional, extraneuronal uptake continues to act as a sink for agonist during the relaxation process as well as during exposure to the amine. By blocking the intracellular penetration of amines their volume of distribution in cell water is decreased and their metabolism prevented. Consequently, the effect of GD-131 on the relaxation rate of untreated strips represents accurately the role of extraneuronal uptake in terminating action; that is in removing amine from the region of the receptors after clearance of the muscle chamber by washout. The increase in relaxation times indicates that approximately 40% and 65% of noradrenaline and adrenaline are inactivated by extraneuronal uptake even in the presence of the competing pathway of diffusion into the muscle chambers. Uptake into neuronal structures had a lesser role in the inactivation of these amines based on an analysis of relaxation rates after cocaine, accounting maximally for 30% and 39%. The slight overlap of the effects of the two inhibitors in the case of adrenaline is probably related to the previously reported activity of cocaine as a weak inhibitor of extraneuronal uptake in aortic strips (Kalsner & Nickerson, 1968b; 1969a, b). The above interpretation is supported by the results of experiments with 17β-oestradiol, another known inhibitor of extraneuronal uptake, which had effects on relaxation rates similar to those of the haloalkylamine. In addition, it is consistent with data obtained previously using the technique of oil immersion (Kalsner & Nickerson, 1969a, b).

It thus appears reasonable to maintain that a slowing of relaxation after prior inhibition of extraneuronal uptake represents block of a site of loss and an increase in relaxation a block of the sequence of events of extraneuronal uptake, accumulation and release. Although there may be alternative explanations of certain of the present data, the analysis provided here is consistent with the known action of the haloalkylamines and steroids in blocking extraneuronal transport. No new actions need be invoked. In addition, since these experiments involve fluxes of agonists in the biophase during termination of action which is a physiological event, it is appropriate to use the responding tissue itself as probably the most sensitive index available of the declining agonist concentration in the region of the pertinent tissue receptors.

After completion of the present work, Trendelenburg (1974) published a study on the relaxation of aortic strips after contractions by adrenaline and noradrenaline. His work, using similar methods, demonstrated the importance of extraneuronal efflux in limiting the rate of relaxation after enzyme inhibition. However, Trendelenburg did not take into account the role of tissue sites as sites of loss during the relaxation process and although it is a complicating factor it

is critical to a complete evaluation of the data.

Experiments of other workers using different techniques have also provided definitive evidence of an extraneuronal uptake process for noradrenaline in vascular tissue (Avakian & Gillespie, 1968; Gillespie, Hamilton & Hosie, 1970; Gillespie & Muir, 1970; Burnstock, McCulloch, Story & Wright, 1972). For example, Avakian & Gillespie (1968), using a histochemical technique, demonstrated fluorescence of the smooth muscle cells of the rabbit ear artery after exposure to a high concentration of noradrenaline (100 μg/ml) which was prevented by pretreatment of the tissue with the haloalkylamine antagonist phenoxybenzamine. In contrast, surface binding of noradrenaline to collagen or elastic tissue was not reduced by such treatment. In another approach, Nedergaard & Bevan (1971) demonstrated a phenoxybenzamine-sensitive uptake of [^{3}H]-noradrenaline (10^{-5} M) into the media of rabbit aorta.

The experiments described here show the influence on relaxation rates of extraneuronal uptake of both noradrenaline and adrenaline at the probably physiological concentrations of 1.8 and 1.6×10^{-7} M, concentrations which produce about half-maximal contractions of aortic strips. It is clear from the present experiments that extraneuronal uptake has a key role in transporting even these low concentrations of adrenaline and noradrenaline to sites of inactivation in effector cells and that the usual fate of the transported amine is inactivation by *O*-methylation and deamination.

The work of other investigators suggests that a carrier system is involved in the extraneuronal transport of noradrenaline in vascular tissue (Gillespie *et al.*, 1970; Gillespie & Towart, 1973) and the present experiments provide strong support for a specific carrier site for biogenic amines which is inhibited competitively by GD-131 and 17β-oestradiol. Blockade of the extraneuronal transport of amines by GD-131 could be prevented by incubation of the tissue with the haloalkylamine in the presence of normetanephrine. Thus, in all probability, GD-131 combines with the same site that is used to transport the amines. Similarly, 17β-oestradiol protects effectively against inactivation of the transport mechanism by the haloalkylamine, pointing again to a common site of action of all these agents.

The present data indicate that the extraneuronal uptake process is not specific for adrenaline and noradrenaline in the control of relaxation rates but serves also to transport other biogenic amines to intracellular loci of enzymatic inactivation and storage. GD-131 speeded the relaxation of strips contracted by histamine and 5-hydroxytryptamine, after inhibition of intrinsic pathways of disposition, results similar to those obtained with the catecholamines. The relaxation of untreated strips contracted by histamine was also increased by the haloalkylamine indicating that extraneuronal accumulation of intact histamine occurs even in the presence of functional pathways of inactivation, and at a concentration of histamine which produces responses of only moderate amplitude in aortic strips. 17β-Oestradiol also increased the relaxation rate of strips contracted by histamine confirming the previous report that this steroid inhibits the extraneuronal inactivation of histamine in vascular tissue (Kalsner, 1970a). The finding that extraneuronal uptake plays a critical role in the inactivation of a variety of amines in vascular tissue suggests that derangement of the extraneuronal transport system should be considered as a possible factor in experimental and clinical conditions in which enhanced responses to or altered metabolism of biogenic amines are observed.

I wish to thank Mr Robert Frew and Mr Gregory Smith for their excellent technical assistance. This work was supported by the Ontario Heart Foundation and the Medical Research Council of Canada.

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(Revised August 20, 1974)