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THE ACTION OF TRYPSIN ON BLOCKADE BY 2-HALOGENOALKYLAMINES: SPECULATION ON THE NATURE OF THE ALPHA RECEPTOR FOR CATECHOLAMINE

BY

J. D. P. GRAHAM AND H. AL KATIB

From the Department of Pharmacology, The Welsh National School of Medicine, Cardiff

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The noradrenaline-sensitive receptor for catecholamine may be blocked by 2-halogenoalkylamine compounds (of which dibenamine was the first). This process is demonstrable as an antagonism to some of the actions of noradrenaline which is at first surmountable, and partially reversible *in vitro*—i.e., competitive—but progresses with increasing concentration of the blocking drug or duration of exposure of the tissue to that drug to an insurmountable antagonism which is irreversible *in vitro* and of variable but frequently prolonged duration *in vivo* (Graham, 1962, p. 139, Table 4.4, compounds Nos. 1, 3 and 5; also Table I of present paper). Dibenamine occupies these receptors slowly but firmly, 2-bromo-*N*-ethyl-*N*-(α-naphthylmethyl)ethylamine (SY28) more quickly but less firmly, and *N*,*N*-dimethyl-2-bromo-2-phenethylamine hydrobromide (compound L_2; Graham & James, 1961) quickest but least firmly. The insurmountable and prolonged block has been attributed to inactivation of the receptor, involving alkylation (Harvey & Nickerson, 1954). It is postulated here that subsequent regeneration *in vivo* is the result of an enzymatic process, the speed and extent of which will vary with the firmness of the alkylation of the receptor substance, which is a function of the physicochemical properties of the blocking molecule. A candidate for the enzymatic role is trypsin; should this prove to be so, consideration of the relation of this enzyme, the 2-halogenoalkylamine, the agonist and the tissue may be expected to throw some light on the attributes of the receptor.

Trypsin and chymotrypsin added to a bath containing smooth muscle are at first stimulant and synergistic with stimulants, in particular with bradykinin, and ultimately depressant and antagonistic to stimulants (Lu, 1952; Rocha e Silva, 1955; Edery, 1964).

METHODS

Pressor tests

Male rats of >250 g wt. were anaesthetized with 7 ml./kg of a 25% (w/v) solution of urethane subcutaneously, and the carotid blood pressure recorded. Noradrenaline, 1.0 μg/kg (as bitartrate), was injected intravenously at 10 min intervals. Fresh solutions of 2-halogenoalkylamine were made

for each experiment by weighing, dissolving in 0.2 ml. of 1% *N*-hydrochloric acid in 70% (v/v) ethanol: water, and diluting as required with 0.9% saline. A dose-response curve was obtained, all given doses being reckoned as cumulative. From it an ED50 was determined. Ten rats were used for this purpose for each blocking drug. The drugs were dibenamine, SY28 and compound L_2.

The ED50 was then administered, allowed the known interval of time to exert its action (Graham, 1962) and the standard injection of noradrenaline repeated until spontaneous full recovery of the pressor response was judged to have occurred. In the case of compound L_2 this was a direct measurement in ten rats; in the case of SY28, it was extrapolated from a time-recovery curve measured over 10 hr in ten rats and further checked in five after 24 hr as for dibenamine; for the latter compound 20 male rats were made drowsy by inhalation of ether and the drug injected into the vein on the dorsum penis. Urethanization and test of pressor response were carried out for 4 hr on each of days 1, 2, 3, and 4, in groups of five rats. The point of recovery was taken as the attainment of a stable response which did not increase over the last 3 hr of testing and which lay within two standard errors of the mean control value previously measured on 57 urethanized rats of the same strain.

Isolated smooth muscle

Pairs of stripped vasa deferentia from guinea pigs weighing 250 to 350 g (in which the organ is most consistent and sensitive in its responses to the conventional agonists) were suspended in 10 ml. baths of Hukovič's solution (1961) at 37° C, gassed with 95% O_2–5% CO_2, and contractions recorded with an isotonic lever, magnification ×4 and load 0.5 g. At intervals of 5 min one of the agonists—adrenaline, noradrenaline, dopamine, histamine, acetylcholine, bradykinin, and occasionally potassium—was added for a contact period of 45 sec and a dose-response curve determined. A submaximal dose of agonist was selected and applied at 5 min intervals after 20 min rest until three equal and consecutive responses were obtained.

(1) *pA_2 values.* These values of the three compounds for 2 and 20 min time of contact were determined for noradrenaline, dopamine, acetylcholine and histamine. Mustine was included as a reference compound and synthetic ethanolamines related to the haloalkylamines used as a control. The pA_2 value gives an indication of the specificity during the initial competitive phase of the antagonism exerted by these compounds.

(2) *Effect of hydrolases on vas deferens and its response to agonists.* This effect was examined by applying the submaximal dose of agonist repeatedly and then interrupting the application by adding enzymes in a range of concentrations and for varying times. The enzymes applied were *trypsin* (a) which was used for the majority of the tests—crystalline, salt free, Koch-Light, 10,000 benzoyl arginine ethyl ester (BAEE) u/mg; (b) crystalline, British Drug Houses, 8,000 BAEE u/mg; (c) crystalline, Boehringer, 5 u/mg protein; (d) lyophilized, salt-free, Serovac Ltd., 8,000 BAEE u/mg); α-chymotrypsin (a) crystalline, salt-free, Koch-Light, 10,000 acetyl-tyrosine ethyl ester (ATEE) u/mg; (b) crystalline, British Drug Houses, 9,000 ATEE u/mg; phosphatase-alkaline, Koch-Light, 6 u/mg; phosphatase-acid, Koch-Light, 800 u/mg; phosphodiesterase, lyophilized powder Koch-Light, approximately 0.3 u/mg; crude, dried Naja Naja venom; papain, crystalline, British Drug Houses, in 0.05 M sodium acetate. The potencies of the enzymes are in all cases as stated by the manufacturer. The Boehringer unit for trypsin records specific activity according to Kunitz (1947). This enzyme was applied in the same amount by weight as Koch-Light trypsin, in three experiments only.

(3) *Effect of halogenoalkylamines on agonists, and of hydrolases on block.* (a) An equiactive dose of a haloalkylamine that would irreversibly and insurmountably antagonize the action of an amount of noradrenaline, dopamine, histamine or acetylcholine that produces a submaximal response was established from dose-response curves and an extension of the pA_2–20 min determinations. (b) To the control vas deferens of a pair this ED100 was added and after an interval for establishment followed repeatedly by the agonist; in the test vas deferens the block was also established but the application of agonist interrupted by adding one of the enzymes in a concentration and for a time which has no effect on the responses to the agonists (see Table 3). (c) In another series the blocked

vas deferens was treated with 2×10^3 BAEE units of trypsin for 1 min, washed, and the agonist applied. This cycle was repeated. In this and the following series, noradrenaline 5×10^{-7} g/ml. was the only agonist used. (d) In another series using trypsin, the vas deferens was blocked, the enzyme applied in various concentrations and for various times, washed, tested and the blocker re-applied and the cycle repeated. (e) In another series specific protection tests were attempted by treating the vas deferens with a range of doses of trypsin and adding SY28 (10^{-7} g/ml.) one minute later for another 4 min in the presence of the enzyme, washing and testing for blockade. (f) In another series the blocked vas deferens was treated with trypsin and an equal amount by weight of ovomucoid; or with a boiled solution of trypsin. (g) Synthetic ethanolamines related to the 2-halogenoalkylamines were used as a control in all tests.

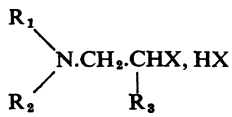
RESULTS

Duration of blocking dose in rat

The pressor response to intravenous injection of 1 μg/kg noradrenaline in the urethanized hooded rat (158 records from 57 rats) is 40 ± 2.2/mm Hg. The approximate ED50 for three 2-halogenoalkylamines in this preparation and the duration of action of the ED50 is given in Table 1. For compound L_2 this was read directly from a series of

TABLE 1

STRUCTURE OF THREE HALOGENOALKYLAMINES, THE ED50s IN μMOLE OF ACTIVE BASE/KG WT. AND APPROXIMATE DURATION OF ANTAGONISM OF THIS DOSE TO PRESSOR ACTION OF 1 μG/KG OF NORADRENALINE IN URETHANIZED RATS



Name	R_1	R_2	R_3	X	ED50	Duration (hr)
Dibenamine	$PhCH_2$	$PhCH_2$	H	Cl	41	Approx. 60
SY28	Naphth-1-yl-methyl	Et	H	Br	14	25
L_2	Me	Me	Ph	Br	5	3

time-recovery curves. For compound SY28 it was extrapolated from similar curves and checked, but for dibenamine the endpoint can only be approximate as these animals could not be used as their own control. The method used is illustrated in Fig. 1 where the mean pressor response in groups of five rats tested at selected periods of time after injection of dibenamine was plotted and the point of recovery selected as the time at which a pressor response equal to the mean plus two standard errors (based on 57 rats) was consistently attained. For dibenamine this gives a figure of not less than 48 and not more than 72 hours. As was previously found in spinal rats with a different endpoint (Graham, 1962) the longest acting blocker is dibenamine and the shortest is compound L_2.

Isolated smooth muscle (vas deferens of guinea-pig)

The pA_2 values are given in Table 2 from which it may be seen that the ethanolamines are inactive but that mustine exerts a detectable antagonism to the two catecholamines. Compound L_2 is the most specific of the compounds in that it is a thousand times more active against the catecholamines than against histamine or acetylcholine. In this test there

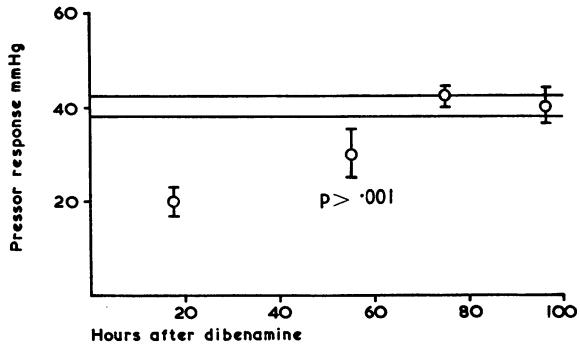


Fig. 1. Method of determining an approximate duration of action of an ED50 of dibenamine in rats on pressor response to 1 μg/kg of intravenous noradrenaline. Horizontal area represents mean pressor response found in 158 trials in 57 rats ± twice the SEM. The points each represent mean pressor response in five rats at time stated after intravenous injection of 41 μmole/kg of dibenamine. Full recovery in all cases takes more than 48 hours.

TABLE 2

pA_2 VALUES AT 2 AND 20 MIN OF THREE MONOHALOGENOALKYLAMINES AND THEIR ANALAGOUS ETHANOLAMINES, AND OF A BIFUNCTIONAL HALOGENOALKYLAMINE, OBTAINED ON ISOLATED VAS DEFERENS OF GUINEA-PIG

	Agonists							
	Noradrenaline Time (min)		Dopamine Time (min)		Acetylcholine Time (min)		Histamine Time (min)	
Antagonist	2	20	2	20	2	20	2	20
Dibenamine	7·5	8·3	6·9	8·0	4·6	5·0	7·2	8·0
—OH	inact	inact	inact	inact	inact	inact	inact	inact
SY28	7·8	8·6	7·9	8·5	5·1	6·3	8·4	9·2
—OH	inact	inact	inact	inact	inact	inact	inact	inact
L_2	7·9	8·6	7·9	8·4	4·7	5·2	4·8	5·3
—OH	inact	inact	inact	inact	inact	inact	inact	inact
Mustine	3·7	4·6	3·0	4·3	inact	inact	inact	inact

is little difference in potency between it and SY28 against dopamine at 2 min/contact but a superiority of approximately ten times over dibenamine. In all cases potency increases with time of contact, a well-established feature of these drugs, and there is little or no difference between the pA_2-20 min of any of them against noradrenaline and dopamine.

Effect of hydrolases on response to submaximal concentrations of agonists

The effects of the enzymes and the doses and times of exposure of the tissue to them are stated in Table 3; also the effect on the response of the vas deferens to repeated exposure to the seven agonists in selected submaximal concentrations which vary with the sensitivity of each tissue.

Trypsin. Crystalline salt-free trypsin when added to the bath in a concentration of 1 to 5×10^3 ABEE u/ml. produces an initial depression of tone followed in 10–20 sec time by a slow contraction. It is unlikely therefore that the initial relaxation is due to the presence of Mg^{2+} as suggested by Lu (1952). The contraction is insensitive to atropine or to mepyramine in concentrations of 10^{-6} g/ml. or to addition of Ca^{2+} in a concentration of 18 m-equiv./l. Responses to acetylcholine are reduced by exposure of the tissue to

TABLE 3

EFFECT OF HYDROLASES ON GUINEA-PIG VAS DEFERENS AND ITS RESPONSE TO SELECTED AGONISTS

Column 2 reports the response of the vas deferens to addition of the enzyme. (a) Is the initial response observed within 20 sec of addition of the enzyme; (b) is the subsequent response. The submaximal dose of the agonist was selected from a preliminary dose-response curve; in all cases except K^+ it was between 0·2 and 1 μg/ml.

r=relaxation c=contraction 0=no effect ab=response abolished −=not tested incr=increased >=greater than dim=diminished

Enzyme	Action on vas (a)(b)	Concn. (u/ml.)	Time of contact (min)	Submaximal dose of agonist and effect on response						
				NA	Ad	Dop	ACh	Hist	Brady	K^+
Trypsin	r C	10^3 BAEE								
		10–2·0	3–5	0	0	0	dim	0	incr	0
		3·0	5	dim	dim	dim	dim	dim	incr	dim
		5·0	10	ab	ab	ab	ab	ab	dim	ab
α-Chymo-trypsin	0 c	10^3 ATEE								
		1–2	3–5	dim	dim	dim	dim	dim	incr	0
		3–4	>10	ab	ab	ab	ab	ab	incr	dim
Phosphatase-alkaline	0 c	0·3	3–5	0	0	0	incr	0	—	—
		0·6	3–5	dim	dim	dim	incr	dim	—	—
		>0·6	>5	dim	dim	dim	dim	dim	—	—
Phosphatase-acid	0 c	40–160	3–5	0	0	0	0	0	—	—
		>160	>5	dim	dim	dim	dim	dim	—	—
Phospho-diesterase	0 c	0·015–0·03	3–5	0	0	0	0	0	—	—
		0·03	>5	dim	dim	dim	dim	dim	—	—
Naja venom	c r	20–60 μg	3–5	dim	dim	dim	0	0	—	—
		>60 μg	>5	ab	ab	ab	dim	dim	—	—
Papain	0 0	50–200 μg	5	0	0	0	0	0	0	0
	0 C	>200 μg	>5	incr	incr	incr	incr	incr	incr	incr

1–2 × 10^3 BAEE units for 3 to 5 min, while the response to histamine is not affected. Particular note may be taken of the fact that a concentration of 2 × 10^3 BAEE units does not alter the response of this tissue to catecholamine, while potentiating the effect of bradykinin. This effect was obtained with purified trypsin from four commercial sources. Higher concentrations of this enzyme or longer periods of contact depress the response to histamine and catecholamine and shift the dose-response curves to the right. A concentration of 5 × 10^3 units for 10 min or more abolishes the responses to all agonists except K^+, which is markedly reduced.

Chymotrypsin. The addition of more than 10^3 ATEE u/ml. of this enzyme produces after 10 to 20 sec a slight contraction of the vas deferens which is prevented by previous addition of 10^{-6} g/ml. of mepyramine. This enzyme in concentrations of 1 to 2 × 10^3 ATEE u/ml. for 3 to 5 min reduces the responses of the vas deferens to catecholamine and histamine and greatly potentiates bradykinin. A concentration of more than 4 × 10^3 u/ml. for 10 min or more abolishes the response to all agonists including K^+.

Alkaline phosphatase. The addition of 0.3 u/ml. of this enzyme for 3 to 5 min does not affect the responses to catecholamine or histamine but increases the response to

acetylcholine. Higher concentrations of the enzyme for longer periods of contact shift the dose-response curves to the right.

Acid phosphatase. Concentrations of 40 to 160 u/ml. of this enzyme have no effect on the responses to catecholamine, histamine or acetylcholine. Higher doses or longer periods of contact depress the responses to these agonists.

Phosphodiesterase (orthophosphoric diester). A concentration of 0.03 u/ml. of this enzyme for 3 to 5 min has no effect on the responses to the agonists but higher doses for longer periods reduce the responses.

Naja naja venom. This venom contains adenosine-triphosphatase and a specific 5'-monophosphatase. Crude dried venom in a dose of 20 to 60 μg/ml. for 3 to 5 min markedly reduces the responses of the vas deferens to the catecholamines but not to histamine or acetylcholine. Higher doses or longer periods of contact abolish the responses to the catecholamines and antagonize the action of histamine and acetylcholine.

Papain. In concentrations of 50 to 200 μg/ml. for 5 min this enzyme has no effect on the vas deferens and the response to the agonists is not altered. Higher concentrations or for a longer time initiate irregular clonic and tonic spasm of the muscle and appear to increase the response to all the agonists. It is difficult to distinguish the two effects. The addition of 18 m-equiv./l. of Ca^{2+} to the bath immediately abolishes the spasm and the apparent potentiation.

Effect of hydrolases on block

Blocking doses in vas deferens

A. The approximate minimal concentrations of the three compounds (as salts) needed to establish insurmountable and irreversible block of the vas deferens in 20 min contact are shown in Table 4. Accordingly, as a matter of convenience only, in all further tests on noradrenaline or dopamine responses, a dose of 0.01 μg/ml. of bath fluid (approximately the ED100 of all three antagonists against these two agonists) was used routinely. For histamine and acetylcholine the ED100 stated in Table 4 was applied routinely.

TABLE 4

APPROXIMATE ED100 IN MOL/L. OF THREE HALOGENOALKYLAMINES IN GUINEA-PIG VAS DEFERENS FOR FOUR AGONISTS; CONTACT TIME 20 MIN

	Agonists			
Compound	Noradrenaline	Dopamine	Histamine	Acetylcholine
Dibenamine	$2{\cdot}6\times10^{-8}$	$2{\cdot}7\times10^{-8}$	$3{\cdot}0\times10^{-7}$	$1{\cdot}6\times10^{-5}$
SY28	$2{\cdot}0\times10^{-8}$	$2{\cdot}0\times10^{-8}$	$2{\cdot}3\times10^{-8}$	$1{\cdot}0\times10^{-6}$
L_2	$2{\cdot}3\times10^{-8}$	$2{\cdot}4\times10^{-8}$	$1{\cdot}5\times10^{-5}$	$1{\cdot}4\times10^{-5}$

B. *Trypsin.* As shown in Table 5 and Fig. 2, trypsin, in concentrations which do not affect the response of the vas deferens to adrenaline, noradrenaline or dopamine, removes the blockade of these three catecholamines but not the blockade of the other agonists. It is easier to restore the response of a blocked vas deferens to adrenaline and noradrenaline by trypsin than the response to dopamine. The blockade exerted by dibenamine is less easy to reverse than that due to compound L_2 (see Fig. 3), requiring

TABLE 5

EFFECT OF TRYPSIN ON BLOCKADE OF GUINEA-PIG VAS DEFERENS BY AN ED100 OF 2-HALOGENOALKYLAMINE FOR 20 MIN

0=no effect. R=partial or complete reversal of the block.

Enzyme Trypsin	Blocker	Agonists					
		Ad	NA	Dop	Hist	ACh	K^+
2×10^3 BAEE u/ml. for 2–5 min	Dibenamine	R	R	R	0	0	0
	SY28	R	R	R	0	0	0
	L_2	R	R	R	0	0	0

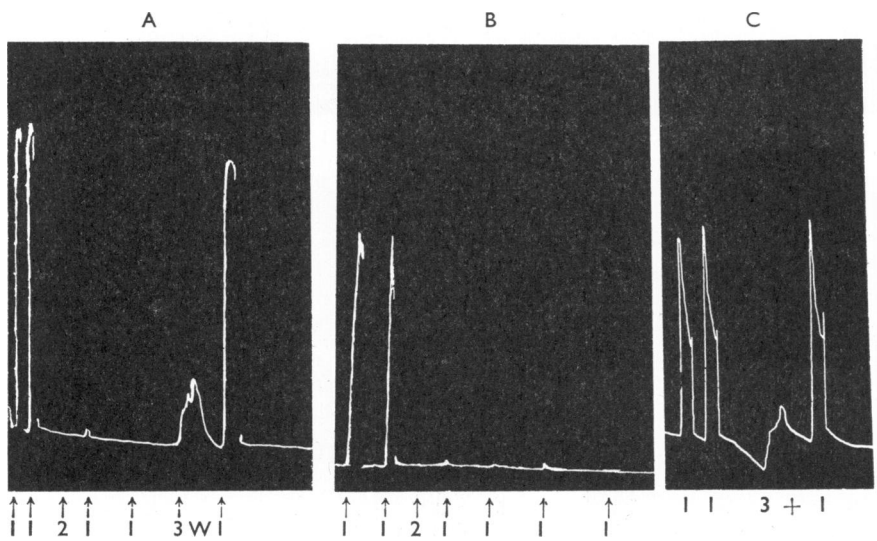


Fig. 2. Isolated vas deferens (A) stimulated with noradrenaline (1), an ED100 of SY28 (2) applied for 20 min, resulting block demonstrated (1), trypsin 2×10^3 u/ml. (3) applied for 2 min, washed out (W), and abolition of block revealed (1). Control vas deferens of pair (B) to which no trypsin was applied remains blocked. (C) Noradrenaline (1) is fully active in presence of trypsin (3).

higher concentrations of trypsin for longer times of exposure in order to produce partial restoration of the response to noradrenaline. This relation is difficult to quantitate meaningfully but undoubtedly the order of ease of reversibility is L_2>SY28>dibenamine.

C. If the concentration of trypsin remains fixed and the initial concentration of blocker is high—i.e., for dibenamine an ED100, for SY28 and L_2 ten times that amount—the reversal may be accomplished in a series of steps, increasing after each application of the enzyme (see Fig. 3*b*).

D. If the vas deferens is blocked and trypsin added so that it becomes unblocked, it will be re-blocked by a second exposure to halogenoalkylamine. It may then be unblocked by trypsin once more and the process repeated a small number of times. The experiment is usually terminated by loss of the reactivity of the muscle to its agonist and the enzyme-blocker relation becomes less certain as one continues. Nevertheless, it is clear that the cycle is repeatable and this is demonstrated in Fig. 4.

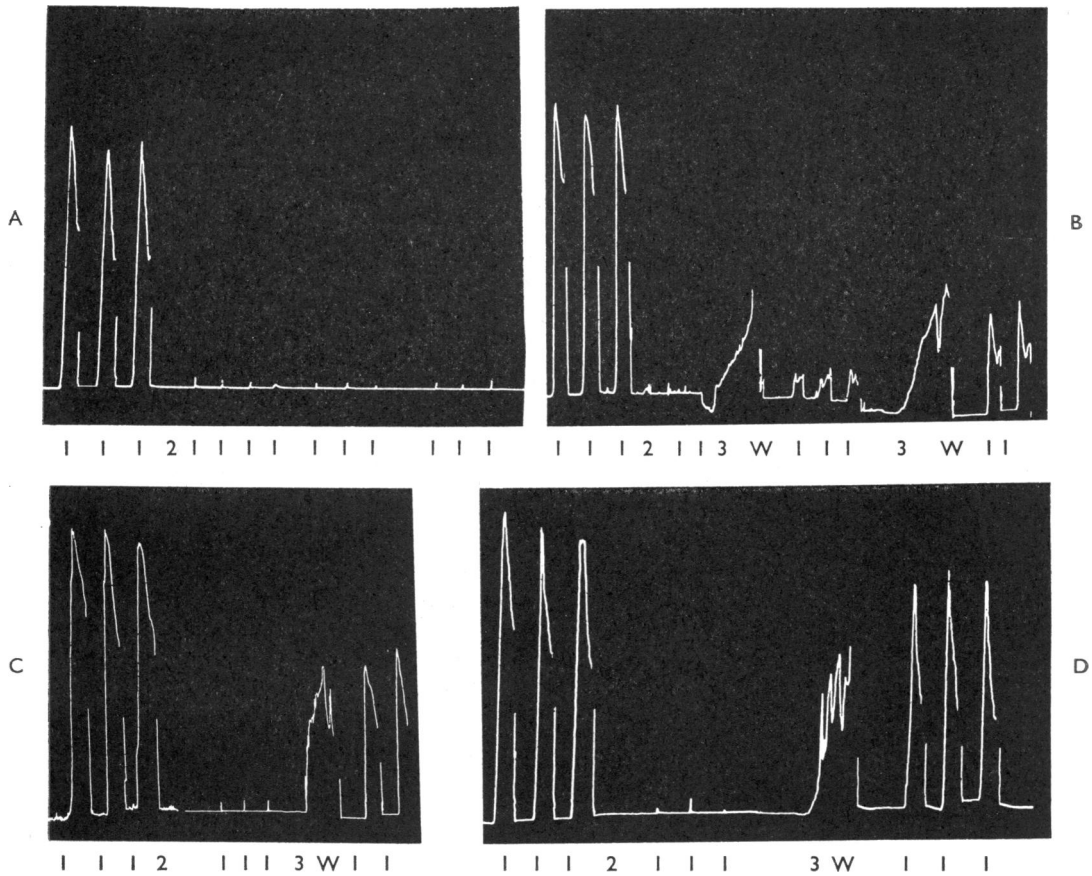


Fig. 3. Isolated vas deferens of guinea-pig. A. Control stimulation with noradrenaline (1), an ED100 of dibenamine (2) added and block proved (1). B. As in A but after block is established trypsin 2×10^3 BAEE u/ml. (3) is added for 2 min and then washed out and (1) repeated. Trypsin (3) is repeated, washed (W) and followed by (1). There is step-like reversal of block. C and D. An ED100 of SY28 (C) and L_2 (D) used as blockers (2). The same concn. of trypsin (3) as in B reveals relative ease of reversal of block to be L_2>SY28>dibenamine.

E. If trypsin is added to an isolated vas deferens in the range of concentrations from 10^2–2×10^3 BAEE u/ml. for 1 min and SY28 added in a concentration of 10^{-7} g/ml. in the presence of the trypsin for a further 4 min and then washed out, there is a marked reduction of the expected block as seen in the control. At the end of the experiment, block may be established by adding fresh SY28. The relation between the concentration of trypsin and the degree of protection is shown for SY28 in Fig. 5. It is a linear one.

F. Ovomucoid in an equal weight to trypsin and mixed with it before adding to the bath prevents the reversal. Boiled trypsin does not reverse the block.

G. *Other hydrolases*

Chymotrypsin. This enzyme, in doses of less than 10^3 ATEE u/ml. for 3 min (which amount does not affect the response of the vas deferens to catecholamine) has no effect

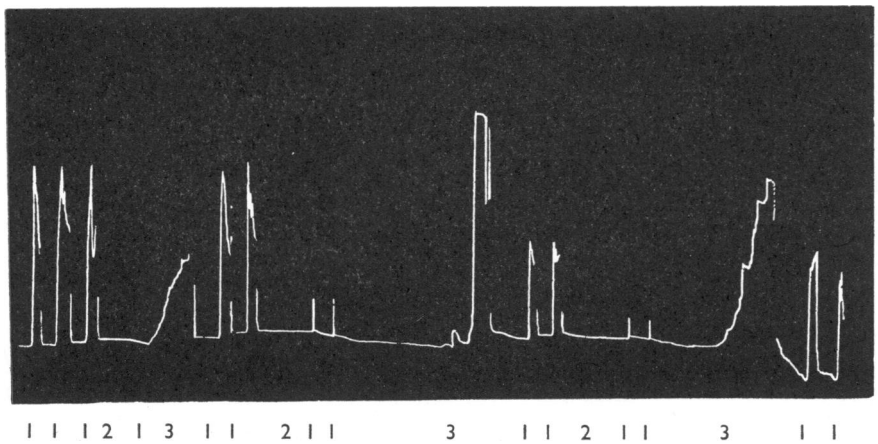


Fig. 4. Isolated vas deferens of guinea-pig, stimulated with noradrenaline (1) and blocked with an ED100 of SY28 (2). Trypsin (3) reverses block which may be re-induced and unblocked repeatedly.

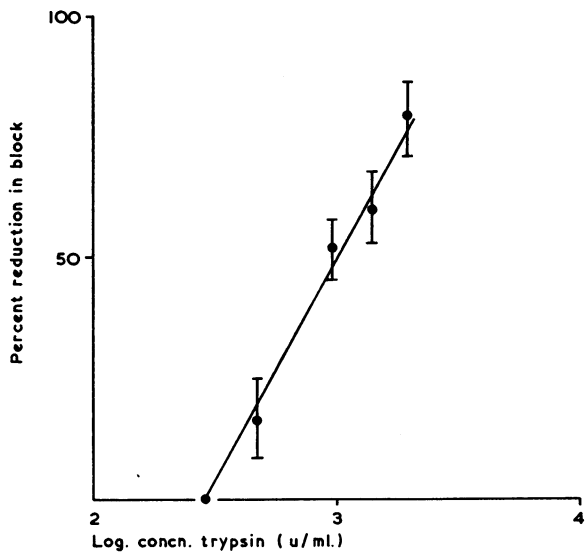


Fig. 5. Isolated vas deferens of guinea-pig stimulated with noradrenaline. Linear relation between log concn. trypsin added for 1 min and followed by 10^{-7} g/ml. SY28 for further 4 min. There is linear relation between the concn. of trypsin present and degree of protection it affords.

on the blockade by any of the three antagonists. Higher concentrations for longer periods of contact, which depress the responses to catecholamine, histamine and acetylcholine, do not affect the blockade.

Alkaline phosphatase. In amounts of 0.3 u/ml., which has no effect on the response to catecholamine or histamine and increases the response to acetylcholine, this enzyme does not affect the blockade exerted by any of the halogenoalkylamines. Higher concentrations do not alter the blockade.

Acid phosphatase. This has no effect on the blockade of any agonist, whether in concentrations up to 160 u/ml. for 5 min, which do not affect the response to the agonists, or in higher concentrations.

Phosphodiesterase. In concentrations up to 0.03 u/ml. for 5 min, which do not affect the responses to the agonists, this has no effect on the blockade. Higher concentrations do not affect the blockade.

Naja naja venom. A crude mixture which contains a number of phosphatases, this is a potent inhibitor of the response of the vas deferens to catecholamine but does not reverse the blockade of noradrenaline nor of histamine and acetylcholine.

Papain. In concentrations of 100 to 200 μg/ml. for 5 min contact time this has no effect on the blockade of any of the agonists. In higher concentrations (over 200 μg/ml.) for a longer time of contact (more than 5 min) it produces irregular spasm which makes it difficult to determine whether or not a degree of recovery from the blockade occurs. Any apparent recovery is probably an artefact in that (1) it is displayed equally towards all the agonists; (2) it only occurs in about half the trials, irregularly; (3) there is no difference in this respect between the three halogenoalkyamines and (4) unlike the initial contraction of the vas deferens caused by trypsin it is prevented or abolished by the addition to the bath of Ca^{2+} in a concentration of 18 m-equiv./l.

H. The synthetic ethanolamines related to the three haloalkylamines are not active as blocking agents and their use as control in all experiments establishes that the activities and relations described are related to the parent halogenoalkylamine or its ethyleneiminium ion (E^+).

DISCUSSION

The estimate of duration of action of an ED50 of the three halogenoalkylamine compounds, dibenamine; 2-bromo-*N*-ethyl-*N*-(α-napthylmethyl)ethylamine hydrobromide (SY28); and *N*:*N*-dimethyl-2-bromo-2-phenylethylamine (L_2) confirms and quantitates the belief that equiactive doses of these compounds exert an antagonism to the pressor response to noradrenaline *in vivo* which is of widely differing duration. The determination of pA_2 values on isolated guinea-pig vas deferens confirms the belief (Graham, 1962) that specificity among them varies. All antagonize the motor effect of dopamine as well as that of adrenaline and noradrenaline, and the equiactive concentrations required to exert the preliminary surmountable antagonism against noradrenaline, *in vitro*, are not widely different at equilibrium time. The bishaloalkylamine mustine exerts a similar antagonism but one which is 10,000 times weaker than that of SY28, a point which has previously escaped notice. The reversal of an established insurmountable block *in vitro* by trypsin appears to be of possible pharmacological significance. It is accomplished only by this enzyme of those examined, in concentrations which do not affect the response to the agonists tested, in particular the catecholamines examined. It follows that the unoccupied " alpha receptor " is not damaged by these concentrations of trypsin; but higher concentrations inhibit the response to all agonists and may therefore be held to affect a site or mechanism common to all of them. It follows that the noradrenaline-receptor complex is not specifically susceptible to trypsin except when occupied by 2-halogenoalkylamine. It is further to be noted that it is easier for trypsin

to reverse *in vitro* the block established by L_2 or by SY28 than by dibenamine. This order of ease of reversibility coincides with the order of ease of spontaneous recovery *in vivo* from established block. The postulate that the reversal of established block by trypsin is a consequence of a specific application of some part of the acknowledged pattern of activity of this enzyme is supported by the specific protection test which gives a result in accordance with the criteria of Furchgott (1954). Noradrenaline protects alpha receptors from 2-halogenoalkylamines (Furchgott, 1954; Nickerson, 1955). Trypsin protects the vas deferens from blockade by halogenoalkylamine (Fig. 5). This we interpret as indicating that trypsin occupies "the same part of the alpha receptor complex" as does 2-halogenoalkylamine and noradrenaline. The reversal of block by trypsin can be brought about in steps; it therefore involves a permanent, if partial, removal of the block from the site. In some few cases, those in which the trypsin treatment was inadequate, repeated washing has been followed by a renewal of the block but in the majority of cases the reversal of block is maintained and may increase with washing and time. This again indicates a permanent removal by enzyme of the blocked part of the alpha site, leaving an intact receptor capable of reacting at once to noradrenaline, or leaving a deficient receptor which can be very speedily restored to full functional activity.

Speculation on nature of alpha site

The specificity of trypsin is stated in the standard texts to be directed towards the hydrolysis of peptides, amides and esters at bonds which involve the carboxyl groups of L-arginine or L-lysine; the ester linkage splits more easily than amide or peptide bonds. The availability of active trypsin in plasma is the subject of controversy but the consensus of opinion seems to be that specific ester splitting activity (trypsin-like activity) does exist in plasma (Ronwin, 1962; Sardesai & Provido, 1965). It has been shown that 2-halogenoalkylamines owe their blocking properties to the formation of an ethyleneiminium ion when in buffered solution (Graham, 1962) by an internal rearrangement. This ion may interact electrostatically with an anionic receptor site through its quaternary nitrogen which would account for an initial competitive phase of antagonism. Subsequent rearrangement bringing an electrophilic carbon sufficiently close to the anionic site might result in alkylation of the latter (Belleau, 1960). The preferred reacting group for the formation of the ion-pair has been the subject of speculation. Harvey & Nickerson (1954) suggested sulphydryl while not excluding carboxylate. Belleau (1963) revived the notion of a phosphate anion and elaborated a detailed theory which involved a bonding with enzyme co-factor which includes one or more phosphate groups in its structure as an essential part of receptor occupancy. Recently Kimelberg & Triggle (1965) have argued on chemical grounds in favour of carboxyl as the anion, basing their preference partly on the variable stability of 2-halogenoethylamine carboxylates in aqueous solution, which correlates with the durability of the blockade. The known instability of this ester of the ethyleneiminium ions of bishaloalkylamines (Goodlad, 1957) would certainly account for the feebleness and brevity of the antinoradrenaline action of mustine if carboxyl is the site of bonding. The effectiveness of trypsin in reversing block by 2-halogenoalkylamine in a dose which does not affect the response to noradrenaline implies that 2-halogenoalkylamines esterify the anionic site of the alpha

receptor and in so doing mask it. Variation in the intrinsic efficacy of blockers of this series could then be accounted for by the physico-chemical effects of the molecular structure on the rapidity, complementarity and exactness of apposition of the constituent parts of the substituents on the iminium nitrogen to the rest of the alpha site. Higher concentrations of trypsin which antagonize noradrenaline, histamine, acetylcholine and potassium may be expected to disrupt peptide bonds as does chymotrypsin, and this activity impairs the reactive part common to all the stimulant agonists tested. Triggle (1966) argues in favour of an intramolecularly facilitated hydrolysis of the alkylated apha receptor as its mode of recovery, on grounds which have been reviewed in detail by Capon (1964). This would depend purely on nucleophilic catalysis for the hydrolysis of the carboxylic ester. The probability of an enzyme-catalysed process renders this mechanism of secondary importance.

We believe that the alpha receptor site is part of the cell membrane because trypsin is known not to penetrate living cells (Weiss, 1963); that it consists at least in part of a peptide chain; that arginine and/or lysine is a part of this peptide with the carboxylate freely reactive. This is the anionic group with which the cationic head of noradrenaline ion or of a 2-halogenoalkyliminium or carbonium ion reacts in the way that Belleau (1963) suggested; in the case of noradrenaline electrostatically and evanescently, in the case of the blocker firstly electrostatically (reversible phase) and subsequently by esterification of the free carboxyl group which is the accepting anion and an essential part of the receptor. This carboxyl group is, moreover, specific to L-arginine and L-lysine or both. Recovery from the long block which ensues (see Table 1) is not possible *in vitro* but is accomplished *in vivo* by hydrolysis of the ester bond by trypsin. This splits off the 2-halogenoalkylamine and leaves the alpha receptor with its free carboxyl anion intact and ready for instant activation by noradrenaline (see Fig. 2). Trypsin exerts a "specific protection" by unbonding—i.e., displacing—2-halogenoalkylamine as fast as the esterification of carboxyl occurs.

If the acceptor onion is a phosphate (Belleau) or a sulphydryl group (Nickerson) and if trypsin acts on amide or peptide bonds to remove the whole fragment (including the acceptor occupied by the 2-halogenoalkylamine) an essential part of the alpha receptor would be lost and the receptor no longer fit to react with added noradrenaline, unless it were repaired very rapidly. This fragment must be an essential part of the receptor because occupancy of it by the blocker inactivates the receptor. The response of the tissue to noradrenaline is not impaired in the presence of block-reversing concentrations of trypsin (see Fig. 2*c*). The removal of the whole fragment would apply only if the preceding amino acid were lysine or arginine and in that case a free arginine or lysine carboxyl would be left behind which might still act as an acceptor if the remaining structural pattern were suitable.

One may argue that these enzymes when disrupting an amide or peptide bond polarize the peptide link, which may exert an effect on the anionic acceptor, weakening its attachment to the rest of the acceptor and with it the attached compound, thus possibly reducing the latter from an irreversible to a reversible blocker. If this were so (apart from inherent chemical difficulties) it would not be possible non-competitively to block a receptor which had been pre-treated with enzyme and then washed (see Fig. 4). If the disruption here were total an essential part of the receptor would be lost and the

response to the agonist lost. This is found with high-concentration treatment and with chymotrypsin for all agonists. The peptide structure must be common to all the receptors for amines and for acetylcholine. The receptor for catecholamine displays a qualitative difference from those for histamine, acetylcholine, etc., in that reversibility of halogenoalkylamine block by trypsin is confined to it.

SUMMARY

1. In urethanized rats the duration of antagonism by equiactive doses of dibenamine, 2-bromo-*N*-ethyl-*N*-(α-naphthylmethyl) ethylamine hydrobromide (SY28), and *N*,*N*-dimethyl-2-bromo-2-phenylethylamine (L_2), to the pressor action of injected noradrenaline was found to be approximately 60, 25 and 3 hr respectively.

2. The effect of these compounds on the stimulant action of noradrenaline, dopamine, histamine and acetylcholine on isolated guinea-pig vas deferens was estimated as a pA_2 value at 2 and at 20 min as a measure of specificity. Mustine displays some activity.

3. In the vas deferens the effect of hydrolases—viz., trypsin, α-chymotrypsin, alkaline and acid phosphatase, phosphodiesterase, Naja venom, and papain—on the action of the stimulants adrenaline, noradrenaline, dopamine, histamine, acetylcholine, bradykinin, and K^+ was examined and the effect of these enzymes on smooth muscle blocked by the three 2-halogenoalkylamines.

4. In concentrations which do not affect the response of the vas deferens to the agonists, trypsin (alone of the hydrolases examined) reverses the blockade, and to catecholamine only of the agonists tested.

5. The ease of reversal is in the order L_2>SY28>dibenamine.

6. The reversal may be accomplished in steps by multiple exposure to trypsin. Boiled trypsin is inactive. Ovomucoid prevents the action.

7. Unblocked vas deferens may be blocked once more by re-application of compound.

8. There is a linear relation between the concentration of trypsin which may be added to a bath before 2-halogenoalkylamine and inhibition of the expected degree of block.

9. The ethanolamines derivable from the 2-halogenoalkylamines are inactive.

10. The de-blocking action of trypsin suggests that this enzyme may catalyse the recovery of alkylated alpha receptors. This it could do by action at an ester linkage on L-arginine or L-lysine, whereas chymotrypsin, or high concentrations of trypsin which desensitize the vas deferens to all agonists may rupture peptide and amide bonds.

11. It follows that the anionic acceptor site in the alpha receptor is a free carboxyl rather than a phosphate, and that the receptor is in part an amino acid chain containing arginine, lysine or both.

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