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STUDIES OF THE CHEMICAL NATURE OF THE α-ADRENERGIC RECEPTOR—V

VALIDITY OF THE RECEPTOR PROTECTION APPROACH*

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Abstract—The uptake of [^{14}C]phenoxybenzamine into rabbit aortic strips has been measured at bath concentrations sufficient to abolish epinephrine-induced contractions. The relationship between the degree of protection conferred by different doses of epinephrine against [^{14}C]dibenamine blockade of α-adrenergic receptors and the count differences between protected strips and unprotected controls has been investigated. The results support the view that epinephrine combines with a large number of non-specific sites as well as α-adrenergic receptors.

FURCHGOTT[1] identified four distinct sets of contraction receptors in the smooth muscle of rabbit aorta, which are specific for adrenergic drugs, 5-hydroxytryptamine, histamine and acetylcholine respectively. He showed that when this preparation is exposed to epinephrine bitartrate for 5 min and dibenamine hydrochloride added, the latter drug combines with the 5-hydroxytryptamine, histamine and acetylcholine receptors, while the adrenergic receptor is protected, at least partially, from combination with this drug. On the basis of these studies, information on the nature of the α-adrenergic receptors has been sought as follows: α-adrenergic receptors of smooth muscle preparations were partially protected with epinephrine while other receptors were labeled with [^{14}C]dibenamine hydrochloride. As a control, the labeling experiment was repeated with the omission of epinephrine. It was anticipated that the control strip, unprotected by epinephrine, would contain an increment in radioactivity over the protected strip. This increment would represent that portion of the [^{14}C]dibenamine hydrochloride combined with sites occupied by epinephrine in the protected strip. It was assumed that the bulk of the sites occupied by epinephrine would be α-adrenergic receptor sites. This method has been applied to the study of α-adrenergic receptors of rabbit aorta tissue by Yong *et al.*[2–4] and to the α-adrenergic receptors of rat seminal vesicle by Lewis and Miller.[5] The latter workers found consistent differences in [^{3}H]phenoxybenzamine uptake between preparations in which α-adrenergic receptors had been protected with norepinephrine or phentolamine and unprotected control preparations. Moran *et al.*, however, using *N*-(2-bromoethyl)-*N*-ethyl-*N*-1-naphthyl-methylamine (^{3}H-SY 28) as the antagonist and employing a variety of preparations and receptor-protecting agents, were unable to demonstrate consistent count differences between protected and unprotected preparations.[6]

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In our studies using the intact rabbit aortic strip preparation, we have shown consistent count differences between strips in which α-adrenergic receptors have been protected against [^{14}C]dibenamine with epinephrine and unprotected control preparations.[2–4] When the protection is carried out using phentolamine (1×10^{-5}), similar results are obtained. However, when the concentration of the protecting agent is reduced by a factor of ten, no significant count differences are obtained, although good protection of the contractile response is still achieved.[4] In strips which have been denervated by removal of the adventitia,[4] epinephrine will provide both protection of the contractile response and consistent count differences between experimental and control preparations when [^{14}C]dibenamine is employed as the antagonist. Under similar conditions, phentolamine provides good protection of the contractile response which is not associated with any such count differences.

The discrepancy between the results of experiments where the protection is carried out with epinephrine and those where phentolamine is employed could arise in one of two ways: either the count difference obtained with epinephrine provides an accurate representation of receptor population, in which case phentolamine is protecting the receptor by some means which does not lead to a difference in counts, or the count difference obtained when epinephrine is used arises only in small part from protection of receptor material and is caused largely by the occupation of sites which are not associated with the contractile response. In the second case phentolamine must be assumed to occupy a smaller number of such nonreceptor sites, although we would have no grounds for believing that it is completely selective for the α-receptor. The second hypothesis appears more probable and to obtain further information on the nature of the sites occupied by epinephrine, labeling studies were carried out with [^{14}C]phenoxybenzamine, which is known to antagonise the α-adrenergic response in lower concentrations than dibenamine. Information as to the nature of the sites occupied by epinephrine has also been sought by studies of the relationship between the degree of protection conferred by different doses of epinephrine against [^{14}C]dibenamine blockade of α-adrenergic receptors and the count differences between protected strips and unprotected controls.

EXPERIMENTAL

Infra-red spectra were obtained using a Perkin–Elmer 137 spectrophotometer and the samples were prepared as a 1% disc with potassium chloride. The melting point is uncorrected. The following compounds have been employed in this work: [^{14}C]benzyl chloride, specific activity 5 mc/m-mole (New England Nuclear), phenoxybenzamine hydrochloride and *N*-(phenoxyisopropyl) ethanolamine (SKF 931) (both compounds kindly donated by Smith Kline & French); [^{14}C]dibenamine hydrochloride, specific activity 0·48 mc/m-mole,[2] and *l*-epinephrine bitartrate (Nutritional Biochemicals). P values were obtained using a one-tailed *t*-test.

Synthesis of [^{14}C]*phenoxybenzamine hydrochloride*

[^{14}C]phenoxybenzamine hydrochloride was prepared by the method of Nikawitz and Gump.[7] [^{14}C]benzyl chloride (50·6 mg; 0·4 m-mole) dissolved in benzene (0·542 ml) and *N*-(phenoxyisopropyl) ethanolamine (156 mg; 0·8 m-mole) were boiled under reflux with anhydrous sodium carbonate (0·45 mg; 0·84 m-equiv.) in dry ethanol

(1·5 ml) for 24 hr. The solvent was evaporated under reduced pressure, water (0·5 ml) was added and the mixture was extracted three times with 2-ml aliquots of ether. The combined ether extracts were dried (sodium sulfate), the ether was removed under reduced pressure and the residue was distilled in a bulb tube. The fraction boiling between 155° and 180° was collected and dissolved in chloroform (2 ml), which had been saturated with dry hydrogen chloride. This solution was boiled under reflux with thionyl chloride (1 ml) for 3 hr. The solvent and excess thionyl chloride were removed by evaporation under reduced pressure and the residue was redissolved in benzene (3 ml) and again evaporated to dryness. The latter process was repeated. The oily residue was induced to crystallize by trituration with ether and the solid was recrystallized from a mixture of acetone and ether. The solid obtained was recrystallized repeatedly from a mixture of ethanol and ether. The recrystallized product still contained small quantities of colored impurities and because insufficient material was available for further purification the following procedure was adopted: the light-colored mother liquors from the second recrystallization from the ethanol–ether mixture were combined with unlabeled phenoxybenzamine hydrochloride (50 mg) and from this solution [^{14}C]phenoxybenzamine hydrochloride (30·1 mg; 0·29 mc/m-mole) was obtained as a fine white powder, m.p. 137°–137·5° (Nikawitz and Gump[7] give 137°–140°). The mixed melting point of this material with an authentic sample of phenoxybenzamine hydrochloride was not depressed and the infra-red spectra of the two samples were identical. The [^{14}C]phenoxybenzamine hydrochloride was found to be indistinguishable from an authentic sample in its biological activity, and it was shown to be radiochemically pure by the technique of reverse isotope dilution. This sample of [^{14}C]phenoxybenzamine hydrochloride has been employed in the experiments discussed below.

Preparation of rabbit aortic strips

Rabbits weighing between 1·5 and 2·5 kg were killed by dislocation of the neck and the aorta was excised, freed from adipose and connective tissue, and cut spirally as described by Furchgott and Bhadrakom.[8] Four strips were obtained from each aorta. The strips were suspended in organ baths of 15-ml working volume containing Krebs bicarbonate solution[2] aerated with 95% oxygen and 5% carbon dioxide and maintained at 37°. The contractions were recorded using force and displacement transducers (Grass FT 03) connected to a four-channel Grass model 5P1 polygraph. The initial resting tension of the strips was set at between 1 and 2 g. The drugs were administered as a 0·2-ml aliquot of a solution in normal saline in the case of epinephrine and in normal saline containing 0·01 M hydrochloric acid in the case of the β-haloalkylamines. The final drug concentrations are expressed as grams of salt per milliliter of bath fluid.

Determination of radioactivity in aortic strips

The radioactivity in the strips was determined in a liquid scintillation counter (Nuclear Chicago Unilux model 6850). Each tissue was removed from the bath, lightly blotted, and freeze-dried at −70° and 0·3 mm for at least 4 hr. The dry tissue was then weighed, placed in a counting vial and dissolved by treatment with 5 N potassium hydroxide (0·2 ml) at 70° for 1 hr. Methanol (5·3 ml) was then added and the

total volume made up to 18 ml with scintillation fluid comprising 6 g 2,5-diphenyloxazole (PPO) and 0·1 g 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP) per liter of toluene. The samples were stored in the dark for 2 days and then counted for 40 min in two channels. In two cases the net count rate was so low that a standard deviation of less then 5 per cent for the corrected count could not be obtained in this or in a considerably extended time. The efficiency of counting was obtained by the channels ratio method and the final results are expressed as dis. per min per mg dry weight of tissue. The use of the Olivetti Programma 101 greatly facilitated calculation and further treatment of the data.

Uptake of [^{14}C]phenoxybenzamine into rabbit aortic strips

The relationship between the concentration of phenoxybenzamine hydrochloride, exposure time and degree of blockade of epinephrine-induced contractions of rabbit aortic strips was first determined using the unlabeled compound. Aortic strips were allowed to equilibrate at 37° for 2 hr and cumulative dose–response curves to epinephrine were constructed. The tissues were then washed at frequent intervals until they had relaxed to the resting tension. Phenoxybenzamine hydrochloride was then added and, after the required exposure time had elapsed, the tissues were washed twice, and thereafter washed at 15-min intervals for 2 hr. Further cumulative dose–response curves were then constructed. The data from these experiments are shown in Table 1.

TABLE 1. RELATIONSHIP BETWEEN CONCENTRATION OF PHENOXYBENZAMINE, EXPOSURE TIME AND REMAINING RESPONSE TO EPINEPHRINE IN RABBIT AORTIC STRIPS

Phenoxybenzamine conc. (g/ml)	Exposure time (min)	$\frac{\text{Max. response 2·5 hr after blockade}}{\text{Max. response before blockade}} \times 100$
1×10^{-7}	1	80–100*
1×10^{-7}	3	0–15
1×10^{-7}	5	0
5×10^{-8}	1	>100
5×10^{-8}	3	40–60
5×10^{-8}	5	0–10
1×10^{-8}	1	>100
1×10^{-8}	3	60–70
1×10^{-8}	5	40–60

* Range is shown for at least three experiments.

This procedure was then repeated using [^{14}C]phenoxybenzamine, and the uptake of this drug under various conditions is shown in Table 2.

Relationship between receptor protection and uptake of [^{14}C]dibenamine in rabbit aortic strips

Four rabbit aortic strips were allowed to equilibrate at 37° for 2 hr and the responses to epinephrine (1×10^{-8}, 5×10^{-8}, 1×10^{-7} and 1×10^{-6}) were recorded. The strips were then washed every 15 min until the tissues had relaxed to the resting tension. Two of the strips were then treated with epinephrine (1×10^{-6}) for 5 min and

TABLE 2. UPTAKE OF [^{14}C]PHENOXYBENZAMINE HYDROCHLORIDE INTO RABBIT AORTIC STRIPS*

Dose of [^{14}C]phenoxybenzamine hydrochloride (g/ml)	Exposure time (min)	dis./min/mg dry wt. (± S.D.)
5×10^{-8}	5	†
1×10^{-7}	5	†
1×10^{-6}	5	12·85 ± 2·1
3×10^{-6}	20	368·8 ± 30·3

* Each value represents the mean of at least four experiments.
† Too small to be determined.

[^{14}C]dibenamine hydrochloride (3×10^{-6}) was added. After a further 20-min period, the strips were washed with Krebs bicarbonate solution containing epinephrine (1×10^{-6}) and this process was repeated at 15-min intervals. When the strips had been washed three times with this medium, successive washings with Krebs bicarbonate solution were carried out at 15-min intervals until the strips had relaxed to the resting tension (approximately 3 hr). Strips treated in this manner were designated experimental strips. As controls, the experiment was repeated using the remaining two strips and omitting the protecting dose of epinephrine. This experiment was repeated four times. The radioactivity in these strips was determined as described above. These experiments were repeated using different protecting doses of epinephrine and the results of these studies are shown in Table 3.

TABLE 3. RELATIONSHIP BETWEEN DEGREE OF PROTECTION CONFERRED BY DIFFERENT DOSES OF EPINEPHRINE AGAINST [^{14}C]DIBENAMINE (3×10^{-6}) BLOCKADE OF α-ADRENERGIC RECEPTORS AND THE COUNT DIFFERENCES BETWEEN PROTECTED STRIPS AND UNPROTECTED CONTROLS*

Protecting dose of epinephrine (g/ml)	Remaining sensitivity to epinephrine (% of original ± S.D.)†	dis./min/mg of dry wt. of strip	
		Protected strip	Unprotected strip
1×10^{-6}	4·2 ± 5·1	333 ± 57·6	329 ± 29·9
3×10^{-6}	30 ± 23·8	260 ± 25·2	259 ± 47·0
1×10^{-5}	59 ± 13·5	300 ± 35·6	328 ± 47·7
3×10^{-5}	77 ± 6·3	288 ± 32·0‡	334 ± 41·0‡
1×10^{-4}	86 ± 11·6	324 ± 72·0	383 ± 75·0

* Figures given are the means obtained in five separate experiments.
† Remaining sensitivity to epinephrine was calculated by means of the following equation: $P = R^1/R \times 100$, where P = per cent of original response to epinephrine; R = tension (g) developed by strips to a dose of epinephrine prior to protecting dose of epinephrine and [^{14}C]dibenamine (3×10^{-6}); R^1 = tension (g) developed by strips to the same dose of epinephrine after the protecting dose of epinephrine and [^{14}C]dibenamine (3×10^{-6}).
‡ The difference between the counts in protected and unprotected strips is significant with this protecting dose of epinephrine ($P < 0.05$).

RESULTS AND DISCUSSION

The remaining response to epinephrine in rabbit aortic strips after blockade with phenoxybenzamine under various conditions is shown in Table 1. The values are expressed as the percentage of the original maximum response remaining 2·5 hr after blockade. Although the strips were allowed to equilibrate at 37° for 2 hr prior to initial testing with epinephrine, some increase in sensitivity to epinephrine was frequently observed during the course of the experiment, giving rise to some values in excess of 100 per cent. A concentration of 1×10^{-7} phenoxybenzamine hydrochloride and an exposure time of 5 min will abolish the epinephrine-induced contractions. Under these conditions the uptake of [^{14}C]phenoxybenzamine was too small to be determined in our system.

The significance of this observation is apparent when considered in relation to our findings with [^{14}C]dibenamine hydrochloride. From the data in Table 3, it is apparent that epinephrine (3×10^{-5}) prevents the uptake of an amount of [^{14}C]dibenamine hydrochloride equivalent to 46 dis./min/mg dry weight of strip. Since the average dry weight of the strips used was 6 mg, epinephrine (3×10^{-5}) has prevented the uptake of 276 dis./min per aortic strip. Assuming epinephrine has protected only α-adrenergic receptor sites, it can be calculated, taking into account differences in specific activity between [^{14}C]dibenamine hydrochloride and [^{14}C]phenoxybenzamine hydrochloride, that at least 172 dis./min of [^{14}C]phenoxybenzamine should be taken up by a strip when complete α-adrenergic blockade is produced. This is clearly not the case (phenoxybenzamine, 1×10^{-7} for 5 min; Tables 2 and 3). Even when used at a concentration many times higher than that necessary for complete α-adrenergic blockade (1×10^{-6} for 5 min, Table 3) the uptake per strip does not reach a figure of 172 dis./min. It therefore follows that epinephrine used in concentrations normally employed in receptor protection studies will protect numerous sites other than α-adrenergic receptor sites.

Further evidence for this view is provided by the data in Table 3. When epinephrine (3×10^{-5}) was used as a protecting dose, contractile protection of 77 per cent was observed, associated with a count difference of 46 dis./min/mg dry weight of strip. On the other hand, when epinephrine (3×10^{-6}) was used as protecting dose, the contractile protection of 30 per cent observed was not associated with any count difference between control and experimental strips. Thus there is no direct correlation between contractile protection and count differences between experimental and control strips. The probable explanation is that higher doses of epinephrine protect nonspecific as well as α-adrenergic receptor sites. Recently Avakian and Gillespie[9] used fluorescence techniques to show that maximal constriction of the rabbit ear artery could be obtained when the smooth muscle had taken up only a small proportion of the norepinephrine of which it was capable. This experiment indicates that norepinephrine is taken up by sites in smooth muscle other than α-adrenergic receptor sites.

It is clear from these studies that our suggestions made previously[4] as to the mechanism by which epinephrine can give rise to large uptake differences of dibenamine between protected and unprotected preparations are substantially correct. Epinephrine cannot be used successfully as a receptor-protecting agent in this preparation owing to its considerable uptake into nonreceptor sites. The nature of the nonreceptor sites which are occupied by epinephrine is unknown, but certain conclusions can be

drawn as to their properties. First, such sites cannot be spare receptors, since May *et al.*[10] have provided convincing evidence that there is no significant receptor reserve in this system. Equally, such sites cannot be structurally identical to the receptor, since phentolamine is capable of distinguishing many if not all nonreceptor sites from α-adrenergic receptors. If the affinity of epinephrine for nonspecific sites were greater than for receptors, conditions would arise whereby significant count differences could be obtained in the absence of any protection of the contractile response. It can be seen from Table 3 that no such conditions have been encountered. Moreover, a certain amount of protection of the contractile response can be achieved in the absence of significant count differences between protected and unprotected preparations. It is thus likely that the affinity of nonreceptor sites for epinephrine is substantially lower than that of the receptors for the same drug.

Several years ago when we embarked on our studies of the α-adrenergic receptor, it appeared that the major problem would be the isolation of the drug–receptor complex after specific labeling of the receptor. It now appears that the major problem in this field is to achieve selective labeling of the α-adrenergic receptor. On the basis of our experience, it is clear that prior to undertaking the isolation of a drug–receptor complex, it is essential to establish whether there is a correlation between the degree of receptor protection achieved with a series of agonists and reversible antagonists, and the difference in radioactivity observed between receptor-protected and unprotected preparations. Furthermore, it will be helpful in these studies to utilize several labeled β-haloalkylamines of the highest specific activity attainable.

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