Biochemical Pharmacology, Vol. 18, pp.1619–1626. Pergamon Press. 1969. Printed in Great Britain

# STUDIES OF THE CHEMICAL NATURE OF THE α-ADRENERGIC RECEPTOR—IV

## LABELING STUDIES ON NERVE-FREE RABBIT AORTIC STRIPS*

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(*Received* 2 *July* 1968; *accepted* 6 *December* 1968)

**Abstract**—Since epinephrine protects uptake sites in sympathetic nerve terminals in addition to α-adrenergic receptors from combination with $^{14}$C-Dibenamine hydrochloride a procedure was developed for denervation of rabbit aorta. With epinephrine as protecting agent in these nerve-free rabbit aortic strips the significant increment in radioactivity associated with unprotected strips as compared to protected strips was confined to the lipid-free residue. This observation provided further evidence that the α-adrenergic receptor is not lipid in nature. In intact aortic strips phentolamine ($1 \times 10^{-5}$) conferred complete protection against blockade by $^{14}$C-Dibenamine hydrochloride ($3 \times 10^{-6}$) and a significant difference was found in the radioactivity of unprotected and protected strips. Although phentolamine ($1 \times 10^{-5}$) conferred 100 per cent protection against blockade by $^{14}$C-Dibenamine hydrochloride ($3 \times 10^{-6}$) in nerve-free rabbit aortic strips no significant difference was detected in the radioactivity of unprotected and protected strips.

A COMPLICATION in our previous attempts to label the α-adrenergic receptors of rabbit aortic strips arose from the fact that epinephrine in the high concentration used to protect these receptors probably also protected the uptake sites in sympathetic nerve terminals from combination with $^{14}$C-Dibenamine hydrochloride.[1, 2] For this reason a method was sought to remove the sympathetic nerves from rabbit aortic strips. Recent studies by several groups of workers have suggested a method by which this may be accomplished. Histochemical studies of blood vessels reveals that the terminal sympathetic effector plexus is confined to the adventitio-medial junction.[3] Moreover, removal of the adventitia from rabbit aortic strips removes the bulk of the sympathetic nerve terminals and concomitantly the capacity of the tissue to bind $^{3}$H-norepinephrine.[4] In this paper we have repeated our labeling studies with epinephrine as a protecting agent in rabbit aortic strips from which the adventitia had been removed.

Since the reversible α-adrenergic receptor blocking agent, phentolamine, protects the receptors from combination with Dibenamine hydrochloride when present in a much lower concentration than epinephrine[2] experiments have been carried out using phentolamine as a protecting agent in intact and nerve-free rabbit aortic strips and the results compared with those obtained using epinephrine as a protecting agent.

* This investigation was supported by a grant from the Alberta Heart Fund.
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## EXPERIMENTAL

Rabbits weighing between 1·5 and 3 kg were killed by a blow on the back of the neck. The descending thoracic aorta was quickly removed and placed in a Petri dish containing Krebs bicarbonate solution[5] aerated with 95% oxygen and 5% carbon dioxide. After removing the adipose and connective tissue from the aorta, a helical strip was prepared according to Furchgott and Bhadrakom[6] and mounted as single strips in organ baths of 15 ml working volume with the aid of thread and stainless-steel hooks. The responses of the tissue were recorded by means of force and displacement transducers (Grass FT·03) connected to a Grass model 5P1 polygraph. The loading tension applied to the strips was 2 *g*. The volume of Krebs bicarbonate solution in the organ bath was 14·8 ml. Drugs were made up to a final volume of 0·2 ml before addition to the bath. The following drugs were used: *l*-epinephrine bitartrate (Nutritional Biochemicals); norepinephrine bitartrate (Nutritional Biochemicals); tyramine hydrochloride (Nutritional Biochemicals); phentolamine methanesulfonate (Rogitine, Ciba); and $^{14}$C-Dibenamine hydrochloride (sp. act. 0·48 mc/m-mole). The final drug concentrations, unless otherwise specified, are expressed as grams of salt per milliliter bath fluid.

The radioactivity in lipid and lipid-free residues of the strips was determined in a liquid scintillation counter (Nuclear Chicago unilux model 6850). Full details of the procedure have been described previously.[5] The counting efficiency of lipid extracts was found to be in the range of 60–75 per cent, while that of lipid-free residues was found to be in the range of 40–50 per cent.

*Phentolamine as a protecting agent in labeling studies.* The responses of rabbit aortic strips to several concentrations of epinephrine were recorded. The strips were treated with phentolamine methanesulfonate ($1 \times 10^{-5}$) for 5 min and $^{14}$C-Dibenamine hydrochloride ($3 \times 10^{-6}$) added. After a further 20-min period the strips were washed for 3 hr at 15-min intervals with Krebs bicarbonate solution and the responses to various concentrations of epinephrine redetermined. As controls, the experiments were repeated with the omission of the protecting dose of phentolamine methanesulfonate ($1 \times 10^{-5}$).

The above experiment was repeated several times keeping all the factors constant but varying the phentolamine concentration. Thus, in the second experiment phentolamine methanesulfonate ($3 \times 10^{-7}$) was used, and in the third experiment phentolamine methanesulfonate ($3 \times 10^{-6}$) was used.

*Effect of removal of adventitia on the response of strips to epinephrine, norepinephrine and tyramine.* The response of rabbit aortic strips to several doses of epinephrine and norepinephrine was recorded. From these results a cumulative dose–response curve was constructed for epinephrine (Fig. 1 A) and norepinephrine. The tension produced by a single dose of tyramine ($1 \times 10^{-5}$) was recorded and found to be 0·6 *g*.

The strips were placed with their adventitial surface facing upward on a piece of paper towel moistened with Krebs bicarbonate solution. The adventitial layer of the strips was peeled off the media with great care by means of a fine curved forceps. During this procedure the strips were flooded continuously with Krebs bicarbonate solution. The modified strips were mounted in organ baths and the responses to several doses of epinephrine and norepinephrine were again determined. The response was expressed as a percentage of the maximal response attainable in the corresponding

intact strips. The results are shown in Fig. 1 B for epinephrine. Similar results were found with norepinephrine. The strips were found to be unresponsive to tyramine ($1 \times 10^{-5}$). In order to shorten subsequent descriptions of this procedure, aortic strips from which the adventitia has been removed will be referred to as nerve-free aortic strips.

*Labeling studies with nerve-free rabbit aortic strips.* (a) Epinephrine as protecting agent. Nerve-free aortic strips were mounted in organ baths and the responses of the strips to several concentrations of epinephrine were recorded. The strips were exposed to epinephrine bitartrate ($1 \times 10^{-4}$) for 5 min and $^{14}$C-Dibenamine hydrochloride ($3 \times 10^{-6}$) was added. After a further 20-min period, the strips were washed for 3 hr at 15-min intervals and the responses of strips to epinephrine redetermined. As controls, the above experiments were repeated with the omission of epinephrine bitartrate ($1 \times 10^{-4}$).

(b) Phentolamine as a protecting agent. The procedure described in (a) was repeated by substituting phentolamine methanesulfonate ($1 \times 10^{-5}$) for epinephrine bitartrate ($1 \times 10^{-4}$) as an α-adrenergic receptor protecting agent.

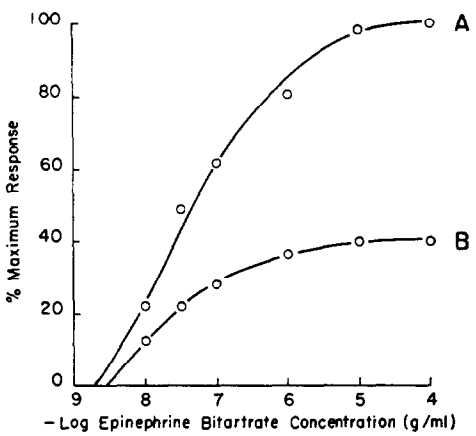


FIG. 1. Relationship between the dose of epinephrine and the contractile response in A, intact rabbit aortic strips and B, nerve-free rabbit aortic strips. Each point represents the mean of four to eight independent observations.

## RESULTS AND DISCUSSION

Bevan and Verity[7] used the following procedure for the preparation of nerve-free rabbit aortic strips: aortic strips were placed in flat-bottomed grooves cut in a plastic block and the adventitia projecting above the surface of the block was destroyed by means of a vibrating razor blade. The bulk of the adventitia was shown to have been removed by this procedure. We have explored the possibility of obtaining nerve-free rabbit aortic strips by carefully peeling off the adventitia with fine curved forceps. It was of interest to compare the responses to epinephrine, norepinephrine and tyramine in our nerve-free aortic strips to those observed by Bevan and Verity[7] in their nerve-free aortic strips. In both preparations the contractions elicited by tyramine ($1 \times 10^{-5}$) in the intact aortic strips were not observed in the nerve-free

strips. Since tyramine produces contractions by liberation of norepinephrine from sympathetic nerve terminals in the adventitia it is clear that in these nerve-free preparations the sympathetic nerve terminals are almost completely absent. Our nerve-free strips responded to a maximum dose of norepinephrine with a contraction which was 45 per cent of that observed in the intact strips. In similar experiments Bevan and Verity[7] found the responses to a maximum dose of norepinephrine in nerve-free strips to be 65 per cent of that observed in intact strips. Recently Maxwell *et al.*[8] have obtained nerve-free rabbit aortic strips by a procedure closely similar to the method we have used. The properties described by the authors for their nerve-free strips were similar to those we have found.

The results of the labeling studies with nerve-free rabbit aortic strips using epinephrine ($1 \times 10^{-4}$) as the protecting agent are recorded in Table 1. A significant

TABLE 1. DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF CONTROL AND EXPERIMENTAL NERVE-FREE RABBIT AORTIC STRIPS USING EPINEPHRINE AS A PROTECTING AGENT*

| Aortic strip | Labeling conditions | Dry weight of aortic strip (mg) | Remaining sensitivity to epinephrine (% of original)† | Dpm/mg of dry weight of strip ± S.D. | | |
|---|---|---|---|---|---|---|
| | | | | Lipid-free residue | Lipid extract | Total |
| Control | $^{14}$C-Dibenamine HCl $3 \times 10^{-6}$ g/ml | 6·4 ± 2·1 | | 646 ± 54 | 98 ± 14 | 562 ± 63 |
| Experimental | Epinephrine bitartrate $1 \times 10^{-4}$ g/ml | | | | | |
| | $^{14}$C-Dibenamine HCl $3 \times 10^{-6}$ g/ml | 6·9 ± 1·4 | 56 ± 10 | 400 ± 72 | 88 ± 25 | 488 ± 91 |
| P value | | | | < 0·05 | > 0·05 | < 0·05 |

* The total dpm/mg dry weight of strip is the sum of the dpm/mg of the lipid extract and lipid-free residue. Figures given are the means obtained in fifteen separate experiments.

† Remaining sensitivity to epinephrine was calculated by means of the following equation: $P = R'/R \times 100$, where P = per cent of original response to epinephrine; R = tension (*g*) developed by strips to a dose of epinephrine prior to epinephrine ($1 \times 10^{-4}$) and $^{14}$C-Dibenamine ($3 \times 10^{-6}$); R′ = tension (*g*) developed by strips to the same dose of epinephrine after epinephrine ($1 \times 10^{-4}$) and $^{14}$C-Dibenamine ($3 \times 10^{-6}$).

increment in radioactivity ($P < 0{\cdot}05$) was noted in the lipid-free residues of control strips when compared to experimental strips. However, no significant difference in radioactivity was noted on comparing the lipid extracts of control and experimental strips ($P > 0{\cdot}05$). With intact strips a significant increment in radioactivity was noted in both lipid extracts and lipid-free residues of control as compared to experimental strips.[5, 9] In comparing the results of these two experiments we concluded that the significant increment in radioactivity associated with the lipid-free residue probably represents the $^{14}$C-Dibenamine hydrochloride molecules combined with α-adrenergic receptor sites. On the other hand, the significant increment in the radioactivity associated with lipid extracts in intact aortic strips probably represents the $^{14}$C-Dibenamine hydrochloride molecules combined with uptake sites in nerve terminals

and other nonspecific sites in the adventitia. On the basis of this experiment it appeared that the lipid portion of the rabbit aortic cell could be eliminated as a candidate for the α-adrenergic receptor. This result is in agreement with that reported by Lewis and Miller[10] using a similar approach to study the α-adrenergic receptors of the smooth muscle cells of rat seminal vesicle.

In our next experiments phentolamine methanesulfonate ($1 \times 10^{-5}$) was used to protect the α-adrenergic receptors of intact rabbit aortic strips. A significant increment in radioactivity was noted in both lipid extracts ($P < 0.05$) and lipid-free residues ($P < 0.05$) of the control strips as compared to the corresponding fractions of experimental strips (Table 2). This corresponded to our previous observations using epinephrine ($1 \times 10^{-4}$) as a protecting agent in intact strips.[5, 9] The results reported in

TABLE 2. DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF CONTROL AND EXPERIMENTAL RABBIT AORTIC STRIPS USING PHENTOLAMINE AS PROTECTING AGENT*

| Aortic strip | Labeling conditions | Dpm/mg of dry weight of strip ± S.D. | | |
|---|---|---|---|---|
| | | Lipid-free residue | Lipid extract | Total |
| Control | $^{14}$C-Dibenamine HCl $3 \times 10^{-6}$ g/ml | 373 ± 54 (9)† | 68 ± 10 (9) | 441 ± 59 (9) |
| Experimental | Phentolamine methanesulfonate‡ $1 \times 10^{-5}$ g/ml | | | |
| | | 266 ± 74 (10) | 48 ± 12 (10) | 314 ± 86 (10) |
| | $^{14}$C-Dibenamine HCl $3 \times 10^{-6}$ g/ml | | | |
| P value | | < 0.05 | < 0.05 | < 0.05 |

* The total dpm/mg dry weight of strip is the sum of the dpm/mg of the lipid extract and lipid-free residue.

† Numbers enclosed by parentheses indicate the number of experiments performed.

‡ Remaining sensitivity to epinephrine was 100 per cent of the original with this protecting dose of phentolamine methanesulfonate. The remaining sensitivity to epinephrine was calculated as follows: $P = R'/R \times 100$, where P = per cent of original response to epinephrine; R = tension (*g*) developed by strips to a dose of epinephrine prior to phentolamine methanesulfonate ($1 \times 10^{-5}$) and $^{14}$C-Dibenamine ($3 \times 10^{-6}$); R′ = tension (*g*) developed by the strips to the same dose of epinephrine 3hr after phentolamine methanesulfonate ($1 \times 10^{-5}$) and $^{14}$C-Dibenamine ($3 \times 10^{-6}$).

Table 3 show that phentolamine at a concentration of $3 \times 10^{-6}$ afforded complete protection against blockade by $^{14}$C-Dibenamine hydrochloride ($3 \times 10^{-6}$) in this tissue. However, using phentolamine at a concentration of $3 \times 10^{-7}$ the degree of protection dropped to less than 8 per cent. It was surprising that despite complete receptor protection with phentolamine ($3 \times 10^{-6}$) a significant difference in radioactivity between control and protected strips was not observed. The results of the labeling studies with nerve-free aortic strips using phentolamine methanesulfonate ($1 \times 10^{-5}$) as the protecting agent are recorded in Table 4. No significant increment in radioactivity ($P > 0.05$) was noted in either the lipid extracts or the lipid-free residues of control strips as compared to experimental strips. With intact aortic strips significant increments in radioactivity were noted in both lipid extracts and lipid-free residues of control strips when compared to experimental strips. This finding was

unexpected since phentolamine ($1 \times 10^{-5}$) had conferred almost 100 per cent protection against blockade by $^{14}$C-Dibenamine hydrochloride in both intact and nerve-free strips. In comparing the results obtained with epinephrine and phentolamine as protecting agents it is of interest to consider the molar ratios of these substances employed relative to $^{14}$C-Dibenamine hydrochloride. Thus the molar ratio of epinephrine to $^{14}$C-Dibenamine hydrochloride was 33:1, while the molar ratio of phentolamine to $^{14}$C-Dibenamine hydrochloride was 2·6:1. Bearing this point in mind the

TABLE 3. INHIBITION OF $^{14}$C-DIBENAMINE HYDROCHLORIDE UPTAKE IN RABBIT AORTIC STRIPS BY PHENTOLAMINE METHANESULFONATE

| Concentration of phentolamine methanesulfonate (g/ml) | Remaining sensitivity to epinephrine (% of original) | Dpm/mg dry weight of strip ± S.D. | |
|---|---|---|---|
| | | Lipid extract | Lipid-free residue |
| 0 | — | 64 ± 18 (10)* | 333 ± 93 (10) |
| $3 \times 10^{-7}$ | < 8 | 54 ± 12 (5) | 277 ± 49 (5) |
| $3 \times 10^{-6}$ | 100 | 67 ± 22 (5) | 323 ± 92 (5) |
| $1 \times 10^{-5}$ | 100 | 44 ± 6† (4) | 229 ± 15† (4) |

* Numbers enclosed by parentheses indicate the number of experiments performed.
† Significantly different from control at 0·05 level.

TABLE 4. DISINTEGRATIONS PER MINUTE DETECTED IN THE LIPID EXTRACTS AND LIPID-FREE RESIDUES OF CONTROL AND EXPERIMENTAL NERVE-FREE RABBIT AORTIC STRIPS USING PHENTOLAMINE AS A PROTECTING AGENT*

| Aortic strip | Labeling conditions | Dry weight of aortic strip (mg) | Dpm/mg of dry weight of strip ± S.D. | | |
|---|---|---|---|---|---|
| | | | Lipid-free residue | Lipid extract | Total |
| Control | $^{14}$C-Dibenamine HCl $3 \times 10^{-6}$ g/ml | 5·8 ± 2·8 | 361 ± 144 | 92 ± 23 | 495 ± 137 |
| Experimental | Phentolamine methanesulfonate† $1 \times 10^{-5}$ g/ml + $^{14}$C-Dibenamine HCl $3 \times 10^{-6}$ g/ml | 6·3 ± 2·1 | 433 ± 87 | 87 ± 20 | 518 ± 105 |
| P value | | | > 0·05 | > 0·05 | > 0·05 |

* The total dpm/mg dry weight of strip is the sum of the dpm/mg of the lipid extract and lipid-free residue. Figures given are the means obtained in 14 separate experiments.
† Remaining sensitivity to epinephrine was 100 per cent of the original with this protecting dose of phentolamine methanesulfonate.

following appears to be the most likely explanation for the different results obtained when using phentolamine instead of epinephrine as a receptor protecting agent: Phentolamine ($1 \times 10^{-5}$) protects the total receptor population from combination with $^{14}$C-Dibenamine hydrochloride. However, the total number of receptors is so small that no significant difference in radioactivity is detectable between control

and experimental nerve-free strips by our technique. The significant difference in radioactivity between control and experimental nerve-free strips observed with epinephrine would then be explained by the fact that epinephrine ($1 \times 10^{-4}$) protects large numbers of nonspecific sites as well as α-adrenergic receptor sites from combination with $^{14}$C-Dibenamine hydrochloride. The following facts support the above interpretation: Lewis and Miller[10] used $^{3}$H-phenoxybenzamine of very high specific activity and observed significant differences in radioactivity between phentolamine protected and unprotected rat seminal vesicles. From the difference in lipid-free residue radioactivity between phentolamine protected and unprotected preparations these workers estimated that there are $1{\cdot}7 \times 10^{13}$ α-adrenergic receptors per gram wet weight of rat seminal vesicle. May *et al.*,[11] by means of a different technique, estimated that there are $1{\cdot}15 \times 10^{15}$ α-adrenergic receptors per gram dry weight of rabbit aorta. Assuming the data of Lewis and Miller[10] to be a reasonable estimate of the number of receptors in the aortic strip it can be calculated that $2{\cdot}5 \times 10^{-10}$ *g* of $^{14}$C-Dibenamine (0·48 mc/m-mole) would be taken up by the α-adrenergic receptors of one aortic strip (30 mg wet weight). This amount of $^{14}$C-Dibenamine corresponds to approximately 1 dpm. On the basis of the data of May *et al.*[11] it can be calculated that an amount of $^{14}$C-Dibenamine corresponding to 20 dpm would be taken up by the α-adrenergic receptors of one aortic strip. From the data in Table 1, it is apparent that epinephrine ($1 \times 10^{-4}$) prevents the uptake of an amount of $^{14}$C-Dibenamine hydrochloride equivalent to 74 dpm/mg dry weight of strip. Since the average dry weight of the strips used was 6 mg, epinephrine ($1 \times 10^{-4}$) has prevented the uptake of 444 dpm per aortic strip. It is thus likely from the data of May *et al.*[11] and Lewis and Miller[10] that the sites protected by epinephrine ($1 \times 10^{-4}$) from combination with $^{14}$C-Dibenamine ($3 \times 10^{-6}$) are mainly nonspecific sites. It is therefore desirable that the experiments using phentolamine as a protecting agent be repeated with $^{14}$C-Dibenamine hydrochloride or $^{14}$C-phenoxybenzamine hydrochloride of higher specific activity. It is essential for further progress in this field to establish conclusively whether or not there is a correlation between the degree of receptor protection achieved with agonists and reversible antagonists and the difference in radioactivity observed between protected and unprotected strips. This is especially necessary in view of the fact that Moran *et al.*[12] appear to have found no correlation between the protective ability of a variety of amines and their direct or indirect sympathomimetic activities.

Investigators in the cancer field[13] studying the mechanism of action of alkylatng agents are beset by similar difficulties to those encountered in these studies when trying to decide which of many different sites alkylated leads to tumor inhibition. By comparing the cellular sites alkylated with two different labeled nitrogen mustards and correlating alkylation with tumour inhibition they were able to distinguish nonspecific from specific alkylation. We believe that a similar approach using $^{14}$C-Dibenamine and $^{14}$C-phenoxybenzamine could be adapted to determine which alkylation sites in rabbit aortic strips lead to α-adrenergic receptor blockade.

*Acknowledgement*—We are indebted to Dr. J. A. Bevan, Department of Pharmacology, The University of California, Los Angeles, Calif. for his suggestion which led to the work described in this paper.

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