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Further biochemical characterization of [^{3}H]dihydroazapetine binding to α-adrenoceptor-related proteins from the rat vas deferens*

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The binding of [^{3}H]dihydroazapetine, a reversible blocker of the α-adrenoceptor, has been characterized in a membrane preparation from the rat vas deferens. Binding is rapid, reversible and shows evidence of saturation. Postganglionic sympathetic denervation of the tissue does not decrease binding to the subcellular fraction. Drugs known to act on the α-adrenoceptor are specific inhibitors of [^{3}H]dihydroazapetine binding while those acting on other receptors are without effect. Scatchard plot analysis suggests that two specific binding components are present, one of which has a similar dissociation constant for [^{3}H]dihydroazapetine to that determined pharmacologically. [^{3}H]Dihydroazapetine binding is lowest in the physiological pH range and tends to decrease with increasing temperature in the range of 25 to 60°. Calcium, magnesium, sodium and potassium inhibit binding of [^{3}H]dihydroazapetine with the divalent ions being approximately 100 times more potent. Studies with group selective reagents indicate that a disulphide bridge, and sulphydryl and carbonyl groups, are at or near the binding site and are necessary for binding. The site is protein possibly with involvement of phospholipids. Specific binding of [^{3}H]dihydroazapetine is inhibited by low concentrations of local anaesthetics suggesting that the binding site of the antagonist may be related to the 'calcium mobilization' site of the α-adrenoceptor.

Recently, we have studied the binding of the tritiated α-adrenoceptor antagonist, dihydroazapetine, to a membrane fraction from the rat vas deferens (Ruffolo, Fowble & others, 1976). [^{3}H]Dihydroazapetine binds to membrane protein from this tissue with many characteristics that would be expected for the α-adrenoceptor. Binding was saturable and to two distinct sites, one of which showed similar affinity for the antagonist as that determined pharmacologically (Ruffolo, Fowble & others, 1977a). There was also good correlation between known affinities for the receptor of various antagonists, and those agonists of the imidazoline class, and ability to inhibit specific binding of [^{3}H]dihydroazapetine (Ruffolo & others, 1976). Stereoselectivity for isomers of α-adrenoceptor antagonists was also observed. Agonists of the phenethylamine class, however, caused a stereoselective increase in [^{3}H]-dihydroazapetine binding which was postulated to be the result of an allosteric interaction consistent with the findings of Kalsner (1970, 1973).

The present investigation was undertaken to further characterize the binding of [^{3}H]dihydroazapetine to a membrane preparation from the rat vas deferens.

MATERIALS AND METHODS

Details of the procedure for isolation of membrane protein and the method used to study the binding of [^{3}H]dihydroazapetine have been reported elsewhere (Ruffolo & others, 1976) and will only be briefly described below. Vasa deferentia were obtained from male albino rats (200–300 g, Sprague-Dawley), cleaned and then homogenized in 0·13 M sodium phosphate buffer (pH 7·4). After filtration through muslin (by suction), the homogenate was centrifuged at 17 000 *g* for 30 min at 4°. The pellet was resuspended in distilled water and centrifuged at 50 000 *g* for 20 min. The supernatant was discarded and the pellet resuspended again in distilled water, except where indicated, and used directly in binding experiments. Protein content was determined by the method of Lowry, Rosebrough & others (1951).

Aliquots (500 μl) of the crude membrane preparation were incubated (37°) for at least 20 min with [^{3}H]dihydroazapetine (49 Ci mmol^{-1}) alone as a control or after 30 min exposures to various chemicals and/or treatments. The concentration of [^{3}H]-dihydroazapetine was 10 nM except where indicated. The binding reaction was terminated by pipetting a

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450 μl aliquot of the incubation mixture through Whatman GF/C glass fibre filters with the aid of suction supplied by a sink aspirator. The filters were washed once with 5 ml distilled water (0°) and placed in 20 ml scintillation vials containing 0·5 ml of 5% Triton X-100. The vials were shaken for at least 60 min after which 10 ml of a toluene-based scintillation cocktail were added. The samples were counted in a Beckman Liquid Scintillation Spectrometer (LS-345) for 10 min and corrections made for quench with the aid of an automatic external standard.

Data are expressed either as total or specific [^{3}H]-dihydroazapetine binding. Specific binding is the difference between total [^{3}H]dihydroazapetine bound, and that binding which occurs in the presence of 10 μM phentolamine. Appropriate corrections were made for [^{3}H]dihydroazapetine retained by the filters in the absence of protein.

In another series of experiments, binding was determined after chronic unilateral surgical denervation (5–7 days) of the rat vas deferens as described by Kasuya, Goto & others (1969), the contralateral tissue served as control. After the appropriate time the tissues were mounted in isolated organ baths equipped to monitor isotonic drug-induced contractions (Patil, Burkman & others, 1972). Only those tissues not responding to 0·3 mM tyramine (an indirectly-acting sympathomimetic amine) but still contracting maximally to 0·1 mM phenylephrine (a directly-acting sympathomimetic amine) were used.

Observations were repeated at least 3 times. Differences between two means were tested for statistical significance ($P < 0.05$) by Students' t-test for paired or unpaired observations. All straight lines were drawn by the method of least squares (Sokal & Rohlf, 1969).

Drugs and buffers were prepared fresh daily in triple distilled demineralized water. [^{3}H]Dihydroazaptine was prepared by the Amersham-Searle Corp. according to our specifications (Ruffolo & others, 1977a). The following drugs and chemicals were obtained: (−)-alprenolol tartrate (AB Hassle), atropine sulphate (Mallinckrodt), azapetine phosphate (Hoffmann-LaRoche), carbamylcholine chloride (Aldrich Chem. Co.), (+)-chlorpheniramine maleate (Schering Corp,), dithiothreitol (Calbiochem), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Aldrich Chem. Co.), *N*-ethylmaleimide (Pierce Chemical Co.), phenoxybenzamine hydrochloride (Smith Kline & French), phentolamine hydrochloride (Ciba-Geigy), oxymetazoline hydrochloride (Schering Corp.), (−)-soterenol hydrochloride (Mead Johnson & Co.), trypsin (Calbiochem.) and phospholipase A and C (Sigma).

RESULTS

Specific binding of [^{3}H]dihydroazapetine is proportional to the quantity of membrane protein added, up to at least 90 μg. Binding rapidly reaches equilibrium (<10 min) and is completely reversible as evidenced by rapid dissociation upon dilution of the incubation mixture or the addition of phentolamine (10 μm). Binding is inhibited 80% by the α-adrenoceptor agonist, oxymetazoline (10^{-7} M), and the α-antagonists, azapetine and phenoxybenzamine (98, 75%; 10^{-6} M), but not by relatively high concentrations of those agents known to interact with other receptors (alprenolol, atropine, chlorpheniramine, soterenol 10^{-6} M, carbamylcholine 10^{-5} M; n = 6–9).

Total binding (i.e., absence of phentolamine) of [^{3}H]dihydroazapetine increases with ligand concentration showing evidence of saturation (Ruffolo & others, 1976). Scatchard analysis (Scatchard, 1949) of the binding data (Fig. 1) indicate that more than one binding site is present. The Scatchard plot approaches, asymptotically, a limiting bound/free ratio (lim B/F in Fig. 1a). This suggests the presence of a non-specific, nonsaturable binding site (Chamness & McGuire, 1975) which represents most of the total binding at higher concentrations of the ligand. Correction of the curve for non-specific binding according to the technique of Chamness & McGuire (1975) results in another curve (Fig. 1b) which may be resolved into two linear components by the method of Rosenthal (1967). These components possibly represent two distinct binding sites with dissociation constants of 5×10^{-9} and 4×10^{-7} M, for the higher and lower affinity sites, respectively, and binding capacities of 5 and 40 pmol mg^{-1}, respectively. These values obtained by the present method are in good agreement with those reported previously (Ruffolo & others, 1976).

Binding of [^{3}H]dihydroazapetine is inhibited by Ca^{2+}, Mg^{2+}, Na^+ and K^+ in a dose-dependent manner (Fig. 2). Both divalent cations are equipotent in this effect (IC50 $\sim$ 10 μM) as are the univalent ions (IC50 $\sim$ 1 mM). The binding of [^{3}H]dihydroazapetine is low, though still detectable, at physiological concentrations of the ions.

Specific binding of [^{3}H]dihydroazapetine varies with pH over approximately 3 pH units (Fig. 3). There is a minimum in the binding at pH 7·35 whereas binding increases rapidly above this value, suggesting that perhaps the unionized form of dihydroazapetine has higher affinity for the site. The slight

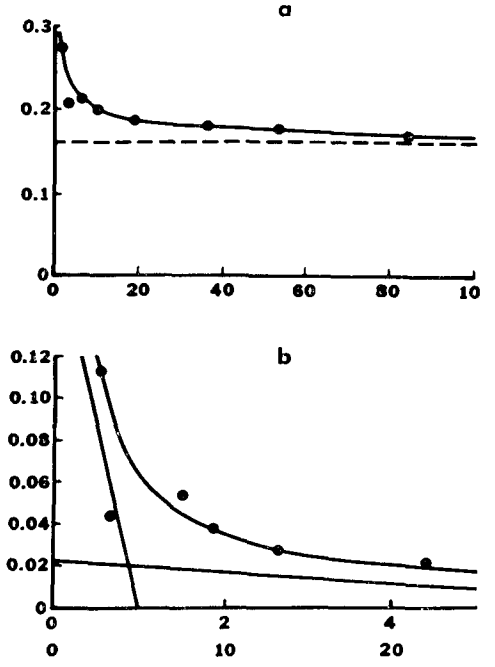

FIG. 1. a. Scatchard plot for total binding of [^{3}H]dihydroazapetine (^{3}H-DHA) to membrane fragments from the rat vas deferens. The curve asympotically approaches a limiting bound/free ratio (lim B/F = 0·16) indicating the presence of a nonsaturable, non-specific binding site. b. Scatchard plot for ^{3}H-DHA binding after correction for the non-specific component (see Results). The curve has been resolved into two linear components which represent higher and lower affinity sites whose dissociation constants are K_1 and K_2, respectively, with binding capacities of B_{max1} and B_{max2}, respectively. $K_1 = 5 \times 10^{-9}$ M; $B_{max1} = 5$ pmol mg^{-1}. $K_2 = 4 \times 10^{-7}$ M; $B_{max2} = 40$ pmol mg^{-1}. Ordinate: [^{3}H-DHA] bound/[^{3}H-DHA] free. Abscissa: [^{3}H-DHA] bound.

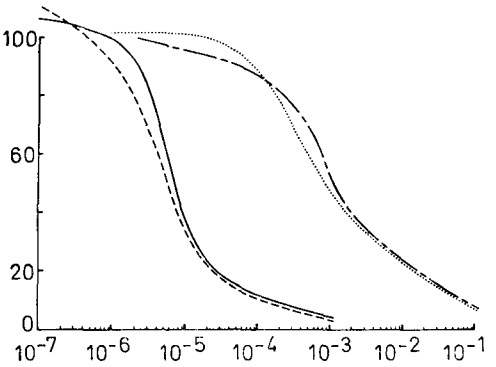

FIG. 2. Effects of univalent and divalent ions on the specific binding of ^{3}H-DHA to membrane fragments from the rat vas deferens. Each line was determined from a minimum of 15 experimental points which have been eliminated for the sake of clarity. - - - Mg^{2+}; —— Ca^{2+}; . . . . Na^+; — - — K^+. Ordinate: Total ^{3}H-DHA bound (% of control). Abscissa: [ion] (M).

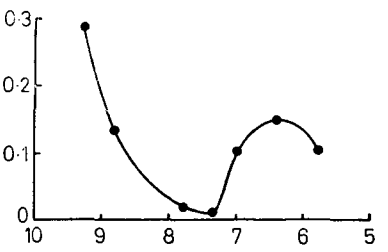

FIG. 3. Effects of pH on specific ^{3}H-DHA binding to membrane fragments from the rat vas deferens. Each point is the mean of 4 to 5 observations. Standard errors were less than 4 percent of the mean and have been omitted for simplicity. Tris-HCl buffer (50 mM) was used and the pH was varied by changing the relative proportions of tris and hydrochloric acid. Ordinate: Specific ^{3}H-DHA bound (pmol mg^{-1}). Abscissa: pH.

increase in binding near pH 6 might possibly reflect a change in ionization at or near the binding site since dihydroazapetine is nearly completely ionized in this range (pKa approximately 9).

Specific binding of the labelled ligand is extremely sensitive to changes in incubation temperature. There is a progressive decrease in [^{3}H]dihydroazapetine binding as the temperature is raised from 25 to 60° (at 25° the extent was 35, at 40° 14 and at 60° 5×10^3 d min^{-1}; n = 8, s.e. <5%). This effect is probably not related to denaturation since it occurs throughout the physiological temperature range.

The effects of various group selective reagents is presented in Table 1. As may be seen, dithiothreitol and *N*-ethylmaleimide, agents that reduce disulphide bridges and irreversibly alkylate free sulphydryl groups, respectively, both significantly inhibited[^{3}H]-dihydroazapetine binding. *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, which irreversibly alkylates free carboxyl groups, also significantly reduced binding.

Urea (4 M), guanidine HCl (0·9 M), glycerol (20% v/v) and trichloroacetic acid (5% w/v), all of which denature proteins, significantly inhibited ($P < 0.05$) specific binding of [^{3}H]dihydroazapetine (Table 1). This effect was dose-dependent (data not shown). Similarly, phospholipase A (0·5 mg ml^{-1}) and C (0·5 mg ml^{-1}) and proteolytic enzyme, trypsin (0·015 mg ml^{-1}), completely inhibited specific binding of [^{3}H]-dihydroazapetine (Table 1).

To demonstrate that specific binding of [^{3}H]-dihydroazapetine represents binding to postjunctional elements, and not to structures located prejunctionally, such as the 'amine-pump' or presynaptic α-adrenoceptors, specific binding was measured in both normal and chronically denervated vasa deferentia. Surgical denervation did not decrease

Table 1. *Effect of various group selective reagents, proteolytic enzymes and protein denaturants on binding of* [^3H]*dihydroazapetine*[a].

Reagents	Concn	Specific [^{3}H]dihydroazapetine bound (% of control)
Control		100 ± 2·2
Group selective reagents		
Dithiothreitol	10^{-3} M	74 ± 2·3[b]
N-Ethylmaleimide	10^{-3} M	56 ± 2·6[b]
N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)	10^{-3} M	56 ± 1·2[b]
Denaturants		
Urea	4M	0[c]
Guanidine HCl	0·9 M	0[c]
Glycerol	20% v/v	40 ± 2·7[b]
Trichloroacetic acid	5% w/v	0[c]
Enzymes		
Trypsin	15 μg ml^{-1}	10 ● 0·5[b]
Phospholipase A	50 μg ml^{-1}	0[c]
Phospholipase C	0·5 mg ml^{-1}	0[c]

[a] Each mean ± s.e.m. was obtained from 4–6 observations.
[b] Significant difference from control ($P < 0.05$).
[c] Complete inhibition of specific as well as partial or complete inhibition of non-specific binding resulted.

specific binding. These results suggest that the major component of [^{3}H]dihydroazapetine binding is to postjunctional structures.

Local anaesthetics produced a concentration-dependent inhibition of specific [^{3}H]dihydroazapetine binding and are approximately equipotent in this effect (IC50 s: 10–30 μM) (Fig. 4). The effect of cocaine is attributed to its local anaesthetic action since dihydroazapetine appears not to interact with the uptake mechanisms located prejunctionally.

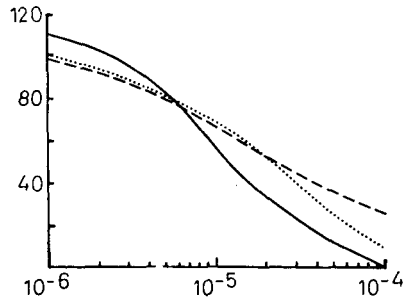

FIG. 4. Effects of three local anaesthetics on specific binding of ^{3}H-DHA to membrane fragments from the rat vas deferens. Each line was determined from at least 12 experimental points which have been eliminated for the sake of clarity. No significant difference ($P > 0.05$) exists between any two lines. —— Tetracaine; – – – lidocaine; . . . . cocaine. Ordinate: Specific ^{3}H-DHA bound (% of control). Abscissa: [Local anaesthetic] (M).

DISCUSSION

Consistent with our previous observations (Ruffolo & others, 1976), [^{3}H]dihydroazapetine binds to protein from the rat vas deferens with many characteristics expected for the α-adrenoceptor. Binding is rapid, reversible and saturable, with one of the binding sites having an affinity similar to that for the receptor in an isolated tissue preparation (Ruffolo & others, 1977a). The binding site(s) shows preference for drugs that act specifically on the α-adrenoceptor.

Observations with group selective reagents indicate that an intact disulphide bridge and free sulphydryl and carboxyl groups, are present at or near the binding site and are required for maximal binding. Salman, Chai & others (1976) have shown that *N*-ethylmaleimide markedly affects the α-adrenergic response of the rabbit aorta suggesting the existence of a free sulphydryl group, and they proposed a critical disulphide bridge at or near the α-adrenoceptor. Lippert & Belleau (1973) made similar conclusions.

It is believed that the active site of the α-adrenoceptor possesses a negative cnarge which is available for interaction with the protonated nitrogen of phenethylamine agonists (Swamy & Triggle, 1972). The source of the negative charge has not been established, but free phosphate and/or carboxyl groups have been postulated. A free carboxyl function is indicated for the binding of [^{3}H]dihydroazapetine by our present findings and is thus possibly associated with the receptor.

The actions of the protein denaturants and trypsin indicate that the [^{3}H]dihydroazapetine binding site(s) is, at least in part, protein. Furthermore, the effects of phospholipase A and C on the binding suggest involvement of phospholipids which is consistent with the model of the α-adrenoceptor proposed by DeRobertis (1975) and with the findings of Dikstein & Sulman (1965).

More than one site of interaction has been proposed for agonists and antagonists on the α-adrenoceptor (Ariëns, 1966, Kalsner, 1970, 1973; Swamy & Triggle, 1972; Ruffolo & others, 1977a; Ruffolo, Turowski & Patil, 1977b). Swamy & Triggle (1972) also proposed that the phenethylamine recognition site of the α-adrenoceptor is allosterically coupled with a 'calcium-mobilization' site which may represent the 'pore' through which the Ca^{2+} traverses

during a phenethylamine-induced response. Since the α-adrenoceptor antagonists tested, and those agonists of the imidazoline class, inhibit specific binding of [^{3}H]dihydroazapetine at concentrations that correlate well with their known affinities for the receptor (Ruffolo & others, 1976), the labelled ligand could be interacting with some part of the receptor. The interaction between phenethylamines and [^{3}H]dihydroazapetine appears to be allosteric (Ruffolo & others, 1976) and it may be that the ligand is interacting at the 'calcium-mobilization' site of Swamy & Triggle (1972). In support of this, local anaesthetics, which are known to inhibit calcium fluxes, inhibit binding of [^{3}H]dihydroazapetine. This finding is also consistent with that of Fleisch & Titus (1973) who observed that local anaesthetics, at similar concentrations to those used herein inhibited α-adrenergic responses in the rabbit aorta, presumably by interfering with the mobilization of calcium.

Kalsner (1970, 1973) has proposed that the interaction between phenethylamines and the non-competitive antagonist, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), is also allosteric. There is similarity between this interaction and that observed between phenethylamines and dihydroazapetine. Since EEDQ inhibits binding of [^{3}H]dihydroazapetine, it may be that both of these compounds are interacting at the same site on the α-adrenoceptor and this could be the 'calcium-mobilization' site where calcium has been proposed to bind to a negatively charged phosphate moiety (Swamy & Triggle, 1972). Since the source of the negative charge has not been conclusively established, it may well be that it is supplied by a free carboxyl moiety with which EEDQ would readily interact (Hoare & Koshland, 1967; Belleau, Martel & others, 1968). The potent inhibitory effects of calcium on [^{3}H]-dihydroazapetine binding observed by us might then be explained by competition of these two species for an identical negative charge supplied by a free carboxyl group. This would be consistent with the model of the α-adrenoceptor proposed by Swamy & Triggle (1972).

Swamy and Triggle (1972) and Janis & Triggle (1971) have also proposed that the longlasting blockade of the α-adrenergic response by dibenamine is due to interaction of the antagonist at the 'calcium-mobilization' site. The structural similarity between dibenamine and dihydroazapetine also supports the contention that [^{3}H]dihydroazapetine may be interacting, at least to some extent, with this site.

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