Biochemical Pharmacology, Vol. 45, No. 8, pp. 1667–1675, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

EXPRESSION OF NON-ADRENERGIC IMIDAZOLINE SITES IN CHROMAFFIN CELLS AND MITOCHONDRIAL MEMBRANES OF BOVINE ADRENAL MEDULLA

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(*Received* 7 *August* 1992; *accepted* 21 *December* 1992)

Abstract—We sought to characterize the non-adrenergic binding site for imidazolines, the imidazoline receptor in whole membranes and subcellular compartments of chromaffin cells of bovine adrenal medulla. [^{3}H]Idazoxan exhibited saturable and high affinity (K_D = 5 nM) binding to chromaffin cell membranes fully displaceable by idazoxan and cirazoline but not by epinephrine or rauwolscine. Binding sites were highly enriched in mitochondrial but not plasma membranes and absent from nuclear fractions. The rank order of potency for displacement of [^{3}H]idazoxan from mitochondrial membranes was: cirazoline > idazoxan > naphazoline > amiloride > detomedine > clonidine ≫ phentolamine > cimetidine = imidazole 4-acetic acid > *p*-iodoclonidine = epinephrine = norepinephrine = rauwolscine. Binding was also inhibited with high affinity by the purported endogenous ligand clonidine-displacing substance and by K^+ and the K^+-channel antagonists 4-aminopyridine and tetraethylammonium bromide but not Na^+. We conclude that: (a) adrenal chromaffin cells express imidazoline receptors but not α_2-adrenergic receptors; (b) these sites are predominantly localized to adrenal medullary mitochondria; and (c) imidazoline receptors conform to an idazoxan preferring (I-2) rather than the clonidine preferring (I-1) subclass and are amiloride sensitive. The data support the view that α_2-adrenergic and imidazoline receptors are distinct receptor species and that adrenal chromaffin cells would be a useful cultured cell system, expressing only imidazoline receptors, for further molecular and functional studies of the receptors.

Imidazoline agents such as clonidine and idazoxan bind not only to α_2-adrenergic receptors but also to newly recognized non-adrenergic binding sites in brain and other organs [1–5]. This non-adrenergic binding site has been proposed to represent a novel receptor which mediates the central anti-hypertensive [6], neuroprotective [7] and some renal tubular actions of clonidine and allied agents [8]. It has been variously named an imidazole receptor [1], imidazoline receptor [3], imidazoline-guanidinium receptive site (IGRS) [9], and imidazoline preferring receptor [10]. We refer to it as the imidazoline receptor (I-receptor†). Evidence that the I-receptor and α_2-adrenergic receptors are distinct, however, has been inferential since the I-receptor has yet to be purified, sequenced, and cloned.

Although pharmacological characterization of non-adrenergic binding sites for imidazoline agents has been reported in many tissues, the crucial evidence linking the binding site to a cellular response is clearly missing. We have discovered recently that exposure of chromaffin cells of the bovine adrenal medulla to a range of agents interacting with I-receptors including clonidine, oxymetazoline, and naphazoline stimulate the influx of $^{45}Ca^{2+}$ [11] and/or expression of the epinephrine synthesizing enzyme, phenylethanolamine *N*-methyl transferase [12]. Moreover, the absence of any cellular responses in second messenger systems associated with activation of α_2-adrenergic receptors raises the possibility that chromaffin cells might selectively express I-receptors in the absence of α_2-adrenergic receptors. If so, this finding would not only provide further evidence for the independence of the two receptors but also identify a tissue which could serve as a unique cellular source for analyzing the cellular mechanisms associated with I-receptor occupancy and for the isolation and cloning of I-receptor.

In the present study, therefore, we have characterized the binding of various ligands that interact with I-receptors and/or α_2-adrenergic receptors in membranes of primary cultures of bovine adrenomedullary chromaffin cells. We sought to determine their subcellular distribution, to identify the class and subclass of receptors to which the ligands bind, and to analyze the effects of Na^+ and K^+ on binding. We report that bovine adrenal chromaffin cells expressed I-receptors but not α_2-adrenergic receptors, that they preferentially localized to mitochondrial membranes, that they corresponded to a subtype of I-receptor preferring the imidazoline idazoxan, and that binding was inhibited by K^+ but not by Na^+.

MATERIALS AND METHODS

Isolation and culturing of adrenal chromaffin,

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† Abbreviations: I-receptor, imidazoline receptor; and CDS, clonidine-displacing substance.

neuroblastoma and PC12 cells. Adrenal chromaffin cells were isolated from bovine adrenal glands as described [11]. Briefly, after receiving adrenal glands from a local slaughterhouse within 4 hr post-mortem, they were perfused with Hepes–phosphate buffer (pH 7.4) containing collagenase (2 mg/mL) and DNAse (50 μg/mL) and incubated at 37° for 1 hr. The medulla was dissected from cortex, minced, and incubated in collagenase/DNAse containing buffer at 37° for 40–60 min to digest completely connective tissue. The resulting suspension was filtered through a 105 μm wire sieve and centrifuged at 1000 *g* for 10 min. The pelleted cells were washed twice in buffer, and the final suspension was loaded onto a step gradient of 15% and 7.5% Renografin and centrifuged for 20 min at 10,000 *g*. Chromaffin cells were collected from the interface gradient, washed twice in buffer, and frozen at −80° before preparing membranes for binding assay.

Neuroblastoma cell lines, SKN-MC and SKN-BE2, and PC12 cells were cultured using the standard protocol described earlier [13]. When the cells were confluent, they were harvested and washed in phosphate-buffered saline and frozen at −80°. Membranes were prepared from these cells as described below.

Subcellular fractionation of adrenal medulla. Subcellular fractions were prepared from whole adrenal medulla by differential centrifugation [14]. Adrenal medulla was separated from cortex after perfusion and incubation with collagenase containing buffer as described above. Combined medullae were then homogenized in Hepes–sucrose buffer (pH 7.4) using Teflon–glass homogenizer and centrifuged at 600 *g* for 10 min at 4°. The supernatant was centrifuged at 6500 *g* for 15 min, and the resulting pellet (crude mitochondrial fraction) was used for membrane preparation and enzyme assays. The supernatant from this step was centrifuged at 30,000 *g* for 20 min to give the pellet enriched in plasma membrane. To obtain a crude membrane fraction, the supernatant from the first centrifugation step (600 *g*) was centrifuged at 30,000 *g* for 20 min and the pellet (crude fraction) used for membrane preparation and enzyme assays. Typically, about 60 mg protein of crude mitochondrial fraction gave about 28 mg protein in the plasma membrane fraction.

Ligand binding studies. Ligand binding assays were performed with washed membranes prepared from various fractions as described [15]. Specifically, intact cells (chromaffin cells, SKN-MC, SKN-BE2 or PC12 cells) or mitochondrial, membrane plasma membrane or crude membrane fractions from adrenal medulla were resuspended in ice-cold 50 mM Tris–HCl buffer (pH 7.4) with 5 mM EDTA and homogenized using a Polytron (setting 6) for 10 sec. The homogenate was centrifuged at 40,000 *g* for 30 min. The resulting pellet was washed three times in buffer by resuspension and recentrifugation. The final washed membrane pellet was resuspended in Tris–HCl buffer (pH 7.4) to give the required protein concentration in a final incubation volume of 1 mL. For saturation binding assays, membranes (50–300 μg protein) were incubated in 1 mL of Tris–HCl buffer (pH 7.4) with 0.1 to 20 nM [^{3}H]idazoxan at 25° for 45 min. For drug displacement assays, [^{3}H]-idazoxan (concentration approximately equal to K_D) was incubated in the presence of various concentrations of drugs. Non-specific binding was defined by either 10 μM unlabelled idazoxan or cirazoline. The binding of [^{3}H]*p*-aminoclonidine (10 nM) was measured under similar assay conditions using 10 μM phentolamine to define non-specific binding. The binding of [^{3}H]rauwolscine (0.1 to 30 nM) was measured using 10 μM rauwolscine to define non-specific binding. Incubations were terminated by rapid vacuum filtration over Whatman GF/B filters, pretreated with 0.1% polyethylamine, using a modified cell harvester (Brandel). The filters were washed with 10 mL of ice-cold buffer and then were suspended in 5 mL of scintillation fluid. Radioactivity was determined by liquid scintillation counting. The binding data were analyzed in detail using LIGAND (Elsevier Biosoft), a parametric nonlinear regression analysis. The protein content was measured by the method of Bradford [16] using bovine serum albumin as standard.

Marker enzyme assays. The activities of marker enzymes such as cytochrome oxidase (for mitochondria) and 5′-nucleotidase (plasma membrane) were measured in crude, mitochondrial and plasma membrane fractions by well-established methods. Cytochrome oxidase activity was measured by the method of Yonetani and Ray [17], based on the principle that initial oxidation of ferrocytochrome *c* by cytochrome oxidase can be evaluated by measuring the decrease in the absorption at 550 nm. 5′-Nucleotidase activity was measured using a commercially available kit (Sigma), measuring the hydrolysis of adenosine 5′-monophosphate to yield adenosine and inorganic phosphorus. The inorganic phosphorus formed was measured by the reduction of acid molybdate in the presence of amino naphthol sulfonic acid. The absorbance of the blue-colored phosphomolybdate was read at 700 nm.

Isolation of clonidine-displacing substance (CDS). CDS was prepared by methods developed in this laboratory [18], with subsequent refinement of procedures to obtain CDS from brain synaptosomes [19]. Briefly, a crude P_2 pellet was prepared from bovine brain and was osmotically shocked with 7.5 vol. of distilled water using a Polytron (setting 4, 5 sec). The suspension was centrifuged at 37,000 *g* for 15 min. The supernatant was then heat-denatured and centrifuged at 100,000 *g* for 30 min. The resulting supernatant was lyophilized and dialyzed (1000 molecular weight cut-off). The diffusate was freeze-dried and then extracted with methanol. Samples of redried methanol extract containing CDS were stored at −70° and reconstituted in buffer just before use. One unit of CDS is defined as the amount of extract that produces 50% inhibition of [^{3}H]*p*-aminoclonidine (1 nM) binding to membranes from bovine brain frontal cortex in a total volume of 1 mL.

Materials. Neuroblastoma cell lines were obtained from Dr. J. Biedler, Memorial Sloan-Kettering Cancer Center, New York. [^{3}H]Idazoxan (43 Ci/mmol) and [^{3}H]rauwolscine (81 Ci/mmol) were purchased from the Amersham Corp. and [^{3}H]PAC (50 Ci/mmol) was from New England Nuclear.

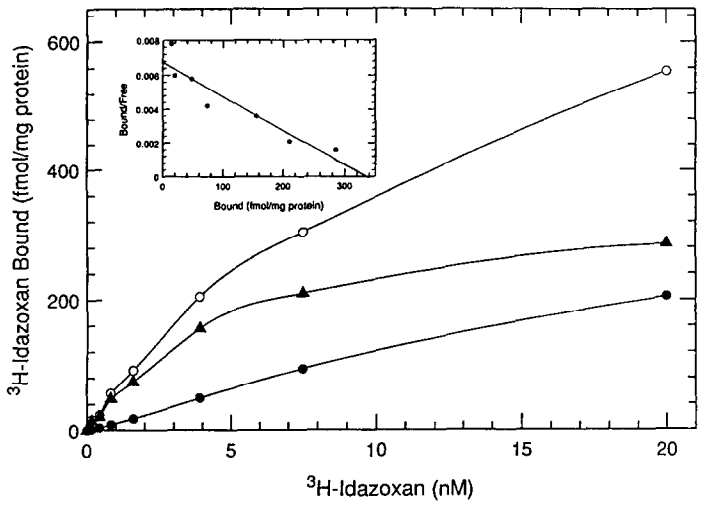

Fig. 1. Specific binding (▲—▲) to chromaffin cell membranes calculated from saturation binding of [^{3}H]idazoxan in the presence (●—●) and absence (○—○) of 10 μM idazoxan. The specific binding was further analyzed by a Scatchard plot (inset). Shown are representative data from three experiments done in triplicate.

Idazoxan, clonidine and rauwolscine were purchased from Research Biochemical Inc. (Boston, MA). Cirazoline and SKF-86466 (6-chloro-*N*-methyl-2,3,4,5-tetrahydrol-1*H*-3-benzapine) were gifts from Synthelabo (Paris) and Smith Kline & French (King of Prussia, PA), respectively. All other chemicals were from the Sigma Chemical Co.

RESULTS

In a pilot experiment, we compared the binding of [^{3}H]idazoxan and [^{3}H]*p*-aminoclonidine to chromaffin cell membranes. [^{3}H]Idazoxan (10 nM) exhibited substantial binding which was potently displaced (about 75%) by 10 μM unlabeled idazoxan. On the other hand [^{3}H]*p*-aminoclonidine (10 nM) showed little binding which was not displaced (less than 20%) even by a 100 μM concentration of unlabeled clonidine. Therefore, subsequent studies were carrried out using [^{3}H]idazoxan as radioligand.

Saturation and displacement of binding to membranes of adrenal medullary chromaffin cells and allied cell lines. Saturation binding was performed by incubating membranes with various concentrations of [^{3}H]idazoxan (0.1 to 20 nM). Non-specific binding was defined by 10 μM unlabeled idazoxan.

The binding of [^{3}H]idazoxan to chromaffin cell membranes was specific, saturable and of high affinity (Fig. 1). The binding constants obtained from Scatchard analysis (inset Fig. 1) (K_D 5 ± 1.1 nM; B_{max} 380 ± 45 fmol/mg protein) were comparable to non-adrenergic binding sites for [^{3}H]idazoxan in brain (K_D 3.7 nM; Ref. 20), kidney (K_D 3.0 nM; Ref. 21) and platelets (K_D 10.8 nM; Ref. 5). Moreover, when a two-site fit was tried against a single-site model, no significant difference in the goodness of fit (no significant decrease in sum of squares) was observed, suggesting that [^{3}H]idazoxan binds to a single site.

Cirazoline and idazoxan, agents interacting at I-receptors, potently and completely inhibited [^{3}H]-idazoxan binding with K_i values of 1 and 5 nM, respectively (Fig. 2A). On the other hand, epinephrine and the non-imidazoline α_2-adrenergic antagonist rauwolscine displace less than 20% of total specific binding even at 10 μM (Fig. 2A). These results demonstrate that the [^{3}H]idazoxan binding to adrenal chromaffin cells is primarily restricted to non-adrenergic I-receptors.

We also examined the binding of [^{3}H]idazoxan to membranes prepared from rat pheochromocytoma (PC12) and human neuroblastoma (SKN-MC and SNK-BE2) cell lines in order to identify a continuous cell line expressing I-receptors. The specific binding of [^{3}H]idazoxan (5 nM) was lower in PC12 (1700 dpm total vs 1000 dpm blank) and SKN-BE2 (1500 dpm total vs 980 dpm blank) cell membranes (100 μg protein) compared to chromaffin cells, while no specific binding was observed in SKN-MC cell membranes. However, as shown in Fig. 2B, the binding of [^{3}H]idazoxan to SKN-BE2 and PC12 cell membranes was displaced completely by both epinephrine and rauwolscine. This finding indicates that SKN-BE2 and PC12 cells do not express I-receptors but may express α_2-adrenergic receptors having lower affinity for rauwolscine (K_i = 100 nM) and that cells of the SKN-MC line express neither.

Binding of [^{3}H]rauwolscine to chromaffin cell membranes. To confirm further whether α_2-adrenergic sites are present in chromaffin cells, we investigated the binding of [^{3}H]rauwolscine, a highly selective α_2-adrenergic ligand, to membranes of chromaffin cells. As shown in Fig. 3, there was no significant difference between total and non-specific binding of [^{3}H]rauwolscine in the concentration ranging from 0.1 to 30 nM. These findings, along with the earlier observation with [^{3}H]idazoxan and [^{3}H]*p*-aminoclonidine, further support the absence

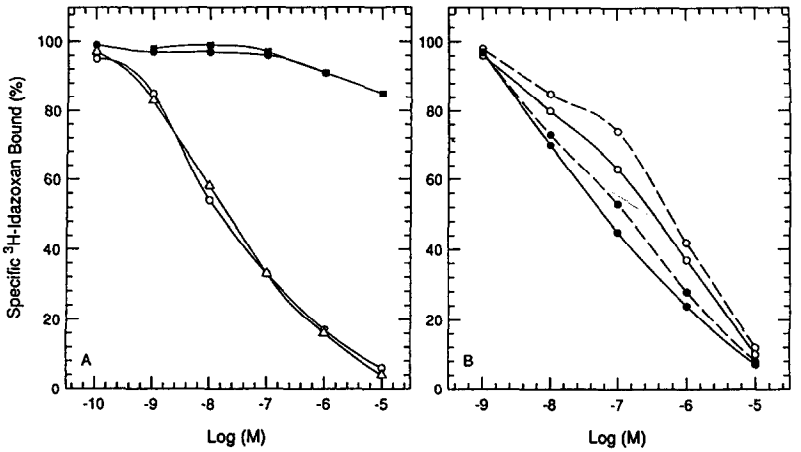

Fig. 2. (A) Displacement of binding of [^{3}H]idazoxan to chromaffin cell membranes by cirazoline (△), idazoxan (○), epinephrine (■) and rauwolscine (●). Membranes (100–150 μg protein) were incubated in Tris–HCl buffer at 25° for 30 min with 5 nM [^{3}H]idazoxan. The total radioactivity ranged between 2500 and 3000 dpm, and the non-specific binding, defined using 10 μM idazoxan, was about 30% of total binding. (B) Displacement of [^{3}H]idazoxan binding to PC12 (solid lines) and SKN-BE2 (broken lines) cell membranes by epinephrine (○) and rauwolscine (●). Binding assay conditions are described in Materials and Methods.

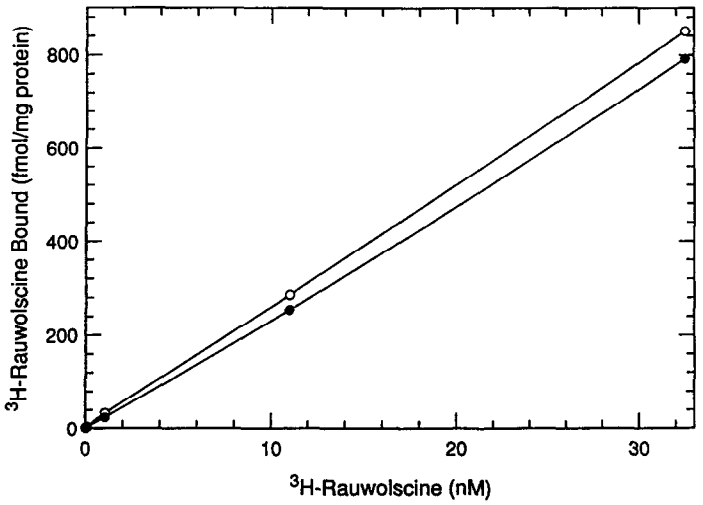

Fig. 3. Saturation binding of [^{3}H]rauwolscine to chromaffin cell membranes in the presence (●—●) and absence (○—○) of 10 μM rauwolscine. Values are from three experiments each done in triplicate.

of any α_2-adrenergic receptors in adrenal chromaffin cells.

Saturation binding of [^{3}H]*idazoxan to subcellular fractions of adrenal medulla.* Since I-receptors have been demonstrated to be enriched in mitochondria of liver [22] and on intracellular sites in platelets [23], we investigated the distribution of I-receptors in subcellular fractions of bovine adrenal medullae (since technical limitations did not allow for fractionation of chromaffin cells).

In a pilot experiment we measured binding of [^{3}H]idazoxan to subcellular fractions of nuclear, mitochondrial and plasma membranes of adrenal medullae. Binding to the nuclear fraction was negligible and hence only mitochondrial and plasma membrane fractions were analyzed in detail.

Crude, mitochondrial and plasma membrane fractions were prepared. The relative enrichment of the fractions was tracked by measurement of the activities of the cytochrome oxidase and 5′-nucleotidase, marker enzymes for mitochondrial and plasma membrane fractions, respectively. Comparisons were made with the entire (crude) membrane preparation.

The binding of [^{3}H]idazoxan was specific, saturable and of high affinity in all three fractions. Scatchard

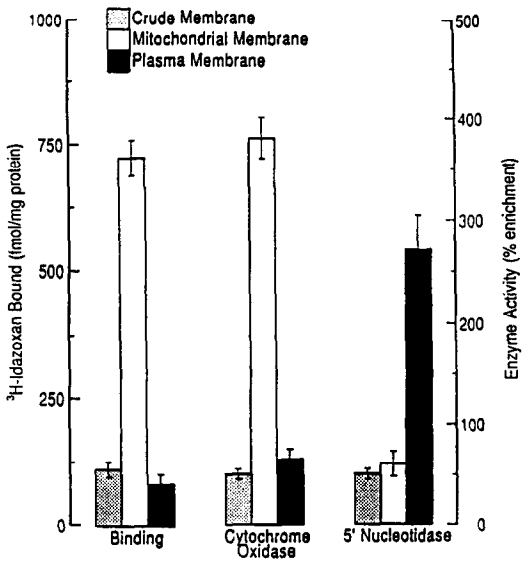

Fig. 4. Binding of [^{3}H]idazoxan and activities of marker enzymes in subcellular fractions of adrenal medulla. The number of binding sites (B_{max}) was calculated from Scatchard analysis of specific binding to each fraction. Enzyme activities in crude membrane fractions were: cytochrome oxidase, 0.601 ± 0.051 U/mg protein and 5′-nucleotidase, 2.8 ± 0.24 U/mg protein. The enrichment of enzyme activities in mitochondrial and plasma membrane fractions were calculated as a percent over the crude membrane fraction. The binding data are means ± SEM of three experiments done in triplicate.

analysis indicated that the binding affinities in membranes in the crude (K_D 5 ± 1.1 nM), mitochondrial (K_D 3 ± 0.9 nM) and plasma membrane (K_D 7 ± 1.5 nM) fractions did not differ significantly. However, the number of binding sites (B_{max}) were markedly different with mitochondrial membranes expressing the most (710 ± 55 fmol/mg protein) and plasma membranes the fewest (95 ± 15 fmol/mg protein) binding sites (Fig. 4). Moreover, the number of binding sites in crude adrenal medullary membranes (125 ± 18 fmol/mg protein) was less than half compared to chromaffin cell membranes (380 fmol/mg protein), indicating that these are the major cells expressing I-receptor in adrenal medulla. [^{3}H]Idazoxan binding sites were enriched 4-fold in mitochondria compared with other fractions (Fig. 4). Thus, the preponderant localization of I-receptors in adrenal medullary chromaffin cells is concentrated in mitochondria.

Displacement of [^{3}H]*idazoxan binding to mitochondrial membranes.* Displacement studies were performed on mitochondrial membranes prepared from adrenal medulla. As with membranes prepared from the entire chromaffin cell, the imidazolines idazoxan and cirazoline completely displaced [^{3}H]-idazoxan (K_i of 1 and 3 nM, respectively) whereas rauwolscine and epinephrine did not (Fig. 5A). Amiloride, a guanidium, also potently displaced [^{3}H]idazoxan with a K_i of 45 nM (Fig. 5A and Table 1). The inhibition constants (K_i) for these and various other adrenergic and non-adrenergic agents tested for the displacement of [^{3}H]idazoxan binding to chromaffin cell membranes or mitochondrial membranes of adrenal medulla are shown in Table 1. All agents were initially tested in chromaffin cell membranes and only agents that showed potent displacement ($K_i < 1\ \mu M$) were further tested in mitochondrial membranes.

The rank order of potency for displacement was: cirazoline > idazoxan > naphazoline > amiloride > detomedine > clonidine ⩾ phentolamine > cimetidine = imidazole 4-acetic acid > *p*-iodoclonidine = epinephrine = norepinephrine = rauwolscine. Thus,

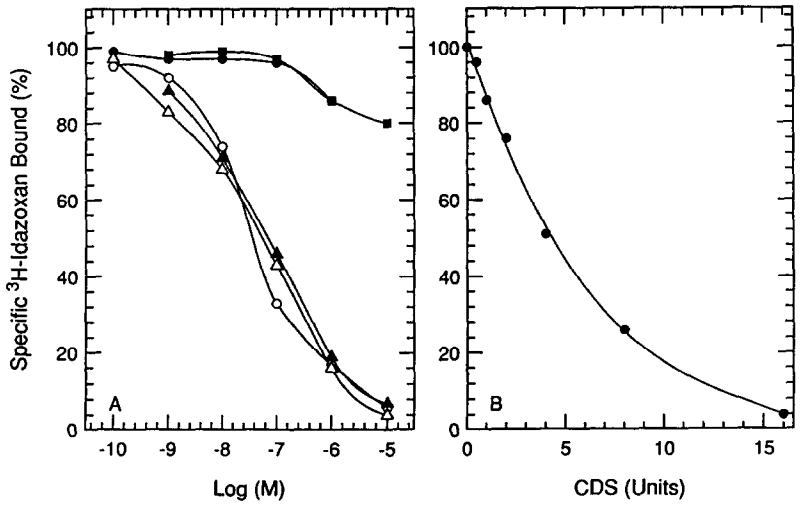

Fig. 5. Displacement of [^{3}H]idazoxan binding to adrenal medullary mitochondrial membranes (A) by cirazoline (△), idazoxan (○), amiloride (▲), epinephrine (■) and rauwolscine (●), and (B) by CDS. The data are from one representative experiment done three times in triplicate. The inhibition constants (K_i, Table 1) were calculated from all experiments.

Table 1. Displacement of [^{3}H]idazoxan binding by various imidazoline and non-imidazoline agents

High affinity (mitochondrial membrane) ($K_i < 1$ μM)		Low or no affinity (chromaffin cell membrane) ($K_i > 1$ μM)	
Cirazoline	3 ± 1.5 nM	Phentolamine	5 ± 1.0 μM
Idazoxan	5 ± 2 nM	Cimetidine	50 ± 8.5 μM
Naphazoline	10 ± 2.5 nM	Imidazole 4-acetic acid	50 ± 7.5 μM
Amiloride	45 ± 10 nM	Epinephrine	>10 μM
Detomedine	80 ± 14 nM	Rauwolscine	>10 μM
Clonidine	200 ± 21 nM	*p*-Iodoclonidine	>10 μM
		Norepinephrine	>10 μM

The binding of [^{3}H]idazoxan was measured in the presence of various concentrations of these agents (10^{-9} to 10^{-4} M). All agents were initially tested using chromaffin cell membranes and only agents that were potent in displacing the binding ($K_i < 1$ μM) were further tested in adrenal medullary mitochondrial membranes. The K_i values for agents showing high affinity ($K_i < 1$ μM, left column) are from mitochondrial membrane binding, whereas values for agents showing low or no affinity ($K_i > 1$ μM, right column) are from chromaffin cell membrane binding. Values are means ± SEM from three experiments done in triplicate.

some imidazolines have higher affinity, other imidazolines and imidazoles lower affinity and non-imidazolines no affinity for these binding sites. This potency profile is consistent with the I-receptor of the mitochondria (and, thus, of the whole adrenal chromaffin cell) being of the idazoxan-preferring receptor subclass [24]. Hence, it is similar to I-receptors of rabbit kidney and liver [9, 20]. Moreover, the slopes of the inhibition curves in mitochondrial (Fig. 5A) as well as in chromaffin cell (Fig. 2) membranes were shallow with Hill coefficients between 0.5 and 0.8. Similar findings in guinea pig kidney have been reported recently by Wikberg *et al.* [25], who suggested that I-receptor may exist in two interconvertible forms.

We also investigated whether the purported endogenous ligand CDS, isolated from bovine brain (see Materials and Methods), would compete for binding of the radioligand on adrenal medullary mitochondrial membranes (Fig. 5B). CDS potently inhibited binding with an IC_{50} of 3 units and with 15 units achieving complete displacement. The result is in agreement with observations that CDS will inhibit the binding of [^{3}H]idazoxan to the whole membrane preparation of chromaffin cells [15].

Effect of K^+ *and* Na^+ *on* [^{3}H]*idazoxan binding to mitochondrial membranes.* The binding of [^{3}H]-idazoxan to I-receptors in liver and kidney is inhibited by K^+ [21, 26]. We therefore investigated whether K^+ and/or Na^+ would inhibit binding of the radioligand to mitochondrial membranes of chromaffin cells. As shown in Fig. 6, K^+ but not Na^+ inhibited binding (K_i 70 mM). Binding was also inhibited by the K^+ channel blockers 4-aminopyridine and tetraethylammonium bromide although 4-aminopyridine was more potent (K_i 20 μM), and, interestingly, inhibited binding at concentrations which block K^+ channels.

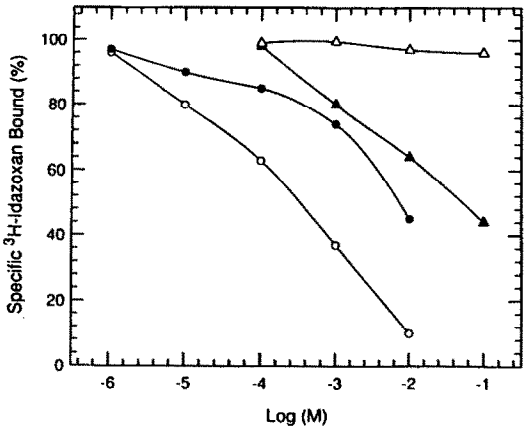

Fig. 6. Inhibition of [^{3}H]idazoxan binding to mitochondrial membranes of adrenal medulla by 4-aminopyridine (○), tetraethylammonium bromide (●), KCl (▲) and NaCl (△). Representative data are shown, and the experiment was done two times in triplicate.

DISCUSSION

In the present study we have demonstrated that [^{3}H]idazoxan binds specifically and with high affinity to a single binding site in membranes of chromaffin cells of the bovine adrenal medulla. The fact that [^{3}H]idazoxan could not be displaced by catecholamines and the failure of binding by [^{3}H]-PAC indicate that the binding site in chromaffin cell membrane is not an adrenergic receptor. The highly selective α_2-adrenergic ligand, [^{3}H]rauwolscine, did not exhibit any specific binding to these cell membranes, further confirming the absence of α_2-adrenergic sites. These findings are in agreement with physiological and pharmacological evidence that imidazoline agents modulate the release of catecholamines from adrenal chromaffin cells by an action on a non-adrenergic receptor [27–30]. It is also consistent with our inability to detect activation of

adrenergically-coupled signal transduction pathways [11] following exposure of adrenal chromaffin cells to clonidine and allied imidazolines. The fact that [^{3}H]idazoxan could be displaced completely in chromaffin cells by some imidazolines indicates that the receptor to which these agents bind is an I-receptor. The results indicate that adrenal chromaffin cells may express I-receptors but not α_2-adrenergic receptors and may settle the long-standing controversy of whether adrenal chromaffin cells express α_2-adrenergic receptors [26, 30].

By examining ligand binding in different subcellular fractions we have discovered that I-receptors of adrenal medulla, like those of liver [22], are markedly enriched in a mitochondrial fraction. Since chromaffin cells constitute over 80% of adrenal medullary tissue, mitochondria from adrenal medulla would be mostly from chromaffin cells. The presence of modest binding to plasma membranes might either indicate incomplete physical separation of the two fractions with cross-contamination or that a minority of binding sites are contained on plasma membranes. Clearly, however, the mitochondrial receptor is the major contributor to the binding profile established in the whole membrane preparation of chromaffin cells and, thus, the one which defines the receptor subclass of the cell in the usual ligand-binding analysis. The only other mitochondrial receptor to which drugs and/or transmitters bind is the peripheral benzodiazepine receptor which is present in many steroid-synthesizing tissues including adrenal cortex [14]. However, I-receptors have not been detected in adrenal cortex and imidazoline agents did not inhibit the binding of [^{3}H]PK 11195, a high affinity ligand for peripheral type benzodiazepine receptors (data not presented), indicating that the two receptors are not the same.

Although I-receptors were originally viewed as a uniform population of binding sites, there is increasing evidence that, like many other receptors, they exist in multiple forms [24]. The evidence, obtained by analysis of the order of potency of membrane binding by various ligands, suggests the presence of two principal subtypes. One is preferentially labeled by [^{3}H]clonidine or [^{3}H]*p*-aminoclonidine [1], the other by [^{3}H]idazoxan [9]. The idazoxan-preferring I-receptor itself may be subclassified depending upon sensitivity to displacement by the guanidinium, amiloride. Thus, an amiloride-sensitive idazoxan-preferring I-receptor has been found in rabbit liver and kidney [9, 19] while amiloride-insensitive idazoxan-preferring I-receptors have been detected in brain (guinea pig, cow), liver (rat), and kidney (human, rat) [5, 20, 26]. The view that all these three sites are actually subtypes and not distinct receptors is supported by the findings that the partially purified purported endogenous ligand CDS exhibits high affinity for all three subtypes of I-receptor [19, 24, 25]. While a uniform nomenclature for the subclasses of I-receptors has not been adopted, it has been proposed to designate the clonidine-preferring and idazoxan-preferring I-receptors, respectively, as I-1 and I-2 subclasses [31]. The amiloride-sensitive I-2 receptor can be designated as an I-2a and the amiloride-insensitive as an I-2b receptor. By these criteria, the I-receptor of adrenal chromaffin cells is of the idazoxan-preferring and amiloride-sensitive I-2a subclass sharing a pharmacological profile with I-receptors of rabbit liver and kidney [9, 22].

The binding of [^{3}H]idazoxan to chromaffin cell membranes was inhibited by K^+ and by the K^+ channel blockers 4-aminopyridine and tetraethylammonium bromide and insensitive to Na^+. The interaction with K^+ is similar to that described for amiloride-insensitive I-2b receptors of rat liver [26] and amiloride-sensitive I-2a receptors of rabbit kidney [21]. The failure of Na^+ to modulate binding in chromaffin cells is consistent with the absence in the tissue of adrenergic receptors, which are regulated by Na^+ but not K^+ [32]. The finding also suggests that both I-2a and I-2b subtypes of I-receptors are regulated by K^+ and may have a common signal transduction mechanism involving K^+ channel modulation. In this context, it should be noted that many imidazoline agents exhibit a modulation of ATP-regulated K^+ channels in pancreatic β-cells that is not related to adrenergic activity [33]. Moreover, imidazolines such as idazoxan, cirazoline and the endogenous CDS have been shown to modulate the influx of $^{22}Na^+$ in rabbit kidney, independent of the stimulation of α_2-adrenergic receptors [8], which could be a secondary action to their blockade of K^+ channels and depolarization of the cell.

The functional importance of the mitochondrial I-2 receptors in adrenal chromaffin cells is not known. It probably does not regulate cellular respiration as studies of the I-2a receptor of the outer mitochondrial membrane of rabbit liver have shown [22]. On the other hand, mitochondria are major intracellular Ca^{2+} storage sites and the movement of Ca^{2+} in and out of mitochondria is coupled to movement of Na^+ and K^+ [34]. We have shown earlier that in chromaffin cells the binding of ligands to I-receptors does not modulate soluble second messenger systems. However, they elicit a smaller and long-lasting influx of $^{45}Ca^{2+}$ into the cell [11]. These findings suggest that imidazolines may modulate intracellular concentrations of cations in adrenal chromaffin cells by interacting with mitochondrial I-receptors possibly on a mitochondrial K^+ transporter and as a consequence initiate a secondary influx of Ca^{2+} into the cytoplasm. Such a mechanism could explain how CDS releases catecholamines from adrenal chromaffin cells [15] since, as demonstrated here, CDS potently binds to mitochondrial I-2a receptors. The localization of I-receptors on mitochondrial membranes also suggests that agents which interact with them, including CDS, should be lipid soluble (hydrophobic) and capable of penetrating the cell membranes. Thus, while *in vitro* binding studies to mitochondrial membranes eliminate cell membrane barriers, it is evident that *in vivo* agents which rapidly penetrate the cell membranes will be the most efficacious in reaching and activating I-receptors.

In conclusion, our findings that [^{3}H]idazoxan bound only to I-receptors of the I-2a subtype in adrenal chromaffin cells, and that CDS from brain bound to these sites further support the existence of a unique ligand/receptor system in mammalian

tissues. The exact role of this receptor, with its unusual localization to the mitochondrial membrane, in cell function is not clear. That it is a functional receptor is strongly suggested by the findings that stimulation of adrenal medulla with agents which interact with I-receptors elicits release of catecholamines and influx of $^{45}Ca^{2+}$ [11]. The fact that chromaffin cells express only I-receptors adds additional support to the view that I-receptors and α_2-adrenergic receptors are distinct. However, the final proof of the structural uniqueness of the I-receptor will only come from the cloning and sequencing of the receptor. This study indicates that cultured chromaffin cells may serve as a source of I-receptor associated molecules, uncontaminated with those associated with the α_2-adrenergic receptor, for further molecular and functional characterization.

Acknowledgement—We thank Dr. M. Gavish, Technion Institute of Technology, Haifa, Israel, for conducting the [^{3}H]PK 11195 binding assays.

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