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Nonadrenergic [^{3}H]Idazoxan Binding Sites Are Physically Distinct from α_2-Adrenergic Receptors

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SUMMARY

We have recently demonstrated that the α_2-adrenergic radioligand [^{3}H]idazoxan also labels additional sites that do not recognize catecholamines but bind with high affinity several chemically distinct drugs previously assumed to be highly selective for α-adrenergic receptors [*Mol. Pharmacol.* 35:324–330 (1989)]. We now have used three approaches to distinguish the nonadrenergic [^{3}H]idazoxan sites from α_2-adrenergic receptors. (a) No nonadrenergic [^{3}H]idazoxan binding sites were found in COS-7 cells transfected with the genes for the two known α_2-adrenergic receptor subtypes. (b) The ratio of α_2-adrenergic and nonadrenergic [^{3}H]idazoxan sites in human platelet membranes varied considerably between various donors. (c) Highly purified platelet plasma membranes were enriched for α_2-adrenergic receptors but did not contain any nonadrenergic [^{3}H]idazoxan binding sites. We conclude that the nonadrenergic [^{3}H]idazoxan binding sites are not co-expressed with α_2-adrenergic receptors and at least in human platelets may be located in an intracellular compartment.

IDA is a high affinity α_2-adrenergic antagonist (1). Because the selectivity over α_1-adrenergic receptors exceeds even that of rauwolscine and yohimbine, [^{3}H]IDA has been used to label α_2-adrenergic receptors in radioligand binding studies. Recently, however, it became clear that [^{3}H]IDA not only labels α_2-adrenergic receptors but also binds with high affinity and pharmacological specificity to nonadrenergic sites (2–4). There may be even more than one additional binding site for [^{3}H]IDA, because the pharmacological specificity of the nonadrenergic [^{3}H]IDA binding site in rabbits differs considerably from that in rats, pigs, and humans (4).

The nonadrenergic [^{3}H]IDA binding site, which we have recently characterized in rat and human renal cortical membranes, is not recognized by epinephrine or phentolamine but has high affinity for a number of drugs previously believed to be quite selective for α_2-adrenergic receptors, e.g., UK 14,304, guanabenz, SK&F 104,078, and tolazoline (3). Although this nonadrenergic site identified with [^{3}H]IDA was clearly not recognized by catecholamines, two pieces of data suggested coexpression with α_2-adrenergic receptors (3). First, saturation studies with [^{3}H]IDA indicated that the nonadrenergic site is present in five tissues that also have α_2-adrenergic receptors (rat kidney, human kidney, human myometrium, human platelets, and the human erythroleukemia cell line) but absent in three cell lines lacking α_2-adrenergic receptors (BC_3H_1 MDCK, and Jurkat cells). Second, the affinities of several drugs (with imidazoline, guanidino, and benzazepine structures) for the nonadrenergic [^{3}H]IDA sites were very similar to those for α_2-adrenergic receptors.

We now have used three approaches to ask whether the nonadrenergic [^{3}H]IDA binding sites are co-expressed with α_2-adrenergic receptors, (a) investigating the presence of nonadrenergic [^{3}H]IDA binding sites in COS-7 cells expressing cloned human α_2-adrenergic receptor subtypes; (b) comparing the ratio of nonadrenergic and α_2-adrenergic [^{3}H]IDA binding sites in platelet membranes obtained from several blood donors; and (c) comparing the subcellular localization of the nonadrenergic [^{3}H]IDA binding sites with that of α_2-adrenergic receptors.

Materials and Methods

The α_2-adrenergic receptor subtypes, α_2-C4 and α_2-C10, were expressed transiently in COS-7 cells, as recently described (5). The pBCα_2-C4 and pBCα_2-C10 plasmid DNA were used at a concentration of 5 μg/ml in the transfection mix and the cells were harvested 3 days after transfection, with medium changes every day. Membranes were prepared as described (5), frozen in liquid nitrogen, and stored at −70°.

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ABBREVIATION: IDA, idazoxan.

Two separately prepared batches of transfected cells were used with similar results.

Crude platelet membranes were prepared from the blood of several donors, as described (6). In some cases blood from more than one donor was pooled in order to yield sufficient amounts of protein. Membranes were frozen in liquid nitrogen and stored at −70°.

Two fractions of platelet membranes were prepared from pooled platelet-rich plasma obtained from blood donors according to published techniques (7), a light plasma membrane fraction, which is enriched 3–5-fold for α_2-adrenergic receptors and forskolin-stimulated adenylate cyclase, and a heavy granule/mitochondrial fraction. These fractions have been characterized previously (8). Membranes were frozen in liquid nitrogen and stored at −70°.

[^{3}H]IDA was obtained from Amersham, and [^{3}H]rauwolscine from New England Nuclear. Unless otherwise indicated, 25–35 nM [^{3}H]IDA and 4–6 nM [^{3}H]rauwolscine were used in the competition binding studies. Details of the binding assay for the membrane preparations and sources of the other chemicals used have been described (3).

Results and Discussion

We first investigated whether COS-7 cells transfected with α_2-adrenergic receptor genes acquire the nonadrenergic [^{3}H]IDA binding site. [^{3}H]Rauwolscine binding to membranes prepared from COS-7 cells transfected with the vector alone was not competed for by epinephrine (100 μM), phentolamine (10 μM), UK 14,304 (10 μM), guanabenz (100 μM), SK&F 104,078 (100 μM), or tolazoline (100 μM; data not shown). These data confirm that COS-7 cells lack α_2-adrenergic receptors. [^{3}H]IDA binding to the vector-transfected cells was also not competed for by epinephrine, phentolamine, UK 14,304, guanabenz, or SK&F 104,078 at the same concentrations (data not shown). Tolazoline, however, competed for some [^{3}H]IDA binding. The "specific" binding (difference between total [^{3}H]IDA binding and that in the presence of 100 μM tolazoline) increased linearly with ligand concentration (up to 150 nM), suggesting that the tolazoline-competable [^{3}H]IDA binding was nonsaturable and of low affinity. We have previously observed similar low affinity, nonsaturable, tolazoline-competable [^{3}H]IDA binding in MDCK cells (3). Thus, vehicle-transfected COS-7 cells lack both α_2-adrenergic receptors and high affinity nonadrenergic [^{3}H]IDA binding sites. The nature of the low affinity, nonsaturable, tolazoline-sensitive [^{3}H]IDA binding was not further investigated. The existence of this site complicates the interpretation of [^{3}H]IDA binding experiments in which tolazoline is used to define nonspecific binding.

Transient expression of recombinant DNAs encoding the α_2-C10 and α_2-C4 adrenergic receptor subtypes was obtained in COS-7 cells (5). The α_2-C10 subtype corresponds to the human platelet α_2-adrenergic receptor and is also equivalent to the α_{2A}-subtype. The α_2-C4 subtype appears to represent the α_{2B}-adrenergic receptor (5); however, this assignment is still uncertain. The C10 and C4 designations refer to the chromosomal localization of the genes for the α_2-adrenergic receptor subtypes. In membranes prepared from cells expressing either the α_2-C10 or the α_2-C4 subtype, total [^{3}H]rauwolscine binding was competed for to a similar extent by epinephrine, phentolamine, UK 14,304, guanabenz, SK&F 104,078, and tolazoline (Ref. 5 and data not shown). In COS-7 cells expressing either gene, epinephrine and phentolamine, which do not recognize the nonadrenergic [^{3}H]IDA binding sites in kidneys, competed for the same amount of total [^{3}H]IDA binding as did UK 14,304, guanabenz, and SK&F 104,078, which do recognize the nonadrenergic [^{3}H]IDA binding sites in kidneys (Fig. 1). The specific binding of both [^{3}H]IDA and [^{3}H]rauwolscine was greater in cells transfected with α_2-C10 receptors than in cells transfected with the α_2-C4 receptors, due to a greater expression of the former. We do not know the reason for the stronger expression of the C10 clone but have observed this consistently. In cells transfected with either gene, tolazoline competed for more [^{3}H]IDA binding sites than did the other drugs (Fig. 1); the additional competition probably represents the low affinity [^{3}H]IDA binding site described above. Thus, transfected cells expressed α_2-adrenergic receptors (which are detected with either [^{3}H]rauwolscine or [^{3}H]IDA) but lack the high affinity nonadrenergic [^{3}H]IDA binding sites.

Even if the nonadrenergic [^{3}H]IDA binding sites are not part of the α_2-adrenergic receptor itself, it is possible that they exist on molecules that are co-expressed and possibly physically associated with the α_2-receptors. In this case, the ratio of nonadrenergic [^{3}H]IDA sites to α_2-adrenergic receptors would be constant in different individuals. We measured the two sites in human platelets, because they have nonadrenergic [^{3}H]IDA

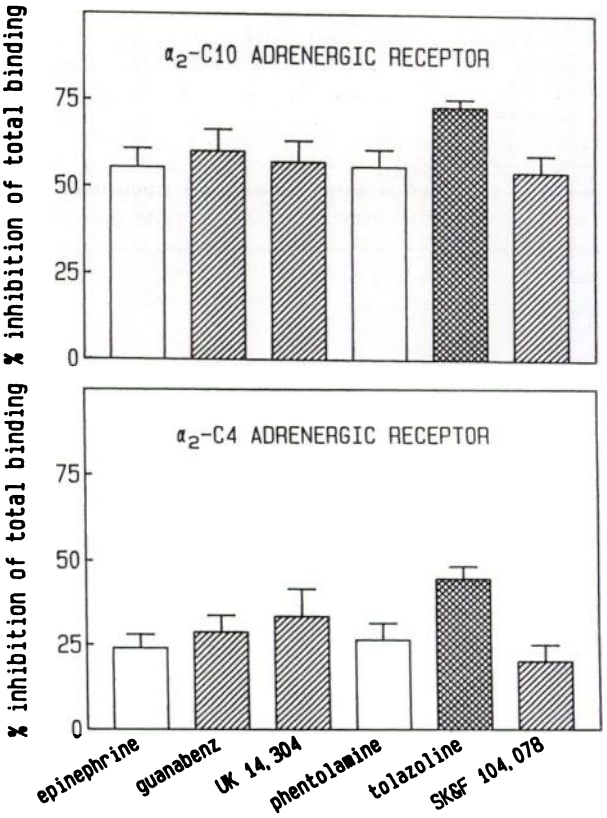

Fig. 1. Binding of [^{3}H]IDA to membranes from COS-7 cells transfected with the genes for the human α_2-C10 (corresponding to α_{2A}) (*upper*) or α_2-C4 adrenergic receptor (probably corresponding to α_{2B}) (*lower*). □, Drugs that do not compete for the nonadrenergic [^{3}H]IDA binding in rat or human renal membranes; ▨, drugs that do (3). ■, Tolazoline (100 μM), which also competed for low affinity [^{3}H]IDA binding to vehicle-transfected COS-7 cells. Data are mean ± standard error of four to six experiments, in which two separate preparations of membranes from transfected cells were used. The amount of competition by epinephrine (100 μM), guanabenz (100 μM), UK 14,304 (10 μM), phentolamine (10 μM), and SK&F 104,078 (100 μM) did not differ significantly as analyzed by one-way analysis of variance. The [^{3}H]IDA concentrations in these experiments was 35 nM for α_2-C10 and 45 nM for α_2-C4 adrenergic receptors. Total [^{3}H]IDA binding averaged 3600 cpm (α_{2A}) and 2900 cpm (α_{2B}); about 1000 cpm represent binding of ligand to the filters.

binding sites (3) and because the number of platelet α_2-adrenergic receptors varies considerably among individuals (9, 10). We quantitated α_2-adrenergic receptors by the amount of [^{3}H]IDA binding competed for by epinephrine or phentolamine. Both drugs competed for similar amounts of [^{3}H]IDA binding (28 ± 4% and 25 ± 5%, respectively) and we based our further calculations on their average. UK 14,304 and guanabenz competed for a significantly greater percentage of total [^{3}H]IDA binding (42 ± 3% and 40 ± 6%, respectively), confirming the presence of nonadrenergic IDA sites in human platelets. Because UK 14,304 and guanabenz competed for a similar amount of [^{3}H]IDA binding, we averaged them for our further calculations. The amount of nonadrenergic IDA sites was defined as the difference of binding competed for by UK 14,304 and guanabenz minus that competed for by epinephrine and phentolamine. The ratio of nonadrenergic to α_2-adrenergic [^{3}H]IDA binding sites varied considerably among the various preparations (0.19–1.18) (Fig. 2). The quantities of the two sites did not significantly correlate (r = 0.0446, 10 experiments, p = 0.9135) (data not shown). Because these studies were performed with a rather high [^{3}H]IDA concentration [3 times the previously reported K_d (3)], we consider it unlikely that this variability is caused by affinity rather than B_{max} alterations. Thus, we conclude that α_2-adrenergic receptors and nonadrenergic [^{3}H]IDA binding sites are not co-regulated in human platelets. Moreover, we have previously shown that the ratio of the two binding sites varies widely between different human tissues (3).

Finally, we studied whether subcellular fractionation could separate the nonadrenergic [^{3}H]IDA binding sites from α_2-adrenergic receptors in human platelets. For this purpose, two membrane fractions were prepared. The heavy membrane fraction (P-fraction of Ref. 7) is a granule/mitochondria-enriched fraction and is slightly depleted of α_2-adrenergic receptors, compared with washed platelet membranes. The light plasma membrane fraction (I-fraction of Ref. 7) is 3–5-fold enriched in α_2-adrenergic receptors. In the P-fraction, UK 14,304 or guanabenz competed for a significantly larger percentage of [^{3}H]IDA binding than did epinephrine or phentolamine (Fig. 3, *left*). In the I-fraction, however, UK 14,304 and guanabenz competed for the same amount of [^{3}H]IDA binding as did epinephrine and phentolamine (Fig. 3, *right*). Thus, purified plasma membranes from human platelets contain α_2-adrenergic receptors but not the nonadrenergic [^{3}H]IDA sites, whereas the nonadrenergic [^{3}H]IDA sites appear to be located elsewhere, i.e., some intracellular compartment or maybe a particular domain of the plasma membrane that is recovered together with the intracellular membranes. Other data suggest that the [^{3}H]IDA binding sites in the rabbit, which are pharmacologically distinct from those in rats, pigs, and humans (4), can also be physically separated from α_2-adrenergic receptors (11).

In conclusion, three types of data show that the nonadrenergic [^{3}H]IDA binding sites can be distinguished from α_2-adrenergic receptors. (a) Cells transfected with DNA encoding genes for α_2-adrenergic receptor subtypes lack the nonadrenergic binding sites. (b) The ratio of the two sites varies considerably in platelets from various donors. (c) α_2-Adrenergic receptors are preferentially located in plasma membranes of platelets, whereas the nonadrenergic binding site is not found in these membranes. It remains puzzling that the nonadrenergic [^{3}H]IDA binding sites are found in many α_2-adrenergic receptor-containing tissues but, nevertheless, can be separated from these receptors in various ways. The possible intracellular localization of the nonadrenergic [^{3}H]IDA binding sites is a further mystery. Many of the drugs binding to this site are quite lipophilic and thus should have access to intracellular binding sites. Because their affinity for the nonadrenergic [^{3}H]IDA binding site and α_2-adrenergic receptors are similarly high, our data point to the intriguing possibility that clinically used

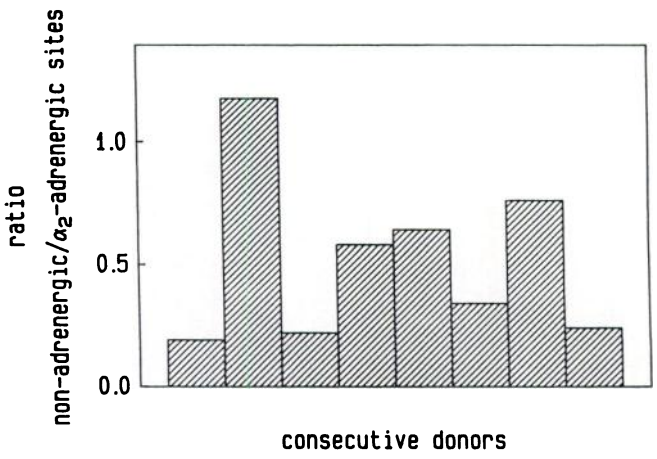

Fig. 2. Binding of [^{3}H]IDA to crude membranes from human platelets of various donors. Shown is the ratio of binding to nonadrenergic sites to that to α_2-adrenergic sites. We used 30–40 nM [^{3}H]IDA and defined the combined specific binding (i.e., to both sites) as the average of binding competed for by 10 μM UK 14,304 and 100 μM guanabenz. The binding to α_2-adrenergic receptors was defined as the average of binding competed for by 100 μM epinephrine and 10 μM phentolamine, and binding to nonadrenergic sites as the difference between combined specific and α_2-adrenergic binding. Each *bar* is the ratio from one membrane preparation using platelets from one to three donors. Two other preparations were excluded from analysis because binding to one of the sites was less than 100 cpm; the ratio in one of these preparations was higher than that of any other and the other had a ratio lower than any other.

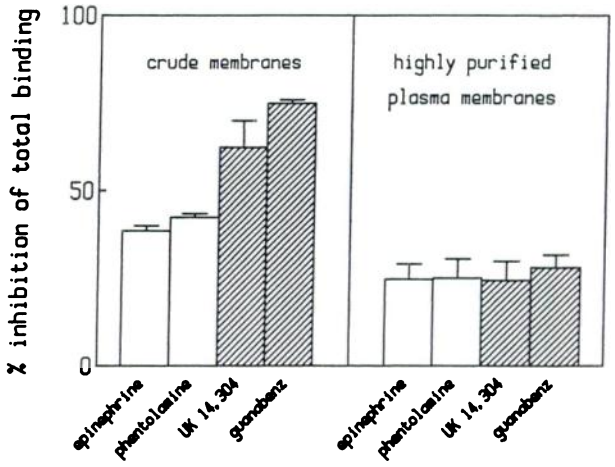

Fig. 3. Binding of [^{3}H]IDA to crude (granule/mitochondrial) and highly purified plasma membranes from human platelets. Membranes were prepared according to Ref. 7; the P-fraction was used for crude and the I-fraction for highly purified plasma membranes. The [^{3}H]IDA concentration was 30–40 nM, and total binding averaged 12,000 and 1,900 cpm in the two fractions, respectively. The different amounts of specific [^{3}H]IDA binding in the two fractions are due to different protein contents and to high filter binding of the ligand (approximately 1000 cpm). Data are mean ± standard error of three experiments (performed in quadruplicate) with membranes prepared from the pooled platelets of 42 donors. Similar data were obtained in three subsequent experiments in a second membrane batch independently prepared from the pooled platelets of 50 donors.

drugs such as guanabenz, idazoxan, or tolazoline might have potent effects that are not mediated by α_2-adrenergic receptors.

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