0026-895X/93/061165-06$03.00/0

MOLECULAR PHARMACOLOGY, 44:1165–1170

Selective Irreversible Binding of Chloroethylclonidine at α_1- and α_2-Adrenoceptor Subtypes

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Received January 28, 1993; Accepted September 20, 1993

SUMMARY

We have determined the alkylating effects and affinity of chloroethylclonidine at α_1- and α_2-adrenoceptor subtypes in saturation and competition radioligand binding studies. Treatment with chloroethylclonidine (10 μM, for 30 min at 37°, with subsequent washout) abolished [^{3}H]prazosin binding to α_{1B}-adrenoceptors in rat spleen almost completely and reduced specific binding in rat kidney and cerebral cortex by a percentage comparable to the known α_{1B}-adrenoceptor content of these tissues. Chloroethylclonidine treatment also markedly reduced [^{3}H]rauwolscine binding to human platelet and kidney membranes but did not affect [^{3}H]rauwolscine binding to rat kidney. Similar chloroethylclonidine treatment (10 μM, 20 min at 37°) reduced the number of detectable α_2-adrenoceptors in cell lines transfected with the α_2-C10 or α_2-C4 gene but not in those transfected with α_2-C2 adrenoceptors. In concentration-response experiments, higher chloroethylclonidine concentrations were required for inactivation of human platelet α_{2A}-adrenoceptors, compared with rat spleen α_{1B}-adrenoceptors, and a smaller maximal inactivation was achieved. The lack of inactivation of rat α_{1A}- and α_{2B}- and human α_2-C2-adrenoceptors was not due to a lack of chloroethylclonidine binding, because the affinity of chloroethylclonidine at these subtypes, as determined in competition binding experiments, was at least as high as the apparent affinity at the alkylated subtypes. α_{2A}-Adrenoceptor alkylation by chloroethylclonidine treatment was functionally relevant, because it significantly reduced α_{2A}-adrenoceptor-mediated Ca^{2+} elevations in HEL cells. We conclude that chloroethylclonidine binds to all major α-adrenoceptor subtypes and irreversibly inactivates not only α_{1B}-adrenoceptors but also α_{2A}- and α_{2C}-adrenoceptors, whereas α_{1A}- and α_{2B}-adrenoceptors are relatively resistant to its alkylating action, although they can bind chloroethylclonidine.

Pharmacological and molecular cloning studies have demonstrated that α_1- and α_2-adrenoceptors cannot be considered as homogeneous entities but rather represent distinct subfamilies within the superfamily of G protein-coupled heptahelical receptors (1, 2). Whereas the properties of the pharmacologically defined α_2-adrenoceptors agree reasonably well with those of the cloned receptors, the classification of α_1-adrenoceptor subtypes remains somewhat controversial (1–3). This controversy stems at least in part from the lack of selectivity of the available experimental tools. For example, WB 4101 and phentolamine have only limited selectivity among α_1-adrenoceptor subtypes (4), and 5-methylurapidil and (+)-niguldipine not only are α_1-adrenoceptor antagonists but also are a serotonin 5-hydroxytryptamine type 1A receptor agonist (5) or a Ca^{2+} entry blocker (6), respectively. Therefore, the irreversible α-adrenoceptor ligand chloroethylclonidine (7), which inactivates α_{1B}-adrenoceptors and inactivates α_{1A}-adrenoceptors much more weakly (8–10), has been used in most physiological studies to discriminate α_1-adrenoceptor subtypes.

This work was supported in part by the Deutsche Forschungsgemeinschaft.

Chloroethylclonidine was originally introduced into pharmacology by Leclerc *et al.* (7), as a compound that has a >500-fold or >20-fold higher pD_2 value, compared with noradrenalin or clonidine, respectively, for the *in vitro* contraction of isolated rat aorta and *in vivo* vasoconstriction in the pithed rat; in contrast to the noradrenalin- or clonidine-induced contraction of rat aorta, the chloroethylclonidine-induced contraction did not abate rapidly upon washout and was maintained for >90 min. In a series of elegant papers in 1987 and 1988, Minneman and co-workers (8–10) found that chloroethylclonidine acts irreversibly only on a subset of α_1-adrenoceptors, which have rather low affinity for WB 4101 and were defined as α_{1B}-adrenoceptors (11). In many subsequent studies chloroethylclonidine has proven to be a useful tool for the discrimination of α_{1A}- and α_{1B}-adrenoceptors.

On the other hand, the original paper by Leclerc *et al.* (7) had already indicated that chloroethylclonidine, similarly to clonidine and other derivatives thereof, may have considerable affinity for α_2-adrenoceptors; this indication was based on the ability of chloroethylclonidine to compete for noradrenalin-sensitive [^{3}H]clonidine binding to a rat brain synaptosomal

ABBREVIATIONS: PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

preparation, with an affinity of 1650 nM. Therefore, we have reinvestigated the α-adrenoceptor subtype selectivity of chloroethylclonidine in various model systems of rat and human α_1- and α_2-adrenoceptor subtypes, using radioligand binding. Our studies have separately investigated the alkylating effects of chloroethylclonidine treatment in saturation binding studies and the apparent chloroethylclonidine affinity in competition binding studies.

Materials and Methods

Tissue preparation. Human renal cortex was obtained from patients undergoing nephrectomy because of hypernephroma; only tissue samples from macroscopically tumor-free sections were used. Human platelets were obtained from healthy drug-free blood donors. Rat kidney, liver, spleen, and cerebral cortex were obtained from male Wistar rats (Lippische Versuchstierzucht, Extertal, Germany). Crude membrane fractions from these tissues were prepared by standard homogenization and centrifugation procedures (centrifugation twice for 20 min at 50,000 × *g*) as described previously (12). A somewhate purified membrane preparation from liver was prepared as described by Clarke *et al.* (13).

Cell culture. HEL cells were grown in suspension culture in chemically defined CG medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and were maintained at a density of 400,000–800,000 cells/ml by daily dilutions. Cells were washed by centrifugation at 200 × *g* for 10 min and were resuspended in Ca^{2+} measurement buffer (composition given below).

LM(tk$^-$) cells stably transfected with the α_2-C10 or α_2-C4 gene were grown in suspension in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum and were harvested by centrifugation at 200 × *g* for 2 min. The cell pellets were suspended in 5 ml of PBS and were incubated with and without chloroethylclonidine; subsequent washouts were performed with 10 ml of PBS each.

NIH/3T3 cells stably transfected with the α_2-C2 receptor gene were grown as monolayers in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum, as described before (14). The culture medium was replaced by 5 ml of PBS and the cells were incubated in the presence of chloroethylclonidine; the incubation medium was then removed and the cells were washed twice with 10 ml of PBS, scraped into 5 ml of ice-cold PBS, and centrifuged at 200 × *g* for 2 min.

Pellets of the transfected cells were suspended in ice-cold buffer (20 mM Tris·HCl, 5 mM EDTA, pH 7.4) and homogenized by sonication for 7 sec. The cell lysates were centrifuged at 200 × *g* for 5 min at 4°. The supernatants were then centrifuged at 40,000 × *g* for 20 min at 4°. The resulting pellets were washed once in the homogenization buffer and suspended in binding buffer (see below).

Radioligand binding. α_1-Adrenoceptors were identified by [^{3}H]prazosin binding (30 min at 25°) as described previously (15). α_2-Adrenoceptors in human and rat tissues were identified by [^{3}H]rauwolscine binding (60 min at 25°) as described previously (12, 15). α_2-Adrenoceptor binding experiments with membranes from transfected cells were also performed with [^{3}H]rauwolscine but incubations were extended to 90 min at 23°. α_1- and α_2-adrenoceptor binding to native tissues was performed in 50 mM Tris, 0.5 mM EDTA, pH 7.5, whereas α_2-adrenoceptor binding to transfected cells was performed in 25 mM glycylglycine, pH 7.6. Incubations were terminated by rapid vacuum filtration over GF/C (native tissues) or GF/B (transfected cells) glass fiber filters. In all experiments nonspecific binding was defined using 10 μM phentolamine.

Ca^{2+} experiments. The free intracellular Ca^{2+} concentration in HEL cells was determined using the fluorescent indicator dye fura-2 and the double-wavelength ratio method, as described previously (16). These experiments were performed at room temperature in a buffer containing 120 mM NaCl, 5 mM KH_2HPO_4, 1 mM magnesium acetate, 1 mM $CaCl_2$, 20 mM HEPES, and 1 mg/ml glucose, at pH 7.4.

Chemicals. [^{3}H]Prazosin and [^{3}H]rauwolscine (≈80 Ci/mmol each) were obtained from New England Nuclear; chloroethylclonidine was from Research Biochemicals Inc. (Natick, MA). Phentolamine was a gift from Ciba Geigy (Basel, Switzerland).

Data analysis. The number and affinity of α_1- or α_2-adrenoceptors were determined from saturation binding experiments using six concentrations of the ligand; B_{max} and K_d values were obtained by fitting rectangular hyperbolic functions to the experimental data, using computer-assisted iterative nonlinear regression analysis. Competition binding experiments were also analyzed by nonlinear regression analysis, which fitted mono- or biphasic sigmoidal functions to the experimental data; a biphasic fit did not result in a significantly improved *F* test in any of our experiments (data not shown). Thus, all competition data presented are from monophasic fits with floating Hill coefficients, which always were close to unity (data not shown). Data are expressed as mean ± standard error of *n* experiments. In the chloroethylclonidine alkylation experiments, the statistical significance of differences was determined using paired two-tailed *t* tests, with $p < 0.05$ being considered significant.

Results and Discussion

The present studies can be divided into three parts. In the first part we tested the alkylating effects of chloroethylclonidine at α_1- and α_2-adrenoceptor subtypes in native tissues and in transfected cell lines expressing the genes of the human α_2-adrenoceptor subtypes. In the second part we determined the apparent affinity of chloroethylclonidine in competition binding studies in various model systems of α_1- and α_2-adrenoceptors. In the third part we investigated the functional consequences of α_{2A}-adrenoceptor alkylation by chloroethylclonidine in a human cell line where α_{2A}-adrenoceptor stimulation leads to elevation of intracellular Ca^{2+} (17).

Receptor inactivation studies. Chloroethylclonidine has been primarily used in experimental pharmacology because of its alkylating effects, which are selective for α_{1B}-adrenoceptors, relative to α_{1A}-adrenoceptors (8–10). To validate our experimental conditions, we first compared the effect of chloroethylclonidine treatment (10 μM, 30 min at 37°) and subsequent duplicate washout (centrifugation at 50,000 × *g* at 4° for 20 min each) on the number of [^{3}H]prazosin binding sites in rat spleen, kidney, and cerebral cortex. As expected, chloroethylclonidine almost completely abolished specific [^{3}H]prazosin binding to spleen membranes and reduced the number of [^{3}H]prazosin binding sites in rat kidney by 56% and in rat cerebral cortex by 66% (Table 1). These reductions in [^{3}H]prazosin binding sites correspond well to the amount of α_{1B}-adrenoceptors previously determined using competitive antagonists in the three tissues (4, 10, 15, 18, 19). The decrease in [^{3}H]prazosin binding site number was accompanied by slightly reduced affinities of the remaining sites in rat kidney (from 334 ± 48 to 700 ± 111 pM, $p = 0.0798$) and cerebral cortex (from 74 ± 9 to 170 ± 31 pM, $p = 0.0369$); the [^{3}H]prazosin affinity of the very few remaining α_1-adrenoceptors in spleen membranes could not be quantitated reliably after chloroethylclonidine treatment. Similar increases of the apparent K_d values for the radioligand after chloroethylclonidine treatment have also been seen by other investigators, regardless of whether [^{3}H]prazosin or ^{125}I-BE 2254 was used as the radioligand (10, 20). Such minor reductions in apparent affinity of the radioligand could be attributed to incomplete washout of the alkylating agent or to pseudoirreversible binding of chloroethylclonidine; preliminary data in our laboratory indicate that more vigorous washouts do not prevent these reductions in apparent affinity.

TABLE 1

Effect of chloroethylclonidine treatment on the density of α-adrenoceptor subtypes in rat and human tissues

Membranes were treated with chloroethylclonidine (10 μM) (CEC-treated) or vehicle (Control) for 30 min at 37° and were then washed twice by centrifugation. Density of α_1- and α_2-adrenoceptors was determined by saturation binding experiments using [^{3}H]prazosin and [^{3}H]rauwolscine, respectively. Data are given as mean ± standard error of *n* experiments.

Tissue	Subtype	*n*	α-Adrenoceptors	
			Control	CEC-treated
			fmol/mg of protein	
Rat spleen	α_{1B}	4	36 ± 3	2 ± 1[a]
Rat kidney	Mixed α_1	4	88 ± 13	39 ± 6[b]
Rat cerebral cortex	Mixed α_1	5	137 ± 16	47 ± 9[a]
Human platelets	α_{2A}	9	270 ± 10	88 ± 15[a]
Rat kidney	α_{2B}	4	108 ± 10	107 ± 8
Human kidney	Mixed α_2	7	44 ± 5	25 ± 6[b]

[a] $p < 0.01$, compared with vehicle-treated membranes in a paired two-tailed *t* test.
[b] $p < 0.05$.

TABLE 2

Effect of chloroethylclonidine treatment on cloned human α_2-adrenoceptor subtypes

Intact stably transfected cells were incubated for 20 min at 37° in the presence (CEC-treated) or absence (Control) of 10 μM chloroethylclonidine, followed by washout and membrane preparation. Data are mean ± standard error of three independent experiments, where the numbers of α_2-adrenoceptors were each determined by a triplicate saturation binding experiment.

Subtype	α-Adrenoceptors	
	Control	CEC-treated
	fmol/mg of protein	
α_2-C10 (α_{2A})	232 ± 26	148 ± 24[a]
α_2-C2 (α_{2B})	368 ± 45	368 ± 62
α_2-C4 (α_{2C})	569 ± 58	269 ± 50[a]

[a] $p < 0.05$.

We next tested the ability of chloroethylclonidine to inactivate α_2-adrenoceptors from human kidney (previously classified as containing mostly α_{2A}-adrenoceptors but also being the source from which the α_2-C4 gene has been cloned) and platelets (homogeneously α_{2A}) and from rat kidney (mostly if not exclusively α_{2B}). Chloroethylclonidine treatment reduced the number of α_2-adrenoceptors in human kidney membranes by 43% and that of α_{2A}-adrenoceptors in human platelet membranes by 67% (Table 1). In both tissues a minor but not statistically significant reduction in the [^{3}H]rauwolscine affinity of the remaining sites was detected (from 4.4 ± 1.6 to 5.4 ± 1.5 nM in kidney, $p = 0.6351$; from 1.73 ± 0.29 to 4.38 ± 1.51 nM in platelets, $p = 0.1245$). In contrast, treatment of rat kidney membranes did not reduce the number of detectable [^{3}H]rauwolscine binding sites (Table 1) or their affinity for the radioligand (2.05 ± 0.22 nM in control and 2.20 ± 0.23 nM in treated membranes, $p = 0.6519$).

Chloroethylclonidine treatment also reduced the number of detectable α_2-adrenoceptors in cell lines hosting the cloned receptor subtypes. As observed in the native tissues, chloroethylclonidine treatment was effective against α_{2A}-adrenoceptors (α_2-C10) but not against α_{2B}-adrenoceptors (α_2-C2); chloroethylclonidine treatment also readily inactivated α_{2C}-adrenoceptors (α_2-C4) (Table 2). For all three subtypes a slight decrease in the apparent affinity of the radioligand was observed; however, the decrease failed to reach statistical significance (α_2-C10, 0.34 ± 0.08 versus 2.21 ± 0.49 nM; α_2-C2, 0.49 ± 0.07 versus 1.18 ± 0.10 nM; α_2-C4, 0.09 ± 0.01 versus 0.37 ± 0.12 nM). Although the reduction in the apparent [^{3}H]rauwolscine affinity after chloroethylclonidine treatment did not reach statistical significance with the given number of experiments in any model system, we observed it quite consistently in almost all tissues and cell lines. However, we are confident that this does not affect our conclusion, because an increase in apparent affinity should, if anything, lead to an overestimation of the number of detectable binding sites.

To obtain a more quantitative estimate of the chloroethylclonidine sensitivity of α_{2A}-adrenoceptors, relative to α_{1B}-adrenoceptors, we performed concentration-response experiments on the alkylating effects of chloroethylclonidine at rat spleen α_{1B}-adrenoceptors and human platelet α_{2A}-adrenoceptors. In rat spleen membranes, 0.1 μM chloroethylclonidine reduced the number of α_1-adrenoceptors by 36% and 10 μM chloroethylclonidine almost completely inactivated them; thus, the EC_{50} for the α_{1B}-adrenergic receptor-alkylating effects of chloroethylclonidine appears to be between 0.1 and 1 μM (Fig. 1). Chloroethylclonidine treatment also concentration-dependently inactivated human platelet α_{2A}-adrenoceptors. Although 1 μM chloroethylclonidine inactivated approximately 50% of α_{2A}-adrenoceptors, even increases up to 100 μM chloroethylclonidine did not cause complete inactivation (Fig. 1). Thus, both α_{1B}- and α_{2A}- and α_{2C}-adrenoceptors can be inactivated by chloroethylclonidine but considerably higher concentrations are required for the inactivation of α_{2A}-adrenoceptors, compared with α_{1B}-adrenoceptors. In contrast, α_{1A}- and α_{2B}-adrenoceptors appear to be relatively resistant to alkylation by chloroethylclonidine.

Studies on apparent chloroethylclonidine affinity. Because chloroethylclonidine inactivated only certain subtypes of α_1- and α_2-adrenoceptors, we have tested whether its lack of alkylating effects towards the other subtypes might be due to a lack of affinity. Therefore, we determined the ability of chloroethylclonidine to bind to various subtypes of α-adrenoceptors in competition binding studies, using [^{3}H]prazosin and [^{3}H]rauwolscine as the α_1- and α_2-adrenergic ligands, respectively. Rat kidney and cerebral cortex were used as sources of mixed populations of α_{1A}- and α_{1B}-adrenoceptors (15, 21) and rat liver and spleen were used as sources of α_{1B}-adrenoceptors (4, 22);

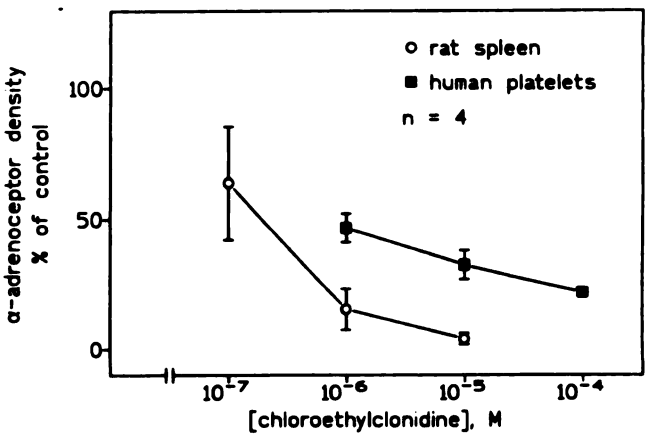

Fig. 1. Effects of chloroethylclonidine treatment (30 min at 37°, followed by two washout centrifugations at 50,000 × *g*) on the number of rat spleen α_{1B}-adrenergic receptors (O) and human platelet α_{2A}-adrenergic receptors (■). Data are mean ± standard error of four experiments and are expressed as percentage of receptor number in vehicle-treated membranes. Receptor number in each experiment was determined in a saturation binding experiment with six ligand concentrations.

membranes from rat kidney and cerebral cortex obtained after pretreatment with chloroethylclonidine (10 μM, 30 min at 37°) and subsequent washout served as a source of α_{1A}-adrenoceptors (23). Rat kidney also was used as a source of α_{2B}-adrenoceptors (24), and human platelets were used as a model for α_{2A}-adrenoceptors (24); human kidney was used as a source of mixed α_2-adrenoceptors in which α_{2A}-adrenoceptors predominate (24), but this tissue has also been the source for the cloning of the α_2-C4 gene (25). Finally, cell lines stably transfected with the genes for human α_2-C10, α_2-C2, or α_2-C4 adrenoceptors served as homogeneous preparations of the respective receptors.

In all rat and human tissues, chloroethylclonidine addition to the binding assay inhibited specific [^{3}H]prazosin or [^{3}H]rauwolscine binding sites with steep and monophasic competition curves (Fig. 2). The apparent affinity of chloroethylclonidine was similar at mixed α_1-adrenoceptors in rat kidney and cerebral cortex, at α_{1A}-adrenoceptors in both tissues after chloroethylclonidine pretreatment, and at α_{1B}-adrenoceptors in rat liver and spleen (Fig. 2; Table 3). Thus, chloroethylclonidine showed no selectivity in its potency to bind to α_{1A}- or α_{1B}-adrenoceptors. It should be noted, however, that true affinities for an irreversible ligand cannot be determined from competition binding studies. Indeed, we found that treatment of spleen membranes with 1 μM chloroethylclonidine (which is slightly more than its apparent affinity) under conditions that mimic those in the competition binding assay (30 min at 25°) reduced the number of α_{1B}-adrenoceptors, as determined in saturation

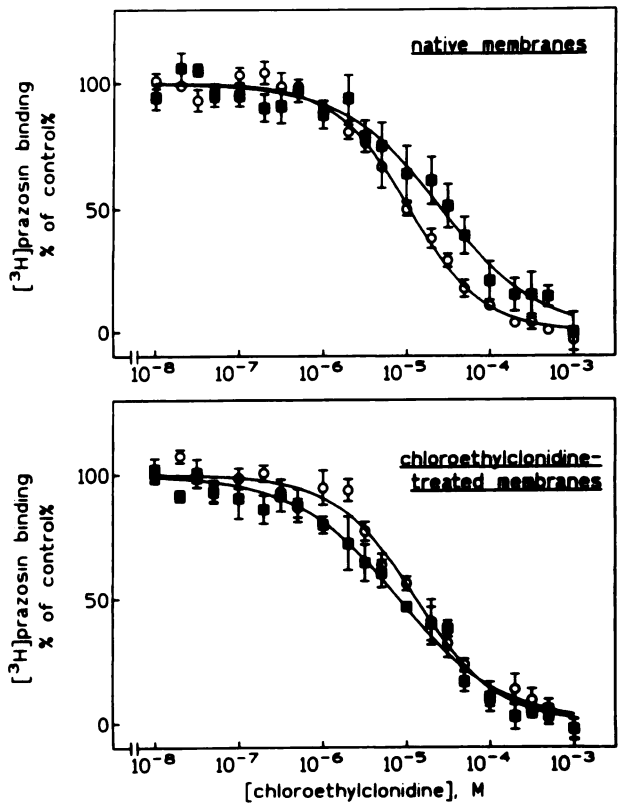

Fig. 2. Competition binding of chloroethylclonidine at rat kidney (■) and cerebral cortex (○) α_1-adrenoceptors. Binding experiments were performed in native membranes (*upper*) and in membranes that had been pretreated with 10 μM chloroethylclonidine for 30 min at 37°, followed by two washout centrifugations (*lower*). Data are mean ± standard error of three to seven experiments. [^{3}H]Prazosin concentrations in the assays ranged between 700 and 3000 pM.

TABLE 3

Apparent chloroethylclonidine affinities at tissue α_1- and α_2-adrenoceptor subtypes

Apparent chloroethylclonidine affinities at α_1- and α_2-adrenoceptor subtypes were determined in competition binding studies with [^{3}H]prazosin and [^{3}H]rauwolscine, respectively, and are expressed as −log IC_{50}. Data are mean ± standard error of *n* experiments. CEC, membranes had been pretreated with 10 μM chloroethylclonidine for 30 min at 37° and then washed twice before the chloroethylclonidine competition experiments.

Tissue	Subtype	*n*	−log IC_{50}
Rat kidney	Mixed α_1	7	4.74 ± 0.22
Rat cerebral cortex	Mixed α_1	3	4.97 ± 0.08
Rat kidney (CEC)	α_{1A}	5	4.90 ± 0.06
Rat cerebral cortex (CEC)	α_{1A}	4	5.89 ± 0.02
Rat liver	α_{1B}	4	4.89 ± 0.17
Rat spleen	α_{1B}	3	4.83 ± 0.14
Human platelets	α_{2A}	3	6.14 ± 0.07
Human kidney	Mixed α_2	5	5.61 ± 0.07
Rat kidney	α_{2B}	3	5.61 ± 0.25

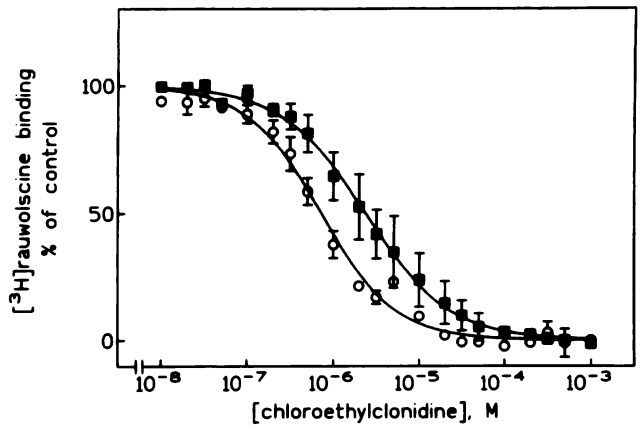

Fig. 3. Competition binding of chloroethylclonidine at rat kidney α_{2B}-adrenoceptors (■) and human platelet α_{2A}-adrenoceptors (○). Data are mean ± standard error of three experiments. [^{3}H]Rauwolscine concentrations in the assays ranged between 2 and 4 nM.

binding studies, by ≈70% (data not shown). Thus, the apparent chloroethylclonidine affinities at rat spleen and liver α_1-adrenoceptors and possibly at kidney and cerebral cortex α_1-adrenoceptors may represent a composite estimate of binding affinity and alkylating effects and, therefore, are questionable. On the other hand, the simultaneous presence of inactivation and competition binding should, if anything, produce overestimation of the chloroethylclonidine affinity at the α_1-adrenoceptor subtype that becomes alkylated. Thus, the apparent chloroethylclonidine affinities were quite similar at all α_1-adrenoceptor subtypes. Because the α_{1A}-adrenoceptors were resistant to alkylation (Table 1), we could calculate chloroethylclonidine affinities (−log K_i) at α_{1A}-adrenoceptors in rat kidney and cerebral cortex of 5.74 ± 0.16 and 5.89 ± 0.02, respectively. We conclude that the chloroethylclonidine affinity at α_{1A}-adrenoceptors is at least as high as that at α_{1B}-adrenoceptors and thus the lack of alkylation of α_{1A}-adrenoceptors by chloroethylclonidine is not due to a lack of binding to this subtype.

Chloroethylclonidine also had high apparent affinities in competition binding experiments at rat kidney α_{2B}-adrenoceptors, human platelet α_{2A}-adrenoceptors, and human kidney mixed α_2-adrenoceptors (Fig. 3; Table 3). These affinities are quite similar to that originally determined by Leclerc *et al.* (7) using noradrenalin-sensitive [^{3}H]clonidine binding in rat brain synaptosomes (1650 ± 400 nM). Chloroethylclonidine also

bound with relatively high apparent affinity to the cloned human α_2-adrenoceptor subtypes (Table 4). The detected affinities for chloroethylclonidine and those for [^{3}H]rauwolscine were somewhat greater in the transfected cell lines than in the native α_2-adrenoceptor-containing tissues; this may be related to the expression environment or the different buffer system used. It should be noted again that the determined apparent affinities at the α_2-adrenoceptor subtypes that are alkylated by chloroethylclonidine may be composite measures of affinity and alkylating effects and, therefore, questionable (see above). Specifically, we found that treatment of human platelet membranes with 1 μM chloroethylclonidine (which is slightly more than its apparent affinity determined in the competition studies) under conditions mimicking those in the competition studies (60 min at 25°) reduced α_{2A}-adrenoceptor density by ≈50% (data not shown). On the other hand, this should also, if anything, have resulted in an overestimation of the affinity of chloroethylclonidine in the competition studies. Thus, the chloroethylclonidine affinities at α_{2B}-adrenocpetors are similar to those at α_{2A}-adrenoceptors, and therefore the lack of alkylation of the α_{2B}-adrenoceptor cannot be attributed to a lack of chloroethylclonidine binding to this subtype. For the nonalkylated rat kidney α_{2B}-adrenoceptor and the cloned human α_2-C2 adrenoceptor, affinities ($-\log K_i$) of 6.18 ± 0.22 and 6.81 ± 0.14, respectively, could be determined.

Functional studies. Finally, we determined the functional relevance of chloroethylclonidine treatment-induced reductions in α_{2A}-adrenoceptors. For this purpose we determined the effect of chloroethylclonidine treatment on adrenalin-stimulated Ca^{2+} elevations in HEL cells, which we have previously shown to be mediated via α_{2A}-adrenoceptors (17). Chloroethylclonidine treatment of intact cells (10 μM, 37° for 30 min), followed by two washout centrifugations (200 × g for 10 min), did not significantly alter basal Ca^{2+} concentration (96 ± 7 versus 89 ± 5 nM, n = 17) and did not nonspecifically impair 100 nM neuropeptide Y-stimulated Ca^{2+} mobilization (252 ± 22 versus 234 ± 13 nM, n = 8). The Ca^{2+}-mobilizing effect of 1 μM adrenalin, however, was consistently reduced by chloroethlclonidine treatment, with an average reduction of 38% (Fig. 4). On one hand, these data indicate that the alkylating effects of chloroethylclonidine were restricted to the receptor site, because there was no modification of the cellular responsiveness of HEL cells with regard to Ca^{2+} mobilization. Furthermore, these data also indicate that chloroethylclonidine effects were restricted to the α_2-adrenoceptors, because cloroethylclonidine did not modify the response to neuropeptide Y. This is important, because other irreversible α-adrenoceptor ligands, such as phenoxybenzamine and benextramine, also inactivate serotonin and neuropeptide Y receptors, respectively (26, 27).

On the other hand, these data demonstrate that the reduction of detectable α_2-adrenoceptors seen in membrane preparations

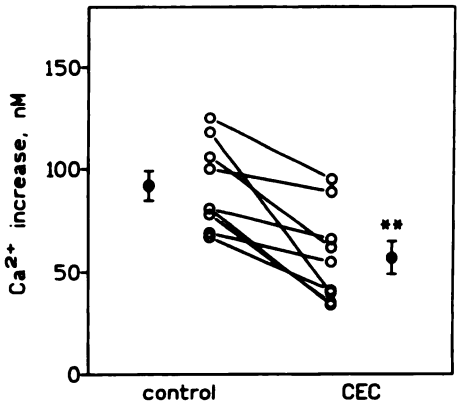

Fig. 4. Effects of chloroethylclonidine treatment (10 μM, for 30 min at 37°, followed by two washout centrifugations at 200 × g) on 1 μM adrenalin-stimulated Ca^{2+} elevations in HEL cells. ○, Data from individual experiments in vehicle (control) or chloroethylclonidine (*CEC*)-treated cells; paired data from the same experiment are connected by a *line*. ●, Mean ± standard error of all experiments. **, $p < 0.01$, versus vehicle-treated cells, in a paired two-tailed t test.

of tissues and cell lines after chloroethylclonidine treatment is of functional relevance. This idea is also supported by data from other investigators, who found that chloroethylclonidine is an irreversible agonist at presynaptic α_2-adrenoceptors in rat vas deferens (28). Because many functional responses such as vasoconstriction can be elicited by α_1- or α_2-adrenoceptor stimulation (29, 30) and because many agonists used in physiological model systems, including the endogenous catecholamines noradrenalin and adrenalin, show limited selectivity for α_1- versus α_2-adrenoceptors (31), our data suggest that it is invalid to base conclusions regarding the involvement of α_{1B}-adrenoceptors in a given physiological response solely on data obtained using chloroethylclonidine. Because chloroethylclonidine treatment inactivates α_{1B}-adrenoceptors at concentrations lower than those needed for α_{2A}-adrenoceptors (Fig. 1), however, such conclusions may be possible if an appropriate selectivity window is selected. In contrast, radioligand binding data are less prone to ambiguous interpretations, because radioligands show greater receptor specificity than do the agonists used in functional studies. Therefore, our chloroethylclonidine data may help to resolve some of the previously noted inconsistencies between pharmacologically defined α-adrenoceptor subtypes in radioligand binding and functional studies (3).

In addition to bringing into question the usefulness of chloroethylclonidine as a pharmacological tool, our observation that chloroethylclonidine recognizes subtypes of α_1- and α_2-adrenoceptors with similar affinities has several implications. Firstly, the alkylating effects of chloroethylclonidine differ within but not between α-adrenoceptor subclasses, despite a greater homology within the α_1- and α_2-adrenoceptor subclasses. Secondly, with regard to the molecular events involved in the α-adrenoceptor/ligand interaction, the irreversible action of chloroethylclonidine appears to be a two-step process, in which a covalent modification presumably follows ligand binding to the receptor site. Distinct structure-activity relationships are likely to exist for each of these steps, because the reactivity of chloroethylclonidine towards the different α-adrenoceptors seems to be unrelated to its binding affinity. Additional studies of these structure-activity relationships should lead to a better understanding of the molecular aspects of α-adrenoceptor function and aid in the development of more specific drugs.

TABLE 4

Apparent chloroethylclonidine affinities at cloned human α_2-adrenoceptor subtypes

Apparent chloroethylclonidine affinities at α_2-adrenoceptor subtypes were determined in competition binding studies with [^{3}H]rauwolscine and are expressed as $-\log IC_{50}$. Data are mean ± standard error of n experiments.

Subtype	Host cell line	n	$-\log IC_{50}$
α_2-C10 (α_{2A})	LM(tk$^-$)	3	6.93 ± 0.05
α_2-C2 (α_{2B})	LM(tk$^-$)	3	6.39 ± 0.13
α_2-C4 (α_{2C})	NIH/3T3	3	6.37 ± 0.06

Acknowledgments

We thank Dr. H. Rübben and his colleagues at the Department of Urology, University of Essen, for providing the human kidney samples and Dr. R. Weinshank (Synaptic Pharmaceutical Corp.) for providing the cloned α_2-adrenoceptor cell lines.

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