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Kinetics of alkylation of cloned rat α_1-adrenoceptor subtypes by chloroethylclonidine

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Abstract

We quantified and compared the rates at which chloroethylclonidine (CEC) inactivated cloned rat α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors. Membranes from cells transfected with one of the three cloned α_1-adrenoceptors were incubated for various intervals with 100 μM chloroethylclonidine at 10°C, 25°C or 37°C. The fraction of receptors alkylated by chloroethylclonidine was determined by [^{3}H]prazosin binding. Chloroethylclonidine fully inactivated each α_1-adrenoceptor subtype via a first order reaction. Alkylation by chloroethylclonidine was markedly slower for the α_{1A}-adrenoceptor vs. the other two subtypes (rate constants in 10^{-3} min^{-1} at 10°C: 0.99 ± 0.01 (α_{1A}), 7.26 ± 0.15 (α_{1B}), and 7.01 ± 0.12 (α_{1D})). Despite differences in rate, activation energies for alkylation were similar among subtypes, suggesting a similar binding sites for chloroethylclonidine. Computer simulations of kinetic data in mixed receptor populations and experiments with membranes from rat brain showed that nonlinear curve fitting could distinguish relative proportions of α_{1A}-adrenoceptor vs. the other two subtypes. We conclude that measurement of the rate of alkylation by chloroethylclonidine, rather than the total amount of alkylation, is most useful in distinguishing the relative proportion of α_{1A}-adrenoceptor in tissues. © 1998 Elsevier Science B.V.

Keywords: α_1-Adrenoceptor subtype; Chloroethylclonidine; Alkylation; (Rat); Kinetics

1. Introduction

α_1-Adrenoceptors mediate the actions of the neurotransmitter norepinephrine in most tissues innervated by the sympathetic nervous system and within the central nervous system. Three distinct α_1-adrenoceptor subtypes (α_{1A}, α_{1B} and α_{1D}) have been identified through pharmacological (Morrow and Creese, 1986; Johnson and Minneman, 1987; Han et al., 1987; Minneman et al., 1988) and molecular cloning experiments (Cotecchia et al., 1988; Schwinn et al., 1990; Lomasney et al., 1991; Perez et al., 1991). Each of these receptors have been shown to have a discrete pattern of distribution among body tissues (Minneman et al., 1988; Cotecchia et al., 1988; Schwinn et al., 1990; Lomasney et al., 1991; Price et al., 1994; Scofield et al., 1995). Although the specific functions of each of the subtypes are largely unknown, emerging evidence suggests that the α_1-adrenoceptor subtypes also discretely act in the mediation of sympathetic responses in tissues (Bylund et al., 1994; Hieble et al., 1995; Graham et al., 1996). The identification of which functions are mediated by each subtype has relied on the use of selective competitive antagonists and alkylating agents such as chloroethylclonidine and SZL-49 (prazobind) (Piascik et al., 1989, 1991; Michel et al., 1993; Perez et al., 1991, 1994).

At the time of the original subclassification of the α_1-adrenoceptor subtypes, short incubations with chloroethylclonidine was found to rapidly alkylate some, but not all α_1-adrenoceptors (reviewed in Minneman, 1988). These 'chloroethylclonidine-insensitive' (α_{1A}-adrenoceptors) and 'chloroethylclonidine-sensitive' (α_{1B}-adrenoceptors) populations were found to correspond to the previously discovered heterogeneity of α_1-adrenoceptors observed for affinity to antagonists such as 2-(2,6-Dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane also known as WB 4101 (Morrow and Creese, 1986). Starting from this time chloroethylclonidine alkylation (usually single concentrations for short incubation periods) was used in many studies in various species to classify the subtype of α_1-adrenoceptor that mediated a particular function in vivo or in vitro.

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The use of chloroethylclonidine was extended to characterize cloned α_1-adrenoceptor subtypes of various species. These experiments produced results that confused previously established ideas of chloroethylclonidine specificity. For example, the bovine α_{1A}-adrenoceptor was found to be sensitive to chloroethylclonidine, leading in part to its original classification as the α_{1C}-adrenoceptor (Schwinn et al., 1990). When the human (Hirasawa et al., 1993) and rat (Laz et al., 1994; Perez et al., 1994) α_{1A}-adrenoceptor subtypes were cloned, they were found to be much less sensitive to chloroethylclonidine than reported for the bovine α_{1A}-adrenoceptor. The third cloned α_1-adrenoceptor subtype, the α_{1D}, was found to be sensitive to chloroethylclonidine but with a more complex pattern of inactivation. In these experiments, chloroethylclonidine completely inactivated α_{1B}-adrenoceptors but could not inactivate all of the α_{1D}-adrenoceptors (Lomasney et al., 1991; Perez et al., 1991, 1994). This led to the designation of the α_{1D}-adrenoceptor as 'partially' sensitive to chloroethylclonidine (Perez et al., 1991, 1994; Bylund et al., 1994). This evidence suggested to us that some unique aspect of α_{1D}-adrenoceptor structure may produce partial chloroethylclonidine sensitivity. One could hypothesize that the α_{1D}-adrenoceptor is expressed in multiple conformations, consisting of chloroethylclonidine-sensitive and chloroethylclonidine-insensitive populations. Alternatively, it could be hypothesized that chloroethylclonidine alkylates the α_{1D}-adrenoceptor with complex kinetics.

Thus, the current state of knowledge with respect to chloroethylclonidine alkylation is that the α_{1A}-adrenoceptor is 'insensitive' to chloroethylclonidine, the α_{1B}-adrenoceptor is 'sensitive' and the α_{1D}-adrenoceptor is 'partially sensitive.' It is surprising to note that despite the reliance of many investigators on the use of chloroethylclonidine to classify α_1-adrenoceptors, a detailed investigation of the kinetics of chloroethylclonidine alkylation has never been published. Therefore it is possible that previous observations of differences among receptor subtypes in chloroethylclonidine sensitivity after fixed incubation times reflect only differences in the rates of inactivation of the receptors by chloroethylclonidine. In the present study we hypothesized that all α_1-adrenoceptor subtypes are sensitive to alkylation by chloroethylclonidine, but at different rates. To test this hypothesis we measured for the first time the kinetics of alkylation by chloroethylclonidine in each cloned rat α_1-adrenoceptor subtype.

2. Materials and methods

2.1. Materials

QIAGEN plasmid kits were purchased from QIAGEN (Chatsworth, CA). Chloroethylclonidine HCl and phentolamine mesylate were purchased from RBI (Natick, MA). EDTA was purchased from Sigma (St. Louis, MO). Tris hydroxymethyl aminomethane hydrochloride and Tris hydroxymethyl aminomethane were purchased from Fisher Scientific (Fair Lawn, NJ). DEAE-dextran was purchased from Pharmacia Biotech (Piscataway, NJ). [^{3}H]prazosin was purchased from DuPont NEN (Boston, MA). Dulbecco's modified Eagle's media, antibiotic–antimycotic, trypsin EDTA, geneticin and fetal bovine serum were purchased from Gibco BRL Lifetechnologies (Grand Island, NY).

2.2. cDNA plasmid purification

Cloned rat α_{1A}-adrenoceptor cDNA (kindly provided by Dr. Dianne Perez) was subcloned into the modified eukaryotic expression plasmid pMT2′. Cloned rat α_{1B}-adrenoceptor cDNA (kindly provided by Dr. Robert Lefkowitz) was subcloned into the plasmid pcDV1R. The plasmids from the gene fusion constructs were harvested and purified from transfected *Escherichia coli* using the QIAGEN mega plasmid purification protocol. Briefly, transfected *E. coli* were incubated and multiplied overnight in Luria–Bertani medium with 50 μg/ml ampicillin at 37°C in a humidified incubator under an atmosphere containing 5% CO_2. The transfected *E. coli* cells were pelleted by centrifugation at $6000 \times g$ for 15 min at 4°C, resuspended and lysed. The lysed *E. coli* solution was centrifuged at $20\,000 \times g$ for 30 min at 4°C, the supernatant removed and applied to a QIAGEN-tip 2500 column. The cDNA plasmids were eluted from the column and were precipitated by addition of isopropanol with immediate centrifugation at $15\,000 \times g$ for 30 min at 4°C. The pellet was washed briefly in 70% ethanol and recentrifuged. After air drying for 5 min, the cDNA plasmid pellet was redissolved in 100–200 μl of 10 mM Tris buffer containing 100 mM NaCl, pH = 7.5. The yield of cDNA plasmid was determined by measuring the DNA concentration in a Beckman DU 650 UV spectrophotometer (Beckman Instruments, Fullerton, CA) followed by analysis on an 4% agarose gel. The cDNA concentration of α_{1B}- and α_{1A}-adrenoceptor cDNA plasmid solutions were 2.567 and 1.178 μg/ml, respectively. The cDNA plasmid solution was stored at −70°C until use.

2.3. Cell transfection and cell culture

COS-1 cells (American Type Culture Collection, Rockville, MD) were transiently transfected with 100 μg cDNA plasmids per 150 mm^2 culture dish, containing either pMT2′ α_{1A} or pcDV1R α_{1B} by the DEAE-dextran method (Cullen, 1987). Cells were harvested 48–72 h after transfection. Human embryonic kidney (HEK) 293 cells stably transfected with rat α_{1D}-adrenoceptor were kindly provided by Dr. Kenneth Minneman and Dr. Timothy Esbenshade. The cloned rat α_{1D}-adrenoceptor cDNA had been subcloned into the Epstein–Barr virus-based expression vector pREP9 and transfected into 293 cells (Minne-

man et al., 1994). Geneticin-resistant cells were selected and propagated in culture media containing 50 μg/ml Geneticin. All the cells were maintained in Dulbecco's modified Eagle's media (DMEM) with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin, at 37°C in a humidified incubator under an atmosphere containing 5% CO_2. Media were changed every other day. Confluent cells were removed from culture flasks by washing twice with ice-cold phosphate-buffered saline (PBS) solution and twice scraping the cells from each flask. The flasks were washed with PBS solution and all the cells were pooled. The intact cells were pelleted by centrifugation at $1500 \times g$ for 10 min at 4°C. The cells were resuspended and lysed in 50 mM Tris buffer containing 2 mM EDTA (pH = 7.4), and homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY).

2.4. Tissue isolation

Male Sprague–Dawley rats (Sasco, Omaha, NE) weighing 250 to 300 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and decapitated. Whole brains were rapidly removed and dissected in ice-cold 50 mM Tris buffer containing 2 mM EDTA (pH = 7.4). The tissue was immediately homogenized with a Polytron homogenizer in 10 ml ice-cold 50 mM Tris buffer.

2.5. Cell membrane preparation

The homogenates from either transfected cells or rat brain were centrifuged at $1260 \times g$ for 5 min at 4°C and the pellet was discarded. The crude cell membranes were isolated by centrifugation of the supernatant fraction at $50\,000 \times g$ for 30 min at 4°C. The final membrane pellets were stored at −70°C and were used within one week. Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.6. Radioligand saturation binding assays

The densities of each expressed α_1-adrenoceptor subtype and the rat brain were determined using saturation binding assays with [^{3}H]prazosin (0.02–2.0 nM, 79.8 Ci/mmol). The non-specific binding was determined in the presence of 100 μM phentolamine. Membrane pellets were resuspended in 50 mM Tris buffer containing 2 mM EDTA (pH = 7.4). The final incubation volume was 1 ml and each incubation was allowed to proceed to equilibrium at 37°C in duplicate for 30 min. Following equilibration, ice-cold 50 mM Tris buffer (2 mM EDTA, pH = 7.4) was added and membranes were filtered onto S&S #32 glass fiber filters (Schleicher and Schuell, Keene, NH) in a Brandel MB-48R cell harvester (Brandel, Gaithersburg, MD). Filters were washed four times with 5 ml ice-cold Tris buffer, and bound radioactivity on the filters was determined by a Beckman LS 5000 TD liquid scintillation counter (Beckman Instruments, Fullerton, CA). Binding data were analyzed by the iterative curve-fitting program LIGAND (Munson and Rodbard, 1980).

2.7. Kinetic studies

The time course of alkylation of each expressed α_1-adrenoceptor subtype by chloroethylclonidine was measured at three different temperatures, 10°C, 25°C and 37°C. Aliquots of cell membranes expressing a single α_1-adrenoceptor subtype were thus incubated with 100 μM chloroethylclonidine for a series of time intervals, 0 to 8 h (10°C), 0 to 120 min (25°C) and 0 to 60 min (37°C). In additional studies, aliquots of cell membranes from rat brain were incubated with 100 μM chloroethylclonidine at 10°C for a series of time intervals from 0 to 7 h. Each aliquot of cell membranes contained approximately 360 to 400 fmol of one of the three α_1-adrenoceptor subtypes, and was incubated in a final volume of 200 μl 50 mM Tris buffer containing 2 mM EDTA (pH = 7.4). The reactions were stopped by rapid dilution with 1.4 ml ice cold 50 mM Tris buffer containing 2 mM EDTA (pH = 7.4), followed by rapid centrifugation at $86\,000 \times g$ for 5 min at 4°C. The pellets were resuspended and this washing step was repeated twice. The fraction of receptors remaining in the non-alkylated state at each time point was determined by measuring specific [^{3}H]prazosin binding using a single concentration (0.6 nM) of the radioligand. Nonspecific binding was determined with 100 μM phentolamine. Incubations were performed in a volume of 1 ml at 37°C in duplicate for 30 min. Incubations were terminated and radioactivity was quantified as described above in radioligand saturation binding assays. To account for possible receptor degradation during the course of incubation, a time control group was included in each experiment in which membranes containing each α_1-adrenoceptor subtype were incubated for the maximal incubation time without chloroethylclonidine. At each temperature, the fraction of receptors of each subtype remaining in these control groups at the maximum incubation time was greater than 90% vs. pre-incubation values.

2.8. Data analysis

The fraction of receptors of each subtype remaining was plotted as a function of chloroethylclonidine treatment time and the experimental data were analyzed with the curve fitting program MicroMath Scientist (MicroMath Scientific Software, Salt Lake City, UT). Inactivation of α_1-adrenoceptor subtypes by alkylating agents would be predicted to follow the following equation, assuming α_1-adrenoceptors have n independent binding sites for that

alkylating agent (Adamson, 1979; Banker and Rhodes, 1979):

$$N = \Sigma N_{n0} * e^{-kn*t}$$

where N_{n0} is the number of adrenoceptor sites of type n at time $t = 0$ (before alkylating agent treatment); N is the α_1-adrenoceptor number remaining at alkylating agent treatment time t; k_n is the rate constant for alkylation of site n. Rate constants for alkylation of each subtype at each temperature were calculated from the fitted data.

The calculated rate constants for each subtype at each temperature were used to calculate the activation energies for chloroethylclonidine alkylation. The mathematical relationship between reaction temperature and rate constant can be described by the Arrhenius equation (Adamson, 1979; Banker and Rhodes, 1979):

$$k = A * e^{-E_a/RT}$$

where: A is a constant of integration; E_a is activation energy of the binding site for alkylation; R is the universal gas constant; k is the rate constant for alkylation at temperature T in Kelvin. Thus the rate constants for chloroethylclonidine alkylation were plotted vs. temperature^{-1} and the activation energy (E_a) of each subtype was determined from linear regression analysis of the data.

All the results are presented as mean ± standard error of the mean (S.E.M.) from at least four individual experiments performed in duplicated. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test, using InStat (GraphPAD Software, San Diego, CA) or SigmaPlot (Jandel, San Rafael, CA). Means were considered as significantly different if $P < 0.05$.

3. Results

3.1. Saturation studies

The K_d values of prazosin at expressed rat α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors were similar, 0.40 ± 0.11, 0.21 ± 0.05 and 0.33 ± 0.07 nM, respectively. The B_{max} values for expressed α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors were 2.4 ± 0.5, 2.6 ± 0.7 and 0.5 ± 0.1 pmol/mg protein, respectively. Since the B_{max} values for α_{1A}- and α_{1B}-adrenoceptors were much higher than those of the α_{1D}-adrenoceptor, α_{1A}- and α_{1B}-adrenoceptor protein densities were adjusted to approximate those of the α_{1D}-adrenoceptor in subsequent kinetic experiments. This was done by mixing (with sonication) cell membranes from COS-1 cells transfected with α_{1A}- or α_{1B}-adrenoceptors with non-transfected COS-1 cell membranes to yield a final density of 0.5 ± 0.1 pmol/mg protein for all subtypes. This procedure had no effect on the affinity of [^{3}H]prazosin for the α_{1A}- or α_{1B}-adrenoceptors. The final protein concentration was about 0.8 ± 0.2 mg/ml.

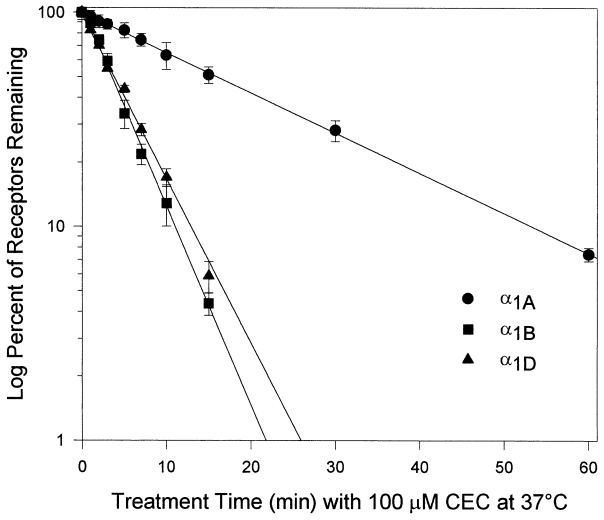

Fig. 1. Alkylation of α_1-adrenoceptor subtypes by 100 μM chloroethylclonidine at 37°C. Cell membranes were incubated with chloroethylclonidine at time intervals from 0 to 60 min ($n = 6$). After each incubation time, the fraction of receptors remaining was determined by measuring specific [^{3}H]prazosin binding. Rate constants (slopes) among the three α_1-adrenoceptor subtypes were significantly different from each other by one-way ANOVA, $P < 0.05$.

3.2. Kinetic studies

Each of the α_1-adrenoceptor subtypes were irreversibly inactivated by 100 μM chloroethylclonidine. α_{1B}- and α_{1D}-adrenoceptors were most susceptible to chloroethylclonidine alkylation, but α_{1A}-adrenoceptors were also alkylated by chloroethylclonidine, albeit at a markedly slower rate. Figs. 1–3 demonstrate the alkylation of the three α_1-adrenoceptor subtypes with 100 μM chloroethyl-

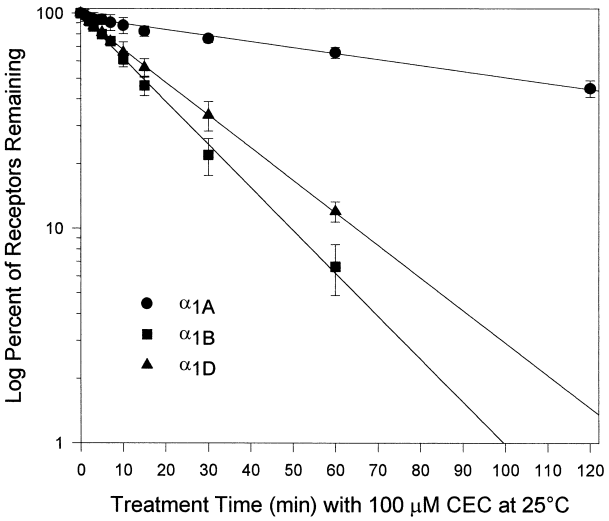

Fig. 2. Alkylation of α_1-adrenoceptor subtypes by 100 μM chloroethylclonidine at 25°C. Cell membranes were incubated with chloroethylclonidine at time intervals from 0 to 120 min ($n = 6$). After each incubation time, the fraction of receptors remaining was determined by measuring specific [^{3}H]prazosin binding. Rate constants (slopes) among the three α_1-adrenoceptor subtypes were significantly different from each other by one-way ANOVA, $P < 0.05$.

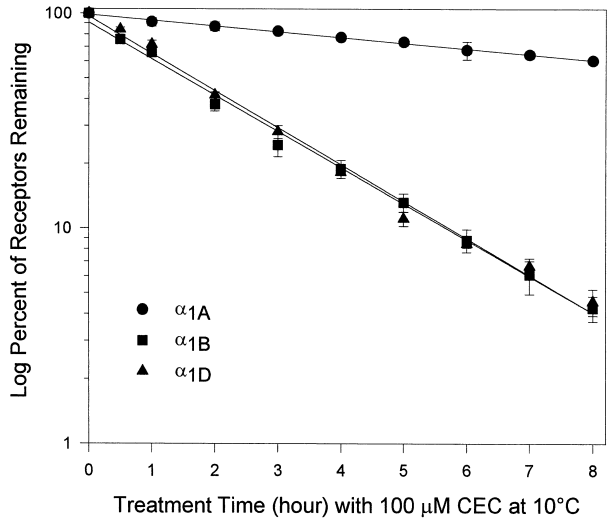

Fig. 3. Alkylation of α_1-adrenoceptor subtypes by 100 μM chloroethylclonidine at 10°C. Cell membranes were incubated with chloroethylclonidine at 10°C at time intervals from 0 to 8 h ($n = 4$). After each incubation time, the fraction of receptors remaining was determined by measuring specific [^{3}H]prazosin binding. The rate constant (slope) for α_{1A}-adrenoceptor was significantly different from rate constants of α_{1B}-adrenoceptors and α_{1D}-adrenoceptors by one-way ANOVA, $P < 0.05$. Rate constants of α_{1D}- and α_{1B}-adrenoceptors were not significantly different from each other by one-way ANOVA, $P > 0.05$.

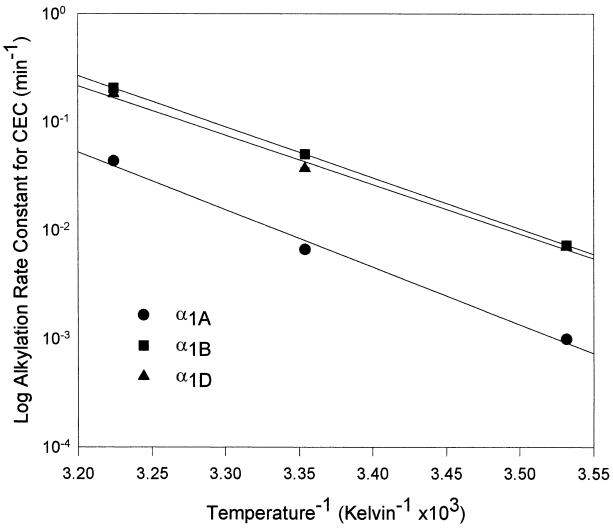

Fig. 4. Rate constants for chloroethylclonidine alkylation of the three α_1-adrenoceptor subtypes as a function of incubation temperature. The slope indicates the activation energy (E_a) of each α_1-adrenoceptor subtypes (see Table 1). The slopes were not significantly different among the three subtypes by one-way ANOVA, $P > 0.05$.

clonidine at 37°C, 25°C and 10°C. The log-linear nature of the data indicated a first order reaction and the rate constants for alkylation of each subtype (in 10^{-3} min^{-1}) at each temperature were determined by linear regression analysis. Rate constants k and reaction half times $t_{1/2}$ (min) for each receptor subtype are shown in Table 1. The rate constants for chloroethylclonidine alkylation of each subtype were plotted as a function of temperature (Fig. 4) and the activation energy E_a of each subtype was determined by linear regression analysis (Table 1). The calculated activation energies for alkylation by chloroethylclonidine of each of the three rat α_1-adrenoceptors were not significantly different.

3.3. Computer simulations

In tissues with mixed populations of α_1-adrenoceptors, the amount of alkylation of α_1-adrenoceptors by chloroethylclonidine within a short time interval has been often used to determine the fraction of α_{1A}-adrenoceptors in tissues. Since our data showed that this method may be inaccurate, we sought to determine whether measurement of the rate of alkylation by chloroethylclonidine, combined with nonlinear curve fitting, could be useful in distinguishing the relative amounts of α_{1A}-adrenoceptors vs. α_{1B}- and α_{1D}-adrenoceptors. Our previous results showed that chloroethylclonidine could not be used to distinguish α_{1B}- from α_{1D}-adrenoceptors because the k values for alkylation are similar (Table 1). Fig. 5 is a simulation of data in which chloroethylclonidine (100 μM at 10°C) is used to inactivate a combination of α_1-adrenoceptors which contain 50% α_{1A}- and 50% α_{1B}-adrenoceptors. To perform this simulation, we used the equation derived from the nonlinear regression analysis of our data from alkylation of cloned rat α_1-adrenoceptors:

$$Y = N_{(\alpha 1A)0} * e^{-K_{(\alpha 1A)} * X} + \left(1 - N_{(\alpha 1A)0}\right) * e^{-K_{(\alpha 1B)} * X}$$

where $K_{(\alpha 1A)} = 0.99 \pm 0.01$, $K_{(\alpha 1B)} = 7.26 \pm 0.15$ (10^{-3}

Table 1
Rate constants k (in 10^{-3} min^{-1}) and half time $t_{1/2}$ (min) for alkylation of cloned α_1-AR subtypes by 100 μM chloroethylclonidine in transfected cell membranes and activation energy E_a (in kJ · mol^{-1}) calculated for each receptor subtype[a]

	10°C ($n = 4$)		25°C ($n = 6$)		37°C ($n = 6$)		
	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	E_a
α_{1A}	0.99 ± 0.01^c	700.15 ± 7.00^c	6.63 ± 0.16^c	105.02 ± 2.46^c	43.46 ± 0.37^c	15.93 ± 0.13^c	100.57 ± 5.26
α_{1B}	7.26 ± 0.15^b	95.47 ± 1.93^b	$50.17 \pm 0.52^{b,c}$	$13.81 \pm 0.14^{b,c}$	$205.61 \pm 5.39^{b,c}$	$3.37 \pm 0.09^{b,c}$	90.18 ± 0.01
α_{1D}	7.01 ± 0.12^b	98.88 ± 1.66^b	36.98 ± 0.44^b	18.73 ± 0.22^b	182.02 ± 2.87^b	3.81 ± 0.06^b	87.05 ± 3.91

[a]Data are expressed as mean ± S.E.M. of the number (n) of experiments shown, each performed in duplicate. Data were analyzed by one-way ANOVA followed by the Student–Newman–Keul test. The difference was considered as significant if $P < 0.05$.
[b]$P < 0.05$ for comparisons with α_{1A}-AR.
[c]$P < 0.05$ for comparisons with α_{1D}-AR.

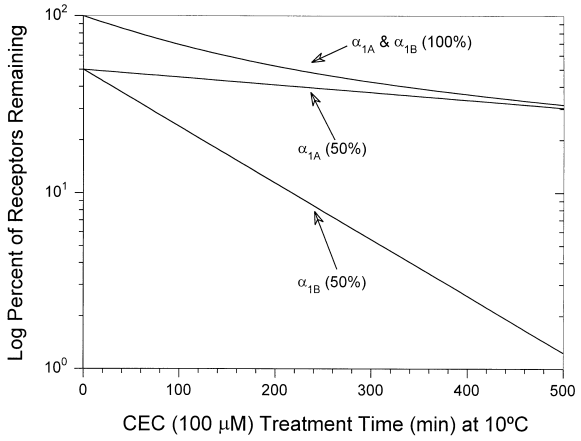

Fig. 5. Computer simulation of a combination of 50% α_{1A}-adrenoceptors and 50% α_{1B}-adrenoceptors inactivated with 100 μM chloroethylclonidine at 10°C. The upper curve is the computer predicted curve in which a combination of 50% α_{1A}-adrenoceptors and 50% α_{1B}-adrenoceptors is inactivated over time with 100 mM chloroethylclonidine at 10°C. The curves of the two component subtypes (α_{1A}- and α_{1B}-adrenoceptors) inactivated with 100 μM chloroethylclonidine each individually at 10°C can be extracted and are shown as the lower two curves (see text for details).

min^{-1}), X is the chloroethylclonidine treatment time, Y is the percentage of receptors remaining at time X and $N_{(\alpha 1A)0}$ is the percentage of α_{1A}-adrenoceptor in the mixture before chloroethylclonidine treatment. The result is a curvilinear plot from which the K values and relative percentages of each component can be extracted by nonlinear curve fitting (MicroMath Scientist). The curves of the two component subtypes (α_{1A}- and α_{1B}-adrenoceptors) inactivated with 100 μM chloroethylclonidine can be extracted and are shown as the lower two curves, using the equation $Y = 50 * e^{-K * X}$, where K value is either 0.99 or 7.26 (10^{-3} min^{-1}). Fig. 6 is a computer simulation of

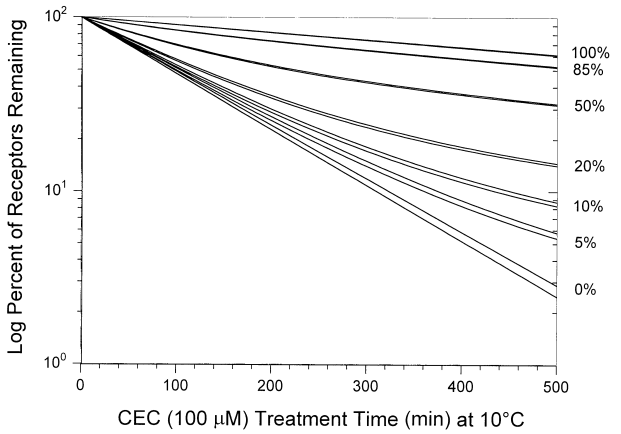

Fig. 6. Computer simulation of chloroethylclonidine alkylation of different proportions of α_{1A}-adrenoceptor and α_{1B}-adrenoceptor inactivated with 100 μM chloroethylclonidine at 10°C. Curves are plotted as the mean ± S.E.M. for each composition. Each curve is marked as the proportion of α_{1A}-adrenoceptor.

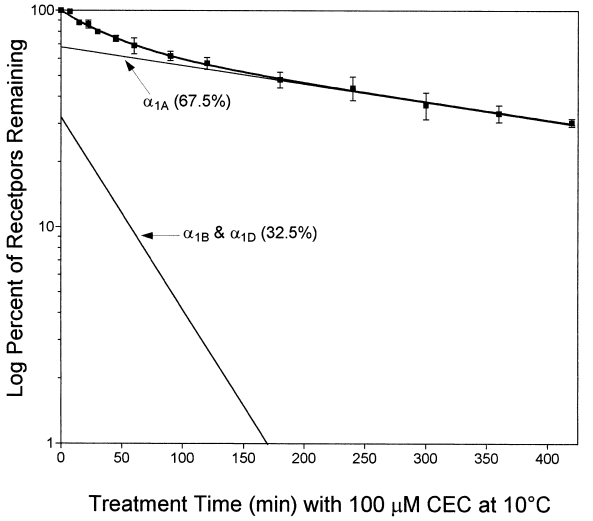

Fig. 7. Alkylation of α_1-adrenoceptor subtypes in rat brain by 100 μM chloroethylclonidine at 10°C. Cell membranes from rat brain were incubated with chloroethylclonidine at 10°C at time intervals from 0 to 7 h ($n = 4$). After each incubation time, the fraction of receptors remaining was determined by measuring specific [^{3}H]prazosin binding. The upper curve shows the alkylation of α_1-adrenoceptor subtypes in rat brain overtime by chloroethylclonidine. Data were analyzed by nonlinear regression and the curves for inactivation of the two component groups (α_{1A}-adrenoceptor, and α_{1B}- and α_{1D}-adrenoceptors) were extracted and are shown as the lower two curves.

different proportions of α_{1A}-adrenoceptor and α_{1B}-adrenoceptor inactivated with 100 μM chloroethylclonidine at 10°C. This method was also applied to the combination of α_{1A}-adrenoceptor and α_{1D}-adrenoceptor (not shown). Because the rate of alkylation for α_{1B}-adrenoceptor and α_{1D}-adrenoceptor was not different at 10°C, the curves in Figs. 5 and 6 are similar to a simulation with α_{1A}-adrenoceptor and α_{1D}-adrenoceptor, (or α_{1A}-adrenoceptor and any combination of α_{1B}- and α_{1D}-adrenoceptor).

3.4. Relative α_{1A}-adrenoceptor density in rat brain

The methods used in the above simulations were tested in rat brain. Fig. 7 demonstrates the alkylation of α_1-adrenoceptors in rat brain with 100 μM chloroethylclonidine at 10°C. The data were analyzed by nonlinear regression and indicated a second order reaction as predicted. The results are consistent with our data for cloned α_1-adrenoceptors in that there are two groups of α_1-adrenoceptors in rat brain (α_{1A}-adrenoceptors, and α_{1B}- and α_{1D}-adrenoceptors). The proportion of α_{1A}-adrenoceptors in rat brain is estimated from these data to be 67.5 ± 4.7%.

4. Discussion

In the present study, we measured the rate of alkylation of α_1-adrenoceptor subtypes of a single species in an attempt to clarify some of the conflicting data and ideas

about the action of chloroethylclonidine. These studies produced three major findings: First, we found that the cloned rat α_{1A}-adrenoceptor is sensitive to chloroethylclonidine, but at a markedly slower rate than the other two subtypes. The activation energy for alkylation of the α_{1A}-adrenoceptor is similar to that of the other two subtypes. Second, we found that the rat α_{1D}-adrenoceptor is fully susceptible to alkylation by chloroethylclonidine, at a rate that is similar to that of the α_{1B}-adrenoceptor. Third, we found that the relative density of α_{1A}-adrenoceptor in a mixed population of α_1-adrenoceptor subtypes can be calculated by nonlinear curve fitting of the data obtained from a kinetic analysis of chloroethylclonidine inactivation of [^{3}H]prazosin binding sites. This method was tested and found to be useful in tissue by using cell membranes from rat brain.

Our findings challenge several notions regarding α_1-adrenoceptor subtypes. As stated above, many investigations have used chloroethylclonidine to attempt to distinguish among α_1-adrenoceptor populations or responses. Our data suggest that this may not be possible when using a single chloroethylclonidine concentration and incubation (or infusion) time, since all three subtypes are sensitive to chloroethylclonidine and the rates of alkylation in the α_{1B}-adrenoceptor and α_{1D}-adrenoceptor are nearly identical. Reports (Lomasney et al., 1991; Perez et al., 1991, 1994) that the α_{1D}-adrenoceptor is only partially sensitive to chloroethylclonidine are also challenged by our work. Perez et al. (1991, 1994) found that following chloroethylclonidine treatment, the cloned rat α_{1D}-adrenoceptor appeared less sensitive to chloroethylclonidine than the α_{1B}-adrenoceptor and that at least 25% of the α_{1D}-adrenoceptor binding sites remained (as measured by the antagonist 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl]tetralone also known as [^{125}I]HEAT), regardless of incubation time. Based on these reports, we expected to find that chloroethylclonidine inactivates the α_{1D}-adrenoceptor at a rate slower than the α_{1B}-adrenoceptor; we also expected a biphasic pattern of inactivation of the α_{1D}-adrenoceptor subtype by chloroethylclonidine. However, in our experiments, we found that chloroethylclonidine rapidly and completely inactivated the α_{1D}-adrenoceptor subtype in a first-order reaction in a manner similar to that seen for the α_{1B}-adrenoceptor. Thus we found no evidence for α_{1D}-adrenoceptor heterogeneity or unusual kinetics of inactivation by chloroethylclonidine. We can not currently explain the discrepancy between our data and those of other investigators. It is possible that experimental differences (e.g., [^{3}H]prazosin vs. [^{125}I]HEAT), may have played a role in our different observations. However, in studies with chloroethylclonidine, Han et al. (1994) and Esbenshade et al. (1995) found that similar concentration of chloroethylclonidine (100 μM) produced similar amount of inhibition of the α_{1B}- and α_{1D}-adrenoceptor subtypes of different species, supporting our observation that this reaction proceeds at identical rate for both subtypes.

Many investigators have reported that rat α_{1A}-adrenoceptors are 'insensitive' to chloroethylclonidine. These reports led us to speculate that the resistance to alkylation could be the result of a structural difference among the α_1-adrenoceptor subtypes, most likely due to a difference in the amino acid sequence of the receptors. However, our experiments revealed that the α_{1A}-adrenoceptor is completely alkylated by chloroethylclonidine, albeit at a markedly slower rate than α_{1B}- or α_{1D}-adrenoceptors. To determine whether differences in chloroethylclonidine alkylation rates were due to actions at different binding sites, we calculated the activation energies for alkylation of each receptor using the Arrhenius equation, which describes the mathematical relationship between reaction temperatures and rate constants. The calculated activation energies for alkylation by chloroethylclonidine of the three rat α_1-adrenoceptors were not significantly different, implying that the three subtypes may have similar or identical binding sites for chloroethylclonidine (Adamson, 1979; Banker and Rhodes, 1979). Another explanation for differences in chloroethylclonidine sensitivity was recently advanced by Hirasawa et al. (1997), who found that the α_{1A}-adrenoceptor has a greater intracellular distribution vs. α_{1B}-adrenoceptors expressed in COS cells. These authors suggest that differences in cellular distribution of α_1-adrenoceptors results in the reduced sensitivity of the α_{1A}-adrenoceptor to chloroethylclonidine. However, in our experiments, which used isolated plasma membranes rather than whole cells, we observed a slower alkylation rate of α_{1A}-adrenoceptors vs. the other subtypes. This suggests that inherent differences in the alkylation rate of the α_{1A}-adrenoceptor exist irrespective of cellular α_1-adrenoceptor distribution.

One of the difficulties in using chloroethylclonidine to measure relative α_1-adrenoceptor densities is that any treatment protocol will result in a fraction of α_{1A}-adrenoceptor inactivation prior to complete inactivation of the α_{1B}- and α_{1D}-adrenoceptor subtypes. However, during the course of our studies we recognized that the relative density of α_{1A}-adrenoceptors vs. the other two subtypes could be determined from a mixed population in kinetic experiments similar to those used in our protocols. This can be accomplished because the difference in the rates of inactivation between the α_{1A}-adrenoceptor and the other two subtypes are sufficiently different to resolve multiple sites through non-linear curve fitting analysis of the kinetic data. Further, the analysis is uncomplicated since chloroethylclonidine alkylates each of the subtypes at a single site by a first-order reaction. If we consider the receptor subtypes in two groups, the α_{1A}-adrenoceptors (slowly alkylated), and $\alpha_{1B/D}$-adrenoceptors (rapidly alkylated), we can determine the proportion of α_{1A}-adrenoceptors in a mixed population by using a single kinetic assay (Figs. 5 and 6). In our simulations, we changed the constitution of α_1-adrenoceptor subtypes to different proportions and determined the utility of the model and its sensitivity.

To test the utility of this method, cell membranes from rat brain were incubated with 100 μM chloroethylclonidine at 10°C for different periods of time. Rat brain is a tissue that reportedly contains all three α_1-adrenoceptor subtypes (Price et al., 1994; Scofield et al., 1995). The data indicated a second order reaction with 67% of the total α_1-adrenoceptors of the α_{1A} subtype. This result is consistent with a previous report in the same tissue (Johnson and Minneman, 1987). The rate constants (in 10^{-3} min^{-1}) for the α_{1A}-adrenoceptor (1.93 ± 0.24) and α_{1B}/α_{1D}-adrenoceptor (20.61 ± 3.72) calculated by nonlinear regression in rat brain at 10°C are somewhat different to those calculated in transfected COS-1 cells ($K_{(\alpha 1A)} = 0.99 \pm 0.01$, $K_{(\alpha 1B/\alpha 1D)} = 7.26 \pm 0.15$). The reason for the small differences in the calculated rate constants between the transfected cells and rat brain is unknown, but the relative differences among the subtypes were preserved in the native tissue. This result confirms that this method is useful in accurately resolving the proportion of α_{1A}-adrenoceptor in a mixed population of α_1-adrenoceptor subtypes. We have also recently employed this method to help characterize a cloned mouse α_1-adrenoceptor subtype (Xiao et al., 1998). In these studies, chloroethylclonidine fully alkylated the cloned mouse α_{1A}-adrenoceptor at a rate that was similar to that seen for the rat α_{1A}-adrenoceptor subtype, confirming its identity as a mouse α_{1A}-adrenoceptor.

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References

Adamson, A.W., 1979. A Textbook of Physical Chemistry, 2nd edn., Academic Press, New York, pp. 562–568.

Banker, G.S., Rhodes, C.T., 1979. Modern Pharmaceutics. Marcel Dekker, New York, pp. 244–248.

Bylund, D.B., Eikenberg, D.C., Hieble, J.P., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Molinoff, P.B., Ruffolo, R.R., Trendelenburg, U., 1994. International union of pharmacology nomenclature of adrenoceptors. Pharmacol. Rev. 46, 121–136.

Cotecchia, S., Schwinn, D.A., Randall, R.R., Lefkowitz, R.J., Caron, M.G., Kobilka, B.K., 1988. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. Proc. Natl. Acad. Sci. USA 85, 7159–7163.

Cullen, B.R., 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. 152, 684–704.

Esbenshade, T.A., Hirasawa, A., Tsujimoto, G., Tanaka, T., Yano, J., Minneman, K.P., Murphy, T.J., 1995. Cloning of the human α_{1d}-adrenergic receptor and inducible expression of three human subtypes in SK-N-MC cell. Mol. Pharmacol. 47, 977–985.

Graham, R.M., Perez, D.M., Hwa, J., Piascik, M.T., 1996. α_1-adrenergic receptor subtypes molecular structure, function, and signaling. Circ. Res. 78, 737–749.

Han, C., Abel, P.W., Minneman, K.P., 1987. Heterogeneity of α_1-adrenergic receptors revealed by chloroethylclonidine. Mol. Pharmacol. 32, 505–510.

Han, C., Hollinger, S., Theroux, T.L., Esbenshade, T.A., Minneman, K.P., 1994. ^{3}H-tamsulosin binding to cloned α_1-adrenergic receptor subtypes expressed in human embryonic kidney 293 cells: antagonist potencies and sensitivity to alkylating agents revealed. Pharmacol. Commun. 5, 117–126.

Hieble, J.P., Bylund, D.B., Clarke, D.E., Eikenberg, D.C., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Ruffolo, R.R., 1995. International union of pharmacology: X. Recommendation for nomenclature of α_1-adrenoceptors: consensus update. Pharmacol. Rev. 47, 267–270.

Hirasawa, A., Horie, K., Tanaka, T., Takagaki, K., Murai, M., Yano, J., Tsujimoto, G., 1993. Cloning, functional expression and tissue distribution of human cDNA for the α_{1C}-adrenergic receptor. Biochem. Biophys. Res. Commun. 195, 902–909.

Hirasawa, A., Sugawara, T., Awaji, T., Tsumaya, K., Ito, H., Tsujimoto, G., 1997. Subtype-specific differences in subcellular localization of α_1-adrenoceptors: chloroethylclonidine preferentially alkylates the accessible cell surface α_1-adrenoceptors irrespective of the subtype. Mol. Pharmacol. 52, 764–770.

Johnson, R.D., Minneman, K.P., 1987. Differentiation of α_1-adrenergic receptor linked to phosphatidylinositol turnover and cyclic AMP accumulation in rat brain. Mol. Pharmacol. 31, 239–246.

Laz, T.M., Forry, C., Smith, K.E., Brad, J.A., Vaysse, P.J.-J., Branchek, T.A., Weinshank, R.L., 1994. The rat homologue of the bovine α_{1C}-adrenergic receptor shows the pharmacological properties of the classical α_{1A} subtype. Mol. Pharmacol. 46, 414–422.

Lomasney, J.W., Cotecchia, S., Lorenz, W., Leung, W.Y., Schwinn, D.A., Yang-Feng, T.L., Brownstein, M., Lefkowitz, R.J., Caron, M.G., 1991. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor. J. Biol. Chem. 266, 6365–6369.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 264–275.

Michel, M.C., Kerker, J., Branchek, T.A., Forray, C., 1993. Selective irreversible binding of chloroethylclonidine at α_1- and α_2-adrenoceptor subtypes. Mol. Pharmacol. 44, 1165–1170.

Minneman, K.P., Han, C., Abel, P.W., 1988. Comparison of α_1-adrenergic receptor subtypes distinguished by chloroethylclonidine and WB 4101. Mol. Pharmacol. 33, 509–514.

Minneman, K.P., 1988. α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. Pharmacol. Rev. 40, 87–119.

Minneman, K.P., Theroux, T.L., Hollinger, S., Han, C., Esbenshade, T.A., 1994. Selectivity of agonists for cloned α_1-adrenergic receptor subtypes. Mol. Pharmacol. 46, 929–936.

Munson, P.J., Rodbard, D., 1980. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107, 220–239.

Morrow, A.L., Creese, I., 1986. Characterization of α_1-adrenergic receptor subtypes in rat brain: A reevaluation of [^{3}H]WB4101 and [^{3}H]prazosin binding. Mol. Pharmacol. 29, 321–330.

Perez, D.M., Piascik, M.T., Graham, R.M., 1991. Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA. Mol. Pharmacol. 40, 876–883.

Perez, D.M., Piascik, M.T., Graham, R.M., 1994. Cloning, expression, and tissue distribution of the rat homolog of the bovine α_{1C}-adrenergic receptor provide evidence for its classification as the α_{1A} subtype. Mol. Pharmacol. 46, 823–831.

Piascik, M.T., Butler, B.T., Kusiak, J.W., Pitha, J., Holtman, J.R., 1989.

Effect of an alkylating analog of prazosin on α_1-adrenoceptor subtypes and arterial blood pressure. J. Pharmacol. Exp. Ther. 251, 878.

Piascik, M.T., Sparks, M.S., Pruitt, T.A., 1991. Interaction of imidazolines with alkylation sensitive and resistant α_1-adrenoceptor subtypes. J. Pharmacol. Exp. Ther. 258, 158–165.

Price, D.T., Chari, R.S., Berkowitz, D.E., Meyers, W.C., Schwinn, D.A., 1994. Expression of α_1-adrenergic receptor subtype mRNA in rat tissue and human SK-N-MC neuronal cells: implications for α_1-adrenergic receptor subtype classification. Mol. Pharmacol. 46, 221–226.

Schwinn, D.A., Lomasney, J.W., Lorenz, W., Szklut, P.J., Fremeau, R.T. Jr., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J., Cotecchia, S., 1990. Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. J. Biol. Chem. 265, 8183–8189.

Scofield, M.A., Liu, F., Able, P.W., Jeffries, W.B., 1995. Quantification of steady state expression of mRNA for α-1 adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. J. Pharmacol. Exp. Ther. 275, 1035–1042.

Xiao, L., Scofield, M.A., Jeffries, W.B., 1998. Molecular cloning, expression and characterization of cDNA encoding a mouse α_{1a}-adrenoceptor. Br. J. Pharmacol., in press.