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Rat pineal α_1-adrenoceptor subtypes: studies using radioligand binding and reverse transcription-polymerase chain reaction analysis

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1 The pharmacological characteristics of α_1-adrenoceptor binding sites in rat pineal gland membranes, detected by use of a selective α_1-adrenoceptor antagonist ([^{125}I]-iodo-2-[β-(4-hydroxyphenyl) ethylaminomethyl]tetralone, [^{125}I]-HEAT), were investigated with the alkylating agent, chloroethylclonidine (CEC), and in competition experiments with a number of adrenoceptor agonists and antagonists.

2 Chloroethylclonidine (CEC) treatment (10 μM, 10 min) of rat pineal membranes inactivated ~70% of specific [^{125}I]-HEAT binding sites. Higher concentrations of CEC (up to 100 μM) or longer treatment periods (upto 40 min) were no more effective.

3 Adrenoceptor agonists and antagonists competitively inhibited [^{125}I]-HEAT binding with Hill coefficients close to unity indicating a single α_1-adrenoceptor subtype is present. The affinity (K_i) of subtype selective agonists (oxymetazoline, SDZ NVI-085) and antagonists (5-methylurapidil, WB4101, benoxathian, phentolamine) was consistent with binding to an α_{1B}-adrenoceptor subtype.

4 The (−)- and (+)-enantiomers of niguldipine had an equal and low affinity for α_1-adrenoceptor binding sites both in untreated (log K_i −6.66 and −6.90 respectively) and CEC-treated membranes in which ~70% of sites had been inactivated (log K_i −6.41 and −6.86 respectively). This indicates that the small proportion of α_1-adrenoceptors insensitive to CEC are not α_{1A}-adrenoceptors.

5 mRNA was isolated from rat pinealocytes, cDNA was synthesized and then amplified by the polymerase chain reaction with α_1-adrenoceptor subtype specific primers. These experiments identified both α_{1A}- and α_{1B}-adrenoceptor mRNA, but not α_{1D}-mRNA in rat pinealocytes, although all three adrenoceptor subtypes were readily identified in rat brain cortex.

6 These data indicate that although both α_{1A}- and α_{1B}-adrenoceptor mRNAs are present in the pineal the major subtype of α_1-adrenoceptor expressed is the α_{1B}.

Keywords: Pineal; α_1-adrenoceptor subtypes; mRNA expression; reverse transcription-polymerase chain reaction (RT–PCR)

Introduction

Pharmacological and molecular cloning experiments have shown that distinct subtypes of α_1-adrenoceptors exist (Bylund *et al.*, 1994; Minneman & Esbenshade, 1994). Functional studies indicate that two pharmacologically distinct α_1-adrenoceptor subtypes (designated α_{1A}- and α_{1B}-) can be distinguished (Minneman, 1988; Ruffolo *et al.*, 1991; Garcia-Sainz, 1993; Bylund *et al.*, 1994). Molecular cloning has isolated three α_1-adrenoceptor subtypes. The α_{1b}-adrenoceptor cDNA, which was isolated from a hamster smooth muscle cell line, encodes a receptor with the properties and tissue expression expected of the pharmacologically defined native, classical α_{1B}-adrenoceptor (Cotecchia *et al.*, 1988). The rat homologue of the hamster α_{1b}-adrenoceptor cDNA has also been obtained (Voigt *et al.*, 1990). A second subtype initially designated, α_{1c}, was originally cloned from bovine brain (Schwinn *et al.*, 1990) and more recently from several rat sources (Laz *et al.*, 1994; Stewart *et al.*, 1994; Perez *et al.*, 1994). The pharmacological properties of the expressed cloned rat α_{1c}-adrenoceptor and its mRNA distribution suggest that the α_{1c}-adrenoceptor gene actually encodes the native classical α_{1A}- subtype identified in many functional studies. A third subtype has been cloned (Lomasney *et al.*, 1991) which was originally thought to correspond the α_{1A}-adrenoceptor. A more rigorous pharmacological analysis has shown clear differences with the classical native α_{1A}-subtype (Schwinn & Lomasney, 1992) and it is now agreed that this is a novel subtype identified as the α_{1d}-adrenoceptor.

The major functional consequence of a α_1-adrenoceptor activation in most cells is an increase in intracellular Ca^{2+}. Early studies suggested that native α_{1A}- and α_{1B}-adrenoceptors increased Ca^{2+} by different mechanisms (Minneman, 1988); it was suggested that α_{1A}-adrenoceptors gated Ca^{2+} influx through voltage-sensitive channels while α_{1B}-adrenoceptors mobilized intracellular Ca^{2+}. It is generally agreed that activation of the α_{1B}-adrenoceptor increases phospholipase C generating the formation of diacylglycerol (DAG) which can activate protein kinase C, and inositol (1,4,5)trisphosphate which mobilizes Ca^{2+} from intracellular stores. The α_{1B}-adrenoceptor can also activate Ca^{2+} influx (Klijn *et al.*, 1991), although voltage-gated channels are not usually involved. Many studies have found that α_{1A}-adrenoceptor activation increases the influx of extracellular Ca^{2+} through voltage-dependent channels (Minneman & Esbenshade, 1994), but an increase in inositol phosphate formation may also occur (Wilson & Minneman, 1990).

α_1-Adrenoceptors are of importance in pineal regulation because the synthesis of the pineal gland hormone, melatonin, and thus circulating serum melatonin concentration, is precisely regulated by noradrenaline (NA). This transmitter is released into the pineal perivascular space from sympathetic nerve fibres. The adrenergic projection conveys information about the photoperiod from the retina to the pineal via a polysynaptic pathway which includes the suprachiasmatic nuclei of the hypothalamus and adrenergic perikarya in the superior cervical ganglia. In an alternating light: dark cycle the neural signals reaching the gland release NA only at night, restricting melatonin synthesis to the nocturnal hours. In pinealocytes NA increases the synthesis of intracellular ade-

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nosine 3':5'-cyclic monophosphate (cyclic AMP), the key second messenger needed to induce the nocturnal synthesis of the pineal-specific, rate-limiting enzyme, 5-hydroxytryptamine *N*-acetyltransferase. The elevation in pinealocyte cyclic AMP is mediated by an action of NA on β- and α_1-adrenoceptors; α_1-adrenoceptor activation amplifies β-adrenoceptor-mediated stimulation of cyclic AMP synthesis dramatically (Vanecek *et al.*, 1985). The mechanism involves α_1-adrenoceptor-mediated elevation of intracellular Ca^{2+} (Sugden *et al.*, 1986) and hydrolysis of phospholipase C (Smith *et al.*, 1979) leading to activation of protein kinase C (Sugden *et al.*, 1985). *In vitro* studies on isolated pinealocytes and pineal gland explants have shown that α_1-adrenoceptor agonists enhance melatonin synthesis (Klein *et al.*, 1983) and *in vivo* experiments with adrenoceptor antagonists have shown that α_1-adrenoceptor activation contributes to the nocturnal elevation in melatonin synthesis (Sugden, 1995).

Previous studies have identified α_1-adrenoceptor sites on pineal gland membranes by use of radioligand binding assays (Sugden & Klein, 1984; Sugden *et al.*, 1995) and α_1-mediated elevation in intracellular Ca^{2+} (Sugden *et al.*, 1986) and phosphatidylinositol hydrolysis (Ho *et al.*, 1988) but there has been no attempt to characterize the pineal α_1-adrenoceptor subtype(s). The present study used radioligand binding assays to establish the pharmacological characteristics of the pineal membrane α_1-adrenoceptor and the reverse transcription-polymerase chain reaction to identify the α_1-adrenoceptor subtype specific mRNA in pinealocytes.

Methods

Radioligand binding studies

Radioligand binding studies were carried out as described previously (Sugden & Klein, 1984). Briefly, pineal glands were collected from adult Sprague-Dawley rats during the daytime. Approximately 25 glands were briefly sonicated (3×5 s) on ice in 250 μl of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA (buffer B) containing 1 mM phenylmethylsulphonyl fluoride. The homogenate was diluted with 7 ml of buffer B and membranes were collected by centrifugation (100 000 $\times g$, 20 min, 4°C) and used in radioligand binding assays. [^{125}I]-HEAT, an α_1-adrenoceptor antagonist with comparable affinity for the native and cloned α_1-adrenoceptor subtypes (Minneman *et al.*, 1994) was incubated (25°C, 20 min) with membranes (3–5 μg/tube). Incubations were terminated by rapid vacuum filtration through glass-fibre filters (Whatman GF/C) soaked in polyethyleneimine (1% v/v) to separate free and bound radioactivity. Filters were washed twice with 5 ml of ice-cold buffer B. Saturation assays used a range of concentrations of [^{125}I]-HEAT (2 to 900 pM). Competition studies were done with a fixed concentration of [^{125}I]-HEAT (40 to 50 pM) and dilutions of various subtype selective α_1-adrenoceptor agonists and antagonists. Niguldipine enantiomers were dissolved and diluted in dimethylsulphoxide to avoid adsorption to tubes and pipette tips (Boer *et al.*, 1989). All binding studies with adrenoceptor agonists were done in the presence of GTP (100 μM). Phentolamine (10^{-5} M) was used to define non-specific binding.

In some experiments, chloroethylclonidine (CEC) was pre-incubated (10 μM, 10 min 37°C) with membranes in a hypotonic buffer (10 mM HEPES, pH 7.6). Membranes were then washed three times in 50 volumes of buffer to remove reversibly bound CEC, before they were used in saturation assays. In each CEC experiment, an aliquot of the same rat pineal membrane preparation was treated in an identical manner except that buffer, instead of CEC, was added to the incubation. In one experiment, the concentration of CEC used (0.1–100 μM) and the duration of incubation (10–40 min) were varied and specific binding determined with a single [^{125}I]-HEAT concentration (200 pM).

Data analysis

Saturation curves were analyzed by non-linear regression analysis with the ENZFITTER programme (Leatherbarrow, 1987) which uses the equation

$$B = B_{max} \cdot F/(K_d + F)$$

where B = the concentration of ligand bound to the receptor, F = the concentration of free ligand, K_d = the equilibrium dissociation constant and B_{max} = the maximal concentration of binding sites. IC_{50} values were determined in competition experiments by use of the ALLFIT programme (De Lean *et al.*, 1978) with the four parameter logistic equation

$$Y = \frac{A - D}{1 + (X/C)^B} + D$$

where X is the concentration of the competing compound, Y is percentage inhibition of specific binding, A is the maximal binding in the absence of competitor, B is the slope factor, C is the IC_{50} and D is the minimal binding (nonspecific binding). K_i values were determined by the Cheng-Prusoff equation (Cheng & Prusoff, 1973). Statistical comparisons were made by use of Student's *t* test.

Cell culture

Pinealocytes were prepared from adult male Sprague-Dawley rat pineal glands by trypsinization (Buda & Klein, 1978) and maintained in suspension culture (1 to 1.5×10^5 cells ml^{-1} in Dulbecco's Modified Eagle's medium containing foetal calf serum (10% v/v) under a humidified atmosphere of 95% air/5% CO_2 at 37°C for 20 to 24 h before use. The yield of cells was between 1.6 and 0.4×10^5 cells/gland. The viability of pinealocytes was assessed by trypan blue 1 and 24 h after cell preparation was >95% in all experiments.

Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was extracted from rat pinealocytes ($1-1.5 \times 10^5$ cells) by the acid guanidinium isothiocyanate method of Chomczynski & Sacchi (1987). Briefly, cells were collected by centrifugation (5,000 *g*, 1 min), washed with Dulbecco's phosphate-buffered saline, re-centrifuged and the pellet lysed immediately in 200 μl of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% v/v sarcosyl, 0.1 M 2-mercaptoethanol) containing dextran (10 μg) as a carrier. RNA was extracted once with 0.1 vol of 2 M sodium acetate, pH 4, 1 vol of water-saturated phenol and 0.2 vol of chloroform-isoamyl alcohol (49:1). RNA was precipitated with isopropanol (−20°C, 2 h) and the pellet washed with ice-cold ethanol (70% v/v). The RNA pellet was dissolved in diethylpyrocarbonate-treated water and treated (37°C, 15 min) with RQ1-DNase (5 u, Promega, Southampton); it was re-extracted with acid/phenol, and reprecipitated with ethanol. Rat brain cortex was sonicated in solution D and total RNA extracted as described above for rat pinealocytes.

First strand cDNA was made immediately. Oligo $dT_{(15)}$ (25 ng) was added to RNA (~0.5 μg) and the mixture heated (80°C, 5 min) to remove secondary RNA structure then cooled immediately on ice. Dithiothreitol (20 mM, DTT), 0.5 mM each dATP, dCTP, dTTP, dGTP, 40 u of ribonuclease inhibitor (RNasin, Promega) and 15 u of avian myoblastosis virus-reverse transcriptase (AMV-RT; Promega) were added and incubated at 42°C for 1 h. AMV-RT was inactivated by heating at 99°C for 5 min. A 10 fold dilution of the RT reaction mixture containing ~2.4 ng of cDNA was amplified in a Hybaid Omnigene thermal cycler in a reaction (20 μl) containing 100 μM each deoxynucleoside 5'-triphosphate, 50 mM KCl, 10 mM Tris HCl, pH 8.3, 0.5% v/v glycerol, 0.1% v/v Triton X-100, 0.5 μM each PCR primer and 1 u of *Taq* DNA

polymerase (Promega). The concentration of $MgCl_2$ used for amplifying α_{1a}- and α_{1d}-adrenoceptors was 1 mM, and 1.5 mM for α_{1b}-adrenoceptors. Thermal cycling conditions were 1 min at 94°C, 1 min at 55°C and 2 min at 72°C for 35–40 cycles with a final extension of 10 min at 72°C. Primer pairs used were designed with the aid of the PRIME programme on the Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984) from published sequences to amplify regions within the cytoplasmic tail of the α_{1b}-adrenoceptor (nucleotides 1424–1764, Voigt *et al.*, 1990), spanning predicted transmembrane regions VI and VII of the α_{1a}-adrenoceptor (nucleotides 797–1220, Stewart *et al.*, 1994; Laz *et al.*, 1994), and the 3'-untranslated region of the α_{1d}-adrenoceptor (nucleotides 2432–2921, Lomasney *et al.*, 1991).

α_{1b}-Adrenoceptor sense primer: 5'-TGG AGA GAT CAC AGT CGC-3'; antisense primer: 5'-CAT GTT GCT CTT GAA GCC-3'; α_{1a}-Adrenoceptor sense primer: 5'-GAC TCA CTT CTC AGT GAG GC-3'; antisense primer: 5'-ACA GAC TCC ATC TGT CTT GG-3'; α_{1d}-Adrenoceptor sense primer: 5'-CTC CTA GTG TCT AAG CAG AAG G-3'; antisense primer: 5'-GAA GAA GGA GGC TCT ATA TGC-3'.

The predicted size of the α_{1b} PCR product was 341 bp, the α_{1a} product 424 bp and the α_{1d} product 490 bp. PCR reaction products were resolved by agarose gel electrophoresis (2.0% w/v) and stained with ethidium bromide (0.5 μg ml^{-1}). The identity of the PCR reaction products was determined by overnight digestion with the following restriction endonucleases (all Promega): *Sau3AI* (10 u, 37°C); *HinfI* (10 u, 37°C); *SmaI* (10 u, 30°C).

Drugs

5-Methylurapidil hydrochloride, WB4101 hydrochloride, benoxathian hydrochloride and chloroethylclonidine dihydrochloride (CEC) were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). (−)- and (+)-enantiomers of niguldipine hydrochloride were a generous gift from Dr Rainer Boer, Byk Gulden Lomberg GmbH, Konstanz, Germany. Cirazoline was provided by Synthelabo (Paris, France) and SDZ NVI-085 by Sandoz (Basel, Switzerland). All other reagents including adrenoceptor agonists and antagonists, GTP, polyethylenimine, phenylmethylsulphonyl fluoride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [^{125}I]-iodo-2-[β-(4-hydroxyphenyl) ethylaminomethyl] tetralone ([^{125}I]-HEAT, specific activity 2200 Ci $mmol^{-1}$) was obtained from DuPont (Stevenage, U.K.).

Results

Radioligand binding studies

Saturation experiments on 5 different rat pineal gland membrane preparations gave an equilibrium dissociation constant (K_d) of 94.8±13.1 pM and a maximal number of binding sites of 324.5±27.8 fmol mg^{-1} protein (mean±s.e.mean). The K_d and B_{max} values agree well with those obtained previously for rat pineal gland membranes (Sugden & Klein, 1984; 1985a, b).

Competition experiments with adrenoceptor agonists were done in the presence of GTP (100 μM) in an attempt to ensure that all α_1-adrenoceptor binding sites in the pineal membrane preparation were in a single low affinity agonist binding state. However, subsequent experiments failed to reveal a significant shift in K_i for NA when competition assays were done in the absence and presence of GTP (−GTP, $K_i = -5.67 \pm 0.11$; +GTP, $K_i = -5.58 \pm 0.17$; mean±s.e.mean, $n=3$). Specific [^{125}I]-HEAT binding was competitively inhibited by a series of adrenoceptor agonists with Hill coefficients (n_H) which were close to one (Table 1). Adrenoceptor agonists show limited subtype selectivity (Minneman & Esbenshade, 1994; Minneman *et al.*, 1994). However, SDZ NVI-085 has been found to show a 300 fold selectivity for α_{1a}-adrenoceptors in binding assays on canine brain membranes (Renaud *et al.*, 1991) and oxymetazoline a 50 to 100 fold higher affinity at native and cloned rat α_{1A}-adrenoceptors compared to α_{1B}-adrenoceptors (Minneman & Esbenshade, 1994; Laz *et al.*, 1994; Perez *et al.*, 1994). Oxymetazoline has only very weak affinity for the cloned rat α_{1d}-subtype (Laz *et al.*, 1994; Perez *et al.*, 1994). In rat pineal gland membranes, SDZ NVI-085 was a very weak inhibitor of [^{125}I]-HEAT binding with an affinity (log $K_i = -5.41$) much closer to that expected for α_{1B}-adrenoceptors (log $K_i = -5.06$) than α_{1A}-adrenoceptors (log $K_i = -7.62$; Renaud *et al.*, 1991). Likewise the affinity of oxymetazoline in rat pineal membranes (log $K_i = -7.11$) was quite similar to its affinity at native rat α_{1B}-adrenoceptors (log $K_i = -6.71$; Minneman & Esbenshade, 1994) and at cloned rat α_{1b}-adrenoceptors expressed in COS cells (log $K_i = -6.74$; Laz *et al.*, 1994), but quite different from its affinity at the native (log $K_i - 8.51$) and cloned (log $K_i = -8.22$) rat α_{1a}- or cloned α_{1d}-adrenoceptor (log $K_i = -5.68$).

Specific [^{125}I]-HEAT binding was competitively inhibited by WB4101, phentolamine, 5-methylurapidil and benoxathian. K_i values for these compounds (Table 2) were very similar to those obtained with the native α_{1B}-adrenoceptor (Boer *et al.*, 1989; Minneman & Esbenshade, 1994; Ford *et al.*, 1994) and at cloned rat α_{1b}- subtypes expressed in COS cells (Laz *et al.*, 1994). These antagonists have a 20 to 100 fold higher affinity for the α_{1A}- than α_{1B}-adrenoceptor.

Niguldipine is a 1,4-dihydropyridine which exists as two enantiomers which have different affinities at α_{1A}- and α_{1B}-adrenoceptor subtypes (Boer *et al.*, 1989; Robinson & Kendall, 1990). (+)-Niguldipine has a very high affinity for the native α_{1A}-subtype (log $K_i x = -10.28$) while (−)-niguldipine (log $K_i = -8.65$) has an affinity some 50 fold lower (Boer *et al.*, 1989); both enantiomers have a similar, relatively weak, affinity at α_{1B}-sites (log $K_i \approx -7.3$). Niguldipine enantiomers inhibited pineal [^{125}I]-HEAT binding equally well with relatively low affinity (log $K_i = -6.88$ for the (−)-enantiomer, log

Table 1 Comparison of the affinities of various α_1-adrenoceptor agonists for rat pineal α_1-adrenoceptors

Drug	$-log\ K_i$ (M)	n_H
(−)-Adrenaline	5.95±0.05	1.00±0.06
(−)-Noradrenaline	5.68±0.08	0.92±0.02
Phenylephrine	5.54±0.11	0.89±0.06
Methoxamine	3.74±0.06	0.96±0.08
Cirazoline	6.77±0.08	1.04±0.04
Oxymetazoline	7.11±0.10	1.01±0.03
SDZ NVI-085	5.41±0.13	1.05±0.09

Values given are the negative logarithm of the K_i values and slope (n_H) of the competition curves. Data are the mean±s.e.mean of 4 separate experiments.

Table 2 Comparison of the affinities of various subtype selective α_1-adrenoceptor antagonists for rat pineal α_1-adrenoceptors

Drug	$-log\ K_i$ (M)	n_H
Phentolamine	7.25±0.06	0.99±0.06
WB 4101	7.74±0.03	1.22±0.13
5-Methylurapidil	6.94±0.06	1.25±0.09
Benoxathian	8.12±0.09	1.00±0.16
(−)-Niguldipine	6.88±0.04	1.15±0.11
(+)-Niguldipine	6.92±0.05	1.12±0.10

Values given are the negative logarithm of the K_i values and slope (n_H) of the competition curves. Data are the mean±s.e.mean of 5 separate experiments except for benoxathian which is a single experiment. Errors given for benoxathian are the computer-derived estimates obtained from curve fitting.

$K_i = -6.92$ for the (+)-enantiomer), consistent with an action at α_{1B}-adrenoceptors (Table 2). Competition curves with all adrenoceptor antagonists gave Hill coefficients (n_H) which were not significantly different from unity.

Treatment of rat pineal gland membranes with CEC (10 μM; 10 min) resulted in a marked loss of α_1- binding sites (Table 3). In 5 separate experiments the mean B_{max} was significantly reduced by 67% (range 53 to 80%) while the mean K_d was not significantly changed. Pre-treatment (10 min) with different concentrations of CEC showed a concentration-related inactivation of specific [^{125}I]-HEAT binding with a maximal loss occurring with 3 μM CEC; higher concentrations of CEC (upto 100 μM) were no more effective (Figure 1a). With a CEC concentration of 10 μM the inactivation of α_1-adrenoceptor sites was maximal in 10 min; longer pre-treatment times did not produce further inactivation (Figure 1b).

Table 3 Effect of chloroethylclonidine (CEC) on [^{125}I]-HEAT binding in rat pineal membranes

		−CEC	+CEC
K_d	(pM)	94.8 ± 13.1	103.4 ± 6.0
B_{max}	(fmol mg^{-1} protein)	324.5 ± 27.8	107.3 ± 12.7*

K_d (pM) and B_{max} (fmol mg^{-1} protein) values were determined in saturation experiments with [^{125}I]-HEAT (2 to 900 pM) after buffer (−CEC) or chloroethylclonidine (+CEC; 10 μM, 10 min., 37°C) pretreatment of membranes. Values given are the mean ± s.e.mean of 5 saturation experiments. *$P<0.005$ compared to −CEC group by Student's t test.

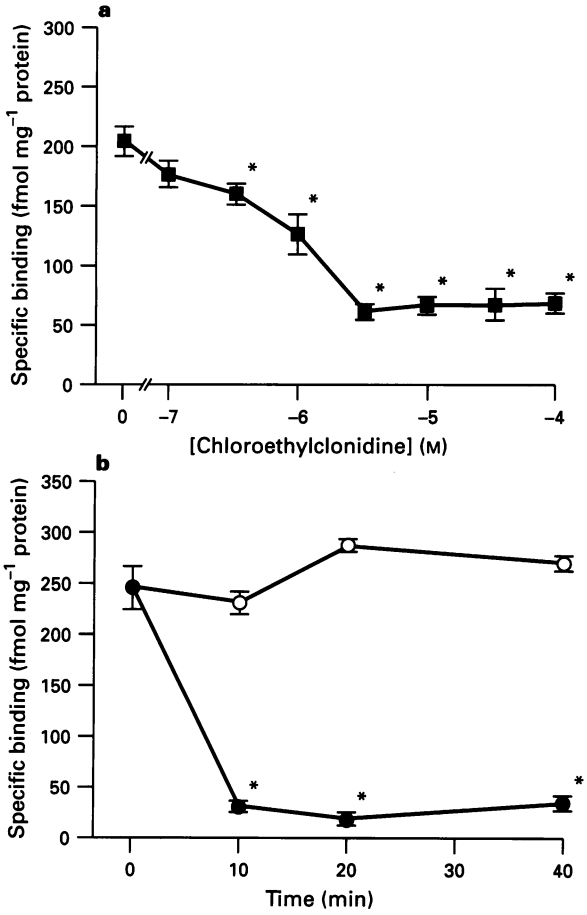

Figure 1 (a) Concentration-related irreversible inactivation of specific [^{125}I]-HEAT binding by chloroethylclonidine (CEC) treatment. Membranes were incubated with the concentration of CEC indicated in hypotonic buffer (10 min, 37°C). After thorough washing to ensure removal of free CEC, α_1-adrenoceptor density was measured with [^{125}I]-HEAT (200 pM). (b) Time-course of irreversible inhibition of specific [^{125}I]-HEAT binding by CEC. Rat pineal membranes were incubated with buffer (○) or CEC (●, 10 μM, 37°C) for the time indicated. Membranes were collected by centrifugation and washed extensively. α_1-Adrenoceptor density was measured with [^{125}I]-HEAT (200 pM). *$P<0.05$ compared to membranes pretreated with buffer instead of CEC.

The affinities of (−)- and (+)-niguldipine were compared in buffer pretreated rat pineal membranes and CEC-treated membranes (10 μM, 10 min, 37°C). As expected, CEC-treatment reduced specific [^{125}I]-HEAT binding by ~70%. The affinity of (−)- and (+)-niguldipine for the remaining sites was not altered (Table 4).

RT–PCR analysis

In rat brain cortex, a single product was observed for each α_1-adrenoceptor primer pair at the expected size for α_{1a}-, α_{1b}- and α_{1d}-adrenoceptor subtypes (i.e. 424, 341 and 490 bp respectively; Figure 2a, c and e). PCR products corresponding to α_{1a}- and α_{1b}-subtypes were also present in rat pinealocytes, but an α_{1d}-PCR product was not seen (Figure 2b, d and f). The identity of each PCR product was confirmed by restriction digest analysis. Each PCR product was digested by each of the restriction enzymes (*Sau3AI, HinfI, SmaI*) exactly as predicted from the published sequence of each α_1-adrenoceptor subtype (Figure 2).

Discussion

The radioligand binding data presented here support the interpretaion that the majority of α_1-adrenoceptors detected with [^{125}I]-HEAT in rat pineal gland membranes are the α_{1B}-subtype. First, in competition experiments all of the adrenoceptor agonists and antagonists inhibited [^{125}I]-HEAT binding with Hill coefficients close to unity suggesting that a single α_1-adrenoceptor subtype is present. Second, the affinity of the subtype-selective adrenoceptor agonists (SDZ NVI-085, oxymetazoline) and antagonists (WB4101, 5-methylurapidil, phentolamine and benoxathian) is consistent with an α_{1B}-subtype. Third, the (−)- and (+)-enantiomers of niguldipine had equal, and relatively low, affinity (log $K_i = -6.9$); the enantiomers have approximately equal affinity for α_{1B}-sites, but the (+)-enantiomer is a very potent inhibitor at α_{1A}-adrenoceptors (Boer *et al.*, 1989). Fourth, CEC, which preferentially inactivates α_{1B}-adrenoceptors, irreversibly inactivated a large fraction (70 to 80%) of specific [^{125}I]-HEAT binding sites.

Although the majority of α_1-adrenoceptors in the pineal appear to be of the α_{1B}-subtype, it is possible that a population of CEC-insensitive α_1-adrenoceptors is also present. α_1-Adrenoceptor subtypes transiently expressed in COS-7 cells show a differential sensitivity to inactivation by CEC, with the α_{1b}-

Table 4 Comparison of the affinities of (−)- and (+)-niguldipine enantiomers for rat pineal α_1-adrenoceptors in untreated and chloroethylclonidine (CEC) - treated membranes

Drug	−log K_i (M)	
	−CEC	+CEC
(−)-Niguldipine	6.41 ± 0.11	6.66 ± 0.08
(+)-Niguldipine	6.86 ± 0.09	6.90 ± 0.08

Specific binding in untreated membranes was 188.3 fmol mg^{-1} protein and in CEC-treated membranes 61.2 fmol mg^{-1} protein. The concentration of [^{125}I]-HEAT used was 86 pM. Values given are the negative logarithm of the K_i values with the error determined from fitting of the competition curves.

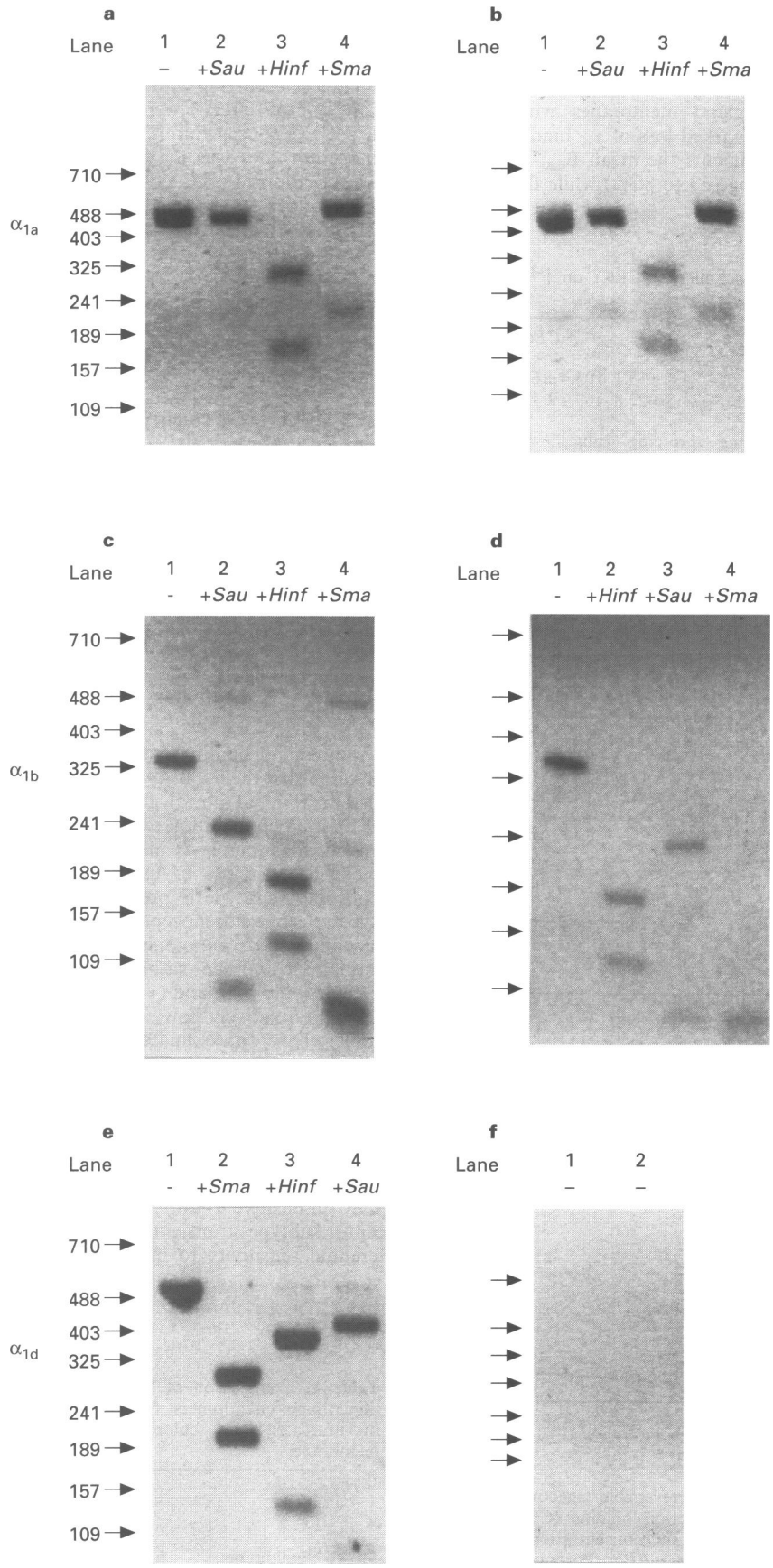

Figure 2 Detection of α_1-adrenoceptor subtypes by RT–PCR. Brain cortex (a, c and e) or rat pinealocyte (b, d and f) cDNA was amplified for 35 (α_{1b}-) or 40 (α_{1a}- and α_{1d}-) cycles. In (f), lanes 1 and 2 refer to two separate cDNA preparations. Aliquots of each PCR reaction were incubated overnight in the absence of restriction enzyme (–) or with the restriction enzymes indicated (*Sau3AI*, 10 u, 37°C; *HinfI*, 10 u, 37°C; *SmaI*, 10 u, 30°C). The position of DNA size markers (bp) are indicated by arrows. Products were separated by agarose gel (2.0%) electrophoresis as described in Methods. The predicted size of authentic α_{1a} product is 424 bp, α_{1b} product 341 bp and α_{1d} 490 bp. The digestion fragments produced are expected to be: *Sau3AI*; α_{1a}-402, 22 bp; α_{1b}-236, 100, 5 bp; α_{1d}-393, 97 bp: *HinfI*; α_{1a}-264, 154 bp; α_{1b}-132, 185, 24 bp; α_{1d}-358, 132 bp: *SmaI*; α_{1a}-none; α_{1b}-105, 62, 97, 77 bp; α_{1d}-301, 189 bp.

subtype most readily inactivated although α_{1d}- and even α_{1a}-subtypes can be inactivated especially when a high concentration (100 μM) of CEC is used (Perez *et al.*, 1994; Laz *et al.*, 1994). In several experiments it was not possible to inactivate completely all pineal [^{125}I]-HEAT binding sites. This was not due to an insufficient CEC concentration or incubation time because higher concentrations or longer pretreatment times did not enhance CEC inactivation (Figure 1). These results agree with observations made in other tissues which show that 10 μM CEC incubated with membranes for 10 min in a hypotonic buffer gives optimal inactivation (Han *et al.*, 1987). In tissues which are considered to contain only α_{1B}-sites, such as the liver and spleen, treatment with CEC inactivates 85 to 95% of [^{125}I]-HEAT binding sites (Han *et al.*, 1987; Minneman *et al.*, 1988).

As competition experiments with subtype-selective antagonists may not reveal a second receptor subtype present in low abundance, another approach was used to detect the presence of α_{1A}- or α_{1D}-sites. CEC was used to inactivate the majority of binding sites in pineal membranes (presumed α_{1B}-sites) thus enriching the proportion of a potential second receptor site, then competition assays were done on the remaining sites with a subtype-specific antagonist. The antagonists used were the niguldipine enantiomers because of the antagonists available (+)-niguldipine shows the greatest specificity for the α_{1A}-adrenoceptors; no α_{1D}-adrenoceptor subtype-selective antagonist is available. Although CEC treatment reduced specific binding substantially as expected, competition assays with the niguldipine enantiomers gave low affinity constants very similar to those determined in membranes which were not treated with CEC. Thus no evidence was found for the expression of an α_1-adrenoceptor with high affinity for (+)-niguldipine, i.e. α_{1A}-adrenoceptor. Thus the CEC-resistant sites may represent α_{1B}-adrenoceptors which escape inactivation perhaps because they are inaccessible to this hydrophilic compound.

Our data from RT–PCR experiments indicate that both α_{1b}- and α_{1a}-adrenoceptor mRNA are present in cultured pinealocytes. The relative abundance of α_{1a}- and α_{1b}-adrenoceptor mRNA is not known and cannot be estimated by simply comparing the density of the α_{1a}- and α_{1b}-PCR products (Figure 2). The identity of the PCR fragments amplified was confirmed by restriction enzyme digestion with three different enzymes. Although an α_{1d}-PCR product was readily detected in rat brain cortex and confirmed by restriction digest analysis, no such product could be detected in pinealocyte cDNA. The α_{1a}-adrenoceptor PCR product clearly originated from cDNA rather than genomic DNA as the primers used were designed to span the single large intron of the α_{1a}-adrenoceptor gene (Perez *et al.*, 1994), total RNA was digested with DNase before synthesis of cDNA and no product was detected if the reverse transcription step was omitted. α_{1a}-mRNA (and α_{1b}- but not α_{1d}-mRNA) was also detected in cDNA prepared from rat pineal glands as well as from rat isolated pinealocytes indicating that the expression of α_{1a}-mRNA is not simply induced upon dissociation and culture of pinealocytes (data not shown).

It will be interesting to examine the distribution of α_{1a}- and α_{1b}-mRNA in pineal gland sections and cultured pinealocytes by *in situ* hybridisation. The pineal gland is often regarded as an essentially homogeneous organ consisting of cells, pinealocytes, specialised for the synthesis and secretion of the hormone, melatonin. Although morphological evidence suggests multiple types of pinealocytes may exist, immunocytochemical studies have shown preparations of cultured pinealocytes to consist almost entirely of cells expressing the pineal specific markers such as S-antigen and hydroxyindole-O-methyltransferase (Wicht *et al.*, 1993; Kuwano *et al.*, 1983). Although the proportion of non-pinealocytes which 'contaminate' the preparation is expected to be very low, the ability of PCR to amplify rare mRNA species suggests that it is conceivable that the α_{1a}-adrenoceptor mRNA detected did not originate in pinealocytes. Alternatively, both mRNA species may be expressed in the same pinealocytes or in different populations of pinealocytes. A recent analysis of α_1-adrenoceptor-mediated stimulation of intracellular Ca^{2+} in single cultured pinealocytes loaded with Fura-2 described a heterogeneity in the pattern of responses (Schaad *et al.*, 1993). Another possibility is that the α_{1a}-adrenoceptor mRNA, whether present in pinealocytes or in other cell types in the gland, may not be translated into a functional receptor protein.

Activation of the pineal α_1-adrenoceptor by NA released at night from the sympathetic neurones which innervate the gland contributes to the nocturnal synthesis and secretion of melatonin. The mechanism involves α_1-adrenoceptor mediated elevation of intracellular Ca^{2+} and activation of phosphatidylinositol hydrolysis leading to PKC activation and a dramatic potentiation of β-adrenoceptor stimulation of cyclic AMP synthesis (Sugden *et al.*, 1985; Sugden, 1989). The subtype of α_1-adrenoceptor which mediates the Ca^{2+} elevation and increase in phosphatidylinositol turnover and the enhanced β-adrenoceptor mediated cyclic AMP response has not been investigated. Further studies are needed to determine the senstivity of these second messenger changes triggered by NA to subtype-selective α_1-adrenoceptor antagonists. Such studies will identify the pinealocyte α_1-adrenoceptor subtype mediating the potentiation of β-adrenoceptor stimulation of melatonin synthesis, and may clarify the relative importance of intracellular Ca^{2+} and phosphatidylinositol turnover in the α_1-adrenoceptor-mediated potentiation mechanism.

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