Differences between the α_2 -adrenoceptor in rat submaxillary gland and the α_{2A} - and α_{2B} -adrenoceptor subtypes

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1 The α_2 -adrenoceptors of rat submaxillary gland, labelled with [³H]-rauwolscine, were characterized by use of a range of subtype selective ligands and were compared to rabbit spleen α_{2A} -adrenoceptors and rat kidney α_{2B} -adrenoceptors.

2 In rat submaxillary gland, [³H]-rauwolscine labelled an apparently homogeneous population of binding sites with relatively low affinity ($K_d = 11.65$ nM) compared to the affinity in rat kidney ($K_d = 2.18$ nM) and rabbit spleen ($K_d = 4.64$ nM).

3 In competition studies using 16 ligands the α_2 -adrenoceptors in rat submaxillary gland appeared to differ from both the α_{2A} -adrenoceptor of rabbit spleen ($r = 0.62$) and also the α_{2B} -adrenoceptor of rat kidney ($r = 0.28$).

4 The affinity data obtained with benoxathian, imiloxan and WB 4101 indicated the presence of an α_{2B} -adrenoceptor in rat submaxillary gland. However, data for chlorpromazine, oxymetazoline, spiroxatrine and xylometazoline indicated that submaxillary gland α_2 -adrenoceptors were of the α_{2A} subtype. The affinity estimate for prazosin in rat submaxillary gland was intermediate between its affinity at the α_{2A} - and α_{2B} -adrenoceptors while affinity estimates for idazoxan and phentolamine in rat submaxillary gland were greater than those obtained at either the α_{2A} - or α_{2B} -adrenoceptor.

5 These data indicate that rat submaxillary gland α_2 -adrenoceptors differ from the α_{2A} - and α_{2B} -adrenoceptors found in rabbit spleen and rat kidney, respectively.

Introduction

Recent studies on the ligand binding properties and molecular biology of the α_2 -adrenoceptor have indicated that α_2 -adrenoceptor subtypes exist. Regan *et al.* (1988) have cloned two genes that encode α_2 -adrenoceptors and have shown that these two receptors can be differentiated on the basis of their affinity for a range of ligands. These molecular biological studies complement the data obtained from ligand binding studies conducted in several laboratories (Nahorski *et al.*, 1985; Bylund, 1985; 1988) which have demonstrated that α_2 -adrenoceptors can be separated into α_{2A} and α_{2B} adrenoceptor subtypes depending upon their affinity for a range of ligands. According to the present classification scheme, α_{2A} -adrenoceptors display high affinity for oxymetazoline and WB 4101 and low affinity for prazosin

and chlorpromazine, while α_{2B} -adrenoceptors display the converse selectivity for these agents (Bylund, 1988).

We have previously demonstrated that rabbit spleen and rat kidney possess α_2 -adrenoceptors that display the pharmacology of the α_{2A} - and α_{2B} -adrenoceptor, respectively (Michel *et al.*, 1989). In the present study we extend our characterization of the rabbit spleen and rat kidney α_2 -adrenoceptors and report on the characterization of an α_2 -adrenoceptor subtype present in rat submaxillary gland that displays significant differences from the α_{2A} - and α_{2B} -adrenoceptors described to date.

Methods

Membrane preparation

All membrane preparation procedures were conducted at 4°C and used ice cold buffers. Kidneys and

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submaxillary glands from adult Sprague-Dawley rats and spleens from adult New Zealand White rabbits were obtained from Pel-Freez (Arkansas, U.S.A.). Tissues were homogenized for 30 s in 3 volumes (w/v) of 50 mM Tris, 5 mM EDTA homogenizing buffer (pH 7.4 at 4°C) in a Waring Blender at maximum setting. The homogenates obtained were further homogenized in a Polytron P10 tissue disrupter (setting 10; two 10 s bursts) and filtered through a double layer of cheesecloth. The homogenates were centrifuged at 500 *g* for 10 min and the supernatants reserved. The pellets were resuspended in 2 volumes of homogenizing buffer and rehomogenized in the polytron P10 tissue disrupter and centrifuged at 500 *g* for 10 min. The supernatants from this step were combined with the original supernatants and centrifuged for 12 min at 43,000 *g*. The crude membrane pellets obtained were washed by resuspending in homogenizing buffer and centrifuging at 43,000 *g* for 12 min. The pellets from this step were washed twice, in a similar manner, but with ice cold 50 mM Tris, 0.5 mM EDTA assay buffer (pH 7.4 at 4°C). The final pellets were resuspended in assay buffer (pH 7.4 at 25°C) and stored under liquid nitrogen until required.

Ligand binding assays

Membranes, [³H]-rauwolscine and competing drugs were incubated in a final volume of 0.25 ml of assay buffer (pH 7.4 at 25°C) for 45 min at room temperature (22°C). In competition studies the final assay concentration of radioligand was 1–2 nM while in saturation studies the concentration of [³H]-rauwolscine was varied between 0.1 and 105 nM. In all experiments non-specific binding was defined by use of 10 μ M phentolamine. Incubations were terminated by vacuum filtration over 0.1% polyethyleneimine pretreated glass fibre filters using a Brandel cell harvester. The filters were washed for 10 s with ice cold 0.1 M NaCl and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Protein was determined by the dye binding method (Bio-Rad) with bovine gamma globulin as standard.

Data analysis

Competition and saturation binding data were analysed as described previously by use of iterative curve fitting techniques (Michel & Whiting, 1988). IC₅₀ values were converted to K_i values using the Cheng-Prusoff approximation (Cheng & Prusoff, 1973).

Materials

[³H]-rauwolscine (Specific activity 82 Ci mmol⁻¹) was obtained from New England Nuclear. Chlorpromazine, noradrenaline and oxymetazoline were obtained from Sigma chemical company as were all chemicals and reagents used. Phentolamine was obtained from Ciba-Geigy while prazosin was obtained from Pfizer. Benoxathian, spiroxatrine and WB 4101 (2-(2,6-dimethoxyphenoxyethyl) amino-methyl-1,4-benzodioxane) were obtained from Research Biochemical Inc. Idazoxan, imiloxan, UK 14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) and SKF 104078 (6-chloro-9-[(3-methyl-2-butenyl)oxyl-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine) were synthesized by Dr R. Clark, IOC, Syntex.

Results

Saturation studies

Results of saturation binding studies conducted in rat kidney, rat submaxillary and rabbit spleen membranes are presented graphically in Figure 1. In rat submaxillary gland, [³H]-rauwolscine labelled a single population of binding sites ($B_{max} = 137.5 \pm 12.2$ fmol mg⁻¹ protein; $n = 4$) with low affinity ($K_d = 11.65 \pm 1.0$ nM). The K_d value obtained in this preparation was significantly higher than the K_d values obtained in either rat kidney ($K_d = 2.18 \pm 0.46$ nM; $n = 4$) or rabbit spleen ($K_d = 4.64 \pm 0.78$ nM; $n = 4$).

Competition studies

Affinity estimates for a range of compounds at α_2 -adrenoceptors of rat submaxillary gland were determined in competition studies. These results are presented in Table 1 along with affinity estimates obtained in both rat kidney and rabbit spleen. Results in rat kidney and rabbit spleen for several of the ligands have been described previously (Michel *et al.*, 1989). Representative displacement isotherms for idazoxan, spiroxatrine and WB 4101 in the three tissues are shown in Figure 2.

Displacement isotherms for most compounds displayed Hill coefficients that were close to unity and the data could only be described assuming the presence of a single population of binding sites. For WB 4101 and oxymetazoline in rabbit spleen, and for oxymetazoline in rat kidney, Hill coefficients were less than unity and we have previously shown that these data are better described by assuming the presence of two populations of binding sites (Michel *et al.*, 1989). WB 4101 and oxymetazoline displayed

pK_i values of 8.71 and 8.60, respectively, for 70–75% of the α_2 -adrenoceptors labelled using [³H]-rauwolscine in rabbit spleen.

The α_2 -adrenoceptor of rat submaxillary gland displayed relatively high affinity for imiloxan with the pK_i value being similar to that obtained at the α_{2B} -adrenoceptor of rat kidney. For benoxathian and WB 4101, affinity estimates in rat submaxillary gland were also similar to those obtained at the α_{2B} -adrenoceptor of the rat kidney.

For oxymetazoline, spiroxatrine and xylometazoline, affinity estimates in rat submaxillary gland were similar to those obtained at the α_{2A} -adrenoceptor in the rabbit spleen.

Finally, for idazoxan and phentolamine, affinity estimates at the rat submaxillary gland α_2 -adrenoceptor were significantly higher than those obtained at either the α_{2A} - or α_{2B} -adrenoceptors. For prazosin, the affinity estimate in rat submaxillary gland was intermediate between affinity estimates obtained at the α_{2A} - and α_{2B} -adrenoceptor.

Correlation between affinity estimates obtained in rat kidney, rat submaxillary gland and rabbit spleen

In Figures 3, 4 and 5 correlations between affinity estimates obtained in the three preparations are presented. As can be seen from Figure 3, there was a poor correlation ($r = 0.066$) between affinity estimates obtained in rat kidney and rabbit spleen. Furthermore, 7 of the compounds examined displayed greater than 10 fold selectivity for one or the other of the α_2 -adrenoceptors compared (Table 1, column A).

Rat submaxillary gland α_2 -adrenoceptors differed substantially ($r = 0.276$) from the α_{2B} -adrenoceptor of rat kidney (Figure 4) with 4 compounds displaying greater than 10 fold selectivity for either the rat kidney α_{2B} -adrenoceptor or rat submaxillary gland α_2 -adrenoceptor (Table 1 column C).

The rat submaxillary gland α_2 -adrenoceptor displayed some similarity to the rabbit spleen α_2 -adrenoceptor ($r = 0.62$) although 4 of the compounds displayed greater than a 10 fold difference in affinity between these two α_2 -adrenoceptors (Figure 5; Table 1, column B).

Figure 1 Saturation binding of [³H]-rauwolscine in rabbit spleen (a), rat kidney (b) and rat submaxillary gland (c). The figures show total (●), specific (○) and nonspecific binding (□) of [³H]-rauwolscine plotted as a function of the free ligand concentration. In the rabbit spleen, rat kidney and rat submaxillary gland, protein was present at 214, 198 and 138 μ g, respectively, B_{max} values were 132, 141 and 140 fmol mg⁻¹ protein, respectively, and K_d values were 4.6, 2.8, and 12.7 nM, respectively. In all cases the lines were drawn by hand and do not correspond to the best fit regression line.

Table 1 Affinity of ligands for α_2 -adrenoceptors of rabbit spleen, rat kidney and rat submaxillary gland

Ligand	Rabbit spleen pK _i /nH	Rat kidney pK _i /nH	Rat submaxillary pK _i /nH	Selectivity ratio		
				A	B	C
Benoxathian	7.65 (0.10) 0.88 (0.07)	6.37 (0.01) 1.12 (0.05)	6.50 (0.04) 0.99 (0.04)	0.05	14.1	0.74
Chlorpromazine	6.06 (0.02) 1.01 (0.05)	7.28 (0.03) 1.06 (0.03)	5.98 (0.15) 1.02 (0.07)	16.6	1.2	20.0
Clonidine	6.81 (0.12) 0.83 (0.04)	7.24 (0.04) 0.83 (0.04)	7.20 (0.06) 0.83 (0.02)	2.7	0.41	1.1
Corynanthine	6.36 (0.03) 1.02 (0.02)	6.53 (0.01) 0.99 (0.08)	5.81 (0.05) 0.87 (0.01)	1.5	3.5	5.2
Idazoxan	7.12 (0.02) 0.96 (0.11)	7.29 (0.02) 0.83 (0.06)	8.25 (0.07) 0.79 (0.10)	1.5	0.07	0.11
Imiloxan	5.52 (0.03) 0.92 (0.05)	7.26 (0.03) 0.97 (0.02)	7.01 (0.03) 0.96 (0.12)	55.0	0.03	1.8
Noradrenaline	6.37 (0.10) 0.81 (0.05)	6.98 (0.03) 0.78 (0.04)	6.22 (0.01) 0.70 (0.04)	4.1	1.4	5.8
Oxymetazoline	8.11 (0.04) 0.74 (0.04)	6.60 (0.02) 0.71 (0.02)	8.06 (0.04) 0.78 (0.07)	0.03	1.1	0.03
Phentolamine	7.78 (0.06) 0.92 (0.04)	7.97 (0.09) 1.02 (0.04)	8.47 (0.09) 0.88 (0.06)	1.5	0.20	0.32
Prazosin	5.33 (0.08) 0.93 (0.08)	7.22 (0.07) 0.96 (0.04)	6.34 (0.06) 1.05 (0.04)	77.6	0.10	7.6
Rauwolscine	8.06 (0.10) 0.92 (0.11)	8.46 (0.10) 0.86 (0.07)	7.73 (0.01) 0.89 (0.02)	2.5	2.1	5.4
SKF 104078	7.00 (0.04) 1.03 (0.06)	6.96 (0.05) 1.22 (0.03)	6.51 (0.16) 0.97 (0.04)	0.91	3.1	2.8
Spiroxastrine	7.03 (0.18) 0.90 (0.06)	8.47 (0.13) 0.85 (0.04)	6.60 (0.10) 0.94 (0.04)	27.5	2.7	74.1
UK 14304	7.46 (0.08) 0.76 (0.04)	6.61 (0.01) 0.86 (0.07)	6.80 (0.02) 0.87 (0.12)	0.14	4.6	0.6
WB 4101	8.17 (0.09) 0.67 (0.04)	7.46 (0.04) 1.02 (0.06)	7.22 (0.09) 0.96 (0.03)	0.19	8.9	1.7
Xylometazoline	7.98 (0.06) 0.84 (0.17)	6.60 (0.03) 0.76 (0.04)	7.70 (0.04) 0.78 (0.02)	0.04	1.9	0.08

Values are the mean ($\pm$ s.e.mean) of the Hill coefficient (nH) or the negative logarithm of the affinity constant (pK_i) from between 3 and 9 experiments. A = Antilog (pK_i rat kidney - pK_i rabbit spleen). B = Antilog (pK_i rabbit spleen - pK_i rat submaxillary gland). C = Antilog (pK_i rat kidney - pK_i rat submaxillary gland).

Discussion

The main finding of the present study was that the α_2 -adrenoceptor found in rat submaxillary gland differed from the α_{2A} - and α_{2B} -adrenoceptor subtypes already identified in ligand binding studies (Nahorski *et al.*, 1985; Bylund, 1985). The evidence for this was based upon paradoxical differences between ligand affinities obtained in rat submaxillary gland and those obtained in rabbit spleen and rat kidney.

In saturation studies, [³H]-rauwolscine bound with low affinity to a homogeneous population of binding sites in rat submaxillary gland. The demonstration that the K_d value obtained in this preparation was significantly higher than the K_d obtained in either rabbit spleen or rat kidney would suggest that rat submaxillary gland α_2 -adrenoceptors

differ from the α_{2A} - and α_{2B} -adrenoceptor subtypes identified in rabbit spleen and rat kidney. More definitive evidence for differences between the rat submaxillary gland α_2 -adrenoceptor and the α_{2A} - and α_{2B} -adrenoceptor subtypes was obtained in competition studies.

In rat submaxillary gland, the affinity estimates obtained for imiloxan, benoxathian and WB 4101 (ligands which can clearly distinguish the α_{2A} - and α_{2B} -adrenoceptor subtypes) were similar to their respective affinities for the rat kidney α_{2B} -adrenoceptor. While these data would suggest that the rat submaxillary gland α_2 -adrenoceptor was of the α_{2B} -subtype, affinity estimates for the subtype selective ligands oxymetazoline, xylometazoline and chlorpromazine indicated the presence of an α_{2A} -adrenoceptor.

To complicate further the classification of rat sub-

Figure 2 Displacement of [³H]-rauwolscine binding from rabbit spleen (●), rat kidney (○) and rat submaxillary gland (■) by either idazoxan, spiroxatrine or WB 4101. Data for idazoxan (a); data for spiroxatrine (b); data for WB 4101 (c). In all cases membranes, [³H]-rauwolscine (1–2 nM), and various concentrations of the competitors were incubated for 45 min at 22°C before separating bound and free ligand by vacuum filtration. The data are from a single representative experiment. In rabbit spleen, total binding was 1109 d.p.m and nonspecific binding (NSB) was 245 d.p.m. In rat kidney total binding was 1287 d.p.m and NSB was 267 d.p.m. In rat submaxillary gland total binding was 980 d.p.m and NSB was 375 d.p.m. In all cases the lines were drawn by hand and do not correspond to the best fit regression line.

Figure 3 Correlation between ligand affinity estimates obtained at the α₂-adrenoceptors of rat kidney and rabbit spleen labelled with [³H]-rauwolscine. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.0461x + 6.881$ ($r = 0.066$).

Figure 4 Correlation between ligand affinity estimates obtained at the α₂-adrenoceptors of rat kidney and rat submaxillary gland labelled with [³H]-rauwolscine. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.216x + 5.690$ ($r = 0.276$).

Figure 5 Correlation between ligand affinity estimates obtained at the α_2 -adrenoceptors of rabbit spleen and rat submaxillary gland labelled with [3 H]-rauwolscine. The dashed line represents the line of identity, while the solid line represents the regression line. The data were best described by the expression $y = 0.691x + 2.199$ ($r = 0.62$).

maxillary gland α_2 -adrenoceptors, the affinity estimate for prazosin in this tissue was intermediate between that obtained at the α_{2A} - and α_{2B} -adrenoceptor while for phentolamine and idazoxan, affinity estimates in rat submaxillary gland exceeded those obtained at both the α_{2A} - and α_{2B} -adrenoceptor.

While the simplest explanation for these differences is that the rat submaxillary gland α_2 -adrenoceptor differs from the rabbit spleen α_{2A} - and the rat kidney α_{2B} -adrenoceptor, several alternatives should be considered briefly.

It is possible that during the membrane preparation procedure modifications to the receptors could have occurred. This appears to be unlikely since such a problem has not been widely reported for any other neurotransmitter receptor system. Furthermore, with respect to the α_2 -adrenoceptors in rat cortex and human platelet Kawahara & Bylund (1985) have shown that such a phenomenon does not occur.

The anomalous binding affinities obtained in rat submaxillary gland could have been due to the presence of a heterogeneous mixture of α_{2A} - and α_{2B} -adrenoceptors in this preparation or even to the presence of receptor heterogeneity in the other two tissues.

Heterogeneity of the α_2 -adrenoceptors present in

rat submaxillary gland seemed unlikely for several reasons. Firstly, the K_d of [3 H]-rauwolscine was higher in rat submaxillary gland than in either rabbit spleen or rat kidney indicating that rat submaxillary gland did not contain a heterogeneous mixture of the α_2 -adrenoceptor subtypes found in rat kidney or rabbit spleen. The additional finding that idazoxan and phentolamine were more potent in rat submaxillary gland than in rabbit spleen or rat kidney would also support this contention. While the intermediate affinity of prazosin for rat submaxillary gland α_2 -adrenoceptors may indicate possible heterogeneity of the α_2 -adrenoceptors in this preparation, the finding that Hill coefficients for prazosin in all three preparations were close to unity would tend to rule out this possibility. Furthermore, ligands such as spiroxatrine, imiloxan and chlorpromazine which display greater than 10 fold selectivity for either the α_{2A} - or the α_{2B} -adrenoceptor, did not display an intermediate affinity for rat submaxillary gland α_2 -adrenoceptors as would be expected if this preparation did in fact contain a heterogeneous mixture of α_{2A} - and α_{2B} -adrenoceptor subtypes.

These findings do not exclude the possibility that the rat kidney or rabbit spleen could contain a heterogeneous population of binding sites. Certainly, the data obtained with oxymetazoline appear to indicate heterogeneity of binding in rat kidney and rabbit spleen. Thus, in rabbit spleen two populations of [3 H]-rauwolscine site that differed in affinity for oxymetazoline by 30–100 fold (Michel *et al.*, 1989) could be identified. While this may indicate heterogeneity of α_2 -adrenoceptors in rabbit spleen several other observations would tend to argue against this.

Most notably, chlorpromazine, imiloxan and prazosin which were equally as selective as oxymetazoline (in terms of their affinity for rat kidney as opposed to rabbit spleen α_2 -adrenoceptors) did not identify similar heterogeneity in rabbit spleen to that identified by oxymetazoline. Furthermore, benoxathian, which was as selective as oxymetazoline for α_{2A} - as opposed to α_{2B} -adrenoceptors, did not identify the same heterogeneity in rabbit spleen.

The reason for the apparent heterogeneity of binding identified by oxymetazoline is unknown at present. It should be noted that it was probably not due to affinity states of the α_2 -adrenoceptors since the non-hydrolyzable guanidine triphosphate (GTP) analogue, Gpp(NH)p, did not modulate the binding of oxymetazoline to the three α_2 -adrenoceptors examined in the present study (unpublished observations).

On the basis of these findings, and with the possible exception of the data obtained with oxymetazoline, it appears unlikely that tissue-related factors, receptor heterogeneity or agonist affinity states could be easily invoked to account for the differences in the

pharmacology of the α_2 -adrenoceptors in rat kidney, rat submaxillary gland and rabbit spleen. It therefore appears that the three tissues examined express different α_2 -adrenoceptors.

In this respect it is interesting to note that additional evidence for three subtypes of the α_2 -adrenoceptor has also been presented. In ligand binding studies Bylund (1988) has recently reported that the α_2 -adrenoceptor present in the opossum kidney OK cell line (Murphy & Bylund, 1988) differ from the α_{2A} - and α_{2B} -adrenoceptor subtypes and may therefore represent an α_{2C} -adrenoceptor. Furthermore, and as mentioned by Bylund (1988), studies on the molecular biology of the α_2 -adrenoceptor by Kobilka *et al.* (1987) have identified three regions of hybridization in southern blots probed at reduced stringency using a DNA probe directed against the α_2 -adrenoceptor of human platelet. Two of these regions have been identified as corresponding to genes that encode α_2 -adrenoceptors. While the third region of hybridization may represent a third α_2 -adrenoceptor, it should be noted that since a DNA probe for the β -adrenoceptor identified the 5-HT_{1A}-receptor (Fargin *et al.*, 1988), it may also be possible that the third putative α_2 -adrenoceptor gene localized to chromosome 2 does not represent an α_2 -adrenoceptor.

Comparison of the three receptor binding sites identified in the present study with the pharmacology of the α_2 -adrenoceptor gene products and the three binding sites identified by Bylund (1988) indicates several features of similarity and several disparities.

In a previous study (Michel *et al.*, 1989) we have shown that the rabbit spleen and rat kidney α_2 -adrenoceptors correspond closely to the α_{2A} - and α_{2B} -adrenoceptor subtypes, respectively, identified by Bylund (1988). The rat submaxillary gland α_2 -adrenoceptor does not, however, appear to correspond to the α_{2C} -adrenoceptor present in the OK cells. Firstly, the affinity of rauwolscine and WB 4101 for the α_{2C} -adrenoceptor (Murphy & Bylund, 1987) is higher than that at the α_{2A} - or α_{2B} -adrenoceptor (Bylund *et al.*, 1988) whereas in our study, the rat submaxillary gland α_2 -adrenoceptors displayed the lowest affinity for these ligands. In addition, prazosin and chlorpromazine displayed high affinity for both the α_{2B} - (Bylund *et al.*, 1988) and α_{2C} -adrenoceptors (Murphy & Bylund, 1988) whereas they displayed lower affinity for the rat submaxillary gland α_2 -adrenoceptor than for the α_{2B} -adrenoceptor in our study.

The cloned α_2 -adrenoceptors identified by Regan *et al.* (1988) were initially thought to correspond to the α_{2A} - and α_{2B} -adrenoceptor subtypes. However, this has been questioned by Bylund (1988) who has suggested that while the α_2 -adrenoceptor of chromo-

some 10 (α_2 -C10) appears to correspond to the α_{2A} -adrenoceptor, the pharmacology of the α_2 -adrenoceptor of chromosome 4 (α_2 -C4) differs from that of the α_{2A} -, α_{2B} - or α_{2C} -adrenoceptors. The results from the present study also indicate difficulties in comparing ligand binding data on the α_{2A} - and α_{2B} -adrenoceptors to binding data obtained using the cloned α_2 -C4 and α_2 -C10 adrenoceptors. Thus, imiloxan, which was an α_{2B} -adrenoceptor selective ligand in our study, did not differentiate between α_2 -C4 and α_2 -C10 (Regan *et al.*, 1988). Furthermore, WB 4101 which displays up to a 10 fold higher affinity for α_{2A} - as opposed to α_{2B} -adrenoceptors (Cheung *et al.*, 1982; Bylund *et al.*, 1988) displayed an 8 fold lower affinity for the α_2 -C10 adrenoceptor than for the α_2 -C4 adrenoceptor.

Clearly, further study is required to correlate definitively the α_2 -adrenoceptors identified in ligand binding studies with those identified from molecular biological studies. In addition, further study is required to identify functional correlates for the increasing number of α_2 -adrenoceptor binding sites that are being characterized in ligand binding studies.

This is especially relevant since, on the basis of existing reports, at least 6 α_2 -adrenoceptor subtypes could be present. The α_{2A} - and α_{2B} -adrenoceptor subtypes identified in ligand binding studies are now widely accepted (Bylund, 1988). The α_2 -adrenoceptors of the rat submaxillary gland identified in the present study and the α_{2C} -adrenoceptors of the OK cell line (Murphy & Bylund, 1988) also appear to represent two further distinct α_2 -adrenoceptor subtypes. Given that the cloned α_2 -C4 adrenoceptor may differ from either the α_{2A} -, α_{2B} - or α_{2C} -adrenoceptors identified to date (Bylund, 1988), the α_2 -C4 adrenoceptor may represent a fifth subtype of α_2 -adrenoceptor. Finally, since SKF 104078 distinguishes between pre- and postsynaptic α_2 -adrenoceptors (Ruffolo *et al.*, 1988) and a ligand binding correlate for this receptor has not been identified to date, presynaptic α_2 -adrenoceptors may form a possible sixth subtype of the α_2 -adrenoceptor.

While such extensive heterogeneity is not without precedent [for instance, 5 muscarinic receptor genes have been identified by Bonner *et al.* (1988) despite the fact that only three functional and ligand binding sites were previously thought to exist (Hammer *et al.*, 1987)], the situation with regard to α_2 -adrenoceptor subtypes is complicated by the heavy reliance on ligand binding data. In this respect the identification of additional subtype selective ligands such as imiloxan (Michel *et al.*, 1989) and spiroxatrine in the present study should aid in identifying functional correlates for the α_2 -adrenoceptor subtypes identified in ligand binding sites.

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