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Perspective

α- and β-Adrenoceptors: From the Gene to the Clinic. 1. Molecular Biology and Adrenoceptor Subclassification

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Introduction

Blockade of the actions of the sympathetic neurotransmitter norepinephrine and the neurohormone epinephrine at α- and β-adrenoceptors has represented an important mechanism in drug therapy for decades, and a vast array of antagonists have been identified. Likewise, many synthetic agonists capable of activating either α- or β-adrenoceptors have been synthesized. These α- and β-adrenoceptor agonists and antagonists have been developed as therapeutic agents and represent important therapeutic classes, such as β-adrenoceptor antagonists for the treatment of hypertension and angina and the secondary prevention of acute myocardial infarction, β_2-adrenoceptor agonists as bronchodilators in asthma and other bronchospastic conditions, and α_2-adrenoceptor agonists as well as α_1-adrenoceptor antagonists for hypertension.

Subtypes of each of the major classes of adrenoceptors have been known for many years, with β-adrenoceptors first being subdivided in 1967[1] and α-adrenoceptors initially subdivided in 1973.[2] Recently, the identification of additional α- and β-adrenoceptor subtypes has stimulated increased interest in the design of agents that interact selectively with the adrenoceptors. For example, the discovery of the β_3-adrenoceptor offered the opportunity for new therapeutic agents for the treatment of diabetes and obesity, and the further subdivision of α_1-adrenoceptors has presented the opportunity to block selectively prostatic α_1-adrenoceptors to relieve urethal obstruction without the symptoms commonly associated with antagonism of vascular α_1-receptors.

In some cases, the discovery of additional adrenoceptor subtypes was facilitated by the availability of molecular biological techniques (*e.g.*, the demonstration of additional subtypes of α_1- and α_2-adrenoceptors). In other cases, such as the β_3-adrenoceptor, the newer receptor subtypes were discovered using classical pharmacological techniques, and selective agonists and antagonists were subsequently synthesized and evaluated in human subjects before the sequence of the novel receptors was identified.

The identification of subtype-selective agonists and antagonists has often resulted in more efficacious drugs. Thus, α-adrenoceptor antagonists were not found to be particularly useful as antihypertensive drugs until the discovery of agents having selectivity for the α_1-adrenoceptor, such as prazosin. It seems likely, therefore, that the recent proliferation of α- or β-adrenoceptor subtypes will also give rise to improvements in therapy.

Molecular Biology of the Adrenoceptors

1. **Characterization of Cloned α-Adrenoceptors.** **A. α_1-Adrenoceptors.** The cloning and expression of α_1-adrenoceptors has confirmed the presence of multiple subtypes of this receptor family and, indeed, has lead to the recognition of the functional and therapeutic importance of a particular subtype, the α_{1A}-adrenoceptor.

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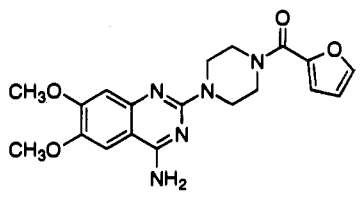
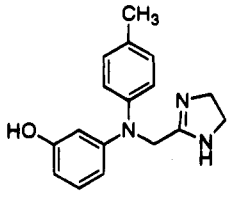
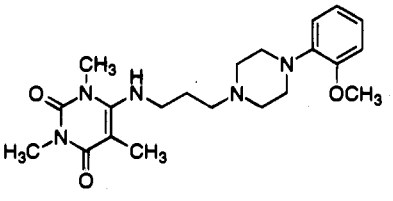
The first α_1-adrenoceptor to be cloned was the α_{1b}-adrenoceptor from a hamster vas deferens cell line (DD_1-MF2).[3] (Throughout this review, lower case subscripts will be used to refer to recombinant receptors and upper case subscripts for native receptors.) The expressed clone has properties consistent with the pharmacological profile of α_{1B}-adrenoceptors in native tissues, having a high affinity for prazosin (1) and a low affinity for phentolamine (2), 5-methylurapidil (3), and yohimbine (4). Also consistent with the α_{1B}-adrenoceptor assignment was a high sensitivity to irreversible alkylation by (chloroethyl)clonidine (5). Using a cDNA probe derived from this hamster α_{1b}-adrenoceptor, the rat homolog was identified and shown to have a similar pharmacologic profile to and greater than 98% amino acid identity with the hamster α_{1b}-adrenoceptor.[4,5] Northern analysis of rat tissues showed mRNA expression for this clone to occur in a variety of tissues predicted to possess the α_{1B}-adrenoceptor including liver, spleen, heart, and cerebral cortex. A similar approach has been used to identify the human α_{1b}-adrenoceptor[6] which, like the rat homolog, had a high degree of amino acid identity (98%) with the hamster α_{1b}-adrenoceptor and virtually identical affinity for prazosin, WB-4101 (6), phentolamine, and 5-methylurapidil as observed for the hamster clone.



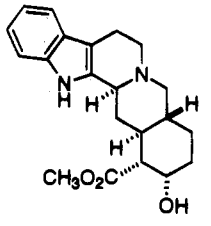
(1) Prazosin



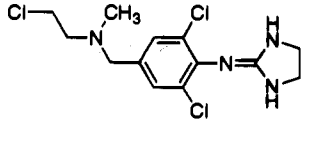
(2) Phentolamine



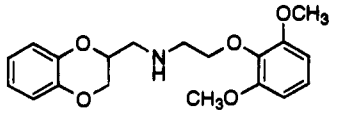
(3) 5-Methylurapidil



(4) Yohimbine



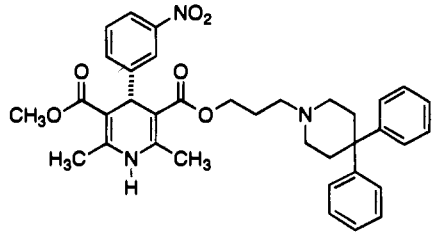
(5) Chloroethylclonidine



(6) WB-4101

A clone has also been identified from the dog which appears to represent an α_1-adrenoceptor but is missing approximately 100 amino acids from the amino terminus.[7] On the basis of a comparison of the amino acid sequence of this receptor with those of human, rat, and hamster α_{1b}-adrenoceptors, this fragment appears to represent the canine α_{1b}-adrenoceptor.[6]

Screening of a rat brain library with a cDNA probe prepared from the hamster α_{1b}-adrenoceptor revealed a clone for yet a different α_1-adrenoceptor subtype.[8] Northern analysis of the tissue distribution of mRNA transcribed by this clone suggested a distribution similar to that anticipated for the α_{1A}-adrenoceptor, and the expressed receptor also had a high affinity for WB-4101. This led to the conclusion that this novel from a rat brain library represented the α_{1A}-adrenoceptor. However, Perez *et al.*,[9] studying an identical clone which was also isolated from rat brain, confirmed the high affinity for WB-4101, but affinity for the more selective α_{1A}-adrenoceptor antagonists 5-methylurapidil and (*S*)-niguldipine (7) was unexpectedly low. Furthermore,

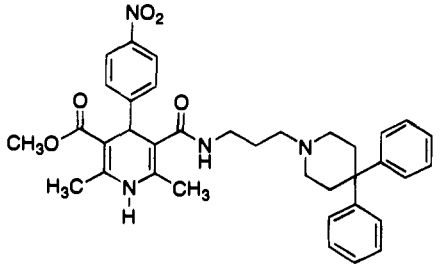


(7) (S)-Niguldipine

although this cloned receptor was more resistant to alkylation by (chloroethyl)clonidine than was the α_{1b}-adrenoceptor expressed in the same system, 72% receptor inactivation could be produced by 100 μM (chloroethyl)clonidine in 10 min at 37 °C. On the basis of these findings, Perez *et al.*[9] concluded that a clone for a novel α_1-adrenoceptor subtype had been isolated and denoted it as the α_{1d}-adrenoceptor. Because the expressed cDNA from this clone has pharmacological properties that were substantially different from those found for the α_{1A}-adrenoceptor in native tissues, it had been proposed that the cloned receptor should be designated as the $\alpha_{1A/D}$-adrenoceptor,[10] which is now simply referred to as the α_{1d}-adrenoceptor[11] (Table 1). A human analog of this receptor was cloned from a hippocampus cDNA library.[12] The clone identified by Bruno *et al.*[12] had a high degree of amino acid identity between the human and rat receptors in the central portion of the molecule (>95%), with a much lower similarity (*ca.* 50% identity) at the amino and carboxyl termini. It has recently been shown[13] that both the 3′ and 5′ ends of the nucleotide sequence reported by Bruno *et al.*[12] are incorrect and that the correct human sequence shows a high degree of amino acid identity (at least 90%) with the rat receptor over the entire molecule. Recently published results of antagonist affinity[13,14] on a cloned human α_1-adrenoceptor when compared with that from the rat α_{1d}-adrenoceptor demonstrated an excellent correlation (Table 2).

On the basis of homology screening of a bovine cerebral cortex cDNA library using a probe derived from the hamster α_{1b}-adrenoceptor, an additional cDNA clone was identified, which apparently encoded for a novel α_1-adrenoceptor subtype.[15] The protein expressed by this clone is distinct from the α_{1b}-adrenoceptor, with only a 65% amino acid identity in the membrane-spanning domains. The pharmacological profile of this receptor is also distinct from either the α_{1B}- or α_{1D}-adrenoceptors, having a relatively high affinity for 5-methylurapidil (but less than that observed in most α_{1A}-adrenoceptor test systems), a high affinity for WB-4101, and high sensitivity to irreversible inactivation by (chloroethyl)-clonidine. In view of its distinct pharmacologic profile,

Table 1. Nomenclature for the Adrenoceptors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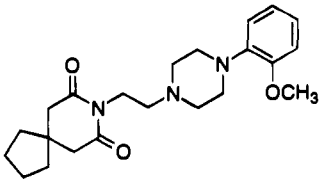
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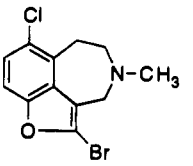
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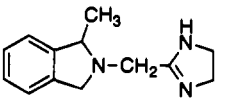
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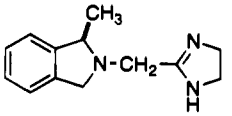
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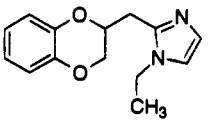
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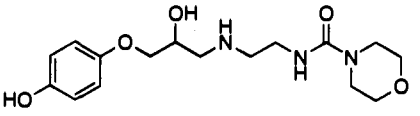
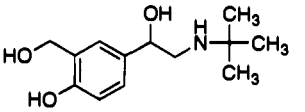
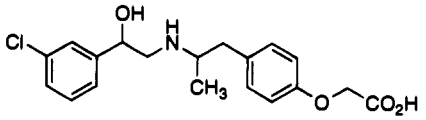

Xamoterol


Salbutamol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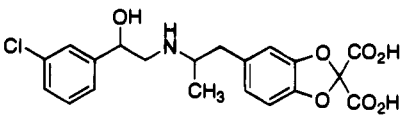
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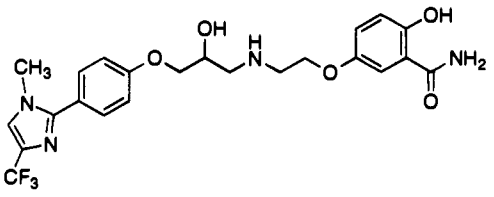
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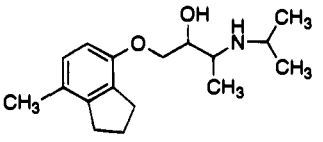
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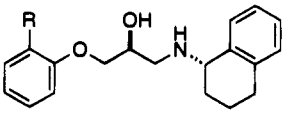

SR 58894 (R = $CH_2CH{=}CH_2$)
SR 59230 (R = CH_2CH_3)

native	cloned: new[a]	cloned: historical	human chromosome location	functional response	tissue location[b]	(chloroethyl)clonidine sensitivity	selective antagonist
α_{1A}	α_{1a} L31774	α_{1c}	C8	perfused rat kidney	rabbit liver rat submaxillary gland	±[e]	SNAP-5089 (*S*)-niguldipine
α_{1B}	α_{1b} L31773	α_{1b}	C5	rat spleen[d]	rat liver rat spleen	++++	
α_{1D}	α_{1d} L31772	$\alpha_{1a/d}$ α_{1a}	C20	rat aorta[e]		++	BMY 7378 SK&F 105854
α_{2A}	α_{2a} M18415	α_2C10	C10	platelet aggregation canine saphenous vein (contraction)	human platelet CNS	+	BRL 44408 BRL 48962[f]
α_{2B}	α_{2b} M 34041	α_2C2 RNG (rat)	C2		neonatal rat lung CNS, liver	−	imiloxan[g]
α_{2C}	α_{2c} J 03853	α_2C4 RG10 (rat)	C4	rat caudal artery (contraction?)	opossum kidney human kidney CNS	+	
α_{2D}	α_{2d} M 62372	α_{2a} (rodent) RG20 (rat)			rat submaxillary gland bovine pineal		

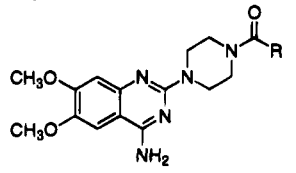
native	cloned	functional response	tissue location	selective agonist	selective antagonist
β_1	β_1 J 03019	increase in cardiac force	myocardium CNS	xamoterol[h]	CGP 20712
β_2	β_2 J 02960	rat uterus (relaxation)	human lymphocyte CNS	salbutamol	ICI 118,551
β_3	β_3 M 29932	rat adipocyte (lipolysis) rat colon (relaxation)	rat adipocyte	BRL 37344 CL 316,243	SR 58894 SR 59230

[a] Includes GenBank accession number of most recently reported human sequence (α_{2d}-rat sequence). [b] As identified using radioligand binding assays in native tissues. [c] (Chloroethyl)clonidine sensitivity of the α_{1A}-adrenoceptor may be species dependent. [d] Prazosin-sensitive component. [e] May not be a homogeneous population.[252,253] [f] BRL 48962 is the *R* enantiomer of BRL 44408. [g] Imiloxan is selective for α_{2B}- versus α_{2A}-receptors. Affinity for other subtypes has not been evaluated. [h] Partial agonist.

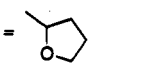
this receptor was originally designated as the α_{1C}-adrenoceptor. The human homolog of this receptor has now been cloned.[13,14,16] Like the bovine receptor, the human homolog has relatively high affinity for both WB-4101 and 5-methylurapidil, and the available data (Table 2)[14] suggest similar pharmacologic profiles exist for the bovine and human receptors.

Northern analysis, using a probe derived from the putative bovine α_{1c}-adrenoceptor, demonstrated a limited tissue distribution for the receptor, with hybridiza-

Table 2. Comparison of the Affinity of α_1-Adrenoceptor Antagonists for Expressed α_1-Adrenoceptor Clones



Terazosin R =



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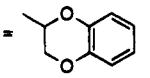
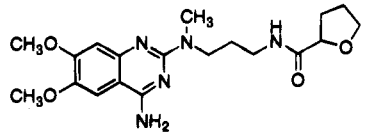
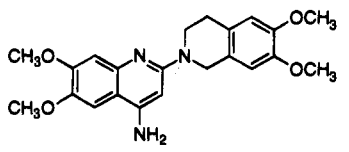
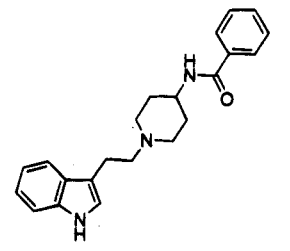




Alfuzosin



Abanoquil



Indoramin

compd	clone[a] (K_i (nM)					
	$\alpha_{1d}{}^H$	$\alpha_{1d}{}^R$	$\alpha_{1b}{}^H$	$\alpha_{1b}{}^{Hm}$	$\alpha_{1a}{}^H$	$\alpha_{1a}{}^B$
prazosin	0.32	0.79	0.32	0.32	0.51	0.17
terazosin	3.5	35	2.2	30	7.3	26
doxazosin	1.2		0.74		2.6	
alfuzosin	3.6	3.5	2.8	12	8.2	23
5-methylurapidil	21	186	92	535	2.3	4.4
abanoquil	0.04	0.03	0.08	0.11	0.04	0.03
WB-4101	0.8	1.2	9.9	37	0.37	0.35
indoramin	110	611	29	25	3.7	12

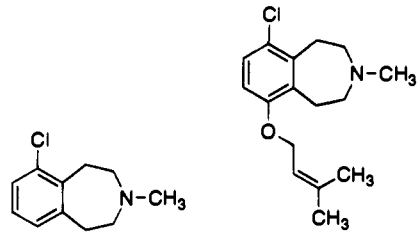
[a] α_1-Adrenoceptor clones expressed in CHO, COS-7, or rat fibroblast cells. H superscript denotes human clones (mean values of data from refs 13, 254, 256); Hm, R, or B superscript denotes hamster, rat, or bovine clones, respectively (mean values of data from refs 255, 254, 18).

tion detected only in rabbit liver and human hippocampus, and no evidence for receptor expression in any rat tissue.[17] Despite these results, a rat homolog to the bovine receptor has recently been cloned and expressed.[18,19] Interestingly, the cloned rat homolog shows a relatively low sensitivity to alkylation by (chloroethyl)clonidine and an affinity profile for competitive antagonists that is consistent with the α_{1A}-adrenoceptor in native tissues.[19,20] Comparison of the α_{1c}-adrenoceptor clones from rat, human, and bovine sources shows the bovine receptor to be more sensitive to alkylation by (chloroethyl)clonidine than the rat or human receptors.[21] The bovine receptor cloned by Schwinn *et al.*[15] was originally designated as a novel subtype (α_{1C}), rather than α_{1A}-adrenoceptor, based primarily on its sensitivity to (chloroethyl)clonidine and its lack of expression in rat tissues. Because subsequent studies have clearly shown that the degree of (chloroethyl)clonidine sensitivity observed in this bovine α_1-adrenoceptor clone may not be typical of this subtype, and considering that the homologous receptor has indeed been found in the rat, it now seems certain that this recombinant receptor corresponds to the α_{1A}-adrenoceptor, as defined pharmacologically in native tissues. This has led to some confusion in nomenclature, and it is now generally accepted that the α_{1c}-adrenoceptor designation be dropped and that the three cloned α_1-adrenoceptors be referred to as α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptors[11,20] (see Table 1).

B. α_2-Adrenoceptors. An α_2-adrenoceptor cDNA was first isolated from human platelet[22] and designated as α_2C10, based on its location on human chromosome 10. Southern analysis with a fragment of the α_2C10 cDNA revealed the presence of related genes on chromosomes 2 and 4. These genes have subsequently been cloned and expressed and are designated as α_2C2 and α_2C4.[23,24] The pharmacological characteristics of these three receptor proteins are consistent with α_2-adrenoceptors. A porcine analog of α_2C10, showing greater than 93% amino acid identity with the human receptor and similar pharmacologic characteristics, has also been isolated.[25,26]

Three α_2-adrenoceptors have also been cloned from the rat. One, designated as RNG, clearly appears to be the rat homolog of α_2C2, on the basis of similar pharmacologic profiles, as well as on the unique lack of consensus sequences for N-linked glycosylation on the amino terminus (although showing only 82% amino acid identity to the human receptor). Another receptor, designated as either pA_2d[27] or RG10,[28] appears to be a species homolog of the human α_2C4-adrenoceptor, with 88% identity of primary structure. The third rat clone, designated as cA_2-47[29] or RG20,[28] shares 89% amino acid identity with the α_2C10, as well as key similarities in pharmacologic profile, such as a low affinity for prazosin. However, some investigators suggest that, rather than being a rat homolog of the human α_2C10, the RG20 clone represents a distinct α_2-adrenoceptor

Table 3. Comparison of the Affinity of α_2-Adrenoceptor Antagonists for Expressed α_2-Adrenoceptor Clones

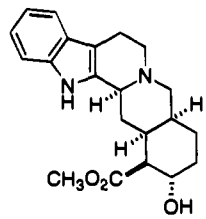


SK&F 86466 SK&F 104078

compd	clone[a] K_i (nM) α_{2a}^H	α_{2d}^R	α_{2b}^H	α_{2c}^H
rauwolscine	3.5	35	4.6	0.6
yohimbine	1.6	52	7.2	1.1
SK&F 86466	9.4		15.8	19.8
SK&F 104078	114[b]	313	142[b]	64[b]
phentolamine	2.6	4.7	7.6	8.4
prazosin	2133	1668	365	95
terazosin	3702		418	213
doxazosin	729		>5000	280
5-methylurapidil	612		406	131
WB-4101	3.5	46	28	0.8
indoramin	2240		528	476

[a] α_2-Adrenoceptor clones expressed in CHO (human) or COS (rat) cells. H superscript denotes human clones (Hieble and Naselsky, unpublished data); R superscript denotes rat clone (mean values of data from refs 26, 28, 38). [b] Mean values of data from human or rat (RNG, RG10 clones) as reported by O'Rourke *et al.*;[26] Lanier *et al.*;[28] Xia *et al.*;[163] Harrison *et al.*[39]

subtype. This is based primarily on the low affinity of the expressed RG20 clone for yohimbine and rauwolscine (8) (Table 3).



(8) Rauwolscine

Three α_2-adrenoceptors have also been cloned from the mouse, having correspondingly high degrees of amino acid identity with the human α_2C2, α_2C4, and α_2C10. The mouse homologs of α_2C2[30] and α_2C4[31] have similar pharmacological profiles to the human clones. As observed in the rat, the mouse homolog of the human α_2C10 has a lower affinity for yohimbine and rauwolscine.[31] An α_2-adrenoceptor has recently been cloned from OK cells;[32] this receptor shows lower amino acid identity with the human α_2C4 receptor than the corresponding rat homolog.

Several clear correlations between the α_2-adrenoceptor subtypes identified in native tissues and cell lines have been established with the receptor proteins expressed from the cDNA clones. However, a disparity remains in that only three α_2-adrenoceptors have been cloned, although four subtypes have been identified through radioligand binding correlations. Correlations between cloned and native α_2-adrenoceptors are supported by hybridization of the α_2-adrenoceptor clones in tissues known to contain a particular α_2-adrenoceptor subtype. The α_2C10 clone was isolated from human platelets. The expressed receptor has radioligand binding characteristics that are in good agreement with those of the platelet α_{2A}-adrenoceptor, and it is now certain that the α_2C10 clone corresponds to the α_{2A}-adrenoceptor. Although the α_2C4 clone was initially thought to correspond to the α_{2B}-adrenoceptor, on the basis of a high affinity for prazosin, comparison of affinities for an extensive series of antagonists shows this clone to correspond more closely to the α_{2C}-adrenoceptor.[33] Northern analysis shows a strong signal when the α_2C4 gene is hybridized with mRNA prepared from OK cells, the source from which the α_{2C}-adrenoceptor subtype was identified.[34] The rat RG10 clone also appears to have the pharmacologic characteristics of the α_{2C}-adrenoceptor.[35,36] Finally, the α_2C2 clone and the corresponding RNG clone correspond well to the α_{2B}-adrenoceptor, on the basis of protein structure and pharmacologic profile. Furthermore, this clone is detected in neonatal rat lung and adult rat kidney, tissues known to possess the α_{2B}-adrenoceptor.[24,37]

The assignment of the rat RG20 clone has been more controversial. As noted above, this clone has 89% amino acid identity with the human α_2C10; however, the pharmacologic profile of the RG20, unlike that of α_2-C10, does not clearly correspond to the α_{2A}-adrenoceptor. A consistent characteristic of the RG20 clone is a relatively low affinity (K_D = 10 nM) for yohimbine and rauwolscine. This has led some investigators to assign this clone to the α_{2D}-adrenoceptor subtype which was identified through radioligand binding correlations (see above), where these antagonists have been observed to have lower affinity than at the other three α_2-adrenoceptor subtypes. Other groups consider the low affinity for rauwolscine to represent a species variation and have therefore assigned the RG20 clone, like the α_2C10 clone, to the α_{2A}-adrenoceptor subtype. One group has observed a relatively low affinity (K_i = 531 nM)[28] of the RG20 clone for SK&F 104078 (9), a novel antagonist that can functionally discriminate between some α_2-adrenoceptors. This fact was used to suggest that the RG20 clone represented the prejunctional, neuronal α_2-adrenoceptor, which has also been postulated to have α_{2D}-adrenoceptor characteristics.[38] However, other investigators have not observed any substantial difference in the affinity of SK&F 104078 between rat α_2-adrenoceptor clones.[39]

Although the RG20 (rat) and α_2C10 (human) clones have distinct pharmacologic profiles, the corresponding clones from murine[31] and bovine (D. B. Bylund, unpublished data) sources appear to resemble closely the rat RG20 clone. The low affinity of the RG20 clone and its mouse homolog for yohimbine and rauwolscine has been postulated to result from a serine at position 201, as opposed to a cysteine in the human α_2C10 clone and its porcine homolog[31] (see below). Hence, molecular evidence is accumulating to support the notion that the α_{2A}- and α_{2D}-adrenoceptors are simply species variants, with the α_{2A}-adrenoceptor, but not the α_{2D}-adrenoceptor, being present in human and porcine tissues and, conversely, the α_{2D}-adrenoceptor, but not the α_{2A}-adrenoceptor, being present in rat, mouse, and bovine tissues. The only data that are perhaps inconsistent with this hypothesis are the assignment of the prejunctional α_2-adrenoceptor in the rabbit to the α_{2A}-adrenoceptor subtype[40] and the adipocyte α_2-adrenoceptor in

Table 4. Radioligand Binding Affinities of Antagonists to α_2-Adrenoceptor Subtypes

source	K_i (nM)[a]				affinity ratio			ref
	YOH	RAU	PRZ	OXY	PRZ/OXY	OXY/YOH	PRZ/YOH	
human platelet	0.9	1.3	540	3.5	154	3.8	600	38, 257–259
HT-29 cell	1.8	1.2	2000	2.2	923	1.2	1128	33, 260–261
human adipocyte	3	4	2200	16	137	5	733	262
canine adipocyte	4		2700	16	158	4	675	263
α_2C10	3.8	4.7	1600	10.5	153	2.8	423	8, 28, 33
α_2-clone (porcine)	4.4		4100	21	197	5	941	25
neonatal rat lung	1	0.4	5	52	0.1	52	5	264
rat kidney	5	2.5	51	220	0.2	44	10	265
NG-108 cell	0.6	0.8	13	132	0.1	220	22	158, 260
α_2C2	4.1	3.9	108	1000	0.1	245	26	5, 33, 266
RNG (rat)	8.7	9.6	46	610	0.1	70	5.3	39, 163
opossum kidney	0.4	0.1	36	73	0.5	182	90	159
OK cell	0.6	0.3	34	26	1.3	43	57	33, 157
Y-79 cell		0.4	123	14	8.8	35[b]	307[b]	157
α_2C4	0.6	0.8	40	73	0.5	122	67	5, 24, 33
Mα_2-4H (mouse)	3.8	0.8	97	109	0.9	29	26	31
RG10 (rat)	2.4	0.9	53	140	0.4	58	22	27, 28, 36, 39, 267
hamster adipocyte	33		2260	3	753	0.1	68	268
rabbit adipocyte	35			14	642	0.4		269
rat adipocyte	70		1800	54	34	0.7	27	270
rat enterocyte	54	45	1900	10	190	0.2	35	271
rat submaxillary		18	457	8	57[b]	0.4[b]	25[b]	157, 160
RINm5F cell	104	83	1900	121	16	1.2	18	272
bovine pineal	3.6	3.4	106	1.5	71	0.4	29	160
RG20 (rat)	52	51	2065	28	74	0.5	40	28, 39
Mα_2-10H (mouse)	54	53	2150	33	65	0.6	40	31

[a] K_i for inhibition of the binding of [^{3}H]yohimbine, [^{3}H]rauwolscine, [^{3}H]RX 821002, or [^{3}H]MK-912 to membrane homogenates. [b] Oxymetazoline/rauwolscine or prazosin/rauwolscine.

this species to the α_{2D}-adrenoceptor subtype (Table 4). If these assignments are correct, then it appears that the rabbit can express both α_{2A}- and α_{2D}-adrenoceptors.

2. β-Adrenoceptor Subtypes. The molecular pharmacology of the β-adrenoceptor has been studied in detail, with the goals of relating protein structure to agonist/antagonist affinity and coupling to second messengers. As compared to the molecular pharmacology of α-adrenoceptors (described above), there is less controversy regarding the correspondence of native and recombinant receptors.

For both the β_1- and β_2-adrenoceptors, there exists an excellent correlation between the properties of the expressed receptor clones and the corresponding receptor as found in native tissues. This correlation is found with respect to both the ability to inhibit radioligand binding and the ability of agonists to stimulate adenylylcyclase activity.

A. β_2-Adrenoceptors. Screening of a hamster genomic library with oligonucleotides complementary to peptide fragments of the purified hamster lung β_2-adrenoceptor yielded a clone which, when expressed in a variety of systems, had functional and radioligand binding properties that were consistent with those of the β_2-adrenoceptor.[41] Using probes derived from this cDNA, β_2-adrenoceptors from mouse, rat, and human sources have been cloned. There are only minor species differences between these clones, with 87–93% overall amino acid identity.

B. β_1-Adrenoceptors. It proved difficult to clone the β_1-adrenoceptor because the human β_2-adrenoceptor cDNA did not cross-hybridize with the β_1-adrenoceptor, even when the full length coding sequence was used. Using the β_2-adrenoceptor as a probe, a related receptor was isolated,[42] which proved to be the 5-HT$_{1A}$ receptor.[43] Using the coding region of the 5-HT$_{1A}$ receptor DNA to probe a human placental cDNA library, the β_1-adrenoceptor clone was finally identified.[44] The overall amino acid identity of human β_1- and β_2-adrenoceptors is only 54%, although the amino acid identity between these receptors increases to 71% in the hydrophobic regions postulated to represent the membrane-spanning domains.

Again using purified receptor protein as a source of oligonucleotide probes, the avian β-adrenoceptor of turkey erythrocyte was cloned.[45] This clone resembles the mammalian β_1-adrenoceptor closely (69% overall amino acid identity with the human receptor and 84% amino acid identity in the transmembrane-spanning regions).

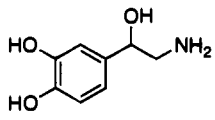
β_1-Adrenoceptor proteins have been cloned from the rat[46,47] and the mouse.[48] Rat and mouse β_1-adrenoceptors show 98% overall amino acid identity with each other and 92% overall identity with the human β_1-adrenoceptor. Species-related differences between rodent and human receptors are concentrated in the third cytoplasmic loop.[48]

C. β_3-Adrenoceptors. Although the presence of "atypical" β-adrenoceptors having characteristics differing from either β_1- or β_2-adrenoceptors was suggested by functional pharmacological assays,[49,50] the existence of a third β-adrenoceptor subtype was not universally accepted until the cloning of this novel receptor protein.

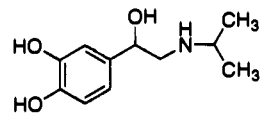
Screening of a human genomic library with probes prepared from the avian β-adrenoceptor and the human β_2-adrenoceptor cDNA resulted in the identification of a novel clone, termed the β_3-adrenoceptor, having the predicted sequence for a G-protein-linked receptor.[51] The identity in primary structure for the β_3-adrenoceptor was only 40–50% *vis a vis* the β_1- or β_2-adrenoceptors (64–69% in the putative transmembrane-spanning regions). A homologous β_3-adrenoceptor was cloned from rat brown adipose tissue;[52,53] this rat β_3-adrenoceptor has 80% overall amino acid identity with the

human β_3-adrenoceptor. Probing a murine genomic library with the entire coding region of the human β_3-adrenoceptor led to the identification of the mouse β_3-adrenoceptor, which shows 82% overall amino acid identity with the human β_3-adrenoceptor.[54] By analogy to the β_1- and β_2-adrenoceptors, the β_3-adrenoceptor gene was assumed to have no introns; however, studies mapping the rat gene have shown the presence of an intron near the end of the coding block.[53,55,56] The presence of similar splicing sites in the rat, mouse, and human β_3-adrenoceptor genes suggests that the mouse and human genes may also contain introns and that the complete β_3-adrenoceptor protein may contain 6 (human) or 12 (rat, mouse) additional amino acids at the carboxy terminus.[53,56] It is possible, therefore, that two forms of the β_3-adrenoceptor exist due to splicing variants at this intron.

The pharmacologic properties of the recombinant β_3-adrenoceptor, expressed in mammalian cells, appear to correspond closely to those previously defined for the atypical β-adrenoceptor. The selective agonist BRL 37344 (**10**) produces potent activation of adenylate cyclase, with a maximum response equivalent to that obtained with norepinephrine (11) or isoproterenol (**12**).[51-54] The characteristics of rodent and human β_3-



(11) Norepinephrine



(12) Isoproterenol

adrenoceptors appear to be qualitatively similar, although the rodent receptors are less sensitive to activation by the endogenous catecholamines[52,57] and the human receptor is less sensitive to the selective β_3-adrenoceptor agonist BRL 37344.[58] Recent studies have shown that both agonists and antagonists can show substantial selectivity between mouse and human β_3-adrenoceptors.[57] Comparison of the pharmacological profile of the truncated (402 amino acids) and full length (408 amino acids) human β_3-adrenoceptors shows no substantial differences.[56]

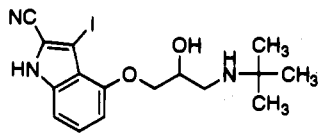
Messenger RNA hybridizing with the recombinant human β_3-adrenoceptor was found in rat tissues where "atypical" β-adrenoceptor-mediated responses have been shown to occur, such as adipose tissue, liver, and skeletal muscle.[51] In contrast, Muzzin *et al.*[52] detected β_3-adrenoceptor mRNA only in rat white and brown adipose tissue, using a probe derived from rat β_3-adrenoceptor cDNA. Conflicting data have been presented with respect to β_3-adrenoceptor expression in human tissues. Krief *et al.*[59] found β_3-adrenoceptor mRNA in adipose tissue and gall bladder, and Granneman *et al.*[56] found β_3-adrenoceptor for mRNA in adipose and intestinal tissues. In contrast, Thomas and Liggett[60] could not detect β_3-adrenoceptor mRNA in any human tissue, including adipose tissue, even when using a sensitive reverse transcriptase PCR assay.

Molecular Characterization of Ligand–Receptor Interaction and Coupling of the Adrenoceptors to Signal Transduction Processes

1. **Physical Structure of the Adrenoceptor Proteins.** Both α- and β-adrenoceptors have been isolated by detergent solubilization and purified by affinity chromatography;[61-63] however, studies on the structure of these receptor proteins, their mode of interaction with the cell membrane, and their interaction with receptor ligands and effector systems were only made possible by the molecular biological techniques which allowed for the cloning and expression of these receptors (see Molecular Biology of the Adrenoceptors). When the β_2-adrenoceptor was first cloned,[41] it was noted that, although there was no substantial amino acid identity with the visual protein rhodopsin, the distribution of hydrophobic and hydrophilic amino acids (hydropathy analysis) within the primary sequence of the β_2-adrenoceptor was remarkably similar to that of rhodopsin. Hydropathy analysis had previously been used to suggest that the amino acid chain of rhodopsin passed through the cell membrane seven times, with the amino and carboxyl termini located in the extracellular space and cytoplasm, respectively, and the segments that spanned the cell membrane were amino acid arranged in an α-helical orientation. The seven α-helices proposed for the β_2-adrenoceptor are radically arranged around a central "pore", in which the receptor ligands bind (see review by Findlay and Pappin, 1986). While this postulated structure is consistent with data from spectroscopic analysis and chemical labeling with photoactivated probes,[64,65] it should be noted that the only membrane protein for which the "seven-transmembrane structure" has been rigorously proven to exist by electron diffraction is bacteriorhodopsin,[66] a protein functioning as a light-driven proton pump in *Halobacterium halobium*. Although rhodopsin and bacteriorhodopsin both respond to light activation through isomerization of a covalently bound retinal molecule, they have no discernible sequence homology and act through completely different second-messenger and effector systems. Hence, although hydropathy analysis assigns many neutrotransmitter and hormone receptors, including all of the adrenoceptors, to the seven-transmembrane-spanning model, and this model is used as the basis for all of the ligand–receptor and G-protein–receptor interaction studies described below, this assignment is based on analogy to a rather dissimilar membrane protein rather than on direct physical evidence.

2. **Interaction of Agonists and Antagonists with Adrenoceptor Proteins. A. β-Adrenoceptors.** Nearly all of the studies directed toward elucidating the mode of interaction of agonists and antagonists with adrenoceptor proteins have been based on the β-adrenoceptor, most commonly the β_2-adrenoceptor subtype. The β-adrenoceptor subtypes range from 388 to 477 amino acids in length. Most models proposed for these receptors assume an orientation of seven transmembrane-spanning helices similar to that demonstrated for bacteriorhodopsin and assumed to occur for mammalian rhodopsin. This is based on several analogies between these molecules, including the proposed ligand–receptor interaction site, where the ligand is hypothesized to bind

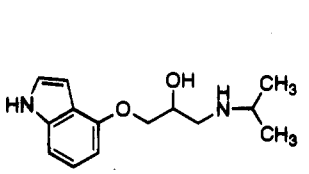
to several of the transmembrane domains of the receptor protein, in a pocket created by the circular array of transmembrane helices. Coexpression of two β_2-adrenoceptor fragments, one comprising transmembrane helices 1–5 and the other providing helices 6 and 7, resulted in a functional membrane receptor capable of binding the β-adrenoceptor radioligand [^{125}I]iodocyanopindolol ([^{125}I]ICYP) (**13**) and mediating activation of adenylate cyclase, in spite of the fact that neither fragment alone recognized a β-adrenoceptor ligand.[67]



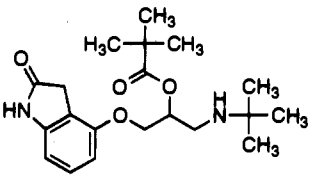
(13) Iodocyanopindolol

Site-directed mutagenesis has demonstrated that an aspartic acid residue, Asp^{113}, located in the third transmembrane-spanning helix, is required for both agonist and antagonist binding to the β_2-adrenoceptor. It is postulated that the free carboxyl group of this amino acid interacts with the protonated amino group of the adrenergic ligands. This is consistent with the observation that aspartic acid is found at this position in all of the adrenoreceptors. Mutation of Asp^{113} to glutamic acid substantially reduces the affinity of the catecholamine neurotransmitters for the β_2-adrenoceptor, suggesting that the resulting change in position of the carboxyl group unfavorably influences ligand–receptor interaction.[68,69] However, this mutation apparently enhances the ability of some β-adrenoceptor partial agonists to produce receptor activation (see below).

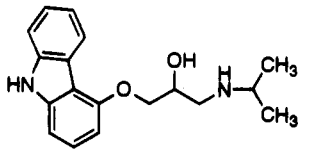
The *m*- and *p*-hydroxyl groups of the catecholamine agonists are postulated to form hydrogen bonds to Ser^{204} and Ser^{207}, respectively, which are located in the fifth transmembrane-spanning helix.[70] Mutagenesis of these serines markedly reduces the affinity of agonist, but not antagonist, ligands. Binding to both Ser^{204} and Ser^{207} appears to be required for full agonist activity at the β-adrenoceptors and, by interfering with these interactions, either by mutation-induced removal of a serine or by removal of a catechol hydroxyl group from the agonist, results in reduced affinity of agonists for the β-adrenoceptor.[68,69] Although many β-adrenoceptor antagonists lack any functional groups capable of hydrogen bonding to Ser^{204} or Ser^{207}, a molecule such as pindolol (14) may be capable of some interaction with Ser^{207} through the indole nitrogen, perhaps explaining its partial agonist activity in many *in vitro* and *in vivo* models.[71] A compound with a more acidic hydrogen in this position, Sandoz 23–784 (**15**), would be expected to form a stronger hydrogen bond to Ser^{207} and therefore has greater intrinsic activity at the β-adrenoceptor than pindolol.[72] This additional site for binding may also contribute to the higher affinity of β-adrenoceptor antagonists containing a nitrogen atom in this position, such as pindolol and carazolol (**16**), compared to propranolol (**17**) or 1-phenoxy-3-(isopropylamino)-2-propanol (1).[73] Such an interaction could also explain the enhanced efficacy and affinity of ring-chlorinated phenylethanolamines, such as dichloroisoproterenol (**19**), compared to analogs with no ring substituents.



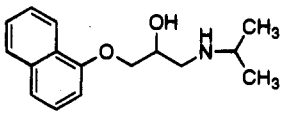
(14) Pindolol



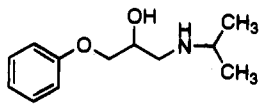
(15) SDZ 23-784



(16) Carazolol



(17) Propranolol



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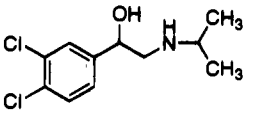
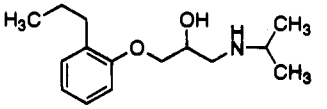


(19) Dichloroisoproterenol

The potential binding site for the β-hydroxyl group of the catecholamine neurotransmitters has not been investigated extensively. Maloney-Huss and Lybrand[74] have proposed a β_2-adrenoceptor model differing from that commonly proposed, in which the transmembrane-spanning helices are arranged in a "mirror-image" orientation to that found in bacteriorhodopsin. This model will accommodate the binding of epinephrine to Asp^{113}, Ser^{204}, and Ser^{207}, as proposed by the other models, but shows a potential interaction between the β-hydroxyl group of the naturally occurring *R* enantiomer of epinephrine with Ser^{319} in the seventh transmembrane-spanning helix. Site-directed mutagenesis has shown Ser^{319} to play a potential role in agonist binding to the β_2-adrenoceptor.[75] Other β_2-adrenoceptor models, based on the bacteriorhodopsin orientation, predict an interaction of the β-hydroxyl group with Ser^{165} of transmembrane helix 4[76] or Asn^{293} of transmembrane helix 6.[77]

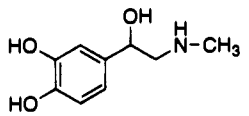
Several other amino acids have been shown to be important in the binding of agonists or antagonists to the β_2-adrenoceptor. It has been postulated that the phenyl ring of Phe^{290}, in the sixth transmembrane-spanning helix, participates in binding of the aromatic ring of agonist ligands.[68,69] An interaction of epinephrine with this amino acid is also predicted by the model of Maloney-Huss and Lybrand.[74] Asn^{312}, in the seventh transmembrane-spanning helix, appears to be important in the binding of phenoxypropanolamine antagonists, since its replacement by alanine or phenylalanine abolishes the ability of [^{3}H]dihydroalprenolol (**20**) to bind to the β_2-adrenoceptor.[78] It is postulated that a



(20) Dihydroalprenolol

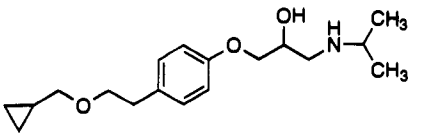
hydrogen bond is formed between the amide group of asparagine and the ether oxygen of the phenoxypropanolamine. A β_2-adrenoceptor mutant where Asn^{312} is replaced by glutamine has affinity for phenoxypropanolamines that is similar to that observed for the wild type; the corresponding threonine analog, which would be expected to form a weaker hydrogen bond, retains some affinity.

The β_1-adrenoceptor would be predicted to have the same structural orientation as the β_2-adrenoceptor and contains aspartic acid (Asp^{138}) and serine residues (Ser^{229}, Ser^{232}) in corresponding positions to those shown to be important for the binding of agonists to the β_2-adrenoceptor. It has not been possible to localize any specific changes in the receptor protein responsible for the relatively subtle differences in pharmacology that exist between the β_1- and β_2-adrenoceptor subtypes. The ability of epinephrine (**21**) and norepinephrine to inhibit [^{125}I]ICYP binding to a series of chimeric receptors that combine the β_1 and β_2-adrenoceptor structures was compared.[79] Consistent with their known pharmacologi-



(21) Epinephrine

cal effects in native tissues, the two catecholamines were equipotent in the wild type β_1-adrenoceptor preparation, and epinephrine was 15-fold more potent than norepinephrine in the wild type β_2-adrenoceptor. Replacing the β_1-adrenoceptor transmembrane-spanning helices 1, 1 and 2 or 1, 2, and 3 with those of the β_2-adrenoceptor had no substantial effect on the relative affinities of the two catecholamines. A chimeric receptor containing helices 1–4 from the β_2-adrenoceptor and helices 5–7 from the β_1-adrenoceptor resulted in a chimeric β-adrenoceptor that resembled pharmacologically the β_2-adrenoceptor. Hence, the structural determinants for agonist affinity appear to be located in transmembrane-spanning helix 4. This is consistent with the observation that helices 4 and 7 show the least amino acid identity between the two β-adrenoceptor subtypes (14 of 25 amino acids identical), while helices 3 and 5, which are postulated to contain the primary catecholamine binding sites, are the most identical (20–22 of 25 amino acids identical). Replacing helices 4 and 5 of the β_1-adrenoceptor with the corresponding elements of the β_2-adrenoceptor appeared to have less of an effect on the epinephrine/norepinephrine affinity ratio.[80] When the relative affinities of the subtype-selective antagonists betaxolol (**22**) and ICI 118551 (**23**) were compared, progressive replacement of transmembrane-spanning helices of the β_1-adrenoceptor with those of the β_2-adrenoceptor produced a progressive change in pharmacological character from β_1 to β_2.[79] The regions that determine the selectivity of CGP 20712 (**24**)for the β_1-adrenoceptor subtype did not appear to be located in transmembrane-spanning helix 4 since the chimeric receptor in which helices 4 and 5 of the β_1-adrenoceptor were replaced had high affinity for this antagonist (K_i = 3.6 nM), equal to that of the wild type β_1-adrenoceptor (K_i = 3.3 nM), and the β_2-adrenoceptor with helices 4 and 5 replaced by those of the β_1-adrenoceptor had low



(22) Betaxolol

affinity (K_i = 2300 nM), comparable to that of the wild type β_2-adrenoceptor (K_i = 4900 nM).[80] Because replacement of individual transmembrane-spanning helices have differential effects on different agonists and antagonists, which would each be expected to interact with the receptor in a slightly different manner, it is likely that the pharmacological differences between β_1 and β_2-adrenoceptors are due to subtle changes in orientation of the primary binding sites, resulting in a slightly different binding pocket, rather than to specific amino acid substitutions.

The human β_3-adrenoceptor protein contains aspartic acid (Asp^{117}) and serine (Ser^{209}, Ser^{212}) residues in positions corresponding to those found in the other β-adrenoceptor subtypes. The rat β_3-adrenoceptor also contains these amino acids (Asp^{114}, Ser^{206}, Ser^{209}). Both human and rat β_3-adrenoceptors are relatively insensitive to stimulation by the endogenous catecholamines and differ markedly from the β_1- and β_2-adrenoceptors with respect to the affinity and efficacy of several synthetic agonists.[58] Whether any relationship exists between these pharmacological properties and the molecular structure of the β_3-adrenoceptor protein has not yet been established.

B. α-Adrenoceptors. Hydropathy analysis suggests that the α-adrenoceptors also have a similar molecular structure to the β-adrenoceptors, with seven transmembrane-spanning helices. The α_1-adrenoceptors, particularly the α_{1b}- and α_{1d}-subtypes, are somewhat larger than the β-adrenoceptors (501–560 amino acids), with longer extracellular amino and cytoplasmic carboxyl termini. The α_2-adrenoceptors are of comparable size to the β-adrenoceptors (450–461 amino acids) but differ from α_1- and β-adrenoceptors by having relatively short amino and carboxyl termini and very long third intracellular loops. In contrast to the many studies employing site-directed mutagenesis to characterize the interactions of agonists and antagonists with the β-adrenoceptors, little specific information is available about the influence of structural alteration of α-adrenoceptors on ligand–receptor interaction.

Kobilka *et al.*[67] constructed chimeric α_2/β_2-adrenoceptors and showed that the substitution of transmembrane-spanning helix 7 from the α_2-adrenoceptor was sufficient to confer specific [^{3}H]yohimbine binding to the β_2-adrenoceptor while at the same time eliminating [^{125}I]ICYP binding. However, this chimeric receptor bound [^{3}H]yohimbine only weakly, and the addition of transmembrane-spanning helices 1–4 from the α_2-adrenoceptor was required to form a chimeric receptor that had relatively high affinity for the α_2-adrenoceptor radioligand. Conversely, substitution of transmembrane-spanning helices 6 and 7 from the β_2-adrenoceptor conferred high-affinity specific binding for the β-adrenoceptor radioligand [^{125}I]ICYP and eliminated [^{3}H]yohimbine binding, although this receptor had low affinity for α- and β-adrenoceptor agonists. Adding an ad-

ditional transmembrane helix (helix 5) from the β_2-adrenoceptor enhanced β-adrenoceptor agonist affinity by at least 1 order of magnitude.

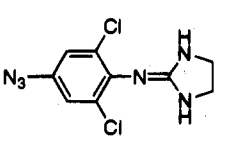
Several other observations are consistent with transmembrane-spanning helix 7 playing an important role in the binding of ligands to the α_2-adrenoceptor. If Phe^{412} of the α_{2a}-adrenoceptor is mutated to asparagine, the affinity for several α_2-adrenoceptor antagonists is reduced by several orders of magnitude,[81] and if Asn^{312} of the β_2-adrenoceptor, which is located in a position corresponding to Phe^{412} of the α_{2A}-adrenoceptor, is mutated to glutamine or threonine, the affinity of yohimbine was increased 11–15-fold and *p*-aminoclonidine, a selective α_2-adrenoceptor agonist, could activate adenylate cyclase.[78] A β_2-adrenoceptor mutant with Asn^{312} replaced by phenylalanine did not bind yohimbine. Although the wild type α_2-adrenoceptor contains phenylalanine at this position, other structural differences between the α_2- and β_2-adrenoceptors could alter the ligand–receptor interaction.

A chimeric receptor containing transmembrane-spanning helices 1–5 of the α_{2c}-adrenoceptor and helices 6 and 7 (plus the third intracellular loop) from the m3 muscarinic receptor does not bind [^{3}H]rauwolscine.[82] Interestingly, if this receptor is coexpressed with the complementary chimera containing helices 1–5 from the m3 muscarinic receptor and helices 6 and 7 from the α_{2c}-adrenoceptor, specific [^{3}H]rauwolscine binding is restored, showing that, as observed for the β_2-adrenoceptor,[67] two different protein molecules can associate within the membrane to form a complex which mimics the intact α_2-adrenoceptor, at least with respect to binding properties.

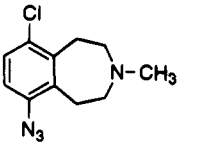
As in the β-adrenoceptors, an aspartic acid in transmembrane helix 3 is required for specific binding of ligands to α_2-adrenoceptors. Mutation of Asp^{113} to asparagine eliminated specific binding of [^{3}H]yohimbine to the α_{2a}-adrenoceptor.[83] However, the interaction of a catecholamine agonist with transmembrane helices 3 and 5 may be slightly different between the α_2- and β_2-adrenoceptors, since mutation studies suggest an interaction of Ser^{204} (which corresponds to Ser^{207} of the β_2-adrenoceptor) with the *p*-hydroxyl group but not between Ser^{200} (corresponding to Ser^{204} on the β_2-adrenoceptor) and the *m*-hydroxyl group.[83] These investigators postulate an interaction of the *m*-hydroxyl group of the catecholamine agonists with Cys^{201} of the α_2-adrenoceptor. It is interesting to note that agonist requirements differ between α_2- and β_2-adrenoceptors, with many structural classes (*e.g.*, phenethylamines, imidazolines, azepines) being capable of activating the α_2-adrenoceptor, while only phenethylamines bearing hydroxyl groups or hydroxyl-mimicking groups in positions corresponding to the catecholamine hydroxyl groups show potent agonist activity at the β_2-adrenoceptor. The structural diversity of α-adrenoceptor antagonists is also much greater than for β-adrenoceptor antagonists, which nearly exclusively contain a phenethylamine moiety.

As noted previously, one of the primary differences between the human and porcine α_{2a}-adrenoceptors and their mouse and rat homologs, which, despite a high degree of amino acid identity, have α_{2D}-adrenoceptor pharmacology, is the lower affinity of the rodent receptors for yohimbine and rauwolscine. Preparation of chimeric mouse/human α_{2a}-adrenoceptors showed that the substantial differences in the structure of these receptors at the junction between transmembrane-spanning helices 6 and 7 were not likely to be responsible for the differences in yohimbine affinity. This might result, in part, from the difference in a single amino acid at position 201 in transmembrane-spanning helix 5 (cysteine in human and porcine receptors, serine in rat and mouse). Although mutation of Ser^{201} in the mouse receptor to cysteine increased the affinity for yohimbine toward the level observed with the human receptor,[31] evaluation of the affinity of rauwolscine and several other antagonists for this mutant receptor showed no differences from the wild type mouse receptor, and substantial differences were still observed from the human α_{2a}-adrenoceptor.[84] Hence, the differences in pharmacology between the human α_{2a}-adrenoceptor and its mouse homolog may relate to subtle changes in structure of the binding pocket, involving additional structural differences between the two receptor proteins.

Studies with photoaffinity probes have suggested that both partial agonist and antagonist ligands can interact with transmembrane-spanning helix 4 of the α_{2a}-adrenoceptor. When photoactivated, the imidazoline partial agonist *p*-azidoclonidine (**25**) and the 3-benzazepine antagonist SK&F 102229 (**26**) formed covalent bonds to a partially purified preparation of platelet α_2-adrenoceptors (α_{2A}-adrenoceptor subtype). Enzymatic degradation of the labeled receptor showed that both probes attached to an amino acid within the fourth transmembrane-spanning helix, although the precise location of the attachment could not be determined.[85]



(25) p-Azidoclonidine

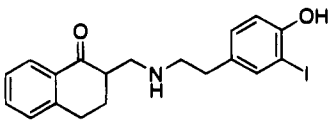


(26) SK&F 102229

Mutations of aspartic acid to asparagine at position 79, 130, or 432[86] or Cys^{442} to alanine or serine[87] do not substantially modify the ability of agonists or antagonists to inhibit the binding of [^{3}H]yohimbine to the porcine α_{2a}-adrenoceptor. Deletion of 75 amino acids from the third intracellular loop does not infuence the affinity of [^{3}H]yohimbine for the human α_{2a}-adrenoceptor or the ability of epinephrine to inhibit yohimbine binding.[88]

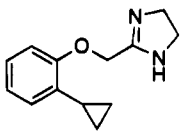
Mutagenesis studies have only recently been directed toward analysis of the interaction of ligands with the α_1-adrenoceptors. The aspartic acid and serine residues shown to be important for agonist/antagonist interaction with β- and α_2-adrenoceptors are conserved in all of the α_1-adrenoceptor subtypes, and the mode of interaction of catecholamines with the α_1-adrenoceptor would be predicted to be similar to that suggested for the other adrenoceptors. Interestingly, the α_{1b}- and α_{1d}-adrenoceptors, like the β-adrenoceptors, but in contrast to the α_2-adrenoceptors, have only two amino acids intervening between the serines thought to be important in binding the catecholamine hydroxyl groups. The α_{1a}-adrenoceptor, like the α_2-adrenoceptors, has three intervening amino acids. Mutation of Ser^{208} of the α_{1b}-adrenoceptor

to alanine does not influence the ability of norepinephrine to inhibit binding of [^{125}I]IBE-2254 (**27**) to the receptor.[89] This suggests thatthere may be only one

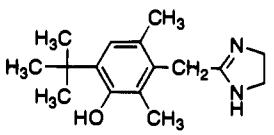


(27) IBE-2254

binding site for the catechol hydroxyl groups, as opposed to two in the β_2-adrenoceptor. Mutation of Ala^{204} to valine or Leu^{314} to methionine increases the affinity of the α_{1b}-adrenoceptor for the selective α_{1a}-adrenoceptor agonists oxymetazoline and cirazoline. Both of these mutations change a nonconserved amino acid of the α_{1b}-adrenoceptor to that present in the α_{1a}-adrenoceptor. Mutation of both Ala^{204} and Leu^{314} to valine and methionine, respectively, results in a mutant α_{1b}-adrenoceptor having affinity for cirazoline (**28**) and oxymetazoline (**29**) equivalent to that of the native α_{1A}-adrenoceptor. Conversely, mutation of Val^{185} and Met^{293}



(28) Cirazoline



(29) Oxymetazoline

of the α_{1a}-adrenoceptor reduces its affinity for these two agonists to that found for the native α_{1B}-adrenoceptor.[89] These data suggest that the selectivity of oxymetazoline for the α_{1A}-adrenoceptor results from a hydrophobic interaction between the *o*-methyl groups and Met^{293}, while the aromatic ring interacts with Val.[185]

Mutant α_1-adrenoceptors have been prepared to study the molecular interactions between receptors and second-messenger systems. Elimination of 47 amino acids from the cytoplasmic carboxyl terminus of the hamster α_{1b}-adrenoceptor had no effect on the binding affinity of [^{125}I]IBE-2254 or the potency of norepinephrine in either inhibiting radioligand binding or stimulating phosphatidylinositol hydrolysis.[90] Replacing 1−13 amino acid segments of the third intracellular loop of this receptor with the corresponding segments from the β_2-adrenoceptor had no effect on the affinity of [^{125}I]IBE-2254 and did not reduce the functional potency of norepinephrine. Indeed, in some cases a dramatic increase in the affinity of norepinephrine, as reflected in both radioligand binding and functional assays, was produced, even by the substitution of a single amino acid. For example, replacement of Ala^{293} by leucine produced a 100-fold increase in the affinity of norepinephrine.[90] Because these amino acids, located in a large cytoplasmic loop, are unlikely to participate directly in agonist binding, changes in this region may induce subtle changes in the overall receptor conformation, thereby indirectly influencing the geometry of the agonist binding pocket.

3. Interaction of Adrenoceptor Proteins with Second-Messenger Systems. The functional responses produced by activation of α- and β-adrenoceptors are produced through the interaction of the receptor proteins with one or more guanine nucleotide regulatory proteins (G-proteins). The G-proteins, which bind and hydrolyze GTP, are physically and functionally interposed between the receptor and its effector system.[91] G-Proteins are heterotrimeric, with subunits designated as α, β, and γ. Agonist binding to an adrenoceptor activates a G-protein by catalyzing the release of GDP from the α-subunit and its replacement by GTP. Following separation from the β- and γ-subunits, the α-subunit hydrolyzes the bound GTP.[92] There is structural diversity in each of these subunits, leading to a large number of distinct G-proteins. The adrenoceptors interact preferentially with three classes of G-proteins: G_s (β-adrenoceptor) mediating activation of adenylate cyclase, G_i (α_2-adrenoceptors) mediating inhibition of adenylate cyclase, and G_q (α_1-adrenoceptors) mediating activation of phospholipase C. Adrenoceptors can be coupled to other effector systems, such as ion channels,[93,94] perhaps through G_o,[95] and a single adrenoceptor subtype can be coupled to multiple effector systems, presumably through different G-proteins.[96] Furthermore, under some conditions, a given receptor can interact with several different G-proteins or effector systems, depending on either agonist concentration[97] or the characteristics of the particular cell in which it is expressed.[98] Within the major classes of adrenoceptors, there appears to be subtype selectivity in the efficiency of coupling to a particular G-protein.[97,99] It is also possible for different receptor subtypes to preferentially interact with different G-proteins. For example, the α_{2C}-adrenoceptor may interact with G_o rather than with G_i as observed with the other α_2-adrenoceptor subtypes.[100]

Site-directed mutagenesis and the preparation of chimeric receptors have established that the receptor and G-protein interact through the third intracellular loop. This portion of the adrenoceptor structure represents the site of greatest diversity between the adrenoceptor classes and between subtypes within each class. It appears that the character of this loop determines to which G-protein the receptor is preferentially coupled. Replacement of the fifth and sixth transmembrane-spanning helices of the α_2-adrenoceptor (and the intervening third intracellular loop) with those of the β_2-adrenoceptor results in a chimeric receptor which recognizes α_2-adrenoceptor ligands but can activate, rather than inhibit, adenylate cyclase, suggesting a preferential interaction with G_s.[67] Likewise, replacing the third intracellular loop of the β_2-adrenoceptor with that of the α_{1b}-adrenoceptor results in a chimeric receptor that couples to phospholipase C.[90]

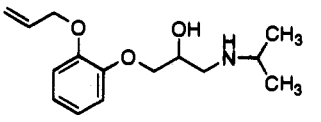
Replacing only a 27-amino acid segment of the N-terminus of the third intracellular loop by the α_{1b}-sequence resulted in a β_2-adrenoceptor mutant capable of activating both phospholipase C and adenylate cyclase.[101] Liggett *et al.*[102] reported that replacement of a 22-amino acid segment of the N-terminal region of the third intracellular loop of the human β_2-adrenoceptor with the corresponding portion of the α_{2a}-adrenoceptor did not affect the ability of the receptor to interact with G_s; however, conservative replacement of only four hydrophobic amino acids in this region of the hamster β_2-adrenoceptor resulted in a substantial reduction in the ability of the mutant receptor to mediate adenylate cyclase activation.[103] Interestingly, replacement of the

four basic residues in this portion of the receptor by serine did not substantially reduce the ability of the β_2-adrenoceptor to couple to adenylate cyclase.[103] Replacement of 12 amino acids from the C-terminal portion of the third intracellular loop of the β_2-adrenoceptor markedly attenuates the ability of isoproterenol to activate adenylate cyclase.[102] In the α_{1b}-adrenoceptor, several regions of the third intracellular loop are important for coupling to phospholipase C, including the N-terminus and several central regions within the loop, but not the C-terminus.[90] Replacement of 13 amino acids from the proximal portion of the cytoplasmic tail of the β_2-adrenoceptor only slightly attenuated the ability of isoproterenol to activate adenylate cyclase, but this mutation, when combined with that on the C-terminal portion of the third intracellular loop, totally abolished agonist-induced adenylate cyclase activation. Combination of these two mutations with that on the N-terminal portion of the loop (N- and C-termini of the third intracellular loop and proximal cytoplasmic tail) resulted in a chimeric receptor that preferentially coupled to G_i but was still capable of interacting with G_s when G_i was inactivated by pertussis toxin.[102] Removal of an 8-amino acid segment from the N-terminus of the third intracellular loop abolished the ability of the β_2-adrenoceptor to activate adenylate cyclase, as did removal of a 13-amino acid segment from the carboxyl terminus of this loop;[94] segments of equivalent length could be removed from the central portion of the loop without influencing adenylate cyclase activation. None of these mutations influenced the ability of the receptor to stimulate sodium–hydrogen exchange, showing that this effector system is coupled through a different portion of the receptor molecule.

Coexpression of the third intracellular loop of the α_{1b}-adrenoceptor inhibited the G-protein-linked coupling of the intact α_{1b}- or α_{1a}-adrenoceptor with phospholipase C.[104]

Several recent studies show that point mutations in the carboxyl terminus of the third intracellular loop can result in a receptor which can activate its effector, through G-protein interaction, without the necessity of agonist binding. Mutation of Ala^{293} of the α_{1b}-adrenoceptor to any of the 19 possible amino acids resulted in a receptor which stimulated basal inositol phosphate hydrolysis.[105] Depending upon the amino acid that replaced Ala^{293}, basal inositol phosphate hydrolysis was increased by 20% (serine) to 200% (glutamic acid). Interestingly, the basic amino acids, arginine and lysine, produced an effect that was nearly equivalent to that of glutamic acid, while aspartic acid was one of the least effective substitutions (30% increase over control). Mutation at this position also enhanced the ability of epinephrine to inhibit the binding of [^{125}I]IBE-2254 to the expressed receptor, without affecting the affinity of this antagonist radioligand. These results can best be explained by a change in receptor conformation which allows production interaction of the receptor and G-protein, thereby allowing activation of the effector system, without the necessity of an agonist-induced change in the secondary structure of the receptor. Small peptides derived from the intracellular loops of the adrenoceptors have been shown to bind and activate G-proteins.[106,107] Hence it is possible that in the native receptor, these regions may be shielded from interacting with the G-protein until agonist–receptor interaction induces an allosteric change in the conformation of this region.[108] Mutation of this key region allows some receptor–G-protein interaction to occur in the basal state, depending upon the character of the amino acid replacement. Mutation of four amino acids in the carboxy terminus of the third intracellular loop of the β_2-adrenoceptor produces a similar effect, yielding a receptor capable of activating adenylate cyclase without agonist occupation and having enhanced agonist affinity.[108] Agonist independent inhibition of adenylate cyclase was induced by replacement of Thr^{373} of the α_{2a}-adrenoceptor by phenylalanine, alanine, glutamic acid, cysteine, or lysine.[109] The greatest enhancement of basal activity was observed in the cysteine and lysine analogs; these amino acids were among those most effective in producing a constitutively active α_{1b}-adrenoceptor,[105] although glutamic acid substitution was less effective on the α_{2a}-adrenoceptor than on the α_{1b}-adrenoceptor.

In addition to the third intracellular loop, other portions of the adrenoceptor molecule contribute to the interaction with G-proteins. Asp^{79}, located in the second transmembrane-spanning helix, is highly conserved in G-protein coupled receptors. Studies with the α_2-adrenoceptor suggest that mutation at this site interferes with G-protein dependent agonist–receptor interaction.[83,86] The location of this amino acid is consistent with an influence on receptor–G-protein interaction since, according to receptor modeling, this site should be accessible from the cytoplasmic compartment. It was noted above that Asp^{113} is a key element in the interaction of both agonists and antagonists with the adrenoceptors, presumably interacting with the protonated amino nitrogen found on catecholamine agonists and β-adrenoceptor antagonists. This site also appears to participate in agonist-induced changes in receptor conformation, since mutation of Asp^{113} to glutamic acid, which would modify the geometry of the ligand binding pocket, produces a substantial enhancement of the ability of several (aryloxy)propanolamine β-adrenoceptor antagonists, such as pindolol or oxyprenolol (**30**), to induce β_2-adrenoceptor-mediated activation of adenylate cyclase.[68,69] This enhancement in intrinsic activity is not



(30) Oxyprenolol

observed for several other β-adrenoceptor antagonists. Although the catecholamines could still activate the mutant β_2-adrenoceptor, their affinities were severalfold lower than for the wild type β_2-adrenoceptor, suggesting differences in the interaction of phenethylamines and (aryloxy)propanolamines with the β-adrenoceptor.

Mutation of Tyr^{350} of the β_2-adrenoceptor, located in the cytoplasmic tail, interferes with coupling of the receptor to G_s.[110] As noted above, the proximal portion of the cytoplasmic tail appears to contribute to receptor–G-protein interaction.[102] Removal of the last 47 amino acids from the α_{1b}-adrenoceptor did not influence its ability to activate phospholipase C.[89]

4. **Modulation of Ligand Receptor Affinity and Receptor Downregulation**. Receptor occupation by an adrenoceptor ligand influences the coupling of the adrenoceptor protein with the associated guanine nucleotide regulatory protein (see above). Conversely, the association of the adrenoceptor with this G-protein can influence the affinity of adrenoceptor agonists for the receptor. This interaction appears to be dependent on Asp^{79}, in the second transmembrane-spanning helix. Similarly, monovalent cations can interfere with agonist binding to both α- and β-adrenoceptors.[111,112] Asp^{79} is also required for this modulation to occur, inasmuch as mutation of this site to asparagine eliminates the ability of sodium ion to modulate binding to the cloned α_{2a}-adrenoceptor, without modifying basal affinity for agonists or antagonists.[86] Analogs of amiloride can also influence ligand binding to the α_2-adrenoceptor, as demonstrated by allosteric facilitation of the dissociation of yohimbine from the receptor. This effect is not influenced by mutation of Asp^{79} to asparagine.[86]

Prolonged interaction of agonists with adrenoceptors generally results in receptor desensitization. This desensitization can result from either a reduction in agonist–receptor affinity, a reduction in cell-surface receptor number through receptor internalization, or an impairment of coupling of receptor to its effector system. The initial desensitization process results from the phosphorylation of serine and threonine residues in the cytoplasmic tail or third intracellular loop by several protein kinases, including β-adrenergic receptor kinase (βARK) and cAMP dependent protein kinase (protein kinase A). Although identified in the β-adrenoceptor system, βARK can also phosphorylate the α_2-adrenoceptor.[88,113] Agonist occupation of the receptor promotes phosphorylation by βARK. βARK-mediated desensitization can be facilitated by the association of a specific protein, β-arrestin, with the phosphorylated receptor.[114] Binding of β-arrestin to the phosphorylated receptor results in inactivation by a mechanism not yet understood.

There are subtype related differences in the susceptibility of both α- and β-adrenoceptors to phosphorylation-induced desensitization. The β_3-adrenoceptor is resistant to this desensitization process, presumably as a result of the relative lack of serine and threonine residues in the cytoplasmic tail. Replacement of the cytoplasmic tail of the β_3-adrenoceptor with the corresponding region of the β_2-adrenoceptor, which contains the necessary amino acids for phosphorylation, causes the β_3-adrenoceptor to undergo phosphorylation and desensitization.[115] Deletion of a segment of the third intracellular loop of the α_{2a}-adrenoceptor containing nine potential phosphorylation sites eliminated agonist-induced rapid desensitization.[88] α_{2a}-Adrenoceptors are less susceptible to agonist-induced desensitization than the other two α_2-adrenoceptor subtypes, as determined either by responses mediated by G_i[116] or by G_s following inactivation of G_i with pertussis toxin.[117]

Tyrosine residues in the cytoplasmic tail may be involved in the receptor internalization and degradation responsible for the slower desensitization process associated with long-term receptor activation. Mutation of both Tyr^{350} and Tyr^{354} of the β_2-adrenoceptor attenuates long-term agonist-induced receptor downregulation.[110]

The cytoplasmic tail of both α- and β-adrenoceptors appears to be anchored to the inner surface of the plasma membrane via a palmitic acid group attached to Cys^{341} (β_2-adrenoceptor) or Cys^{442} (α_{2a}-adrenoceptor). β_2-Adrenoceptor activation has been shown to increase the incorporation of labeled palmitic acid, suggesting an involvement with signal transduction.[118] Mutation at this site so as to eliminate the possibility of fatty acid linkage resulted in a β_2-adrenoceptor that was highly susceptible to phosphorylation and unable to couple effectively to G_s.[119] In contrast, a similar mutation to the α_{2A}-adrenoceptor does not alter the ability to couple to G-proteins,[87] although susceptibility to phosphorylation was not specifically examined.

Subclassification of Adrenoceptors Based on Correlation of Radioligand Binding Affinities

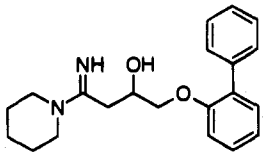
1. **α-Adrenoceptors. A. α_1-Adrenoceptor Subclassification**. Morrow and Creese[120] demonstrated that the α-adrenoceptor antagonist WB-4101 would produce a biphasic displacement of [^{3}H]prazosin binding to rat brain. They proposed that prazosin was binding to two α_1-adrenoceptor subtypes having differential affinity for WB-4101 and designated the sites having higher affinity for WB-4101 as α_{1A}-adrenoceptors and the lower affinity sites as α_{1B}-adrenoceptors. Accordingly, they found the density for high-affinity binding sites for [^{3}H]WB-4101 to be approximately one-half of the density of [^{3}H]prazosin sites. Phentolamine was also found to discriminate between these sites, with a 20-fold selectivity for the α_{1A}-adrenoceptors. On the basis of the affinity ratio between prazosin and phentolamine in either radioligand binding or functional assays, Morrow and Creese[120] assigned the α_1-adrenoceptor-mediated responses in a variety of tissues to either the α_{1A}- or α_{1B}-adrenoceptors.

Jagadeesh and Deth[121] also observed biphasic displacement of [^{3}H]prazosin binding by WB-4101 and phentolamine, using membranes prepared from bovine aorta. However, the affinities of these antagonists were substantially different from those determined by Morrow and Creese[120] in the brain. Jagadeesh and Deth[121] interpreted their [^{3}H]prazosin binding data as showing two distinct binding sites for prazosin. Other investigators[122–128] have suggested the presence of multiple [^{3}H]prazosin binding sites in a variety of tissues. However, the affinity of prazosin for the two postulated sites often differs by less than 1 order of magnitude,[121,129] and/or one site represents only a relatively low percentage (<15%) of total binding;[121,127] hence, the heterogeneity in prazosin binding observed in these studies may not have apparent functional significance. Many other studies have detected only one [^{3}H]prazosin binding site, and the biphasic binding of this radioligand cannot always be reproduced in the tissues where it has been previously observed.[130]

However, several studies[125–127,131] have detected [^{3}H]-prazosin binding sites with dissociation constants of 3–10 nM, which are substantially lower than is commonly observed for this radioligand and which potentially correlate with the functional α_1-adrenoceptors having lower affinity for prazosin (see α-Adrenoceptors below). Also consistent with functional experiments is the relative insensitivity of the low affinity [^{3}H]prazosin binding sites to irreversible alkylation by phen-

oxybenzamine.[131-132] One of the tissues postulated to have low affinity for prazosin is the canine prostate.[127] While the dissociation constant obtained by Sulpizio *et al.*[133] for [^{3}H]prazosin in this tissue (1.5 nM) is lower than that observed by Ohmura *et al.*[127] (9.3 nM), prazosin has been found to be nearly 10-fold less potent in binding to membranes from canine prostate compared to similar experiments in canine aorta (K_D = 0.17 nM; J. P. Hieble, unpublished data). These binding data are consistent with the lower functional potency of prazosin in blocking norepinephrine-induced contraction in canine prostatic strips (K_B = 18 nM).[134] Thus, it appears that prazosin binding sites which correspond to the α_{1L}-receptor (see α_1-Adrenoceptor Subclassification Based on Prazosin Affinity) are not commonly detected due to the use of insufficiently high radioligand concentrations, or the high nonspecific binding commonly observed as the radioligand concentration range is extended.

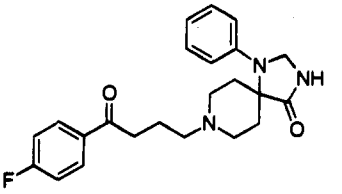
Although the demonstration of multiple binding sites for [^{3}H]prazosin is controversial, it is clear that certain antagonists can selectively inhibit the high affinity binding of this and other radioligands to α_1-adrenoceptor subtypes. The subclassification arising from this selective inhibition is generally consistent with the α_{1A}/α_{1B}-adrenoceptor subdivision based on the affinity of [^{3}H]-WB-4101 or with functional experiments using this or other antagonists (see Molecular Characterization of Ligand–Receptor Interaction and Coupling of the Adrenoceptors to Signal Transduction Processes). WB-4101 generally produces biphasic displacement of [^{3}H]-prasozin[135,136] or [^{125}I]IBE-2254,[137,138] and 5-methylurapidil and the dihydropyridine calcium channel blocker (*S*)-niguldipine show up to 100-fold selectivity for the α_{1A}-adrenoceptor as reflected by differences in their high- and low-affinity dissociation constants.[136,139-141] Studies in vascular membranes from several species show that 5-methylurapidil consistently produces biphasic displacement of [^{3}H]prazosin binding.[142] Highly selective competitive antagonists of the α_{1B}-adrenoceptor have only recently been identified, *e.g.*, AH1110A (**31**),[143] and not yet extensively characterized; spiperone has been reported to show moderate selectivity for this subtype.[135] Radioligand binding affinities for these subtype-selective antagonists in representative α_{1A}- and α_{1B}-adrenoceptor models are shown in Table 5.



(31) AH 11110A

The subdivision of α_1-adrenoceptors into the α_{1A}- and α_{1B}-adrenoceptor subtypes was supported by Johnson and Minneman[144] who showed that the alkylating agent (chloroethyl)clonidine, regardless of concentration, would only inactivate approximately 60% of the [^{125}I]IBE-2254 binding sites in rat cortex. (Chloroethyl)clonidine has been a useful tool for the differentiation of α_{1A}- and α_{1B}-adrenoceptors, both in radioligand binding assays and in the functional experiments described below. Other irreversible α-adrenoceptor antagonists, including phenoxybenzamine (**32**),[145] dibenamine (**33**), benextramine (**34**), and EEDQ (**35**),[144]alkylate the entire receptor population of α_1-adrenoceptor subtypes, with no evidence for selective interaction with any particular subpopulation. α_1-Adrenoceptors in different brain regions differ in their sensitivities to receptor inactivation by (chloroethyl)clonidine, with the hippocampus showing nearly complete resistance. WB-4101 and

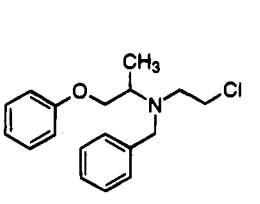
Table 5. Pharmacological Tools Used To Subclassify and Characterize the α_1-Adrenoceptor Subtypes



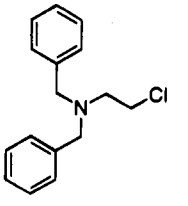
Spiperone

compd	α_{1A}[a]	α_{1B}[b]
WB 4101	0.08 ± 0.02 (5)	8.0 ± 2.8 (11)
5-methylurapidil	0.7 ± 0.1 (5)	96 ± 26 (11)
spiperone	7.2 (1)	1.3 ± 0.72 (2)
(chloroethyl)clonidine[c] (irreversible)	–	+++

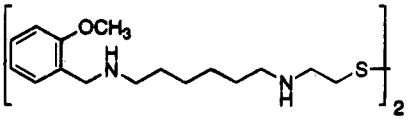
[a] Affinity for α_{1A}-adrenoceptors was determined in tissue homogenates. K_i values for WB-4101 and 5-methylurapidil represent either the high-affinity component of binding displacement in tissues containing both α_{1A}- and α_{1B}-adrenoceptors (*e.g.*, rat cortex, rat vas deferens, human cortex) or the overall binding displacement in tissues containing only the α_{1A}-adrenoceptor subtype (rat submaxillary gland). Affinity represents mean ± SEM of several reported values. The number of experimental values used for the mean determination is noted in parentheses following the mean K_i. Data from refs 135, 139, 147. [b] Affinity for α_{1B}-adrenoceptors was determined either in tissue homogenates or to membranes from cells expressing the rat, hamster, or human α_{1b}-adrenoceptor. K_i values for WB-4101 and 5-methylurapidil represent either the low-affinity component of binding displacement in tissues containing both α_{1A}- and α_{1B}-adrenoceptors (*e.g.*, rat cortex, rat vas deferens, human cortex) or the overall binding displacement in tissues containing only the α_{1A}-adrenoceptor subtype (rat spleen, rat liver). Data presented as for the α_{1A}-adrenoceptor, obtained from refs 135, 139, 147, 13, 273, 6, 17. [c] Sensitivity to irreversible receptor inactivation by (chloroethyl)clonidine determined by the degree of reduction in B_{max} for α_1-adrenoceptor binding or reduction in maximum response to an α_1-adrenoceptor agonist following treatment of tissue homogenates or intact tissue segments.



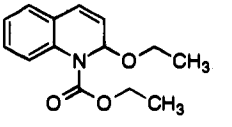
(32) Phenoxybenzamine



(33) Dibenamine



(34) Benextramine

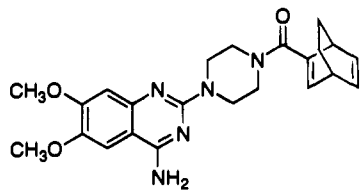


(35) EEDQ

phentolamine were slightly more potent inhibitors of [^{125}I]IBE-2254 binding in hippocampus compared to cortex. Following treatment of cortical membranes with (chloroethyl)clonidine, the potency of WB-4101 was observed to increase, becoming equivalent to that in hippocampus. These data suggested that (chloroethyl)-clonidine selectively alkylated the α_1-adrenoceptor subpopulation that was less sensitive to WB-4101, which corresponded to the α_{1B}-adrenoceptor of Morrow and Creese.[120]

The degree of α_1-adrenoceptor inactivation by (chloroethyl)clonidine varies substantially between tissue preparations, with rat spleen and liver being the most sensitive and rat vas deferens and caudal artery being resistant.[137,146] In many cases, the high sensitivity of an α_1-adrenoceptor population to alkylation by (chloroethyl)clonidine correlates with a receptor population having affinities for selective competitive antagonists that are consistent with the α_{1B}-adrenoceptor subtype (*e.g.*, low affinity for 5-methylurapidil or WB-4101).[137,140,141,147] However, (chloroethyl)clonidine cannot completely eliminate low affinity sites for 5-methylurapidil, particularly in rad cardiac membranes,[141] and treatment with (chloroethyl)clonidine does not enhance the ability of WB-4101 to inhibit [^{3}H]prazosin binding in rabbit aorta.[128] α_1-Adrenoceptors having high sensitivity to (chloroethyl)clonidine as well as relatively high affinity for WB-4101 and 5-methylurapidil have been identified and may represent α_{1D} adrenoceptors. Furthermore, it is likely that an α_1-adrenoceptor, analogous to the α_{1L}-receptor described in functional assays, which is insensitive to (chloroethyl)clonidine and has low affinity for the selective α_{1A}-adrenoceptor antagonists, can be detected in radioligand binding assays.[128]

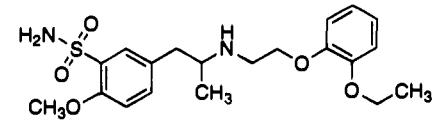
SZL-49 (**36**) is a chemically reactive analog of prazosin which appears to alkylate a subpopulation of [^{3}H]-prazosin binding sites,[148] although the functional properties of this compound do not conform to those of phenoxybenzamine, (chloroethyl)clonidine, or other irreversible α-adrenoceptor antagonists. Administration



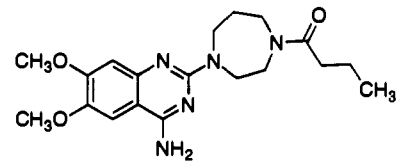
(36) SZL-49

of SZL-49 to rats has been reported to abolish the component of [^{3}H]prazosin binding in ventricular membranes showing a high affinity for WB-4101.[129] These results suggest that SZL-49 selectively inactivates the α_{1A}-adrenoceptor subtype. This hypothesis is also consistent with the failure of SZL-49 to produce a substantial reduction in the density of [^{3}H]prazosin binding sites in rat spleen,[148] a tissue where the predominant α_1-adrenoceptor subtype is the α_{1B}-adrenoceptor. However, the high-affinity component of WB-4101 only represented 7% of the total [^{3}H]prazosin binding sites in the ventricle under control conditions, and the dissociation constant for WB-4101 (6 pM) is at least 1 order of magnitude lower than that generally observed for this antagonist at the α_{1A}-adrenoceptor. Comparison of the affinity of SZL-49 for α_{1A}- and α_{1B}-adrenoceptors in rat hippocampus and rat liver, respectively, showed no subtype selectivity, and an equivalent reduction in the density of [^{125}I]IBE-2254 binding sites was produced in the two tissues.[149] Hence, the ability of SZL-49 to alkylate a subpopulation of α_1-adrenoceptor sites in radioligand binding assays does not appear to be a consequence of the compound's selectivity for the α_{1A}-adrenoceptor, and as such, molecular basis underlying the existence of SZL-49-sensitive and -insensitive sites remains to be determined.

Several other radiolabeled α_1-adrenoceptor antagonists have been shown to bind to additional prazosin-insensitive sites. However, these additional sites have not been conclusively shown to represent an α-adrenoceptor population. In the rat hippocampus, but not in rat spleen, [^{3}H]YM-617 (tamsulosin) (**37**) labels both an α_1-adrenoceptor and a site that is insensitive to prazosin and bunazosin (**38**).[150] 5-Methylurapidil and phentolamine inhibit [^{3}H]YM-617 binding to both sites,although



(37) Tamsulosin

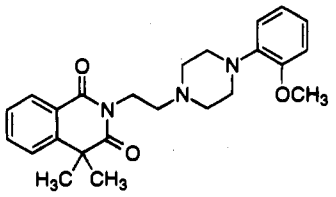


(38) Bunazosin

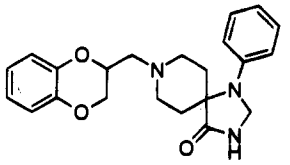
the shallow displacement curves for these antagonists suggest differential affinity for the two binding sites. Interestingly, WB-4101 apparently does not discriminate between the two sites, as evidenced by an inhibition curve with a Hill coefficient of 0.96. Although little detail, and no chemical structure of the radioligand, was presented, a similar pattern may be present with [^{3}H]-IK29, which is another radiolabeled α_1-adrenoceptor antagonist that can bind to both prazosin-sensitive and -insensitive sites in rabbit prostate and rabbit aorta.[151]

B. α_2-Adrenoceptors. The first suggestion that α_2-adrenoceptors could be subdivided was provided by the finding that prazosin, previously thought to interact only with the α_1-adrenoceptor, could produce relatively potent inhibition (K_i < 100 nM) of the binding of [^{3}H]-rauwolscine in certain tissues, such as neonatal rat lung and rat kidney, as well as in some cell lines, such as NG-108 cells. In other cells, such as human platelets, or in the HT-29 cell line, the K_i of prazosin against [^{3}H]-rauwolscine was >1000 nM. The prazosin-insensitive receptor was designated as the α_{2A}-adrenoceptor and the prazosin-sensitive receptor as the α_{2B}-adrenoceptor (see review by Bylund, 1992).[152] Analysis of competition data for inhibition of [^{3}H]rauwolscine binding by prazosin demonstrated that other tissues, such as rat[153] and human[154] cortex, had mixed populations of α_{2A}- and α_{2B}-adrenoceptors, showing that this α_2-adrenoceptor subclassification did not simply represent a species difference in α_2-adrenoceptor characteristics. Other antag-

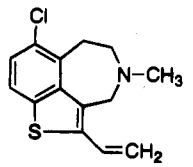
onists have been identified having a selective action at α_{2B}-adrenoceptors (ARC 239 (**39**), spiroxatrine (**40**), SK&F 104856 (**41**)). Interestingly, most selective α_{2B}-adrenoceptor antagonists are also potent α_1-adrenoceptor antagonists, with the only currently known exception being imiloxan (**42**).[155]



(39) ARC 239



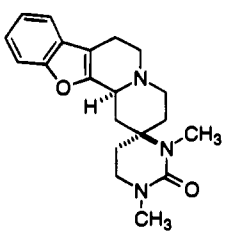
(40) Spiroxatrine



(41) SK&F 104856

In the initial subclassification of α_2-adrenoceptors, it was noted that the partial agonist oxymetazoline had the opposite selectivity profile to prazosin, interacting preferentially with the α_{2A}-adrenoceptor. Another imidazoline-containing molecule, BRL 44408 (**43**), has been shown to be a selective α_{2A}-adrenoceptor antagonist.[156,157]

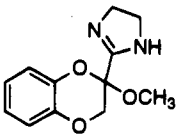
Because a variety of antagonists having selectivity for α_2-adrenoceptors are now available, a more precise mechanism for receptor characterization is possible, based on correlation of inhibitory potency against radioligand binding to α_2-adrenoceptors for a series of antagonists between different receptor sources. This type of analysis has been used to demonstrate the presence of two additional α_2-adrenoceptor subtypes. The α_{2C}-adrenoceptor was initially shown to be present in a tissue culture cell line derived from the opossum kidney[158] and has now been found to exist in native opossum kidney[159] as well as in a human retinoblastoma cell line.[157] Recent studies using a new α_2-adrenoceptor radioligand, [^{3}H]MK 912 (**44**) have demonstrated a mixed α_2-adrenoceptor population in rat cortex and spinal cord, with one component being the α_{2C}-adrenoceptor subtype.[36] Interestingly, this radioligand, like



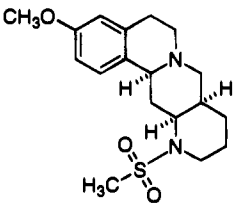
(44) MK 912

rauwolscine, appears to have 10-fold higher affinity for the α_{2C}-adrenoceptor compared to the α_{2B}-adrenoceptor. The differences between α_{2C}- and α_{2B}-adrenoceptors are subtle, with no one antagonist showing clear selectivity. The principal distinguishing characteristics of the α_{2C}-adrenoceptor are a very high affinity for rauwolscine and a higher prazosin/oxymetazoline affinity ratio (Table 4). As can be seen from Table 4, the characteristics of these two subtypes are sufficiently close to make assignment ambiguous based only on the affinity of yohimbine, rauwolscine, prazosin, and oxymetazoline. However, when the affinities of a more extensive series of antagonists are correlated, the differences between these subtypes become more apparent. Furthermore, results with the human α_2-adrenoceptor clones (see above) support this subclassification.

A fourth α_2-adrenoceptor subtype, designated as the α_{2D}-adrenoceptor, has been found in bovine pineal and rat submaxillary gland.[160] This subtype has characteristics that are similar to the α_{2A}-adrenoceptor but has a lower affinity for rauwolscine and yohimbine than the other subtypes, resulting in a decreased prazosin/yohimbine potency ratio (Table 4). This decreased affinity appears to be peculiar to rauwolscine, with other α-adrenoceptor antagonists, such as RX 821002 (**45**) and phentolamine, having similar affinities for other α_2-adrenoceptor subtypes. The dissociation constants for the binding of [^{3}H]RS-15385-197 (**46**) to α_{2A}-, α_{2B}-, and α_{2D}-adrenoceptors do not differ from one another.[161] The low affinity of the α_{2D}-adrenoceptor for yohimbine and rauwolscine, relative to the other α_2-adrenoceptor subtypes, is also reflected as a decrease in inhibitory potency against other radioligands that bind to the α_2-adrenoceptors.[36,161]



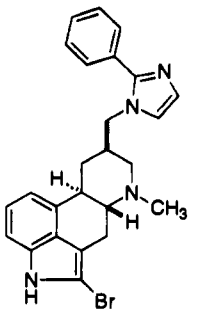
(45) RX 821002



(46) RS-15385-197

Several other tissues possess an α_2-adrenoceptor that has low affinity for yohimbine and rauwolscine, including adipocytes from several species and rabbit jejunal enterocytes. On the basis of the data shown in Table 4, it is likely that these tissues represent additional examples of the α_{2D}-adrenoceptor. Studies in the rat brain using two highly potent and selective α_2-adrenoceptor radioligands, [^{3}H]RS-15385-197[161] and [^{3}H]MK-912,[36] show the existence of a site having low affinity for yohimbine and rauwolscine, consistent with an α_{2D}-adrenoceptor. Other than yohimbine and rauwolscine, only BAM 1303 (**47**) shows moderate selectivity between α_{2A}- and α_{2D}-adrenoceptors, but the two subtypes can be distinguished when the potency ratios for several antagonist pairs are compared.[160]

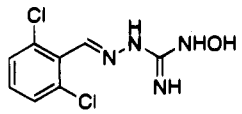
Considering adipocyte α_2-adrenoceptors, it appears that there are species dependent differences in subtype, with human and dog having α_{2A}-adrenoceptors and rat, rabbit, and hamster having α_{2D}-adrenoceptors (Table 4). Species dependent assignment of pineal tissue between α_{2A}- (chicken) and α_{2D}- (bovine) adrenoceptors has also been made, on the basis of a 10-fold difference in affinity



(47) BAM 1303

of yohimbine and rauwolscine.[160] The possibility of these two α_2-adrenoceptor subtypes being species homologs is consistent with both functional and molecular data presented below.

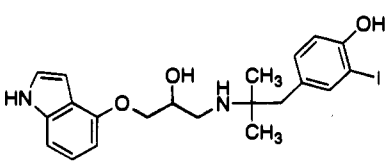
Analysis of the binding of [^{3}H]RX-821002, [^{3}H]MK-912, and [^{3}H]yohimbine in several tissues, such as rat kidney and rat spleen, suggested that the α_{2A}- or α_{2B}-adrenoceptors in this tissue could be subdivided further into two populations, based on differential affinity of guanoxabenz (48).[162,163] However, it was recently shown that these results were likely to be a consequence of conversion of guanoxabenz to a metabolite having higher affinity for the α_2-adrenoceptors, by an enzyme present in these tissues.[164]



(48) Guanoxabenz

2. β-Adrenoceptors. The primary division of β-adrenoceptors into the β_1- and β_2-adrenoceptor subclasses was initially performed using functional assays[1] prior to the development of radioligand binding techniques. Hence, in contrast to the α-adrenoceptors, radioligand binding studies on the β-adrenoceptors have been used primarily to evaluate the distribution of β-adrenoceptor subtypes in tissues, rather than in receptor subclassification.

The ability of a series of β-adrenoceptor agonists and antagonists to inhibit the binding of ([^{125}I]iodohydroxybenzyl)pindolol (49) to turkey erythrocyte membranes has been compared to the corresponding values derived from mammalian tissues containing predominantly β_1- (guinea pig heart, cat ventricle) and β_2- (rat lever, cat soleus muscle) adrenoceptors. When affinities for the erythrocyte β-adrenoceptor were compared to those for either β_1- or β_2-adrenoceptors, a poor correlation was obtained for agents showing subtype selectivity but not for the nonselective agents.[165] These results provided



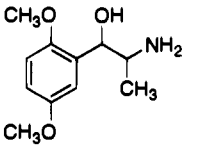
(49) Iodohydroxybenzylpindolol

evidence that different pharmacological characteristics existed for the avian β-adrenoceptor than for either the mammalian β_1- or β_2-adrenoceptors, consistent with its distinct molecular structure (see above).

More recently, a similar type of experiment was used to show that the β-adrenoceptor populations in rat soleus muscle and brown adipose tissue were similar to each other and consistent with that predicted for the β_3-adrenoceptor. The ability of β-adrenoceptor agonists or antagonists to inhibit the binding of [^{125}I]ICYP in membranes prepared from these tissues correlated well, despite a markedly lower affinity for the agonists than would be predicted by functional data.[166]

Subclassification of Adrenoceptors Based on Functional Studies

1. α-Adrenoceptors. A. α_1-Adrenoceptor Subclassification Based on Prazosin Affinity. Although prasozin is a potent and highly selective α_1-adrenoceptor antagonist, many investigators have noted differences in the ability of prazosin to antagonist α_1-adrenoceptor-mediated responses. Medgett and Langer[167] observed that the receptor dissociation constant for prazosin against norepinephrine-induced contraction in the rat caudal artery was concentration dependent, suggesting that norepinephrine could interact with two α_1-adrenoceptor sites, with *ca.* 10-fold difference in affinity for prazosin. Furthermore, their data suggested that the synthetic α_1-adrenoceptor agonist methoxamine (**50**) could only interact with the site having higher affinity for prazosin. Comparison of dissociation constants for prazosin against norepinephrine-induced contraction in a variety of smooth muscletissues, both vascular and nonvascular, shows a

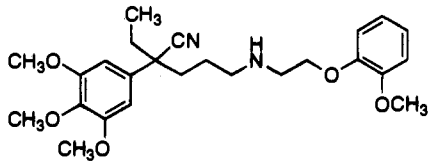


(50) Methoxamine

range of at least 100-fold.[168-170] While at least some of this variation can be explained by differences in experimental conditions, consistent differences in the potency of prazosin have been observed, which are highly suggestive of α_1-adrenoceptor heterogeneity.

Recently, the ability of prazosin to inhibit norepinephrine-induced responses has been used to subclassify α_1-adrenoceptors by Muramatsu and co-workers,[10,126-128,171-175] building upon a postulate originally presented by Flavahan and Vanhoutte.[176] Studying blood vessels from several species, these authors subdivided α_1-adrenoceptors into three subtypes, the α_{1H}-adrenoceptor having high affinity (K_B < 1 nM) for prazosin, the α_{1L}-adrenoceptor having a lower affinity for prazosin (K_B > 2 nM) as well as low affinity for yohimbine (K_B > 300 nM), and the α_{1N}-adrenoceptor, which has an affinity for prazosin that is comparable to the α_{1L}-adrenoceptor but a higher affinity for yohimbine (K_B < 100 nM). The prazosin-insensitive α_1-adrenoceptors can also be differentiated by their affinity for HV-723 (**51**), which has higher affinity for the α_{1N}-adrenoceptor (K_B = 0.4–1 nM) than for the α_{1L}-adrenoceptor (K_B = 2–7 nM).[172] These α_1-adrenoceptor sub-

types appear to make differential contributions to the responses of different blood vessels to both exogenous agonists and sympathetic nerve stimulation.[171]



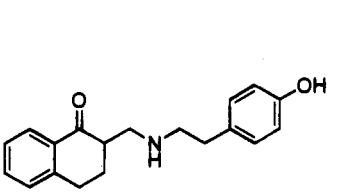
(51) HV-723

Careful Schild analysis of the blockade by prazosin of norepinephrine-induced contraction in the rabbit aorta and rabbit carotid artery can detect two receptor dissociation constants, differing by approximately 1 order of magnitude.[128,171-173] Other α_1-adrenoceptor antagonists did not produce a biphasic Schild plot against norepinephrine, and blockade by prazosin of methoxyamine-induced contraction in the aorta resulted in only one dissociation constant that correspond to the low-affinity component observed against norepinephrine. These data suggest that norepinephrine can activate both α_{1H}- and α_{1L}-adrenoceptors in the rabbit aorta, with methoxamine having selectivity for the α_{1L}-adrenoceptor. This potential selectivity for methoxamine is consistent with that observed by Flavahan and Vanhoutte[176] who compared the dissociation constants of prazosin against clonidine and methoxamine in the rabbit pulmonary artery (also implying that clonidine has selectivity for the α_{1H}-adrenoceptor) but is in contrast to the results of Medgett and Langer[167] in the rat caudal artery (see above).

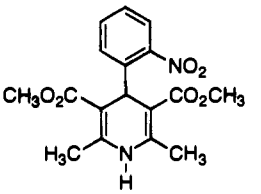
This subclassification scheme may explain the earlier results in canine splenic artery[177] where both prazosin (K_B = 9 nM) and rauwolscine (K_B = 91 nM) produced competitive blockade of the constrictor response to norepinephrine.

The above functional subclassification scheme also appears applicable to nonvascular smooth muscle. The contractile response to norepinephrine in the canine prostate is postulated to result from activation of an α_{1L}-adrenoceptor,[127] consistent with previous data in this tissue showing a dissociation constant of 18 nM for prazosin.[134] In the rat spleen, prazosin produces a biphasic Schild plot, suggesting the presence of two α_1-adrenoceptor populations, with receptor dissociation constants of 0.6 and 25 nM.[178] Treatment of the tissue with phenoxybenzamine eliminates that component of the contractile response showing high affinity for prazosin.

B. Functional Evidence for Subclassification of Prazosin-Sensitive α_1-Adrenoceptors. There is strong evidence for the subclassification of prazosin-sensitive α_1-adrenoceptors, perhaps representing the α_{1H}-adrenoceptors described above,[126] into at least two subclasses, which have been designated as α_{1A}- and α_{1B}-adrenoceptors. This subclassification is supported by functional data, as well as by the radioligand binding and molecular biology studies. WB-4101 has a moderate degree of functional selectivity, blocking norepinephrine-induced contraction in rat vas deferens (K_B = 0.3 nM) with higher affinity than in the rat spleen (K_B = 5.4 nM).[146] 5-Methylurapidil shows similar selectivity in these tissues, with dissociation constants values of 6 and 110 nM in vas deferens and spleen, respectively.[133] Several other α-adrenoceptor antagonists, such as BE 2254 (**52**), yohimbine, and phentolamine, are equipotent in vas deferens and spleen, suggesting that the selectivity observed with WB-4101 and 5-methylurapidil is indeed based on selective interaction of these antagonists with distinct α_1-adrenoceptor subtypes. While differences in the potency of WB-4101 as a competitive α_1-adrenoceptor antagonist have been observed between different blood vessels,[179,180] these differences cannot be consistently related to α_1-adrenoceptor subtype distribution as predicted by sensitivity to irreversible blockade by (chloroethyl)clonidine or sensitivity to the calcium channel blocker nifedipine (**53**).

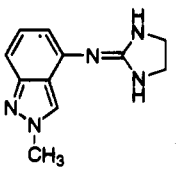


(52) BE 2254



(53) Nifedipine

C. Functional Classification via Sensitivity to Irreversible Blockade by (Chloroethyl)clonidine (CEC) and Other Alkylating Agents. Several early experiments[180,181] suggested that the irreversible α-adrenoceptor antagonists phenoxybenzamine and benextramine could selectively alkylate an α_1-adrenoceptor population in the rat anococcygeus. These observations were based on selective attenuation of the response to SGD 101/75 (**54**), an imidazoline α_1-adrenoceptor agonist. However, additional experiments in this tissue suggested that the results could be explained by the partial agonist activity of SGD 101/75 and that there was no convincing evidence that SGD 101/75 was acting at a different α_1-adrenoceptor from norepinephrine.[182,183]



(54) SGD 101/75

As observed in radioligand binding assays, CEC shows α_1-adrenoceptor subtype selectivity in its ability to produce irreversible blockade of the contractile response to α-adrenoceptor agonists in functional *in vitro* experiments. The selectivity of this alkylating agent for the α_{1B}-adrenoceptor observed in radioligand binding assays has been confirmed in functional assays, with no effect of CEC being produced on norepinephrine-induced contraction in the vas deferens using a treatment regimen which produces a rightward shift and suppression of maximum response in the rat spleen.[146] Sensitivity to CEC has been used to clarify the α_1-adrenoceptor subtype mediating contraction of several rat blood vessels.[179] In these vessels, the ability of CEC to suppress the maximum response to norepinephrine correlated inversely with the potency of WB-4101 as a competitive antagonist of norepinephrine-induced con-

traction, suggesting that in these blood vessels, the sensitivity to CEC was related to the participation of the α_{1B}-adrenoceptor in the contractile response.

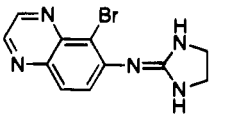
CEC selectively attenuates the response to norepinephrine in blood vessels where α_1-adrenoceptors have been classified as α_{1H}-adrenoceptors[171,172] and eliminates the component of the contractile response showing a high affinity for prazosin in rabbit carotid artery[171] or rabbit aorta.[128,173] The data of Oriowo and Ruffolo[180] in the rabbit aorta, showing that CEC had no effect on the potency of prazosin as an antagonist of norepinephrine-induced contraction, are also consistent with that of Muramatsu and co-workers, since under their experimental conditions a component of the response showing high affinity for prazosin would not have been observed. These results have been used to suggest that the α_{1H}-adrenoceptor-mediated component of norepinephrine-induced contraction in the rabbit aorta represents an α_{1B}-adrenoceptor.[128] It has been postulated that both α_{1A}- and α_{1B}-adrenoceptors, as well as the other α_1-adrenoceptors having high affinity for prazosin identified through molecular biological techniques, represent subgroups of the α_{1H}-adrenoceptor.[126]

However, there are some inconsistencies in the use of CEC for functional subclassification of α_1-adrenoceptors. In aortae from several species, the tissue showing greatest sensitivity to CEC (rat aorta) was also the most sensitive to competitive blockade by WB-4101[180] which is inconsistent with the α_{1A}- and α_{1B}-adrenoceptor classification schemes. Divergent effects of CEC are observed in the rat aorta, ranging from an apparently parallel shift in the norepinephrine concentration–response curve, with no effect on maximum response,[180] to a nearly complete abolition of the norepinephrine response.[179] Wenham and Marshall[184] observe a concentration-related reduction in the response to norepinephrine at concentrations up to 300 μM, with a secondary response, reaching nearly the original maximum, at 1 mM norepinephrine. The contractile response to norepinephrine in canine prostate is sensitive to irreversible blockade by CEC, but the receptor dissociation constant for 5-methylurapidil in this tissue (5 nM) is less than that predicted for an α_{1B}-adrenoceptor-mediated response, with no evidence for α_1-adrenoceptor subtype heterogeneity based on Schild analysis of the interaction between 5-methylurapidil and norepinephrine.[185] Some of these inconsistencies may be explained by the existence of additional α_1-adrenoceptor subtypes which have not yet been fully characterized in functional studies. Indeed, recent data suggest that the contractile response of the rat aorta is mediated by the α_{1D}-adrenoceptor.[186]

SZL-49 has widely divergent effects on the functional response to α_1-adrenoceptor activation. In the rabbit aorta, an apparently parallel rightward shift in the norepinephrine concentration–response curve is produced, with no reduction in the maximum response. However, pretreatment with SZL-49 markedly increased (>100-fold) the dissociation constant for prazosin as an antagonist of norepinephrine-induced contraction.[124] This suggests that SZL-49 produces a functional inactivation of a portion of the α_1-adrenoceptor population. The ability of yohimbine to block norepinephrine-induced contraction was also attenuated by SZL-49, eliminating the possibility that treatment with this agent was unmasking an α_2-adrenoceptor-mediated contractile response. In the rat aorta, a nonparallel shift in the concentration–response curve to phenylephrine or norepinephrine was produced by SZL-49, with an apparent reduction in maximum response being overcome at very high agonist concentrations.[148] The rat aorta was more sensitive to SZL-49 than the rabbit aorta. In human prostate, relatively low concentrations of SZL-49 (30–100 nM) produced concentration-related reductions in the maximum response to norepinephrine, with a 65% reduction occurring at 100 nM.[187] The human epigastric artery was even more sensitive, with the maximum contraction being reduced by 90% by SZL-49 at a concentration of 100 nM.[274] These differences in tissue sensitivity and in characteristics of the blockade produced may relate to α_1-adrenoceptor reserve and the presence of nonadrenergic receptors that are activated by high concentrations of norepinephrine[188] in addition to potential tissue differences in α_1-adrenoceptor subtype distribution. The relationship between functional sensitivity to SZL-49 and the α_1-adrenoceptor subtype profile determined by CEC or by sensitivity to the selective competitive antagonists has yet to be established.

2. Functional Subclassification of α_2-Adrenoceptors. Although the differentiation of α_2-adrenoceptors into the α_{2A}-, α_{2B}-, α_{2C}-, and α_{2D}-adrenoceptors has been based primarily on radioligand binding studies, there is some support for this classification scheme from functional studies. Clearly, most "classical" α_2-adrenoceptor-mediated responses fall into the α_{2A}- or α_{2D}-adrenoceptor groups, since they were typically defined as being "α_2-adrenoceptor-mediated responses" as a result of their lack of sensitivity to prazosin blockade.

The ability of prazosin and ARC-239 to produce functional blockade of UK-14304 (**55**)-mediated inhibition of adenylate cyclase in cells containing homogeneous populations of α_{2A}- (HT-29) or α_{2B}- (NG-108) adrenoceptors correlates with their ability to inhibit [^{3}H]-rauwolscine binding.[189] ARC-239 was 100-fold more



(55) UK 14,304

potent in the NG-108 cells, with a dissociation constant of 10 nM, while yohimbine and phentolamine were equipotent in the two cell lines. Hence, inhibition of the actions of this α_2-adrenoceptor agonist on adenylate cyclase in the NG-108 cell appear to be mediated by the α_{2B}-adrenoceptor.

In a similar experiment, prazosin was shown to be effective in inhibiting UK 14,304-induced inhibition of adenylate cyclase in OK cells, a response presumably mediated by the α_{2C}-adrenoceptor.[158] Although a dissociation constant was not calculated in these experiments, prazosin appeared to be a less potent antagonist of the action of UK 14,304, compared to yohimbine, than in the NG-108 cells, consistent with binding affinity ratios of α_{2B}- and α_{2C}-adrenoceptors (Table 4).

The functional characteristics of presynaptic α_2-adrenoceptors at a variety of sites have been studied

extensively. Most prejunctional α_2-adrenoceptors have low sensitivity to prazosin and appear to be either α_{2A}- or α_{2D}-adrenoceptors, depending upon the species.[40] Presynaptic α_2-adrenoceptors of rat cerebral cortex[40] and submaxillary gland[190] appear to have α_{2D}-adrenoceptor characteristics. Although prejunctional α_2-adrenoceptors of the rat vas deferens were assigned to the α_{2A}-adrenoceptor subtype,[191] the results are perhaps more consistent with an α_{2D}-adrenoceptor subtype.[190] In spite of the fact that several reports initially suggested that the presynaptic receptor of the rat atrium might represent an α_{2B}-adrenoceptor subtype,[191,192] comparison of functional dissociation constants for a series of antagonists as inhibitors of UK 14,304-induced inhibition with binding affinities for the four α_2-adrenoceptor subtypes shows closest similarity with the α_{2D}-adrenoceptor, although subtle differences do exist, suggesting the possibility of mixed receptor populations. Hence it has been postulated[40] that most presynaptic α_2-adrenoceptors in the rat may be of the α_{2D}-adrenoceptor subtype.

In contrast to these results, presynaptic α_2-adrenoceptors of the rabbit may be of the α_{2A}-adrenoceptor subtype, based on studies in cortex[40] and pulmonary artery.[193] The differences in subtype of presynaptic α_2-adrenoceptor between rat and rabbit are based primarily on the higher relative potency of phentolamine versus rauwolscine in rat tissues. The use of this ratio to differentiate α_{2A}- and α_{2D}-adrenoceptors is consistent with the low potency of rauwolscine for inhibition of binding to the α_{2D}-adrenoceptor (see above).

It has recently been suggested that the prejunctional α_2-adrenoceptor of the human kidney may represent an example of the α_{2C}-adrenoceptor subtype, on the basis of correlation of the potency of a series of α_2-adrenoceptor antagonists for potentiation of stimulation-induced norepinephrine overflow with their binding affinities at α_2-adrenoceptor subtypes.[194] However, both prazosin and ARC-239 have relatively low potency, and the antagonist potencies correlate well ($r = 0.96$) with corresponding values for these antagonists[190] in the rat atrium. Hence, as in the rat atrium, the presynaptic α_2-adrenoceptor of the human kidney may not correspond precisely to any of the α_2-adrenoceptor subtypes identified through radioligand binding techniques.

Different presynaptic α_2-adrenoceptors existing in a single tissue, modulating the release of different neurotransmitters, may have different characteristics. Prazosin appears to block those prejunctional α_2-adrenoceptors controlling stimulation-evoked release of norepinephrine in guinea pig ileum while having no effect on prejunctional α_2-adrenoceptors controlling acetylcholine release from the same tissue. Conversely, oxymetazoline activated only those α_2-adrenoceptors controlling acetylcholine release.[195] However, further studies in this test system with additional antagonists will be required to establish whether these differences result from differences in subtypes of α_2-adrenoceptors on different neuronal populations.

Little data are available regarding the functional assignment of postjunctional α_2-adrenoceptors to the α_2-adrenoceptor subtypes identified through radioligand binding techniques. Although it has been suggested that the postjunctional α_2-adrenoceptor of the human saphenous vein has α_{2B}-adrenoceptor characteristics, on the basis of the correlation of dissociation constants for a series of antagonists against norepinephrine-induced contraction with their radioligand binding affinities,[196] most studies in this tissue show prazosin to be a very weak antagonist of norepinephrine-induced contraction.[197] Furthermore, some contribution of α_1-adrenoceptor blockade to the antagonist action of prazosin and ARC-239 in this tissue cannot be eliminated.

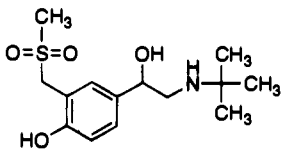
Another functional α_2-adrenoceptor subclassification scheme based on the profile of some novel α-adrenoceptor antagonists has been postulated. SK&F 104078 and SK&F 104856 are capable of blocking some α_2-adrenoceptor-mediated responses, such as constriction of peripheral blood vessels, while having no effect on the neuroinhibitory actions of α_2-adrenoceptor agonists in atria from several species or in guinea pig ileum.[198,199] In several *in vivo* models, neither SK&F 104078 nor SK&F 104856 produced evidence of prejunctional α_2-adrenoceptor blockade. It was initially suggested that these compounds could differentiate between pre- or postjunctional α_2-adrenoceptors. However, in the field-stimulated rat vas deferens, SK&F 104078 can produce relatively potent blockade of the neuroinhibitory action of some, but not all, α_2-adrenoceptor agonists.[200,201] It is probably more appropriate to assume that these antagonists can discriminate between α_2-adrenoceptors on a functional, rather than anatomical, basis. SK&F 104078 has equal affinity for α_{2A}- and α_{2B}-adrenoceptors, as well as a relatively high affinity for the expressed human[5,202,203] and rat[39] α_2-adrenoceptor clones. It has been suggested that the prejunctional α_2-adrenoceptor has α_{2D}-adrenoceptor characteristics, since SK&F 104078 has relatively low affinity against [^{3}H]rauwolscine binding in the bovine pineal gland.[38] However, SK&F 104078 has higher affinity in other test systems assigned to the α_{2D}-adrenoceptor subtype, and the other functionally selective α_2-adrenoceptor antagonist, SK&F 104856, has relatively high affinity for the α_{2D}-adrenoceptor in the bovine pineal or rat submaxillary gland.[160] Hence, no relationship can yet be established between the functional α_2-adrenoceptor subclassification produced by SK&F 104078 and SK&F 104856 and the subclassification established by molecular and radioligand binding assays.

3. Functional Subclassification of β-Adrenoceptors. The following section will briefly describe functional studies showing the contribution of β_1- and β_2-adrenoceptors to a variety of physiological responses. The primary aim is to illustrate the methods that can be used to determine the relative contribution of the different β-adrenoceptor subtypes.

The initial subclassification of β-adrenoceptors by Lands *et al.*[1] was based on the differential potency of phenethylamine agonists in different tissue preparations. The ability to produce selective activation of β-adrenoceptor subtypes has been confirmed in many studies, and this selectivity has been utilized in the design of more effective therapeutic agents, for example, bronchodilators with less cardiac stimulation.

Many of the functional studies subclassifying β-adrenoceptors have compared β_1-adrenoceptor-mediated inotropic and/or chronotropic activity in cardiac (commonly atrial) preparations with β_2-adrenoceptor-mediated relaxation of pulmonary (commonly tracheal) smooth muscle. Several classes of agonists have been identified

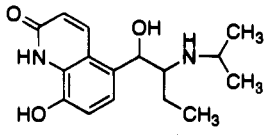
which will produce potent relaxation of tracheal smooth muscle at concentrations having no effect on atrial rate. For example, sulfonterol (**56**)has an EC_{50} of 17 nM for



(56) Sulfonterol

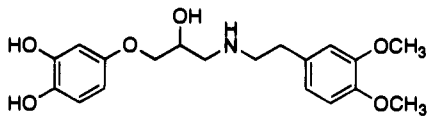
relaxing spontaneously contracted guinea pig trachea, but over 1,000-fold higher concentrations (EC_{50} = 28 μM) are required for stimulating the rate of contraction in isolated guinea pig right atrium.[204] However, one must be cautious in interpreting this agonist selectivity, since the functional response produced by an agonist is dependent on both its affinity and efficacy at the receptor.[205] Further investigation of sulfonterol showed the compound to be a partial agonist at the β_2-adrenoceptor, as reflected by a lower degree of relaxation than was produced by isoproterenol,[206] a lower maximal stimulation of adenylylcyclase in cultured muscle cells containing β_2-adrenoceptors,[207] and a failure to produce any adenylylcyclase activation in these cells following partial β_2-adrenoceptor desensitization.[207] In addition, sulfonterol has moderate affinity, but low efficacy, at the β_1-adrenoceptor, as evidenced by its ability to block isoproterenol-induced increases in contractile force in guinea pig papillary muscle. Comparison of the pA_2 values for sulfonterol as an antagonist of isoproterenol-induced tracheal relaxation (6.15) and increases in papillary muscle contraction (5.82)[206] shows that the affinity of sulfonterol for β_1- and β_2-receptors is actually quite similar.

Similar results, where apparent β_2-adrenoceptor selectivity is primarily a result of decreased efficacy at the β_1-adrenoceptor, have been shown for other agonists as well. However, agonists having true selectivity for the β_2-adrenoceptor have been identified, one example being procaterol (OPC-2009) (**57**) which has a high intrinsic activity at the β_2-adrenoceptor[206-208] and does not interact with β_1-adrenoceptors (to produce blockade of isoproterenol-induced responses) until concentrations approximately 100-fold higher than the EC_{50} at β_2-adrenoceptors are attained.[206,209] The selective affinity of procaterol for β_2-adrenoceptors has been confirmed in radioligand binding studies (see following section).



(57) Procaterol

While many functionally selective β_2-adrenoceptor agonists have been identified by comparing potencies in atrial and tracheal preparations, it has been difficult to demonstrate the converse (*i.e.*, agonists which will stimulate the atrial but not the tracheal β-adrenoceptors). This may be due to the presence of a functional β_1-adrenoceptor subpopulation in guinea pig trachea, the most commonly used preparation. Ro 363 (**58**) produces positive inotropic and chronotropic responses in guinea pig left and right atria, respectively, with a maximum response comparable to that produced by isoproterenol.[210] This agonist is several orders of magnitude less potent in producing β_2-adrenoceptor-mediated relaxation in potassium-depolarized guinea pig

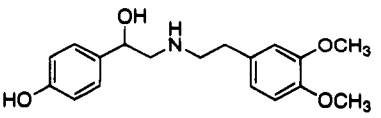


(58) Ro 363

uterus, and the compound also has a low intrinsic activity in this tissue. However, Ro 363 appears to be a full agonist in guinea pig trachea, where its potency relative to isoproterenol is comparable to that observed for producing β_1-adrenoceptor-mediated responses in the atria. The effect in both atria and trachea can be blocked by practolol, a moderately selective β_1-adrenoceptor antagonist; equivalent dissociation constants in these tissues would suggest that both responses are mediated by β_1-adrenoceptors. Radioligand binding studies have been used to show that, although the β_2-adrenoceptor subtype predominates, the guinea pig trachea does contain a significant population of β_1-adrenoceptors (see the following section) which can be activated by a selective agonist.

Functional responses mediated by both β-adrenoceptor subtypes have also been demonstrated in isolated strips of human right atrial appendage.[211] Ro 363 produced an inotropic response in this tissue with an intrinsic activity of approximately 0.8 relative to isoproterenol. This response was inhibited by selective β_1-adrenoceptor antagonists. Conversely, procaterol produced a similar maximum inotropic response, which was sensitive to inhibition by selective β_2-adrenoceptor antagonists.

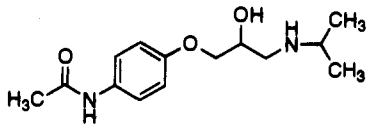
Although still less extensively studied than the selective β_2-adrenoceptor agonists, several other agents are available which show β_1- versus β_2-adrenoceptor selectivity comparable to norepinephrine, without having the α-adrenoceptor agonist activity associated with the endogenous catecholamine. These include denopamine (**59**)[212] and xamoterol (**60**).[213] However, as with Ro 363, both of these agents are partial agonists at the β_1-adrenoceptor, and some of the apparent *in vivo* β_1-adrenoceptor selectivity may be the result of their low intrinsic activities.[214] Hence, when selective *in vitro* stimulation of β_1-adrenoceptors is desired, norepinephrine in the presence of an α-adrenoceptor antagonist may still be the best pharmacological tool.



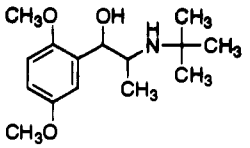
(59) Denopamine

The availability of highly selective antagonists has facilitated the analysis of the functional roles of β_1- and β_2-adrenoceptors. Soon after the initial subclassification

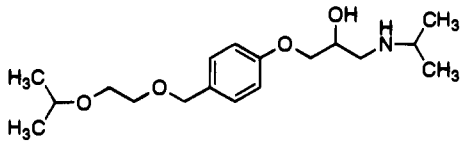
of the β-adrenoceptors, several antagonists having moderate selectivity for either β-adrenoceptor subtype, such as practolol (**61**) (β_1) and butoxamine (**62**) (β_2), were identified.[215-217] However, these compounds had neither high potency nor high selectivity. Currently, the selective antagonists most commonly used are CGP 20712A, which has a dissociation constant below 1 nM at the β_1-adrenoceptor and a β_1/β_2-adrenoceptor selectivity ratio of 10 000.[218,219] Another selective β_1-antagonist commonly employed as a tool is bisoprolol (**63**), which has a dissociation constant of 2–3 nM at the β_1-adrenoceptor and a β_1/β_2-adrenoceptor selectivity of approximately 100-fold.[220] For the β_1-adrenoceptor, ICI 118,551 is a potent antagonist, with a dissociation constant below 1 nM and a β_2/β_1-adrenoceptor selectivity of over 100-fold.[221,222]



(61) Practolol

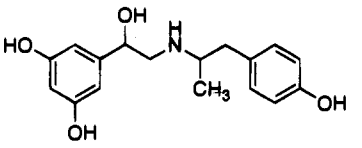


(62) Butoxamine

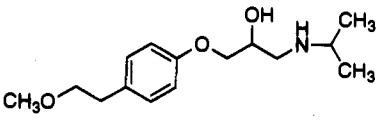


(63) Bisoprolol

Careful analysis of agonist/antagonist interactions can be used to show that both β_1- and β_2-adrenoceptor activation contribute to many functional responses. For example, Kaumann and Lemoine[220] analyzed the Schild plots for antagonism by bisoprolol of the effects of norepinephrine and epinephrine in feline right atrium, left atrium, and papillary muscle. In each tissue, nonlinear regression lines demonstrated that epinephrine was acting on two receptors, having dissimilar affinities for bisoprolol. The contribution of the low-affinity site, presumed to be the β_2-adrenoceptor, was greatest for the chronotropic response in the right atrium. It was estimated that a β_2-adrenoceptor-mediated effect in the atrium was responsible for 24% of the chronotropic response to epinephrine. In contrast, the Schild plots for norepinephrine were linear, or nearly so, in all three tissues, consistent with the low affinity of norepinephrine for the β_2-adrenoceptor. A similar analysis, employing ICI 118,551, was used to show that in guinea pig trachea 97% of the relaxing effect of epinephrine is mediated via β_2-adrenoceptors but only 7% of norepinephrine-induced relaxation was due to a β_2-adrenoceptor-mediated effect.[222] These authors also used this technique to confirm that a selective β_2-adrenoceptor agonist, fenoterol (**64**), could produce a chronotropic response in guinea pig right atrium, which was mediated predominantly (76%) via β_2-adrenoceptors. In the presence of the highly selective β_1-adrenoceptor antagonist CGP 20712A, the chronotropic response to epinephrine in rat right atrium became clearly biphasic. The component of the response that was insensitive to CGP 20712A, representing approximately 25% of the total response, was sensitive to inhibition by ICI 118,551, confirming that a functional β_2-adrenoceptor-mediated effect could be demonstrated in this tissue.[223] This contrasts with the results obtained in



(64) Fenoterol



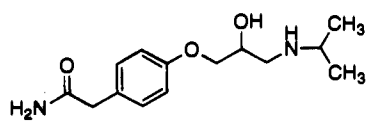
(65) Metoprolol

rat atria by Juberg *et al.*[224] who blocked β_1-adrenoceptors with metoprolol (**65**), which has a much lower β_1-adrenoceptor selectivity than CGP 210712A, which may explain why the β_2-adrenoceptor-mediated component was not observed. Interestingly, Juberg *et al.*[224] found that ICI 118,551, although much less effective than metoprolol, produced a greater (4.2-fold) shift in the chronotropic response to procaterol compared to its effect on the inotropic response to this agonist (1.3-fold shift).

The greater contribution of β_2-adrenoceptors to chronotropic *vis a vis* inotropic responses has been observed by several other groups[225-227] and is one of the explanations for the chronotropic selectivity of agents such as procaterol and salbutamol (**66**).[227]

In a similar fashion, analysis of the effects of ICI 118,551 on catecholamine-induced relaxation of guinea pig trachea has been used to show that epinephrine acts almost exclusively (97%) through β_2-adrenoceptors while norepinephrine acts primarily through the β_1-adrenoceptor, with only a minor component (7%) being sensitive to inhibition by selective β_2-adrenoceptor antagonists.[222]

Only a few functional responses appear to be mediated entirely by β_1- or β_2-adrenoceptors. For example, both isoproterenol and norepinephrine relax the human saphenous vein through a β_2-adrenoceptor-mediated effect.[228] Likewise, both atenolol (**67**) (β_1) and ICI 118,551 (β_2) produced equivalent dissociation constants that were consistent with their β_2-adrenoceptor affinities, against isoproterenol, norepinephrine, and procaterol in the rat costouterine muscle.[229]



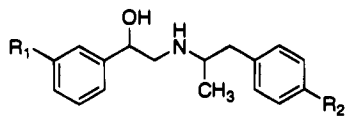
(67) Atenolol

Conversely, ICI 118,551 is a weak antagonist of the effects of either isoproterenol- or norepinephrine-induced relaxation of bovine coronary artery, suggesting

that both agonists act only on β_1-adrenoceptors in this tissue.[230] Even where a substantial β_2-adrenoceptor population has been shown to be present by radioligand binding studies (see the following section), functional analysis using a subtype-selective antagonist can show the response to be mediated entirely by the β_1-adrenoceptor. Other examples of this selectivity pattern are the canine coronary artery,[231] cat atrium,[220] and rabbit atrium.[232] These differences may reflect different efficiencies of β_1- *vis a vis* β_2-adrenoceptor coupling to the effector response.

The strongest functional evidence for the existence of a β-adrenoceptor that is distinct from either the β_1- or β_2-adrenoceptors is provided by studies in rat adipose tissue. Isoproterenol-induced lipolysis in rat adipocytes is relatively insensitive to blockade by either propranolol ($pA_2 = 6.6$), ICI 188,551 ($pA_2 = 5.5, 5.8$), practolol ($pA_2 = 4.9$), or CGP 20712A ($pA_2 = 4.8$).[50,233] Furthermore, propanolol and several other β-adrenoceptor antagonists show a lower degree of stereoselectivity for blocking the lipolytic response than in blocking other β-adrenoceptor-mediated effector systems.[234] This novel β-adrenoceptor has now been clearly shown to represent a third subtype, designated as the β_3-adrenoceptor. In contrast to rat adipocytes, the human adipocyte appears to have classical β_1-adrenoceptors, with respect to both antagonist potency and stereoselectivity,[235] although the β_3-adrenoceptor may also mediate a component of the lipolytic response in human tissue as well.[236]

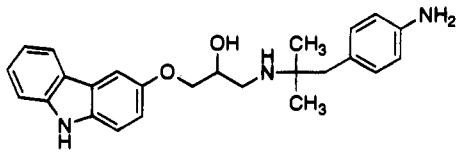
The identification of novel agonists, such as BRL 28410 (**68**), BRL 35135 (**69**), and BRL 37344, which are potent agonists at the rat adipocyte β_3-adrenoceptor, with substantially lower activity in blocking β_1- and β_2-adrenoceptor-mediated responses in rat atria, uterus, or trachea,[50] provides further support for the existence of adipocyte β_3-adrenoceptor.



(68) BRL 28410 (R_1 = H, R_2 = CO_2H)

(69) BRL 35135 (R_1 = Cl, R_2 = $OCH_2CO_2CH_3$)

The rat adipocyte clearly contains a mixed β-adrenoceptor population, with some sites having classical β_1-adrenoceptor characteristics, as determined by high-affinity [^{3}H]dihydroalprenolol or [^{125}I]iodocyanopindolol binding displaceable by β_1-adrenoceptor antagonists.[237,238] However, functional experiments comparing the potency of CGP 20712A and ICI 118,551 against epinephrine-induced cyclic AMP accumulation[238] did not support a functional β_1-adrenoceptor-mediated lipolytic effect.[233] A lack of correlation between functional lipolytic activity and receptor binding affinity in the rat white adipocyte may occur for BRL 28410.[239] This is consistent with the observation that irreversible alkylation of the β-adrenoceptor by a photoactive intermediate derived from (*p*-aminobenzyl)carazolol (**70**) will reduce the number of [^{125}I]iodocyanopindolol binding sites in both rat reticulocytes and rat adipocytes but reduces isoproterenol-induced adenylylcyclase activation only in the reticulocyte.[240]

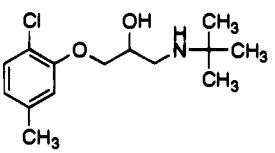


(70) p-Aminobenzylcarazolol

A similar situation exists regarding the β-adrenoceptor population in the rat brown adipocyte. Activation of β-adrenoceptors on the specialized adipose tissue also stimulates lipolysis, which results in a thermogenic response.[241] These receptors are likely to play an important role in diet-induced heat generation.[242] As in the rat white adipocyte, the stimulation of lipolysis in the rat brown adipocyte is less sensitive to inhibition by propranolol than are the classical β_1- and β_2-adrenoceptor-mediated responses, and the selective β_3-adrenoceptor agonists BRL 37344 and BRL 35135 can selectively activate lipolysis and stimulate metabolic rate.[49]

Direct measurement of metabolic rate *in vitro* confirms that BRL 37344 is a potent agonist of the β_3-adrenoceptor and that this response is relatively resistant to blockade by nonselective (propranolol), β_1-adrenoceptor-selective (atenolol), and β_2-adrenoceptor-selective (ICI 118,551) antagonists.[243]

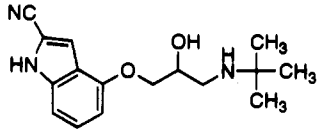
In guinea pig right atrium, in addition to a β_2-adrenoceptor-mediated component, high concentrations of (−)-pindolol will produce a chronotropic response that is insensitive to inhibition by either β_1-adrenoceptor blockade with bisoprolol or β_2-adrenoceptor blockade with ICI 118,551. This response could be antagonized by high concentrations of the nonselective β-adrenoceptor antagonist bupranolol (**71**) and may correspond to the binding site for [^{3}H]bupranolol that has low affinity ($pK_D = 4.9$) for (−)-pindolol.[244] A detailed analysis of the cardiac stimulant action (chronotropic, inotropic, and/or adenylylcyclase activation) of several partial β-adrenoceptor agonists supports an action in an atypical cardiac β-adrenoceptor having similar characteristics to the β_3-adrenoceptor that exists on the rat adipocyte.[245]



(71) Bupranolol

Lower concentrations of BRL 37344 produce relaxation of histamine-contracted longitudinal strips of guinea pig ileum.[246] Concentrations of propranolol and nadolol sufficient to produce profound blockade of β_1- and β_2-adrenoceptor-mediated responses in other tissues produced only small rightward shifts of the relaxation induced by either BRL 37344 or isoproterenol in guinea pig ileum. In order to produce additional blockade, higher antagonist concentrations (up to 1 mM) were required. These results suggest a minor β_1-adrenoceptor-mediated, propranolol-sensitive component, with the majority of the response being mediated by the β_3-adrenoceptor. In the presence of propranolol to saturated the β_1- and β_2-adrenoceptor populations, atenolol

produces competitive blockade of the inhibitory effect of isoproterenol in transmurally stimulated guinea pig ileum ($pA_2 = 6.5$).[247] Under these conditions, cyanopindolol (**72**) is an even more potent antagonist of the propranolol-insensitive response to isoproterenol in this tissue ($pA_2 = 7.63$).[248]



(72) Cyanopindolol

In rat colon, another series of novel β-adrenoceptor agonists, which can be considered as hybrids of the phenylethanolamine and aminotetralin structures, produces inhibition of spontaneous contraction which is sensitive to inhibition by nonselective β-adrenoceptor antagonists (alprenolol, pindolol, propranolol) but is resistant to either selective β_1- (atenolol) or β_2h (ICI 118,551) adrenoceptor blockade.[249,250] The pA_2 values for pindolol (6.5) and propranolol (6.2) against these selective agonists indicate substantially weaker β-adrenoceptor blockade than observed against the typical β_1- and β_2-adrenoceptors and of a similar potency to that observed against isoproterenol, BRL 28140, or BRL 35135 on the rat adipocyte β_3-adrenoceptor ($pA_2 = 6.2-7.0$).[50,235] Isoproterenol is also active in this preparation, and the inhibition by β-adrenoceptor antagonists of this response suggests an action on both typical and atypical β-adrenoceptors.[249] In rat distal colon contracted with high potassium, both isoproterenol and BRL 37344 induce concentration dependent relaxation.[251] The interaction of propranolol with isoproterenol suggested an action on a mixed β-adrenoceptor population, while the response to BRL 37344 was insensitive to inhibition by propranolol.

In addition, the β-adrenoceptors mediating stimulating of lactate formation and inhibition of glycogen synthesis in rat soleus muscle may have characteristics similar to both the β_3- and β_2-adrenoceptors.[239] However, in this case, only a relatively small (10-fold) difference is observed in the potency of propranolol against the inhibitory effect of isoproterenol and BRL 28410 on glycogen synthesis.

Hence, although there are quantitative differences between the various responses described above, there is sufficient evidence to infer that the rat adipocyte (white and brown), guinea pig ileum, rat colon, and cardiac tissue from several species all may possess β_3-adrenoceptors, most likely as a component of a mixed β-adrenoceptor population. A different proportion of β_3-adrenoceptors *vis a vis* β_1- and β_2-adrenoceptors may provide the explanation for the differences observed between these tissues.

Biographies

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Robert R. Ruffolo, Jr. received his B.S. and Ph.D. degrees in pharmacology from The Ohio State University. After postdoctoral training with Marshall Nirenberg at the National Institutes of Health, Dr. Ruffolo joined Eli Lilly in 1978. He joined SmithKline Beecham in 1984 as Director of Cardiovascular Pharmacology where he is presently Vice-President and Director of Pharmacological Sciences, US, U.K., and Europe. In 1988, Dr. Ruffolo was awarded the prestigous Abel Award in Pharmacology by the American Society for Pharmacology and Experimental Therapeutics for his many contributions to the fields of receptor theory, adrenoceptors, and cardiovascular pharmacology.

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