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Localization of the Fourth Membrane Spanning Domain as a Ligand Binding Site in the Human Platelet α_2-Adrenergic Receptor†

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ABSTRACT: The human platelet α_2-adrenergic receptor is an integral membrane protein which binds epinephrine. The gene for this receptor has been cloned, and the primary structure is thus known [Kobilka et al. (1987) *Science 238*, 650–656]. A model of its secondary structure predicts that the receptor has seven transmembrane spanning domains. By covalent labeling and peptide mapping, we have identified a region of the receptor that is directly involved with ligand binding. Partially purified preparations of the receptor were covalently radiolabeled with either of two specific photoaffinity ligands: [^{3}H]SKF 102229 (an antagonist) or *p*-azido[^{3}H]clonidine (an agonist). The radiolabeled receptors were then digested with specific endopeptidases, and peptides containing the covalently bound radioligands were identified. Lysylendopeptidase treatment of [^{3}H]SKF 102229 labeled receptor yielded one peptide of M_r 2400 as the product of a complete digest. Endopeptidase Arg-C gave a labeled peptide of M_r 4000, which was further digested to the M_r 2400 peptide by additional treatment with lysylendopeptidase. Using *p*-azido[^{3}H]clonidine-labeled receptor, a similar M_r 2400 peptide was obtained by lysylendopeptidase cleavage. This M_r 2400 peptide corresponds to the fourth transmembrane spanning domain of the receptor. These data suggest that this region forms part of the ligand binding domain of the human platelet α_2-adrenergic receptor.

α_2-Adrenergic receptors bind endogenous epinephrine and norepinephrine and are involved in the regulation of a variety of physiological processes (Bylund & U'Prichard, 1983). At the cellular level, the signal transduction mechanism involves inhibition of the regulatory enzyme adenylyl cyclase (Jakobs, 1979). The α_2-adrenergic receptor has been purified from human platelets (Regan et al., 1986a) and has been shown to functionally interact with the inhibitory guanine nucleotide binding protein, G_i (Cerione et al., 1986). The gene for the human platelet α_2-adrenergic receptor has also been cloned (Kobilka et al., 1987). The deduced primary structure of this receptor shows that it is a member of a larger family of G-protein-coupled receptors which includes the β-adrenergic receptors (Lefkowitz & Caron, 1988), the muscarinic receptors (Kubo et al., 1986; Peralta et al., 1987), the receptor for substance K (Masu et al., 1987), and rhodopsin (Nathans &

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Hogness, 1983), the receptor for light. A general model (Dohlman et al., 1987) for these receptors includes the following basic features: a glycosylated amino terminus which is extracellular; a carboxyl terminus which is intracellular; and a peptide backbone which loops back and forth through the plasma membrane 7 times. These 7 regions that cross the membrane are each believed to consist of 20–25 predominantly hydrophobic amino acids in an α-helical conformation. The α-helical, transmembrane spanning domains are numbered consecutively 1–7, starting from the amino terminus. Variable stretches of hydrophilic amino acids connect the membrane spanning regions and are believed to form loops. There are a total of six loops, three of which project into the cytoplasm and three into the extracellular space.

The arrangement of these membrane spanning regions and loops to form the ligand binding domains of the adrenergic receptors is of great interest. In vitro mutagenesis studies suggest that transmembrane segments 2, 3, and 7 of the β_2-adrenergic receptor are involved with ligand binding (Dixon et al., 1987; Strader et al., 1987). Moreover, transmembrane segments 2 of the β_2-adrenergic receptor (Dohlman et al., 1988) and 7 of the turkey β_1-adrenergic receptor (Wong et al., 1988) have been implicated as sites of ligand binding. Thus, specific covalent labeling of the second and seventh transmembrane spanning domains was demonstrated using radioactive and chemically reactive β-adrenergic antagonists.

Current information concerning the ligand binding domain of the α_2-adrenergic receptor derives from chemical modification studies. Modification of sulfhydryl residues in purified human platelet α_2-adrenergic receptors resulted in a loss of ligand binding (Regan et al., 1986a). This loss could be prevented if the chemical modification was done in the presence of α_2-adrenergic ligands, suggesting the possible involvement of cysteine residues in ligand binding. Similarly, tyrosine residues were implicated in the binding of ligands to human platelet α_2-adrenergic receptors (Nakata et al., 1986). Through molecular cloning, the complete amino acid sequence of the α_2-adrenergic receptor is known; however, regions of the receptor which may be involved with ligand binding remain undefined. Toward this end, we have now used photoaffinity labeling and peptide mapping to identify a specific region of the α_2-adrenergic receptor that is involved with ligand binding.

Materials and Methods

Materials. Sources of the chemicals were as follows: molecular weight standards, Pharmacia; lysylendopeptidase, Wako Chemicals USA, Inc.; endopeptidase Arg-C, Boehringer Mannheim; *p*-azido[^{3}H]clonidine and [^{3}H]SKF 102229, New England Nuclear–Dupont. α_2-Adrenergic receptors were purified to a specific activity of 2–3 nmol/mg of protein by successive steps of yohimbinic acid–agarose (Repaske et al., 1987), heparin–agarose, and wheat germ agglutinin–agarose chromatographies (Regan et al., 1986a). Sources of other chemicals and drugs have been described previously (Regan et al., 1986a).

Photoaffinity Labeling. α_2-Adrenergic receptors ($\sim$100 pmol) were incubated with 100–200 nM [^{3}H]SKF 102229 in 2.0 mL of 50 mM HEPES, pH 7.4, 1 mM EDTA, 300 mM GlcNac, and 0.05% digitonin. Just prior to photolysis, dithiothreitol (DTT)[1] was added to a final concentration of 1 mM. Photolysis was conducted as described previously (Regan et al., 1986b). After photolysis, free ligand was removed by repeated concentration and dilution using a 10-mL Amicon stirred cell with a YM-30 membrane and buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.02% digitonin. Finally, samples were concentrated to 200–400 pmol/mL and were stored at −80 °C until use. Labeling with *p*-azido[^{3}H]clonidine was done in the same manner only with a final concentration of 200–300 nM.

Endopeptidase Treatments. Reaction conditions for the treatment of photolabeled α_2-adrenergic receptors are described in the figure legends.

Electrophoresis and Autoradiography. Electrophoresis was performed using the urea/SDS–PAGE (9% acrylamide/8 M urea) system of Swank and Munkres (1971). Samples, which usually had been frozen at −80 °C, were thawed and then mixed with the urea/SDS–PAGE sample buffer. The samples were heated at 60 °C for 10 min and after cooling were loaded onto the gels. After electrophoresis, the gels were fixed in methanol/acetic acid/H_2O (50:10:40) for at least 4 h and were washed 3 times in methanol/acetic acid/H_2O (10:10:80) to remove urea. The gels were then treated with Enlightning (NEN–Dupont) according to the manufacturer's instructions. Exposures were made for 3–30 days. For some experiments, photolabeled α_2-adrenergic receptor samples were analyzed by 10% SDS–PAGE and processed for fluorography as described (Regan et al., 1986b).

Electroelution. Electroelution of photolabeled α_2-adrenergic receptors was performed according to Jacobs and Clad (1986) using an Elutrap (Schleicher & Schuell). Photolabeled receptors were first analyzed by 10% SDS–PAGE, followed by fluorography. Bands of interest were excised from the dried gel and applied to the electroelution chamber directly. The electroeluted samples were concentrated and diluted repeatedly with a Centricon-30 (Amicon) to exchange the electroelution buffer with 20 mM Tris-HCl buffer, pH 7.5, and 0.02% digitonin.

Analysis. The apparent molecular weights (M_r) of radiolabeled peptides were determined as follows. Radiolabeled bands were identified by fluorography following polyacrylamide gel electrophoresis. The relative mobility of these bands was determined and was used to calculate a molecular weight on the basis of a regression of log M_r versus relative mobility for a set of standards. All standards were used in the regression.

Results

Previous studies have established the utility of using the α_2-selective antagonist [^{3}H]SKF 102229 to photoaffinity label α_2-adrenergic receptors (Regan et al., 1986b). These studies showed that the photolabeled receptor migrates as a broad band with a molecular weight centered at $\sim$67 000. Covalent incorporation of [^{3}H]SKF 102229 into this band was blocked in the presence of an excess of phentolamine, an α-adrenergic antagonist, indicating the specificity of photolabeling. One of the advantages of using [^{3}H]SKF 102229 is that it gives a very high yield ($\sim$70%) of covalent insertion into the receptor. The site of this covalent photoaffinity labeling was mapped by using two endopeptidases of differing substrate specificities.

Lysylendopeptidase (EC 3.4.21.50) is a trypsin-like protease with a strict specificity for lysine residues (Masaki et al., 1981). It cleaves on the carboxyl-terminal side of lysine–X bonds, and it is active under denaturing conditions. Figure 1 shows a model of the human platelet α_2-adrenergic receptor with the lysine residues identified by shading. There are a total of 16 lysines, and complete cleavage would yield 15 peptides ranging

[1] Abbreviations: DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; pBABC, [*p*-(bromoacetamido)benzyl]iodocarbazolol; CYP, cyanopindolol.

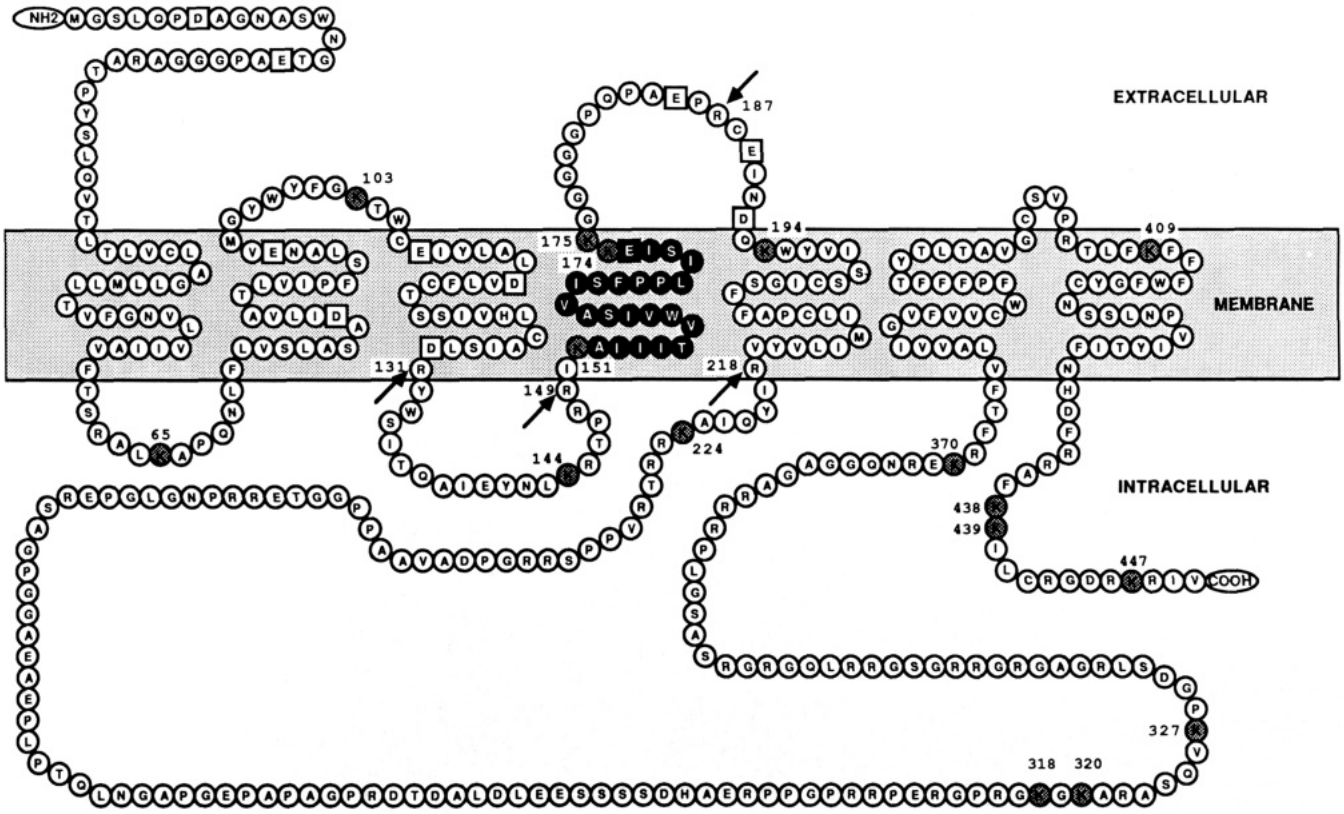

FIGURE 1: Amino acid sequence and model of the secondary structure of the human platelet α_2-adrenergic receptor. Amino acids are denoted by the single-letter code. Intracellular, extracellular, and membrane domains are indicated. The black circles show the sequence of a limit peptide of lysylendopeptidase cleavage that appears to be involved with ligand binding. Lysine residues are shaded, and some arginine residues of interest are indicated by arrows. Acidic residues present in extracellular regions, or in the membrane domains, are squared.

in size from 2 to 94 residues plus 2 free lysines (Figure 2).

Urea/SDS–PAGE was used to fractionate digests after peptidase treatment because of the greater resolving power for low molecular weight peptides as compared to regular SDS–PAGE. Figure 3 shows a urea/SDS–polyacrylamide gel of the time course of lysylendopeptidase treatment of [^{3}H]SKF 102229 labeled α_2-adrenergic receptors. Uncleaved receptor (lane 1) migrates primarily as two bands near the top of the gel. The highest molecular weight band represents aggregated receptor at the interface of the stacking and separating gels, and the second band is the intact receptor (M_r ~67 000). In addition, there are some minor degradation products below the receptor which accumulate during storage. After 1 and 6 h of lysylendopeptidase treatment (lanes 2 and 3), bands with the following molecular weights are observed: 6000, 5000, 3500, and 2400. After 18 h (lane 4), all of the labeled material was present in the M_r 2400 peptide, and no further digestion took place with 24 h (lane 5) of enzyme treatment. Even with the addition of fresh lysylendopeptidase, and another 24 h of treatment, the M_r 2400 peptide remained as the only labeled band (data not shown). It is noted that the dye front was located approximately 3 cm below the M_r 2560 standard and cannot be seen in this figure. This figure and the others in this paper were cropped just above and just below the highest and the lowest radiolabeled bands that were present in the gel. Using this urea/SDS–PAGE gel system, it is possible to resolve peptides with molecular weights as low as 1600 (Dohlman et al., 1988).

An examination of Figure 2 shows that the labeled M_r 2400 peptide would most likely represent Ala^{152}-Lys^{174} (M_r 2510). In addition to the similarity in apparent molecular weight, labeled peptides that were observed early in the digestion can be predicted if it is assumed that [^{3}H]SKF 102229 labeled Ala^{152}-Lys^{174}. Thus, the M_r 6000 band would represent Arg^{145}-Lys^{194}, and the M_r 5000 and 3500 bands would represent Ala^{152}-Lys^{194} and Arg^{145}-Lys^{174}, respectively. On the basis of apparent size, it is also conceivable that the labeled M_r 2400 peptide might represent Gly^{176}-Lys^{194} (M_r 1910). However, if such an assignment is made, there is no theoretical peptide corresponding to the observed M_r 3500 peptide. For example, if Gly^{176}-Lys^{194} were labeled by [^{3}H]SKF 102229, the next largest peptide would be Lys^{175}-Lys^{194} (M_r 2038) followed by Ala^{152}-Lys^{194} (M_r 4530).

Endopeptidase Arg-C was also used to localize the region of the α_2-adrenergic receptor photolabeled by [^{3}H]SKF 102229. Endopeptidase Arg-C is specific for arginine residues and cleaves on the carboxyl-terminal side of the peptide bond (Schenkein et al., 1977). Figure 4 shows time courses for the treatment of [^{3}H]SKF 102229 labeled receptors either with Arg-C alone (lanes 3–5) or with Arg-C followed by lysylendopeptidase (lanes 6–8). Treatment with endopeptidase Arg-C, alone, generated a limit peptide of M_r 4000. Further treatment for 24 h with fresh enzyme did not result in further digestion of the labeled M_r 4000 peptide (data not shown). Further treatment with lysylendopeptidase, however, generated the same M_r 2400 peptide that was previously observed following treatment of the intact receptor with lysylendopeptidase. Therefore, the sequence Ala^{152}-Lys^{174}, which represents the M_r 2400 peptide, must also be present in the M_r 4000 peptide generated by treatment of the labeled receptor with endopeptidase Arg-C. Interestingly, a peptide of M_r 3941 is predicted by the specific cleavage of arginine residues in the α_2-adrenergic receptor. This peptide, Ile^{150}-Arg^{187}, also contains the sequence Ala^{152}-Lys^{174} and thus seems likely to represent the M_r 4000 band. The only other predicted arginine-specific cleavage product of comparable molecular weight would be Cys^{188}-Arg^{218} (M_r 3480). The latter peptide, however, is considerably smaller than the observed M_r 4000 peptide

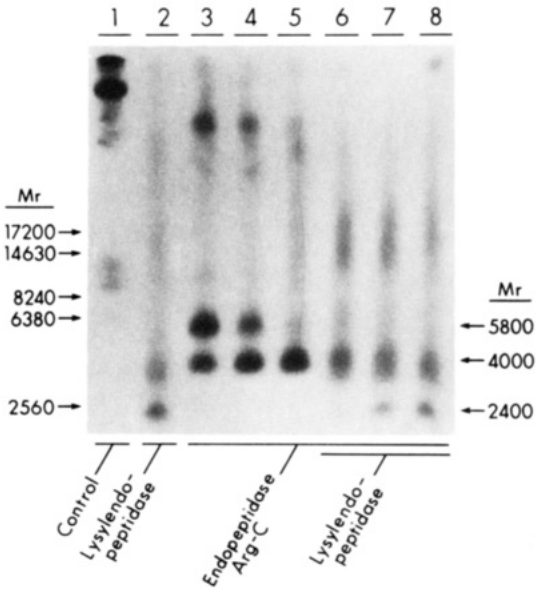

FIGURE 4: Fluorograph of labeled peptides generated by treatment of [^{3}H]SKF 102229 labeled α_2-adrenergic receptors with endopeptidase Arg-C or by combined treatment with endopeptidase Arg-C and lysylendopeptidase. [^{3}H]SKF 102229 labeled α_2-adrenergic receptors (~200 nM) in 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.025% digitonin were treated with endopeptidase Arg-C (325 μg/mL) at 37 °C for 1, 6, and 24 h (lanes 3, 4, and 5, respectively). For combined enzymatic treatments, the receptors were first treated with endopeptidase Arg-C at 37 °C for 24 h. The mixture was adjusted to 50 mM Tris-HCl, pH 9.0, 1 mM EDTA, 1 mM DTT, 1 M urea, and 0.025% digitonin and was incubated with lysylendopeptidase (325 μg/mL) at 37 °C for an additional 1, 6, and 24 h (lanes 6, 7, and 8, respectively). In lane 1, α_2-adrenergic receptors were incubated at 37 °C for 24 h without enzyme, and in lane 2, α_2-adrenergic receptors were treated with lysylendopeptidase, alone, as described (Figure 3) for 24 h. Markers on the right indicate the molecular weights of the labeled peptides. The positions of the molecular weight standards are shown on the left.

FIGURE 2: Histogram of the peptides that could theoretically be generated from a complete digest of human platelet α_2-adrenergic receptors by lysylendopeptidase. The various limit peptides are arranged according to the peptide's position, indicated by the amino acid residue numbers on the left, in the complete amino acid sequence of the α_2-adrenergic receptor. The lengths of the peptides are shown on the x axis, and the calculated molecular weights of the peptides are shown on the right. Molecular weights were calculated on the basis of the actual amino acid composition and are corrected for peptide bonds. The molecular weights of [^{3}H]SKF 102229 and of p-azido-[^{3}H]clonidine are 243 and 275, respectively.

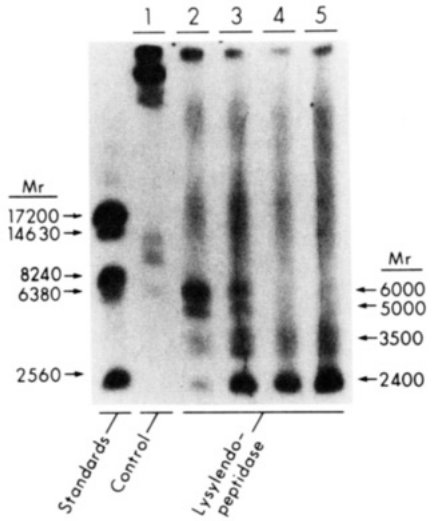

FIGURE 3: Time course of lysylendopeptidase treatment of [^{3}H]SKF 102229 labeled α_2-adrenergic receptors. [^{3}H]SKF 102229 labeled α_2-adrenergic receptors (200 nM) in 25 mM Tris-HCl, pH 9.0, 1 mM EDTA, 1 mM DTT, 2 M urea, and 0.025% digitonin were treated with lysylendopeptidase (325 μg/mL) at 37 °C for 1, 6, 18, and 24 h (lanes 2, 3, 4, and 5, respectively). In lane 1, receptors were incubated at 37 °C for 24 h without enzyme. Samples were analyzed by urea/SDS–PAGE (9% acrylamide/8 M urea) as described under Materials and Methods. Markers on the right give the molecular weights of the labeled peptides. The positions of the standards are shown on the left.

and contains a predicted lysylendopeptidase cleavage product, Trp^{195}-Arg^{218} (M_r 2781), which is larger than the observed M_r 2400 peptide.

p-Azido[^{3}H]clonidine was used to examine the incorporation of an agonist photoaffinity ligand into the α_2-adrenergic receptor. Previous studies with p-azido[^{3}H]clonidine have demonstrated reversible binding to rat brain α_2-adrenergic receptors; however, evidence for covalent labeling was lacking (Kawahara et al., 1985). We have found that the affinity of p-azidoclonidine for partially purified human platelet α_2-adrenergic receptors is low. p-Azidoclonidine in competition for the binding of [^{3}H]yohimbine yielded a K_i of 170 nM (data not shown). Therefore, high concentrations of both p-azido-[^{3}H]clonidine and receptor were used for the present studies. Figure 5 shows a fluorograph of a 10% SDS–PAGE gel after photoaffinity labeling of human platelet α_2-adrenergic receptors with p-azido[^{3}H]clonidine. Like [^{3}H]SKF 102229, p-azido[^{3}H]clonidine specifically labels a broad band of M_r ~67 000. Unlike [^{3}H]SKF 102229, however, the yield of covalent labeling of the receptor by p-azido[^{3}H]clonidine is very low, less than 5%. Photoincorporation of p-azido[^{3}H]-clonidine into the M_r 67 000 band was prevented with appropriate α_2-adrenergic ligands. Thus, phentolamine, yohimbine, and p-aminoclonidine (lanes 2, 3, and 5) all prevented labeling whereas the α_1-selective antagonist prazosin did not (lane 4).

To see if the covalent labeling of α_2-adrenergic receptors by p-azido[^{3}H]clonidine could be localized, the labeled receptors were treated with lysylendopeptidase. For these studies, it was first necessary to electroelute the p-azido-[^{3}H]clonidine-labeled receptor from a 10% SDS–PAGE gel. The reason for this, which can be seen in Figure 5, is that in

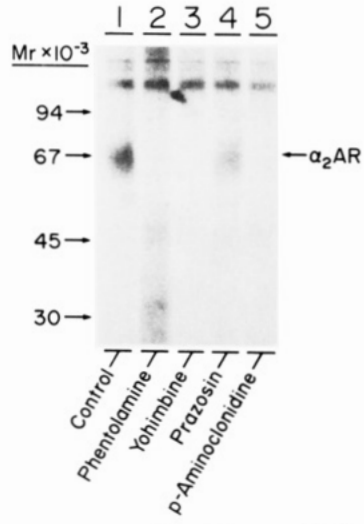

FIGURE 5: Photoaffinity labeling of human platelet α_2-adrenergic receptors by *p*-azido[^{3}H]clonidine. Partially purified receptors (~10 pmol) were incubated at 4 °C for 4 h with 200 nM *p*-azido[^{3}H]-clonidine in the absence of competitor (lane 1) or in the presence of 2 μM phentolamine (lane 2), 2 μM yohimbine (lane 3), 2 μM prazosin (lane 4), or 20 μM *p*-aminoclonidine (lane 5). The samples were photolyzed, and free ligands were removed by gel filtration over Sephadex G-50. The samples were lyophilized and redissolved in SDS–PAGE sample buffer and were electrophoresed on 10% polyacrylamide gels. The gels were prepared for fluorography, and exposures were made for 6 weeks at −80 °C.

addition to the α_2-receptor other proteins were nonspecifically labeled by *p*-azido[^{3}H]clonidine as well. Thus, electroelution was effectively used as a purification step prior to enzymatic digestion. Figure 6B shows a fluorograph of a urea/SDS–polyacrylamide gel obtained after lysylendopeptidase treatment of *p*-azido[^{3}H]clonidine-labeled α_2 receptors. Although the resolution is poor, the molecular weight of the limit peptide is 2400 (lane 5). A broad band is also present with a molecular weight of approximately 5000. Similar results were also obtained with [^{3}H]SKF 102229 labeled α_2-adrenergic receptors that were electroeluted from SDS–PAGE gels (Figure 6A). Interestingly, the [^{3}H]SKF 102229 labeled receptors were resistant to cleavage with endopeptidase Arg-C. For this reason, no attempts were made to cleave *p*-azido[^{3}H]clonidine-labeled receptors with this enzyme.

Attempts to further pinpoint the labeled residue proved unsuccessful. These approaches included additional treatment with different endopeptidases and isolation of the labeled peptide by reverse-phase HPLC. Additional enzymatic treatments of the M_r 2400 peptide were tried with carboxypeptidases A, B, and Y, post-proline-cleaving enzyme, and α-chymotrypsin. Although potential cleavage sites are present for all of these enzymes, the M_r 2400 peptide was resistant to further digestion. Similar resistance to further proteolytic cleavage has also been noted for a tryptic fragment of a β_1-adrenergic receptor peptide that contains membrane spanning domains (Wong et al., 1988). We assume that the extreme hydrophobicity of the peptide and/or factors associated with its secondary structure preclude further enzymatic attack. Attempts to purify the M_r 2400 peptide by reverse-phase HPLC resulted in irreversible adsorption to the columns. These results are consistent with the hydrophobic nature of a membrane spanning peptide.

Discussion

This study documents by photoaffinity labeling and peptide mapping a region of the α_2-adrenergic receptor which is directly involved with ligand binding. Using both antagonist

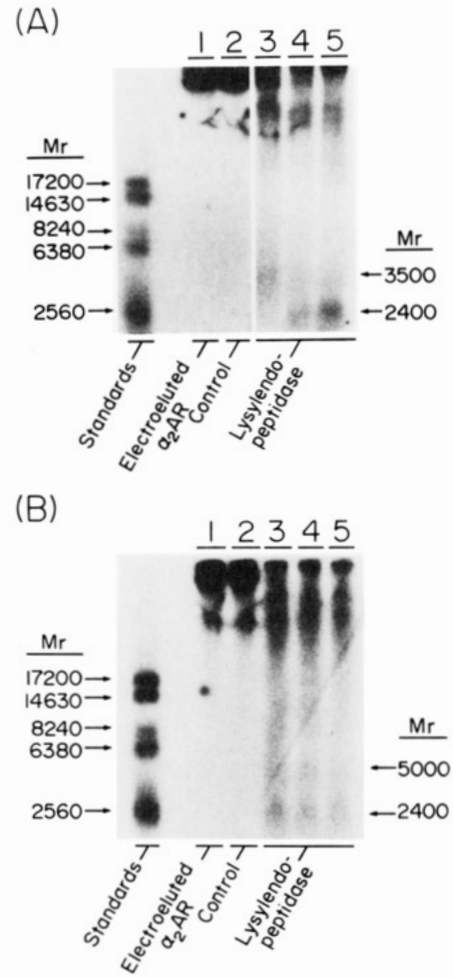

FIGURE 6: Lysylendopeptidase treatment of electroeluted α_2-adrenergic receptors photoaffinity labeled with either [^{3}H]SKF 102229 (A) or *p*-azido[^{3}H]clonidine (B). Photoaffinity labeling, SDS–PAGE, and electroelution were conducted as described under Materials and Methods. Lysylendopeptidase treatment was done as described in Figure 4, except that the urea concentration was 2 M. Treatment was for 1, 6, and 24 h (lanes 3, 4, and 5, respectively). Lane 1 is the electroeluted receptor, and lane 2 is the electroeluted receptor incubated at 37 °C for 24 h. The molecular weights of the labeled peptides are on the right, and the molecular weight standards are on the left.

and agonist photoaffinity probes, we observed a labeled M_r 2400 peptide as the final product of lysylendopeptidase cleavage. The amino acid sequence of the α_2-adrenergic receptor that corresponds to this peptide is Ala^{152}-Lys^{174}. This sequence is outlined in Figure 1, and it represents the fourth transmembrane spanning region of the α_2-adrenergic receptor.

It is interesting that both [^{3}H]SKF 102229, an antagonist, and *p*-azido[^{3}H]clonidine, an agonist, appeared to label the same region of the α_2-adrenergic receptor. Although they represent different chemical classes of compounds, and have opposing functional effects, they do resemble each other in terms of the location of the photoreactive azido group. Thus, in both cases, the azido group is attached directly to the aromatic ring system of the parent ligand. Since the aromatic rings of these ligands are structurally equivalent to the aromatic ring of a catecholamine, it is possible that the fourth membrane spanning segment interacts directly with the catechol moiety of epinephrine during binding to the α_2-adrenergic receptor.

Other regions of the receptor may be important for recognizing the other functional groups present in catecholamines. For example, the amino group of epinephrine might interact with the acidic residues present in the second and third

membrane spanning domains of the α_2-adrenergic receptor. It is noted that these acidic residues are conserved in both the β_1- and β_2-adrenergic receptors and in the muscarinic receptors (Kobilka et al., 1987). In this regard, Dohlman et al. (1988) labeled the second membrane spanning segment of the β_2-adrenergic receptor using ^{125}I-pBABC. The location of the reactive group of this affinity ligand is equivalent to a position which is adjacent to the amino group of a catecholamine, i.e., opposite to the location of the reactive groups of SKF 102229 and *p*-azidoclonidine. Thus, both the second and fourth membrane spanning domains could be simultaneously involved with the binding of epinephrine by having separate recognition sites for the amino and catechol groups, respectively.

Site-directed mutagenesis studies also suggest that the aspartate residues present in the second and third membrane spanning regions of the β_2-adrenergic receptor are important for ligand binding (Strader et al., 1987). Additionally, the integrity of an asparagine residue in the seventh transmembrane region was determined to be important for agonist binding (Strader et al., 1987). While these mutagenesis studies provide useful information, it is difficult to establish whether or not a particular amino acid is directly involved with binding. For example, the mutation of amino acids on the cytoplasmic side of the receptor can also abolish ligand binding even though they probably do not interact with the ligand (O'Dowd et al., 1988).

An approach involving the construction of chimeric α_2-β_2-adrenergic receptors indicates that the seventh membrane spanning domain is a crucial determinant of ligand binding specificity (Kobilka et al., 1988). Thus, a chimeric receptor in which the first six membrane spanning segments were β_2-adrenergic, and the seventh α_2-adrenergic, bound the α_2-selective antagonist [^{3}H]yohimbine but did not bind the β-selective antagonist ^{125}I-CYP. As noted previously with respect to the mutagenesis studies, a role for a physical interaction of ligands with the seventh membrane spanning segment cannot be assessed by this method. Recently, however, the seventh membrane spanning domain of the turkey β_1-adrenergic receptor has been affinity labeled with two different radiolabeled antagonist ligands (Wong et al., 1988). Clearly, this region contributes significantly to ligand binding. It is likely that many or all of the membrane spanning regions, as well as some of the loops, contribute to the three-dimensional conformation needed for ligand binding. The extensive sequence homology in the transmembrane regions of the different G-protein-coupled receptors suggests this to be the case. The role of each of the transmembrane regions in the formation of the ligand binding domain should emerge by combining biochemical and genetic approaches.

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