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Characterization of α_2-Adrenergic Receptor Subtype-Specific Antibodies

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SUMMARY

Subtypes of α_2-adrenergic receptors have been defined pharmacologically in a variety of mammalian tissues. The α_{2A}, α_{2B}, α_{2C}, and most recently α_{2D} subtypes have been characterized by their affinities for selective receptor antagonists and agonists. The genes that may encode the α_{2A}, α_{2B}, and α_{2C} subtypes have been identified in human and rat. In human these genes are termed α_2-C10, α_2-C2, and α_2-C4, respectively, based on their chromosomal localization, whereas three genes, designated RG20α_2, RNGα_2, and RG10α_2, are thought to be the corresponding rat homologues. These assignments were based on the pharmacology of the cloned receptor genes expressed in transfected cells and on the detection of homologous mRNAs by Northern blot analyses in cell lines or tissues with pharmacologically defined α_2-adrenergic receptors. However, the subtype assignment of cloned genes has not been fully resolved by these means. To help clarify the subtype assignment, we have raised antibodies against sequences from the divergent third intracellular loop of the human and rat α_2-adrenergic receptors. These antibodies were found to be subtype specific in immunoprecipitating either the cloned receptors expressed by DNA transfection or the pharmacologically defined receptors prepared from various tissues. Our immunological data corroborate the assignments of α_2-C2/RNGα_2 as encoding the α_{2B} subtype in NG108-15 cells and rat neonatal lung and of α_2-C4/RG10α_2 as encoding the α_{2C} subtype in opossum kidney cells. Furthermore, antibodies against α_2-C10 and RG20α_2 but not α_2-C2/RNGα_2 or α_2-C4/RG10α_2 were both found to recognize α_2-adrenergic receptors expressed in rat submaxillary glands and in bovine pineal gland, two tissues with α_{2D} pharmacology. Because three genes were identified in the rat and human genome, these data suggest that the pharmacologically defined "α_{2D} receptor" is genetically of the α_{2A} subtype.

Pharmacological subtypes of α_2-ARs have been defined by their relative affinities for a variety of α_2-AR antagonists (1). For example, the α_{2B} subtype found in many rat tissues and cell lines was distinguished from the prototypic α_{2A} subtype characterized in human platelets based on differences in their relative affinities for oxymetazoline and prazosin (2). The α_{2A} subtype exhibits high affinity for oxymetazoline and low affinity for ARC-239, chlorpromazine, and prazosin. The α_{2B} subtype exhibits low affinity for oxymetazoline and high affinity for prazosin. A third pharmacologically defined subtype, the α_{2C}, was later identified in the opossum kidney and in the OK cell line. The α_{2C} subtype exhibits a pharmacology similar to but distinct from that of the α_{2B} subtype. The ratio of the affinity of α_{2C} for two selective drugs (oxymetazoline and prazosin) is intermediate between those of α_{2A} and α_{2B} (3). Most recently, an α_{2D} subtype has been proposed based on the pharmacology of the α_2-ARs found in rat submaxillary gland and bovine pineal gland; it exhibits pharmacological characteristics similar to those of α_{2A} but with low affinity for rauwolscine, yohimbine, and SKF104078 (4, 5). Thus, each distinct subtype has become associated with characteristic source tissues in which their pharmacological properties have been defined.

The proliferation of pharmacologically defined subtypes has been paralleled by the cloning of multiple α_2-ARs from a variety of species, predominantly human and rat. So far, three genes have been cloned from human and designated as α_2-C10, α_2-C2, and α_2-C4, according to their chromosomal localization (6-9). Rat α_2-AR homologues have also been isolated by a number of laboratories; we refer to the three rat genes isolated as RG20α_2, RNGα_2, and RG10α_2 (10-14). The question of which gene corresponds to which specific pharmacologically defined subtype has been difficult to sort out, in part because the nucleotide and amino acid sequence homologies of α_2-AR genes within a species can be similar to those of the same gene across

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ABBREVIATIONS: AR, adrenergic receptor; OK cell, opossum kidney cell; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE 1

Summary of pharmacological and molecular biological classification of α_2-AR subtypes

Subtype	Pharmacologically defined tissue or cell type	Cloned receptors		Northern blot
		Human	Rat	
α_{2A}	HT-29 cells (2)[a] Human platelets (2)	α_2-C10 (6)	cA2-47[b] (11)	Rat brain (15) HT-29 cells (15)
α_{2B}	NG108-15 cells (2) Rat neonatal lung (2)	α_2-C2 (8) Clone 5A (9)	RNGα_2 (12)	Rat kidney (15) No NG108-15 cells (15) Rat neonatal lung (12)
α_{2C}	OK cells (3)	α_2-C4 (7)	RG10α_2 (10) RBα2B (13) A2d (14)	NG108-15 cells (15) Rat brain (15) OK cells (15)
α_{2D}	Rat submaxillary gland (4) Bovine pineal gland (5)		RG20α_2[b] (10)	Rat salivary gland (10)

[a] Numbers in parentheses are reference numbers.
[b] Two clones are identical, but one group concludes that the RG20α_2 clone is different from the rat homologue of α_2-C10, based on pharmacology (10).

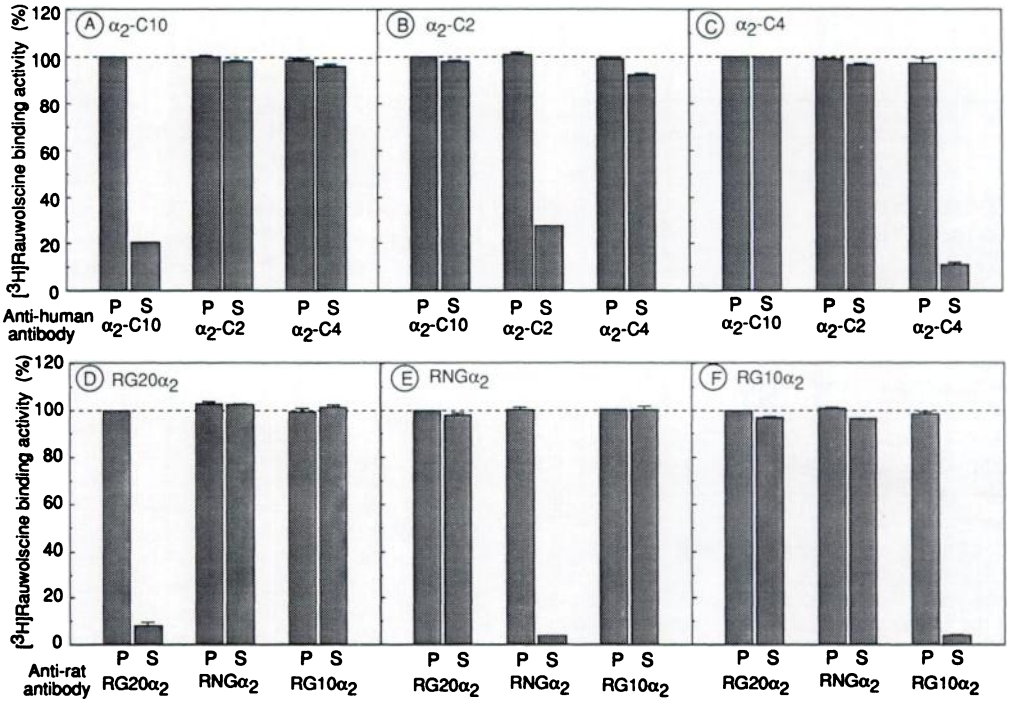

Fig. 1. Specificity of antibodies for cloned human and rat α_2-AR genes expressed by COS cell transfection. Solubilized receptors were prepared from the membranes of COS cells transfected with either the human α_2-C10 (A), α_2-C2 (B), or α_2-C4 (C) genes or the rat RG20α_2 (D), RNGα_2 (E), or RG10α_2 (F) genes. These receptor preparations were incubated with either preimmune serum (*P*) or antisera (*S*) against α_2-C10, α_2-C2, and α_2-C4 (for expressed human genes) or RG20α_2, RNGα_2, and RG10α_2 (for expressed rat genes) and were immunoprecipitated with Pansorbin. α_2-AR binding of [^{3}H]rauwolscine remaining in the supernatant was measured as described in Experimental Procedures. Values were normalized to either the preimmune serum of the rabbit immunized with the GST-α_2-C10 fusion protein, set as 100% (A, B, and C), or the preimmune serum of the rabbit immunized with GST-RG20α_2 fusion protein, set as 100% (D, E, and F). The data shown here are the mean ± standard error of three separate experiments. Receptor concentrations in the antibody incubations were determined to be 0.99–1.01 nM (A), 0.69–0.73 nM (B), 0.93–1.48 nM (C), 1.70–3.32 nM (D), 1.36–1.78 nM (E), and 1.97–2.49 nM (F) in these experiments.

species. This issue has been addressed by a variety of approaches, including 1) characterizing the pharmacology of the cloned and expressed receptor gene and 2) determining whether the cloned gene is expressed in a pharmacologically defined source tissue or cell line, usually by Northern blot analyses. The results of such analyses are summarized in Table 1. However, several significant discrepancies have not been resolved. Northern blot analyses have often been ambiguous; for example, mRNAs from NG108–15 cells that express the α_{2B} subtype hybridized with an α_2-C4 probe but not an α_2-C2 probe (15). Based on sequence similarity and limited pharmacological data, the RG20α_2 was proposed to be the rat homologue of human α_2-C10 (11). However, analyses of the pharmacology of the expressed RG20α_2 gene revealed differences from that of the human α_{2A}-AR and similarities to the properties of the rat submaxillary gland α_2-AR, leading Lanier *et al.* (10) to suggest that RG20α_2 encodes the α_{2D} subtype.

To help reconcile the assignment of cloned rat and human genes to pharmacologically defined subtypes, we have raised subtype-specific antibodies against the divergent sequences of the third intracellular loop of these receptors. The specificities of these antibodies were demonstrated by their immunoprecipitation only of receptors prepared from transfected cells expressing the gene product against which they had been raised. Subsequently, these antibodies were used to determine the

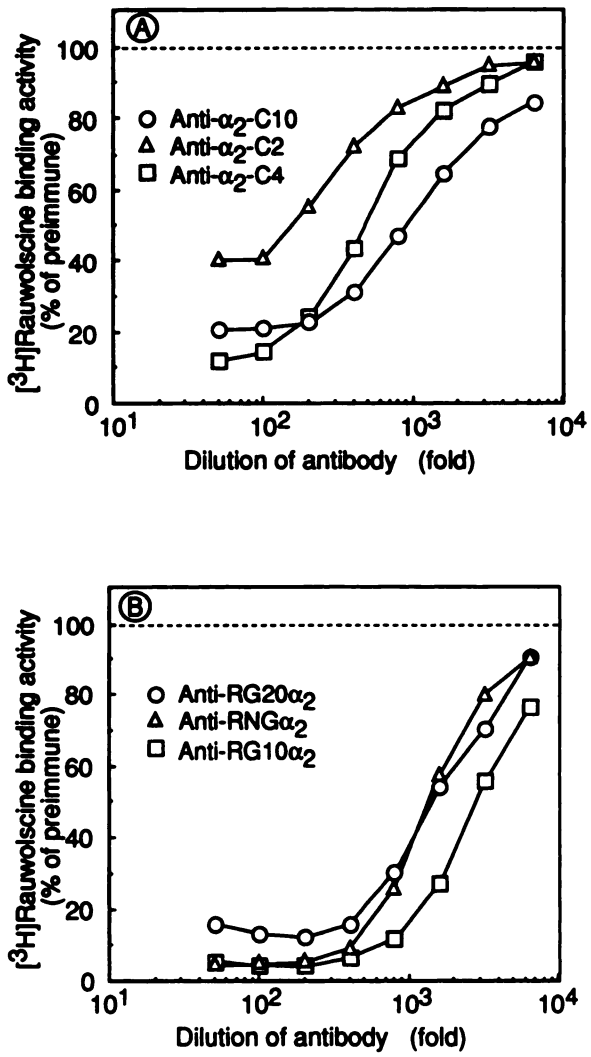

Fig. 2. Titration curves for anti-human and -rat α_2-AR antibodies. Solubilized α_2-ARs from the membranes of COS cells transfected with α_2-C10, α_2-C2, or α_2-C4 genes (A) or with RG20α_2, RNGα_2, or RG10α_2 (B) genes were incubated with either the indicated dilution of preimmune serum or antisera against α_2-C10 (○), α_2-C2 (△), and α_2-C4 (□) (for solubilized human α_2-ARs) (A) or RG20α_2 (○), RNGα_2 (△), and RG10α_2 (□) (for solubilized rat α_2-ARs) (B) and were immunoprecipitated with Pansorbin. α_2-AR binding activity in the supernatant was determined as described in Experimental Procedures, and values were normalized to preimmune serum, set as 100%. The data shown here are representative of two separate experiments. Receptor concentrations in the antibody incubations were determined to be 1.00 nM (α_2-C10), 1.02 nM (α_2-C2), 0.78 nM (α_2-C4), 0.28 nM (RG20α_2), 1.20 nM (RNGα_2), and 1.64 nM (RG10α_2) in these experiments.

receptor subtype expressed in a variety of tissues with pharmacologically defined α_2-ARs.

Experimental Procedures

Materials. Sources for materials were as follows: [^{3}H]rauwolscine (78.4–82.3 Ci/mmol), deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate (1176–1346 Ci/mmol), and Replinase, DuPont-New England Nuclear (Boston, MA); digitonin, Gallard-Schlesinger (Carle Place, NY); isopropyl-β-D-thiogalactoside and low melting point agarose, GIBCO BRL (Gaithersburg, MD); Pansorbin cells, Calbiochem (La Jolla, CA); glutathione-agarose (sulfur-linked), PMSF, leupeptin, aprotinin, and glutathione (reduced form), Sigma Chemical Co. (St. Louis, MO); *Thermus aquaticus* DNA polymerase, Perkin Elmer Cetus (Norwalk, CT); yohimbine, Aldrich (Milwaukee, WI); and deoxynucleotides, deaza-T7 sequencing kit, PTZ18R, Sephadex G-50, and pGEX-2T, Pharmacia (Piscataway, NJ).

Construction of expression plasmids. Portions of the third intracellular loop of α_2-C10 (nucleotides 715–1058, numbered as originally reported), α_2-C2 (nucleotides 637–1029), α_2-C4 (nucleotides 715–1122), RG20α_2 (nucleotides 799–1101), RG10α_2 (nucleotides 721–1044), and RNGα_2 (nucleotides 992–1264) were amplified by PCR with oligonucleotide primers of 31–37 nucleotides in length. Primers encoded a *Bam*HI restriction site (5′ end) or *Eco*RI restriction site (3′ end) to facilitate the subcloning of PCR products, and a stop codon (TGA) was introduced before the *Eco*RI site to terminate the reading frame. PCR reactions were performed with Replinase in the presence of 10% dimethylsulfoxide, except for the α_2-C10 product, which was amplified with *T. aquaticus* polymerase in the presence of 10% dimethylsulfoxide. Conditions for PCR were 1 min at 94°, 2 min at 55°, and 3 min at 72° for 25 cycles, followed by a 10-min extension at 72°. PCR fragments were subcloned into the *Bam*HI and *Eco*RI sites of PTZ18R for sequencing by the Sanger chain-termination method (16). The sequences of PCR products from α_2-C10, α_2-C2, and RNGα_2 were identical to the original published sequences. The sequence of the RG10α_2 PCR fragment corresponded to that reported by others (13, 14), as did the sequence of RG20α_2 (11). In the case of α_2-C4, the sequence of the PCR fragment showed one extra cytidine between nucleotides 847 and 848, one guanosine between nucleotides 925 and 926, and one guanosine between nucleotides 934 and 935, and nucleotide 1020 was a guanosine instead of a cytidine. We resequenced this portion of the original clone (pBCα_2-C4) and found that the sequence of the PCR fragment was correct. The PCR products were ligated into the *Bam*HI and *Eco*RI sites of pGEX-2T to produce in-frame GST fusion proteins (17).

Constructs for eukaryotic expression of α_2-C10, α_2-C2, and α_2-C4 were as described (6–8). For the expression of RG20α_2, RG10α_2, and RNGα_2, the *Nco*I-*Sal*I fragment of pBCα_2-C10 was replaced with either the *Nco*I-*Hind*III fragment of pGEM7-RG20α_2 or pGEM7-RG10α_2 or the *Nco*I-*Apa*I fragment of pGEM7-RNGα_2, respectively. These α_2-AR constructs were transiently expressed in COS-7 cells transfected by the DEAE-dextran method as described (18).

Fusion protein induction and purification. *Escherichia coli* strains NM522 or BL21(DE3)pLysS harboring the pGEX-2T-derived plasmids were used for the production of α_2-AR fusion proteins. Overnight cultures were diluted 10-fold into Luria broth and incubated for 1 hr at 37°. Expression of fusion proteins was induced with 1 mM (NM522) or 0.4 mM [BL21(DE3)pLysS] isopropyl-β-D-thiogalactoside for 2 hr. The induced NM522 cells were resuspended in PBS plus 10 mM EDTA and incubated with 0.2 mg/ml lysozyme for 20 min on ice. After centrifugation at 39,000 × *g* for 30 min at 4°, supernatants were applied to a 0.5-ml column of glutathione-agarose, washed two or three times with 10 ml of PBS plus 10 mM EDTA, and eluted with 2 ml of 5 mM glutathione in 50 mM Tris, pH 8.0. Induced BL21(DE3)pLysS cells were resuspended in PBS plus 10 mM EDTA and homogenized with a Dounce homogenizer after freeze/thaw. After centrifugation at 39,000 × *g* for 30 min at 4°, the supernatants were combined with 0.5 ml of glutathione-agarose and gently rotated for 2 hr at 4°. The glutathine-agarose gel was collected by centrifugation at 1500 rpm for 5 min, washed two or three times with 40 ml of PBS plus 10 mM EDTA, and poured into a column for elution as described above. Affinity-purified fusion proteins were dialyzed against 100 mM HEPES, pH 7.3, and used for immunizations.

Immunization of rabbits. Two or three female New Zealand white rabbits were immunized for each of the fusion proteins. Each rabbit received 0.3 mg of affinity-purified fusion protein for the primary and booster injections. Blood was obtained 7–10 days after a second booster injection and the antisera were stored at −80°. Aliquots were kept at 4° in the presence of 0.02% NaN_3.

Solubilized receptor preparations. Rat submaxillary glands and bovine pineal glands were obtained from Pel-Freeze (Rogers, AR). Rat

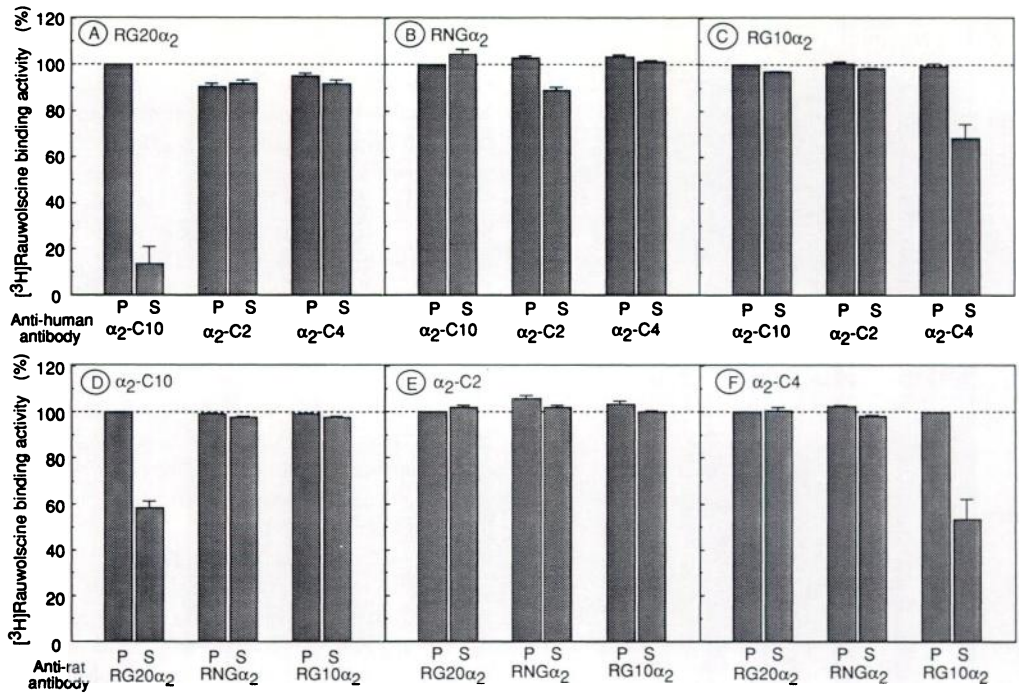

Fig. 3. Cross-species reactivity of anti-human and -rat α_2-AR antibodies. Solubilized α_2-ARs from the membranes of COS cells transfected with RG20α_2 (A), RNGα_2 (B), or RG10α_2 (C) genes or with α_2-C10 (D), α_2-C2 (E), or α_2-C4 (F) genes were incubated with either preimmune serum (*P*) or antisera (*S*) against α_2-C10, α_2-C2, and α_2-C4 (for solubilized rat α_2-ARs) or RG20α_2, RNGα_2, and RG10α_2 (for solubilized human α_2-ARs) and were immunoprecipitated with Pansorbin. α_2-AR binding activity in the supernatant was determined as before, and values were normalized to the preimmune sera of rabbits immunized with the GST-α_2-C10 fusion protein, set as 100% (A, B, and C), or the preimmune sera of rabbits immunized with the GST-RG20α_2 fusion protein, set as 100% (D, E, and F). The data shown here are the mean ± standard error of three separate experiments. Receptor concentrations in the antibody incubations were determined to be 1.51–1.86 nM (A), 1.29–1.77 nM (B), 1.33–2.35 nM (C), 0.89–1.24 nM (D), 0.53–0.81 nM (E), and 0.61–0.81 nM (F) in these experiments.

neonatal lungs were dissected from 3-day-old rats and were frozen in liquid nitrogen. Tissues were stored at −80° until homogenization in cold 50 mM Tris, pH 7.5, 5 mM EDTA, supplemented with 0.2 mM PMSF, 2 μg/ml leupeptin, and 0.5 μg/ml aprotinin (homogenization buffer), by using a Polytron tissue disrupter (Brinkman Instruments, Westbury, NY). Homogenates were centrifuged at 750 × *g* for 10 min at 4°, and the pellets were rehomogenized as before. After centrifugation at 750 × *g* for 10 min at 4°, the supernatants were combined and filtered through three layers of cheesecloth. The resulting filtrates were centrifuged at 39,000 × *g* for 20 min at 4°, and the pellets were resuspended in solubilization buffer at a protein concentration of 5 mg/ml. Solubilization buffer contained 20 mM HEPES, pH 8.0, 5 mM EDTA, 2 mM EGTA, 1% digitonin, 0.2 mM PMSF, 2 μg/ml leupeptin, and 0.5 μg/ml aprotinin. The membranes were solubilized overnight at 4°, and after centrifugation at 39,000 × *g* for 1 hr at 4° the supernatant was used as solubilized receptor. Solubilized human and rat α_2-AR subtypes expressed in transfected cells were prepared as follows. COS-7 cells were transfected with each of the α_2-AR eukaryotic expression constructs as described (18). COS cells do not express detectable endogenous [^{3}H]rauwolscine binding sites. Membranes were harvested into homogenization buffer and homogenized by using the Polytron. Homogenates were centrifuged at 39,000 × *g* for 20 min at 4° and the homogenization and centrifugation were repeated twice. The pellets were resuspended in solubilization buffer at a protein concentration of 3 mg/ml and solubilized as described above. Solubilized receptors were frozen and stored at −80° until used. OK cells and NG108–15 cells were grown as described (15), and the membrane pellets were treated the same way as were COS-7 cells.

Immunoprecipitations. Solubilized receptors were incubated for 6 hr at 4° with 20 μl of preimmune or immune serum in a total volume of 1 ml. Immunocomplexes were precipitated by addition of 180 μl of a 10% Pansorbin suspension, incubation for 2 hr at 4°, and centrifugation. Pansorbin cell suspensions were made fresh, with 1 mg/ml bovine serum albumin, before use. After removal of immunocomplex by centrifugation, the supernatants were assayed for binding. Binding assays were performed with 10 nM or 15 nM [^{3}H]rauwolscine as described (18). Nonspecific binding was determined in the presence of 10 μM yohimbine. We used a preabsorption method in which immunoprecipitation of α_2-ARs was assayed by the removal of [^{3}H]rauwolscine binding sites from the supernatant, compared with preimmune serum controls. This preabsorption assay was required because of the high rate of dissociation of [^{3}H]rauwolscine from the RG20α_2 gene product and from rat submaxillary gland α_2-ARs (4, 10). A digitonin solution (0.3%) was used for dilution of and preequilibration of Sephadex G-50 columns for receptors prepared from rat submaxillary gland, bovine pineal gland, rat neonatal lung, NG108–15 cells, and OK cells.

Results

Subtype-specific antibodies were produced against a series of bacterially expressed fusion proteins in which amino acid sequences from the cloned third intracellular loop of human and rat α_2-ARs were expressed in conjunction with GST. The specificities of the antibodies were first determined by immunoprecipitation of receptors solubilized from transfected COS cells expressing only one cloned α_2-AR gene product. Receptor preparations solubilized from the membranes of COS cells expressing a specific α_2-AR gene were incubated with either preimmune serum (as a control) or immune serum and then precipitated with the addition of Pansorbin. As shown in Fig. 1, each antibody immunoprecipitated 70–90% of the α_2-AR gene product against which it had been raised but showed limited cross-reactivity with other receptor proteins. For ex-

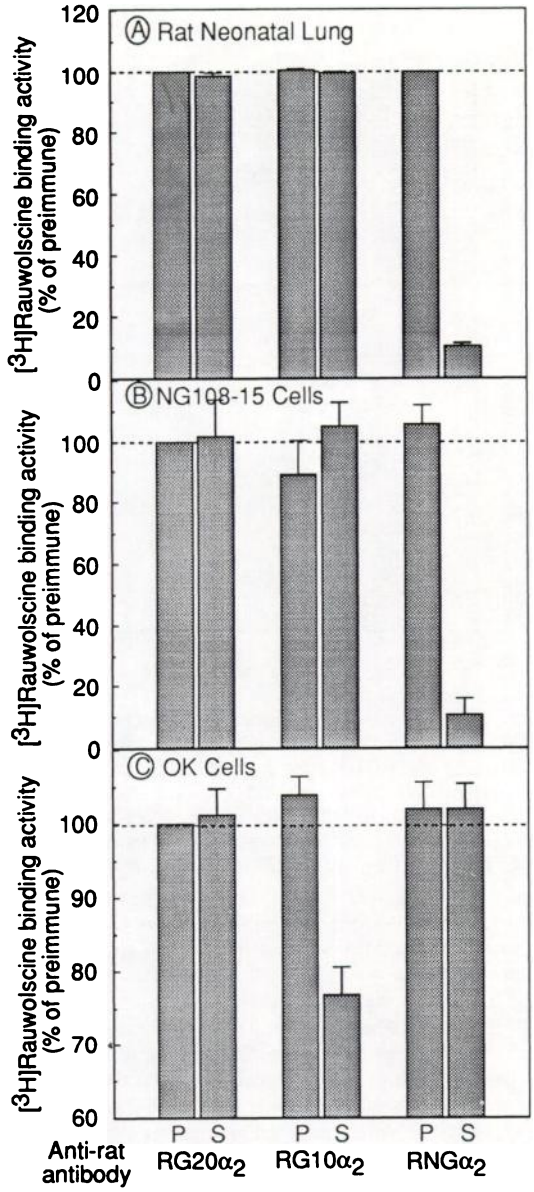

Fig. 4. Immunoprecipitation of α_2-ARs from rat neonatal lung, NG108–15 cells, and OK cells with antibodies to rat α_2-AR genes. Receptors prepared from rat neonatal lung (A), NG108–15 cells (B), or OK cells (C) were incubated with either preimmune serum (*P*) or antisera (*S*) against the cloned rat α_2-AR gene products. The α_2-AR binding activity remaining in the supernatant after Pansorbin treatment was determined as before, and values were normalized to the preimmune serum of the rabbit immunized with the GST-RG20α_2 fusion proteins, set as 100%. Data shown are the mean ± standard error of three separate experiments. Receptor concentrations in the antibody incubations were determined to be 0.16–0.30 nM (A), 0.13–0.22 nM (B), and 0.26–0.37 nM (C).

ample, COS cell-expressed α_2-C10 receptors were immunoprecipitated by the antibody against α_2-C10 but not by antibodies against α_2-C2 or α_2-C4 (Fig. 1A). These results demonstrate the utility of fusion proteins containing the third intracellular loop region for producing antibodies specific for each α_2-AR subtype. Furthermore, these antibodies provide an alternative method for determining which α_2-AR gene product is expressed in a tissue containing a pharmacologically defined subtype.

The titer of each serum was also determined. As shown in Fig. 2, EC_{50} values for anti-human or -rat α_2-AR antibodies were 400–600-fold or 1000–2000-fold dilutions, respectively. These results show that the lesser ability of the anti-α_2-C2 antibody to immunoprecipitate the α_2-AR encoded by the α_2-C2 gene was not due to the lower titer of the antiserum (Fig. 2A). Each of the antibodies showed maximal effects at 1/100 to 1/200 dilution. We used a 1/50 dilution of antisera for the subsequent experiments.

Because we used human antibodies to rat α_2-ARs, and rat antibodies to human α_2-ARs in some experiments, we determined the cross-species reactivity of the antibodies. Anti-α_2-C10 antibodies recognized rat RG20α_2 as well as human α_2-C10 (Fig. 3A). Anti-α_2-C4, RG20α_2, and RG10α_2 antibodies immunoprecipitated about 40–50% each of the corresponding homologues (Fig. 3, C, D, and F). Anti-α_2-C2 antibodies immunoprecipitated small but significant amounts of rat homologue RNGα_2 (about 11%). However, we could not detect significant immunoprecipitation of human α_2-C2 with rat RNGα_2 antibody (Fig. 3, B and E). Sequence similarity of the third intracellular loop between α_2-C2 and RNGα_2 clones is only 68%, and the lower reactivity may reflect this fact. Under the same assay conditions, we detected essentially no cross-immunoprecipitation of human β_1- and β_2-ARs (data not shown).

The α_{2B} subtype has been pharmacologically characterized in rat neonatal lung and in the neuroblastoma × glioma hybrid cell line NG108–15. The α_{2B} subtype of both tissue and cell line is thought to be encoded by the mouse or rat homologue of the rat RNGα_2 gene and human α_2-C2 gene, because α_2-ARs from these sources are not glycosylated and RNGα_2 and α_2-C2 do not have glycosylation sites (8, 9, 12, 19, 20). The anti-RNGα_2 antibody but not antibodies to either RG20α_2 or RG10α_2 immunoprecipitated the rat lung α_{2B} subtype (Fig. 4A), demonstrating that only the RNGα_2 antibody specifically recognizes the α_{2B} subtype. A similar assay of solubilized receptors from NG108–15 cells then found that specific preabsorption of the α_{2B} subtype was accomplished only with the anti-RNGα_2 antibody (Fig. 4B). We could not detect any significant immunoprecipitation with anti-α_2-C2 antibodies, because the ability of this antibody to immunoprecipitate the rat homologue was weak (about 11%; see Fig. 3B) and the level of expression of α_2-AR in NG108–15 cells and rat neonatal lung was too low for detection of small differences (data not shown). Because the α_2-AR of OK cells was pharmacologically defined as the α_{2C} subtype, we determined which antibody could immunoprecipitate the OK cell α_2-AR. Only anti-RG10α_2 antibody could immunoprecipitate small but significant amounts of this α_2-AR (Fig. 4C). Anti-human antibodies against α_2-C10, -C2, or -C4 genes did not immunoprecipitate the OK cell α_2-AR (data not shown).

The human platelet α_2-AR is the prototype of the α_{2A} subtype (1, 2). Antibodies against α_2-C10 but not α_2-C2 or α_2-C4 were found to immunoprecipitate the human platelet α_2-AR (Fig. 5A). This same subtype was also specifically recognized by the antibody against RG20α_2 (Fig. 5D). It was recently proposed that the α_2-AR expressed in rat submaxillary gland and bovine pineal gland might constitute a fourth pharmacologically defined subtype, α_{2D} (1, 4, 5). These tissues were solubilized and antibodies to the human and rat genes were used to immunoprecipitate their α_2-ARs. As shown in Fig. 5, the receptors from these tissues were recognized only by antibodies against the rat RG20α_2 gene and the human α_2-C10 gene. The preimmune serum of the α_2-C2 antibody showed some degree of cross-reactivity (Fig. 5C); however, this apparent reduction of [^{3}H]

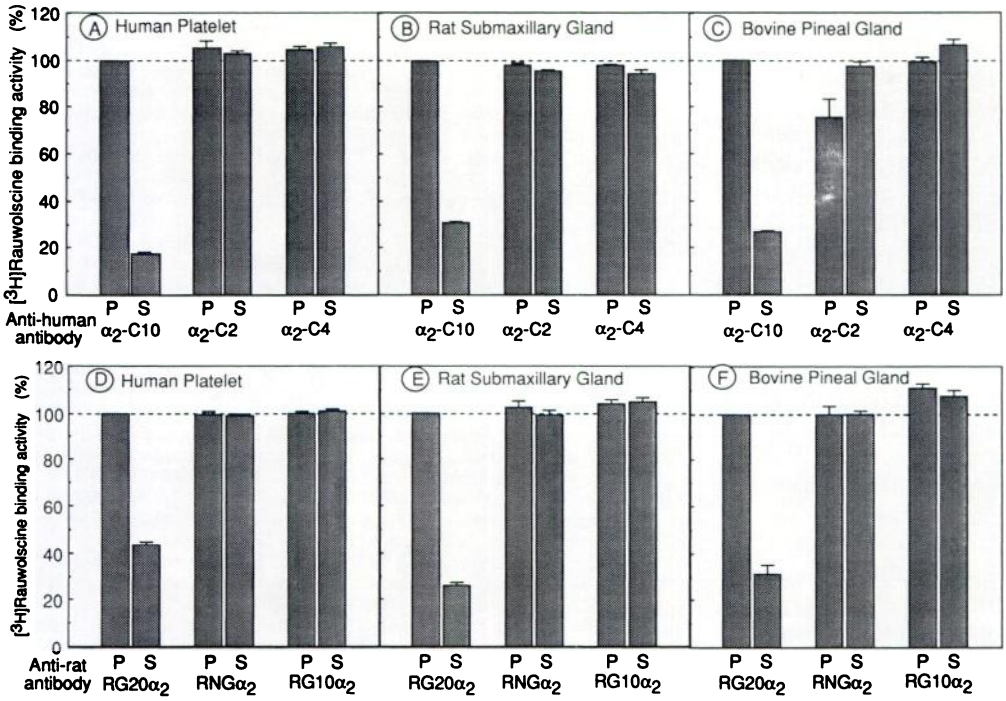

Fig. 5. Immunoprecipitation of α_2-ARs from human platelets, rat submaxillary gland, and bovine pineal gland with antibodies against cloned human and rat α_2-AR genes. Solubilized receptors were prepared from either human platelets (A and D), rat submaxillary gland (B and E), or bovine pineal gland (C and F) and were incubated with either preimmune serum (*P*) or antisera (*S*) against the cloned human (A, B, and C) or rat (D, E, and F) α_2-AR genes. Values were normalized to either the preimmune serum of the rabbit immunized with the GST-α_2-C10 fusion protein, set as 100% (A, B, and C), or the preimmune serum of the rabbit immunized with GST-RG20α_2 fusion protein, set as 100% (D, E, and F). The data shown here are the mean ± standard error of three separate experiments. Receptor concentrations in the antibody incubations were determined to be 0.37–0.41 nM (A), 0.44–0.45 nM (B), 0.056–0.084 nM (C), 0.52–0.82 nM (D), 0.34–0.40 nM (E), and 0.10–0.11 nM (F).

rauwolscine binding activity remaining in the supernatant after Pansorbin treatment reflected increased nonspecific binding, without a change in total binding activity (data not shown). This activity was found only in this rabbit and it was not present in the immune serum after immunization with the GST-α_2-C2 fusion protein. These results demonstrate that α_2-ARs expressed in the rat submaxillary gland and the bovine pineal gland share the immunological epitopes of the α_{2A} subtype.

Discussion

The α_2-AR subtypes are closely related members of the guanine nucleotide-binding protein-coupled receptor superfamily (1, 21, 22). Pharmacologically and structurally very similar α_2-AR subtypes mediate diverse physiological responses in a variety of tissues; examples include the contraction of smooth muscle, inhibition of neurotransmitter release, and inhibition of insulin secretion. Subtypes of these functionally similar molecules can, however, be defined by their distinct affinities for α_2-AR agonists and antagonists. The molecular cloning of α_2-AR genes has largely substantiated these pharmacological distinctions as representing discrete gene products. However, only three α_2-AR genes have been isolated from human and rat, and Southern blot analyses of human and rat genomic DNA suggest that these genes comprise the α_2-AR subfamily (6, 23). These data, then, must be reconciled with four proposed pharmacologically defined α_2-AR subtypes.

The assignment of each cloned gene as encoding a particular subtype has in some respects been straightforward. The pharmacology of the human α_2-C10 gene product is consistent with that of the human platelet α_{2A} subtype; the human α_2-C2 and rat RNGα_2 genes exhibit high amino acid homology (82%), and they both have the pharmacological characteristics of the α_{2B} subtype. The properties of the human α_2-C4 gene and its rat homologue RG10α_2 are similar but not identical to the α_{2C} subtype defined in OK cells, but the small differences in pharmacology may be attributable to variations in the same gene between species. The major unresolved issue is the identity of RG20α_2, which exhibits very high sequence homology with the human α_2-C10 gene (89% amino acid identity in the entire coding region and 98% identity in transmembrane regions) but, with regard to its lower affinity for rauwolscine, yohimbine, and SKF104078, is pharmacologically similar to the α_{2D} subtype described in rat submaxillary gland and in bovine pineal gland.

We assumed that a fourth subtype of α_2-AR, if existent, should be significantly different from the three known subtype clones, because only three genes were identified in the human and rat genome with Southern blot analyses and each was specifically hybridized with one of the probes from human and rat α_2-AR clones (6–8, 23). Based on this assumption, we have taken the approach of using antibodies specific for each cloned gene product to determine whether the α_{2D} subtype in rat submaxillary gland and in bovine pineal gland is distinct from the RG20α_2 and α_2-C10 genes or whether the α_{2D} subtype is immunologically indistinguishable from the RG20α_2 and α_2-C10 gene products. Antibodies generated against fusion proteins expressing divergent, and therefore characteristic, sequences from each cloned receptor were found to be specific in their ability to interact with each receptor subtype. This property of subtype specificity was demonstrated using a defined source of each gene product, i.e., receptors expressed by transfection of a single cloned gene product into COS cells and then solubilized from their membranes. Each antibody recognized only the gene product corresponding to sequences against which it had been raised (Fig. 1). It was clearly demonstrated that the RNGα_2/α_2-C2 clone corresponds to the α_{2B} subtype in neonatal rat lung and NG108–15 cells (Fig. 4). Our immunochemical data are also supported by biochemical data, which showed that α_2-ARs in NG108–15 cells and rat neonatal lung are not glycosylated (19, 20). Relatively small amounts (about 25%) of α_2-ARs of OK cells could be immunoprecipitated by anti-rat RG10α_2 antibody (Fig. 4). Although information on the se-

quence of the third intracellular loop of the α_2-AR in OK cells is not available, Blaxall *et al.* (24) reported that the sequence within transmembrane regions II and VII of the OK cell α_2-AR showed only 63% similarity to that of the human α_2-C4 and rat RG10α_2 clones, whereas similarity in this region between human α_2-C4 and rat RG10α_2 is 87%. Anti-RG10α_2 antibody immunoprecipitated only 47% of the α_2-AR encoded by α_2-C4 but >90% of the α_2-AR encoded by RG10α_2 (Figs. 1F and 3F). These results suggest that the lower reactivity of anti-RG10α_2 antibody is the result of the limited sequence similarity within the third intracellular loop. These immunochemical results agree with the recent classification by pharmacological approaches, which analyzed the correlation of dissociation constants of 12 α_2-AR-selective drugs for α_2-ARs from pharmacologically defined tissues or cell lines and cloned human α_2-ARs (25). All of these data are consistent with the notion that the pharmacologically defined α_{2D} subtype in rat submaxillary gland and in bovine pineal gland is genetically of the α_{2A} subtype (Fig. 5).

These results lead to the conclusion that, although pharmacologically defined subtypes often reflect distinct gene products, it can also be the case that pharmacological differences are the result of subtle differences in the same gene between species. A precedent for this observation is the distinct pharmacology of the A_1 adenosine receptors in rat and cow, which share 97% amino acid identity in transmembrane regions (26). Recently, Link *et al.* (27) cloned and expressed the mouse homologue of the human α_2-C10 gene, which had pharmacological characteristics similar to those of the rat RG20α_2 clone (i.e., lower affinity for rauwolscine and yohimbine). They showed that the change of a single amino acid residue in the fifth transmembrane region of the mouse homologue was responsible for the decrease in affinity of the mouse α_2-AR for yohimbine (27).

In conclusion, we have raised subtype-specific antibodies against human and rat α_2-AR genes and applied them to pharmacologically defined subtype classification. NG108–15 cells and rat neonatal lung express the α_{2B} subtype encoded by RNGα_2 in rat and α_2-C2 in human. OK cells expresses the α_{2C} subtype encoded by RG10α_2 in rat and α_2-C4 in human. It is strongly suggested that the α_2-ARs from rat submaxillary gland and bovine pineal gland correspond to the α_{2A} subtype encoded by the rat RG20α_2 gene and the bovine homologue of the human α_2-C10 and rat RG20α_2 genes.

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