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ARYLAZIDE PHOTOAFFINITY PROBE FOR α_2-ADRENOCEPTORS

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Abstract—An arylazide photoaffinity probe for α_2-adrenoceptors has been developed and characterized. The compound, 3-methyl-6-chloro-9-azido-1*H*-2,3,4,5-tetrahydro-3-benzazepine (SKF 102229), had a K_i for the human platelet α_2-adrenoceptor of ~40 nM. Upon exposure to ultraviolet light, SKF 102229 irreversibly blocked the binding of [^{3}H]yohimbine to both membrane bound and solubilized, partially purified, receptors. The extent of α_2-adrenoceptor blockade was dependent upon both the length of exposure to ultraviolet light and the concentration of SKF 102229. Typically, a 60% decrease in α_2-adrenoceptor number is obtained following 8 min of photolysis in the presence of 100 nM SKF 102229. The pharmacologic characteristics of the irreversible blockade produced by SKF 102229 were those of an α_2-adrenoceptor. Thus, photodependent, irreversible blockade of α_2-adrenoceptors by SKF 102229 was prevented by the concomitant presence of phentolamine or *p*-aminoclonidine but not by prazosin. Given its specificity and efficient blockade of the ligand binding site, SKF 102229 should prove useful for studies of the structure and function of α_2-adrenoceptors.

Understanding the biochemistry and pharmacology of receptors has been advanced by the development of ligands which are capable of forming covalent bonds with the receptor [1]. These irreversible, or "affinity", ligands generally fall into two classes, those whose chemical reactivity depends upon the presence of light and those whose chemical reactivity depends upon variables that are difficult or impossible to control. The advantages of the former group, i.e. the photoaffinity ligands, are obvious since the formation of the reactive species can be specifically induced after equilibrium conditions have been established. This control generally results in a greater ratio of specific to nonspecific labeling.

Although a number of useful affinity ligands have been developed for β- and α_1-adrenoceptors [2–6], there has been less progress made with affinity ligands for α_2-adrenoceptors. An isothiocyanate derivative of clonidine, clonidine-NCS, has been used for the irreversible blockade of α_2-adrenoceptors [7]. However, as a consequence of its chemically reactive nature, and its rather low affinity for α_2-adrenoceptors, the utility of clonidine-NCS may be limited. Recently, [^{3}H]phenoxybenzamine has been used as an affinity label for α_2-adrenoceptors [8]. Although it was successfully used to identify the subunit size of the α_2-adrenoceptor, it was only effective after partial purification of the receptor. In addition to the problems associated with its chemical reactivity, phenoxybenzamine suffers from a lack of α-adrenergic specificity. This report describes the development of an arylazide photoaffinity probe for α_2-adrenoceptors. The ligand, SKF 102229, has good affinity, is specific, and upon photolysis can produce an irreversible blockade of α_2-adrenoceptors.

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EXPERIMENTAL

Synthesis of 3-methyl-6-chloro-9-azido-1H-2,3,4,5-tetrahydro-3-benzazepine. A solution of 630 mg (3 mmoles) of 3-methyl-6-chloro-9-amino-1*H*-2,3,4,5-tetrahydro-3-benzazepine [9] in 2 ml of concentrated sulfuric acid and 10 ml of water was stirred in an ice bath while 276 mg (4 mmoles) of sodium nitrite in 5 ml of water was added dropwise. After addition was completed, the ice bath was removed and the mixture was stirred at room temperature for 1 hr, after which 60 mg of solid urea was added. After 15 min, 325 mg (5 mmoles) of solid sodium azide was added and the mixture was stirred at room temperature overnight. Nitrogen was evolved and a white precipitate formed. The precipitate was removed by filtration and was washed with a small amount of cold ethanol followed by diethyl ether to give 545 mg (55%) of white crystals. This was recrystallized from ethanol to give 390 mg (39%) of white crystals which gradually decomposed above 175°. ^{1}H NMR (DMSO-D_2O) δ 7.52 (d, 2H, J = 9 Hz), 7.26 (d, 2H, J = 9 Hz), 3.6 (m, 4H), 2.9 (s, 3H), 2.8 (m, 4H); M^+/e 236; Anal. Calc. for $C_{11}H_{13}N_4Cl \cdot H_2SO_4 \cdot 0.25H_2O$: C, 38.94; H, 4.72; N, 16.51. Found: C, 38.85; H, 4.40; N, 16.55.

α_2-Adrenoceptor preparation. The preparation of human platelet membranes and their solubilization with 1% digitonin were conducted as previously described [10]. α_2-Adrenoceptors were partially purified from the solubilized membranes by sequential chromatography over an α_2-adrenoceptor-affinity gel and over a lectin gel. The application and

elution of material from the affinity gel were qualitatively the same as described previously [8] except that the column was larger (2.6 × 17 cm) and a different elution buffer was employed. Thus, following the wash, the affinity column was eluted with 2 bed volumes of the following buffer: 10 mM sodium phosphate, 145 mM NaCl, pH 7.2 (PBS), containing 50 μM phentolamine and 0.1% digitonin. Peak fractions of α_2-adrenoceptor activity which eluted from the affinity gel were then applied directly to a column of wheat germ agglutinin-agarose (1.5 × 12 cm). Typically, volumes of 100–150 ml, containing 30–40 pmoles of binding activity, were applied to the WGA column. The WGA column was then washed with 3–4 bed volumes of PBS–0.1% digitonin and then eluted with the latter buffer containing 500 mM *N*-acetylglucosamine. The peak binding activity eluted from the WGA column in a volume of 4–8 ml and with a 70% yield. All operations were conducted at 4–6°.

Photolysis. Unless otherwise indicated, the following protocol was used for all of the photolysis experiments. The partially purified receptor preparations (10–13 pmoles/mg protein) were diluted 5- to 6-fold with PBS-0.1% digitonin and 400-μl aliquots were placed in 12 × 75 mm polystyrene test tubes. Ligands were dissolved in 1 mM HCl and were added to give a final volume of 500 μl. The mixture was allowed to equilibrate for 2 hr at 0–4° and photolysis was initiated using a 4 W mercury-vapor lamp (Mineralight UVSL-25). With the lamp on "short wave" (254 nm) the samples were exposed from directly overhead, the distance being 6.5 cm from the lamp to the surface of the solution. Following photolysis, free ligands were separated from the receptor by gel filtration over 3.4 ml columns (6.3 × 110 mm) of Sephadex G-50 (fine). Material eluting in the void volume (1.0 ml) was assayed for the binding of [^{3}H]-yohimbine. The columns were pre-equilibrated and eluted with PBS-0.1% digitonin and were run in the cold (4–6°).

Binding assays. The binding of [^{3}H]yohimbine was determined in duplicate at a final concentration of 10 nM and in a total volume of 500 μl. To determine nonspecific binding a final concentration of 10 μM phentolamine was included. Assays were equilibrated for 3 hr at 0–4°, and bound and free [^{3}H]-yohimbine were separated by gel filtration as described in the preceding section. Radioactivity present in the void volume was determined by liquid scintillation spectroscopy (counting efficiency 45%). Binding data from competition and saturation experiments were analysed by computer using a non-linear least squares regression for a model describing the interaction of one or more ligands with a single class of binding sites [11].

Materials. [^{3}H]Yohimbine, sp. act. 75 Ci/mmole, was from New England Nuclear. WGA-agarose was from E-Y Laboratories, Inc. (San Mateo, CA). Sephadex G-50 and Sepharose CL-4B were from Pharmacia Fine Chemicals. HEAT (BE2254, 2-[β-(4-hydroxyphenyl)-ethyl-aminomethyl]-tetralon) was from Beiersdorf AG (Hamburg, FRG). Other drugs were from the same sources as reported previously [8]. All other reagents were of the highest available quality.

Other. Proteins were determined by the method of Schaffner and Weissmann [12]. Spectra were recorded on a Cary model 219, dual-beam spectrophotometer.

RESULTS

Figure 1 shows ultraviolet absorption spectra for SKF 102229 following increasing times of exposure to ultraviolet light. The uppermost curve, which represents the absorption spectrum of SKF 102229 prior to photolysis, shows a strong absorption maximum at 257 nm (E = 16,000 $cm^{-1}M^{-1}$). This peak is characteristic of simple arylazides that absorb strongly in this region. Following increasing time of exposure to short wavelength ultraviolet light, there was a progressive decrease in the absorption at 257 nm. This decrease reflects the decomposition of the azido group as it was converted into a highly chemically reactive nitrene upon exposure to the light. Assuming a simple exponential decay, the half-time ($T_{\frac{1}{2}}$) for the initial decomposition of SKF 102229

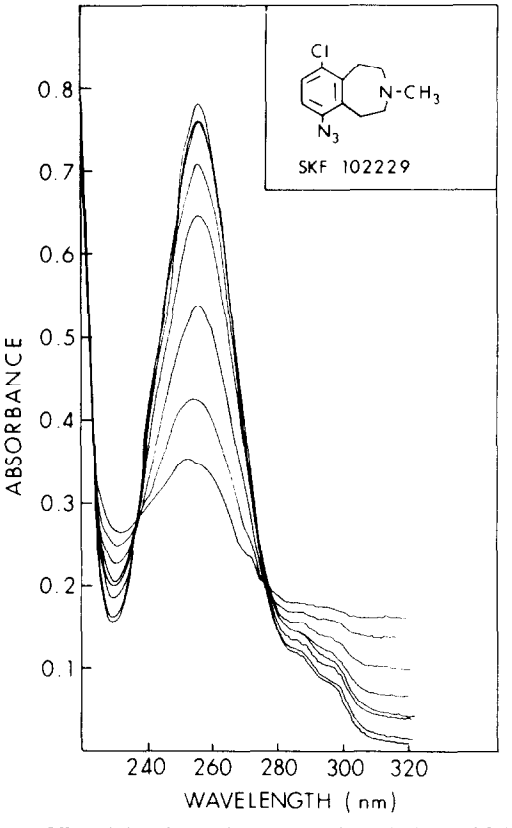

Fig. 1. Ultraviolet absorption spectra for solutions of SKF 102229 before and after various lengths of exposure to a source of short wavelength ultraviolet light. The uppermost curve represents the absorption spectrum of a 50 μM solution of SKF 102229 before exposure to ultraviolet light. The remaining curves (in order of decreasing absorption at 257 nm) represent the following time points: 0.25, 0.5, 1, 2, 4, 8, and 16 min. Solutions were made up with 1 mM HCl, and exposures were made from a distance of 6.5 cm. The manufacturer's rated output at 254 nm is ~130 μW/cm^2 at a distance of 15 cm. The inset shows the structure of SKF 102229.

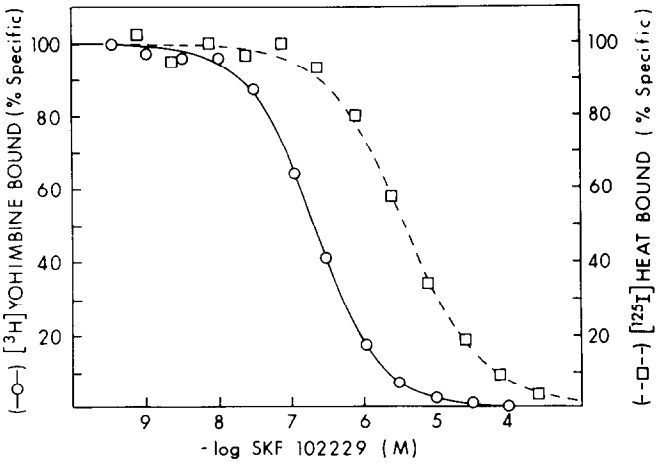

Fig. 2. Competition curves for the inhibition of [^{3}H]yohimbine binding (○) and [^{125}I]HEAT binding (□) by SKF 102229 in soluble receptor preparations derived from human platelet and rat liver respectively. Partially purified α_2-adrenoceptors were prepared from human platelets as described in Experimental. The K_i for the inhibition of [^{3}H]yohimbine binding by SKF 102229 was 43 ± 9 nM ($\bar{x}$ ± S.D., N = 2). Solubilization of rat liver α_1-adrenoceptors and their measurement using [^{125}I]HEAT were done according to the methods described by Wikberg *et al* [14]. The K_i for the inhibition of [^{125}I]HEAT binding by SKF 102229 was 2400 ± 800 nM (N = 2). All assays were done under conditions of low, indirect, artifical light.

was 8.9 min. Using light of a longer wavelength (366 nm), the decomposition was slower with a $T_{\frac{1}{2}}$ of 69 min (data not shown).

The parent compound from which SKF 102229 is derived, i.e. 3-methyl-6-chloro-1*H*-2,3,4,5-tetrahydro-3-benzazepine, shows high affinity and selectivity for α_2-adrenoceptors [13]. To investigate whether these characteristics were retained by SKF 102229, competition studies were performed using the α_2-selective ligand [^{3}H]yohimbine and the α_1-selective ligand [^{125}I]HEAT. The results of these studies are presented in Fig. 2. Using partially purified preparations of α_2-adrenoceptors, the inhibition of [^{3}H]yohimbine binding by SKF 102229 yielded a K_i of 43 nM. This K_i represents a slight (2.5-fold) decrease in affinity as compared with the parent compound but it nevertheless denotes high affinity for the α_2-adrenoceptor. In contrast, the affinity of SKF 102229 for α_1-adrenoceptors was markedly lower. In solubilized rat liver membranes, the K_i of SKF 102229 for the inhibition of [^{125}I]-HEAT binding was 2400 nM. Thus, SKF 102229 showed roughly a 55-fold selectivity for α_2-adrenoceptors as compared with α_1-adrenoceptors. This high selectivity compares favorably with the parent compound which, in bioassays, shows a 53-fold selectivity for α_2-adrenoceptors [13].

The ability of SKF 102229 to cause a light-dependent inactivation of α_2-adrenoceptors was investigated, and the results are shown in Fig. 3. As reflected by the decrease in [^{3}H]yohimbine binding, this figure shows that, in the presence of 100 nM SKF 102229, there was a progressive inactivation of α_2-adrenoceptors with increasing time of exposure to ultraviolet light. The well-known capacity of short wave ultraviolet light to damage proteins was also evident from the decrease in [^{3}H]yohimbine binding obtained following photolysis of the receptor preparation alone; however, this decrease was markedly less than that which occurred in the presence of SKF 102229. Figure 3 also shows that decreases in α_2-adrenoceptor number resulting from photolysis in the presence of SKF 102229 were specific and involved occupation of the ligand binding site. Thus, exposure of α_2-adrenoceptors to ultraviolet light in the presence of both SKF 102229 and an excess of phentolamine prevented the decrease in [^{3}H]yohimbine binding that occurred as a consequence of photolysis in the presence of SKF 102229 alone. The

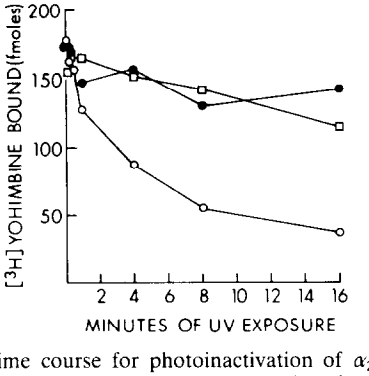

Fig. 3. Time course for photoinactivation of α_2-adrenoceptors following exposure to short wavelength ultraviolet light. Partially purified human platelet α_2-adrenoceptors were preincubated either alone (□), in the presence of 100 nM SKF 102229 (○), or in the presence of 100 nM SKF 102229 plus 1 μM phentolamine (●). The samples were then exposed to ultraviolet light for the indicated lengths of time and were chromatographed over Sephadex G-50. Following gel filtration, the α_2-adrenoceptor concentration was determined by the binding of [^{3}H]yohimbine. Each point represents the average of two determinations with an average variation of less than 3%. The experiment was repeated with similar results.

time course of α_2-adrenoceptor inactivation caused by photolysis in the presence of SKF 102229 was rapid between 0.5 and 8 min exposure to ultraviolet light. After 8 min exposure, the rate of α_2-adrenoceptor blockade decreased. Assuming a simple exponential process for the initial receptor blockade, the $T_{\frac{1}{2}}$ for the net decrease in α_2-adrenoceptor number was 8.4 min. These results show a good correlation with the data obtained in Fig. 1 on the time course of the direct photolysis of SKF 102229 in the absence of receptor. Therefore, in keeping with the properties expected of a photoaffinity ligand, these data demonstrate the specificity of α_2-adrenoceptor blockade by SKF 102229, the requirement of ultraviolet light, and a time dependence for receptor inactivation which parallels the photolytic decomposition of the azide.

The requirement of the azido group in SKF 102229 for the photolytic blockade of α_2-adrenoceptors was examined by comparing the ability of its des-azido analogue (SKF 86466) to cause a similar blockade. As shown in Fig. 4, photolysis of partially purified α_2-adrenoceptors in the presence of 100 nM SKF 86466 did not result in any receptor inactivation above that normally obtained by photolysis in the absence of ligands. This result was not due to lower occupancy since the affinity of SKF 86466 (K_d = 17 nM) was actually greater than that of SKF 102229 for the α_2-adrenoceptor [9]. As was the result in the previous figure, 8 min of photolysis in the presence of 100 nM SKF 102229 produced a significant 60% loss of [^{3}H]yohimbine binding activity. These data also show that decreases in [^{3}H]yohimbine binding were not due to incomplete removal of the ligands. Thus, in the absence of exposure to ultraviolet light, preincubation of α_2-adrenoceptors with either 100 nM SKF 102229 or 100 nM SKF 86466, followed by gel filtration, did not result in any loss of [^{3}H]-yohimbine binding as compared with preincubation in the absence of ligands.

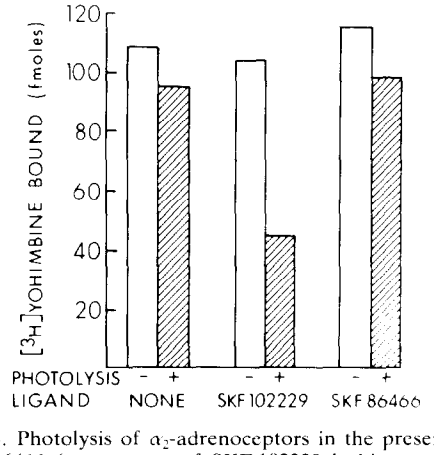

Fig. 4. Photolysis of α_2-adrenoceptors in the presence of SKF 86466 (a congener of SKF 102229 lacking an azido group). Duplicate aliquots of partially purified α_2-adrenoceptors were preincubated with either nothing, 100 nM SKF 102229, or 100 nM SKF 86466 (3-methyl-6-chloro-1*H*-2,3,4,5-tetrahydro-3-benzazepine). One set (shaded bars) was then exposed to ultraviolet light for 8 min while the other set (open bars) was kept in the dark. All of the samples were then desalted by gel filtration, and the binding of [^{3}H]yohimbine was assessed. The experiment was repeated with identical results.

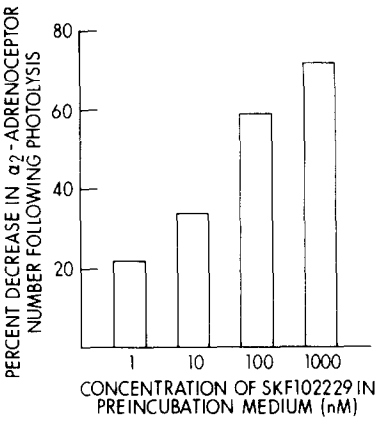

Fig. 5. Concentration dependence for the photoinactivation of α_2-adrenoceptors by SKF 102229 following exposure to short wavelength ultraviolet light. Aliquots of partially purified human platelet α_2-adrenoceptors were preincubated with the indicated concentrations of SKF 102229 and were then exposed to the ultraviolet light (8 min). Following gel filtration over Sephadex G-50, the binding of [^{3}H]yohimbine was assessed. One hundred percent equals 135 fmoles of [^{3}H]yohimbine binding activity. Each point represents the average of two determinations with a variance of less than 3%. The experiment was done once.

In Fig. 5 the results of an experiment are shown in which the time of ultraviolet exposure was held constant (at 8 min) and the concentration of SKF 102229 was varied. Consistent with a receptor–ligand interaction, the photolytic inactivation of α_2-adrenoceptors by SKF 102229 was concentration dependent. At the lowest concentration, 1 nM, the decrease in α_2-adrenoceptor number approximated the decrease expected due to direct inactivation of the receptor by ultraviolet light. The extent of the SKF 102229-dependent photoinactivation of the α_2-adrenoceptor between 10 and 1000 nM is in reasonable agreement with the receptor occupancy that one could expect based upon the affinity of SKF 102229 for the α_2-adrenoceptor.

To be sure that decreases in [^{3}H]yohimbine binding reflected decreases in α_2-adrenoceptor number and not competitive or allosteric effects, saturation isotherms were run following photolysis in the presence of SKF 102229. The results of experiments using human platelet membranes are shown in Fig. 6. These Scatchard plots reveal that there was a 46% decrease in receptor density (B_{max}) following photolysis in the presence of 100 nM SKF 102229 with no change in affinity. When this experiment was repeated using partially purified α_2-adrenoceptors, a 63% decrease in B_{max} was recorded with no significant change in K_d (see legend, Fig. 6). These data are in good agreement with previous results obtained using a single, saturating, concentration of [^{3}H]-yohimbine to assess α_2-adrenoceptor number.

The pharmacology of α_2-adrenoceptor photoinactivation by SKF 102229 was studied, and the results are expressed in Fig. 7. In the absence of any

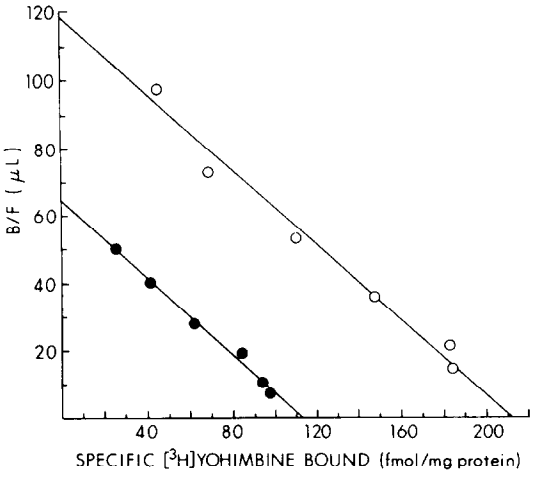

Fig. 6. Scatchard plots showing the binding of [^{3}H]yohimbine to α_2-adrenoceptors in human platelet membranes following photolysis in the presence (●) and absence (○) of 100 nM SKF 102229. Ten milliliters of human platelet membranes (2.1 mg protein/ml) was placed in a Petri dish and exposed to short wavelength ultraviolet light as previously described. The membranes were diluted to 40 ml with phosphate-buffered saline (PBS) and were centrifuged at 43,000 *g* for 20 min. The latter was repeated, and the final pellet was resuspended in 10 ml of PBS. Aliquots (0.3 ml) were assayed in duplicate at six concentrations of [^{3}H]yohimbine between 0.5 and 10 nM and in a final volume of 0.5 ml. Assays were terminated by filtration through Whatman GF/C filters followed by four 4-ml washes with cold PBS. All other conditions or operations were the same as described in Experimental for [^{3}H]yohimbine binding to solubilized receptors. The original data were analysed as described in Experimental and the following parameter estimates ± their standard errors were obtained. For the control (photolysis in the absence of SKF 102229), the $B_{max} = 268 \pm 11$ pM and the $K_d = 1.1 \pm 0.1$ nM; for photolysis in the presence of 100 nM SKF 102229, the $B_{max} = 140 \pm 9$ pM and the $K_d = 1.0 \pm 0.1$ nM. The experiment was repeated using partially purified α_2-adrenoceptors, and the following parameter estimates were obtained. For the control, the $B_{max} = 81 \pm 5$ pM and the $K_d = 3.4 \pm 0.4$ nM; for photolysis in the presence of 100 nM SKF 102229, the $B_{max} = 30 \pm 3$ pM and the $K_d = 1.9 \pm 0.4$ nM.

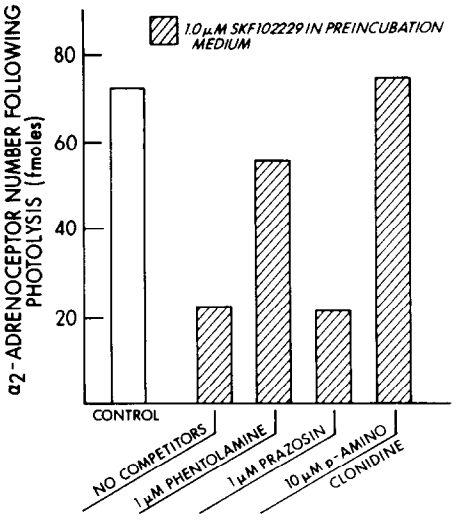

Fig. 7. Pharmacology of the photoinactivation of α_2-adrenoceptors by SKF 102229. Aliquots of partially purified human platelet α_2-adrenoceptors were preincubated with either no drugs (control), or with 1 μM SKF 102229 alone, or with 1 μM SKF 102229 and a competitor. All samples were then exposed to the ultraviolet light and afterwards were chromatographed over Sephadex G-50. α_2-Adrenoceptor binding activity was measured using [^{3}H]yohimbine as described in Experimental. The experiment was repeated three additional times with very similar results.

competitors, photolysis in the presence of 1 μM SKF 102229 resulted in a 69% decrease in α_2-adrenoceptor number relative to control. The concomitant presence of the nonselective α-adrenoceptor antagonist, phentolamine, during the preincubation period protected the α_2-adrenoceptor, as only a 22% decrease was recorded following photolysis. On the other hand, prazosin, an α_1-selective antagonist, was unable to prevent α_2-adrenoceptor photoinactivation by SKF 102229. Using the partial agonist *p*-aminoclonidine (α_2-selective), complete protection was afforded against the photolysis-induced irreversible binding of SKF 102229 to the α_2-adrenoceptor.

DISCUSSION

Evidence has been presented which shows SKF 102229 to be a potent and specific photoaffinity probe for the α_2-adrenoceptor. The inactivation of α_2-adrenoceptors by SKF 102229 was clearly dependent on exposure to ultraviolet light and on the concentration of SKF 102229 present during photolysis. The receptor blockade was most likely due to the light-induced transformation of the azido group to the corresponding nitrene with subsequent formation of a covalent bond between this species and the ligand binding site of the α_2-adrenoceptor. This conclusion is supported by the known mechanism of photolytic arylazide decomposition, by the data that show a dramatic decrease in receptor density following photolysis, and by the irreversible nature of the α_2-adrenoceptor blockade.

The maximum extent of α_2-adrenoceptor blockade obtained by photolysis in the presence of SKF 102229 approached 80%. This compares favorably with the 60–80% decreases that have been found in similar studies using nonradiolabeled photoaffinity ligands to the β-adrenoceptor [3, 4]. It is interesting that, when these same β-adrenergic photoaffinity ligands were radiolabeled, the maximum efficiency of incorporation of the radiolabel was only about 15%. This may be related to the very high affinity of these ligands for the β-adrenoceptor (20–200 pM) and their extreme hydrophobicity. Thus, it is possible that some of the apparent blockade obtained using the nonradiolabeled isomers may have been due to incomplete removal of the ligand and not to the formation of covalent bonds with the β-adrenoceptor. With respect to the present studies this possibility is less likely. The affinity of SKF 102229 for the α_2-adrenoceptor is in a range where the half-time for dissociation is minutes, not hours, and SKF

102229 is a moderately hydrophilic compound which means that it is easier to remove from the lipophilic environment of membranes. This is supported by the data in Fig. 4 which show that, in the absence of photolysis, preincubation of α_2-adrenoceptors with SKF 102229, followed by gel filtration, did not result in any subsequent inhibition of [^{3}H]yohimbine binding. Additionally, the data in Fig. 6 show that, following photolysis in the presence of SKF 102229, the decrease in [^{3}H]yohimbine binding was due to a decrease in receptor density rather than to a decrease in affinity. This finding is contrary to what would be expected if the removal of any free SKF 102229 was incomplete. Nevertheless, to determine the actual extent of the covalent incorporation will require a radiolabeled isomer or congener of SKF 102229.

Photosensitized destruction is another means of receptor inactivation that may occur following photolysis in the presence of a ligand [15]. This could explain the discrepancy noted previously between the apparent labeling of the β-adrenoceptor by unlabeled and radiolabeled isomers of some β-adrenergic photoaffinity ligands. Although photosensitized destruction seems to be a greater theoretical concern, as opposed to one that is commonly encountered, several experiments addressed this phenomenon. First, in the pharmacology experiments, controls were done (data not shown) to make sure that the competitors did not have the intrinsic ability to induce photoinactivation of α_2-adrenoceptors. In no case was this observed, intimating that simple occupation by an agonist or antagonist does not make the α_2-adrenoceptor more susceptible to direct photolytic inactivation. The data in Fig. 4 address the possibility that α_2-adrenoceptor inactivation might have been due to photosensitized destruction via the benzazepine chromophore of SKF 102229, as opposed to a specific inactivation by the azido group. Thus, photolysis of α_2-adrenoceptors in the presence of SKF 86466 (in which the azido group is replaced by a hydrogen atom), did not cause a decrease in receptor number above that normally obtained by photolysis alone.

The data that have been presented show SKF 102229 to be a potent photoaffinity ligand and strongly suggest that, upon photolysis, SKF 102229 is capable of a high degree of covalent insertion into human platelet α_2-adrenoceptors. Experience has shown that chemical modifications of the aryl moiety of SKF 102229, and other benzazepine-based α_2-adrenergic ligands, can produce dramatic changes in their affinity for α_2-adrenoceptors ([9] and unpublished results). It would thus seem likely that the azido group of SKF 102229 is intimately associated with the ligand binding site of the α_2-adrenoceptor. Future studies should establish the utility of SKF 102229 for understanding the physiology and biochemistry of α_2-adrenoceptors.

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