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PHOTOAFFINITY LABELLING OF β-ADRENERGIC RECEPTORS OF C_6 GLIOMA CELLS

PRESENCE OF A NUCLEOPHILIC GROUP IN THE RECEPTOR*

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Abstract—(±) 1-[1-(*p*-Nitrophenoxy), 2-methyl, 2-propylamino], 3-(α-naphtyloxy), 2-propanol (PNP) was synthesized and found to be a potent photoaffinity label for β-adrenergic receptors of C_6 glioma cells.

In the dark, PNP displaced all the [^{3}H]DHA binding sites on C_6 glioma cell membranes ($K_D = 5.5 \times 10^{-8}$ M).

Upon photolysis on isolated C_6 glioma cell membranes: (a) PNP reduced in a dose-dependent manner maximally stimulated β-adrenergic sensitive adenylate cyclase. After extensive washing of the membranes, the maximal β-adrenergic stimulation was reduced without change in the apparent affinity for isoproterenol. A 62% decrease in activity was obtained with 10^{-5} M PNP without any change in basal and NaF stimulated adenylate cyclase activities. (b) PNP also irreversibly reduced in a dose-dependent manner the total number of [^{3}H]DHA binding sites without changing the affinity of the remaining sites. The effects of PNP on adenylate cyclase and [^{3}H]DHA binding were suppressed in the presence of (−)alprenolol. Upon photolysis on intact C_6 glioma cells, PNP inactivated β-adrenergic receptors coupled with adenylate cyclase without any change in basal, NaF and Gpp(NH)p stimulated adenylate cyclase activities.

These results indicate that PNP photolabelling occurred on the β-adrenergic receptors. Furthermore, as PNP was shown to react with model nucleophiles upon photolysis, this labelling implies the presence of a nucleophilic group in the β-adrenergic receptor.

The properties of β-adrenergic receptors have mainly been studied using reversible ligands [1–5], but to purify these receptors and to study their turnover, irreversible or long lasting ligands are needed.

Beta-adrenergic receptors have been labelled with alkylating agents [6–8] or with light activated [9–11] and long lasting compounds [12–14]. For irreversible blockade, photoaffinity probes offer some advantages over alkylating agents since, in the absence of light, they can reach the binding site without decomposition or hydrolysis. They can act as reversible ligands and the characteristics of their association with the receptor can be established before photoactivation.

Photoaffinity labelling of β-adrenergic receptors has been performed with antagonists containing an aromatic azide moiety. This chemical group is completely inert in the dark but, upon irradiation, is converted into a reactive nitrene able to create a covalent bond with any molecule present (proteins, buffers, etc.) [15, 16]. Thus, an azide derivative from acebutolol was reported to irreversibly and specifically inhibit the β-adrenergic activated adenylate cyclase of rat reticulocyte membranes upon photolysis [9]. More recently, radioactive azide derivatives were used to label the β-adrenergic receptor of duck [10] and frog [11] erythrocyte membranes.

Besides azides, another class of photolabels—nitrophenyl ethers—has been described [17]. The use of these reagents is based on their well documented nucleophilic aromatic photosubstitution reactions [18]. These labels have some advantages over aromatic azides since they give high yield reactions upon photolysis in the presence of nucleophiles, and no reaction in their absence [18]. Such a property can be useful for labelling receptors which may contain nucleophilic groups like the β-adrenergic receptor of C_6 glioma cells [19]. For this purpose we synthesized a nitrophenyl ether derivative of propranolol PNP 1 (Fig. 1), and examined the labelling of β-adrenergic receptors of C_6 glioma cells with this new photoaffinity probe.

MATERIALS AND METHODS

Synthesis of nitrophenyl ethers

(±) *1-[1-(p-Nitrophenoxy), 2-methyl, 2-propylamino], 3-(α-naphtyloxy), 2-propanol: PNP 1.* One equivalent of (*p*-nitrophenoxy), 2-amino, 2-methyl propane obtained from 2-amino, 2-methyl propanol and *p*-nitrochlorobenzene [20] was heated overnight in the dark under nitrogen with one equivalent of α-naphtyloxy, 2,3-oxirane, 1-propane. The solvent was removed under vacuum and the crude solid twice recrystallized from 2-propanol, leading to yellow crystals of PNP (F = 114–116°, yield = 62%). TLC: (silica gel) R_f = 0.65 ($AcOEt/CH_3OH/NH_4OH$ 90/10/1, v/v/v). HPLC: (μCN) retention volume =

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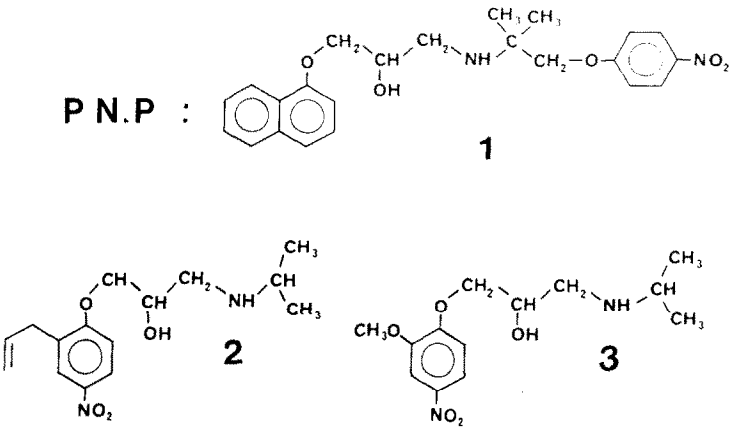

Fig. 1. Structure of PNP: 1: (±) 1-[1-(*p*-nitrophenoxy), 2-methyl, 2-propylamino], 3-(α-naphtyloxy), 2-propanol. 2: (±) 1-(isopropylamino), 3-(2-allyl, 4-nitrophenoxy), 2-propanol. 3: (±) 1-(isopropylamino), 3-(2-methoxy, 4-nitrophenoxy), 2-propanol.

10.4 ml ($CH_3OH/H_2O/TFA$, 500/500/3, v/v/v), 2 ml/min. u.v.: (CH_3OH): $\lambda = 214$ and 230 nm (shoulder), $\lambda = 295$ nm ($\varepsilon = 15{,}600$). PMR: (DMSO d_6) δ (ppm): $C(CH_3)_2 = 1.13$; $O—CH_2—C—N = 3.80$; $O—CH_2—C—OH = 4.03$; —CH— = 3.96 and $N—CH_2 = 2.80$. Analysis: $C_{23}H_{26}N_2O_5$: (C, H, N).

(±) *1-(Isopropylamino), 3-(2-allyl, 4-nitrophenoxy), 2-propanol: PNP 2.* The chlorhydrate of this compound was prepared by conventional methods [21] from 2-allyl, 4-nitrophenol which was obtained from 4-nitrophenyl allyl ether by Claisen rearrangement [21]. $F = 133$–135°C. TLC: (silica gel) $R_f = 0.26$ ($CH_3OH—CH_3CO_2C_2H_5$, 8/2, v/v).

(±) *1-(Isopropylamino), 3-(2-methoxy, 4-nitrophenoxy), 2-propanol: PNP 3.* This compound was prepared from 4-nitro gaiacol and epichlorhydrin [21] followed by the reaction of isopropylamine with the resulting crude oxirane. $F = 161$–163° (2-propanolol). TLC: (silica gel) $R_f = 0.32$ ($CH_3CH_2OH—CH_3CO_2H$, 95/5, v/v).

Photolysis conditions

Photolysis was performed using a 366 nm lamp (USVL-25 mineral lamp, Ultraviolet Products, San Gabriel, CA). No significant reduction of binding sites was observed when membranes were irradiated for 30 min. When cells were irradiated under the same conditions, they were still able to grow and membranes prepared from these cells showed no significant reduction of binding sites and no reduction of isoproterenol adenylate cyclase stimulation, compared to membranes prepared from non-irradiated cells.

C_6 glioma cell membrane labelling by PNP

Membranes (0.5–2.0 mg protein/ml) prepared as previously described [23] were mixed in polypropylene Eppendorf-conic tubes with PNP to the desired concentration. The buffer (25 mM Tris–HCl, pH 8; 2.5 mM $MgCl_2$) was deoxygenated for 30 min by bubbling nitrogen in the buffer at 4°. The volume of the tubes was adjusted to 0.5 ml and the preparations were incubated at 30° for 10 min in the dark. Tubes were then cooled in an ice bath for 2 min and positioned vertically at 0.5 cm from the lamp. Photolysis was performed at 4° for a total period of 30 min during which the tubes were cooled every 10 min in the dark. After the irradiation, the tubes were again cooled and 0.5 ml of cold buffer, with or without 5×10^{-5} M (−)alprenolol (see figure captions), was added before centrifugation (1 min, 12,000 rpm). The supernatant was discarded and the pellet was suspended in 0.5 ml of cold buffer (25 mM Tris–HCl, pH 8; 5 mM EDTA) and adenylate cyclase or (^{3}H)DHA binding experiments were performed as previously described [23].

C_6 glioma cell labelling by PNP

C_6 glioma cells were cultured in 25 cm^2 Corning flasks [23]. Before each experiment the culture medium was changed to Hanks medium (5 ml). Flasks were incubated in the dark for 20 min at 37° with the desired PNP concentration. Cells were then photolyzed for 30 min at room temperature at a distance of 2.5 cm from the bottom of the flask. Cells were washed with 5 × 5 ml of Hanks medium, further incubated and again irradiated. This procedure was completed 4 times (total irradiation time = 2 hr). After the last irradiation, cells were washed thoroughly 9 times at room temperature with 5 ml Hanks buffer, and twice with 5 ml 0.9% NaCl, lysed, suspended in cold hypotonic buffer, and disrupted in a Potter homogenizer (10 strokes). The homogenate was centrifuged at 5000 *g* for 30 min at 4°. The pellet was suspended in the same hypotonic buffer and adenylate cyclase assays were performed as previously described [23].

Calculations

The isoproterenol dissociation constant (K_D) for β-adrenergic receptors was determined experimentally from the isoproterenol concentration that inhibited [^{3}H]DHA binding by 50%: $IC_{50} = K_D[1 + (S)/K_D^*]$ where (S) is the [^{3}H]DHA concentration and K_D^* the dissociation constant of [^{3}H]DHA for its binding sites. $K_A$app is the agonist activation constant for the adenylate cyclase system and is equal

to the agonist concentration yielding 50% maximal activation.

Chemicals

(−) [^{3}H]Dihydroalprenolol[^{3}H]DHA, cyclic [^{3}H]AMP and [α^{32}P]ATP were purchased from the NEN Corp. (−)Isoproterenol was obtained from Sigma. (−)Alprenolol was kindly supplied by the Ciba-Geigy Laboratories. *N*-α Boc lysine was a gift from Dr S. Lavielle (Paris). Isobutyl mercaptan was obtained from Aldrich. Hanks medium was supplied by Gibco and Phosphate Buffered Saline (PBS) was obtained from Eurobio.

RESULTS

PNP photolysis in the presence of model nucleophiles. Like other nitrophenyl ethers, PNP remained stable in the dark, even in the presence of high concentrations of nucleophiles and, as expected, decomposed upon irradiation (Fig. 2). For these experiments, two types of nucleophiles were used: *N*-α Boc lysine, which, like in protein side-chains, contains an ε-NH_2, and isobutyl mercaptan, a thiol nucleophile related to cysteine. PNP decomposition was easily monitored by HPLC, the amount of the remaining PNP being evaluated after different photolysis times (Fig. 2). Preparations were irradiated in two buffers: PBS (pH 7.4), a non-nucleophile buffer used in cell cultures and 25 mM Tris–HCl, pH 8, a nucleophilic one required for adenylate cyclase stability. The complete photodecomposition of 5.10^{-5} M PNP in the presence of 5.10^{-4} M *N*-α Boc lysine occurred after 65 min in the first buffer and 30 min in the second, with decomposition half-times of 7 and 3 min, respectively. The PNP photodecomposition was about twice as fast in Tris–HCl as in PBS buffer. This difference might be due to the higher pH value of the Tris–HCl buffer and/or to the expected PNP photoreaction with the nucleophilic buffer. PNP decomposition was observed in Tris–HCl buffer in the absence of *N*-α Boc lysine but with a velocity 10 times lower than in its presence (50% decomposition in about 30 min). Slight decomposition was also observed with the PBS buffer in the absence of *N*-α Boc lysine (50% decomposition in about 72 min). This reaction could be due to the presence in the structure of PNP of an amino group able to react intermolecularly during photolysis with another PNP molecule. Results similar to those obtained with *N*-α Boc lysine were also observed with isobutyl mercaptan (data not shown).

Like other nitrophenyl ethers, PNP irradiation in the presence of a primary amine led to a nitroaniline derivative. Figure 3 shows the absorption spectrum of PNP before irradiation and after complete decomposition in the presence of *N*-α Boc lysine. Nitrophenyl ether absorption at 295 nm was progressively attenuated upon photolysis, and a new absorption band appeared at 415 nm in Tris–HCl buffer (410 nm in PBS buffer and 405 nm in methanol). This band is characteristic of nitroaniline derivatives and its presence showed that at the wavelength used (366 nm) the photoreaction occurred as expected.

PNP and prephotolyzed PNP binding to the β-adrenergic receptor of C_6 glioma cell membranes in the dark. [^{3}H]DHA was displaced from its binding sites with PNP and with prephotolyzed PNP, i.e. PNP photolyzed for 2 hr in 0.4 M Tris–HCl, pH 8. The displacement curves for [^{3}H]DHA binding by PNP gave a dissociation constant for PNP of 5.5×10^{-8} M. PNP was able to displace all specific [^{3}H]DHA binding, showing that this compound interacted with all β-adrenergic sites (Fig. 4A). Inhibition of the isoproterenol stimulated adenylate cyclase by PNP allowed calculation of its inhibition

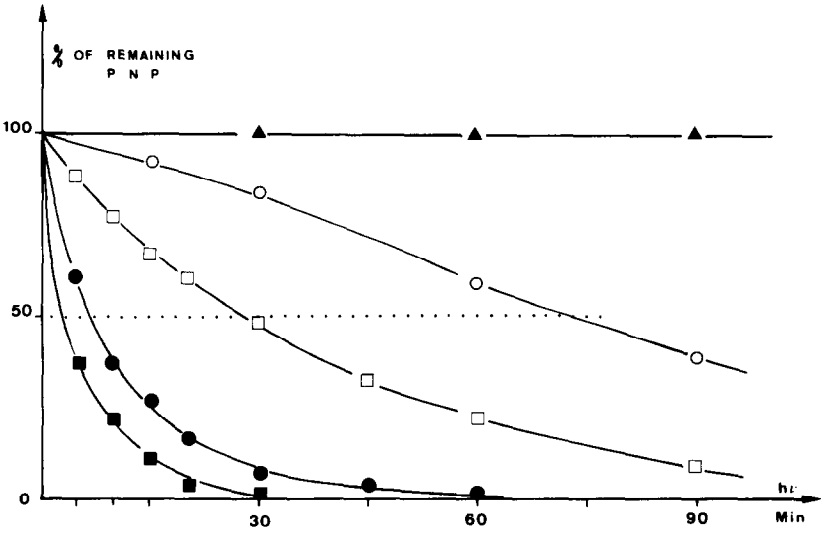

Fig. 2. Photolysis of PNP at 366 nm. 5×10^{-5} M PNP in 20 mM PBS (pH 7.4) (○, ●) or in 25 mM Tris–HCl (pH 8) (□, ■) was photolyzed as described in Materials and Methods, without (○, □) or with (●, ■) 5×10^{-4} M *N*-α Boc lysine. A control experiment was performed in the absence of light: 5×10^{-5} M PNP was mixed in the dark with 5×10^{-3} M *N*-α Boc lysine in Tris–HCl or PBS (▲). The amount of the remaining PNP at different photolysis times was monitored by HPLC.

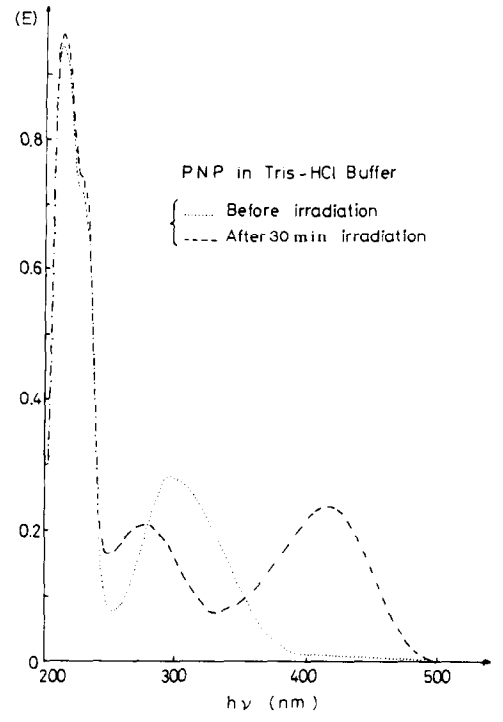

Fig. 3. u.v.–visible absorption spectrum of PNP and photolyzed PNP in the presence of *N*-α Boc lysine. A mixture of 2×10^{-5} M PNP and 2×10^{-5} M *N*-α Boc lysine were added to 25 mM Tris–HCl buffer (pH 8) and the spectrum was recorded before (...) and after (---) 30 min irradiation at 366 nm (room temperature, quartz cuvettes).

constant, K_I (10^{-7} M) (data not shown) which was close to its K_D. Prephotolyzed PNP (Fig. 4B) also displaced all specifically bound [^{3}H]DHA but with a 15 times higher dissociation constant than that of PNP ($K_D = 7.8 \times 10^{-7}$ M).

Inhibition of isoproterenol-stimulated adenylate cyclase by PNP upon photolysis. When membranes were incubated for 10 min at 30° with various PNP concentrations and photolyzed for 30 min, the maximal isoproterenol-stimulated adenylate cyclase activities were 77, 69, 38 and 3% for PNP concentrations of 5×10^{-8} M, 10^{-7} M, 10^{-5} M and 10^{-4} M, respectively. PNP concentrations up to 10^{-5} M did not affect basal or fluoride adenylate cyclase activities. Complete inhibition of isoproterenol-stimulated adenylate cyclase occurred at 10^{-4} M PNP, but the fluoride and basal adenylate cyclase activities dropped by about 50%. Two facts indicate that the decline in isoproterenol stimulation was not due to competitive inhibition of isoproterenol binding by PNP: firstly, we obtained the same adenylate cyclase activities with two supramaximal isoproterenol concentrations: 5×10^{-5} M and 10^{-4} M. Secondly, after a photolysis experiment with 5×10^{-7} M PNP followed by extensive washing of the membranes, we observed (Fig. 5B) a decrease of about 50% in the maximally stimulated isoproterenol adenylate cyclase with no change in the $K_A$app. The blockade of isoproterenol stimulation by 5×10^{-7} M PNP was mediated by β-adrenergic receptors since it was suppressed when photolysis was done in the presence of (−)alprenolol (5×10^{-5} M). At such an alprenolol concentration, it was calculated from the respective

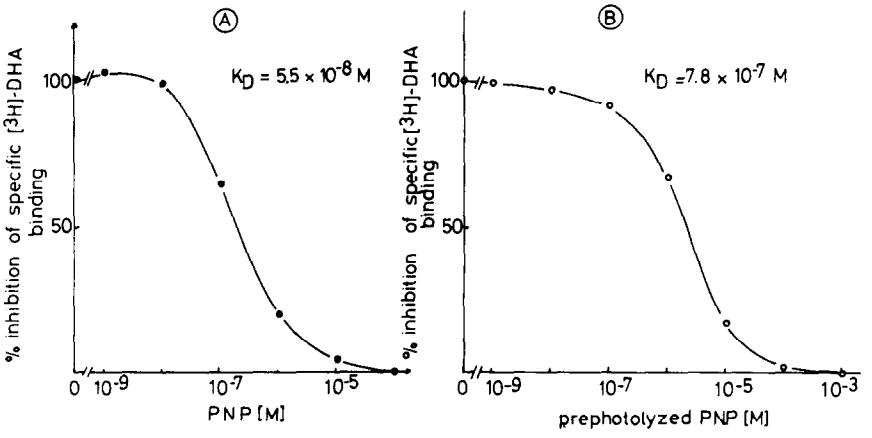

Fig. 4. PNP and prephotolyzed PNP affinities for β-adrenergic receptors in C_6 glioma cell membranes. (A) Specific [^{3}H]DHA binding displacement by PNP: membranes at a final concentration of 0.56 mg protein/ml were incubated for 10 min at 30° in the dark with [^{3}H]DHA (6.8 nM) and increasing PNP concentrations. [^{3}H]DHA binding was determined as described [22]. Each point is the mean of duplicate determinations. The maximal amount of specific [^{3}H]DHA bound to membranes was 247 fmole/mg protein. The PNP dissociation constant, K_D, for the β-adrenergic receptor was calculated as described in Materials and Methods, using a [^{3}H]DHA concentration of 6.8 nM and a [^{3}H]DHA dissociation constant (K_D^*) of 3 nM. (B) Specific [^{3}H]DHA binding displacement by prephotolyzed PNP; PNP (10^{-3} M) was photolyzed in 0.4 M Tris–HCl for 2 hr. HPLC evaluation showed that more than 98% of the PNP was photolyzed under these conditions. Membranes at a final concentration of 0.6 mg protein/ml were incubated for 10 min at 30° in the dark with [^{3}H]DHA (5.6 nM) and increasing prephotolyzed PNP concentrations, [^{3}H]DHA binding was then determined. Each point is the mean of duplicate determinations. The maximal amount of specific [^{3}H]DHA bound to membranes was 232 fmole/mg protein. The prephotolyzed PNP dissociation constant (K_D) was calculated with a [^{3}H]DHA concentration of 5.6 nM and a [^{3}H]DHA dissociation constant (K_D^*) of 3 nm. These results are representative of two other experiments.

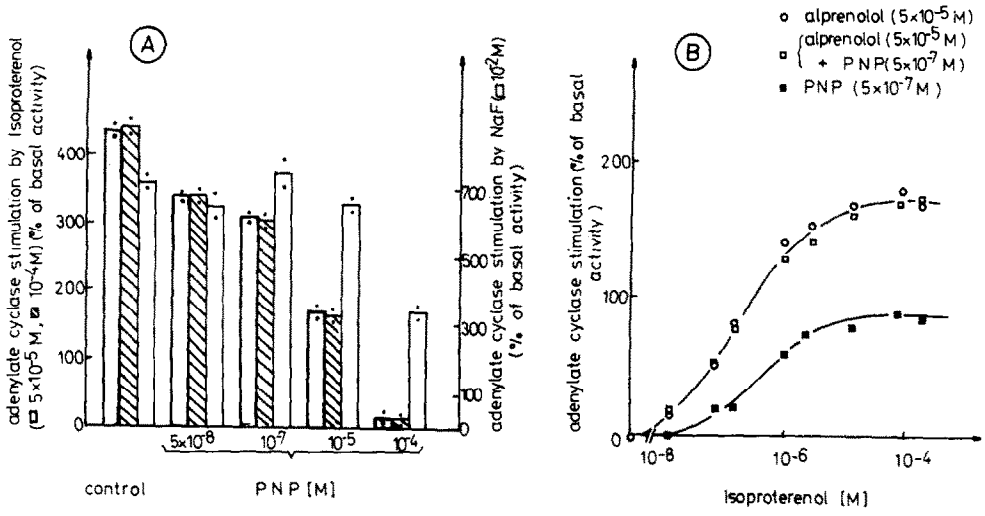

Fig. 5. Inactivation of β-adrenergic receptors of C_6 glioma cell membranes by photoaffinity labelling and its protection with (−)alprenolol. (A) Dose-dependent inactivation of β-adrenergic receptors by photoaffinity labelling with PNP. C_6 glioma cell membranes at a final concentration of 1.9 mg protein/ml were incubated in the dark at 30° for 10 min in deoxygenated buffer (25 mM Tris–HCl, pH 8; 2.5 mM $MgCl_2$) with increasing PNP concentrations. In control experiments membranes were treated without PNP but with (−)alprenolol (10^{-6} M). After incubation, membranes were photolyzed for 30 min at 4°. Ten μl of these membranes were used for determination of basal, isoproterenol- and NaF-stimulated adenylate cyclase activities (see Materials and Methods). Basal adenylate cyclase activities were 52 pmole/15 min/mg protein in the controls and 49, 47, 43 and 27 pmole/15 min/mg protein in the PNP treated series (5×10^{-8} M, 10^{-7} M, 10^{-5} M and 10^{-4} M, respectively). (B) Protection from inactivation by photoaffinity labelling with (−)alprenolol. C_6 glioma cell membranes at a final concentration of 1.2 mg protein/ml were incubated in degassed buffer (25 mM Tris–HCl, pH 8; 2.5 mM $MgCl_2$) at 30° for 10 min in the dark with (a) 5×10^{-5} M (−)alprenolol (○), (b) 5×10^{-5} M (−)alprenolol plus 5×10^{-7} M PNP (□) and (c) 5×10^{-7} M PNP (■). After 30 min irradiation 0.5 ml of degassed Tris–HCl buffer was added together with 5×10^{-5} M (−)alprenolol in series (■) and without alprenolol in series (○) and (□). The tubes were centrifuged and washed five times with 1 ml cold buffer. Isoproterenol dose–response experiments were performed as described in Materials and Methods. Basal adenylate cyclase activities were 70, 83 and 75 pmole/15 min/mg protein in series (○), (□) and (■), respectively. These results are representative of two other experiments.

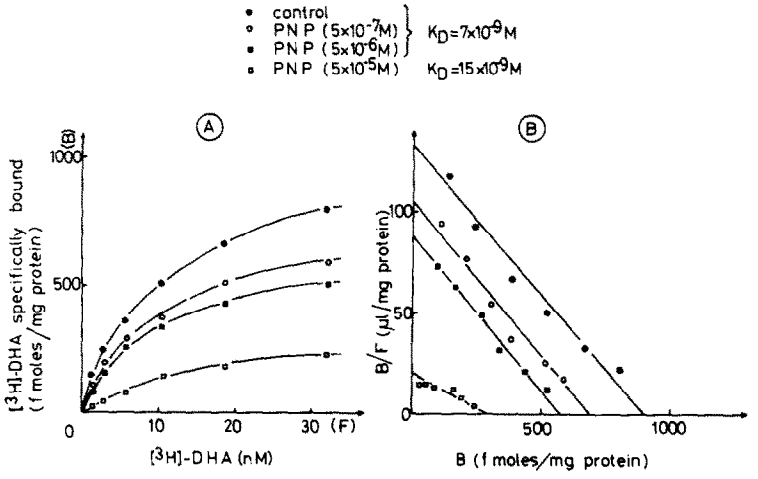

Fig. 6. Dose-dependent blockade of β-adrenergic receptors by PNP in C_6 glioma cell membranes. (A) Specific [^{3}H]DHA binding: membranes (1.4 mg protein/ml) were incubated for 10 min at 30° in the dark in 0.5 ml degassed Tris–HCl buffer (25 mM, pH 8; 2.5 mM $MgCl_2$) containing 5×10^{-5} M (−)alprenolol plus 5×10^{-7} M PNP (●) control; 5×10^{-7} M PNP (○); 5×10^{-6} M PNP (■) and 5×10^{-5} M PNP (□). After incubation, all four series were irradiated for 30 min at 4° as described in Materials and Methods. After photolysis 0.5 ml of Tris–HCl buffer was added together with (−)alprenolol (5×10^{-5} M) to series (○), (■), (□) and without alprenolol to series (●). The four series were washed six times in Tris–HCl buffer and resuspended in 25 mM Tris–HCl, pH 8; 5 mM EDTA. Two separate experiments gave the same results. Each point is the mean of triplicate determinations. (B) Scatchard plot of the data of (A).

K_D of PNP (5.5×10^{-8} M) and alprenolol (3×10^{-9} M) that more than 99% of the binding sites were occupied by alprenolol (Fig. 5B).

Photoaffinity labelling by PNP of β-adrenergic receptors of isolated membranes. Particulate fractions of C_6 glioma cells were incubated with various PNP concentrations for 10 min at 30° in the dark, photolyzed and then extensively washed. [^{3}H]DHA binding experiments were performed as described in Materials and Methods. As in the adenylate cyclase experiments, binding data indicated that β-adrenergic receptors are blocked by PNP during photolysis. This was found to intensify with increasing PNP concentrations, thus, 25, 37 and 68% blockade were observed with 5×10^{-7} M, 5×10^{-6} M and 5×10^{-5} M PNP, respectively (Fig. 6A). The blockade of the β-adrenergic receptors was reflected by the drop in the total number of binding sites. The only change in the dissociation constant of [^{3}H]DHA occurred at the highest PNP concentration (5×10^{-5} M). [^{3}H]DHA K_D is doubled (Fig. 6B). This could be due either to slight contamination of the membranes by PNP or to less accurate determination of specific binding when low concentrations of binding sites are present in the membrane.

In a separate experiment performed in the dark, without photolysis, the same number of [^{3}H]DHA binding sites were obtained for membranes incubated without PNP (820 fmole/mg protein) and for membranes incubated with 10^{-6} M PNP (850 fmole/mg protein).

Blockade of β-adrenergic receptors after treatment of intact C_6 glioma cells with PNP. C_6 glioma cells were incubated with 5×10^{-7} PNP for 20 min at 37° in the dark and then photolyzed at room temperature for 30 min. Cells were extensively washed and particulate fractions prepared. Three separate experiments indicated that, at two supramaximal concentrations of isoproterenol (10^{-5} M and 5×10^{-5} M), adenylate cyclase stimulation diminished by 32%. Control experiments were conducted with cells incubated and photolyzed under the same conditions but without PNP (Fig. 7). When the same concentration of PNP was used and the same treatment (time, photolysis and washing procedures) repeated 4 times, the maximal stimulation of adenylate cyclase dropped by 63%, compared to control membranes. There was no significant modification of basal Gpp(NH)p or NaF-stimulated adenylate cyclase activities (Fig. 7).

DISCUSSION

Photoaffinity labelling is an effective method for irreversible blockade of receptors or active sites in enzymes [24, 25]. Two affinity labels for β-adrenergic receptors were recently used for this purpose: both are aromatic azides, one derived from acebutolol [9] and the other from benzyl pindolol [10]. Azide derivatives often lead to low yields of receptor covalent fixation [26]; in this connection, Rashidbaigi and Ruoho, who used iodoazidobenzyl pindolol,

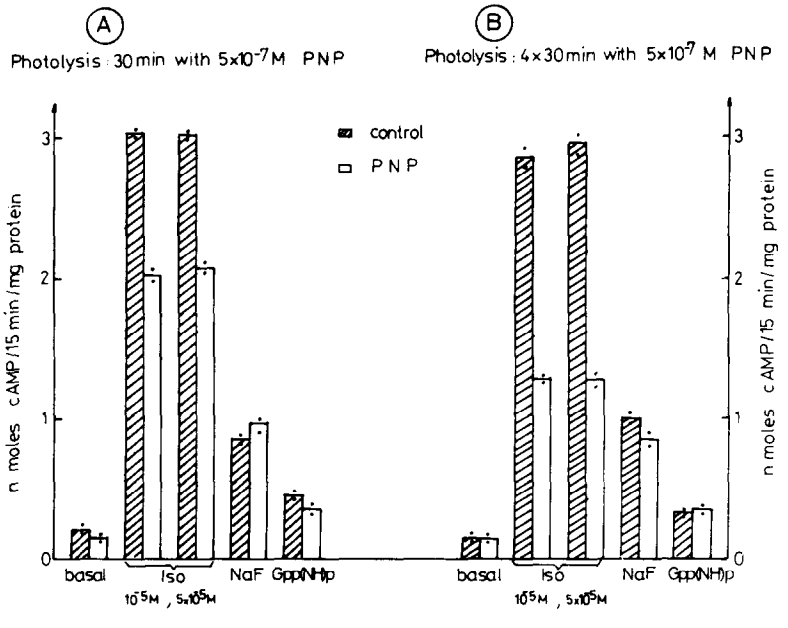

Fig. 7. *In vivo* inactivation of β-adrenergic receptors of C_6 glioma cells by photoaffinity labelling. C_6 glioma cells were incubated at 37° for 20 min in the dark in Hanks medium with 5×10^{-7} M PNP or without PNP (control). Cultures were then irradiated for 30 min at room temperature and washed five times with Hanks medium (A). For the experiment in (B), incubation (with PNP or without PNP) and irradiation followed by extensive washing as above were repeated four times (total irradiation time 2 hr). After the last photolysis, cultures were washed nine times with Hanks medium and rinsed twice with 5 ml 0.9% NaCl at room temperature. Cells were then lysed in 5 ml cold hypotonic medium (25 mM Tris–HCl, pH 8; 5 mM EDTA) resuspended, homogenized and centrifuged. Pellets were resuspended in the same cold hypotonic medium prior to determination of isoproterenol adenylate cyclase stimulation. Each value is the mean of duplicate determinations. Adenylate cyclase activities were also stimulated by 10^{-2} M sodium fluoride and 10^{-5} M Gpp(NH)p. This experiment was done three times.

reported that only 8–13% of the total β-adrenergic receptor population was labelled [10]. It has been suggested that such weak labelling might be due to the electrophilic nature of nitrenes which allow preferential reactions with water of buffer components [16] and thus reduce the chances of reaction with the target.

A higher level of receptor covalent fixation may be expected with nitrophenyl ether derivatives since these compounds are known to react upon photolysis in the presence of nucleophiles only [18]. The aromatic photosubstitution occurs via a short lived species with a half-live of 10^{-7}–10^{-9} sec [27] compared with 10^{-2}–10^{-4} sec for azides [28]. This ensures that once in its binding site the photolabel does not wander away [24, 29] before reacting with a nucleophilic group of the receptor. Furthermore, the chemical yields of these reactions involving nitrophenyl ether derivatives should be high, since non-productive deactivation of the light-excited state regenerates the starting compound.

As shown in model reactions, PNP, a nitrophenyl ether derivative of propranolol, gives rise to photoreactions with NH_2 or SH nucleophiles. PNP affinity for β-adrenergic receptors ($K_D = 5.5 \times 10^{-8}$ M) is higher than that of the prephotolyzed product ($K_D = 7.8 \times 10^{-7}$ M). Adenylate cyclase and binding measurements performed on C_6 glioma cell membranes showed that PNP blocked the β-adrenergic receptor in a non-competitive manner upon photolysis. This blockade was concentration dependent. Total blockade of isoproterenol-stimulated adenylate cyclase was obtained with 10^{-4} M PNP. However, at this concentration a 50% reduction of basal and NaF-stimulated activities was observed suggesting that a non-specific effect can occur at high PNP concentrations. At a lower concentration (10^{-5} M), 63% of the maximal isoproterenol-stimulated adenylate cyclase activity was blocked without any change in basal or fluoride activities. This blockade was specific for β-adrenergic receptors since complete protection of binding sites was observed in the presence of (−)alprenolol. Similar findings in binding experiments confirmed the specificity of the label ling. Lavin *et al.* also reached the same maximal blockade on frog erythrocyte membranes with an azido derivative of carazolol [11].

Upon photolysis PNP also labelled receptors on intact cells. C_6 glioma cells were not affected by 2 hr irradiation in the absence of PNP, since no reduction in the total number of binding sites or in isoproterenol adenylate cyclase activity was detected. Furthermore, cells continued to grow after such treatment. As on isolated membranes, a 66% reduction of isoproterenol adenylate cyclase stimulation was achieved, with four irradiations in the presence of 5×10^{-7} M PNP. This reduction was obtained without any drop in basal, fluoride or Gpp(NH)p stimulations.

Due to the mechanism of the nitrophenyl ether photoreaction, our data implied that a nucleophilic group was located close to the active site of the β-adrenergic receptor. In that case the group would react with PNP upon photolysis and form a covalent bond with the nitrophenyl moiety, with concomitant elimination of the other portion of the molecule. This nucleophile could be an NH_2 or an SH group, possibly located near the amino alkyl side chain binding site. We previously reported that an SH group was probably located in such an area, since a thiol derivative of propranolol blocked β-adrenergic receptors of C_6 glioma cells in a non-competitive manner [19]. We did not find any other nucleophile associated with a different binding area of the antagonist, since with other nitrophenyl ether β-adrenergic antagonists synthesized such as compound 2, a nitro derivative of alprenolol ($K_D = 5 \times 10^{-7}$ M) or compound 3 ($K_D = 3 \times 10^{-7}$ M) (see Materials and Methods) we did not observe any significant blockade of β-adrenergic receptors when these compounds were irradiated in the presence of membranes.

Consequently PNP, a new photoaffinity label, irreversibly blocked β-adrenergic receptors of C_6 glioma cells. The extent of this labelling opens up hopeful prospects for the study of β-adrenergic receptor turnover, and the relationship between the catecholamine activation of the adenylate cyclase and the number of β-adrenergic receptors in C_6 glioma cells.

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