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LNP 906, the first high-affinity photoaffinity ligand selective for I_1 imidazoline receptors

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1 The hypotensive effect of imidazoline-like drugs, such as clonidine, was attributed both to α_2-adrenergic receptors and nonadrenergic imidazoline receptors, which are divided into I_1, I_2 and I_3 subtypes.

2 We have recently synthesized a derivative of (2-(2-chloro-4-iodo-phenylamino)-5-methyl-pyrroline (LNP 911), the first high-affinity and selective ligand for I_1 receptors (I_1R), with a photoactivable function (LNP 906).

3 This work aims to test whether this derivative retained the binding properties of LNP 911 and bound irreversibly to I_1R.

4 Binding studies showed that LNP 906 exhibited nanomolar affinity for I_1R and was selective for I_1R over I_2 receptors and α_2-adrenergic receptors (α_2Ars).

5 Upon exposure to u.v. light, LNP 906 irreversibly blocked the binding of [^{125}I]-paraiodoclonidine (PIC) to I_1R, time- and dose-dependently, on PC12 cell membranes and interacted with I_1R in a reversible and competitive manner in the absence of light. Pharmacological studies showed that this blockade was prevented by the concomitant presence of rilmenidine (a well-known I_1 agonist), but not by rauwolscine (an α_2 antagonist).

6 Finally, LNP 906 clearly antagonized the decrease in forskolin-stimulated cAMP level induced by rilmenidine, but not by melatonin.

7 These results indicate that LNP 906 is the first high-affinity and selective photoaffinity ligand for I_1R and that it behaves as an I_1R antagonist.
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Abbreviations: α_2ARs, alpha$_2$-adrenoceptors; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; I_1R, I_1 receptor; I_2BS, I_2-binding site; LNP 911, (2-(2-chloro-4-iodo-phenylamino)-5-methyl-pyrroline); PBS, phosphate buffer saline; PIC, para-iodoclonidine

Introduction

There is a large body of evidence which supports the view that the hypotensive action of clonidine involves α_2-adrenoreceptors (for a review, see Bousquet & Feldman, 1999). However, the central hypotensive effect of clonidine and related compounds was shown to be mediated through nonadrenergic imidazoline receptors also (Bousquet *et al*., 1984; Ernsberger *et al*., 1990; Molderings *et al*., 1993; Piletz & Sletten, 1993). It is now widely recognized that mammalian imidazoline-binding sites can be divided into I_1 and I_2 subtypes, and recently a third class, I_3 sites, has also been proposed (Regunathan & Reis, 1996; Ernsberger, 1999; Bousquet *et al*., 2000). Cloning strategies and biochemical studies have assigned I_2-binding sites (I_2BS) to a modulatory site on monoamine oxidases A and B (Gargalidis-Moudanos & Parini, 1995; Tesson *et al*., 1995). The existence of I_3 receptors (non-I_1 non-I_2) has been suggested according to the insulin release modulatory properties of some imidazolines in β pancreatic cells (Chan *et al*., 1994; Zaitsev *et al*., 1996). The I_1 receptor (I_1R) is a plasma membrane receptor protein (Heemskerk *et al*., 1998), which has been also detected in human platelets, bovine chromaffin cells and PC12 cells (Molderings *et al*., 1993; Piletz *et al*., 1996; Greney *et al*., 2000). Transduction pathways have already been associated with this receptor in the PC12 cells: activation of a phosphocholine-specific phospholipase C (Separovic *et al*, 1996) and inhibition of an adenylate cyclase (Greney *et al*., 2000). Moreover, pharmacological studies have shown that this receptor is involved in several functions such as regulation of the cardiovascular function (Bousquet *et al*., 1984; Ernsberger *et al*., 1990), modulation of the ocular pressure (Ogidigben *et al*., 2001) and renal sodium excretion (Smyth & Penner, 1999).

So far, all the radioligands used to characterize the I_1 receptors were 'hybrid' molecules able to bind with similar affinities both to I_1 receptors and to α_2-adrenoceptors (Molderings *et al*., 1993; Piletz *et al*., 1996). In an effort to fully characterize these receptors, we developed a series of new imidazoline analogs with high selectivity and affinity for I_1 receptors. We have recently synthesized a series of pyrroline analogs with no detectable affinities for I_2BS and for α_2-adrenoceptors (Schann *et al*., 2001). Among these pyrroline compounds, 2-(2-chloro-4-iodo-phenylamino)-5-methyl-pyrroline

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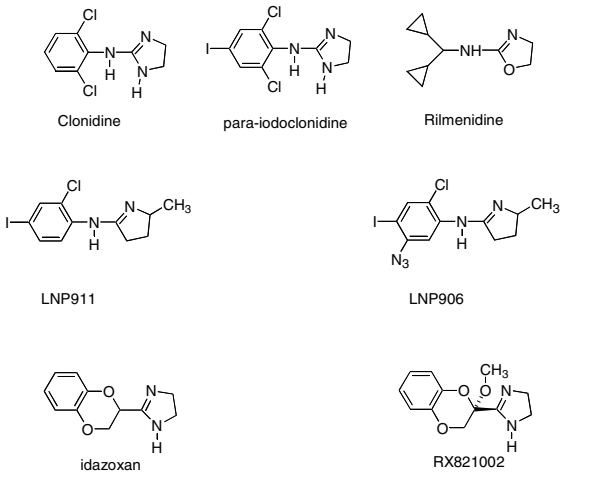
(LNP 911) was found to be highly selective for I_1R and interestingly exhibited nanomolar affinity for these receptors (Urosevic *et al*., 2002).

A ligand capable of binding irreversibly at I_1R is important to investigate the molecular characteristics of this receptor; however, no such ligand is currently available. To fill this gap, we have synthesized a derivative of LNP 911 with a photoactivable function (2-(5-azido-2-chloro-4-iodo-phenylamino)-5-methyl-pyrroline or LNP 906). In this report, we tested whether this derivative retained the binding properties of LNP 911 (i.e. high affinity and selectivity for I_1R) and demonstrated that LNP 906 binds irreversibly to this receptor. The results showed that LNP 906 binds to I_1 receptors with high affinity and high selectivity. Moreover, LNP 906 interacts with I_1 receptors in a reversible and competitive manner in the dark, but binds irreversibly to I_1 receptors after exposure to u.v. light (254 nm) of membrane preparations of rat PC12 cells. In this study, PC12 cells were used because it was previously shown in binding and hybridization studies that these cells lack α_2-adrenoreceptors and I_2-binding sites (Piletz *et al*., 1991; Bricca *et al*., 1994; Separovic *et al*., 1996; Greney *et al*., 2000). Therefore, PC12 cells represent an interesting model to study the I_1 receptors in the absence of α_2-adrenoreceptors and I_2-binding sites. The inactivation of I_1 receptors by LNP 906 was clearly dependent on the time of exposure to UV light and on the concentration of LNP 906 present during photolysis. LNP 906 was able to prevent the decrease in forskolin-stimulated cAMP levels induced by agonist on I_1R; it therefore behaved as an I_1 antagonist. Thus, LNP 906 is the first highly selective photoaffinity ligand exhibiting a nanomolar affinity for I_1R, and should prove useful for studies of the structure and function of I_1 receptors.

Table 1 Chemical structures of imidazoline and pyroline compounds



Methods

LNP 906 synthesis

LNP 906 was synthesized from LNP 911, which was previously synthesized in our laboratory (Table 1) and characterized as the first high-affinity ligand selective for the I_1 receptors (Urosevic *et al*. , 2002). LNP 906 is an analog of LNP 911 with an azido group on C5 of the aromatic ring. Briefly, LNP 906 was obtained by reaction of (2-(5-amino-2-chloro-4-iodo-phenylamino)-5-methyl-pyrroline) with $NaNO_2$ in the presence of HCl 4 N at 0°C. After agitation of 45 min at the same temperature, NaN_3 was added slowly to the intermediate at room temperature, and the compound was then extracted with CH_2Cl_2 in the presence of K_2CO_3; the azido compound was finally obtained after washing and drying of the organic phase and separation by chromatography.

Radioligand-binding assays

I_1-binding sites PC12 cell membrane preparation and binding assays with [^{125}I]-PIC on PC12 cell membranes were performed as described elsewhere (Greney *et al*., 2000). Binding assays with [^{125}I]-LNP 911 on PC12 cell membranes were all performed at 25°C and were as follows. Incubation was initiated by the addition of membranes (20–50 μg of protein) in a final volume of 250 μl of Tris–HEPES buffer (50 mM Tris–HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA and 0.5 mM $MgCl_2$) and was carried out at 25°C during 60 min (equilibrium conditions). The reaction was stopped by rapid vacuum filtration through GF/B glass fibre filters with a Brandel harvester, followed by three rapid washes of the filters with 3 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4. Radioactivity retained on the dried filters was determined in a Minaxi gamma counter (Packard, Meriden, CT, U.S.A.). Nonspecific binding was defined as [^{125}I]LNP 911 binding in the presence of 100 μM PIC, and accounted for about 10% of the total radioactivity when 0.2 nM [^{125}I]-LNP 911 was used. The choice of 100 μM PIC came from pilot experiments showing that, at this concentration, the residual binding obtained with PIC was similar to that obtained with all the other drugs tested (clonidine, par-aminoclonidine and LNP 911). Competition studies were performed using 0.2 nM [^{125}I]LNP 911 ($0.1K_D$) and 11–13 different concentrations of the unlabelled ligand under investigation, ranging from 10^{-10} to 10^{-3} M. Kinetic dissociation experiments were carried out with 0.2 nM [^{125}I] LNP 911 and was initiated by the addition of 100 μM LNP 911 after 60 min incubation of the membranes with the radioligand. Reactions were stopped at time points between 10 s and 60 min by rapid filtration and washing with ice-cold buffer.

I_2-binding sites Rabbit kidney membranes preparation and affinities of drugs were performed as described (Pigini *et al*., 1997). Nonspecific binding was determined with 10 μM cirazoline for [^{3}H]-idazoxan binding. Radioactivity retained on the filters was determined in a beta TriCarb counter (Packard).

α_2-binding assays CHO membrane preparation is as described in the following (Piletz *et al*., 1996). For [^{3}H]-RX 821002-binding assays in CHO cells, membranes (30 μg ml^{-1} for CHO-α_{2A}, CHO-α_{2B} and 100 μg ml^{-1} for CHO-α_{2c}) were incubated for 1h at room temperature in binding buffer (33 mM Tris, pH 7.5 containing 1mM EDTA)

in a final volume of 500 μl containing 0.8, 1 or 2 nM [^{3}H]-RX821002, respectively, for hα_{2A}-, hα_{2B}-, hα_{2c}-AR. At these concentrations, [^{3}H]-RX821002 was shown to label exclusively α_2ARs (Chan *et al*., 1994). Nonspecific binding was defined with 10 μM phentolamine.

Photoaffinity labelling

u.v. light characteristics of LNP 906 were determined and stability of the probes was assessed by hourly sequential u.v. measurement over a period of 4 h in the binding buffer at 25°C. The LNP 906 is stable for up to 4 h in the absence of light and shows spectral characteristics appropriate for direct irradiation photoactivation (Goeldner & Hirth, 1980; Peng *et al*., 1994) with a strong absorption at 254 nm. To investigate the photoaffinity labelling, the incubation was performed in greater volumes (minimum 2 ml tubes) at 25°C. The photoaffinity assay was performed as follows: after ~60 min incubation in the dark (which is the period required for the binding of an I_1 ligand to reach equilibrium on I_1R) under dim red light with LNP 906 20 μM, the membranes were placed on ice in an opened glass Petri dish and irradiated for 15 min at a distance of 2 cm with a Bioblock VL-6LC UV 12 W transilluminator. These photoactivable ligand concentrations and the photolysis times were found to give an optimal photolabelling yield in pilot experiments. These conditions and the photolabelling technique was adapted from that described elsewhere (Kawahara *et al*., 1985; Ernsberger & U'Prichard, 1986; Lanier *et al*., 1993). For the pharmacological studies of the irreversible binding, membranes were pre-incubated with the ligand at a concentration of 20 μM for 60 min at 25°C, in the presence or absence of the photoactivable ligand. After irradiation, the samples were then washed in 25 ml of an ice-cold buffer (5 mM Tris–HEPES, pH 7.7, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM $MgCl_2$ and NaCl 100 mM), homogenized with 10 strokes of a glass Dounce homogenizer, and centrifuged at 60,000 × *g* for 10 min at 4°C. The pellet was washed twice in 25 ml of cold Tris–HEPES buffer and centrifuged. Pellets were at least resuspended in 2 ml of the same buffer and used in receptor-binding assays.

The washing procedure was validated in pilot experiments, that is, membranes were incubated in the dark up to equilibrium with or without 20 μM LNP 906 (control) for 60 min, but were not irradiated. The samples were then washed several times by centrifugation with the binding buffer at 4°C.

cAMP experiments

cAMP experiments were conducted as previously described, but in the dark (Greney *et al*., 2000). In antagonism experiments, LNP 906 (10^{-5} M) was added to the medium 10 min before adding other drugs. The radioreceptor assay kit for measuring cAMP levels (Amersham, France) was used according to the instructions of the manufacturer.

Drugs and supplies

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies (Cergy-Pontoise, France). Rilmenidine was a gift from Laboratories Servier (Courbevoie, France). Melatonin and all other compounds were purchased from Sigma (L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France). [^{3}H]-idazoxan (45 Ci mmol^{-1}), [^{125}I]-LNP 911 (2000 Ci mmol^{-1}) and [^{3}H]-RX821002 (59 Ci mmol^{-1}) were obtained from Amersham Life Science (Orsay, France). [^{125}I]-PIC (2200 Ci mmol^{-1}) was purchased from New England Nuclear (Paris, France).

PC12 cells were kindly provided by Dr G. Rebel (IRCAD, Strasbourg, France) and CHO cells by Dr A.D. Strosberg (Paris, France).

Protein assay

Protein concentrations were determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Data analysis

Data from kinetic, saturation and competition experiments were analysed using the least-square-fitting program GraphPad Prism (GraphPad Software Inc.). IC_{50} values were calculated according to Cheng and Prusoff equation (Cheng & Prusoff, 1973). In binding experiments, the significance of the improvement of fit obtained by a two-site equation over one-site equation was analysed by F-statistics (partial F-test). The statistical significance of differences was analysed by Student's *t*-tests. Results are expressed as the mean values ± s.e.m.

For all other cases, the statistical significance of differences was analysed by a one-way factorial ANOVA with *post hoc* tests of Bonferroni (Statsview Software, Abacus Concepts), and was reached when $P<0.05$, n denoted the number of experiments.

Results

Binding characteristics of unlabelled LNP 906

The binding characteristics of LNP 906 at the I_1-, I_2- and α_2-binding sites were measured in the dark.

The binding affinities of LNP 906 were determined in PC12 cell membranes (I_1R) and kidney membranes (I_2BS) by displacement of [^{125}I]-LNP 911 and [^{125}I]-PIC specific binding and [^{3}H]-idazoxan binding, respectively.

As shown in Figure 1, [^{3}H]-idazoxan-specific binding to I_2BS, in rabbit kidney membrane preparations, was displaced by LNP 906 with a low affinity ($IC_{50}=133{,}000\pm84.14$ nM, $n=3$). In contrast with this result, LNP 906 proved able to displace both the [^{125}I]-PIC and the [^{125}I]-LNP 911 specific binding to I_1R in PC12 cell membrane preparations with two affinities ($IC_{50}=6.03\pm0.69$ nM (41% of total sites) and 2034 ± 706 nM, $n=11$ and $IC_{50}=9.96\pm1.65$ nM (31% of total sites) and 8076 ± 1105 nM, $n=4$, respectively).

The affinity of LNP 906 for α_2-adrenergic receptors was measured in human α_{2A}-, α_{2B}-, or α_{2C}-adrenoceptor-transfected CHO cell membranes. The results are summarized in Table 2. LNP 906 displaced the [^{3}H]-RX 821002-specific binding with very low affinity ($K_i>10^{-6}$ M) for the three subtypes of α_2-adrenoceptors expressed in CHO cells.

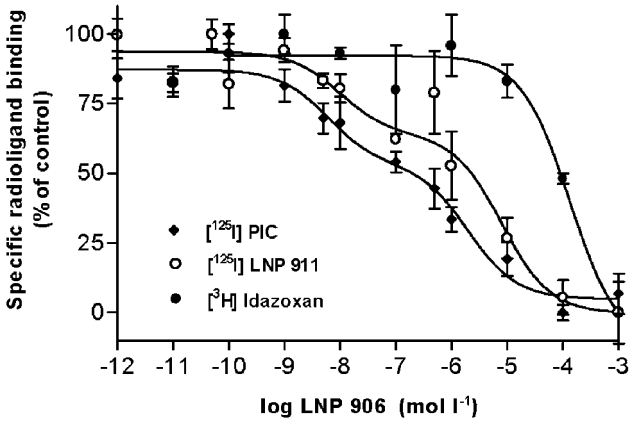


Figure 1 Test of the specific binding of LNP 906 for I_1 and I_2 receptors. Competition assays are performed as described in Methods either with 0.2 nM [^{125}I]-LNP 911 or with 5 nM [^{125}I]-PIC in PC12 membranes with increasing concentrations of LNP 906; assays on I_2 receptors were performed with 5 nM [^{3}H]-idazoxan in rabbit kidney membranes. In these studies, nonspecific bindings were determined either by 100 μM PIC for [^{125}I]-LNP 911 and [^{125}I]-PIC or by 100 μM cirazoline for [^{3}H]-idazoxan. Each point is the mean $\pm$ s.e.m. of three to 11 experiments performed in triplicate and using different membrane preparations. Curves were analysed using the iterative nonlinear least-squares curve-fitting program GraphPad.

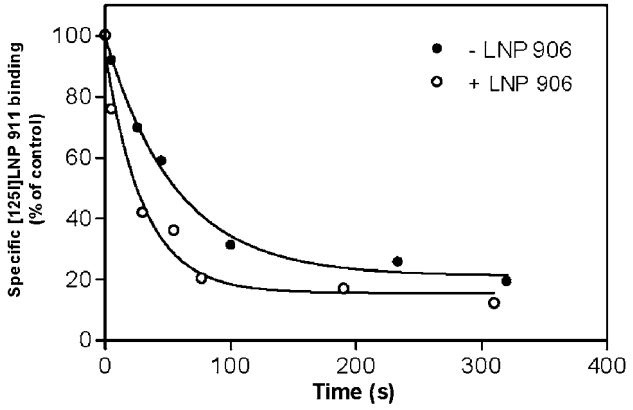


Figure 2 Influence of LNP 906 on the kinetic dissociation of [^{125}I]-LNP 911 binding to PC12 cell membranes at 25°C. Kinetic dissociation at 25°C of the radioligand was performed in the absence or presence of 50 μM LNP 906. Dissociation was started with the addition of LNP 911 (100 μM) after 60 min incubation at 25°C (as described in Methods). Nonspecific binding of [^{125}I] LNP 911 was determined by 100 μM PIC in triplicate at different times. The apparent rate constants of dissociation (k_{-1}) were 0.019 and 0.033 for the dissociation with LNP 911 or with LNP 911 in the presence of LNP 906, respectively. Binding at $t=0$ (100%) represented, respectively, 3917 and 4185 c.p.m. in the absence and presence of LNP 906. The result shown is representative of four separate experiments.

Table 2 Inhibition constants (K_i (nM)) of LNP 906 for the α_{2A}-adrenergic receptors in CHO-transfected cells

Compound	*CHO-α_{2A}*	*CHO-α_{2B}*	*CHO-α_{2C}*
LNP 906	2240 $\pm$ 445	3740 $\pm$ 545	8380 $\pm$ 30

Competition-binding studies were performed at 25°C in CHO cell membranes with [^{3}H]-RX821002 (0.8, 1 or 2 nM, respectively, for hα_{2A}-, hα_{2B}-, hα_{2c}-AR) and increasing concentrations (10^{-10}–10^{-3}) of LNP 906. Nonspecific binding was defined with 10 μM phentolamine. Data are presented as means $\pm$ s.e.m. errors of three experiments.

Allosteric modulation of [^{125}I] LNP 911 binding by LNP 906 in PC12 cells

The question of whether LNP 906 could allosterically modulate the binding of [^{125}I] LNP 911 has been addressed. To check this hypothesis, the effects of 50 μM of LNP 906 on the [^{125}I]LNP 911 dissociation kinetic at 25°C were studied (Figure 2). Compared to the k_{-1} value measured in the absence of drug ($k_{-1}=0.019\pm0.001\,min^{-1}$ ($n=5$)), LNP 906 led to a significant increase of the k_{-1} ($0.033\pm0.003\,min^{-1}$ ($n=5$) ($P<0.05$, t test)). These results indicate that 50 μM LNP 906 accelerated the dissociation rate by about 1.7.

Influence of varying exposure to u.v. light on irreversible LNP 906 binding

PC12 cell membranes were chosen as a system for future analysis of covalent LNP906-I_1 receptor complexes. Preliminary evaluation of the photoactive ligand was carried out as follows: PC12 membranes were incubated with LNP 906 20 μM for 60 min (this concentration was chosen in order to saturate nearly all sites of the I_1 receptors, based on the binding properties of LNP906) and the irreversible labelling upon u.v. irradiation was assessed by measuring [^{125}I]-PIC binding, after dissociation of non-covalently bound ligand by several washings at 4°C (see Methods).

The results showed that the UV irradiation (254 nm) by itself only slightly altered the binding sites at 5 and 15 min (96 $\pm$ 2% of the control ($P=0.77$, $n=4$) and 92 $\pm$ 3% of the control ($P=0.58$, $n=4$), respectively) and moderately at 30 and 45 min (85 $\pm$ 4% of the control ($P=0.28$, $n=4$); 75 $\pm$ 5% of the control ($P=0.073$, $n=4$, respectively), suggesting that imidazoline-binding sites themselves are, to some extent, sensitive to these conditions of irradiation (Figure 3). Membranes photolysed in the presence of the azido compound LNP 906 displayed a progressive decreased binding capacity for [^{125}I]-PIC, with increasing duration of exposure to u.v. light (30 $\pm$ 10% of the control at 45 min irradiation, $P<0.005$, $n=4$) (Figure 3). Therefore, photolysed LNP 906 appeared to bind in a covalent manner to the I_1 receptors, leading to an efficient inhibition of the specific [^{125}I]-PIC binding. In the absence of exposure to UV light, preincubation of I_1 receptors with 20 μM LNP 906, followed by washing, did not result in any loss of [^{125}I]-PIC binding, as compared with preincubation in the absence of the ligand.

Effect of the concentration of LNP 906 on the photolysed I_1 receptors

In Figure 4, the results of several experiments are shown, in which the duration of ultraviolet exposure was held constant (15 min) whereas the concentration of LNP 906 was changed. Consistent with a receptor–ligand interaction, the photolytic inactivation of I_1 receptors by LNP 906 was concentration dependent (88 $\pm$ 7% of the control ($P=0.13$, $n=5$), 40 $\pm$ 7% of the control ($P<0.01$, $n=5$) and 17 $\pm$ 6% of the control ($P<0.01$, $n=5$) at 2, 20 and 200 μM, respectively). No

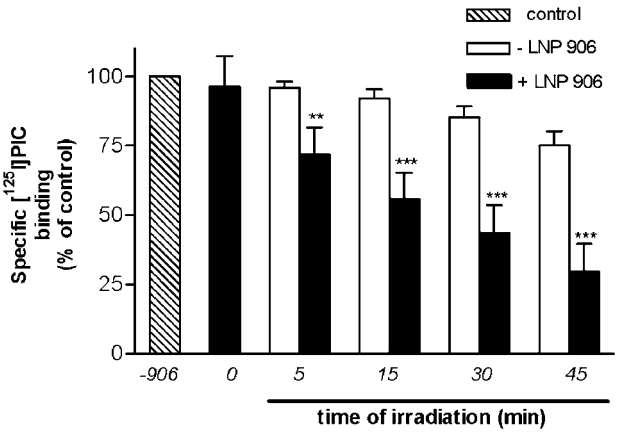


Figure 3 Effect of varying exposure to ultraviolet irradiation on irreversible binding of LNP 906 to PC12 membranes. Membranes (20–50 μg) were preincubated for 60 min in the dark in the absence or presence of 20 μM LNP 906, then irradiated at 25°C for 5–45 min, followed by washing and determination of [^{125}I]-PIC binding. Control membranes were free of LNP 906 and were not irradiated. Specific binding of [^{125}I]-PIC was determined with 100 μM PIC at different times indicated. Means ± s.e.m. of four independent experiments performed in triplicate. Significance of the differences between control experiments (dark, without LNP 906) and photolysed membranes treated with LNP 906 are given. $^{**}P<0.01$, $^{***}P<0.005$.

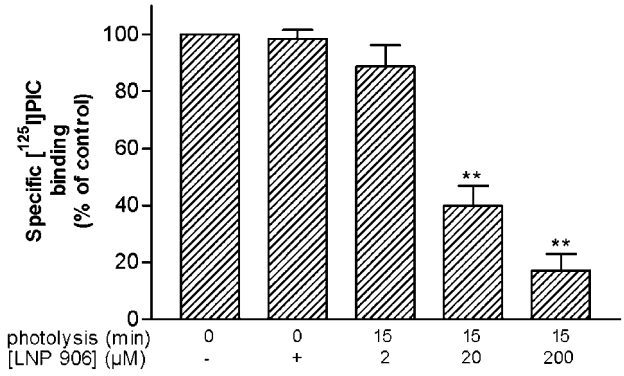


Figure 4 Concentration dependence for the photoinactivation of I_1 receptors by LNP 906 following exposure to short-wavelength ultraviolet light. Membranes (20–50 μg) were incubated as described in experimental procedures, without and with increasing concentrations of LNP 906 (2–200 μM) for a period of 60 min, and then irradiated at 25°C for 15 min. Then, the binding of [^{125}I]-PIC was assessed. Control membranes were free of LNP 906 and were not irradiated. Specific binding of [^{125}I]-PIC was defined with 100 μM PIC in triplicate. Values are means means ± s.e.m. of five independent experiments performed in triplicate. $^{**}P<0.01$.

significant change in [^{125}I]-PIC-specific binding was observed following pretreatment with 20 μM LNP 906 in the dark, indicating that the washing steps were sufficient to eliminate non-covalently bound ligand. At lower concentrations, 2–200 nM, the decrease in [^{125}I]-PIC-specific binding approximated the decrease due to direct inactivation of the receptor by UV light (data not shown). Thus, a concentration of 20 μM LNP 906, reaching a satisfactory photolabelling yield (40 ± 7% of the control, $P<0.01$, $n=5$), was used in all our photolabelling experiments.

Protection against the loss of the [^{125}I]-PIC-specific binding on photolysed I_1 receptors

The pharmacology of I_1 receptor inactivation by LNP 906 was studied, and the results are expressed in Figure 5. In these experiments, the receptors were exposed to LNP 906 alone or to combinations of LNP 906 and other ligands. Subsequently, the free and reversibly bound ligands were removed by washing, and the specific binding of [^{125}I]-PIC was assessed. If LNP 906 is capable of specific irreversible binding to I_1 receptors, one would expect a decrease in [^{125}I]PIC binding that should be prevented by co-incubation of appropriate I_1-imidazolinic selective ligands. Figure 5 shows that LNP 906 alone decreased specific [^{125}I]-PIC binding to 36 ± 11% of the control ($P<0.01$, $n=4$) as compared with the controls (preincubation without any drugs). The co-incubation with the selective I_1-receptor agonist rilmenidine affords complete protection against the loss of [^{125}I]PIC-specific binding. On the other hand, rauwolscine, an α_2-selective antagonist, was unable to prevent I_1-receptor photoinactivation by LNP 906, as a specific [^{125}I]-PIC binding of 29 ± 8% of the control ($P<0.01$, $n=4$) was recorded following photolysis. A complete dissociation was observed for control nonphotolysed azido compound or when membranes were irradiated in the presence of the two tested drugs in the absence of LNP 906, confirming the validity of our washing conditions for eliminating non-covalently bound ligand. This ability of an I_1-selective agent (but not of an α_2-selective agent) to prevent the loss of specific [^{125}I]-PIC binding is consistent with the known pharmacology of I_1-imidazoline receptors.

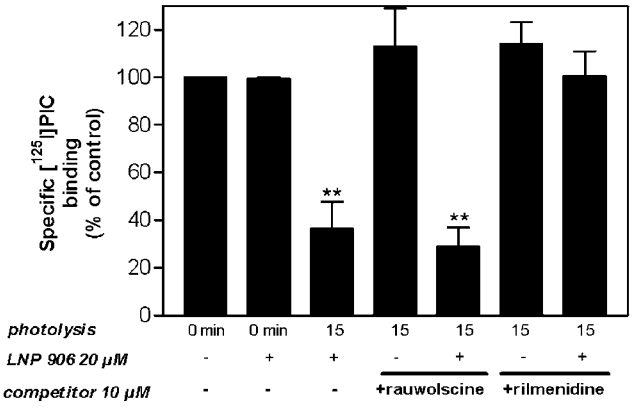


Figure 5 Specificity of the photolabelling of I_1 receptors by LNP 906. Membranes were preincubated for 60 min, with 20 μM LNP 906, in the presence or absence of competitors (rilmenidine, rauwolscine) in a final volume of 0.25 ml. The competitors and the final concentrations used are listed in the figure. Following preincubation, samples were irradiated for 15 min and the remaining I_1 receptor activity was then assayed by the specific binding of [^{125}I]-PIC. Control membranes were free of LNP 906 and were not irradiated. Controls represent identical samples of I_1 receptors, which were carried through the same preincubation and binding steps as those samples, which were preincubated with LNP 906 plus competitor. Specific binding of [^{125}I]-PIC was defined with 100 μM PIC in triplicate. Each point is the mean of four ± s.e.m. experiments performed in triplicate. $^{**}P<0.01$.

LNP 906 is a functional I_1R antagonist

We tested the effect of LNP 906 on the cAMP transduction pathway associated with the I_1 receptors in the PC12 cells (Greney *et al*., 2000). In a first series of experiments, we tested the effects of LNP 906 at 10^{-5} M alone or in combination with rilmenidine, an I_1 selective ligand (Figure 6a). LNP 906 (10^{-5} M) had no significant effect on its own on the basal (82 ± 7 and 81 ± 7 fmol min^{-1}/10^5 cells ($P=0.17$, $n=4$) in the absence and presence of LNP 906, respectively) and forskolin-stimulated cAMP level (222 ± 19 and 226 ± 21 fmol min^{-1}/10^5 cells ($P=0.5$, $n=4$) in the absence and presence of LNP 906, respectively) in the cells, as expected for an antagonist. On the other hand, rilmenidine proved able to decrease slightly, but significantly and dose-dependently, the forskolin-stimulated accumulation of cAMP in the cells (12 ± 4 ($P=0.1$, $n=4$), 15 ± 1 ($P<0.05$, $n=4$), 21 ± 2 ($P=0.01$, $n=4$) and $38\pm9\%$ ($P<0.01$, $n=4$) of inhibition only for the concentration range of 10^{-7}–10^{-4} M of rilmenidine, respectively, in the absence of LNP 906), acting thus as agonist on this pathway (Figure 6a) (as previously shown, Greney *et al*., 2000). In all, 100% of the control represents the value in the presence of forskolin, which was only 222 ± 19 fmol min^{-1}/10^5 cells. However, when LNP 906 (10^{-5} M) was added 10 min before rilmenidine, it prevented the decrease in forskolin-stimulated cAMP level obtained with this agonist: 1 ± 0.4 ($P=0.064$, $n=4$), 2 ± 0.5 ($P<0.05$, $n=4$), 8 ± 2 ($P<0.05$, $n=4$) and $12\pm3\%$ ($P<0.01$, $n=4$) of inhibition in the presence of LNP 906.

In order to demonstrate the specificity of action of LNP 906, we tested whether LNP 906 has antagonist properties towards melatonin, a neuromodulator proved able to suppress the PC12 cell growth and death by inhibiting the cAMP formation at MT_1-specific receptors on cell surface (Roth *et al*., 1997; 2001). As expected, melatonin significantly decreased the cAMP levels up to $12\pm4\%$ of inhibition at 10^{-6} M ($P<0.01$, $n=4$) and $32\pm4\%$ of inhibition at 10^{-5} M ($P<0.01$, $n=4$). This effect of melatonin was not significantly antagonized by LNP 906 (12 ± 4 of inhibition at 10^{-6} M of melatonin ($P=0.95$, $n=4$) and 34 ± 6 of inhibition at 10^{-5} M of melatonin ($P=0.69$, $n=4$) in the presence of LNP 906) (Figure 6b).

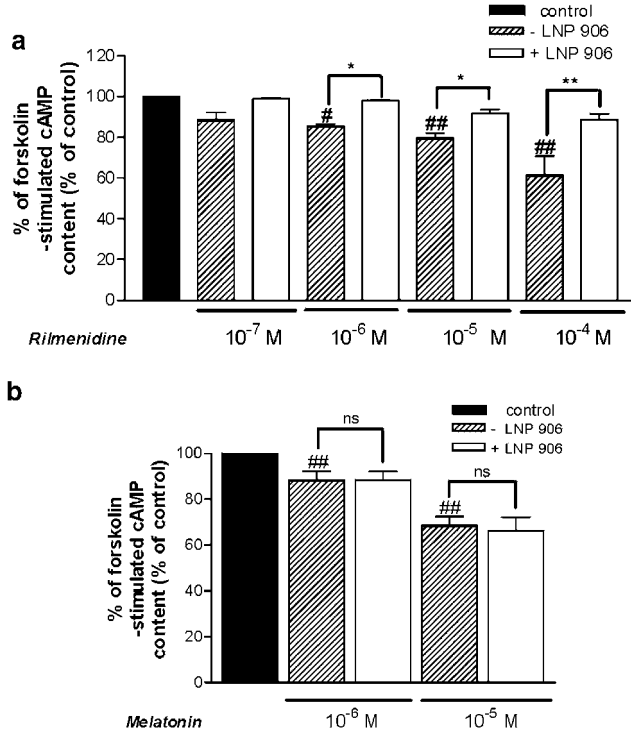


Figure 6 Antagonist activity of LNP 906 on the forskolin-stimulated cAMP level response of I_1R agonists in PC12 cells. Cells (10^5) were first preincubated in the presence of LNP 906 (10^{-5} M) for 10 min at 37°C, then increasing concentrations of rilmenidine (a) or melatonin (b) were added with 1 μM forskolin for a period of 30 min at the same temperature in the dark. cAMP level was measured as described in Methods. Results represent the average of at least four experiments performed in triplicate, and are expressed as the percentage of inhibition $\pm$ s.e.m. of the control (100% represents the value in the presence of forskolin only, which was 222 ± 19 fmol min^{-1}/10^5 cells, full column). * or $^{\#}P<0.05$, ** or $^{\#\#}P<0.01$ (* represents the significance of the differences between cells treated with or free of LNP 906; # represents the significance between cells treated with rilmenidine or melatonin and controls (free of rilmenidine)).

Discussion and conclusions

So far, the most common radiolabelled radioligands used in the field of imidazoline receptors are [^{3}H]-clonidine and [^{125}I]-PIC (Table 1) (Piletz *et al*., 1991; Molderings *et al*., 1993; Bricca *et al*., 1994); unfortunately, they bind to both α_2 and I_1 receptors. Therefore, highly selective ligands were needed for these receptors.

One of them was synthesized and characterized in our laboratory: LNP 509 exhibited a high selectivity for the I_1R as compared to α_2-ARs and decreased blood pressure (Schann *et al*., 2001). Nevertheless, its affinity for I_1 receptors was not high enough to use it as a tool relevant for molecular studies of the I_1 receptors. Later on, in a series of pyrroline compounds, the LNP 911 has been selected as a high-affinity I_1 receptor ligand (Table 1). The radioiodinated derivative of LNP 911 was shown to be the first radioligand highly selective for I_1 receptors (Greney *et al*., 2002).

Therefore, LNP 911 appeared as a prototypic pharmacological tool. Nevertheless, in the range of the tools necessary for studying the I_1 receptors, a photoaffinity ligand was still missing. In order to obtain such a photoaffinity ligand, we prepared the azido derivative of LNP 911, LNP 906. We report here that LNP 906 exhibited high affinity for I_1-binding sites of PC12 membrane preparations. Photoaffinity labelling has been useful in the identification of α_1-adrenoreceptors (Hess *et al*., 1983; Leeb-Lundberg *et al*., 1984), α_2-adrenoreceptors (Regan *et al*., 1985), β-adrenoreceptors (Lavin *et al*., 1981; Rashidbaigi & Ruoho, 1981) or I_2 receptors (Lanier *et al*., 1993; Dontenwill *et al*., 1997; Coates *et al*., 2000). Irreversible ligands allow complete knockout of one particular site. Moreover, irreversible ligands act as antagonists at these receptors.

The present study demonstrates that the novel compound LNP 906 is the first potent photoaffinity probe selective for the I_1 receptors, which interacts with I_1 receptors in a reversible and competitive manner in the absence of light, but interacts with the I_1 receptor in an irreversible manner when exposed to ultraviolet light. The inactivation of I_1 receptors by LNP 906 was clearly dependent on exposure to u.v. light and on the concentration of LNP 906 present during photolysis.

The selectivity of LNP 906 for I_1R *versus* I_2BS and α_2-adrenoceptors was checked by different ways in the absence of

light: (1) in rabbit kidney membranes, where I_2BS are largely expressed (Coupry *et al*., 1990), unlabelled LNP 906 proved unable to displace [^{3}H]-idazoxan with a high affinity; (2) in $\alpha_{2A,B,C}$-adrenoceptor transfected CHO cell membranes, unlabelled LNP 906 displaced [^{3}H]-RX 821002 with $IC_{50} > 10^{-6}$ M (data not shown); (3) unlabelled LNP 906 proved able to displace the specific binding of [^{125}I]-PIC and [^{125}I]-LNP 911 to I_1R on PC12 cell membranes with a nanomolar affinity; (4) the selectivity of the irreversible interactions is demonstrated, since preincubation with rauwolscine, an α_2 antagonist, had no influence on the effect of LNP 906, whereas it was totally prevented when an I_1 agonist such as rilmenidine was added in the preincubation medium; (5) LNP 906 antagonized the decrease cAMP levels induced by rilmenidine, an I_1 agonist, but not that induced by melatonin. These results confirm the selectivity of LNP 906 for I_1R over α_2-adrenergic receptors and I_2BS and also over foreign receptors expressed in PC12 cells, such as melatonin receptors, having the same signal transduction pathways.

LNP 906 displaced both the agonist [^{125}I] PIC and the antagonist [^{125}I] LNP 911 in a biphasic manner. LNP 911 was previously shown to bind to allosteric modulatory sites (Greney *et al*., 2002). It was important to establish that LNP 906 and LNP 911 are able to bind to the same allosteric modulatory sites of the I_1R. To check this hypothesis, the effect of 50 μM LNP 906 on the kinetic dissociation of the [^{125}I] LNP 911 binding was studied. Unlabelled LNP 911 dissociated the binding of [^{125}I] LNP 911 as previously described (Greney *et al*., 2002). This rapid dissociation rate observed for [^{125}I]LNP 911-binding sites is in agreement with previous kinetic data concerning I_1 receptors (Piletz & Sletten, 1993). Like moxonidine, LNP 906 significantly increased the dissociation parameter (k_{-1}) of [^{125}I]LNP 911 (Greney *et al*., 2000). Therefore, LNP 906 and moxonidine have the same modulatory effect on the [^{125}I] LNP 911 binding on I_1R, suggesting that both drugs bind to the same site at the I_1R.

In fact, the pharmacological mechanism of action of unphotolysed LNP 906 was also explored in functional experiments in which rilmenidine and melatonin exhibited agonist properties by decreasing the cAMP level in the cell (Greney *et al*., 2000; Roth *et al*., 2001). It is interesting to note that rilmenidine decreased cAMP level (present data), whereas clonidine was inactive in the same assay (Greney *et al*., 2000). As far as several transduction pathways are associated with a unique receptor, it is not uncommon that a particular drug acts as an agonist on one pathway but as an antagonist on another one *et vice versa* of course (for discussion on this point, see Greney *et al*. (2000) and Gerhardt *et al*. (1999)). When LNP 906 (10^{-5} M) was added 10 min before these compounds (10^{-5} M), it prevented the decrease in forskolin-stimulated cAMP level caused by rilmenidine for the concentration range of 10^{-7}–10^{-4} M. In these cells, rather high concentrations of rilmenidine are needed to significantly reduce the cAMP level; this is preferably due to the fact that the maximal inhibitory effect of the imidazoline compounds on the cAMP pathway is quite weak (Greney *et al*., 2000).

However, LNP 906 did not influence the decrease in forskolin-stimulated cAMP level induced by melatonin, the natural agonist for melatonin MT_1 receptors. Moreover, LNP 906 was inactive by itself on the adenylate cyclase transduction pathway, suggesting that unphotolysed LNP 906 is a selective antagonist on the cAMP-dependent cell surface I_1 receptors.

We have previously shown that the low-affinity sites labelled by rilmenidine belong to the I_1R. These low-affinity sites were shown to correspond to the G-protein uncoupled state of the receptor (Greney *et al*., 2000, 2002). In the present work, we show that LNP 906 prevented the effects of rilmenidine on the cAMP pathway and that rilmenidine conversely prevented the binding interaction between LNP 906 and [^{125}I] PIC. Therefore, as far as I_1R of PC12 cells are defined as receptors on which rilmenidine, moxonidine and other imidazoline compounds are able to bind, one can assume that the low-affinity sites of rilmenidine and LNP 906 are or, at least, belong to the I_1 receptor.

When irradiated with u.v. light, LNP 906 is highly photosensitive. It completely decomposes over 5 min irradiation as determined on thin layer chromatography, forming spots distinct the nonphotolysed form (data not shown). Therefore, LNP 906 has the features of a relevant photoaffinity probe (Regan *et al*., 1985; Kotzyba-Hibert *et al*., 1996). The maximum I_1 receptor blockade obtained by photolysis in the presence of LNP 906 by photolysis reached 70%. This feature compares favourably with the 60–80% decreases that were reported in similar studies using nonradiolabelled photoaffinity ligands of β-adrenoreceptors (Lavin *et al*., 1981; Burgermeister *et al*., 1982). Extensive washing of membranes preincubated with 20 μM of LNP 906 did not change the specific binding of [^{125}I]-PIC. This result indicates that the binding of LNP 906 is reversible in the absence of light. The results obtained for control nonphotolysed cells are also indicative of a good dissociation of the ligand under the experimental conditions used, so that the decrease in [^{125}I]-PIC binding was not due to incomplete removal of the ligands.

The irreversibility of this interaction in the presence of u.v. light is indicated by the decreased [^{125}I]-PIC specific binding in treated membranes. This decrease could not be reversed by extensive washing. It is unlikely that the observed decrease in binding could be due to the persistence of LNP 906 or photochemical reaction products in PC12 membranes, since, following preincubation with an equal concentration of [^{125}I]-PIC, in the absence of photolysis, 99.8% of the original specific binding was recovered by the washing procedure. Nevertheless, measurement of the actual extent of the covalent incorporation would require a radiolabelled derivative of LNP 906. The inactivation of I_1 receptors by LNP 906 was clearly dependent on exposure to UV light with a maximal effect at 45 min of irradiation. A 15 min duration of irradiation was then used in all our photolabelling assays because, at this time, a 50% blockade of I_1R was observed and because the membranes were only slightly sensitive to these conditions of ultra-violet light exposure. A concentration dependence of the photoinactivation of I_1 receptors by LNP 906 following exposure to short wavelength ultraviolet light was also observed. So far we cannot explain the discrepancy observed between the nanomolar IC_{50} obtained when LNP 906 was used as a competitor in the absence of light and the micromolar concentration of LNP 906, which was required to irreversibly block the binding at I_1Rs after irradiation. A possibility is that the photolysis may alter to some extent the interaction between the ligand and the receptor protein. Such a photolysis-induced phenomenon has already been reported with various phoaffinity probes (Ernsberger & U'Prichard, 1986; Dontenwill *et al*., 1997). Therefore, these data demonstrate the specificity of I_1 receptor labelling

by LNP 906, the requirement of ultraviolet light, and a time and dose dependence for receptor inactivation, which parallels the photolytic decomposition of the azide.

In conclusion, LNP 906 is the first highly selective photoaffinity ligand exhibiting a nanomolar affinity for I_1R. Our results indicate that, upon photolysis, LNP 906 irreversibly labelled the I_1-binding sites with efficacy and specificity. The blocking effect of photolysed LNP 906 on the [^{125}I]-PIC-specific binding was time and concentration dependent. LNP 906 blocked the decrease of the forskolin-stimulated cAMP level induced by rilmenidine but not by melatonin, suggesting that it acted as a selective I_1 antagonist. Taken together, these results are consistent with LNP 906 being of a photoaffinity probe. It will be useful to identify and investigate in detail the structure and functions of I_1 receptors even in tissues or cells expressing different imidazoline-binding proteins (α_2-adrenoceptors or I_2BS). A radiolabelled form of LNP 906 may prove useful in isolating the I_1 receptor.

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