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Polyclonal anti-idiotypic antibodies to idazoxan and their interaction with human brain imidazoline binding sites

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Abstract

Polyclonal antibodies were raised in rabbits against purified polyclonal anti-idazoxan antibodies. The anti-idiotypic antibodies thus obtained, proved able to inhibit [^{3}H]idazoxan specific binding to anti-idazoxan antibodies. Applied to human nucleus reticularis lateralis membrane preparations, these antibodies (20 μg) inhibited about 50 and 70% of the imidazoline specific binding of [^{3}H]idazoxan and [^{3}H]clonidine, respectively. Furthermore, they specifically immunoprecipitated 50% of [^{3}H]idazoxan binding activity of imidazoline binding sites solubilized from the same tissue. [^{3}H]Rauwolscine binding to α_2-adrenoceptors in rat cortex was not significantly affected by these antibodies. The antibodies labeled a 43 kDa protein in Western blots of partially purified imidazoline binding sites from human brain. In conclusion, these anti-idiotypic antibodies recognize imidazoline binding sites from human brain and allow the detection of a 43 kDa binding protein associated with or representing the imidazoline receptor expressed in human brain.

Keywords: Idazoxan; Imidazoline receptor; Anti-idiotypic antibody; Brain, human

1. Introduction

Idazoxan, a benzodioxan bearing an imidazoline ring, was shown to antagonize the hypotensive effect of clonidine and related compounds involving imidazoline receptors located in the brainstem (Bousquet et al., 1984; Tibirica et al., 1991). Therefore, [^{3}H]idazoxan was used to label specific imidazoline binding sites in various human brain regions such as the cortex, the nucleus reticularis lateralis/rostral ventrolateral medulla region and the striatum (Bricca et al., 1993; De Vos et al., 1994). Despite extensive pharmacological characterization, such binding sites have not yet been purified from human brain. Purification of [^{3}H]idazoxan binding proteins from peripheral tissues have been reported, i.e. rabbit kidney (Limon et al., 1992) and bovine adrenal glands (Wang et al., 1992). Heterogeneity of [^{3}H]idazoxan binding sites between tissues and species has already been described according to their sensitivity to amiloride. [^{3}H]Idazoxan binding to rabbit kidney and to chromaffin cells membranes was shown to be sensitive to amiloride whereas in human brain it was not (Limon et al., 1992; Wang et al., 1992; Greney et al., 1994a).

In our laboratory, the purification of the human cerebral imidazoline receptors was undertaken using affinity chromatography procedures. Specific antibodies could be valuable tools in the purification procedure of these receptors and to gain further insight into their biochemical characterization. Because a homogeneous imidazoline receptor preparation from human brain was not avalaible as an antigen, an alternative approach was put forward to prepare specific antibodies. This approach is derived from the idiotypic network theory of Jerne (1974); it consists in generating anti-idiotypic antibodies directed at the combining site of antibodies raised against a ligand of the receptor. Among these anti-idiotypic antibodies, antibodies able to react with the binding site of the receptor can be raised. This approach has been successfully used in the preparation of polyclonal or monoclonal antibodies to several receptors including β-adrenoceptors (Guillet et al., 1985), dopamine receptors (Elazar et al., 1988), nicotinic receptors (Bjercke and Langone, 1989), thromboxane A_2 receptors (Wai and Tai, 1993), PAF receptors (Wang and Tai, 1990), opioid receptors (Cupo et al., 1992).

First, we developed, in the rabbit, polyclonal anti-idazoxan antibodies which, like imidazoline receptors, recognized imidazolines and not catecholamines (Bennai et

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al., 1993). We took advantage of this characteristic, common to anti-ligand antibodies and imidazoline receptors, to develop anti-idiotypic antibodies able to specifically cross react with imidazoline receptors. Thus, we injected purified anti-idazoxan antibodies into rabbits with the aim to generate anti-idiotypic antibodies recognizing the imidazoline receptors.

Here we describe the procedure of production and characterization of anti-idiotypic antibodies able to inhibit the binding of [^{3}H]idazoxan to both anti-idazoxan antibodies and human brain imidazoline receptors. With such antibodies, the receptor binding activity was immunoprecipitated and protein bands were labeled on immunoblots. Using this procedure, a 43 kDa protein which is a good candidate to be an imidazoline specific binding protein of the human brain was revealed.

2. Materials and methods

2.1. Materials

[^{3}H]Idazoxan (43 Ci/mmol) was from Amersham, [^{3}H]clonidine (65.5 Ci/mmol) and [^{3}H]rauwolscine (85 Ci/mmol) were from New England Nuclear. Idazoxan was from Research Biochemicals Incorporated. Aminolink columns were from Pierce and PD10 columns from Pharmacia. Protein A-Trisacryl, 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium and alkaline phosphatase linked anti-rabbit immunoglobulins antibodies were from Sigma. Molecular weight standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Biorad. Cirazoline was a gift from Synthelabo (France). Human brains were obtained during autopsies, 12–24 h after death from patients devoided of neurological diseases (Bricca et al., 1993; De Vos et al., 1994).

2.2. Immunization

Polyclonal anti-idazoxan antibodies were prepared and purified as previously described (Bennai et al., 1993). The purified antibodies kept the specificity spectrum determined in the gamma-globulins fraction (Bennai et al., 1993). These purified antibodies (100 μg) were injected intradermally at multiple sites in rabbits. The immunization efficiency was periodically controlled by testing the development of anti-idazoxan anti-idiotypic antibodies by radioimmunoassay (RIA) in the rabbit's sera.

2.3. Radioimmunoassay

The presence of anti-idiotypic antibodies was first screened in the whole serum of immunized rabbits by a RIA. The protocol of this RIA used to detect and to determine the specificity of anti-idazoxan antibodies was already described elsewhere (Bennai et al., 1993). Rabbit anti-idazoxan antibodies (100 μl, dilution 1/1000, 1.5–2.5 μg protein) were incubated in 50 mM Tris-HCl buffer pH 7.2 with [^{3}H]idazoxan (20 μl, 1 nM) and various dilutions of anti-idiotypic antibodies or normal rabbit serum (100 μl). After 2 h of incubation at room temperature, the precipitation of immunoglobulins was initiated by the addition of 50% (v/v) of saturated ammonium sulfate in the presence of bovine γ-globulins (300 μl – 5 mg/ml). Bound [^{3}H]idazoxan was separated from the free ligand by centrifugation in Eppendorf tubes (12 000 $\times$ g – 15 min). Pellets were counted directly in a Packard Tri-carb scintillation counter after removal of the supernatant and dissolving it in 50 μl of 50 mM Tris-HCl buffer. Non-specific binding of [^{3}H]idazoxan to anti-idazoxan antibodies was determined with 10 μM cold idazoxan and never exceeded 10% of the total binding.

2.4. Immunoaffinity columns preparation

Purified anti-idazoxan antibodies (200 μg) were covalently bound to Aminolink column (1 ml of gel) according to the manufacturer's instructions (Pierce).

2.5. Purification of anti-idiotypic antibodies

Immunoglobulins were precipitated from whole serum of immunized rabbits or preimmune rabbits with 50% (v/v) saturated ammonium sulfate. After extensive dialysis against distilled water, immunoglobulins fractions were applied on an Aminolink-anti-idazoxan antibodies column. After washing with 0.01 M phosphate buffer, pH 7.2, until all unbound proteins were removed, anti-idiotypic antibodies were eluted with 10 ml of 0.1 M sodium acetate buffer, pH 2.5. and the fractions immediately neutralized with 1.2 ml Tris HCl buffer, pH 9.6. The fractions were then lyophilised, dissolved in 2.5 ml distilled water and buffer exchanged achieved by a passage through a PD10 column equilibrated in 50 mM Tris-HCl buffer, pH 7.2. Alternatively, fractions were extensively dialyzed against 3 liters of distilled water (with 3 changes), lyophilised and dissolved in 50 mM Tris-HCl buffer, pH 7.2. The two methods led to similar results.

2.6. SDS-PAGE and immunostaining

SDS-PAGE was performed according to the method of Laemmli (1970) using 10% polyacrylamide minigels, under reducing (with β-mercaptoethanol) conditions. The gels were either stained with $AgNO_3$ (Biorad silver stain plus kit) or processed for blotting on nitrocellulose membranes. Electrotransfer of proteins on nitrocellulose membranes was achieved at 40 mA for 12 h. Unreacted sites were blocked with phosphate buffered saline (PBS, 10 mM Na phosphate, 120 mM NaCl, pH 7.2) containing 5% non-fat dry milk. The membranes were then processed for immunostaining. The blots were probed with anti-idiotypic

antibodies or non-immune immunoglobulins (0.5–2 μg/ml) followed by a goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (1/1000). Enzyme activity was revealed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) as substrate.

2.7. Receptor binding studies

Human brain (nucleus reticularis lateralis) and rat cerebral cortex membranes were prepared as previously described (Bricca et al., 1993; Cheung et al., 1982). Membranes (100 μl – 0.1 to 0.2 mg proteins) were incubated overnight at 4°C with purified anti-idiotypic antibodies (300 μl) or preimmune serum at different dilutions. After the addition of labeled ligands and incubation at 25°C for 45 min, membranes were filtered through Whatman GF/B filters under vacuum followed by washing of the filters 3 times with 4 ml of ice cold 50 mM Tris-HCl buffer, pH 7.5. After drying, 10 ml Packard scintillator were added and the radioactivity counted in a Packard Tri-Carb scintillation counter with 58% efficiency. [^{3}H]Idazoxan (30 nM) and [^{3}H]clonidine (30 nM) imidazoline specific binding were determined in presence of 10 μM (−)-norepinephrine in order to mask α_2-adrenoceptors. Non-specific binding of the two radioligands on human nucleus reticularis lateralis membranes was determined with 10 μM cirazoline. Non-specific binding of [^{3}H]rauwolscine (2 nM) in rat cortex membranes was determined with 10 μM phentolamine.

2.8. ELISA tests

Microtiter wells (96 wells plates-Nunc) were coated overnight at 37°C with 10 μg/well of either human nucleus reticularis lateralis or rat cerebellum membranes in 100 μl of 50 mM Tris-HCl buffer, pH 7.4. Wells were rinsed and then incubated for 1 h at 37°C with 100 μl of PBS containing 0.1% Tween 20 and 5% (w/v) non-fat dry milk to prevent non-specific adsorption of antibodies. Wells were rinsed with PBS-Tween (200 μl) and incubated overnight at 4°C with 100 μl of anti-idiotypic antibodies or preimmune immunoglobulins. After rinsing, bound antibodies were detected using a secondary goat anti-rabbit immunoglobulin G antibody conjugated to peroxidase (1/1000 – 100 μl). Peroxidase assay was initiated by the addition of 0.05% (w/v) *o*-phenylenediamine, 0.03% H_2O_2 in 0.1 M sodium citrate, pH 5.5. The reaction was stopped by the addition of 50 μl 2 N H_2SO_4 and absorbance read at 492 nm in a multiscan Metertech photometer.

2.9. Immunoprecipitation

Imidazoline receptors from human nucleus reticularis lateralis were solubilized with 13 mM CHAPS as described elsewhere (Greney et al., 1994b). Briefly nucleus reticularis lateralis membranes (2–3 mg protein/ml) were stirred for 45 min on ice in 50 mM Tris-HCl, pH 7.5, 13 mM CHAPS in the presence of protease inhibitors. After centrifugation at 120 000 × *g* for 60 min, supernatant was collected and solubilization procedure repeated once. Protein A-Trysacryl (30 μl) washed trice in 50 mM Tris-HCl buffer pH 7.2 containing protease inhibitors, was incubated for 4 h at 4°C with 20 μg purified anti-idiotypic antibodies or non-immune rabbit immunoglobulins. Saturation of the remaining Protein A active sites was then achieved with an excess of normal rabbit immunoglobulins. After centrifugation, 30 fmol (15–30 μg protein) solubilized imidazoline receptors were added on Protein A pellets and the incubation performed during 12 h at 4°C with gentle agitation. Trisacryl beads were pelleted by centrifugation (3000 × *g* – 10 min). Imidazoline specific [^{3}H]idazoxan binding was determined in 200 μl supernatant as previously described.

2.10. Preparation of imidazoline binding site enriched fractions.

Fractions enriched in imidazoline binding sites were prepared either by gel filtration chromatography on a sephacryl S200 column as described previously (Greney et al., 1994b) or by affinity chromatography on a Tris-NH_2 blocked epoxy-activated sepharose CL6B column as shown elsewhere (Greney et al., 1994a,b). Briefly, gel filtration chromatography was performed in 50 mM Tris-HCl buffer, pH 7.5 supplemented with 1 mM phenylmethylsulfonylfluoride, 0.01% CHAPS, 0.02% NaN_3. Proteins eluting in a retarded fraction corresponding to an apparent molecular weight of 35 000 Da including [^{3}H]idazoxan and [^{3}H]clonidine imidazoline specific binding sites (Greney et al., 1994a,b) were then subjected to SDS-PAGE and immunostained. Solubilized imidazoline binding sites from human nucleus reticularis lateralis membranes (2–5 pmol) were loaded on a Tris-NH_2 blocked epoxy-activated Sepharose CL6B column, and after extensive washing, the elution performed using 1 M KCl (Greney et al., 1994a). These eluted proteins, containing [^{3}H]idazoxan and [^{3}H]clonidine imidazoline specific binding sites, were subjected to SDS-PAGE and immunostained.

3. Results

The presence of anti-idiotypic antibodies in the sera of immunized rabbits was tested with an RIA as described in Materials and methods. 100 μl of whole immune serum were able to inhibit about 50% of specific [^{3}H]idazoxan binding to polyclonal anti-idazoxan antibodies (Ab_1) whereas the same volume of preimmune serum did not affect significantly this binding. Thus anti-idiotypic activity was detected in immunized rabbits. These antibodies were then purified by immunoaffinity chromatography

through an anti-idazoxan antibodies-Aminolink column. Preimmune serum was treated in the same way and the eluates tested in parallel in all the tests as controls. The purified proteins were analyzed by SDS-PAGE and bands characteristic of immunoglobulins (50 and 25 kDa) were observed. The effects of purified anti-idiotypic antibodies on [^{3}H]idazoxan binding on Ab_1 are shown in Fig. 1. As with whole serum, about 20 μg of immunopurified antibodies were able to inhibit 50% of the [^{3}H]idazoxan binding to Ab_1. These results suggest that these purified anti-idiotypic antibodies to idazoxan (at least part of them) exhibit internal image properties classifying them as Ab_{2B} or antigen inhibitable antibodies and that they were directed towards or close to the combining site of the idiotypic antibodies. In control experiments, we checked that non-immune immunoglobulin G-Aminolink affinity chromatography column did not retain any protein material capable of [^{3}H]idazoxan binding inhibition.

Should these antibodies be the internal image of idazoxan, they should then be able to interact with [^{3}H]imidazoline specific binding sites. To determine whether they could interact specifically with proteins from the human nucleus reticularis lateralis region, ELISA tests were first performed. As depicted in Fig. 2, a specific binding of antibodies to human nucleus reticularis lateralis membranes immobilised on ELISA plates was observed whereas preimmune serum gave values near the background. In the same assay, the binding of anti-idiotypic antibodies to rat cerebellar membranes was close to that observed with preimmune serum (Fig. 2). In ELISA tests, cirazoline, idazoxan and clonidine added before the antibodies, proved unable to inhibit the interaction between antibodies and nucleus reticularis lateralis membranes up to 1 mM. In fact, in some cases we observed an increase in the optical density in the presence of 1 mM cirazoline rather a decrease (data not shown).

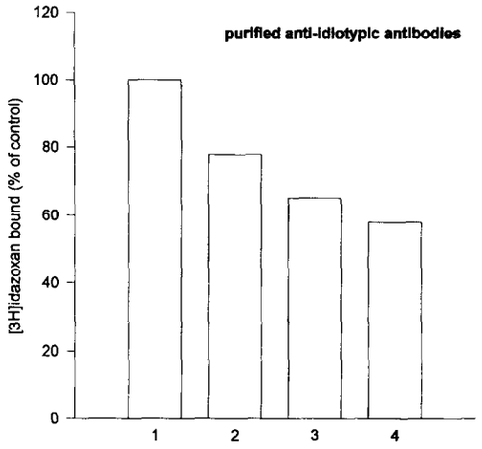

Fig. 1. Effect of affinity-purified anti-idiotypic antibodies on [^{3}H]idazoxan binding to polyclonal anti-idazoxan antibodies. Pools of purified anti-idiotypic antibodies have been systematically tested for inhibition of [^{3}H]idazoxan binding (1 nM) to anti-idazoxan antibodies (dilution 1/1000). A representative experiment with one pool is shown; each point was determined in triplicate. Other pools gave similar qualitative results. (1) Normal rabbit immunoglobulins (20 μg). (2–4) Purified anti-idiotypic antibodies: 4 μg, 8 μg and 16 μg.

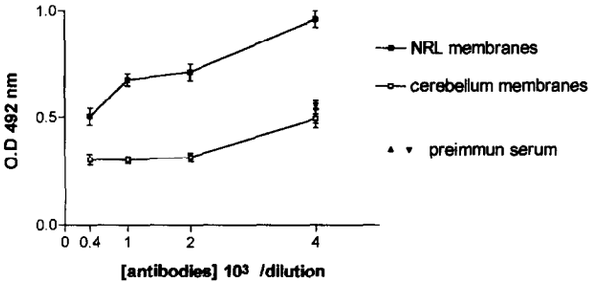

Fig. 2. Binding of anti-idiotypic antibodies to membrane preparations of human nucleus reticularis lateralis or rat cerebellum in ELISA. Microtiter plates were coated with human nucleus reticularis lateralis membranes or rat cerebellum membranes (10 μg/well) overnight at 37°C. Unoccupied sites were blocked with non-fat dry milk. Experiments were performed in triplicate with either increasing amounts of anti-idiotypic antibodies or non-immune immunoglobulins. Representation of means ± S.D. of 3 determinations is shown. A background value of 0.268 ± 0.02 O.D. ($n = 6$) and of 0.206 ± 0.02 O.D. was substracted from nucleus reticularis lateralis and cerebellum membranes values respectively, corresponding to incubation with anti-rabbit immunoglobulin G-antibodies coupled to peroxidase alone.

Thus, in order to determine if some purified anti-idiotypic antibodies were able to bind to ligand binding sites of human nucleus reticularis lateralis imidazoline receptors, inhibition experiments of [^{3}H]idazoxan specific binding to imidazoline receptors were performed. As shown in Fig. 3, a dose dependent inhibition was observed; 50% inhibition was achieved with about 15 μg antibodies.

Since we also used [^{3}H]clonidine to label imidazoline receptors of human nucleus reticularis lateralis membranes, we investigated the effect of anti-idiotypic antibodies on [^{3}H]clonidine imidazoline specific binding. Fig. 4 shows that 20 μg of purified antibodies led to 72% inhibition of this [^{3}H]clonidine specific binding whereas non-immune immunoglobulins failed to inhibit the binding up to 10%. Thus, imidazoline binding sites labeled either with [^{3}H]clonidine or [^{3}H]idazoxan (with the specific bindings determined in the presence of an excess of (−)-norepinephrine to mask α_2-adrenoceptors) in the human nucleus reticularis lateralis membranes, were both sensitive to these anti-idiotypic antibodies.

Clonidine like idazoxan were first described as α_2-adrenoceptor ligands. Therefore anti-idiotypic antibodies raised against anti-idazoxan antibodies might recognize these adrenoceptors. But this was not the case since our antibodies were unable to specifically inhibit the binding of [^{3}H]rauwolscine to adrenoceptors in rat cortex: 14 ± 5% inhibition and 10 ± 9% inhibition with 20 μg purified anti-idiotypic antibodies and non-immune immunoglobulins respectively.

As the anti-idiotypic antibodies proved able to inhibit [^{3}H]idazoxan binding to imidazoline receptors in human nucleus reticularis lateralis membranes, it should be possi-

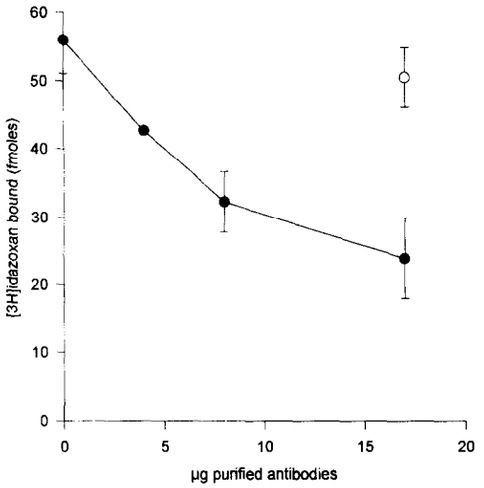

Fig. 3. Inhibition of the binding of [^{3}H]idazoxan to human brain nucleus reticularis lateralis membranes with purified anti-idiotypic antibodies. [^{3}H]Idazoxan imidazoline specific binding (30 nM) was performed in human nucleus reticularis lateralis membrane preparations (100 µg). Data are means ± S.D. of 3 experiments in triplicate with 3 different pools of affinity purified anti-idiotypic antibodies (filled circles) or preimmune immunoglobulins (open circles). Preimmune serum was passed over an affinity column and the eluates tested as control. Total specific binding of [^{3}H]idazoxan was determined in the presence of 10 µM (−)-norepinephrine to mask α_2-adrenoceptors and non-specific binding was defined with 10 µM cirazoline.

ble to immunoextract the imidazoline binding proteins with these antibodies and to lower the binding activity. Table 1 shows the results of the immunoprecipitation experiments. Preimmune immunoglobulins failed to immunoextract any [^{3}H]idazoxan binding activity whereas anti-

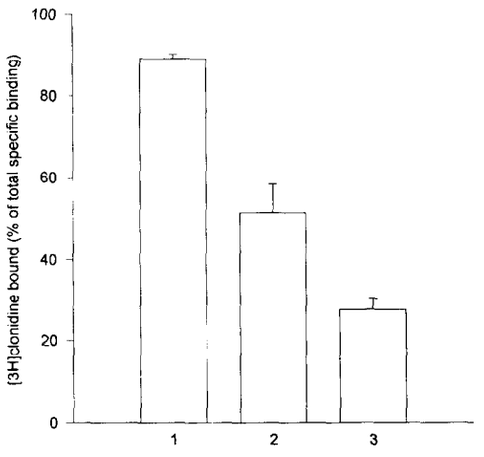

Fig. 4. Inhibition of the binding of [^{3}H]clonidine to human brain nucleus reticularis lateralis membranes with purified anti-idiotypic antibodies. [^{3}H]Clonidine (30 nM) imidazoline specific binding was performed in the presence of 10 µM (−)-norepinephrine in order to mask α_2-adrenoceptors. Cirazoline (10 µM) was used to define non-specific binding. Data are means ± S.D. of 3 experiments in triplicate with 3 different pools of purified antibodies or preimmune immunoglobulins. (1) Preimmune immunoglobulins (20 µg); (2) Purified anti-idiotypic antibodies (10 µg); (3) purified anti-idiotypic antibodies (20 µg).

Table 1
Immunoprecipitation of [^{3}H]idazoxan binding sites from human nucleus reticularis lateralis membranes solubilized with 13 mM CHAPS

	[^{3}H]Idazoxan binding
Buffer	100%
Preimmune IgG (20 µg)	123 ± 16.9%
Anti-idiotypic antibodies (20 µg)	43.5 ± 13.0%

Anti-idiotypic antibodies or preimmune serum (20 µg protein) were incubated overnight, with protein A Trisacryl beads at 4°C. Solubilized [^{3}H]idazoxan binding sites (30 fmol) were then added. [^{3}H]Idazoxan specific binding remaining in the supernatant was determined as described in Materials and methods. The results are means ± S.E. of 2 experiments in triplicate with 2 different pools of purified antibodies. Specific counts were: with buffer, 432 ± 2 dpm; with preimmune IgG, 533 ± 71 dpm; and with anti-idiotypic antibodies, 188 ± 55 dpm. Neither anti-idiotypic antibodies nor preimmune IgG modified the non-specific binding.

idiotypic antibodies proved able to deplete about 55% of this activity. In parallel experiments, preimmune immunoglobulins were passed over the anti-idazoxan antibodies-Aminolink column and the eluates were tested for immunoprecipitation. No such activity was found with these samples thus ruling out the possibility of a contamination by anti-idazoxan antibodies to explain the results

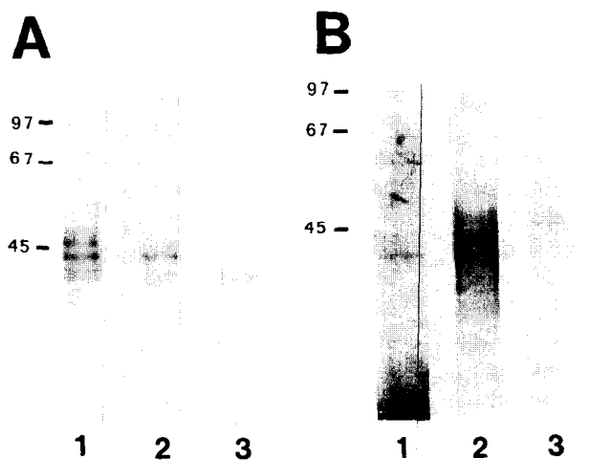

Fig. 5. Western blots of human nucleus reticularis lateralis imidazoline binding sites using the anti-idiotypic antibodies. Proteins were resolved on 10% SDS-PAGE, electroblotted onto nitrocellulose and immunostained as described in Materials and methods. (A) (1) Solubilized proteins (80 µg/160 fmol receptor) from nucleus reticularis lateralis membranes immunostained with 0.5 µg/ml purified anti-idiotypic antibodies, (2) and (3) fractions immunostained as in (1), obtained after a gel filtration chromatography of these solubilized proteins on a S200 sephacryl column, (2) fraction containing 150 fmol imidazoline binding sites (about 5 µg protein), (3) fraction devoid of any binding activity (5 µg protein). (B) Human brain imidazoline binding proteins eluted with 1 M KCl from a Tris-NH_2 blocked epoxy-activated sepharose column; (1) and (2) fraction containing imidazoline specific binding activity (1.5 µg protein/100–150 fmol receptor) was either silver stained (1) or immunostained (2) with 2 µg/ml purified anti-idiotypic antibodies. (3) Fraction (1.5 µg protein) without imidazoline binding activity immunostained as in (2).

obtained with purified anti-idiotypic antibodies. Moreover, no anti-idazoxan activity was detected in these purified anti-idiotypic antibodies as assessed by RIA.

The purified anti-idiotypic antibodies were used as probes in immunoblotting assays with solubilized human nucleus reticularis lateralis membranes bearing imidazoline receptors (Fig. 5A, lane 1) and in samples enriched in imidazoline binding sites ([^{3}H]idazoxan as well as [^{3}H]clonidine binding sites) obtained either by gel filtration chromatography on an S200 sephacryl column (Fig. 5A, lane 2 and 3) or by affinity chromatography on a Tris NH_2 blocked epoxy-activated sepharose column (Fig. 5B, lane 1, 2 and 3). When solubilized proteins were immunoblotted (Fig. 5A, lane 1), several bands were labeled with anti-idiotypic antibodies. In the fractions enriched in imidazoline binding sites by either chromatography (Fig. 5A, lane 2 and Fig. 5B, lane 2), only one protein was immunostained with a molecular weight of about 43 kDa whereas no band was stained in fractions devoid of binding activity (Fig. 5A, lane 3 and Fig. 5B, lane 3). Control experiments were done with an immunoglobulin G fraction of preimmune serum or with the same fraction after passing through an anti-idazoxan antibodies-Aminolink column; no bands were revealed in both controls (data not shown).

4. Discussion

The idiotypic network theory of Jerne (1974) proposes that the variable regions of antibodies (the idiotypes), may give rise to a secondary set of anti-idiotypic antibodies. If the idiotypic antibody is directed against a ligand of a receptor, then some of the anti-idiotypic antibodies can react with the combining site of the receptor. This approach has been successfully used to raise antibodies against numerous receptors (Guillet et al., 1985; Elazar et al., 1988; Bjercke and Langone, 1989; Wai and Tai, 1993; Wang and Tai, 1990; Cupo et al., 1992) and is particularly adequate when completely purified receptors are not available. As the human brain imidazoline receptor has not yet been purified to homogeneity, the strategy in this study was to use purified anti-idazoxan antibodies as immunogens to obtain anti-idiotypic antibodies able to crossreact with imidazoline receptors.

Although idazoxan displays a high affinity for α_2-adrenoceptors in the rat brain cortex (Pimoule et al., 1983), in the guinea pig cerebral cortex (Wikberg and Uhlen, 1990) and in ovine myometrium (Vass-Lopez et al., 1990), [^{3}H]idazoxan is used as a radioligand to label imidazoline receptors. In fact, [^{3}H]idazoxan only labeled imidazoline specific binding sites (insensitive to catecholamines) in some other tissues and/or species including human liver (Tesson et al., 1991), smooth muscle of rabbit urethra (Yablonsky et al., 1988), rabbit adipocytes (Langin and Lafontan, 1989) and in our hands in human brain membranes (Greney et al., 1994b). Thus, anti-idazoxan antibodies raised in rabbits (Bennai et al., 1993), were used to produce anti-idiotypic antibodies able to recognize the imidazoline receptors. These polyclonal antibodies, like the receptor, exhibited an affinity in the low nanomolar range for [^{3}H]idazoxan and showed a selectivity spectrum providing similarities with the one of the receptor (i.e. recognition of imidazolines and no recognition of catecholamines). In order to reduce the production of immunoglobulins not directed against the idiotype, we used an homologous system of immunization (rabbit's immunoglobulins injected in rabbits). Here we report the successful production of anti-idiotypic antibodies after immunizations of rabbits with purified anti-idazoxan antibodies.

The first criterion of the anti-idiotypic nature of the immunoglobulins was their inhibitory activity on [^{3}H]idazoxan binding to polyclonal anti-idazoxan antibodies. At least part of these anti-idiotypic antibodies proved also able to recognize the imidazoline receptors from human brain as assessed by the different tests: (1) specific recognition of proteins in human nucleus reticularis lateralis membranes (ELISA tests), (2) dose dependent inhibition of [^{3}H]imidazoline specific binding in nucleus reticularis lateralis membranes, (3) positive immunostaining of proteins in fractions containing binding activity and (4) immunoprecipitation of imidazoline specific binding activity. Normal rabbit serum in all the tests failed to show any significant activity.

The inhibition of [^{3}H]idazoxan and [^{3}H]clonidine specific binding by the anti-idiotypic antibodies that we observed, is in favour of a direct interaction with the ligand binding site of the receptor. However, small ligands such as cirazoline, clonidine and idazoxan proved unable to prevent the binding of antibodies to human nucleus reticularis lateralis membranes in ELISA tests. Yet, our ELISA reaction appeared specific since we checked that our anti-idiotypic antibodies behave quite similarly compared to the preimmune serum in a tissue known to express almost no imidazoline specific binding proteins, the rat cerebellum (Kamisaki et al., 1990). It must be pointed out here, that the order of addition of ligands and antibodies was opposite in the two experiments and that the size differences beetween ligands and antibodies might also account for differences in the results obtained in binding and ELISA assays respectively. An alternative explanation might be that the antibodies recognize an epitope close to the binding site as described by others (Elazar et al., 1988).

In the human nucleus reticularis lateralis region, both [^{3}H]clonidine and [^{3}H]idazoxan binding sites (tentatively named I_1 and I_2 imidazoline receptor subtypes) have been described (Bricca et al., 1993; Greney et al., 1994b). Although it has been demonstrated that there was non-competitive interaction beetween idazoxan and clonidine (Bricca et al., 1993; De Vos et al., 1994), binding studies in the human brain have shown that these sites display both similar affinities for imidazoline derivatives (Greney

et al., 1994b) and analogous ion dependency (De Vos et al., 1994). Some structural analogies might be expected for the two binding sites which in turn would explain that both are inhibited by the anti-idiotypic antibodies. In fact, the recognition of different receptor subtypes by anti-idiotypic antibodies has already been reported by others (Gramsch et al., 1988).

In solubilized human nucleus reticularis lateralis membranes, our anti-idiotypic antibodies proved able to cross-react with several proteins; these data support the hypothesis of the existence of multiple proteins bearing imidazoline binding sites (Michel and Insel, 1989) even in the central nervous system (Bricca et al., 1993). On the other hand, these anti-idiotypic antibodies proved able to reveal a 43 kDa protein solubilized and isolated from the human nucleus reticularis lateralis region by two different chromatography procedures (Greney et al., 1994a,b). That this protein is related to an imidazoline binding protein from human nucleus reticularis lateralis is supported by the facts that [^{3}H]clonidine and [^{3}H]idazoxan imidazoline specific bindings were recovered in the fractions containing the 43 kDa protein and that no immunostaining was observed with these antibodies in fractions devoid of imidazoline specific binding activity. This 43 kDa protein appeared to be different from the [^{3}H]idazoxan binding proteins purified from peripheral tissues (Limon et al., 1992; Wang et al., 1992) because of their molecular weights and their insensitivity to clonidine and could be a good candidate for the I_1 receptor of the human brain. In this way, results obtained by Escriba et al. (1994) are very interesting. In fact, these authors were able to detect immunoreactivities in different human brain area with an anti-imidazoline binding protein antibody which was raised against the 70 kDa idazoxan binding protein from bovine adrenal glands (Wang et al., 1993). Interestingly, immunoreactive proteins of 43–47 kDa were only clearly detected in the human medulla oblongata containing the nucleus reticularis lateralis area (Escriba et al., 1994).

In conclusion, we present in this paper the first polyclonal anti-imidazoline receptor antibodies obtained via the anti-idiotypic procedure. With these polyclonal anti-idiotypic antibodies the existence, at least in the human nucleus reticularis lateralis region, of a 43 kDa protein related to an imidazoline binding site was identified. These antibodies represent useful tools to further characterize the imidazoline receptors and in particular to achieve their purification by immunoaffinity chromatography.

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