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Immunological studies on the rat peripheral-type benzodiazepine acceptor

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Photoaffinity labelling of rat adrenal mitochondrial preparations with [^{3}H]PK 14105 resulted in a single ^{3}H-labelled band on SDS/PAGE gels with an apparent-molecular-mass peak of 18 kDa. This represents a polypeptide associated with the peripheral-type benzodiazepine-binding site. Solubilization of photoaffinity-labelled membranes with 6 M-guanidine hydrochloride, followed by gel filtration and reversed-phase h.p.l.c. of the solubilized material, resulted in the purification to homogeneity of the [^{3}H]PK 14105-labelled polypeptide. This purified polypeptide was used to raise a rabbit polyclonal antiserum which recognized the immunogen in pure form and exclusively recognized it in a crude preparation of rat adrenal mitochondria as judged by immunoblotting. By the same analysis the antiserum identified the corresponding polypeptide from rat kidney and salivary gland, demonstrating its cross-reactivity. Subsequent immunocytochemical studies localized the polypeptide to the cortex of the adrenal gland, the distal tubules of kidney, the interstitial cells of testis, the biliary epithelium of liver and the choroid plexus and ependyma cells within the brain. This selective localization within organs may provide an insight into the physiological role of the peripheral-type benzodiazepine acceptor.

INTRODUCTION

Benzodiazepines are believed to exert their psychopharmacological effects through the benzodiazepine receptor associated with the neuronal 4-aminobutyrate-gated chloride channel [1–3]. The physiological relevance of a second type of benzodiazepine-binding site, located primarily in peripheral tissues [4], is still unknown. This latter type, known as the peripheral-type benzodiazepine acceptor, is pharmacologically distinct from its neuronal counterpart [4]. In addition, it does not appear to be coupled to the 4-aminobutyrate-gated chloride channel [5]. Furthermore, it also binds isoquinoline carboxamides (chemically unrelated to the benzodiazepines), including PK 11195 [6]. The nitrophenyl derivative PK 14105 has been shown to be a selective photoaffinity label for the peripheral-type benzodiazepine-binding site [7].

The peripheral-type benzodiazepines and their acceptor have been suggested to play roles in a kaleidoscope of biological phenomena such as inhibition of cell proliferation [8], alteration in cardiac action potentials [9], alterations of proto-oncogene expression [10], inhibition of mitochondrial respiratory control [11] and stimulation of steroidogenesis [12,13]. The verification or otherwise of these roles may be helped by analysing the location of the peripheral-type acceptor both at the subcellular and the tissue level. Subcellular-fractionation studies provide evidence for a mitochondrial-outer-membrane location [14–16]. Localization studies in various tissues have been performed by using light-microscopic autoradiographic techniques [17,18] based on the binding of a radioactive ligand which is selective for the peripheral-type acceptor. By this approach, differential localization of the binding site has been reported in various tissues, including the adrenal gland, where it is proposed that the site is present exclusively in the cortex [17], the testis, where it is most prominent in the interstitial tissue [17], the kidney, where it is primarily present in the distal convoluted tubules and the ascending limb of the loop of Henle [18], and the brain, where it is chiefly present in the choroid plexus and ependyma cells and to a lesser extent the glomerular layer of the olfactory bulb [19].

A potential disadvantage of the latter technique is that endogenous ligands [20,21] could inhibit the binding of the radioactive probe, thus presenting a false impression of the binding-site distribution. An alternative approach which bypasses this potential problem is immunocytochemical analysis of tissue slices. A polyclonal antiserum raised against the purified polypeptide containing the binding site would be a successful probe irrespective of the presence of endogenous inhibitor because it should recognize epitopes separate from or additional to those forming the binding site. Another significant advantage over the autoradiographic technique would be its speed and practical simplicity.

This paper describes a novel purification of the polypeptide containing the peripheral-type benzodiazepine-binding site, exploiting [^{3}H]PK 14105 as a specific photolabel. The purified polypeptide allowed the production of a polyclonal antiserum which was subsequently used in immunocytochemical studies.

MATERIALS AND METHODS

Materials

[^{3}H]PK 11195 (90 Ci/mmol) was from N.E.N., Dreieich, Germany. [^{3}H]PK 14105 (87 Ci/mmol) was from C.E.A., Gif-sur-Yvette, France. PK 11195 was a gift from Dr. Gerard Le Fur (Pharmuka Laboratories, Gennevilliers, France). Acro L313 disposable filters (0.45 μm pore size) were from Gelman International, Dublin, Ireland. Aquapore reversed-phase RP-300 columns (30 mm × 2.1 mm) were from Brownlee Labs., Santa Clara, CA, U.S.A. Horseradish-peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad Laboratories, Watford, Herts., U.K. VECTASTAIN ABC Kit was from Vector Laboratories, Peterborough, U.K.

Preparation of rat adrenal mitochondrial fraction

Wistar rats (200 g) were killed by cerebral dislocation and subsequent decapitation. Adrenal glands were immediately removed and decapsulated. All subsequent steps were performed at 4 °C. The glands were washed superficially in ice-cold 50 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA and 0.32 M-sucrose, and homogenized in 10 vol. of the same buffer with 10 strokes in a Thomas glass homogenizer fitted with a motor-

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driven Teflon pestle. The homogenate was centrifuged at 2500 *g* for 10 min. The supernatant was decanted and retained, and the pellet was homogenized in 5 vol. of the initial homogenization buffer and treated as above. The combined first and second supernatants were centrifuged at 48000 *g* for 15 min. The supernatant was discarded, the pellet resuspended as above in about 10 vol. of homogenization buffer and centrifuged at 48000 *g* for 15 min. The supernatant was discarded and the pellet homogenized in 50 mM-Tris/HCl buffer, pH 7.4, to a final protein concentration of 2–5 mg/ml as determined by the method of Kakiuchi *et al.* [22].

Measurement of [^{3}H]PK 11195 binding to rat adrenal mitochondrial fractions

Rat adrenal mitochondrial fractions (15 μg of protein) were incubated with 0.2–40 nM-[^{3}H]PK 11195 (13.32 Ci/mmol) in 50 mM-Tris/HCl buffer, pH 7.4, in a total volume of 1 ml at 4 °C. All samples were incubated in triplicate for 60 min. The incubation mixtures were then rapidly diluted with 1 ml of ice-cold incubation buffer, immediately filtered through Whatman glass-fibre filters (GB/B, 2.5 cm diam.), presoaked in 0.1% (w/v) polyethyleneimine, followed by rapid rinsing of the test tubes with 1 ml of ice-cold incubation buffer. Washing of the filters twice with 5 ml of ice-cold incubation buffer rapidly followed (total time for filtration and washing 15 s). When dried, the filters were counted for radioactivity by scintillation counting. Total binding and non-specific/non-saturable binding (i.e. in the presence of 10 μM unlabelled PK 11195) were determined as the mean (±S.E.M.) of three replicates. Specific/saturable binding was calculated from the difference between total and non-saturable binding. Mean values for specific binding and free [^{3}H]PK 11195 concentration were fitted to an equation describing a rectangular hyperbola by weighted non-linear regression [23].

Photoaffinity labelling of rat adrenal mitochondrial fractions with [^{3}H]-PK 14105 [5]

Rat adrenal mitochondrial fractions (20 mg of protein) were preincubated with 25 nM-[^{3}H]PK 14105 (90 Ci/mmol) or 6.25 nM-[^{3}H]PK 14105 (for determining non-specific incorporation; see below) in 50 mM-Tris/HCl buffer, pH 7.4, in a total volume of 4 ml at 4 °C for 1 h in the dark, with (for non-specific incorporation) or without (for sum of specific and non-specific incorporation) 10 μM-PK 11195. The mixture was then irradiated 10 cm from a Philips TL 40 W/08 black-light blue lamp (360 nm) for 1 h. This lamp at 20 cm yields an intensity of 65 mJ·cm^{-2}·min^{-1} at the surface of the mixture. After illumination, the mitochondrial fraction was harvested from the incubation mixture by addition of 50 mM-Tris/HCl buffer, pH 7.4, to a final volume of 40 ml, followed by centrifugation at 48000 *g* for 20 min. The supernatant was discarded and the pellet resuspended in 20 ml of 50 mM-Tris/HCl buffer, pH 7.4, containing 10 μM-PK 11195, by using an Ultra-Turrax homogenizer (model no. 263612). After a 1 h incubation on ice in the dark, the suspension was centrifuged at 48000 *g* for 20 min and the pellet washed in 20 ml of buffer.

Purification of a polypeptide associated with the peripheral-type benzodiazepine-binding site

Photolabelled mitochondrial membrane pellets were resuspended (5 mg of protein/ml), by Ultra-Turrax, in 6 M-guanidine hydrochloride. The suspensions were agitated gently for 45 min at 4 °C, followed by ultracentrifugation at 100000 *g* for 1 h. The supernatants constituting soluble extracts were subjected to Sephacryl S-200 gel-filtration chromatography as described in the legend to Fig. 1. The relevant pooled fractions (indicated by the bar in Fig. 1*a*) were subsequently filtered through a poly-(vinylidene difluoride) ACRO L313 filter (0.45 μM pore size). This filter retained 70% of the radioactivity, which could then be eluted with methanol (1 ml), an effective means of concentrating the sample. The organic extract was evaporated to dryness with a gentle stream of nitrogen. The residue remaining was subsequently dissolved in 6 M-guanidine hydrochloride (0.2 ml) and subjected to reversed-phase h.p.l.c. as described in the legend to Fig. 2.

Analysis of protein samples

Eluate fractions from the reversed-phase column were subjected to SDS/PAGE analysis by the method of Laemmli [24] on 20% (w/v) polyacrylamide slab gels. Proteins were detected by silver staining [25]. Appropriate gel lanes were subsequently cut into 2 mm slices, digested with 0.4% (w/v) 2,5-diphenyloxazole in Triton X-100/toluene/NCS tissue solubilizer/Hyamine hydroxide (50:100:1:1, by vol.) at room temperature for 48 h and counted for radioactivity.

Preparation of antisera

A quantity of the eluate component from the reversed-phase column, constituting approx. 10 μg of purified 18 kDa protein, was freeze-dried. Protein content was calculated crudely by comparing the area of the absorbance peak representing purified 18 kDa protein (indicated by the bar in Fig. 2) with the peak areas of resolved standard proteins (myoglobin, RNAase and lysozyme) of known quantity.

The freeze-dried material was dissolved in 1 ml of phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.2) containing 0.5% (w/v) SDS and emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected intradermally into 20 sites in the back of a male New Zealand White rabbit. A booster injection prepared by mixing equal volumes (1 ml) of antigen (as above) in Freund's incomplete adjuvant was injected intramuscularly into the hind legs 21 days after the initial injection. A second booster injection, identically prepared, was similarly injected 10 days later. Blood was collected by cardiac puncture 1 week after the second booster. The blood was left to clot at room temperature for 1 h in glass containers and then stored overnight at 4 °C to allow the clot to retract. Serum was decanted and centrifuged at 1000 *g* for 10 min. The supernatant constituted polyclonal antiserum and was stored at −20 °C until further use. A blood sample (5 ml) collected before immunization was similarly treated to prepare preimmune serum.

Western immunoblotting

SDS/PAGE was performed as described above, on 20% (w/v) polyacrylamide gels (8 cm × 5 cm). After separation the proteins were electrophoretically transferred (0.1A, 25 min) from the gels to nitrocellulose sheets by using a Hoefer TE 70 Semiphor semi-dry transfer unit. Transfer buffer [0.192 M-glycine/25 mM-Tris, pH 8.3, containing 1.3 mM-SDS and 15% (v/v) methanol] was as recommended by the manufacturer. After transfer, the nitrocellulose sheets were treated for 1 h with a solution containing 3% (w/v) powdered milk (Marvel) in 0.5 M-NaCl/0.05% (v/v) Tween 20/20 mM-Tris/HCl buffer, pH 7.5 (TTBS), to block non-specific binding. The blots were then incubated for 1 h in a 1:100 dilution of sera (either before or after immunization) in TTBS containing 1% Marvel. The blots were subsequently washed three times (10 min each) with TTBS before incubation for 1 h with a 1:500 dilution of horseradish-peroxidase-conjugated goat anti-rabbit IgG in TTBS containing 1% Marvel. After a further three washes (10 min each) in TTBS, immunoreactive bands were detected by incubating the blots in a

solution obtained by mixing freshly prepared 4-chloronaphthol (60 mg) in 20 ml of methanol with 60 μl of H_2O_2 in 100 ml of 0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.2.

After immunodetection, some of the lanes on the blots were analysed to determine the location of ^3H. In these cases the nitrocellulose lanes were cut into 2 mm horizontal slices, added to scintillation cocktail and counted for radioactivity.

Immunocytochemistry

Preparation of tissue slices. Tissues were removed from Wistar rats immediately after cerebral dislocation and placed in Bouin's fixative [1% (w/v) saturated picric acid/40% (v/v) formaldehyde/acetic acid (15:5:1, by vol.)] for 3 h. They were then transferred to 70% ethanol and subsequently dehydrated overnight in a graded ethanol series (70%, 96%, 100%), followed by clearance in two changes of chloroform. The tissues were then embedded in paraffin wax, and serial sections (5 μm) were cut with an '820' Spencer rotary microtome. The sections were placed on glass slides previously washed in chromic acid. The sections were then allowed to dry before use.

Immunocytochemical staining. Paraffin-embedded sections of tissues were stained with the VECTASTAIN ABC Kit as recommended by the manufacturer. This is based on the method of Hsu *et al.* [26]. In all cases sera were used at a dilution of 1:500.

RESULTS

Purification of peripheral-type benzodiazepine acceptor

Measurement of specific binding of [^3H]PK 11195 to four different preparations of rat adrenal mitochondrial membranes showed the density of the peripheral-type benzodiazepine acceptor to be 76 ± 7 pmol/mg of protein (mean ± S.E.M.). This high degree of specific binding, consistent with a previous report [17], makes the adrenal gland the source of choice for purification studies. The initial fractionation steps comprised guanidine hydrochloride solubilization of photolabelled adrenal mitochondrial membranes, followed by Sephacryl S-200 gel filtration. Photolabelling with [^3H]PK 14105 in the absence of PK 11195 (allowing both specific and non-specific incorporation) produced a gel-filtration elution profile consisting of three major peaks of radioactivity (Fig. 1*a*), one of which was eluted at the void volume and another of which was eluted at the total volume (presumably unbound [^3H]PK 14105 and/or low-molecular-mass photolytic products). Photolabelling in the presence of PK 11195 (allowing non-specific incorporation only), however, produced a profile (Fig. 1*b*) consisting of two peaks of radioactivity which were coincident with the two peaks described above. Thus it is proposed that the first radioactive peak in Fig. 1(*a*) represents non-specific incorporation of [^3H]PK 14105, the second peak represents specific incorporation of radioligand and the third represents free radioligand.

Fractions constituting this second peak were pooled and prepared for the next fractionation step, reversed-phase h.p.l.c. Fortunately the photolabelled component after gel filtration bound to 0.45 μm filters used to remove particulate matter before h.p.l.c. The binding of material to poly(vinylidene difluoride) filters may be possibly due to its hydrophobic nature, which might also explain the elution and solubility of this material in methanol. Although not completely understood, this binding allowed for easy concentration of the sample, which was subsequently subjected to reversed-phase h.p.l.c. Several A_{208} peaks were observed, one of which was coincident with the major radioactive peak (Fig. 2). Electrophoretic analysis of this component demonstrated the purity of a polypeptide species of molecular mass 18 kDa (Fig. 3*a*). Gel slicing and counting of radioactivity demonstrated its photolabelled nature (Fig. 3*b*). The yield of purified polypeptide was calculated to be 10–15% relative to the initial crude mitochondrial preparation, based on comparing the magnitude of the u.v. absorbance (208 nm) of the product with that of standard proteins as described in the Materials and methods section.

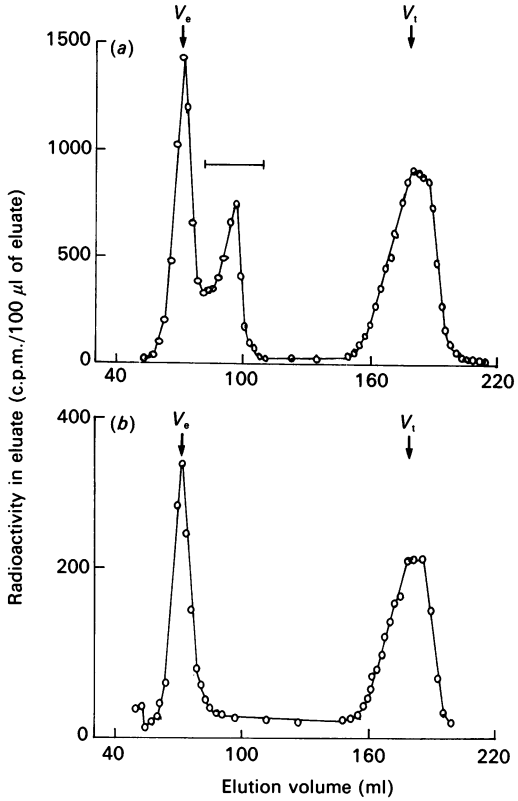

Fig. 1. Sephacryl S-200 gel filtration of guanidine hydrochloride-soluble extracts of photolabelled adrenal mitochondrial membranes

Guanidine hydrochloride-soluble extracts of adrenal mitochondrial membranes (20 mg of protein) previously photolabelled in (*a*) the absence and (*b*) the presence of 10 μM-PK 11195 were applied at a flow rate of 13 ml/h to a Sephacryl S-200 column (76 cm × 1.8 cm), equilibrated with 6 M-guanidine hydrochloride. Portions (0.1 ml) of fractions (2.5 ml) were counted for radioactivity by liquid-scintillation counting. The two arrows represent the void (V_e) and total (V_t) volumes as determined by chromatography of Blue Dextran and pyridoxal phosphate respectively. Fractions that were pooled and used for further analysis are indicated by the bar.

The nature of the minor radioactive peak eluted earlier on in the gradient (Fig. 2) is unknown. It is not free [^3H]PK 14105, which is eluted between the radioactive peaks in Fig. 3 (results not shown), nor does it contain associated protein, as judged by absence of a coincident absorbance peak and also by absence of protein-staining bands when subjected to SDS/PAGE (results not shown).

Subsequent studies have shown this novel purification procedure to be applicable to other tissues. For instance, when either salivary gland or kidney, tissues with relatively high levels of peripheral-type benzodiazepine-binding site [17], are subjected to the same regime, a photolabelled homogeneous 18 kDa protein which chromatographed identically with the adrenal form on reversed-phase h.p.l.c. is obtained.

Immunoblotting

The immunoreactivity of anti-(purified 18 kDa protein)-

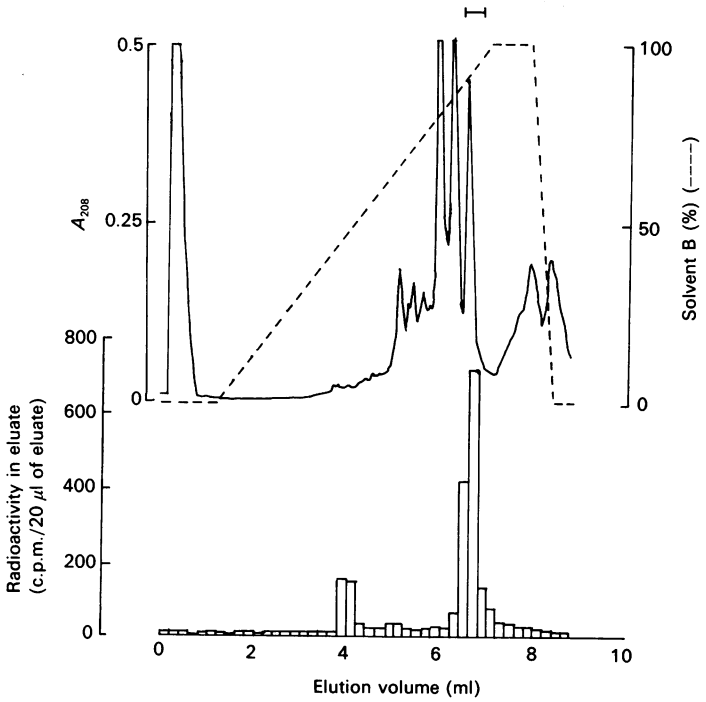

Fig. 2. Reversed-phase h.p.l.c. of eluate fractions from Sephacryl S-200 column

Pooled fractions from the Sephacryl S-200 column prepared as described in the Materials and methods section were applied to an Aquapore reversed-phase column (30 mm × 2.1 mm) pre-equilibrated in 0.1% (w/v) trifluoroacetic acid (solvent A) at a flow rate of 0.2 ml/min. A linear gradient to 100% solvent B (acetonitrile/water/trifluoroacetic acid, 700:200:0.9, by vol.) was introduced over 30 min as indicated by the dashed line. The flow rate remained at its initial magnitude throughout. The A_{208} of the eluate was continuously monitored (continuous line), and portions (20 μl) of fractions (0.2 ml) were counted for radioactivity (histogram). Pooled fractions constituting purified photolabelled 18 kDa protein are indicated by the bar.

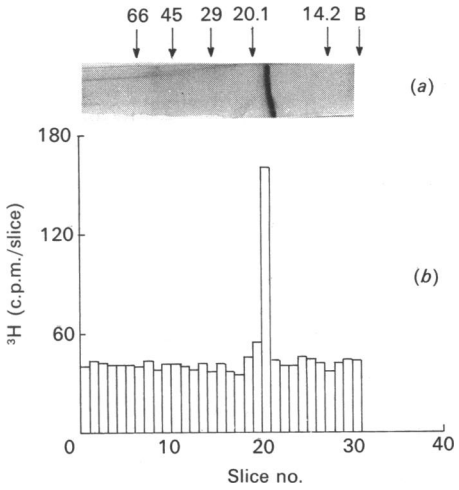

Fig. 3. Electrophoretic analysis and photolabelled nature of purified 18 kDa protein

A portion (20 μl) of the pooled fractions from the reversed-phase column (indicated by the bar in Fig. 2) was electrophoresed in a 20% (w/v)-polyacrylamide slab gel. Silver staining (*a*) demonstrated a single band representing a polypeptide of 18 kDa. Radioactivity analysis (*b*) as described in the Materials and methods section reveals the selective localization of ^{3}H in this band. The migration of the Bromophenol Blue dye front (B) and of standard proteins of known molecular mass (in kDa) are indicated by arrows.

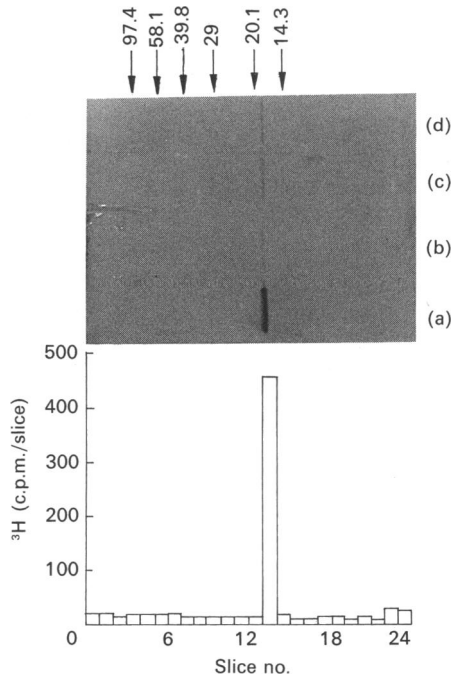

Fig. 4. Western blotting of 18 kDa protein from rat tissues

Top: purified photolabelled 18 kDa protein from rat adrenal (lane a, 1.5 μg), rat adrenal mitochondria (lane b, 200 μg of protein) and purified photolabelled 18 kDa protein from rat kidney (lane c, 0.5 μg) and salivary gland (lane d, 0.5 μg) were subjected to SDS/PAGE, transferred to nitrocellulose filters and treated with anti-(purified 18 kDa protein) antiserum as described in the Materials and methods section. Bottom: to demonstrate the photolabelled nature of the recognized proteins, ^{3}H was localized on the various lanes as described in the Materials and methods section. Lane b is shown as a representative sample, but similar profiles were obtained with the other lanes. The arrows indicate migration of standard proteins of known molecular mass (in kDa).

antiserum was assessed by immunoblotting. It is clear from Fig. 4 (lanes *a* and *b*) that not only did the antiserum recognize purified photolabelled 18 kDa protein (the immunogen) from rat adrenals, but it exclusively identified the same polypeptide from a crude adrenal mitochondrial preparation. Similar immunoblotted samples treated with preimmune serum exhibited no reactive bands. The antiserum also recognized purified photolabelled 18 kDa protein from rat kidney and salivary gland (Fig. 4, lanes *c* and *d*), demonstrating the cross-reactivity of the antiserum with respect to different tissues from the same species. This suggested its suitability for immunocytochemical staining of various rat tissues.

Immunocytochemical staining

Various rat tissues were examined immunocytochemically with anti-(purified 18 kDa protein)antiserum as probe. Preimmune serum was also used to act as a control and to define non-specific staining. All of the tissues examined displayed localized reactivity with anti-(purified 18 kDa protein) antiserum relative to preimmune serum. Sections from adrenal gland (Fig. 5*a*) showed uniform staining of the cortex (c), with no staining of the medulla (m). Both the preimmune serum and the antiserum produced intense staining of small dot-like structures, the nature of which is unknown. They may represent some residual endogenous peroxidase activity. Kidney sections (Fig. 5*b*) displayed reactivity which was localized to the distal convoluted tubules (dct).

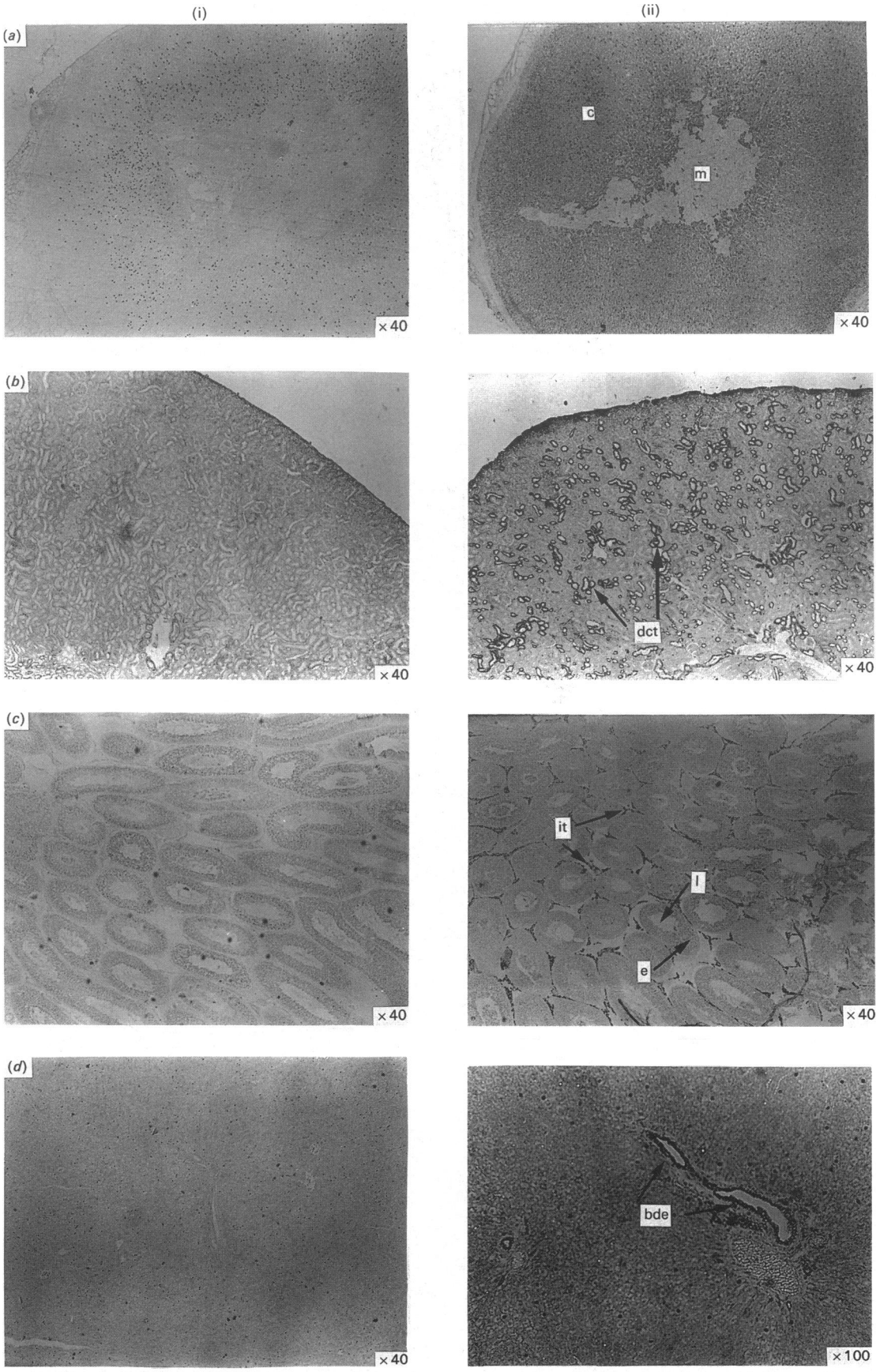

Fig. 5. Immunocytochemical staining of rat peripheral tissues with anti-(purified 18 kDa protein) antiserum

Sections of rat (*a*) adrenal, (*b*) kidney, (*c*) testes and (*d*) liver were immunostained with either (i) preimmune serum or (ii) anti-(purified 18 kDa protein) antiserum as described in the text. Letterings are also explained in the text. Magnifications are indicated.

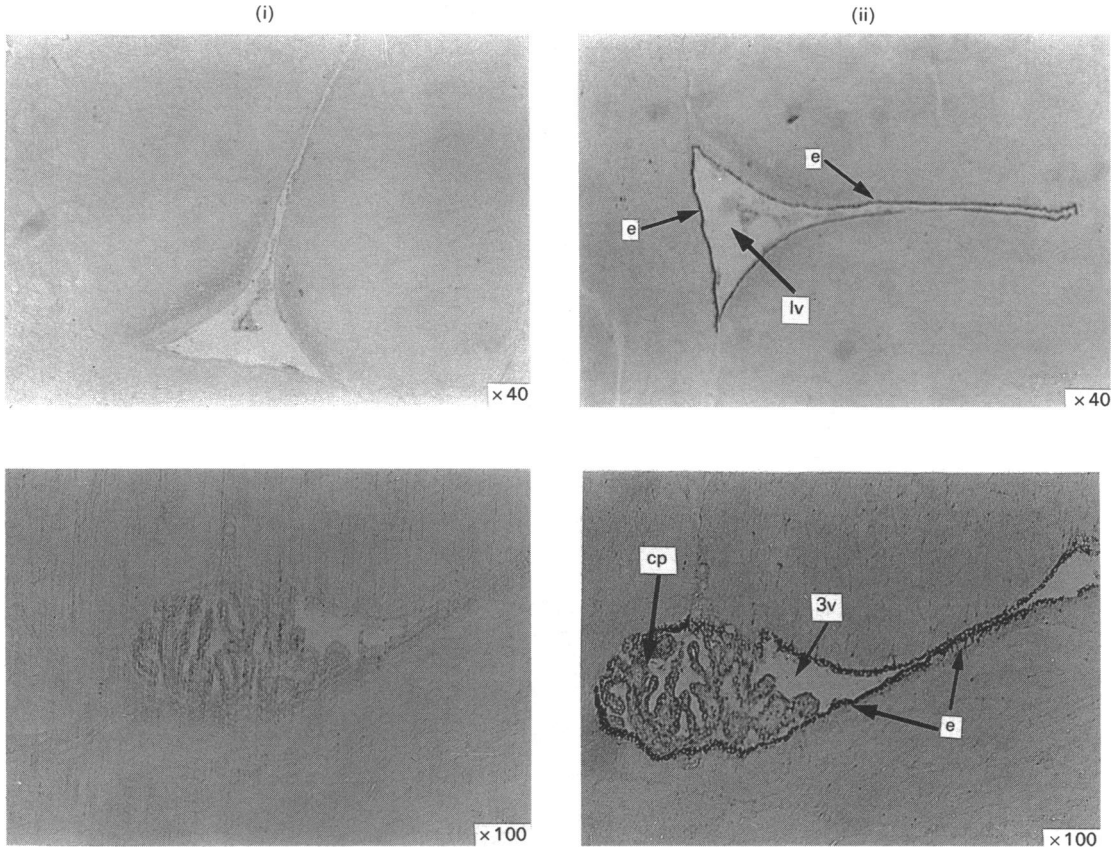

Fig. 6. Immunocytochemical staining of rat brain with anti-(purified 18 kDa protein) antiserum

Coronal sections of rat brain were immunostained with either (i) preimmune serum or (ii) anti-(purified 18 kDa protein) antiserum as described in the text. Note the heavy specific staining of the ependymal cells (e) lining the lateral (lv) and third (3v) ventricles and of the choroid plexus (cp) associated with the third ventricle. Magnifications are indicated.

Sections from testis (Fig. 5*c*) exhibited localized staining to the interstitial tissue (it), with no significant staining of the epithelium (e) or the lumen (l) of the seminiferous tubule. Liver sections (Fig. 5*d*) showed very discrete staining of the bile-duct epithelium (bde), with negligible staining of the surrounding hepatocytes. Brain sections (Fig. 6) revealed predominant staining of the choroid plexus and ependymal cell lining of the ventricles. In contrast with the autoradiographic study [19], no staining was detected in the olfactory bulb, which might reflect the lower sensitivity of the immunocytochemical approach.

DISCUSSION

This paper describes the rapid purification to electrophoretic homogeneity of a polypeptide associated with the peripheral-type benzodiazepine-binding site from the rat adrenal gland and the subsequent raising of a polyclonal antiserum which was used in immunocytochemical analysis, the first such study performed. A novel purification scheme, also applicable to other rat tissues, was employed which utilized harsh denaturing conditions, including solubilization with 6 M-guanidine hydrochloride. The use of a chaotropic agent rather than a detergent for the latter purpose theoretically allows for gel filtration of superior resolving power because of the absence of interfering micellar structures [27]. This interference is more prominent for detergents of higher aggregation number [27], necessitating other forms of fractionation, as was the case in a previous study [28] which also describes the purification of the peripheral-type acceptor. The latter study involved solubilization of adrenal mitochondrial membranes (previously photolabelled with [^{3}H]PK 14105) with digitonin and a subsequent combination of ion-exchange chromatography and reversed-phase h.p.l.c. to purify the acceptor. This protein is probably identical with the purified material described in the present work because of common features such as both being specifically photolabelled by [^{3}H]PK 14105, similar molecular masses as determined by SDS/PAGE and, more significantly, shared amino acid sequence as judged by partial amino acid sequencing of a CNBr-derived peptide from the purified polypeptide obtained in the present study (results not shown) and comparing the sequence with that of the previously purified protein [29]. Both purification processes give comparable yields of purified materials, but the versatility of the present system with respect to purification from different sources is attractive.

The antigen dose (10 μg) used in the immunization regime was at the lower end of the generally recommended dose range (10–50 μg), and the antigen was thus injected in denatured form, in an attempt to increase its immunogenicity by exposing new epitopes.

The immunoblot studies demonstrated the reactivity of the antiserum towards purified polypeptide, but also emphasized its selectivity in that only this single polypeptide was recognized from a crude mitochondrial preparation. The cross-reactivity of the antiserum towards the corresponding polypeptides from rat kidney and salivary gland, coupled with their identical behaviour during the purification process, indicates some structural conservation of this polypeptide, at least with respect to different tissues from the same species. This suggestion prompted the use

of the antiserum to screen various rat tissues for the peripheral-type benzodiazepine-binding site by immunocytochemical means, thus possibly localizing it to specific subpopulations of cells in different tissues.

Immunocytochemical staining of rat adrenal, kidney, testis and brain produced staining patterns similar to those obtained from the autoradiographic studies [17–19]. The only notable difference is that immunocytochemical analysis showed uniform reactivity in the adrenal cortex, whereas one of the autoradiographic studies [17] demonstrated differential reactivity within the cortex, in that the outer zona glomerulosa showed more intense staining than the middle zona fascilulata and inner zona reticularis. The presence, or higher levels, of an endogenous ligand/modulator for the binding site in the last two regions could account for the differences between the autoradiographic and immunocytochemical studies. The immunocytochemical approach was also applied to liver, which had not previously been probed by the autoradiographic technique. This localized the peripheral-type benzodiazepine acceptor to the biliary epithelium, a novel finding, which explains the relatively low density of the acceptor [17] in the tissue as a whole.

The most obvious unifying feature common to all of the cells to which the acceptor has been localized is their epithelially derived nature, possibly suggesting some role in secretory activity for the acceptor.

The immunocytochemical approach will facilitate a more wide-ranging screening of tissues which contain the peripheral-type benzodiazepine acceptor, e.g. salivary gland, intestine, heart etc. The localization of the acceptor to specific subpopulations of cells on this scale may increase our understanding of its physiological role.

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