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Tissue distribution of a novel neurotensin-degrading metallopeptidase

An immunological approach using monospecific polyclonal antibodies

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A monospecific polyclonal antiserum was raised against a recently purified rat brain neurotensin-degrading metallopeptidase. The purified IgG fraction immunoprecipitated the peptidase and inhibited its proteolytic activity. Western blot analyses revealed that the immune fraction recognizes only one protein in rat brain homogenates, and this corresponds closely to the purified enzyme. The IgG displayed a restricted specificity towards the peptidase from murine origin. In the rat, the neurotensin-degrading enzyme was widely distributed throughout peripheral organs with the noticeable exception of the duodenum. In addition, the peptidase was detected in various cell lines or membrane preparations of neural or extraneural origin in which it had been previously characterized by means of biochemical methods. In light of this widespread distribution, the putative role of the peptidase in the metabolism of neuropeptides is discussed.

INTRODUCTION

The elucidation of the mechanisms by which neuropeptides are physiologically inactivated is an important challenge which has inspired a growing number of reports. Some of these studies have led to the detection and the purification of novel peptidases which were shown to hydrolyse a variety of neuropeptides *in vitro*. However, the determination of the exact role of these enzymes in the modulation of neuropeptide concentrations *in vivo* often appears difficult due to technical limitations and is always subject to the development of specific tools.

We have previously examined the process of neurotensin inactivation by rat brain synaptic membranes (Checler *et al.*, 1983). Later, studies on the catabolism of this peptide were extended to membrane or cell preparations containing high-affinity neurotensin receptor sites whose occupancy was directly linked to a modulation of subcellular events (Amar *et al.*, 1985, 1986; Bozou *et al.*, 1986). Although the patterns of neurotensin inactivation varied according to the tissue source (Checler *et al.*, 1988), in all cases a primary inactivating cleavage occurred at the Pro^{10}-Tyr^{11} bond, yielding the biologically inactive fragments neurotensin-(1–10) and neurotensin-(11–13). The ubiquitous activity responsible for this cleavage appeared insensitive to specific inhibitors developed against other already isolated peptidases (Checler *et al.*, 1984). This was the first indication of the presence of a novel enzyme, and this finding was later corroborated by the purification and complete biochemical characterization of the peptidase from rat brain (Checler *et al.*, 1986*a*; Barelli *et al.*, 1988*a*). Here we describe the properties of a polyclonal antiserum raised against the brain neuropeptidase and its use as a probe to confirm the presence of this enzyme in tissues from dual central and peripheral origins.

MATERIALS AND METHODS

Purification of the peptidase

The peptidase was purified from 100 rat brains according to the procedure previously described (Checler *et al.*, 1986*a*). After the last purification step, the enzyme was submitted to preparative SDS/polyacrylamide gel electrophoresis (SDS/PAGE) and the bands corresponding to the activity were sliced and electroeluted (4 °C, 10 h, 200 V). An aliquot of the electroeluate was then submitted to SDS/PAGE analysis and coloured with Coomassie Blue in order to estimate the amount of peptidase recovered.

Immunization procedure

Purified peptidase (30 μg) in complete Freund's adjuvant was injected directly into the lymph nodes in the legs of a rabbit. Three weeks later, the rabbit was boosted intramuscularly and subcutaneously with 60 μg of peptidase in incomplete Freund's adjuvant and two additional boosters (50 μg of peptidase) were administered at three week intervals. The final injection (20 μg) occurred after a further month. The rabbit was first bled one month after the first injection and blood was then collected every week. The beginning of the immunological response was detected about seven weeks after the initial injection.

Purification of IgG

The presence of several plasma proteases in the preimmune serum precluded the possibility of examining directly the putative anticatalytic properties of the immune serum. Therefore, the purified IgG fractions from both immune and preimmune sera were obtained after treatment with octanoic acid according to the procedure previously described (Steinbuch & Audran, 1969).

Abbreviations used: SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; TFA, trifluoroacetic acid; TEA, triethylamine.

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Anticatalytic assay

Purified peptidase (0.1 μg) was preincubated for 18 h at room temperature with various dilutions of immune or preimmune IgG fractions in a final volume of 100 μl of 100 mM-Tris/HCl, pH 7.5, containing 0.02% bovine serum albumin. Neurotensin (2 nmol) was then added and incubations carried out for 1 h at 37 °C, being terminated by acidification (10 μl of 1.25 M-HCl). Neurotensin hydrolysis was monitored by h.p.l.c. using the conditions previously described (Checler *et al.*, 1988). Briefly, analyses were performed by means of a 35 min linear gradient of 0.1% trifluoroacetic acid (TFA), 0.05% triethylamine (TEA)/0.1% TFA, 0.05% TEA in acetonitrile from 90:10 to 65:35 (v/v) (Barelli *et al.*, 1988*a*). Elutions were carried out at room temperature at a flow rate of 1 ml/min and absorbance was monitored at 230 nm.

Immunoprecipitation

Purified peptidase (0.1 μg) was preincubated in the conditions described above with 20 μl of preimmune (8 mg/ml) or immune (10 mg/ml) IgG fractions. Protein A–Sepharose (100 μl, 100 mg/ml) was added and incubated for 2 h at room temperature under vigorous stirring. Incubations were then centrifuged (10 min, 10000 *g*) and the supernatants were tested for neurotensin degradation as described above.

Preparation of tissues, cell lines and membranes

Brains and other organs were homogenized by Polytron (setting = 6) for 15 s in 10 vols. of 10% sucrose in the presence or absence of a mixture of inhibitors comprising EDTA (10 mM), iodoacetamide (1 mM), phenylmethanesulphonyl fluoride (1 mM) and pepstatin (1 μM), and then centrifuged for 20 min at 800 *g*. The supernatants were removed, and centrifuged for 20 min at 3000 *g*, and the resulting pellets were resuspended with 2 ml of 20 mM-Tris/HCl, pH 7.5, with or without the mixture of inhibitors.

Primary cultured neurones, neuroblastoma N1E115 and HT29 cells were cultured as previously described (Checler *et al.*, 1986*b*,*c*). Cells were spun for 5 min at 800 *g* then resuspended and homogenized in 5 mM-Tris/HCl, pH 7.5. Homogenates were centrifuged for 5 min at 1500 *g* and both supernatant and pellet fractions were kept for further analysis.

The following highly purified membrane preparations were kindly provided by Dr. Jo-Ann Fox (School of Nursing, McMaster University, Hamilton, Canada). Plasma membranes from dog mesenteric arteries and rat fundus were prepared as described (Kwan *et al.*, 1979; 1982). Plasma membranes from circular and longitudinal dog ileum smooth muscle were purified according to previously reported procedures (Ahmad *et al.*, 1987; Kostka *et al.*, 1987).

SDS/PAGE and Western blot analysis

Aliquots of tissue homogenates were suspended in 100 μl of sodium phosphate buffer, pH 7.5, containing 2% SDS and 5% β-mercaptoethanol. Samples were boiled and electrophoresed overnight (15 mA/gel) according to a classical procedure (Laemmli, 1970) in 8% acrylamide gels. Proteins were then blotted onto nitrocellulose sheets according to the procedure described by Towbin *et al.* (1979). Efficiency of the blotting was checked by revealing the transferred proteins with 0.2% Ponceau Red in 3% trichloroacetic acid. Nitrocellulose was then destained with 140 mM-NaCl/20 mM-Tris/HCl, pH 7.5 (buffer A), containing 0.2% Tween 20, and incubated for 4 h at room temperature in buffer A containing 3% bovine serum albumin. Nitrocellulose was then exposed overnight to a 1:1000 dilution of the preimmune or immune IgG fractions (in buffer A containing 1% bovine serum albumin). Sheets were rinsed (5 × 5 min) with buffer A containing 0.2% Tween 20 then exposed for 3 h to a 1:800 dilution of goat anti-(rabbit immunoglobulins) coupled to peroxidase according to the manufacturer's recommendations (Pharmacia). Nitrocellulose was finally rinsed as above and the IgG–antigen complexes were revealed with 100 ml of methanol/buffer A (1:9, v/v) containing 30 mg of 4-chloro-1-naphthol and addition of 100 μl of H_2O_2.

RESULTS

The presence in the preimmune serum of several plasma proteases able to hydrolyse neurotensin precluded the possibility of studying the anticatalytic properties of the immune serum. The treatment of both sera with octanoic acid (see Materials and methods) produced immune and preimmune purified IgG fractions totally devoid of catalytic activities (results not shown). The rat brain neurotensin-degrading peptidase was inhibited in a dose-dependent manner by the immune IgG, while the

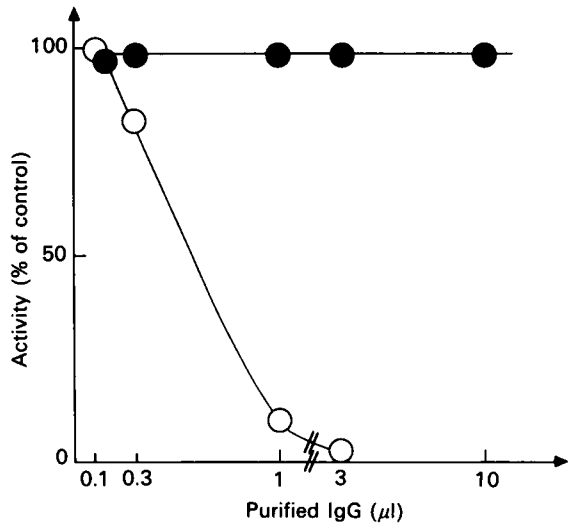

Fig. 1. Anticatalytic activity of the immune IgG fraction directed towards the neurotensin-degrading peptidase

Purified neurotensin-degrading metallopeptidase (0.1 μg) was preincubated for 18 h at room temperature in the absence (control) or in the presence of the indicated volumes of preimmune (●) or immune (○) IgG-purified fractions as described in Materials and methods. Neurotensin (2 nmol) was added and incubations carried out for 1 h at 37 °C, being terminated by acidification. Activity was monitored by following the formation of neurotensin(1–10) by h.p.l.c. and quantified by comparing the absorbance at 230 nm with known amounts of synthetic neurotensin(1–10) run in the same conditions. Data are expressed as the percentage of control activity (obtained in the absence of antibodies).

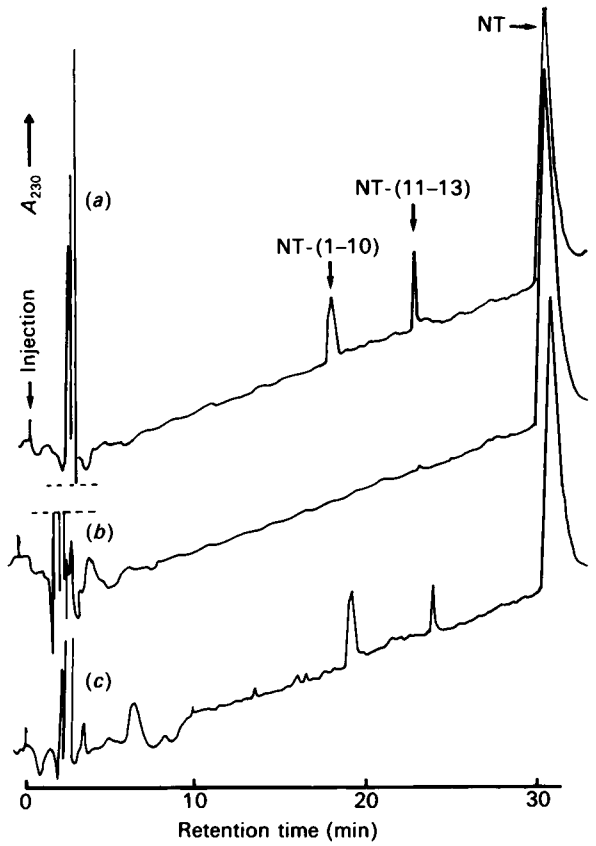

Fig. 2. Immunoprecipitation of the neurotensin-degrading metallopeptidase by the immune IgG fraction

The enzyme (0.1 μg) was preincubated in the absence (*a*) or with 20 μl of immune (*b*) or preimmune (*c*) IgG for 18 h at room temperature in the conditions described in Materials and methods. Protein A–Sepharose (100 μl, 100 mg/ml) was added for a further 2 h incubation; the incubation mixtures were then centrifuged (10 min, 10000 *g*). Supernatants were tested for neurotensin degradation and analysed by h.p.l.c. as described in Materials and methods. Arrows indicate the retention times of neurotensin (NT) synthetic fragments run in the same conditions.

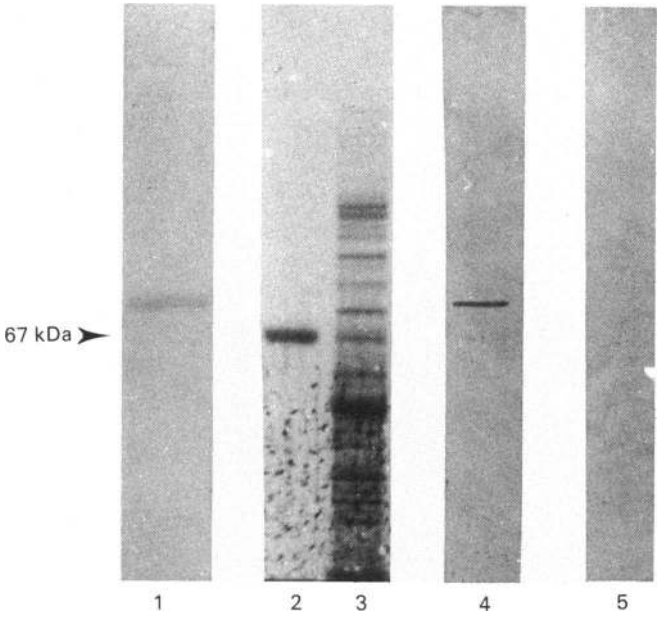

Fig. 3. Western blot analysis of proteins from rat brain homogenate

Rat brain homogenate was prepared as described in Materials and methods. A sample corresponding to 100 μg was electrophoresed (15 mA, overnight) in an 8% acrylamide gel (see Materials and methods). Proteins were then blotted onto nitrocellulose and stained with 0.2% Ponceau Red. Nitrocellulose sheets were destained and exposed to immune and preimmune purified IgG fractions. Antigen–IgG complexes were revealed, with goat anti-(rabbit immunoglobulins) coupled to peroxidase. Lane 1, SDS/PAGE analysis of purified rat brain neurotensin-degrading peptidase; lanes 2 and 3, Ponceau Red staining of electrophoresed and blotted bovine serum albumin and rat brain homogenate proteins, respectively; lanes 4 and 5, immunoblot analyses of homogenate proteins with immune and preimmune IgG fractions, respectively.

preimmune fraction was totally devoid of anticatalytic properties (Fig. 1). In agreement with this finding, immunoprecipitation experiments clearly established the presence of IgG capable of interacting with the rat brain peptidase in the immune fraction but not in the preimmune fraction (Fig. 2). Finally, immunoblots of total proteins of rat brain homogenates revealed that only one band was specifically recognized by the immune immunoglobulins and that the position of the stain closely corresponded to that of the purified neurotensin-degrading peptidase (Fig. 3).

The specificity of the immune IgG allowed us to examine the tissue-specificity of the neurotensin-hydrolysing activity in the rat. In fact the peptidase was detected in most of the rat peripheral organs except the duodenum (Fig. 4). In some organs such as kidney and ileum, an additional label was observed with a slightly lower apparent molecular weight. Interestingly, when a mixture of classical peptidase inhibitors was added during the homogenization procedure (see Materials and methods) this latter staining was strongly reduced while the high molecular weight protein was more densely labelled, indicating that the smaller protein probably represented a degradation product generated by proteolytic activities present in the homogenates (results not shown).

The evidence for a ubiquitous localization of the neurotensin-degrading peptidase in tissues from central and peripheral origins was further corroborated by the immunological detection of the enzyme in cell lines from neural and extraneural sources (Fig. 5). The peptidase was detectable in both the supernatant and pellet fractions obtained from pure primary cultured neurones of mouse embryo cerebral hemispheres as well as in the neuroblastoma clone N1E115 and was also present in the extraneural cell line A7R5 derived from rat aorta (Fig. 5). By contrast, no positive immunoreaction was observed when the human colon adenocarcinoma cell line HT29 was examined (Fig. 5).

Among a series of highly purified membrane preparations of muscular and nervous origin (see Materials and methods), the only label was obtained with plasma membranes from rat gastric fundus (results

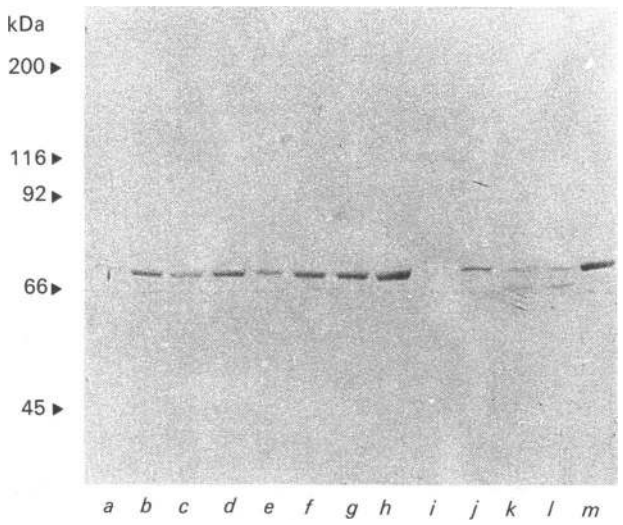

Fig. 4. Western blot analysis of proteins from various rat organs

Homogenates of various rat organs were prepared, electrophoresed and blotted onto nitrocellulose sheets as detailed in Materials and methods. Transferred proteins were incubated with a 1:1000 dilution of the purified immune IgG fraction and the immunopositive reaction was revealed with goat anti-rabbit IgG coupled to peroxidase. No reaction was observed after incubation with the preimmune IgG. Lanes: *a*, lung; *b*, spleen; *c*, stomach; *d*, liver; *e*, heart; *f*, testis; *g*, colon; *h*, caecum; *i*, duodenum; *j*, skeletal muscle; *k*, kidney; *l*, ileum; *m*, brain.

not shown). This was apparently in contradiction with the fact that the neurotensin-degrading peptidase activity was previously detected in plasma membranes from circular and longitudinal dog ileum smooth muscle (Checler *et al.*, 1987) as well as in the nervous layers innervating these tissues (Barelli, H., Checler, F. & Vincent, J.-P., unpublished data). These discrepancies led us to examine the species-specificity of the immune purified IgG fraction. Fig. 6 clearly demonstrates that the immune fraction only stained the enzyme located in the brains of animals belonging to the murine family.

DISCUSSION

The present paper describes the properties of a polyclonal antiserum raised against a recently purified rat brain neurotensin-degrading metallopeptidase and its use to examine the presence of the enzyme in various tissues and membrane preparations.

The IgG-purified fraction obtained from the immune serum precipitated the neurotensin-degrading metallopeptidase and also displayed anticatalytic activity. In addition, immunoblotting experiments revealed that the IgG recognized only one protein present in a rat brain homogenate and that the position of the stain closely corresponded to that of the purified enzyme. None of the above properties was shared by the preimmune fraction. Altogether, this indicated that the IgG-purified fraction could be used as a specific probe to detect the presence of the peptidase.

We recently purified a metallopeptidase from rat ileum that was able to hydrolyse a variety of neuropeptides

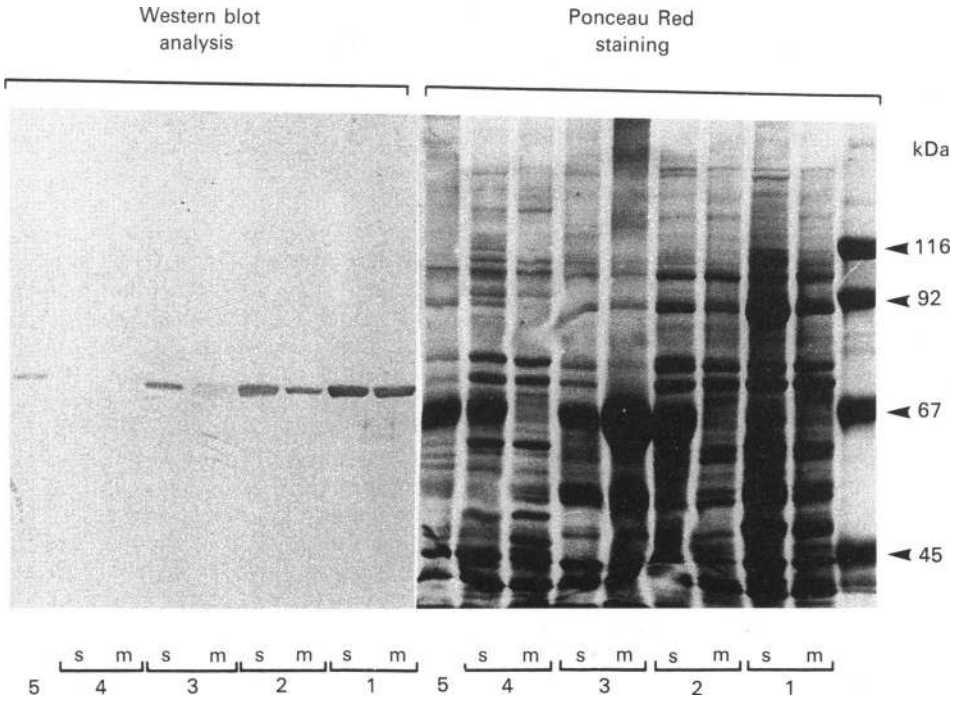

Fig. 5. Western blot analysis of proteins from various cell preparations

Soluble (s) and 'membrane-bound' (m) fractions were prepared from various cell lines as described in Materials and methods. Samples of proteins (about 100 μg) were electrophoresed, blotted onto nitrocellulose and revealed. Lanes 1 and 2, differentiated and non-differentiated neuroblastoma N1E115 cell line, respectively; lane 3, pure cultured neurones from mouse embryo cerebral hemispheres; lane 4, human adenocarcinoma HT29 cell line; lane 5, rat aorta A7R5 cell line homogenate.

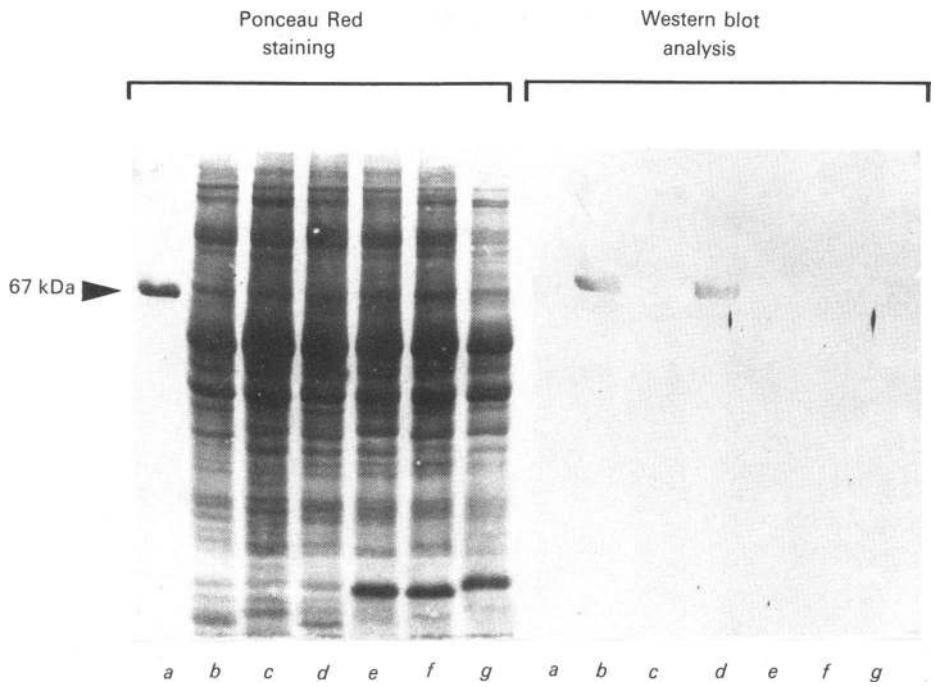

Fig. 6. Western blot analysis of proteins from brains of various species

Brain homogenates were prepared according to the procedure described in Materials and methods. Samples of proteins (about 150 μg) were electrophoresed, transferred onto nitrocellulose sheets and exposed to the immune IgG fraction. Antigen–IgG complexes were revealed by means of goat anti-(rabbit immunoglobulins) coupled to peroxidase. Lanes: *a*, bovine serum albumin; *b*, mouse; *c*, chicken; *d*, rat; *e*, guinea-pig; *f*, rabbit; *g*, pig.

including neurotensin (Barelli *et al.*, 1988*b*). The biochemical characterization of this enzyme indicated that it displayed the same affinity and specificity towards the neurotensin sequence, hydrolysed the same natural peptides and exhibited the same physical characteristics (molecular weight and behaviour on chromatographic supports) as the brain enzyme. Altogether, this indicated that the ileal enzyme probably corresponded to the peripheral counterpart of the brain peptidase. This was confirmed by means of the present purified IgG fraction, which labelled the activity in both an ileum homogenate (Fig. 4) and a purified fraction derived from this tissue (Fig. 7). The fact that the central and peripheral enzymes appeared to share common antigenic determinants encouraged us to examine the tissue-specificity in the rat. A widespread distribution was observed since all the organs tested except the duodenum contained the peptidase.

The catabolism of neurotensin was previously examined in cells from neural origins (N1E115 neuroblastoma clone and pure cultured neurons, Checler *et al.*, 1986*b*,*c*). These cell cultures provided interesting models with which to study the physiology of neurotensin since receptor occupancy by the peptide had been shown to modulate various subcellular events including cyclic nucleotide levels and phosphatidylinositol turnover (Amar *et al.*, 1985, 1986). In these cells, a neurotensin(1–10)-generating activity was detected that was ascribed to the neurotensin-degrading metallopeptidase since this cleavage was not inhibited by a series of specific peptidase inhibitors developed against other well-characterized cerebral peptidases (Checler *et al.*, 1986*a*). The presence of this enzyme was confirmed by direct immunopositive reaction observed with the immune IgG fraction. Interestingly, the peptidase was recovered in both the supernatant and particulate fractions. This confirmed the biochemical detection of the peptidase in the 100000 ***g*** supernatant fraction of a rat brain synaptic membranes homogenate (results not shown) and suggested that the peptidase was partly cytosoluble but could also exist in a membrane-associated form as has already been reported for another metallopeptidase, namely endopeptidase 24.15 (Acker *et al.*, 1987).

The use of the purified IgG fraction appeared to be clearly limited to the detection of the peptidase in tissues or membrane preparations of murine origin (Fig. 6). This restricted specificity could explain the discrepancies observed between the biochemical detection of the peptidase in the human adenocarcinoma HT29 cell line (Checler *et al.*, 1986*c*) as well as in the circular and longitudinal dog ileum smooth muscle plasma membranes (Checler *et al.*, 1987), and the total lack of staining obtained after exposure of these preparations to the immune IgG-fraction (Fig. 5; other results not shown).

Since neither specific inhibitor nor fluorimetric substrate have been yet developed against the neurotensin-degrading metallopeptidase, the IgG fraction appears to be the only tool available with which to inhibit or detect the enzyme. These antibodies will also allow us to establish the distribution of this novel proteolytic activity in the rat brain by immunohistochemical studies in order to assess whether the enzyme is closely co-localized with neurotensin receptors or more widely distributed throughout the central nervous system.

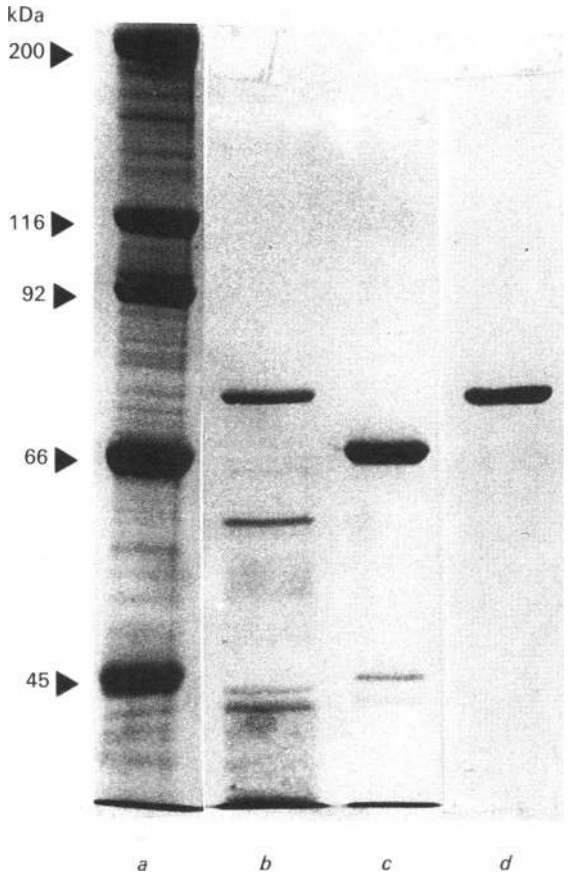

Fig. 7. Western blot analysis of proteins recovered at the last purification step of the rat ileum neurotensin-degrading metallopeptidase

Proteins (20 μg) recovered after the last purification step (gel permeation on ACA34) were dried and resuspended in phosphate buffer containing 2% SDS, 5% β-mercaptoethanol and 10% glycerol. Samples were boiled and electrophoresed overnight in an 8% acrylamide slab gel. Proteins and markers were stained with Coomassie Blue and destained in 7% acetic acid. Lanes *a* and *c*, standards; lane *b*, post-ACA34 proteins. Western blot analysis of post-ACA34 proteins (lane *d*) was carried out as described under Materials and methods.

The latter possibility together with the fact that the peptidase displays the ability to hydrolyse a variety of biologically active peptides would support a putative general role of the enzyme in the physiological inactivation of neuropeptides.

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