Membrane peptidases in the pig choroid plexus and on other cell surfaces in contact with the cerebrospinal fluid

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A comprehensive survey of 11 peptidases, all of which are markers for renal microvillar membranes, has been made in membrane fractions prepared from pig choroid plexus. Two fractionation schemes were explored, both depending on a MgCl_2 -precipitation step, the preferred one having advantages in speed and yield of the activities. The specific activities of the peptidases in the choroid-plexus membranes were, with the exception of carboxypeptidase M, lower than in renal microvillar membranes: those of aminopeptidase N, peptidyl dipeptidase A ('angiotensin-converting enzyme') and γ -glutamyltransferase were 3–5-fold lower, those of aminopeptidase A and endopeptidase-24.11 were 12–15-fold lower, and those of dipeptidyl peptidase IV and aminopeptidase W were 50–70-fold lower. Carboxypeptidase M had a similar activity in both membranes. Alkaline phosphatase and $(\text{Na}^+ + \text{K}^+)$ -activated ATPase were more active in the choroid-plexus membranes. No activity for microsomal dipeptidase, aminopeptidase P and carboxypeptidase P could be detected. Six of the peptidases and $(\text{Na}^+ + \text{K}^+)$ -activated ATPase were also studied by immunoperoxidase histochemistry at light- and electron-microscopic levels. Endopeptidase-24.11 and $(\text{Na}^+ + \text{K}^+)$ -activated ATPase were uniquely located on the brush border, and the other two peptidases appeared to be much more abundant on the endothelial lining of microvessels. Dipeptidyl peptidase IV and aminopeptidase W were also detected in microvasculature. Pial membranes associated with the brain and spinal cord also stained positively for endopeptidase-24.11, aminopeptidase N and peptidyl dipeptidase A. The immunohistochemical studies indicated the subcellular fractionation did not discriminate between membranes derived from epithelial cells (i.e. microvilli) and those from endothelial cells. The possible significance of these studies in relation to neuropeptide metabolism and the control of cerebrospinal fluid production is discussed.

INTRODUCTION

The choroid plexuses in the ventricles of the brain are the major source of the cerebrospinal fluid (CSF), which fills the subarachnoid space (see, e.g., Coxon, 1968). The plexus is a much folded organ, being divided into branching tufts that are covered on the ventricular surface with a polarized epithelium resting on a vascular core. The apical membrane of the epithelial cells takes the form of a brush border comparable in some ways with those of the kidney, intestine and placenta. Fractions enriched in choroid-plexus microvilli have been prepared by methods developed for these other tissues and such membrane vesicles exhibit similar functions, such as amino acid transport, and also appear to possess some of the same markers, such as alkaline phosphatase and γ -glutamyltransferase (Langenbeck & Kinne, 1980; Ross & Wright, 1984). There is, however, one crucial difference in the polarity of the choroid-plexus epithelium in that $(\text{Na}^+ + \text{K}^+)$ -ATPase has an apical rather than a basolateral membrane distribution (Masuzawa *et al.*, 1980, 1981, 1984; Ernst *et al.*, 1986) and is therefore a marker for the brush border.

There is much interest in the factors that might modulate CSF production. Various receptors have been identified in choroid plexus preparations, including H_2

receptors for histamine (Crook *et al.*, 1986), 5-HT_{1c} receptors for 5-hydroxytryptamine (Pazos *et al.*, 1985; Conn *et al.*, 1986; Hoyer *et al.*, 1986; Yagaloff & Hartig, 1986) as well as β -adrenergic receptors (Nathanson, 1979, 1980). There also are several reports of receptors for peptides. Vasoactive intestinal peptide, secretin and glucagon increase cyclic AMP in the epithelial cells, with the first-named being much more potent than the two related peptides (Crook & Prusiner, 1986). Atrial natriuretic factor (as the 28-amino acid-residue peptide, α -ANP) binds to the membranes of the plexus with high affinity (McCarty & Plunkett, 1986; Lynch *et al.*, 1986) and has also been localized by autoradiography to the epithelial cells of the plexus (Bianchi *et al.*, 1986). Intraventricular injections of α -ANP lead to a decrease in CSF production (Steardo & Nathanson, 1987) and, in kaolin-induced experimental hydrocephalus, an increase in α -ANP binding has been observed (Tsutsumi *et al.*, 1988). α -ANP may therefore be involved in regulating CSF production.

Some of the peptidases of renal and intestinal microvilli are present on the choroid plexus and may therefore play a role in the metabolism of neuropeptides at that site. Endopeptidase-24.11 (E-24.11) is the enzyme responsible for initiating the hydrolysis of many neuropeptides when they are incubated with renal microvillar membranes.

Abbreviations used: CSF, cerebrospinal fluid; Z-, benzyloxycarbonyl-; Bz-, benzoyl-; -NH-Mec, 7-amino-4-methylcoumarylamide; Dip-F, diisopropyl phosphorofluoridate; ACE, peptidyl dipeptidase A ('angiotensin-converting enzyme'); AP-N, aminopeptidase N; DPP-IV, dipeptidyl peptidase IV; E-24.11, endopeptidase-24.11; $(\text{Na}^+ + \text{K}^+)$ -ATPase, $(\text{Na}^+ + \text{K}^+)$ -activated ATPase; α -ANP, 28-amino acid-residue peptide of atrial natriuretic factor.

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The susceptible peptides include substance P, atrial natriuretic peptide, bradykinin, the angiotensins and oxytocin (Stephenson & Kenny, 1987a,b). E-24.11 has been located by immuno-peroxidase methods to the choroid plexus of the pig as well as to pial and ependymal membranes (Matsas *et al.*, 1986; Barnes *et al.*, 1988a). The hydrolysis of α -ANP (labelled in the C-terminal Tyr-28 residue) by a crude membrane fraction of human choroid plexus is in part dependent on the activity of E-24.11 (Deschodt-Lanckmann *et al.*, 1988). Peptidyl dipeptidase A ('angiotensin-converting enzyme', ACE) appears to be very abundant (Arregui & Iversen, 1978; Rix *et al.*, 1981) and in rat and human has been localized immunohistochemically to the apical membrane (Defendini *et al.*, 1983). γ -Glutamyltransferase is enriched in membrane fractions of choroid plexus (Langenbeck & Kinne, 1980) and aminopeptidase N (AP-N) has been immunochemically identified in rat choroid plexus (Solhonne *et al.*, 1987). However, the present information on these cell-surface peptidases in the choroid plexus is somewhat fragmentary, rarely quantitative and sometimes leaves room for doubt concerning the specificity of the assay. In the present paper we have attempted a comprehensive survey of the peptidases in membrane fractions prepared from pig choroid plexuses and have correlated some of these data with the results of immunoperoxidase studies at light- and electron-microscopic levels.

EXPERIMENTAL

Materials

Peptidase assay materials were obtained as noted in sources describing the assay methods. Peptidase inhibitors were from the suppliers noted in Stephenson & Kenny (1987a) and Jackson *et al.* (1988). The reagents used for immunohistochemistry were obtained as described previously (Barnes *et al.*, 1988a,b).

Enzyme assays

The peptidase substrates used were: E-24.11 (EC 3.4.24.11), 125 I-labelled insulin B chain; AP-N (EC 3.4.11.2), Ala-NH-Mec; aminopeptidase A (EC 3.4.11.7), α -Glu-NH-Mec; γ -glutamyltransferase (EC 2.3.2.2), γ -Glu-NH-Mec; aminopeptidase W, Glu-Trp; DPP-IV (EC 3.4.14.5), Gly-Pro-NH-Mec; ACE (EC 3.4.15.1), Bz-Gly-His-Leu; microsomal dipeptidase (EC 3.4.13.11), Gly-D-Phe; aminopeptidase P (EC 3.4.11.9), Gly-Pro-Hyp; carboxypeptidase P (EC 3.4.17.-), Z-Pro-Phe; carboxypeptidase (Skidgel, 1988), Bz-Gly-Lys. The assay methods have been described by Fulcher & Kenny (1983) and Hooper *et al.* (1987), except those for aminopeptidase W (Jackson *et al.*, 1988), ACE (Hooper & Turner, 1987), alkaline phosphatase (EC 3.1.3.1) (Bessey *et al.*, 1946) and ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)-ATPase (EC 3.6.1.3) (Jørgensen & Skou, 1969). Assays using h.p.l.c. were used for carboxypeptidase P (Hedeager-Sørensen & Kenny, 1985), aminopeptidase P and carboxypeptidase M (Hooper & Turner, 1988a).

All enzyme units are expressed as nmol of substrate hydrolysed/min in the defined assay conditions. Where specific inhibitors were available to discriminate between the named and other enzymes that might hydrolyse the assay substrate, the reported activities are those observed to be sensitive to the inhibitor, preincubated for 15 min at 37 °C (45 min at 37 °C for Dip-F) before addition of

the substrate. Complete inhibition (> 98%) may be predicted for E-24.11 by 1 μM -phosphoramidon, AP-N by 10 μM -amastatin, ACE by 1 μM -captopril, DPP-IV by 0.1 mM-Dip-F and aminopeptidase A by 50 μM -amastatin. In the case of ($\text{Na}^+ + \text{K}^+$)-ATPase, 1 mM-ouabain was used to discriminate between this and other ATPases. An immunoradiometric assay for aminopeptidase W (Gee *et al.*, 1985; Gee & Kenny, 1985) was used to confirm the presence of this enzyme in choroid plexus.

Tissue and subcellular fractionation

Fresh pig brains were obtained from the slaughterhouse (kindly donated by ASDA Farm Stores, Lofthousegate, West Yorkshire, U.K.) and the choroid plexuses were immediately removed from the lateral ventricles, washed in ice-cold 0.9% NaCl (method A) and stored at -70 °C.

Two procedures for preparing microvilli-enriched preparations were used, both of which depended on a MgCl_2 precipitation step (Scheme 1). Method A used well-washed choroid plexuses as the starting material and was identical with that for renal microvilli (Booth & Kenny, 1974) except that the tissue (20 g) was homogenized (2 $\times$ 15 s) in an Ultra-Turrax homogenizer (Janke und Kunkel G.m.b.H., Staufen, Germany) and the first MgCl_2 pellet (P1) was resuspended and re-treated with 10 mM- MgCl_2 , then re-centrifuged and the supernatant pooled with supernatant S1 before continuing with the high-speed centrifugation step and second MgCl_2 treatment giving pellet P2, the whole of which was resuspended for the steps leading to the final pellet, designated P4.

Method B was modified in the light of the results with the first method. The tissue was frozen immediately on removal from the brain and not washed in saline before homogenization, but in other respects the procedure was that of method A up to the first high-speed centrifugation step (15000 g_{av} for 15 min), generating pellet P2 and supernatant S2. S2 was then centrifuged at 31000 g_{av} for 120 min to give pellet P2a, and the supernatant was re-centrifuged at 65000 g_{av} for 120 min to give pellet P2b. A Sorvall SS34 rotor was used for each step except for the last, which required a Beckman Al-30 rotor. The pellets from both methods were resuspended in the mannitol/Tris homogenization buffer at 1 mg of protein/ml and stored in small portions at -70 °C.

The preparation of kidney microvillar membranes (P4) was as described previously (Booth & Kenny, 1974).

Immunohistochemistry

The tissues were from piglets (aged 1-4 weeks) obtained from the University Field Station. The animals were premedicated with Immobilon (Reckel and Colman), anaesthetized with Halothane (if perfused-fixed) or killed by exsanguination as previously described (Matsas *et al.*, 1986). Regions of the brain including the lateral ventricles were quickly removed from the unfixed brains, cut into suitable blocks, embedded in 3% CM-cellulose and frozen in isopentane cooled by liquid N_2 and subsequently stored in liquid N_2 . The preparation of cryostat sections and their immuno-peroxidase staining, by using the biotin-streptavidin system, was as described previously (Matsas *et al.*, 1986). For electron microscopy the choroid plexuses from perfused-fixed brains were immunostained before being embedded as previously described (Barnes

Scheme 1. Summary of steps in preparation of membrane fractions of choroid plexuses by method A (P4) and the further centrifugation of S2 in method B to give P2a and P2b

et al., 1988b) with the use of diaminobenzidine for the peroxidase reaction, following the methods of Priestley & Cuello (1983). Blocks of spinal cord were obtained from unperfused piglets and treated similarly for light microscopy, except that CM-cellulose was not required in the preparation of the frozen blocks.

Primary antibodies

Polyclonal antibodies were generated in rabbits after immunization with purified preparations of the peptidases. RP123 was specific for E-24.11, RP133 for AP-N and RP127 for ACE. The first two had been affinity-purified by elution from a column of the antigen and the specificity of all three had been established by immunoblotting (Barnes *et al.*, 1988a). The polyclonal antibodies to DPP-IV (RP143) and aminopeptidase W (RP147) had also been affinity-purified. The former revealed a single band of M_r 130000 in immunoblotting against pig kidney microvillar-membrane proteins. The latter also revealed a strong band of M_r 130000, and some fainter bands of lower M_r . Pre-absorption by purified antigens (E-24.11, AP-N, ACE, aminopeptidase W and microsomal dipeptidase) of the appropriate antibody abolished immunostaining.

The antibody to $(Na^+ + K^+)$ -ATPase was monoclonal antibody (TK-3B5) generated after immunizing a mouse with a pig renal basolateral-membrane preparation. The antibody was produced as ascites fluid after intraperitoneal injection of the hybridoma cells and used at 1:100 dilution. In pig kidney cryostat sections it stained basolateral membranes of both proximal and distal tubules. Immunoaffinity purification of the antigen from Triton X-100-solubilized pig kidney medullary

membranes yielded a protein of two subunits (M_r 56000 and 96000) on SDS/polyacrylamide-gel electrophoresis. Confirmation of the identity of the TK-3B5 antigen was obtained from inhibition studies. The ouabain-sensitive $(Na^+ + K^+)$ -ATPase activity in a pig microsomal-membrane preparation was strongly inhibited (83%) by overnight treatment at 4 °C with TK-3B5, compared with controls, including other monoclonal antibodies.

Determination of protein

Protein was determined by the Lowry method, with bovine serum albumin as standard.

RESULTS

Stability of peptidase activities in choroid plexus stored at -70 °C

All the subcellular fractionations were performed on stocks of frozen plexuses, this being necessary in order to accumulate sufficient material for a membrane preparation. Batches of frozen choroid plexuses were homogenized and the homogenates were stored at -70 °C for a period of 6 weeks. These were compared with homogenates of fresh samples, not subjected to freezing. For most hydrolase activities the homogenates gave comparable values; thus for E-24.11, AP-N, DPP-IV, γ -glutamyltransferase and $(Na^+ + K^+)$ -ATPase the assays were within $\pm 7\%$. In two cases, ACE and aminopeptidase A, the activities were substantially higher (+33 and +50%) in the frozen sample, and in two others, aminopeptidase W and alkaline phosphatase, they were about 40% lower.

Table 1. Fractionation of choroid plexus by method A

See the experimental section for details. The results are the mean values for two preparations, each with 8 g of tissue. Recoveries are shown as percentages of the homogenate value. Enrichments are the ratio of specific activities (fraction/H). The tissues used in these preparations had been very extensively washed in saline before homogenization.

	Distribution of activity (units)					Specific activity (units/mg of protein)				
	H	P4	Recovery (%)	S2	Recovery (%)	H	P4	Enrichment	S2	Enrichment
Protein (mg)	601	2.1	(0.4)	132	(22)	—	—	—	—	—
Endopeptidase-24.11	37.4	1.6	(4.3)	10.1	(27)	0.06	0.76	(12.7)	0.08	(1.3)
Peptidyl dipeptidase A	3420	91.5	(2.7)	—	—	5.4	45	(8.3)	—	—
Dipeptidyl peptidase IV	1620	74	(4.6)	—	—	2.5	36	(14.4)	—	—
Aminopeptidase N	13050	522	(4.0)	—	—	21.7	249	(11.5)	—	—
Alkaline phosphatase	200000	5900	(3.0)	—	—	330	2880	(8.7)	—	—

Enzyme activities in membranes prepared by method A

Table 1 summarizes the results of two experiments. The yields of the five hydrolases assayed were in the range 2–5 % in the P4 fraction. This is low in comparison with the same procedure applied to rabbit kidney cortex (Booth & Kenny, 1974), where 8–13 % of the microvillar markers were found in P4. The enrichment values (range 6–14-fold) were generally similar to those for kidney cortex (11–17-fold). An immunoradiometric assay also revealed the presence of aminopeptidase W, but the absorption curve did not permit a precise value of the activity to be computed. The most unsatisfactory feature of the fractionation was the high proportion (27 %, compared with 10 % for kidney cortex) of E-24.11 activity that had not pelleted in the P2 fraction and was not available for the second $MgCl_2$ treatment that led to the final P4 pellet. For this reason the protocol was modified (method B) to include two steps at higher centrifugal forces in order to recover the membranes in S2.

Enzyme activities in membranes prepared by method B

In this protocol the S2 fraction was further centrifuged at 31000 *g* for 120 min to yield pellet P2a. Another centrifugation step (65000 *g* for 120 min) of this supernatant gave pellet P2b. However, very little enzyme activity was detected in P2b and the results in Table 2 are restricted in P2 and P2a. The recoveries of hydrolase activity in P2 were considerably higher (3–21 %) than those in Table 1, and in most instances an approximately equal amount was recovered in the P2a fraction. The enrichments in P2 for all but one of the hydrolases were comparable (4–8-fold) with the same fraction in method A. However, the specific activities were very much higher in the P2a fraction and the enrichment values were in the range 10–25-fold. The exception was carboxypeptidase M, which was poorly enriched in both P2 and P2a, suggesting that the homogenate contained significant

activity in the cytosol (carboxypeptidase N) hydrolysing Bz-Gly-Lys. In comparison with the P4 fraction obtained by method A, the specific activities of the hydrolases in P2a were about 50 % lower. However, method B had two advantages: it was quicker and the increased yield enabled more enzymes to be assayed on each batch.

Effect of peptidase inhibitors on activities in the membrane fractions

As noted in the Experimental section, the principal peptidase activities (E-24.11, AP-N, ACE, DPP-IV and aminopeptidase A) reported above had been corrected by assaying in the presence and in the absence of specific inhibitors. Table 3 shows that in a few cases some inhibitor-insensitive residual activity was observed. Thus about 10 % of the total endopeptidase activity with ^{125}I -labelled insulin B chain was phosphoramidon-insensitive in P2a, although 60 % was insensitive in the homogenate. About 4 % of the aminopeptidase activity hydrolysing Ala-NH-Mec was insensitive to amastatin. The 20 % residual activity for the hydrolysis of α -Glu-NH-Mec in the presence of 10 μM -amastatin was also observed with renal microvillar membranes and probably reflects the lower potency of the inhibitor with aminopeptidase A compared with aminopeptidase N. When 50 μM -amastatin was used in this assay only 2 % residual activity was observed. The activities hydrolysing Gly-Pro-NH-Mec and Bz-Gly-His-Leu were fully inhibited, consistent with their attributions to DPP-IV and ACE.

Immunoperoxidase staining of peptidases in the choroid plexus

Of the six peptidases for which specific polyclonal antibodies were available, only three revealed intense immunostaining of the choroid plexus, as shown in Fig. 1. The staining for E-24.11 was very strong over the

Fig. 1. Immuno-peroxidase staining of hydrolases in choroid plexus

See the Experimental section for details. Cryostat sections (10 μm) from fresh tissue were stained with polyclonal antibodies. (a) RP123 to endopeptidase-24.11 (E); (b) RP127 to peptidyl dipeptidase A (ACE); (c) RP133 to aminopeptidase N (APN). These sections were from the same block and were lightly counterstained with Harris haematoxylin to reveal nuclei. (d) was from another block and was stained with TK-3B5, a monoclonal antibody recognizing $(Na^+ + K^+)$ -ATPase. Closed arrows indicate the surface of the epithelial cells and the open arrows the vascular core. All are at same magnification. Bar = 50 μm .

Table 2. Fractionation of choroid plexus by method B

See the Experimental section for details. The results (with some exceptions) are the means of three preparations, each with 20 g of tissue (the variation indicated as $\pm$ S.E.M.). Those for aminopeptidases A, P and W, carboxypeptidases M and P, γ -glutamyltransferase and ($\text{Na}^+ + \text{K}^+$)-ATPase are from a single preparation. Recoveries are shown as percentages of the homogenate value. Enrichments are the ratios of the specific activities P2/H and P2a/H. The tissues used in these preparations were not washed before homogenization.

	Distribution of activity (units)				Specific activity (units/mg of protein)					
	H	P2	Recovery (%)	P2a	Recovery (%)	H	P2	Enrich- ment	P2a	Enrich- ment
Protein	2070	25.3 ± 7.8	(1.2)	10.3 ± 1.9	(0.5)	0.0236	0.16 ± 0.0013	(6.8)	0.320 ± 0.053	(14)
Endopeptidase-24.11	45.3	3.90 ± 1.07	(8.6)	3.05 ± 0.221	(6.7)	1.65	8.07 ± 1.8	(4.9)	25.0 ± 7.7	(15)
Peptidyl dipeptidase A	3250	225 ± 109	(6.9)	228 ± 44	(7.0)	0.93	6.18 ± 0.28	(6.7)	12.7 ± 3.2	(14)
Dipeptidyl peptidase IV	1830	164 ± 56	(9.0)	119 ± 15	(6.5)	6.59	53.6 ± 10	(8.1)	151 ± 37	(23)
Aminopeptidase N	13400	1490 ± 704	(11.1)	1430 ± 156	(10.7)	0.64	2.65	(4.2)	10.4	(16)
Aminopeptidase A	1320	109	(8.3)	82.7	(6.3)	0.10	0.79	(7.9)	1.65	(17)
Aminopeptidase W	200	19.7	(9.9)	13.0	(6.5)	—	—	—	—	—
Aminopeptidase P	N.D.*	—	—	—	—	—	—	—	—	—
Carboxypeptidase P	N.D.†	—	—	—	—	—	—	—	—	—
Carboxypeptidase M	1380	54.3	(3.9)	26.3	(1.9)	0.69	1.3	(1.9)	3.3	(4.8)
Microsomal dipeptidase	N.D.‡	—	—	—	—	—	—	—	—	—
γ-Glutamyltransferase	16800	3530	(21)	1680	(10)	8.38	86.1	(10.3)	210	(25)
Alkaline phosphatase	188000	6330 ± 601	(3.4)	13200 ± 1420	(7.0)	91.7	427 ± 52	(4.7)	1410 ± 347	(15)
(Na ⁺ + K ⁺)-ATPase	5340	625	(12)	214	(4.0)	2.67	15.3	(5.7)	26.7	(10)

* Not detectable after 3 h incubation.

† Not detectable after 4 h incubation.

‡ Not detectable after 6 h incubation.

Table 3. Effects of inhibitors on peptidases in choroid plexus

The inhibitors were preincubated with the membranes for 15 min (except Dip-F, which was preincubated for 45 min) at 37 °C before addition of the assay substrate. The results are the means $\pm$ S.E.M. for three preparations except those for aminopeptidase A (one preparation).

Enzyme	Inhibitor	Relative activity (%)		
		H	P2	P2a
Endopeptidase-24.11	1 μ M-Phosphoramidon	63 $\pm$ 4	14 $\pm$ 3	9 $\pm$ 2
Peptidyl dipeptidase A	1 μ M-Captopril	0	0	0
Dipeptidyl peptidase IV	0.1 mM-Dip-F	0	0	0
Aminopeptidase N	10 μ M-Amastatin	10 $\pm$ 2	7 $\pm$ 2	4 $\pm$ 1
Aminopeptidase A	10 μ M-Amastatin	17	16	21
	50 μ M-Amastatin	14	3	2

apical surface of the epithelial layer of the plexus (Fig. 1a, marked E), the vascular core being unstained. Fig. 1(b) shows, in contrast, intense staining for ACE in the vascular components with little or no staining visible in this section at the apical surface. AP-N staining (Fig. 1c, marked APN) is present on the epithelial surface and in the vascular elements. For comparison, the immunostaining with the monoclonal antibody (TK-3B5) to (Na⁺ + K⁺)-ATPase showed this antigen to be restricted at the apical surface (Fig. 1d, marked ATPase).

Although the light micrographs in Fig. 1 failed to reveal significant epithelial surface staining for ACE, electron micrographs of the apical pole of the epithelial cells after immuno-peroxidase staining (Fig. 2a) demonstrated clearly that all three of these peptidases, E-24.11, ACE and AP-N, are present in the brush border, where they are restricted to the membrane, being absent from the core of the microvilli. Our inability to observe brush border ACE by light microscopy indicates that the amount of the antigen is below the sensitivity of the method. The immunostaining for electron microscopy was done on free-floating pieces of tissue and the peroxidase reaction with diaminobenzidine was allowed to proceed until the staining was very intense.

Immunostaining for DPP-IV revealed weak staining of the vascular core of the plexus with none visible at the epithelial surface. Immunostaining for aminopeptidase W was near the limit of detection, but also appeared to be associated with endothelial cells (results not shown). Antibodies to other peptidases were not available. When perfused-fixed choroid plexus was examined, immuno-peroxidase staining for E-24.11, ACE and AP-N was still readily demonstrated, but that for dipeptidyl peptidase IV became very weak and none was visible for aminopeptidase W.

Immunoperoxidase staining of peptidases in the meninges

We have previously reported the presence of E-24.11, ACE and AP-N in pial membranes of pig brain (Matsas *et al.*, 1986; Barnes *et al.*, 1988a). In Fig. 2(b) sections of the leptomeninges surrounding the cervical spinal cord are shown. The three peptidases can be seen in the cord pia, as well as in the strands of membrane that occupied the subarachnoid space between the pia and arachnoid membranes, which, in the absence of CSF, was collapsed in these preparations. Vascular staining in the cord and leptomeninges for ACE and AP-N was observed and the

latter enzyme was also detected in the arachnoid membrane (Fig. 2b, APN).

The strong staining for these three peptidases of the pial membrane continued along the whole length of the cord. At the point at which a lumbar dorsal root emerges (Fig. 3) it can be seen to carry with it a sheath of pia that stained positively for E-24.11 and AP-N, as did the surface at the area of contact between the root and the cord. Staining for ACE was not noted, except in the vasculature. In the lumbar region of the cord, where many bundles of myelinated nerves ran parallel to the cord, the strong pial staining for these three peptidases was evident only where the cord was not in contact with these bundles (Fig. 4a), but here the staining for ACE was mainly in the microvasculature, with little or none in the pia. Where the section revealed the nerve bundles in close contact with the cord, no immunostaining of the pia was observed (Fig. 4b) but it was detected at the surfaces of the outermost bundles (not shown in this Figure).

Some staining for peptidases was also seen at the luminal surface of ependymal cells in the ventricles and central canal of the spinal cord. For E-24.11 and ACE any staining was weak and sporadic, for AP-N rather more positive cells were seen, whereas DPP-IV staining was much stronger and more consistent than the other peptidases (results not shown).

DISCUSSION

Peptidase activities in choroid plexus

It is noteworthy that most of the same peptidases present in the kidney microvillar membrane were identified in the choroid-plexus-membrane preparations. Their activities in the two tissues are compared in Table 4, where, with the exception of carboxypeptidase M, which was similar in the two membranes, the specific activities of choroid-plexus peptidases in P2a are distinctly lower than in pig kidney P4. For AP-N, ACE and γ -glutamyltransferase they are up to 5-fold less, but are about 15-fold less for E-24.11 and aminopeptidase A and are about 50-fold less for DPP-IV and aminopeptidase W. Microsomal dipeptidase, carboxypeptidase P and aminopeptidase P were undetectable in the choroid plexuses. It is noteworthy that two other hydrolases, alkaline phosphatase and (Na⁺ + K⁺)-ATPase, have higher activities in the choroid-plexus P2a fraction, the former 40% higher and the latter being undetectable in

Fig. 2. Immuno-peroxidase staining for endopeptidase-24.11, peptidyl dipeptidase A and aminopeptidase N in choroid plexus (a) and spinal-cord meninges (b)

See the Experimental section for details. Series (a) are electron micrographs of the apical region of epithelial cells of the choroid plexus. The immuno-peroxidase staining and OsO_4 treatment had been completed before embedding and sectioning. The primary antibodies were: E, RP123 for endopeptidase-24.11; ACE, RP127 for peptidyl dipeptidase A; APN, RP133 for aminopeptidase N. In each micrograph the brush border (arrow) shows positive staining, which is confined to the microvillar membrane. Also visible is part of the nucleus (N) and Golgi apparatus (G). No intracellular immunostaining is seen. Bar = 2 μm. Series (b) are light micrographs of adjacent sections (10 μm) of the ventral median fissure of the cervical spinal cord with associated leptomeninges (the block was dissected away from the dura mater) immunostained with RP123 for endopeptidase-24.11 (E), RP127 for peptidyl dipeptidase A (ACE) and RP133 for aminopeptidase N (APN). The sections are counterstained with Harris haematoxylin. The subarachnoid space has collapsed in the absence of the cerebrospinal fluid, but SA indicates its location. Positive immuno-peroxidase staining for all three peptidases can be seen at the pial surface of the spinal cord (solid arrows) and also in fibrous strands of membranes that *in vivo* traversed the subarachnoid space. Both peptidyl dipeptidase A and aminopeptidase N were readily detected in blood vessels (open arrows) and the latter also was prominent at the outer surface of the section where the arachnoid is associated with the connective-tissue elements of the epidural space. Bar = 50 μm.

Fig. 3. Immuno-peroxidase staining for endopeptidase-24.11 (E), peptidyl dipeptidase A (ACE) and aminopeptidase N (APN) in leptomeninges of lumbar spinal cord

See the Experimental section for details. The three adjacent sections show the leptomeninges at the point of emergence of a dorsal root. SC, spinal cord; DR, dorsal root. The root is ensheathed by the pia (P), which shows immunostaining in E and AP-N, as, too, do the remnants of the arachnoid (A); these aspects are missing in ACE. Note the abrupt transition (arrow) as the root acquires the characteristics of a peripheral nerve: beyond that point Schwann-cell staining can be detected in E and perineurium staining in AP-N.

Fig. 4. Immunoperoxidase staining for endopeptidase-24.11 (E), peptidyl dipeptidase A (ACE) and aminopeptidase N (AP-N) on structures adjacent to the lumbar spinal cord

See the Experimental section for details. The two series (*a*) and (*b*) at the same level of the cord each show three adjacent sections. In (*a*) strong staining for E and AP-N can be seen in the pia (closed arrows). This is absent for ACE, but this peptidase, like AP-N, is revealed in vessels (open arrows). In (*b*) many nerve bundles are seen immediately adjacent to the cord. At the area of contact (arrows), no staining of the pia is visible. Vascular staining can be seen for ACE and AP-N and the latter is also seen in the perineurium. Bar = 50 μ m.

Table 4. Comparison of hydrolases in renal microvillar and choroid-plexus membranes in the pig

All values for choroid plexus, and those for kidney marked with an asterisk (*), were determined during this study; the other values for kidney are from Hooper & Turner (1988a,b). Abbreviation: N.D., not detectable.

Enzyme	Specific activities (units/mg of protein)		Ratio K/CP
	Choroid- plexus membranes (CP) (fraction P2a)	Kidney microvillar membranes (K) (fraction P4)	
Endopeptidase-24.11	0.32	8.0*	15
Peptidyl dipeptidase A	25	131*	5.2
Dipeptidyl peptidase IV	13	606	47
Aminopeptidase N	151	524	3.5
Aminopeptidase A	10.4	126	12
Aminopeptidase W	1.7	120*	71
Aminopeptidase P	N.D.	68	—
Carboxypeptidase P	N.D.	5.9	—
Carboxypeptidase M	3.3	3.7	1.1
Microsomal dipeptidase	N.D.	121	—
γ -Glutamyltransferase	210	650*	3.1
Alkaline phosphatase	1410	880*	0.6
(Na ⁺ + K ⁺)-ATPase	27	N.D.	—

this preparation of kidney P4, in line with the atypical apical location of the ATPase in choroid plexus.

Previous studies of ACE in choroid-plexus membranes have indicated it to be much more abundant than in tissues, such as lung and kidney, from which the enzyme is often purified (Arregui & Iversen, 1978; Rix *et al.*, 1981). Thus in the rat the specific activity appeared to be 6–40-fold higher than in the lung and 1700-fold higher than in the kidney, in striking contrast with our results, which show kidney activity to be 5-fold higher. However, there may be a trivial explanation why lung and kidney values were underestimated in these earlier studies, the assays for which depended on the determination of His-Leu. This dipeptide is rapidly hydrolysed by microsomal dipeptidase, which is abundant in kidney and lung but not detectable in choroid plexus. Our assay depended on the detection of Bz-Gly by h.p.l.c. and was therefore unaffected by any further hydrolysis of His-Leu.

Heterogeneity of the P2a fraction

Most reports on receptors and enzymes in the choroid plexus have been on unfractionated membranes. Others, such as those of Langenbeck & Kinne (1980), Ross & Wright (1984) and the present study, have included a precipitation step with Mg²⁺ or Ca²⁺ ions, based on methods devised earlier for the preparation of microvillar-membrane vesicles (Schmitz *et al.*, 1973; Booth & Kenny, 1974). In regard to pig choroid plexuses, it is clear from our immunohistochemical studies that some peptidases have a dual localization in both epithelial and endothelial cell surfaces. Yet the enrichment values for E-24.11 and (Na⁺ + K⁺)-ATPase in P4 or P2a fractions were not higher than those for AP-N and ACE, even though the first pair are unique to the brush border,

whereas the latter pair appear to be more abundant in the vascular core. Thus endothelial-cell membranes are a major component of these fractions and the finding of a high activity of any enzyme in such a fraction cannot be taken as evidence of its location on the surface of the epithelium. Since the surfaces of the two cell types are in contact with either CSF (in the case of epithelial cells) or blood (in the case of the endothelium), the potential substrates of the peptidases and their possible physiological roles may be quite different. An improved method of fractionation is necessary and it might be fruitful to exploit immunochemical approaches that can discriminate epithelial from endothelial surfaces.

Functional significance

An important finding of these studies is that the cell surfaces in contact with CSF are richly endowed with peptidases. E-24.11 is present on virtually the entire surface bathed by CSF, and so too is AP-N. ACE on the other hand is present on the epithelium of the choroid plexus and on the brain pial membrane, but is not a significant enzyme in the cord pia. The arachnoid was in part removed with the dura before the blocks were sectioned, but often some remnants persisted and these too were positively stained for E-24.11 and AP-N. As in the brain (Matsas *et al.*, 1986; Barnes *et al.*, 1988a), E-24.11 was not observed on endothelial surfaces, in contrast with ACE and AP-N. Thus susceptible peptides on the vascular side of the blood/brain barrier may be degraded by ACE and AP-N, but not by E-24.11. In contrast, any CSF peptides will be potential targets for all three peptidases and, since E-24.11 has a large repertoire of potential neuropeptide substrates, this enzyme may play a key role in regulating neuropeptide concentrations in the CSF. The potential substrates include tachykinins, enkephalins, bradykinin, cholecystokinin octapeptide (Matsas *et al.*, 1983, 1984a,b), angiotensins and atrial natriuretic peptide (Stephenson & Kenny, 1987a,b), and, in the case of the last-named, functional receptors have been detected in the choroid plexus. In addition, E-24.11 and other peptidases, in contact with the CSF, might exert a protective role by ensuring that peptides that gain access to the CSF from within the CNS would be prevented from reaching, by the circulation of the CSF, distant but possibly responsive sites. In support of this possibility, radioimmunoassays of atrial natriuretic peptide and substance P have indicated that the concentrations of these peptides are an order of magnitude lower in the CSF than in plasma (Pernow, 1983; Roesler *et al.*, 1986; Marumo *et al.*, 1987).

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