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Immunohistochemical Localization of Aminopeptidase M in Rat Brain and Periphery: Relationship of Enzyme Localization and Enkephalin Metabolism

LOUIS B. HERSH,[1] NABIL ABOUKHAIR AND STANLEY WATSON

Department of Biochemistry, University of Texas Health Science Center at Dallas, Dallas, TX 75235 and Mental Health Research Institute, University of Michigan School of Medicine, Ann Arbor, MI 48109

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HERSH, L B , N. ABOUKHAIR AND S WATSON *Immunohistochemical localization of aminopeptidase M in rat brain and periphery Relationship of enzyme localization and enkephalin metabolism.* PEPTIDES 8(3) 523–532, 1987 —An antiserum specific for rat aminopeptidase M has been used for the immunohistochemical localization of the enzyme in rat brain and peripheral tissues. The enzyme in brain is localized exclusively on blood vessels. Within the pituitary the enzyme was associated with the vasculature in the posterior lobe, on the surface of the intermediate lobe and on the surface of some cells in the anterior lobe In the liver, fine cell staining was observed between parenchymal cells, in the ileum the entire lumenal surface was stained, while in the kidney both proximal tubular and a central tubular staining was detected In each tissue aminopeptidase M is localized such that it can limit diffusion across specific barriers Aminopeptidase M activity in brain has been proposed to function in the degradation of synaptically released enkephalins. Its localization on blood vessels requires that enkephalins diffuse prior to degradation, a concept not in concert with current hypotheses Based on these studies it is proposed that diffusion away from enkephalinergic synapses plays a key role in terminating enkephalin action

Aminopeptidase M Immunohistochemistry Enkephalin degradation in brain

IT is currently believed that the physiological action of synaptically released enkephalins is terminated by enzymatic degradation [12, 21, 22]. Two peptidase activities have been identified in synaptic membrane preparations which could act as "enkephalinases" *in vivo*. One of these is a neutral metalloendopeptidase which cleaves enkephalins at the Gly-Phe bond, while the other is an aminopeptidase which cleaves at the Tyr-Gly bond of the enkephalins [12, 21, 22].

Two membrane bound aminopeptidases were identified in rat synaptic membrane preparations and designated MI and MII [11,13]. Aminopeptidase MII exhibits a broad substrate specificity with regard to amino acid beta naphthylamides and has been shown to represent the major enkephalin degrading aminopeptidase activity in brain homogenates [11]. Its distribution in rat brain approximates the distribution of enkephalin receptors [13]. The enzyme is inhibited by the classical aminopeptidase inhibitor bestatin but can be distinguished from other membrane aminopeptidases by its sensitivity to inhibition by puromycin [11,13].

Although this enzyme appeared to represent the major aminopeptidase which could act as an "enkephalinase" *in vivo*, several studies have raised questions concerning the role of aminopeptidase MII in degrading synaptically released enkephalins. Using brain slices it was shown that individually the neutral endopeptidase inhibitor thiorphan and the general aminopeptidase inhibitor bestatin could protect endogenous enkephalins, released by potassium depolarization, against degradation [5,6]. Together these inhibitors afforded almost complete protection against enkephalin degradation. In addition these inhibitors alone or in combination produced an analgesic effect in mice However, the aminopeptidase MII inhibitor puromycin, which is relatively specific for this enzyme, was ineffective in protecting synaptically released enkephalins from degradation and did not elicit an analgesic response in experimental animals [3, 5,

[1]Requests for reprints should be addressed to Louis B. Hersh, Department of Biochemistry, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235

6] However in another study [10] puromycin produced a dose-related, naloxone reversible analgesia in rats which was accompanied by an increase in striatal enkephalin levels.

An apparent solution to this confusion was the recent finding of aminopeptidase M activity in rat brain synaptic membrane preparations [8, 9, 17]. This enzyme was separated from aminopeptidase MII by either chromatography on conA-sepharose [8,9] or by phase separation using Triton X-114 [17]. Aminopeptidase M (APM) activity comprised approximately 5% of the total membrane aminopeptidase activity. The enzyme hydrolyzes enkephalins with a Km of 45 μM It is potently inhibited by bestatin (Ki=4 μM), but poorly inhibited by puromycin (Ki=100 μM). By comparison aminopeptidase MII exhibits a Ki of 0.5 μM for bestatin and a Ki of 1 μM for puromycin. Both of these aminopeptidases can be distinguished from aminopeptidase MI [11] The latter enzyme is specific for basic aminoacid beta naphthylamides, is considerably less sensitive to puromycin (Ki=1 mM), and does not hydrolyze enkephalins at an appreciable rate [8].

Thus if aminopeptidase M could be shown to exist at enkephalinergic synapses or in the neuropile of enkephalin rich areas its involvement in the degradation of synaptically released enkephalins would be more firmly established. In the present paper we report the results of immunohistochemical studies on the localization of aminopeptidase M in rat brain and selected peripheral tissues.

METHOD

Purification of Aminopeptidase M

Aminopeptidase M was purified from rat kidney using a modification of the procedure used by Gros *et al.* [8]. Rat kidneys were homogenized in 10 volumes of 50 mM Tris-HCl buffer, pH 7.5. The membrane fraction was obtained as described [8] and solubilized by resuspension in a volume of 50 mM Tris-HCl, pH 7.5 containing 1% Triton X-100 equal to the original weight of tissue. After stirring for 1 hour at room temperature, the material was centrifuged at 100,000 × g for 1 hour. To the 100,000 × g supernatant was added $MgCl_2$, $MnCl_2$, and $CaCl_2$ to a final concentration of 1 mM and 2-mercaptoethanol to a final concentration of 5 mM. The enzyme was then chromatographed on Con A-Sepharose and DEAE-cellulose as described by Gros *et al.* [8] Starting with 50 g or rat kidney 1.6 mg of essentially homogeneous enzyme was obtained.

Assay of Aminopeptidase Activity

Aminopeptidase activity was measured with alanine beta-naphthylamide as substrate as previously described [13]. Reaction mixtures contained 0.2 mM alanine beta-naphthylamide, 50 mM Tris-HCl buffer, pH 7.4, and enzyme in a final volume of 250 μl. Protein was determined by the method of Bradford [1].

Aminopeptidase M Antisera

The anti-rat kidney aminopeptidase M antisera used in these studies was that prepared by Tsao and Curthoys [24] and provided to us as a generous gift.

Immunohistochemistry

Aminopeptidase M immunohistochemistry was conducted by standard procedures [23]. Male Sprague-Dawley rats were either untreated or injected with colchicine (200 μg/10 μl of normal saline) in the lateral ventricle. After 48 hours of colchicine treatment the rats were perfused with 4% neutral buffered formaldehyde. The brains were removed and postfixed for 1 hour in 4% formaldehyde, immersed in 15% sucrose overnight and then frozen in liquid nitrogen. The tissue was cryostat sectioned at 10 μM and mounted onto gelatin subbed slides. Aminopeptidase M antiserum was used at titers ranging from 1/100 to 1/1,000 on whole coronal sections taken throughout rat brain. Sections were taken in a similar fashion from pituitary, ileum, kidney and liver Tissue sections were incubated overnight at 4°C with the APM antisera, then washed in phosphate buffered saline (PBS). Goat anti rabbit serum was applied to the tissue and sections incubated overnight at 4°C The tissue was washed in PBS, incubated at 37°C for 40 minutes with rabbit anti horseradish peroxidase (HRP), washed again in PBS and then incubated at 37°C for 40 minutes with 4 μg/ml HRP enzyme. After completion of this step the slides were immersed in a solution of 0.125 mg/ml diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 for 15 minutes The tissue was then washed for 30 minutes in water immersed in 2% OsO_4 briefly, dehydrated and mounted in Permount. Observations were made on a Leitz Orthoplan Microscope.

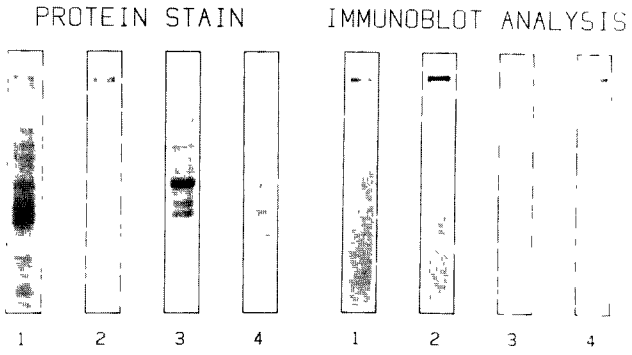


FIG 1 Immunoblot detection of rat kidney and rat brain aminopeptidase M Aminopeptidase M from various samples of rat kidney or rat brain was subjected to SDS-PAGE and either stained for protein with Coomassie Blue or electrophoretically transferred to nitrocellulose and visualized with anti-aminopeptidase M antisera The samples tested were as follows Lane 1—20 μg of crude rat kidney homogenate Lane 2—2 μg of purified rat kidney APM Lane 3—20 μg of rat brain homogenate Lane 4—10 μg of rat brain aminopeptidase M eluted from con A-sepharose

Blocking competition studies, using purified rat kidney aminopeptidase M enzyme (2.5 mg/ml), were carried out at two antiserum concentrations (1/100 and 1/1,000) and at several enzyme concentrations (12.5 μg, 2.5 μg, 0.5 μg, 0.25 μg, 0 025 μg, per 100 μl incubation volume). Thirty minutes prior to addition of serum to the section, enzyme solution was added to antiserum. A parallel control study with enzyme buffer only was carried out with the same volumes as the 12.5 μg concentration of enzyme.

Other Methods

SDS-PAGE and immunoblot analysis was conducted as previously described [2].

RESULTS

The presence of aminopeptidase M activity in brain has

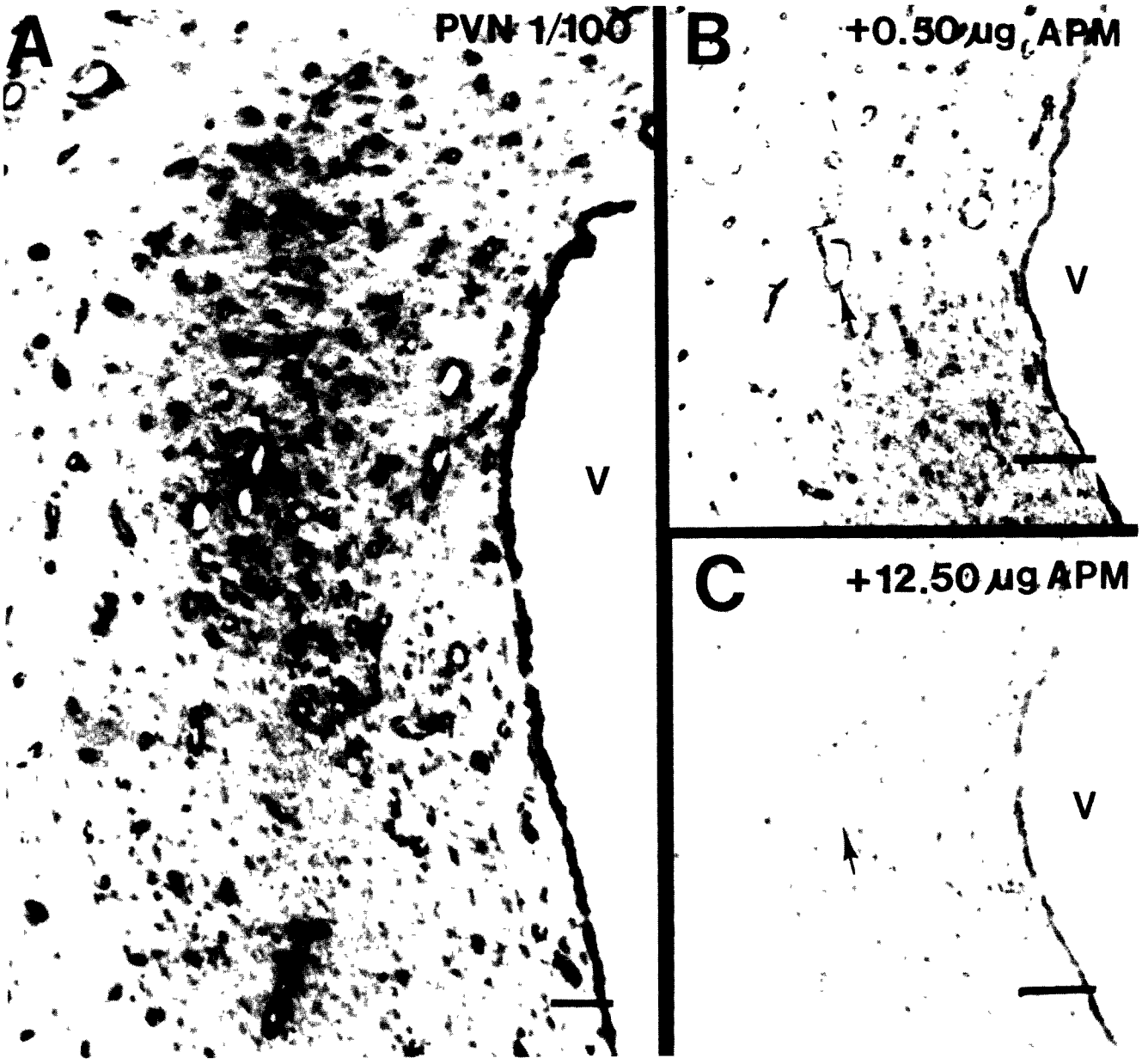


FIG. 2. Paraventricular nucleus of hypothalamus (PVN) was stained by APM antiserum at 1/100 dilution. Panel A shows four distinct features—a darkly stained ependymal lining, darkly stained blood vessels and lightly stained neuropile and nuclei Panels B and C show blocking with increasing concentrations of purified APM protein (0 5 μg and 12.5 μg of APM protein). Neither blocking control shows changes in nuclear or neuropile staining, while partial loss of ependymal and complete loss of vascular staining is found in Panel C. Calibration bar Panel A=50 μm, Panels B and C=200 μm (v=third ventricle)

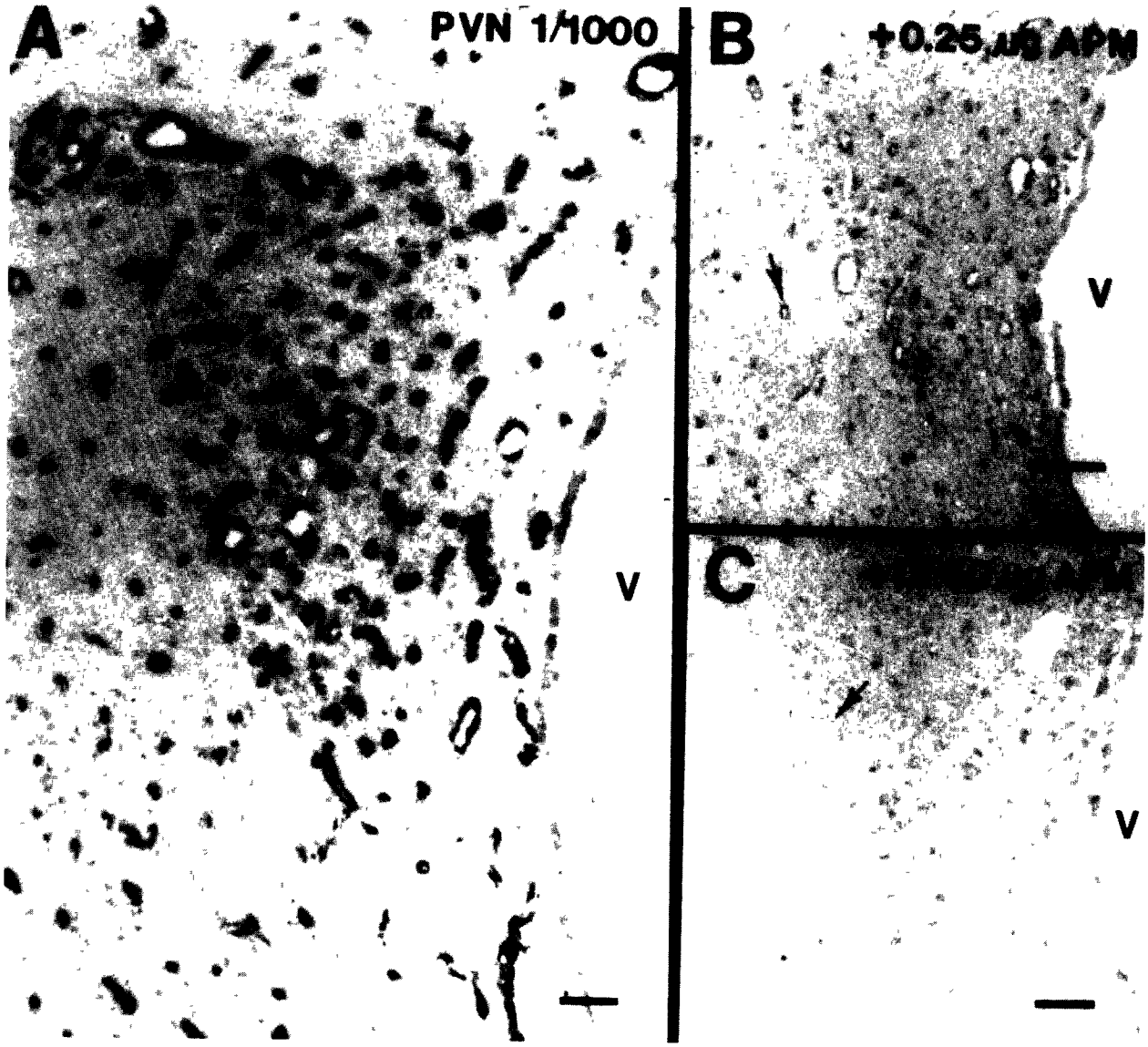


FIG 3 Same treatment as Fig 1 except that the PVN is stained with APM antiserum at 1/1,000 dilution At this concentration, Panel A shows only vascular staining, while 0 25 μg and 12 5 μg of APM protein (Panels B and C) increasingly blocked that stain Calibration bar Panel A=50 μm (v=third ventricle)

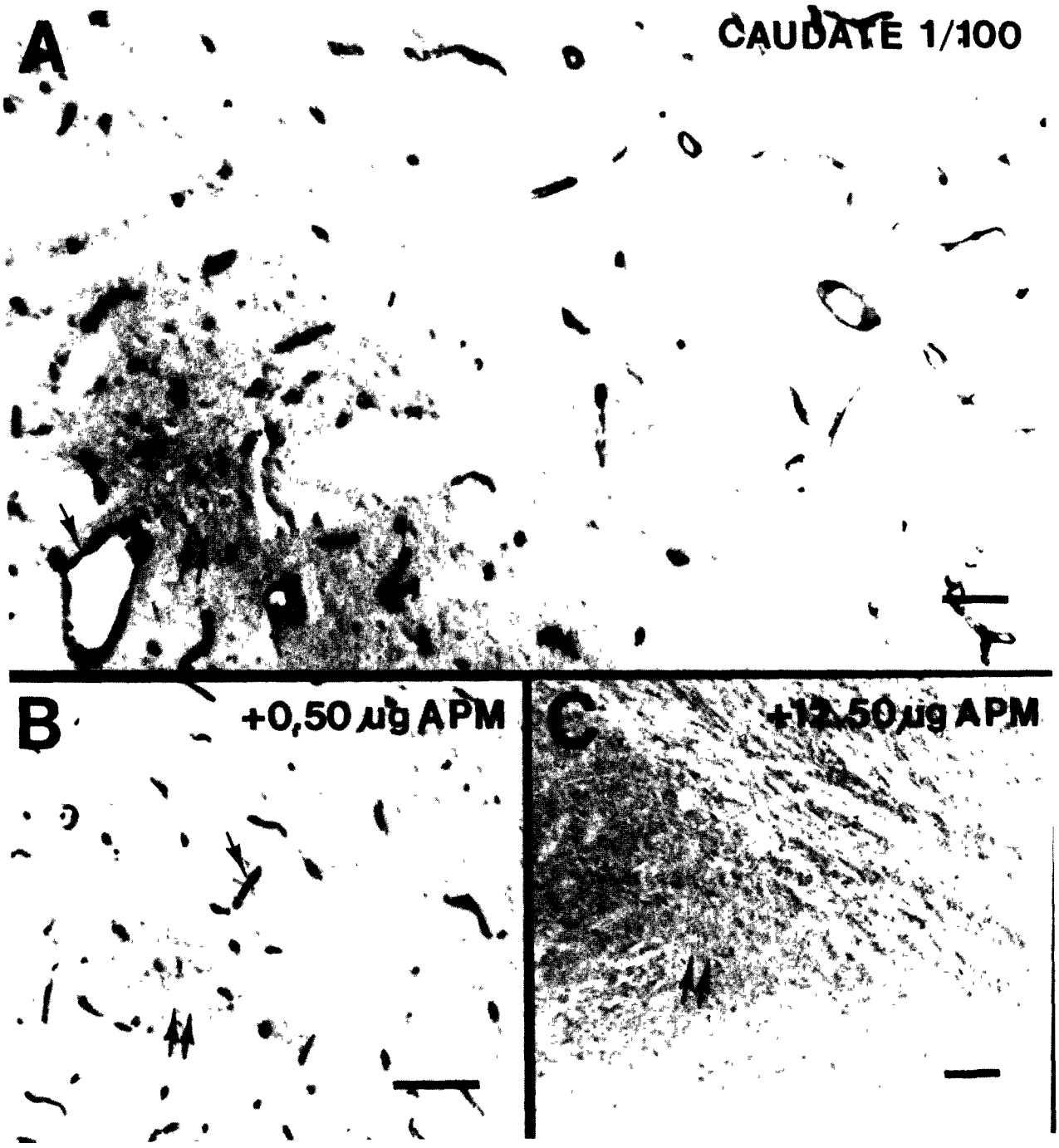


FIG 4. Caudate and corpus callosal areas are stained by APM antiserum at 1/100 dilution Panel A shows heavy vascular (single arrow) and light neuropile (double arrow) staining As in Figs 1 and 2, 0.5 μg and 12.5 μg of APM protein increasingly blocked the vascular stain but not that found in the neuropile Calibration bar Panel A=50 μm, Panel B=100 μm, Panel C=50 μm

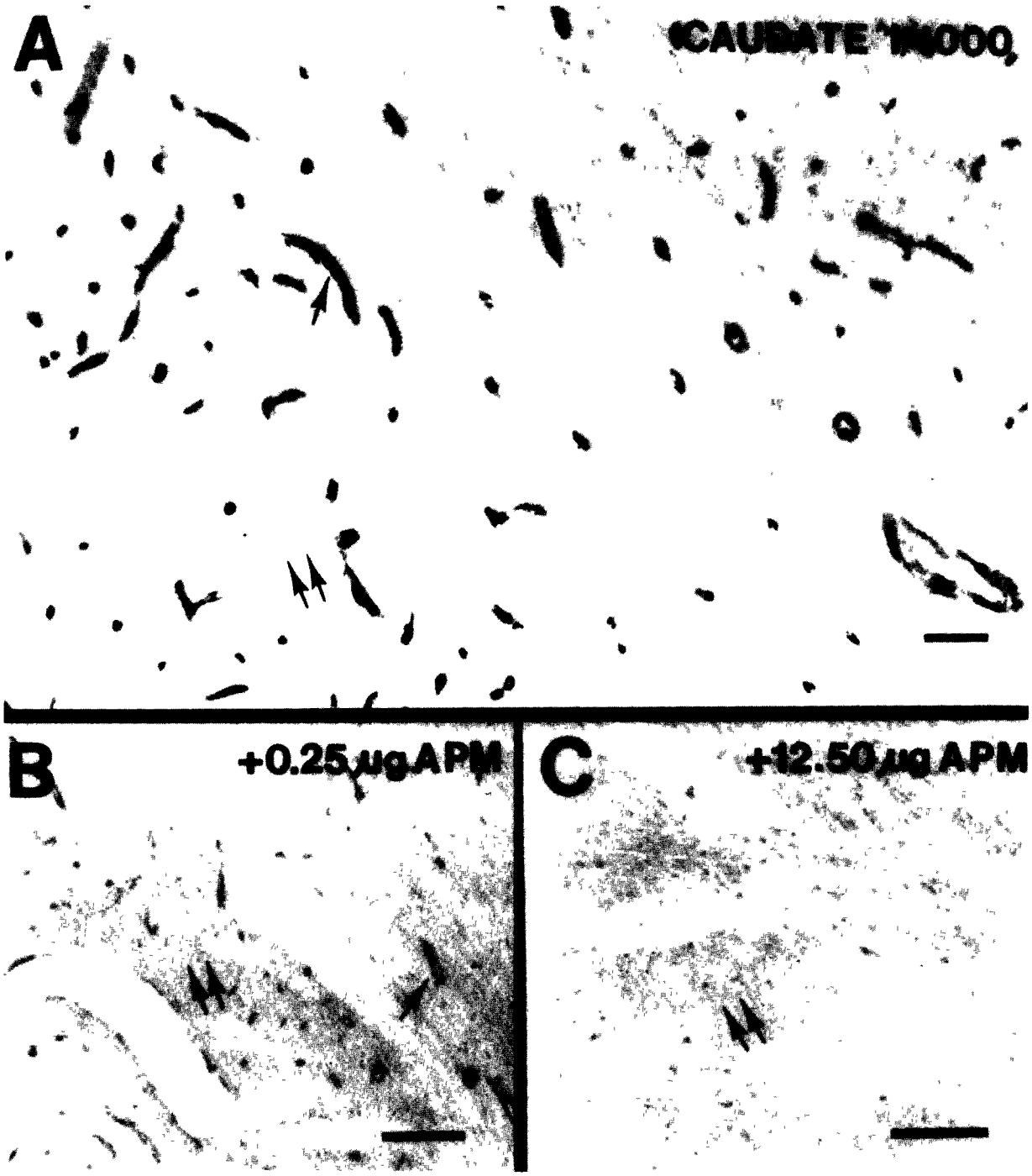


FIG. 5 Caudate and corpus callosal areas stained by APM antiserum at 1/1000. Panel A shows the light neuropile (double arrows) and heavy vascular stain (single arrow). Panel B and C show increasing blockade of the vascular staining, but not the neuropile, with APM protein. Calibration bar Panel A=50 μm, Panel B and C=100 μm.

recently been demonstrated by Gros *et al.* [8,9] and by Matsas *et al.* [17]. We have confirmed this observation using Con A-Sepharose to resolve aminopeptidase M from aminopeptidase MII activity as described by Gros *et al.* [8]. Aminopeptidases MI and MII do not bind to Con A-Sepharose, while aminopeptidase M activity is retained by Con A-Sepharose and subsequently eluted with 0.2 M methyl alpha-D-mannoside. Approximately 3% of the total aminopeptidase activity obtained from a Triton extract of rat brain membranes is attributable to aminopeptidase M activity when assayed with alanine beta-naphthylamide as a substrate. To confirm that the aminopeptidase activity bound to Con A-Sepharose is indeed aminopeptidase M, the ability of an anti-rat kidney aminopeptidase M antiserum to inhibit this activity was examined. This antisera showed essentially the identical inhibition curve with rat kidney aminopeptidase M as with the putative rat brain aminopeptidase M activity. No inhibition of aminopeptidase MII activity was observed with this antisera. Adsorption of the antisera with purified rat kidney aminopeptidase M abolished both the ability of the antisera to inhibit both the brain and kidney activities as well as the ability to visualize either protein by immunoblotting. In addition 25 μM puromycin inhibited aminopeptidase MII activity more than 90% while aminopeptidase M activity was inhibited less than 20% at this concentration of puromycin. Both kidney and brain aminopeptidase M activities were inhibited greater than 90% with 15 μM bestatin. These results serve to establish the identity of the kidney and brain aminopeptidase M.

The specificity of the anti-aminopeptidase M antisera was examined by immunoblot analysis. As shown in Fig. 1 this antiserum binds to only a single protein in a crude rat kidney homogenate which corresponds to the purified enzyme. The level of aminopeptidase activity in brain is too low to be detected in a crude homogenate by immunoblot analysis. However we were able to visualize a partially purified preparation of the rat brain enzyme. The absence of any detectable immunoreactivity with a crude rat brain homogenate as well as the finding that only aminopeptidase M reacts with the antiserum in a crude kidney homogenate demonstrates that this antiserum is monospecific for aminopeptidase M and exhibits no cross-reactivity with any proteins found in rat brain. Based on the above experiments this antiserum was judged suitable for immunohistochemical visualization of the enzyme in rat brain.

The anti aminopeptidase M antiserum stained rat brain at concentrations up to (1/1,000). At 1/100 the staining of brain was composed of three types of signals, modest nuclear and cytoplasmic stains throughout the neuropile, and a denser stain surrounding all blood vessels (Figs. 2 and 4). There was no obvious preference of particular brain regions, as all seemed positive. At 1/1,000, only the vascular staining could be detected (Figs. 3 and 5). Because colchicine pretreatment was indistinguishable from untreated rats, all data are on normal, untreated rats.

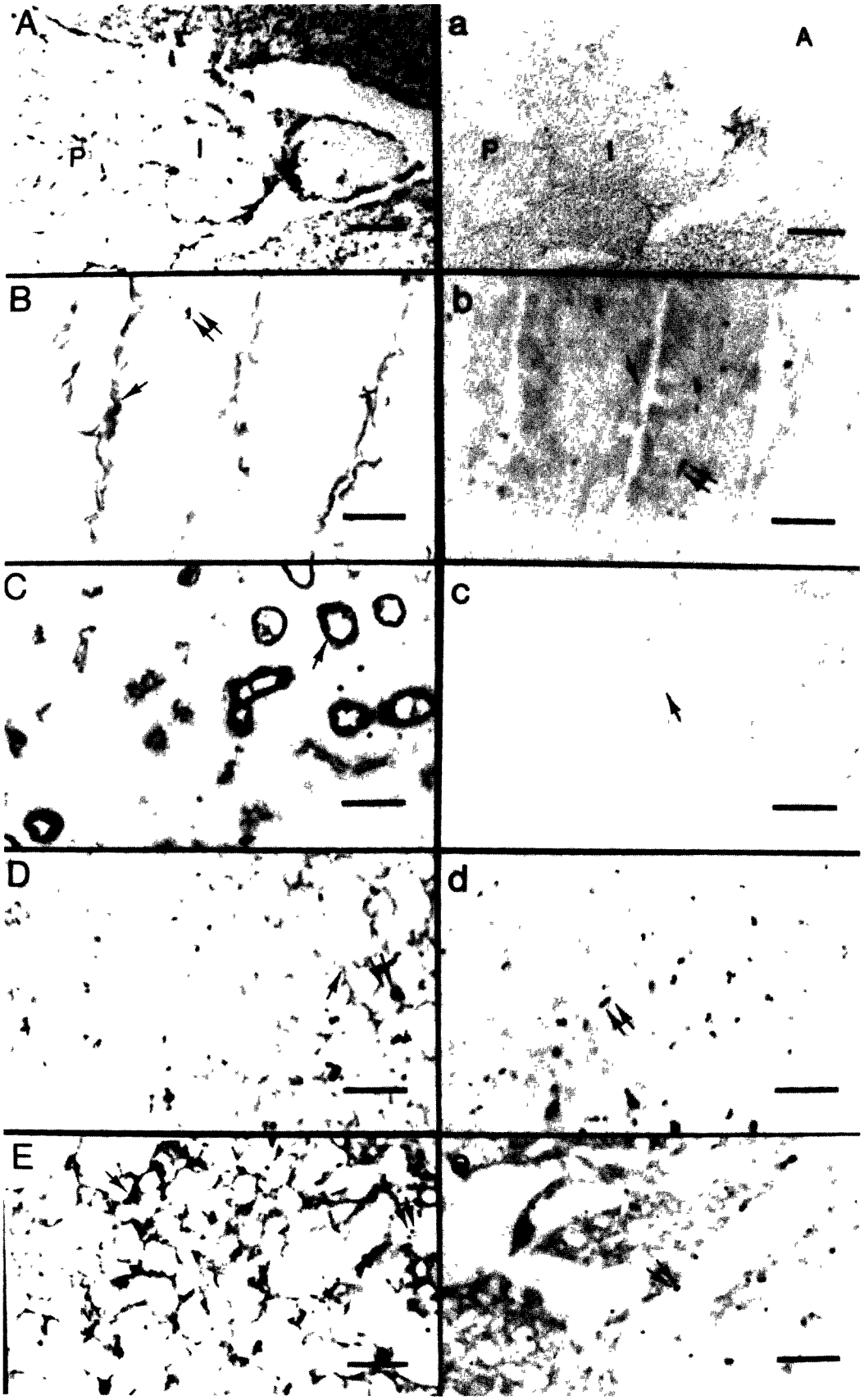
Because it was unclear which of the staining patterns was specific (vascular, nuclear or cytoplasmic) we carried out solution-phase competition studies at 1/100 and at 1/1,000 antibody dilution. At 1/100 the enzyme buffer solution did not alter the staining patterns. However 12.5 μg of APM enzyme (and 2.5 μg of APM enzyme) completely blocked all vascular staining without obviously effecting the nuclear and cytoplasmic staining (Figs. 2C and 4C). At 0.5 μg of APM enzyme, return of faint vascular staining was detected (Figs 2B and 4B), with increasingly clear staining as the concentration of antigen dropped (0.25 μg, 0.025 μg). At 1/1,000 titer the buffer had no effect on the only staining in the section (around the vasculature). All staining was blocked at 12.5 μg, 2.5 μg and 0.5 μg of APM enzyme (Figs. 3C and 5C) beginning to return at 0.25 μg (Figs. 3B and 5B) and increasing as the antigen concentration dropped. Thus specific, blockable staining was only detected in the region of brain vasculature and not in the nuclei, cytoplasm or the neuropile. Beyond the vasculature the other set of apparently stained structures included the choroid plexus, ependyma and dura. After blocking it became clear the choroid plexus was not specifically stained. However, both the dura and ependyma were only partially blocked suggesting the possibility of a modest amount of APM in those tissues.

The pituitary showed blockable staining in all three lobes at 1/1,000 titer (Fig. 6A and a). In the posterior pituitary the staining again appeared to be associated with vasculature. In the intermediate lobe only its surface was immunopositive. Small regions of patchy immunoreactivity, apparently at the cell surface, were seen in the anterior lobe. In the liver fine cell surface staining could be detected between parenchymal cells (Fig. 6D). In the rat ileum the entire lumen was positively stained over the surface of the villus projections (Fig. 6B). In the kidney two specific staining patterns could be seen (Fig. 6C). One is a very heavy proximal tubular stain and the second is a lighter more central tubular stain. No glomeruli were stained. In the adrenal medulla patches of APM were detected (Fig. 6E) in the cortex a lower level of staining was also seen.

DISCUSSION

The results of these studies clearly demonstrate that aminopeptidase M activity in rat brain is localized primarily if not exclusively on blood vessels. Beyond this vascular pattern in the brain the localization of APM on proximal tubules in the kidney, between parenchymal cells of the liver, on some cell surfaces in the anterior pituitary and adrenal medulla, on the luminal surface of the small gut—all suggest a unique physiological role for APM. In each case this enzyme is localized so that it can limit diffusion of peptides across certain barriers. The broad substrate specificity of aminopeptidase M is consistent with its role in hydrolyzing a broad spectrum of peptide substrates which differ amongst different tissues. APM in brain vessels might limit contact between blood born materials and neurons; in kidney APM in the tubules may degrade peptides and thereby prevent their reentry into the vasculature. In the gut, APM on the surface of the lumen might also control access of consumed substances to the vasculature. In a similar fashion APM on liver parenchyma, adrenal medulla and anterior pituitary cell surface may help limit peptide diffusion through these sinusoidal systems. It should be noted that APM's vascular localization in other tissues has previously been demonstrated [19].

Given this general distribution pattern, the evidence that aminopeptidase M acts as an "enkephalinase" in brain must be reconciled with its barrier type localization. It has been assumed that the analgesic effect of the aminopeptidase inhibitor bestatin is directly related to its inhibition of the enzyme [8,9]. Inhibition of the enzyme would decrease the rate at which endogenously released enkephalins are inactivated. Enkephalin levels would increase, which in turn would lead to a greater occupancy of enkephalin receptors. Support for this hypothesis comes from the observation that


A
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bestatin increases the level of intact enkephalin which can be recovered after potassium depolarization of brain slices [5,6] or after substance P induced enkephalin release from rat spinal cord [4]. A tacit assumption of this model is that the enkephalin degrading peptidases which act as "enkephalinases" *in vivo* are located at synaptic clefts between enkephalinergic neurons analogous to the localization of acetylcholinesterase at cholinergic synapses.

The current light microscopic study offers little support for aminopeptidase M localization in the neuropile and therefore does not support synaptic region localization. The heavily vascular localization in the brain reported here is a very broad CNS pattern and is not distributed in a fashion unique to opioid receptors or enkephalin terminals. It has in fact been seen in all brain areas studied and has been shown to be an enzyme with rather broad peptide actions [19]. Thus the model that enkephalin degrading peptidases which act as "enkephalinases" *in vivo* are located at enkephalinergic synapses is not supported by this study. Instead a model in which enkephalins diffuse away from enkephalinergic synapses and are then degraded distal to the receptor appears more consistent with our findings.

The neutral endopeptidase called "enkephalinase" has also been implicated as acting as an "enkephalinase" *in vivo* [12, 21, 22]. This enzyme has recently been found to be localized on glial cells and not on neurons [15,16]. Thus, like aminopeptidase M, this enzyme could not easily exert its "enkephalinase" action directly at enkephalinergic synapses.

The question that remains to be answered is how the enkephalin degrading peptidases can act as "enkephalinases" *in vivo*, and yet be located outside enkephalinergic synapses. One possibility is that a minor fraction of these enzymes does in fact exist in the neuropile at enkephalinergic synapses. If only a small fraction of APM was located at enkephalin synapses it is unlikely that the current methods could detect it. Multiple forms of the neutral endopeptidase "enkephalinase" have been observed [7,20], and one of these subforms could represent a synapse specific form of the enzyme. However no such multiple forms of aminopeptidase M have been reported, and no evidence that the multiple "enkephalinase" forms have different cellular localization has been obtained.

A model that can be considered is one in which the levels of synaptically released enkephalins exceed that required by the enkephalin receptors. Diffusion of free enkephalin away from the vicinity of its receptor might reduce its concentration below the Kd of the receptor. However, the extracellular space through which the enkephalins diffuse is of a limited finite volume. The concentration of free enkephalins would initially be rapidly decreased as they move through a large concentration gradient, but would remain relatively high as the concentration of free enkephalins distal to the receptor increases. The resultant effect would be a pool of free enkephalins which exists for a relatively large time in the vicinity of enkephalin receptors. However, if enkephalin degrading peptidases act in proximity to enkephalinergic synapses the diffusion of enkephalins away from their receptor would be facilitated by the maintenance of a large concentration gradient. The function of "enkephalinases" thus *might be* to maintain a concentration gradient to ensure the efficient diffusion of enkephalins away from their receptors.

The functioning of peptidases in this manner would explain how they serve as "enkephalinases" with their localization being distal to enkephalinergic synapses. In addition, the lack of specificity known to exist in all of the peptidases believed to act as "enkephalinases" *in vivo* would not be inconsistent with such a model. The action of these peptidases distal to enkephalinergic receptors would not require the high degree of specificity seen in an enzyme such as acetylcholinesterase, and would permit these peptidases to service a variety of peptidergic systems. For example angiotensin converting enzyme [12,21] and the enkephalin degrading aminopeptidases M and MII [14] hydrolyze opioid as well as non-opioid peptides. The neutral endopeptidase "enkephalinase" acts on a variety of opioid as well as non-opioid peptides [14] and it has been proposed that this enzyme is the major substance P degrading peptidase in brain [18].

Thus we propose that "enkephalinases" function not by inactivating enkephalins at enkephalinergic synapses, but rather in rough proximity to these synapses. The role of these peptidases may be to facilitate the diffusion of enkephalins away from their receptors.

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FIG 6. Several other tissues showed signs of specific APM staining at 1/1,000. pituitary (A, a); ileum (B, b), kidney (C, c), liver (D, d), and adrenal medulla (E, e) The positive stains are seen in panels indicated by capital letters and the APM blocked controls by panel with lower case letters Blocking was carried out at 12.5 μg of APM protein (all calibration bars=100 μm) Panels A and a All three lobes of pituitary show specific APM staining. In the posterior lobe (P) it seems to be mainly vascular In the intermediate lobe (I) it is on the superficial aspects of the lobe. In the anterior lobe (A) scattered positive structures are seen. Panels B and b The superficial aspect of the villus processes (projecting into the gut lumen) of ileum shows strong positive APM staining (single arrow) Double arrows point to unblocked RBCs. Panels C and c The inner aspect of proximal tubules of the kidney were heavily positive (single arrow) for APM, but glomeruli were not. Panels D and d: Liver parenchymal cells (single arrow) show specific stains in the region of cell-cell contacts. Double arrows indicate non-specific residual peroxidase staining in red cells Panels E and e: Adrenal medullary cells (single arrow) showed specific cell surface staining. Double arrows indicate non-specific peroxidase staining.

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