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New Fluorescent Histochemical Method for the Visualization of Aminopeptidase N activity

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New fluorogenic substrate for aminopeptidase N – L-Ala-6-hydrazido-2-butyl-1,8-naphthalimide is synthesized and used for the histochemical localization of the enzyme in tissue sections of rat organs. The enzyme activity liberates the fluorochrome N-butyl-6-hydrazino-1,8-naphthalimide, which reacts simultaneously with some aromatic aldehyde, presented in the incubation medium to form a perfectly water-insoluble hydrazone. The last compound precipitates on the places of enzyme activity and marks them by an intensive red fluorescence. The method permits precise enzyme localization at a very low or none fluorescent background noise. It is considered promising for future applications in the histochemical studies not only of aminopeptidase N, but also of other peptidases after synthesizing the appropriate substrates.

Key words: Aminopeptidase N, Naphthalimide derivatives, Fluorescent methods, Enzyme histochemistry.

Introduction

Development of new fluorescent methods is presently an important direction in the progression of enzyme histo- and cytochemistry, which is due to their high sensitivity and selectivity. However, the fluorescent methods for peptidases are very scarce and are usually applied with success only for the visualization of enzymes in cultured cells, but not in tissue sections. Since many peptidases are used as marker enzymes for cell growth and differentiation as well as for different disorders, it is of great importance to develop new fluorescent methods for their study in situ.

Aminopeptidase N (APN, EC 3.4.11.2) is a membrane-bound enzyme, which cleaves single amino acids from the amino terminus of peptides. The enzyme acts preferably on alanine moieties. Its pH-optimum is broad (6.0 – 8.0) and depends on the substrate used. APN belongs to the enkephalin-degrading enzyme family [6, 8]. It is identical to CD 13 marker in monocyte membrane [12] and plays an important role in the anti inflammatory processes [7]. Histochemical visualization of APN is performed using chromogenic methods, since the only available fluorescent method is not accepted for routine applications due to the numerous artifacts involved [5].

In the present study we present a newly synthesized fluorogenic substrate for APN

– L-Ala-6-hydrazido-2-butyl-1,8-naphthalimide (Ala-HBNI) and a novel fluorescent method for the histochemical localization of the enzyme, based on it. Using the here presented histochemical method we managed to visualize the enzyme in tissue sections of rat organs.

Materials and Methods

Synthesis of the fluorochrome N-butyl-6-hydrazino-1,8-naphthalimide (BHNI). All the chemicals are from Merck (Darmstadt, Germany). BHNI was synthesized by a four-step procedure as follows. Acenaphthene was brominated by N-bromosuccinimide in dimethylformamide after Ross et al. [11]. The obtained 6-bromoacenaphthene was oxidized to 6-bromonaphthalanhydride routinely with sodium dichromate in acetic acid. 6-Bromo-N-butyl-1,8-naphthalimide was synthesized from the last compound and *n*-butylamine by boiling in toluene according to Chang et al. [2]. Finally, the desired fluorochrome – BHNI was obtained from the brom-containing compound by hydrazine monohydrate in methoxy ethanol after Gan et al. [4].

Synthesis of the substrate. The APN substrate – L-Ala-6-hydrazido-2-butyl-1,8-naphthalimide (Ala-HBNI) was synthesized from BHNI and benzyloxycarbonyl-L-alanine (Z-Ala, Bachem, Switzerland) by dicyclohexylcarbodiimide method in tetrahydrofurane and the Z-protection was cleaved using HBr in acetic acid [1].

Tissue treatment and incubation. Mature Wistar rats were decapitated under ether anesthesia. Pieces of kidney and small intestine were extracted and frozen immediately in liquid nitrogen. Ten µm thin sections were cut on cryotome Reichert Jung (Germany) at –22°C and mounted on gelatinized glass slides or on visking dialyzing tubing 27/32 (Serva, Germany). The sections on glass slides were incubated in substrate solutions, consisting of 0,5 mM substrate (Ala-HBNI) and 1 mg/ml aromatic aldehyde (anisaldehyde, piperonal or 2-hydroxy-4-methoxy benzaldehyde) in 0,1 M phosphate buffer, pH 7.2, for 30–45 min at 37°C. The sections on dialyzing membranes were incubated in half-dry media, prepared from 0,8 % type VI low-melting agarose (Serva), containing the same components as described above. In this case the incubation lasted 40–90 minutes. After the incubation, all the samples were post-fixed in 4 % neutral formaldehyde and embedded in glycerol-jelly. Control sections were incubated only in buffered aldehyde (in the lack of substrate).

The sections were studied under fluorescent microscope OPTON IM 35 by filter combination G 546, LP 590. The microphotographs were made using Konica Minolta VX 100 multicolor films.

Results

Control sections did not show any background fluorescence when observed using the above filter combination. Non-specific precipitates were not observed on them. The novel APN substrate – Ala-HBNI was readily hydrolyzed by the enzyme to liberate the fluorochrome BHNI. The primary reaction product reacted quickly with the aromatic aldehyde in the medium to give practically water insoluble fluorescent hydrazones (the histochemical principle is shown on Fig. 1 in the case of 2-hydroxy-4-methoxybenzaldehyde as a coupling reagent). All the three hydrazones (obtained with the three aldehydes) fluoresce in red when excited by green light (520–580 nm). Using the sections mounted on glass slides and incubated in liquid media, the APN activity was not strictly localized. The final reaction product diffused from the places of its origin and was scat-

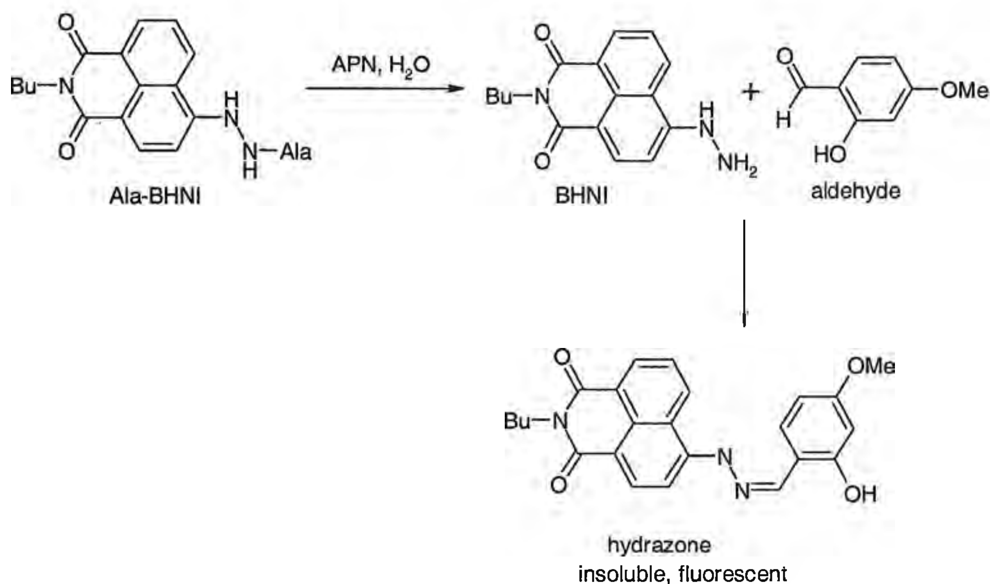

Fig. 1. Histochemical principle for the visualization of APN using the newly synthesized APN substrate – APN-HBNI and 2-hydroxy-4-methoxy benzaldehyde as coupling reagent. Bu – n-butyl moiety

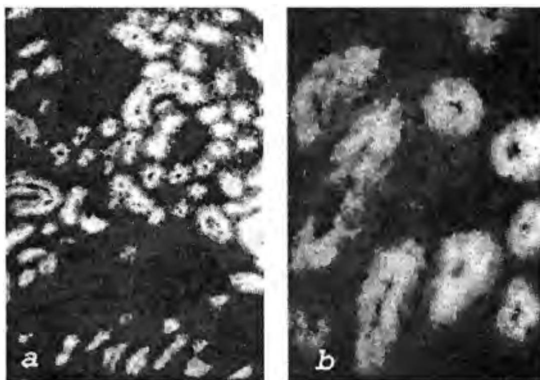

Fig. 2. APN in rat kidney. Ala-HBNI and 2-hydroxy-4-methoxybenzaldehyde. The enzyme activity is restricted to the brush border of proximal convoluted tubules. The glomeruli are negative (a – × 200; b – × 500)

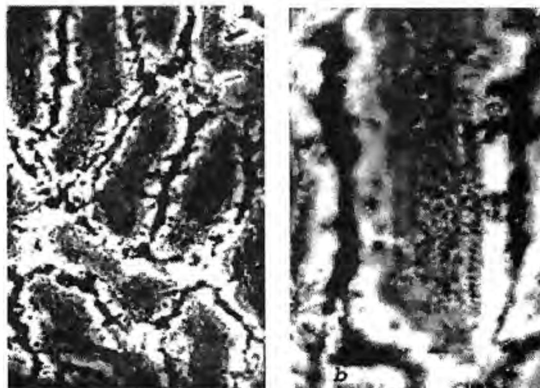

Fig. 3. APN activity in small intestine. Ala-HBNI and piperonal. The enzyme reaction is visible in the brush-border of enterocytes (a – × 200; b – × 500)

tered, which might be due to the slight water solubility of BHNI. Thus, the liquid incubation was rejected for further use. Oppositely, the sections mounted on dialyzing membranes and incubated in half-dry media showed the enzyme locations precisely. The more viscous incubation medium prevented the diffusion of BHNI and permitted APN localization strictly on the places, where it is known to be active. The quality of the final reaction product did not depend on the aldehyde used. Only in the case of anisaldehyde, the precipitated hydrazone had a slightly granular appearance, but was still strictly localized. For the visualization of APN in the organs, where its activity is high, only 40 minutes of incubation was necessary, whereas the enzyme activity in heart muscle was detected in 90 minutes. APN was localized in the brush-border of proximal convoluted tubules in kidney (Fig. 2 a, b) and in the brush border of small intestinal enterocytes (Fig. 3 a, b). The renal glomeruli were negative.

Discussion

Aminopeptidase N (CD 13) is presently a subject of a vast interest due to the number of functions it has in the organism. For example, it is regarded as an inactivator of inflammatory mediators such as neuropeptide hormones, kinins and chemotactic substances [12] and is thought to play role in cell surface antigen processing [7]. Its levels are found to change in some diseases, e.g. malignant lymphoma [10] and renal cancer [13]. So, a precise fluorescent histochemical method is much needed for the enzyme studies. The highest APN activities found by chromogenic methods are restricted to the proximal tubule brush-border in kidney, in the brush borders of small intestinal enterocytes and in the capillary endothelium of some organs [5].

The search for new fluorescent compounds, which can be used as bases for the synthesis of enzyme substrates usually starts from already known fluorescent textile or plastic dyes or fluorescent compositions of inks or enamels. Considering that, the N-alkylnaphthalimides are well known as fluorescent dyes for plastic materials, liquid crystals, etc. The compound BHNI, which is used here, is synthesized more recently and is shown to possess good potential for use in different applications [3]. Whereas BHNI fluoresces in green when excited by blue light (λ_{excit} = 460 nm, λ_{emiss} = 525 nm), its hydrazones with aromatic aldehydes, possessing donor group at 4-position, fluoresce in red when excited by green light (λ_{excit} = 500–530 nm, λ_{emiss} = 600–660 nm) [3]. So, the fluorescent histochemical procedure, developed by us on the bases of the newly synthesized APN substrate, had to use aromatic aldehydes in the incubation solutions for two main reasons. First, because BHNI has some water solubility and diffuses from the places of its origin, and second, because BHNI fluorescence almost coincides with tissue autofluorescence. Nevertheless, gel incubation prevents the diffusion of BHNI and the obtained hydrazones with either one of the three aromatic aldehydes fluoresce in such an area of the spectrum, where tissue autofluorescence is invisible. Thus, we managed to localize APN at the lack of background noise. The only other fluorescent method for peptidases described by now, uses amino acid amides of 4-methoxy-2-naphthylamine as substrates and 5-nitrosalicylaldehyde as simultaneous coupling reagent. The histochemical principle very closely resembles the one, which we use here. However, whereas BHNI is only slightly water-soluble and can be hold in place by the use of gel incubation, methoxynaphthylamine is well soluble in water and diffuses in gel media as well [5]. Another advantage of the here proposed method is that the final reaction product fluoresces in red and permits the enzyme visualization in the lack of background fluorescence. Oppositely, the hydrazone formed in the old procedure fluoresces in yellow and is difficult to tell from the tissue fluorescence [5, 9].

Thus, the newly developed method escapes the disadvantages of the method in use and permits a precise APN localization in sections of rat organs. It can be useful also for the visualization of other peptidases after synthesizing their appropriate substrates on the bases of the same fluorescent naphthalimide.

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