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Distribution and Properties of an Adenosine Triphosphatase in the Tanycyte Ependyma of the IIIrd Ventricle of the Rat*

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Summary. The distribution, histochemical properties and ultrastructural localization of adenosine triphosphate (ATP) hydrolyzing enzymes in the tanycyte ependyma of the third ventricle have been studied in female Wistar rats.

Using a calcium-cobalt procedure and a lead capture technique, splitting of ATP could be demonstrated in perikarya and processes of tanycytes in the region of the ventromedial nucleus. The reaction showed no dependence on magnesium or sodium ions, did not occur with other mono-di-, and tri-phosphates as substrates, and was inhibited by p-chlormercuribenzoate (PCMB) and sodium fluoride, but not by ouabain. With the calcium-cobalt method the highest intensity of reaction was found at pH 9.4, whereas the lead method gave optimal results at pH 6–8.

At the ultrastructural level, the reaction product was found at the outer surface of the plasma membranes of tanycytes and reached its highest concentrations in the region of the apical microvilli.

From the findings it is concluded that splitting of ATP in tanycytes is due to a true ATPase. The enzyme might be involved in an active transport of substances by tanycytes.

Introduction

The basal part of the wall of the third ventricle in mammals is formed by a specialized ependyma which differs morphologically and histochemically from that found in other regions of the brain (Colmant, 1967; Schachenmayr, 1967a, b; Bock and Goslar, 1969; Goslar and Bock, 1970, 1971; Bleier, 1971, 1972; Millhouse, 1971, 1972; Fleischhauer, 1972; Knowles, 1972; Akmayev et al., 1973; Pilgrim, 1974). It consists almost entirely of tanycytes (Horstmann, 1954), the processes of which traverse the nervous tissue and terminate on

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blood vessels in the ventromedial and arcuate regions of the hypothalamus, on the basal surface of the hypothalamus near the median eminence, or on the capillary loops of the hypophysial portal vascular system within the median eminence. Hypothalamic tanycytes therefore provide a morphological connection of cerebrospinal fluid, nerve cells and blood vessels, and could possibly mediate exchange of information among them. The presence of numerous microvilli at the ventricular surfaces of the tanycytes (Leonhardt, 1966; Kobayashi et al., 1970; Rodriguez, 1972) is a further indication that they may receive humoral signals by uptake of substances from cerebrospinal fluid, subsequently modulating the activity of neural or vascular structures. The uptake and transportation by tanycytes of substances injected into the cerebrospinal fluid has been demonstrated by several groups of workers (Luppa and Feustel, 1971; Kobayashi et al., 1972; Joseph et al., 1973; Léranth and Schiebler, 1974; Scott et al., 1974; Wagner and Pilgrim, 1974). Since the cellular mechanisms responsible for active transport of substances across membranes are thought to derive energy from the enzymatic hydrolysis of adenosine triphosphate (ATP) the histochemical detection of adenosine triphosphatase (ATPase) activity in tanycytes of the third ventricle of the rat hypothalamus (Schachenmayr, 1967a, b) is of considerable interest. The aim of the present investigation is to characterize this tanycyte ATPase histochemically and to study its ultrastructural localization.

Materials and Methods

Female Wistar rats, weighing 150 g–200 g, were used for all experiments. They were killed by anaesthesia with Nembutal (250 mg/kg body weight); brains were removed immediately after death and frozen with solid carbon dioxide.

HISTOCHEMICAL DEMONSTRATION OF ATPases

A. Light Microscopy

Fresh, air-dried cryostat sections cut at 20 µm were fixed in 10% formalin (buffered to pH 7.4 with sodium acetate/acetic acid) for one min at 4° C. Two methods were used for the demonstration of ATPases:

1. A Calcium-Cobalt Procedure, modified after Padykula and Herman (1955a). Sections were incubated for one h at 37° C in a medium containing 0.03 M sodium barbiturate, 0.02 M calcium chloride and 0.0023 M disodium ATP, adjusted to the required pH. Subsequently sections were rinsed in three changes of 1% calcium chloride, transferred to 2% cobalt chloride for 3 min, washed in distilled water for 1 min, immersed briefly in dilute ammonium sulphide, rinsed in distilled water and mounted in glycerine jelly.

2. A Lead Capture Method based on the technique of Wachstein and Meisel (1957a, b). Sections were incubated for one h at room temperature in a medium containing 0.003 M lead nitrate, 0.27 M glycine, 0.03 M Tris and 0.001 M disodium ATP, adjusted to the required pH. After rinsing in distilled water and brief treatment with dilute ammonium sulphide, sections were mounted in glycerine jelly.

Using these two methods as a basis, the following investigations were carried out:

a) Cation Dependence

Using the calcium-cobalt medium adjusted to pH 9.0 with 1 N sodium hydroxide, the effects of magnesium chloride and sodium chloride over concentration ranges of 0.01–0.05 M and 0.03–0.17 M respectively were tested. The tests were repeated using the lead capture method at pH 7.4 and using both acetates and chlorides of magnesium or sodium, pH adjustment being carried out with the appropriate acid.

Control sections were incubated in the same media adjusted to the appropriate pH, but without addition of magnesium or sodium salts.

b) pH Dependence

The effects of changes in pH were examined over the range 7.5–9.4 for the calcium-cobalt medium and 5.5–9.0 for the lead medium containing 0.01 M magnesium acetate. Adjustment of pH was made using 1 N acetic acid or 1 N sodium hydroxide in both systems.

c) Substrate Specificity

Tests were carried out using the lead capture method only. The medium contained 0.01 M magnesium acetate in all cases, and the pH was adjusted to 7.4 with 1 N acetic acid. ATP was not included in the medium, being replaced by the following substrates, all at a concentration of 25 mg/50 ml medium: p-nitrophenylphosphate; α-naphthyl phosphate; β-glycerophosphate; glucose-1-phosphate; glucose-6-phosphate; fructose-1,6-diphosphate; riboflavine-5-monophosphate; thiamine pyrophosphate; adenosine-5′-monophosphate; adenosine-5′-diphosphate; 5′-adenylyl-imidodiphosphate; inosine-5′triphosphate.

d) Specific Inhibitors

Tests were carried out in either the calcium-cobalt basic medium at pH 9.0, or in the lead medium containing 0.01 M magnesium acetate at pH 7.8. The inhibitors tested by addition to the media were 0.01 M ouabain, 0.003 M p-chlormercuribenzoate (PCMB) and 0.03 M sodium fluoride. Control sections were incubated in the appropriate media without inhibitors.

B. Electron Microscopy

For electron microscopical localization of ATPase, the ventral hypothalamus was excised and fixed for 90 min in 3% glutaraldehyde, buffered with 0.1 M sodium cacodylate-HCl, pH 7.4, at 4° C. The block was then trimmed to the region required, embedded in 7% ion agar and cut at 50 µm on a tissue chopper. The sections were rinsed twice in 0.1 M Tris-HCl buffer, pH 7.4, then incubated at room temperature and pH 7.4 for 30 min in the modified Wachstein-Meisel medium containing 0.01 M magnesium acetate. After incubation the sections were rinsed in several changes of Tris-HCl buffer and postfixed in 1% osmium tetroxide, buffered to pH 7.4 with 0.1 M cacodylate-HCl, for 1 h at 0° C. The tissue was processed through alcohols and propylene oxide, and embedded in Araldite. Silver sections were lightly counterstained with uranyl acetate and examined in an AEI 6B electron microscope.

Results

The characteristic distribution of ATP-splitting enzymes in the rat hypothalamus is shown in Figure 1. They are found in the tanycyte ependyma and the adjacent

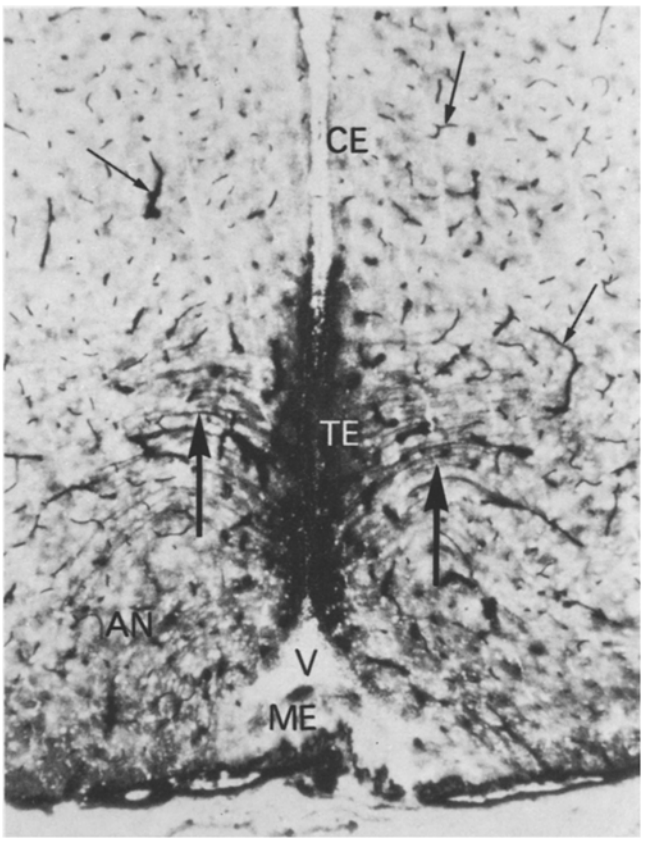

Fig. 1. Distribution of ATP-splitting activity in the rat hypothalamus. Lead capture technique. Magn. ×40. Strong activity in the dorsal part of the tanycyte ependyma (*TE*), the adjacent subependymal layer, and blood vessels (thin arrows). Less intense reaction in the neuropil of the region of the arcuate nucleus (*AN*). Thick arrows point to individual reactive tanycyte processes. In the ciliated ependyma (*CE*) no hydrolysis of ATP is to be observed. *ME* median eminence; *V* lumen of the 3rd ventricle. In the region of the dorsal tanycyte ependyma the ventricular lumen is filled with reaction product, due to the strong ATP-splitting activity in the apical parts of the tanycyte perikarya

subependymal layer, in blood vessels and, at a lower activity, in the neuropil of the arcuate region.

Not all of the tanycyte ependyma shows ATPase activity: a positive reaction is confined to its more dorsal part, which is adjacent to the ventromedial nucleus. The tanycytes found in the arcuate region and in the median eminence remain unstained. In the reactive tanycytes the highest activity occurs in the perikarya, especially in their apical parts. In sections incubated in the lead medium individual tanycyte processes can be seen at varying distances from the third ventricle.

In general, the morphological picture is similar regardless of whether the calcium-cobalt or lead capture technique is used. However, the lead method gives higher staining intensity and better localization of the reaction product.

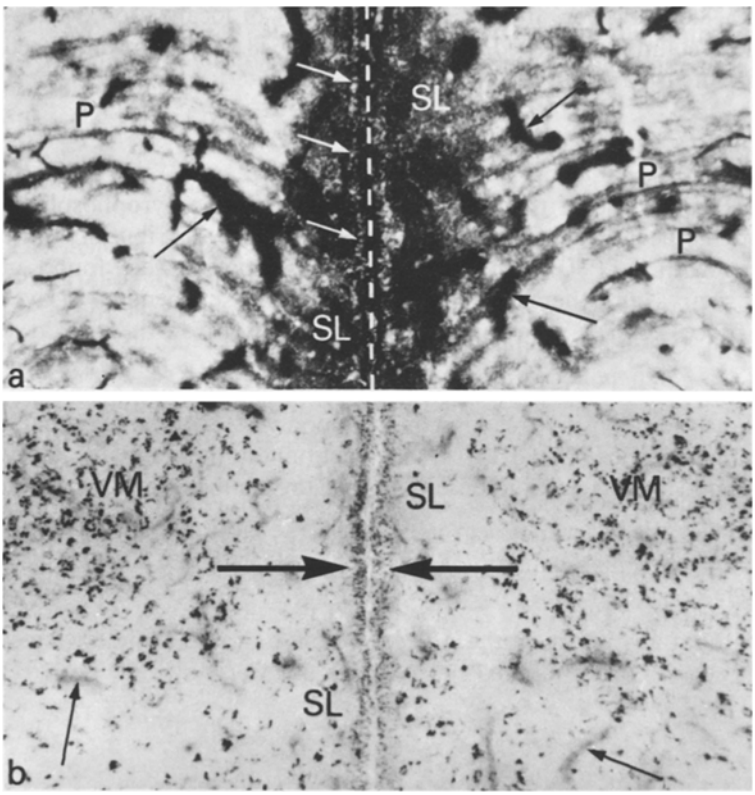

Fig. 2. Different distribution of ATP- (Fig. 2a) and thiamine pyrophosphate-splitting activity (Fig. 2b) in the tanycyte ependyma and in the adjacent nervous tissue. Lead capture technique. Magn. ×100. Figure 2a shows a part of Figure 1. The broken line marks the course of the ventricular cleft which is filled with reaction product. A strong reaction occurs in the tanycyte perikarya, the position of which on the left side of the ventricular wall is clearly indicated by their unstained cell nuclei (thin white arrows), and in blood vessels (thin black arrows). Less intense reaction in the subependymal layer (*SL*) and in tanycyte processes (*P*). Figure 2b shows the same region as Figure 2. The ventricular cleft does not contain any reaction product and therefore appears as a narrow light zone between the tanycyte ependyma (thick black arrows) of both sides of the ventricular wall. Note the granular reaction in the tanycyte perikarya and in nerve cells of the ventromedial nucleus (*VM*). No staining occurs in the subependymal layer and in tanycyte processes. A very weak reaction is to be observed in blood vessels (small black arrows)

Cation Dependence

Addition of sodium or magnesium at a range of concentrations seemed to have no effect on the distribution or intensity of the reaction. This observation applies to both the calcium-cobalt and the lead capture methods.

pH Dependence

Using the calcium-cobalt method, the highest intensity of reaction was found at pH 9.4. The lead capture method gave a uniform intensity of reaction over

the pH range 5.5–8.0; however, at pH 5.5 the specificity of localization deteriorated and nuclear staining appeared. In the pH range 8.5–9.0 a pronounced drop in reaction intensity occurred.

Substrate Specificity

Apart from ATP, only one of the substrates tested, thiamine pyrophosphate, gave a reaction in the tanycytes. However, this showed a distribution quite unlike that seen with ATP (Fig. 2): small granular deposits were found in the tanycyte perikarya and there was no reaction in the subependymal layer or in tanycyte processes. The granular deposits appeared also in the perikarya of neurons.

All the substrates tested, except riboflavine-5-monophosphate and adenosine-5′-monophosphate gave a positive reaction on the blood vessels of the hypothalamus and median eminence. In all cases the reaction on the capillary loops of the median eminence was more intense than that on the hypothalamic blood vessels.

Specific Inhibitors

Ouabain was without any effect on the ATPase reaction of either the tanycytes or the blood vessels. PCMB inhibited the tanycyte reaction but did not affect

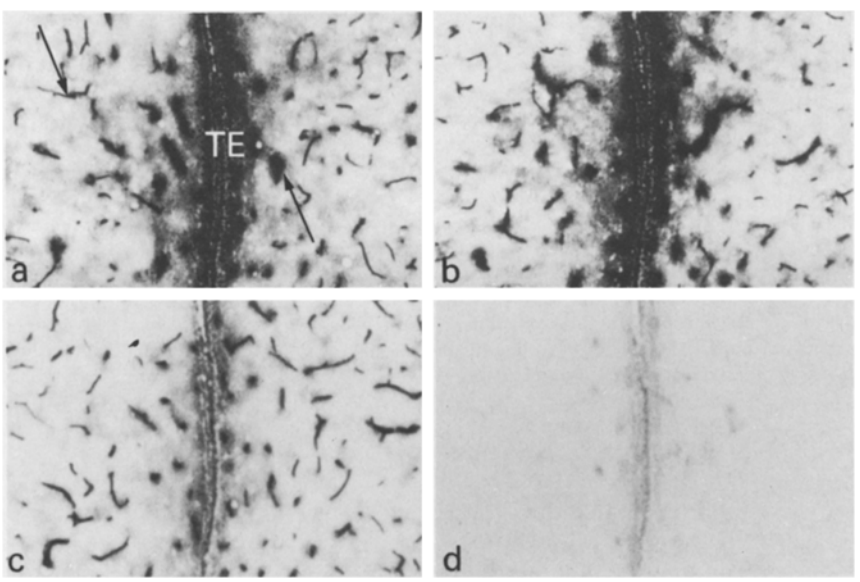

Fig. 3a–d. Action of specific inhibitors on the ATP-splitting activity in tanycyte ependyma and blood vessels of parallel sections of rat hypothalamus. Lead capture technique. Magn. ×60. (a) Control section. Strong reaction in tanycyte ependyma (*TE*) and blood vessels (small black arrows). (b) Ouabain 0.01 M. No inhibition of the reaction. (c) p-Chloromercuribenzoate 0.003 M. Marked inhibition of the reaction in tanycytes, no influence on the reaction in blood vessels. (d) Sodium fluoride 0.03 M. Strong inhibition of the reaction in tanycytes and blood vessels

the intensity or distribution of the vascular reaction. Sodium fluoride inhibited reaction at all hypothalamic sites. These effects are illustrated in Figure 3. The action of the inhibitors was the same in both the calcium-cobalt and the lead capture systems.

Electron Microscopy

The general pattern of cytochemically demonstrable ATPase activity in the rat hypothalamus seen by electron microscopy was consistent with that found by light microscopy. Strong reaction was associated with the tanycytes and blood vessels, and weaker reaction with the neuropil.

The tanycytes in the region of the ventromedial nucleus showed reaction over their entire surfaces. Dense deposits were present on the microvilli at the ventricular surface, and also on the interdigitations of the tanycytes with one another and with ciliated ependymal cells (Fig. 4). The basal processes were also reactive, being outlined by electron-dense reaction product along their entire length (Fig. 5). The vascular reaction was associated with the external aspect of the endothelial cells and with adjoining glial foot-processes. Reaction

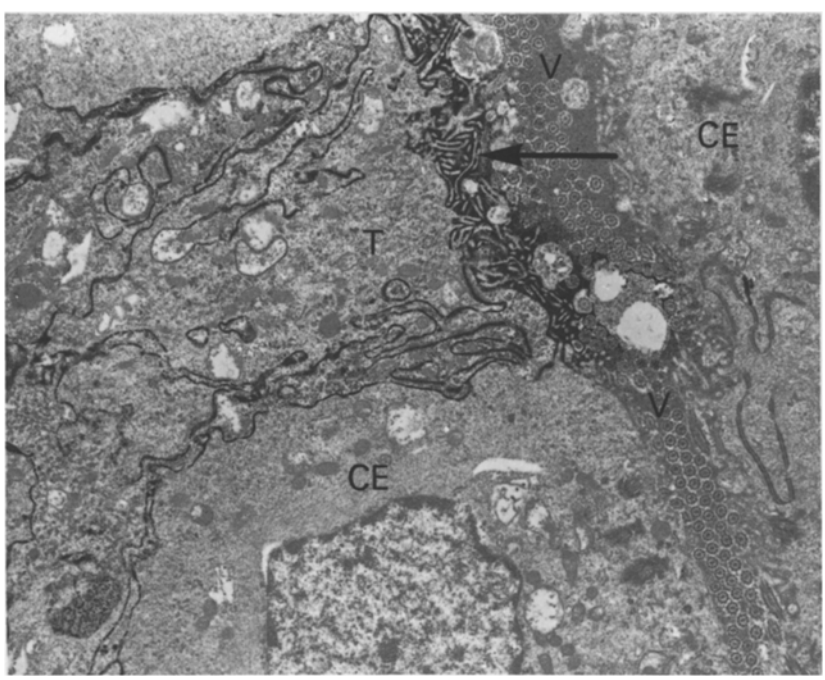

Fig. 4. Electronmicroscopical demonstration of ATP-splitting activity in a tanycyte (*T*) of the dorsal part of the tanycyte ependyma. The reaction product is localized on the plasma membrane. The apical microvilli (arrow) are intensely stained. No reaction occurs in the adjoining ciliated ependymal cells (*CE*). In the ventricular cleft (*V*) many cilia are to be seen, which, too, do not show any ATP-splitting activity. Magn. ×6,000

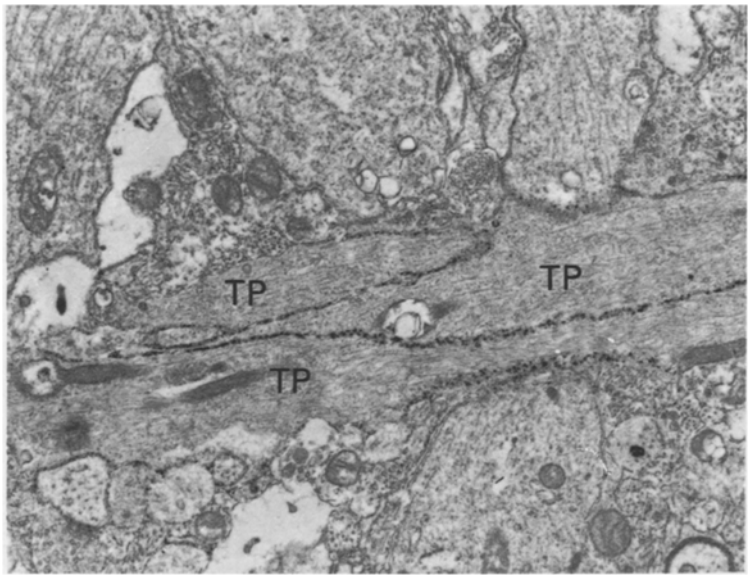

Fig. 5. Electronmicroscopical demonstration of ATP-splitting activity on the surfaces of basal tanycyte processes (*TP*). Reaction in the adjoining nerve cells is very weak or absent. Magn. ×17,000

in the neuropil was somewhat inconsistent and weak, but mainly appeared to be associated with oligodendrocytes.

It is apparent from Figure 4 that ciliated ependymal cells are non-reactive; they are only associated with reaction product at their areas of contact with tanycytes.

Discussion

In recent years lead salt and other metal capture techniques for the localization of ATPase have been subjected to serious criticism, much of it probably valid. For example, it seems to be firmly established that ATPases, and especially Na-K-ATPase, can be very severely inhibited by most, if not all, fixatives (Novikoff et al., 1958; Moses et al., 1966). Lead and calcium ions can inhibit the enzymes, both in fresh and fixed tissues, and can also catalyze non-enzymatic hydrolysis of ATP and other nucleoside di-and triphosphates (Lowenstein, 1958; Novikoff et al., 1958; Tetas and Lowenstein, 1963; Moses et al., 1966; Rosenthal et al., 1966). In addition, there is a risk of confusion with a less specific polyphosphatase and with nonspecific alkaline phosphatases which also can split ATP (Freiman and Kaplan, 1960; Firth and Marland, 1975). None of these factors invalidate the methods, but all of them underline the need for very careful controls and cautious interpretation of results. Our reasons for considering the tanycyte reaction to indicate the presence of a true ATPase are as follows:

1. The incubation temperature used for the lead capture method was low enough for non-enzymatic hydrolysis of ATP by lead ions to be very slight

(Rosenthal et al., 1966, 1969; Novikoff, 1967, 1970). The calcium-cobalt method, which could be more suspect owing to the higher incubation temperature, gave the same distribution of reaction product as the lead method.

2. The likelihood of non-enzymatic splitting of ATP being significant is further reduced by the inability of other nucleoside phosphates to replace ATP in the reaction (compare Rosenthal et al., 1966), and by the results obtained with inhibitors, notably PCMB and sodium fluoride (see Novikoff, 1967). Inhibition by fluoride cannot be attributed to precipitation of lead from the reaction mixture (Moses and Rosenthal, 1968) as structures in the same sections known to be rich in alkaline phosphatases, such as the pia mater, show an undiminished intensity of reaction in the presence of fluoride. Additionally, any fall in the lead concentration to a marginal level might be expected to produce diffusion artefacts, but no such effect has been observed.

3. Ciliated ependyma adjacent to tanycytes remained unstained. It is therefore unlikely that staining is due to an unspecific binding of lead by plasma membranes.

4. Several lines of evidence indicate that the enzyme being studied is a specific ATPase. It is unlikely that a nonspecific alkaline phosphatase would retain high activity at pH 5.5 as the tanycyte enzyme appears to do. In addition, the substrate specificity of the enzyme is very high: the only other substrate to give a tanycyte reaction was thiamine pyrophosphate, but in this case the distribution of the reaction product was related to the Golgi complex rather than to the plasma membrane. This is consistent with the general distribution of thiamine pyrophosphatase, which is regarded as a Golgi complex marker enzyme in many tissues, including nervous tissue (Goldfischer et al., 1964; Lazarus and Wallace, 1964; Shantaveerappa and Bourne, 1965). The occurrence of a thiamine pyrophosphatase in tanycytes of the 3rd ventricle of the rat has already been described by Luppa and Feustel (1971).

The tanycyte ATPase evidently differs significantly from the activity found in the blood vessels of the hypothalamus and median eminence. This latter enzyme has a far wider range of substrate specificity, and its activity is not inhibited by PCMB. It seems likely that this belongs to the group of "less specific polyphosphatases" described by Freiman and Kaplan (1960) in blood vessels of dog kidney and jejunum.

It now remains to consider to which class of ATPases the tanycyte enzyme should be assigned, and what function it might have. It is evident that it is not one of the Na-K-dependent transport ATPases: it shows no dependence on the presence of sodium ions, it is not inhibited by ouabain (Post and Sen, 1967; Bonting, 1970), and it can be demonstrated in the presence of lead ions (Moses et al., 1966).

Inhibition by sodium fluoride and PCMB are regarded as characteristic features of ATPases (Kalckar, 1951; Hoffmann-Ostenhof, 1954; Kielly, 1955, 1961; Padykula and Herman, 1955b; Perry, 1955; Tice and Barnett, 1960; Lazarus and Barden, 1962; Barden and Lazarus, 1964; Torack and Markey, 1964; Post and Sen, 1967). The finding that PCMB is capable of producing strong inhibition even without preincubation suggests that the tanycyte ATPase belongs to the group of "sulfhydryl enzymes", which require for their activity

free SH-groups in their active centres (Madsen, 1963). The inhibition by sodium fluoride might suggest that the enzyme is magnesium-dependent (Hewitt and Nicholas, 1963), although we have been unable to demonstrate any influence of magnesium ions on its activity. It is worth observing that other workers have commented on their inability to demonstrate cation dependence of ATPase in certain freeze-dried (Pratt, 1954) or formalin fixed tissues (Novikoff et al., 1961). It seems likely that some physical and chemical treatments may be able to alter the properties of the enzyme with respect to cations.

Using the lead capture technique the optimum pH range for the demonstration of tanycyte ATPase was about 6.0–8.0, which is consistent with the findings of Novikoff et al. (1961) on formol-calcium fixed rat kidney, cerebellum and uterus. Below pH 6.0 diffusion artefacts occur which can be explained by inadequate trapping of phosphate. Regarding the upper limit of optimal pH there is a difference between the lead method and the calcium-cobalt procedure, the latter providing an optimal demonstration of tanycyte ATPase at pH 9.4. This observation is difficult to interpret. It seems unlikely that the two methods demonstrate different enzymes in view of the identical distributions of reaction products and identical responses to inhibitors. Perhaps part of the answer is that the efficiency of phosphate trapping by calcium falls sharply as the pH is reduced from 9.4 (see Pearse, 1968, p. 499), making it necessary for the calcium-cobalt method to be used at a pH well above the optimum of the enzyme. On the other hand, the rather sharp deterioration in efficiency of the lead method above pH 8.0 could be due to an increase of the inhibitory action of lead on the ATPase at higher pH values. It cannot be caused by a loss of lead ions from the medium due to precipitation of lead hydroxide (Novikoff et al., 1961), as in the same sections demonstration of the ATP-splitting blood vessel enzyme is unaffected. In view of its histochemical properties the tanycyte ATPase described here should be classed with the plasma membrane ATPases studied by Novikoff et al. (1961). At the electron microscopic level these enzymes are typically localized on the external surface of the plasma membrane. This is also the case for the tanycyte ATPase. In contrast with this, the nitrophenyl phosphate-strontium method for Na-K-ATPases generally gives localizations on the internal aspect of plasma membranes (Ernst, 1972; Firth, 1974). However, it is probably advisable to be cautions about the interpretation of such data, as Leuenberger and Novikoff (1974) have described an external surface localization for Na-K-ATPase, and have suggested that the precise position of the reaction product in relation to the membrane may depend on many factors other than the actual position of the catalytic sites.

Interpretation of the functional role of the tanycyte ATPase is very difficult at present. Its localization on the plasma membrane and its especially high activity in the region of the microvilli suggest that it may be involved in transport processes. Knigge and Silverman (1972) showed that the uptake of labelled substances by median eminence tissue in vitro can be inhibited by Na-K-ATPase blockers. Although it is far less certain that other plasma membrane ATPases have a transport role, it is possible that the histochemically demonstrable tanycyte ATPase is also concerned with active uptake of substances from cerebrospinal fluid.

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