HISTOCHEMICAL METHOD FOR LOCALIZATION OF CYCLIC 3', 5'-NUCLEOTIDE PHOSPHODIESTERASE

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Summary. A histochemical method has been described for demonstration of cyclic 3', 5'-nucleotide phosphodiesterase in tissues. 5'-AMP is formed due to splitting of substrate cyclic 3', 5'-AMP by cyclic 3', 5'-AMPase. The 5'-AMP is split into adenosine and phosphate by the 5'-nucleotidase from added snake venom. Endogenous tissue 5'-nucleotidase would contribute to this activity. The phosphate was in turn visualized by conversion to the lead salt in the presence of lead acetate and finally into brownish-black lead sulphide by treatment with yellow ammonia sulphide. Control studies with and without substrate and snake venom, as well as inhibition by theophylline, indicate the test to be specific for cyclic 3', 5'-AMPase. In the eye the conjunctiva, ciliary process, choroid and retina all showed strongly positive activity. In the kidney the proximal and distal tubules both ascending and descending and the loop of Henle show strongly positive activity — the rest of the elements being negative. The cardiac and skeletal muscle exhibited very little positive activity. The villi of the small intestine showed strongly positive activity at the apical part of the cells. Neurons showed very little positive activity in either the cerebral cortex or the cerebellum. On the other hand, the molecular layer in the cerebellum and the plexiform layer of the cerebral cortex showed strongly positive activity. The significance of these findings are briefly discussed.

Introduction

A wide variety of biochemical functions are believed to be mediated by cyclic 3', 5'-adenosine monophosphate (cyclic 3', 5'-AMP). Much of this work has been summarized by SUTHERLAND, et al. (1965). The enzyme which forms cyclic 3', 5'-AMP, adenylyl cyclase, appears to be associated with cell membranes. Its response to hormone may in some way even depend on membrane integrity. Concentration of cyclic 3', 5'-AMP is also influenced by the rate of breakdown via cyclic 3', 5'-nucleotide phosphodiesterase (cyclic 3', 5'-AMPase). This enzyme has been isolated from many different sources and, in fresh homogenates, is associated mainly with the particulate fraction (SUTHERLAND et al., 1962). However, NAIR (1966) found most of the activity in the 105,000 g supernatant fraction from tissue which had been frozen and then thawed.

The present paper describes a method for localization of cyclic 3', 5'-nucleotide phosphodiesterase in tissue sections using exogenous 5'-nucleotidase from snake venom.

Materials and Methods

The procedure used in this study is based mainly on GOMORI's (1939, 1950) phosphatase method. The localization of cyclic 3', 5'-AMPase has been accomplished by incubating the tissue sections with cyclic 3', 5'-AMP in the presence of snake venom and lead acetate with a suitable buffer. Under these conditions, adenosine monophosphate (5'-AMP) is formed by

* T. R. SHANTHAVEERAPPA — in previous publications.
the action of cyclic 3', 5'-AMPase. This 5'-AMP is further split into adenosine and phosphate by the combined action of 5'-nucleotidase from the tissues and from added venom of Crotalus atrox (Richard's et al., 1965). The phosphate in turn reacts with lead acetate to form lead phosphate which converts to brown or brownish black deposits following treatment with dilute yellow ammonium sulphide.

New Zealand White rabbits (2-4 kg) maintained on Purina rabbit chow were used in this study. The rabbits were either killed by decapitation or by air embolism. Tissue (liver, kidney, cerebellum, cerebral cortex, heart, skeletal muscle, small intestine, eye and lacrimal gland) were removed from the animal as quickly as possible. (The eyes were bisected anteroposteriorly after removal.) Small pieces of these tissues were immediately frozen by wrapping in aluminum foil and burying in dry ice. Sections 10 μ thick were cut on a cryostat at −15°C, mounted on a cover slip and then stored at −20°C until used.

Immediately before incubation, sections were dried under a fan for 3-5 minutes and then they were incubated in Columbia jars containing 10 ml of a freshly prepared mixture with the following concentrations:

i. Cyclic 3', 5'-AMP (1.44 × 10⁻³ M);
ii. Tris-maleate buffer (5 × 10⁻³ M);
iii. Magnesium chloride (1.0 × 10⁻² M);
iv. Lead acetate (2 × 10⁻³ M);
v. Snake venom¹ (1 mg added to the above mixture).

Tris-maleate buffer prepared at pH 7.62 before addition of the other constituents gave a final pH of 7.50 ± 0.01. The final pH was always checked and adjusted if necessary by adding Tris base or maleic acid. (This adjustment never required more than 3 × 10⁻³ mmoles of either acid or base.) Sections were incubated at 37°C for various periods from 30 minutes to 3 hours.

Following incubation, the sections were briefly rinsed in 3 changes of distilled water, placed in dilute yellow ammonium sulfhide for 1-2 minutes, thoroughly washed with distilled water and mounted with aquamount.

**Optimum pH for Incubation**

The optimum pH for activity was determined by incubations with the medium adjusted to pH 6.25, 6.43, 7.21, 7.5, 7.67 and 8.1.

**Buffer Solutions**

Tris-maleate was the buffer pair of choice due to the absence of precipitation with lead and to a buffering capacity sufficient for maintenance of pH in the optimum range following addition of substrate, snake venom and lead acetate. Buffers of sodium succinate or tris-hydrochloride proved unsatisfactory.

**Controls**

The histochemical procedures were evaluated by a number of controls: i. Omission of substrate in the final incubating solution; ii. Omission of both substrate and snake venom; iii. Omission of snake venom only; iv. Pretreatment of sections using an autoclave for 30 minutes at 10 lb pressure; v. Zero time incubation; vi. Incubation of sections at 0°C.

**Inhibition Tests**

The sections were pretreated for 30-40 minutes with 0.1 M theophylline ethylenediamine mixed with buffer and then incubated in the normal incubating medium. Alternatively, 0.01 M theophylline or theophylline ethylenediamine was added to the usual incubating medium and tissues were not pretreated. Sections were processed in the normal fashion following incubation.

In order to inhibit endogenous 5'-nucleotidase, some sections were pretreated 30 minutes in solutions which contained 0.05 M tris-maleate buffer and 0.1 M sodium fluoride, final pH 7.50 (Heppel and Hilmore, 1961).

Throughout these studies, slides of the same tissue were simultaneously incubated in the normal incubating medium for comparison with inhibited slides.

¹ Crotalus atrox — purchased from Sigma Chemical Co.