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CHARACTERISTICS OF PHOSPHODIESTERASE OF CYCLIC NUCLEOTIDES AND CALMODULIN IN THE MUCOSA OF THE RABBIT SMALL INTESTINE

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KEY WORDS: mucosa of small intestine; phosphodiesterase; calmodulin.

Cyclic nucleotides play a decisive role in the development of certain acute intestinal infections. However, mechanisms of action of intestinal toxins have been studied chiefly on enzyme and membrane preparations of tissues such as the brain, heart, and erythrocytes [13]. This is because the enzymes of cyclic nucleotide metabolism have been well studied in those tissues but practically not at all in the intestinal mucosa. The difficulties of isolating these enzymes from the mucosa, the absence of sufficiently accessible, simple, and reproducible methods of purifying the mucosal membranes, the small quantities of this tissue available, and various other technical difficulties have led to the result that the cyclase system of the mucosal cells has been studied much less thoroughly than in other tissues. Cyclic nucleotide metabolism is closely bound up with the Ca^{++} concentration in the cell. Cyclic AMP increases the passive permeability of the outer cytoplasmic membrane of the cell for Ca^{++} [12] and accelerates active Ca^{++} transport by the intracellular cisterns of the endoplasmic reticulum [4]. In turn, Ca^{++} activates cyclic AMP synthesis [1, 6] and the hydrolysis of this nucleotide [3, 5, 10]. All these effects of Ca^{++} are mediated through its binding with a special regulator protein, namely calmodulin [1, 3, 5, 6, 10].

In the present investigation, the action of Ca^{++} on phosphodiesterase (PDE) of cyclic nucleotides was studied and the properties of calmodulin determined in a preparation of mucosa of the rabbit jejunum and ileum.

EXPERIMENTAL METHOD

After decapitation of a rabbit weighing 1 kg the corresponding parts of the small intestine were quickly removed and washed with physiological saline, after which the mucosa was curetted and homogenized for 3 min in a Potter's homogenizer in 50-100 ml of a cold solution of 20 mM Tris-HCl and 1 mM EDTA, pH 7.4, at 4°C. The homogenate was filtered through three layers of gauze and centrifuged at 4000g for 15 min. The residue was discarded and the supernatant used for isolation of PDE and calmodulin.

Dry ammonium sulfate was added to the supernatant up to 60% saturation, mixed for 1 h, and the mixture was then centrifuged at 10,000g for 20 min. The supernatant obtained after centrifugation was used to obtain the Ca-dependent protein regulator, and the residue, which contained PDE, was dissolved in the minimal volume of a solution containing: 10 mM Tris-HCl,

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TABLE 1. Effect of Ca^{++} on PDE Activity (in nmoles cyclic AMP/mg protein/min) of Mucosa (M ± m)

Tissue and fraction	EGTA 2mM	Ca^{2+}, 10^{-5} M
Jejunum		
homogenate	0,20±0,01	0,21±0,01
cytosol	2,54±0,04	2,80±0,08
Ileum		
homogenate	0,13±0,02	0,13±0,01
cytosol	2,09±0,05	2,36±0,04

TABLE 2. Effect of Thermostable Protein Fraction of Jejunal Mucosa and Homogeneous Brain Calmodulin (in nmoles cyclic AMP/mg protein/min) on Ca-Sensitive Form of PDE from Jejunum and Ileum (M ± m)

PDE	Without additives	Thermostable fraction	Brain regulator
Jejunum	4,31±0,41	18,3±0,8	20,1±1,7
Ileum	6,04±1,3	21,1±1,2	20,4±2,0

Legend. Activity measured in the presence of 10^{-5} M Ca^{++}.

0.1 mM dithioerythritol, and 50 mM NaCl (pH 8.0), after which it was dialyzed twice against 10 mM Tris-HCl, pH 8.0. The dialyzed solution of PDE was applied to a column (1.5 × 10 cm) with DEAE-cellulose, previously equilibrated with buffer containing 0.1 mM EDTA and 10 mM Tris-HCl, pH 8.0, and rinsed with the same buffer until the optical density of the eluted solution fell to zero.

The enzyme was eluted by a linear ionic strength gradient: 100 ml of a solution containing 10 mM Tris-HCl and 0.1 mM dithioerythritol (pH 8.0), against 100 ml of the same solution, containing 0.6 M NaCl additionally. Fractions of 7 ml were collected and PDE activity was measured in them in a medium of the following composition: 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0, 0.2 μCi ^{3}H-3',5'-AMP, 10^{-5} M unlabeled cyclic AMP. The reaction was started by the addition of a protein solution of PDE (2-10 μg protein), conducted at 37°C for 15 min, and stopped by application of 5 μl of the incubation mixture to a "Silufol UV-254" plate (Czechoslovakia) at the same points as those to which nonradioactive solutions of 5 × 10^{-3} M cyclic AMP and 5'-AMP had previously been applied as witnesses. The hydrolysis products were separated by chromatography in a mixture of solvents isopropanol—water—ammonia (7:2:1). The "spots" of the nucleotides were identified in UV light, the AMP "spot" was cut out and placed in scintillation flasks, to which 0.5 ml H_2O was added, and 12-14 h later 10 ml of scintillation mixture made up in toluene and Triton X-100 was added to each flask and radioactivity was measured on a Mark III or SL-4000 liquid scintillation counter.

The protein regulator was isolated from bovine brain by the method described in [2].

To isolate calmodulin from the mucosa the supernatant obtained after salting out the PDE with ammonium sulfate was used. The pH of the solution was adjusted to 4.0, and after 1 h centrifugation was carried out at 10,000g for 20 min. The supernatant was discarded and the residue dissolved in a solution containing 30 mM histidine, 1 mM magnesium acetate, and 170 mM NaCl, pH 7.5. The protein solution was placed in a boiling water bath for 2 min and then cooled to 4°C. The resulting suspension was centrifuged for 20 min at 20,000g, after which the supernatant was dialyzed against 30 mM histidine buffer, pH 7.5, and kept in the frozen state. The calmodulin preparation thus obtained had an activating effect on the Ca-dependent form of PDE.

Gel filtration of the calmodulin was carried out as follows. To a column (1.0 × 65 cm) with Sephadex G-100, 2 ml (about 1 mg protein) of a solution of the protein regulator was added, and elution was carried out with a solution containing 30 mM histidine, 1 mM magnesium acetate, and 170 mM NaCl. Fractions of 1 ml were collected and the calmodulin concentration in them determined by the ability of these fractions to activate PDE in the presence of 10^{-5} M Ca^{++}. To rule out any possible effect of the eluting solution of PDE, in all cases control measurements of the action of the fractions on the PDE preparation were made in the presence of 1 mM EGTA, i.e., under conditions when the calmodulin could not interact with the enzyme.

EXPERIMENTAL RESULTS

It will be clear from Table 1 that Ca^{++} ions had practically no effect on PDE activity in the mucosal homogenate. Removal of structural components of the cell by centrifugation led to a marked increase in specific PDE activity, but the effect of Ca^{++} on the enzyme did not become substantially stronger.

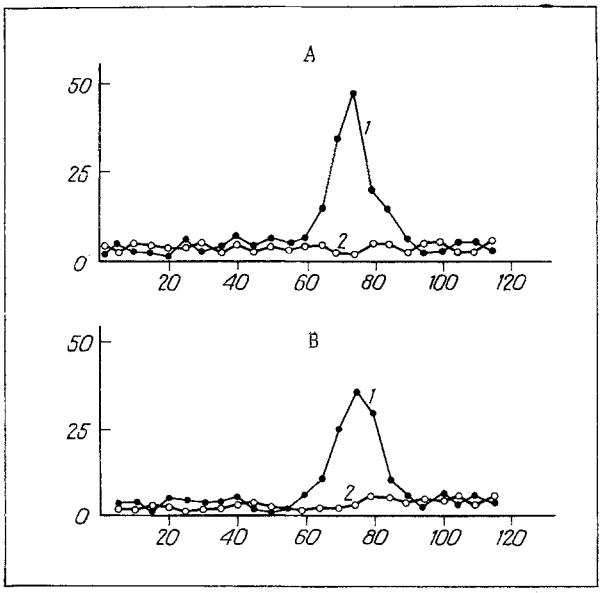

Fig. 1. Gel filtration of Ca-dependent protein regulator on column with Sephadex G-100. A) Ca-dependent brain protein regulator, B) thermostable fraction of jejunal mucosa. Abscissa, volume of eluate (in ml); ordinate, PDE activity (in nmoles cyclic AMP/mg protein/min). 1) PDE activity in presence of 2 mM EDTA, 2) the same, in the presence of 10^{-5} M Ca^{++}.

After chromatography of the cytosol on a column with DEAE-cellulose, an activating effect of calmodulin on PDE could be found in some fractions. Total PDE activity in these fractions was much lower than total activity in fractions containing PDE in a form insensitive to calmodulin.

As Table 2 shows, the thermostable fraction of the mucosa had the same activating effect on the Ca-sensitive form of PDE as the best studied homogeneous brain calmodulin.

As Fig. 1 shows, the calmodulin of the mucosa has the same molecular weight as brain calmodulin. This regulator protein shows neither tissue nor species-specificity [2, 14] and evidently has the same amino acid composition in all tissues [9]. This regulator protein participates in the mechanism of action of cholera toxin on adenylate cyclase (AC). It has been shown on brain preparations that in the absence of calmodulin cholera toxin cannot activate AC [13]. It is also important to note that calmodulin makes a whole series of enzymes, unconnected with the synthesis or hydrolysis of cyclic nucleotides, sensitive to Ca^{++}: protein kinases [7, 8], phospholipase A_2 [15], and the process of assembly of microtubules [11].

These experiments showed that calmodulin can activate only certain fractions of mucosal PDE. This is in agreement with data obtained on other tissues: Forms of PDE sensitive and insensitive to calmodulin exist [2, 3].

The homogenate and cytosol of the mucosa contain calmodulin and a form of PDE sensitive to this regulator protein, but minor quantities of this form of the enzyme can bring about activation by Ca^{++} ions of the total velocity of cyclic AMP hydrolysis by not more than 10% (Table 1). Such a weak activating effect cannot be explained by a deficiency of calmodulin in the given tissue, for the addition of brain calmodulin did not potentiate the effect of Ca^{++} on mucosal PDE activity. The Ca-sensitive and Ca-insensitive forms of PDE possibly function in different cell populations of the intestinal mucosa.

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ACTION OF HUMAN CHORIONIC GONADOTROPHIN ON PROLIFERATION OF HYPATOCYTE ORGANELLES FROM THE NORMAL AND PATHOLOGICALLY CHANGED RAT LIVER

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One manifestation of intracellular regeneration is proliferation of the cytoplasmic organelles [9]. Previous investigations [5-7] have shown an increase in the intensity of proliferation of the rough endoplasmic reticulum (RER), the smooth endoplasmic reticulum (SER), and mitochondria (M) of the hepatocytes of the pathologically changed human and animal liver under the influence of the human placental hormone chorionic gonadotrophin (CG). On injection of CG into albino rats with experimental chronic hepatitis, the stimulating action of the hormone lasted throughout the period of observation (60 days). A marked increase in the number of structures in the treated animals receiving the stimulator was observed 48 h after two injections of the hormone. The character of the proliferative processes was pulsed. The stages of proliferation of the organelles was preceded by a state of their hypertrophy. Hypertrophy of the structures was the starting stage for the measurement of proliferation. It was postulated on this basis that the healthy and pathologically changed organ respond differently to the action of the hormone. The direction of action of CG on hepatocytes of the intact liver is unknown.

The purpose of this investigation was a comparative study of proliferation of the reticulum and mitochondria of hepatocytes of the normal and pathologically changed rat liver under the influence of CG.

EXPERIMENTAL METHOD

Experiments were carried out on 30 noninbred albino rats weighing 180-200 g, divided into two groups: 1) healthy animals, 2) animals with chronic hepatitis induced by CCl_4. The animals of both groups were given CG in a dose of 150 i.u. per rat subcutaneously at 8 a.m. on 2 consecutive days. The rats were decapitated 0, 12, and 48 h after the beginning of CG injections. Before decapitation the animals were given trimeperidine in a dose of 10 mg/kg body weight. Pieces of tissue were fixed by Caulfield's method at pH 7.2 (the working solution of the fixative was made up as required), stained with uranyl acetate, dehydrated in alcohols, and embedded in Araldite by the usual methods. Serial sections were cut on the LKB-800 ultramicrotome, counterstained by Reynolds' method, and examined in the UÉMV-100K

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