INHIBITION OF ADENYLATE CYCLASE BY Ca^{++} IONS IN THE JEJUNAL MUCOSA OF RABBITS

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Adenylate cyclase (ACase) activity is inhibited in various tissues by Ca^{++} ions (10^{-4}-10^{-3} M). This effect evidently takes place through allosteric binding of Ca^{++} with the enzyme [12, 14]. The inhibitory action of Ca^{++} is unchanged during solubilization of ACase and also during further purification of the enzyme, which is accompanied by removal of its regulatory proteins from ACase [6]. A biphasic effect has been found in tissues of the brain [10] and heart [1]: Ca^{++} in low concentrations (10^{-7}-10^{-6} M) causes activation of ACase and in higher concentrations inhibits the enzyme. Activation of ACase by Ca^{++} ions takes place through the intervention of a thermostable Ca-binding protein. In some tissues Ca^{++} acts on hormonal regulation of ACase activity. For example, adrenal ACase is sensitive to the activating action of ACTH only in the presence of Ca^{++}, which does not affect the binding of this hormone with the specific receptor, although it participates in the transmission of the hormonal signal from receptor to ACase [8]. The data given above are evidence of the organ-specific action of Ca^{++} on ACase.

There are no data in the literature on the character of the action of Ca^{++} on ACase of the intestinal mucosa. However, besides its theoretical interest, the study of this problem may also be of practical importance. In various intestinal infections bacterial toxins (and, in particular, cholera exotoxin), by activating ACase in the mucosa of the small intestine, cause a marked rise in the cyclic AMP level, which probably plays a key role in the development of diarrhea. In the search for effective inhibitors of ACase in the intestinal mucosa, attention must be concentrated first on Ca^{++}, a widely available and nontoxic agent with an inhibitory action on cyclic AMP synthesis in many animal tissues.

This paper describes an analysis of the action of Ca^{++} ions on ACase in the jejunal mucosa of rabbits.

EXPERIMENTAL METHOD

The jejunal mucosa of adult rabbits was homogenized for 3-4 min at 4°C in a Potter's homogenizer in 50 ml of a solution containing 20 mM Tris-HCl and 1 mM EDTA, pH 7.5 (4°C). The homogenate was filtered through four layers of gauze and centrifuged twice for 15 min each time at 4000g. The final residue of the membranes was used as membrane ACase preparation. In special experiments the membrane preparation was preincubated for 15 min at 37°C in the presence of EGTA (1 mM), after which it was washed with isolation medium and centrifuged for 20 min at 4000g. In another variant the membrane preparation was treated additionally with a solution of high ionic strength. In this case the final residue of the membrane was incubated for 15 min (4°C) in a solution containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M KCl, and 0.25 M sucrose, then centrifuged for 30 min at 4000g, and the residue was washed with isolation medium. The control membrane preparation was treated in the same way in isolation medium. To discover whether the membrane extracts contained CA-dependent thermostable regulator, the supernatant obtained during centrifugation of the membranes was heated for 1.5 min on a boiling water bath, cooled, centrifuged (15 min, 4000g), and tested for its ability to activate phosphodiesterase (PDE) in the presence of Ca^{++}. PDE isolated from rabbit heart by the method in [7], with certain modifications [4], was used. PDE activity was measured by the method in [4] and ACase activity by the method in [13]. The concentration of the original

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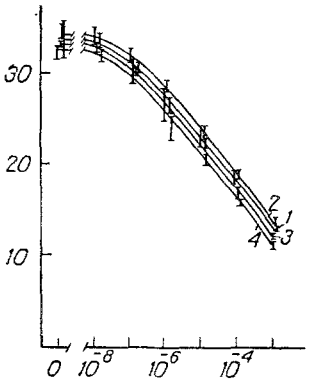

Fig. 1. Dependence of ACase activity on Ca^{++} ion concentration. Abscissa — concentration of Ca^{++} in incubation medium (in M); 0) Ca^{++} concentration 10^{-9} M. Ordinate — ACase activity (in pmoles cyclic AMP/mg protein/min). 1) In absence of hormones; 2) in presence of isoproterenol (10^{-4} M); 3) serotonin (10^{-4} M); 4) histamine (10^{-4} M).

TABLE 1. Effect of Thermostable Proteins Extracted from Membranes of the Jejunal Mucosa on Cyclic AMP PDE Activity (in counts/10 min/12 µg Protein of PDE) in Absence and Presence of Ca^{++} Ions (M ± m)

Incubation conditions	Ca^{2+}	
	10^{-9} M	10^{-3} M
Without addition of thermostable protein	417±37	540±90
The same + thermostable protein No. 1	400±40	817±32
The same + thermostable protein No. 2	450±36	1285±100

Note: Thermostable protein No. 1 was obtained by extraction of membranes with EDTA solution with low ionic strength, thermostable protein No. 2 was obtained with the same solution with 1 mM KCl and 0.25 M sucrose. The two extracts were preincubated (1.5 min, 100°C) and thereafter they possessed no PDE activity.

solutions of $MgCl_2$ and $CaCl_2$ was determined by complexometric titration in the presence of Eriochrome black T or murexide [3]. The necessary concentrations of Ca^{++} in the incubation medium were created by means of Ca^{++}-EGTA buffer. To calculate the concentrations of free Ca^{++} ions, constants of complex formation given in [2] were used. The protein concentration was determined by Lowry's method [9].

EXPERIMENTAL RESULTS

As Fig. 1 shows, ACase of the rabbit jejunal mucosa is inhibited by Ca^{++} ions over a wide range of concentrations (from 10^{-7} to 10^{-3} M). A 50% inhibition effect was obtained with Ca^{++} in a concentration of 10^{-4} M. Isoproterenol (10^{-4} M), histamine (10^{-4} M), and serotonin (10^{-4} M) did not affect mucosal ACase activity. The action of Ca^{++} on ACase was practically unaffected by the presence or absence of hormones. Unlike ACase from brain [10] and heart [1], mucosal ACase was not activated by Ca^{++} ions in concentrations of 10^{-8}-10^{-3} M.

Preincubation of the membranes with EGTA, which usually leads to removal of the regulator protein from enzymes controlled by it, did not change the character of inhibition of Acase by Ca^{++} ions. This can be explained by the absence of Ca-dependent protein regulator of ACase, which participates in manifestation of the activating effects of Ca^{++} in the brain [10] and heart [1], in membranes of the mucosa. However, since this protein does not possess

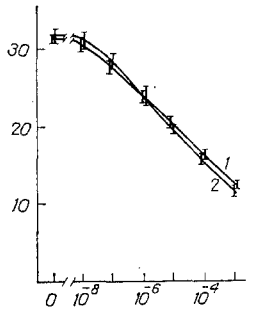

Fig. 2

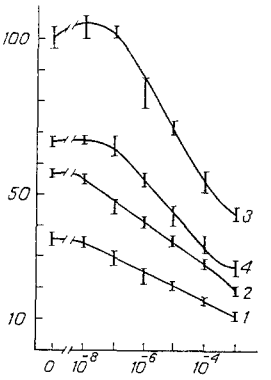

Fig. 3

Fig. 2. Adenylate cyclase activity as a function of Ca^{++} ion concentration after extraction of membrane preparation by EDTA solution with low (1) and high (2) ionic strength. Legend as in Fig. 1.

Fig. 3. ACase activity as a function of Ca^{++} concentration. 1) In absence of activators; 2) in incubation medium containing 10^{-4} M GIDP; 10^{-2} M NaF; 4) after preincubation of membranes with 10^{-4} M GIDP followed by washing membranes to remove activator. Remainder of legend as in Fig. 1.

strict enzyme specificity and since it can be conveniently tested on Ca-dependent activation of PDE, the corresponding tests were carried out. It will be clear from Table 1 that the thermostable protein extracted from membranes of the mucosa by EDTA solutions activates PDE only in the presence of Ca^{++} ions. The close similarity of its properties to those of Ca-dependent regulator of brain [10] and heart [1] membranes is also reflected in its thermostability. Extraction of the membranes by solutions of high ionic strength likewise did not change the character of inhibition of the mucosal ACase by Ca^{++} ions (Fig. 2). Consequently, proteins and structures bound to membranes by weak van der Waals forces do not participate in the mechanism of this effect.

To study the character of the inhibitory action of Ca^{++} on ACase we used guanylyl imidodiphosphate (GIDP; 10^{-4} M), a GTP analog that does not undergo hydrolysis. As Fig. 3 shows, Ca^{++} inhibited ACase equally effectively in the absence and in the presence of GIDP, ACase, preincubated with GIDP, followed by rinsing of the membrane preparation, also was inhibited by Ca^{++} ions. A similar effect of inhibition also was seen in the presence of NaF (10^{-2} M). Since there were no significant differences between the effects of Ca^{++} on basal ACase, or on ACase preincubated with GIDP and measured in the presence of GIDP and NaF, it can be concluded that the inhibitory effect of these Ca^{++} ions is evidently manifested on the catalytic process itself — on the reaction of conversion of ATP into cyclic AMP — regardless of the initial state of ACase activity. GIDP irreversibly fixes ACase in a highly active state, for this nucleotide cannot be hydrolyzed by specific membrane GTPase [5]. ACase is fixed in this same state also by cholera toxin [5]. The results suggest that activation of ACase by cholera toxin will also be reduced by Ca^{++}.

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EFFECT OF DITHIOTHREITOL ON TISSUE-SPECIFIC UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY RAT LIVER MITOCHONDRIA

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The present writers have shown that the liver [4] as well as the kidney, heart, lungs, and thymus of rats [3] contain regulators which can selectively depress the coupling of oxidative phosphorylation (OP) by mitochondria from homologous tissue. A study of the properties of a tissue-specific uncoupler (TSU) from the soluble phase of liver cells showed that this regulator is a low-molecular-weight compound with mol. wt. under 5000, and with a negative charge at alkaline pH values. A combination of gel filtration with ion-exchange chromatography on DEAE-cellulose enabled it to be purified about 200 times relative to protein [6]. However, further fractionation of the TSU was much more difficult because of its inactivation during purification. The necessity thus arises for a study of the mechanism of this process. One of the most widespread causes of inactivation of bioorganic compounds is autooxidation of the sulfhydryl groups which they contain [1]. In some cases it is reversible and the activity of some compounds can be restored by simultaneous incubation with thiol compounds, such as dithiothreitol (DTT) [7].

No data could be found on the existence of active SH-groups in the regulator which we discovered. Experiments were therefore carried out to study the action of DTT on the activity of the TSU, which had been lost in the course of the preparative procedures, with a view to establishing whether the regulator may contain sulfhydryl groups.

EXPERIMENTAL METHOD

Experiments were carried out on male Wistar rats weighing 200-250 g. The fraction of mitochondria from liver and kidney was isolated by Schneider's method with modifications. The soluble phase of liver cells was obtained by simultaneous sedimentation of all organelles from the homogenate by ultracentrifugation at 105,000g (1 h). Activity of TSU in this fraction was determined polarographically by measuring the selective increase in uptake of oxygen in the active metabolic state by liver mitochondria (ΔO_{act}). Data on the effect of the test fraction on the corresponding parameter of kidney mitochondria served as the control. The soluble phase of the cells was subjected to gel filtration on a column with Sephadex G-25 [6]. By means of this procedure the regulator could be purified 15-16-fold relative to protein. Thermal fractionation was carried out by heating the soluble phase to 90°C for 10 min. The denatured proteins were removed by centrifugation. DTT used in the experiment was from Calbiochem. Incubation with DTT continued for 1 h at 32°C. The results given in the paper are average results of five experiments.

EXPERIMENTAL RESULTS

To begin with, it was established that addition of DTT in a concentration of 10 mM to intact liver and kidney mitochondria reduced the value of ΔO_{act} of these mitochondria by 8-

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