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ACTIVATION AND PHOSPHORYLATION OF CARBONIC ANHYDRASE BY ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASES

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SUMMARY

Two isozymes of carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) from bovine erythrocyte were obtained on DE 52 cellulose column chromatography, one of which was activated in the presence of ATP and Mg^{2+} by adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase from hog muscle, or bovine brain, and the other one was not. The activation of the enzyme by the protein kinase was dependent on preincubation time and concentration of protein kinase, cyclic AMP and ATP. The concentrations of cyclic AMP and ATP required to give half-maximal activation of carbonic anhydrase by protein kinase were about $1.6 \cdot 10^{-6}$ and $1.5 \cdot 10^{-5}$ M, respectively. The activation of carbonic anhydrase was also observed in the presence of the cyclic 3′,5′-monophosphate derivatives of inosine, guanosine, uridine and cytidine as well as with N^6-2′-*O*-dibutyryladenosine 3′,5′-monophosphate by using a much higher concentration. Protein kinase modulator inhibited the activation of carbonic anhydrase by protein kinase. The protein kinase was no longer capable of activating carbonic anhydrase when it was boiled previously.

Carbonic anhydrase was phosphorylated by the protein kinase, and its reaction was cyclic AMP dependent. Phosphorylation of carbonic anhydrase was dependent on incubation time and protein concentration.

The results indicate that carbonic anhydrase is activated by cyclic AMP-dependent protein kinase, and that its activation is associated with phosphorylation of the enzyme protein.

INTRODUCTION

The hypothesis that the diverse actions of adenosine 3′,5′-monophosphate (cyclic AMP) are mediated through activation of protein kinases has been proposed [1, 2]. According to this concept, the effects of cyclic AMP are brought about by the

Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; cyclic IMP, inosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; cyclic CMP, cytidine 3′,5′-monophosphate; dibutyryl cyclic AMP, N^6-2′-*O*-dibutyryladenosine 3′,5′-monophosphate; cyclic UMP, uridine 3′,5′-monophosphate.

protein kinase in a tissue, and by the phosphorylation of the substrates of the protein kinase in that tissue. Several substrates for protein kinase which may possibly play the important role in physiological mechanisms have been identified. These include phosphorylase kinase [3], glycogen synthetase [4], histone [5], ribosomes [6], hormone-sensitive lipase [7, 8], RNA polymerase [9], neurotubule protein [10] and several membrane proteins [11–15].

We have reported that gastric acid stimulants such as carbachol, tetragastrin and histamine increased the cyclic AMP level in the gastric mucosa of theophylline-treated rat, stimulated gastric juice secretion [16], and increased the Mg^{2+}-ATPase and carbonic anhydrase activities [17]. In addition, N^6-2′-*O*-dibutyryladenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) stimulated gastric acid secretion, and in vitro addition of cyclic AMP to the supernatant fraction of rat gastric mucosa caused a substantial increase of carbonic anhydrase activity [16]. Bersimbaev et al. [18] have also observed that histamine had a stimulatory effect on the adenylate cyclase activity of rat stomach. Salganik et al. [19] have found that gastrin pentapeptide, histamine and cyclic AMP stimulated the carbonic anhydrase activity of gastric tissue. These data have suggested a series of reactions which regulate gastric acid secretion. That is, gastric stimulants activate adenylate cyclase in gastric mucosa which increases the cyclic AMP level. The cyclic nucleotide formed, in turn, activates the protein kinase which phosphorylates carbonic anhydrase with the concomitant activation in the tissue.

This paper shows that cyclic AMP-dependent protein kinase from hog muscle or bovine brain activates and phosphorylates carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) from bovine erythrocyte. This system is unique in that it has been possible to study the effect of the protein kinase on the carbonic anhydrase which has been purified to homogeneity as shown on analytical disc gel electrophoresis.

MATERIALS AND METHODS

Materials

Bovine erythrocyte carbonic anhydrase was obtained from Boehringer Mannheim. DE 52 cellulose (Whatman, microgranular preswollen) was purchased from W. and R. Balston, Ltd. Cyclic AMP, guanosine 3′,5′-monophosphate (cyclic GMP), inosine 3′,5′-monophosphate (cyclic IMP), cytidine 3′,5′-monophosphate (cyclic CMP), uridine 3′,5′-monophosphate (cyclic UMP) and dibutyryl cyclic AMP were purchased from Boehringer Mannheim. [^{32}P]Orthophosphate (carrier free) was purchased from Japan Radioisotope Association. Other materials used were as reported previously [16, 17, 20].

Preparation of carbonic anhydrase and assay for its activity

Carbonic anhydrase commercially obtained was further purified according to the method of Carter and Parsons [21] with a slight modification. One hundred mg of bovine erythrocyte carbonic anhydrase were dissolved in 20 ml of 40 mM triethanolamine–HCl buffer, pH 7.8, and applied to a 3.5 cm × 30 cm column of DE 52 cellulose which had been equilibrated with the same buffer. The enzyme proteins were then eluted from the column with a linear gradient of triethanolamine–HCl buffer (40–75 mM), pH 7.8, in a total volume of 2 l. The flow rate was about 5 ml per 7 min. Two

active enzyme peaks, designated carbonic anhydrase I and II, respectively, were obtained. The active fraction from each peak were pooled and dialysed overnight against running deionized water.

The specific activity of the carbonic anhydrase I preparation increased about 5-fold in comparison to that of the enzyme preparation before being applied to the DE 52 cellulose column chromatography. In contrast, the specific activity of the carbonic anhydrase II preparation increased about 2.5 times greater than that of the original preparation. The enzyme preparation of carbonic anhydrase I showed a single protein band on analytical disc gel electrophoresis which was carried out at pH 9.5 according to the methods of Orstein [22] and Davis [23], whereas that of carbonic anhydrase II showed a major protein band and another broad band on the electrophoresis. The specific activity of carbonic anhydrase I and carbonic anhydrase II were 9.0 and 4.5 kunits/mg protein at 0 °C, respectively.

Carbonic anhydrase activity was assayed as described by Philpot and Philpot [24] with a slight modification [17]. Definition of the enzyme unit: If the reciprocal of the reaction time is plotted against the amount of the enzyme, the result is a straight line within a reasonable range. Thus, the number of the enzyme units in solution giving a reaction time t is $K(t_0/t-1)$, where t_0 is the reaction time of the blank and K is a constant equal to 17.7.

Activation of carbonic anhydrase by protein kinase

The standard conditions of carbonic anhydrase activation by protein kinase were carried out in a preincubation volume of 1.0 ml. The standard reaction mixture, unless otherwise indicated, contained 100 mM Tris–HCl buffer, pH 7.6, 1 mM ATP, 10 mM $MgCl_2$, 10 μM cyclic AMP, 10 μg of carbonic anhydrase I or II and 100 μg of hog muscle or bovine brain protein kinase. The mixture was preincubated at 30 °C for 10 min in a shaking water bath. After preincubation, the test tubes of samples were placed in an ice bath and then the assay of carbonic anhydrase activity was started by addition of Na_2CO_3 solution containing $NaHCO_3$ as described above.

Preparation of protein kinase and assay for its activity

Cyclic AMP-dependent protein kinases were purified through the DEAE-cellulose column chromatography step from hog muscle and bovine brain according to the method of Miyamoto et al. [20]. The specific activity of the hog muscle and bovine brain protein kinases was 19.3 and 13.5 nmoles, respectively, per mg protein per 5 min at 30 °C.

Protein kinase activity was measured according to the method of Miyamoto et al. [20]. One unit of the enzyme activity was defined as that amount of enzyme which transferred 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered protein in 5 min at 30 °C in the standard system.

Preparation of protein kinase modulator

Protein kinase modulator was prepared from bovine brain through the step of trichloroacetic acid precipitation essentially as described by Donnelly et al. [25].

Other methods

[γ-^{32}P]ATP was prepared by the method of Post and Sen [26]. Protein was

measured by the method of Lowry et al [27], with bovine serum albumin as the protein standard.

RESULTS

Effect of varying preincubation time on activation of carbonic anhydrase

The activation of the carbonic anhydrase by increasing the preincubation time is shown in Fig. 1. The activity of carbonic anhydrase I was proportional to the pre-

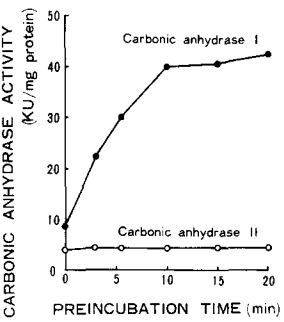

Fig. 1. Effect of preincubation time on the activation of carbonic anhydrase. The protein kinase used was obtained from hog muscle. Preincubation and incubation conditions were as described in the text, except for the variation in preincubation time. KU, Kilounits.

incubation time for 10 min, and then almost reached a plateau. In contrast, the activity of carbonic anhydrase II showed no increase for the 20 min of preincubation time tested.

Effect of varying the amount of protein kinase on the activation of carbonic anhydrase

The effect of varying the amount of hog muscle protein kinase on the activation of carbonic anhydrase is shown in Fig. 2. The protein kinase, which contained no

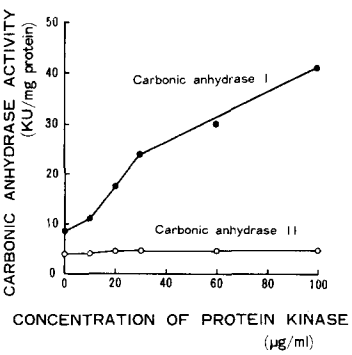

Fig. 2. Effect of protein kinase concentration on the activation of carbonic anhydrase. The protein kinase used was obtained from hog muscle. Preincubation and incubation conditions were as described in the text, except for the variation in protein kinase concentration. KU, Kilounits.

carbonic anhydrase activity itself, activated carbonic anhydrase I almost proportionally up to 100 μg/ml, and its activation fold was about 4.6 at the highest concentration (100 μg/ml) used. In contrast, carbonic anhydrase II was not activated even in the presence of 100 μg of the protein kinase.

Effect of varying concentration of cyclic AMP

The relationship between carbonic anhydrase activity and cyclic AMP concentration is shown in Fig. 3. Carbonic anhydrase I was activated by increasing the amount

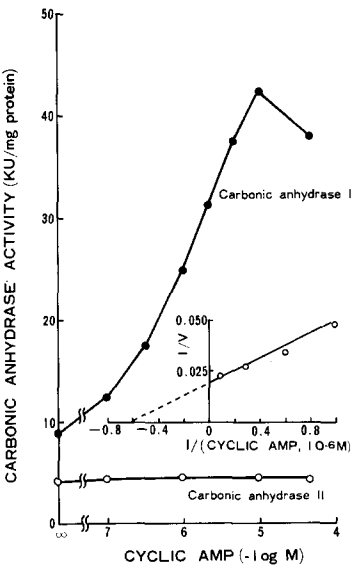

Fig. 3. Effect of cyclic AMP concentration on the activation of carbonic anhydrase. The hog muscle protein kinase was used. Preincubation and incubation conditions were as described in the text, except for the variation in concentration of cyclic AMP as indicated. KU, Kilounits.

of cyclic AMP. The maximal activity was obtained in the presence of 10^{-5} M cyclic AMP. Increasing the concentrations of cyclic AMP above 10^{-5} M caused a progressively lower activation of carbonic anhydrase, and, thus, 10^{-4} M cyclic AMP decreased it up to 75% of the maximal activity. Cyclic AMP alone, at a concentration of 10^{-5} M, was ineffective in activating carbonic anhydrase I in the absence of protein kinase. In contrast, cyclic AMP had no effect on the activation of carbonic anhydrase II at the concentration up to 10^{-4} M even in the presence of protein kinase.

A double-reciprocal plot of the activity of carbonic anhydrase I with respect to cyclic AMP concentration gave a linear relationship. The concentration of cyclic AMP required to give half-maximal activation of carbonic anhydrase I was determined to be about $1.6 \cdot 10^{-6}$ M.

Effect of cyclic AMP analogues

The effect of several cyclic nucleotides on the activation of carbonic anhydrase I is shown in Table I. Cyclic AMP was more effective in activating carbonic anhydrase I than any of its analogues. Cyclic IMP was the second best of the cyclic nucleotides

TABLE I

EFFECT OF VARIOUS CYCLIC NUCLEOTIDES ON THE ACTIVATION OF CARBONIC ANHYDRASE I

The protein kinase used was obtained from hog muscle. Preincubation and incubation conditions were as described in the text, except for the variation in kind and concentration of cyclic nucleotides. The various cyclic nucleotides were present in the final concentration indicated.

Cyclic nucleotide	Carbonic anhydrase activity (kunits/mg protein)					
	Concentration of cyclic nucleotides (M):					
	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
None	10					
Cyclic AMP		12	31	43	35	
Cyclic IMP			15	31	22	
Cyclic GMP				12	30	33
Cyclic UMP				18	21	29
Cyclic CMP				18	26	31
Dibutyryl cyclic AMP				16	28	33

tested in activating the enzyme. However, the maximal activation obtained with cyclic IMP was lower than that with cyclic AMP. Interestingly, cyclic GMP, cyclic UMP, cyclic CMP and dibutyryl cyclic AMP were capable, at higher concentrations (approx. 10^{-4}–10^{-3} M), of causing the same level of the activation as obtained with cyclic IMP in the range of concentrations tested.

Effect of varying concentration of ATP

The activity of carbonic anhydrase as a function of ATP concentration in the presence of 10^{-5} M cyclic AMP is shown in Fig. 4. The activity of carbonic anhydrase I increased by raising the concentration of ATP, and reached the maximum at 10^{-4} M. Increasing the concentrations of ATP above 10^{-3} M caused a progressively lower activation of the carbonic anhydrase I. Thus, 10^{-3} M ATP caused only 70% of the maximal activation obtained with 10^{-4} M ATP. From a double-reciprocal plot, the concentration of ATP required to give half-maximal activation of carbonic anhydrase I was determined to be about $1.5 \cdot 10^{-5}$ M. The presence of protein kinase was absolutely required for the activation of carbonic anhydrase I by ATP. Thus, 10^{-4} M ATP alone was ineffective in activating carbonic anhydrase I in the absence of protein kinase. ATP, however, was incapable of activating carbonic anhydrase II at the concentrations tested even in the presence of protein kinase.

Effect of protein kinase modulator and of boiled protein kinase on the activation of carbonic anhydrase I

Addition of 100 μg of protein kinase modulator, obtained from bovine brain, to 1.0 ml of the reaction mixture of preincubation resulted in 48% inhibition of the activation of carbonic anhydrase I by the protein kinase. Increasing the amount of the modulator to 400 μg almost completely abolished the activation of the enzyme.

The effect of the protein kinase on the activation of carbonic anhydrase I disappeared upon boiling the preparation of the protein kinase before use.

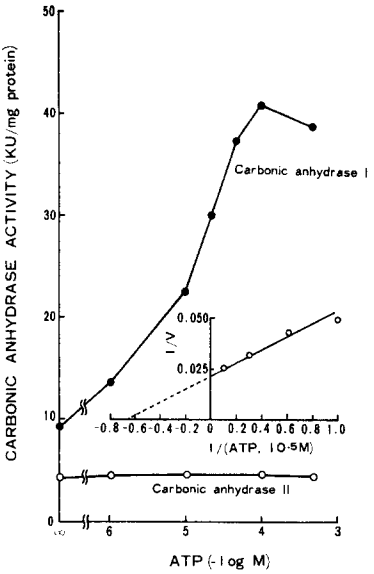

Fig. 4. Effect of ATP concentration on the activation of carbonic anhydrase. The hog muscle protein kinase was used. Preincubation and incubation conditions were as described in the text, except for the variation in concentration of ATP as indicated. KU, Kilounits.

Effect of the bovine brain protein kinase on the activation of carbonic anhydrase

When carbonic anhydrase I was preincubated in the presence of 100 μg of the bovine brain protein kinase instead of the hog muscle protein kinase with the other components for the enzyme activation, and assayed, its activity was 38 kunits/mg protein. This value was 3.8 times higher than that determined in the absence of the protein kinase. Thus, the protein kinase from bovine brain was also capable of activating carbonic anhydrase I as observed in the case of the hog muscle protein kinase. The protein kinase from bovine brain was incapable of activating carbonic anhydrase II.

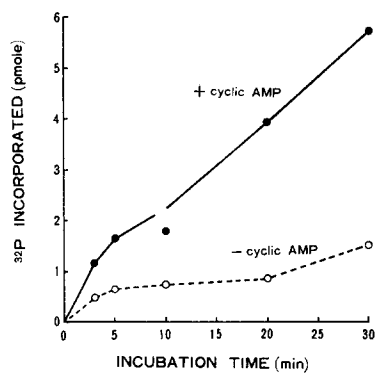

Fig. 5. Effect of incubation time on phosphorylation of carbonic anhydrase I. The hog muscle protein kinase (6.8 μg) was used. Incubation conditions were as described in the text, except for the variation in incubation time. Values for the incorporation of phosphate were corrected for those determined at each time as indicated without added substrate in the absence and presence of 1 μM cyclic AMP.

Cyclic AMP-dependent phosphorylation of carbonic anhydrase

The effect of incubation time on the phosphorylation of carbonic anhydrase I is shown in Fig. 5. The amount of phosphate incorporated into carbonic anhydrase I was proportional to the reaction time for 5 min, and increased markedly in the presence of 1 μM cyclic AMP.

The effect of varying the amount of carbonic anhydrase I on the activity of the protein kinase is depicted in Fig. 6. The amount of phosphate incorporated into

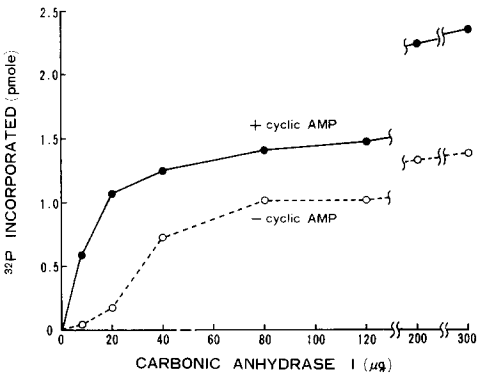

Fig. 6. Effect of carbonic anhydrase I concentration on its phosphorylation. The hog muscle protein kinase (6.8 μg) was used. Incubation conditions were as described in the text, except for the variation in concentration of carbonic anhydrase I. Values for incorporation of phosphate were corrected for 0.21 and 1.99 pmoles determined without added substrate in the absence and presence of 1 μM cyclic AMP, respectively.

carbonic anhydrase I almost reached a plateau at 200 μg per 0.2 ml of reaction mixture. The concentration of carbonic anhydrase I required to reach half-maximal velocity was 24 μg per 0.2 ml of reaction mixture, determined in the presence of 1 μM cyclic AMP. Interestingly, the phosphorylation of carbonic anhydrase I was more stimulated by cyclic AMP in the range up to 40 μg of the substrate per 0.2 ml of reaction mixture.

The ability of carbonic anhydrase I and II to serve as substrate was compared to that of other proteins including arginine-rich histone, casein and phosvitin. Arginine-rich histone was the best substrate for the protein kinase among the proteins tested, and the phosphorylation of carbonic anhydrase I was found to be less than that of casein and better than that of phosvitin. Carbonic anhydrase II also showed incorporation of phosphate into the enzyme protein. These results which are similar to the hog muscle protein kinase were obtained by using the bovine brain protein kinase.

DISCUSSION

The results described in this communication indicate that one of the carbonic anhydrase isozymes (carbonic anhydrase I) is activated by cyclic AMP-dependent protein kinase, obtained from hog muscle or bovine brain in the presence of ATP and Mg^{2+}. The activation of carbonic anhydrase was dependent on preincubation time (Fig. 1)

and the concentrations of protein kinase (Fig. 2), cyclic AMP (Fig. 3), ATP (Fig. 4) or other cyclic nucleotides (Table I). The ability or the order of the effect of cyclic nucleotides to activate carbonic anhydrase was similar to that of the nucleotides to stimulate protein kinase as described previously [28, 29], although the concentration of cyclic AMP or other cyclic nucleotides required to give half-maximal activation of carbonic anhydrase was about 10 times higher than that of cyclic AMP or other cyclic nucleotides for the protein kinase. The results suggest that the activation of carbonic anhydrase I is well correlated with that of protein kinase by cyclic AMP. Moreover, protein kinase modulator obtained from bovine brain inhibited the protein kinase to activate carbonic anhydrase I, and the heat-treated protein kinase was no longer capable of activating carbonic anhydrase I. These results indicate that the activation of carbonic anhydrase I is mediated through the protein kinase.

Carbonic anhydrase I was phosphorylated by the protein kinases and the reaction was dependent on cyclic AMP, incubation time (Fig. 5) and the substrate concentration (Fig. 6). The results suggest that the activation of carbonic anhydrase I results from the phosphorylation of the enzyme molecule, and that carbonic anhydrase I is another substrate for cyclic AMP-dependent protein kinase in association with the activation of the enzyme.

In contrast to the results of carbonic anhydrase I, carbonic anhydrase II was not activated by the protein kinase under the conditions used. The enzyme preparation of carbonic anhydrase II was phosphorylated by the protein kinases. It may be possible that the contaminating protein in the carbonic anhydrase II preparation is phosphorylated, since two protein bands were observed on analytical disc gel electrophoresis.

In this investigation, the protein kinase from hog muscle and bovine brain were used for the activation of carbonic anhydrase from bovine erythrocyte. In view of the similarity of substrate specificity for protein kinases obtained from various sources of mammalian tissues, it seems likely that carbonic anhydrase from gastric mucosa may serve as a substrate for the protein kinase in its tissue. One of the mechanisms, even if not the only, by which cyclic AMP stimulates the gastric juice secretion may involve the activation of carbonic anhydrase by cyclic AMP-dependent protein kinase in gastric mucosa.

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