Crayfish abdominal muscle adenylate cyclase

Studies on the stimulation by a Ca^{2+} -binding protein

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A plasma-membrane preparation of crayfish muscle showed an adenylate cyclase activity which is inhibited to about 80% of its original activity by $100\mu\text{M}$ -EGTA. Measurements of the enzyme activity in the presence of $100\mu\text{M}$ -EGTA and various concentrations of Ca^{2+} revealed an increase in enzyme activity of about 400%, indicating an adenylate cyclase which is dependent on Ca^{2+} for activity. Fluphenazine (1 mM), a blocker of the Ca^{2+} -binding protein calmodulin, decreased enzyme activity to zero. The enzyme can be re-activated by the addition of certain concentrations of calmodulin to the assay medium. This suggests that crayfish muscle adenylate cyclase is dependent on Ca^{2+} and calmodulin for activity.

Adenylate cyclase, the enzyme that produces cyclic AMP, together with phosphodiesterase regulates the intracellular cyclic nucleotide concentration. Generally, Ca^{2+} has been regarded as an inhibitor of adenylate cyclase. However, there seem to exist adenylate cyclases, especially from brain, which show a biphasic response to Ca^{2+} , in that Ca^{2+} concentrations in the micromolar range activate the enzyme, whereas higher concentrations inhibit it (Johnson & Sutherland, 1973; Brostrom *et al.*, 1975; Cheung *et al.*, 1975; Morishima, 1979; Piascik *et al.*, 1980, 1981). This suggests that some adenylate cyclases may be controlled by Ca^{2+} as well as by the calcium-binding protein, calmodulin. Although calmodulin is present in all tissues and species examined, a specific physiological role has not yet been established in any case (Marx, 1980; Cheung, 1980, 1981; Means, 1981; Scharff, 1981; Means *et al.*, 1982). Indeed, only a few adenylate cyclases are known to be regulated by calmodulin, for example those from brain (Piascik *et al.*, 1980), from lung membranes (Ofulue & Nijjar, 1981), from pancreatic islets (Valverde *et al.*, 1979; Sharp *et al.*, 1980) and from intestinal epithelial cells (Amiranoff *et al.*, 1982).

Reports about adenylate cyclases in invertebrates are more or less limited to insects, but to our knowledge there is only one investigation about the influence of Ca^{2+} and a calcium-binding protein on an adenylate cyclase (silkworm fat-body; Morishima, 1979).

We are interested in crayfish adenylate cyclase in connection with the mode of action of the crustacean hyperglycaemic hormone ('CHH'), which seems to act via cyclic AMP as a second messenger (Sedlmeier & Keller, 1981; Sedlmeier, 1982). The properties of crayfish adenylate cyclase in the presence of Mg^{2+} , Mn^{2+} and NaF are comparable with those of other adenylate cyclases (G. Dieberg & D. Sedlmeier, unpublished work). The studies reported in the present paper yield information about the existence of a Ca^{2+} -calmodulin-dependent adenylate cyclase in crayfish muscle. This is based on the effects of EGTA and fluphenazine dihydrochloride, an anti-psychotic drug, which is thought to act specifically on calmodulin (Levin & Weiss, 1978, 1979).

Materials and methods

Animals

Crayfish (*Orconectes limosus*) were collected from the Havel river near Berlin-Spandau. They were kept in large tanks, which were supplied by running tap water (about 12°C). Only males in intermoult stages were used for experiments. Live weight was about 20 g. The animals were fed on dry cat food.

Chemicals

$[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 410 Ci/mmol) and cyclic $[\text{H}^3]\text{AMP}$ were obtained from Amersham-Buchler, Braunschweig, Germany. Dowex AG-50

X4 was supplied by Serva, Heidelberg, Germany, and neutral alumina was from Woelm Pharm, Eschwege, Germany. All other chemicals were produced in pure grade by Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany) or Serva. Fluphenazine dihydrochloride was a gift from Dr. J. Becker, Bonn. Bovine brain calmodulin was prepared by Mr. H. G. Hergenbahn in our laboratory.

Enzyme preparation

Animals were killed by a cut through the ventral nerve cord. Abdominal muscle was dissected and homogenized, in 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol and 0.25 mM-sucrose, with a Potter-Elvehjem homogenizer with Teflon pestle. The homogenate was filtered through cheese-cloth and centrifuged (40000 g at 2°C) for 20 min. The supernatant was discarded and the pellet dissolved in 5 vol. of the homogenization buffer. This fraction was used for the enzyme assays.

Adenylate cyclase assay

Adenylate cyclase activity was assayed as described by Salomon (1979). The reaction volume of 50 μ l contained: phosphocreatine, 5 mM; creatine kinase, 50 units/ml; Tris/acetate, 25 mM; dithiothreitol, 1 mM; cyclic AMP, 50 μ M; GTP, 10 μ M; magnesium acetate, 15 mM; bovine serum albumin, 0.1 mg/ml; ATP, 75 μ M, with [α - 32 P]ATP, 1 μ Ci; and about 40 μ g of protein. The reaction was started by the addition of the enzyme, and incubations were performed at 30°C for 15 min. A solution of 2% (v/v) sodium dodecyl sulphate, 45 mM-ATP and 1.3 mM-cyclic AMP in a volume of 100 μ l was used for termination of the reaction. The nucleotides were separated on ion-exchange columns and columns of neutral alumina. Cyclic AMP production was linear with the incubation time and with protein up to 50 μ g per assay.

Protein determination

Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Crayfish muscle adenylate cyclase activity is inhibited by the addition of Ca^{2+} in concentrations higher than 10 μ M (Fig. 1). Various concentrations of EGTA were added to the incubation medium to investigate whether there is an activation by Ca^{2+} in the micromolar range that is hidden by the Ca^{2+} concentrations present in the preparation. We observed an inhibition of cyclase activity with EGTA concentrations higher than 1 μ M (Fig. 2). Maximal inhibition was reached with 100 μ M-EGTA. Measurement of cyclase activity with different Ca^{2+} concentrations in the presence of 100 μ M-EGTA produced a biphasic curve. Ca^{2+} concentrations

Fig. 1. Adenylate cyclase activity of a membrane fraction after exogenously added Ca^{2+}

O, No addition of Ca^{2+} ; ●, with Ca^{2+} . Results are means $\pm$ S.E.M. for six determinations. For details see the text.

Fig. 2. Inhibition curve of adenylate cyclase activity after addition of various concentrations of EGTA

●, Activity with EGTA; O, activity without EGTA. The values represent the means $\pm$ S.E.M. for six determinations. For details see the text.

below 10 μ M did not cause any change in activity, whereas 100 μ M- Ca^{2+} stimulated adenylate cyclase activity 2-fold. Concentrations higher than 1 mM- Ca^{2+} were inhibitory (Fig. 3).

To determine whether crayfish adenylate cyclase is regulated by calmodulin we used an anti-psychotic drug, fluphenazine, which can bind to Ca^{2+} -binding proteins and thereby inhibit activities of various

Fig. 3. Adenylate cyclase activity in the presence of 100 μ M-EGTA and various concentrations of Ca^{2+} . O, Activity in the presence of 100 μ M-EGTA only; ●, activity in the presence of 100 μ M-EGTA plus the given concentrations of Ca^{2+} . Results are means $\pm$ S.E.M. for six to nine determinations. For details see the text.

Fig. 5. Re-activation of adenylate cyclase activity inhibited half-maximally by 100 μ M-fluphenazine by means of addition of bovine brain calmodulin. ■, Activity without any treatment; O, activity after treatment with 100 μ M-fluphenazine; ●, activity after treatment with 100 μ M-fluphenazine and various concentrations of calmodulin. Results shown are from a representative experiment performed in triplicate. For details see the text.

Fig. 4. Influence of different concentrations of fluphenazine on the activity of an adenylate cyclase preparation. O, Activity in the absence of fluphenazine; ●, activity in the presence of fluphenazine. Each point represents the mean $\pm$ S.E.M. for three experiments in triplicate. For details see the text.

enzymes induced by calmodulin. With different concentrations of fluphenazine a strong inhibition of adenylate cyclase activity was observed (Fig. 4): 10 μ M-fluphenazine and higher inhibited the enzyme, with 1 mM-fluphenazine providing complete inhibition. Adenylate cyclase, half-maximally inhibited by fluphenazine, could be re-activated by the addition of bovine brain calmodulin (Fig. 5). Calmodulin (1 ng)

started the re-activation, and 100 ng was sufficient to reach the activity values from untreated membrane extracts. Higher concentrations activated the enzyme beyond the value measured without fluphenazine.

Discussion

The present results indicated that a Ca^{2+} -calmodulin-dependent adenylate cyclase exists in crayfish abdominal muscle. EGTA (100 μ M) added to the assay medium decreased the activity of the enzyme to about 20%; higher concentrations had no further effect. Ca^{2+} added in various concentrations in the presence of EGTA produced a biphasic curve. Concentrations of Ca^{2+} below 100 μ M activated the enzyme, whereas Ca^{2+} in excess of EGTA was inhibitory. This response of adenylate cyclases to Ca^{2+} has been described previously, especially from brain tissues (Brostrom *et al.*, 1975; Lynch *et al.*, 1977; Potter *et al.*, 1980), but there is also a report on such behaviour in the fat-body of an insect (Morishima, 1979).

This biphasic curve also means that only a small range of Ca^{2+} concentrations are able to act in a stimulatory manner on the enzyme. This raises some possibilities of regulation of adenylate cyclase activity by variation of the Ca^{2+} concentration in the

cell. Probably one has to look at this in connection with the activity of phosphodiesterase, the enzyme that degrades cyclic AMP to 5'-AMP. From vertebrates we know that this enzyme is activated by Ca^{2+} and calmodulin, a Ca^{2+} -binding protein. However, whether crayfish muscle phosphodiesterase is regulated by calmodulin is unknown. Fluphenazine, which is an anti-psychotic drug, is used as an inhibitor of calmodulin, and it is thought to act specifically on this molecule (Levin & Weiss, 1978, 1979; Weiss & Wallace, 1980). Crayfish adenylate cyclase is nearly completely inhibited by the action of this drug, but we were able to re-activate the enzyme by addition of bovine brain calmodulin in excess of fluphenazine. From this we conclude that the enzyme is modifiable by calmodulin and that the inhibition of the enzyme is due to the blockade of calmodulin and not to any effects of the drug on the enzyme itself.

The fact that bovine brain calmodulin and not calmodulin from crayfish muscle was used in this experiment is not a complication. Calmodulin was found in every species examined (Waisman *et al.*, 1975), and calmodulins of various species show only small differences in amino acid composition (Kakiuchi *et al.*, 1981). In the meantime we have also detected calmodulin in the muscle of the crayfish *Orconectes limosus* (H. G. Hergenbahn & D. Sedlmeier, unpublished work).

If it is possible to demonstrate a regulation of crayfish phosphodiesterase by Ca^{2+} and calmodulin, then one may speculate that cyclic AMP concentrations in crayfish muscle may be regulated by changes in cellular concentrations of Ca^{2+} , with both enzymes having different responses to Ca^{2+} . This enables one enzyme to be switched on while the other is switched off or at least is working on a smaller scale.

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