Protein Kinase Activity Stimulated by Adenosine 3':5'-Cyclic Monophosphate in Synaptic-Membrane Fragments from Ox Brain

INHIBITION OF INTRINSIC ACTIVITY BY FREE AND MEMBRANE-BOUND CALCIUM IONS

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1. Cyclic AMP-stimulated protein kinase activity phosphorylating intrinsic substrates in preparations of synaptic-membrane fragments from ox cerebral cortex was examined in relation to (a) the content of membrane-bound Ca^{2+} in the preparations and (b) added Ca^{2+} in the assay medium. 2. Centrifugal washing of synaptic-membrane fragments with buffered ethane dioxibis(ethylamine)tetra-acetate solutions decreased bound Ca^{2+} from 2.8 ± 0.4 (S.D.) to 0.9 ± 0.3 nmol/mg of protein. In washed preparations basal protein kinase activity was increased by about 40% and the cyclic AMP-stimulated activity by about 15%. Addition of Ca^{2+} in the concentration range 5–50 μM to the assay medium progressively inhibited the kinase activity of the washed preparations; in this range of Ca^{2+} concentration the basal activity was inhibited more than the stimulated activity. 3. In unwashed preparations concentrations of Ca^{2+} above 100 μM inhibited the cyclic AMP-stimulated activity more than the basal activity. 4. The inhibitory effect of several concentrations of Ca^{2+} was examined in relation to cyclic AMP concentration; no evidence for competition between Ca^{2+} and cyclic AMP for a site on the enzyme was observed.

Membrane fragments prepared from cerebral cortex of several species contain cyclic AMP-stimulated protein kinase activity and protein phosphatase activity which together catalyse the turnover of intrinsic protein-bound serine phosphate in the membrane (Rodnight & Lavin, 1966; Weller & Rodnight, 1971, 1973a; Johnson *et al.*, 1971; Maeno & Greengard, 1972; Ueda *et al.*, 1973). When measured *in vitro* the rate of the kinase is faster than that of the phosphatase; consequently incubation of membrane fragments with saturating concentrations of ATP eventually results in the complete phosphorylation of the membrane phosphoprotein (Weller & Rodnight, 1971). Experiments *in vivo*, however, showed that in the intact brain the membrane protein is only partially phosphorylated (Weller & Rodnight, 1973b). It appears likely therefore that the state of phosphorylation of membrane protein *in vivo* is regulated by factors that are either absent or only partly operate in the situation *in vitro*.

One factor which may play a part in controlling protein phosphorylation *in vivo* is the concentration of Ca^{2+} in the vicinity of the membrane. Ca^{2+} inhibits protein kinase activity *in vitro* at concentrations that have little or no activity on the phosphatase (Weller & Rodnight, 1971). However,

in the present work, the lowest concentration of Ca^{2+} found to inhibit the kinase was 50 μM , which is several times higher than the intracellular concentration of free Ca^{2+} in neural tissues (Baker, 1972; Rasmussen *et al.*, 1972). Since membrane fragments contain bound Ca^{2+} (Goldfarb & Rodnight, 1970), it appeared possible that kinase activity in normal membrane preparations may be partly inhibited by membrane-bound Ca^{2+} . The present study confirms this hypothesis and shows that bound Ca^{2+} inhibits the basal protein kinase activity to a greater extent than the activity stimulated by cyclic AMP. A preliminary report of the work has already appeared (Weller & Rodnight, 1973c).

Experimental

Materials

EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] was obtained from Fison's Scientific Apparatus Ltd., Loughborough, Leics., U.K. Calcein (fluorescein-3,3'-bismethyliminodiacetic acid) was from Hopkins and Williams, Chadwell Heath, Essex, U.K. [^{32}P]ATP was prepared as the Tris salt by the procedure of Rodnight & Lavin (1966). Other chemicals were of the purest grade available. Solutions were made up in de-ionized water.

Methods

Membrane preparations. Preparations rich in

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synaptosomal-membrane fragments were made by the procedure of Rodnight *et al.* (1969) from ox brain cerebral cortex. The preparations were stored at -20°C in a 4mM-imidazole buffer (adjusted to pH7.0 with HCl) at a protein concentration of about 10mg/ml and used within 40 days. Where indicated the cation content of the preparations was decreased by centrifugal washing with 1mM-EGTA adjusted to pH7.0 (at 2°C) with Tris base. An appropriate quantity of a preparation was suspended in 1mM-EGTA at a protein concentration of 2mg/ml and centrifuged at 12500g for 15min at 0°C in a Sorvall RC-2B centrifuge. The pellet was washed twice by resuspension in 1mM-EGTA followed by centrifugation at the same speed. Finally the washed preparation was resuspended in 1mM-EGTA at a protein concentration of 4mg/ml.

Determination of calcium. An adaptation of the method of Kepner & Hercules (1963) was used. All reagents were made up in freshly de-ionized water with a resistance greater than $4\text{M}\Omega/\text{cm}$ and were stored in plastic bottles. The calcein reagent consisted of 12mg of calcein dissolved in 2ml of 4M-KOH and made up to 400ml with water. The reagent was found to have appreciable fluorescence in the absence of added Ca^{2+} . To decrease this, 1mM-EGTA (pH7.0) was added slowly until 0.2ml samples added to 1ml of 0.4M-NaOH gave no appreciable fluorescence at 511nm in the Aminco-Bowman spectrophotofluorimeter with an activation wavelength of 405nm. It was important to avoid adding excess of EGTA solution as this would have interfered with the determination of low concentrations of Ca^{2+} .

To determine Ca^{2+} in the membrane preparations, samples were heated in platinum crucibles in a muffle furnace at 100°C until dry and then ashed at 700°C . For a sample containing 20mg of protein 3h at this temperature was required. The ash was dissolved in about 1ml of 0.1M-HCl for each 10mg of protein in the original sample and neutralized with 1M-KOH. Appropriate samples of the solution were transferred to spectrophotofluorimeter tubes and made up to 1ml with water; then 0.1ml of 4M-NaOH followed by 0.1ml of calcein reagent were added. Fluorescence was measured in an Aminco-Bowman spectrophotofluorimeter at 511nm with an activation wavelength of 405nm. The Ca^{2+} content of the sample was determined by comparison with a linear standard curve prepared from pure fresh solutions of CaCO_3 (1–10nmol). Similar results were obtained by using internal standards; recovery of added Ca^{2+} through the whole procedure was about 80%.

Determination of intrinsic protein kinase activity. The method of Weller & Rodnight (1971) was used. All reactions were done at 20°C in a reaction mixture containing 1mM- MgSO_4 , 1mM-Tris- $[\text{}^{32}\text{P}]\text{ATP}$,

30mM-Tris-HCl (pH7.4) and other additions as indicated; incubation time was 1min. The cyclic AMP-stimulated protein kinase activity is defined as the activity measured with the nucleotide present less the activity observed in its absence.

Determination of protein. The method of Miller (1959) was used with bovine plasma albumin as standard.

Stabilization of low concentrations of Ca^{2+} . The Ca^{2+} -EGTA buffer method of Portzehl *et al.* (1964) (see also Baker, 1972) was used. The ratio total Ca^{2+} /EGTA was always adjusted so the mixture had maximum buffering capacity for Ca^{2+} . No allowance was made for the presence of ATP or Mg^{2+} as their effect would be insignificantly small (Portzehl *et al.*, 1964).

Results

Inhibition of kinase activity by membrane-bound Ca^{2+}

The membrane fragments were known to contain bound Ca^{2+} (Goldfarb & Rodnight, 1970). To examine whether this was affecting kinase activity, several membrane preparations were exposed in preliminary experiments for 30min at 0°C to either Tris-EGTA or Tris-EDTA solutions (10mM) before determining kinase activity in the presence of 1mM concentrations of the same chelating agents. Exposure to EDTA lowered activity both with and without cyclic AMP equally. By contrast EGTA slightly stimulated the basal activity and inhibited the stimulated activity, resulting in a statistically significant decrease in the percentage stimulation of activity given by cyclic AMP. Addition of CaCl_2 in equimolar concentrations to the EGTA concentration (resulting in an estimated free Ca^{2+} concentration of 20–30 μM) inhibited the basal activity more than the stimulated activity and restored the percentage cyclic AMP-stimulation to its control value.

These results suggested that bound Ca^{2+} was involved in the stimulation of kinase activity by cyclic AMP, and it was therefore decided to examine kinase activity in a membrane preparation that had been centrifugally washed with EGTA solutions to remove bound Ca^{2+} . The washing procedure decreased bound Ca^{2+} in the preparations from $2.8 \pm 0.4\text{nmol/mg}$ of protein (s.d., eight observations) to $0.9 \pm 0.3\text{nmol/mg}$ of protein. Basal kinase activity in the washed preparations was increased by about 40% and stimulated activity by about 15%; the apparent stimulation of activity on a percentage basis by cyclic AMP was therefore decreased by the treatment. When Ca^{2+} , in the concentration range 5–100 μM , was added to the assay medium both components of the kinase activity were inhibited. However, in the range 5–50 μM - Ca^{2+} the basal activity was markedly more sensitive to inhibition by Ca^{2+} than was the stimulated activity,

Fig. 1. Inhibition of protein kinase activity by Ca^{2+} in an EGTA-washed membrane preparation

EGTA-washed membrane fragments were prepared as described in the Experimental section. Protein kinase activity was determined in the presence of 0.1 mM-Tris-EGTA (added with the membrane preparation, as indicated in the Experimental section) and sufficient CaCl_2 to give the stated concentration of free Ca^{2+} (Portzehl *et al.*, 1964). Each point is the mean of six observations; vertical bars indicate ± 1 s.e.m. Enzyme activity is expressed as a percentage of activity in the unwashed preparation. ●, Activity without cyclic AMP; ■, activity with 10 μM -cyclic AMP minus basal activity.

thus restoring the original quantitative relation between the two components of the total activity (Fig. 1). In unwashed preparations, however, concentrations of Ca^{2+} of 100 μM or higher inhibited the stimulated activity to a greater degree than the basal activity (Fig. 2).

Inhibition of kinase activity by Ca^{2+} in relation to cyclic AMP concentration

The above results suggested the possibility that cyclic AMP may stimulate kinase activity in the membrane fragments by displacing inhibitory bound Ca^{2+} by a competitive mechanism. This interpretation was shown to be untenable by determining the degree of inhibition of kinase activity by Ca^{2+} in washed and unwashed preparations in the presence of various concentrations of cyclic AMP. Figs. 3(a) and 3(b) show that the inhibition of both the total activity and the cyclic AMP-stimulated activity by Ca^{2+} was not overcome by increasing the concentration of cyclic AMP in the assay; very high concentrations of cyclic AMP were in fact inhibitory.

Fig. 2. Inhibition of protein kinase activity by Ca^{2+} in an unwashed membrane preparation

Ca^{2+} was added as CaCl_2 without buffering with EGTA. Each point is the mean of four to six observations; vertical bars indicate ± 1 s.e.m. ●, Activity without cyclic AMP; ■, activity with 100 μM -cyclic AMP minus basal activity.

It is noteworthy that the cyclic AMP-stimulated activity of both the EGTA-washed preparations and normal preparations assayed in the presence of Ca^{2+} was less sensitive to low concentrations of cyclic AMP than was the same activity in untreated preparations.

Discussion

These results show that membrane preparations from ox cerebral cortex contain sufficient bound Ca^{2+} to inhibit intrinsic protein kinase activity in the membrane. Thus decreasing the bound Ca^{2+} by only 2 nmol/mg of protein resulted in an increase of kinase activity of about 40%. In a reaction volume of 2.5 ml this decrease in bound Ca^{2+} is equivalent to a concentration of free Ca^{2+} of 0.8 μM , which compares with the concentrations (30–50 μM) that are required to inhibit kinase activity in washed preparations to the value of rates observed in unwashed preparations (Fig. 1). It appears likely therefore that part at least of the bound Ca^{2+} normally present in membrane fragments is bound to specific sites controlling kinase activity. It is noteworthy that in washed preparations inhibition of kinase activity was observed with Ca^{2+} concentrations comparable with the intracellular concentration of free Ca^{2+} in nervous tissue (Baker, 1972; Rasmussen *et al.*, 1972). Coupled with the obser-

Fig. 3. Sensitivity of protein kinase activity to cyclic AMP at various concentrations of Ca^{2+}

Ca^{2+} was added as CaCl_2 without buffering with EGTA. Each point is the mean of six observations or in the case of ▼ the mean of duplicates. ●, Unwashed preparation, no added Ca^{2+} ; ▲, unwashed preparation with 0.1 mM- Ca^{2+} ; ▼, unwashed preparation with 0.25 mM- Ca^{2+} ; ■, EGTA-washed preparation. (a) Total kinase activity. (b) Activity with cyclic AMP minus basal activity.

vation that EGTA increases protein phosphorylation in respiring slices of cerebral cortex (Williams, 1974) the results suggest that Ca^{2+} bound to the membrane may be a factor controlling the activity of this enzyme in the intact tissue. However, the possibility cannot be excluded that some of the bound Ca^{2+} inhibiting the enzyme in membrane fragments may have become bound to the cell membrane as a result of disruption of the cell structure and redistribution of tissue Ca^{2+} .

The present study does not permit any firm conclusions to be made regarding the mechanism of the inhibition of kinase activity by Ca^{2+} , nor why the basal activity is more sensitive to inhibition by the cation than is the cyclic AMP-stimulated activity. However, the observation that inhibition by Ca^{2+} was not overcome by increasing the concentration of cyclic AMP excludes competition between the nucleotide and Ca^{2+} for a common site in the enzyme system. Further speculation on the basis of the present data would be premature in view of several factors. First, the heterogeneity of the membrane preparation makes it uncertain whether the basal and cyclic AMP-stimulated activities represent components of the same enzyme system; secondly, the presence of associated protein phosphatase activity in the membrane fragments, which limits the rate of ^{32}P labelling (Weller & Rodnight, 1971), precluded measurement of the maximum rates of protein phosphorylation; thirdly, because the subunit structure of the membrane-bound kinase

system is not known. With regard to the latter it is particularly relevant to know whether the membrane-bound system consists of regulatory and catalytic units, as has been shown for other protein kinases from the cytosol (Walsh *et al.*, 1972).

The inhibitory effect of Ca^{2+} on intrinsic kinase activity observed in the present work contrasts with the reports of Johnson *et al.* (1971) and Ueda *et al.* (1973), that Ca^{2+} supports endogenous protein phosphorylation in the absence of Mg^{2+} and cyclic AMP in synaptic membrane fragments from rat brain. In our experience with the ox brain preparation no phosphorylation is observed if Mg^{2+} is replaced by Ca^{2+} and if Mg^{2+} is present Ca^{2+} is always inhibitory. Other relevant discrepancies between our results and those of Greengard's group (Ueda *et al.*, 1973) were mentioned in a previous paper (Weller & Rodnight, 1973a).

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