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Zinc causes tyrosine phosphorylation of hippocampal p60$^{c\text{-}src}$

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Zinc cations at concentrations of 0.2 mM and greater catalyzed specific phosphorylation, by ATP, of two membrane-associated proteins from rat hippocampus. These proteins, corresponding to molecular weights of 60 and 49 kDa, were phosphorylated primarily at tyrosine residues. The 60-kDa protein was identified as p60$^{c\text{-}src}$ by immunoprecipitation using two different p60^{src}-specific monoclonal antibodies. The 49-kDa protein co-immunoprecipitated with p60$^{c\text{-}src}$. Cyanogen bromide cleavage of p60$^{c\text{-}src}$ and the 49-kDa protein phosphorylated in the presence of Zn^{2+} gave different patterns of phosphopeptides. It is suggested that tyrosine phosphorylation of p60$^{c\text{-}src}$ and the p60$^{c\text{-}src}$-associated 49-kDa protein may be a way of zinc participation in hippocampal neurotransmission.

Zinc; Protein phosphorylation; Phosphotyrosine; p60$^{c\text{-}src}$; Rat hippocampus

1. INTRODUCTION

Zinc is considered to play an important role in the central nervous system [1] where this element is concentrated primarily in the hippocampus. The concentration of zinc in mossy fibers of human and rat hippocampus has been estimated at 0.22–0.30 mM [2]. Zn^{2+} is actively taken up and released from the mossy fibre terminals during stimulation of nerve fibre tracts [3,4], suggesting that it may act as a neuromodulator. However, the functional significance of zinc for neurotransmission is currently unknown.

Zinc has been shown to modulate protein kinase C [5] and calmodulin-stimulated protein kinase II [6] activities. Zn^{2+}, acting as the sole bivalent cation, induced tyrosine kinase activities of human and sheep platelet membranes [7,8]. One of us has observed zinc-induced tyrosine phosphorylation of p56^{lck} (a protein kinase of the *Src* family) in particulate fraction from lymphoma cells [9]. Keeping in mind the significant role of protein phosphorylation in intercellular communication, we have analyzed in this study the ability of Zn^{2+} to induce phosphorylation of membrane-associated proteins from rat hippocampus.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (> 5,000 Ci/mmol) was purchased from Amersham. The anti-*Src* monoclonal antibody, 327, was a gift of J. Brugge (University of Pennsylvania). The anti-*Src* monoclonal antibody, LAO, was from NCI Repository Microbiological Associates (Bethesda).

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2.2. *Preparation of membrane fraction from rat hippocampus*

Six male Wistar rats (2–3 months old; 200–300 g) were decapitated, hippocampi were removed and homogenized on ice with a glass–Teflon Potter homogenizer in 5 vols. of 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM PMSF. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was centrifuged at 100,000 × *g* for 60 min. The precipitate was homogenized in 50 mM HEPES-NaOH buffer, pH 6.9, containing 5 mM EDTA and 1 mM PMSF, and centrifuged at 100,000 × *g* for 60 min. The pellet was washed with 50 mM HEPES buffer, pH 6.9, containing 1 mM PMSF, resuspended after centrifugation in the same buffer and frozen at −80°C. The protein concentration of the fractions was measured by the method of Bradford [10].

2.3. *Protein phosphorylation assay*

The standard reaction mixture (20 μl) contained 50 mM HEPES buffer, pH 6.9, 0.01–1.0 mM $ZnCl_2$ or the salt of another bivalent cation, 10^{-7}–10^{-4} M [γ-^{32}P]ATP (1–10 Ci/mmol) and membrane fraction proteins (1–2 mg/ml). After incubation for 1 to 10 min at 37°C the reaction was terminated by the addition of 7 μl of sample buffer, containing 10% SDS and 20% β-mercaptoethanol, and boiling for 5 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% gels, which were then stained with Coomassie blue, dried and exposed for autoradiography.

2.4. *Phosphoamino acid analysis*

After phosphorylation and electrophoresis the proteins were electrophoretically transferred from the gel to an Immobilon (Millipore) membrane [11]. The membrane was exposed for autoradiography. Pieces of Immobilon, containing individual phosphoproteins, were excised and subjected to acid hydrolysis by 200 μl of 6.0 N HCl for 2 h at 110°C. The hydrolysate was analyzed by electrophoresis at 800 V on a cellulose plate at pH 3.5 [12].

2.5. *Immunoprecipitation*

Proteins of hippocampal membranes (120–150 μg) were phosphorylated in a mixture containing 50 mM HEPES, pH 6.9, 0.5 mM $ZnCl_2$ and 0.1 mM [γ-^{32}P]ATP (5 Ci/mmol) for 5 min at 37°C and spun for 10 min at 11,000 × *g* at 4°C. Proteins were extracted from the precipitated membranes with a buffer containing 50 mM Tris-HCl, pH 8.1, 0.5 M KCl, 2% Nonidet P40, 10% glycerol, 0.2 mM Na_3VO_4, 1 mM EDTA and 2 mM dithiothreitol, and immunoprecipitated ac-

cording to [13] using either Sepharose–monoclonal anti-phosphotyrosine (Amersham) or one of the antibodies indicated in the legend to Fig. 4, followed by Protein A–Sepharose. Immunoprecipitated proteins were eluted from washed Sepharose beads by boiling with electrophoresis sample buffer, and subjected to electrophoresis.

2.6. *Cyanogen bromide cleavage analysis*

After phosphorylation and electrophoresis dried gel bands containing individual phosphoproteins were excised and subjected to cyanogen bromide treatment by the method of Takeya et al. [14], loaded onto 18.75% SDS-polyacrylamide gels containing 6 M urea prepared as described by Kadenbach et al. [15] and analyzed by electrophoresis.

3. RESULTS AND DISCUSSION

Incubation of hippocampal membranes with ATP and Zn^{2+} at concentrations greater than 0.2 mM led to phosphorylation of 60- and 49-kDa membrane-associated proteins (Fig. 1). This phosphorylation was observed at ATP concentrations starting at 0.01 mM, and reached a plateau in 5 min at 37°C. The pattern of protein phosphorylation caused by Zn^{2+} was specific in comparison with the patterns of phosphorylation induced by Mg^{2+}, Mn^{2+} and Ca^{2+} (Fig. 1), as well as by Fe^{2+}, Ni^{2+}, Co^{2+} and Cd^{2+} (data not shown). Magnesium cations had no significant influence on the protein phosphorylation induced by Zn^{2+} (Fig. 1, lane 9). Phosphoamino acid analysis has determined that the 60- and 49-kDa proteins were phosphorylated in the presence of zinc cations primarily at tyrosine and, to some extent, serine residues (Fig. 2). Both proteins bound to phosphotyrosine-specific Sepharose-bound monoclonal antibody, and the binding was inhibited by pre-incubation of the antibody with phosphotyrosine (Fig. 3). Vanadate (an inhibitor of protein tyrosine phosphatases) had no effect on the phosphorylation of the proteins in the presence of Zn^{2+} (Fig. 1, lane 7), as was found in the absence of this cation. These results

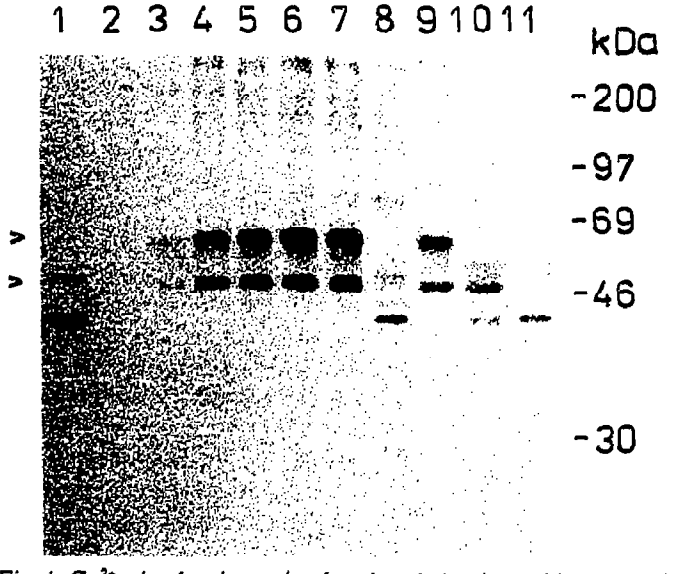

Fig. 1. Zn^{2+}-stimulated protein phosphorylation in rat hippocampal membranes washed with EDTA. Phosphorylation by 0.1 mM [γ-^{32}P]ATP was performed for 5 min in the absence of bivalent cations (lane 1) and in the presence of $ZnCl_2$ at concentrations of 0.1 mM (lane 2), 0.2 mM (lane 3), 0.3 mM (lane 4), 0.4 mM (lane 5), 0.5 mM (lane 6), or in the presence of 0.5 mM $ZnCl_2$ and 0.1 mM Na_3VO_4 (lane 7), 1.0 mM $MgCl_2$ (lane 8), 0.5 mM $ZnCl_2$ and 1.0 mM $MgCl_2$ (lane 9), 1.0 mM $MnCl_2$ (lane 10), or 1.0 mM $CaCl_2$ (lane 11). Arrows mark the positions of the 60- and the 49-kDa proteins.

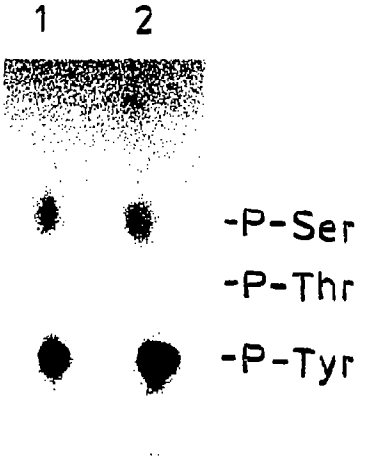

Fig. 2. Phosphoamino acid analysis of the 47-kDa protein (lane 1) and the 60-kDa protein (lane 2) phosphorylated in the presence of $ZnCl_2$.

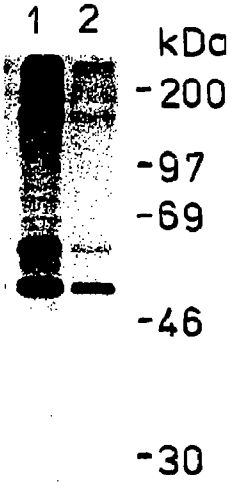

Fig. 3. Determination of phosphotyrosine-containing proteins bound to Sepharose-bond monoclonal antibody to phosphotyrosine after phosphorylation of hippocampal membranes by 0.1 mM [γ-^{32}P]ATP in the presence of 0.5 mM $ZnCl_2$. Antibody was not pre-incubated (lane 1) or was pre-incubated with 10 mM phosphotyrosine (lane 2).

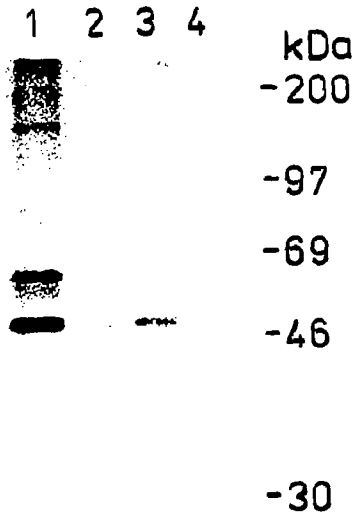

Fig. 4. Identification of the proteins immunoprecipitated from the membranes phosphorylated by Zn[γ-^{32}P]ATP with p60^{src}-specific monoclonal antibodies 327 (lane 1) and LAO (lane 3), or with the control monoclonal antibodies to leukocyte common antigen (lane 2) and to synaptophysin (lane 4).

show that Zn^{2+} alone, as well as in the presence of Mg^{2+} at physiological concentrations, induces tyrosine phosphorylation of two proteins associated with membranes of hippocampus.

Brain, particularly hippocampal pyramidal cells, contains a high level of the p60$^{c\text{-}src}$ [16,17], 60-kDa membrane-associated *Src* proto-oncogene product which is phosphorylated at tyrosine residues [12,14]. To elucidate whether the 60-kDa protein phosphorylated in the presence of Zn^{2+} is p60$^{c\text{-}src}$ we performed immunoprecipitation with two different p60^{src}-specific monoclonal antibodies. Both antibodies immunoprecipitated the 60-kDa phosphoprotein (Fig. 4), therefore we concluded that this protein is the p60$^{c\text{-}src}$. The 49-kDa phosphoprotein co-immunoprecipitated with p60$^{c\text{-}src}$ (Fig. 4). To determine whether 49-kDa phosphoprotein is a degradation product of p60$^{c\text{-}src}$, or a p60$^{c\text{-}src}$-associated protein, both p60$^{c\text{-}src}$ and the 49-kDa protein were subjected to cyanogen bromide cleavage analysis. Different patterns of phosphopeptides for these two proteins (Fig. 5) led us to conclude that the 49-kDa protein phosphorylated at tyrosine residues in the presence of Zn^{2+} is not a product of p60$^{c\text{-}src}$ proteolysis but a p60$^{c\text{-}src}$-associated protein.

The presented results demonstrate that zinc cations, at physiological concentrations, stimulate tyrosine phosphorylation of p60$^{c\text{-}src}$ and the 49-kDa protein associated with p60$^{c\text{-}src}$ in rat hippocampal membranes. This is of particular interest because the hippocampal pyramidal cells are the major site of p60$^{c\text{-}src}$ localization [17] and have an uptake system for the endogenous zinc released from mossy fibers in post-synaptic dendrites [18]. Therefore we propose that Zn^{2+} may participate in hippocampal neurotransmission via protein-tyrosine phosphorylation involving phosphorylation of p60$^{c\text{-}src}$ and the p60$^{c\text{-}src}$-associated 49-kDa protein.

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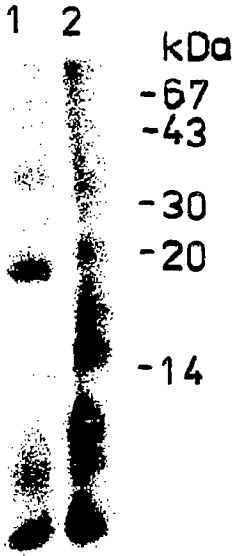

Fig. 5. Cyanogen bromide cleavage analysis of p60$^{c\text{-}src}$ (lane 1) and the 49-kDa protein (lane 2) phosphorylated in the presence of $ZnCl_2$.

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