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QUARTERLY

In vitro phosphorylation of α-preprotachykinin by protein kinase C

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The undecapeptide substance P (SP) is the main mammalian member of the family of tachykinins. Extensive studies have led to the conclusion that SP and other biologically active peptides are of the greatest importance as neurotransmitters or modulators involved in neurotransmission [1].

It is generally accepted that a small biologically active peptide, such as substance P, is initially synthesized as a larger precursor — α-preprotachykinin (α-PPT) (M_r 13000) [2]. In addition to α-PPT, in mammalian tissues, β-PPT and γ-PPT were also found. Differential RNA splicing of the mammalian SP gene primary transcript was recently reviewed [3]. Precursors of regulatory peptides, such as α-PPT, undergo a maturation process, which includes proteolytic processing. In the cells peptides are produced from precursors through cleavage at pairs of basic amino acids (Lys-Lys or Arg-Arg). Although enzyme activities specific for cleavage at adjacent basic amino acids within mammalian peptide precursors have been recently reported (subtilisin-related proteinases called furin, PC2, PC1/3, PC4, PACE4) [4], up to now, little or nothing has been known about the regulation of this process. Reversible modifications of α-PPT, e.g. phosphorylation might be one of many factors which are of the significance in the regulation of SP release from α-PPT. However, nothing has been known so far about posttranslational modifications of intact α–PPT molecules, mainly because metabolic turnover of such proteins as α-PPT is extremely rapid and it is very difficult or even impossible to isolate the intact SP precursor from mammalian cells.

The SP gene expression responsible for the production of α-PPT takes place mainly in the nervous tissue. It has also been shown that the nervous tissue is the richest source of protein kinases, and phosphorylation/dephosphorylation of brain proteins are of primary significance in neurotransmission [5]. Therefore, using the recombinant α-PPT obtained previously in an efficient *Escherichia coli* expression system by a procedure involving fusion to β-galactosidase [6], we have examined phosphorylation *in vitro* of the substance P precursor protein. The reaction mixture for phosphorylation of α-PPT contained in a final volume of 50 µl: 50 mM Tris/HCl buffer, pH 7.5, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 2 µM SC-10 ([*N*-(*n*-heptyl)-5-chloro-1-naphthalenesulfonamide]), 50 µg/ml phosphatidylserine, 5 µM [γ-^{32}]ATP (sp. act. 20 Ci/mmol), 40 ng of protein kinase C (Calbiochem, sp. act. 2500 picomolar units/µg) and 600 ng of α-PPT.

The incubation was carried out at 37°C for 1 h. Then the reaction was stopped by the addition of an equal volume of the buffer containing 0.125 M Tris/HCl, pH 6.8, 6% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were boiled for 2 min and 50 µl aliquots were applied on 18% polyacrylamide-SDS gels. Proteins were visualized by silver staining (Fig. 1A), the gels were dried and the

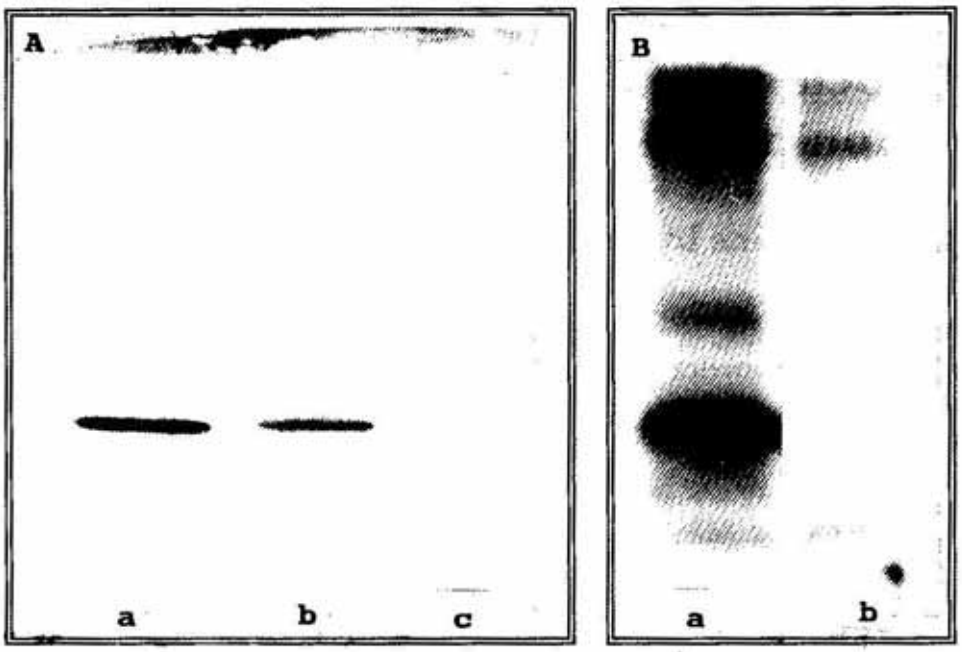

Fig. 1. SDS-polyacrylamide gel electrophoresis of the substance P precursor (α-PPT) preparation phosphorylated by protein kinase C in vitro. A. Silver stained gel: lane a, lysozyme (marker); lane b, phosphorylated α-PPT; lane c, control (incubation without α-PPT). B. Autoradiogram; lanes a and b correspond to lanes b and c, respectively, of Fig. 1A.

radioactive proteins were detected by autoradiography on X-ray film (Fig. 1B).

The recombinant precursor of substance P (α-PPT) prepared from *E. coli* cells was readily phosphorylated by protein kinase C (PKC) from rat brain (Fig. 1B; lane a). To our knowledge, phosphorylation *in vitro* of α-PPT is here reported for the first time.

PKC is found at high levels in the brain and has a broad protein substrate specificity. Although there has been as yet limited information on physiological targets of PKC, the sequence of a number of substrates of PKC has been determined *in vitro* with the use of synthetic peptide substrates or pseudosubstrates. It was found that PKC requires for its action basic residues surrounding the target serine or threonine [7], a feature common to a number of serine/threonine-specific protein kinases. The presence of positively charged amino acids on C-terminal tail of α-PPT suggests that two or three different seryl residues might serve as phosphorylation sites. However, precise identification of the phosphorylation sites requires further investigation.

Highly specific proteolysis at sites bearing basic amino acids is an important requirement for maturation of a large number of protein precursors. Assuming that good peptide substrates for PKC are characterized by a cluster of positively charged residues surrounding serine or threonine, we suppose that proteolytic processing of α-PPT might be controlled *via* reversible phosphorylation/dephosphorylation reactions. Perhaps negatively charged phosphate groups introduced into seryl residues placed near the clusters of basic amino acids are likely to alter the conformation state of α-PPT and its susceptibility to proteolysis. Therefore, the phosphorylation state of α-PPT may serve as a mechanism regulating its molecular stability.

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