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Activation of protein kinase C by the 14-3-3 proteins homologous with Exo1 protein that stimulates calcium-dependent exocytosis

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The 14-3-3 proteins are a family of acidic proteins found mainly in the brain and are suggested to have a role in monoamine synthesis based on their ability to activate tyrosine and tryptophan hydroxylases in the presence of type II Ca^{2+}/calmodulin-dependent protein kinase. Recently, however, it has been demonstrated that a member of the 14-3-3 family, termed Exo1, stimulates Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells, suggesting that this protein family may influence the protein kinase C-mediated control of Ca^{2+}-dependent exocytosis. Here we show that the 14-3-3 proteins activate protein kinase C at about 2-fold more than the known level of the activated protein kinase, i.e. the activity of protein kinase C in the presence of Ca^{2+} and phospholipids. This raises the possibility that the cellular activity of protein kinase C is regulated by diverse members of the 14-3-3 family and that the reported ability of Exo1 to reactivate Ca^{2+}-dependent exocytosis is based on its stimulatory effect on protein kinase C activity. The 14-3-3 family, therefore, appears to be a multifunctional regulator of cell signalling processes mediated by two types of Ca^{2+}-dependent protein kinase, protein kinase C and type II calmodulin-dependent protein kinase.

Signal transduction; Ca^{2+}-dependent exocytosis; Protein kinase C

1. INTRODUCTION

The 14-3-3 proteins are a family of acidic, dimeric proteins with a subunit mol. wt. of ~30 kDa [1]. This protein family is distributed mainly in the brain especially in neurons, and is axonally transported to the nerve terminals [2–4]. The 14-3-3 proteins purified from brain or reconstituted in vitro from isolated polypeptides activate tyrosine and tryptophan hydroxylase (TH and TRH) in the presence of type II calmodulin-dependent protein kinase (PKII) [1,5–7]. They are also essential for activation of TRH by cAMP-dependent protein kinase [8]. Due to this activity and neuronal distribution this protein family has been suggested to have a role in monoamine synthesis. However, evidence is now accumulating that a subset of the family is also expressed in various mammalian tissues [7,9], transformed human epithelial amnion cells [10], *Drosophila melangaster* [11], and in plants [12,13], suggesting the widespread distribution of this family among eukaryotic cells. In proliferating fibroblasts, the synthesis of one of the members of the protein family is down-regulated compared to SV40 transformed cells [14].

Of the seven polypeptide types found in the bovine brain (termed α to η) three (β, γ, and η) have been sequenced through direct protein analysis or by cloning their complementary DNAs [1,7]. The human [15], *Drosophila* [11], and plant [12,13] homologues have also been cloned. These polypeptides exhibit extraordinarily high sequence conservation and have similar characteristics to a highly acidic C-terminal region that may be responsible for the kinase-dependent activation of TH and TRH [1]. The sequences of the 14-3-3 proteins are unique, however, Aitken et al. [16,17] recently found a strong homology of the sequence of the bovine 14-3-3 protein with a sheep protein, termed KCIP, which is purported to inhibit the enzymatic activity of protein kinase C (PKC). More recently, Morgan and Burgoyne [18] isolated endogenous protein factors that stimulate Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells, and identified one of these proteins, termed Exo1, as a member of the 14-3-3 family. They suggested that Exo1 may influence the PKC-mediated control of Ca^{2+}-dependent exocytosis.

In this report we have examined the effect of the 14-3-3 proteins on the activity of PKC and found that

Abbreviations: PKII, Ca^{2+}/calmodulin-dependent protein kinase type II; TH, tyrosine hydroxylase; TRH, tryptophan hydroxylase; HPLC, high performance liquid chromatography.

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the 14-3-3 proteins activate PKC in the presence of known ligands of this protein kinase.

2. EXPERIMENTAL

The ζ chain was isolated by reverse-phase HPLC of bovine brain 14-3-3 protein and was renatured as in [7] for activity measurements. The renatured ζ-chain formed a dimer with a relative molecular mass of ca. 60,000 as examined by two-dimensional electrophoresis without chaotrophic agents [9]. PKC was purified from rat brain extracts essentially as in [19]. PKC activity was assayed in a mixture (100 μl) containing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mg/ml histone (type IIIs, Sigma), 50 μM [γ-^{32}P]ATP (1,000 cpm/pmol, Amersham Corp.), and either 1 mM EGTA or 0.5 mM $CaCl_2$, 50 μg/ml phosphatydylserine, 1 μg/ml diolein, and appropriate amounts of the renatured ζ_2 protein. The reaction was initiated at 30°C for 3 min and was stopped by the addition of 10% (w/v) trichloroacetic acid (1 ml). The mixture was poured onto a nitrocellulose disk (TM-2; Toyo Membrane Filters, Tokyo) and the disk was washed and counted.

The amino acid sequence of the ζ chain was determined by the analysis of seven CNBr fragments and sixteen fragments derived by lysylendopeptidase digestion of pyridylethylated ζ chain (1 mg each), as well as of several subfragments produced by secondary chymotryptic cleavage of the CNBr or lysylendopeptidase fragments. The fragments were isolated by reverse-phase chromatography on a Phenyl-5PWRP column (4.6 × 75 mm; Tosoh, Tokyo) or on a Lichrosorb RP-8, select B column (4.6 × 250 mm; Merck, Germany) by elution with a linear gradient of CH_3CN (10–60%, v/v) in 0.1% (v/v) trifluoroacetic acid. Where necessary, the fragments were further purified by re-chromatography using 0.08% (v/v) heptafluorobutyric acid in place of trifluoroacetic acid. The amino acid sequence of isolated fragments was determined with a model 477A automated sequencer (Applied Biosystems, Foster City, CA).

3. RESULTS AND DISCUSSION

Among the seven polypeptide types, α–η, of the 14-3-3 protein [1] we have chosen the ζ chain for analysis because it is most abundant in our bovine brain preparation. Thus, the effect of the dimeric ζ_2 protein on the activity of PKC was examined by an assay system similar to that used for the identification of KCIP (Fig. 1 and its legend), which was reported to be a potent inhibitor of PKC with an amino acid sequence highly homologous with 14-3-3 [16,17]. Despite the sequence similarity with KCIP, the ζ_2 protein added to the assay mixture stimulated the activity of PKC to about 2-fold more than the level of the activated PKC measured in the presence of known ligands of this protein kinase. The effect was dose-dependent, and the concentration of 14-3-3 necessary for half-maximal activation ($V_{max}/2$) of PKC was 0.77 μM. This concentration is almost equal to the reported concentration at which KCIP exhibits 50% inhibition of PKC activity [17]. The ζ_2 protein also activated the phosphatylserine-activated PKC in the absence of diolein. However, the activation did not occur in an assay mixture without Ca^{2+} or without phospholipids, where the activity of PKC remained at a basal level even in the presence of excess amount of the ζ_2 protein (Fig. 1). Subsequent phosphoprotein analysis of the assay mixture by reverse-phase HPLC (Fig. 2) or by

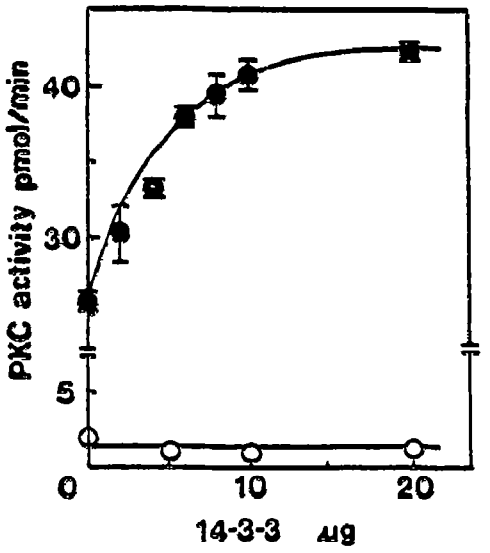

Fig. 1. Effect of the ζ_2-type 14-3-3 protein on the activity of PKC in the presence (●) and absence (○) of Ca^{2+} and phospholipids. The data are from triplicate determinations.

autoradiography following polyacrylamide gel electrophoresis demonstrated heavy incorporation of ^{32}P into the substrate, histone, but no significant radioactivity was detected in the ζ chain. Because direct phosphoryl-

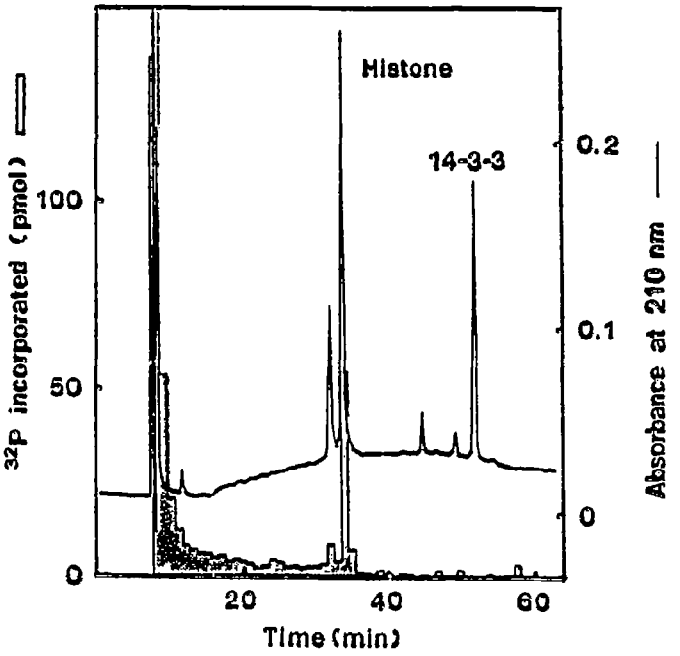

Fig. 2. Analysis of phosphorylated protein. A PKC assay mixture containing the ζ_2 protein (10 μg) was prepared as described in section 2, incubated for 3 min at 30°C in the presence of Ca^{2+} and phospholipids, and a portion of the reaction mixture was applied to a reverse-phase Vydac C_4 column (6.0 × 150 mm). The column was eluted with a linear gradient of CH_3CN (10–60%, v/v) in 0.1% trifluoroacetic acid and 1 ml aliquots were collected and counted for ^{32}P. Peaks were identified from the retention time of each component as well as by amino acid analysis of polypeptides recovered from HPLC.

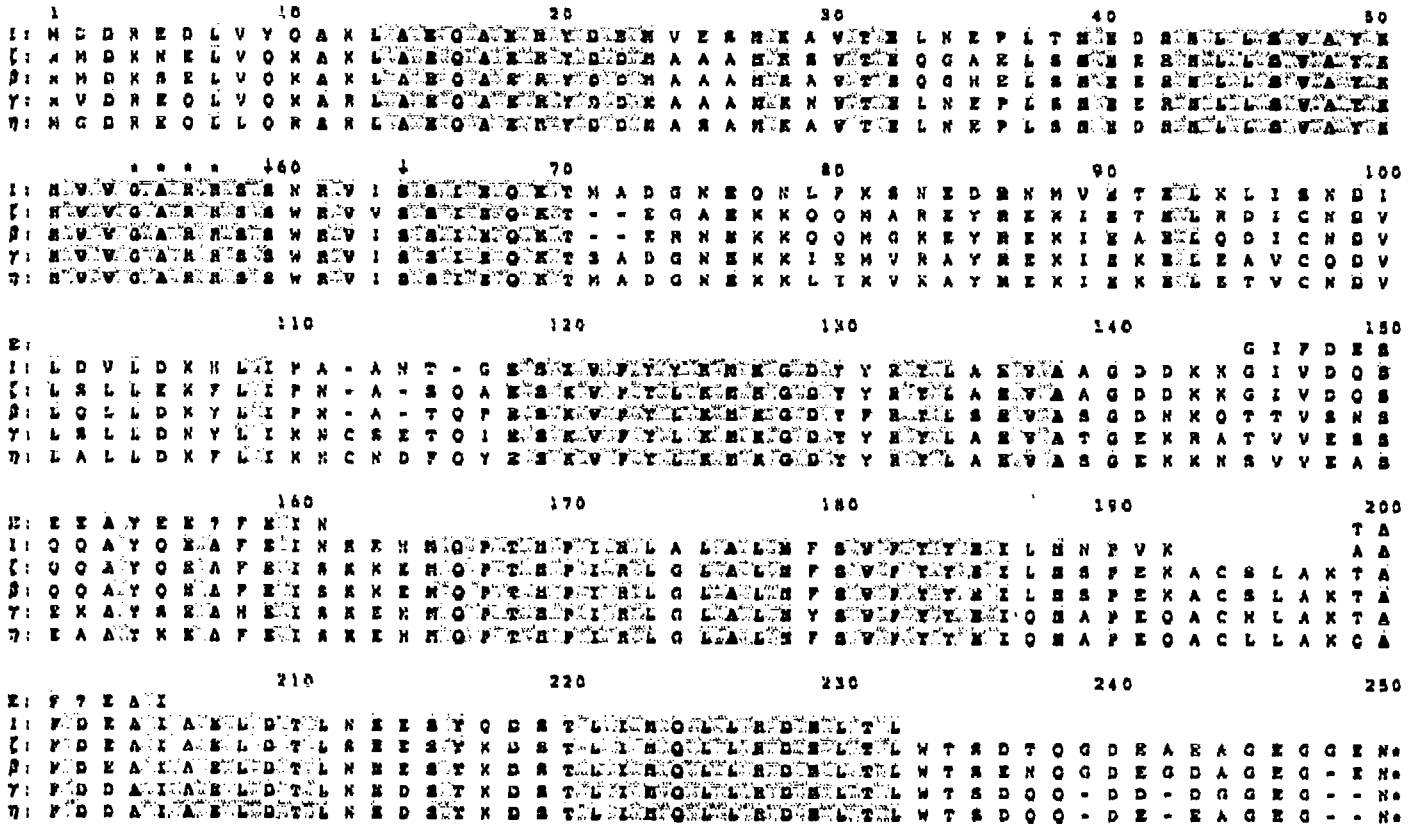

Fig. 3. Alignment of the amino acid sequences (single-letter notation) of 14-3-3 proteins with Exo1 and KCIP. The sequences of the 14-3-3 β-, γ-, and η chain (β, γ, η) were from [1,?] and the partial sequences of Exo1 (E) and KCIP (I) were from [18] and [16], respectively. Amino acid sequences are numbered from the N-terminus of the η chain. x refers to a blocking group of the N-terminus. Amino acids identical in all polypeptides are shaded. Note that in all of the polypeptide chains there are serine residues that have the potential for PKC-, PKII-, and cAMP/kinase-dependent phosphorylation (indicated by arrows), although these residues were not phosphorylated by PKII [7] or PKC (present study), at least under the conditions employed. Also found in the sequences is a 'pseudo-substrate' sequence, GARR, which was assumed to be a site by which KCIP may exert its inhibitory effect on PKC [16] (indicated by asterisks).

ation experiments of the ζ_2 protein with PKC also indicated that the ζ_2 protein is not a substrate of PKC, we concluded that the increased PKC activity is due to activation of PKC by the 14-3-3 ζ_2 protein.

To characterize the ζ chain in further detail we have determined its amino acid sequence (Fig. 3). As expected from its known activity towards TRH [7], the ζ chain is highly homologous (74–87% identity) with the other polypeptides found in the 14-3-3 proteins, thereby confirming that the ζ chain is a member of the 14-3-3 family. The alignment also demonstrates the high degree of sequence homology among bovine 14-3-3, sheep KCIP, and Exo1. Despite the difference in animal source, the 14-3-3 proteins share 69–74% identical residues with KCIP, which are comparable to the structural variations within the different 14-3-3 chains. We examined the activities of two other isoforms, β_2 and ε_2, one of which is closely related and the other most distantly related to the ζ_2 isoform in amino acid sequence (Fig. 3 and Isobe et al., unpublished results), and found that both proteins activate PKC to a similar extent as the ζ_2 protein (results not shown).

Besides the activity reported here, previous observations also suggested that the 14-3-3 proteins have a more diverse physiological function than the predicted role in monoamine synthesis: (i) 14-3-3 represents ca. 1% of total cytosolic protein in the brain [3] which is apparently more abundant than that expected from rather small populations of monoamine synthetic neurons in the brain; (ii) a low level of 14-3-3 has been detected in a wide variety of tissues and cells, including those in which the activity of TH or TRH is not detectable at a significant level [3,5,9]; (iii) the different types of 14-3-3 probably arise from different genes the expression of which is controlled differently among the tissues [7]. In addition to these observations, in situ hybridization histochemistry using a rat cDNA encoding the η chain revealed that the η chain is expressed, not only in the monoamine-synthetic neurons, but also in many other neurons which do not synthesize either catecholamine or serotonin [20]. These observations, coupled with the results described above, suggest that the diverse members of the 14-3-3 family are involved in PKC-mediated signal transduction processes through their

stimulatory or inhibitory effect on the enzymatic activity of PKC, although we have currently been unable to identify a KCIP equivalent of 14-3-3 that inhibits the PKC activity. We note that the tissue distribution of 14-3-3 almost parallels the known distribution of PKC [9].

The present study demonstrates that some, if not all, of the isoforms of the 14-3-3 family activate PKC. Because activation of PKC increases Ca^{2+}-dependent exocytosis in chromaffin cells [20–22], we propose that Exo1 exerts its effect on Ca^{2+}-dependent exocytosis by activation of PKC. Thus, in monoamine synthetic cells, such as chromaffin cells and some populations of neurons, 14-3-3 can accelerate Ca^{2+}-dependent exocytosis by dual pathways, one by stimulation of exocytosis through activation of PKC and the other by stimulation of monoamine synthesis through activation of TH or TRH in concert with the action of PKII.

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