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DIFFERENTIAL ACTIVATION OF PKC ISOZYMES BY 14-3-3 ζ PROTEIN

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SUMMARY: 14-3-3 proteins are ubiquitous in eukaryotes associated with many fundamental functions in signal transduction pathways and cell cycle regulation. Protein kinase C comprises a large family of serine/threonine protein kinases that are involved in cell growth and differentiation. Different protein kinase C isozymes have distinct roles in signal transduction pathways; protein kinase C ε is of particular interest because its overexpression leads to oncogenic transformation. The 14-3-3 protein has been reported to regulate the activity of protein kinase C, although the nature of its effect is equivocal. In this study we report the differential activation of various protein kinase C isoforms by 14-3-3 ζ protein. The classical isozymes show approximately a twofold activation, protein kinase C δ shows no significant increase in activity, whereas protein kinase C ε, another novel isozyme, is highly activated. This activation shows strong positive cooperativity with a Hill coefficient of 6.1 ± 0.2.

The 14-3-3 proteins, first isolated almost 30 years ago (1), have attracted renewed attention on the basis of their emerging role as important regulatory molecules. The 14-3-3 proteins are acidic, existing as dimers in solution with a monomer molecular weight around 30 kDa (2). The recently solved X-ray structure reveals that the dimer contains a large, negatively charged channel with invariant residues lining the interior. This channel is thought to be the site of interaction between the 14-3-3 proteins and their partners (3,4). So far eight mammalian brain isoforms have been described, named α-η after their respective elution positions on HPLC (5). Five of the eight isoforms have been sequenced and two, the α and δ isoforms, proved to be identical in primary structure to the β and ζ isoforms, respectively, but differ in post-translational modification (6).

14-3-3 proteins are highly enriched in brain but are expressed in most tissues (5,7,8). They have been isolated from a broad range of organisms, including amphibians, insects, yeast and plants,

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and are often functionally interchangeable between widely divergent species (8). This conservation suggests a fundamental role of these proteins in basic cellular processes.

14-3-3 proteins have a diverse range of activities. Of relevance to the current study, 14-3-3 proteins have been reported to regulate the enzymatic activity of protein kinase C (PKC), a major family of serine/threonine kinases involved in cell growth and differentiation. Toker et al. first isolated a 14-3-3 protein (which they named KCIP1) as a potent inhibitor of protein kinase C (2,9,10). In contrast, other groups reported that 14-3-3 activates protein kinase C (5,11,12). 14-3-3 proteins play a role in determining the timing of mitosis in fission yeast (13) and associate with polyomavirus middle tumor antigen, a protein involved in the regulation of cell proliferation (14). In yeast 14-3-3 protein was reported to be essential in Ras - Raf coupling and necessary for the activation of Raf by Ras (15,16).

The role of 14-3-3 proteins in the regulation of PKC activity has so far remained unclear, with reports claiming either inhibition or activation of PKC by 14-3-3 protein (2,5,9-12). In our experiments we examined the effect of recombinant 14-3-3 ζ protein on the activity of different recombinant PKC isozymes in the absence of anionic phospholipids. We found that 14-3-3 ζ differentially activates different protein kinase C isozymes with the greatest effect on protein kinase C ϵ.

MATERIALS AND METHODS

Expression of recombinant PKC isozymes
The expression of the different protein kinase C isozymes was performed as described previously by our group (17).

Purification of protein kinase C
Protein kinase C was partially purified from mouse brain as described by Jeng et al. (18).

Kinase assay
Protein kinase C activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) into substrates, (as described previously (19)) in the presence and absence of 14-3-3 ζ protein (Upstate Biotechnology Inc, Lake Placid, NY) that was used in final concentrations ranging from 0.214 μM to 3.42 μM. The assay buffer contained 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μM leupeptin, 10 μg/ml aprotinin (all purchased from Sigma, St. Louis, MO) and where specified 100 μg/ml phosphatidylserine (PS) (Sigma) and 1 μM phorbol 12-myristate 13-acetate (PMA) (LC Laboratories, Woburn, MA). The reaction was started by the addition the ATP (50 μM final concentration, 1.5 μCi) and substrate and was stopped by chilling on ice. A 25 μl aliquot was spotted onto phophocellulose disks (Gibco BRL Life Technologies, Gaithersburg, MD) and the disks were washed three times in 0.5% phosphatidic acid and three times in distilled water. The bound radioactivity was measured by liquid scintillation counting. Incubation was at 30°C for 10 min. The kinase assay was linear with time over this incubation period. We used PKC-α pseudosubstrate peptide (RFARKGSLRQKNV) (Gibco BRL Life Tech.) and histone H1 (from calf thymus) (Sigma)

as substrates. Phosphorylation of the substrates (30-50 μM) was linear with protein over the concentrations of PKC isozymes used in the assays. Computer analysis of the saturation curve of PKCε was performed by fitting it to the Hill equation: $B = (B_{max} x\ L_H^n)/(K_d^n + L_H^n)$.

Western blotting and autoradiography

The reaction mixture from the kinase assay was treated with an equal volume of SDS sample buffer containing 10% 2-mercaptoethanol and boiled for 5 min. The samples were subjected to SDS-PAGE electrophoresis according to Laemmli (20), transferred to nitrocellulose membranes and subjected to autoradiography.

RESULTS

We tested the effect of recombinant 14-3-3 ζ protein on the activity of individual protein kinase C isozymes in an *in vitro* kinase assay under linear conditions in the absence of phospholipid. We found that various enzymes are differentially activated by 14-3-3 ζ isoform. The classic PKC isozymes (α,β and γ) showed approximately the same level of activation (twice the control). The novel isozyme PKCδ showed no significant increase in activity, while the novel isozyme PKCε was strongly activated. At the same time, all the classical and novel isozymes were highly activated (8-10 times) in the presence of phosphatidylserine and phorbol 12-myristate 13-acetate, as expected for intact PKC (data not shown). The atypical PKCζ isoform behaved like PKCδ as far as activation was concerned, although its basal activity is very high and it could not be activated with PS and PMA (Fig. 1)

Over a 16-fold range of concentrations (0.214 μM - 3.42 μM) of the 14-3-3 protein we got practically a similar pattern of PKC isozyme selectivity (Fig. 2). Computer analysis of the saturation curve of PKCε showed strong cooperativity with a Hill coefficient of 6.1 ± 0.2. The concentration of 14-3-3 necessary for half-maximal activation of PKCε was 0.5 μM ± 0.03 (Fig. 3).

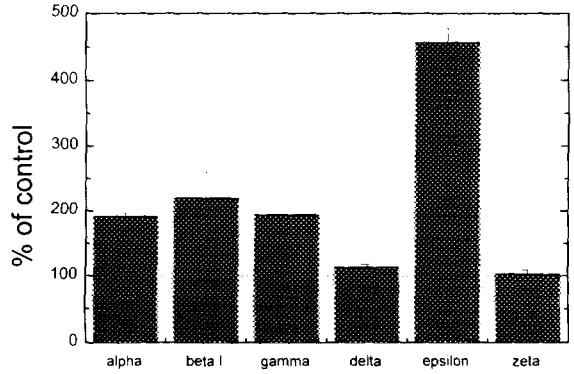

Fig. 1. Activity of different protein kinase C isozymes in the presence of 14-3-3 ζ protein. Activity of different PKC isozymes was measured by incorporation of radioactive phosphate into histone protein or PKC-α pseudosubstrate peptide in the presence of 1 μM 14-3-3 ζ protein. Columns represent the average of at least three independent experiments with duplicate determinations of activity in each experiment.

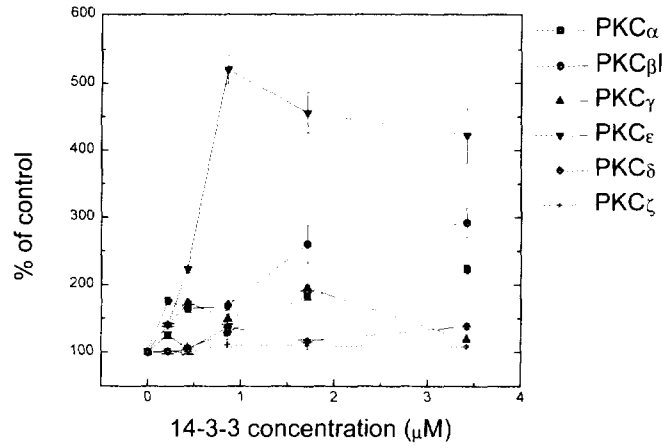

Fig. 2. Activation of different PKC isozymes by increasing concentrations of 14-3-3 ζ protein. PKCα(-■-), PKCβI (-●-), PKCγ (-▲-), PKCε (-▼-), PKCδ (-◆-) and PKCζ (-+-) isoforms were used at 0.01 μM concentration. Values represent the average of at least three experiments, with duplicate determinations under each condition in each experiment.

In accordance with previous reports, 14-3-3 protein was phosphorylated by protein kinase C (2,10). Phosphorylation was saturated at a low concentration of the 14-3-3 protein (0.1 μM) (data not shown).

DISCUSSION

We examined the effect of 14-3-3 ζ protein on the activity of various PKC isozymes in vitro. We found that 14-3-3 ζ protein activated protein kinase C, inducing a modest, about 2-fold activation in the classical isozymes whereas the ε subtype of the novel isozymes was activated about 5-fold.

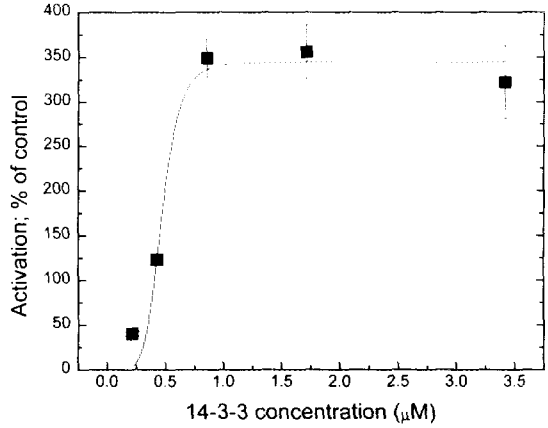

Fig. 3. Activation of PKC ε by increasing concentrations of 14-3-3 ζ protein. Fitting the saturation curve to the Hill equation yielded the following parameters: Kd: 0.5 ± 0.03, Hill coefficient: 6.1 ± 0.2. Values represent the average of three independent experiments with duplicate determinations of activity in each experiment.

This activation occurs in the absence of the usual PKC stimulators, PS and PMA. The effect was dose-dependent and the concentration of 14-3-3 necessary for half maximal activation of PKC ϵ was 0.5 μM. This concentration is similar to the concentration found by Isobe et al. using PKC isolated from rat brain (0.77 μM) and the concentration at which KCIP inhibited PKC activity by 50% (1.7 μM) (9,11). Isobe et al. found activation of PKC in the absence of diolein (a physiological activator of PKC) but failed to find activation in an assay mixture without Ca^{2+} or without phospholipids (11). Since brain tissue contains mostly classical isozymes (α,β,γ) (unpublished observation), the dependence on Ca^{2+} is not surprising. In our experiments the activation of PKCϵ was independent of the presence of Ca^{2+} (data not shown) suggesting that the interaction of 14-3-3 with PKC per se is Ca^{2+}-independent. Both Isobe et al. and Tanji et al. found about two-fold activation of PKC isolated from brain (rat and bovine, respectively). We could show the same increase, in the presence of 14-3-3, in the activity of PKC isolated from mouse brain and assayed in the presence of phosphatidylserine and phorbol 12-myristate 13-acetate (data not shown).

Chen and Wagner suggested that binding of 14-3-3 to histone was responsible for effects on PKC phosphorylation of histone, since they found no effect on phosphorylation of a PKC specific peptide substrate (21). In our experiments we obtained stimulation with either histone or α-peptide as substrates for PKC. 14-3-3 protein was phosphorylated by PKC under our assay conditions with saturation occurring at low concentrations (0.1 μM). Previous papers report phosphorylation of 14-3-3 by PKC but not by a wide range of protein kinases, likewise with low stoichiometry (2,10). Whether 14-3-3 itself is regulated directly by phosphorylation in vivo is unknown. It is known, however, that 14-3-3 protein is phosphorylated in vivo (22,23) and also that phosphorylation of 14-3-3 at a protein kinase C consensus site increases its affinity for protein kinase C in vitro (4,24).

14-3-3 proteins share sequence homology with several membrane proteins (RACKS, that stands for receptors for activated C kinase) that are reported to act as intracellular receptors for activated PKC (25). On this basis Leffers et al. proposed that the different isoforms of 14-3-3 may be binding proteins for corresponding isoforms of PKC (26). The X-ray structure of 14-3-3 proteins provides further insight into protein kinase C binding to the 14-3-3 protein. The authors propose that the basic Zn-finger domain of PKC binds to the acidic channel lining 14-3-3 and involves in particular residues 123 - 138 of 14-3-3 (3).

The discrepancies concerning the effect of 14-3-3 on PKC activity are perplexing. One possible explanation might be different effects of different 14-3-3 subtypes. The 14-3-3 τ isoform was the isoform used to inhibit PKC (10), while the studies showing activation of PKC, like the present study, were performed with 14-3-3 ζ isotype (11,12). A protein has also been isolated that copurifies with 14-3-3, unless steps are taken to eliminate it, and that causes 14-3-3 to inhibit PKC

(12). Differencies in the isolation method might thus also contribute to the diverse results. We tried to circumvent this problem by using recombinant 14-3-3 protein for our experiments.

Protein kinase C consists of a family of at least 10 closely related isozymes. The various isozymes show considerable diversity in their structure, regulatory properties and biological effects (27,28). Of all the isozymes only PKC ϵ exhibits full oncogenic potential (29,30). PKCδ has been reported to have the opposite effect on cell growth, i.e. overexpression of PKCδ causes arrest in cell division (31). The differential activation of these isozymes by 14-3-3 protein could thus have important biological consequences.

The tissue distribution of 14-3-3 almost parallels the known distribution of PKC (32). Some members of the 14-3-3 protein family are localized in the Golgi apparatus (7), while others can be detected in the cytoplasm (26). PKCϵ is abundant in the Golgi apparatus and can modulate Golgi function (33).

14-3-3 protein family members are involved in the activation of the protein kinase Raf (15,16). Since protein kinase C appears to be involved in phosphorylating Raf and 14-3-3 interacts with PKC, a role for 14-3-3 in coupling this phosphorylation cascade seems very appealing.

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