Brain Research, 379 (1986) 196–199
Elsevier

BRE 21683

Protein kinase C in human brain and its inhibition by calmodulin

TSUNAO SAITOH and KAREN R. DOBKINS

Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, CA 92093 (U.S.A.)

(Accepted April 8th, 1986)

Key words: postmortem human brain — phosphorylation — phorbol ester — phosphatidylserine — calmodulin

Protein kinase C is a ubiquitous enzyme which is especially concentrated in brain tissue and which has been reported to have a key role in the regulation of neuronal activity. Substrates for this kinase were studied in frozen postmortem human cortex. Two major protein substrates with mol. wts. of 86,000 and 67,000 Da were found in the cytosol fraction. Calmodulin was found to inhibit phosphorylation of these two proteins.

Calcium/phosphatidylserine-dependent protein kinase (protein kinase C) appears to be universal in the animal kingdom. Every animal phylum studied has been shown to contain this enzyme[9,16]. This enzyme also appears to be ubiquitous in its tissue distribution. Thus, it has been found in every animal tissue examined[9,14]. Interestingly, brain and peripheral nervous tissues contain higher levels of the enzyme than do most non-nervous tissues[5,7,9,14]. However, the human brain remains one of the few tissues on which no biochemical study of this kinase or its substrates has been made. It has been believed that activation of protein kinase C requires the presence of both calcium and phospholipid[7,20,22]. Takai et al.[21] have demonstrated that micromolar concentrations of diacylglycerol are required for protein kinase C to be fully activated in the presence of phosphatidylserine (PS) and physiological concentrations of calcium. Further, they have reported[3] that certain tumor-promoting phorbol esters increase protein kinase C activity. Other investigators have shown that the activation of kinase C by phorbol esters does not require the presence of elevated levels of calcium[4]. This finding raised the possibility of measuring protein kinase C activity without employing calcium in vitro, which makes the assay reliable. Protein kinase C has been reported to be activated by calcium-activated proteases[8,13]. Inclusion of calcium as activator in the protein kinase C assay may confound the result because of contaminating calcium-activated proteases. Calcium-activated proteases are rather abundant enzymes in nervous tissue[6,18] and they are important constituents of neurons because they play important roles in neuronal function[2,12]. Thus, it is desirable to exclude calcium from the protein kinase C assay. Here, we report a successful demonstration of kinase C activity in human cortex cytosol fraction in the absence of calcium using the combination of phorbol ester (PMA) and PS as activators.

Postmortem human brains (postmortem times were 1.5, 2, 11 and 14 h for 4 brains, aged 71, 72, 82 and 90 years, respectively) were frozen and kept at −70 °C until dissection. Before dissection, brains were kept 4 h at 4 °C and sections ca. 1 cm thick were sliced while brains were still frozen. Midfrontal cortex was dissected from these sections on a glass plate cooled from below by a bed of powdered dry ice. Dissected cortex, brought back to −70 °C and kept frozen at −70 °C until homogenization, was placed in 10 vols. of the homogenization buffer (0.32 M sucrose; 5 mM HEPES, pH 8.0; 5 mM benzamidine; 2 mM β-mercaptoethanol; 3 mM EGTA; 0.5 mM $MgSO_4$; 0.5 mM $ZnSO_4$; 0.1 mM pMSF; 0.1 mg/ml leupeptin; 0.05 mg/ml pepstatin and 0.1 mg/ml aprotinin) and

Correspondence: T. Saitoh, Department of Neurosciences; School of Medicine (M-024), University of California, San Diego, La Jolla, CA 92093, U.S.A.

homogenized by two 5-s strokes of a Polytron homogenizer (Brinkmann, Westbury, N.Y.). This homogenate was centrifuged 8 min at 2500 *g* at 2 °C to precipitate nucleus and cytoskeleton. The 2500 *g* supernatant was then centrifuged 1 h at 100,000 *g* at 2 °C to separate the membrane-rich fraction (100,000 *g* pellet) from the cytosol. The 100,000 *g* pellet was reconstituted to the original volume with the homogenization buffer, aliquoted, frozen in an ethanol/dry-ice bath and stored at −70 °C until used for the phophorylation reaction.

To measure stimulation of phosphorylation by PMA and PS, 10 μl of each fraction in the homogenization buffer containing 6.3 μg protein was mixed with 15 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.6 (all concentrations are for final 25 μl), 10 mM $MgSO_4$, 100 μg/ml protein kinase A inhibitor (Sigma, St. Louis, MO), 5 mM β mercaptoethanol, 10 μM ATP, 1 μCi/tube [γ-^{32}P]ATP (ICN, Irvine, CA) and 0.2 mM EDTA. Kinase C activity was detected by the addition of a reaction mixture containing 0.1 μM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) and 50 μg/ml PS (Sigma, St. Louis, MO) in addition to all the above constituents. PMA was stored at −20 °C at a concentration of 0.1 mM in methanol. PS dispersion was kept at −20 °C in water at a concentration of 1 mg/ml and sonicated just before it was used. The phosphorylation reaction was 12 min at 30 °C. The reaction was stopped by the addition of 6.3 μl of a buffer containing 40% glycerol, 25% β mercaptoethanol, 12% SDS, 0.31 M Tris-HCl (pH 6.8), 25 mM EDTA, and 0.1% Bromophenol blue. The samples were then subjected to 6.5–12.5% polyacrylamide gel (1 mm thickness) electrophoresis with 2-cm stacking gel[10] at a current of 20 mA per gel. The electrophoresis was stopped when the tracking dye reached 1 cm before the edge of the gel. Gels were fixed 1 h in 10% acetic acid containing 15% isopropyl alcohol, stained 1 h with 0.2% Coomassie brilliant blue in 10% acetic acid and 40% isopropyl alcohol, and destained in 10% acetic acid and 15% isopropyl alcohol. Destained gel was rinsed 1 h with water, dried and exposed about 10 h to X-Omat RP film (Kodak) with Hi Plus intensifying screen (Du Pont) at −70 °C. Protein amounts were determined using the method of Lowry et al.[11] with plasma globulin (Bio-Rad, Richmond, CA) as the standard.

To measure the influence of calcium/calmodulin on phosphorylation, the cytosol fraction containing 6.3 μg protein was subjected to the phosphorylation reaction as described in the previous paragraph, without activators or in the presence of 1.5 mM $CaCl_2$ and 20 μg/ml calmodulin. EGTA, 1.2 mM (final concentration), is carried over from the homogenization buffer to the final phosphorylation reaction. Thus the addition of 1.5 mM $CaCl_2$ gives a concentration of 10^{-5} M free calcium.

Inclusion of PMA and PS in the in vitro phosphorylation reaction stimulated the phosphorylation of two cytosol proteins which have mol. wts. of 86,000 and 67,000 Da, although it did not change the degree of phosphorylation of any proteins in the 100,000 *g* pellet fraction (Fig. 1). Quantification of the results (Table I) shows that the phosphorylation of the 86,000-Da protein was stimulated 2.4-fold in the presence of PMA and PS. Phosphorylation of the 67,000-Da protein was not quantificated because of its low radioactivity.

Calmodulin was previously found to inhibit the calcium/phospholipid-dependent phosphorylation of an endogenous substrate, called the 87-kDa protein, in a crude extract prepared from rat brain synaptosom-

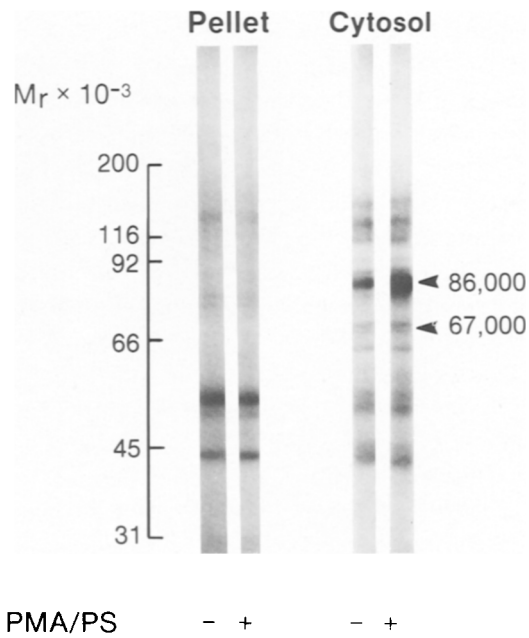

Fig. 1. Stimulation of phosphorylation of 86,000-Da and 67,000-Da proteins in cytosol by PMA and PS. An autoradiogram of ^{32}P-labeled proteins from membrane and cytosol obtained in the presence or absence of protein kinase C activators (PMA/PS) is shown.

TABLE I

Stimulation and inhibition of the phosphorylation of the 86,000-Da protein

The phosphorylation reactions were with 6.3 μg of protein from the cytosol fraction, either in the presence or in the absence of PMA/PS and calcium/calmodulin (Ca^{2+}/CaM) as described in the text. The 86,000-Da protein as well as the remaining portion of the lane were dissected out from dried gels and counted in liquid scintillation cocktail with a liquid scintillation counter. The proportion of radioactivity recovered in the 86,000-Da band as compared to radioactivity recovered in the entire lane is shown. Results are from 4 independent experiments using 4 different individuals. Total radioactivity was between 2500 and 3500 cpm.

Addition	*Proportion of radioactivity in 86,000-Da protein (% of total radioactivity)*	*Ratio to control*
-	4.3 ± 0.6	1.0
PMA/PS	10.3 ± 3.1	2.4
Ca^{2+}/CaM	1.3 ± 0.9	0.3

al cytosol[1,23]. It is probable that the 87-kDa protein observed in the rat brain is the homologue of the M_r 86,000 -protein that we observed in human brain. Thus, we studied the effect of calmodulin on the phosphorylation of the 86,000-Da and 67,000-Da proteins in the cytosol fraction. As shown in Fig. 2, in the presence of calcium and calmodulin, the phosphorylation of these proteins as well as minor phosphorylation of proteins between 150,000 and 100,000 was essentially abolished, although the phosphorylation of other proteins, which were not stimulated by the presence of phorbol ester and phosphatidylserine, was not influenced by the presence of calcium and calmodulin except for a 65,000-Da protein whose phosphorylation was stimulated 3,2-fold in the presence of calcium/calmodulin. The quantification of the results (Table I) shows that the inhibition of 86,000-Da protein phosphorylation by calcium/calmodulin was as much as 70%. Again, the result suggests that the human 86,000-Da protein that we observed is the homologue of the 87-kDa protein which has been previously described in rat brain. It is somehow surprising that calcium and calmodulin did not stimulate phosphorylation of any proteins around 50,000 Da under our conditions, as calcium and calmodulin have been known to stimulate the autophosphorylation of soluble calcium/calmodulin-dependent protein kinase II which has a mol. wt. close to

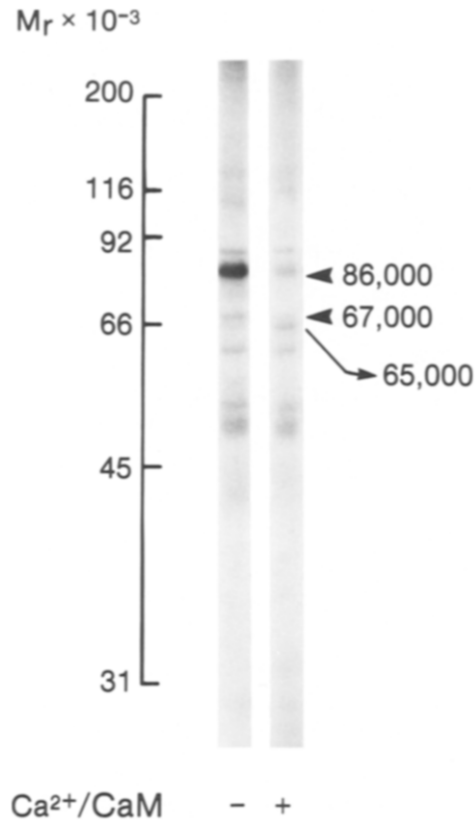

Fig. 2. Inhibition of phosphorylation of 86,000-Da and 67,000-Da proteins and stimulation of phosphorylation of 65,000 protein by calcium/calmodulin (CaM). An autoradiogram of dried gel is shown. Two arrowheads show calcium/calmodulin-inhibited phosphoproteins and an arrow shows a calcium/calmodulin-stimulated phosphoprotein.

50,000 Da[15,19]. This lack of stimulation seems to be caused by zinc which we used in the homogenization buffer; we found intense inhibition of purified *Aplysia* calcium/calmodulin-dependent protein kinase II by zinc (unpublished data).

Here, we report the presence of protein kinase C activity and kinase C substrates in postmortem human brain. This is important because now we can confidently use frozen postmortem human brain tissue to study kinase C activity as well as its substrates under experimental circumstances. However, it should be stressed that there is the possibility that postmortem changes occur. We find a decline of total kinase activity in human frontal cortex as a function of postmortem time ($t^{1/2}$ = ca. 12 h). Furthermore, we could not detect any protein kinase C activity in a brain which had been left unfrozen for 10 days. Thus,

to be meaningful, especially to compare brains from different individuals, the factor of postmortem changes must be kept in mind.

Endogenous protein kinase C activity detected by phosphorylation of the 86,000-Da protein appears to be inhibited in the presence of calcium and calmodulin. Because of the implied importance of both calcium and kinase C in controlling neuronal activity[17], it will be most interesting to compare kinase C activity and its substrates among postmortem brains from different neurologically disordered patients.

We thank Dr. R. Terry for the brain tissue. This research was supported by research grants from the McKnight Foundation and the National Institutes of Health (AG05386 and AG05131).

1 Albert, K.A., Wu, W.C.-S., Nairn, A.D. and Greengard, P., Inhibition by calmodulin of calcium/phospholipid-dependent protein phosphorylation, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 3622–3625.

2 Baxter, D.A., Johnston, D. and Strittmatter, W.J., Protease inhibitors implicate metalloendoprotease in synaptic transmission at the mammalian neuromuscular junction, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 4174–4178.

3 Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y., Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters, *J. Biol. Chem.*, 257 (1982) 7847–7851.

4 DeVirgilio, F., Lew, D.P. and Pozzan, T., Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels, *Nature (London)*, 310 (1984) 691–693.

5 Girard, P.R., Mazzei, G.J., Wood, J.G. and Kuo, J.F., Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localization of the enzyme in rat brain, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 3030–3034.

6 Kamakura, K., Ishiura, S., Sugita, H. and Toyokura, Y., Identification of Ca^{2+}-activated neutral protease in the peripheral nerve and its effects on neurofilament degeneration, *J. Neurochem.*, 40 (1983) 908–913.

7 Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y., Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties, *J. Biol. Chem.*, 257 (1982) 13341–13348.

8 Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y., Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease, *J. Biol. Chem.*, 258 (1983) 1156–1164.

9 Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W., Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of the animal kingdom and comparison of effects of phospholipid, calmodulin, and trifluoperazine, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 7039–7043.

10 Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature (London)*, 227 (1970) 680–685.

11 Lowry, O.H., Rosenbrough, N.J., Farr, A.G. and Randall, R.J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 193 (1951) 265–275.

12 Lynch, G. and Baudry, M., The biochemistry of memory: a new and specific hypothesis, *Science*, 224 (1984) 1057–1063.

13 Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. and Horecker, B.L., Binding of protein kinase C to neutrophil membranes in the presence of Ca^{2+} and its activation by a Ca^{2+}-requiring proteinase, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 6435–6439.

14 Minakuchi, R., Takai, Y., Yu, B. and Nishizuka, Y., Widespread occurrence of calcium-activated, phospholipid-dependent protein kinase in mammalian tissues, *J. Biochem.*, 89 (1981) 1651–1654.

15 Nairn, A.C., Hemmings, H.C., Jr. and Greengard, P., Protein kinases in the brain, *Annu. Rev. Biochem.*, 54 (1985) 931–976.

16 Nishizuka, Y., The role of protein kinase C in cell surface signal transduction and tumour promotion, *Nature (London)*, 308 (1984) 693–698.

17 Routtenberg, A., Phosphorylation of memory formation: enhancement and control of synaptic plasticity by protein kinase C and protein F1, *Ann. N.Y. Acad. Sci.*, 444 (1985) 203–211.

18 Saitoh, T., Oswald, R., Wennogle, L.P. and Changeux, J.-P., Conditions for the selective labeling of the 66,000 dalton chain of the acetylcholine receptor by the covalent non-competitive blocker 5-azide-(^{3}H)trimethiosoquin, *FEBS Lett.*, 116 (1980) 30–36.

19 Saitoh, T. and Schwartz, J.H., Phosphorylation-dependent subcellular translocation of a Ca^{2+}/calmodulin protein-dependent kinase produces an autonomous enzyme in *Aplysia* neurons, *J. Cell Biol.*, 100 (1985) 835–842.

20 Schatzman, R.C., Raynor, R.L., Fritz, R.B. and Kuo, J.F., Purification to homogeneity, characterization, and monoclonal antibodies of phospholipid-sensitive Ca^{2+}-dependent protein kinase from spleen, *Biochem. J.*, 209 (1983) 435–443.

21 Takai, Y., Kishimoto, A., Kikkawa, U., Terutoshi, M. and Nishizuka, Y., Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system, *Biochem. Biophys. Res. Commun.*, 91 (1979) 1218–1224.

22 Wise, B.C., Raynor, R.L. and Kuo, J.F., Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. I. Purification and general properties, *J. Biol. Chem.*, 257 (1982) 8481–8488.

23 Wu, W.C.-S., Walaas, S.I., Nairn, A.C. and Greengard, P., Calcium/phospholipid regulates phosphorylation of a M_r '87K' substrate protein in brain synaptosomes, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 5249–5253.