Protein kinase C and Simulated Ischemia Possible Aberrations of Signal Transduction during Ischemia

Kazuo IRITA, Matthew G. HEBDON*, Pedro CUATRECASAS* and Jun-ichi YOSHITAKE

ATP depletion is always associated with prolonged ischemia. It was found that ATP affected calcium- and phospholipid-dependent activation of protein kinase C without hydrolysis of the nucleotide when the activation was monitored by an assay for [^{3}H] 4-β-phorbol-12, 13-dibutyrate binding activity in a reconstitution system having physiological concentrations of free calcium. When the ATP level was low, an increase in the free calcium concentration could not activate the enzyme. A decrease in pH exacerbated the depressed activation. The concentration of magnesium also affected the activation. On the other hand, free fatty acids, which increase during ischemia, were able to activate the enzyme at a low concentration of ATP in the absence of phorbol ester and phosphatidylserine. These results suggest that calcium- and phospholipid-dependent activation of protein kinase C is suppressed during ischemia, and that fatty acids in turn activate the enzyme. It is possible that ischemia interferes with normal signal transduction via the protein kinase C pathway and causes unusual protein phosphrylation.
(Key words: ATP, protein kinase C, phorbol ester binding, signal transduction, ischemia)

(Irita K, Hebdon MG, Cuatrecasas P et al.: Protein kinase C and simulated ischemia: Possible aberrations of signal transduction during ischemia. J Anesth 3: 172–177, 1989)

Depletion of intracellular ATP is always associated with prolonged ischemia and induces a variety of responses including changes in magnesium concentration, a rise in cytosolic free calcium, calcium-activated proteolysis, activation of phospholipases, accumulation of diacylglycerol and free fatty acids, and lactic acidosis, all of which are known to affect protein kinase C activity[1].

Protein kinase C is now believed to play important roles in signal transduction in almost all mammalian cells[2]. The enzyme is particularly abundant in the brain and exerts widespread effects on synaptic transmission by affecting transmitter release, receptor sensitivity and ion channel activity[3]. The activation of protein kinase C is thought to occur in two stages. The first stage, called "priming", is the binding of the enzyme to phosphatidylserine at the cell membranes in the presence of a resting level of free calcium. In the second stage, the intercalation of diacylglycerol or phorbol ester into this ternary complex makes the enzyme active[4]. The binding of [^{3}H] 4β-phorbol-12, 13-dibutyrate (PDBu) to protein kinase C in a reconstitution system also requires the presence of phosphatidylserine and calcium, which implies that the binding activity represents a

*Department of Anesthesiology, University Hospital of Kyushu, Fukuoka, Japan and *Glaxo Research Laboratories, North Carolina, USA*

Address reprint requests to Dr. Irita: Department of Anesthesiology, University Hospital of Kyushu, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812 Japan

Table 1. Effects of adenine nucleotides on phorbol ester binding to protein kinase C.

Nucleotides	Bound [^{3}H]PDBu (cpm)
Without adenine nucleotide	3080 ± 293
"Normal"	
ATP 2.5 mM	8560 ± 139
ADP 0.4 mM	2976 ± 195
AMP 0.05 mM	2881 ± 176
ATP (2.5 mM) + ADP (0.4 mM) + AMP (0.05 mM)	9017 ± 51
"Ischemia"	
ATP 0.2 mM	1537 ± 168
ADP 0.2 mM	2664 ± 113
AMP 0.8 mM	2636 ± 116
ATP (0.2mM) + ADP (0.2 mM) + AMP (0.8mM)	2469 ± 45

[^{3}H]PDBu binding assay was performed for 4 min at 1 μM free calcium with or without adenine nucleotide(s). "Normal" means the normal concentrations of the nucleotides and "Ischemia" means the concentrations of those nucleotides which are observed in gerbil brains after 5 min of ischemia, reported in ref. 7. Numbers are the means ± S.E. for n = 3–4.

large part of the activation process of the enzyme. Free fatty acids also activate the enzyme, but the physiological significance of this type of activation is unknown[5].

Although the Km of the enzyme for ATP with histone H1 as a substrate is reported to be around 6 μM[6], we found that its value was around 2 mM when assessed at physiological concentrations of free calcium[7]. Because intracellular ATP concentration in normal and ischemic conditions are reported to be about 2.5 mM and 0.2–0.7 mM, respectively[8], it is possible that changes in ATP concentration within a physiological range affect the phosphotransferase activity of the enzyme. Furthermore, we found that ATP modulated [^{3}H]PDBu binding activity without hydrolysis of the nucleotide[7,9]. ATP had both an inhibitory and a stimulatory effect on phorbol ester binding activity, suggesting that ATP has multiple sites of action for the activation of protein kinase C. Above 0.5 mM ATP, binding was directly proportional to ATP concentration. These observations suggest that a fall in ATP level is accompanied by a decrease in membrane association of the enzyme, possibly resulting in an interruption of the signal transduction via the protein kinase C pathway. In this paper, we examine [^{3}H]PDBu binding as functions of free calcium concentration, magnesium concentration, and pH. We also study fatty acid-activation of the enzyme in the presence of a physiological concentration of free calcium. Physiological relevance of the findings is discussed in terms of aberrations of signaling during ischemia.

Methods

The details of the methods will be published elsewhere[7,9]. Briefly, the enzyme was partially purified from the particulate fraction of rat forebrain by extraction with EDTA/EGTA followed by DEAE-cellulose chromatography. The reaction mixture of the assay for [^{3}H]PDBu binding activity consisted of 0.1 M Hepes-HCl (pH 7.2), 3.1 mM $MgCl_2$, 1 mM EGTA, ATP, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 40 μg/ml phosphatidylserine and 40 nM[^{3}H]PDBu (10.2 Ci/mmol). The reaction mixture of the assay for phosphotransferase activity consisted of 0.1 M Hepes (pH 7.2), 3.1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, phospholipid or free fatty acid, PDBu, 250 μg/ml Histone H1, 0.1 mM ATP and 1 μCi[γ-^{32}P]ATP. $CaCl_2$ was added to give the indicated free calcium concentration. The reaction mixture was incubated at 30°C for 4 min.

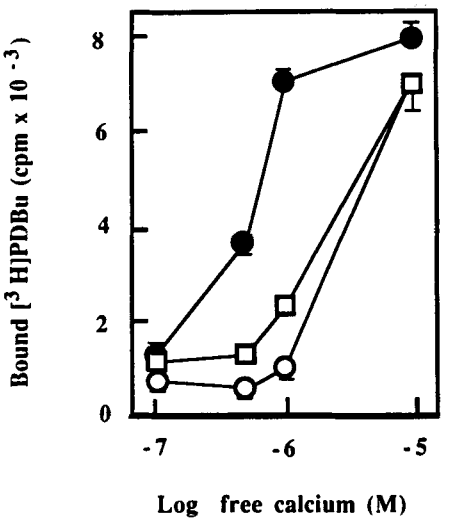

Fig. 1. Phorbol ester binding as a function of the concentration of free calcium.

[^{3}H]PDBu binding assay was performed in a reconstitution system in the presence of various concentrations of free calcium. □, Without ATP; ○, with 0.1 mM ATP; ●, with 2.5 mM ATP. Values are means ± S.E. for n = 3–4.

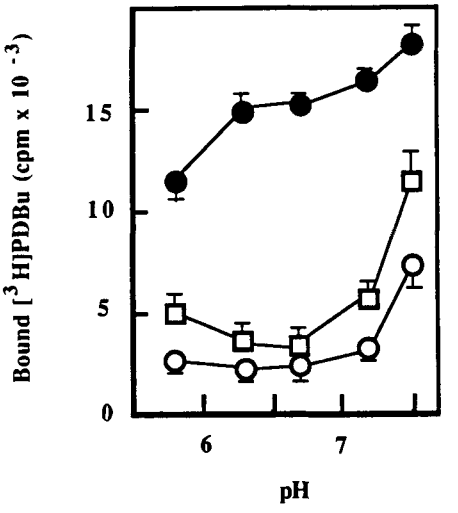

Fig. 2. Phorbol ester binding as a function of pH.

[^{3}H]PDBu binding assay was performed at a free calcium concentration of 1 μM in the presence of 0.1 M Hepes-HCl buffer at varying pH. Values are means ± S.E. for n = 3–4. Symbols are the same as in figure 1.

Results

Table 1 shows [^{3}H]PDBu binding activity monitored at a free calcium concentration of 1 μM in the presence and in the absence of adenine nucleotide(s). The adenine nucleotide concentrations used are the normal values and those values observed 5 min after brain ischemia produced by bilateral carotid artery occlusion in gerbils[8]. ATP had both a stimulatory and an inhibitory effect on the binding activity. ATP at 2.5 mM enhances the binding activity, while ATP at 0.2 mM inhibits the activity. Above 0.5 mM ATP, the binding activity is directly proportional to ATP concentration (data not shown). Neither ADP nor AMP affects the binding activity. ADP and AMP have little effect on the binding activity modified by ATP.

Next, the effects of free calcium, magnesium, and pH on the binding activity are examined. Raising the free calcium concentration from 100 nM to 500 nM - 1 μM, which simulates the increase in the free calcium concentration observed in a cell stimulated by some physiological stimuli[10] and in a cell under ischemia[11,12], increases the binding activity in the presence of 2.5 mM ATP but not in the absence or presence of 0.1 mM ATP (fig. 1). This indicates that the activation of protein kinase C within a cell requires the presence of normal concentrations of ATP. These changes produced by ATP are observed at physiological concentrations of free calcium but not at higher concentrations which could be observed in an irreversible stage of ischemia. Figure 1 also suggests the possibility that an extreme increase in cytosolic free calcium concentration (higher than 10 μM) induces an uncontrolled, non-specific activation of protein kinase C.

The optimal pH for a phosphotransferase activity of protein kinase C determined in the presence of 10μM ATP is reported to be around 7.5[6]. Figure 2 shows that a decrease in the binding activity produced by lowering pH from 7.5 is more marked in the presence and in the absence of 0.1 mM ATP than in the presence of 2.5 mM ATP. Intracellular pH is thought to be around 7.1 in normal condition, and it decreases to 5.4–6.0 during

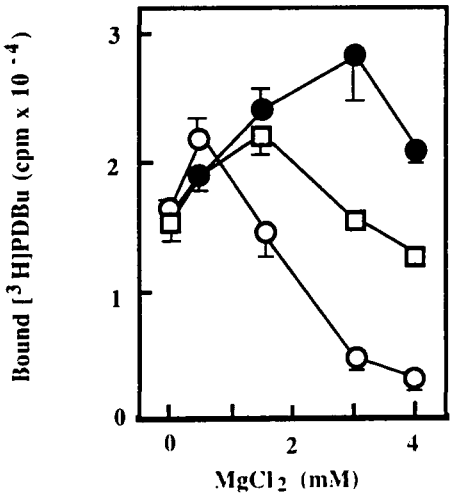

Fig. 3. Phorbol ester binding as a function of the concentration of $MgCl_2$.

[^{3}H]PDBu binding assay was performed at 1 μM free calcium, pH 7.2 in the presence of various concentrations of $MgCl_2$. Symbols are the same as in figure 1. Values are means ± S.E. for n = 3–4.

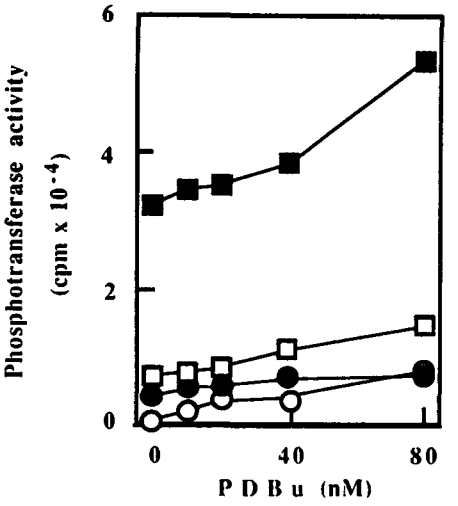

Fig. 4. Phosphotransferase activity of protein kinase C stimulated by phosphatidylserine or free fatty acids as a function of the concentration of PDBu.

Phosphotransferase activity was determined at 1 μM free calcium and 0.1 mM ATP in the presence of various concentrations of PDBu. The incubation was done for 4 min. ○, With phosphatidylserine 40 μg/ml; ●, with stearic acid 400 μM; □, with oleic acid 50 μM; ■, with oleic acid 400 μM. Values are means ± S.E. for n = 2. S.E. is within the range of symbols.

ischemia[13]. Between pH 5.8–7.2, the binding activity in the presence of 2.5 mM ATP is always higher than that in the presence and in the absence of 0.1 mM ATP, suggesting that a restoration of ATP level could activate protein kinase C even in the presence of intracellular acidosis.

Figure 3 shows that magnesium concentration is also an important regulatory factor of the binding activity. At the magnesium concentration of 3 mM, which coincides with the reported normal values of total magnesium within a cell, the binding activity is most obviously influenced by changing the ATP concentration.

Because the level of cytosolic free fatty acids during ischemia is known to become extremely high[14], we finally examined the effect of high concentrations of free fatty acids on phosphotransferase activity of protein kinase C (fig. 4). The assay was done in the presence of 1 μM free calcium and 0.1 mM ATP. Phosphatidylserine-dependent activation of the enzyme absolutely requires the presence of PDBu, but stearic and oleic acids are able to activate the enzyme in the absence of PDBu and phosphatidylserine. The activity induced by 400 μM oleic acid is remarkably high.

Discussion

Previous papers from our laboratory showed an impairment in the normal activation of protein kinase C at low concentrations of ATP, suggesting that ischemia interferes with the normal signal transduction via the protein kinase C pathway[7]. This hypothesis is further supported by the present observation that a rise in free calcium, a decline of pH, and a fall in total magnesium[15], all of which are observed during ischemia, do not affect the changes produced by ATP. Preliminary experiments with Chinese hamster ovary cells showed that a part of protein (de)phosphorylation induced by phorbol 12-myristate 13-acetate was inhibited during anoxia (unpublished observation).

Free fatty acids at high concentrations

were shown to activate the enzyme effectively even in the absence of PDBu and phosphatidylserine, indicating that fatty acid-dependent activation of the enzyme does not necessarily need the production of diacylglycerol and the association of the enzyme with membranes. Activation of protein kinase C by fatty acids might suggest a possible activation of the enzyme in the cytosol. Because the presence of cytosolic substrates for protein kinase C are reported[16], the substrate specificity of the fatty acid-stimulated enzyme might be different from that of the phosphatidylserine-stimulated form.

Several reports have appeared which describe an altered second messenger system during ischemia. Matthys et al. reported the elevation of diacylglycerol during renal ischemia[17] and Hochachka speculated that the increased level of diacylglycerol disturbed normal signal transduction[18]. Present findings, however, indicate that normal activation of protein kinase C can not occur even in the presence of diacylglycerol and increased cytosolic calcium (up to 1 μM), when the ATP level is low (fig. 1). Recently excitatory amino acid neurotransmission has been thought to play important roles in the pathogenesis of ischemic brain damage, and protein kinase C is reported to be involved in glutamate-mediated responses[19]. The enzyme is also reported to be involved in biological effects of endotoxin[20,21]. Focusing on the derangement of the protein kinase C pathway in ischemia and shock, therefore, seems to be a promising approach. Chin et al. hypothesized that increased calcium-protease activity inactivated calmodulin-dependent protein kinase during ischemia[22]. Chaudry and Baue reported decreases in the cAMP concentration in the rat liver, kidney, muscle and brain during hemorrhagic shock[23], while Kobayashi et al. reported the elevated cAMP level in the ischemic gerbil brain[8]. Systematic investigation of the changes in second messenger systems and protein kinases is required to elucidate perturbations in the signal transduction during ischemia and to develop effective pharmacological management of ischemic diseases.

In conclusion, regulation of protein kinase C by ATP may lead to new insights into the mechanism for activation of the enzyme and into a cellular dysfunction during ischemia and shock.

Acknowledgement: We would like to thank Steven Sabotta for reading the manuscript.

(Received Nov. 11, 1988, accepted for publication Mar. 1, 1989)

References

1. Raichle ME: The pathophysiology of brain ischemia. Ann Neurol 13:2–10, 1983
2. Nishizuka Y: The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665, 1988
3. Baraban JM: Phorbol esters: Probes of protein kinase C function in the brain. Trends Neuro Sci 10:57–58, 1987
4. Ganong BR, Loomis CR, Hannun YA, Bell RM: Specificity and mechanism of protein kinase C activation by *sn*-1, 2-diacylglycerols. Proc Natl Acad Sci USA 83:1184–1188, 1986
5. Sekiguchi K, Tsukada M, Ogita K, Kikkawa U, Nishizuka Y: Three distinct forms of rat brain protein kinase C: Differential response to unsaturated fatty acids. Biochem Biophys Res Commun 145:797–802, 1987
6. Kikkawa U, Takai Y, Minakuchi R, Inohara S, Nishizuka Y: Calcium-activated, phospholipid-dependent protein kinase from rat brain: Subcellular distribution, purification, and properties. J Biol Chem 257:13341–13348, 1982
7. Irita K, Cuatrecasas P, Hebdon GM: Regulation of protein kinase C by ATP concentration. submitted.
8. Kobayashi M, Lust WD, Passonneau JV: Concentrations of energy metabolites and cyclic nucleotides during and after bilateral ischemia in the gerbil cerebral cortex. J Neurochem 29:53–59, 1977
9. Irita K, Cuatrecasas P, Hebdon GM: Fatty acid substitution for phosphatidylserine in a reconstitution of phorbol binding to protein kinase C from rat brain: Dissociation of phorbol binding from the activation of protein kinase C. submitted.
10. Nakagawara M, Takeshige K, Takamatsu J, Takahashi S, Yoshitake J, Minakami S: Inhibition of superoxide production and

Ca^{2+} mobilization in human neutrophils by halothane, enflurane, and isoflurane. Anesthesiology 64:4–12, 1986
11. Cheung JY, Bonventre JV, Malis CD, Leaf A: Calcium and ischemic injury. N Eng J Med 314:1670–1676, 1986
12. Uematsu D, Greenberg JH, Reivich M, Kobayashi S, Karp A: In vivo fluorometric measurement of changes in cytosolic free calcium from the cat cortex during anoxia. J Cereb Blood Flow Metab 8:367–374, 1988
13. Chopp M, Frinak S, Walton DR, Smith MB, Welch KMA: Intracellular acidosis during and after cerebral ischemia: *In vivo* nuclear magnetic resonance study of hyperglycemia in cats. Stroke 18:919–923, 1987
14. Rehncrona S, Westerberg E, Åkesson B, Siesjö BK: Brain cortical fatty acids and phospholipids during and following complete and severe incomplete ischemia. J Neurochem 38:84–93, 1892
15. Vink R, McIntosh TK, Demediuk P, Weiner MW, Faden AI: Decline in intracellular free Mg^{2+} is associated with irreversible tissue injury after brain trauma. J Biol Chem 263:757–761, 1988
16. Turner RS, Raynor RL, Mazzei GJ, Girard PR, Kuo JF: Developmental studies of phospholipid-sensitive Ca^{2+}-dependent protein kinase and its substrate and of phosphoprotein phosphatases in rat brain. Proc Natl Acad Sci USA 81:3143–3147, 1984
17. Matthys E, Patel Y, Kreisberg J, Stewart JH, Venkatachalam M: Lipid alterations induced by renal ischemia: Pathogenic factor in membrane damage. Kidney Int 26:153–161, 1984
18. Hochachka PW: Defense strategies against hypoxia and hypothermia. Science 231:234–241, 1986
19. Vaccarino F, Guidotti A, Costa E: Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebellar neurons. Proc Natl Acad Sci 84:8708–8711, 1987
20. Wightman PD, Raetz CRH: The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J Biol Chem 259:10048–10052, 1984
21. Spitzer JA, Turco ER, Deaciuc IV, Roth BL: Perturbation of transmembrane signaling mechanisms in acute and chronic endotoxemia. Prog Clin Biol Res 236A:401–418, 1987
22. Chin JH, Buchholz TM, DeLorenzo RJ: Calmodulin and protein phosphorylation: Implications in brain ischemia. Prog Brain Res 63:169–184, 1985
23. Chaudry IH, Baue AE: Depletion and replenishment of cellular cyclic adenosine monophosphate in hemorrahgic shock. Surg Gynecol Obstet 145:877–881, 1977