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Mode of Inhibitory Action of Bilirubin on Protein Kinase C

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ABSTRACT. Ca^{2+} -activated, phospholipid-dependent protein kinase (protein kinase C) is widely distributed in mammalian tissues. Accumulating evidence has revealed that protein kinase C as well as cAMP-dependent protein kinase plays important roles in various cellular functions. The purpose of this study is to examine the effect of bilirubin on protein kinase C and cAMP-dependent protein kinase activity in a cell-free system as a cause of bilirubin toxicity to the central nervous system. Bilirubin inhibited protein kinase C activity in a dose-dependent manner. This effect was markedly diminished by the addition of human serum albumin at a molar ratio of bilirubin to albumin of less than 1.0. Kinetic analysis revealed that bilirubin did not compete with phospholipid, diacylglycerol, or calcium. Bilirubin also inhibited cAMP-dependent protein kinase, but did not compete with cAMP. The inhibitory effect of bilirubin on protein kinase C seems to be irreversible because removal of bilirubin by Sephadex G-25 column chromatography did not restore the protein kinase C activ-

ity. Observations reported herein suggest that bilirubin, especially in its free form, induces an irreversible change to the catalytically active site of protein kinase C. (*Pediatr Res* 19: 587-590, 1985)

Abbreviation

Protein kinase C, calcium-activated, phospholipid-dependent protein kinase

Bilirubin is toxic to the central nervous system, causing encephalopathy, especially in low birth weight premature infants with hyperbilirubinemia and acidosis. The toxic mechanism of bilirubin has been extensively investigated. Several enzymes relating to oxidative phosphorylation and to metabolisms of carbohydrate, amino acid, and lipid are affected by bilirubin (for a review see Reference 1). However, no specific enzyme has been singled out as a target of bilirubin toxicity.

Recent evidence has revealed that protein phosphorylation is a very important pathway in various cellular functions including those of the nervous system (for a review see Reference 2). To

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date, there are four known types of protein kinases, namely, cAMP-dependent protein kinase, cGMP protein kinase, Ca^{2+} /calmodulin dependent protein kinases, and Ca^{2+} -activated, phospholipid-dependent protein kinase (protein kinase C). Among these kinases, the role of protein kinase C in various cellular functions has been extensively studied (for a review see Reference 3). In neuronal tissues, several proteins are reported to be substrates of protein kinase C including tyrosine hydroxylase, nicotinic acetylcholine receptor, microtubule-associated protein-2, myelin basic protein, GABA-modulin, Synapsin I, and 87K protein (2). Recently Constantopoulos and Matsaniotis (4) reported that bilirubin inhibits cAMP-dependent protein kinase *in vitro*. The present study was designed to investigate the effect of bilirubin on protein kinase C in a cell free system as a possible cause of bilirubin toxicity to brain cells.

EXPERIMENTAL PROCEDURES

Materials and chemicals. A homogeneously purified protein kinase C from rat brain was donated by Dr. Kikkawa, Department of Biochemistry, Kobe University School of Medicine (5). This homogeneous preparation of protein kinase C was used in all experiments except that summarized in Table 1 in which we used protein kinase C partially purified through DEAE-cellulose and Sephadex G 150 gel column chromatography as described by Kikkawa *et al.* (5). Calf thymus H1 histone was prepared by the method of Hashimoto *et al.* (6). Phospholipid mixture was extracted from bovine brain by the method of Folch *et al.* (7) and fractionated on a silicic acid column. Diolein was purchased from Nakarai Chemicals. (γ - ^{32}P)ATP was prepared by the method of Glynn and Chappell (8). Bilirubin, human serum albumin, cAMP-dependent protein kinase from bovine heart were the products of Sigma. Prepacked sephadex G25 column (PD-10) was obtained from Pharmacia. Other chemicals were obtained from commercial sources.

Enzyme assay. Protein kinase C was assayed by measuring the incorporation of ^{32}P into H1 histone from (γ - ^{32}P) ATP. The standard reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium chloride, 50 μg of H1 histone, 2.5 nmol of (γ - ^{32}P) ATP (1.0 to 1.5×10^5 cpm/nmol), and 0.5 μg of protein kinase C. Phospholipid, diolein, bilirubin, and Ca^{2+} were added as indicated in each experiment. cAMP-dependent protein kinase was similarly assayed except that 50 μg of whole histone was used as substrate and cAMP was added instead of Ca^{2+} , phospholipid, and diolein. Bilirubin was dissolved in 0.1 N NaOH and then adjusted to pH 8.0 with 0.2 N HCl in the dark. All reactions were carried out in plastic tubes. After incubation for 3 min at 30°C , the reactions were stopped by the addition of 25% trichloroacetic acid. The acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore

size, 0.45 μm). The radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer, model 3320. Protein was determined by the method of Lowry *et al.* (9) with bovine serum albumin as a standard.

RESULTS

Protein kinase C requires Ca^{2+} and phospholipid for its activation. A small amount of unsaturated diacylglycerol such as diolein markedly increases the affinity of protein kinase C for Ca^{2+} and phospholipid (10, 11). Figure 1A shows that bilirubin-inhibited protein kinase C in a dose-dependent manner when the enzyme was assayed in the presence of Ca^{2+} , phospholipid, and diolein. Bilirubin also inhibited cAMP-dependent protein kinase in the presence of 1.0×10^{-7} M cAMP as shown in Figure 1B, which confirms previous report of Constantopoulos and Matsaniotis (4). The concentrations of bilirubin needed for half maximum inhibition of the enzymes were approximately 40 μM for protein kinase C and 60 μM for cAMP-dependent protein kinase.

Most of the bilirubin in the plasma is bound firmly to albumin (12). Only unbound bilirubin is thought to cross the blood-brain barrier where it could possibly impair damage brain function. Therefore, in the next experiments we added human serum albumin to the reaction mixture to test this concept in our cell free system. As shown in Figure 2, addition of albumin diminished inhibitory effect of bilirubin on protein kinase C. But it should be noted that bilirubin still significantly inhibited protein kinase C in the presence of albumin when the bilirubin/albumin molar ratio was 2.0. In the case of cAMP-dependent protein kinase, the effect of albumin could not be examined because cAMP bound to albumin and cAMP stimulated kinase activity could not be measured. But albumin did prevent the basal activity of cAMP-dependent protein kinase from inhibition by bilirubin (data not shown).

Several phospholipid-interacting drugs such as chlorpromazine, dibucaine, and tetracaine have been shown to inhibit pro-

Fig. 1. Inhibition of protein kinase C (A) and cAMP dependent protein kinase (B) by bilirubin. A, protein kinase C was assayed in the reaction mixture (0.25 ml) which contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium chloride, 50 μg of calcium chloride, 50 μg of H1 histone, 2.5 nmol of (γ - ^{32}P) ATP (1.5×10^5 cpm/nmol), 4 μg of phospholipid, 0.5 μg of diolein, 0.5 μg of protein kinase C, and various amounts of bilirubin as described in "Experimental procedures". The activity is expressed as pmol ^{32}P incorporated/min/tube. Calcium-dependent basal activity is subtracted from each value. B, cAMP-dependent protein kinase was assayed in the reaction mixture (0.25 ml) which contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium chloride, 50 μg of whole histone, 2.5 nmol of (γ - ^{32}P) ATP (1.5×10^5 cpm/nmol), 25 pmol of cAMP, 10 μg of cAMP-dependent protein kinase, and various amounts of bilirubin as described in "Experimental procedures". The results represent three different experiments.

Table 1. Irreversibility of inhibitory effect of bilirubin on protein kinase C*

Treatment	Before G25	After G25
	column chromatography	column chromatography
	pmol P/min	
Vehicle	6150 (100%)	5870 (100%)
Bilirubin	1620 (26%)	1470 (25%)

* Partially purified protein kinase C (100 μg) was preincubated with either 100 μM of bilirubin or vehicle for 5 min at 4°C , then subjected to G25 column (PD 10) and eluted with 20 mM Tris/HCl at pH 7.5 containing 50 mM 2-mercaptoethanol and 0.5 mM EGTA. An aliquot (0.8 μg protein) was subjected to protein kinase C assay before and after column chromatography as described in "Experimental procedures." The activity is expressed as total activity (pmol ^{32}P incorporated/min). The percentage activity is shown in parentheses where total activity of control is expressed as 100%. The results represent the mean of triplicate samples of a single experiment.

tein kinase C activity by competing with phospholipid (13). It has been reported that bilirubin binds reversibly to phospholipids of mitochondria, intact cells, and cell fragments (14). Therefore, we examined whether or not bilirubin inhibits protein kinase C through interaction with phospholipids. Unlike cationic amphiphilic drugs, the inhibitory effect of bilirubin was not abolished by increasing the amount of phospholipid as shown in Figure 3. Moreover, increasing the amount of diolein or Ca^{2+} in the

Fig. 2. Effect of the molar ratio of bilirubin to albumin on the inhibitory action of bilirubin on protein kinase C. Protein kinase C was assayed under conditions similar to those specified in the legend to Figure 1 except that various amounts of human serum albumin and bilirubin were added as indicated. The concentration of albumin was fixed, and that of bilirubin was varied. Protein kinase activity without bilirubin is expressed as 100%. Δ , with 4.7 mg/ml of albumin; $\square$, with 9.4 mg/ml of albumin. The results represent three different experiments.

Fig. 3. Effect of phospholipid concentration on the inhibitory action of bilirubin on protein kinase C. Protein kinase C was assayed under conditions similar to those specified in the legend to Figure 1 except that various concentrations of phospholipid were added as indicated. Calcium-dependent basal activity is subtracted from each value. $\circ$ — $\circ$, without bilirubin; $\bullet$ — $\bullet$, with bilirubin (34 μM). The results are representative for two different experiments.

Fig. 4. Effect of cAMP concentration on the inhibitory action of bilirubin on cAMP-dependent protein kinase. cAMP-dependent protein kinase was assayed under conditions similar to those specified in the legend to Figure 1 except that various concentrations of cAMP were added as indicated. $\circ$ — $\circ$, without bilirubin; $\bullet$ — $\bullet$, with bilirubin (68 μM). The results represent two different experiments.

reaction mixture did not affect inhibitory action of bilirubin (data not shown). Increasing the amount of cAMP also failed to prevent cAMP-dependent protein kinase activity from inhibition by bilirubin as shown in Figure 4. Therefore, bilirubin seems to inhibit both protein kinase C and cAMP-dependent protein kinase through interaction with catalytically active sites rather than through competition with their activators.

The inhibitory effect of bilirubin on protein kinase C seems to be irreversible as shown in Table 1. Once protein kinase C was preexposed to 100 μM of bilirubin, the activity of protein kinase C significantly decreased despite a reduction in the final concentration of bilirubin in the reaction mixture to 8 μM . Furthermore, the activity of protein kinase C was not restored by removal of bilirubin by Sephadex G25 gel column chromatography.

DISCUSSION

The activation process of protein kinase C is tightly coupled to phosphatidylinositol turnover (3, 11) which is provoked by a wide variety of extracellular messengers including neurotransmitters and many biologically active substances (15). Protein phosphorylation is presumed to be a final common pathway of signal transduction, and actually several substrates of protein kinase C in nervous tissue have been reported (2).

Our present results demonstrate that bilirubin inhibits both protein kinase C and cAMP-dependent protein kinase in a cell-free system. Protein kinase C activity was not totally inhibited even when high concentration of bilirubin was added as shown in Figure 1A. The exact reason of this is unclear at present, but when protein kinase C was preincubated with bilirubin in the absence of lipids, bilirubin totally inhibited protein kinase C at 200 μM (data not shown). This result suggests that lipids might somehow protect protein kinase C as reported in the case of Ca^{2+} -dependent inactivation of this enzyme (5).

The binding activity of bilirubin to phospholipid is considered to be a cause of bilirubin toxicity (14, 16); however, our results suggest that bilirubin inhibits protein kinase C through interacting with its catalytically active site(s) rather than competing with phospholipids. The results shown in Table 1 suggest that bilirubin might cause covalent modification or conformational change on the catalytic site(s) of protein kinase C.

Whether or not the inhibitory action of bilirubin on protein

kinase C *in vitro* is directly related to the state of kernicterus remains unclear from our present results. We failed to demonstrate an *in vivo* effect of bilirubin on the specific activity of protein kinase C in adult Sprague-Dawley rats (data not shown). However, kernicterus is confined to the very early neonatal period or low birth weight infants; acidosis or hypoalbuminemia also tend to occur in such patients. Acidosis or hypoalbuminemia increases unbound bilirubin levels allowing the accumulation of bilirubin in central nervous tissues (17). Therefore, experiments using premature or very early neonatal animals with hyperbilirubinemia are necessary in order to clarify precisely the role of protein kinases in kernicterus.

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Unsuccessful Attempts to Induce Peroxisomes in Two Cases of Zellweger Disease by Treatment with Clofibrate

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ABSTRACT. The cerebro-hepato-renal syndrome of Zellweger is a fatal hereditary disease and most of the affected infants die before the age of 6 months. Most probably the fatal outcome of the disease is due to an apparent complete lack of peroxisomes in the liver, kidneys, and brain. Treatment with clofibrate is known to increase drastically the number of peroxisomes in mammalian liver. We therefore treated two infants with the Zellweger syndrome with clofibrate (30 and 45 mg/kg body weight, respectively) for 3-4 wk. No clinical effect of the treatment was observed in any of the two cases, and the pattern of abnormal bile acids in serum did not change. No peroxisomes could be

detected by electron microscopy of liver biopsies taken immediately after the treatment. Our failure to induce peroxisomes in the two Zellweger patients is in accord with the hypothesis that the protein missing in this autosomal recessive disease is absolutely essential for the formation of peroxisomes. (*Pediatr Res* 19: 590-593, 1985)

The Zellweger syndrome is a fatal hereditary disease characterized by craniofacial malformations, hypotonus, liver enlargement, renal cysts, central nervous disturbances, and mental retardation (1). In general affected infants die before 6 months of age. The most characteristic histological finding in this disease is an apparently complete absence of peroxisomes in the liver and kidneys (2). Goldfischer *et al.* (2) have also demonstrated

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