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Inhibition of Brain Protein Kinase C Subtypes by Lead

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ABSTRACT

Protein kinase C (PKC) is an important enzyme in mediating cellular signal transduction and neuronal plasticity. Extremely low concentrations (picomolar range) of Pb^{++} have been reported to activate partially purified PKC from rat brain (Markovac and Goldstein, 1988). However, the lead activation of PKC at such low concentrations is still a matter of discussion (Simons, 1989). To clarify this point, we have examined the lead effect on highly purified PKC subtypes. Pb^{++} was found to be a potent inhibitor for all three PKC subtypes (types I, II and III) with IC_{50} of 2 to 10 μM. Characterization of this lead inhibition of PKC suggests that 1) the inhibition is not due to the competition with Ca^{++}, 2) the site of action of lead is on the catalytic domain of PKC, 3) the inhibition is not dependent on the mode of activation (phosphatidylserine/diacylglycerol *vs.* *cis*-unsaturated fatty acid) and 4) the inhibition is totally reversible.

The neurotoxicity of lead has been well documented. Lead acts on the central and peripheral nervous systems at low doses. A variety of clinical studies in children indicate that there is a close association between lead exposure and behavioral disorders, cognitive development and impairment of learning processes (for review, Silbergeld, 1985; Needleman, 1991).

A growing body of evidence indicates that the phosphorylation of brain proteins by PKC plays an important role in the regulation of neuronal plasticity, a physiological basis of learning and memory (for review, Routtenberg, 1991). Activation of PKC by phorbol ester or DAG modulates a variety of ion channels in neuronal cells and controls the excitability of neurons (for review, Shearman *et al.*, 1989). PKC is now known to be a large family of protein kinases with multiple subtypes and currently ten of them (α, βI, βII, γ, δ, ϵ, ζ, η, θ, and λ) have been identified (Nishizuka, 1988, 1992; Osada *et al.*, 1990). Three distinct isoforms of brain PKC, types I, II and III, have been purified chromatographically (Huang *et al.*, 1986; Jaken and Kiley, 1987). The types I, II and III PKC have been shown to correspond to γ, βI+βII and α-PKC, respectively (Kikkawa *et al.*, 1987; Ono *et al.*, 1987).

It has been reported that extremely low concentrations of Pb^{++} (10^{-14}–10^{-10} M) can activate brain PKC in the presence of PS and DAG (Markovac and Goldstein, 1988). Activation of PKC by Pb^{++} was shown to be highly selective and potent; the Pb^{++}-induced PKC activity is more than 10 times higher than that by Ca^{++} or by other metal ions. Their study suggests that PKC may be the potential target of lead toxicity in biological systems, particularly in the central nervous system. However, the PKC preparation used in their experiment is relatively crude, and it may contain various subtypes of PKC and possibly other protein kinases. Thus, the activation of PKC by Pb^{++} at such low concentrations needs further evaluation. We have purified three PKC subtypes (types I, II and III) from the rat brain to apparent homogeneity and examined the effect of Pb^{++} on the PKC activity.

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Methods

Purification of PKC subtypes. Twenty-five rat brains from male Sprague Dawley rats (Harlan, 150–250 g) were used as a source of PKC. A whole brain (including cerebral cortex and limbic system as well as cerebellum) was used to obtain a sufficient amount of highly purified PKC subtypes for the study. Rats were maintained in our light-dark controlled (12-h cycle) animal facility, usually for 3 days before sacrifice. PKC was purified by a four-step liquid chromatography as described elsewhere (Chen and Murakami, 1992). Briefly, PKC was isolated from 100,000 × *g* supernatant of brain homogenate using DEAE-cellulose, phenylsepharose, AcA34 gel filtration chromatography and phenyl-5PW high-performance liquid chromatography. This PKC fraction was separated further into three fractions (types I, II and III) using hydroxyapatite column connected to an FPLC system (Pharmacia). We have used three different PKC preparations for the study. The specific activities of these fractions were 800 to 2000 nmol/min/mg of protein. The apparent homogeneity was confirmed by silver staining.

PKC assay in the presence of heavy metals. Chelators such as EGTA were not used in the PKC assay because they chelate not only Ca^{++} but also Pb^{++} with a much higher affinity (Bartfai, 1979), thus making it difficult to control free concentrations of Pb^{++} and Ca^{++} in

ABBREVIATIONS: PKC, protein kinase C; PS, phosphatidylserine; cFA, *cis*-fatty acid; DAG, diacylglycerol; DiC8, 1,2-dioctanoyl-*sn*-glycerol; LTP, long-term potentiation; NMDA, N-*methyl*-D-aspartate.

the assay system. To reduce contamination of Ca^{++} and other metal ions as much as possible, deionized distilled water was treated further with chelex-100 followed by AG501-X8 column. Test tubes used in the experiments were treated with 0.1 N HCl, followed by an extensive wash with chelex-100/AG501-X8-treated deionized distilled water.

PKC activity was assayed by measuring $^{32}P_i$ incorporation from [γ-^{32}P]ATP into lysine-rich histone (type III-S, Sigma) at 30°C as previously described (Murakami *et al.*, 1986). During the 10-min incubation period, the enzymatic velocity was linear with time. The basic reaction mixture contained 300 μg/ml of histone, 30 μg/ml of PS, 1 μM DiC8, 5 mM magnesium acetate, 60 μM [γ-^{32}P]ATP (100–300 cpm/pmol) and appropriate concentrations of metal ions (acetate salt) in a final volume of 260 μl in 20 mM Tris-acetate, pH 7.4. In some experiments, 100 μM oleic acid was used instead of PS and DiC8. Because Cl^- significantly affects the solubility of lead, acetate salts were used in the assay.

The reversibility of the Pb^{++} effect was tested as follows. PKC was treated with or without 10 μM Pb^{++} for 30 min at 4°C. After the incubation, the lead-treated and untreated PKC samples were chelated for Pb^{++} with 2 mM EGTA, and the reversibility of the Pb^{++} effect was examined using a synergistic PKC activation mode. We used this mode for studying the reversibility because it does not require Ca^{++} for the activation of PKC (Chen and Murakami, 1992), and the chelation of Pb^{++} by EGTA does not interfere with the measurement of PKC activity.

Limited proteolysis and protein kinase M activity measurement. Purified type I PKC was preincubated at 30°C for 1 min and treated with 0.6 μg/ml of trypsin from bovine pancreas to generate a catalytic fragment of PKC, protein kinase M (Huang *et al.* 1989). After 5 min of the trypsin treatment, proteolysis was stopped by soybean trypsin inhibitor (final concentration, 8 μg/ml). Protein kinase M obtained by the limited proteolysis was diluted 10 times with 20 mM Tris-acetate, pH 7.4, and the activity was tested in the presence or absence of 10 μM lead acetate.

Results

Pb^{++} effect on PKC activity. Heavy metal ions such as Zn^{++} have been shown to modulate PKC activity, phorbol-ester binding and membrane-interacting properties of PKC (Murakami *et al.*, 1987; Csermely *et al.*, 1988; Forbes *et al.*, 1990). It has been reported that Pb^{++} can strongly activate brain PKC using partially purified preparations (Markovac and Goldstein, 1988). To test whether low concentrations of Pb^{++} can directly activate PKC, we have examined the effect of Pb^{++} on PS/DAG-induced PKC activity using highly purified subtypes. As shown in figure 1, Pb^{++} at low concentrations ($<10^{-8}$ M) did not mimic Ca^{++} for the activation of PKC regardless of the subtypes. However, Pb^{++} was found to potently inhibit the PS/Ca^{++}-dependent PKC activity. IC_{50} of the Pb^{++} inhibition is 5 μM for types I and II and 10 μM for type III PKC.

Brain PKC also can be activated by cFAs (Murakami *et al.*, 1986; Sekiguchi *et al.*, 1987). We have shown previously that metal ions act differently on phospholipid/Ca^{++}-dependent and cFA-dependent PKC activity (Murakami *et al.*, 1987). We therefore examined the Pb^{++} effect on cFA-dependent PKC activity to test whether Pb^{++} inhibition is specific to the mode of activation (fig. 2). cFA-dependent PKC activity also was inhibited by Pb^{++} with IC_{50} values of 4, 2 and 5 μM for types I, II and III PKC, respectively. This result indicates that its inhibitory effect is not dependent on activation mode. Because there is no significant difference in Pb^{++} inhibition of the PKC subtypes tested, type I PKC, which has been shown to be a brain-specific subtype (Nishizuka, 1988), was studied in the following experiments for the further characterization of Pb^{++} inhibition.

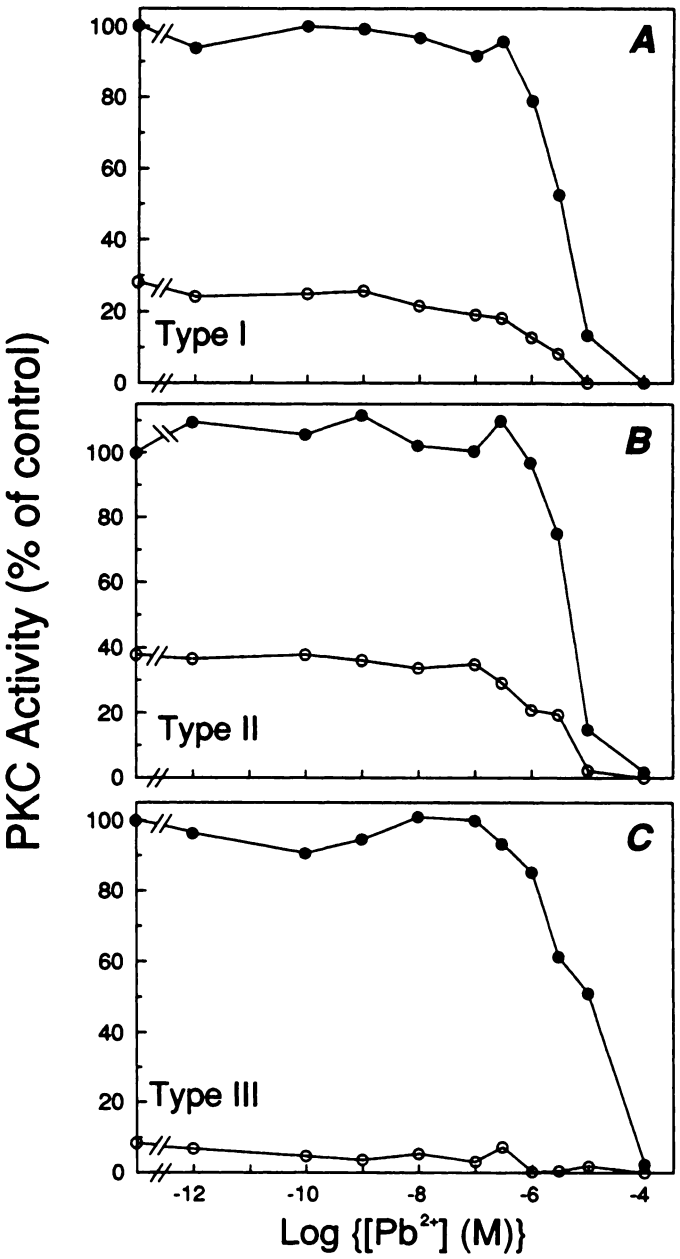

Fig. 1. Effect of Pb^{++} on PS/DAG-induced activation of PKC subtypes. Dose-dependency of Pb^{++} inhibition of PKC activity was examined at 30 μg/ml of PS, 1 μM DiC8 and various concentrations of Pb^{++} in the presence (●) or absence (○) of 100 μM Ca^{++}. The activity was normalized to that induced by 30 μg/ml of PS, 1 μM DiC8 and 100 μM Ca^{++} in the absence of Pb^{++}. A, type I; B, type II; C, type III. The figure is a representative result from three separate experiments. Each data point was determined by duplicated measures.

Characterization of PKC inhibition by Pb^{++}. Figure 3 shows the dose-response curve of Ca^{++} for type I PKC in the presence of different concentrations of Pb^{++}. The maximal velocity of PKC was greatly reduced by Pb^{++} in a concentration-dependent manner. One could expect a large excess of Ca^{++} would overcome the Pb^{++} inhibition if Ca^{++} competes with Pb^{++} for the same site; however, our result indicates that the Pb^{++} inhibition is not due to competition with Ca^{++}.

Biochemical and molecular biological studies have revealed that all PKC subtypes are a single polypeptide that consists of a regulatory domain and a catalytic domain at N- and C-

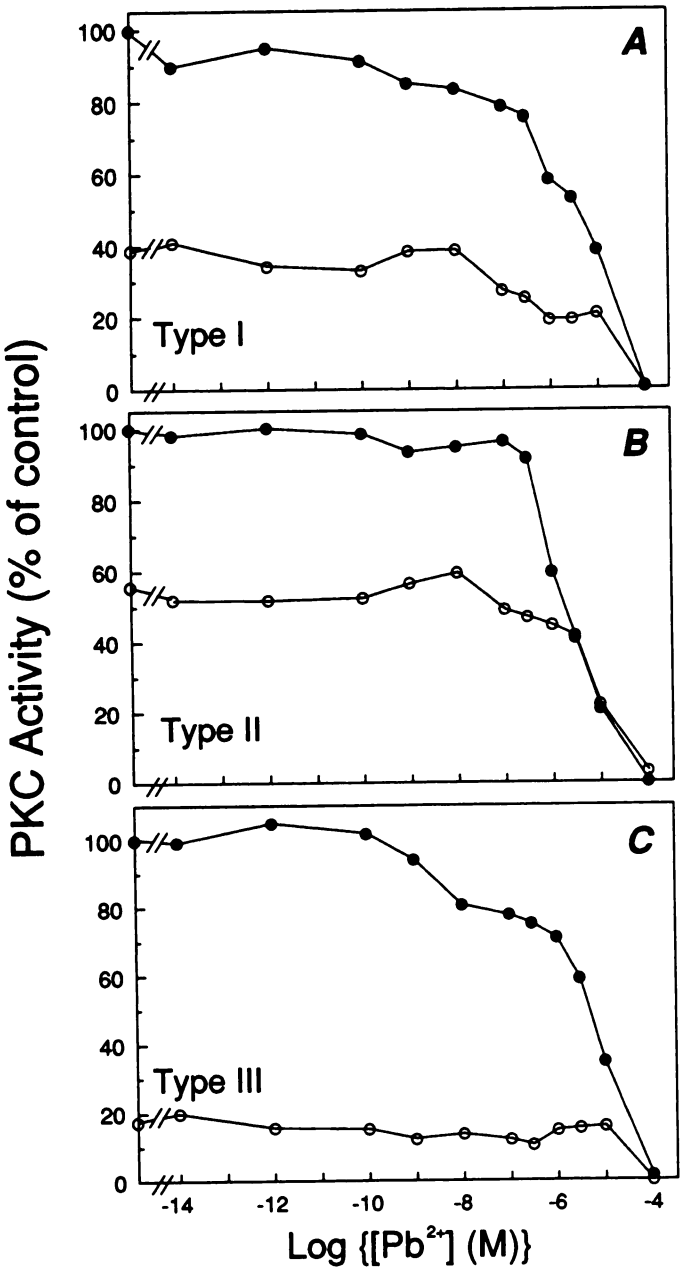

Fig. 2. Effect of Pb^{++} on *cis*-fatty acid-induced activation of PKC subtypes. Dose-dependency of Pb^{++} inhibition of PKC activity was examined at 100 μM oleic acid and various concentrations of Pb^{++} in the presence (●) or absence (○) of 100 μM Ca^{++}. The activity was normalized to that induced by 100 μM oleic acid and 100 μM Ca^{++} in the absence of Pb^{++}. A, type I; B, type II; C, type III. The figure is a representative result from three separate experiments. Each data point was determined by duplicated measures.

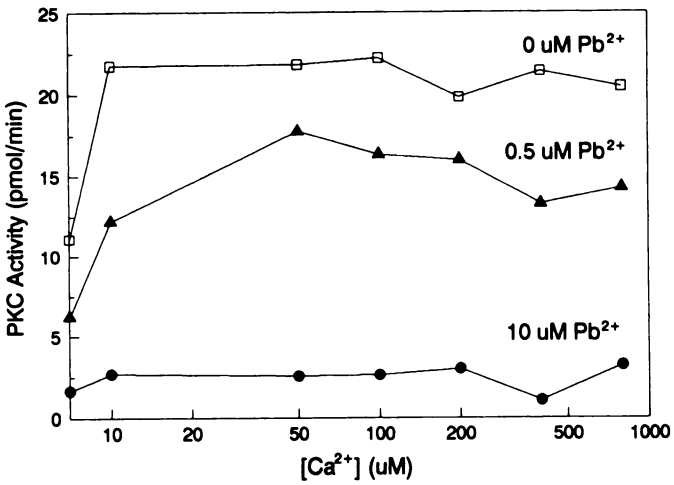

Fig. 3. Effect of Pb^{++} on Ca^{++} dose-response curve. Type I PKC activity was determined at different concentrations of Ca^{++} in the presence of 0 μM (□), 0.5 μM (▲), and 10 μM (●) Pb^{++}. The concentrations of PS and DiC8 were fixed at 30 μg/ml and 1 μM, respectively. The figure is a representative result from three separate experiments. Each data point was determined by duplicated measures.

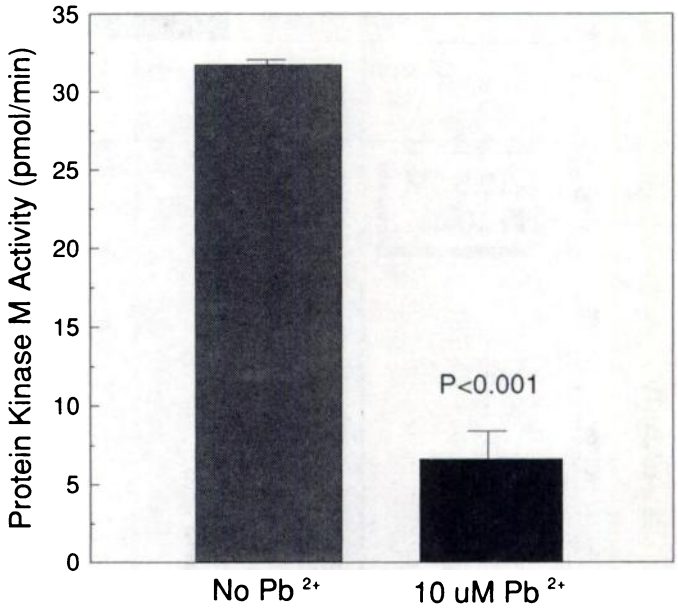

Fig. 4. Effect of Pb^{++} on protein kinase M activity. Type I PKC was subjected to limited proteolysis to generate protein kinase M as described in "Methods." The protein kinase M activity was assayed in the absence (no Pb^{++}) or presence (10 μM Pb^{++}) of Pb^{++}. Because protein kinase M was constitutively active, the kinase assay was performed in the absence of any lipid activators and Ca^{++}. Data were presented as means ± S.D. of four determinations.

termini, respectively (Nishizuka, 1988). Limited proteolysis of PKC has been shown to generate a catalytic domain of PKC that is constitutively active, called protein kinase M (Takai *et al.*, 1977; Huang, *et al.*, 1989). If the site of Pb^{++} action resides on the catalytic domain, Pb^{++} should inhibit protein kinase M, whereas if it is on the regulatory domain, then Pb^{++} should have no effect. Figure 4 shows the Pb^{++} effect on the proteolytically generated catalytic fragment activity. 10 μM of Pb^{++} significantly inhibited this protein kinase M activity to a similar extent to that observed in intact PKC (figs. 1 and 2). Pb^{++} inhibition of this constitutively active protein kinase indicates that Pb^{++} may act directly on the catalytic domain of PKC.

We have tested whether the Pb^{++} inhibition is reversible. Type I PKC was incubated with 10 μM Pb^{++} for 30 min, and the effect of chelation on the PKC activity was observed (fig. 5). Incubation of PKC with Pb^{++} strongly inhibited its activity. However, chelation of Pb^{++} by EGTA completely restored the PKC activity, suggesting that the Pb^{++} effect is totally reversible.

It has been shown that other metal ions also can inhibit PS/Ca^{++}-dependent PKC activity (Murakami, *et al.*, 1987). Therefore, we compared the potency of Pb^{++} inhibition to that by other heavy metals. As shown in figure 6, among the heavy metal ions tested in the experiment, Pb^{++} was the most potent

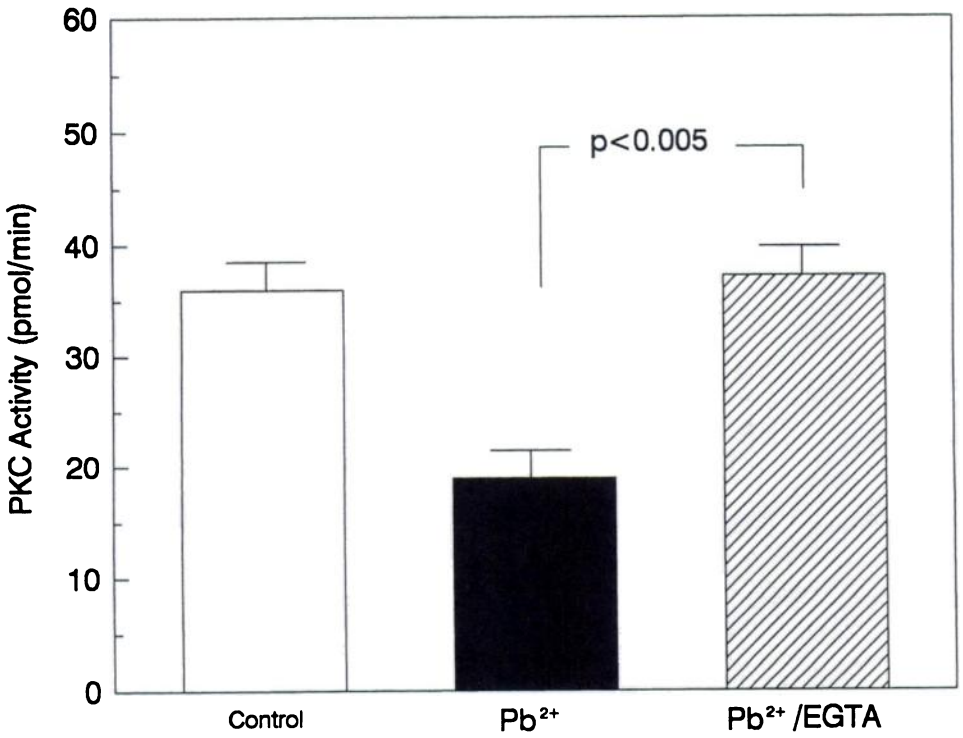

Fig. 5. Reversibility of Pb^{++} inhibition. Type I PKC was incubated with or without 10 μM Pb^{++} at 4°C for 30 min. For Pb^{++}-treated samples, the PKC activity was assayed in the absence of EGTA (Pb^{++}), or in the presence 2 mM EGTA (Pb^{++}/EGTA). The samples incubated without Pb^{++} were assayed in the absence of EGTA (control). The PKC activity was measured in the presence of 30 μg/ml PS, 1 μM DiC8 and 100 μM oleic acid in the absence of Ca^{++}. Other conditions are the same as described in "Methods." Data were presented as means ± S.D. of four determinations.

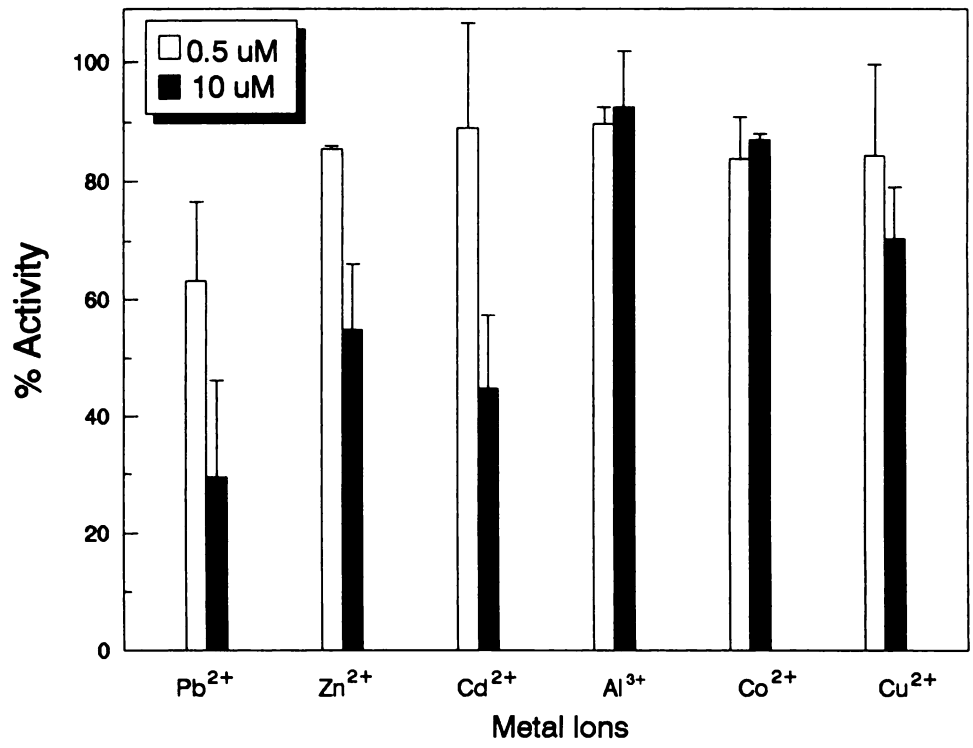

Fig. 6. Effect of various metal ions on PKC activity. The activity of Type I PKC was assayed in the presence of 30 μg/ml of PS, 1 μM DiC8 and 100 μM Ca^{++} (control), or in the presence of additional metal ions (Pb^{++}, Zn^{++}, Cd^{++}, Al^{+++}, Co^{++}, Cu^{++}) at 0.5 and 10 μM. The results were normalized with the control and presented as means ± S.E.M. from two to four separate experiments.

inhibitor, followed by Cd^{++} and Zn^{++}. Al^{+++}, Co^{++} and Cu^{++} had no effect on the PKC inhibition under the conditions tested.

Discussion

This study showed that Pb^{++} has a potent inhibitory effect on PKC subtypes purified from rat brain. Micromolar concentrations of Pb^{++} inhibit both PS/DAG-dependent and cFA-dependent PKC activation. This contrasts with the previous report that low concentrations of Pb^{++} could strongly activate partially purified brain PKC (Markovac and Goldstein, 1988). This discrepancy may be attributed to the preparations of PKC. Because crude preparation could contain other proteins and kinases, it is possible that the observed increased histone phosphorylation by Pb^{++} is due to the activation of other protein kinases or an indirect activation of PKC.

The activity of proteolytically generated catalytic fragment

of PKC activity also can be inhibited by Pb^{++}. This suggests that the site of action of Pb^{++} is at the catalytic domain of PKC. Studies of the Ca^{++}-requirement of phorbol ester binding indicate that the Ca^{++} binding site is in the C2 region in the regulatory domain of PKC, (Ono *et al.*, 1989; Kaibuchi *et al*, 1989). Thus, the interaction site of Pb^{++} appears to be different from the Ca^{++} interaction site. This notion is supported further by the Ca^{++} dose-dependent experiment at different Pb^{++} concentrations (fig. 3), which clearly shows that the Pb^{++} effect on PKC is not due to the competitive inhibition with Ca^{++}.

Neurotoxic effects of Pb^{++} have been studied extensively. Recent studies indicate that a low-level chronic lead exposure in childhood causes a deficit in central nervous system functioning and behavioral disorder, including learning disabilities (Needleman *et al.*, 1990; Lyngbye *et al.*, 1990). Collins *et al.*, (1982) have shown that a low dose of lead (25 μg/kg daily) administered to rats resulted in a preferential accumulation of this metal in the hippocampus, a brain region that is involved in information storage and memory. The LTP paradigm in the hippocampus has been studied extensively as a model system of neuronal plasticity in the brain (Teyler and DiScenna, 1987). It has been shown that the activation of an NMDA subclass of glutamate receptors in dentate gyrus and the CA1 region of the hippocampal formation plays an important role in the induction of LTP (Collingridge and Bliss, 1987), whereas PKC activation is necessary for the persistent expression of LTP (Lovinger *et al.*, 1987). Recently, 2 to 50 μM concentrations of Pb^{++} were shown to block NMDA current in rat hippocampal neurons without affecting non-NMDA currents (Alkondon *et al.*, 1990). Here we showed that a similar concentration range (2–10 μM) of Pb^{++} inhibits brain PKC subtypes, including brain-specific type I. These observations raise a possibility that the learning disorder associated with lead poisoning could result from the interaction with PKC, an important component for neuronal plasticity.

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