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# The interaction of aluminium with soluble protein kinase C from mouse brain

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**Abstract.** The interaction of aluminium ion species with soluble protein kinase C, $Ca^{2+}$/phospholipid-dependent protein kinase, from mouse brain has been examined in vitro. The activity of protein kinase C was increased by addition of $Ca^{2+}$ displaying an $EC_{50}$ value of $10.3 \pm 1.1 \times 10^{-6}$ M. The Al species inhibited the activity with an $IC_{50}$ values of $8.6 \pm 0.5 \times 10^{-5}$ M and $2.7 \pm 0.3 \times 10^{-5}$ M in the presence of 0.5 mM $Ca^{2+}$ and absence of $Ca^{2+}$, respectively. Concerning the $EC_{50}$ for $Ca^{2+}$ activation, this was increased by the Al species in a dose-dependent manner. Moreover, the inhibition was of a non-competitive type with respect to H1 histone and of a mixed type with respect to ATP. It is likely that the inhibition was caused by 1) the blocking of $Mg^{2+}$ binding to ATP, 2) the blocking of $Ca^{2+}$ binding to protein kinase C. Our results suggested that protein kinase C was involved in neurotoxicity of Al.

**Key words:** Protein kinase C – Aluminium – Cerebrum – Carboxyl groups

## Introduction

Aluminium (Al) has recently been recognized as a causative agent in dementia, anemia and osteomalacia occurring in chronic hemodialysis (Wills and Savory 1983; Alfrey 1984) and in Alzheimer's disease (Perl 1985). Al has also been implicated in neurotoxicity associated with amyotrophic lateral sclerosis, and in a form of Parkinsonism-dementia complex, in the indigenious population of Guam, where soils are high in $Al^{3+}$ and low in $Mg^{2+}$ and $Ca^{2+}$ (Garruto et al. 1984).

On the other hand, protein kinase C has been implicated in transmembrane signaling to regulate many cellular functions and proliferation (for reviews, refer to Nishizuka 1984, 1986, 1988). Recently, much attention has been paid to its role in neuronal signal transduction, since the highest concentration of protein kinase C has been found in nervous systems (Kuo et al. 1980; Minakuchi et al. 1981; Ashendel et al. 1983) and activation of protein kinase C causes an increase in neurotransmitter release (Shuntoh and Tanaka 1986; Tanaka et al. 1986; Nichols et al. 1987). In order to clarify whether protein kinase C is involved in neurotoxicity of heavy metal cations, our laboratory has been investigating the effects of divalent cations on protein kinase C. In brief, cations were divided into three groups based on their effects on the activity of soluble protein kinase C, that is, 1) as substitutes for $Ca^{2+}$ to activate; $Sr^{2+}$ or $Ba^{2+}$, 2) essentially no effect; $Co^{2+}$, $Mn^{2+}$, etc., and 3) as inhibitors; $Hg^{2+}$, $Pb^{2+}$, $Cd^{2+}$, etc. (Saijoh et al. 1988). In particular, mercurials impaired protein kinase C at micromolar concentrations due to SH blocking (Inoue et al. 1988). These findings suggest that ionic circumstances may affect the activity of protein kinase C. Accordingly, the question that arises now is whether protein kinase C can be affected by trivalent cations like $Al^{3+}$, as well as divalent cations, viz, whether protein kinase C is involved in the neurotoxicity of Al. The present study was undertaken to evaluate the effect of Al ion species on the enzymatic activity of soluble protein kinase C.

## Materials and methods

*Materials.* The following compounds were used in this study: [$\gamma$-$^{32}$P] ATP (8 Ci/mmol, New England Nuclear), leupeptin and ATP (Sigma), diolein (Serdary), phosphatidylserine (Tokyo Kasei), Pipes, phenylmethylsulfonyl fluoride, 2-mercaptoethanol, EGTA, 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDAC), dicyclohexyl carbodiimide (DCCD), N-ethylmaleimide (NEM), and metal salts [$CaCl_2$, $AlCl_3$ and $HgCl_2$; of highest purity (99.99%)] (Nakalai tesque). H1 histone was a generous gift from Dr U Kikkawa and Dr K Ogita, Department of Biochemistry, Kobe University School of Medicine. All other compounds used were of analytical quality.

*Buffer composition.* Preparation of protein kinase C and assay of its enzymatic activity were performed essentially according to the method of Kikkawa et al. (1986) as described below. However, Pipes/NaOH (pH 6.8) was used instead of Tris/HCl (pH 7.4) in order to examine the effect of carbodiimide modification on protein kinase C. The slightly lower pH should favor the reaction of carbodiimide with the carboxyl group (Means and Feeny 1971). No significant change was found in the activity of protein kinase C between Pipes and Tris buffers.

*Removal of contaminating metals.* As described previously (Saijoh et al. 1988), $Ca^{2+}$ and other metal ions in Pipes/NaOH (pH 6.8) and histone were removed using Chelex-100 column (Bio-Rad) which reduced $Ca^{2+}$ in the solution

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to less than 0.2 μM. Metals contained in the protein kinase C preparation were removed during the purification described below. According to the method of Chao et al. (1984), $Ca^{2+}$-free solutions were stored in polyethylene containers that had been treated with dilute HCl, maintained at about 95° C for 5 min, and then rinsed several times in deionized water obtained from Mili-Q (Millipore), containing less than 0.1 μM $Ca^{2+}$. Since the concentration of $Ca^{2+}$ was critical, plastic ware was used throughout the experiment.

*Partial purification of protein kinase C.* Whole mouse brain (Jcl: ICR, female, 6 week old), excluding the olfactory bulb and cerebellum, was homogenized with 10 vol homogenate buffer [10 mM Pipes/NaOH (pH 6.8), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol and 0.01% leupeptin]. The homogenate was centrifuged at 100000 *g* for 1 h. Thereafter, the supernatant was further centrifuged with 120000 *g* for 45 min. Soluble protein kinase C was partially purified over DEAE-cellulose (DE-52, Whatman Inc.) column. Following passage through ultrafiltration membrane YM100 (partition molecular weight: 100000, Amicon), the partially purified protein kinase C was washed ten times with 10 vol EGTA- and 2-mercaptoethanol-free homogenate buffer using ultrafiltration membrane cone CF25 (partition molecular weight: 25000, Amicon) in order to remove EGTA and 2-mercaptoethanol. $Ca^{2+}$ content in the partially purified protein kinase C was less than 0.01 ppm, equal in effect to less than 0.2 μM. In order to measure the irreversible effects of carbodiimides and NEM, the enzyme was treated with 1 mM EDAC, DCCD and NEM for 1 h on ice.

*Enzymatic activity of protein kinase C.* Enzymatic activity was assayed by measuring the incorporation of $^{32}P$ into H1 histone from [$\gamma$-$^{32}P$] ATP, according to the method of Kikkawa et al. (1986). However, 2-mercaptoethanol, EGTA and EDTA were not used in order to avoid binding to cations. A 70 μl aliquot (final; 25 μg protein/ml) of protein kinase C preparation was added to the reaction mixture in a total volume of 180 μl, containing 10 mM Pipes/NaOH (pH 6.8), [$\gamma-^{32}P$] ATP (10 μM; 30000 cpm/nmol), 8 μg/ml H1 histone, 5 mM magnesium acetate, 0–1 mM $CaCl_2$, 8 μg/ml phosphatidylserine and 0.8 μg/ml diolein with or without metal salts, carbodiimides and NEM. The mixture without diolein served as a blank. For kinetic analysis, various concentrations of ATP (1.7–20 μM) and H1 histone (40–400 μg/ml) were used. The reaction was terminated by addition of 1 ml 25% trichloroacetic acid and the precipitate was collected and washed by filtration over a membrane filter. The radioactivity was determined using a scintillation spectrophotometer.

*Other methods.* Protein was measured using Bio-Rad protein assay kit. Statistical analysis was performed using Student's *t*-test or Tukey's multiple comparison test.

## Results

*Effect of Al species on the enzymatic activity of protein kinase C.* In the absence of any Al species, addition of $Ca^{2+}$ increased the enzymatic activity of protein kinase C in a concentration-dependent manner ($EC_{50}$: 10.3 ± 1.1 $\times 10^{-6}$ M). This activity was maximally achieved at 0.1–0.2 mM $Ca^{2+}$ (Fig. 1), as reported previously (Saijoh et al. 1988). The activity was suppressed by the Al species both in the presence and absence of $Ca^{2+}$. In the presence of 0.5 mM $Ca^{2+}$, the Al species showed no detectable effect up to $4 \times 10^{-5}$ M, although the activity of protein kinase C was impaired with an $IC_{50}$ value of $8.6 \pm 0.5 \times 10^{-5}$ M. In the absence of $Ca^{2+}$, the Al species inhibited the activity of protein kinase C with an $IC_{50}$ of $2.7 \pm 0.3 \times 10^{-5}$ M (Table 1). On the contrary, addition of the Al species to the assay mixture increased an $EC_{50}$ for $Ca^{2+}$ as well as decreased the basal and maximum activities (Fig. 1).

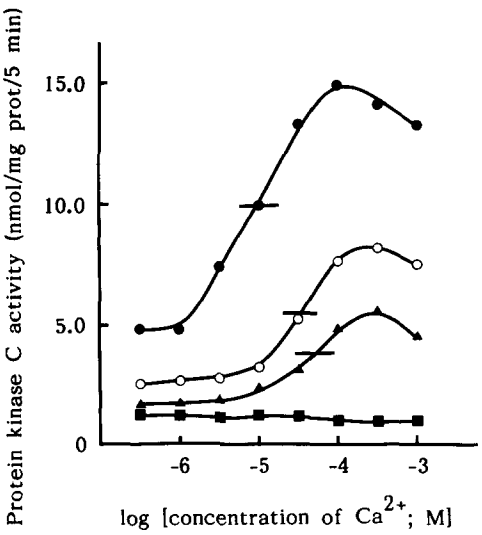


**Fig. 1.** Effect of Al species on the activity of protein kinase C at varying concentrations of $Ca^{2+}$. Each *point* represents the mean of at least four determinations. *Horizontal bar* indicates the $EC_{50}$ value. (●) Control; (○) $8 \times 10^{-5}$ M Al species, almost identical with the $IC_{50}$ value as described in Table 1; (▲) $10^{-4}$ M; (■) $2 \times 10^{-4}$ M. Significant differences in $EC_{50}$ values; control – $8 \times 10^{-5}$ M and control – $10^{-4}$ M ($p < 0.05$, Tukey's multiple comparison test)

**Table 1.** Effect of aluminium on enzymatic activity of protein kinase C

| | Activity (nmol/mg prot/5 min) | $IC_{50}$ for Al species (M) |
|---|---|---|
| (−) $Ca^{2+}$ | 5.0 ± 0.8 | $2.7 \pm 0.3 \times 10^{-5}$ |
| (+) $Ca^{2+}$ | 15.6 ± 1.0* | $8.6 \pm 0.5 \times 10^{-5}$* |

Values were the means ± SE of six determinations. *$p < 0.05$ (Student's *t*-test)

*Kinetic analysis of inhibition.* $Al^{3+}$ has been reported to inhibit enzyme systems involving both ATP and $Mg^{2+}$ (Harrison et al. 1972; Martin 1986). In order to determine the type of inhibition, the activity of protein kinase C was determined over ranges of ATP and histone concentrations in the presence of $Ca^{2+}$. Double-reciprocal plots showed that the Al species displayed a mixed type of inhibition with respect to ATP, whereas a non-competitive type was

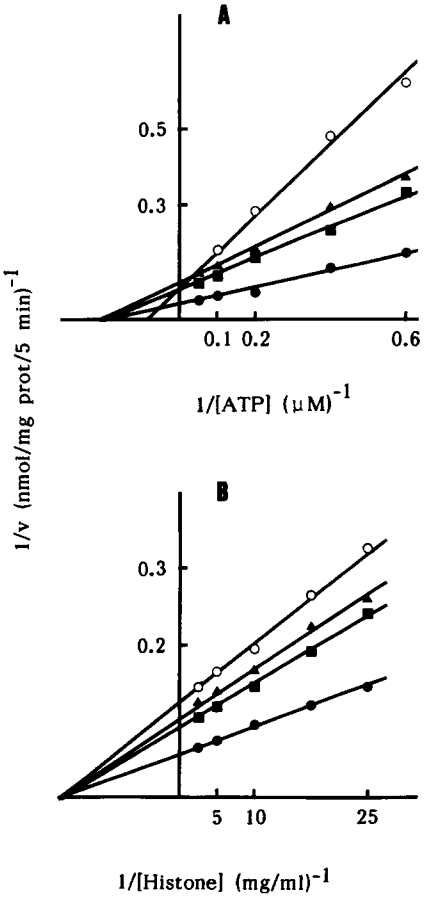


**Fig. 2. A, B** Kinetic analysis of inhibition for protein kinase C activity over concentration ranges of ATP (**A**), and histone (**B**), in the presence of 0.5 mM $Ca^{2+}$. Each *point* represents the mean of at least four determinations. (●) Control; (○) $8 \times 10^{-5}$ M Al species, almost identical with the $IC_{50}$ value; (■) $1 \times 10^{-7}$ M $Hg^{2+}$; (▲) $8 \times 10^{-4}$ M NEM. The concentrations of $Hg^{2+}$ and NEM used were almost identical to the $IC_{50}$ values as reported previously (Inoue et al. 1988). For the effect of NEM, the preparation was priorly incubated with NEM for 1 h on ice. Significant differences; (**A**) $V_{max}$: control-Al species, $Hg^{2+}$ and NEM and $K_m$: control-Al species and (**B**) $V_{max}$: control-Al species, $Hg^{2+}$ and NEM ($p < 0.05$, Tukey's multiple comparison test)

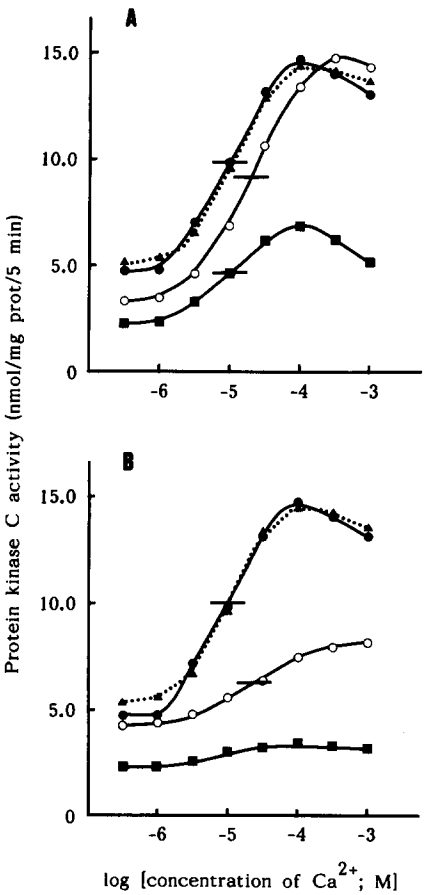


**Fig. 3. A, B** Effects of carbodiimides, $Hg^{2+}$ or NEM on the activity of protein kinase C over ranges of $Ca^{2+}$ concentrations. (●) Control. **A** (○) 1 mM EDAC, (▲) 1 mM DCCD and (■) $1 \times 10^{-7}$ M $Hg^{2+}$ were added to the assay mixture. **B** Protein kinase C was priorly incubated with (○) 1 mM EDAC, (▲) 1 mM DCCD and (■) $8 \times 10^{-4}$ M NEM for 1 h on ice. Horizontal bar indicates the $EC_{50}$ value. Each *point* represents the mean of at least four determinations. Significant differences in $EC_{50}$ values; (**A**) control – EDAC, DCCD – EDAC and $Hg^{2+}$ – EDAC and (**B**) control – EDAC and DCCD – EDAC ($p < 0.05$, Tukey's multiple comparison test)

shown by $Hg^{2+}$ and NEM (Fig. 2*A*). With respect to histone, on the other hand, the Al species as well as $Hg^{2+}$ and NEM displayed a non-competitive type of inhibition (Fig. 2*B*). Different kinds of SH-blocking reagents, such as 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), *p*-chloromercuribenzoic acid (PCMB), etc. showed the same types of inhibition as with $Hg^{2+}$ and NEM (data not shown).

*Chemical modification of protein kinase C.* The effect of modification of carboxyl groups on protein kinase C was compared with the effect of the Al species essentially according to the method of Marquis and Black (1984). To measure the reversible competitive effects of EDAC and DCCD on enzymatic activity, EDAC or DCCD was added to the assay mixture. EDAC, a water-soluble carbodiimide, increased the $EC_{50}$ for $Ca^{2+}$ without changing both the basal and maximum activities, whereas DCCD, a lipid-soluble carbodiimide, showed no apparent effect (Fig. 3*A*). $HgCl_2$ did not alter the $EC_{50}$ for $Ca^{2+}$ but decreased both the basal and maximum activities. Thereafter, prior incubation of protein kinase C with carbodiimides and NEM was carried out (Fig. 3*B*) in order to irreversibly block the carboxyl and SH groups, respectively. Treatment with EDAC induced a reduction in the basal activity and increased the $EC_{50}$ for $Ca^{2+}$. In contrast, DCCD still showed no effect on the activity. NEM reduced the basal activity to one half and abolished the activation by $Ca^{2+}$.

## Discussion

The findings obtained in the present study indicated that the Al species inhibited the enzymatic activity of protein kinase C with an $IC_{50}$ value of $2.7 \times 10^{-5}$ M (almost equal to 0.7 ppm) without 0.5 mM $Ca^{2+}$ and $8.6 \times 10^{-5}$ M (almost equal to 2.3 ppm) with 0.5 mM $Ca^{2+}$. This inhibition is possibly caused by the interaction of the Al species and $Ca^{2+}$ with the same anionic site of protein kinase C, as

well as by the competition of $Al^{3+}$ with $Mg^{2+}$ for the phosphate site of ATP.

The concentration of the Al species necessary to inhibit the enzymatic activity of protein kinase C was higher than other divalent cations such as $Hg^{2+}$, $Cd^{2+}$, $Pb^{2+}$, etc. and trivalent cations such as $Tb^{3+}$, $Sm^{3+}$ etc., which inhibited that at micromolar order concentrations. However, the inhibition caused by the Al species was more potent vis-a-vis $Mn^{2+}$, $Ni^{2+}$ etc. (Inoue et al. 1988; Saijoh et al. 1988). The usual value of Al in normal human brain is within the range of 0.9–2.2 mg/kg dry weight and in the dialysis encephalopathy patient brain 12.4–24.5 mg/kg dry weight (Arieff et al. 1979). Moreover, no Al was detectable in the control rat brain and 8.6 mg/kg wet weight (= 8.6 ppm) of Al was accumulated in the rat brain 14 days after administration of an $LD_{50}$ value (14 days) of Al nitrate (Llobet et al. 1987). Within physiological pH, the highest permissible $Al^{3+}$ concentration is calculated to be approximately $10^{-8}$ M with a total allowable Al ion concentration of about $10^{-5}$ M. The predominant water-derived complex is $Al(OH)_4^-$. The remainder of the added $Al^{3+}$ has either been complexed by other ligands or formed into insoluble $Al(OH)_3$ (Martin 1986). Polynuclear species may also be formed in a time-dependent manner (Smith 1971). In the present study, relatively low concentrations of $AlCl_3$ were dissolved prior to use, in order to avoid forming any time-dependent polymerization. Such a bio-inorganic chemistry of Al makes it difficult to interpret the findings, although it is reasonable to speculate that protein kinase C is involved in the neurotoxicity of Al.

Sequence studies revealed that there were at least three major functional sites in the catalytic domain of protein kinase C, viz, the MgATP binding site, the protein substrate binding site and the catalytic site, and at least two major functional sites in the regulatory domain, that is, the diacylglycerol or phorbol ester binding site and the $Ca^{2+}$ binding site (Coussens et al. 1986; Parker et al. 1986; Ohno et al. 1987; Ono et al. 1987). However, Kikkawa et al. (1987) reported that no obvious structure for the binding of $Ca^{2+}$, such as the EF-hand that is found in $Ca^{2+}$ binding proteins like calmodulin, was apparent in the molecule. As reported previously (Inoue et al. 1988), SH-blocking reagents including mercurials suppressed the activity of soluble protein kinase C, probably due to inhibition of the catalytic site as well as inhibition of diacylglycerol binding. In the present study, kinetic analysis was performed in order to clarify the mechanism of inhibition by the Al species.

The Al species showed a mixed type of inhibition with respect to ATP, which was different from SH-blocking reagents. Martin (1986) reported that $Al^{3+}$ could not replace $Ca^{2+}$ in proteins without substantial changes in liganding groups, but bound almost $10^7$ times more strongly to ATP than does $Mg^{2+}$. It is considered possible that the effect of $Al^{3+}$ on protein kinase C activity is via the substitution for $Mg^{2+}$ rather than for $Ca^{2+}$. On the other hand, the $IC_{50}$ value was increased in the presence of $Ca^{2+}$, indicating that protein kinase C was protected from the Al species inhibition in high $Ca^{2+}$ media. Moreover, a water-soluble carbodiimide, but not a lipid-soluble carbodiimide, showed an increase in $EC_{50}$ for $Ca^{2+}$ not only when it was added to the assay mixture but also after it irreversibly blocked the carboxyl group of protein kinase C. These findings are taken to suggest that carboxyl group played an important role in $Ca^{2+}$ binding to protein kinase C. A similar interaction between the Al species and $Ca^{2+}$ was reported in case of acetylcholinesterase (Marquis and Black 1984). Although Richardt et al. (1985) has claimed that the Al species in toxicologically relevant concentrations did not interact with calmodulin, Siegel and Haug (1983) reported that the ability of the Al species to inhibit calmodulin was correlated with Al toxicity. These controversies seemed to be due to the complex bio-inorganic chemistry of Al, as described above. The possibility that the observed effects were due to insoluble $Al(OH)_3$ or polynuclear species affecting the enzyme or H1 histone cannot be fully ruled out. Still, it seems likely that, in the $10^{-5}$–$10^{-4}$ M range, the Al ion species, like $Ca^{2+}$, may bind to the anionic sites of protein kinase C which involves a carboxyl group that can be blocked by carbodiimides.

Consequently, the Al species inhibited protein kinase C, probably not only due to competition with $Mg^{2+}$ for ATP but also due to blocking of $Ca^{2+}$ binding to protein kinase C. It is reasonable to assume that the Al species showed neurotoxicity by inhibiting protein kinase C and/or calmodulin, both of which played important roles in $Ca^{2+}$-mediated intracellular signal transduction. In this line of thought, the modulation of Al neurotoxicity by intracellular $Ca^{2+}$ should also be considered.

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