# Aluminum interaction with plasma membrane lipids and enzyme metal binding sites and its potential role in Al cytotoxicity

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**Abstract** **The trivalent cation aluminum can cause chronic cytotoxicity in plants, animals and microorganisms. It has been suggested that Al interaction with cell membranes and enzyme metal binding sites may be involved in Al cytotoxicity. In this study, the binding of Al to microsomes and liposomes was found to be lipid dependent with the signal transduction element phosphatidylinositol-4,5-bisphosphate having the highest affinity for Al with an Al:lipid stoichiometry of 1:1. Al binding was only reduced in the presence of high concentrations of $Ca^{2+}$ (>1 mM). Both citrate and, to a lesser extent, malate were capable of preventing Al lipid binding, which is consistent with the involvement of these organic acids in a recently described Al detoxification mechanism in plants. The effects of $AlCl_3$, Al-citrate and $ZnSO_4$ on metal-dependent enzyme activities (enolase, pyruvate kinase, $H^+$-ATPase, myosin, Calpain, proteinase K, phospholipase $A_2$ and arginase) was assayed in vitro. While $Zn^{2+}$ was capable of inhibiting all the enzymes except the $H^+$-ATPase, $AlCl_3$ and Al-citrate had minimal effects except for with phospholipase $A_2$ where an interaction with $AlCl_3$ occurred. However, this could be negated by the addition of citrate. The results indicate that, contrary to current hypotheses, the toxic mode of Al is not through an interaction with enzymatic catalytic metal binding sites but may be through the interaction with specific membrane lipids.**

*Key words:* Aluminium; Cytotoxicity; Enzyme activity; Lipid binding; Metal binding; Zinc

## 1. Introduction

Aluminium (Al) is a highly cytotoxic metal to both plants and animals and is responsible for both significant losses in world agricultural crop production and a range of neurological disorders [1–3]. However, to date the causes of Al toxicity, particularly in plants, have remained elusive and controversial [1]. Recently it has been proposed that one primary site of toxicity may be associated with the insertion of Al in metal binding domains of enzymes and lipids, causing a disruption in cell metabolism and signalling [4–7]. Although it has been shown that the addition of cations such as $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Ba^{2+}$ are capable of inhibiting metal-dependent enzymes and changing lipid fluidity via substitution in $Ca^{2+}$ and $Mg^{2+}$ binding domains [8], there is little evidence for similar inhibitions by the cations $Al^{3+}$, $Al(OH)^{2+}$ or $Al(OH)_2^+$. One study had previously indicated that $Al^{3+}$ was capable of blocking the $Ca^{2+}$–calmodulin-dependent enzyme phosphodiesterase via an occupation of the calmodulin $Ca^{2+}$ binding site [9]. However, subsequent studies with electron paramagnetic resonance have indicated that these earlier findings may be incorrect [10,11]. The only other evidence for Al substitution in enzymes comes from recent studies on the effects of Al on the phosphoinositide signalling pathway where it has been shown that Al specifically inhibits the $Ca^{2+}$-dependent enzyme phospholipase C which acts on the lipid substrate phosphatidylinositol-4,5-bisphosphate [12,13]. In an effort to resolve the controversy concerning Al/Ca interactions in enzymes, the activities of a range of purified enzymes with known $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ binding domains were screened in the presence of $AlCl_3$ and Al-citrate with $ZnSO_4$ used as a positive control. In addition, the binding characteristics of Al to microsomal and liposomic membranes was also determined to determine the affinity of certain lipids for Al and to what extent potential cellular (e.g. excretion of organic acids [14]) or environmental (increased external ($Ca^{2+}$) [1]) detoxification mechanisms are able to counteract Al binding.

## 2. Materials and methods

### 2.1. Preparation of microsomal membranes from wheat

Seeds of the Al-resistant winter wheat (*Triticum aestivum* L.) cultivar Atlas 66 were grown under hydroponic culture conditions and microsomes harvested as described in [12]. The microsomal lipids are composed of ≈65% phospholipid of which ≈60% is phosphatidylcholine (PC) [15].

### 2.2. Enzymatic assays

$Ca^{2+}$-activated neuronal protease (Calpain, EC 3.4.22.17; rabbit skeletal muscle) activity was assayed according to the method of [16]. Assays were performed in the presence of 10 mM Tris-HCl (pH 7.4), 1 mg $mL^{-1}$ *N,N*-dimethylated casein, 300 μM $Ca^{2+}$ and 0.1 U of enzyme. After 5 min, the proteins were TCA precipitated and proteolytic activity measured by the change in absorbance at 240 nm. Proteinase K (EC 3.4.21.64; *Tritirachium album*) was measured in an identical manner to Calpain except in the presence of 0.5 U of enzyme.

Enolase (EC 4.2.1.11; *Saccharomyces cerevisiae*) which catalyses the conversion of 2-phospho-D-glycerate (PGA) to phospho(enol)pyruvate (PEP) was assayed according to the method of [17]. The assay medium contained 50 mM Tris-HCl (pH 7.4), 0.15 mM $MgSO_4$, 1 mM PGA, 0.1 mM KCl and 0.25 U of enolase and activity was measured by the change in absorbance at 240 nm over a 60 s period.

Pyruvate kinase (EC 2.7.1.40; rabbit muscle) which converts PEP to pyruvate was assayed according to the method of [18]. The assay medium contained 10 mM Tris-HCl (pH 7.4), 1 mM PEP, 2 mM NADH, 10 mM ADP, 0.1 mM $MgSO_4$, 5 U lactic dehydrogenase (EC 1.1.1.28; *Leuconostoc mesenteroides*) and 5 U of pyruvate kinase and activity was assessed by measuring the change in absorbance at 340 nm over a 60 s period.

Plasma membrane $H^+$-ATPase (EC 3.6.1.3) which catalyses the conversion of ATP to ADP was assayed according to the method of [19] using microsomal membranes isolated from wheat. The assay solution contained 25 mM Mes-Tris buffer (pH 6.8), 0.4% Brij-58, 10 mM ammonium molybdate, 250 mM KCl, 30 mM ATP, 0.2 mM $MgSO_4$ and 1 mg $ml^{-1}$ microsomal membranes. After 15 min the production of $P_i$ was assayed according to the method of [21].

$Ca^{2+}$-dependent ATPase (myosin, EC 3.6.1.32; rabbit muscle) ac-

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tivity was assayed according to the method of [20]. The assay medium consisted of 50 mM Tris-HCl (pH 7.4), 300 mM KCl, 2 mM $CaCl_2$, 2 mM ATP and 0.01 U of myosin and $P_i$ production measured after 30 min by the method of [21].

Phospholipase $A_2$ ($PLA_2$, EC 3.1.1.4; *Naja naja atra*) which catalyzes the hydrolysis of 2-acyl groups in phospholipids was assayed according to the method of [22]. The assay medium consisted of 50 mM Hepes-KOH (pH 7.0), 5 μM 6:0-*N*-1-acyl-2-[6-(7-nitro-1,3-benzoxadiazol-4-yl)amino]caproyl phosphocholine (NBD-PC; Avanti Polar Lipids, Alabaster, AL), 20 μM $CaCl_2$ and 1 U of $PLA_2$. The release of fluorescent NBD was continuously measured over a 5 min period.

Arginase (EC 3.5.3.1; bovine liver) which catalyses the formation of ornithine and urea from arginine was assayed according to the method of [23]. The assay medium contained 10 mM Tris-HCl (pH 7.4), 125 mM arginine and 10 U of $Mn^{2+}$-activated arginase and the reaction was run for 30 min.

To assess the metal dependency of enzyme action, increasing concentrations of either $CaCl_2$ or $MgSO_4$ were added to the assay medium. To assess the inhibition of the enzymes by Al and Zn, either buffer alone, $ZnSO_4$, $AlCl_3$ or Al-citrate were added to the medium 2 min before the start of the assay. $AlCl_3$ (0–100 μM) solutions were made 5 min before the start of the assay to prevent long-term precipitation whilst higher concentration Al solutions (100–1000 μM; pH 7.2) were prepared 60 min before the start of the experiment and kept as dilute gel suspensions. All assays were started by the addition of enzyme and carried out at 25°C in triplicate. No metal interference in any of the assay procedures (e.g. absorbance, colour development) was observed when $AlCl_3$, Al-citrate or $ZnSO_4$ were added after the completion of control reactions. All enzymes except $H^+$-ATPase were obtained in a purified form (metal free) from Sigma or Calbiochem. Arginase was bought in $Mn^{2+}$-activated state.

### 2.3. Preparation of liposomes

Lipids (1–5 mg) were dissolved in chloroform/methanol (9:1 v/v), dried down under a gentle stream of $N_2$, 1 mL of assay buffer (AB) added (2 mM $CaCl_2$, 1 mM HomoPipes, pH 4.45) and liposomes formed by sonication with an ultrasonic probe (25% power for two 10 s pulses; Branson Ultrasonics, Danbury, CT). Liposomes were made fresh on the day of experimentation and the binding studies performed within 8 h of formation over which period no reduction in Al binding was observed. Liposomes were made from SB (crude lipid extract from soybean), DB (crude lipid extract from dog brain), PC (bovine brain phosphatidylcholine; MW = 768), PG (egg yolk phosphatidylglycerol; MW = 770), PA (egg yolk phosphatidic acid; MW = 697), PI (soybean phosphatidylinositol; MW = 857) and $PIP_2$ (bovine brain phosphatidylinositol 4,5-bisphosphate; MW = 1082). Lipids were obtained from Sigma (SB, DB, PC, PG, PA; Poole, Dorset, UK), Avanti Polar Lipids (PI, Alabaster, AL) and Calbiochem ($PIP_2$; San Diego, CA).

### 2.4. Binding studies

Microsomes and liposomes were diluted in AB to concentrations ranging from 0 to 100 μg protein $ml^{-1}$ and 0–500 μg lipid $ml^{-1}$, respectively. $AlCl_3$ (1 mM) was then added to give a final Al concentration of 25 μM and volume of 1 ml and the tubes incubated at 25°C for 20 min. Controls were performed without $AlCl_3$. After centrifugation (125 000 × *g*, 30 min, 25°C), 0.5 ml of lipid-free supernatant solution was removed for Al analysis. The efficiency of microsome/liposome pelleting during centrifugation was assessed using the lipophilic fluorescent dye Nile Red (2 μg $ml^{-1}$; excitation 660 ± 10 nm, emission 323 ± 10 nm). The centrifugation procedure was found to be greater than 95% efficient in separating lipid from the supernatant.

Identical Al binding studies to those described above were also performed except with the addition of increasing concentrations of $CaCl_2$ (0–10 mM), malate (0–100 μM, pH 4.45) or citrate (0–100 μM, pH 4.45) to the assay mixture prior to the addition of Al. Due to the problems of Al speciation and precipitation at neutral pH, the effect of pH on trivalent metal binding to liposomes and microsomes was assayed as described above using $GdCl_3$ and $LaCl_3$ (25 μM) in the presence of 2-(*N*-morpholino)ethanesulfonic acid (Mes)-Tris buffer (5 mM, pH range 4.0–7.0). All experiments were replicated twice.

Table 1
Stoichiometry of Al–liposome binding reaction

| Lipid composition | Lipid–Al binding (nmol lipid/nmol Al) |
|---|---|
| Mixed | |
| SB | 8.28 ± 0.29 |
| DB | 5.71 ± 0.72 |
| Single | |
| PA | 3.10 ± 0.02 |
| PC | 16.3 ± 0.16 |
| PG | 2.12 ± 0.54 |
| PI | 2.04 ± 0.28 |
| $PIP_2$ | 0.98 ± 0.02 |

Values represent means ± SE ($n = 2$).
Assay conditions = 2 mM $CaCl_2$, 1 mM HomoPipes buffer (pH 4.45), 25 μM $AlCl_3$, ≈25 μM lipid.

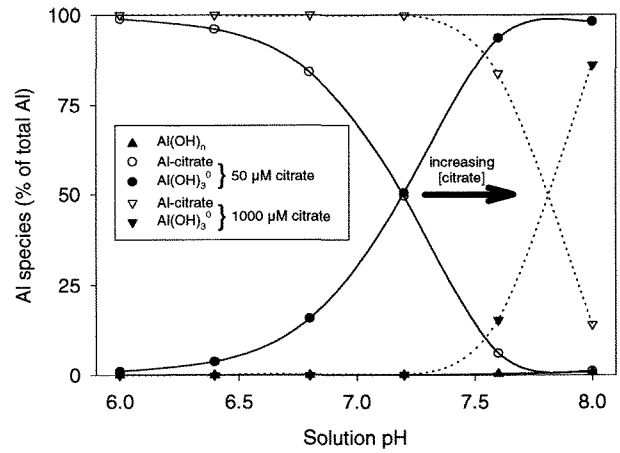


Fig. 1. Speciation of Al at cytosolic pH in the presence of increasing concentrations of citrate as predicted with chemical equilibria speciation program GEOCHEM-PC [24]. The concentration of the Al species $Al(OH)_n$ (▲), Al-citrate (○) and $Al(OH)_3^0$ (●) at 50 μM citrate (solid lines) and $Al(OH)_n$ (▲), Al-citrate (▽) and $Al(OH)_3^0$ (▼) at 1000 μM citrate (dashed lines) are presented. The total Al concentration was set at 50 μM in an ionic background of 100 mM KCl. $Al(OH)_n$ is defined as the sum of $Al^{3+}$, $Al(OH)^{2+}$, $Al(OH)_2^{1+}$ and $Al(OH)_4^{1-}$. With increasing citrate concentration there is a gradual shift in the maximum amount of Al complexable by citrate (denoted by arrow).

### 2.5. Chemical analysis

Metals were analysed by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS; Elan Instruments; detection limits ≈0.05 μM for Al, Gd and La). P content of microsomes was determined after digestion in 50% $HClO_3/HNO_3$ (v/v) at 140°C for 6 h. Theoretical estimates of metal–ligand complex formation in solution was made using GEOCHEM-PC v2.0 [24] using constants detailed in [25].

## 3. Results

### 3.1. Effect of Al on metal-dependent enzyme activity

Aluminium exhibits a complex speciation chemistry which is highly dependent on pH and the presence of complexing ligands and is known to precipitate at pH values greater than 4.5 [24,25]. It has been speculated therefore that organic acids (lactate, citrate and aconitate) are the primary carriers of Al within the cytoplasm [5,12] as these suppress $Al(OH)_3^0$ precipitation and due to their low molecular weight nature, permit relatively free movement within the cell. Chemical equilibria predictions made using GEOCHEM-PC indicate that at solution pH values greater than 6.0, and in the absence of complexing ligands, that less than 0.03% of Al in solution is present as the $Al^{3+}$ ion with the rest in the $Al(OH)_2^{1+}$,

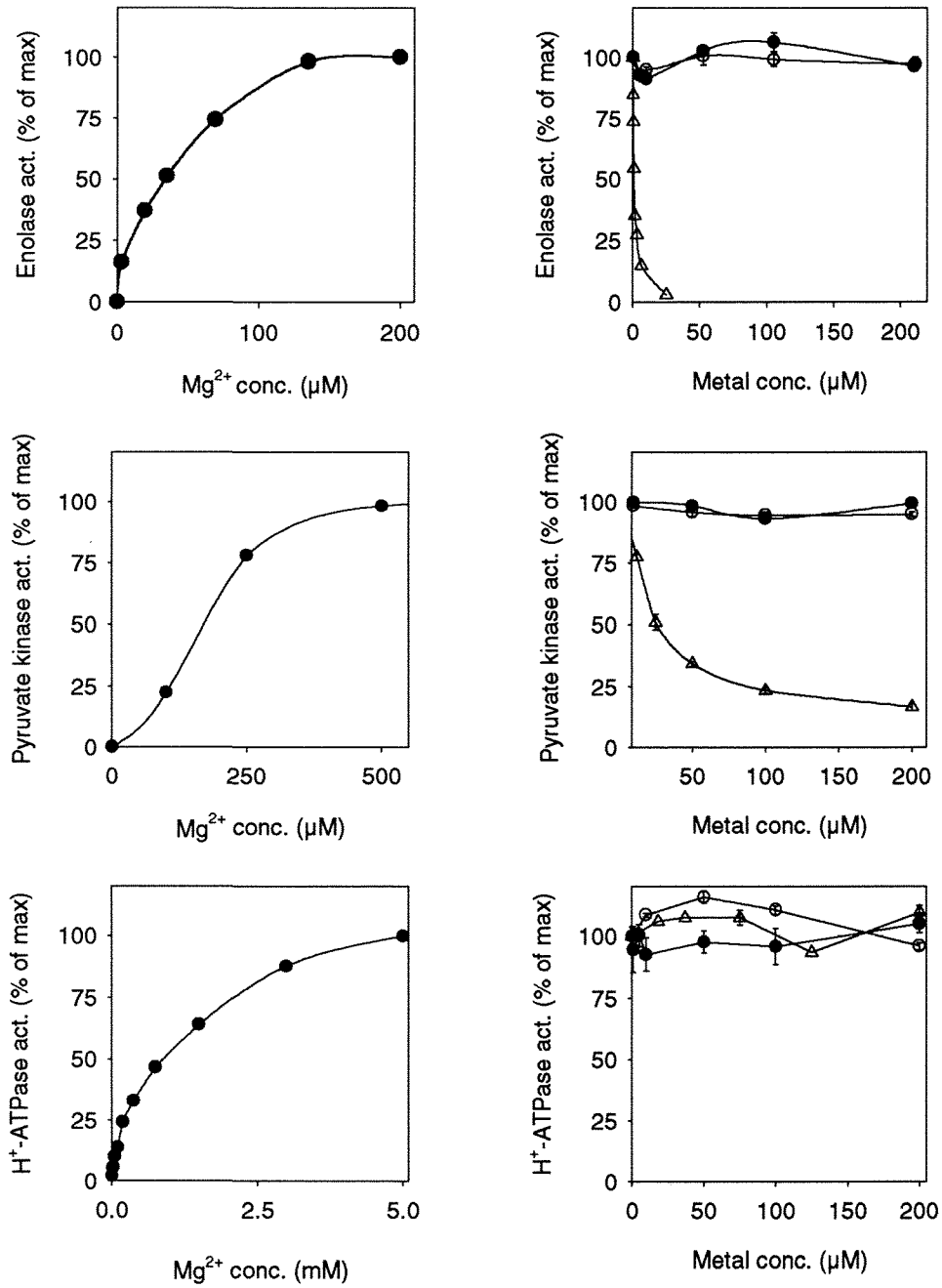


Fig. 2. Effect of Al and $Zn^{2+}$ on the $Mg^{2+}$-dependent enzymes enolase, pyruvate kinase and $H^+$-ATPase. The left hand graphs show the $Mg^{2+}$ dependency of the enzyme reaction (●), whilst the right hand graphs show the effects of $ZnSO_4$ (Δ), $AlCl_3$ (●) and Al-citrate (○) on enzyme activity at a fixed $Mg^{2+}$ concentration. For details of the assay conditions see Section 2. The maximum activity of the enzymes was: enolase, 146 μmol PEP $U^{-1}$ $h^{-1}$; pyruvate kinase, 1 μmol pyruvate $U^{-1}$ $h^{-1}$; $H^+$-ATPase, 5.7 μmol P $mg^{-1}$ prot. $h^{-1}$. Values are means ± SE ($n = 3$).

$Al(OH)_3^0$ and $Al(OH)_4^{1-}$ forms (data not presented). Equilibria predictions with Al at a physiologically relevant concentration for plants (root epidermal cells) and animals (blood serum) of 50 μM [5,26], and citrate concentrations of between 50 and 1000 μM, indicate that citrate is a competent Al complexer at pH values around 7.0, with the degree of Al-citrate complexes formed determined by the excess of free citrate (Fig. 1). If citrate is not present then particulate $Al(OH)_3^0{}_{(s)}$ is the predominant form of Al present at cytosolic pH (Fig. 1). As citrate is known to be present in the cytoplasm and serum at high concentrations (0.1–5 mM; [5,27], citrate was used as a model Al carrier in the enzymatic assays.

The effects of Al on enzymes with catalytic $Mg^{2+}$ metal binding domains (enolase, pyruvate kinase) or which require $Mg^{2+}$-ATP as a substrate ($H^+$-ATPase) are shown in Fig. 2. As expected, enzyme activity showed saturable metal activation kinetics; however, none of the enzymes tested were inhibited by $AlCl_3$ or Al-citrate. In contrast, $Zn^{2+}$, which can occupy the $Mg^{2+}$ binding domain [8], was capable of inhibiting both enolase and pyruvate kinase whilst having no effect on $H^+$-ATPase activity.

The effect of Al on $Ca^{2+}$-dependent enzymes is shown in Fig. 3. Whilst $Zn^{2+}$ was capable of inhibiting the $Ca^{2+}$-ATPase, myosin, again $AlCl_3$ and Al-citrate had no effect at con-

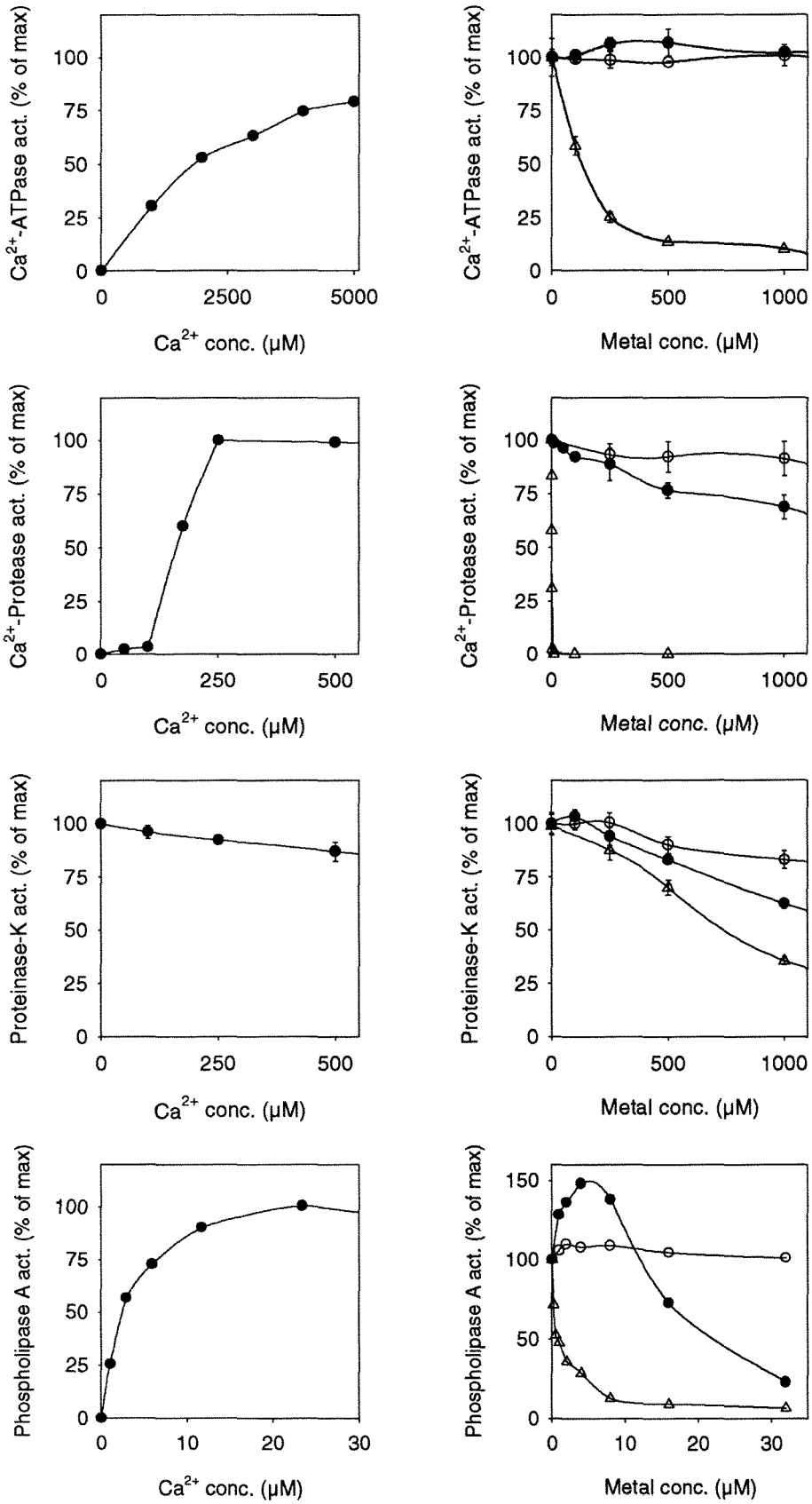
Ca2+-ATPase act. (% of max)
Ca2+ conc. (μM)
Metal conc. (μM)
Ca2+-Protease act. (% of max)
Proteinase-K act. (% of max)
Phospholipase A act. (% of max)

Fig. 3. Effect of Al and $Zn^{2+}$ on the $Ca^{2+}$-dependent enzymes myosin ($Ca^{2+}$-ATPase), $Ca^{2+}$-activated neural protease (Calpain) and phospholipase $A_2$, and on the non-$Ca^{2+}$ activated enzyme, proteinase K. The left-hand graph shows the $Ca^{2+}$ dependency of the enzyme reaction (●), whilst the right-hand graph shows the effects of $ZnSO_4$ (Δ), $AlCl_3$ (●) and Al-citrate (○) on enzyme activity at a fixed $Ca^{2+}$ concentration. For details of the assay conditions see Section 2. The maximum activity of the enzymes was: $Ca^{2+}$-ATPase, 110 μmol $P_i$ $U^{-1}$ $h^{-1}$; Calpain, Δ3.0 $Abs_{280}$ $U^{-1}$ $h^{-1}$; phospholipase $A_2$, 3.6 μmol NBD-PC $mg^{-1}$ $h^{-1}$; proteinase K, Δ1.4 $Abs_{280}$ $U^{-1}$ $h^{-1}$. Values are means ± SE ($n=3$).
←

centrations up to 1 mM. The $Ca^{2+}$ dependent neural protease, Calpain, was inhibited at very low $Zn^{2+}$ concentrations (< 10 μM) whilst significant inhibition by $AlCl_3$ and Al-citrate only occurred at higher metal concentrations (≥1 mM). To assess whether this was an effect on the $Ca^{2+}$ binding domain, control experiments were carried out on the non-$Ca^{2+}$ dependent protease, proteinase K. Both $AlCl_3$ and Al-citrate had a similar effect to that seen for Calpain indicating that the inhibition was unrelated to $Ca^{2+}$ binding and was probably related to Al binding to the protease substrate, casein. Phospholipase $A_2$ was activated at low $Ca^{2+}$ concentrations (1–5 μM) and was inhibited by low ($Ki_{50}$ 1 μM) levels of $Zn^{2+}$. The effects of $AlCl_3$ on $PLA_2$ were stimulatory at low Al concentrations (1–10 μM) whilst inhibitory at higher $AlCl_3$ concentrations ($Ki_{50}$ 22 μM). These effects, however, were negated in the presence of citrate. As Al readily binds to lipids (see below) it is possible that Al binds to the $PLA_2$ substrate NBD-PC blocking $PLA_2$ access, rather than an occupation of the $Ca^{2+}$ binding domain. The effect of Al on the $Mn^{2+}$-dependent enzyme arginase is shown in Fig. 4 and again no inhibition by Al was observed.

### 3.2. Al binding to microsomal and liposomal membranes

Al toxicity in plant root cells becomes manifest when the external solution pH falls below 4.50. As predicted by GEOCHEM-PC, under the pH conditions employed here (pH 4.45) most Al is in the free $Al^{3+}$ form (76.0%), with smaller amounts present as Al–OH complexes ($Al(OH)^{2+}$, 19.6%; $Al(OH)_2^{1+}$, 4.4%; $Al(OH)_3^0$, < 0.1%) [24]. Kinetic binding studies carried out over a 60 min period with Al and microsomal membranes indicated that the Al binding reaction was extremely rapid with 81.5 ± 0.5% of the solution Al becoming bound to the microsomes within the first 60 s compared to that bound after 60 min (Fig. 5A). The Al binding curves for microsomal membranes and either single or mixed liposomes are shown in Fig. 5B. As expected, increasing the concentration of lipid increased the amount of Al bound until all the solution Al had been depleted (total Al in solution 25 nmol), with the affinity for Al being lipid specific. PC had the lowest affinity for Al, probably because it has a neutral head group, whilst lipids with a net negatively charged headgroup (PI, PG, PA, $PIP_2$) had greater affinities for Al. The affinity of Al for the mixed lipid liposomes isolated from soybean leaves and dog brain tissue was moderate in magnitude reflecting the large proportion of PC within these samples (personal communication, Sigma Chemical Co.). The stoichiometry of the Al lipid binding reaction was estimated from the linear portion of the plots in of Fig. 5B. The results indicate a 1:1 binding between the signal transduction element $PIP_2$ and Al, 2:1 and 3:1 for PI and PA respectively and 6:1 to 8:1 for the mixed liposomes (SB, DB) (Table 1). With respect to the microsomal membranes, it can be estimated from the binding studies that the Al binding capacity is 1.19 nmol Al μg prot.$^{-1}$. If it assumed that the majority of microsomal P is associated with the phospholipid component then the binding of Al to microsomal membranes can be estimated at 0.66 nmol Al mg membrane$^{-1}$.

Recently it has emerged that Al-resistant cultivars of wheat and maize release Al-chelating organic acids specifically from the root apical cells upon exposure to toxic levels of Al. This has been shown to be an Al detoxification mechanism which is Al specific, Al inducible and is under the control of a single gene locus [28]. From mathematical models of organic acid diffusion around root cells, the concentration of organic acids at the cell surface has been estimated at around 80 μM [29]. The effect of increasing malate and citrate concentrations (0–100 μM) on Al binding to liposomes and microsomal membranes is shown in Fig. 6. Citrate was highly effective in preventing Al sorption to liposomes and could effectively remove approximately 80% of the bound Al from mixed microsomes and liposomes at a citrate concentration of 80 μM, while malate was only capable of removing ≈40% of the Al bound to the membrane.

High levels of external $Ca^{2+}$ have also been shown to relieve Al cytotoxicity [30]. Al binding experiments performed on both microsomes and liposomes in the presence of increasing amounts of $Ca^{2+}$ (0.1–10 mM) indicated that only high concentrations of $Ca^{2+}$ (> 1 mM) were capable of displacing Al from lipid binding sites (Fig. 7) and that the binding strength of Al is ≈300 greater than that for $Ca^{2+}$.

Phospholipid charge is largely associated with the polar head group whose charge is variable and controlled by solution pH and the type of head group involved. Binding experiments performed over an extended pH range (pH 4.0–7.0)

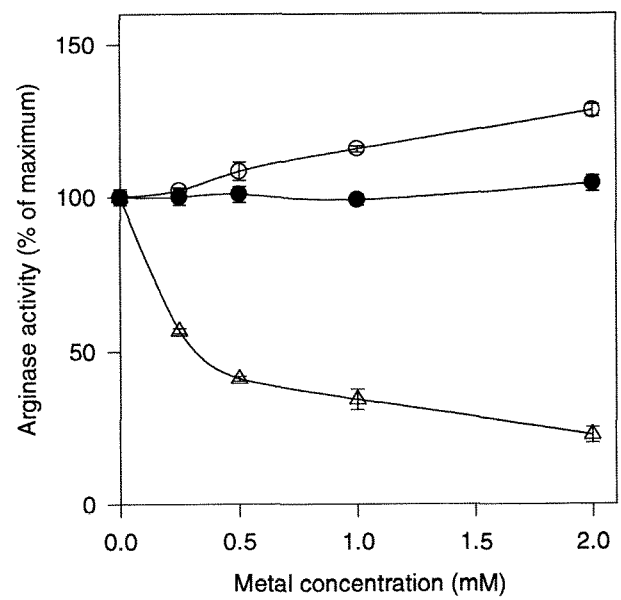


Fig. 4. Effect of $ZnSO_4$ (Δ), $AlCl_3$ (●) and Al-citrate (○) on the $Mn^{2+}$-dependent enzyme arginase. For details of the assay conditions see Section 2. Maximum arginase activity was 0.2 μmol urea $U^{-1}$ $h^{-1}$ under these assay conditions. Values are means ± SE ($n$=3).

with the trivalent cations $La^{3+}$ and $Gd^{3+}$ indicated that Al binding to the lipids is probably little affected by solution pH.

## 4. Discussion

It is likely that $Al^{3+}$ is present at sub-nanomolar levels in solution with pH values of the cytoplasm (pH 7.0–7.5) and that given the typical levels of organic acids in cells, most Al in the cytoplasm will probably be present as Al–organic acid complexes [5]. Despite many reviews stating that Al may occupy enzymatic metal binding domains [1,3,5,31–33], there is little or no evidence to support this. Of the results presented to date on the inhibition of enzyme activity by Al, inhibition via binding of Al to the substrate is rarely if ever considered. As many enzyme substrates contain exposed phosphate (e.g. lipid substrates) or carboxylic groups (e.g. protease substrates) for which Al has an extremely high affinity [33], it is clearly possible that binding of Al to these groups may block access, preventing catalysis. Further, most studies to date have failed to provide controls in the form of non-metal activated enzymes or in the presence of alternative chelators (e.g. citrate). These results, alongside those presented for calmodulin-activated phosphodiesterase and phospholipase C indicate that Al probably does not occupy $Ca^{2+}$ binding sites [10,11,13]. This is supported by the fact that very little $Al^{3+}$ is present in solution and that the ionic size of hexadentate $Al^{3+}$ (54 pm) is much smaller than that for the octadentate $Ca^{2+}$ (110 pm)

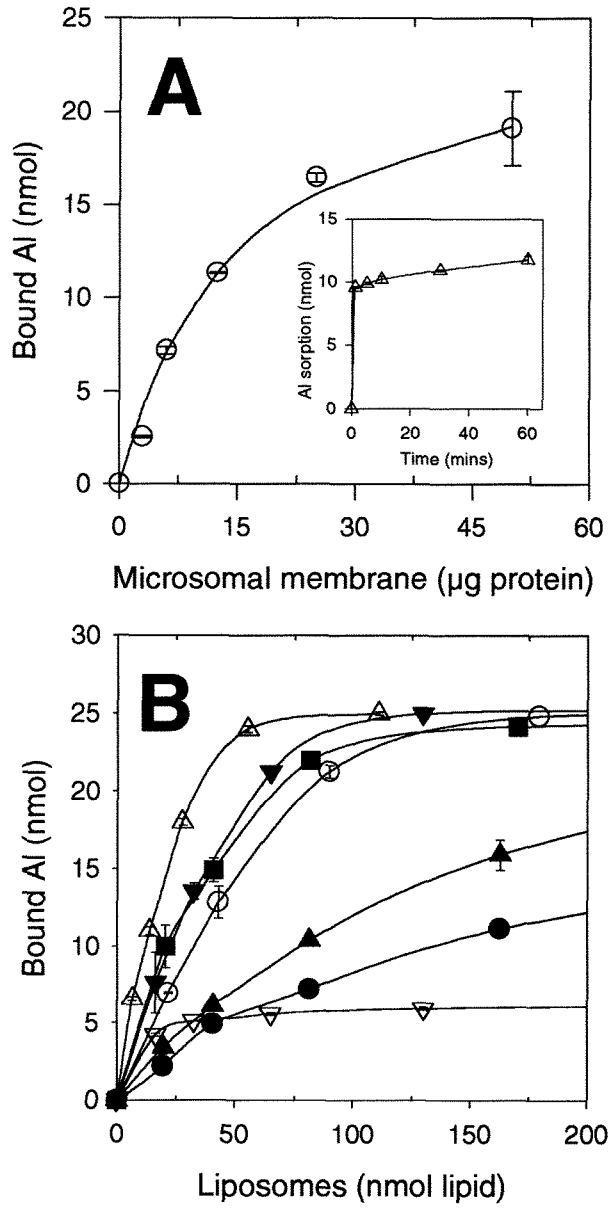


Fig. 5. Binding of aluminum (25 μM, pH 4.45) to wheat root microsomal membranes and liposomes. (A) Binding of Al to increasing concentrations of microsomes after 20 min. (Inset A) Time course showing the rapid binding of Al to microsomal membranes. (B) The binding of Al to increasing concentrations of either mixed or single lipid composition liposomes after 20 min (SB, ●; PA, ○; DB, ▲; $PIP_2$, Δ; PG, ▼; PC, ∇; PI, ■). The assay medium in all experiments was 2 mM $CaCl_2$, 25 μM Al (25 nmol total) and 1 mM HomoPipes buffer (pH 4.45). Values represent means ± SE ($n = 2$).

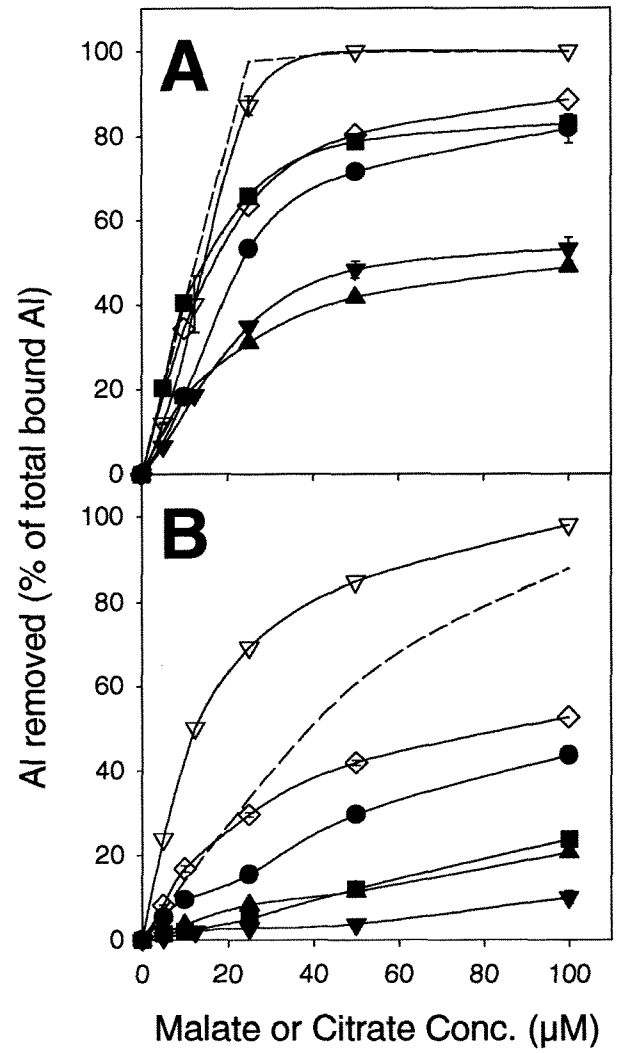


Fig. 6. Effect of increasing concentrations (0–100 μM) of citrate (A) and malate (B) on the removal of Al from microsomal and liposomal membranes (microsomal (MS), ◇; SB, ●; $PIP_2$, ▲; PG, ▼; PC, ∇; PI, ■). Dotted lines reflect theoretical predictions of Al–citrate and Al–malate complex formation made using the chemical equilibria speciation program GEOCHEM-PC [24], assuming that Al bound to the membrane is freely available. Differences between theoretical and experimental curves reflect the amount still retained by the membrane. Values represent means ± SE ($n = 2$).

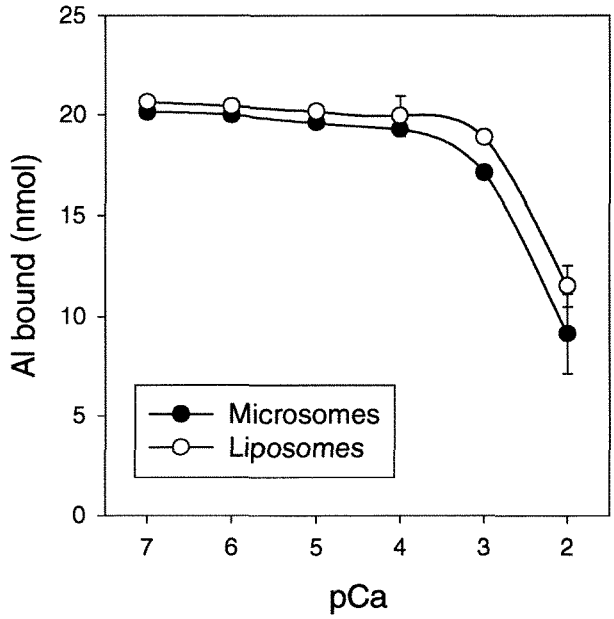


Fig. 7. Effect of increasing $Ca^{2+}$ concentration on Al binding to wheat microsomal membranes (●) and soybean liposomes (○). Values represent means ± SE ($n = 2$).

ion. In addition, the evidence presented here for Al effects on $Mg^{2+}$- and $Mn^{2+}$-activated enzymes, suggests that Al displacement of $Mg^{2+}$ and $Mn^{2+}$ from enzymes is not a mechanism of toxicity even though the $Al^{3+}$ (54 pm) and $Mg^{2+}$ (57 pm) ions are of similar size. Further, the concentration of $Mg^{2+}$ in the cytoplasm is around 1 mM [34] indicating that Al at typical cellular concentrations (10–50 μM) will probably have a minimal effect. Until direct evidence for occupation of metal binding domains by Al can be definitively proved using techniques such as electron paramagnetic resonance, we should assume that the cytotoxic action of Al is not through the occupation of metal binding domains.

The Al lipid binding experiments indicate there is a strong interaction between Al and the lipid components of the plasma membrane, with Al interacting most strongly with the phosphoinositide signal transduction element, $PtdInsP_2$, even in the presence of high concentrations of $Ca^{2+}$ (2 mM). Previous research with the phosphorescent analogue of $Al^{3+}$, $Tb^{3+}$, has also indicated a strong interaction between trivalent cations and the protein component of the plasma membrane [33]. However, the evidence presented above shows that exposure of both soluble and membrane-bound enzymes to $AlCl_3$ and Al-citrate (0–100 μM), causes no changes in enzymatic activity. These findings, in addition to reports that Al affects vesicle fusion and alters membrane permeability [35,36], indicates that the plasma membrane and not enzymatic binding domains is the most likely site of Al toxicity in plants.

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