Aluminium perturbs oscillatory phosphoinositide-mediated calcium signalling in hormone-stimulated hepatocytes

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Aluminium is known to be toxic to cells from bone, brain and bone marrow but the molecular target(s) affected by Al^{3+} are not known. We show here that Al^{3+} disrupts the oscillatory free Ca^{2+} responses of hepatocytes exposed to the Ca^{2+} -mobilizing agonist phenylephrine. Al^{3+} initially increases the frequency of the oscillations and later induces broad Ca^{2+} spikes lasting several minutes. These broad spikes persist after removal of both agonist and Al^{3+} from the medium. In the absence of agonist, Al^{3+} has no effect on free Ca^{2+} . The data suggest that some component(s) of the receptor-phosphoinositide- Ca^{2+} signalling pathway might be the site at which Al^{3+} exerts toxic effects.

INTRODUCTION

Aluminium is the causative agent in the anaemia, osteodystrophy and encephalopathy associated with long-term dialysis using aluminium-rich diffusate [1–3]. Aluminium has been implicated in the aetiology of Alzheimer's disease, being found, associated with silicon, in the senile plaques characteristic of this disorder [4–6]; its environmental availability has been implicated in the epidemiology of Alzheimer's disease [7]. The release of aluminium into the environment under acidic conditions is implicated in the toxic effect of acid rain on fish and plants [8,9]. Moreover, aluminium has been found to affect a variety of biological systems both *in vivo* and *in vitro* [10–13]. The exact molecular mechanisms involved in the varied toxicology of aluminium are not known but are generally attributed to the ability of Al^{3+} to displace Mg^{2+} at binding sites where Mg^{2+} is an essential catalytic cofactor. The much stronger binding of Al^{3+} and the 10^5 times slower ligand exchange rate are then responsible for the inhibition of activity. Citrate is a strong complexing agent for Al^{3+} which is able to prevent the binding of Al^{3+} by ATP [11] and by the phosphate groups of phospholipid vesicles [14]. However, it has been proposed by one of us [15] that Al^{3+} will be especially strongly bound by vicinal phosphate groups on inositol, for example in inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], perhaps altering the kinetics of its metabolism and, possibly, the binding of this molecule to receptor sites. The computed energy of binding of Al^{3+} to $\text{Ins}(1,4,5)\text{P}_3$ is 3-fold greater than that of Al^{3+} to ATP, representing at least a 10^6 -fold higher affinity of Al^{3+} for $\text{Ins}(1,4,5)\text{P}_3$ than for ATP, and suggesting that Al^{3+} will bind to $\text{Ins}(1,4,5)\text{P}_3$ in the presence of a large excess of Mg^{2+} and citrate. Since micromolar or less $\text{Ins}(1,4,5)\text{P}_3$ mobilizes intracellular Ca^{2+} , the effects of Al^{3+} binding could be pronounced. Here we show that repetitive free Ca^{2+} transients which are induced in liver cells by Ca^{2+} -mobilizing hormones acting through the phosphoinositide signalling system [16] are disrupted in a reproducible fashion by micromolar concentrations of aluminium citrate in the superfusing medium.

MATERIALS AND METHODS

As a model system to investigate Al^{3+} effects on Ca^{2+} mobilization by phosphoinositide-mediated hormones, we have used the isolated rat hepatocyte, which generates repetitive free Ca^{2+} transients in response to agonists such as phenylephrine and vasopressin [16]. Hepatocytes were isolated by collagenase perfusion and microinjected with aequorin as described before [16,17]. The experimental medium was Williams Medium E gassed with 5% CO_2 in air at 37 °C. AlCl_3 (10 mM) was premixed for at least 2 h with sodium citrate (50 mM), and portions were added to the medium shortly before superfusion over the cell. Citrate releases Al^{3+} only very slowly, avoiding problems of Al^{3+} precipitation by phosphate [18]. The concentration of citrate in the medium (approx. 50 μM) was comparable with normal plasma concentrations. Control experiments using 50 μM -sodium citrate had no effect on free Ca^{2+} responses to phenylephrine. Desferrioxamine (supplied by Ciba Laboratories, Horsham, Sussex, U.K.), was stored at 5 °C. Unfortunately, EGTA binds Al^{3+} avidly [13], making calibration of aequorin *in vitro* at known free Ca^{2+} levels in the presence of known free Al^{3+} concentrations extremely difficult to achieve. However, Al^{3+} is prevented from binding to calmodulin when excess citrate is present [19], so aequorin is unlikely to be affected directly by Al^{3+} in these experiments. All other aspects of the technique were as previously described [16,17].

RESULTS

Fig. 1 shows that repetitive free Ca^{2+} transients induced by phenylephrine were modified by superfusion of medium containing 10 μM - Al^{3+} /50 μM -citrate. The earliest detectable effect was an increase in the frequency of the transients, starting within 5 min after the addition of aluminium citrate. This was a consistent effect (also visible in Fig. 3). The decrease in peak free Ca^{2+} (Fig. 1a) was not consistent (Fig. 1b), being seen in only three out of 11 cells. After 5–10 min in aluminium citrate, the

Abbreviation used: G-protein, guanine-nucleotide-binding protein.

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Fig. 1. Effects of aluminium citrate on phenylephrine-induced Ca^{2+} transients

Aequorin signals from single isolated liver cells calibrated as free Ca^{2+} (nM) from (a) a cell exposed to phenylephrine (Phe; $0.8 \mu\text{M}$) plus aluminium citrate ($10 \mu\text{M-AlCl}_3/50 \mu\text{M-citrate}$), and (b) a cell exposed to phenylephrine ($0.6 \mu\text{M}$) plus aluminium citrate. Time-constants for the plot were 10 s at resting free Ca^{2+} levels and 1 s during transient production.

basal level of free Ca^{2+} (between transients) started to rise and the spikes became broader and often irregular. Removal of aluminium citrate from the medium usually did not prevent these irregular, often prolonged, spikes of free Ca^{2+} from being generated. Furthermore, removal of the agonist usually failed to stop these irregular spikes. Fig. 2(a) shows that the effects of Al^{3+} depended upon the presence of an agonist. However, when agonist was added to a cell after a period of 0.5 h in aluminium citrate, no response to phenylephrine was found (Fig. 2b). In the experimental protocol depicted in Fig. 1, eleven out of sixteen cells showed responses to aluminium citrate; in the remaining five out of sixteen cells no effects were recorded at Al^{3+} concentrations up to $100 \mu\text{M}$ over periods approx. 30 min (results not shown). In the protocol depicted in Fig. 2(a), all cells (ten out of ten) failed to undergo any free Ca^{2+} changes during about 30 min of exposure to aluminium citrate in the absence of agonist. When agonist was added after the exposure to aluminium citrate alone for around 30 min (e.g. Fig. 2b), five out of eight cells show no phenylephrine-induced spiking; the remaining three cells spiked normally. In studies in which aluminium was added in a large excess of fluoride in order to generate the fluoroaluminate anion, an activator of guanine-nucleotide-binding proteins (G-proteins), a similar proportion of hepatocytes failed to show a response [20]. Control experiments in which sodium citrate alone ($50 \mu\text{M}$) was added to the medium showed no effects whether or not phenylephrine was present (five cells; results not shown). Since removal of extracellular Al^{3+} failed to reverse its effects (Fig. 1b), we attempted to chelate intracellular Al^{3+} using desferrioxamine, which has a high affinity for Al^{3+} (log stability constant = 22 [21]) and which may be cell-permeant [22]. Fig. 3 shows three experiments, representative of six in total, none of which convincingly showed desferrioxamine to be capable of restoring free Ca^{2+} to the stable resting free Ca^{2+} level typical of an unperturbed unstimulated cell. Large irregular free Ca^{2+} spikes still occurred after 30 min of exposure to desferrioxamine. Of these six cells, that depicted in Fig. 3(b) showed the most pronounced recovery. Early addition of desferrioxamine, at the point where Al^{3+} had accelerated transient frequency but had not yet induced the large irregular spikes (Fig. 3c) failed to prevent

abnormal Ca^{2+} spikes. These data eliminate the possibility that the residual effects of Al^{3+} are extracellular, but we cannot definitively say that desferrioxamine will have chelated residual intracellular Al^{3+} .

DISCUSSION

These data indicate that Al^{3+} interferes with the generation of repetitive free Ca^{2+} transients that are induced by hormone receptors linked to the phosphatidylinositol signalling pathway [16,17]. Fig. 2 clearly shows that in the absence of a Ca^{2+} -mobilizing agonist, free Ca^{2+} remains at resting levels. When an agonist is present, addition of aluminium citrate leads initially to an increase in the frequency of the repetitive free Ca^{2+} transients (Figs. 1 and 3). The maximum frequency observed is approx. 2 min^{-1} , corresponding to a period of around 30 s. This is similar to the maximum frequency observed (in the absence of Al^{3+}) in cells exposed to higher concentrations of phenylephrine ($10 \mu\text{M}$ and above [16,17]). Thus at this point the effect of Al^{3+} appears to potentiate rather than block the effects of agonists on free Ca^{2+} spiking. We have proposed that the intertransient interval represents a period in which a GTP-liganded G-protein is accumulating, under the influence of agonist-occupied receptor, towards a threshold level of GTP-G-protein sufficient to trigger a free Ca^{2+} transient [23–25]. In view of evidence that Al^{3+} can inhibit the hydrolysis of GTP by G-proteins such as transducin [13], one possibility is that the increased frequency of free Ca^{2+} transients is caused by a decrease of GTP hydrolysis by G-proteins, leading to a more rapid attainment of the threshold GTP-G-protein level. In the absence of agonist, Al^{3+} has also been shown to inhibit the ability of receptors to activate G-proteins [13]; this may be the explanation for the failure of phenylephrine to induce normal free Ca^{2+} transients in cells pretreated with Al^{3+} (Fig. 2b).

Another mechanism for the generation of abnormal free Ca^{2+} responses is suggested by compelling theoretical evidence predicting the avid binding of Al^{3+} to the vicinal phosphates of $\text{Ins}(1,4,5)\text{P}_3$ and, presumably, of $\text{PtdIns}(4,5)\text{P}_2$ [15,26]. A prolongation of the half-life of $\text{Ins}(1,4,5)\text{P}_3$, potentiation of its ability

Fig. 2. Dependence of Al³⁺ effects on presence of agonist

(a) Removal of phenylephrine (Phe) shortly before addition of aluminium citrate (5 μM/25 μM) shows that Al³⁺ alone has no effect on free Ca²⁺ over at least 30 min. Control experiments (results not shown) indicated that sodium citrate (50 μM) alone had no effect on free Ca²⁺. (b) Pretreatment with aluminium citrate (10 μM/50 μM) before addition of phenylephrine abolished spiking activity.

to release Ca²⁺ from stores, or an enhanced hydrolysis of Al³⁺-liganded PtdIns(4,5)P₂ by phosphoinositidase C could all conceivably enhance Ca²⁺ mobilization. The inability of desferrioxamine to reverse the effects of aluminium citrate added in the presence of agonist, even over periods of approx. 30 min (Fig. 3) may indicate that Al³⁺ is binding to intracellular targets with very slow off-rates, the vicinal phosphates of Ins(1,4,5)P₃ or PtdIns(4,5)P₂ being prime candidates [15,26]. However, we do not know how fast desferrioxamine penetrates hepatocytes.

The total Al³⁺ concentration used in these experiments (5 or 10 μM) is approx. 10–20-fold higher than normal human plasma levels [27], although within the range found in the plasma of patients in severe dialysis syndrome (5–8 μM [28]). In most pathological conditions Al³⁺ appears to have chronic effects rather than acute toxicity. The abnormal free Ca²⁺ responses reported here represent a subtle but persistent disruption of a signalling pathway involving free Ca²⁺ transients, rather than an elevation of free Ca²⁺ to overtly toxic levels. We suggest, in view of its wide distribution in cells from virtually all tissues, that the phosphoinositide–Ca²⁺ signalling pathway might encompass the

Fig. 3. Effects of Al³⁺ chelation on Ca²⁺ transients

Persistent irregular free Ca²⁺ rises are not abolished by superfusion of desferrioxamine, which avidly chelates Al³⁺ (log *K*_s = 22 [21]). Desferrioxamine (500 μM) had no effect on transients induced by phenylephrine (Phe) (results not shown).

common target for Al³⁺, capable of explaining this element's varied toxicology.

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