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The perturbation, by aluminium, of receptor-generated calcium transients in hepatocytes is not due to effects of Ins(1,4,5)P_3-stimulated Ca^{2+} release or Ins(1,4,5)P_3 metabolism by the 5-phosphatase and 3-kinase

The pursuit of the mechanism underlying toxic effects of Al^{3+} has led to the demonstration that Al^{3+} may compete for Mg^{2+}-binding sites on certain enzymes even when the free Mg^{2+} concentration is as much as 10^8-fold in excess (Miller *et al.*, 1989). Moreover, a recent publication in this journal has highlighted the possibility of an interaction between aluminium and the phosphoinositide-mediated calcium signalling pathway (Schöfl *et al.*, 1990); in hepatocytes, hormone-stimulated repetitive Ca^{2+} transients (Woods *et al.*, 1986), were perturbed by superfusion of the cells with as little as 5–10 μM-Al^{3+} (as Al^{3+} citrate). Amongst possible explanations for these effects are the proposals that Ins(1,4,5)P_3 might bind Al^{3+} particularly avidly, thereby affecting Ins(1,4,5)P_3 metabolism or Ca^{2+} mobilization (Birchall & Chappell, 1988; Schöfl *et al.*, 1990). Therefore we decided to look at possible effects of aluminium on (i) calcium movements in isolated rat liver microsomes, and (ii) Ins(1,4,5)P_3 metabolism by the 5-phosphatase and 3-kinase. It should be emphasized that in view of the uncertainties surrounding the kinetics of Al^{3+} exchange between various chelators (Miller *et al.*, 1989), the media used in our experiments were pre-equilibrated for several hours in plastic containers.

The preparation of rat liver microsomes and the measurements of Ca^{2+} uptake and release using a Ca^{2+}-sensitive electrode were as described by Dawson *et al.* (1987). Aluminium was solubilized by mixing $AlCl_3$ (10 mM) and sodium citrate (50 mM) for at least 2 h prior to use. Rat liver microsomes were preincubated for 2 h at 0–4 °C with 300 μM-Al^{3+}/1.5 mM-citrate (to give 10 μM-Al^{3+} in the final assay medium) and, separately, 0.5 mM-Ins(1,4,5)P_3 with 5 mM-Al^{3+}/25 mM-citrate for up to 5 h. Al^{3+}-pretreated microsomes behaved exactly like control microsomes (i.e. plus citrate alone) in terms of Ca^{2+} uptake, GTP-enhancement of Ins(1,4,5)P_3 sensitivity and Ins(1,4,5)P_3-stimulated Ca^{2+} release. Similarly, Al^{3+}-pretreated Ins(1,4,5,)P_3 behaved just like control Ins(1,4,5)P_3.

For the studies of Ins(1,4,5)P_3 metabolism, 1 mM-$AlCl_3$ was stirred with 25 mM-sodium citrate for 3–4 h. Ins(1,4,5)P_3 5-phosphatase was assayed in 0.5 ml aliquots of buffer containing 2 μM-[^{3}H]Ins(1,4,5)P_3 (1 nCi/ml), 100 mM-KCl, 1 mM-$MgSO_4$, 10 mM-Hepes (pH 7.2 with KOH) and saponin (0.2 mg/ml). Ins(1,4,5)P_3 3-kinase was assayed in similar buffer, except for the addition of 5 mM-Na_2ATP plus 5 mM-$MgSO_4$. Either 40 μM-Al^{3+} (or the equivalent citrate concentration as the control) was added to each of the buffers, which were preincubated overnight at 0–4 °C before being brought to 37 °C and mixed with 25 μl of a 1% (w/v) liver homogenate prepared in 0.25 M-sucrose/5 mM-Hepes (pH 7.2 with KOH). Ins(1,4,5)P_3 metabolism was then analysed as described by Shears (1989). There was no significant effect of 40 μM-Al^{3+} on either the 3-kinase or the 5-phosphatase. For example, in representative triplicate determinations (% substrate metabolized/min) the 5-phosphatase activity was 1.75 ± 0.04 (control) or 1.67 ± 0.1 (Al^{3+}); the 3-kinase activity was 0.13 ± 0.02 (control) or 0.14 ± 0.005 (Al^{3+}).

These results indicate that aluminium does not interact with Ins(1,4,5)P_3 by perturbing either its metabolism or its Ca^{2+}-releasing properties. It would therefore seem more profitable to pursue the idea that the profound effects of aluminium upon hepatic calcium transients are due to either (*a*) modulation of receptor-activated release of Ins(1,4,5)P_3, through an interaction with either phospholipase C or G-proteins (Miller *et al.*, 1989; Schöfl *et al.*, 1990) or (*b*) interactions with other aspects of the Ca^{2+} transport system such as Ins(1,3,4,5)P_4 (Irvine *et al.*, 1988) and Ca^{2+}-induced Ca^{2+} release (Berridge *et al.*, 1988).

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On the use of EGTA to assess the effect of Ca^{2+} on liver microsomal glucose-6-phosphatase

In a recent report, Waddell *et al.* (1990) claimed that: "..EGTA cannot be used as a buffer to study the effects of Ca^{2+}.." on liver microsomal glucose-6-phosphatase because they found that metal chelators such as EGTA and nitrilotriacetic acid (NTA) caused (1) disruption of the microsomal membrane and (2) loss of enzyme activity in disrupted microsomes. The intactness of the membrane, as assessed by the measurement of mannose-6-phosphatase before and after treatment of the vesicles with a detergent, fell in their hands from 96% to 65% when

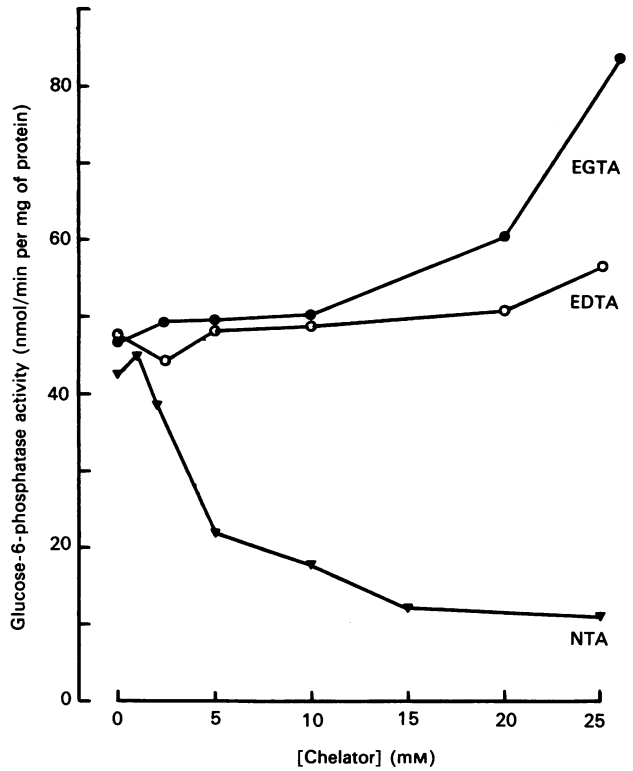

Fig. 1. Differential effects of chelators on liver microsomal glucose-6-phosphatase activity

Glucose-6-phosphatase was measured at 30 °C with 0.5 mM-[U-^{14}C]glucose 6-phosphate during 1 min in fasted rat liver microsomes after treatment with 0.4% deoxycholate as described previously (van de Werve, 1989), in the presence of varying concentrations of chelators kept at pH 7.3 with 50 mM-Tris/HCl, as indicated.

EGTA was added to the preparation at a final concentration of 25 mM.

The first of the observations of Waddell *et al.* (1990) is however in complete contradiction with our prior finding that 25 mM-EGTA did not cause disruption of the microsomal membrane as indicated by a 90% latency of mannose-6-phosphatase we found in that condition (see Fig. 3B in van de Werve, 1989). This evidence is routinely found in our laboratory by measurements (in the presence of 25 mM-EGTA) of the intactness of the microsomal membrane which was 90±8% (mean value±S.D. for 72 different liver microsomal preparations) and has also been confirmed by Mithieux *et al.* (1990).

Regarding the second observation of the Dundee group, if glucose-6-phosphatase is inhibited by NTA in deoxycholate-disrupted microsomes, a property we were aware of, it is definitely not the case with EGTA nor with EDTA. Indeed, in our experimental conditions, one can see (Fig. 1) that EGTA and EDTA were not inhibitory to glucose-6-phosphatase whereas NTA caused a dose-dependent loss of activity of the enzyme in disrupted microsomes. Not only is EGTA not inhibitory, but it actually stimulates glucose-6-phosphatase at concentrations higher than 10 mM, a property which fits with the chelation of an inhibitory ion such as Ca^{2+}. Moreover, the kinetic parameters (K_m and $V_{max.}$) of glucose-6-phosphatase, measured in the presence of 25 mM-EGTA, that we published (van de Werve, 1989) were in the normal range reported by other workers in the field.

Methodological differences have to be taken into account to tentatively explain the discrepancy between the report of Waddell *et al.* (1990) and ours (van de Werve, 1989). These include the pH and nature of the buffers used (cacodylate at pH 6.5 versus Tris at pH 7.3), the product measured (P_i versus glucose) and the incubation time (10 min versus 1 min) for the assay. The duration of the incubation might be critical in the assay of the enzyme in the presence of EGTA in disrupted microsomes. Indeed, the same group showed before (Burchell *et al.*, 1985) that a 21 kDa Ca^{2+}-binding protein stabilized glucose-6-phosphatase in disrupted structures. In the presence of 25 mM-EGTA, the protection against inactivation of the enzyme might be less effective and short incubation times would be required. We verified that in all our experimental conditions the rate of the reaction was linear and an incubation time of 1 min was chosen. Another methodological difference is the product measured in the assay, but it does not explain the discrepancy because we found similar activities of the enzyme with 25 mM-EGTA whether glucose or P_i were assayed. Finally, it cannot be excluded that at a lower pH (6.5 versus 7.3) in the incubation medium, leading to different proportions of the ionized forms of EGTA (see e.g. Kim & Padilla, 1978), the chelator might affect the glucose-6-phosphatase system.

Whatever the causes for the different findings of Waddell *et al.* (1990), it is clear that in our experimental conditions, EGTA can indeed be used to assess the effects of Ca^{2+} on glucose-6-phosphatase without disrupting the microsomes nor affecting the stability of the enzyme. By using EGTA–Ca buffers, we found a marked inhibition of glucose-6-phosphatase in the physiological (submicromolar) Ca^{2+} concentration range (Fig. 3A in van de Werve, 1989). This finding was recently confirmed by others (Mithieux *et al.*, 1990) when using a EGTA–Ca buffer of sufficient capacity (> 10 mM-EGTA); not unexpectedly, glucose-6-phosphatase inhibition by Ca^{2+} was reversed when EGTA was diluted, i.e. when the capacity of the buffer was reduced. A simple calculation of a buffer capacity (β) (Segel, 1976) indicates that when a 25 mM-EGTA–Ca buffer (set at 1 μM-free Ca^{2+}) is diluted 25-fold, the buffer capacity decreases by about 95% ($\beta = 52$ μM), a value unlikely to maintain 1 μM free Ca^{2+} against the high buffering capacity of the microsomal membranes.

We also showed that at higher unphysiological (millimolar) Ca^{2+} concentrations this inhibition was reversed (Fig. 3A in van de Werve, 1989), indicating complex effects of Ca^{2+} on the system, indeed compatible with the small stimulatory effect first reported by Yamaguchi *et al.* (1989) and subsequently confirmed by others (Waddell *et al.*, 1990; Mithieux *et al.*, 1990). As to the illogicality of glucose-6-phosphatase being inhibited in conditions of increased Ca^{2+}-induced glycogenolysis, this apparent paradox can be resolved by our hypothesis (Vidal & van de Werve, 1990) that glucose-6-phosphatase could be inhibited by intraluminal endoplasmic reticulum Ca^{2+} rather than by cytosolic Ca^{2+}. Such a property would fit with the subcellular location of this enzyme and that of the hormone-mobilizable Ca^{2+} pool.

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