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The toxic effects of organometals on the Lands cycle in HL-60 cells*

H F Krug
Kernforschungszentrum Karlsruhe, Institute of Genetics and Toxicology, PO Box 3640, D-7500 Karlsruhe 1, Federal Republic of Germany

The concentration of free fatty acids within cells is mainly dependent upon the following enzyme activities: liberation by phospholipase A_2 (PLA_2), activation of free acids by acyl-CoA-synthetase and re-esterification by lysophospholipid acyltransferase (LAT). In many cell types, especially those of the haematopeotic system, this deacylation–reacylation cycle ('Lands cycle') plays an important role in the regulation of free fatty acid concentration, above all that of arachidonic acid.

We have shown here that heavy-metal compounds affect this cycle mainly at two points and thereby lead to an increase of free fatty acids. On the one hand, organometals cause an inhibition of the reacylation of lysophospholipids; and on the other, the induction of PLA_2 activity produces the same result. All compounds investigated such as methylmercury chloride (MeHgCl), diethyl-, triethyl-, and trimethyl-lead chloride (Et_2PbCl_2, Et_3PbCl, Me_3PbCl) as well as trimethyltin chloride (Et_3SnCl) and di-*t*-butyltin dichloride (*t*-Bu_2SnCl_2) show at least one of these effects. In the case of Et_3PbCl, the use of PLA_2-inhibitors or pertussis toxin causes a drastic decrease in the amount of arachidonic acid liberated. These experiments demonstrate that the organometallic compounds inhibit the reacylation and/or stimulate the deacylation of fatty acids that are involved in many important biological or pathological mechanisms. The results suggest that in differentiated HL-60 cells the organometal compounds stimulate the Lands cycle by increasing the activity of the PLA_2, possibly via a signal-transduction mechanism, and this effect is intensified via an inhibition of re-esterification.

Keywords: Organolead, organomercury, organotin, toxicity, lipid metabolism, arachidonic acid, Lands cycle, cell culture, HL-60 cells

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INTRODUCTION

Metals are ubiquitously distributed toxicants and lead is still at the head of the world-wide emissions of all trace elements.[1] Although the organic lead antiknock motor fuel additives and other organic metal compounds have been used for a long time, most of the information about their toxic effects has only appeared during the last decade. Organo-lead and -mercury were found in human brains[2,3] and organo-lead, -mercury, and -tin affect cytoskeletal structures such as microtubules and intermediate filaments.[4–7] Moreover, organometals, such as Et_3SnCl, MeHgCl, and Et_3PbCl, are demonstrated to induce aggregation of human blood platelets.[8–10] This reaction is associated with the liberation of arachidonic acid and eicosanoid formation.[9,10]

The cascade of arachidonic acid liberation and its metabolism becomes more and more important within physiological and pathological processes. With regard to inflammatory reactions or immunological alterations, there is some evidence for the involvement of xenobiotics in this mechanism.[11–13] As heavy metals accumulate in the environment[1] and the biosphere,[2,3,14] the effects of these compounds and their ability to increase lipid mediators of inflammatory or immunological reactions are of great interest. Et_3PbCl and MeHgCl exhibit a strong effect on the distribution of fatty acids within the lipid classes. Additionally, our experiments gave a greater insight into the mechanism by which Et_3PbCl and possibly other organometals enhance the concentration of free arachidonic acid within cells.

MATERIALS AND METHODS

Chemicals

Quinacrine and *p*-bromophenacyl bromide (*p*BPB) were obtained from Serva (Heidelberg, FRG), RPMI medium, foetal calf serum and

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other medium additives from Gibco (Eggenstein, FRG). The calcium ionophore A 23187, pertussis toxin and fMet-Leu-Phe (fMLP) were from Sigma (Munich, FRG), the pertussis toxin B subunit was from List Biological Laboratories (Campbell, USA) and the SIL G Polygram thin-layer plates were from Macherey & Nagel (Düren, FRG). The [1-^{14}C]arachidonic acid (2.07 GBq mmol^{-1}) and [^{3}H]arachidonic acid (3.66 TBq mmol^{-1}) were purchased from Amersham (Braunschweig, FRG) and the organometals from Ventron Alpha Products (Karlsruhe, FRG). The compounds were used without further purification. All other chemicals were of analytical grade and solvents for HPLC were obtained from Promochem (Wesel, FRG).

Cell culture

Incubation of HL-60 cells

HL-60 cells were grown in suspension culture in RPMI 1640 medium supplemented with 15% foetal calf serum, 1.5% glutamate (200 mmol dm^{-3} in water), 1% non-essential amino acid solution, 1% sodium pyruvate (100 mmol dm^{-3} in water), and 0.5% of a mixture of streptomycin (1000 μg cm^{-3}) and penicillin (1000 IU cm^{-3}). The cells were induced to differentiate to mature granulocytes by the addition of 1.3% dimethyl sulphoxide for five days. They were harvested by centrifugation, washed once with RPMI without any additives and finally resuspended in medium containing 1% dimethyl sulphoxide and 3.3% foetal calf serum at a concentration of 1×10^{7} cells cm^{-3}. Experiments were started after a rest period of 30 min. The cell suspensions (3 cm^{3}) were then incubated at 37 °C with 10 μmol dm^{-3} calcium ionophore A 23187 or the organometals as indicated. In the case of radioactive prelabelling, [^{14}C]arachidonic acid was dissolved in dimethyl sulphoxide, added at day 4 (92.5 kBq per 50 cm^{3}) to the culture medium and the cells were incubated overnight. The labelled cells were washed twice with RPMI and resuspended as described above.

Incubation of human blood platelets

Fresh human blood from healthy donors (3.8% citrate/blood; 1:9, v/v) was centrifuged at 340 *g* for 10 min at 22 °C. The platelet-rich plasma obtained was incubated with [^{3}H]arachidonic acid for 2 h at 35 °C under constant stirring. The labelled platelets were washed twice[10] and experiments were started 45 min after final resuspension. Platelets were incubated with Et_3PbCl or MeHgCl for 15 min or 5 min, respectively, before incubations were stopped.

Viability

The viability of HL-60 cells was investigated by Trypan Blue exclusion. The dye was dissolved in 0.9% sodium chloride solution to a final concentration of 0.5%. After mixing of the cell suspension with the dye solution, the number of blue cells were estimated using a Neubauer chamber. The alternative method of lactate dehydrogenase leakage was omitted because an enzyme inhibition by the organometallic compounds within the test could not be excluded.

Lipid extraction and separation of lipid classes

After incubation of the cell suspensions, the lipids were extracted as reported earlier.[15] The extract was dried under nitrogen, taken up in chloroform, spotted on to SIL G polyester plates (20 cm × 20 cm) and separated by thin-layer chromatography. In the case of platelet lipids a single solvent system[10] and for HL-60 cells a double system was used.[15] These systems give good separation of eicosanoids or phospholipids, free fatty acids and neutral lipids, respectively. The R_f values for the lipid classes were determined by comparison of their migration with that of commercial standards. Radioactive lipids were localized by scanning, cut out, and counted for radioactivity in a liquid scintillation counter.

RESULTS AND DISCUSSION

Organometal-induced lipid metabolism

Human blood platelets can be stimulated *in vitro* with thrombin to liberate arachidonic acid and produce its metabolites, the eicosanoids. Compared with this physiological inducer, organomercury and organolead compounds induce *per se* the aggregation of human blood platelets and the arachidonic acid cascade of [^{3}H]arachidonic-acid-labelled platelets (Table 1). As reported earlier, Et_3PbCl induces this effect down to a concentration of 5 μmol dm^{-3} when incubated for 3 h.[10] However, human blood platelets have only a short lifetime after isolation from

Table 1 Stimulation of platelet aggregation and arachidonic acid metabolism by MeHgCl and Et_3PbCl

Compound	Aggregation[a] (%)	Radioactivity within arachidonic acid metabolites (cpm)		
		TXB_2[b]	12-HHT[b]	12-HETE[c]
Control[d]	0	490 ± 210	390 ± 200	310 ± 50
Thrombin[e]	60	2210 ± 680	2560 ± 820	1270 ± 1030
100 μmol dm^{-3} Et_3PbCl	90	7630 ± 1820	17 010 ± 2640	14 590 ± 3340
50 μmol dm^{-3} MeHgCl	90	9740 ± 800	13 799 ± 3380	5860 ± 710

[a] Aggregation was measured with washed platelets (thrombin) or in platelet-rich plasma (organometals) by use of an Elvi aggregometer. [b] TXB_2 (thromboxane B_2) and 12-HHT (12-hydroxy-5,8,10-heptadecatrienoic acid) are cyclo-oxygenase products. [c] 12-HETE (12-hydroxy-5,8,10,14-eicosatetraenoic acid) is a lipoxygenase product. [d] Vehicle-treated control incubations. [e] 2 U cm^{-3} of platelet suspension were used for stimulation.

Statistical significance: all values from stimulated platelets were different from the corresponding control values ($P < 0.01$; $n = 5–7$).

blood. Thus, stimulation of the arachidonic acid cascade by lower concentrations of the organometal compounds has to be carried out with long-lived cell types that can be incubated for longer periods of time.

HL-60 cells, differentiated with dimethyl sulphoxide to mature granulocytes, exhibit a mechanism comparable with that of blood platelets; whereas platelets metabolize nearly 100% of the liberated arachidonic acid into three main metabolites (Table 1), the HL-60 cells produce eicosanoids to a lesser degree. The main fraction that could be measured is the free arachidonic acid. These cells were labelled for 24 h in the presence of [^{14}C]arachidonic acid. Although nearly 80% of the label was incorporated during the first 2 h, an equilibrium in arachidonic acid distribution within the lipid classes was first reached after 24 h (results not shown). When equilibrium was reached, more than 90% of the label taken up was esterified into phospholipids, with the remainder being incorporated into triacylglycerols. Free arachidonic acid could be detected in only trace amounts (≤1%). The distribution of the radioactivity within the cellular lipids was determined after 24 h and compared with that of cells treated with the calcium ionophore A23187 or organometal. It is obvious that after short-time treatment with high concentrations of the alkyl-tin and -lead compounds liberation of arachidonic acid takes place (Table 2). These results implied that investigations in more detail would be merited for the most toxic compound, Et_3PbCl, in comparison with the effect of the calcium ionophore. Figure 1 shows the alterations of arachidonic acid content within the main lipid classes of HL-60 cells.

Whereas stimulation with A23187 induces an identical loss of label within phosphatidylcholine (PC) as well as in phosphatidylethanolamine (PE), treatment with Et_3PbCl affects PE more than PC. These two phospholipids represent the substrata for the intracellular phospholipase A_2.[16] Moreover, phosphatidylinositol (PI) is not affected (diacylglycerol or phosphatidic acid was not detectable) indicating no participation of phospholipase C (data not shown).

Heavy-metal compounds, especially organometals, impair cell viability at very low concentrations.[17–19] In this connection, the type of organic moieties as well as the different metal centres affect the cytotoxic potency of these compounds to a comparable extent.[17,18] Important effects should be binding to membrane proteins[20] and the disintegration of cell membranes.[21,22] It has been demonstrated that the stimulated liberation of a substantial portion of the polyunsaturated fatty acids from membrane phospholipids leads to structural alterations of membrane-associated components[22,23] or to cell death.[24] This is due to the decrease in necessary phospholipids and to increasing amounts of free fatty acids as well as lysophospholipids that have detergent-like activity.[25] Treatment with calcium ionophore, for instance, results in a total loss of cell viability within 15–30 min. In the case of the organometals investigated, increase in free fatty-acid concentration precedes the loss in cell viability. For both

alkyltins the viability is comparable with control incubations (Fig. 2; open symbols; 60 min) whereas the amount of free arachidonic acid increases significantly (Table 2). The alkyl-lead compounds, on the other hand, are more toxic than the tin compounds. The values for free arachidonic acid increase immediately before cell viability decreases (Fig. 2; Table 2).

After treatment of HL-60 cells with lower concentrations of Et_3PbCl, the cells showed no loss in viability up to an incubation period of 5 h for concentrations of 1 and 5 μmol dm^{-3}, just as for 24 h incubations and concentrations ≤1 μmol dm^{-3} (data not shown). These low concentrations, however, induce a shift of arachidonic acid from phospholipids to the triacylglycerol fraction (Fig. 3).

Inhibitors of phospholipase A_2

Quinacrine and *p*-bromophenacylbromide (*p*BPB) are known to inhibit the liberation of arachidonic acid from cellular phospholipids.[25] It could be demonstrated by the use of these inhibitors that the Et_3PbCl-induced effects are dependent on fatty-acid liberation from phospholipids. HL-60 cells, differentiated with dimethyl sulphoxide to mature granulocytes, were incubated for 24 h in the presence of [^{14}C]arachidonic acid. Preincubation of HL-60 cells with either quinacrine or *p*BPB almost completely prevented the appearance of arachidonic acid after stimulation with A23187 or Et_3PbCl (Fig. 4), as shown earlier for blood platelets and the inhibitor quinacrine.[10]

Inhibition of incorporation of exogenous arachidonic acid by Et_3PbCl

Incubation of HL-60 cells with exogenous [^{14}C]arachidonic acid for 60 min resulted in nearly 75% uptake of the fatty acid, one-third into the neutral lipids and two-thirds into the phospholipids (the bulk was found in PC).

As compared with vehicle-treated control cells, Et_3PbCl inhibited the incorporation of exogenous arachidonic acid into various lipid classes (Fig. 5). It is clear that the label is reduced mainly within phosphatidylcholine, phosphatidylethanolamine and the neutral lipids by 79, 68 and 94%, respectively, whereas other lipids such as PI or phosphatidylserine are unaffected during the incubation time. The incorporation of fatty acids into lysophospholipids could be prevented by the inhibition of two enzymes, the acyl-CoA-synthetase and/or the lysophospholipid acyltransferase. Moreover, organomercury compounds affect these enzymes in a way comparable with that described for ethylmercurithiosalicylate,[15,26] $MeHgCl$[27] and *p*-hydroxymercurisalicylate.[28]

Table 2 Stimulation of arachidonic acid liberation in HL-60 cells by various organometallic compounds

Compound	Concentration (μmol dm^{-3})	Time (min)	Free arachidonic acid[a] (% of incorporated label)
Control[b]	—	30	1.0 ± 0.1
A23187[c]	10	30	20.3 ± 0.9
Et_2PbCl_2	100	30	3.5 ± 0.9
Et_2PbCl_2	500	30	4.9 ± 0.7
Et_3PbCl	50	30	5.7 ± 0.5
Et_3PbCl	100	30	8.7 ± 1.4
Me_3PbCl	500	30	6.8 ± 0.7
Me_3PbCl	1000	30	9.6 ± 1.5
Me_3SnCl	500	60	2.3 ± 0.9
t-Bu_2SnCl_2	500	60	6.3 ± 1.3

[a] These values represent the sum of free arachidonic acid and the eicosanoids formed.

[b] Vehicle-treated control incubations. [c] Maximum stimulation with calcium ionophore A 23187.

Statistical significance: all values from stimulated HL-60 cells were different from the corresponding control value ($P<0.05$; $n=4$–7).

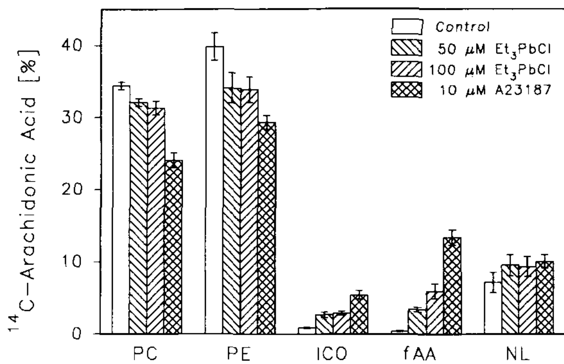

Figure 1 Liberation of arachidonic acid in HL-60 cells after stimulation with calcium ionophore A23187 or different concentrations of Et_3PbCl. Suspensions of differentiated HL-60 cells were prelabelled with [^{14}C]arachidonic acid and incubated at 37 °C for 30 min, in RPMI 1640 medium with calcium ionophore A 23187, Et_3PbCl or with vehicle only (control). After incubation with either A23187 or Et_3PbCl, cellular lipids were extracted and separated by thin-layer chromatography. Radioactive spots were located by scanning, cut out, and counted for radioactivity in a liquid scintillation counter. Values are the mean of 4–13 experiments ± SEM. PC, phosphatidylcholine; PE, phosphatidylethanolamine; ICO, eicosanoids; fAA, free arachidonic acid; NL, neutral lipids.

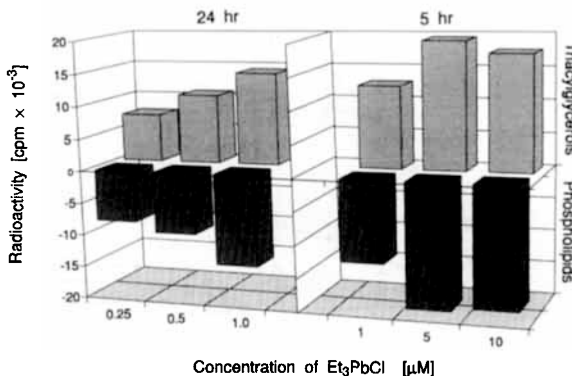

Figure 3 Alteration of arachidonic acid composition within cellular lipids of HL-60 cells induced by low concentrations of Et_3PbCl. Differentiated HL-60 cells were treated with Et_3PbCl as indicated on the abscissa, or with vehicle only. The columns represent the decrease or increase in [^{14}C]arachidonic acid content within the phospholipids or triacylglycerols, respectively, as difference from the vehicle-treated control incubations. During the period of incubation a loss in cell viability could be detected only for the highest concentration of 10 μmol dm^{-3} (10 μM). Statistical significance: alterations were different from the corresponding value of vehicle-treated control incubations ($P < 0.001$; $n = 4$–6).

Effects of pertussis toxin and its B-oligomer on Et_3PbCl stimulated liberation of arachidonic acid

Enzymes at the cytosolic side of the membrane are often coupled to receptors on the outside of the cell and are thereby regulated by external triggers. Signal transmission through the cell membrane is accomplished in many cases via GTP-binding proteins, so-called G-proteins. During the last few years, more and more evidence has shown that PLA_2 is possibly linked to

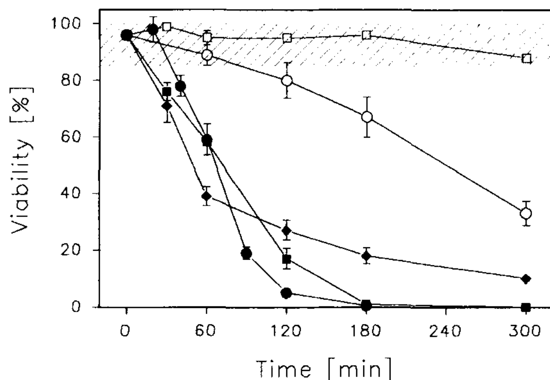

Figure 2 Viability of HL-60 cells after incubation with five different organometal compounds. Suspensions of differentiated HL-60 cells were treated with the organometals at 37 °C in RPMI 1640 medium. At the indicated times aliquots were measured by the Trypan Blue exclusion test for viability. The hatched area indicates the viability of vehicle-treated control cells. Values are the mean of 4–7 experiments ± SEM. ●, 100 μmol dm^{-3} Et_3PbCl; ■, 500 μmol dm^{-3} Et_2PbCl_2; ♦, 500 μmol dm^{-3} Me_3PbCl; ○, 500 μmol dm^{-3} t-Bu_2SnCl_2; □, 1 mmol dm^{-3} Me_3SnCl.

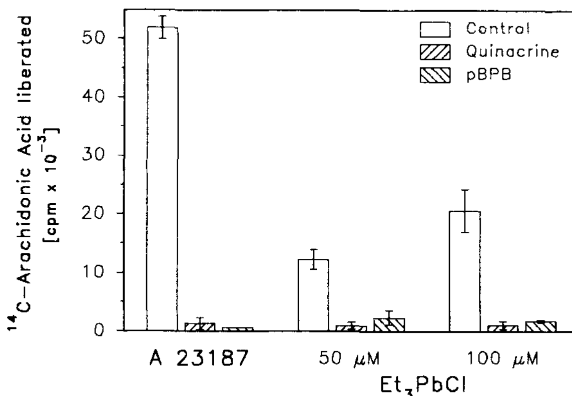

Figure 4 Effect of phospholipase inhibitors quinacrine and p-bromophenacylbromide (pBPB) on A23187- or Et_3PbCl-stimulated liberation of arachidonic acid. Prelabelled HL-60 cells were preincubated with 1 mmol dm^{-3} quinacrine (5 min) or 50 μmol dm^{-3} (30 min) before calcium ionophore A23187 or Et_3PbCl was added and the incubation was continued for 30 min. Extraction and separation of cellular lipids were as described in Fig. 1. Values are the mean of four experiments ± SEM.

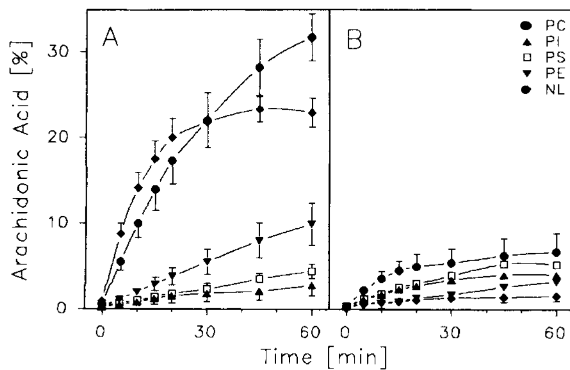

Figure 5 Inhibition of arachidonic acid incorporation into lipids of HL-60 cells after preincubation with Et_3PbCl. Suspensions of differentiated HL-60 cells were vehicle-treated (A) or preincubated with 50 μmol dm^{-3} Et_3PbCl for 30 min (B) before radioactively labelled arachidonic acid was added to the incubation mixtures. The cells were then incubated for the indicated times. Cellular lipids were extracted, and the amount of incorporated label in the various lipid classes was estimated. Values are the mean of four experiments ± SEM. ●, Phosphatidylcholine; ▲, phosphatidylinositol; □, phosphatidylserine; ▼, phosphatidylethanolamine; ⧫, neutral lipids.

cellular receptors via such a G-protein.[29] To determine whether a direct stimulation or a receptor-coupled effect takes place, we carried out a series of experiments with the G-protein inhibitor pertussis toxin. HL-60 cells prelabelled with [^{14}C]arachidonic acid were incubated for 3 h at 37 °C with 500 or 1000 ng cm^{-3} of the holotoxin or an equivalent amount of its B-oligomer, the membrane binding subunit. During this period of time no alteration of incorporation and distribution of [^{14}C]arachidonic acid within the lipid classes could be detected (data not shown). Figure 6 shows that pertussis toxin treatment prevented the ability of the cells to release arachidonic acid from phospholipids after stimulation with the chemotactic peptide f-Met-Leu-Phe (fMLP). Furthermore, Et_3PbCl stimulation is to a high degree sensitive to pertussis toxin, even at high concentrations of the lead compound (Fig. 6). Equivalent amounts of the pertussis toxin noncatalytic subunit (B-oligomer) have only little or no effect on fMLP- as well as on Et_3PbCl-induced arachidonic acid liberation.

CONCLUSIONS

The quantity of free unsaturated fatty acids within cells is very low and strictly regulated.[30] However, many cell types respond to exogenous stimuli, e.g. thrombin, collagen, A23187, or fMLP, with a rapid increase above all of free arachidonic acid. This is an important metabolic pathway and, thus, these cells are provided with an efficient regulatory mechanism in controlling free fatty acid concentration. Involved in these processes are the fatty-acid-liberating enzymes, phospholipase C and diacylglycerol lipase or phospholipase A_2, and the reacylating enzymes, acyl-CoA synthetase, lysophospholipid acyltransferase and diacylglycerol acyltransferase.[30]

In various cell types, the thiol-blocking activity of heavy metals leads to an inhibition of the reacylation of free fatty acids into phospholipids.[26-28] Similarly to these organic mercury compounds, Et_3PbCl inhibits the incorporation of exogenously added [^{14}C]arachidonic acid into cellular lipids. However, the liberation and subsequent redistribution of fatty acids is still induced at very low concentrations that were not able to inhibit the reincorporation (Fig. 3).[15]

The substrate specificity for arachidonic acid at the *sn*-2 position of PC and PE and the prevention by phospholipase A_2 inhibitors indicate a central role of this enzyme. Moreover, the inhibitory effect of pertussis toxin on Et_3PbCl-induced lipid metabolism points to a G-protein-dependent mechanism.

Phospholipases are important enzymes within regulatory processes inducible by external signals. Their products are second messengers with a

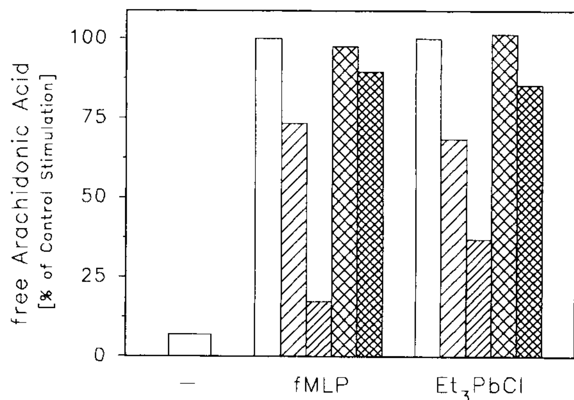

Figure 6 Effect of pertussis toxin and its B-oligomer on Et_3PbCl-induced arachidonic acid liberation in HL-60 cells. [^{14}]Arachidonic-acid-prelabelled and differentiated HL-60 cells were not preincubated (open bars), preincubated for 3 h with 500 or 1000 ng cm^{-3} pertussis toxin (hatched bars) or equivalent amounts of its B-oligomer (cross-hatched bars) before the cells were stimulated with fMLP or 100 μmol dm^{-3} Et_3PbCl (20 min). Lipids were extracted and separated as described in Fig. 1.

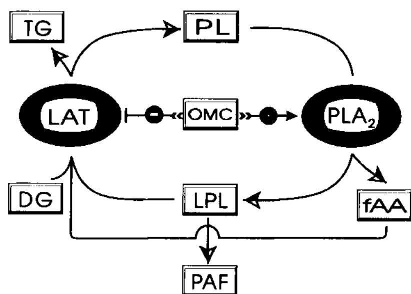

Figure 7 Simplified scheme of the effects of organic metal compounds within the Lands cycle. Shown here are the effects of organic metal compounds (OMC) on the deacylation–reacylation cycle of fatty acids. Free arachidonic acid (fAA) will be produced by the activity of a phospholipase A_2 (PLA_2) from phospholipids (PL). The resulting lysophospholipids (LPL) are precursors for the production of platelet activating factor (PAF). To reduce the concentration of free fatty acids, different lysophospholipid acyltransferases (LAT) reincorporate acyl-CoA into LPL and diacylglycerol (DG) to give PL or triacylglycerol (TG).

multitude of functions, intra- as well as intercellular. Especially, neutrophilic granulocytes are able to interact with various cell types, such as macrophages, mast cells, platelets, polymorphonuclear leukocytes and many others, e.g. via their products of the phospholipase A_2 cascade.[31] In these cell types the deacylation–reacylation cycle, the 'Lands cycle'[32] plays an important role in the regulation of free arachidonic acid concentration, the precursor of eicosanoid synthesis. The results presented here demonstrate that the organometals, especially Et_3PbCl, affect this cycle at two points, therefore leading to an increase in free fatty acids (Fig. 7). Firstly, they may cause an inhibition of lysophospholipid acyltransferase, preventing the reacylation of fatty acids into lysophospholipids; and secondly, they enhance the activity of PLA_2, possibly via a signal-transduction mechanism. These effects lead to an increase in lipid precursors, the eicosanoids and the platelet-activating factor, which are discussed as potent mediators of inflammatory, allergic and pseudo-allergic reactions.[31,33]

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