Effects of Organometals on Cellular Signaling. I. Influence of Metabolic Inhibitors on Metal-induced Arachidonic Acid Liberation

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Organic lead and tin compounds stimulate an increase of free arachidonic acid (AA) in HL-60 cells. This fatty acid is involved in numerous health problems and physiological mechanisms. Three major pathways result in a liberation of AA from membrane phospholipids and there is evidence that G-proteins serve as couplers within all three pathways. Therefore we investigated the influence of pertussis toxin (PT) on the organometallic-induced AA liberation. The effect of all studied compounds (organotin and organo-lead) was diminished by PT. We conclude that the organometals activate PLA₂ to some extent via a PT-sensitive pathway. The ionophor A23187 (1–10 μM) led to an increase of free AA by raising the intracellular Ca²⁺ level. One of the postulated ways of AA release is via Ca²⁺ channel activation; phospholipases are Ca²⁺ dependent. Thus, we examined the necessity of free intracellular Ca²⁺ for the organometallic effect. The Ca²⁺ chelator EGTA inhibited the increase of free AA induced by organometals. This is true also for verapamil, a Ca²⁺ channel blocker. Quinacrine, which is thought to be an inhibitor of phospholipase A₂ (PLA₂), prevented the AA liberation from membrane phospholipids induced by organometals. This could be due to the inhibition of PLA₂, but it could also be the result of an inhibited Ca²⁺ influx. — Environ Health Perspect 102(Suppl 3):325–330 (1994).

Key words: organolead, organotin, lipid metabolism, arachidonic acid, pertussis toxin, quinacrine, verapamil, calcium, HL-60 cells

Introduction

Many organometallic products are directly applied to the environment, e.g., as biocides, as antifouling paints, or as gasoline additives (1). Thus, they are ubiquitously distributed toxins, some of which can accumulate in the body (2). However, less attention has been paid to chronic, nonfatal exposures to such toxic compounds.

Arachidonic acid (AA) and a number of its biologically active products are generated in response to many physiologic and pathologic stimuli and also in response to several metallic and organometallic compounds (3–7). The importance of AA in the induction of long-term potentiation, in the modulation of signal transduction, in the regulation of enzyme activities, and in intracellular Ca²⁺ homeostasis is well known (8). In most cells, including HL-60 cells, AA is not normally found in a free form. The liberation of AA from its sn-2 position in membrane phospholipids limits the synthesis of eicosanoids and other biologically active metabolites. The concept that changes in phospholipase activity are responsible for controlling free AA levels is generally accepted (9).

There are three physiologic pathways to increase free AA. First, the activation of phospholipase C (PLC) hydrolyzes phosphatidylinositol (PI) to inositoltrisphosphate (IP₃) and diacylglycerol (DAG). In a second step, diacylglycerol lipase liberates AA from DAG. IP₃ and DAG as second messengers increase the cytosolic Ca²⁺ concentration, and activate protein kinase C. Second, the activation of phospholipase A₂ (PLA₂) directly leads to an increase of free AA and subsequent metabolism. Third, activated Ca²⁺ channels increase the intracellular Ca²⁺ concentration. Most of phospholipases are Ca²⁺ dependent, but that does not necessarily mean they are Ca²⁺ controlled. In neutrophils, the second pathway (via PLA₂) has been thought to be primarily responsible for the liberation of AA (10). There is evidence to suggest that G-proteins serve as couplers within all three mentioned pathways. Several studies have shown recently that treatment with pertussis toxin (PT) inhibits AA release in various cells, suggesting a G-protein-mediated stimulation of AA liberation (11,12).

The present study was undertaken to gain more insight into the mechanism(s) of organometallic-induced AA release. The first step was to investigate the influence of PT. This exotoxin, produced by Bordetella pertussis, is one of the A-B toxins. The B-oligomer (binding subunit) binds to the cell surface to permit the A-proteomer (catalytic subunit) to reach the intracellular site. The A-proteomer catalyzes ADP-ribosylation of the α-subunit of G-proteins. Thereby, G-proteins lose their function. PLA₂ is known to require Ca²⁺ for activity. Thus, we wanted to clarify whether there is any effect of Ca²⁺ availability on the organometallic-induced AA liberation. We examined the necessity of free extracellular Ca²⁺ by the use of EGTA-containing medium, and the involvement of Ca²⁺ channels by blocking them with verapamil, a substance which is in pharmacologic use. Quinacrine is used as an inhibitor of PLA₂ (13,14). Lacking a real PLA₂ inhibitor (15), we examined the influence of quinacrine on the organometallic-induced AA liberation, keeping in mind that this substance alters Ca²⁺ homeostasis also.

Materials and Methods

Chemicals

All organometals were obtained from Ventrion Alfa Products (Karlsruhe, Germany). Dibutyltin (Bu₂SnCl₂) and trimethyllead (Me₃PbCl) were used without further purification; triethyllead...
(Et₃PbCl) has been purified. Pertussis toxin, verapamil, fMLP, and calcium ionophore A 23187 were from Sigma (Munich, Germany) and the SIL G-Polygram thin-layer plates from Macherey & Nagel (Düren, Germany). EGTA, nitro blue tetrazolium chloride, trypan blue dye, and quinacrine were purchased from Serva (Heidelberg, Germany). The [1-14C]-arachidonic acid (2.07 GBq/m mole) was purchased from Amersham (Braunschweig, Germany). The pertussis toxin β-oligomer was from List Biological Laboratories (Campbell, CA). All other chemicals were of analytic grade.

**Cell Culture and Incubation**

The HL-60 cells were expanded from stocks frozen in liquid nitrogen and were grown in suspension culture in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum. The suspensions were split twice a week to maintain cell number between 2 × 10⁵ and 2 × 10⁶ cells/ml. They were induced to differentiate into mature granulocytes by the addition of 1.3% dimethyl sulfoxide for 5 to 6 days.

The percentage of differentiated cells was quantified using the nitroblue tetrazolium reduction method as described (16); if the extent of differentiation exceeded 70%, the cells were used for the experiments.

[1-14C]-Arachidonic acid (92.5 kBq) was added at day 5 to 50 ml culture medium, and 3 × 10⁸ cells were incubated overnight (7, 17). The labeled cells were harvested by centrifugation, washed once with RPMI without additives, and resuspended in medium containing 3.3% heat-inactivated fetal calf serum (and, if necessary 5 mM EGTA) at a concentration of 0.75 × 10⁷ cells/ml. Experiments were started after a 30-min resting time. The cell suspensions (2 ml) were then incubated at 37°C with the different organometal compounds, fMLP or A 23187 and additionally PT, β-oligomer, EGTA, verapamil, or quinacrine in the concentrations indicated. Verapamil and quinacrine were added to the labeled cells 5 to 10 min before the organometals. In the case of PT- or PT-β-oligo-pretreatment of cells, the organometals were added 3 hr later.

Stock solutions of Et₃PbCl, Me₃PbCl, quinacrine, and verapamil (20 mM each) were prepared in deionized water, whereas Bu₃SnCl₂, A 23187, and fMLP had to be dissolved in dimethyl sulfoxide (20 mM). The final concentration of dimethyl sulfoxide within the incubation mixture did not exceed 1.3%. EGTA was dissolved in the incubation medium (see above), and [1-14C]-Arachidonic acid was dissolved in dimethyl sulfoxide.

**Viability**

Cell viability was determined by trypan blue exclusion. The number of blue cells was estimated using a Neubauer-chamber subsequently mixing the cell suspension with the dye solution. The organometal concentrations within the incubation mixtures did not decrease cell viability below 80% of control during the incubation time.

**Figure 1.** Influence of PT and its β-oligomer on organometal-induced arachidonic acid release. Suspensions of radioactive labeled, differentiated HL-60 cells were preincubated with PT or its β-oligomer for 3 hr before fMLP, A 23187 or organometals were added as indicated on the abscissa. Dotted line: control stimulation without pretreatment. Cellular lipids were extracted after the times indicated and arachidonic acid was separated by thin-layer chromatography. Identification of lipids was performed by use of commercial lipid standards. The amount of liberated arachidonic acid was compared to that of vehicle-treated HL-60 cells (100%). Values are the mean of 7 to 12 experiments ± SD.

**Figure 2.** Inhibition of organometal-induced arachidonic acid release by the Ca²⁺ chelator EGTA. [14C]-arachidonic acid prelabeled, differentiated HL-60 cells were resuspended in EGTA-containing medium (5 mM) and incubated with different organometals as indicated. At the end of the incubation period (indicated at the abscissa) cellular lipids were extracted and separated by thin-layer chromatography. The amount of AA was expressed as percent of total incorporated radioactivity. Values are the mean ± SEM of five to seven experiments. Statistical significance: ** = 0.0005 > p, compared with vehicle treated controls, # = 0.005 > p > 0.0005, # = 0.05 > p > 0.001, compared with control stimulations (unpaired t test).
Lipid Extraction and Separation

After incubation of the cell suspensions (2 ml) they were extracted according to the method of Krug and Cullig (7) by addition of 7.5 ml chloroform/methanol (1:2) and phase separation was induced with further 2.5 ml chloroform and 2.5 ml 0.2% formic acid. After 5 min on ice, this mixture was centrifuged at 300g to 400g for 5 min. The organic phase was dried under nitrogen and taken up in a small volume of chloroform.

The lipid extracts were spotted onto SIL G polyester plates and separated into lipid classes by thin-layer chromatography (TLC) as described earlier (7). Labeled products were located by autoradiography, and the appropriate regions of the plates were cut out and counted for radioactivity in a 1219 Rackbeta liquid scintillation counting system (LKB Freiburg, Germany).

Results

HL-60 cells, differentiated with 1.3% dimethyl sulfoxide into mature granulocytes, were incubated for 24 hr in the presence of [14C]-arachidonic acid. An equilibrium in arachidonic acid distribution within the lipid classes was reached after 24 hr (7,17).

Upon stimulation with A 23187, fMLP, and various organometals, HL-60 cells showed increased levels of free arachidonic acid (18–20). The effect of different substances on the organometal-induced AA liberation was examined.

Effect of PT and Its β-Oligomer on Organometal Stimulation of HL-60 Cells

Pretreatment with PT (1500 ng/ml in the case of stimulation with organometals, 500 ng/mL in the case of stimulation with fMLP or A 23187) led to a reduced effect of organometals regarding the increase of free AA in dimethyl sulfoxide differentiated HL-60 cells (Figure 1, crosshatched bars). Preincubation of the cells with PT led to a 44% reduction of AA liberation as compared to treatments with Me3PbCl (500 μM; 20 min) alone. The effect of Et3PbCl (100 μM; 20 min) and Bu2SnCl2 (500 μM; 60 min) was about 30% reducible by pretreatment with PT, whereas stimulation with fMLP (1 μM; 10 min) was decreased to 20% of the control stimulation. A 23187-induced (10 μM; 10 min) AA release, on the other hand, was only 25% diminished by PT (Figure 1).

The hatched bars in Figure 1 show the influence of β-oligomer (equimolar to PT), the noncatalytic subunit of PT, on the stimulation of AA liberation. This allowed an estimation of how much the simple binding of a protein to the plasma membrane alters the level of free AA in HL-60 cells. The AA liberation induced by the different stimuli was reduced, in any case, by not more than 10%.

Influence of EGTA, Verapamil, and Quinacrine

The effect of EGTA (5 mM), a Ca2+ chelator, is shown in Figure 2. The Ca2+ depletion by EGTA decreased the AA liberation induced by organometals. Two of the compounds, Me3PbCl and Et3PbCl, lost their AA liberation potency completely in the presence of EGTA. On the contrary, the effect of Bu2SnCl2 was only half diminished (52% reduction).

Not only EGTA but also verapamil, a Ca2+ channel blocker, alters the organometal-induced AA release (Figure 3). Verapamil (100 μM) could prevent 67, 65, and 43% of the AA release induced by Me3PbCl, Et3PbCl, or Bu2SnCl2, respectively.

Quinacrine (1 mM), which is used as a PLA2 inhibitor, also influences the AA lib-
eration in response to organometals (Figure 4). The effect of the organolead compounds (Me,PbCl and Et,PbCl) on the AA metabolism nearly disappeared when cells were pretreated with quinacrine. Moreover, the effect of the tin compound was decreased by this substance only to 54%. This inhibitory effect of quinacrine was similar to the results obtained with Ca\(^{2+}\) chelating by EGTA.

**Discussion**

Because the use of organometals is still very widespread and the environmental pollution is increasing, the effects within living organisms have not been sufficiently investigated. It is possible that some intracellular effects of alkylmetals are indirect due to altered characteristics of the plasma membrane and correlated enzymes like G-proteins, adenylate cyclase, and phospholipases. Within the last 20 years the importance of phospholipases in biological processes has been recognized. In particular, PLA\(_2\) has attracted much attention because of its ability to produce substrates for the generation of inflammatory lipid mediators. The sources of these potent, biologically active substances (like eicosanoids, paf-acether, lysophospholipids) are phospholipids found in the cellular membranes. Investigations into the influence of organometals on eicosanoid metabolism in platelets or cultured cells have been reported by several groups (3-7,20).

G-protein regulation has been demonstrated for several membrane-associated signal-transducing-systems, including phospholipases and ion channels [phospholipases (12,21); ion channels (22,223)]. The bacterial toxin PT has been widely used as a tool to demonstrate the involvement of G-proteins in signal transduction. To estimate if G-proteins are involved in the organometal-induced AA liberation in dimethyl sulfoxide differentiated HL-60 cells, we examined the inhibitory potency of PT and its \(\beta\)-oligomer on this effect (Figure 1). We found that the release of AA from membrane phospholipids induced by organometals could be reduced by pretreatment with PT as described earlier (7). The inhibition of the signal transduction by PT points to an involvement of a G-protein in this mechanism, although the overall inhibitory effectiveness varies over a wide range within cell cultures of different origin (7) (Figure 1). It clearly shows that the effect of organometals on eicosanoid metabolism is not only due to a direct activation of either PLA\(_2\) or PLC. Although the relative contribution of the possible pathways to the liberation of AA (direct activation of PLA\(_2\), indirect via activation of PLC, or rising Ca\(^{2+}\) level) is still obscure, PLA\(_2\) is the most likely candidate responsible for AA release (8). The lack of an inhibitory action of the \(\beta\)-oligomer showed that this effect mediated by the bacterial holotoxin really resulted from the ADP ribosylation of G-proteins and not merely from unspecified alterations of membrane (function) by intercalation of the toxin.

G-proteins may play a role in PLA\(_2\) control (8), but many other factors are also capable of modulating the activity of the enzyme (8,24,25). Therefore it seems likely that there is more than one mechanism that leads to an AA release after incubation with organometals. Since phospholipases are dependent on Ca\(^{2+}\), and Ca\(^{2+}\) channels are known to be regulated by G-proteins, compounds that influence cellular levels of Ca\(^{2+}\) were examined for inhibitory activity in this context. Brooks et al. (26) suggest a model for PLA\(_2\) regulation in which the enzyme is functionally associated with plasma-membrane Ca\(^{2+}\) channels. A sustained increase of intracellular Ca\(^{2+}\) concentration is often linked to the onset of cytotoxicity. Several biochemical mechanisms are stimulated by an increase of Ca\(^{2+}\) and can directly mediate cell death by causing disruption of the cytoskeleton, by DNA fragmentation, and by extensive damage to other cell components (27). By increasing free intracellular Ca\(^{2+}\), organometals might initiate chains of events exciting and finally killing the cell. Therefore we studied the effect of EGTA and verapamil.

In contrast to the slight but clear effect of PT, the depletion of extracellular Ca\(^{2+}\) led to total disappearance of the organolead (Et,PbCl, Me,PbCl)-induced AA release in HL-60 cells (Figure 2). All phospholipases are known to be Ca\(^{2+}\)-dependent and Ca\(^{2+}\) is a prime candidate for being a PLA\(_2\) regulator because a Ca\(^{2+}\) binding site has been identified on PLA\(_2\) (28). Alkylmetals cause a linear increase in intrasynaptosomal Ca\(^{2+}\) after an initial rise (29). Ca\(^{2+}\) appears to mediate the neurotoxicity due to cyanide, lead, methyl mercury, chlordecone, and organotin compounds (30). Addition of thimerosal to human platelets causes a sharp rise in intracellular Ca\(^{2+}\) (5). The EGTA-inhibited AA liberation by organolead could therefore be explained by an inability to raise intracellular Ca\(^{2+}\) to levels sufficient for phospholipase activity. The observation that the Bu\(_3\)SnCl\(_2\)-induced AA liberation could only be inhibited to about 50% by EGTA leads to speculation that this is due to the release of Ca\(^{2+}\) from intracellular stores by the organotin compound (Figure 2). This was confirmed by measuring the intracellular Ca\(^{2+}\) concentration after Bu\(_3\)SnCl treatment using EGTA-containing medium, which led to an intracellular increase of Ca\(^{2+}\) (31). In the absence of extracellular Ca\(^{2+}\) (chelated by 1 mM EGTA), thimerosal still induced a significant increase in the intracellular Ca\(^{2+}\) (5). However, the increase of the Ca\(^{2+}\) level during physiologic activation of phospholipases is below that needed in vitro; and the Ca\(^{2+}\) dependency of an enzyme does not automatically mean that it is under Ca\(^{2+}\) control. On the other hand, sustained Ca\(^{2+}\) influx across the plasma membrane could generate very high Ca\(^{2+}\) concentrations in the vicinity of membrane-associated PLA\(_2\), thus leading to its activation. Intracellular Ca\(^{2+}\) is regulated by channels, pumps, exchangers, and stores at both plasma membrane and intracellular sites. Thus, the mechanism by which organometals cause an increase in cytosolic Ca\(^{2+}\) could be at least 2-fold: first, by activating Ca\(^{2+}\) channels, and second, by non-specific effects on the membrane characteristics. Both could be followed by an activation of PLA\(_2\) and/or PLC.

The activation of a Ca\(^{2+}\) channel could be direct or via a G-protein (22,23). Verapamil, an antihypertensive agent, is known to block slow Ca\(^{2+}\) channels (32). Fernandez and Balsinde (33) reported that verapamil persistently inhibits AA release in macrophages. They suggest the involvement of voltage-gated Ca\(^{2+}\) channels in the control of PLA\(_2\) activation. Treatment with verapamil diminished the organometal effect, but did not abolish the AA liberation totally (Figure 3). This could be due to the inhibition of only certain (voltage-gated) Ca\(^{2+}\) channels. Organotin-induced AA liberation could not be inhibited in the same range as the organolead-induced AA liberation, perhaps due to an additional activation of intracellular Ca\(^{2+}\) stores by Bu\(_3\)SnCl. The remaining increase in free AA in HL-60 cells could be explained by an additional and more direct activation of phospholipases (directly on the enzyme or via a G-protein coupled to the phospholipase) by organometals. The Ca\(^{2+}\) antagonist verapamil is also discussed as a PLA\(_2\) inhibitor. This has been attributed to the formation of nonspecific drug-phospholipid complexes rather than to blockage of slow Ca\(^{2+}\) channels (28). The difference between the effect of organolead and organotin compounds could be explained
by differences in solubility. Whereas Et3PbCl and Me3PbCl can be dissolved in water, Bu4SnCl has to be dissolved in organic solvents like dimethyl sulfoxide or ethanol. This is due to the higher lipophilicity of Bu4SnCl compared to the organolead compounds. This lipophilicity may allow the Bu4SnCl to reach the intracellular stores and to mobilize Ca2+, whereas the other substances may exhibit their effects almost exclusively at the plasma membrane.

Short pretreatment with quinacrine, which is thought to be a PLA2 inhibitor (13,14) led to significantly lower AA liberation induced by organometals. Nakashima et al. (12) report that quinacrine effectively suppresses the paf-induced liberation of [3H]-AA from phospholipids in human polymorphonuclear neutrophils. In this case, extracellular Ca2+ is not required for AA release. The results discussed here (Figure 4) obviously show the same pattern as the inhibition of the AA release by the Ca2+ chelator EGTA. We therefore conclude that the antimalarial agent quinacrine operates as a Ca2+ antagonist rather than as a PLA2 inhibitor. Stokke et al. (34) reported the negative inotropic effects of quinacrine in the heart. They concluded that quinacrine binds to or close to slow Ca2+ channels.

The inhibition of PLA2 activity by quinacrine could possibly lead to a diminished Ca2+ availability and not to a direct inhibition of the enzyme itself.

Once activated, PLA2 can mediate a variety of pathophysiologic reactions either directly action or through subsequent transformation of its products into several potent, biologically active substances such as prostaglandins, leukotrienes, and paf. Lysophospholipids, the coproducts of PLA2 activation, are cytotoxic substances and membrane fusions, and have been implicated in several human inflammatory conditions.

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