Phosphorylation of membrane proteins in erythrocytes treated with lead

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In immature rat microvessels, endothelial cells and glioma cells, exposure to lead results in an increase in the level of protein kinase C in membranes. In this paper we have extended these studies to human erythrocytes and, in addition, studied the phosphorylation of membrane proteins. A significant increase in the phosphorylation of membrane cytoskeletal proteins of molecular mass 120, 80, 52 and 45 kDa was observed in human erythrocytes treated for 60 min with lead acetate at concentrations greater than 100 nM. These same proteins were phosphorylated when erythrocytes were treated for 10 min with 50 nM phorbol 12-myristate 13-acetate (PMA). Similarly, protein kinase C activity was elevated and an increase in the amount of protein kinase C-α was observed in membranes from erythrocytes exposed to concentrations of lead acetate above 100 nM. No changes, however, in the activities of cAMP-dependent protein kinase, protein phosphatases I and IIA or casein kinase were observed. Phosphorylation of these membrane proteins stimulated by lead acetate or by PMA was not observed in erythrocytes depleted of protein kinase C by a 72-h treatment with 500 nM phorbol 12,13-dibutyrate. Finally, no changes in the levels of calcium or diacylglycerol were observed in erythrocytes stimulated with 100 nM lead acetate. These results indicate that, in erythrocytes, lead acetate stimulates the phosphorylation of membrane cytoskeletal proteins by a mechanism dependent on protein kinase C. Since levels of calcium or diacylglycerols did not increase, it appears that lead may activate the enzyme by a direct interaction.

INTRODUCTION

Lead (Pb^{2+}) is a potent toxin that is an occupational hazard and an environmental pollutant. In children, blood Pb^{2+} levels greater than 10 μg/dl correlate with behavioural and learning problems [1,2]. The deleterious effects of Pb^{2+} in the nervous system that occur at these low concentrations may be explained, in part, by its effects on processes that are regulated by calcium (Ca^{2+}) [3–5]. For example, Pb^{2+} binds to calmodulin [6,7] and to Ca^{2+} channels with a higher affinity than does Ca^{2+} [8]. At picomolar concentrations Pb^{2+} can substitute for Ca^{2+} in assays for protein kinase C activity from rat brain [9,10]. Furthermore, in immature microvessels from rat brain, high nanomolar concentrations of Pb^{2+} increase the amount of protein kinase C in the membrane [11]. Also, an increase in the amount of protein kinase C-α in membranes was observed in endothelial cell and glial cell cultures treated with Pb^{2+} [12].

Although Pb^{2+} is known to increase the amount of membrane-bound protein kinase C, we do not know whether exposure to the metal results in the phosphorylation of proteins. It is possible that Pb^{2+} activates other processes, such as protein phosphatases, which will dephosphorylate proteins and mask the effects of protein kinases. Also, the cofactors (Ca^{2+}, diacylglycerol and phosphatidylserine) that are required for protein phosphorylation by classical protein kinase C isoforms (α, β and γ) may not be available to cells exposed to Pb^{2+} [13,14]. Unless Pb^{2+} increases protein phosphorylation, it is difficult to conclude that the detrimental effects of Pb^{2+} are caused by activation of protein kinase C.

To resolve this problem, the phosphorylation of membrane proteins in human erythrocytes exposed to Pb^{2+} was examined. Erythrocytes were chosen because they accumulate large amounts of Pb^{2+}; indeed, erythrocytes contain over 90 % of the Pb^{2+} in blood [15]. Additionally, membranes from erythrocytes are well characterized [16]. Several proteins, including spectrin, ankyrin, actin, adducin, band 3, protein 4.1 and protein 4.9, have been identified and shown to be substrates for protein kinases. With respect to only the serine/threonine protein kinases, casein kinase (I and II) was shown to phosphorylate spectrin, ankyrin, adducin, band 3, protein 4.1 and protein 4.9 [16,17]; cAMP-dependent protein kinase phosphorylates spectrin, ankyrin, adducin, protein 4.1 and protein 4.9 [18–20]; and protein kinase C phosphorylates adducin, protein 4.1 and protein 4.9 [18,19,21].

In the present study, we found that membrane proteins with molecular masses of 120, 20, 55 and 45 kDa were phosphorylated in erythrocytes exposed to Pb^{2+} or stimulated with phorbol 12-myristate 13-acetate (PMA). Furthermore, the concentration of Pb^{2+} that increased the level of protein kinase C in membranes was similar to that which increased protein phosphorylation. Lastly, the phosphorylation of these proteins was lost if erythrocytes were depleted of protein kinase C.

EXPERIMENTAL

Preparation of erythrocytes

Blood was withdrawn from a healthy volunteer into a heparinized syringe and resuspended at a 1:10 (v/v) dilution in Alsever's solution. The blood was stored at 4 °C and used with 2 days.

Phosphorylation of erythrocyte membrane proteins

The suspension of erythrocytes was centrifuged (1000 ***g***) and the cell pellet was washed three times with phosphate-free Dulbecco's

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified essential medium; PDBu, phorbol 12,13-dibutyrate.

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modified essential medium (DMEM). In each wash the buffy coat that contains white blood cells was removed. A 100 ml suspension was concentrated to 20 ml in phosphate-free DMEM and incubated at 37 °C with 200 μCi/ml carrier-free [^{32}P]P_i. After 3 h, the erythrocytes were washed and resuspended in 135 mM NaCl, 3 mM KCl, 5 mM $MgSO_4$, and 20 mM Hepes, pH 7.4 (buffer A). Different concentrations of Pb^{2+} (as the acetate salt) or 50 nM PMA were added and the erythrocytes were incubated at 37 °C for 60 min or 10 min respectively. Cells were centrifuged at 800 ***g*** for 10 min and the cells in the pellet were lysed with a buffer consisting of 10 mM Tris/HCl (pH 7.4), 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 0.01 mM PMSF at a 1:20 (v/v) ratio [21]. The lysate was incubated at 4 °C for 30 min and centrifuged at 29000 ***g*** for 30 min. The pellet was washed until it was white and free from haemoglobin. The fluffy white membrane was transferred to a second tube and the proteinase inhibitors leupeptin, aprotinin and soybean trypsin inhibitor were added to final concentrations of 25 μg/ml, 10 μg/ml and 10 μg/ml respectively. An aliquot of 10 μg of protein was subjected to SDS/PAGE on a 7.5% (w/v) polyacrylamide gel [22]. Gels were stained with Coomassie Blue and dried. Radioactivity in the protein was quantified by autoradiography and computerized densitometry (Molecular Dynamics).

Assay for protein kinase C

Erythrocyte membranes were prepared and homogenized in 20 mM Tris/HCl, pH 7.5, containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, 25 μg/ml leupeptin and 10 μg/ml aprotinin. The reaction mixture (50 μl total volume) contained 20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 0.1 mM PMSF, 25 μg/ml leupeptin, 0.5 mM dithiothreitol, 100 μM [γ-^{32}P]ATP (1000–3000 Ci/mmol) and 300 μM peptide substrate (KRTLRR) [23]. The reaction was initiated by adding substrate. The reaction was stopped after incubation at 25 °C for 5 min by adding 25 μl of 300 mM phosphoric acid, and 10 μl was spotted on to P81 filter paper. The paper was washed with 10 ml of 75 mM phosphoric acid and radioactivity was determined by liquid scintillation spectroscopy. Radioactivity in the presence of 2.5 mM EGTA was subtracted from radioactivity in the presence of 320 μg/ml phosphatidylserine, 30 μg/ml, 1,2-diolein and 100 μM $CaCl_2$. One unit of activity is defined as the amount of enzyme required to transfer 1 pmol of phosphate/min. Data are expressed as units/mg of protein.

Level of protein kinase C in membranes

Erythrocytes were exposed to Pb^{2+} or treated with PMA and membranes were homogenized in buffer (see protein kinase C assay) with 0.5% Triton X-100. Approx. 10 μg of protein was subjected to SDS/PAGE and transferred to nitrocellulose paper by electroblotting. The level of protein kinase C was determined by Western blotting [24] with a monoclonal antibody to the catalytic subunit of the α or β isotype [25]. The antibody–antigen complex was identified with a horseradish peroxidase-linked secondary antibody and chemiluminescence. The intensity of staining was measured by computerized densitometry.

Levels of diacylglycerol

Membranes were prepared from erythrocytes exposed to Pb^{2+} and the amount of protein was determined. Lipids were extracted from an equivalent of 100 μg of protein with methanol/chloroform (2:1, v/v) [26] and concentrated under nitrogen. The lipids were resuspended in chloroform and applied to TLC plates. Diolein (1,2-diacylglycerol) was used as an external standard. The plates were developed in ether/light petroleum (b.p. 35–60 °C)/acetic acid (50:50:1, by vol.) until the solvent front had migrated 2 cm from the top of the plate [27]. The plates were dried, stained with 0.1% Coomassie Blue R250 and destained with 30% methanol [28]. The plates were dried and the spot that migrated the same distance as diolein was analysed by densitometry.

Levels of Ca^{2+}

Levels of Ca^{2+} were measured in erythrocytes by a fluorimetric ratio-imaging method [29]. A 2% (w/v) solution of erythrocytes in buffer A was incubated for 90 min with 3 μM fura 2-acetoxymethyl ester (fura 2-AM). The cells were washed and treated for 20 min at 37 °C. The cells were then centrifuged and lysed with buffer A containing 50 μg of digitonin/ml. The fluorescence emitted at 510 nm after excitation at 340 nm and 380 nm was measured. The concentration of Ca^{2+} was determined using the following equation:

$$[Ca^{2+}] = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

The K_d for the fura 2–Ca^{2+} complex was taken to be 224 nM at 37 °C. In our system R_{max} was 14.3, R_{min} was 0.17 and S_{f2}/S_{b2} was 14.7. R_{max} and R_{min} are the maximal and minimal fluorescence ratios of fura 2, obtained in buffer containing saturating Ca^{2+} (1 mM) and in Ca^{2+}-free buffer with EGTA respectively. S_{f2}/S_{b2} is the ratio of fluorescence values for Ca^{2+} bound/Ca^{2+} free at 380 nM.

Assay for protein phosphatase

Protein serine/threonine phosphatase I and IIA activity was assayed by measuring the ability of erythrocyte membranes to dephosphorylate [^{32}P]phosphorylase *a* [30]. [^{32}P]Phosphorylase *a* was prepared first in a reaction catalysed by phosphorylase *b*. The incubation mixture contained 150 mM Tris/HCl, pH 8.2, 40 mg/ml phosphorylase *a*, 20 mg/ml phosphorylase *b*, 60 mM β-glycerophosphate 0.8 mM EDTA, 30 mM 2-mercaptoethanol, 20% (w/v) glycerol, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM $CaCl_2$ and 5 mCi/ml [γ-^{32}P]ATP (3000–6000 Ci/mmol). After 60 min at 30 °C, phosphorylase *a* was precipitated with 50% ammonium sulphate and isolated by filtration through a Centricon Membrane (molecular mass cut-off 5 kDa). The specific radioactivity was approx. 7×10^5 c.p.m./mg and the protein concentration was adjusted to 3 mg/ml.

Phosphatase activity was measured in erythrocyte membranes homogenized in 50 mM Tris/HCl, pH 7.0, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 25 μg/ml leupeptin, 25 μg/ml aprotonin and 0.5% Triton X-100. The samples could be stored in 20% glycerol at −70 °C. The assay buffer contained 50 mM imidazole chloride, pH 7.6, 5 mM EDTA, 1 mg/ml BSA, 0.5 mM dithiothreitol, 10 mM caffeine and 200 μg/ml [^{32}P]phosphorylase *a*. After 10 min at 30 °C, protein was precipitated with 20% (w/v) ice-cold trichloroacetic acid and radioactivity was determined in an aliquot of the supernatant fraction (free phosphate) by liquid scintillation spectroscopy. The radioactivity was converted to nmol of phosphate, and one unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of phosphate/min. Data are expressed as units/mg of protein.

Assay for cAMP-dependent protein kinase

In this assay, the phosphorylation of kemptide, a highly specific substrate for cAMP-dependent protein kinase, was measured [31]. The membranes were prepared from erythrocytes exposed to Pb^{2+} and homogenized in 50 mM Tris, pH 7.5, 5 mM EDTA and proteinase inhibitors (see assay for protein kinase C). The reaction was carried out in the presence of 50 μM kemptide, 100 μM ATP, 20 μCi/ml [γ-^{32}P]ATP (3000–6000 Ci/mmol), 10 mM $MgCl_2$, 250 μg/ml BSA and 50 mM Tris, pH 7.5. After a 5 min incubation at 30 °C, 20 μl was spotted on to phosphocellulase paper, which was then washed as described for the protein kinase C assay. The radioactivity on the paper was determined by liquid scintillation spectroscopy and converted to pmol of phosphate. One unit of activity is defined as the amount of enzyme required to transfer 1 pmol of phosphate/min. Phosphorylation of kemptide by kinases other than cAMP-dependent protein kinase was determined by measuring phosphorylation in the presence of 1 μM inhibitory peptide. Activated cAMP-dependent protein kinase was determined by subtracting activity in the presence of inhibitor from the activity in the absence of inhibitor. Data are reported in units/mg of protein.

Assay for casein kinase

In this assay, the phosphorylation of α- and β-casein (dephosphorylated forms) was measured [32,33]. The membranes were prepared from erythrocytes exposed to Pb^{2+} and homogenized in 50 mM Tris, pH 7.2, with proteinase inhibitors (see assay for protein kinase C). The reaction was carried out in 50 mM Tris/HCl, pH 7.2, with 10 mM $MgCl_2$, 20 μM ATP, 0.2 mCi of [γ-^{32}]ATP/ml, 120 mM KCl and 250 μg/ml α- and β-casein. The mixture was incubated for 20 min at 22 °C. The reaction was stopped and radioactive casein was separated from [γ-^{32}P]ATP as described for the protein kinase C assay. The background, determined as the radioactivity in the absence of substrate, was subtracted. Data are expressed as c.p.m./mg of protein.

Materials

Alsever's solution, DMEM, phosphorylase *a*, phosphorylase *b*, kemptide and cAMP-dependent protein kinase inhibitor protein were purchased from Life Sciences. [^{32}P]P_i, [γ-^{32}P]ATP, nitrocellulose paper and kits to perform chemiluminescence were purchased from Amersham. The antibody against the catalytic domain of protein kinase C-α was obtained from Upstate Biotechnology. Phospholipase C (*Clostridium perfringens*; type I), 1,2-diolein and all reagents, including chemicals for buffers, proteinase inhibitors, PMA and electrophoresis, were obtained from Sigma. The solvents used in the analysis of lipids were purchased from Fisher, and fura 2-AM was obtained from Molecular Probes. Phosphocellulose paper was obtained from Whatman, and Centricon filters were from Amicon.

RESULTS

Phosphorylation of proteins on erythrocyte membranes

Erythrocytes were labelled with [^{32}P]P_i and exposed to different concentrations of Pb^{2+} for 60 min or to 50 M PMA for 10 min. Membranes were isolated and equal amounts of protein were subjected to SDS/PAGE. Radioactivity was quantified by autoradiography and densitometry. An increase in the phosphorylation of four proteins, of molecular mass 120, 80, 52 and 45 kDa, was observed in erythrocytes stimulated with 50 nM PMA or 200 nM Pb^{2+} for 60 min (Figure 1). Phosphorylation was not observed on exposure to Pb^{2+} for 20 min (results not shown).

Exposure to 100 nM Pb^{2+} was sufficient to increase the level of phosphorylation of each protein (Figure 1C). Phosphorylation of the 52 kDa and 45 kDa proteins reached a plateau at 100 nM Pb^{2+}, but the phosphorylation of the 80 kDa and 120 kDa proteins was optimal at 300 nM Pb^{2+}.

Protein kinase C in membranes

The similarity in the molecular masses of the phosphorylated proteins observed in Pb^{2+}-exposed and PMA-stimulated erythrocytes suggested that Pb^{2+} increased protein phosphorylation by activating protein kinase C. Activation is often associated with an increase in the level of protein kinase C in membranes. The level of protein kinase C activity was 1.5 units/mg of protein in membranes from untreated erythrocytes. Activity increased to 4.9 units/mg of protein and 2.7 units/mg of protein in erythrocytes treated for 10 min with 50 nM PMA or for 60 min with 100 nM Pb^{2+} respectively.

The isoform of protein kinase C in erythrocyte membranes was determined by Western blotting. As expected, a 10 min incubation at 37 °C with 50 nM PMA increased by 10-fold the amount of protein kinase C-α in membranes (results not shown). In addition, a 60 min exposure to 100 nM Pb^{2+} increased by 2-fold the amount of protein kinase C-α in the membrane (Figure 2). Pb^{2+} at 100 nM appeared to be optimal; there was no increase in protein kinase-α with 30 nM Pb^{2+}, but 1000 nM Pb^{2+} gave lower values than did 100 nM. Protein kinase C-β was not detected (results not shown).

Depletion of protein kinase C

The contribution of protein kinase C to the stimulation of protein phosphorylation in erythrocytes by Pb^{2+} was examined by depleting erythrocytes of protein kinase C. Almost 95 % of the kinase activity was removed by incubating erythrocytes with 500 nM phorbol 12,13-dibutyrate (PDBu) for 72 h at 37 °C (results not shown). Erythrocytes were cultured in this manner (depleted), or cultured without PDBu (control) and labelled with [^{32}P]P_i. Pb^{2+} did not stimulate phosphorylation of the 120, 80 or 45 kDa proteins in erythrocytes incubated with PDBu (Table 1). Phosphorylation of the 120, 80 and 45 kDa proteins was observed in erythrocytes not incubated with PDBu. Similarly, PMA did not stimulate the phosphorylation of the 120, 80 and 45 kDa proteins in erythrocytes depleted of protein kinase C, but did stimulate phosphorylation in erythrocytes that contained protein kinase C.

Interestingly, phosphorylation of the 52 kDa protein in cultured erythrocytes stimulated with Pb^{2+} or PMA was not observed. The conditions used to culture erythrocytes may have resulted in degradation of this protein.

Level of diacylglycerol

In the next two experiments we tried to uncover the mechanism for the activation of protein kinase C that was stimulated by Pb^{2+}. Since protein kinase C-α is activated by a rise in the levels of diacylglycerol and Ca^{2+}, these intracellular messengers were measured in erythrocytes exposed to Pb^{2+}. Erythrocytes were exposed for 60 min to 1 μM or 100 nM Pb^{2+}, or remained unexposed. Lipids from membranes were extracted, and diacylglycerols were isolated by TLC and identified using standards by staining with Coomassie Blue. Diacylglycerols were not observed in erythrocytes treated with Pb^{2+} or in controls, but

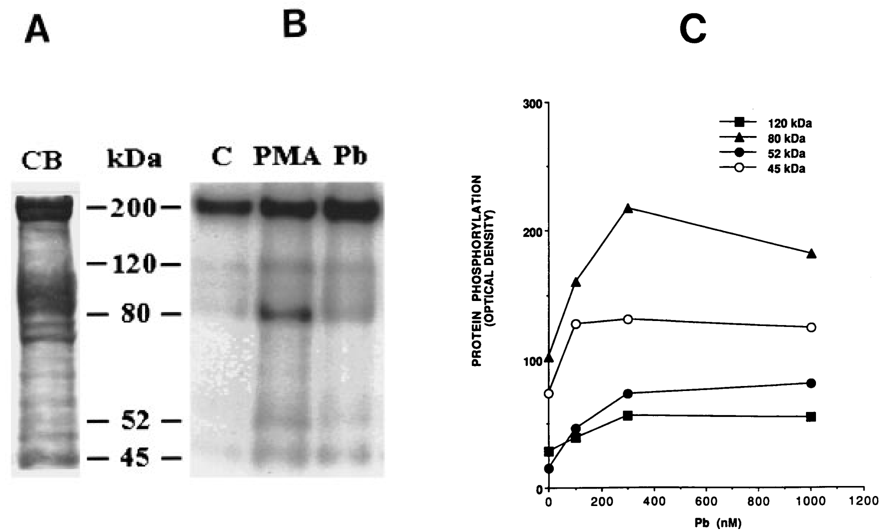

Figure 1 Phosphorylation of erythrocyte membrane proteins by PMA and Pb^{2+}

Erythrocytes were labelled with 200 μCi/ml [^{32}P]P_i for 3 h and then exposed to Pb^{2+} for 60 min or to 50 nM PMA for 10 min. Membranes were prepared as described in the Experimental section, normalized to protein, subjected to SDS/PAGE, and processed for autoradiography (**B**) or stained with Coomassie Blue (**A**). Lane C, Control. The bands on the autoradiograms were quantified by densitometry (**C**). Data from densitometry measurements are given as absorbance (optical density). Duplicate samples for each treatment were analysed, and data are expressed as means ± S.E.M. Molecular mass was determined using external standards.

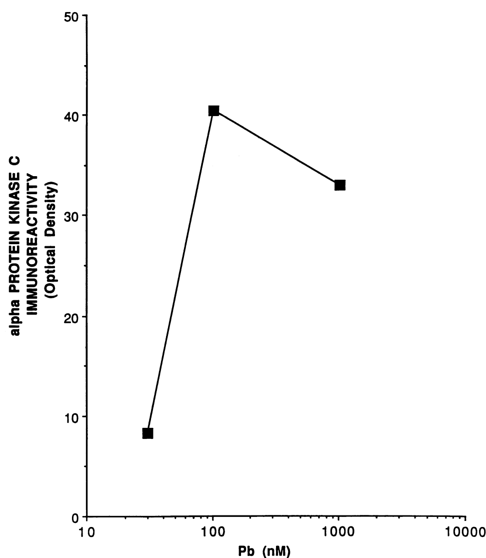

Figure 2 Protein kinase C translocation in erythrocytes exposed to Pb^{2+}

Membranes were prepared from erythrocytes exposed to Pb^{2+}, and equal amounts of protein were subjected to SDS/PAGE and Western blotting. The catalytic subunit of protein kinase C-α was reacted with a monoclonal antibody, and the antibody bound to protein kinase C was located by chemiluminescence and quantified by densitometry. Data from densitometry measurements are given as absorbance (optical density). Duplicate samples for each treatment were analysed, and data are expressed as means ± S.E.M.

they were detected in erythrocytes treated with 0.05 unit of phosphatidylcholine-specific phospholipase C/ml. The absorbance of staining in erythrocytes treated with the enzyme was 0.14 unit. An extract prepared by adding 0.7 μg of 1,2-diolein to erythrocyte membranes during extraction displayed an

Table 1 Phosphorylation in erythrocytes depleted or protein kinase C

Erythrocytes were incubated for 72 h in DMEM with 500 nM PDBu to deplete the protein kinase C, or in DMEM, alone. The erythrocytes were then labelled with [^{32}P]P_i for 4 h and stimulated with 50 nM PMA for 10 min or with 100 nM Pb^{2+} for 60 min. Controls consisted of unstimulated erythrocytes maintained in buffer for the same period of time. Erythrocyte membranes were prepared and proteins were separated by SDS/PAGE on 10% acrylamide gels. The gels were dried and processed for autoradiography, and radioactivity was measured by computerized densitometry. The data are expressed as percent ages of control values.

Protein	Stimulant	Protein phosphorylation (% of control)	
		+ PDBu	− PDBu
45 kDa	Pb^{2+}	95	160
	PMA	98	350
80 kDa	Pb^{2+}	99	210
	PMA	97	460
120 kDa	Pb^{2+}	120	290
	PMA	110	170

absorbance of 0.06 unit. Lower amounts of 1,2-diolein could not be detected.

Level of Ca^{2+}

The effect of Pb^{2+} at 100 nM or 1 μM on levels of intracellular Ca^{2+} was examined in erythrocytes by fluorimetric ratio-imaging with fura 2-AM. The concentration of intracellular Ca^{2+} in unexposed erythrocytes was 160 ± 12 nM. The level of intracellular Ca^{2+} in erythrocytes exposed to 1 μM Pb^{2+} for 60 min was 195 ± 20 nM. In contrast, the level of Ca^{2+} increased to

Table 2 Levels of enzymes in erythrocyte membranes

Membranes were prepared from erythrocytes that were treated for 60 min with 100 nM or 1 μM Pb^{2+}, or which remained untreated (control). Data are reported as means $\pm$ S.E.M. from triplicate samples. Numbers in parentheses refer to the number of replicate experiments. cAMP-dependent protein kinase activity is reported as units/mg of erythrocyte membrane protein. Casein kinase activity is reported as $10^{-3} \times$ c.p.m./mg of erythrocyte membrane protein. The activities of protein phosphatases I and IIA were measured together in one assay and are reported as units/mg of erythrocyte membrane protein.

		Pb^{2+}	
	Control	100 nM	1 μM
cAMP-dependent protein kinase (4)	1104 $\pm$ 81	1069 $\pm$ 76	398 $\pm$ 39
Casein kinase (3)	342 $\pm$ 17	331 $\pm$ 14	221 $\pm$ 11
Protein phosphatase (4)	1.71 $\pm$ 0.15	1.98 $\pm$ 0.21	2.51 $\pm$ 0.31

871 $\pm$ 73 nM in erythrocytes treated with 1 μM ionomycin in the presence of 1 mM $CaCl_2$.

cAMP-dependent protein kinase activity

Erythrocyte membrane proteins are also phosphorylated by cAMP-dependent protein kinase. It is possible that, in addition to its effect on protein kinase C, Pb^{2+} increased the phosphorylation of membrane proteins by activating cAMP-dependent protein kinase. This possibility was investigated by determining whether Pb^{2+} increased this activity. cAMP-dependent protein kinase activity was measured in membranes prepared from erythrocytes exposed for 60 min to 1 μM or 100 nM Pb^{2+}. Erythrocytes were also stimulated with 0.5 mM bromo-cAMP for 10 min as a positive control. The activity of cAMP-dependent protein kinase in erythrocytes treated with or without cAMP was 1609 $\pm$ 81 and 1104 $\pm$ 99 units/mg of protein respectively. The activity in erythrocytes treated with 100 nM and 1 μM Pb^{2+}, however, was similar to control levels (Table 2).

Casein kinase activity

Casein kinase is another protein kinase found in erythrocytes that phosphorylates proteins on erythrocyte membranes. The enzyme was assayed in membranes prepared from erythrocytes treated with Pb^{2+} (100 nM or 1 μM) for 60 min. A significant amount of enzyme activity was detected, but treatment with Pb^{2+} had no effect (Table 2).

Protein phosphatase activity

The level of phosphate on proteins is controlled by protein phosphatases as well as protein kinases. In addition to stimulating protein kinase C, Pb^{2+} may increase phosphorylation of proteins by decreasing protein phosphatase activity. The effect of Pb^{2+} on protein phosphatase activity was examined in membranes prepared from erythrocytes exposed for 60 min to 1 μM or 100 nM Pb^{2+} using an assay that measures the total activity of protein phosphatases I and II. A significant increase in enzyme activity was observed in the group treated with 1 μM Pb^{2+} but not in the group treated with 100 nM Pb^{2+} (Table 2).

DISCUSSION

The purpose of these studies was to determine whether, in human erythrocytes, Pb^{2+} increases the phosphorylation of proteins by activating protein kinase C. Previously our laboratory found that Pb^{2+} activates protein kinase C in immature microvessels from rat brain [11], and in bovine retinal endothelial cells and rat gliomas cells [12]. Although protein phosphorylation is usually the result of activation of protein kinase C, it may not occur after exposure to Pb^{2+}. Other processes might also be affected by Pb^{2+}, e.g. elevated phosphatase activity which would negate the effects of increased protein kinase C activity; alternatively, cofactors required for protein kinase C activity may not be available in Pb^{2+}-exposed cells. Nevertheless, increases in the phosphorylation of four membrane proteins, of molecular mass 120, 80, 52 and 45 kDa, were observed in erythrocytes stimulated with Pb^{2+} or PMA. Evidence from other studies on the phosphorylation of erythrocyte membrane proteins by protein kinase C suggests that, on the basis of molecular mass, the 80 kDa protein may be the erythrocyte membrane protein 4.1 and that the 55 kDa and 45 kDa proteins are subunits of dematin.

Three experiments support our conclusion that Pb^{2+} increased the phosphorylation of proteins of molecular mass 120, 80, 52 and 45 kDa by activating protein kinase C. First, Pb^{2+} and PMA stimulated the phosphorylation of similar proteins. PMA is a specific activator of protein kinase C [34] and several studies have shown the phosphorylation of proteins with similar molecular masses in erythrocytes treated with PMA [19,21]. Secondly, Pb^{2+} increased the amount of protein kinase C in membranes. Similarly, the localization of protein kinase C to the membrane appears to be important for the phosphorylation of other membrane proteins, such as MARCKS in fibroblasts [35] and macrophages [36] and vinculin in fibroblasts [37]. Targeting of protein kinase C to membranes is important for the phosphorylation of membrane proteins, since the enzyme's location probably defines its substrate. These phosphorylated proteins are localized to the membrane and their phosphorylation should require an interaction between protein kinase C and the membrane.

Thirdly, phosphorylation of the 120, 80 and 45 kDa proteins stimulated by Pb^{2+} did not occur if erythrocytes were depleted of protein kinase C by chronic treatment (72 h) with PDBu. Although inhibitors are also used often to demonstrate a role for protein kinase C, many are not specific and are not effective in all types of cells. For example, we were unable to inhibit translocation using the protein kinase C inhibitor chelerythrine chloride (L. Belloni-Olivi and J. P. Bressler, unpublished work). A disadvantage of using cells depleted of protein kinase C is that they recover by resynthesizing more enzyme; however, since erythrocytes do not synthesize proteins the loss of protein kinase C from erythrocytes is irreversible. The mechanism for down-regulation by PMA is not clear. In erythrocytes, down-regulation of protein kinase C is thought to require proteolytic cleavage by m-calpain at the V3 domain of the enzyme [38]. However, recent evidence from studies on expressed protein kinase C-α with amino acid substitutions in the V3 domain suggest that other proteinases besides m-calpain may also play a role [39].

The mechanism by which Pb^{2+} activates protein kinase C-α and increases protein phosphorylation in erythrocytes is not clear. Although protein kinase C-α requires Ca^{2+} and diacylglycerol for activity, we were unable to detect an increase in these intracellular messengers in Pb^{2+}-exposed cells. It is possible that the methods used in this study lacked the sensitivity to detect small changes in the levels of Ca^{2+} or diacylglycerol. On the other hand, activation may require a direct interaction between Pb^{2+} and protein kinase C. Diacylglycerol binds to protein kinase C-α and increases the affinity of the enzyme for Ca^{2+}. Ca^{2+} enables the enzyme to bind to acidic phospholipids [40,41], such as phosphatidylserine, a cofactor required for

enzymic activity. A high concentration of Ca^{2+} alone, however, may be sufficient to activate protein kinase C [34]. Since Pb^{2+} often interacts with proteins that bind Ca^{2+} [6,7], Pb^{2+} may activate protein kinase C-α by binding to this Ca^{2+} site. Additionally, another site on protein kinase C may be involved. There are two cysteine-rich sites in the C1 region of protein kinase C-α that bind zinc [42–44] and are likely to be important for binding diacylglycerol and/or phosphatidylserine. Indeed, an increase in the binding of protein kinase C to membranes was observed when zinc was added to homogenates of lymphocytes [45,46]. Since Pb^{2+} interacts with proteins that bind zinc [47], Pb^{2+} may activate protein kinase C-α in erythrocytes by binding to the zinc site.

Other enzymes, such as cAMP-dependent protein kinase, casein kinase and protein phosphatase, affect the phosphorylation of proteins in erythrocyte membranes. cAMP-dependent protein kinase [18–20] and casein kinase [16,17] phosphorylate proteins with molecular masses that are similar to those observed in this study. However, the activities of these protein kinases did not change in erythrocytes exposed to Pb^{2+}. Protein phosphatases may also affect the phosphorylation of erythrocyte membrane proteins. For example, an increase in the phosphorylation of spectrin was observed in erythrocytes treated with okidaic acid, an inhibitor of protein phosphatases I and IIA [48,49]. In our system, however, the contribution of protein phosphatases was minimal. A 100 nM concentration of Pb^{2+} increased protein phosphorylation but did not change protein phosphatase activity. Although we cannot rule out the possibility that other enzymes that regulate protein phosphorylation were affected by Pb^{2+}, at this time we conclude that Pb^{2+} increases protein phosphorylation only by activating protein kinase C.

In summary, the phosphorylation of four proteins with molecular masses of 120, 80, 55 and 45 kDa by protein kinase C was observed in erythrocytes exposed to Pb^{2+}. Although these studies were done *in vitro*, it is possible that these proteins are phosphorylated in animals exposed to Pb^{2+}. *In vivo* effects of low concentrations of Pb^{2+} in erythrocytes are potentially useful for the development of biomarkers, since a biomarker for exposure to low-level Pb^{2+} has not been reported. In addition, our studies have relevance to the neurotoxicity of Pb^{2+}. Proteins similar to those found on erythrocyte membranes are present in the central nervous system. For example, erythrocyte membrane protein 4.1 is very similar to synapsin [50], and dematin is associated with the synapse in rod photoreceptor cells. It is possible that the learning problems observed in Pb^{2+}-exposed children may be related to the level of phosphorylation of these proteins.

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