Conversion of human placental alkaline phosphatase from a high M_r form to a low M_r form during butanol extraction

An investigation of the role of endogenous phosphoinositide-specific phospholipases

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Alkaline phosphatase in a wide range of tissues has been shown to be anchored in the membrane by a specific interaction with the polar head group of phosphatidylinositol. It has previously been suggested that the production of low M_r alkaline phosphatase during the commonly used butanol extraction procedure may result from the activation of an endogenous phosphoinositide-specific phospholipase C which removes the 1,2-diacylglycerol responsible for membrane anchoring. This conversion process was investigated in greater detail with human placenta used as the source of alkaline phosphatase. M_r and hydrophobicity of the alkaline phosphatase were determined by gel filtration on TSK-250 and partitioning in Triton X-114, respectively. Alkaline phosphatase extracted from human placental particulate fraction with butanol at pH 5.4 or released by incubation with *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C produced a form of alkaline phosphatase of M_r approx. 170 000 and relatively low hydrophobicity. By contrast, the butanol extract prepared at pH 8.3 was an aggregated form of M_r approx. 600 000 and was relatively hydrophobic. The effect of a variety of inhibitors and activators on the amount of low M_r alkaline phosphatase produced during butanol extraction revealed that it was a Ca^{2+} - and thiol-dependent process. Proteinase inhibitors had no effect. [^3H]Phosphatidylinositol hydrolysis by the particulate fraction, unlike low M_r alkaline phosphatase production, was relatively sensitive to heat inactivation, indicating that the phosphoinositide-specific phospholipases C from cytosol and lysosomes were unlikely to be responsible for conversion. A butanol-stimulated activity which removed the [^3H]myristic acid from the variant surface glycoprotein ([^3H]mfVSG) of *Trypanosoma brucei* was detectable in the human placental particulate fraction. Since this activity was acid active, Ca^{2+} - and thiol-dependent and relatively heat stable, it may be the same as that responsible for production of low M_r alkaline phosphatase. The only ^3H -labelled product identified was phosphatidic acid, suggesting that the [^3H]mfVSG-cleaving activity is a phospholipase D. These data strongly support the proposal that production of low M_r alkaline phosphatase during butanol extraction is an autolytic process occurring as the result of an endogenous phospholipase. However, they also suggest that the lysosomal and cytosolic phosphoinositide-specific phospholipases C that have previously been described in many mammalian tissues are not responsible for this process.

INTRODUCTION

Alkaline phosphatase is a member of an unusual and functionally diverse group of membrane proteins which are all anchored to the membrane by a specific interaction with the polar head group of phosphatidylinositol. It has been suggested that the interaction between alkaline phosphatase and phosphatidylinositol is a covalent one (Low & Zilversmit, 1980b), a view that has recently been substantiated by direct chemical studies with other members of this group of membrane proteins (Ferguson *et al.*, 1985b; Futerman *et al.*, 1985; Tse *et al.*, 1985; for review see Low *et al.*, 1986).

A widely used method for solubilizing membrane-bound alkaline phosphatase is extraction of the tissues with butanol. This produces a soluble non-aggregating form of the enzyme (generally, a dimer of M_r approx. 130 000–170 000), which can then be purified by conventional chromatographic techniques in the complete absence of butanol or detergents. In a previous study

(Low & Zilversmit, 1980b) with pig kidney alkaline phosphatase we proposed that the solubilizing effect of butanol was in fact due to an autolytic process in which the butanol facilitated the degradation of the phosphatidylinositol in the hydrophobic membrane anchoring domain by an endogenous enzyme. We suggested that this enzyme might be a phosphoinositide-specific phospholipase C since enzymes of this specificity are widely distributed in mammalian tissues. These preliminary investigations (Low & Zilversmit, 1980b) indicated that this phospholipase C was Ca^{2+} -dependent and thus could be the same as, or related to, the cytosolic phosphoinositide-specific phospholipase C (for references see Shukla, 1982). By contrast, studies with rat liver alkaline phosphatase (Kominami *et al.*, 1985; Miki *et al.*, 1985) suggested that the phospholipase C, responsible for an apparently similar autolytic conversion during butanol extraction, was lysosomal in origin and presumably Ca^{2+} -independent (Irvine *et al.*, 1977, 1978).

Alkaline phosphatase is released from the tissues into

Abbreviation used: mfVSG, membrane form of the variant surface glycoprotein of *Trypanosoma brucei*; PI-PLC, phosphatidylinositol-specific phospholipase C.

the serum in a variety of pathological and physiological situations and in humans the amount and tissue origin of serum alkaline phosphatase is utilized for the clinical diagnosis of several diseases (McComb *et al.*, 1979; Moss, 1982). We therefore suggested (Low & Weglicki, 1981) that the alkaline phosphatase might be released *in vivo* from the tissues into the serum by activation of the same endogenous phospholipase C that appeared to be responsible for conversion during butanol extraction (Low & Zilversmit, 1980b). A similar proposal has recently been made by Ikehara and co-workers (Kominami *et al.*, 1985; Miki *et al.*, 1985). However, in spite of this, it is difficult to visualize how a phosphoinositide-specific phospholipase C could be responsible for releasing alkaline phosphatase from the external surface of the plasma membrane since these phospholipases are believed to be located in the cytoplasmic surface of the plasma membrane, the cytosol or the lysosome (Shukla, 1982).

In the present study we have investigated the proposed involvement of phosphoinositide-specific phospholipase C in the conversion of human placental alkaline phosphatase from a membrane-bound to a soluble, low M_r form during butanol extraction. From detailed studies of the effect of various activators and inhibitors of this process we have confirmed that conversion occurs by the action of an endogenous enzyme. However, we have also obtained evidence to distinguish this activity from both the cytosolic and lysosomal phosphoinositide-specific phospholipases C previously described in mammalian tissues.

MATERIALS AND METHODS

Materials

A crude particulate fraction of human placenta was prepared by a modification of the procedure described by Doellgast *et al.* (1977). An individual human term placenta (frozen immediately after delivery) was thawed, its membranous and fibrous tissue discarded, the remainder chopped with scissors and then homogenized in an equal volume of 0.25 M-sucrose/5 mM-MgCl₂/24 mM-KCl/50 mM-Tris/HCl, pH 7.5 (1 ml/g of tissue), with a Waring blender at full speed for 1 min. The homogenate was centrifuged at 6000 *g* for 10 min, the supernatant filtered through two layers of Miracloth (Calbiochem) and then re-centrifuged at 150 000 *g* for 40 min. The resulting pellet was washed with the homogenizing buffer (0.3 ml/g of tissue) and finally resuspended with the same buffer to give a protein concentration of approximately 25–35 mg/ml. The resuspended pellets were stored at –20 °C until required. The specific activity of alkaline phosphatase was generally in the range 10–20 μ mol/min per mg of protein.

Phosphatidylinositol labelled with [³H]inositol was prepared from rat liver microsomal fraction as described by Low & Zilversmit (1980a). The membrane form of the variant surface glycoprotein (mfVSG) of *Trypanosoma brucei* labelled with [9,10-³H]myristic acid was prepared by the method of Ferguson & Cross (1984) and was a gift from Dr. M. A. J. Ferguson at The Rockefeller University, New York, NY, U.S.A.

Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from *Staphylococcus aureus* by a

Fig. 1. Release of alkaline phosphatase from human placental particulate fraction by phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*

Human placental particulate fraction (0.025–0.035 mg of protein/ml) was incubated in the presence of 20 mM-Hepes/NaOH, pH 7.0, and PI-PLC from *S. aureus* at the concentrations indicated for 30 min at 37 °C (□). Some incubation mixtures also contained 0.15 M-NaCl (■). The incubation mixtures were centrifuged at 150 000 *g* for 40 min and the supernatants assayed for released alkaline phosphatase activity. Released activity is expressed as a percentage of total alkaline phosphatase activity determined in samples of the incubation mixture. Data from a typical experiment are shown, one of three giving similar results. Mean values ($\pm$ S.E.M.) for control (i.e. no PI-PLC) and maximal release of alkaline phosphatase were respectively $6.7 \pm 1.2\%$ and $66.6 \pm 1.7\%$ for incubations in the absence of NaCl; the corresponding values for incubations in the presence of NaCl were $1.6 \pm 0.2\%$ and $68.1 \pm 0.7\%$. In these experiments, changes in total activity as a result of incubation either in the presence or absence of PI-PLC were not observed.

slight modification of the previously published method (Low, 1981). In this paper the term phosphatidylinositol-specific phospholipase C (PI-PLC) is used exclusively for referring to the *Staphylococcus aureus* enzyme, since it has been shown to be able to hydrolyse phosphatidylinositol and its glycosylated derivatives, but is apparently unable to hydrolyse the polyphosphoinositides (Ferguson *et al.*, 1985b). The mammalian phospholipases C which hydrolyse phosphatidylinositol are collectively referred to as phosphoinositide-specific phospholipases C, since the cytosolic and membrane-associated activities from several tissues are also able to hydrolyse the polyphosphoinositides, phosphatidylinositol 4-phosphate and 4,5-bisphosphate, and similar data regarding the specificity of the lysosomal phospholipases C are not currently available. The Amberlite CG-50 eluate (derived from one 4-litres batch of culture supernatant) was mixed with sufficient solid (NH₄)₂SO₄ to give 95% saturation (0.65 g/ml of eluate), stirred for approximately 60 min at 0 °C and centrifuged at 20 000 *g* for 60 min. The pellets were dissolved in 9 ml of 50 mM-Tris/acetate/0.02%

Fig. 2. Gel-filtration chromatography of human placental alkaline phosphatase

Samples of alkaline phosphatase solubilized from human placental particulate fraction was applied to a TSK-250 gel-filtration column, eluted and fractions were assayed for alkaline phosphatase activity as described in the Materials and methods section. Alkaline phosphatase was solubilized by the following procedures: (a) treatment with PI-PLC (*S. aureus*) as described in the legend to Fig. 1 (■); extraction with butanol (at 0 °C for 10 min with 45 mM-Hepes/NaOH, pH 7.0, buffer) as described in the Materials and methods section (□); (b) extraction with butanol at pH 5.4 (■) or 8.3 (□). V_0 is void volume.

(w/v) sodium azide, pH 7.4, and dialysed twice against 500 ml of the same buffer. The dialysate was further purified by gel-filtration chromatography on Sephadex G-75 using two 2.5 cm × 120 cm columns connected together; the flow rate was 12 ml/h and 17 ml fractions were collected.

Substrates, inhibitors and other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise indicated.

Butanol extraction of placental particulate fraction

Placental particulate fraction (1.25–1.75 mg of protein) and buffer in a total volume of 0.65 ml were mixed with 0.35 ml of ice-cold butan-1-ol and incubated at 25 °C for 15 min; unless mentioned otherwise, 40 mM-Tris/maleate buffers were used. The mixture was cooled on ice and centrifuged at 12000 *g* for 30 min. A 0.4 ml sample of the lower, aqueous phase was centrifuged for 5 min at 500 *g* through a 2 ml mini-column of Bio-Gel P6DG (Bio-Rad Laboratories, Richmond, CA, U.S.A.) which had previously been equilibrated with buffer A (0.15 M-NaCl/0.1 mM-MgCl₂/0.01 mM-zinc acetate/10 mM-Hepes/NaOH, pH 7.0) in order to remove the butanol. The extract was then diluted approx. 20–50-fold (depending on the specific activity of the starting material) in buffer A before its properties were determined as described below. Approx. 100% of the original alkaline phosphatase activity was recovered in the aqueous phase of the butanol extraction; no activity (i.e. less than 0.5% of recovered activity) was detectable in the butanol phase. Recovery of alkaline phosphatase in mini-column eluates was generally 50–80% of applied activity. Proteinase and phospholipase inhibitors and other agents were mixed with the placental particulate fraction and buffer before addition of the butanol, except in the case of phenylmethanesulphonyl fluoride, which was dissolved in the butanol. The total amount of alkaline phosphatase activity extracted by butanol in these studies was not substantially affected by the

conditions of extraction (i.e. pH, presence of inhibitors etc.).

Gel-filtration chromatography

Gel-filtration chromatography of alkaline phosphatase (0.1 ml portion appropriately diluted in buffer A) was done at 4 °C using a 7.5 mm × 75 mm Bio-Sil TSK-125 guard column connected to a 7.5 mm × 300 mm Bio-Sil TSK-250 column (both columns from Bio-Rad Laboratories) equilibrated with buffer A. Two elution procedures were used: (i) for high resolution (i.e. Figs. 1 and 2) the column was eluted at a flow rate of 0.2 ml/min and 0.2 ml fractions were collected; (ii) for rapid, lower resolution separations the column was eluted at a flow rate of 0.5 ml/min and 0.6 ml fractions were collected. This latter procedure was utilized for determining the proportion of alkaline phosphatase in soluble form; activities eluting after 6.2–8.6 ml and 8.6–11.0 ml were used to quantify the high and low *M_r* forms of butanol-extracted alkaline phosphatase respectively.

Triton X-114 phase separation

A simplified version of the procedure described by Bordier (1981) was utilized to separate the forms of alkaline phosphatase based on their hydrophobicity. A 0.3 ml portion of alkaline phosphatase (appropriately diluted in buffer A) was mixed with 0.2 ml of 1% (w/v) Triton X-114 (pre-condensed against buffer A; Bordier, 1981) in 1.5 ml conical Microfuge tubes and incubated on ice for 5 min. The tubes were then incubated at 37 °C for 10 min with thorough mixing after 5 min. The two phases were separated by centrifugation at 12000 *g* in a Beckman Microfuge 12 (rotor warmed to 37 °C) for 2 min. A 0.25 ml sample of the upper, aqueous phase was removed and the remainder discarded. The tubes were returned to the 37 °C incubator and the lower, Triton X-114 enriched phase was washed free of residual upper phase by carefully adding 0.4 ml of buffer A (warmed to 37 °C) without mixing. The tubes were re-centrifuged and

the upper phase was discarded. The lower phase (volume approximately 0.03 ml) was redissolved with 0.47 ml of buffer A and incubation (with mixing) at 0 °C for approx. 5 min. Although the cloud point for Triton X-114 is about 20 °C (see Bordier, 1981) we have found it necessary to maintain samples above 30 °C in order to obtain reproducible distribution of alkaline phosphatase between the upper and lower phases. Samples of the upper and lower phase were assayed for alkaline phosphatase as described below, except that it was necessary to add 0.05 ml of 1% sodium deoxycholate (Bordier, 1981) to the samples before measuring absorbance in order to reduce turbidity due to Triton X-114. The presence of Triton X-114 during the assay had no apparent effect on alkaline phosphatase activity.

Alkaline phosphatase assay

Alkaline phosphatase activity was determined by incubating enzyme samples with 5 mM-*p*-nitrophenyl phosphate in 1 mM-MgCl₂/1 M-diethanolamine, pH 10, at 37 °C in a total volume of 0.5 ml. The reaction was stopped with 1.5 ml of 1 M-NaOH/5 mM-EDTA and the amount of *p*-nitrophenol produced was determined by measurement of *A*₄₁₀.

Phospholipase assays

Phospholipase activity was measured in human placental particulate fractions by two procedures. (i) Hydrolysis of [³H]inositol-labelled phosphatidylinositol was determined essentially as described by Low & Weglicki (1983) except that 50 mM-Tris/maleate was used as buffer and no deoxycholate was present; incubation was for 60 min at 25 °C or 37 °C with 0.05–0.14 mg of protein. (ii) Hydrolysis of [³H]myristate-labelled mfVSG was determined by a modified version of the method of Ferguson *et al.* (1985b). Placental particulate fractions (0.05–0.14 mg of protein), 40 mM-Tris/maleate buffer and [³H]mfVSG (approx. 25000 d.p.m./assay) in a total volume of 0.26 ml were mixed with 0.14 ml of butanol and incubated at 25 °C or 37 °C for 60 min. The reaction was stopped with 1.1 ml of toluene/acetic acid (10:1, v/v) and 0.74 ml of water; the phases were separated by centrifugation and a 0.5 ml sample of the upper phase was removed for determination of radioactivity. This assay also contained 80 mM-urea, 0.2 mM-NH₄HCO₃ and 0.01% (w/v) NP-40, components contributed by the buffer used for purification and storage of [³H]mfVSG.

RESULTS

Release of alkaline phosphatase from human placental particulate fraction by phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*

Incubation of human placental particulate fraction with PI-PLC from *S. aureus* (0.01 µg/ml) causes the release of approx. 60% of the total alkaline phosphatase (Fig. 1). The remainder appeared to be resistant to release since increasing the concentration of PI-PLC approx. 100-fold (Fig. 1) did not increase the amount of alkaline phosphatase released by the PI-PLC. The sensitivity of alkaline phosphatase to release was markedly reduced by the inclusion of 0.15 M-NaCl in the incubation mixture (Fig. 1), but this did not have a substantial effect on the maximum amount that could be released. Alkaline phosphatase activity released from human placental particulate fraction by the PI-PLC was

Table 1. Phase separation of human placental alkaline phosphatase in Triton X-114

Various types of alkaline phosphatase preparation were analysed by phase separation in Triton X-114 as described in the Materials and methods section: (i) the untreated placental particulate fraction, (ii) supernatants from placental particulate fractions treated with PI-PLC from *S. aureus* (see the legend to Fig. 1), (iii) butanol extracts prepared from the placental particulate fraction at the pH values indicated, (iv) alkaline phosphatase extracted with butanol at pH 6.0 and separated into the high and low *M_r* forms by gel-filtration chromatography as described in the Materials and methods section. Values (means of duplicate separations) are the activity, expressed as a proportion of the total recovered activity, that was present in the aqueous phase of the Triton X-114 phase separation and are from a typical experiment, one of three giving similar results.

Source of alkaline phosphatase	Alkaline phosphatase in aqueous phase (% of recovered activity)
Placental particulate fraction	2.5
PI-PLC released	99.4
pH 5.4 butanol extract	84.0
pH 6.0 butanol extract	51.5
pH 8.3 butanol extract	< 0.5
High <i>M_r</i> form	9.0
Low <i>M_r</i> form	93.5

analysed by high performance gel-filtration chromatography on a TSK-250 column. The activity eluted from the column as a single peak with an *M_r* of approx. 170000 (Fig. 2).

The hydrophobicity of the released alkaline phosphatase was assessed by determining its distribution in a two-phase Triton X-114/water system (Bordier, 1981). The released alkaline phosphatase was found to partition exclusively into the relatively hydrophilic aqueous phase and the alkaline phosphatase in the untreated particulate fraction was mainly found in the relatively hydrophobic, Triton-enriched phase (Table 1). These data indicate, on the basis of sensitivity to release by PI-PLC (see Shukla, 1982) and the molecular size and hydrophobicity of the released enzyme (Low & Zilvermit, 1980b; Kominami *et al.*, 1985), that human placental alkaline phosphatase is similar to the other major types of alkaline phosphatase. Thus, even though much evidence indicates that these other types are coded by distinct genes (Harris, 1982) the mode of membrane anchoring appears to be essentially the same.

Characteristics of alkaline phosphatase extracted from human placental particulate fraction with butanol

Gel filtration analysis of alkaline phosphatase extracted from human placental particulate fraction by butanol at pH 7.0 (Fig. 2a), revealed the presence of two peaks of activity: one of these peaks eluted close to the void volume (*M_r* approx. 600000), the other eluted with an *M_r* of approx. 170000 (these are subsequently referred to as the high *M_r* and low *M_r* forms of alkaline phosphatase, respectively). The relative amounts of activity detected in these two peaks was markedly dependent on the pH at which the butanol extraction was

Fig. 3. Effect of phosphatidylinositol-specific phospholipase C on M_r of butanol-extracted human placental alkaline phosphatase

Human placental particulate fraction was extracted at pH 8.8 as described in the Materials and methods section. Alkaline phosphatase (0.01 ml), *S. aureus* PI-PLC (14 μ g) and 1 ml of buffer A were incubated at 37 °C for 60 min. A sample (0.1 ml) was applied to the TSK-250 gel-filtration column, eluted and fractions were assayed for alkaline phosphatase activity (■); control incubation with no PI-PLC added (□). V_0 is void volume.

done (Fig. 2b). Thus, after extraction at pH 5.4, most of the activity was in the low M_r form whereas at pH 8.3 most of the activity was in the high M_r form.

The hydrophobicity of the butanol-extracted alkaline phosphatase was determined by using Triton X-114 (Bordier, 1981). With the pH 5.4 butanol extract, most of the activity was in the aqueous phase but, for the pH 8.3 extract, most of the activity was in the detergent phase (Table 1). This result suggested that the high M_r form produced by extraction at high pH is relatively hydrophobic and the low M_r form produced by extraction at low pH is hydrophilic. This suggestion was confirmed in an experiment where the extraction was done at an intermediate pH, to give approximately equal amounts of the two forms (i.e. pH 6.0); these were separated by gel filtration and the hydrophobicity was determined individually (Table 1). As predicted, with the high M_r form the activity was largely found in the detergent phase and with the low M_r form it was in the aqueous phase. Approx. 9% of the activity in the high M_r form (prepared from a pH 6.0 butanol extract) was found in the aqueous phase, compared with less than 0.5% for an unfractionated extract prepared at pH 8.3 (Table 1). This may be due to the presence of a small proportion of hydrophilic alkaline phosphatase of relatively high M_r , e.g. similar to the tetrameric form released from liver plasma membranes by PI-PLC or butanol (Chakrabarty & Stinson, 1985).

The low M_r form of alkaline phosphatase produced by butanol extraction is similar in M_r and hydrophobicity

to the alkaline phosphatase released from membranes by PI-PLC (Fig. 2 and Table 1), and substantial amounts of the high M_r form could, in fact, be converted to the low M_r form by incubation of butanol-extracted alkaline phosphatase with PI-PLC from *S. aureus* (Fig. 3). However, as with direct release of alkaline phosphatase from the placental particulate fraction (Fig. 1), conversion of the high M_r form to the low M_r form by the *S. aureus* PI-PLC was incomplete; approx. 40% of the activity appeared to be resistant to the PI-PLC (Fig. 3). These data suggested that the low M_r form might be produced by the action of an endogenous phosphoinositide-specific phospholipase C on the high M_r form as proposed in previous studies with butanol-extracted alkaline phosphatase from pig kidney (Low & Zilversmit, 1980b) and rat liver (Kominami *et al.*, 1985; Miki *et al.*, 1985).

Identification of the enzyme activity responsible for production of soluble alkaline phosphatase during butanol extraction

In order to assess possible involvement of a phosphoinositide-specific phospholipase C in the conversion of alkaline phosphatase to a low M_r form during

Fig. 4. Effect of pH on butanol extraction of human placental alkaline phosphatase

Samples of human placental particulate fraction were analysed by the following procedures: (i) alkaline phosphatase was extracted with butanol at the pH indicated in the Figure and analysed by gel-filtration chromatography as described in the Materials and methods section. The proportion of the eluted activity that was present as the low M_r form was used as an estimate of the endogenous enzyme responsible for its production and this value is given in the Figure. The lowest pH used was 4.6, since alkaline phosphatase is unstable at low pH. Values (▲) are means ($\pm$ S.E.M.) for four to six experiments done with particulate fractions prepared from a total of six different placentas. Values without error bars were means of two experiments. (ii) [3 H]Phosphatidylinositol hydrolysis (■) and [3 H]mfVSG hydrolysis (●) were determined (see the Materials and methods section) at the pH indicated in the Figure. Values are means of duplicate determinations in a single experiment, one of three giving similar results. For clarity, hydrolysis data in this Figure were normalized with respect to activities obtained at pH 5.0 (●) or pH 5.4 (■), which were arbitrarily set at 100%.

Fig. 5. Effect of preincubation temperature on butanol extraction of human placental alkaline phosphatase

Human placental particulate fraction (diluted 5-fold in water) was preincubated at the temperature indicated for 15 min, cooled on ice and samples were analysed by the following procedures: (i) alkaline phosphatase was extracted with butanol at pH 6.0 or 6.5 (three experiments each) and analysed by gel-filtration chromatography as described in the Materials and methods section (▲). The proportion of the eluted activity that was present as the low M_r form was used as an estimate of the endogenous enzyme activity responsible for its production. The total amount of alkaline phosphatase extracted was not significantly affected by the preincubation, consistent with the high thermal stability of this enzyme. (ii) Hydrolysis of [³H]phosphatidylinositol (■) was assayed at pH 5.0 or 6.5 (two experiments each) or pH 6.0 (one experiment). (iii) Hydrolysis of [³H]mfVSG (●) was assayed at pH 5.0, 37 °C or pH 6.0, 25 °C (two experiments each) in the presence of butanol as described in the Materials and methods section. All values ($\pm$ S.E.M.) in the Figure are expressed as a proportion of that obtained with a control particulate fraction which was preincubated at 0 °C.

butanol extraction, the properties of the two activities were compared. Unfortunately, it was not possible to measure these reactions under identical conditions since the substrate for the phospholipase C assay (i.e. [³H]inositol-labelled phosphatidylinositol) was not hydrolysed by human placental particulate fraction in the presence of butanol. Separate experiments indicated that this was most likely to be due to a combination of the substrate partitioning into the butanol phase and a direct effect of the butanol dissolved in the aqueous phase on the activity of the phospholipase C itself. Consequently, it was necessary to carry out the assays in which [³H]phosphatidylinositol was used as substrate (Fig. 4; Table 2) in the absence of butanol. As a more appropriate substrate, we have made use of the [³H]myristate-labelled variant surface glycoprotein ([³H]mfVSG) of *T. brucei*. This protein, which appears to be anchored to the membrane by a similar mechanism to alkaline phosphatase (Low *et al.*, 1986), has the advantage that it is readily biosynthetically labelled with [³H]myristate and thus can be used as a convenient substrate for comparing phospholipase activity and the conversion of alkaline phosphatase to the low M_r form in the presence of butanol. Incubation of this

substrate with the placental particulate fraction resulted in the release of ³H radioactivity from the [³H]mfVSG into the organic phase. In contrast with the [³H]phosphatidylinositol substrate, hydrolysis of [³H]mfVSG was markedly stimulated by butanol; less than 10% of the maximum activity was detectable in the absence of butanol.

The effect of a variety of experimental conditions on hydrolysis of these two radioactive substrates and on conversion of alkaline phosphatase to the low M_r form were compared (Table 2; Figs. 4 and 5). All three activities were markedly increased at pH values below 7, although [³H]phosphatidylinositol hydrolysis, in contrast with the two other activities, showed a distinct maximum at pH 5.5 (Fig. 4). The activities were also inhibited by *p*-hydroxymercuriphenylsulphonic acid and EGTA. The inhibitory effect of EGTA seemed to be due to Ca²⁺ removal, since addition of this ion in 50% molar excess over the EGTA significantly reduced the inhibition observed. However, in the case of [³H]phosphatidylinositol hydrolysis this Ca²⁺ addition gave significant stimulation over the original control activity; an effect that was not observed in assays with the other two substrates. The effects of trifluoperazine and chlorpromazine were more complex; neither of these agents had an effect on conversion of alkaline phosphatase to a low M_r form but completely inhibited [³H]phosphatidylinositol hydrolysis. The results with [³H]mfVSG hydrolysis were somewhat variable; trifluoperazine either stimulated or had no effect whereas chlorpromazine gave partial inhibition.

Since human placental alkaline phosphatase is a relatively heat stable enzyme, sensitivity to thermal inactivation could be used as an additional method for comparing the different activities (Fig. 5). It was observed that hydrolysis of phosphatidylinositol was markedly reduced by preincubation at temperatures as low as 45 °C and was almost completely inhibited at temperatures of 55 °C and greater. By contrast [³H]mfVSG hydrolysis and conversion of alkaline phosphatase was relatively unchanged at temperatures up to 60 °C. However, after preincubations above this temperature there was a decline in alkaline phosphatase conversion that was not exhibited when [³H]mfVSG hydrolysis was measured (Fig. 5). These results appear to confirm the observations made with the inhibitory agents, i.e. that a substantial proportion of the enzyme activity involved in converting alkaline phosphatase to a low M_r form had properties very similar to the one involved in hydrolysing [³H]mfVSG, but was distinct from the bulk of the activity hydrolysing [³H]phosphatidylinositol.

A wide range of proteinase inhibitors was also tested for their effect on conversion of alkaline phosphatase to the low M_r form, but no inhibition was observed (Table 2). It is possible that other enzymes that are insensitive to these inhibitors are responsible for the conversion. However, the failure of leupeptin to inhibit conversion certainly seems to eliminate the involvement of Ca²⁺-dependent proteinases.

The product of [³H]mfVSG hydrolysis by placental particulate fraction was analysed by thin-layer chromatography on silica gel. In solvent systems for neutral lipids, i.e. light petroleum/diethyl ether/acetic acid (70:30:2 or 50:40:2, by vol.) the ³H radioactivity produced remained at the origin, indicating that the

Table 2. Effect of inhibitors on butanol extraction of placental alkaline phosphatase

Samples of human placental particulate fraction were analysed by the following procedures: (i) the samples were mixed with a variety of inhibitors at the concentrations indicated, alkaline phosphatase was extracted with butanol at pH 6.0 and analysed by gel-filtration chromatography as described in the Materials and methods section. Duplicate extractions were done in each experiment. The proportion of the eluted alkaline phosphatase activity that was present as the low M_r form was used as an estimate of the endogenous enzyme activity responsible for its production. This activity is presented relative to controls (i.e. no additions) done in each experiment; the proportion of low M_r alkaline phosphatase in these controls was in the range of 45–90% of total eluted activity. The mean values ($\pm$ S.E.M.) derived from three or four separate experiments are given in the Table. (ii) [3 H]Phosphatidylinositol and [3 H]mfVSG hydrolysis were determined at pH 6.0 in the presence or absence of inhibitors at the concentrations indicated, as described in the Materials and methods section. Proteinase inhibitors: a mixture containing aprotinin (0.77 mg/ml final concentration), bacitracin (0.3 mg/ml), benzamidine (0.077 mg/ml), pepstatin (0.77 mg/ml) and soybean trypsin inhibitor (0.077 mg/ml). Phenylmethanesulphonyl fluoride (0.34 mg/ml) was also included but it was dissolved in the butanol used for the extraction. Numbers of experiments are shown in parentheses.

Additions	Relative activity (% of control)		
	Conversion of alkaline phosphatase to low M_r form	mfVSG hydrolysis	Phosphatidylinositol hydrolysis
No additions	100	100	100
1.5 mM- <i>p</i> -Hydroxymercuriphenylsulphonic acid	75.9 $\pm$ 9.1 (4)	45.8 $\pm$ 9.6 (3)	10.7
3.0 mM- <i>p</i> -Hydroxymercuriphenylsulphonic acid	45.4 $\pm$ 6.6 (4)	31.6 $\pm$ 10.5 (3)	10.4
2.0 mM-Trifluoperazine	105.3 $\pm$ 13.0 (3)	113.6 $\pm$ 15.2 (3)	0
2.0 mM-Chlorpromazine	97.2 $\pm$ 5.2 (3)	56.6 $\pm$ 18.3 (3)	0
1.6 mM-EGTA	22.7 $\pm$ 3.3 (3)	0	19.2
1.6 mM-EGTA/2.4 mM-CaCl ₂	92.1 $\pm$ 3.5 (3)	63.0 $\pm$ 11.4 (3)	207.5
1.9 mM-Leupeptin	103.6 $\pm$ 6.0 (4)	—	—
Proteinase inhibitors	97.2 $\pm$ 7.7 (4)	—	—

product was not myristic acid, 1,2- or 1,3-dimyristoylglycerol or monomyristoylglycerol (although significant amounts of [3 H]myristic acid were present as a contaminant of the [3 H]mfVSG used in the experiments). In chloroform/methanol/acetic acid/water (75:45:12:4, by vol.) most of the product co-migrated with phosphatidylethanolamine and was well separated from phosphatidylinositol. In this solvent the product had a significantly lower mobility than the phosphatidic acid standard (derived from egg yolk phosphatidylcholine); however, in subsequent experiments it was found that dimyristoyl phosphatidic acid also moved significantly slower than this standard. In chloroform/methanol/acetic acid/water (80:13:8:0.3, by vol.) and chloroform/methanol/30% aq. NH₃ soln. (65:25:4, by vol.) most of the product had a mobility similar to that of the phosphatidic acid standard. As a control in these studies the product of hydrolysis of [3 H]mfVSG by *S. aureus* PI-PLC in the presence of butanol was also analysed by these techniques and found to be exclusively 1,2-diacylglycerol.

The conclusion that [3 H]phosphatidic acid was the product of [3 H]mfVSG hydrolysis by placental particulate fraction was strengthened by the finding that the product was insensitive to nitrous acid or acetic anhydride/pyridine (1:1, v/v) treatment. Nitrous acid has previously been shown to cleave the glycosidic linkage between inositol and glucosamine as a result of a deamination of the free amino group on glucosamine liberating [3 H]phosphatidylinositol (Ferguson *et al.*, 1985b); this was confirmed under the conditions used in the present study. Thus, if the hydrolysis had occurred in the structure linking the inositol ring to the VSG protein, i.e. by action of a glycosidase or proteinase, then

the product of hydrolysis would either be [3 H]phosphatidylinositol itself or be converted to [3 H]phosphatidyl-inositol by a subsequent nitrous acid deamination. Similarly, treatment with acetic anhydride rapidly acetylates the inositol ring of phosphatidylinositol with a consequent increase in mobility. Since neither of these treatments appeared to have an effect on the mobility of the 3 H-labelled product we tentatively conclude that the enzyme is cleaving the phosphodiester linkage to the diacylglycerol by a phospholipase D type hydrolysis. Definitive identification of the initial hydrolysis product is, however, complicated by the possibility of several competing reactions. For example, phospholipases D in higher plants are well known for their ability to transphosphatidylate aliphatic or polar alcohols (Yang *et al.*, 1967). In this situation, the product obtained in our experiments would have been phosphatidylbutanol. However, even though phosphatidic acid and the transphosphatidylated alcohols have been reported to be difficult to resolve by thin-layer chromatography in acidic solvent systems, some difference in mobility would be expected in the basic solvent system utilized here.

Another possibility is the involvement of a phospholipase C in the initial cleavage of [3 H]mfVSG followed by phosphorylation of the 1,2-diacylglycerol produced either by an endogenous diacylglycerol kinase or by the alkaline phosphatase itself operating as a phosphotransferase (McComb *et al.*, 1979). Preliminary experiments in which these competing reactions were blocked by the addition of 1,2-diacylglycerol or 5'-adenylylimidodiphosphate or by preincubation of the particulate fraction at 65 °C for 15 min gave no detectable accumulation of 1,2-[3 H]diacylglycerol. Definitive identification of the site of hydrolysis and substrate specificity

of this phospholipase, however, will require purification in order to free it of these likely contaminating enzyme activities.

DISCUSSION

The results presented here are consistent with our previous studies (Low & Zilversmit, 1980b) made with pig kidney, which showed that alkaline phosphatase extracted from membranes with butanol can exist (after removal of the butanol) in two forms: a hydrophobic, aggregated form of high M_r and a hydrophilic, low M_r form. The high M_r form can be converted to the low M_r form by bacterial PI-PLC or by an endogenous enzyme which becomes active during the butanol extraction process at acidic pH. Recent studies by Ikehara and co-workers (Kominami *et al.*, 1985; Miki *et al.*, 1985) with rat liver alkaline phosphatase suggests the occurrence of a similar process in this tissue also. In their study, the high M_r and low M_r forms were distinguished on the basis of mobility in polyacrylamide gel electrophoresis under non-denaturing conditions. Even though they did not present any data directly relating to the hydrophobicity of the two forms, it seems likely that the observed differences in mobility were due to aggregation of the hydrophobic form. The similarity of the results obtained in these three studies with different tissue sources of alkaline phosphatase further support the claim (Low & Zilversmit, 1980b) that the butanol-extraction technique, which is widely used for alkaline phosphatase solubilization, is an autolytic process. Furthermore, the extreme sensitivity of this process to pH in the acid to neutral range could lead to the production of variable amounts of the two forms of alkaline phosphatase, depending on the exact conditions of the butanol extraction. In this context it is relevant to note that butanol extractions are often done on whole tissue homogenates in which the pH is not controlled.

Miki *et al.* (1985) suggested, on the basis of an acid pH optimum for the production of the low M_r form of rat liver alkaline phosphatase, that the enzyme responsible was the lysosomal phosphoinositide-specific phospholipase C. This conclusion seemed rather premature in view of the fact that the cytosolic phosphoinositide-specific phospholipases C in mammalian tissues also have a relatively acidic pH optimum (Shukla, 1982). In the present study, using a crude particulate fraction, an acid pH optimum for conversion was observed also but since the conversion was Ca^{2+} -dependent as well, the lysosomal phosphoinositide-specific phospholipase C (which is Ca^{2+} -independent; Irvine *et al.*, 1977, 1978) is unlikely to be responsible; in the studies with the liver alkaline phosphatase the effect of Ca^{2+} chelators on conversion from a high M_r form to a low M_r form was not reported (Miki *et al.*, 1985). These investigators did observe converting activity in a lysosomal membrane fraction but since the activity was also present in a plasma membrane fraction the subcellular distribution of this activity is at present difficult to assess.

It is possible that the enzymes responsible for the conversion process are different in the two tissues; however, in this regard it should be noted that in both liver and placenta addition of excess bivalent cations or of cationic drugs had no effect on the conversion process even though these agents have marked effects on the cytosolic and lysosomal phosphoinositide-specific phospho-

lipases C (Shukla, 1982). Furthermore, in our previous study with pig kidney alkaline phosphatase, inhibition of conversion was obtained by inclusion of EDTA (Low & Zilversmit, 1980b); it therefore seems likely that the same enzyme is responsible for conversion in all three tissues. This enzyme is probably present in heart and platelets also, since these tissues have been reported to contain deoxycholate-stimulated activities which convert alkaline phosphatase or acetylcholinesterase from a membrane-bound to a soluble form (Low & Weglicki, 1981; Majumdar & Balasubramanian, 1985).

Previous work with human placental alkaline phosphatase has also revealed that it can exist in two forms which differ markedly with regard to the size of the native enzyme and hydrophobicity. However, SDS/polyacrylamide gel electrophoresis, N-terminal sequence analysis and peptide mapping did not reveal any differences in subunit size or primary structure (Doellgast *et al.*, 1977; Abu-Hasan & Sutcliffe, 1984a,b). Furthermore, no differences were detected in the response of these forms to the proteinase subtilisin (Abu-Hasan & Sutcliffe, 1984b), which cleaves the small (i.e. 2 kDa) domain from the C-terminus that is believed to be responsible for anchoring alkaline phosphatase to the membrane (Jemmerson *et al.*, 1984, 1985). These earlier data and the present findings are quite consistent with the view that alkaline phosphatase is attached to the membrane via covalent linkage with a phosphatidylinositol molecule. Although detailed structural information is not yet available for alkaline phosphatase, it seems likely that this linkage between the protein and the phosphatidylinositol involves an ethanolamine- and glucosamine-containing glycan (for review, see Low *et al.*, 1986). We now propose that the high M_r , hydrophobic form of alkaline phosphatase is an aggregate which forms when the membrane lipids are removed by butanol extraction; the aggregate may also contain other membrane proteins (Doellgast *et al.*, 1977; Abu-Hasan & Sutcliffe, 1984a). The alkaline phosphatase molecules in the aggregate are identical with the original membrane-bound form of the enzyme in that they retain intact the covalently attached phosphatidylinositol molecule. The low M_r form, by contrast, has lost this hydrophobic anchor as the result of enzymic degradation and is most likely to be a dimer of two 67 kDa subunits.

Although the results of the present study are consistent with previous work, the present more detailed analysis has produced two unexpected findings. The first was our ability to inhibit the majority (i.e. more than 95%) of the [^3H]phosphatidylinositol hydrolysing activity in the placental particulate fraction without substantially affecting the production of the low M_r form of alkaline phosphatase or the hydrolysis of [^3H]myristate-labelled mfVSG from *T. brucei*. This suggested that the activity responsible for conversion of alkaline phosphatase to the low M_r form and for hydrolysing mfVSG might be due to the same enzyme. However, at present, the results do not completely exclude the alternative possibility that conversion of alkaline phosphatase to the low M_r form was due to a minor form of phosphoinositide-specific phospholipase C, since this enzyme has been found to exist in multiple molecular forms in many mammalian tissues (Low *et al.*, 1984). The endogenous trypanosomal enzyme that converts mfVSG to the soluble form was previously shown to be a phospholipase C (Ferguson *et al.*, 1985a), and consequently the existence of a similar

enzyme activity in mammalian tissue seemed likely. The observation that the product of mfVSG hydrolysis by the placental enzyme was not 1,2-diacylglycerol was therefore unexpected. The major product was tentatively identified as phosphatidic acid, and since no evidence for phosphorylation of 1,2-diacylglycerol by contaminating enzymes was obtained, the possibility that this placental mfVSG-cleaving enzyme has the bond specificity of a phospholipase D must be considered. If the enzyme in human placenta responsible for conversion of alkaline phosphatase from a high M_r to a low M_r form is also a phospholipase D, it seems to be unrelated to other phospholipases that have previously been described. Phospholipases D and 'base exchange' activities have been reported in mammalian tissues (for references see van den Bosch, 1982) and recent studies implicate a phospholipase D in the hydrolysis of inositol phospholipids in neutrophils stimulated with fMet-Leu-Phe (Cockcroft & Allan, 1985). However, until the alkaline phosphatase converting activity described here has been purified and its specificity determined in the absence of butanol and contaminating lipids and enzymes, its relationship to these other activities and to the phosphoinositide-specific phospholipases C will remain unclear.

This work was supported by a grant from the National Institutes of Health (GM 35873) and by a grant-in-aid from the American Heart Association with funds contributed in part by the Oklahoma Affiliate. A.S.M. also acknowledges the support of the OMRF Fleming Scholar Program. We are grateful to Ms. Chris Leaver for technical assistance, Ms. Julie Butler for artwork, and Dr. M. A. J. Ferguson for invaluable discussions and for supplying [^3H]mfVSG.

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Received 18 March 1986/9 July 1986; accepted 15 August 1986