Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C

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Renal dipeptidase (dehydropeptidase-I, EC 3.4.13.11) was released from pig kidney membrane preparations by treatment with phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* and *Bacillus thuringiensis* and a phospholipase C preparation from *Bacillus cereus* to a similar extent as alkaline phosphatase. Endopeptidase-24.11 and aminopeptidase N were not released by this treatment. After treatment of the membrane fraction with the *S. aureus* phospholipase C the dipeptidase was converted from an amphipathic to a hydrophilic form, as deduced from phase-separation experiments in Triton X-114. It is concluded that renal dipeptidase is anchored to the microvillar membrane by covalently attached phosphatidylinositol.

INTRODUCTION

Several plasma membrane proteins appear to be anchored in the lipid bilayer via a C-terminal glycolipid moiety containing covalently attached phosphatidylinositol (reviewed in Low *et al.*, 1986; Low, 1987). These comprise proteins of widely divergent functions. Some are ectoenzymes: alkaline phosphatase (EC 3.1.3.1), acetylcholinesterase (EC 3.1.1.7), 5'-nucleotidase (EC 3.1.3.5) and trehalase (EC 3.2.1.28) (Low *et al.*, 1986; Low, 1987; Takesue *et al.*, 1986). Others are surface proteins without known enzyme activity, e.g. Thy-1 glycoprotein (Low & Kincade, 1985), a neural-cell adhesion molecule (He *et al.*, 1986), decay-accelerating factor of complement (Medof *et al.*, 1986) and the variant surface glycoprotein of *Trypanosoma brucei* (Ferguson *et al.*, 1985). A characteristic feature of such proteins is the ability of phosphatidylinositol-specific phospholipase C (PI-PLC) to cleave the hydrophobic membrane anchor, releasing selectively a soluble, hydrophilic form of the protein (Futerman *et al.*, 1985; Low *et al.*, 1986). There may also exist endogenous mechanisms for controlling the release of soluble forms of this family of membrane proteins (Malik & Low, 1986).

Renal dipeptidase (dehydropeptidase-I, microsomal dipeptidase; EC 3.4.13.11) is a microvillar membrane enzyme which catalyses the hydrolysis of a wide range of dipeptides (Campbell, 1970; Kozak & Tate, 1982). It also exhibits β -lactamase activity, can hydrolyse the β -lactam antibiotic, imipenem (Kropp *et al.*, 1982) and is characteristically inhibited by cilastatin (Kahan *et al.*, 1983). Like alkaline phosphatase, the dipeptidase is associated with the 'Triton-insoluble fraction' of kidney microvilli (Kim & Campbell, 1983), but its mode of association with the plasma membrane is unknown. Here we show that renal dipeptidase can be released from a pig

kidney microvillar membrane preparation by treatment with PI-PLCs from several bacterial sources. Treatment of the dipeptidase with *Staphylococcus aureus* PI-PLC converted the enzyme from an amphipathic to a hydrophilic form as deduced from phase-separation experiments in Triton X-114. Renal dipeptidase therefore represents the first example of a membrane peptidase that is anchored via covalently attached phosphatidylinositol. In contrast, the ectoenzymes endopeptidase-24.11 (EC 3.4.24.11), and aminopeptidase N (EC 3.4.11.2) were unaffected by treatment with bacterial PI-PLC.

MATERIALS AND METHODS

Materials

Pig kidneys were obtained from ASDA Farm Stores, Lofthousegate, W. Yorkshire, U.K. Cilastatin (MK0791, monosodium Z-S-[6-carboxy-6-[(2,2-dimethyl-(s)-cyclopropyl)carbonyl]amino]-5-hexenyl]-L-cysteine) was a gift from Dr. H. Kropp, Merck Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). PI-PLC from *S. aureus* was purified as described previously (Malik & Low, 1986). PI-PLC from *B. thuringiensis* was purified by a modification (M. G. Low, unpublished work) to the original procedure reported by Taguchi *et al.* (1980). Both preparations were in excess of 90% purity as judged by SDS/polyacrylamide-gel electrophoresis. Units of PI-PLC activity are $\mu\text{mol/min}$. *B. cereus* phospholipase C type III, *Clostridium perfringens* phospholipase C type XII, phospholipase A₂ (bee venom), phospholipase D (cabbage), glycyl-D-phenylalanine and Triton X-114 were purchased from Sigma Chemical Co. Triton X-114 was pre-condensed before use (Bordier, 1981).

Abbreviations used: IC₅₀, concentration of inhibitor producing 50% inhibition; PI-PLC, phosphatidylinositol-specific phospholipase C.

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Subcellular fractionation of pig kidney cortex and solubilization of membrane proteins by phospholipases

All operations were carried out at 4 °C unless stated otherwise. Approx. 100 g of pig kidney cortex was homogenized in 1 litre of 0.33 M-sucrose/50 mM-Hepes, pH 7.5. The resultant homogenate was centrifuged at 8000 *g* for 15 min, the supernatant was decanted off and further centrifuged at 26000 *g* for 2 h. The microsomal pellet was resuspended in 10 mM-Hepes, pH 7.0, and centrifuged at 31000 *g* for 1.5 h. The pellets were resuspended in the appropriate buffer and an aliquot of the phospholipase preparation was added. After solubilizing protein by shaking at 37 °C for 30–120 min, the incubation mixtures were centrifuged at 31000 *g* for 1.5 h.

Enzyme activities were determined in the total incubation mixture after solubilization but before centrifugation, and in the supernatant after centrifugation at 31000 *g*, and the solubilized activity was expressed as a percentage of the total activity in the original incubation mixture.

Enzyme assays

Dipeptidase activity was assayed by a novel h.p.l.c. method using glycyl-D-phenylalanine (Gly-D-Phe, 1.0 mM final concentration) as substrate in 0.1 M-Tris/HCl/0.3 M-NaCl/10 μ M-ZnCl₂, pH 8.3 (100 μ l final reaction volume). After incubating at 37 °C for 10–30 min, samples were heated at 100 °C for 4 min and centrifuged at 15000 *g*. Hydrolysis of Gly-D-Phe was monitored by reverse phase h.p.l.c. on a μ Bondapak C₁₈ column with a u.v. (214 nm) detector at a flow rate of 1.5 ml/min and a 15 min gradient of 4.5–30% (v/v) acetonitrile in 0.08% H₃PO₄ at pH 2.5, followed by 5 min elution at the final conditions. The product, D-

phenylalanine, elutes approx. 2 min earlier than the substrate, Gly-D-Phe, (retention times of approx. 6.20 min and 8.40 min respectively) and can be quantified by calibration from a standard curve. The specific activity of the homogenate was 12.7 nmol of Gly-D-Phe hydrolysed/min per mg, and of the microsomal membrane pellet 41.3 nmol/min per mg.

Alkaline phosphatase was assayed by the spectrophotometric method of Bessey *et al.* (1946) using *p*-nitrophenyl phosphate as substrate. Endopeptidase-24.11 and aminopeptidase N were assayed as described previously (Matsas *et al.*, 1985). Protein was determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

A dipeptidase activity is released from pig kidney membranes by bacterial PI-PLC

A dipeptidase activity hydrolysing Gly-D-Phe was released from pig kidney membranes by purified preparations of PI-PLC from *S. aureus* and *B. thuringiensis* (Figs. 1a and 1c) and by *B. cereus* phospholipase C (Fig. 1b). Alkaline phosphatase was released in a similar manner (Fig. 1) in agreement with previous results (Low & Finean, 1977; Takesue *et al.*, 1986). No release of two other membrane peptidases, endopeptidase-24.11 and aminopeptidase N, was detectable using any of the PI-PLCs (Fig. 1). As reported by Malik & Low (1986), complete release of alkaline phosphatase was not observed. Table 1 shows the effects of other phospholipases, Triton X-100 and trypsin on the release of the various membrane proteins, and demonstrates the relative resistance of the dipeptidase to solubilization by Triton X-100 and trypsin.

Fig. 1. Release of membrane enzymes from a pig kidney membrane fraction by PI-PLCs

Pig kidney membrane fraction (approx. 8.0 mg of protein/ml) was incubated as follows. (a) With 10 mM-Hepes, pH 7.0, and *S. aureus* PI-PLC for 30 min at 37 °C. Data from a typical experiment are shown, one of two giving similar results. The values for control (i.e. no PI-PLC) and maximal (0.1 μ g of PI-PLC/ml) release of alkaline phosphatase varied by 0.05% and 3.15% of total release respectively between the two experiments. (b) With 10 mM-sodium phosphate/0.1 M-NaCl, pH 7.0, and *B. cereus* phospholipase C for 2 h at 37 °C. Data from a typical experiment are shown, one of two giving similar results. The values for control and maximal (0.1 unit of phospholipase C/ml) release of alkaline phosphatase varied by 0.05% and 0.60% of total release respectively between the two experiments. (c) With 10 mM-Hepes, pH 7.0, and *B. thuringiensis* PI-PLC for 30 min at 37 °C. Only one such experiment was performed. In these experiments, no changes in total activity as a result of incubation either in the presence or absence of phospholipase were observed, except for alkaline phosphatase in the case of the *B. cereus* phospholipase C where a decrease in activity was observed in the total incubation mixture (27.5% of control). ○, Alkaline phosphatase; △, dipeptidase; □, endopeptidase-24.11, aminopeptidase N and protein, except in (b) where □, endopeptidase-24.11 and aminopeptidase N; ■, protein.

Table 1. Effect of phospholipases and Triton X-100 on the release of enzymes from a pig kidney membrane fraction

Samples of pig kidney membrane fraction (approx. 8.0 mg of protein/ml) were incubated in the presence of the following components under the conditions stated. Buffers used: (a) 10 mM-Tris/HCl/10 mM-CaCl₂, pH 7.0; (b) 10 mM-sodium acetate/50 mM-CaCl₂/30 mM-NaCl, pH 6.2; (c) 10 mM-sodium phosphate/0.1 M-NaCl, pH 7.0; (d) 10 mM-Hepes, pH 7.0; (e) 10 mM-sodium phosphate, pH 7.0. Except for the Triton X-100, all other components were used at two or more different concentrations, and the maximal release is given. No changes in total activity as a result of the incubations were observed, except in the case of alkaline phosphatase using *B. cereus* PLC (see legend to Fig. 1). Abbreviation: n.d., not determined.

Condition (buffer used in parentheses)	Incubation		Release (% of total membrane activity)				
	Time (min)	Temperature (°C)	Protein	Alkaline phosphatase	Dipeptid- ase	Aminopep- tidase N	Endopeptid- ase-24.11
None	120	37	5.7	0.0	2.5	0.5	0.9
<i>C. perfringens</i> phospholipase C (0.1 units/ml) (a)	120	37	10.3	1.0	3.1	2.8	0.7
Bee venom phospho- lipase A ₂ (5.0 units/ml) (a)	120	37	8.0	0.5	0.0	0.8	0.6
Cabbage phospho- lipase D (5.0 units/ml) (b)	120	37	5.8	3.2	1.1	0.4	0.5
<i>B. cereus</i> phospho- lipase C (0.1 units/ml) (c)	120	37	13.2	85.9	63.4	0.7	3.6
<i>S. aureus</i> PI-PLC (1.0 µg/ml) (d)	60	37	17.9	76.3	82.4	0.6	0.6
<i>B. thuringiensis</i> PI-PLC (10 munits/ ml) (d)	60	37	19.4	79.0	61.7	1.3	n.d.
20% Triton X-100 (e) (7:1 detergent: protein ratio)	60	4	52.5	20.6	27.1	64.1	58.8
Trypsin (1 mg/ml) (e) (1:10 trypsin: protein ratio)	60	37	13.5	0.3	5.7	5.3	0.0

Table 2. Triton X-114 phase-separation of pig kidney membrane enzymes

Pig kidney membranes (470 µg of protein) which had been incubated with 10 mM-Hepes, pH 7.0 for 1 h at 37 °C in either the absence (a) or presence (b) of *S. aureus* PI-PLC (1.0 µg/ml), and the 31000 g supernatant after such treatment with *S. aureus* PI-PLC (c) were made up to 200 µl with 10 mM-Tris/150 mM-NaCl/1.0% Triton X-114, pH 7.4, and subjected to phase-separation at 30 °C for 3 min as described by Bordier (1981). The detergent-rich and detergent-poor phases were separated through a sucrose cushion by centrifugation at 3000 g and assayed in duplicate for enzyme activities. The results are the mean (±S.E.M.) for four phase separations. Activities recovered in the detergent-rich phase are expressed as a percentage of the total activity. Under these conditions, 76% of alkaline phosphatase and 82% of the dipeptidase activities were released into the supernatant. Abbreviation: n.d., not determined.

	Enzyme activity in detergent-rich phase (% of total activity)			
	Alkaline phosphatase	Dipeptidase	Aminopeptidase N	Endopeptidase-24.11
(a) Untreated membranes	94.7 ± 1.1	96.1 ± 1.7	90.1 ± 0.5	88.2 ± 1.0
(b) PI-PLC treated membranes	8.2 ± 1.8	11.1 ± 3.5	91.6 ± 1.1	89.8 ± 1.3
(c) PI-PLC released supernatant	4.2 ± 2.3	3.5 ± 2.1	n.d.	n.d.

Phase-separation of soluble and membrane forms of the dipeptidase in Triton X-114

Samples of pig kidney membranes were analysed by phase-separation in Triton X-114 as described in the Materials and methods section, and the detergent-rich and detergent-poor phases were assayed for enzyme

activities (Table 2). The dipeptidase and alkaline phosphatase activities partitioned predominantly (> 93%) into the detergent-rich phase when pig kidney membranes which had been incubated for 1 h at 37 °C were treated with Triton X-114 (Table 2a). However, on incubating the membranes under similar conditions but in the presence of 1.0 µg of *S. aureus* PI-PLC/ml, both

the dipeptidase and alkaline phosphatase were found to partition predominantly (> 86%) into the detergent-poor phase upon treatment with Triton X-114 (Table 2b). Under identical conditions, endopeptidase-24.11 and aminopeptidase N remained in the detergent-rich phase after PI-PLC treatment and phase-separation (Tables 2a and 2b). The soluble fraction released by *S. aureus* PI-PLC was also subjected to phase-separation in Triton X-114 and assayed for dipeptidase and alkaline phosphatase activities (Table 2c). The soluble forms of both enzymes were now found predominantly (> 95%) in the detergent-poor phase.

Inhibition profile of the dipeptidase activity released by PI-PLC

Gly-D-Phe was selected as a substrate for the dipeptidase since this dipeptide is not hydrolysed by aminopeptidases which are abundant in the microvillar membrane. Table 3 shows the effects of various inhibitors on the Gly-D-Phe hydrolysing activity released by PI-PLC. The activity was that of a metallopeptidase, being inhibited by 1,10-phenanthroline and EDTA. Dithiothreitol also caused substantial inhibition (Kozak & Tate, 1982). The specific inhibitor of renal dipeptidase, cilastatin (Kahan *et al.*, 1983), potently inhibited the dipeptidase activity released by PI-PLC (Table 3 and Fig. 2). The inhibition curves were indistinguishable whether activity was measured in the membrane fraction or in the solubilized preparation and similar IC_{50} values were obtained (1.01 ± 0.08 and $0.67 \pm 0.05 \mu M$ respectively), comparable with the value reported by Kahan *et al.* (1983). By these criteria, therefore, the released activity can be equated with renal dipeptidase.

General conclusions

Several investigators have shown that alkaline phosphatase can be released from kidney membranes by *S. aureus* and *B. thuringiensis* PI-PLC (Low & Finean, 1977; Takesue *et al.*, 1986), the enzyme being converted from an amphipathic to a hydrophilic form with

Fig. 2. Inhibition by cilastatin of dipeptidase released by *S. aureus* PI-PLC

A sample of the supernatant released on incubation of pig kidney membranes with *S. aureus* PI-PLC (1 $\mu g/ml$, 60 min at 37 °C), approx. 1 μg of protein, was incubated with Gly-D-Phe (10 mM) for 15 min at 37 °C in the absence or presence of various concentrations of cilastatin. Data from a typical experiment are shown, one of three giving similar results.

Table 3. Effect of inhibitors on the dipeptidase activity released by *S. aureus* PI-PLC

A sample of the supernatant (approx. 1 μg of protein) released on incubation of pig kidney membranes with *S. aureus* PI-PLC (1 $\mu g/ml$, 60 min at 37 °C), was incubated with Gly-D-Phe (10 mM) for 20 min at 37 °C in the absence and presence of inhibitors. A 10 min preincubation with inhibitor was carried out at room temperature. In the absence of any inhibitors, approx. 10% substrate breakdown was observed. The results are the means ($\pm$ S.E.M.) for four determinations.

Inhibitor	Relative activity (%)
None	100
1.0 mM-1,10-Phenanthroline	52.0 $\pm$ 2.5
10 mM-EDTA	56.8 $\pm$ 5.4
0.1 mM-Dithiothreitol	61.9 $\pm$ 6.3
1.0 mM-Dithiothreitol	13.7 $\pm$ 1.3
1.0 mM- <i>p</i> -Chloromercuribenzoate	114.4 $\pm$ 4.8
0.1 mM-Di-isopropylphosphofluoridate	113.4 $\pm$ 10.6
10 μM -Cilastatin	13.5 $\pm$ 2.5

concomitant loss of the hydrophobic membrane anchor (Low *et al.*, 1986). In this study we have shown that PI-PLC from *S. aureus* and *B. thuringiensis* can also release a dipeptidase from pig kidney membranes in a similar fashion (Fig. 1). This release was apparently specific for PI-PLC, as negligible release was observed with a broad-specificity phospholipase C (*C. perfringens*), phospholipase A₂ or phospholipase D (Table 1). The release of dipeptidase by the commercial preparation of broad-specificity phospholipase C from *B. cereus* is probably due to contamination with the PI-PLC also produced by this bacterium (Ferguson *et al.*, 1985). A similar *B. cereus* phospholipase C preparation has been shown to convert *Torpedo* acetylcholinesterase from an amphipathic to a hydrophilic form (Stieger *et al.*, 1986); this protein is known to be anchored to the membrane by a covalently attached phosphatidylinositol moiety.

The amphipathic or hydrophilic character of untreated and PI-PLC released enzymes was assessed by phase-separation in Triton X-114 (Bordier, 1981; Matsas *et al.*, 1985). Phase-separation of untreated membranes resulted in the majority of the dipeptidase and alkaline phosphatase activities partitioning into the detergent-rich phase (Table 2a), indicative of an amphipathic nature for both enzymes. When the activities released from the membrane by *S. aureus* PI-PLC were treated with Triton X-114, both dipeptidase and alkaline phosphatase now partitioned into the detergent-poor phase (Tables 2b and 2c). This implies that both enzymes had been converted

into soluble, hydrophilic forms consistent with the loss of a hydrophobic membrane-anchoring domain after treatment with PI-PLC.

Throughout this study, endopeptidase-24.11 and aminopeptidase N were monitored in parallel. Both these ectoenzymes are anchored in the membrane through a sequence of hydrophobic amino acids at the *N*-terminus (Kenny & Maroux, 1982; Fulcher *et al.*, 1986). The failure of these two enzymes to be released from the membrane, or converted to a hydrophilic form, by bacterial PI-PLC (Fig. 1 and Table 2) serves to emphasize the specificity of the releasing effects of PI-PLC. Both endopeptidase-24.11 and aminopeptidase N could be solubilized readily by Triton X-100, whereas the dipeptidase was much more resistant to solubilization (Table 1). This apparent resistance of renal dipeptidase to solubilization with Triton X-100 has led Kim & Campbell (1983) to suggest that the enzyme is not located primarily at the luminal surface of the microvillar membrane. However, alkaline phosphatase, an established microvillar membrane ectoenzyme (Kenny & Maroux, 1982), is equally resistant to Triton X-100 solubilization (Table 1). The release of both enzymes by treatment with bacterial PI-PLC suggests that renal dipeptidase, like alkaline phosphatase, is anchored in the microvillar membrane by covalently attached phosphatidylinositol.

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REFERENCES

- Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946) *J. Biol. Chem.* **184**, 321–329
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Campbell, B. J. (1970) *Methods Enzymol.* **19**, 722–729
- Ferguson, M. A. J., Low, M. G. & Cross, G. A. M. (1985) *J. Biol. Chem.* **260**, 14547–14555
- Fulcher, I. S., Pappin, D. J. C. & Kenny, A. J. (1986) *Biochem. J.* **240**, 305–308
- Futerman, A. H., Low, M. G., Michaelson, D. M. & Silman, I. (1985) *J. Neurochem.* **45**, 1487–1494
- He, H.-T., Barbet, J., Chaix, J.-C. & Goridis, C. (1986) *EMBO J.* **5**, 2489–2494
- Kahan, F. M., Kropp, H., Sundelof, J. G. & Birnbaum, J. (1983) *J. Antimicrob. Chemother.* **12** Suppl. D, 1–35
- Kenny, A. J. & Maroux, S. (1982) *Physiol. Rev.* **62**, 91–128
- Kim, H. S. & Campbell, B. J. (1983) *J. Membr. Biol.* **75**, 115–122
- Kozak, E. M. & Tate, S. S. (1982) *J. Biol. Chem.* **257**, 6322–6327
- Kropp, H., Sundelof, J. G., Hajdu, R. & Kahan, F. M. (1982) *Antimicrob. Agents Chemother.* **22**, 62–70
- Low, M. G. (1987) *Biochem. J.* **244**, 1–13
- Low, M. G. & Finean, J. B. (1977) *Biochem. J.* **167**, 281–284
- Low, M. G. & Kincade, P. W. (1985) *Nature (London)* **318**, 62–64
- Low, M. G., Ferguson, M. A. J., Futerman, A. H. & Silman, I. (1986) *Trends Biochem. Sci.* **11**, 212–215
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Malik, A.-S. & Low, M. G. (1986) *Biochem. J.* **240**, 519–527
- Matsas, R., Stephenson, S. L., Hryszko, J., Kenny, A. J. & Turner, A. J. (1985) *Biochem. J.* **231**, 445–449
- Medof, M. E., Walter, E. I., Roberts, W. L., Haas, R. & Rosenberry, T. L. (1986) *Biochemistry* **25**, 6740–6747
- Stieger, A., Carsoso de Almeida, M.-L., Blatter, M.-C., Brodbeck, V. & Bordier, C. (1986) *FEBS Lett.* **199**, 182–186
- Taguchi, R., Yoshinari, Y. & Ikezawa, H. (1980) *Biochim. Biophys. Acta* **619**, 48–57
- Takesue, Y., Yokota, K., Nishi, Y., Taguchi, R. & Ikezawa, H. (1986) *FEBS Lett.* **201**, 5–8

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