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BIS-(4-METHYLUMBELLIFERYL) PHOSPHATE AS A SUBSTRATE FOR THE SURFACE MEMBRANE-ASSOCIATED PHOSPHODIESTERASE ACTIVITY OF PIG PLATELETS

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Summary

It was found that the newly-available compound, bis-(4-methylumbelliferyl) phosphate, could be used as a substrate for the pig platelet surface membrane-associated phosphodiesterase activity, usually assayed with bis-(*p*-nitrophenyl) phosphate. This enzyme activity is distinct from the phosphodiesterase activity towards 5′-dTMP-*p*-nitrophenyl ester, which is probably associated with intracellular membrane structures in platelets. Consequently, the use of the 4-methylumbelliferyl derivative as substrate for the phosphodiesterase activity provides a sensitive, fluorimetric assay for this marker enzyme of the platelet surface membrane.

The platelet surface membrane is known to respond to certain haemostatic stimuli such as thrombin, fibrillar collagen, ADP and 5-hydroxytryptamine. However, little is known of the molecular nature of the membrane-associated receptors and/or transducers for these stimuli. This is due, in part, to the problems involved in isolating pure preparations of platelet surface membranes and to the lack of satisfactory marker enzyme activities for the various membrane structures of platelets; the classically accepted surface membrane marker enzymes (e.g. 5′-nucleotidase, alkaline phosphatase, L-leucine β-naphthylamidase, (Na^+—K^+)-ATPase) are either absent or detectable with only low activities in platelet fractions. However, we have shown previously that a phosphodiesterase activity towards bis-(*p*-nitrophenyl) phosphate was associated with membrane vesicles isolated from pig platelet homogenates [1,2]. Further, this enzyme activity appeared to be associated with the surface membrane, since its distribution in subfractionation experi-

ments was found to correspond to a population of membrane vesicles which could be labelled by specific surface membrane-labelling techniques [3]. This preparation of surface membranes can be clearly distinguished from a second membrane subfraction that appears to be intracellular in origin and has phosphodiesterase activity to 5′-dTMP-*p*-nitrophenyl ester associated with it [2]. Bis-(*p*-nitrophenyl) phosphate phosphodiesterase has also been reported to be associated with the surface membrane of human platelets [4,5].

To investigate a possible role for the surface located phosphodiesterase in the platelet's haemostatic response, we have attempted to solubilise and purify the enzyme activity. As part of this study, we investigated the utility of the newly-available substrate, bis-(4-methylumbelliferyl) phosphate, since the increased sensitivity of a fluorimetric assay for the released 4-methylumbelliferone, compared with the colourimetric assay for *p*-nitrophenol, would allow enzyme purifications to be followed more easily starting from small amounts of membrane protein.

The isolation and homogenisation of pig platelets, and the preparation of a fraction of platelet membranes (M) were carried out as described previously [2]. The separation of M into subfractions MI (surface membrane) and MII (probably intracellular) was carried out by the zonal rotor procedure previously reported [2].

The phosphodiesterase (EC 3.1.4.1) activities using *p*-nitrophenyl derivatives were assayed routinely as follows: up to 0.2 ml of enzyme sample was incubated at 37° C for 30 min –2 h in a final volume of 1.0 ml containing (a) 1.0 μmol bis -(*p*-nitrophenyl) phosphate (Sigma Ltd.) and 0.2 mmol sodium acetate, buffered to pH 5.5 with glacial acetic acid; (b) 1.0 μmol 5′-dTMP-*p*-nitrophenyl ester (Boehringer Corporation) and 0.8 ml of a buffer system containing 3 mmol sodium acetate, 120 mmol sodium succinate, 120 mmol sodium maleate and 120 mmol Tris per l, adjusted with HCl to pH 7.9. Assays were terminated by the addition of 2.0 ml 0.2 M NaOH and the released *p*-nitrophenol was determined spectrophotometrically at 410 nm. Protein was determined by the Folin procedure [6].

The activity using the 4-methylumbelliferyl substrate was assayed routinely by a procedure similar to that described by Robinson and Willcox [7] for acid phosphatase, as follows: up to 0.1 ml of diluted enzyme was incubated at 37° C for up to 30 min in a final volume of 1.0 ml containing 1.0 μmol bis-(4-methylumbelliferyl) phosphate (Koch-Light Laboratories Ltd., Cat. No. 6997 h) and 0.2 mmol sodium acetate, buffered to pH 5.5 with glacial acetic acid. The assay was terminated by the addition of 2.0 ml of 0.5 M glycine/NaOH buffer (pH 10.4) and the fluorescence of the liberated ionised 4-methylumbelliferone (excitation maximum, 360 nm; emission maximum, 448 nm) was measured in a Locarte LF 4 fluorimeter or a Perkin-Elmer model 203 fluorescence spectrophotometer, using quinine sulphate (4 μg/ml in 50 mM H_2SO_4) as a fluorescence standard. Enzyme samples were diluted sufficiently so as to liberate up to 5 nmol of 4-methylumbelliferone.

The pH dependence of the phosphodiesterase activity towards the 4-methylumbelliferyl substrate was determined using platelet homogenates

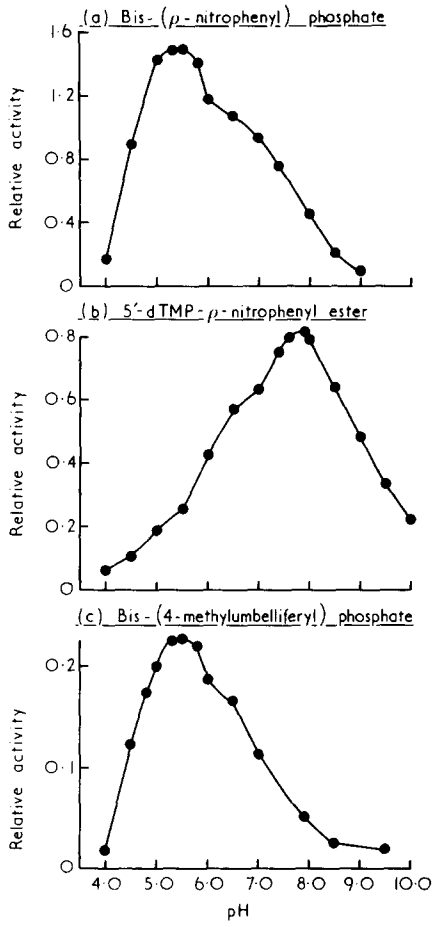

Fig. 1. pH dependence of platelet membrane phosphodiesterase activities. The whole platelet membrane fraction (M) was prepared as described in the text and the buffer used was the acetate/succinate/maleate/Tris·HCl system used for the routine assay of 5'-dTMP-*p*-nitrophenyl ester phosphodiesterase. The activities are expressed as μmol of product released/h per ml of membrane preparation.

and the whole membrane fraction (M) and was found to correspond exactly with that of the bis-(*p*-nitrophenyl) phosphate activity (Fig. 1), i.e. a broad peak of activity between pH 4.5 and 7.0 with the optimum at pH 5.5. The phosphodiesterase activity towards 5'-dTMP-*p*-nitrophenyl ester has a pH optimum of 7.9.

The K_m values for the activities towards bis-(*p*-nitrophenyl) phosphate and bis-(4-methylumbelliferyl) phosphate were determined at pH 5.5, using platelet whole membrane fraction, and were found to be 0.19 mM and 2.55 mM respectively. Bis-(4-methylumbelliferyl) phosphate was found to inhibit competitively the activity towards bis-(*p*-nitrophenyl) phosphate with an apparent K_i of 2.85 mM. Conversely, the 5'-dTMP-*p*-nitrophenyl ester phosphodiesterase activity of the platelet whole membrane fraction was not inhibited by the 4-methylumbelliferyl substrate.

The effects of several inhibitors on the phosphodiesterase activities associated with pig platelet membranes were investigated. It was found that the activities towards bis-(*p*-nitrophenyl)- and bis-(4-methylumbelliferyl) phosphates were inhibited similarly, viz. both activities were inhibited approx. 50% by 40 mM sodium potassium tartrate and 5 mM dithiothreitol (final concentrations) but only slightly by 10 mM sodium fluoride. Conversely, the 5′-dTMP-*p*-nitrophenyl ester phosphodiesterase activity was totally inhibited by 10 mM sodium fluoride (50% inhibition obtained with 0.6 mM fluoride), but was unaffected by tartrate or dithiothreitol.

As described previously [2], the basis for the subfractionation of pig platelet membranes into a surface membrane-enriched fraction (MI) and a second fraction, tentatively identified as intracellular in origin (MII), is the separation of the two phosphodiesterase activities towards bis-(*p*-nitrophenyl) phosphate and 5′-dTMP-*p*-nitrophenyl ester, respectively. The distribution of activity towards bis-(4-methylumbelliferyl) phosphate parallels very closely that of the bis-(*p*-nitrophenyl) phosphate activity in such membrane subfractionations, and, as shown in Table I, there is a very similar degree of enrichment of the two activities in MI with respect to homogenate and whole membrane fraction.

As part of our studies to isolate specific platelet membrane proteins, we have found that the surface membrane-associated phosphodiesterase activity can be solubilised by incubating membrane material (1 mg of protein/ml) at 37°C for 1 h in Triton X-100 (final concentration, 0.5%) in 10 mM Tris·HCl, pH 7.4. Under these conditions, approx. 60% of the membrane protein was not sedimentable after centrifugation at 100 000 × g_{av} for 60 min, whereas 95—100% of the phosphodiesterase activity towards both bis-(*p*-nitrophenyl) phosphate and bis-(4-methylumbelliferyl) phosphate was solubilised.

Our findings, therefore, imply that the same phosphodiesterase is active on both bis-(*p*-nitrophenyl) phosphate and bis-(4-methylumbelliferyl)

TABLE I

SPECIFIC ACTIVITIES OF PHOSPHODIESTERASES ASSOCIATED WITH PIG PLATELET MEMBRANE FRACTIONS

The preparation of the platelet homogenate (H), whole membrane fraction (M) and the two membrane subfractions (MI and MII) is described in the text. Values quoted are specific activities expressed as μmol of product released/h per mg protein; values in parentheses refer to the enrichment of enzyme activity relative to the homogenate.

Substrate	Specific activity			
	H	M	MI	MII
Bis-(4-methylumbelliferyl) phosphate	0.019	0.065 (3.42)	0.138 (7.26)	0.074 (3.89)
Bis-(*p*-nitrophenyl) phosphate	0.118	0.466 (3.95)	0.888 (7.53)	0.451 (3.82)
5′-dTMP-*p*-nitrophenyl ester	0.067	0.202 (3.01)	0.157 (2.34)	0.472 (7.04)

phosphate; this is supported strongly by the close similarity between K_m for the 4-methylumbelliferyl compound and the K_i for its competitive inhibition against the bis-(*p*-nitrophenyl) activity. Recently, we have achieved a partial purification of the enzyme (Taylor, D.G. and Crawford, N., unpublished work), using the Triton X-100-solubilised membrane protein, on Sepharose 4B. The activity towards the two substrates was eluted as a single peak by 10 mM Tris·HCl, pH 7.4 containing 0.5% Triton X-100. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis of this activity peak showed considerable purification over the whole membrane fraction, with the prominent 95 000 dalton band [2] present as the major component.

In conclusion, the use of bis-(4-methylumbelliferyl) phosphate as substrate for the pig platelet membrane phosphodiesterase activity provides a sensitive, fluorimetric means of locating surface membrane fragments in fractionation studies, and preliminary investigations have shown that this enzyme activity has a similar surface location in human platelets. There are several advantages in using the 4-methylumbelliferyl substrate instead of the *p*-nitrophenyl compound, since, although the K_m for the bis-(4-methylumbelliferyl) phosphate is an order of magnitude greater, the increased sensitivity allows the analysis of very small amounts of membrane protein. This substrate is now used routinely in this laboratory in monitoring purification procedures for the surface membrane-associated phosphodiesterase.

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