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ABSENCE OF 5′-NUCLEOTIDASE IN PORCINE POLYMORPHONUCLEAR LEUCOCYTE MEMBRANES

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A fraction enriched in plasma membranes from porcine polymorphonuclear leucocytes, isolated by sucrose density centrifugation was shown to possess considerable AMP hydrolysing activity (150 nmol/min per mg protein). However all of this activity could be inhibited using excess *p*-nitrophenyl phosphate in the incubation medium. Furthermore the hydrolysis of AMP by the membrane was unaffected by the 5′-nucleotidase inhibitor α,β-methyleneadenosine diphosphate and by the lectin concanavalin A, another potent inhibitor of 5′-nucleotidase. An antibody against mouse liver 5′-nucleotidase also did not inhibit the activity. These results suggest that the hydrolysis of AMP by porcine polymorph membranes is not accomplished by a specific 5′-nucleotidase and the necessity for distinguishing between true 5′-nucleotidase and non-specific phosphatase activity is discussed.

Introduction

The enzyme 5′-nucleotidase is the best known and most widely used marker enzyme for the plasma membrane in a large variety of cell types. In the polymorphonuclear leucocyte (PMNL) its existence appears to depend upon the species. In the guinea-pig it has been shown to be exclusively an ectoenzyme, whose activity can be distinguished from other less-specific phosphatases present in the cell membrane [1,2]. PMNLs isolated from the peritoneum of the rabbit [3] also show 5′-nucleotidase activity. There are conflicting results for human PMNLs [4]. Although several reports have appeared describing AMP hydrolysing activity in human polymorphs, the activity may be due to non-specific acid phosphatase [5] or alkaline phosphatase [6]. In more recent work with ^{32}P-labelled AMP as substrate [7] and using a specific inhibitor of 5′-nucleotidase [8] there is evidence that human PMNLs contain little or no 5′-nucleotidase. On the other hand other workers [9] have detected significant 5′-nucleotidase activity in human neutrophil homogenates, although this is possibly, at least in part, due to contaminating mononuclear cells.

There have been no other investigations of 5′-nucleotidase in polymorphonuclear cells from other species. In this paper membranes from porcine PMNLs have been prepared and their endogenous phosphate activity investigated. Using a number of techniques including the inclusion of *p*-nitrophenyl phosphate as a 'substrate divertor', the 5′-nucleotidase inhibitors α,β-methylene ADP and concanavalin A, and an anti-5′-nucleotidase anti-

Abbreviations: PMNL, polymorphonuclear leucocyte; α,β-methylene ADP, α,β-methyleneadenosine 5′-diphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

body, it was concluded that the membrane of porcine PMNLs contains very little or no 5′-nucleotidase activity.

Materials and Methods

Adenosine 5′-monophosphate (AMP) and calf-intestinal alkaline phosphatase were obtained from the Boehringer Corporation (London) Ltd., and [^{3}H]AMP and *N*-succinimidyl[2,3-^{3}H]propionate from both Amersham International Ltd. and the New England Nuclear Corporation. Concanavalin A, *Crotalus* venom 5′-nucleotidase, adenosine 5′-triphosphate, dextran, α,β-methylene ADP, wheat germ agglutinin, ouabain, *p*-nitrophenyl phosphate and 2-amino-2methyl-1-propanol were all obtained from the Sigma Chemical Company Ltd., U.K. Ficol-Paque was purchased from Pharmacia Ltd. Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) was from Pierce and Warriner, U.K. The anti-5′-nucleotidase antibody was a kind gift from Dr. W.H. Evans (Mill Hill, U.K.). All other reagents were of analytical grade.

Isolation of porcine polymorphonuclear leucocytes

All procedures were carried out at room temperature. Fresh pig blood from a local abbatoir, anticoagulated with acid-citrate-dextrose, was mixed with one-half volume of 6% dextran ($M_{r,av}$ 500000) and left to settle at room temperature for 1–2 h. The supernatant was syphoned and centrifuged at $130 \times g$ for 20 min at 20°C. The cells were gently resuspended in a Krebs-Ringer solution containing 10 mM glucose and loaded onto Ficol-Paque in 50 ml centrifuge tubes (10 ml of cell suspension onto 20 ml Ficol-Paque). After centrifugation ($400 \times g$, 30 min, 20°C) the pellet, containing PMNLs and erythrocytes, was resuspended in 2 ml of distilled water and whirlimixed for 1 min before adding Krebs-Ringer to restore isotonicity. The suspension was centrifuged at $130 \times g$ for 20 min at 20°C and the pellet was subjected to one or two more cycles of hypotonic lysis until all erythrocytes had been removed.

Preparation of leucocyte membranes

Freshly prepared PMNLs were resuspended in 4 ml of ice-cold 0.2 M sucrose, 0.5 mM $MgCl_2$ (neutralised with $NaHCO_3$) and immediately centrifuged at $450 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 4 ml of the above sucrose solution and homogenised in a Potter-Elvejhem homogeniser (clearance 0.11–0.15 mm) for 15 passes on ice. The homogenate was centrifuged at $450 \times g$ for 5 min. The loose pellet was resuspended in 2 ml of the same sucrose solution and homogenised again (three passes) and centrifuged as before. The supernatants were combined and loaded onto a discontinuous sucrose gradient prepared as follows: 3.0 ml 50%, 2.0 ml 42%, 3.5 ml 35%, 4.0 ml 32%, 2.5 ml 20% w/w sucrose. All sucrose solutions used were brought to pH 7.4 with $NaHCO_3$. About 4 ml of supernatant was loaded onto each gradient. After centrifugation ($66000 \times g_{av}$, 90 min, 2°C, 3×25 swing-out rotor) the creamy-white membranes were collected at the interface of the 20–32% w/w sucrose layers. They were washed in 50 ml of ice-cold 0.25 M sucrose, 10 mM Hepes, pH 7.4, followed by centrifugation at $137000 \times g_{av}$ for 1 h at 2°C to pellet the membranes. To remove any contaminating soluble proteins, the membranes were resuspended in 5 ml ice-cold 10 mM Hepes, pH 7.4 with a Potter-Elvejhem homogeniser for 1 min, after which an equal volume of ice-cold 0.5 M sucrose, 10 mM Hepes, pH 7.4 was added. The volume was adjusted to 50 ml with ice-cold 0.25 M sucrose, 10 mM Hepes, pH 7.4 and the membranes pelleted as before. The membranes were stored at −20°C in 0.25 M sucrose, 10 mM Hepes, pH 7.4.

Assay of 5′-nucleotidase

The assay used was based on that of Avruch and Wallach [10]. The incubation medium (final volume 0.6 ml) consisted of 83 mM Tris-HCl, pH 7.9, 8.3 mM $MgCl_2$, 0.11 mM AMP, [^{3}H]AMP (about 60000 dpm) and *p*-nitrophenyl phosphate at the concentrations indicated. The membrane fraction (usually 7–9 μg protein) was added to start the reaction. After incubation for 30 min at 36°C, the reaction was stopped with 0.2 ml 0.15 M $ZnSO_4$ followed by 0.2 ml 0.35 M $Ba(OH)_2$. After mixing, the sample was centrifuged at $10000 \times g$ for 2 min and a further 0.1 ml 0.15 M $ZnSO_4$ followed by 0.1 ml 0.35 M $Ba(OH)_2$ added. Without further mixing the suspension was centrifuged at $10000 \times g$ for 2 min. An aliquot of the supernatant was taken for ^{3}H-counting in Fiso-

Fluor multi-purpose cocktail in a Philips PW 4540 scintillation counter. All but 3–4% of the counts were precipitated by the $ZnSO_4/Ba(OH)_2$ treatment in controls lacking the enzyme. In constructing the pH profile of the enzyme, Tris-maleate buffer was used for pH values between 3.65 and 7.0, Tris-HCl between pH 7.0 and 9.0 and 2-amino-2-methyl-1-propanol-HCl for pH values between 9.0 and 11.0. These buffers were found to be suitable for this assay, as they did not interfere with the precipitation of [^{3}H]AMP.

Preparation of rat liver extract

A fresh rat liver was washed in ice-cold 50 mM Tris-HCl, pH 7.4, prior to homogenisation in a Potter-Elvejhem homogeniser in the same buffer, on ice. The homogenate was filtered through a silk stocking and the filtrate stored at −20°C. The protein concentration of the extact was 61 mg/ml.

Miscellaneous

Protein was determined by the method of Lowry et al. [11] using bovine serum albumin as standard.

p-Nitrophenyl phosphatase was measured essentially by the method of DePierre and Karnovsky [1].

$(Na^+ + K^+)$-activated, ouabain-inhibited adenosine triphosphatase activity of PMNL fractions was measured essentially by the procedure of Sha'afi et al. [12], the inorganic phosphate released being determined by the method of Martin and Doty [13].

^{3}H-Labelled wheat germ agglutinin was prepared using *N*-succinimidyl [2,3-^{3}H]proprionate, the free and protein-bound tritium being separated by G-25 column chromatography. Cell-surface labelling, using Iodogen, was performed essentially by the procedure of Salisbury and Graham [14].

Results

Characterisation of porcine PMNL plasma membranes

The membrane material collected at the 20–32% w/v sucrose interface on discontinuous gradient centrifugation was characterised by $(Na^+ + K^+)$-activated, ouabain-inhibited adenosine triphosphatase activity (12) and by labelling the membranes with ^{3}H-wheat germ agglutinin and with ^{125}I using the sparingly soluble chloroamide, Iodogen.

The specific activity of $(Na^+ + K^+)$-activated, ouabain-inhibited adenosine triphosphatase of the particulate fraction at the 20–32% w/w sucrose interface was 17.7 (±) 7.3 nmol/min per mg protein (mean and S.D. for seven preparations) compared to 3. 1 (±1.6) nmol/min per mg protein (mean and S.D. for three preparations) for the homogenate.

An experiment was also made to iodinate the intact cells prior to homogenisation using the sparingly soluble chloroamide, Iodogen. In this experiment 40% of the trichloroacetic acid precipitable radioactivity was associated with the particulate fraction at the 20–32% w/w sucrose interface. This result is comparable to those figures obtained for the plasma membrane of other cells [14].

In another experiment intact cells were exposed to ^{3}H-labelled wheat germ agglutinin for 15 s prior to their homogenisation and subcellular fractionation in the usual way. The association of the lectin with each of the particulate fractions from the gadient was determined. The fraction collected from the 20–32% w/w sucrose interface bound 68% of the lectin associated with all of the particulate fractions.

In other experiments ^{3}H-labelled wheat germ agglutinin-labelled membrane, collected from the 20–32% w/w sucrose interface was treated with digitonin (3 mg/mg membrane protein) and subjected to isopycnic density centrifugation on a continuous sucrose gradient from 18 to 56% w/w sucrose. The digitonin-treated membrane identified by ^{3}H-labelled wheat germ agglutinin counts and by *p*-nitrophenylphosphatase activity migrated to a density of 1.18 (40% w/w sucrose) whereas the control, untreated membrane migrated only to a density of 1.12 (29% w/w sucrose).

The plasma membrane-enriched fraction from porcine PMNL showed considerable phosphatase activity. When AMP was used as a substrate the nucleotide was readily hydrolysed at pH 7.9 with a specific activity of 100 nmol/min per mg of membrane protein at 36°C.

Effect of p-nitrophenyl phosphate on membrane phosphatase activity using AMP as substrate

The phosphatase activity of procine plasma

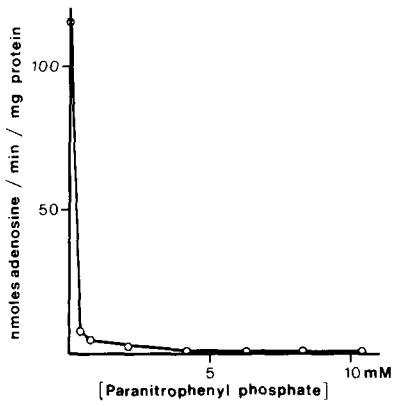

Fig. 1. Porcine PMNL membranes (7.6 μg protein) were incubated with tritiated 0.11 mM AMP as described in Methods in the presence of 0–10.4 mM *p*-nitrophenyl phosphate. After incubation [^{3}H]adenosine was counted after precipitation of [^{3}H]AMP using $ZnSO_4/Ba(OH)_2$ as described.

membrane was markedly inhibited when *p*-nitrophenyl phosphate was included in the incubation medium (Fig. 1). With an AMP concentration of 0.11 mM, *p*-nitrophenyl phosphate at 0.4 mM (i.e. in about 4-fold excess) caused a 93% inhibition of AMP hydrolysis, and at 4.2 mM (about 40-fold excess) the inhibition was 99% complete.

The nature of this inhibition by *p*-nitrophenyl phosphate was investigated by varying the AMP concentration over the range 0.005 to 0.53 mM in the absence and in the presence of 0.167 mM *p*-nitrophenyl phosphate. The results (Fig. 2) when plotted in the double-reciprocal manner indicate that the inhibition is probably competitive in nature. The K_m for the reaction in the absence of *p*-nitrophenyl phosphate was $5 \cdot 10^{-5}$ M.

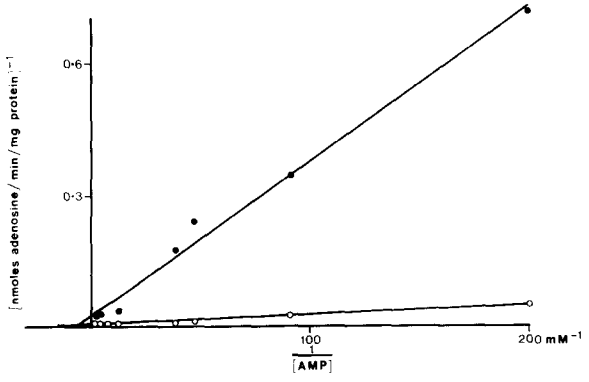

Fig. 2. Porcine PMNL membranes (7.6 μg protein) were incubated with or without 0.167 mM *p*-nitrophenyl phosphate in the presence of 0.005 to 0.53 mM [^{3}H]AMP, and the amount of [^{3}H]adenosine released was counted as described in Methods. A double-reciprocal plot of the results was made, the straight lines being drawn by least-squares regression analysis. ●——●, plus *p*-nitrophenyl phosphate; ○——○, minus *p*-nitrophenyl phosphate.

The possibility that inhibition was caused by phosphate or *p*-nitrophenol being released from *p*-nitrophenyl phosphate was checked by assaying the phosphatase activity without *p*-nitrophenyl phosphate, but in the presence of increasing concentrations of inorganic phosphate and *p*-nitrophenol. Both inorganic phosphate and *p*-nitrophenol inhibit the reaction but not to an extent that could explain the dramatic inhibition by *p*-nitrophenyl phosphate. Even at 0.4 mM (a concentration at which *p*-nitrophenyl phosphate inhibits 93% of the activity) *p*-nitrophenol caused only a 21% inhibition and inorganic phosphate an inhibition of 32%. Since the concentration of free *p*-nitrophenol and inorganic phosphate in the incubation medium containing *p*-nitrophenyl phosphate, is likely to be much lower than this concentration, it was considered that most of the inhibition observed, could not have been caused by *p*-nitrophenol or inorganic phosphate present as a result of *p*-nitrophenylphosphatase activity.

Effect of α,β-methylene ADP on PMNL membrane phosphatase using AMP as substrate

α,β-Methylene ADP, a powerful inhibitor of 5′-nucleotidase, was included in the incubation medium at concentrations ranging from 0.007 to 0.36 mM, with the PMNL membranes as the source of enzyme activity. In control experiments the effects of the ADP analogue on purified 5′-nucleotidase from *Crotalus* venom and on calf-intestinal alkaline phosphatase were examined (Fig. 3).

α,β-Methylene ADP markedly inhibited purified 5′-nucleotidase from *Crotalus* venom; an 80% inhibition of activity was obtained when the α,β-methylene ADP concentration was as low as 0.01 mM. The ADP analogue also inhibited the hydrolysis of AMP by alkaline phosphatase from calf-intestine, the degree of inhibition of this enzyme was much less than that of the purified *Crotalus* venom 5′-nucleotidase, viz. there was only a 35% inhibi-

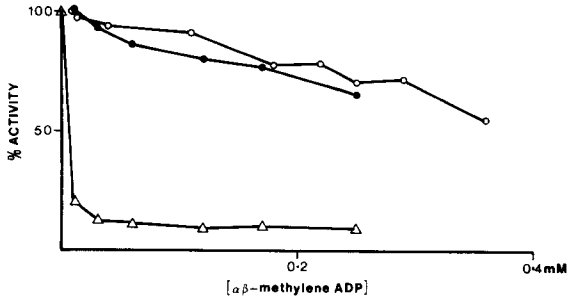

Fig. 3. Varying concentrations of α,β-methyleneadenosine diphosphate (from 0 to 0.36 mM) were incubated with porcine PMNL membranes (7.6 μg protein) or *Crotalus* venom 5′-nucleotidase (50 nmol) or calf-intestinal alkaline phosphatase (0.033 U) in the presence of 0.11 mM [^{3}H]AMP and the amount of [^{3}H]adenosine released was counted in the usual way. ○——○, porcine PMNL membrane; ●——●, calf-intestinal alkaline phosphatase; △——△, *Crotalus* venom 5′-nucleotidase.

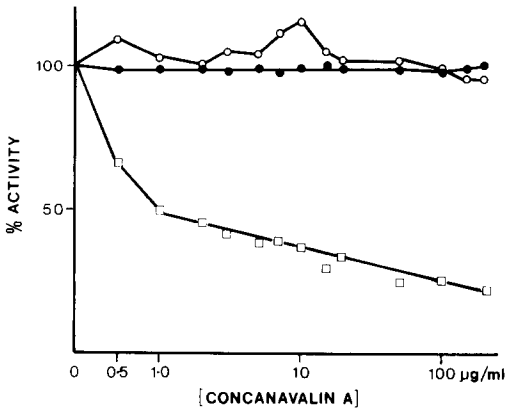

Fig. 4. Varying concentrations of concanavalin A (from 0 to 200 μg/ml) were incubated with porcine PMNL membranes (8.6 μg protein) or *Crotalus* venom 5′-nucleotidase (50 nmol) or calf-intestinal alkaline phosphatase (0.267 U) for 15 min at room temperature. [^{3}H]AMP was then added and the hydrolysis of AMP was assayed as before. ○——○, porcine PMNL membrane; ●——● calf-intestinal alkaline phosphatase; □-□, *Crotalus* venom 5′-nucleotidase.

tion of the alkaline phosphatase activity at 0.25 mM α,β-methylene ADP.

The effect of α,β-methylene ADP on the phosphatase activity of the PMNL membrane fraction, using AMP as a substrate, was similar to its effect on calf-intestinal alkaline phosphatase (Fig. 3). The ADP analogue did inhibit the membrane phosphatase, but only a 30% inhibition was found at 0.25 mM α,β-methylene ADP.

Effect of concanavalin A

It has now been well established that concanavalin A is a potent inhibitor of 5′-nucleotidase from a number of different cell sources. The effect of this lectin on porcine PMNL membrane phosphatase activity was examined by incubating the membrane fraction with various concentrations of concanavalin A (from 0.5 to 200 μg/ml) at room temperature for 15 min, prior to adding [^{3}H]AMP as substrate. For control experiments purified 5′-nucleotidase from *Crotalus* venom and calf-intestinal alkaline phosphatase were treated in an identical manner. Only *Crotalus* 5′-nucleotidase was inhibited by concanavalin A (Fig. 4). Only 50% of the activity in the absence of lectin remained at a concanavalin A concentration of 1 μg/ml, and 30% of the activity remained at 10 μg/ml concanavalin A. Calf-intestinal alkaline phosphatase was unaffected by the lectin. Porcine PMNL membrane phosphatase was not inhibited by concanavalin A, even up to concentrations of 100 μg/ml. Possibly even a slight stimulation of activity was obtained at concentrations up to 10 μg/ml concanavalin A.

Antibody to 5′-nucleotidase

The antibody used was one that has been shown to inhibit specifically the 5′-nucleotidase of various lymphoma cell lines [15]. Porcine PMNL membrane fractions were pre-incubated at 37°C with varying concentrations of the antibody. After 20 min the substrate was added and the hydrolysis of AMP was assayed in the normal way. For control experiments, three other enzyme preparations were used, viz. a crude rat liver extract, purified 5′-nucleotidase from *Crotalus* venom and calf-intestinal alkaline phosphatase. The antibody inhibited the 5′-nucleotidase activity of a crude rat liver extract, but surprisingly it stimulated 5′-nucleotidase from *Crotalus* venom. Neither PMNL membrane phosphatase, nor calf-intestinal alkaline phosphatase were affected by the antibody, even at concentrations which inhibited 86% of rat liver 5′-nucleotidase (Fig. 5).

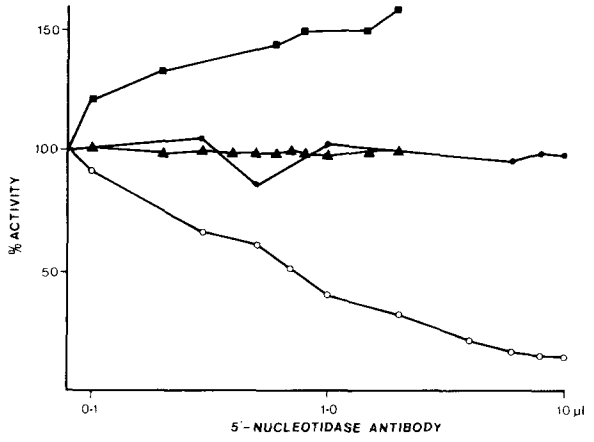

Fig. 5. Varying amounts of 5'-nucleotidase antiserum (from 0.1 to 10 µl) were incubated with porcine PMNL membranes (8.6 µg protein) or *Crotalus* venom 5'-nucleotidase (50 nmol) or calf-intestinal alkaline phosphatase (0.066 U) or rat liver extract (122 µg protein) for 20 min at 36°C prior to the addition of [^{3}H]AMP and the assay of AMP hydrolysis in the usual way. The assay medium for the rat liver extract also contained 1 mM *p*-nitrophenyl phosphate. ●———●, porcine PMNL membrane; ▲———▲, calf-intestinal alkaline phosphatase; ■———■, *Crotalus* 5'-nucleotidase; ○———○, rat liver extract.

Effect of pH on PMNL membrane phosphatase activity using both AMP and p-nitrophenyl phosphate as substrates

The pH profiles for the hydrolysis of AMP and for *p*-nitrophenyl phosphate by porcine PMNL membrane are given in Fig. 6. Very little activity is shown in the acid pH region, suggesting the absence of acid phosphatase. The optimum pH for AMP hydrolysis was pH 8.5 and for *p*-nitrophenyl phosphate was pH 10.3. The specific activities at these optimum pH values were 150 nmol/min per mg protein for AMP hydrolysis and 1.28 µmol/min per mg protein for *p*-nitrophenyl phosphate hydrolysis. The corresponding specific activities at pH 7.9 (at which most of the assays described here were made) were 100 nmol/min per mg protein for AMP hydrolysis and 95 nmol/min per mg protein when *p*-nitrophenyl phosphate was the substrate.

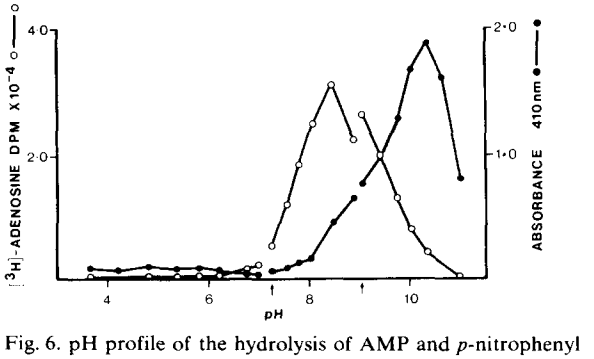

Fig. 6. pH profile of the hydrolysis of AMP and *p*-nitrophenyl phosphate by porcine PMNL membranes. The assays were carried out as described in Methods. ○———○, AMP hydrolysis; ●———●, hydrolysis of *p*-nitrophenyl phosphate. ↑ indicates the position of a buffer change.

Discussion

PMNL plasma membranes are of particular importance since it is at the cell surface that the triggering events for phagocytosis must take place and an understanding of the molecular events that occur in the membrane when it is internalised, should lead to the elucidation of the mechanism of phagocytosis. Studies on the plasma membranes of these cells have been hampered by the lack of a good enzyme marker which can be followed during the course of membrane isolation. The classic marker enzyme for cell membranes is 5'-nucleotidase [16]. This is a glycoprotein present exclusively as an ectoenzyme in many different cell types. Guinea-pig peritoneal PMNLs contain this ectoenzyme and in this species it is suitable as an enzyme marker. However, it is less certain that 5'-nucleotidase is present in human PMNLs [7,8]. It is clear that from the complexity of phosphatase activities present in the cell membranes of polymorphs (acid and alkaline phosphatases and ATPase) quite rigorous analysis has to be performed to identify a specific 5'-nucleotidase.

The membrane material used in this work was believed to be mainly derived from the plasma membrane of the PMNL. The fraction was located at the 20–32% w/w sucrose interface on a discontinuous sucrose gradient, this being the same density at which the plasma membranes of bovine [17] and human PMNL (Geny, B., personal communication) are located. Furthermore this membrane fraction contains most of the $(Na^+ + K^+)$-activated ouabain-inhibited adenosine triphosphatase activity, which has been shown to be a

plasma membrane marker for rabbit [12] and human [18] PMNL plasma membranes. In addition, surface labelling studies with ^{125}I and ^{3}H-labelled wheat germ agglutinin indicate that the fraction is enriched in plasma membrane, and further support for this comes from experiments on its interaction with digitonin [19]. Full results of porcine PMNL subcellular fractionation will be presented elsewhere (Chibber, R. and Castle, A.G., in preparation).

The analogue α,β-methylene ADP is considered to be a powerful inhibitor of 5′-nucleotidase [20], having only a very small inhibitory effect on alkaline phosphatase [21]. In this work α,β-methylene ADP was shown to have a large inhibitory effect on purified 5′-nucleotidase from *Crotalus* venom, but a much smaller effect on the hydrolysis of AMP by calf-intestinal alkaline phosphatase. The analogue had the same small effect on the hydrolysis of AMP by porcine membranes, suggesting that the enzymes(s) involved was not a true nucleotidase.

The lectin concanavalin A has been shown by several workers to inhibit 5′-nucleotidase, both in the membrane-bound form [22,23] and the purified enzyme [24,25]. The inhibition occurs presumably because 5′-nucleotidase is a glycoprotein with sugar residues favoured by the lectin and the association of concanavalin A with the enzyme interferes with the association between 5′-nucleotidase and the cytoskeletal component, actin [26]. The lectin has been reported to have no inhibitory effect on membrane alkaline phosphatase [27]. In the present work concanavalin A was shown to have a marked inhibitory effect on 5′-nucleotidase from *Crotalus* venom but no effect on calf-intestinal alkaline phosphatase using AMP as substrate. The lectin also had little effect on the hydrolysis of AMP by porcine PMNL membranes, once again suggesting the absence of 5′-nucleotidase in the leucocyte membrane.

An antiserum against detergent-extracted mouse liver plasma membrane, in which the major component was 5′-nucleotidase, has been raised in rabbits by Evans and co-workers. This antiserum has been shown to inhibit liver membrane 5′-nucleotidase in both membrane-bound and soluble forms, and to inhibit the 5′-nucleotidase of several lymphoma cell lines [15]. The antibody was shown in the present work to have no effect on porcine PMNL membrane phosphatase activity using AMP as substrate, again being evidence against the presence of such an enzyme in the leucocyte membrane. However, the antibody was shown to be ineffective against purified 5′-nucleotidase from *Crotalus* venom, whereas it inhibited the rat liver enzyme. These antibody results suggest that a membrane-type 5′-nucleotidase similar to that found in liver membranes is absent from pig PMNL membranes.

One possible explanation of the results presented in this paper is that 5′-nucleotidase is an exoenzyme on this cell, but is washed off during the hypotonic lysis steps in water. This explanation is unlikely. If the rat liver extract is treated in the same way as the porcine PMNLs, viz. washed with water for 1 min at the approximate protein:water volume ratios used in the PMNL preparation, then the liver extract still retains its 5′-nucleotidase activity which is inhibited by 5′-nucleotidase antibody. One, two or three such hypotonic washes do not affect the 5′-nucleotidase. Brief hypotonic lysis to remove contaminating red cells from PMNL preparations is commonly used and indeed virtually all membrane studies of blood PMNLs have been made after this step. Furthermore, there is now evidence that at least in certain lymphoma cells, 5′-nucleotidase is a transmembrane protein, i.e. it spans the membrane from the inner, cytoplasmic surface to the outer cell surface [15]. The chances of such a firmly attached enzyme being washed off the membrane by brief hypotonic lysis seems unlikely.

From the experiments with *p*-nitrophenyl phosphate, α,β-methylene ADP, concanavalin A and 5′-nucleotidase antibody, it can be inferred that a classical membrane-bound 5′-nucleotidase is absent from porcine PMNL membranes, and it could be suggested that the hydrolysis of AMP by the membranes takes place by (a) non-specific phosphatase(s), possibly an alkaline phosphatase. To explore this possibility the pH profile of the hydrolysis of AMP by the membranes was compared with that of the hydrolysis of *p*-nitrophenyl phosphate. The pH optima are in fact quite different (Fig. 6) suggesting that more than one enzyme is involved. The enzyme that hydrolyses AMP at pH 7.9 can presumably also utilise *p*-nitrophenyl

phosphate as substrate, leading to the results obtained when these two substrates were used together. The enzyme is probably a non-specific phosphatase and certainly it does not exhibit the characteristics of a classical 5′-nucleotidase.

A number of workers have reported the use of 5′-nucleotidase as a marker during the isolation of PMNL membranes [28] but only one group, using peritoneal guinea-pig cells [1,2] has rigorously established that the enzyme is present in the cells specifically as an ectoenzyme. From the results presented here it is obvious that great care must be made to ensure that AMP hydrolysis by a membrane sample is being accomplished by a true 5′-nucleotidase and not by another non-specific phosphatase. It is suggested that the following criteria are established with respect to a presumptive 5′-nucleotidase: (i) it is inhibited by α,β-methylene ADP (ii), it is inhibited by concanavalin A (iii) a 'substrate divertor' such as *p*-nitrophenyl phosphate is used in excess in the assay medium to prevent AMP hydrolysis by a non-specific phosphatase. In addition the substrate specificity with regard to 5′- and 2′,3′-nucleotides should also be established.

Acknowledgements

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