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Canine neutrophil plasma membrane markers

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The purpose of this investigation was to determine which enzyme activities are true canine neutrophil plasma membrane markers. Three enzymes thought to be present on plasma membranes were chosen for study: 5′-nucleotidase, magnesium-dependent adenosine triphosphatase (Mg^{2+}-ATPase), and leucine aminopeptidase. Both 5′-nucleotidase and Mg^{2+}-ATPase were found to be ectoenzymes in the canine neutrophil but additional Mg^{2+}-ATPase activity was located intracellularly. An endogenous inhibitor of 5′-nucleotidase was found in the cytosol of canine neutrophils. The specific 5′-nucleotidase inhibitor, adenosine 5′-[α,β-methylene] diphosphate also inhibited the canine enzyme in intact cells. Leucine aminopeptidase was located solely in the myeloperoxidase-containing granules of the canine neutrophil. Plasma membrane, as identified by the presence of Mg^{2+}-ATPase and 5′-nucleotidase activities, was separated from other cell organelles by Percoll-density gradient centrifugation of a 10 000 × *g* supernatant of nitrogen cavitated neutrophils.

Introduction

Attempts have been made to isolate plasma membranes from neutrophils of various species [1–5]. Essential to the process of isolation and purification as well as to its use is the identification of plasma membrane markers. Enzymes such as 5′-nucleotidase, magnesium-dependent adenosine triphosphatase (Mg^{2+}-ATPase), alkaline phosphodiesterase, alkaline phosphatase, and leucine aminopeptidase have been used as plasma membrane marker enzymes for the neutrophils of various species [6–13]. The presence of these enzyme activities in neutrophil plasma membranes, however, is not universal and their existence in certain species has been disputed [14–18].

We have identified 5′-nucleotidase and Mg^{2+}-ATPase as marker enzymes of the canine neutrophil plasma membrane. The initial steps involved in the isolation of purified plasma membrane also have been described.

Materials and Methods

Neutrophil isolation. Neutrophils were isolated from normal dogs and purified by separation on Ficoll-hypaque, followed by dextran sedimentation and erythrocyte lysis with ammonium chloride buffer [19]. The final neutrophil preparations had < 2% lymphocyte-monocyte and < 1% platelet contamination.

For experiments which involved measurement of enzyme activity in intact cells or cell sonicates, the neutrophils were resuspended in Hank's balanced salt solution with 2 mM $MgCl_2$ but without calcium, or phenol red (pH 7.3).

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Cell disruption. In some experiments cells ($1 \cdot 10^7$ neutrophils/ml in Hank's balanced salt solution without Ca^{2+} or Mg^{2+}) were sonicated at 4°C with two 30-s bursts at 35% of full power by a Fisher Sonic Dismembrator, Model 300 (Fisher Scientific Co., Pittsburgh, PA) using the microprobe.

Cells which were to be disrupted by nitrogen cavitation were suspended in a disruption buffer consisting of Hank's balanced salt solution with 10 mM $CaCl_2$, 2.5 mM $MgCl_2$ and 1 mM $MgCl_2$, and 10 mM adenosine triphosphate (ATP; disodium trihydrate, Calbiochem-Behring, LaJolla, CA), pH 7.4. The entire procedure was performed on ice or at 4°C. Twenty ml of a cell suspension ($5 \cdot 10^7$/ml) in a plastic beaker with a stirring bar were placed in a Cell Disruption Bomb (Model 4635; Parr Instrument Co., Moline, IL) surrounded by ice. Cells were pressurized with nitrogen at 400 lb/in^2 for 20 min. The pressure was released slowly and the cavitatate was collected dropwise into a beaker containing 0.95 ml of 0.25 M ethylenediaminetetraacetate (EDTA) (pH 7.0). Cell lysis was assessed microscopically, using Evans blue dye (0.1%) to enhance the visibility of nuclei. The cavitated cells were centrifuged at $400 \times g$ for 10 min to remove the unbroken cells and nuclei, and the supernatant was centrifuged at $10\,000 \times g$ for 15 min to remove most of the granules and mitochondria.

Density-gradient centrifugation. The $10\,000 \times g$ supernatant was added either to sucrose-Percoll or saline-Percoll (Pharmacia, Piscataway, NJ) [20]. Sucrose-Percoll gradients were formed by combining equal volumes of $10\,000 \times g$ supernatant and sucrose-isopercoll (90% Percoll, and 10% 2.5 M sucrose). The sucrose-Percoll gradients (12 ml) were centrifuged at $80\,000 \times g$ for 30 min at 4°C in a Beckman Model L Ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA) using a type 40 fixed-angle rotor.

For saline-Percoll gradients, the $10\,000 \times g$ supernatant was mixed with an equal volume of saline-isopercoll (90% Percoll, and 10% 1.5 M NaCl). Saline-Percoll gradients were centrifuged at $40\,000 \times g$ for 30 min at 4°C. The Percoll and the $10\,000 \times g$ supernatant were mixed, not layered. Fractions of the gradients were collected dropwise from the bottom of the tube.

Sucrose-density gradients were prepared as previously described [19].

Enzyme assays. Mg^{2+}-ATPase activity was measured by a modification of the method of DePierre and Karnovsky [7]. The samples were incubated for 30 min in a shaking water bath at 37°C with 2 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate, 14.2 mM KCl and 1 μl (1 μCi) of 0.2 μM [γ-^{32}P]ATP (ICN, Irvine, CA) in Hank's balanced salt solution (pH 7.4) in a total volume of 1 ml. One ml of cold 10% acid washed charcoal in 10% trichloroacetic acid was added. The mixture was centrifuged at $3000 \times g$ for 10 min to pellet the charcoal-bound [^{32}P]ATP, and the amount of ^{32}P in the supernatant was determined by liquid scintillation counting.

5'-Nucleotidase activity was measured by the method of Avruch and Wallach [21]. Samples in Hank's balanced salt solution (pH 7.4) containing 1 mM $MgCl_2$ and 1 μCi of [^{3}H]adenosine monophosphate (0.1 μM; AMP; New England Nuclear, Boston, MA) in a total volume of 1 ml were incubated for 1 h at 37°C in a shaking water bath. The reaction was stopped by the addition of 0.2 ml of 0.25 M $ZnSO_4$. Unreacted substrate was removed by precipitation with 0.2 ml of 0.25 M $Ba(OH)_2$. The ^{3}H-adenosine in the supernatant was quantitated by liquid scintillation counting. In some experiments, adenosine 5'-[α,β-methylene]-diphosphate (p[CH_2]pA, 40 μM; Sigma Chemical Co., St. Louis, MO) was utilized as a specific, and characteristic inhibitor of 5'-nucleotidase activity [22].

Leucine aminopeptidase activity was assayed according to the method of Goldbarg and Rutenburg [23].

NADPH-cytochrome *c* reductase activity (superoxide dismutase-resistant), a marker for endoplastic reticulum, was measured by the method of Sottocasa et al. [24] as modified by Gennaro et al. [25]. Succinate dehydrogenase activity was used as an assay for the presence of mitochondria [26]. Myeloperoxidase and vitamin B_{12}-binding protein activities were measured as previously described [19] to localize the two major granule types.

Results

An important consideration in characterizing enzymes as plasma membrane markers is their

orientation in the membrane. This often can be determined by comparison of the enzyme activities in intact and disrupted neutrophils. An ectoenzyme is located on the external surface of the plasma membrane, therefore its activity should be measurable using intact cells. If the plasma membrane does not allow free passage of the substrate (as with ATP and AMP), and the enzyme is an endoenzyme whose active site is localized totally to the interior of the cell, activity should not be detectable in intact cells. Endoenzyme activity is detectable in disrupted cells. Combinations of ectoenzymes and endoenzymes can also occur. The substrate should be added to the membrane preparation immediately after cell disruption because sealed impermeable vesicles may form.

When canine neutrophils were disrupted by sonication, there was a 36.5 ± 8.5% (mean ± S.E.; $n = 6$) decrease in 5′-nucleotidase activity. This decrease in 5′-nucleotidase activity suggested an ectoenzyme, but the decrease in activity was so great that the possibility of either enzyme destruction or a released cytoplasmic inhibitor was considered.

When the sonicate was centrifuged at 40 000 × g for 30 minutes most of the plasma membrane was pelleted. The cytosol was removed and the pellet resuspended to the original volume of the cell suspension (Table I). This process resulted in more 5′-nucleotidase activity from the pelleted membrane than was detected in the intact cells. This difference may have been caused by a soluble inhibitor present in the intact cell preparation. Passage of a saline-Percoll isolated plasma membrane preparation through a Sephacryl S-1000 (Pharmacia, Piscataway, NJ) column further purified the membrane by removing any residual cytosol. The plasma membranes which were obtained in the void volume fractions of the Sephacryl S-1000 column demonstrated a marked increase in 5′-nucleotidase activity over the other two samples after correcting for sample dilution (Table I). When the 5′-nucleotidase activity was expressed as fmol AMP cleaved per min per mg protein the value for the intact cells became 4.0 while the Sephacryl S-1000 void volume sample was $2.6 \cdot 10^4$. This is an increase of over 6000-fold, a magnitude likely to represent both purification of plasma membranes and loss of a 5′-nucleotidase inhibitor.

TABLE I

5′-NUCLEOTIDASE ACTIVITY

In experiment I $5 \cdot 10^7$ neutrophils or their equivalent were used per 1 ml assay volume. The 40 000 × g pellet consisted of $5 \cdot 10^7$ cells sonicated and centrifuged at 40 000 × g for 30 min at 4°C, and assayed in 1 ml. The Sephacryl S-1000 void volume sample was derived from cavitated neutrophils which were subsequently centrifuged on a Percoll-density gradient. The plasma membrane rich fractions were chromatographed on Sephacryl S-1000 and the void volume fractions were obtained. The 5′-nucleotidase activity of the Sephacryl void volume sample was obtained by multiplying the activity by the dilution factor. In experiment II the samples contain either Percoll-purified plasma membrane vesicle from the Sephacryl S-1000 void volume region; the putative inhibitor from the Sephacryl S-1000 chromatographic fractions containing the soluble protein and small molecule eluate; and/or Tris-buffered saline. The volume of each constituent was 0.45 ml with a total assay volume of 0.90 ml. Standard error is shown; $n = 5$.

Sample	fmol AMP cleaved per min per $5 \cdot 10^7$ neutrophil equivalents
(I) Intact cells	8.9
40 000 × g pellet	87.0
Sephacryl S-1000 void volume	458.0
(II) Plasma membranes + buffer	55.0 ± 0.7
Inhibitor + buffer	< 0.1
Plasma membranes + inhibitor	< 0.1

To evaluate the possibility of a soluble inhibitor, a saline-Percoll isolated plasma membrane preparation was thoroughly dialysed to remove EDTA and ATP, and subsequently chromatographed on Sephacryl S-1000 (Table I). Inhibitory activity was present in the fractions which contained soluble proteins. The inhibitor completely blocked the 5′-nucleotidase activity in the purified plasma membranes obtained in the void volume.

Specific inhibition by adenosine 5′-[α,β-methylene]diphosphate is a characteristic of 5′-nucleotidase activity [22]. The 5′-nucleotidase activity of intact canine neutrophils was inhibited by 40 μM adenosine 5′-[α,β-methylene]diphosphate (81.5 ± 18.5%; mean ± S.E.).

During a 5′-nucleotidase assay of intact cells, 11.0% of the [^{3}H]AMP was converted to [^{3}H]adenosine. Centrifugation of the assay mixture at 400 × g for 10 min at 4°C revealed that 75.8% of the [^{3}H]adenosine was found in the supernatant and 24.2% was cell-associated. This sug-

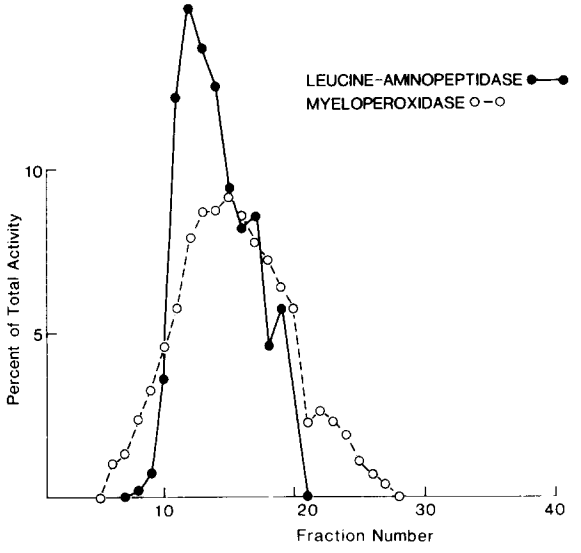

Fig. 1. The distribution of leucine aminopeptidase and myeloperoxidase activities from disrupted canine neutrophils as separated by sucrose-density gradient centrifugation using methods previously described [19]. The high-density fractions are on the left side of the graph. Most of the plasma membranes, as measured by 5′-nucleotidase and Mg^{2+}-ATPase activity, was found in the upper fourth of the sucrose-density gradients (data not shown).

gested that the [^{3}H]AMP was hydrolyzed by an ectoenzyme, 5′-nucleotidase, at the cell surface with release of the product into the supernatant.

Mg^{2+}-ATPase activity was detectable in intact cells (1.8 pmol ATP cleaved per min per 10^7 neutrophils; S.E. $\pm$ 0.1; $n = 5$), indicating an external location of at least some of the enzyme. The activity, however, increased following sonication (2.6 ± 0.1; $n = 5$), showing that some of the enzyme also is located intracellularly. In a separate experiment using intact cells little ATPase activity was detected in the absence of Mg^{2+} (0.6 ± 0.0 pmol ATP cleaved per min per 10^7 neutrophils) when compared with the assay performed in the presence of 2 mM Mg^{2+} (3.1 ± 0.2).

Using sucrose-density gradient centrifugation to separate cell structures following disruption, it was found (Fig. 1) that the leucine aminopeptidase activity was located in the high density region of the gradient in the same fractions as the myeloperoxidase granules.

Nitrogen cavitation was the disruption method chosen to obtain plasma membranes for purification. Percoll-density gradient centrifugation was used to separate neutrophil subcellular structures for evaluation of the use of 5′-nucleotidase and Mg^{2+}-ATPase activities as markers of plasma membranes in organelle isolation procedures.

The cavitation pressure which was chosen (400 lb/in^2) minimized nuclear and maximized cellular disruption. Intact nuclei were removed from cavitated cell preparations by $400 \times g$ centrifugation, as determined by microscopic examination, so that contamination of the Percoll-gradients with nuclear membranes was minimal. Only 23% of the cells from the whole cavitate were viable as determined by Evans Blue dye exclusion. The 77% non-viable cells could have lost part or all of their plasma membrane. The plasma membranes of at least 55.8% of the cells in the cavitate were totally disrupted as determined microscopically; 13% of the cells were completely lysed and 42.8% were free nuclei (nuclei with no visible plasma membrane encompassing them). In addition, some of the 21.1% non-viable cells (cells with Evans Blue dye-stained nuclei but some visible plasma membrane and/or intracellular organelles) appeared to have lost a part of their plasma membrane. Eighty-seven percent of the nuclei in the original cell suspension could be accounted for in the cavitate.

When plasma membrane vesicles from the $10\,000 \times g$ supernatant of cavitated cells were added to Percoll and centrifuged, most of both the Mg^{2+}-ATPase and 5′-nucleotidase activities resolved into a single peak (Fig. 2). The peaks, which were in the low density region of the gradient, coincided. Ninety-five percent of the Mg^{2+}-ATPase activity added to the Percoll-gradients could be accounted for by activity recovered from the Percoll-gradient fractions.

Granules were effectively separated from plasma membranes on saline-Percoll gradients (Fig. 2). Both the myeloperoxidase (primary) granules and the vitamin B_{12}-binding protein (secondary) granules had higher densities than the plasma membranes as determined by 5′-nucleotidase and Mg^{2+}-ATPase activities. Both granule populations were in the lower third of the Percoll-gradient while the plasma membranes were in the upper third. Of the two granule types, the myeloperoxidase granules were more dense, however, there was

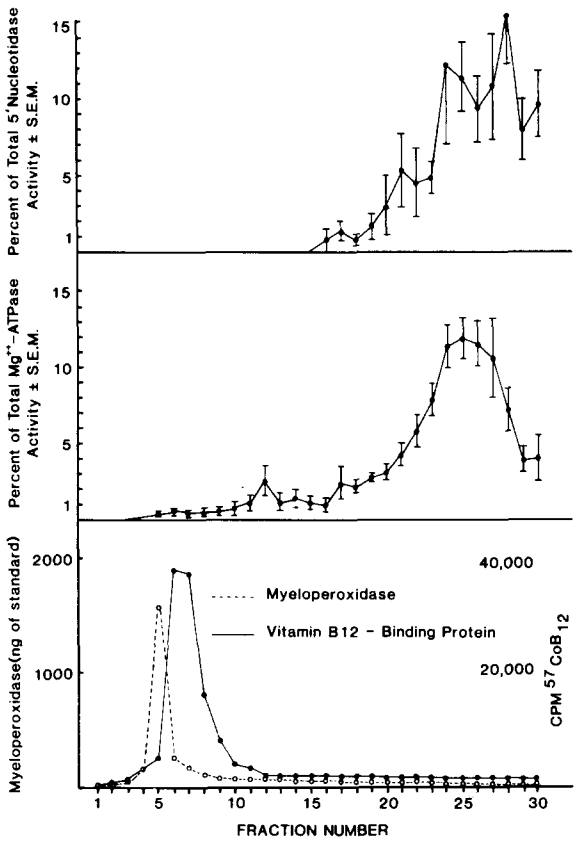

Fig. 2. The distribution of 5′-nucleotidase, Mg^{2+}-ATPase, myeloperoxidase, and vitamin B_{12}-binding protein activities from canine neutrophils disrupted by nitrogen cavitation and centrifuged in a saline-Percoll gradient. The high-density fractions are shown on the left side of the graph.

incomplete separation of the myeloperoxidase from the vitamin B_{12}-binding protein granules.

Succinate dehydrogenase was used as a marker for mitochondria. Most of the activity from cavitated neutrophils was sedimented at $400 \times g$ and $10\,000 \times g$ and no activity was detectable on either sucrose-Percoll or saline-Percoll gradients (data not shown). NADPH-cytochrome *c* reductase, a marker for the presence of endoplasmic reticulum, was not detected in saline-Percoll gradient fractions including those containing 5′-nucleotidase and Mg^{2+}-ATPase activity (data not shown).

Discussion

From the data presented, it is clear that Mg^{2+}-ATPase and 5′-nucleotidase are present in canine neutrophil plasma membranes and can be used as plasma membrane markers. Both are ectoenzymes, although Mg^{2+}-ATPase activity is also present within the cell. Neither of the substrates of these two enzymes should cross the plasma membrane into the cell. Both of the enzyme activities could be measured in intact cells. It is likely, therefore, that substrate hydrolysis takes place at the external cell surface. Three times as much [^{3}H]adenosine was found outside of neutrophils as was cell associated. The small amount which was cell associated could have been due to active transport [27]. The fact that Mg^{2+}-ATPase activity was increased 44% by sonication suggests that the intact plasma membrane prevented substrate ([^{32}P]ATP) interaction at an intracellular site. Accordingly, 56% of the Mg^{2+}-ATPase activity is likely to be an ectoenzyme as described by Banerjee [28]. It is most likely that the latent Mg^{2+}-ATPase activity was located on the cytoplasmic surface of the plasma membrane because there was no second peak of Mg^{2+}-ATPase found on Percoll gradients (Fig. 2). It can also be seen that Mg^{2+}-ATPase and 5′-nucleotidase activities coincided on Percoll gradients (Fig. 2) as would be expected if they were both located on the plasma membrane. If the residual Mg^{2+}-ATPase activity is located on the cytoplasmic surface of the plasma membrane, it could function as a transport ATPase since these enzymes bind intracellular ATP.

A soluble 5′-nucleotidase inhibitor was found in the neutrophil cytosol. Since preparations that were extensively dialyzed continued to show inhibition of plasma membrane-associated 5′-nucleotidase activity, the inhibitor probably has a molecular weight greater than 10000. A 5′-nucleotidase inhibitor also has been identified in the cytoplasm of human leukemic lymphocytes by Sun et al. [29]. The presence of this inhibitor diminishes the value of 5′-nucleotidase as a neutrophil plasma membrane marker, however, it still can be used in later stages of organelle separation when the cytosol is no longer present. In earlier stages of plasma membrane isolation Mg^{2+}-ATPase is the preferred marker.

5′-Nucleotidase is measurable in intact cells and is probably an ectoenzyme because enzyme found on the interior of the cells would be suppressed by the soluble inhibitor. The fact that the enzyme

activity increases in the 40 000 × g pellet of cavitated cells and Sephacryl S-1000 void volume fractions suggests the removal of inhibitor. The increase in 5′-nucleotidase activity in preparations of cytosol-free cell membranes and partially purified plasma membranes, when compared with intact neutrophils may be explained either by a partial release of inhibitor in the intact preparation suppressing externally located enzyme, or the inhibition of internal 5′-nucleotidase. If there is internal 5′-nucleotidase it must be located on the internal surface of the plasma membrane, since all of the activity was associated with that structure. We cannot exclude the possibility of soluble 5′-nucleotidase which was undetected because of the soluble inhibitor, because we did not attempt to separate soluble constituents other than by dialysis.

The specific inhibitor adenosine 5′-[α,β-methylene]diphosphate produced almost complete inhibition of the 5′-nucleotidase activity in intact cells. This shows that the enzyme involved is a true 5′-nucleotidase [22,30].

Leucine aminopeptidase has been used as a plasma membrane marker in human neutrophils [6]. In the present study of canine neutrophils it was found to co-distribute with myeloperoxidase granules rather than the plasma membranes.

Plasma membranes were separable from other membranes by our methods. Nitrogen cavitation achieved a high degree of cell disruption while nuclei remained largely intact and easily removable by centrifugation. Mitochondria and endoplasmic reticulum could not be found in fractions containing the plasma membrane enzymes, Mg^{2+}-ATPase and 5′-nucleotidase.

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