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Ligands of purinergic receptors stimulate electrogenic H^+-transport of neutrophils

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Abstract The possible role of ATP, acting as a ligand on cell surface receptor was investigated in the activation of the electrogenic H^+-transporting pathway of porcine neutrophil granulocytes. (1) ATP brought about 2.1-fold increase in the rate of H^+-efflux. (2) The order of potency of different nucleotides suggests, that ATP acts on P_2 type purinoceptor. (3) The effect of the nucleotides was prevented by inhibition of phospholipase A_2. (4) Inhibition of the metabolism of arachidonic acid (AA) via the cyclooxygenase pathway had no effect, whereas inhibition of the lipoxygenase pathway significantly enhanced H^+-release. This is the first report about activation of the H^+-transporter by physiological stimulator acting on the cell surface.

Key words: Electrogenic H^+-transporter; Adenosine triphosphate; Arachidonic acid; Phopholipase A_2; Neutrophil granulocyte

1. Introduction

In phagocytic cells, the generation of superoxide anion ($O_2^{\bullet -}$) in the extracellular or intraphagosomal space is accompanied by liberation of protons into the cytoplasm resulting in intracellular acidification and a decrease in the membrane potential [1–4]. As a compensating mechanism, the operation of a H^+-transporting pathway has been suggested in the plasma membrane that could allow the efflux of protons in the direction of their electrochemical gradient [3,4]. The existence of an electrogenic H^+-transporting pathway has been demonstrated in the plasma membrane of neutrophil granulocytes [3–7], macrophages [8] and HL-60 granulocytes [9], on the basis of intracellular pH-determinations by fluorescent dyes [3–6] and by whole cell patch clamp measurements [7–9].

Electrogenic H^+-release from phagocytes was elicited through the activation of protein kinase C (PKC) by phorbol esters [5,6], by addition of external arachidonic acid [10–12], by intracellular acidification [7–9,11,12] or by depolarization of the plasma membrane [5–9]. The resulting H^+-movement was inhibited by Cd^{2+} or Zn^{2+} [3–9]. The activating effect of phorbol esters was prevented by BPB, a potent inhibitor of phospholipase A_2, suggesting that PKC acts indirectly, via stimulation of PLA_2. In agreement with this finding, the effect of AA on the H^+-movements of intact cells was not influenced by inhibition of protein kinase C [11], indicating that the effect of AA was not mediated through PKC activation. On the basis of these observations it has been proposed that AA (or one of its metabolites) could be a direct activator of the putative H^+-transporter [10–12].

There are, however, no data available about the activation of the electrogenic H^+-pathway in granulocytes by natural stimulators acting on a receptor of the plasma membrane. Thus it remained to be elucidated whether endogenous AA (or its metabolite) generated in the course of the transduction of an extracellular signal could participate in the regulation of the proton conductance. The aim of the present study was to find conditions where the activity of the electrogenic H^+-pathway could be influenced by a receptor-mediated stimulus and to characterize the signal transducing pathway coupled to physiological stimulation.

2. Materials and methods

2.1. Chemicals and solutions

BCECF/AM and nigericin were obtained from Calbiochem; NDGA, BPB, staurosporine, PMA, ATP (K-salt), ATPγS (Li-salt) and AA from Sigma; valinomycin from Serva; Percoll from Pharmacia, IBP from Aldrich.

The phosphate buffered saline (PBS) medium contained: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 5 mM glucose, pH 7.3. The KCl medium contained: 140 mM KCl, 5 mM Tris-HCl and 5 mM glucose, pH 7.4.

2.2. Cell preparation and loading with fluorescent indicator

Neutrophil granulocytes were isolated from citrated pig blood basically as described in [13] and detailed in [6].

Cells (10^9) suspended in 1 ml of PBS were incubated with 4 μM BCECF/AM at 37°C for 20 min. BCECF-loaded cells were then diluted, washed and resuspended at $5–7 \times 10^8$/ml in PBS medium.

2.3. Manipulation of intracellular pH

The intracellular compartment of BCECF-loaded cells was acidified by using the ammonium pre-pulse technique [14] as detailed in [6]. Cells were finally resuspended in 150 mM choline chloride, 5 mM Tris-HCl and 5 mM glucose, pH 7.4. The internal pH was 6.516 ± 0.0306 ($n = 14$). Acidified cells were kept at 4°C.

2.4. Measurement of intracellular pH

Measurement of intracellular pH was carried out in the presence of high extracellular [K^+] and valinomycin, i.e. when the plasma membrane potential was dissipated and free charge compensation could occur. Under these conditions the function of the Na^+/H^+ exchanger was precluded by the absence of Na^+ in the extracellular space. To prevent the H^+ generation by the NADPH oxidase the enzyme was blocked by IBP. Since the addition of 500 nM bafilomycin, a potent inhibitor of the plasma membrane H^+-ATPase had no effect on the detected H^+-movements, this drug was not routinely added to the medium.

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Abbreviations: AA, arachidonic acid; ATPγS, adenosine5′-*O*-3-thiotriphosphate; BCECF/AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; BPB, *p*-bromophenacyl bromide; CTP, cytidine 5′-triphosphate; DIDS, 4′,4′-diisothiocyanostilbene-2,2′-disulfonate; fMLP, formyl-methionyl-leucine-phenylalanine; IBP, iodonium biphenyl; $K_{0.5}$, half-maximal activating concentration; NDGA, nordihydro-guaiaretic acid; pH_i, intracellular pH; pH_o, extracellular pH; PKC, protein kinase C; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; UTP, uridine 5′-triphosphate.

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A pH gradient of approx. 1 unit favoring H^+-efflux was obtained by acidification of the internal compartment. Typically, BCECF-loaded acidified cells (1.25×10^7) were suspended in 2.5 ml of KCl medium, containing 0.8 μM valinomycin and 400 μM IBP. When the effect of arachidonic acid and ATP was investigated, valinomycin was added after the stimulating agent. Altering conditions are indicated in the legend to the respective figure. Fluorescence was monitored by a Perkin-Elmer 3000 spectrofluorimeter with wavelengths of 485 and 540 nm for excitation and emission, respectively. We used these relatively less sensitive wavelengths to minimize the alteration of the flourescence due to putative shape changes of the cells caused by different stimuli.

Calibration of the signal in terms of pH_i was carried out as described in [6]. No significant change in the baseline fluorescence was observed up to 4 h from the time of loading the cells with the indicator.

3. Results and discussion

3.1. Effect of ATP and other nucleotides on the electrogenic H^+-transporting pathway

The aim of the present study was to test whether a physiological stimulus known to act through a cell surface receptor is able to influence the activity of the putative H^+-channel of porcine neutrophil granulocytes. Porcine neutrophils do not respond to the most potent chemotactic agent, fMLP. Thus we tested the effect of ATP, that has been shown to act on human neutrophil granulocytes through purinergic receptors which is coupled both to phopholipase C and A_2 signal transduction pathway [15].

ATP stimulated the rate of H^+-efflux from porcine granulocytes, in a concentration-dependent manner with half-maximal effect of at approx. 50 μM. When applied in the maximally effective concentration ATP brought about 2.11 ($\pm$ 0.19

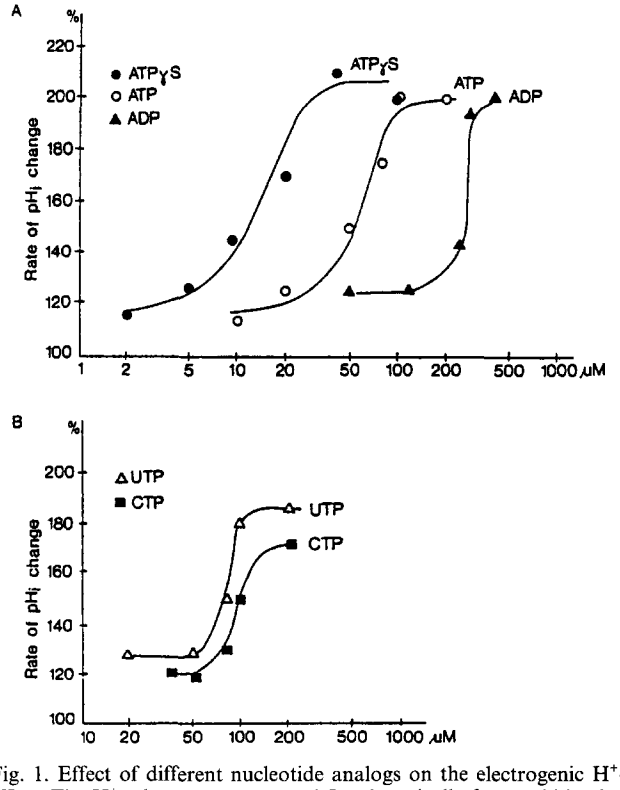

Fig. 1. Effect of different nucleotide analogs on the electrogenic H^+-efflux. The H^+-release was measured fluorimetrically from acid loaded cells as detailed in section 2. 100% corresponds to the basal H^+-efflux of 0.076 $\pm$ 0.011 (S.E.M.) ΔpH/min (n = 7) determined in the presence of valinomycin. This figure shows a representative curve from 3 similar ones.

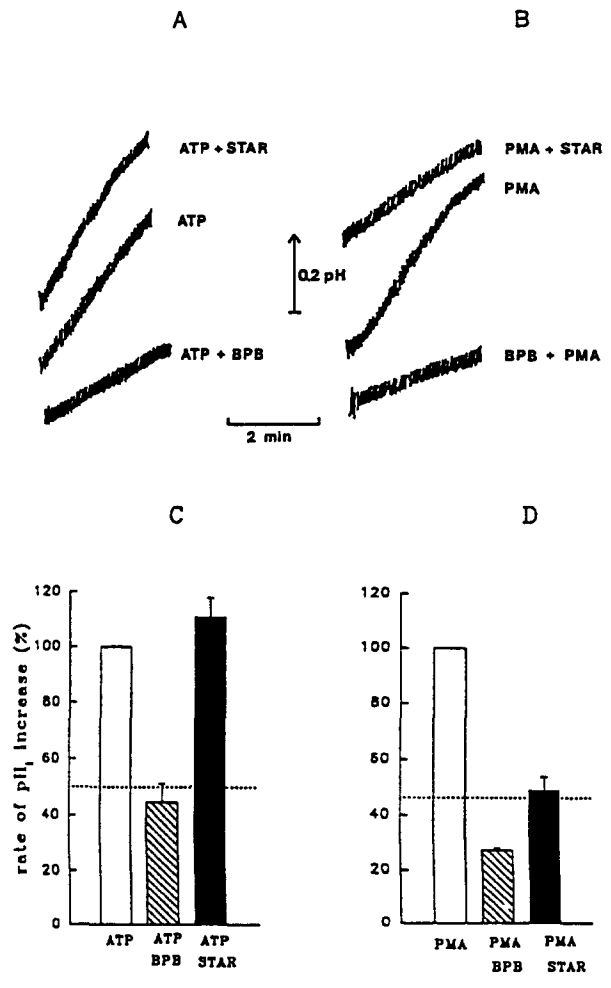

Fig. 2. Inhibitor sensitivity of the ATP- and PMA-induced H^+-efflux. Electrogenic H^+-efflux was stimulated by 200 μM ATP (A,C) or by 10 nM PMA (B,D). Where indicated, 0.2 μM staurosporine (STAR) (black columns) or 10 μM BPB (hatched columns) was also present. (A) and (B) are representative curves, (C) and (D) summarize the results from 5 similar experiments. BPB in these experiments was added immediatelly before the stimulus. The rate of H^+-efflux measured in the presence of the respective stimulator but in the absence of any inhibitor is shown as 100%. The dashed line corresponds to the basal H^+ efflux. The rate of H^+ efflux was 0.140 $\pm$ 0.014 (S.E.M.) ΔpH/min (n = 7) with ATP and 0.132 $\pm$ 0.016 (S.E.M.) ΔpH/min in the case of PMA (n = 7). The basal H^+-efflux was 0.076 $\pm$ 0.011 (S.E.M.) ΔpH/min (n = 7).

S.E.M.)-fold increase in the rate of H^+-efflux (Fig. 1A). ATP did not induce a non-specific increase in the membrane permeability as no leakage of the dye to the extracellular space could be observed (measured as fluorescent activity in the supernatant after pelleting the cells). The H^+-egress induced by ATP could be inhibited by Cd^{2+} and Zn^{2+} in similar concentrations as described earlier [6]. However, it should be noted that nucleoside triphosphates are good chelators of the divalent cations, so it is difficult to assess to which extent the inhibition is due to the decrease in the effective concentration of the stimulant.

However, the ATP concentration causing half-maximal activation was considerably higher in our experiments than reported previously in several other systems [16]. A possible explanation for this increased ATP requirement might be the activity of ectonucleotidase(s), hydrolysing the nucleoside triphosphate. To assess this possibility, we tested the effect of

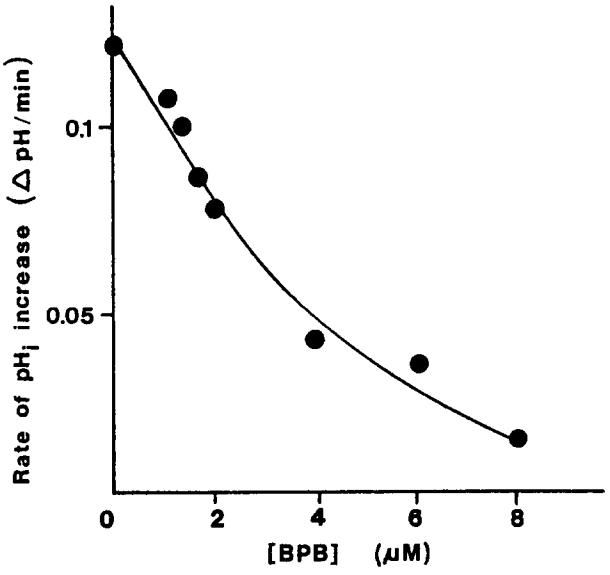

Fig. 3. Inhibitory effect of BPB on H^+-efflux stimulated by ATP. The final concentration of ATP was 240 μM. All other conditions were as described in section 2.

ATPγS, a poorly hydrolysable analogue of ATP [17], which is known to have the same affinity for this receptor. As shown on Fig. 1, ATPγS induced electrogenic H^+-efflux at significantly lower concentrations than ATP. Furthermore, the activity of the ectonucleotidase(s) was also confirmed by direct measurement of the liberation of inorganic phosphate from ATP under our conditions (data not shown). These data suggest that ATP most probably acts on a cell surface receptor.

Our next aim was to get information about the type of the purinergic receptor involved in the effect of ATP. The relative potency to adenosine nucleotides is a characteristic feature of the P_1 and P_2 purinergic receptor classes. In our system ADP was significantly less effective than ATP ($K_{0.5} \approx 300$ μM versus 50 μM) (Fig. 1A) whereas AMP caused almost no increase in the H^+-transport activity (data not shown). The effect of ATP was sensitive to suramin, a blocker of the P_2 purinergic receptor (data not shown). These finding are consistent with the involvement of P_2 purinergic receptors [16].

In order to substanstiate the probable subtype of the receptor responsible for the effect, we tested other nucleotides as well. UTP and GTP (not shown) exerted their effect in the same concentration range as ATP, whereas CTP proved to be less potent (Fig. 1B). This order of potency of various nucleoside triphosphates to stimulate H^+-efflux is similar in pharmacological properties to the purinoceptor described earlier in HL-6O cells and in human granulocytes, most probably P_{2U} type [18,15].

Various types of leukocytes were reported to possess also a pore-forming purinergic receptor (P_{2Z}) that could be inhibited by DIDS [19]. This compound did not influence the ATP-stimulated H^+-release in our system (data not shown). In addition H^+-efflux stimulated by ATP (or UTP or GTP) was strictly dependent on the charge compensation provided by valinomycin indicating that the membrane permeability was selectively increased for protons but not for small alkali cations. These findings together indicate that the ATP does not act through P_{2Z}-receptor mediated pore formation.

Considering all these data, it seems probable that in the porcine granulocytes ATP influences the previously described electrogenic H^+-transporter by acting on purinergic receptors at the cell surface.

3.2. Possible mechanism of ATP action

Our next aim was to investigate the intracellular signaling of purinergic receptors in our system. These receptors were shown to be coupled both to phopholipase C and the A_2 pathways [15]. To assess the relative contribution of these routes in the ATP induced H^+-efflux from porcine PMNs, we tested the effect of the PKC inhibitor staurosporine and a PLA_2 blocker BPB on the electrogenic H^+-movements.

Fig. 2 shows that staurosporine did not influence the ATP-induced H^+-release, whereas it completely abolished the PMA-evoked response, as reported earlier [11]. This finding implies, that the PKC is not essential for the effect of ATP.

In contrast, ATP-stimulated H^+-release was blocked by BPB almost as effectively as the PMA-stimulated H^+-egress (Fig. 2). As shown in Fig. 3, the inhibitory effect of BPB was concentration-dependent and fell in the same range as reported earlier [11] for PMA-stimulation: half-maximal inhibition of ATP and PMA-induced H^+-efflux was attained at 3.5 and 4 μM BPB, respectively. The same quantitative and qualitative inhibitor sensitivities were observed when UTP, GTP or ADP were applied as stimuli (data not shown).

These pharmacological data together with the striking similarity in the capability of various nucleotides to liberate AA

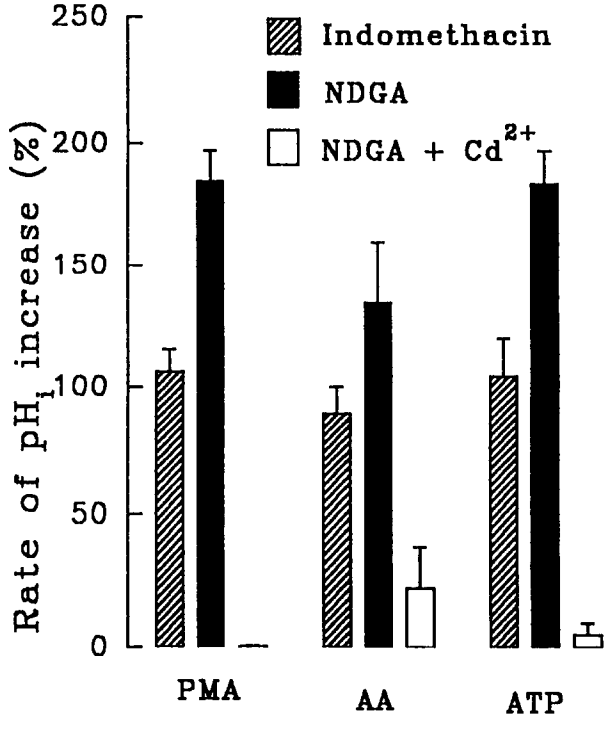

Fig. 4. Effect of inhibition of AA metabolism on the electrogenic H^+-efflux. The medium contained either 10 μM indomethacin (hatched columns), or 20 μM NDGA (black columns) or 20 μM NDGA plus 1 mM Cd^{2+} (open columns). Where indicated, H^+-efflux was stimulated either by 10 nM PMA, by 7.2 μM AA or by 200 μM ATP. 100% corresponds to the transport activities measured in the absence of any inhibitors, which was 0.132 ± 0.016 (S.E.M.) ΔpH/min in case of PMA ($n = 7$); 0.145 ± 0.021 (S.E.M.) ΔpH/min in case of AA ($n = 5$); and 0.140 ± 0.014 (S.E.M.) ΔpH/min in case of ATP ($n = 7$).

from neutrophils [15] and to stimulate H^+-efflux in our system, suggest that the H^+-pathway may be regulated by endogenous AA liberated via receptor-coupled PLA_2 activation.

3.3. Is AA itself or one of its metabolites the effective agent?

AA produced by PLA_2 in neutrophil granulocytes can be metabolized by the cyclooxygenase and the lipoxygenase pathways, resulting in the formation of prostaglandins and leukotrienes, respectively.

Several ion channels of the plasma membrane have been shown to be influenced by AA [20–22]. In most cases AA itself seems to be the effective agent, but the cardiac K^+-channel was only stimulated by leukotrienes and other derivatives produced on the lipoxygenase pathway [22]. To answer the above question in the case of the H^+-channel of granulocytes, we compared the effect of the inhibition of the cyclooxygenase and lipoxygenase metabolic pathways.

Fig. 4 summarizes our results obtained with inhibitors of the arachidonic acid metabolism. We tested the effect of indomethacin and NDGA, on the stimulating action of ATP and of other activators like AA and PMA. The rate of H^+-efflux measured in the presence of the respective stimulator but in the absence of any inhibitor, is represented as 100%. Inhibition of the cyclooxygenase pathway by indomethacin had no significant influence on the H^+-egress in either case. However, addition of NDGA an inhibitor of the lipoxygenase pathway resulted in a remarkable increase of the rate of H^+-release: it was almost doubled when PMA was applied as stimulant and even in the presence of a half-maximally stimulating concentration of external AA an augmentation by 40% could be detected.

NDGA did not alter the rate of H^+-release induced by oleic acid which is not a substrate of the lipoxygenase, or in the presence of BPB, when PLA_2 was inhibited (data not shown) so its direct stimulatory effect to the H^+-transport can be excluded. Similarly to AA and PMA, the effect of ATP was not influenced by indomethacin but it was almost doubled in the presence of NDGA (Fig. 4). Similar results were obtained with the inhibitors, when UTP or ADP were used as a stimulant (data not shown). The H^+-efflux increased by NDGA was fully sensitive to Cd^{2+} (Fig. 4).

Considering our observations that inhibition of the lipoxygenase enzyme produces an enhancing effect and inhibition of the cyclooxygenase is without any effect, we hypothesize that AA itself and not one of its metabolites could be the effective regulator of the H^+-channel. The enhancing effect of NDGA might suggest rapid metabolism of AA by the lipoxygenase enzyme in neutrophils. This view is supported by the fact that stimulated granulocytes liberate considerable amounts of leukotrienes and only a negligible amount of prostaglandins [23].

In our experiments carried out on porcine neutrophil granulocytes, the rate of electrogenic H^+-efflux was significantly increased by ATP and other nucleotides. The profile of effective and non-effective nucleotides suggests that ATP could act on P_2 purinoreceptor. The effect of ATP on the H^+-transport was most probably mediated by the activation of the PLA_2 and liberation of AA. Inhibition of the metabolism of AA on the lipoxygenase pathway has an enhancing effect in both studied cases, activation of protein kinase C and stimulation of a cell surface receptor. It is thus conceivable that several stimulating agents converge on the same final pathway: liberation of AA. In this view AA could serve as a common intracellular regulator of the putative H^+-channel.

This is the first report where the activity of the electrogenic H^+-transporting pathway is shown to be enhanced by an agent acting on a receptor on the cell surface. It is thus possible that regulation of the putative H^+-channel is coupled to other effector reactions (like $O_2^{\bullet-}$ production) induced by physiological stimulations.

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Note added in proof

After this manuscript had been submitted an article by Schumann, M.A., Leung, C.C. and Raffin, T.A. appeared in J. Biol. Chem. 270 (1995) 13124–13132, showing that fMLP and tumor necrosis factor activate H^+-current in human neutrophils.