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The chloride channel blocker 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) uncouples mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells

Gergely L. Lukacs[1,2], Arvind Nanda[1], Ori D. Rotstein[2] and Sergio Grinstein[1]

[1] *Division of Cell Biology, Hospital for Sick Children, 555 University Ave., Toronto M5G 1X8, Canada* and [2] *Department of Surgery, Toronto General Hospital, Toronto M5G 2C4, Canada*

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We present evidence that the potent chloride channel blocker NPPB has protonophoric activity in the mitochondria and across the plasma membrane of phagocytic cells. The resting O_2 consumption of murine peritoneal macrophages was stimulated up to 2.5-fold in the presence of NPPB, with a $K_{0.5}$ of 15 μM. The stimulatory effect of NPPB on O_2 consumption, like that of the classical protonophore CCCP, was prevented by the mitochondrial respiratory chain inhibitors antimycin A, rotenone or cyanide. NPPB also mediated rheogenic proton transport across the plasma membrane of human neutrophils and macrophages in the direction dictated by the electrochemical proton gradient. As a consequence of its protonophoric activity, NPPB uncoupled mitochondrial ATP synthesis, resulting in partial depletion of cellular ATP. These observations indicate that, at the concentrations frequently used for blockade of anion channels, NPPB acts as an effective protonophore, potentially disturbing cytosolic pH and mitochondrial ATP synthesis.

Chloride channel; NPPB; Uncoupler; Cytoplasmic pH; Proton; Mitochondrion

1. INTRODUCTION

The identification and functional characterization of chloride channels has been accomplished primarily by electrophysiological methods in conjunction with the application of chloride channel blockers such as disulfonic stilbene derivatives, diphenylamine-2-carboxylate, anthracene-9-carboxylate and indanyloxyacetic acid derivatives [1–4]. A new family of blockers, typified by 5-nitro-2-(3-phenylpropyl-amino)benzoic acid (NPPB), has been particularly useful for the study of anion channels because of their great potency and selectivity. NPPB and its derivatives were synthesized by Greger and his associates and successfully used to inhibit chloride channels in the thick ascending limb of the loop of Henle [5]. Subsequently, NPPB has also been shown to inhibit chloride channels in other tissues such as rectal gland [6], airway epithelia [7,8], lobster axon [9] and colonic carcinoma cells [10]. The apparent K_i for inhibition of chloride channels by NPPB in these tissues ranges from 0.1 to 100 μM.

As with other pharmacological probes, care must be taken to attribute the functional effect of NPPB to inhibition of its primary target, namely anion-selective channels. Indeed, in the course of studies of endomembrane proton and chloride transport, we obtained evidence that NPPB can also function as an effective protonophore. This effect, which is seemingly unrelated to the ability of the drug to block anion channels, occurs at the concentrations often used for electrophysiological studies and can have a severe impact on the physiology of the cells being analyzed. This report summarizes the evidence that NPPB has protonophoric and therefore uncoupling properties in phagocytic cells.

Abbreviations: NPPB: 5-nitro-2-(3-phenylpropyl-amino)benzoic acid; CCCP: carbonylcyanide *m*-chlorophenylhydrazone; BCECF: 2′,7′,bis(carboxyethyl)-5,6-carboxyfluorescein

Dr. G.L. Lukacs' permanent address is: Department of Physiology, Semmelweis Medical School, Budapest, Hungary

Correspondence address: S. Grinstein, Division of Cell Biology, Hospital for Sick Children, 555 University Ave., Toronto, M5G 1X8 Canada. Fax: (1) (416) 591-5028.

2. METHODS

Thioglycolate-elicited murine macrophages and human blood neutrophils were isolated as described previously [11,12]. Oxygen consumption was measured at 37°C with a Clark oxygen electrode in Na^+ medium (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $CaCl_2$ and 10 mM HEPES, pH 7.35) and calculated using a solubility coefficient of 0.024 ml O_2/ml. Cytoplasmic pH (pH_i) in neutrophils and macrophages was determined spectroscopically after loading the cytoplasm with the fluorescent pH-sensitive dye BCECF as described [11,12]. The cellular ATP content was measured by the luciferin-luciferase assay. Macrophages (10^7 cells/ml) were incubated at 37°C for 8 min in the presence or absence of NPPB (100 μM) or CCCP (10 μM) and centrifuged for 20 s. Pellets were then extracted with 8% per-

chloric acid at 0°C, neutralized with 1 M $NaHCO_3$ and centrifuged to sediment the precipitated material. The ATP content of the supernatants was determined in duplicte using a commercially available assay kit (Calbiochem).

3. RESULTS AND DISCUSSION

3.1. *NPPB stimulates mitochondrial respiration in macrophages*

The rate of oxygen consumption by a suspension of murine macrophages was measured in Na^+ medium using an O_2 electrode. In four experiments the resting O_2 consumption of macrophages averaged 0.96 ± 0.16 nmol/10^6 cells/min. In these cells O_2 consumption was accelerated by $65 \pm 13\%$ ($n = 3$) after the addition of 10 μM NPPB. The rate of O_2 consumption was further increased by a second addition of 10 μM NPPB (Fig. 1a). The half-maximal effect of NPPB on O_2 consumption was observed at 15 μM and maximal stimulation was attained at 50 μM (Fig. 2). The increased O_2 consumption induced by NPPB could be attributed to enhanced mitochondrial respiration for the following reasons. The NPPB-induced oxygen consumption was completely inhibited by antimycin A (Fig. 1b,c), a specific blocker of the respiratory chain that binds to and impairs the function of cytochrome *c* [13]. Moreover, other respiratory chain inhibitors such as rotenone or cyanide, which inhibit the NADH-CoQ reductase and the cytochrome *c* oxidase complex, respectively, also reduced markedly the effect of NPPB (not shown). Further evidence of the mitochondrial site of action of NPPB was obtained using the well characterized uncoupling agents CCCP and SF6874 (see [14] for review). As expected, these protonophores also increased the resting rate of respiration of macrophages (approx. twofold). However, the uncouplers were ineffective when added after maximally stimulating concentrations of NPPB or in the presence of antimycin A (not illustrated). These observations suggest that, similar to the effects of CCCP and SF6874, NPPB increases the proton permeability of the inner mitochondrial membrane, resulting in a compensatory activation of respiration [15].

The hypothesis that NPPB has protonophoric activity is also supported by the apparent structural similarities between the chloride channel blocker and the classical protonophoric uncouplers. Both NPPB and the protonophoric uncouplers have a bulky hydrophobic group which favors solubility of the molecules in the membrane bilayer. Moreover, like protonophoric uncouplers, NPPB has a dissociable proton and a strong electron withdrawing moiety (for structural comparison see Fig. 1 in [1] and Table I in [14]). If stimulation of mitochondrial respiration by NPPB is attributable to its protonophoric properties, the compound might be expected to enhance proton permeability across other membranes as well. This prediction was tested in the experiments described below.

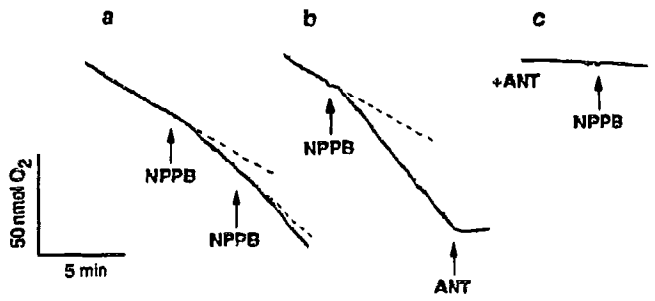

Fig. 1. NPPB stimulates mitochondrial oxygen consumption in macrophages. Macrophages (2×10^6/ml) were suspended in Na^+ medium and O_2 consumption was measured with a Clark electrode. Additions: a, NPPB 10 μM; b, NPPB 100 μM, antimycin A (ANT) 5 μg/ml; c, antimycin A 5 μg/ml was present in the medium prior to addition of cells, NPPB 100 μM added where indicated. Traces are representative of 3–5 experiments.

3.2. *NPPB acts as a protonophore on the plasma membrane of phagocytes*

To determine the effect of NPPB on the proton permeability of the plasma membrane, passive proton movements were followed by measuring the cytoplasmic pH (pH_i) of phagocytes with the fluorescent pH-sensitive dye BCECF. The direction of the proton motive force was altered by manipulating the membrane potential by means of ionophores and extracellular ionic substitution. As shown in Fig. 3a and b, when neutrophils were suspended in depolarizing (K^+-rich) medium in the presence of CCCP or NPPB, the cytosolic pH remained near neutrality, suggesting that metabolic acid generation offsets the tendency for proton (equivalents) to exit the cell down their electrochemical gradient. Under these conditions, passive proton efflux from the cells is limited by the availability of permeable counterions. This can be demonstrated by addition of valinomycin, a rheogenic K^+ ionophore, which promoted a large cytosolic alkalinization. Because the valinomycin-induced alkalinization occurred in the presence of CCCP or NPPB, but not in their

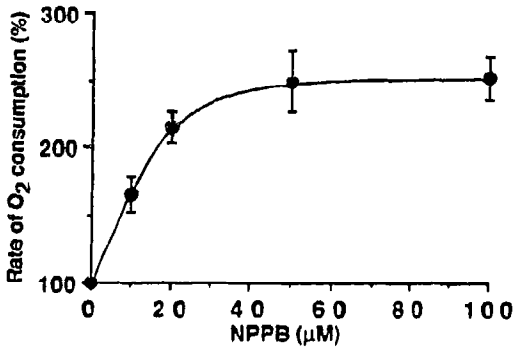

Fig. 2. NPPB stimulates O_2 consumption in macrophages in a dose-dependent manner. The experimental protocol was as in Fig. 1b. The rate of O_2 consumption is expressed as percent of the resting level. Data are means of 3–5 experiments ± SE.

absence (not shown), the pH_i change must be attributed to electrically coupled conductive fluxes of protons and K^+. While addition of valinomycin and protonophores in the reverse order also induced alkalosis (not illustrated), the effects were delayed by the time required for partition of the protonophores.

Sequential addition of CCCP and valinomycin to cells suspended in NMG^+ (low K^+) induced a sizable cytosolic acidosis (Fig. 3c), consistent with electrophoretic proton uptake catalyzed by the protonophore in hyperpolarized cells. A similar, though somewhat smaller acidification was induced by NPPB plus valinomycin (Fig. 3d). As before, the effects of CCCP and NPPB on pH_i in NMG^+ medium were greatly reduced by omission of valinomycin. NPPB also increased the proton permeability of the plasma membrane in macrophages (not shown). Together, these findings support the notion that, like CCCP, the anion channel blocker NPPB can catalyze conductive proton fluxes across the plasmalemma of phagocytes.

As shown in Fig. 4, NPPB increased the rate of transmembrane proton flux in a dose dependent fashion, with significant effects observed at concentrations as low as 25 μM. Comparison of Figs 2 and 4 indicates that lower concentrations of NPPB were required to uncouple respiration than to modify the cytosolic pH. This may reflect differential sensitivity of the plasma and mitochondrial membranes or may indicate that smaller proton fluxes are required for the respiratory effect. Taken together, the results reported above are consistent with the notion that NPPB can act as an effective protonophore in cellular membranes.

3.3. *Effect of NPPB on the cellular ATP content*

Despite continuous utilization, the cellular ATP pool is maintained at a constant level, due largely to resynthesis by the mitochondrial F_1F_0-ATPase. In the presence of uncouplers, the proton motive force across the inner mitochondrial membrane decreases and mitochondrial ATP synthesis is consequently inhibited. Furthermore, under these conditions the F_1F_0-ATPase can itself hydrolyze cytoplasmic ATP in an attempt to regenerate the proton gradient [15]. As a result, the cellular ATP content is expected to decrease upon addition of protonophores. To test whether this effect is induced by NPPB, we compared the ATP content of cells treated with or without the channel blocker. As a positive control, the effects of uncoupling by CCCP were also assessed. As anticipated, in the presence of 10 μM CCCP the ATP content of macrophages dropped to $10 \pm 3\%$ ($n = 5$) of the control level after 8 min. A qualitatively similar effect was observed in the presence of NPPB (100 μM), which decreased cellular ATP content to $65 \pm 5\%$ of control ($n = 7$). It is noteworthy that, while NPPB and CCCP stimulate mitochondrial respiration to a comparable extent, the ATP depletion induced by the anion channel blocker is substantially

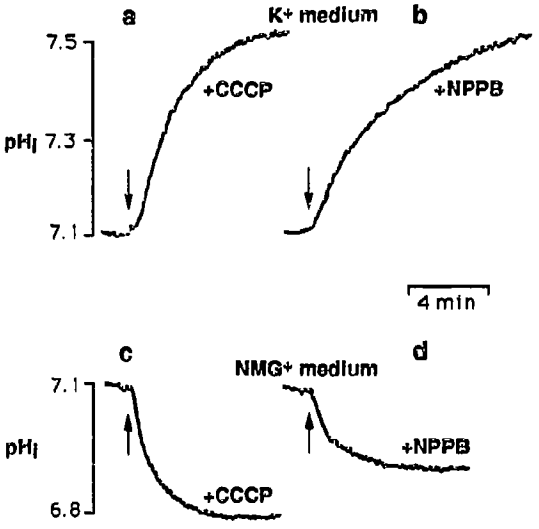

Fig. 3. NPPB and CCCP increase the plasma membrane proton permeability in human neutrophils. Cytoplasmic pH (pH_i) changes were measured using the fluorescent indicator BCECF. BCECF-loaded neutrophils (10^6/ml) were suspended in K^+ medium (140 mM KCl, mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.7; panels a and b) or NMG^+ medium (140 mM NMG-Cl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.7; panels c and d) at 37°C in the presence of 1 μM CCCP (a and c) or 100 μM NPPB (b and d). After a steady-state pH_i was attained, conductive proton movements were induced by increasing the plasma membrane K^+ permeability with 1 μM valinomycin (arrows). Representative of 3-4 experiments.

smaller. This discrepancy could be explained if NPPB simultaneously exerted an inhibitory effect on ATP consumption by blocking the mitochondrial adenine nucleotide carrier, the F_1F_0-ATPase and/or other ATP utilizing processes.

In pancreatic beta cells, NPPB was reported to affect the activity of K^+-selective channels [16]. Because these channels are sensitive to the intracellular level of ATP, the effects of the anion channel blocker were attributed

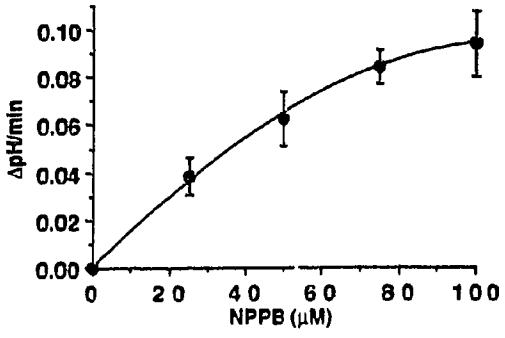

Fig. 4. The rate of conductive proton transport across the plasma membrane of human neutrophils as a function of NPPB concentration. Proton movements were determined in K^+ medium in the presence of the indicated concentration of NPPB as described in Fig. 3b. Data are means of at least 3 experiments ± SE.

to a decreased concentration of the nucleotide. The authors of this report concluded that NPPB might interact with the anion channel of inner mitochondrial membranes and induce uncoupling by a mechanism that was not clearly defined. In light of our results, however, uncoupling can be more simply explained by the direct protonophoric effect of the channel blocker.

In summary, the data presented here indicate that NPPB, a commonly used chloride channel blocker, can increase the conductive proton (equivalent) permeability of biological membranes. When exerted on the plasma membrane, this protonophoric activity can induce cytosolic pH changes. More importantly, at concentrations frequently used for electrophysiological experiments, NPPB can uncouple mitochondria, leading to partial ATP depletion. In view of these findings, caution is recommended when interpreting results obtained using NPPB and related compounds as anion channel blockers in intact cells.

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