Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 312-319

ATP-DEPENDENT H^+ TRANSPORT BY THE TURTLE BLADDER: NBD-Cl PREFERENTIALLY INHIBITS THE VANADATE-INSENSITIVE COMPONENT IN ISOLATED MEMBRANES

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Received April 10, 1989

SUMMARY: We investigated the inhibitory effects of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) on ATP-dependent H^+ accumulation by membrane vesicles prepared from the turtle urinary bladder epithelium. NBD-Cl at 30 μM was found to completely inhibit the vanadate-insensitive component of H^+ transport, with half-maximal inhibition occurring at 4.2 to 5.4 μM. In contrast, the vanadate-inhibitable component was unaffected by 30 μM NBD-Cl. At high concentrations (300 μM), both components were fully inhibited. The results confirm the presence of two distinct H^+ transport processes in turtle bladder membranes and identify selective inhibitors, NBD-Cl and vanadate, for each process.

The urinary bladder of the turtle can acidify or alkalinize the urine and is regarded as an acid-base transport model of the renal collecting duct of mammals (1-4). Acidification occurs by a process that is active and electrogenic, not dependent on sodium (5), and appears to be due to a membrane-bound ATPase which secretes H^+ (or absorbs equivalents of base) (3,6). In accord with this model, it is known that membrane vesicles prepared from the bladder epithelium accumulate H^+ when MgATP is present (7-10). Recently, we have demonstrated that the active transport of H^+ in vesicles has two components, one of which is inhibited by low concentrations of vanadate (apparent I_{50}, 45 nanomolar) and one which is unaffected by concentrations as high as 1×10^{-4} M (11). Similar results are seen with the intact bladder, where the transepithelial acidification current also has vanadate-inhibitable and vanadate-insensitive components (12,13).

To aid the experimental evaluation of the two H^+ transport systems in isolated membranes, it would be of value to be able to selectively inhibit either component. While vanadate clearly inhibits one, no agent has been available to selectively inhibit the other, the vanadate-insensitive process. In the present study, we report

Abbreviations: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; EDTA, ethylenediaminetetraacetic acid; TRIS, tris(hydroxymethyl)aminomethane; I_{50}, concentration yielding 50% inhibition; V_0, initial velocity of fluorescence quenching; ΔF_{Nig}, fluorescence increase upon Nigericin addition.

0006-291X/89 $1.50

the effects of such an inhibitor, NBD-Cl. Our results demonstrate that NBD-Cl at a concentration of 30 micromolar fully inhibits vanadate-insensitive H^+ transport by isolated membranes while having no effect on vanadate-inhibitable transport. The latter is inhibited only at significantly higher concentrations. The results further establish the presence of two distinct ATP-dependent H^+ transport processes in membranes isolated from the turtle bladder epithelium.

MATERIALS AND METHODS

Isolation of the cell homogenate. Membrane vesicles were prepared from the epithelial cells of 12-18 *Pseudemys scripta elegans* urinary bladders. Bladders were filled with a buffer containing (in mM): NaCl, 100; KCl, 2.5; $MgSO_4$, 0.8; KH_2PO_4, 0.16; K_2HPO_4, 1.01; Na_3 EDTA, 0.425; glucose, 11; pH 7.4. Bladders were immersed in this same solution, gassed with 100% O_2, and incubated for one hour at 22°, during which time some epithelial cells separated from the submucosa. At the end of the incubation, more cells were loosened by gently rubbing the bladders and the cell-containing mucosal fluid was removed and centrifuged at 10,000 x g for 20 minutes at 3° to yield a pellet of packed cells. All subsequent isolation steps were done at 0-3°. The pellet was suspended in 30-40 ml of: sucrose, 250 mM; α-methyl-D-mannoside, 2 mM; TRIS-EDTA, 1 mM; and bovine serum albumin, 0.1%, pH 7.4, and homogenized with ten strokes of a Dounce homogenizer (tight pestle) followed by eight strokes with a Potter-Elvehjem homogenizer.

Isolation of membrane fraction. Membranes were separated from other components of the homogenate by centrifugation. After centrifugation of the crude homogenate, twice at 750 x g (10 min. each time), nuclei and unbroken cells were found in the pellet (P-1) which was discarded. The supernatant S-1 was centrifuged at 14,500 x g for 15 minutes. The resulting supernatant S-2 was saved and the pellet P-2 was resuspended in the same medium and rehomogenized by eight strokes of the Potter-Elvehjem homogenizer. The centrifugation and rehomogenization steps were repeated twice more to yield supernatants S-3 and S-4 and a two-layered pellet P-4. P-4 was discarded. S-2, S-3 and S-4 were pooled and centrifuged at 100,000 x g (1 hr) to yield supernatant S-5, which was discarded, and a low-density membrane pellet P-5. These membranes are enriched in ouabain-sensitive ($Na^+ + K^+$)-ATPase activity but lack measurable oligomycin-sensitive ATPase and β-glucuronidase activities (11). Thus, the membrane fraction P-5 is enriched in plasma membranes and devoid of mitochondrial and lysosomal membranes. P-5 was resuspended (to 5-15 mg protein/ml) in 0.5-1.0 M sucrose and stored at −70° until used. The concentration of protein in membrane preparations was determined by the method of Bradford (14).

Assay of proton transport in vesicles. Active proton transport in vesicles was measured by determining the magnitude and rate of ATP-induced quenching of acridine orange (AO) fluorescence, indicative of an inside-acid pH gradient (15,16). This was carried out in Perkin-Elmer 512 or Turner 430 spectrofluorometers using 493 nm excitation/526 nm emission wavelengths. In each assay, an aliquot of membranes (containing 85 µg protein) was suspended in a medium of: 100 mM KCl/3.0 mM $MgSO_4$/15 mM sucrose/10 mM TRIS-Hepes/1.0 µM AO/10^{-4} M ouabain/5-10 µg/ml oligomycin; pH 7.3; final volume 400 µl. That these membranes are present in vesicular form has been shown by their osmotic reactivity (8,9). The vesicles were preincubated in this medium for 5 minutes or until the AO fluorescence reached a steady-state, at which time TRIS-ATP was added to a concentration of 1.0 mM. The response to this addition was a rapidly developing (4-8 min.) decrease (quench) in AO fluorescence to a new steady-state, usually 40-50% of the pre-ATP level. At the end of each experiment, fluorescence was returned to the pre-ATP level by the addition of 3 µM nigericin.

The same assay was carried out with NBD-Cl (1×10^{-6} to 3×10^{-4} M) added prior to ATP, either in the absence or presence of 10^{-5} M sodium orthovanadate (Na_3VO_4, Sigma). In some experiments, vanadate was added at the point of maximum quenching of AO fluorescence. NBD-Cl was used no more than one month after

receipt from the supplier (Sigma). On the day of the experiment, it was made into stock solutions of 50 mM in 100% ethanol and 2 mM in 4% ethanol. The stock solutions were shielded from light, kept at 0°C until used, and discarded after 3 hours. Precautions against vanadate cross-contamination have been described elsewhere (11).

RESULTS

Effects of NBD-Cl on Vanadate-insensitive H^+ Transport. To examine the effects of NBD-Cl on the vanadate-insensitive component of proton transport, the vanadate-sensitive component was first eliminated by pre-incubating vesicle suspensions with a maximally inhibiting concentration of vanadate (10^{-5} M) as determined previously (11). Figure 1 shows the effects of 0, 10, and 30 μM NBD-Cl on ATP-induced quenching of acridine orange fluorescence (proportional to the inside-acid pH gradient formed across vesicle membranes) in three aliquots from a single batch of membranes. It is apparent that NBD-Cl inhibits both the initial velocity of quenching (V_0) observed immediately after ATP addition, and the ultimate total magnitude of quenching (ΔF_{Nig}, the nigericin-induced return to baseline at the end of each experiment).

The figure 2 shows full dose-response inhibition curves for the effect of NBD-Cl (in the presence of vanadate) on V_0 (left panel) and ΔF_{Nig} (right panel) in membranes from a single batch. Both curves are sigmoidal in shape; 50% inhibition

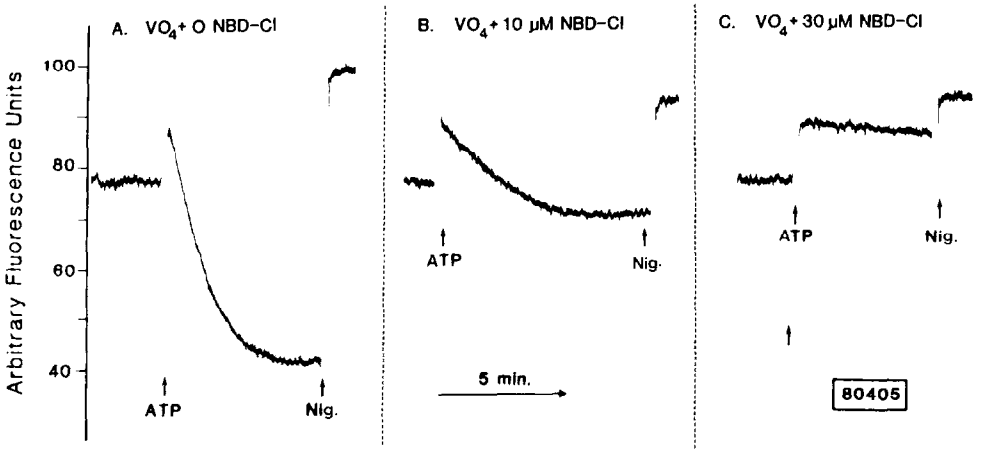

Figure 1. NBD-Cl inhibition of ATP-dependent, vanadate-resistant H^+ transport. Suspensions of membrane vesicles were prepared for acridine orange fluorescence measurements as described in Methods. Sodium orthovanadate (Na_3VO_4), final concentration 1 X 10^{-5} M, was present throughout and NBD-Cl at the indicated concentrations was added at the start of each experiment. Panel A: control, no NBD-Cl; B: 10 μM; and C: 30 μM. MgATP (final concentration 1 mM) was added to initiate pH gradient formation, evidenced by progressive quenching of fluorescence F to a new steady-state F_{min}. At that point the pH gradient was collapsed and F was returned to baseline F_{final} by 3 μM Nigericin. MgATP causes a shift in baseline. Abscissa: time; ordinate: fluorescence in arbitrary units.

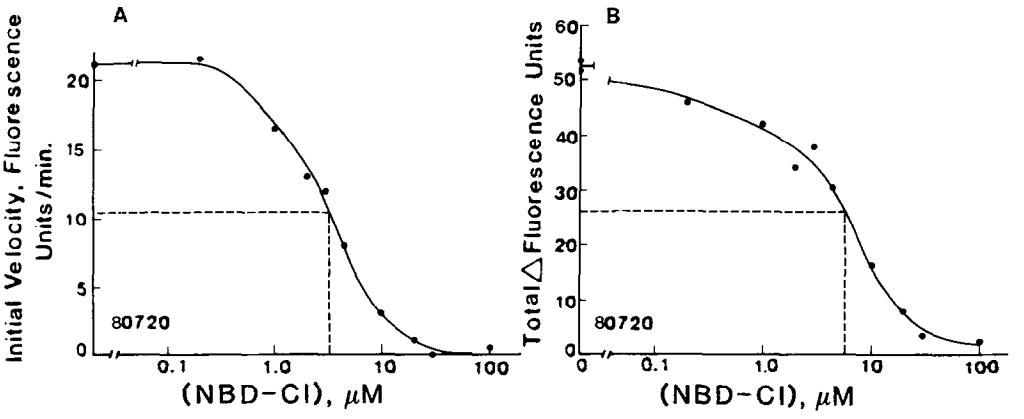

Figure 2. Dose-response inhibition curves for the effects of NBD-Cl on quenching of acridine orange fluorescence in response to MgATP. Individual data points represent experiments of the type shown in Figure 1 and were obtained with aliquots from a single batch of membranes. Panel A: effect on initial velocity of quenching immediately after MgATP addition. Abscissa, concentration of NBD-Cl in μM. Ordinate, initial velocity in arbitrary fluorescence units/min. Panel B: effect on total quenching. Abscissa, concentration of NBD-Cl in μM. Ordinate, fluorescence change (in arbitrary units) upon addition of 3 μM Nigericin at the end of each experiment (F_{final} - F_{min} as defined in legend to Figure 1).

occurs at 3.3 μM NBD-Cl in the case of V_0 and 6.0 μM in the case of ΔF_{Nig}. When the NBD-Cl concentration was raised to 30 μM, 100% of the V_0 and 93% of the ΔF_{Nig} values were eliminated. The results seen in this batch of membranes were typical of others. In five batches, the I_{50} value for V_0 was 4.2 ± 1.3 μM and for ΔF_{Nig}, 5.4 ± 1.1 μM. The difference between the values, 1.2 ± 1.0 μM, was not significant ($p > 0.2$). In the same five batches, 30 μM NBD-Cl in the presence of 10 μM Na_3VO_4 eliminated 99 ± .5% of the V_0 and 90 ± 2% of ΔF_{Nig}. The ΔF_{Nig} remaining is due primarily to the presence, in some preparations, of a small spontaneous gradient present before ATP is added.

Effect of NBD-Cl on Total H^+ Transport and the Vanadate-inhibitable Component. The following experiments were carried out to determine if low concentrations of NBD-Cl, which inhibit vanadate-insensitive acid transport, act similarly on the vanadate-inhibitable transport of protons. Thus, these experiments were not preincubated with vanadate. Figure 3 shows the effects of 0, 10, and 30 μM NBD-Cl on both total (vanadate-inhibitable plus vanadate-insensitive) quenching and on the vanadate-inhibitable component in aliquots from a single membrane batch. The vanadate-inhibitable component was determined by measuring the decay in fluorescence quenching for five minutes after 10^{-5} M vanadate was added at the point of maximum quenching (11). The figure indicates that while NBD-Cl inhibits the total degree of quenching (V_0 or ΔF_{Nig}), the vanadate-inhibitable component is unaltered by 10 or 30 μM NBD-Cl. In four membrane batches, the vanadate-induced decay of quenching in the presence of 30 μM NBD-Cl was 101 ± 6% of that seen in the absence of NBD-Cl ($p > .9$ versus control; control = 17 ± 3 fluorescence units,

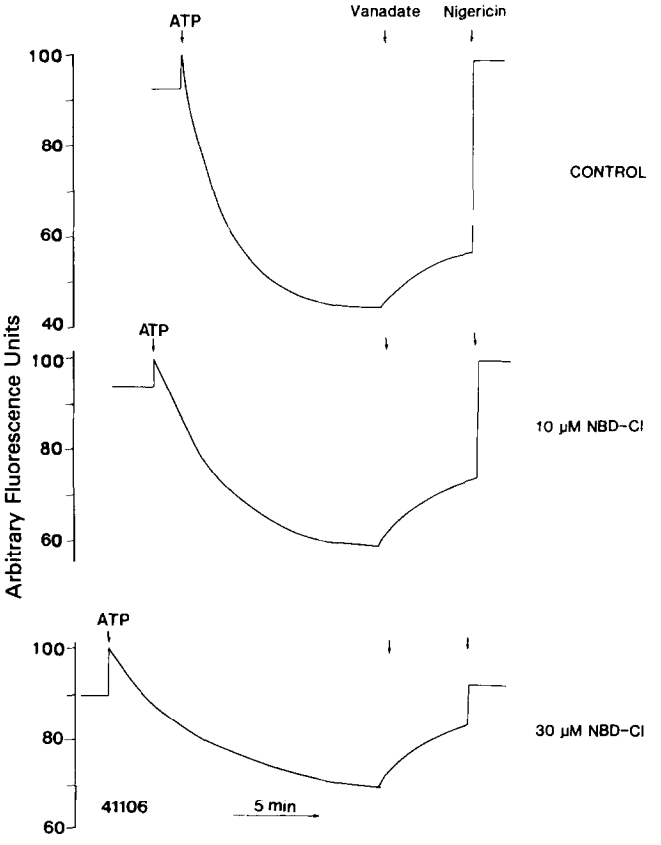

Figure 3. Lack of effect of low NBD-Cl concentrations on formation of pH gradients by vanadate-inhibitable H^+ transport. Experiments were performed as described in the legend to Figure 1, except that sodium orthovanadate was omitted initially. Instead, to assess the amount of vanadate-sensitive H^+ transport present at different NBD-Cl concentrations, vanadate was added (final concentration 1 X 10^{-5} M) after ATP had generated maximally quenched fluorescence. Panel A: control, no NBD-Cl; Panel B: 10 μM NBD-Cl present throughout; Panel C: 30 μM NBD-Cl. Abscissa is time, ordinate is fluorescence in arbitrary units.

FU). Ten μM NBD-Cl actually increased dequenching slightly to 110 ± 5% of control, although the change in mean value did not reach significance ($0.1 > p > .05$; control = 17 ± 3 FU). At a higher concentration, 300 μM, (not shown) essentially all ATP-dependent quenching was eliminated. Taken together, the results indicate that at 30 μM NBD-Cl, vanadate-insensitive transport is maximally inhibited, while vanadate-inhibitable transport is unaffected.

DISCUSSION

Membrane vesicles isolated from the turtle bladder epithelium actively accumulate H^+ in the presence of MgATP (7-10). This suggests that acidification of urine by the bladder *in situ* is due at least in part to a proton-translocating ATPase. We reported recently that the ATP-dependent uptake of H^+ ions in isolated vesicles

is in fact two distinct processes, one of which is inhibited by low concentrations of orthovanadate and one of which is not affected by vanadate (11). To further distinguish and characterize these, we report here the effects of NBD-Cl in isolated membrane vesicles. We find that vanadate-insensitive H^+ transport is selectively eliminated by 30 μM NBD-Cl and that 50% inhibition occurs at 3-6 μM. In contrast, vanadate-inhibitable H^+ transport is not affected by 30 μM NBD-Cl. At a higher concentration, 300 μM, all ATP-dependent H^+ transport is eliminated.

Inhibition of both types of H^+ transport (albeit at different concentrations) is not unexpected. NBD-Cl is an adenine analog (17) that is known to inhibit a variety of ATPases, including the NaK-ATPase (18) and a number of H^+-translocating ATPases. In this report, we have investigated only the effects of NBD-Cl on ATP-dependent proton transport *per se* and have not investigated its enzymatic effects or mechanism of inhibition. NBD-Cl inhibits both proton transport and ATPase activity in other systems, including clathrin-coated vesicles (19-21), the membranes of storage granules in platelets (22) and adrenal chromaffin cells (23), the Golgi apparatus (24), and the vacuolar membranes of yeast (25), fungi (26,27), and higher plants (17). Subsequent studies have shown that NBD-Cl binds in an ATP-protectable manner to tyrosyl hydroxal or cysteinyl sulphydryl groups in or near the ATP binding site of the ATPases (17,25).

The proton pumps described above belong to the proposed class of intracellular proton pump designated "vacuolar," which also includes those found in lysosomes (28,29) and non-clathrin-coated endosomes (30,31), whose sensitivity to NBD-Cl is not yet known. Vacuolar H^+-translocating ATPases also share resistance to oligomycin, which inhibits the coupling-factor H^+-ATPases, and vanadate, which inhibits ATPases which form a phosphorylated intermediate state (20). This suggests that the two H^+ pumps found in turtle bladder membranes correspond to two H^+-ATPases. One of these, vanadate-insensitive but inhibited by low concentrations of NBD-Cl, may be of the vacuolar type. The second type, less sensitive to NBD-Cl but inhibitable by low vanadate concentrations, is likely to be a phosphorylated intermediate-forming or E_1E_2 type. It is notable that both vacuolar and E_1E_2 H^+-ATPases (as well as the mitochondrial H^+-ATPase) are found in the fungus *Neurospora crassa* (26,27,32), the yeast *Saccharomyces cerevisiae* (25), and higher plants (17,33). In each case the phosphorylated intermediate-forming enzyme is located in the plasma membrane.

In summary, the present results further confirm the existence of two distinct ATP-dependent proton transport processes in isolated epithelial cell membranes from the turtle bladder. It seems likely that these processes correspond to the vanadate-insensitive and vanadate-inhibitable portions of the transepithelial acidification current found across the intact bladder (12,13). The present work indicates that one process is inhibited by low concentrations of NBD-Cl but is insensitive to vanadate, and thus could be due to a vacuolar-type H^+-ATPase. This class of enzyme has been

described in a number of intracellular organelles. Proton pump-containing cytoplasmic membrane vesicles, which are thought to be exocytotically inserted into the apical membrane upon stimulation of acidification in the turtle bladder and mammalian collecting duct epithelia (for review, see 34), may be a specialized example. The other process, less sensitive to NBD-Cl but inhibited by low vanadate concentrations, appears analogous to the E1E2-type constitutive plasma membrane H^+ transporters found in other tissues. The availability of inhibitors selective for each process in isolated membranes will aid in determining the role of each of these transport processes in acid secretion by the intact bladder.

ACKNOWLEDGMENTS. This work was supported by grant number DK33743 from the NIH. We are grateful to Sue Chapman for excellent word processing of the manuscript.

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