Quinacrine mustard and lipophilic cations inhibitory to both vacuolar H^+-ATPase and F_0F_1-ATP synthase

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Abstract Various lipophilic cations, such as quinacrine mustard and dequalinium, which are known to inhibit mitochondrial F_1-ATPase, strongly inhibited vacuolar H^+-ATPase purified from bovine adrenal chromaffin granules. Quinacrine mustard bound irreversibly to vacuolar H^+-ATPase subunit *A*, and the 115 kDa accessory polypeptide and dithiothreitol had no effect. The binding was competitively inhibited by chlorpromazine and quinacrine, and these compounds specifically reduced the amount of labeling of subunit *A*. Quinacrine mustard also prevented the binding of [α-^{32}P]ATP to subunit *A* but had no effect on the binding of [^{3}H]*N*-ethylmaleimide to either subunit *A* or the 115 kDa accessory polypeptide. These results suggest that the binding site of quinacrine mustard in subunit *A* is not related to the *N*-ethylmaleimide-binding site(s), which is important for activity.

Key words: Chromaffin granule; Vacuolar H^+-ATPase; Quinacrine mustard; Dequalinium; Lipophilic cation; Inhibitor

1. Introduction

Vacuolar H^+-ATPases (V-ATPases) transport protons into various organelles, including lysosomes, the Golgi apparatus, chromaffin granules and synaptic vesicles (for reviews, see [1–5]). The inside acidic pH and the transmembrane electrochemical proton gradient thus established are essential for many cellular processes, such as digestion of proteins in lysosomes and concentration of amines and amino acids into synaptic vesicles.

The V-ATPases are composed of two different sectors: a hydrophilic V_1 sector containing five different subunits (subunits *A–E*) and a hydrophobic V_0 sector containing at least five subunits [1–6]. It is generally accepted that V-ATPases have a similar subunit organization to F_0F_1 ATP synthase (F-ATPase): subunits *A*, *B* and *c* (16 kDa subunit) of V-ATPases have similar copy numbers and significant amino acid sequence homologies to the β, α and *c* subunits of F-ATPase, respectively [1–5]. Similarities in the catalytic mechanisms of V- and F-ATPases have also been pointed out [7–9]. Moriyama and coworkers observed that chromaffin granule ATPase has ATP binding sites with high and low affinities [7] and is modulated by ADP [8]. Furthermore, yeast V-ATPase shows unisite catalysis, suggesting the presence of similar catalytic cooperativity to that of F-ATPase [9]. A significant functional difference is, however, that V-ATPases have no ability to synthesize ATP coupled with H^+ transport [1,10], whereas most F-ATPases function as ATP synthases. It is important to understand the molecular bases for the similarities and differences between these two types of ATPases.

Inhibitors of both types of ATPases may provide an understanding of their mechanistic similarities and differences, but only limited numbers of such inhibitors are known [2,3]. Therefore, in this study, we tested the effects of various lipophilic cations such as quinacrine mustard and dequalinium, which are known inhibitors of mitochondrial F_1-ATPase [11,12], on V-ATPase purified from bovine adrenal chromaffin granules. We found that the V-ATPase was also sensitive to these F_1-ATPase inhibitors, and studied the mechanism of the inhibition.

2. Experimental

V-ATPase with a specific activity of 3.0 U/mg protein was purified from chromaffin granules and reconstituted into liposomes by the method of Moriyama and Nelson [7,13]. Synaptic vesicles from rat brain [14] and vacuolar membrane vesicles from carrots and potatoes [15] were prepared by established procedures and stored at −70°C. F-ATPase was purified from over-producing *Escherichia coli* cells (DK8/pBWU13) [16]. Quinacrine mustard and other lipophilic cations used in this study were purchased from Sigma. [^{3}H]NEM (40 mCi/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were obtained from New England Nuclear and Amersham, respectively. Other reagents were the highest grade commercially available.

Proteoliposomes containing purified V-ATPase were incubated at 30°C for 10 min in 20 mM MOPS-Tris (pH 7.0) supplemented with 0.1 M NaCl, 2 mM $MgCl_2$ and 30 μM quinacrine mustard, washed three times with the buffer only and suspended in the buffer supplemented with 0.1 M $NaNO_3$, 2 mM $MgCl_2$, and 4 mM ATP. The proteoliposome suspension was kept in an ice bath for 1 h with occasional mixing. This treatment released the hydrophilic V_1 sector from the proteoliposomes [17]. After centrifugation at 279,000 × *g* for 1 h, the supernatant containing V_1 was carefully separated and its fluorescence of quinacrine mustard was measured fluorometrically in 1 ml of 0.2 M MOPS-Tris (pH 8.0) with excitation and emission wave lengths of 275 and 495 nm, respectively.

Published methods were used for measuring ATPase activity [16], ATP-dependent formation of a pH gradient [14], labeling of V-ATPase subunits with [^{3}H]NEM and [α-^{32}P]ATP [7], protein [18] and polyacrylamide gel-electrophoresis in the presence of sodium dodecyl sulfate [7].

3. Results

3.1. Lipophilic cations as V-ATPase inhibitors

Allison and colleagues reported that a series of lipophilic

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Abbreviations: F-ATPase, F_0F_1 ATP synthase; F_1-ATPase, catalytic portion of F-ATPase; MOPS, morpholinopropane sulfonic acid; NEM, *N*-ethylmaleimide; V-ATPase, vacuolar H^+-ATPase; V_1, catalytic portion of V-ATPase.

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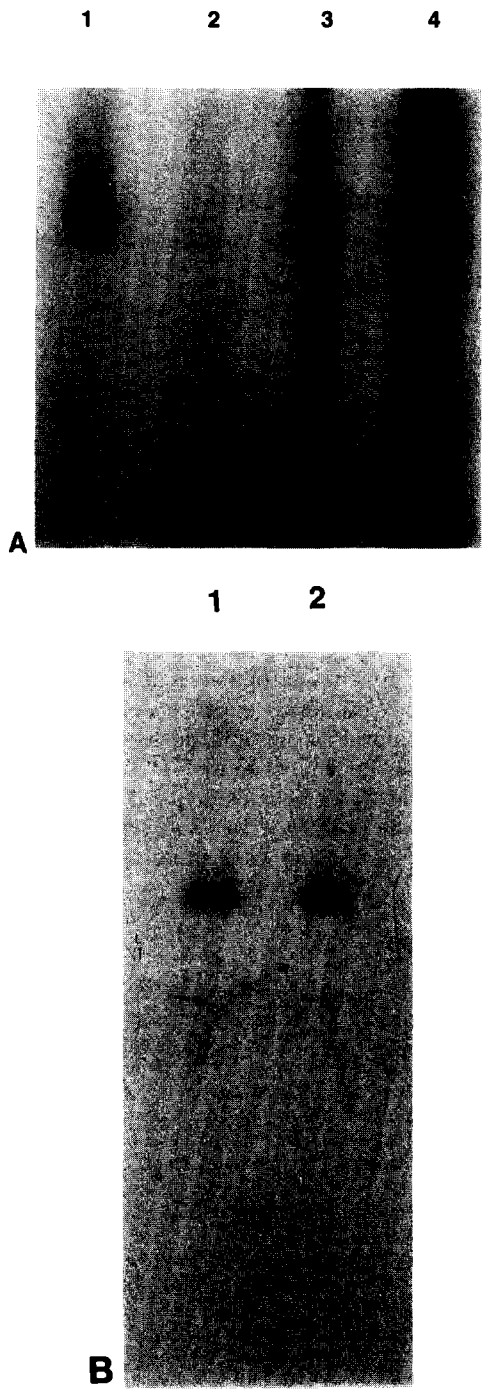

Fig. 1. Effect of quinacrine mustard on chemical modification of V-ATPase with [α-^{32}P]ATP or [^{3}H]NEM. (A) Effect of quinacrine mustard on ATP-binding. Purified V-ATPase (3 μg protein) in 50 μl of 20 mM MOPS-Tris (pH 7.0) buffer containing 5 mM monothioglycerol, 2 mM $MgCl_2$ and 0.3 μM [α-^{32}P]ATP was irradiated with UV light for 2 min in the presence of various inhibitors [4]. After dissociation of the enzyme, the samples were subjected to 10% polyacrylamide gel-electrophoresis in the presence of 0.2% SDS. The gels were fixed and autoradiographed: lane 1, no inhibitor; lane 2, 0.1 mM NEM; lane 3, 20 μM quinacrine mustard; lane 4, 1 μM bafilomycin A1. (B) Effect of quinacrine mustard on NEM-binding. Purified V-ATPase (3 μg protein) was incubated in 50 μl of 20 mM MOPS-Tris (pH 7.0) buffer containing 0.1 M NaCl and 50 μM [^{3}H]NEM in the absence (lane 1) or presence (lane 2) of quinacrine mustard (50 μM). After incubation for 10 min at room temperature, reactions were terminated by addition of the same buffer (10 μl) containing 10% β-mercaptoethanol and 10% SDS and the mixtures were subjected to polyacrylamide gel-electrophoresis. A fluorograph is shown.
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cations inhibited F_1-ATPase [11,12]. We investigated the effects of the lipophilic cations on V-ATPase purified from bovine chromaffin granules in comparison with their effects on bacterial F-ATPase. These compounds inhibited both V- and F-ATPase at essentially the same concentrations and orders of effectiveness (Table 1). This is the first observation that lipophilic cations inhibit V-ATPase.

3.2. *Characteristics of quinacrine mustard-induced inhibition*

Dequalinium and quinacrine mustard were strong inhibitors: 4.4 μM dequalinium and 8.2 μM quinacrine mustard caused 50% inhibition (ID_{50}) of the activity of purified V-ATPase (Table 1). Quinacrine mustard inhibited ATP-dependent proton transport by V-ATPase from rat brain synaptic vesicles and plants vacuoles at similar concentrations (Table 2). However, dequalinium was required at a more than 10-fold higher concentration for 50% inhibition of the transport and ATPase activity in synaptic and plant vesicles (data not shown). Therefore, we concentrated on the mode of inhibition of V-ATPases by quinacrine mustard.

The ATPase activity inhibited by quinacrine mustard was not restored by extensive washing of proteoliposomes with 10 mM MOPS-Tris buffer (pH 7.0) containing 2 mM $MgCl_2$ and 5 mM thioglycerol, indicating that quinacrine mustard bound to the enzyme irreversibly. The other compounds listed in Table 1 inhibited the enzyme reversibly.

Quinacrine and chlorpromazine were much less inhibitory. However, they lowered the inhibition by quinacrine mustard. After incubation of V-ATPase with quinacrine mustard (30 μM) in the presence or absence of quinacrine (5 mM) and chlorpromazine (1 mM), the enzyme was diluted 20-fold and the ATPase activity was measured: 57 and 63% of the activities, respectively remained, whereas only 10% of the activity remained after incubation without quinacrine or chlorpromazine.

Quinacrine mustard prevented the binding of [α-^{32}P]ATP to subunit *A* (Fig. 1A). Similar inhibition of the labeling was observed with NEM, the binding site of which is in subunit *A*, but neither bafilomycin A_1 nor *N,N'*-dicyclohexylcarbodiim-

Table 1
Inhibitions (ID_{50}; μM) of V- and F-ATPases by various lipophilic cations

Reagent	V-ATPase	F-ATPase
Quinacrine mustard	8.2	5.3
Quinacrine	930	580
Dequalinium	4.4	24
Acridine orange	270	68
Brilliant green	170	27
Rhodamine 6G	315	34
Safranine O	450	330
Pyronin Y	78	70
Rhodamine 123	1500	580

The ATPase activities of purified V-ATPase or bacterial F-ATPase were measured with at least seven different concentrations of the listed lipophilic cations, and results are expressed as ID_{50} values (concentrations required for 50% inhibition).

ide, known to bind to V_0 subunits [8,9], inhibited the labeling (Fig. 1A). Quinacrine mustard had no effect on the binding of [^{3}H]NEM to subunit *A* (Fig. 1B) and the inactivation by quinacrine mustard was not affected by dithiothreitol (0.5 mM), indicating that a cysteine residue(s) in subunit *A* is not involved in the inhibition by quinacrine mustard.

3.3. Labeling of subunit A by quinacrine mustard

To confirm that subunit *A* is the binding site(s) for quinacrine mustard, V-ATPase inactivated with 30 μM quinacrine mustard was dissociated and applied to a polyacrylamide gel, and the subunits labeled with quinacrine mustard were located. We found that subunit *A* and a 115 kDa accessory subunit were the proteins predominantly labeled fluorescently with the reagent (Fig. 2, lane 1), whereas essentially no quinacrine mustard fluorescence was detectable in other subunits. Quinacrine (5 mM) (Fig. 2, lane 2) or chlorpromazine (1 mM) (data not shown) reduced the binding of quinacrine mustard to subunit *A* and its inhibition of ATPase activity. These results suggested that the binding of quinacrine mustard to subunit *A*, but not to the 115 kDa polypeptide, inhibited the enzyme.

The labeled V_1 sector was prepared by cold treatment [17], and quinacrine mustard in subunit *A* was measured fluorometrically: about 2.7 nmol of quinacrine mustard bound to 1 nmol of V_1 sector. Quinacrine mustard bound with the 115 kDa polypeptide that remained in the membrane sector, consistent with the intrinsic membrane location of this subunit [7]. These results suggested that 3 mol of quinacrine mustard bound per mol of V_1 sector, suggesting that incorporation of 1 mol of quinacrine mustard per mol of subunit *A* caused complete inactivation.

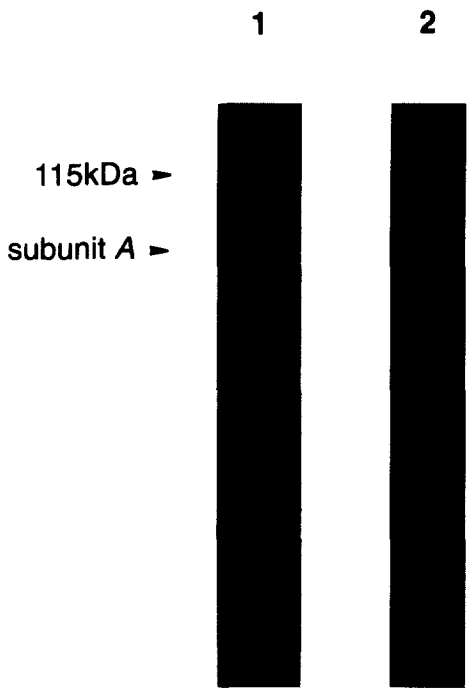

Fig. 2. Detection of V-ATPase subunits labeled with quinacrine mustard. Reconstituted V-ATPase proteoliposomes (3 μg protein) were incubated in 50 μl of 20 mM MOPS-Tris (pH 7.0) buffer containing 0.1 M NaCl, 2 mM $MgCl_2$ and 30 μM quinacrine mustard in the absence (lane 1) or presence (lane 2) of quinacrine (5 mM) for 10 min at room temperature. Reactions were terminated by adding 10 μl of 20 mM Tris-Cl (pH 8.8) containing 10% SDS and the mixtures were subjected to polyacrylamide gel-electrophoresis. The gels were soaked in 0.1 M Tris-Cl (pH 8.8) and quinacrine mustard bound to the subunits was detected under UV light. Positions of subunits were identified in gels stained with Commassie brilliant blue.

4. Discussion

The effects of lipophilic cations on V-ATPase have been estimated previously using organellar membrane preparations. The results were, however, discrepant depending on the preparation, because V-ATPase is not the only enzyme hydrolyzing ATP [19]. For instance, chlorpromazine and tricyclic antidepressant inhibited partially purified chromaffin granule ATPase [20,21], and antimalarial agents such as chloroquine inhibited lysosomal ATPase [22]. However, we showed recently that these lipophilic cations are less inhibitory to purified V-ATPase even at mM concentrations [23]. Here, we found that a group of lipophilic cations, and especially quinacrine mustard, strongly inhibited V-ATPase as well as F-ATPase.

The mode of inhibition of V-ATPases by quinacrine mustard was similar to that of inhibition of mitochondrial F_1-ATPase: the inhibition was irreversible, partially protected by chlorpromazine or quinacrine and was due to stoichiometric binding to the catalytic subunit [11]. These results suggested that quinacrine mustard has similar binding sites in F- and V-ATPases and blocks a catalytic pathway common to the two. Allison et al. reported that quinacrine mustard bound to a cluster of anionic amino acids (DELSEED) in the catalytic β subunit of mitochondrial F-ATPase [24]. This region corresponds to ASLAETD (residues 499–505) in subunit *A* of bovine brain V-ATPase (identical residues underlined) [25], and may be a quinacrine mustard-binding site in subunit *A*. It is noteworthy that this region is different from a putative nucleotide binding domain (bovine residues 250–257) or a cysteine residue(s) conferring NEM sensitivity [25]. Consistent with this possibility, the present results suggest that the binding site for quinacrine mustard is different from that of the ATP or NEM-binding domain in subunit *A*.

This work showed that quinacrine mustard and other lipophilic cations are strong inhibitors of V- and F-ATPases. These compounds will be useful to probe the molecular mechanism common to both types of ATPase. Furthermore, derivatives of these compounds may have selective toxicities on V-ATPases of non-mammalian species such as fungi and protozoa.

Table 2
Inhibitions of V-ATPases from various sources by quinacrine mustard

Vacuolar membranes	ID_{50} (μM)
Rat brain synaptic vesicles	4.8
Carrot vacuolar membrane vesicles	5.0
Potato vacuolar membrane vesicles	3.5

ATP-dependent H^+ transports in various vacuolar membranes were assayed by acridine orange fluorescence quenching in the presence of various concentrations of quinacrine mustard. Results are expressed as ID_{50} values. With plant vacuolar membrane vesicles, vanadate (0.2 mM) and EGTA (0.1 mM) were added to inhibit plasma membrane H^+-ATPase (P-type ATPase) and Ca^{2+}/H^+, respectively, although contaminations of the plasma membrane vesicles were negligible.

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