

Vacuolar ATPase-mediated cellular concentration and retention of quinacrine: a model
for the distribution of lipophilic cationic drugs to autophagic vacuoles

François Marceau, Marie-Thérèse Bawolak, Johanne Bouthillier and Guillaume
Morissette

From Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier
Universitaire de Québec and Department of Medicine, Université Laval, Québec, Canada
G1V 4G2 (F.M., M.-T. B., J.B., G.M.).

Running title page

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Corresponding author: François Marceau, M.D., Ph.D., Centre de recherche en rhumatologie et immunologie, Room T1-49, Centre Hospitalier Universitaire de Québec, 2705 Laurier Blvd., Québec QC Canada G1V 4G2. Tel.: 418-525-4444, ext. 46155. Fax: 418-654-2765. e-mail: francois.marceau@crchul.ulaval.ca

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Abbreviations: LC3, microtubule-associated protein light chain 3; OCT, organic cation transporter; V-ATPase, vacuolar ATPase

Abstract

The anti-protozoal agent quinacrine is a lipophilic cationic drug highly distributed to tissues. It has been used in present experiments to examine whether the vacuolar and autophagic cytopathology induced by organic amines is independent from the therapeutic class. Further, we tested the presence of the concentrated cationic drug itself in the enlarged vacuoles by exploiting the intense green fluorescence of quinacrine. Finally, the influence of lipophilicity on the apparent affinity of amine pseudotransport has been addressed by comparing quinacrine to another substituted triethylamine, procainamide. Quinacrine was concentration-dependently taken up by human smooth muscle cells (cytosolic granular-vacuolar morphology at and above 25 nM; in cell extracts, uptake nearly maximal in 2 h, apparent K_M of 8.7 μM). The vacuolar (V)-ATPase inhibitor bafilomycin A1 prevented quinacrine uptake by cells or released the cell-associated drug in preloaded cells. The lipidated (II) form of microtubule-associated protein light chain 3 (LC3) accumulated at and above a quinacrine concentration of 2.5 μM (4 h), indicating the conserved macroautophagic nature of the vacuolar cytopathology, although vacuole size was modest. The enlarged vacuoles containing quinacrine excluded cherry fluorescent protein; many vacuoles were lined with cherry fluorescent protein-conjugated Rab7, a GTPase associated with late endosomes/lysosomes. Together, these results are compatible with the transition of quinacrine-concentrating vacuoles towards an autophagolysosome identity. Quinacrine is concentrated in cells via V-ATPase-mediated ion trapping with an apparent affinity ~500-fold higher than that of the less lipophilic drug procainamide and, despite the small size of ensuing vacuoles, the macroautophagic signature of this cytopathology was observed.

Introduction

It has been proposed a long time ago that the particular vacuolar cytopathology induced by cationic drugs is due to ion trapping, with the protonation of the drug at low pH in acidic cell organelles, decreased rate of retrodiffusion and subsequent osmotic swelling of the vacuoles (de Duve et al., 1974). Recent progress in this research area supports that the proton pump vacuolar (V)-ATPase is necessary for this form of cationic drug sequestration in intact cultured cells (a form of pseudotransport; Morissette et al., 2005; 2008b). It was once believed that vacuolar cytopathology was of lysosomal origin and amines that caused it were dubbed “lysosomotropic”; however, this is an oversimplification because many of the giant vacuoles originate from the trans-Golgi, which also expresses V-ATPase (Morissette et al., 2005; 2008b). On the other hand, it was recently discovered that the giant vacuoles induced by cationic drugs rapidly become macroautophagic, being labeled with the membrane-bound (lipidated) form of the autophagic effector microtubule-associated protein light chain 3 (LC3 II), and the vacuoles acquire late endosome/lysosome markers, presumably due to lysosomal fusion to autophagosomes (Morissette et al., 2005; 2008b). The intense concentration of the tertiary amine procainamide in human vascular smooth muscle has been the primary model exploited to reach these conclusions, exhibited an apparent K_M of 4.7 mM, was abolished by chemically unrelated V-ATPase inhibitors (bafilomycin A1 or FR 167356) and preceded the formation of multiple large and clear vacuoles (Morissette et al., 2008b). The postulated first step of this form of sequestration is the crossing of plasma and vacuolar membranes by simple diffusion, a process that is inhibited for procainamide by acidifying the extracellular fluid (Morissette et al., 2008b) and that should be greater

for drugs with increased lipophilicity. Consistent with this idea, the anti-arrhythmic agent amiodarone (logP 7.2), which can be considered as a substituted triethylamine like the more hydrophilic agent procainamide (logP 1.13), was concentrated in cell vacuoles and activated LC3 processing (formation of LC3 II) at 5-20 μ M (Morissette et al., 2009). Accumulation of macroautophagic vacuoles has been observed in the skin of a patient with amiodarone-induced skin discoloration (Morissette et al., 2009), a side effect caused by drug deposition in tissues (Ammoury et al., 2008).

The anti-protozoal agent quinacrine can also be considered a substituted triethylamine, has an intermediate lipophilicity (logP 5.67), two protonable functions (pK_a 10.3 and 7.7), an intense green fluorescence and occasionally causes skin discoloration upon chronic dosing (Zuehlke et al., 1981), like amiodarone. Quinacrine has been used in present experiments to further clarify whether the vacuolar and autophagic cytopathology is independent from the therapeutic class, that the cationic drug is itself present in a concentrated form in the enlarged vacuoles and if lipophilicity influences the apparent affinity of the pseudotransport. The effect of this parameter has been isolated by reproducing a procedure previously applied to measure of procainamide uptake into smooth muscle cells (Morissette et al., 2008b).

Methods

Bafilomycin A1 was purchased from LC Laboratories (Woburn, MA), Mitotracker Red CMXRos, from Invitrogen and all other drugs, from Sigma-Aldrich (St. Louis, MO). The institutional research ethics board approved the anonymous use of human umbilical cord segments to initiate primary cultures of human umbilical artery smooth muscle cells, as described (Morissette et al, 2008b), for morphological and biochemical studies related to quinacrine cell uptake. The identity of cellular structures that concentrated quinacrine, identified by its green fluorescence, was established using fluorescent labels in intact cells: Mitotracker Red, Hoechst 33258 and the small G proteins Rab5 and -7 (Morissette et al., 2005; 2008b). Thus, some cells were transfected as described with vectors coding for cherry fluorescent protein-conjugated Rab7 or -Rab5 which were prepared by recloning the insert from the GFP-conjugated constructions (Morissette et al., 2008b) into the appropriate vector. The uptake of quinacrine was established by a variation of a technique previously applied to quantify cell uptake of procainamide in the same cell type (Morissette et al., 2008b): test drugs were added to confluent 25 cm² smooth muscle cell flasks containing 3 ml of serum-containing culture medium according to various schemes and time frames. Quinacrine uptake was determined by rapidly washing each cell flask 3 times with 3 ml of phosphate-buffered saline, pH 7.4, at room temperature and dissolving the cells with 10 ml of 1 N NaOH. Quinacrine was analyzed in the NaOH extract using a FluoroLog tau-3 luminescence spectrophotometer (HORIBA JobinYvon Inc, Edison, NJ) against a standard curve of the authentic drug dissolved in NaOH 1 N (excitation 414 nm, corrected emission 501 nm). Control fluorescence from extracts of untreated cells was systematically verified, of small magnitude and subtracted from experimental values.

Results and Discussion

Quinacrine's green fluorescence is granular and its uptake is concentration-dependent in smooth muscle cells incubated for 4 h with concentrations as low as 25 nM (fig. 1A, epifluorescence microscopy). The specific V-ATPase inhibitor bafilomycin A1 (100 nM) profoundly decreased the granular uptake of the drug over the tested quinacrine concentration range (25 nM-5 μ M). The size of the fluorescent granule was modest and vacuolization was not apparent at 100 \times magnification (phase contrast microscopy, data not shown). Other amines like procainamide cause cell vacuolization and a parallel accumulation of the activated form (II) of the macroautophagic effector LC3 (Morissette et al., 2008b). This has been replicated for a 2.5 mM concentration of procainamide (fig. 1B, 4 h treatment). Under the same conditions, quinacrine shows the conversion of the cytosolic LC3 I (~18 kDa) into the lipidated LC3 II form (~16 kDa) at and above 2.5 μ M (fig. 1B; quinacrine at 10 μ M yielded a signal as strong as procainamide at 2.5 mM), suggesting the lack of resolution of endogenous autophagy by the buffering of the vesicular acidity, the activation of autophagy by vacuolar expansion or both.

Other intact cells were labeled with 3 drugs before observation (fig. 2A): quinacrine (5 μ M, 4 h, green fluorescence), Mitotracker Red (200 nM, red fluorescence) and Hoechst 33258 (25 μ M, blue fluorescence; the 2 last stains applied 15 min before observation). At high magnification, the green fluorescence was granular or vesicular, not nuclear or filamentous. Bafilomycin A1 cotreatment selectively abolished the green labeling of cells without affecting the nuclear labeling by Hoechst 33258 or the mitochondrial staining. A combination of inhibitors that abolishes the mitochondrial inner membrane potential,

antimycin + oligomycin, prevented the uptake of Mitotracker Red with selectivity (fig. 2A). The identity of quinacrine-containing vacuoles was explored in smooth muscle cells (fig. 2B). Cell transfection with a cherryFP-coding vector showed that the enlarged vacuoles excluded the cytosolic fluorescent protein and exhibited various intensities of green fluorescence. In cells expressing cherryFP-conjugated Rab5, a marker of early endosomes, the green vacuoles were rarely lined with red, as opposed to what was seen in cells expressing cherryFP-Rab7, a marker of late endosomes/lysosomes (fig. 2B). Together, these results are compatible with the transition of quinacrine-concentrating vacuoles towards an autophagolysosome identity. Vacuoles induced by procainamide (2.5 mM) were larger and more uniform than those resulting from quinacrine treatment, also lined with cherryFP-Rab7 (fig. 2B). The size difference is possibly related to the higher absolute quantity of drug transported per cell culture surface unit at the used procainamide concentration (Morissette et al., 2008b). Procainamide's presence in vacuoles was not proven owing to its lack of visible fluorescence (fig. 2B).

Quinacrine extracted from confluent, intact smooth muscle cells treated with the drug was quantified, each experimental point corresponding to a 25-cm² flask of cells (fig. 3). A 5 μM drug concentration determined a nearly maximal uptake in approximately 2 h (fig. 3A). Cotreatment of cells with bafilomycin A1 massively inhibited the uptake after an initial retention that is possibly explained by the progressive neutralization of acidic cell compartments following bafilomycin application. In cells that were loaded for 2 h with quinacrine, the cell-associated drug was released following the addition of bafilomycin A1. The bafilomycin-induced quinacrine efflux suggests that continuous proton pumping

by V-ATPase maintains the drug in a cationic and nondiffusible form in the large vacuoles. All these properties of quinacrine uptake were previously observed in the same cell type for the sequestration of procainamide (2.5 mM) (Morissette et al., 2008b). Quinacrine cell uptake in 30 min apparently followed hyperbolic kinetics with an apparent K_M of 8.7 μ M (4.7 mM for procainamide, Morissette et al., 2008b) and a V_{max} of 29 nmol/flask (fig. 3B). The ~500-fold higher apparent affinity of the pseudotransport of quinacrine is likely to be explained by the higher lipophilicity of the drug, both quinacrine and procainamide uptake being mediated by V-ATPase in this system, and clearly supports the clinical relevance of intracellular sequestration for quinacrine. Co-treatment with large concentrations of tetraethylammonium (1 mM), cimetidine (1 mM) or verapamil (20 μ M), respectively a substrate of several organic cation transporters (OCT), an inhibitor of OCT3 and an inhibitor of P-glycoprotein and of OCTN1 and OCTN2, failed to influence quinacrine uptake (5 μ M, 30 min) by smooth muscle cells (relative to a control value of $100 \pm 5.1\%$, values of 131.0 ± 21.5 , 126.3 ± 10.0 and $109.1 \pm 7.2\%$, respectively, $n=3$, nonsignificant by ANOVA). These agents were found to modulate quinacrine uptake in other systems (Miller et al., 1999; Doghu et al., 2004). The expression of drug transporters tends to be limited to organs/tissues specialized in xenobiotic handling (kidney, liver, blood-brain and placental barriers) and, with exceptions, rare outside these organs (Zhang et al., 2006). OCT3 (responsible for catecholamine uptake-2) has been identified in bronchial and vascular smooth muscle cells (Horvath et al., 2007). The classical cation trapping model is based on the simple diffusion of the uncharged form of the secondary or tertiary amines (De Duve et al., 1974). Drug transport or efflux at the plasma membrane level would complicate the

model, but not invalidate it because V-ATPase could mediate the high capacity concentration and retention of the amines in intracellular acidic vesicles. Although other forms of quinacrine interaction with cell constituents have been reported (e.g., DNA binding due to an intercalating action comparable to that of Hoechst 33258, Doglia et al., 1993), nuclear labeling was not significant in our experiments and the dominant mechanism for quinacrine uptake in the μM range was that mediated by V-ATPase.

The recent proposition that quinacrine inhibits prion-induced cell lesions has recently generated interest, but the drug has been found ineffective in ovine scrapie, possibly due to a unfavorable distribution to the central nervous system (Gayraud et al., 2005). The distribution of lipophilic cationic drugs to acidic cell compartments may be more generalized than appreciated and has several potential consequences, such as agent sequestration away from cell surface or from nuclear sites of action for the most lipophilic antihistamines or anthracyclin antibiotics, respectively (Morrissette et al., 2008a; Ouar et al., 2003). In summary, quinacrine is concentrated and retained in cells via V-ATPase-mediated ion trapping and, despite the small size of ensuing vacuoles, it activates the macroautophagic signature of this cytopathology.

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References

- Ammoury A, Michaud S, Paul C, Prost-Squarcioni C, Alvarez F, Lamant L, Launay F, Bazex J, Chouini-Lalanne N, Marguery MC (2008) Photodistribution of blue-gray hyperpigmentation after amiodarone treatment: molecular characterization of amiodarone in the skin. *Arch Dermatol* **144**: 92-96.
- de Duve C, de Barse T, Poole B, Trouet A, Tulkens P, Van Hoof F (1974) Lysosomotropic agents. *Biochem Pharmacol* **23** : 2495-2531.
- Dohgu S, Yamauchi A, Takata F, Sawada Y, Higuchi S, Naito M, Tsuruo T, Shirabe S, Niwa M, Katamine S, Kataoka Y (2004) Uptake and efflux of quinacrine, a candidate for the treatment of prion diseases, at the blood-brain barrier. *Cell Mol Neurobiol* **24**: 205-217.
- Doglia SM, Albinson B, Hiort C, Nordén B, Gräslund A (1993) Quinacrine: spectroscopic properties and interactions with polynucleotides. *Biopolymers* **33**: 1431-1442.
- Gayraud V, Picard-Hagen N, Vigiúé C, Laroute V, Andréoletti O, Toutain PL (2005) A possible pharmacological explanation for quinacrine failure to treat prion diseases: pharmacokinetic investigations in a ovine model of scapie. *Br J Pharmacol* **144**: 386-393.
- Horvath G, Mendes ES, Schmid N, Schmid A, Conner GE, Salathe M, Wanner A (2007) The effect of corticosteroids on the disposal of long-acting β 2-agonists by airway smooth muscle cells. *J Allergy Clin Immunol* **120**: 1103-1109.
- Miller DS, Villalobos AR, Pritchard JB (1999) Organic cation transport in rat choroids plexus cells studied by fluorescence microscopy. *Am J Physiol Cell Physiol* **276**:

C955-C968.

- Morissette G, Ammoury A, Rusu D, Marguery MC, Lodge R, Poubelle PE, Marceau F (2009) Intracellular sequestration of amiodarone: role of V-ATPase and macroautophagic transition of the resulting vacuolar cytopathology. *Br J Pharmacol*. **157**: 1531–1540.
- Morissette G, Lodge R, Bouthillier J, Marceau F (2008a) Receptor-independent, vacuolar ATPase-mediated cellular uptake of histamine receptor-1 ligands: possible origin of pharmacological distortions and side effects. *Toxicol Appl Pharmacol* **229**: 320-331.
- Morissette G, Lodge R, Marceau F (2008b) Intense pseudotransport of a cationic drug mediated by vacuolar ATPase: procainamide-induced autophagic cell vacuolization. *Toxicol Appl Pharmacol* **228**: 364-377.
- Morissette G, Moreau E, C-Gaudreault R, Marceau F (2005) N-substituted 4-aminobenzamides (procainamide analogs): an assessment of multiple cellular effects concerning ion trapping. *Mol Pharmacol* **68**: 1576-1589.
- Ouar Z, Bens M, Vignes C, Paulais M, Pringel C, Fleury J, Cluzeaud F, Lacave R, Vandewalle A (2003) Inhibitors of vacuolar H⁺-ATPase impair the preferential accumulation of daunomycin in lysosomes and reverse the resistance to anthracyclines in drug-resistant renal epithelial cells. *Biochem J* **370**: 185-193.
- Zhang L, Strong JM, Qiu W, Lesko LJ, Huang SM (2006) Scientific perspectives on drug transporters and their role in drug interactions. *Mol Pharmaceutics* **3**: 62-69.
- Zuehlke RL, Lillis PJ, Tice A (1981) Antimalarial therapy for lupus erythematosus: an apparent advantage of quinacrine. *Int J Dermatol* **20**: 57-61.

Footnotes

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Reprint requests to: François Marceau, M.D., Ph.D., Centre de recherche en rhumatologie et immunologie, Room T1-49, Centre Hospitalier Universitaire de Québec, 2705 Laurier Blvd., Québec QC Canada G1V 4G2; e-mail: francois.marceau@crchul.ulaval.ca

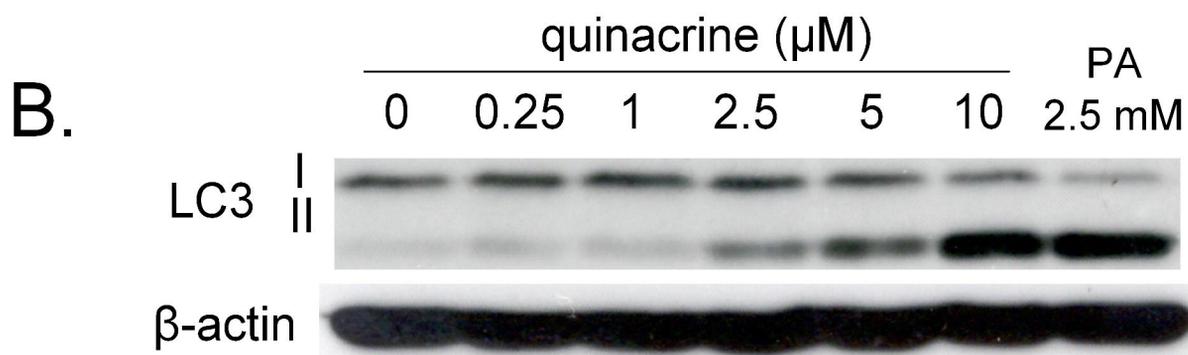
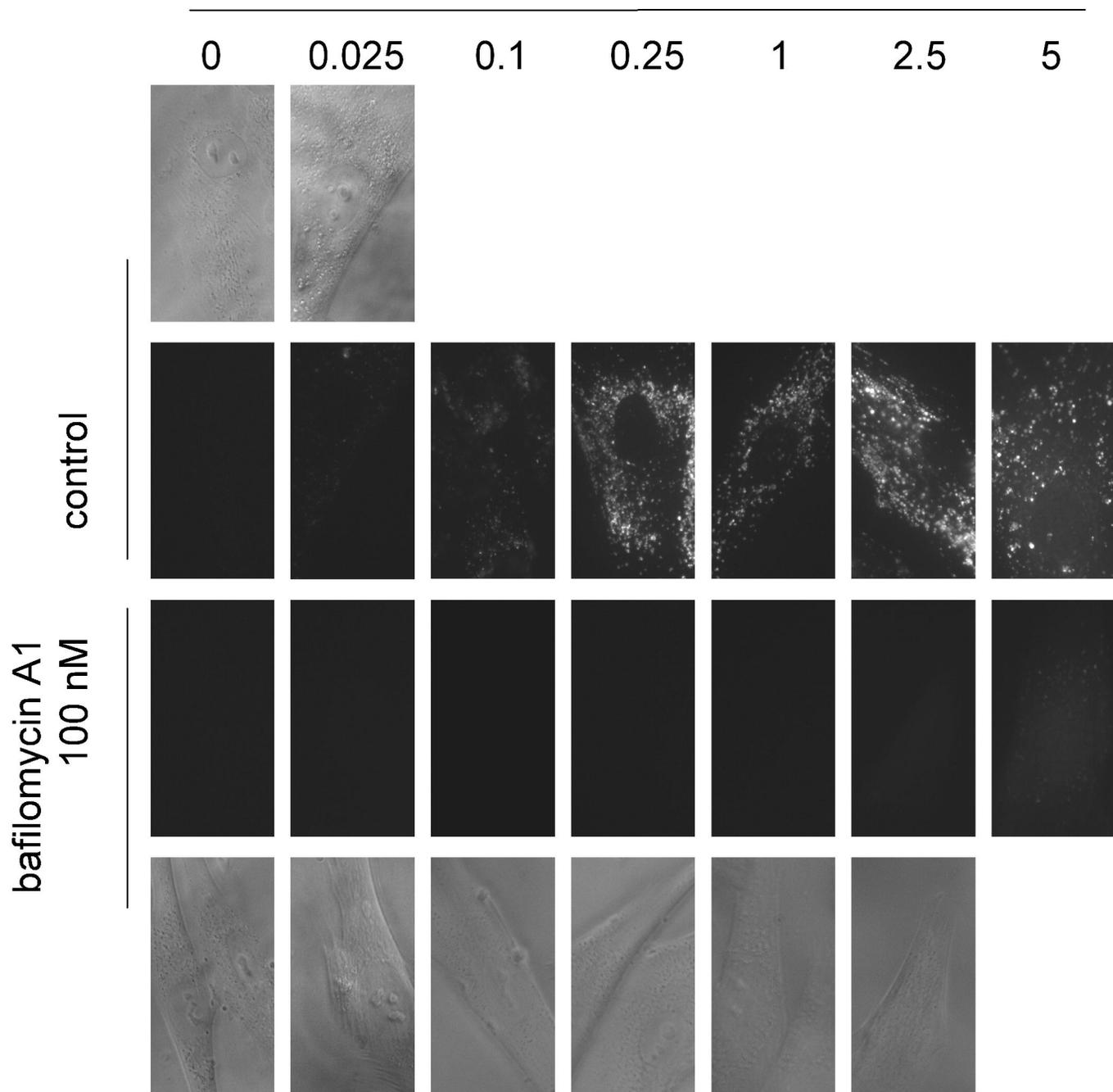
Legends for Figures

Fig. 1. A. Granular uptake of quinacrine (0.025-5 μ M, 4 h) into intact smooth muscle cells and effect of co-treatment with bafilomycin A1 (100 nM) (green epifluorescence; matched phase contrast views are shown for fields with very low fluorescence in the upper and lower rows). Original magnification 100 \times . B. Processing of endogenous LC3 in smooth muscle cells subjected to the indicated treatments and evaluated by immunoblots for LC3 in the total cell extract. (PA, procainamide). Representative results of 2 experiments.

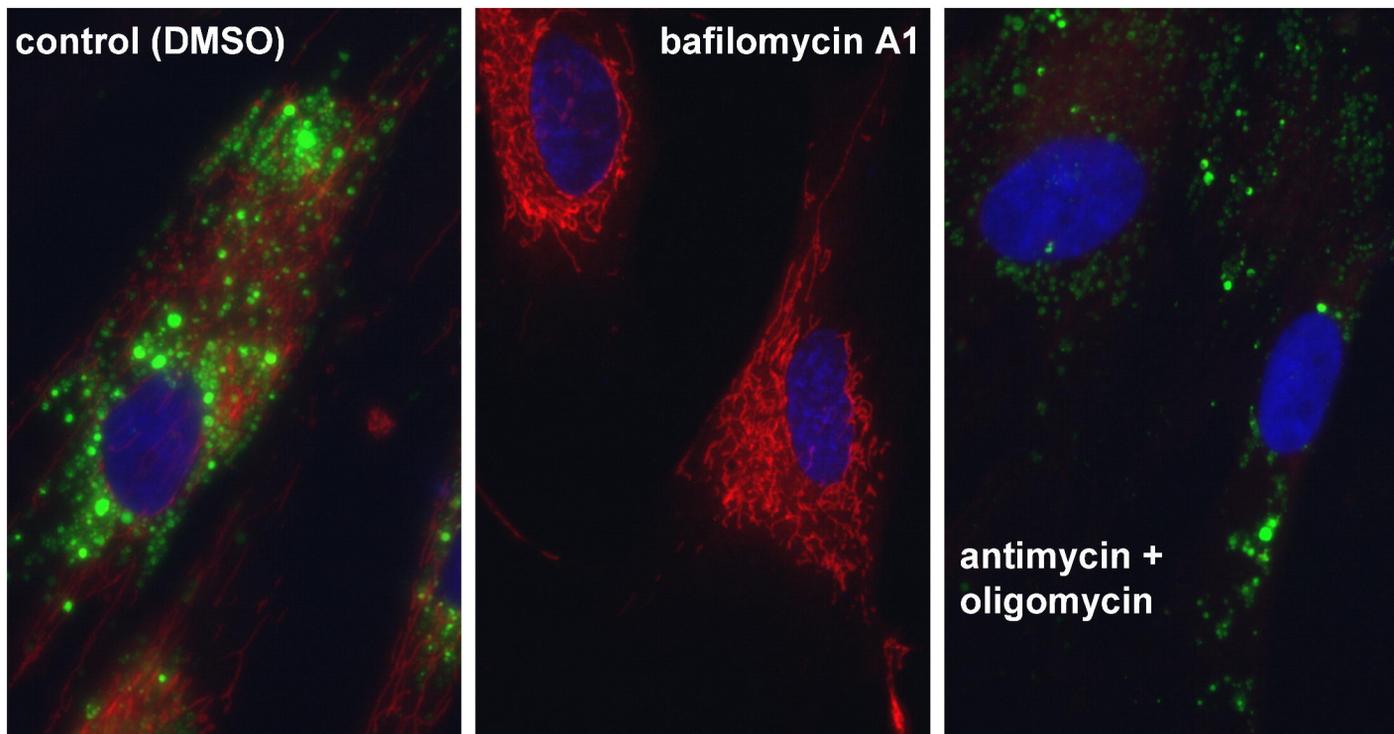
Fig. 2. A. Triple labeling of intact smooth muscle cells by cationic drugs possessing different mechanisms of cell uptake. Quinacrine uptake (5 μ M, applied 4 h before application) is prevented by bafilomycin A1 (300 nM, co treatment), but not that of Mitotracker Red (200 nM) or Hoechst 33258 (25 μ M, both applied 15 min before observation). The mitochondrial inhibitors antimycin and oligomycin (10 and 5 μ M, respectively, 15 min before observation) inhibited selectively Mitotracker Red uptake. B. Morphology of quinacrine-induced vacuoles (green fluorescence) in cells expressing cherry fluorescent protein (FP) or cherryFP-conjugated Rab5 and Rab7. Procainamide-induced vacuoles shown for comparison. Original magnification in A and B, 1000 \times .

Fig. 3. Uptake of quinacrine into confluent human umbilical artery smooth muscle cells as measured using extract-associated fluorescence. Each experimental point is derived from a confluent 25-cm² flask. Values are mean \pm s.e.m. of 3 replicates. A. Time course of drug uptake and drug retention upon bafilomycin A1 (100 nM) addition for a 5 μ M drug concentration. B. Effect of drug concentration on uptake during a 30-min period.

A. quinacrine (μM) x 4 h



A.



B.

