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High affinity capture and concentration of quinacrine in polymorphonuclear neutrophils via vacuolar ATPase-mediated ion trapping: Comparison with other peripheral blood leukocytes and implications for the distribution of cationic drugs

Caroline Roy, Valérie Gagné, Maria J.G. Fernandes, François Marceau *

Centre de recherche en rhumatologie et immunologie, CHU de Québec, and Department of Microbiology-Infectious Diseases and Immunology, Faculty of Medicine, Laval University, Québec, QC, G1V 4G2 Canada

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ABSTRACT

Many cationic drugs are concentrated in acidic cell compartments due to low retro-diffusion of the protonated molecule (ion trapping), with an ensuing vacuolar and autophagic cytopathology. In solid tissues, there is evidence that phagocytic cells, e.g., histiocytes, preferentially concentrate cationic drugs. We hypothesized that peripheral blood leukocytes could differentially take up a fluorescent model cation, quinacrine, depending on their phagocytic competence. Quinacrine transport parameters were determined in purified or total leukocyte suspensions at 37 °C. Purified polymorphonuclear leukocytes (PMNLs, essentially neutrophils) exhibited a quinacrine uptake velocity inferior to that of lymphocytes, but a consistently higher affinity (apparent K_M 1.1 vs. 6.3 μM, respectively). However, the vacuolar (V)-ATPase inhibitor bafilomycin A1 prevented quinacrine transport or initiated its release in either cell type. PMNLs capture most of the quinacrine added at low concentrations to fresh peripheral blood leukocytes compared with lymphocytes and monocytes (cytofluorometry). Accumulation of the autophagy marker LC3-II occurred rapidly and at low drug concentrations in quinacrine-treated PMNLs (significant at ≥2.5 μM, ≥2 h). Lymphocytes contained more LAMP1 than PMNLs, suggesting that the mass of lysosomes and late endosomes is a determinant of quinacrine uptake V_{max}. PMNLs, however, exhibited the highest capacity for pinocytosis (uptake of fluorescent dextran into endosomes). The selectivity of quinacrine distribution in peripheral blood leukocytes may be determined by the collaboration of a non-concentrating plasma membrane transport mechanism, tentatively identified as pinocytosis in PMNLs, with V-ATPase-mediated concentration. Intracellular reservoirs of cationic drugs are a potential source of toxicity (e.g., loss of lysosomal function in phagocytes).

Introduction

Many cationic drugs are concentrated in acidic cell compartments due to low retro-diffusion of the protonated molecule (ion trapping; reviewed by De Duve et al., 1974; Kaufmann and Krise, 2007; Marceau et al., 2012). In examined cultured cells, the driving force of this pseudo-transport is provided by vacuolar (V)-ATPase, a proton pump expressed in the trans-Golgi and derived organelles (endosomes, lysosomes, secretory granules). Thus, specific vacuolar (V-)ATPase inhibitors such as bafilomycin A1 have been extensively used to document the cellular capture and retention of amines, such as triethylamine (Et_3N; Morissette et al., 2005) and drugs from various therapeutic classes that can be considered to be substituted forms of this tertiary amine: in increasing order of lipophilicity, procainamide (Morissette et al., 2008), lidocaine (Bawolak et al., 2010), chloroquine (Zheng et al., 2011), quinacrine (Marceau et al., 2009, in press) and amiodarone (Morissette et al., 2009; Stadler et al., 2008). The threshold concentration for inducing the retention of amines and the characteristic vacuolar morphology that derives from it decreases with increased lipophilicity because the first step of ion trapping is believed to be simple diffusion of the uncharged form though the plasma and vacuolar membranes. Further, it has been consistently observed that the vesicles become autophagosomes, possibly due to the fact that lysosomal enzymes are inhibited at the higher pH determined by the massive trapping of basic molecules; thus, cellular/granular accumulation of macroautophagic effectors, such as microtubule-associated protein light chain 3 (LC3), may indicate the inhibition of the basal autophagic flux rather than the triggering of autophagy (reviewed by Marceau et al., 2012). Morphologically, the amine-induced giant vacuoles evolve toward multilamellar inclusions as a function of time, a cytopathology called phospholipidosis.

Several lines of evidence indicate that cationic drugs may exhibit a tropism for phagocytic leukocytes. In the Et_3N series, for instance, the lipophilic anti-arrhythmic drug amiodarone and its des-ethyl metabolite are well known to be concentrated by peripheral blood neutrophils and

* Corresponding author at: Centre de recherche en rhumatologie et immunologie, Room T1-49, Centre Hospitalier Universitaire de Québec, 2705 Laurier Blvd., Québec, QC, G1V 4G2 Canada. Fax: +1 418 654 2765.
E-mail address: francois.marceau@crchul.ulaval.ca (F. Marceau).

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induce inclusion bodies in these cells (Adams et al., 1986; Somani et al., 1986). Further, the abnormal skin pigmentation seen in many patients under chronic amiodarone therapy is a drug storage disease that essentially concerns dermal histiocytes (Ammoury et al., 2008; Delage et al., 1975). Corneal opacities and hepatic and lung inflammation are also probably related to tissue accumulation of amiodarone (Vassallo and Trohman, 2007). This drug was effectively taken up much more efficiently in vitro by human adherent macrophages vs. cell of mesenchymal origin, vascular smooth muscle cells (Morissette et al., 2009). Systemically administered amiodarone is rapidly concentrated in the liver and lungs of rats, but not efficiently in fat (Wyss et al., 1990), supporting the hypothesis that there may be a tropism of such drugs for macrophage-rich tissues. An alternate lipophilic substituted Et_3N formerly used as an antiprotozoal drug, quinacrine, also concentrates in the liver, lungs and spleen in vivo and induces side effects dependent on tissue accumulation (skin and oral mucosa discoloration, possible role in a reversible hepatitis; Ehsanian et al., 2011). It thus seems possible that phagocytes accumulate cationic drugs more effectively than other cell types, with possible effects on cell functions (e.g., autophagy, alteration of hydrolase activities; Baritussio et al., 2001) and, ultimately, on immunity.

Neutrophils are the only terminally differentiated phagocytes in peripheral blood. We hypothesized that peripheral blood leukocytes could differentially concentrate a fluorescent model cation, quinacrine, depending on their phagocytic competence, and addressed the mechanisms of the capture and retention of the drug, including but not limited to V-ATPase-mediated ion trapping. Quinacrine was used in these studies since it exhibits an intrinsic strong green fluorescence that is well suited for morphological and cell transport studies (Marceau et al., 2009, in press).

Methods

Drugs

Bafilomycin A1 was purchased from LC Laboratories (Woburn, MA) and all other drugs, from Sigma-Aldrich (St. Louis, MO).

Cells

The institutional research ethics board approved the anonymous use of human citrated blood from healthy volunteers to obtain leukocytes. PMNLs (essentially neutrophils) and mononuclear cells were prepared according to Fernandes et al. (2006), with some modifications. Briefly, after sedimentation of red blood cells in 2% dextran, PMNLs were aseptically purified by centrifugation on Ficoll-Paque cushions. Contaminating erythrocytes were removed by hypotonic lysis and PMNLs were resuspended (10^6/ml) in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Mononuclear cells were also recovered in some experiments, washed twice in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS), resuspended in RPMI 1640 supplemented with 10% FBS, plated in plastic 75-cm^2 flasks and incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. The non-adherent cells, predominantly lymphocytes, were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FBS and used in comparative transport and microscopy experiments.

Quinacrine transport

The uptake of quinacrine was established by a variation of a technique previously applied to quantify cell uptake of this drug in adherent smooth muscle cells (Marceau et al., 2009): test drugs were added to 1 ml cell suspensions (10^6 cells) based on the serum-containing culture media described above according to various schemes and time frames. The cells, protected from light, were incubated under agitation at 37 °C (Thermomixer, Eppendorf), rapidly centrifuged (12,500 rpm, 30 s), washed with 1 ml of phosphate-buffered saline, pH 7.4, at room temperature, recentrifuged and the final pellet was dissolved in 1 ml of 1 N NaOH. Quinacrine was analyzed in the NaOH extract using a SLM-AMINCO-Bowman Series 2 luminescence spectrophotometer against a standard curve of the authentic drug dissolved in NaOH 1 N (excitation 414 nm, emission 501 nm). Control fluorescence from extracts of untreated cells was systematically verified, of small magnitude and subtracted from experimental values.

Microscopy and cytofluorometry

Epifluorescence microscopy was used to ascertain the cellular uptake and subcellular distribution of quinacrine in PMNLs or lymphocytes purified as described above and maintained in the indicated culture medium. In addition, total peripheral blood leukocytes, prepared from citrated blood submitted to hypotonic lysis and resuspended in HBSS, were also incubated with quinacrine (30 min), to monitor the cell type into which the associated green fluorescence was associated as a function of drug concentration. The cells were spun, resuspended in HBSS and the drug uptake in cell populations was assessed using the BD SORP LSR II cell analyzer (BD Biociences, Franklin Lakes, NJ; green fluorescence). The results were analyzed using the BD FACS DIVA software.

Fluid phase endocytosis (pinocytosis) was evaluated in pure population of leukocytes or myeloid cell lines by measuring the uptake of dextran (40 kDa) conjugated to rhodamine B (Sigma-Aldrich; final concentration in HBSS 1 mg/ml; adapted from Pataki et al., 1995). After a 30-min incubation at 37 °C, the cells were centrifuged and washed prior to being photographed (green fluorescence and transmission, 1000×). Photoshop software (version 6, Adobe Systems, Mountain View, CA, USA) was used to manually delineate each cell in the photographic record of fluorescence from which the median pixel intensity was derived and statistically analyzed. A variation of the technique involved another conjugated dextran taken up by cells via pinocytosis: LysoSensor Yellow/Blue dextran (Invitrogen) contains a fluorophore that is pH indicator of the endocytic/lysosomal pathway (yellowish at low pH; Diwu et al., 1999; Lee et al., 2010). Leukocyte suspensions (10^6/ml) in their respective culture medium were treated with 1 mg/ml of LysoSensor Dextran for 1 h; then the washed cells were either observed in microscopy (under ultraviolet excitation using an electronic camera that covered all visible wavelengths) or assessed using cytofluorometry (excitation at 355 nm, readings at both 440 and 535 nm wavelengths; Lee et al., 2010).

Immunoblots

The effect of drugs (0–4 h treatments) on autophagic signaling was tested in PMNLs maintained in DMEM + 10% FBS (8×10^6 cells per 25-cm^2 flasks). After treatment, the cells were centrifuged, washed in HBSS, respun, resuspended in sample buffer and boiled as described (Marois et al., 2011). Cell extracts equivalent to 1.6×10^6 cells per lane were run on 12% SDS-PAGE and transferred to PVDF membranes. Anti-human LC3B rabbit polyclonal antibodies (Novus; dilution 1:3000) were used to detect the cytosolic form LC3-I and processed particulate form LC3-II in PMNLs treated with quinacrine for 0–4 h (Morissette et al., 2008). Equal protein loading was verified by immunoblotting with an anti-β-actin antibody. The expression of lysosome associated membrane protein 1 (LAMP1) was compared in leukocyte populations (cell extracts equivalent to 0.8×10^6 cells per lane run on a 9% SDS-PAGE) using monoclonal antibodies from Iowa Developmental Hybridoma Bank (1:1000). The results were corroborated with alternate rabbit polyclonal antibodies (H-228, Santa Cruz Biotechnology). The staining was detected with the appropriate horseradish peroxydase-conjugated secondary antibodies and a luminescent substrate used as directed (Western Lightning, PerkinElmer).