Massive Cell Vacuolization Induced by Organic Amines Such as Procainamide

Guillaume Morissette, Emmanuel Moreau, René C.-Gaudreault, and François Marceau
Centre de recherche de L’Hôtel-Dieu (G.M., F.M.) and Biotechnology and Bioengineering Unit, Hôpital St-François d’Assise (E.M., R.C.-G.), Centre Hospitalier Universitaire de Québec, Québec, Québec, Canada

Received January 26, 2004; accepted March 8, 2004

ABSTRACT

Procaine and some other basic drugs reportedly induce vacuolization of various cell types. We addressed the concentration-effect and structure-activity relationships as well as the mechanism of this effect using three cell lines. Massive vacuolization occurs over several hours in primary cultures of rabbit pulmonary artery smooth muscle cells (SMCs) and COS-1 cells in response to procaine and loosely related amine compounds (procainamide, N-acetyl-procainamide, metoclopramide, lidocaine, triethylamine, nicotine) used at 2.5 mM. Furthermore, chloroquine, propranolol, diphenhydramine, and neutral red are active in this respect at 100 to 250 μM in SMCs and COS-1 cells. Human embryonic kidney 293 cells mildly responded to triethylamine, nicotine, and propranolol only. Tetraethylammonium was uniformly inactive, as well as many other drugs in all three cell types (concentrations up to 2.5 mM). Procainamide does not induce apoptosis in SMCs treated for up to 48 h, although the vacuolization is sustained and proliferation and migration are reduced during this period. Procainamide-induced vacuolization is reversible on drug washing, largely prevented by bafilomycin A1 cotreatment, and has a tentatively identified Golgi origin (uptake of ceramide-C5). Procainamide and neutral red are concentrated in SMCs in a bafilomycin A1-sensitive manner. The preventive effect of bafilomycin A1 suggests that the vacuoles originate from the osmotic swelling of acidic organelles in which the charged basic drugs are trapped at low pH. Drug transport at the plasma membrane may be limiting for this type of response, as suggested by the cell type selectivity of agents and the inhibitory effect of some drugs such as quinidine.

It has been known for a long time that the formation of numerous vacuoles visible in light microscopy is a response of various types of cultured cells to some basic compounds, such as procaine, procainamide, nicotine, atropine, and many others (Belkin et al., 1962; Yang et al., 1965; Finnin et al., 1969; Ohkuma and Poole, 1981; Henics and Wheatley, 1997, 1999). This drug response transcends conventional pharmacological classes, and with the exception of local anesthetics, usually occurs at supratherapeutic concentration levels for the compounds that are used in therapeutics. It has been recently confirmed that procaine and some other local anesthetics at millimolar levels induce massive vacuolization of human skin fibroblasts in culture (Michalik et al., 2003). This reaction was further qualified as resulting from an increased uptake of the extracellular fluid phase, reversible on drug washing and prevented by specific drugs such as caffeine. It is plausible that the organic weak bases enter into acidic organelles of the cells (trans-Golgi, endosome, lysosomes, and perhaps mitochondria) become positively charged at low pH and trapped in these vacuoles (Siebert et al., 2004). Then, an osmotic appeal of water would drive the vacuole enlargement (Ohkuma and Poole, 1981). Some support for this mechanism is notably provided by the presence of neutral red in the giant vacuoles, because this dye (pKₐ of 6.5) should be charged at low vacular pH (Ohkuma and Poole, 1981; Michalik et al., 2003).

It has been previously noted that the potency of organic bases for vacuolization induction is not correlated to simple physicochemical properties, such as the pKₐ (Ohkuma and Poole, 1981). Although the simple diffusion through membranes of the neutral form of a tertiary amine may be very efficient, the recent literature supports that saturable transport systems exist for the uptake of many basic drugs; thus, the ability of a compound to induce vacuolization may be the result of both the presence of a specific transporter and of a tropism for acidic vacuoles. For example, the procaine analog procainamide is transported into some epithelial cells by a
tertiary amine/H$^+$ antiport system (Katsura et al., 2000) or organic cation transporters (OCTs; Urakami et al., 2002). Alternatively, amine drugs could stimulate fluid uptake indirectly via an apparently unrelated mode of action. Procaine and/or procainamide are known to block or stimulate several types of ion channels (Stephenson and Wendt, 1986; Mieyal et al., 1998; Huang et al., 1999; Nakahara et al., 2001); notably, procainamide blocks the delayed rectifier K$^+$ channel human ether-a-go-go-related gene (HERG; Yang et al., 2001; Ridley et al., 2003). Further cellular actions of millimolar levels of procaine and procainamide include DNA demethylation (Villar-Garea et al., 2003).

Wortmannin (250 nM) also induces the formation of giant vacuoles in several cell types and in a definite time window (Chen and Wang, 2001; Houle and Marceau, 2003). The effect of wortmannin is apparently dependent on the simultaneous activation of the Rab5 GTPase and the inhibition of phosphoinositide-3-kinase, notably based on the reconstitution of the production of giant vacuoles by a constitutively activated mutant of Rab5 combined with a more selective phosphatidylinositol-3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY 294002) (Houle and Marceau, 2003). The giant vacuoles induced by wortmannin clearly belong to the endocytic pathway, because they are labeled with the B$_2$ receptor for bradykinin, a G protein-coupled receptor translocated from the cell surface in HEK 293 cells treated with the corresponding agonist (Houle and Marceau, 2003).

We reasoned that vacuolization induced by procaine and similar drugs could also alter the cycling of receptors if the vacuoles originated from the endosomes. We characterized the massive vacuolization induced by specific basic drugs mostly in vascular smooth muscle cells (SMCs) and with procainamide as a prototype compound. Comparisons with other cell types and chemicals have been performed, notably to explore the structure-activity and concentration-effect relationships for drugs that cause vacuolization. The origin of the vacuoles, mechanism of the swelling, and overall toxicity associated with this response have been addressed. Results show that vacuoles induced by basic drug do not originate from the endocytic pathway, but they may have relevance to the formation of a tissue reservoir of drugs via ion trapping (Siebert et al., 2004).

**Materials and Methods**

**Drugs and Drug Treatments.** Neutral red was purchased from Laboratoire MAT (Quebec City, Canada). Cimetidine and metoclopramide were the injectable form (Tagamet, GlaxoSmithKline, Mississauga, ON, Canada; Reglan, A. H. Robins, Montreal, QC, Canada). The remaining drugs were obtained from Sigma-Aldrich (St. Louis, MO). Organic solvents were avoided to make stock solution (1–10 mg/ml) of the drugs; acidification and gentle heating were used when appropriate. Exceptions were bafilomycin A1, wortmannin, and brefeldin A, for which concentrated stock solution were made in dimethyl sulfoxide. Drugs were directly added to the serum-containing culture media, generally in 12-well plates where subconfluent cells were maintained. Experiments involving drug washout were based on the rinsing of cells with fresh serum-containing media.

**Cells and Microscopy.** Primary cultures of SMCs were obtained from explants of deendothelialized rabbit pulmonary arteries maintained in medium 199 supplemented with antibiotics and fetal bovine serum (10% FBS; Invitrogen, Burlington, ON, Canada). Cells at confluence were passaged with a brief trypsin-EDTA treatment and plated on gelatin-treated surfaces; they were used between passages 3 to 6. They expressed the marker α-smooth muscle actin (SmoothAct; Sigma-Aldrich). In addition, HEK 293 and COS-1 cells were used (maintained respectively in α-minimal essential medium supplemented with 5% FBS, 5% horse serum, and 1% penicillin-streptomycin or Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotics; reagents from Invitrogen). A number of observations were carried out using phase contrast microscopy (Nikon Eclipse TE-2000-5 microscope, 200× for the SMCs, 400× for the other cell types); photomicrographs were taken using a digital camera (CoolSnap HQ, Photometrics-Roper Scientific, Trenton NJ). Time-lapse photography was also exploited with the same system equipped with a heated chamber (37°C, 5% CO$_2$, saturated humidity) using cells maintained in 35-mm Petri dishes; one image was generated every 10 min and edited using the software MetaMorph 6.1 (Universal Imaging Corporation, Downingtown, PA).

**Cell Proliferation and Migration Assays.** SMCs (5 × 10$^4$) of rabbit pulmonary origin were seeded on gelatin-coated 35-mm Petri dishes at time 0 in a medium suitable for cell proliferation (medium 199 supplemented with 10% FBS). Some cells were stimulated with procainamide (0.25 or 2.5 mM) from the time 24 h on. Cell counts were obtained either at 48 or 72 h after trypsin-EDTA detachments of cells (thus, after 24 or 48 h of treatment, respectively). For the migration assay, confluent 35-mm Petri dishes of rabbit pulmonary artery SMCs were used. The cells had been seeded 3 to 4 days before the wound scraping. The plastic was denuded of cells along a single line using a disposable 1-ml pipette tip (blue) positioned in a perpendicular manner relative to the plastic surface and moved along a predefined diameter line. Subsequent microscopic observations showed that the width of the “wound” averaged approximately 950 μm. Immediately after, a series of photomicrographs of the wound region was taken using a digital camera (100×, phase contrast, up to eight per Petri dish at predefined sites distributed along the whole diameter). The cell dishes were incubated for an additional 24 h at 37°C in humidified air containing 5% CO$_2$, optionally with procainamide added to the FBS-containing medium, and a second series of photomicrographs was taken at the same sites. The recolonization of denuded plastic was quantified as the migration front displacement from manually aligned micrographs printed on plain paper.

**Apoptosis and Cell Viability.** Flasks (75 cm$^2$) of rabbit pulmonary artery SMCs were maintained in serum-containing culture medium while treated for 0 to 48 h with procainamide (2.5 mM) or for 24 h with actinomycin D (0.4 μM, as a positive control for apoptosis). Low-molecular-weight DNA was isolated using the Suicide-Track DNA Ladder isolation kit (Oncogene Science, Cambridge, MA), used as directed. The presence of a ladder of low-molecular-weight DNA was interpreted as the result of apoptotic events in a cell culture. In other SMC cultures, cell viability change in response to the same treatments was determined by means of the trypan blue dye exclusion.

**Cell Transfection, Organelle Labeling, and Confocal Microscopy.** A pCDNA3 vector containing the human HERG coding sequence (Zhou et al., 1998) was a generous gift from Dr. Gail Robertson (Department of Physiology, University of Wisconsin, Madison, WI). A vector coding for a dominant negative form of Rab5 (Rab5 S34N; Stenmark et al., 1994) was a gift from Dr. M. Zerial (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). Cells grown until 70% confluent in 35-mm Petri dishes were transiently transfected with the HERG coding vector, the pHe Red1-N1 vector (BD Biosciences Clontech, Palo Alto, CA; coding for the red fluorescent protein HIRed), and/or the vector coding from Rab5 S34N using the Ex-Gen 500 transfection reagent (MBI Fermentas Inc., Flamborough, ON, Canada), used as directed by the manufacturer. All transfected cells were maintained in the complete culture medium with serum for 48 h. Other cells were
labeled with the organelle-specific probes: C5-ceramide-BODIPY FL for the Golgi network and LysoTracker Red DND-99 for lysosomes and other acidic organelles (Molecular Probes, Eugene, OR; Invitrogen) (used as 5 μM or 100 nM solutions, respectively, in serum-free medium 199 for 30 min after other cell treatments, followed by rinsing with serum-free medium and observation). The subcellular fluorescence distribution in live cells was observed without fixation using a Bio-Rad 1024 laser beam confocal microscope (60× objective with oil immersion; HcRed and LysoTracker: emission 568 nm, detection above 585 nm; probe for Golgi: emission 488 nm, detection above 510 nm).

**Cellular Uptake of Procainamide.** Confluent Petri dishes containing rabbit pulmonary artery SMCs were washed twice and filled with 2 ml of Earle’s balanced salt solution (EBSS, without phenol red; Invitrogen) containing procainamide (1 mM) and optionally bafilomycin A1 (300 nM). In control dishes, either the cells or procainamide were absent to evaluate any possible nonspecific binding of procainamide to the dish material or the cell release of soluble substances that would interfere with the optical measurement of procainamide concentration, respectively. The dishes were incubated at 37°C in 5% CO₂, 95% air. Samples (100 μl) of the supernatants were periodically removed, diluted 20-fold in EBSS, and the concentration of procainamide was determined by the optical density at 278 nm against a calibration curve of the drug dissolved in EBSS. ¹H NMR (AC Brucker 300, CDCl₃) analysis was applied to supernatants extracted in dichloromethane to ascertain the absence of drug metabolism.

**Results**

**Vacuolization of Three Cell Types in Response to Procaine-Related Drugs.** Both the cell type specificity and the structure-activity relationship of procaine-related drugs were addressed in experiments reported in Fig. 1 (phase contrast observations). A 4-h treatment with procaine or its structural analogs procainamide and metoclopramide (2.5 mM) induced massive vacuolization of the majority of rabbit pulmonary SMCs and COS-1 cells observed, but no morphological effect on HEK 293 cells (Fig. 1). The procainamide metabolite N-acetylprocainamide also induced vacuolization at 2.5 mM, but smaller and fewer vacuoles were produced (especially in COS-1 cells, where some cells apparently remained unaffected). The hydrolysis metabolite of procaine, para-aminobenzoic acid, was not active, nor the topically active local anesthetic benzocaine at 2.5 mM (Fig. 1). As reported (Michalk et al., 2003), lidocaine induces cell vacuolization but less intensely than procaine (Fig. 1). Triethylamine was highly active in SMCs and COS-1 cells and mildly active in HEK 293 cells. Nicotine induced the vacuolization of all three cell types at 2.5 mM (Fig. 1), as reported for the mouse macrophages (Ohkuma and Poole, 1981). The drugs active to induce massive vacuolization in Fig. 1 are tertiary amines. NH₄Cl or the quaternary amine tetraethylammonium chloride (2.5 mM of each; Fig. 1) were inactive, as well as a 0.25 mM concentration of any of the drugs represented in Fig. 1 (data not shown).

The three tested cell types responded differentially to wortmannin (250 nM). The observations were made 90 min after drug application because the vacuolization induced by this drug is temporary, owing to its instability in aqueous solutions (Houle and Marceau, 2003). As reported previously, HEK 293 exhibited large vesicles in response to wortmannin; this also applied to COS-1 cells (Fig. 2), but the effect was much less intense in SMCs, where the size and number of vacuoles induced by procaine and other active organic amines were consistently larger.

Table 1 contains a list of drugs ineffective to induce vacuolization in any of the three cell types over a 4-h observation period. A subset of the agents listed in Table 1 has been chosen on the basis of a similarity to or possible antagonism of a proposed action of procainamide (see Introduction). The chosen concentrations of the agents are well within the active range for the mentioned mode of action. Ruthenium red, a blocker of all ryanodine receptor subtypes (Ozawa, 2001), does not affect cell morphology, as well as a high BaCl₂ concentration that blocks inward rectifier K⁺ channels, including HERG (Paquette et al., 1998; Overholt et al., 2000).

In addition, an antagonist of ryanodine type 1 and 3 receptors, dantrolene (Zhao et al., 2001), failed to induce the vacuolization of COS-1 cells or to inhibit the effect of procainamide (Table 1). E-4031, a class III antiarrhythmic agent recognized to block HERG with high affinity (Tristani-Firouzi and Sanguinetti, 2003), also failed to induce vacuolization in SMCs and to inhibit the effect of procainamide (2.5 mM). Other inactive drugs on the three cell types included cimetidine (a substrate for some OCTs) and colchicine (Table 1). Decynium-22, used at a concentration that blocks the OCT isoforms 1, 2, and 3 (Hayer-Zillgen et al., 2002), also failed to induce vacuolization or to inhibit the effect of procainamide in the SMCs and COS-1 cells. The polyamine spermidine and primary amine putrescine do not induce cell vacuolization at 2.5 mM (Table 1). Additional compounds that failed to inhibit procainamide-induced vacuolization in SMCs are the protein synthesis inhibitor anisomycin, the cyclooxygenase inhibitor diclofenac, and brefeldin A, a drug that inhibits the transfer of newly formed proteins from the endoplasmic reticulum to the Golgi (Helms and Rothman, 1992; Table 1).

The basic drugs chloroquine and propranolol caused some vacuolization of SMCs and of COS-1 cells when applied at 100 μM for 4 h (Fig. 2). Diphenhydramine, a substrate for a tertiary amine/H⁺ antiport system (Katsura et al., 2000), induced some vacuolization at 250 μM in the SMCs and the COS-1 cells (Fig. 2). Of those chemical, only the secondary amine propranolol produced a consistent effect on HEK 293 cells.

**Time-Course and Concentration-Effect Relationship of Procainamide-Induced Vacuolization of Rabbit SMCs.** The 250 μM concentration of procainamide did not induce vacuolization over the whole 48-h observation period, but the 1 mM level has an effect on many cells (Fig. 3). Omission of FBS from the culture medium did not change importantly this concentration-effect relationship (data not shown). Vacuolization is detectable as soon as 1 h after 2.5 mM drug application and continues to progress over 24 h, but without apparent mortality or detachment over 48 h. Prolonged (>4 h) exposure to the drug at 2.5 mM changed the morphology of the cells, with a retraction of the pseudopodia. Vacuolization induced by procainamide-related drugs was slowly reversed by drug washout with fresh culture medium (Fig. 3). A wound closure assay showed that the 250 μM and 2.5 mM concentrations of procainamide are equally effective to reduce the migration of SMCs (Fig. 4), thus dissociating this effect from vacuolization. Time-lapse photography (Video 1) showed the perinuclear origin of many of the large vacuoles formed in response to procainamide (2.5 mM) in SMCs, their appar-
ent absence of coalescence as a mechanism for enlargement, and that migration and mitosis are not totally inhibited (one mitotic event was observed in the field shown). The last part of the video, corresponding to a 24-h washout period, shows that these changes are reversible.

**Apoptosis and Cell Viability and Proliferation.** Relative to the positive control actinomycin D, which induced both apoptosis and the uptake of trypan blue in the majority of cells (Fig. 5, A and B), procainamide (2.5 mM) did not induce apoptosis of adherent, nearly confluent SMCs over a 4- to 48-h period (Fig. 5A) and caused little decrease of viability, although a time effect can be noticed (Fig. 5B). The same drug blocked the proliferation of subconfluent SMCs at 2.5 mM, but not at 250 μM (Fig. 5C).

**Mechanism of Vacuolization Induced by Procainamide.** In Fig. 6, a series of drug treatments were applied to SMCs with or without concomitant procainamide (2.5 mM; 4 h), to detect antagonist effects on the vacuolization. Cells exposed to KCl (60 mM), used as a depolarizing agent, were not morphologically influenced, and the effect of procainamide on vacuolization was not modified. Tetraethylammonium (3 mM), a blocker of several types of K⁺ channels and substrate for the OCTs, did not prevent the vacuolization induced by procainamide (Fig. 6). Bafilomycin A1 (300 nM),
an inhibitor of intracellular vacuole acidification (Dinter and Berger, 1998), was highly effective to prevent the procainamide-induced vacuolization when simultaneously applied to SMCs (Fig. 6). Additional drugs were more or less effective to inhibit the cell vacuolization induced by procainamide (2.5 mM) under the applied conditions. The antibacterial sulfamethoxazole (2.5 mM) and the antiarrhythmic quinidine (250 μM) consistently reduced the effect of procainamide; quinidine is a documented inhibitor of nicotine uptake via a tertiary amine/H^+ antiport (Fukada et al., 2002). Caffeine (30 mM) largely inhibited the vacuolization induced by procainamide (Fig. 6), but the treatment induced a morphology change in many cells (apparent retraction). The actin-depolymerizing agent cytochalasin D (2 μM) also exerted a profound morphologic effect on SMCs, but it did not antagonize procainamide (Fig. 6).

Essentially the same findings were made in COS-1 cells treated with procainamide (2.5 mM; 4 h) and examined in phase contrast, as caffeine, bafilomycin A1, quinidine, and sulfamethoxazole showed inhibitory activity. Caffeine and quinidine produced direct morphological effects (cell retraction) (data not shown). The effect of procainamide (4 h) on COS-1 cells is reversible on washing (data not shown).

The giant vacuoles induced by procainamide treatment exclude the HcRed fluorescent protein in SMCs and COS-1 cells, and therefore show up in negative against a fluorescent cytosolic background (Fig. 7). HEK 293 cells were not responsive; for a positive control, the effect of wortmannin (250 nM; 1.5 h) is shown on each cell type (modest on SMCs). This approach confirms that bafilomycin A1 cotreatment reduces the vacuolization induced by procainamide in SMCs. Cotransfection with a vector coding for Rab5 S34N, a dominant negative form of Rab5 that substantially reduces endocytosis (Stenmark et al., 1994) and the size of wortmannin-induced vacuoles (Houle and Marceau, 2003), failed to inhibit procainamide-induced vacuolization in SMCs (Fig. 7). Cotransfection of HEK 293 cells with a vector for human HERG did not confer the vacuolization response to procainamide (2.5 mM, last 4 h of incubation).

**Cell Concentration of Organic Amines.** Procainamide concentrations measured in the EBSS supernatant of confluent SMCs are shown in Fig. 8. A ~10% decline of the concentration was observed over 2 to 4 h of incubation at 37°C; no uptake occurred in Petri dishes without cells. The cells incubated without procainamide did not release any material interfering with the optical determination of the drug concentration. NMR analysis of the cell supernatants did not reveal any organic substance different from procainamide (specifically no N-acetyl-procainamide), supporting that the concentration decline does not result from

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Tested Cell Types</th>
<th>Cell Types for Which the Compound Failed to Antagonize Procainamide (2.5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaCl₂</td>
<td>0.25–2.5 mM</td>
<td>SMC, COS-1, HEK 293</td>
<td>SMC</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>2.5 mM</td>
<td>SMC, COS-1, HEK 293</td>
<td>NT</td>
</tr>
<tr>
<td>Spermidine</td>
<td>2.5 mM</td>
<td>SMC, COS-1, HEK 293</td>
<td>NT</td>
</tr>
<tr>
<td>Putrescine</td>
<td>2.5 mM</td>
<td>SMC, COS-1, HEK 293</td>
<td>NT</td>
</tr>
<tr>
<td>Ruthenium red</td>
<td>100 μM</td>
<td>SMC, COS-1, HEK 293</td>
<td>NT</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>100 μM</td>
<td>SMC, COS-1, HEK 293</td>
<td>COS-1</td>
</tr>
<tr>
<td>Colchicine</td>
<td>20 μM</td>
<td>SMC, COS-1, HEK 293</td>
<td>NT</td>
</tr>
<tr>
<td>Decynium-22</td>
<td>10 μM</td>
<td>SMC, COS-1</td>
<td>SMC, COS-1</td>
</tr>
<tr>
<td>E-4031</td>
<td>10 μM</td>
<td>SMC</td>
<td>SMC</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>10 μM</td>
<td>SMC</td>
<td>SMC</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>36 μM</td>
<td>SMC</td>
<td>SMC</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>500 nM</td>
<td>SMC</td>
<td>SMC</td>
</tr>
</tbody>
</table>

NT, not tested.
drug metabolism (data not shown). Considering the very small proportion of the dish content (2 ml) occupied by the cells, the cellular uptake of ~0.2 µmol per dish is evidence for a concentration of the drug by cells, and this process was reduced at time 4 h by concomitant treatment with bafilomycin A1 (Fig. 8).

Neutral red has been applied for 4 h to SMCs that were subsequently observed without rinsing using transmission microscopy (Fig. 9). The numerous vacuoles induced by this tertiary amine (25–100 µM) are much more intensely pigmented than the surrounding cytosol or culture medium, providing evidence for an active concentration of the dye into the large vacuoles. The vacuolization process induced by neutral red is virtually abolished by bafilomycin A1 and reduced by quinidine treatment (Fig. 9), like that induced by procainamide. Furthermore, concomitant treatment with procainamide and neutral red inhibits the cellular uptake of the pigment (Fig. 9).

**Subcellular Origin of Procainamide-Induced Vacuoles.** Fluorescent ceramide C5 labels the Golgi of SMCs as ill-defined bright bands mostly with a perinuclear location (Fig. 10). In cells previously treated with procainamide (2.5 mM; 2 h), the membrane of the large vacuoles concentrates the fluorescent lipid, supporting their Golgi origin. For comparison, SMCs treated with wortmannin and further exposed to fluorescent ceramide C5 do not exhibit an enrichment of the fluorescence around the large vacuoles (Fig. 10). The LysoTracker dye strongly labels dispersed and peripheral granules, presumably lysosomes, and more weakly the bands with a perinuclear starting point, presumably the Golgi network. This relative intensity of staining is retained in cells pretreated with procainamide, because the dense peripheral granules seem to be unaffected, but the large perinuclear vacuoles are weakly stained (including their content). In wortmannin-treated cells, the vacuoles are minimally labeled, relative to their counterparts induced by procainamide.

**Discussion**

A collection of drugs used at high concentrations reproduce in SMCs and COS-1 cells the effect of some local anesthetics on human fibroblasts: massive vacuolization readily detectable using light microscopy was observed to develop over a few hours (Michalik et al., 2003). The assortment of drugs active in SMCs and COS-1 cells includes triethylamine and some compounds that could be considered substituted triethylamine derivatives (procaine, lidocaine, procainamide, N-
acetyl-procainamide, and metoclopramide, Fig. 1; chloroquine, Fig. 2). In the subset of substituted triethylamines, a definite structure-activity relationship can be suspected as the bulkier compounds N-acetyl-procainamide, metoclopramide, or chloroquine are slightly less active, equipotent, or more potent than procainamide, respectively (Figs. 1 and 2). Another compound showing activity, nicotine, is a cyclic tertiary amine. Propranolol has some activity, but not spermidine or putrescine; the two first chemicals are secondary amines, the last, a primary amine. The effects on vacuolization cannot be precisely correlated to solubility or to other simple physicochemical properties, such as the pK_a, as noted previously (Ohkuma and Poole, 1981). For instance, N-acetyl-procainamide is less polar than procainamide but nearly as effective as the parent drug. The response is not an ineluctable cytotoxicity, because the vacuolization is reversible on washing as observed previously (Ohkuma and Poole, 1981; Michalik et al., 2003). We have extended this observation by showing that most affected cells do not die or undergo apoptosis over 48 h, based on the DNA fragmentation assay applied, but their proliferation and migration were significantly reduced via unknown mechanisms. In a previous study, procaine at a concentration that produced vacuolization inhibited the mitosis of hepatoma cells and fibroblasts by retarding the late S and G2 phases of the cell cycle (Henics and Wheatley, 1997).

The possibility of indirect modes of action was examined. Some drugs that induce vacuolization have in common the capacity to block the HERG ion channel, but they do so at concentration much inferior to those active in the present assay (IC_{50} of 139 μM for procainamide, Ridley et al., 2003; 2.5 μM for chloroquine, Traebert et al., 2004). The pharmacological promiscuity of the HERG channel has been noted previously (Pearlstein et al., 2003). Some agents, such as
procainamide, are dependent on an open channel to reach their site of binding (Ridley et al., 2003). High [K+] did not mimic or interfere with the effect of procainamide in SMCs or COS-1 cells. Furthermore, some recognized HERG blockers failed to induce or block vacuolization (E-4031, millimolar barium). Finally, transfection of HERG in the unresponsive HEK 293 cells does not confer to them the responsiveness to procainamide.

Procainamide and other basic drugs may be transported in some cells via recombinant OCTs, based on their capacity to compete with cellular uptake of organic cations (Kakehi et al., 2002; Urakami et al., 2002). However, most or all OCTs transport tetraethylammonium (Burckhardt and Wolff, 2000) and are blocked by micromolar concentrations of decynium-22 (Hayer-Zillgen et al., 2002) or by cimetidine (Kakehi et al., 2002); these properties dissociate the OCTs from the capacity to induce massive vacuolization in our systems.

The ability of several drugs to induce massive vacuolization in SMCs and COS-1 cells is better correlated to tertiary amine/H+ antiports systems observed previously in intestinal cells (Katsura et al., 2000; Mizuuchi et al., 2000; Fukada et al., 2002). This form of transport applied to procainamide and diphenhydramine (KeM of 8.3 and 0.8–1 mM, respectively), agents that are active to induce cell vacuolization in SMCs. Procainamide and triethylamine (but not tetraethylammonium or cimetidine) competed for the transport of diphenhydramine (Mizuuchi et al., 2000); triethylamine and diphenhydramine (but not tetraethylammonium) for that of procainamide (Katsura et al., 2000). Nicotine uptake by Caco-2 cells (KeM of 0.9 mM) was inhibited by quinidine but not tetraethylammonium or cimetidine (Fukada et al., 2002).

Our results may support that quinidine blocks the uptake of procainamide and neutral red (Figs. 6 and 9). Thus, the identity of drugs and active drug concentration ranges are similar. Amine drug-induced vacuolization of HEK 293 cells applies only to triethylamine, propranolol, and nicotine in our experiments. The lack of effect of procainamide and of several other agents on HEK 293 cells indirectly supports that drug transport to the cytosol is limiting. The hypothet-
ical plasma membrane transporter(s) are not identified, but probably different from the polyamine transporter that exhibits micromolar affinity for chemicals such as spermidine (Soulet et al., 2002).

The lysosomotropic agent chloroquine has been often used experimentally to neutralize the function of lysosomes. When such a tertiary amine reaches the cytosol, osmotic swelling of specific acidic organelles in which the amines diffuse and become trapped (charged) at low pH is the probable mechanism of the massive vacuolization (Ohkuma and Poole, 1981; Henics and Wheatley, 1999; Fig. 11). This is supported in the present experiments by the inhibitory effect of bafilomycin A1, a blocker of the vacuolar ATPase that acidifies specific intracellular vacuoles (trans-Golgi, endosomes, and lysosomes; Dinter and Berger, 1998) and probably provides the energy for the vacuolization. Procainamide-induced vacuolization may not be driven by endocytosis, unlike that produced by wortmannin, based on the differential effect of Rab5 S34N (Fig. 7; Houle and Marceau, 2003). At variance with Michalik et al. (2003), we did not observe that the large vacuoles induced by procainamide treatment were labeled by the fluid phase uptake marker Lucifer yellow (data not shown). The bafilomycin A1-sensitive vacuolization induced

Fig. 6. Effect of concomitant drug treatments on the SMC vacuolization induced by procainamide (2.5 mM; 4 h). The direct effect of each drug (without procainamide) is shown in the left column. Presentation as in Fig. 1.

Fig. 7. Vacuolization of cells induced by drugs as shown in negative in cells transiently expressing the HcRed fluorescent protein observed using confocal microscopy. A, drug effect on SMCs. Concomitant treatment with bafilomycin A1 or of cotransfection with a dominant negative version of Rab5 S34N (Fig. 7; Houle and Marceau, 2003). B, effect of drugs on COS-1 cells. C, effect of drugs on HEK 293 cells. Whether transfection with a vector coding for human HERG makes the cells responsive to procainamide has been tested. In this figure, square fields have sides of 120 μm for SMCs and of 40 μm for COS-1 or HEK 293 cells.

Fig. 8. Procainamide concentration measured in the supernatant of confluent SMCs as a function of time. Some dishes contained no cells (plastic) and bafilomycin A1 concomitant treatment was applied to others. Values are the means ± S.E.M. of two determinations.
by neutral red supports an active concentration of the amine in the vacuole, based on pigmentation intensity (Fig. 9). Thus, the fluid entry into vesicles of cell treated with basic drugs may be osmotic, as suggested a long time ago (high osmotic pressure in the culture medium reduces procaine-induced cell vacuolization; Finnin et al., 1969). Although basic drugs may be trapped in a various types of acidic organelles, including mitochondria (Siebert et al., 2004), the ones that are submitted to osmotic swelling in our SMC model may originate from the trans-Golgi, on the basis of their labeling with ceramide-C5, perinuclear starting point, the presence of the vesicular ATPase sensitive to bafilomycin A1, and the absence of inhibition by the dominant negative Rab5. In previous studies, the mitochondria remained intact in hepatoma cells or fibroblasts treated with millimolar levels of procaine (Yang et al., 1965; Henics and Wheatley, 1997). It cannot been ruled out that additional organelles expressing the vacuolar ATPase and derived from the trans-Golgi, such as the lysosomes, also concentrate procainamide, as suggested previously for neutral red (Sousa et al., 1996). However, the organic amine LysoTracker, which itself seems to weakly label the Golgi network, still strongly labels a population of morphologically intact lysosomes in procainamide-treated cells (Fig. 10).

Basic lipophilic compounds are characterized by a high volume of distribution as a result of extensive tissue uptake; recent experimental data indicate that ion trapping in acidic cell organelles is a major mechanism of reservoir formation for drugs that exhibit a high pK\(_a\) (Siebert et al., 2004). It is clear that drug morphological effects documented in the present experiments occur at supratherapeutic concentrations for most drugs that are clinically used, but not for procaine and lidocaine used as local anesthetics. Michalik et al. (2003) discussed previous clinical reports of inflammation and cell vacuolization induced at sites where local anesthetic were used; an additional report was based on the well controlled drug administration to rabbits (Atilla et al., 2003). Propranolol is a \(\beta\)-adrenoceptor antagonist active to induce vacuolization at high concentrations in various cell types (Fig. 2; Ohkuma and Poole, 1981). A toxicity experiment conducted in dogs has evidenced cardiomyocyte vacuolization after a lethal dose of propranolol (Whitehurst et al., 1999). Interestingly, rat livers perfused with a solution of propranolol exhibit an extensive drug uptake attributed to ion trapping into acidic cell compartments because it is reduced by monensin treatment (a Na\(^{+}/\)H\(^{+}\) ionophore that disrupts proton gradients in organelles; Siebert et al., 2004). Bafilomycin A1 exerted such a partial inhibitory effect on the procainamide uptake by SMCs (Fig. 8). A radiolabeled derivative of the alternate \(\beta\)-blocker pindolol is similarly concentrated within cultured type II pneumocytes to bafilomycin A1, and the absence of inhibition by the dominant negative Rab5. In previous studies, the mitochondria remained intact in hepatoma cells or fibroblasts treated with millimolar levels of procaine (Yang et al., 1965; Henics and Wheatley, 1997). It cannot been ruled out that additional organelles expressing the vacuolar ATPase and derived from the trans-Golgi, such as the lysosomes, also concentrate procainamide, as suggested previously for neutral red (Sousa et al., 1996). However, the organic amine LysoTracker, which itself seems to weakly label the Golgi network, still strongly labels a population of morphologically intact lysosomes in procainamide-treated cells (Fig. 10).

In conclusion, several weakly basic drugs (mostly tertiary...
amines) have been shown to induce cell vacuolization in the 0.1 to 2.5 mM concentration range; the preventive effect of bafilomycin A1 and other findings suggest that the vacuoles originate from the osmotic swelling of acidic organelles tentatively identified as the trans-Golgi. Drug transport at the plasma membrane may be limiting for this type of response, as suggested by the cell type selectivity of agents and the inhibitory effect of some drugs like quinidine.

Acknowledgments

We thank Dr. Francis Rioux (Université Laval) for critical reading of the manuscript, Johanne Bouthillier for technical help, and André Lévesque for advice on microscopic techniques.

References


Overholt JL, Ficker E, Yang T, Shams H, Bright GR, and Prabhakar NR (2000) HERG-Like potassium current regulates the resting membrane potential in glo-


**Address correspondence to:** Dr. François Marceau, Centre Hospitalier Universitaire de Québec, Centre de recherche, Pavillon l'Hôtel-Dieu de Québec, 11 Côte-du-Palais, Québec, Quebec, Canada G1R 2J6. E-mail: francois.marceau@crhdq.ulaval.ca