Imaging exocytosis of ATP-containing vesicles with TIRF microscopy in lung epithelial A549 cells

Irina Akopova · Sabina Tatur · Mariusz Grygorczyk · Rafał Luchowski · Ignacy Gryczynski · Zygmunt Gryczynski · Julian Borejdo · Ryszard Grygorczyk

Received: 9 March 2011 / Accepted: 16 August 2011 / Published online: 1 September 2011
© Springer Science+Business Media B.V. 2011

Abstract Nucleotide release constitutes the first step of the purinergic signaling cascade, but its underlying mechanisms remain incompletely understood. In alveolar A549 cells much of the experimental data is consistent with Ca^{2+} -regulated vesicular exocytosis, but definitive evidence for such a release mechanism is missing, and alternative pathways have been proposed. In this study, we examined ATP secretion from A549 cells by total internal reflection fluorescence microscopy to directly visualize ATP-loaded vesicles and their fusion with the plasma membrane. A549 cells were labeled with quinacrine or Bodipy-ATP, fluorescent markers of intracellular ATP storage sites, and time-lapse imaging of

vesicles present in the evanescent field was undertaken. Under basal conditions, individual vesicles showed occasional quasi-instantaneous loss of fluorescence, as expected from spontaneous vesicle fusion with the plasma membrane and dispersal of its fluorescent cargo. Hypo-osmotic stress stimulation (osmolality reduction from 316 to 160 mOsm) resulted in a transient, several-fold increment of exocytotic event frequency. Lowering the temperature from 37°C to 20°C dramatically diminished the fraction of vesicles that underwent exocytosis during the 2-min stimulation, from ~40% to ≤1%, respectively. Parallel ATP efflux experiments with luciferase bioluminescence assay revealed that pharmacological interference with vesicular transport (brefeldin, monensin), or disruption of the cytoskeleton (nocodazole, cytochalasin), significantly suppressed ATP release (by up to ~80%), whereas it was completely blocked by *N*-ethylmaleimide. Collectively, our data demonstrate that regulated exocytosis of ATP-loaded vesicles likely constitutes a major pathway of hypotonic stress-induced ATP secretion from A549 cells.

S. Tatur · M. Grygorczyk · R. Grygorczyk (✉)
Research Centre, Centre hospitalier de l'Université
de Montréal (CRCHUM)—Hôtel-Dieu,
3850 St. Urbain St.,
Montreal, QC, Canada H2W 1T7
e-mail: rysard.grygorczyk@umontreal.ca

R. Grygorczyk
Department of Medicine, Université de Montréal,
Montreal, Quebec, Canada

I. Akopova · R. Luchowski · I. Gryczynski · Z. Gryczynski ·
J. Borejdo
Center for Commercialization of Fluorescence Technologies,
Department of Molecular Biology and Immunology,
University of North Texas,
Fort Worth, TX, USA

I. Gryczynski
Department of Cell Biology and Anatomy,
University of North Texas,
Fort Worth, TX, USA

Z. Gryczynski
Department of Physics and Astronomy,
Texas Christian University,
Fort Worth, TX, USA

Keywords Purinergic signaling · ATP release ·
Mechano-sensitive nucleotide release · ATP exocytosis ·
TIRF microscopy

Introduction

Extracellular ATP, UTP, and their degradation products are important autocrine/paracrine mediators in most tissues, including lung epithelia. Through interactions with cell surface purinergic receptors they regulate diverse functions, such as fluid secretion, cell proliferation, stem cell homing, pain sensation, neutrophils chemotaxis, and mucociliary clearance [1, 2]. Despite physiological relevance of the responses triggered by extracellular purines in the lungs,

little is known about their origin on the airway surface and in the alveolus, and their mechanisms of release remain incompletely understood [3]. Mechanical perturbations, namely, cell stretch, shear, and hypo-osmotic swelling, have been shown to elicit significant ATP release from many cell types through a cell-regulated process that does not involve cellular lysis [4–6]. Mechano-sensitive nucleotide secretion is particularly relevant in the lungs, where surface epithelia, during tidal breathing and cough, are exposed to air flow-induced shear stress in the airways and cell stretch/distortion in alveoli. Physical forces are known regulators of lung formation and function [7, 8]; cell distortion, for example, is considered as the predominant physiological stimulus for surfactant secretion in the alveolus [9], which may involve mechano-sensitive release of ATP, a major surfactant secretagogue. Bearing in mind the importance of extracellular ATP in the alveoli, we focused our investigations on the ATP release mechanism in human pulmonary adenocarcinoma A549 cells, which have many features consistent with alveolar type II (AT2) epithelial cells. Morphologically, they retain a cuboidal shape and bear a number of the ultrastructural characteristics, including lamellar bodies, consistent with those reported in AT2 cells in situ. They also manifest similar phospholipid biosynthetic properties and have been found to release and/or express a variety of cytokines and growth factors [10, 11].

Hypo-osmotic stress is often used as a surrogate of mechanical stimulus, with which it shares many similarities including membrane stretch/unfolding, transient cytoskeleton reorganization, and activation of intracellular Ca^{2+} and Rho/Rho kinase signaling pathways. Our earlier investigations disclosed that hypo-osmotic stress-induced ATP release from A549 alveolar cells, and 16HBE14o[−] airway epithelial cells, depends tightly on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), and similar dependence was observed when the release was elicited by cell mechanical deformation at the air–liquid interface. ATP secretion was almost completely inhibited at low temperature (10°C) or by application of *N*-ethylmaleimide (NEM), implicating an exocytotic process [12–14]. Other studies, however, suggested that pannexin 1 channels mediate conductive ATP release from agonist (thrombin)-activated A549 cells, and hypotonic stress-activated A549 and airway epithelial cells [15–17]. The present study was undertaken to further examine the mechanism underlying stress-induced ATP efflux in A549 cells. In addition to examining the effects of pharmacological agents known to interfere with the cytoskeleton or with the exocytotic process, we have also utilized total internal reflection fluorescence (TIRF) microscopy in an attempt to directly visualize ATP-loaded vesicles as well as their recruitment and fusion with the plasma membrane during stimulated ATP release.

Methods

Cells

Human lung carcinoma A549 cells, a model of AT2 cells, were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. ATP efflux was measured from A549 cell monolayers grown to confluence (~ 500 cells/mm²) on 24×60 mm glass coverslips. TIRF imaging experiments were conducted with cells grown at low density (40–60% confluence) on 22×22-mm glass coverslips. To block exocytosis, the cells were pre-treated with 1 mM *N*-ethylmaleimide for 15 min at room temperature, and were examined at 37°C after washing.

ATP efflux assay

ATP efflux was measured with high temporal resolution in a custom-designed, low-volume (325 μL) flow-through chamber similar to that described previously [12]. Briefly, a 24×60-mm coverslip with confluent cell monolayer was mounted in the chamber and perfused with a warm (37°C) solution by in-line SF-28 heater (Warner Instruments Co., Hamden, CT, USA) at 1.3 mL/min. The cells were stimulated with hypotonic saline (HS) of 50% reduced osmolality (see below). The perfusate was collected continuously at 15- to 30-s intervals for later ATP content evaluations. ATP in the samples was quantified by luciferase–luciferin assay, with ATP Assay Mix and ATP Assay Mix Dilution Buffer supplied by Sigma-Aldrich Canada, Ltd. (Oakville, ON). ATP release rates were expressed in pmoles min^{−1} (10⁶ cells)^{−1}. All test compounds that were added to the extracellular solution during the ATP efflux experiments were also examined for their ability to directly interfere with luciferase bioluminescence. To correct for any inhibitory effects, luciferase–luciferin luminescence was always calibrated with ATP standards in the presence of test compounds.

Solutions and chemicals

Physiological isotonic solution (PS) contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, and 10 TES, pH 7.4, adjusted with NaOH. For ATP efflux assay, HS at 50% osmolality was prepared by reducing NaCl concentration to 70 mM. Osmolality of the solutions was checked with a freezing point osmometer (Micro Osmometer 3300, Advanced Instruments Inc., Norwood, MA, USA): it was 316±3 mOsm for PS and 161±3 mOsm for HS. In TIRF experiments, cells were stimulated by hypo-osmotic shock by adding an equal amount of distilled water (0.5 ml) directly to the PS covering the cells in the imaging chamber. All

reagents, including NEM, quinacrine, and brefeldin A, were obtained from Sigma-Aldrich Canada, Ltd. BODIPY FL ATP (adenosine 5'-triphosphate, 2'-(or-3')-O-(N-(2-aminoethyl) urethane)), trisodium salt, 5 mM in buffer, Cat. No. A-12410, was procured from Invitrogen-Molecular Probes (Kingston, ON).

Fluorescent labeling of vesicular ATP

To monitor vesicular ATP, cells were incubated with 5 to 10 μ M quinacrine for 10–20 min at room temperature. Quinacrine often serves as a fluorescent marker for intracellular ATP-enriched vesicles: it has a peak absorption at 420–460 nm, but due to broad absorption spectra, its fluorescence could still be efficiently excited by common laser lines, such as 470 and 488 nm, producing maximum emission (E_m) at 490–510 nm [18–20]. Alternatively, in some experiments, vesicular ATP was visualized by loading cells with the fluorescent ATP analog Bodipy-ATP (E_m 515 nm), 20–30 μ M, for 1–3 h at 37°C. Loading of quinacrine into A549 cells and its intracellular distribution were studied by confocal imaging of cell fluorescence intensity and by fluorescence lifetime imaging microscopy (FLIM). Fluorescence lifetime, the average time that the fluorophore molecule spends in the excited state, is strongly affected by its interactions with the environment. Therefore, FLIM is a suitable method to map different intracellular fluorophore environments and may provide information complementary to fluorescence intensity images. Here, we used an Olympus IX71 inverted laser scanning confocal microscope (water immersion objective $\times 60$, NA1.1) attached to a MT200 fluorescence lifetime imaging system (Picoquant, GmbH, Berlin, Germany). Quinacrine fluorescence was excited with a 470-nm pulsed laser line (20 MHz, 480 ps effective pulse duration including detector response time), operated at 50% power (0.05 μ W at the sample), manufactured by Picoquant. On the detection path, a 30- μ m size pinhole was used. The fluorescence signal was selected with both a 500-nm long pass filter and 510/23 band pass filter. Average lifetime was determined with fast FLIM calculation in the instrument acquisition software Symphotime (Picoquant, <http://www.picoquant.com/>) on the basis of time-correlated, single-photon data collected for each pixel.

TIRF microscopy and analysis

Exocytosis of quinacrine-stained vesicles was studied by time-lapse TIRF imaging with an Olympus IX71 inverted microscope equipped with a $\times 60$ TIRF oil immersion objective (NA 1.45), under through-the-objective evanescent field illumination. Fluorescence was excited with the 488-nm line of an argon air-cooled laser IMA101010-B0S (Melles

Griot); output power was 65 mW. Laser light intensity was reduced by 50–75% with a neutral density filter. Images were acquired via a 512-nm emission filter at the rate of 1 image/s and exposure time of 300–500 ms with a Hamamatsu digital camera (C4742-95) controlled by SimplePCI 6.0 software (Hamamatsu Corp., Sewickley, PA, USA). During off-line analysis with this same software, regions of interest (ROI) were placed manually to encircle individual vesicles present in the evanescent field. In a typical experiment eight to 20 cells were visible in the field of view (FOV), and depending on their size each cell displayed between 20 and 50 quinacrine-stained vesicles under evanescent field illumination. To reduce analysis time, for each experiment a representative pool of 50 to 120 vesicles was chosen within different cells in the FOV. To assure unbiased selection, care was taken to include vesicle that showed different initial fluorescence intensities and were in different locations within the cell outline. Vesicle clusters, often seen as irregularly shaped spots of high intensity, were omitted in the analysis because of difficulty in resolving individual vesicles. The time course of quinacrine fluorescence intensity changes was measured for those vesicles that retained their XY position within the ROI throughout the observation period, typically 1–2 min. Changes in the mean fluorescence intensity of each ROI were displayed in arbitrary units (AU) without background subtraction. Background fluorescence, varied between different experiments and within individual cells, was typically between 500 and 1,000 AU. Exocytotic events were defined as the quasi-instantaneous decreases of ROI fluorescence of >20% between two sequentially acquired images, i.e., within ~ 1 s. Temporal resolution was limited by the image acquisition rate of our system (1 image/s).

Statistical analysis

Statistical significance was determined by two-sample independent *t* test, and $P < 0.05$ was considered significant. Origin 7.5 (OriginLab, Northampton, MA, USA) was used for statistical analysis. The data are presented as mean values $\pm$ SD, and the number of experiments per group is included in the figure legends.

Results

Cytoskeleton disruption and exocytosis modulators affect ATP release To explore the release mechanism, here, we studied ATP efflux in a flow-through chamber and tested the effect of pharmacological agents known to influence cytoskeleton integrity or interfere with various steps of the exocytotic process. Figure 1 demonstrates that peak ATP release, typically observed 2–3 min after stimulation with 50% hypotonic stress [12, 21], was significantly

Fig. 1 Modulators of cytoskeleton and vesicle formation affect ATP release from A549 cells. **a** Examples of ATP release time course measured in a flow-through chamber with A549 cells treated with brefeldin A (BFA) to disrupt the Golgi complex, exocytosis inhibitors monensin and NEM, and cytochalasin D and nocodazole to disrupt the cytoskeleton. They were stimulated with 50% hypotonic stress ($t=0$), and ATP release rate was expressed in pmol/min/10⁶ cells. **b** The peak

rate of release (black bar) and total ATP secreted during 15 min of hypotonic stimulation (gray bar) were inhibited in cells treated with different agents acting on the cytoskeleton or affecting vesicle formation. The data, mean values $\pm$ SD from three to four independent experiments for each drug tested, were normalized to control values. All drugs produced significant inhibition of ATP release, indicated by asterisks (two-sample independent t test, $P<0.05$)

inhibited by the disruption of the actin filaments with cytochalasin D (2 μ M, 18 h) or microtubules with nocodazole (20 μ M, 18 h), $\sim 67\%$ and $\sim 58\%$ inhibition, respectively. A similar effect was produced by disrupting

the Golgi complex with brefeldin A (BFA, 10 μ g/ml, 2.5 h, $\sim 41\%$ inhibition). Interfering with vesicular transport through the Golgi apparatus with the Na⁺ ionophore monensin (10 μ M, 18 h) or impeding the exocytotic

machinery with NEM (1 mM, 15 min) exerted the strongest effects, ~81% and ~95% inhibition, respectively. The inhibitory outcomes were somewhat lower when total ATP release was evaluated (Fig. 1). These results are consistent with the view that regulated exocytosis may be responsible for most ATP release observed in these experiments.

Fluorescent staining of intracellular ATP storage sites

A549 cells were labeled with quinacrine, considered to be the preferred available fluorescent marker for detecting releasable ATP stores [22–24]. Figure 2 shows quinacrine

loading into A549 cells at room temperature, monitored by confocal microscopy as changes of fluorescence intensity or fluorescence lifetime with FLIM. Both intensity and lifetime images revealed non-uniform quinacrine distribution within the cells. Punctate staining, which results from vesicular accumulation of the dye, was seen throughout the cytoplasm but was particularly abundant and intense in the perinuclear region. Quinacrine loading seemed to saturate after 15–20 min (not shown). Similar granular staining and distribution pattern was observed with TIRF microscopy in cells pre-loaded with quinacrine or the fluorescent ATP analog Bodipy-ATP (Fig. 3).

Fig. 2 Quinacrine loading into A549 cells. Images comparing fluorescence intensity (**a**) and fluorescence lifetime (**b**), at time 0 (background autofluorescence; *left-side images*), and 17 min after the addition of 5 μ M quinacrine (*right-side images*). Both intensity and lifetime images show non-uniform granular distribution of quinacrine within the cells. **c** Histograms of fluorescence lifetime τ , the peak on the left, which corresponds to $\tau=1.5$ ns, arises from cell autofluorescence, while in quinacrine-loaded cells a second peak of longer

fluorescence lifetime of $\tau=4.5$ ns is predominant. Images were acquired with a confocal Olympus microscope equipped with a PicoQuant fluorescence lifetime system (see “Methods”), picture resolution 300×300 pixels (area $80 \times 80 \mu\text{m}^2$). Fluorescence intensity and lifetime *scale bars* are shown to the *right of the images*. Note different fluorescence intensity scales for each image, adjusted to disclose a weak background autofluorescence in the absence of quinacrine

Fig. 3 TIRF images of A549 cells. Comparison of TIRF images of A549 cells loaded with 10 μ M quinacrine (*left*) or 30 μ M Bodipy-ATP (*right*). Both images show similar staining pattern. With both fluorophores, fluorescence was excited with a 488-nm laser line and observed at 512 nm, Olympus IX71 inverted microscope with $\times 60$, NA 1.45 TIRF objective

Monitoring exocytosis with TIRF microscopy

To study exocytosis of quinacrine-stained vesicles, fluorescence intensity changes of individual vesicles were analyzed at 37°C under basal conditions and during cell stimulation by hypotonic shock for a total observation time of 1–2 min. Figure 4a represents an example of a single quinacrine-stained cell imaged by TIRF microscopy. Sequential TIRF images in Fig. 4b show the time course of fluorescence intensity changes of vesicles (indicated by arrows) in a small cell region. This is presented graphically in Fig. 4c, giving

three typical examples of single-vesicle fluorescence intensity changes observed during hypotonic shock stimulation. Vesicle (1), which did not undergo exocytosis, showed only gradual loss of the fluorescence due to photobleaching. Vesicle (2) manifested an abrupt loss of fluorescence, as expected from exocytotic quinacrine release and its dispersal by diffusion. In this case, it was preceded by a transient fluorescence increase likely owing to vesicle recruitment towards the plasma membrane at the cell base, i.e., deeper in the evanescent field. Since evanescent field intensity increases exponentially in close proximity to the glass

Fig. 4 Fluorescence intensity changes of quinacrine-containing vesicles during exocytosis. **a** TIRF image of a single quinacrine-stained cell: the cell contour is outlined by a dotted line (*left*). On the *right*, the same cell with regions of interest placed on selected vesicles. **b** Sequential TIRF images of quinacrine-loaded vesicles showing the time course of fluorescence changes within ROI during 60 s. Hypotonic shock was applied at $t=0$. **c** Three examples of fluorescence changes within single vesicles (indicated by arrows in **a** and **b**) during hypotonic shock (*t*) gradual loss of fluorescence signal due to photobleaching, no exocytosis; (2) abrupt loss of fluorescence attributed to exocytotic release of vesicular quinacrine and its dispersal by diffusion that was preceded by a transiently increased fluorescence likely due to vesicle recruitment deeper into the evanescent field; (3) abrupt loss of fluorescence without the preceding intensity increase. Fluorescence intensity is expressed in arbitrary units (AU)

substrate, a stronger fluorescence will be elicited in vesicles near the substrate than those further away. Vesicle (3) also displayed an abrupt loss of fluorescence, but without the preceding intensity increase, and may represent a vesicle that was already docked at the plasma membrane before stimulation. In subsequent analysis, such abrupt fluorescence losses of individual vesicles were interpreted as exocytotic events.

Hypotonic shock transiently stimulates exocytosis of quinacrine-stained vesicles

To examine the effect of hypotonic stimulation, the number of exocytotic events observed in a field of view was counted at different time points before and during stimulation. Figure 5a represents an example of such TIRF image analysis, with several cells in the FOV and with 120 selected ROI. Figure 5b demonstrates that exocytotic events, seen as abrupt losses of fluorescence intensity, take place at different time points during stimulation. These data are presented as histogram of exocytotic events in Fig. 5c. It demonstrates that hypotonic stimulation was associated with a transient, several-fold increase in the number of exocytotic events during the first ~60 s of the stimulus ($t=20$ –80 s), as compared to very few spontaneous events occurring under basal conditions before the stimulation ($t=0$ –20 s). Such transient stimulation compares well with the time course of ATP release shown in Fig. 1a, despite differences in the way the hypotonic stimulus was applied (solution perfusion versus direct addition of water). In these experiments, performed at 37°C, ~40% of vesicles underwent exocytosis during the first 2 min of hypotonic stimulation (146 out of 367; cumulative data from 30 cells in three independent experiments). Interestingly, when similar experiments were performed at 20°C, only 0.6% of vesicles underwent exocytosis during the 2 min of hypotonic stimulation (two vesicles out of 334; cumulative data from 19 cells in three independent experiments), Fig. 5d. Thus, the rate of exocytosis observed by TIRF microscopy was significantly diminished at reduced temperature.

Discussion

Nucleotide release is a key step in the purinergic signaling cascade that affects multiple components of innate lung defense including airway surface liquid homeostasis, ciliary beating, mucin release, and surfactant secretion in the alveoli [25–27]. However, the mechanisms by which ATP is released from non-excitable cells are still being debated [28, 29]. The generally accepted consensus is that several ATP release pathways may be functional in the same cell, depending on the stimulus and physiological/pathological

conditions. In principle, two distinct mechanisms may underlie non-lytic mechano-sensitive ATP release from epithelial cells: (1) exocytosis of ATP-containing vesicles; (2) cytosolic ATP secretion via intrinsic plasma membrane proteins, which could be either channels or transporters (nucleotide transporters or nucleotide/nucleoside exchangers). While exocytosis remains least controversial, much effort has focused on a search for ATP-conducting channels, and the list of possible channel candidates has expanded enormously in the last decade. Possible ATP release pathways and their regulatory mechanisms are discussed in detail in recent excellent reviews [28, 29]. Current understanding of these mechanisms is briefly summarized below to put the results of the present study into the context.

Early reports have suggested that the cystic fibrosis transmembrane conductance regulator (CFTR) and other members of the superfamily of ATP-binding cassette transport proteins serve as a conductive pathway for ATP release, or regulate an associated ATP channels [30, 31]. Re-examination of this hypothesis with the patch clamp, lipid bilayer, and luminometry techniques, has not revealed any detectable CFTR-mediated or CFTR-regulated ATP release [6, 32–37]. It was also found that stretch-activated, gadolinium-sensitive channels are an unlikely pathway for ATP efflux [38]. Volume-regulated anion channels (VRAC) are known to have considerable permeability to cations and large organic anions [39, 40], and several studies have implicated VRAC in conductive ATP release and autocrine regulation of cell volume, or non-synaptic communication between axons and nervous system cells. Not all studies support that view, and until VRAC are identified at the molecular level, evidence for their role as ATP conduits will remain circumstantial [12, 34, 41–44]. Cell swelling has also been found to induce a large-conductance (~400 pS), voltage-dependent Cl^- channels [45] that are permeable to ATP, and to some extent, resemble voltage-dependent anion channels (VDAC, porin, or maxi Cl^- channel) [44, 46]. VDAC mediates ATP movement across the outer mitochondrial membrane [47], and similar channels have occasionally been found in the plasma membrane of several cells [48]. Finally, connexins and the related pannexins currently appear to be the most extensively investigated family of proteins reported to function as ATP conduits in a broad range of cell types [17, 29, 49–52].

In the specific case of hypotonic stress-induced ATP secretion from alveolar A549 cells, evidence exists in support of both conductive and non-conductive (exocytotic) ATP release. Observations that are consistent with an exocytotic release mechanism include the following: (1) ATP secretion is a Ca^{2+} -dependent process tightly correlated with $[\text{Ca}^{2+}]_i$ elevation [12, 21]. (2) There is a limited pool of releasable ATP: typically less than 5% of cell ATP content is liberated, most of it during the first 3–5 min of

Fig. 5 Hypotonic shock stimulates exocytosis of quinacrine-stained vesicles. **a** TIRF image of several cells in a field of view with 120 selected ROIs. **b** Examples of exocytotic events, seen as abrupt losses of fluorescence intensity taking place at different time points during stimulation by 50% hypotonic shock applied at $t=20$ s. **c** Histogram of exocytotic events: hypotonic stimulation was applied at $t=20$ s.

Cumulative data from three independent experiments performed at 37°C; 367 ROI totally. **d** Single-vesicle fluorescence intensity changes during hypotonic shock at 20°C. For clarity, intensity traces from only 13 ROI in one cell are illustrated. Only one trace showed a step-drop of fluorescence (arrow). The data are representative of three experiments: 334 ROI totally

the continuous 15–30-min hypotonic stimulation [12, 21] (Fig. 1a). Such release might be expected for a mechanism that involves a limited pool of readily releasable vesicles, analogous to that in neuroendocrine cells and neurons. (3) The ratios of co-released nucleotides ADP/ATP (1:3) and UDP/UTP (1:2) are markedly higher than the cytosolic ratios of these species, suggesting that a nucleotide diphosphate-rich compartment, e.g., the secretory pathway, contributes to nucleotide release [13]. (4) ATP release is almost completely inhibited at reduced temperature (10–20°C), as expected for an exocytotic process that involves several highly temperature-sensitive steps, such as vesicle trafficking and membrane fusion. Partial reduction of ATP secretion under those conditions is indicative of a conductive (diffusive) release pathway [12]. (5) Laser confocal microscopy experiments have revealed increased FM1-43 staining of the plasma membrane of A549 cells upon hypotonic shock or ionomycin treatment, consistent with enhanced vesicular exocytosis and membrane recycling under such conditions [13].

The present study adds important complementary evidence demonstrating that exocytosis of ATP-loaded vesicles operates in A549 cells and that it is likely the major stress-induced ATP release pathway in these cells. Quinacrine labeling of A549 cells disclosed punctate staining, indicating ATP storage within cytoplasmic granules (Figs. 2, 3, 4, and 5). Similar granular quinacrine staining has been reported in rabbit ciliary epithelial cells [22], human vascular endothelial cells [19], rat pancreatic acini [23], lymphocytes [24], astrocytes [20, 53], and rat hepatoma cells [54]. In the present study, similar staining of A549 cells was also observed with the fluorescent ATP analog Bodipy-ATP, supporting the view that quinacrine labels ATP-containing vesicles (Fig. 3). A similar accumulation pattern of both, fluorescent ATP analogue and quinacrine, was also found in pancreatic [23] as well as parotid and lacrimal gland acini [55]. It should be noted, however, that quinacrine has been shown to bind to a variety of polyanions, not only ATP, and to accumulate within acidic intracellular compartments [56]. Thus, quinacrine fluorescence should be interpreted with caution and serve only as a semiquantitative, indirect tool to monitor vesicular ATP.

By applying TIRF microscopy to quinacrine-stained cells, we were able to directly detect exocytotic events of quinacrine/ATP-loaded vesicles, which were seen as abrupt changes of their fluorescence due to post-exocytotic dispersal of vesicular cargo. Abrupt loss of single-vesicle fluorescence was occasionally preceded by transiently increased fluorescence, which could have resulted from two processes. The first is vesicle movement along the *z*-axis towards the plasma membrane at the cell base, representing vesicle recruitment. The more intense evanescent field at the cell base results in increased fluorescence but it remains localized in well-defined spot until vesicle fusion with the plasma membrane. The second process may derive

from post-exocytotic release of fluorescent cargo towards the intense evanescent field at the glass substrate and could be seen as fluorescence spread followed by a rapid disappearance. Such observations require special conditions because dilution of the dye after exocytotic release rapidly reduces the fluorescence signal. A transient increase of fluorescence intensity could be favored if fluorescent cargo is released in the narrow extracellular space confined between the cell base and the glass substrate, as opposed to the release into open space, e.g., at the cell edge. In our experiments, ~25% of exocytotic events showed such a transient fluorescence increase. The majority of vesicles displayed only a quasi-instantaneous fluorescence decline, suggesting the involvement of pre-docked vesicles, but we may have missed some vesicles showing transiently increased fluorescence due to limited temporal resolution of our image acquisition system. Nevertheless, an abrupt decrease of fluorescence attributed to exocytosis was consistently recorded in all experiments, allowing analysis of the effects of cell stimulation and low temperature on exocytosis. The rate of exocytotic events observed by TIRF microscopy was strongly stimulated at the onset of hypotonic stress (first 60–80 s) and almost completely inhibited by lowering the temperature to 20°C, which parallels similar modulation of ATP release from A549 cells studied previously by luciferase bioluminescence assay [12]. Disruption of the cytoskeleton and interference with vesicle formation strongly diminished ATP secretion, while up to 95% of ATP release was inhibited by NEM, indicating that exocytosis is responsible for most, if not all release observed in these experiments [12]. It should be noted that in TIRF experiments only a thin slice of a cell is illuminated by the evanescent field that extends ~100 nm from the glass substrate, limiting observation of exocytotic events to the cell base. However, due to isotropic nature of the hypo-osmotic stimulus and the fact that A549 cells are not well polarized, similar exocytotic release might be expected to take place at the “luminal” surface. This notion is supported by a reasonably good temporal correlation between kinetics of exocytosis, detected by TIRF, and ATP release studied with luciferase–luciferin assay, in hypotonicity-stimulated A549 cells.

Our conclusion that vesicular exocytosis is responsible for the major part of ATP release is at variance with recent studies on A549 and airway epithelial cells, in which pharmacological inhibitors of pannexin 1 and pannexin 1 knockdown significantly decreased hypotonicity-stimulated ATP secretion [15, 17], leaving only residual ATP release that could be attributed to other pathways, such as exocytosis. The fact that a large part of ATP release was blocked by different approaches, one intended to interfere with pannexin 1 function [15, 17], the other with exocytosis (this study and [12]), might be attributed, at least in part, to the non-specific effect of the pharmacological and bio-

physical (low temperature) tools which may affect both release pathways. Furthermore, both pathways may have common elements that are activated by hypo-osmotic stress, such as cytoskeleton re-arrangements [57]. Nevertheless, a more specific approach of pannexin 1 knockdown provides a strong support for its role in hypotonicity-stimulated ATP release. Whether pannexin 1 functions as ATP-conducting channel, or has indirect role as a regulator of other ATP release pathways, remains unclear. Conductive ATP release mechanisms are still incompletely characterized, and several important questions remain open. For example, regardless of the molecular nature of ATP-conducting channels, the main conceptual difficulty with such a release mechanism is that ATP permeation pore has to be of large dimensions (0.6 to 1.1 nm, [44]), resulting in poor selectivity and large conductance (hundreds of pS) for small ions such as K^+ , Na^+ , Cl^- , and Ca^{2+} . Thus, opening such large, non-selective pores in the plasma membrane will elicit significant influx of Na^+ and Ca^{2+} down their electrochemical gradients into the cytoplasm. Although collapse of transmembrane ionic gradients might be relevant for triggering cell death and apoptosis [58], it may pose a significant challenge to normal cell function and survival. Several mechanisms could be in place to limit the deleterious effects of opening such pores; for example self-inhibition by extracellular ATP and interaction with cytoskeletal components were proposed to control pannexin 1 activity, reviewed in [29]. However, even if the duration of channel activity is strictly controlled, a significant amount of Ca^{2+} will permeate into the cells via open channel pores during ATP release. For hypotonic stress-activated A549 cells, ATP release peaks for about 3–5 min, before it declines to the background level, Fig. 1 [12]. How much extracellular Ca^{2+} could enter into the cell during ATP secretion? Pannexin selectivity has not been studied in as much detail as, e.g., the mitochondrial VDAC [59], but the current literature indicates that pannexin channels have significant Ca^{2+} permeability, comparable to K^+ , i.e., higher than for ATP itself [60, 61]. Making a conservative assumption that only one Ca^{2+} ion gets into the cell for each ATP molecule that goes out, we could estimate that hypotonicity-stimulated, cumulative ATP release from A549 cells ($\sim 10^{-15}$ mole/cell, [12]) corresponds to ~ 0.3 mM Ca^{2+} accumulation in a cell volume of 3 pL [62]—an overwhelming challenge for cellular Ca^{2+} homeostasis mechanisms. Interestingly, Fura-2 fluorescence Ca^{2+} imaging demonstrated that during hypotonic stimulation of A549 cells and 16HBE14o⁻ cells, the peak $[Ca^{2+}]_i$ response coincides with the peak rate of ATP release. It was, however, much lower (~ 100 -fold) than the above estimate suggests [12]. Furthermore, in the case of A549 cells, the response did not involve Ca^{2+} influx

from extracellular spaces, but was entirely due to Ca^{2+} release from intracellular stores [21]. There are at least two possible explanations for this discrepancy: either currently available data greatly over-estimate pannexin 1 Ca^{2+} permeability, or pannexin 1 in A549 cells does not function as plasmalemmal ATP channel, but as ATP release regulator. Pannexin1 has been shown to form associations with other proteins, including P2X₇ purinergic receptor, subunit of the voltage-dependent potassium channel, and actin microfilaments, but its role in these complexes remains unclear [52, 61]. Intracellular roles of pannexins are also emerging, for example, pannexin 3 functions as an endoplasmic reticulum Ca^{2+} channel in osteoblasts, HEK 293, and prostate cancer epithelial LNCaP cells [50, 63], adding to the complexity of understanding their potential role in ATP secretion. Clearly, further studies on pannexin channels are required to clarify this issue and to facilitate more complete knowledge of their functions in cells. While the conductive ATP release mechanisms await more complete characterization, Ca^{2+} -dependent vesicular exocytosis, which represents a safe and well-regulated mechanism available to cells for controlled ATP secretion, offers a more consistent description of our experiments.

In summary, we demonstrated that Ca^{2+} -dependent exocytosis of ATP-loaded vesicles operates in alveolar A549 cells and it likely represents a major pathway of hypotonic stress-stimulated ATP secretion in these cells. Such a mechanism is also consistent with mechano-sensitive ATP release seen with A549 cells stimulated by tension forces at the air–liquid interface [14].

Acknowledgments This study was supported in part by the Canadian Institutes of Health Research, the Canadian Cystic Fibrosis Foundation (RG) and the National Institutes of Health (USA): NIH-5R21CA149897-02 (ZG) and NIH-HL090786 (JB).

References

1. Burnstock G, Fredholm BB, North RA, Verkhratsky A (2010) The birth and postnatal development of purinergic signalling. *Acta Physiol (Oxf)* 199:93–147
2. Burnstock G (2006) Purinergic signalling. *Br J Pharmacol* 147 (Suppl 1):S172–S181
3. Burnstock G (2008) Unresolved issues and controversies in purinergic signalling. *J Physiol* 586:3307–3312
4. Okada SF, Nicholas RA, Kreda SM, Lazarowski ER, Boucher RC (2006) Physiological regulation of ATP release at the apical surface of human airway epithelia. *J Biol Chem* 281:22992–23002
5. Button B, Picher M, Boucher RC (2007) Differential effects of cyclic and constant stress on ATP release and mucociliary transport by human airway epithelia. *J Physiol* 580:577–592
6. Grygorczyk R, Hanrahan JW (1997) CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. *Am J Physiol* 272:C1058–C1066
7. Vlahakis NE, Hubmayr RD (2003) Response of alveolar cells to mechanical stress. *Curr Opin Crit Care* 9:2–8

8. Liu M, Post M (2000) Invited review: mechanochemical signal transduction in the fetal lung. *J Appl Physiol* 89:2078–2084
9. Edwards YS (2001) Stretch stimulation: its effects on alveolar type II cell function in the lung. *Comp Biochem Physiol A Mol Integr Physiol* 129:245–260
10. Foster KA, Oster CG, Mayer MM, Avery ML, Audus KL (1998) Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. *Exp Cell Res* 243:359–366
11. Shapiro DL, Nardone LL, Rooney SA, Motoyama EK, Munoz JL (1978) Phospholipid biosynthesis and secretion by a cell line (A549) which resembles type II alveolar epithelial cells. *Biochim Biophys Acta* 530:197–207
12. Boudreault F, Grygorczyk R (2004) Cell swelling-induced ATP release is tightly dependent on intracellular calcium elevations. *J Physiol* 561:499–513
13. Tatur S, Kreda S, Lazarowski E, Grygorczyk R (2008) Calcium-dependent release of adenosine and uridine nucleotides from A549 cells. *Purinergic Signal* 4:139–146
14. Ram Singh R, Grygorczyk A, Solecki A, Cherkaoui LS, Berthiaume Y, Grygorczyk R (2011) Cell deformation at the air-liquid interface induces Ca^{2+} -dependent ATP release from lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 300:L587–L595
15. Seminario-Vidal L, Okada SF, Sesma JI, Kreda SM, Van Heusden CA, Zhu Y, Jones LC, O'Neal WK, Penuela S, Laird DW, Boucher RC, Lazarowski ER (2011) RHO signaling regulates pannexin 1-mediated ATP release from airway epithelia. *J Biol Chem* 286(30):26277–26286
16. Seminario-Vidal L, Kreda S, Jones L, O'Neal W, Trejo J, Boucher RC, Lazarowski ER (2009) Thrombin promotes release of ATP from lung epithelial cells through coordinated activation of rho- and Ca^{2+} -dependent signaling pathways. *J Biol Chem* 284:20638–20648
17. Ransford GA, Fregien N, Qiu F, Dahl G, Conner GE, Salathe M (2009) Pannexin 1 contributes to ATP release in airway epithelia. *Am J Respir Cell Mol Biol* 41:525–534
18. van der Ploeg M, Ploem JS (1973) Filter combinations and light sources for fluorescence microscopy of quinacrine mustard or quinacrine stained chromosomes. *Histochemie* 33:61–70
19. Bodin P, Burnstock G (2001) Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J Cardiovasc Pharmacol* 38:900–908
20. Striedinger K, Meda P, Scemes E (2007) Exocytosis of ATP from astrocyte progenitors modulates spontaneous Ca^{2+} oscillations and cell migration. *Glia* 55:652–662
21. Tatur S, Groulx N, Orlov SN, Grygorczyk R (2007) Ca^{2+} -dependent ATP release from A549 cells involves synergistic autocrine stimulation by coreleased uridine nucleotides. *J Physiol* 584:419–435
22. Mitchell CH, Carre DA, McGlinn AM, Stone RA, Civan MM (1998) A release mechanism for stored ATP in ocular ciliary epithelial cells. *Proc Natl Acad Sci USA* 95:7174–7178
23. Sorensen CE, Novak I (2001) Visualization of ATP release in pancreatic acini in response to cholinergic stimulus. Use of fluorescent probes and confocal microscopy. *J Biol Chem* 276:32925–32932
24. Yegutkin GG, Mikhailov A, Samburski SS, Jalkanen S (2006) The detection of micromolar pericellular ATP pool on lymphocyte surface by using lymphoid ecto-adenylate kinase as intrinsic ATP sensor. *Mol Biol Cell* 17:3378–3385
25. Lazarowski ER, Tarran R, Grubb BR, Van Heusden CA, Okada S, Boucher RC (2004) Nucleotide release provides a mechanism for airway surface liquid homeostasis. *J Biol Chem* 279:36855–36864
26. Kreda SM, Okada SF, Van Heusden CA, O'Neal W, Gabriel S, Abdullah L, Davis CW, Boucher RC, Lazarowski ER (2007) Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *Journal of Physiology-London* 584:245–259
27. Rooney SA (2001) Regulation of surfactant secretion. *Comp Biochem Physiol A Mol Integr Physiol* 129:233–243
28. Praetorius HA, Leipziger J (2009) ATP release from non-excitable cells. *Purinergic Signal* 5:433–446
29. Lazarowski ER, Sesma JI, Seminario-Vidal L, Kreda SM (2011) Molecular mechanisms of purine and pyrimidine nucleotide release. *Adv Pharmacol* 61:221–261
30. Reisin IL, Prat AG, Abraham EH, Amara JF, Gregory RJ, Ausiello DA, Cantiello HF (1994) The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel. *J Biol Chem* 269:20584–20591
31. Sugita M, Yue Y, Foskett JK (1998) CFTR Cl^- channel and CFTR-associated ATP channel: distinct pores regulated by common gates. *EMBO J* 17:898–908
32. Grygorczyk R, Hanrahan JW (1997) Cystic fibrosis transmembrane conductance regulator and adenosine triphosphate [response]. *Science* 275:1325–1326
33. Grygorczyk R, Tabcharani JA, Hanrahan JW (1996) CFTR channels expressed in CHO cells do not have detectable ATP conductance. *J Membr Biol* 151:139–148
34. Hazama A, Shimizu T, Ando-Akatsuka Y, Hayashi S, Tanaka S, Maeno E, Okada Y (1999) Swelling-induced, CFTR-independent ATP release from a human epithelial cell line: lack of correlation with volume-sensitive Cl^- channels. *J Gen Physiol* 114:525–533
35. Li C, Ramjeesingh M, Bear CE (1996) Purified cystic fibrosis transmembrane conductance regulator (CFTR) does not function as an ATP channel. *J Biol Chem* 271:11623–11626
36. Reddy MM, Quinton PM, Haws C, Wine JJ, Grygorczyk R, Tabcharani JA, Hanrahan JW, Gunderson KL, Kopito RR (1996) Failure of the cystic fibrosis transmembrane conductance regulator to conduct ATP. *Science* 271:1876–1879
37. Watt WC, Lazarowski ER, Boucher RC (1998) Cystic fibrosis transmembrane regulator-independent release of ATP. Its implications for the regulation of P2Y2 receptors in airway epithelia. *J Biol Chem* 273:14053–14058
38. Boudreault F, Grygorczyk R (2002) Cell swelling-induced ATP release and gadolinium-sensitive channels. *Am J Physiol Cell Physiol* 282:C219–C226
39. Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G (1997) Properties of volume-regulated anion channels in mammalian cells. *Prog Biophys Mol Biol* 68:69–119
40. Strange K, Emma F, Jackson PS (1996) Cellular and molecular physiology of volume-sensitive anion channels. *Am J Physiol* 270:C711–C730
41. Hisadome K, Koyama T, Kimura C, Droogmans G, Ito Y, Oike M (2002) Volume-regulated anion channels serve as an auto/paracrine nucleotide release pathway in aortic endothelial cells. *J Gen Physiol* 119:511–520
42. Wang Y, Roman R, Lidofsky SD, Fitz JG (1996) Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. *Proc Natl Acad Sci USA* 93:12020–12025
43. Fields RD, Ni Y (2010) Nonsynaptic communication through ATP release from volume-activated anion channels in axons. *Sci Signal* 3:ra73
44. Sabirov RZ, Okada Y (2005) ATP release via anion channels. *Purinergic Signal* 1:311–328
45. Dutta AK, Okada Y, Sabirov RZ (2002) Regulation of an ATP-conductive large-conductance anion channel and swelling-induced ATP release by arachidonic acid. *J Physiol* 542:803–816
46. Sabirov RZ, Okada Y (2009) The maxi-anion channel: a classical channel playing novel roles through an unidentified molecular entity. *J Physiol Sci* 59:3–21

47. Rostovtseva T, Colombini M (1997) VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function. *Biophys J* 72:1954–1962
48. Bathori G, Parolini I, Szabo I, Tombola F, Messina A, Oliva M, Sargiacomo M, de Pinto V, Zoratti M (2000) Extramitochondrial porin: facts and hypotheses. *J Bioenerg Biomembr* 32:79–89
49. Romanello M, Pani B, Bicego M, D'Andrea P (2001) Mechanically induced ATP release from human osteoblastic cells. *Biochem Biophys Res Commun* 289:1275–1281
50. D'hondt C, Ponsaerts R, De Smedt H, Vinken M, De Vuyst E, De Bock M, Wang N, Rogiers V, Leybaert L, Himpens B, Bultynck G (2010) Pannexin channels in ATP release and beyond: an unexpected rendezvous at the endoplasmic reticulum. *Cell Signal* 23:305–316
51. Dando R, Roper SD (2009) Cell-to-cell communication in intact taste buds through ATP signalling from pannexin 1 gap junction hemichannels. *J Physiol* 587:5899–5906
52. Ma W, Hui H, Pelegrin P, Surprenant A (2009) Pharmacological characterization of pannexin-1 currents expressed in mammalian cells. *J Pharmacol Exp Ther* 328:409–418
53. Pryazhnikov E, Khiroug L (2008) Sub-micromolar increase in $[Ca^{2+}]_i$ triggers delayed exocytosis of ATP in cultured astrocytes. *Glia* 56:38–49
54. Feranchak AP, Lewis MA, Kresge C, Sathe M, Bugde A, Luby-Phelps K, Antich PP, Fitz JG (2010) Initiation of purinergic signaling by exocytosis of ATP-containing vesicles in liver epithelium. *J Biol Chem* 285:8138–8147
55. Novak I, Jans IM, Wohlfahrt L (2010) Effect of P2X(7) receptor knockout on exocrine secretion of pancreas, salivary glands and lacrimal glands. *J Physiol* 588:3615–3627
56. Miller DS, Villalobos AR, Pritchard JB (1999) Organic cation transport in rat choroid plexus cells studied by fluorescence microscopy. *Am J Physiol* 276:C955–C968
57. Blum AE, Walsh BC, Dubyak GR (2010) Extracellular osmolarity modulates G protein-coupled receptor-dependent ATP release from 1321 N1 astrocytoma cells. *Am J Physiol Cell Physiol* 298:C386–C396
58. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS, Isakson BE, Bayliss DA, Ravichandran KS (2010) Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* 467:863–867
59. Tan W, Colombini M (2007) VDAC closure increases calcium ion flux. *Biochim Biophys Acta* 1768:2510–2515
60. Bao L, Locovei S, Dahl G (2004) Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett* 572:65–68
61. Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1 β release by the ATP-gated P2X7 receptor. *EMBO J* 25:5071–5082
62. Groulx N, Boudreau F, Orlov SN, Grygorczyk R (2006) Membrane reserves and hypotonic cell swelling. *J Membr Biol* 214:43–56
63. Ishikawa M, Iwamoto T, Nakamura T, Doyle A, Fukumoto S, Yamada Y (2011) Pannexin 3 functions as an ER Ca^{2+} channel, hemichannel, and gap junction to promote osteoblast differentiation. *J Cell Biol* 193:1257–1274