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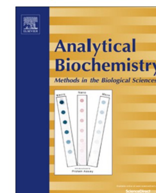

Quantification of potassium levels in cells treated with *Bordetella* adenylate cyclase toxin

Tomas Wald [a,1], Inga Petry-Podgorska [b,1], Radovan Fiser [a,c], Tomas Matousek [b], Jiri Dedina [b], Radim Osicka [a], Peter Sebo [a], Jiri Masin [a,*]

[a] Laboratory of Molecular Biology of Bacterial Pathogens, Cell and Molecular Microbiology Division, Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic
[b] Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, 60200 Brno, Czech Republic
[c] Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Prague 2, Czech Republic

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ABSTRACT

The aim of this study was to compare two methods for quantification of changes in intracellular potassium concentration (decrease from ~140 to ~20 mM) due to the action of a pore-forming toxin, the adenylate cyclase toxin (CyaA) from the pathogenic bacterium *Bordetella pertussis.* CyaA was incubated with stably transfected K1 Chinese hamster ovary cells expressing the toxin receptor CD11b/CD18 and the decrease in potassium concentration in the cells was followed by inductively coupled plasma mass spectrometry (ICP–MS). It is shown that this method is superior in terms of sensitivity, accuracy, and temporal resolution over the method employing the potassium-binding benzofuran isophthalate–acetoxymethyl ester fluorescent indicator. The ICP–MS procedure was found to be a reliable and straightforward analytical approach enabling kinetic studies of CyaA action at physiologically relevant toxin concentrations (<1000 ng/ml) in biological microsamples.

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A decrease in cellular potassium levels as a result of cell permeabilization by the action of pore-forming toxins (PFTs) was shown to be crucial for activation of a variety of cellular signaling cascades [1]. Upon PFT-induced plasma-membrane damage (cell permeabilization), the cells change their metabolism, enter a resting state [2], and activate the autophagy program, as shown for *Staphylococcus aureus* α-hemolysin; *Vibrio cholerae* cytolysin, aerolysin, streptolysin, and listeriolysin and *Escherichia coli* α-hemolysin [2–4]. PFTs also trigger formation of lipid droplets, serving as energy storage devices, and reduce the anabolic activity of cells via a global arrest in protein translation [2]. Both events appear to be triggered by a decrease in cytosolic potassium concentration that leads to, among others, activation of the inflammasome [5–8], a multisubunit complex involved in the activation of caspase 1 [9]. For example, a caspase 1-mediated cell-death pathway called pyroptosis can be triggered by PFTs [10]. Further, downstream targets of caspase 1 are the inflammatory cytokines interleukin (IL) 1β and 18, which are released by cells treated by various PFTs [5,7,11]. Also, activation of caspase 2 (an initiator caspase that subsequently activates caspases 3, 8, and 9) by PFT aerolysin and *S. aureus* α-hemolysin [12] was shown to be dependent on potassium efflux. Further, the master regulators of lipid metabolism, the SREBP transcription factors, were shown to be activated upon action of PFTs [6]. Activation of SREBPs in response to aerolysin and *S. aureus* α-hemolysin promoted cell survival and required potassium-efflux-dependent activation of caspase 1 [6]. Finally, PFTs also activate all three mitogen-activated protein kinase pathways, p38, JNK, and ERK, which promote the recovery of plasma membrane integrity and ion balance [2,3,13–16]. A drop in cellular potassium concentration, hence, clearly emerges as one of the master regulators of the cellular response to PFTs.

By instantaneously disrupting bactericidal functions of host phagocytes, the adenylate cyclase toxin–hemolysin (CyaA) plays a major role in the virulence of pathogenic *Bordetellae* [17]. The toxin primarily targets host myeloid cells expressing the CD11b/CD18 integrin [18], known also as complement receptor 3, Mac-1, or the $\alpha_M\beta_2$ integrin. Upon binding it translocates, directly across the cytoplasmic membrane of cells, its N-terminal adenylate cyclase enzyme (AC) domain, which binds cytosolic calmodulin and paralyzes cellular signaling by unregulated conversion of cytosolic ATP to the key signaling molecule, cAMP. The repeats in toxin (RTX) hemolysin moiety of CyaA (~1300 carboxy-proximal

* Corresponding author. Fax: +420 241 062 152.
E-mail address: masin@biomed.cas.cz (J. Masin).
[1] These authors contributed equally to this work.

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residues) is then functionally independent of the AC domain and is itself capable of forming small cation-selective membrane pores [19,20]. These pores can permeabilize target cell membranes and provoke colloid-osmotic lysis of erythrocytes [21,22]. The RTX moiety of CyaA itself consists of several functional subdomains [23,24]. It contains (i) a hydrophobic pore-forming domain comprising residues 500 to 700 [19]; (ii) an activation domain between residues 800 and 1000, where the posttranslational palmitoylation of CyaA occurs [19,25,26]; and (iii) a typical calcium-binding RTX domain, harboring the nonapeptide repeats of a consensus sequence, X-(L/I/F)-X-G-G-X-G-(N/D)-D, which form numerous (~40) calcium-binding sites [24]. Cooperation and structural integrity of all domains of the hemolysin moiety then appear to be critical for membrane insertion of the toxin and translocation of the AC domain into the target cell cytosol [21,23]. Recently, Dunne and co-workers [5] showed that by eliciting K^+ efflux from CD11b-expressing dendritic cells, the pore-forming activity of CyaA contributes to activation of the NALP3 inflammasome and thereby to induction of an innate IL-1β-mediated proinflammatory immune response. The capacity of CyaA to permeabilize cells for K^+ efflux then depends on the capacity of the toxin to promote Ca^{2+} influx into cells and escape the rapid macropinocytic removal from target cell membrane of phagocytes [27].

Currently, the measurement of intracellular potassium concentration is mostly carried out using a fluorescence method employing the potassium-binding benzofuran isophthalate–acetoxymethyl ester (PBFI–AM) indicator [5,27–32]. This method relies on changes in the spectral properties of the probe induced by potassium binding. However, the selectivity of PBFI–AM for potassium over sodium is not high, which poses a problem in situations in which cells have been permeabilized by pore-forming toxins and entry of extracellular sodium ions into the cells through toxin-formed lesions cannot be excluded or is likely.

Inductively coupled plasma mass spectrometry (ICP–MS) has become in recent years a well-established technique for quantification of elements at trace levels in all kinds of matrices. This very sensitive method can be used to quantify the concentrations of elements in biomatrices as an alternative to other commonly applied approaches. ICP–MS analysis of intracellular K^+ concentrations was previously described; however, the method performance was not discussed [33].

Here we used K1 Chinese hamster ovary (CHO) cells stably transfected with human CD11b/CD18 (CHO-CD11b/CD18) and treated with the pore-forming adenylate cyclase toxin to compare the method of cellular potassium content measurement by the traditional PBFI–AM fluorescence probe to the approach employing ICP–MS.

Materials and methods

Production and purification of CyaA

Recombinant CyaA was produced in *E. coli* XL1-Blue cells transformed with the pT7CACT1 construct [34]. The toxin was purified from urea extracts of insoluble cell fractions by two-step chromatography on DEAE and phenyl Sepharose close to homogeneity in 8 M urea, as previously described [35]. Purification of recombinant CyaA in 8 M urea was used to prevent aggregation of the amphipathic toxin molecules. Prior to addition to the cells, the toxin samples were prediluted from concentrated stocks to 100 times the final indicated toxin concentration using 8 M urea, 50 mM Tris–HCl, pH 8.0, and 0.2 mM $CaCl_2$. To reach the indicated working toxin concentration, aliquots of the prediluted toxin samples were further 100-fold diluted into cell suspensions made in modified HBSS (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 50 mM glucose, 10 mM Hepes–Na, pH 7.4), yielding a final urea concentration of 80 mM. The CyaA toxin was previously shown to spontaneously refold and gain maximal biological activity upon dilution to final 80 mM or lower urea concentration [36].

Cell preparation

cDNAs encoding CD11b and CD18 were a kind gift from D. Golenbock, Boston University School of Medicine (Boston, MA, USA) [37]. Genes for CD11b and CD18 subunits of complement receptor 3 were cloned into pcDNA3 and pcDNA3.1, respectively. One microgram of each construct was mixed with 50 μl of Lipofectamine 2000 (11668-027, Invitrogen, USA) and CHO cells were transfected according to the manufacturer's protocol. To establish stably transfected cell lines expressing CD11b/CD18, the cells were cultured in the presence of 600 μg/ml zeocin (ant-zn-1, Invivogen, USA) and 1000 μg/ml G418 (ant-gn-1, Invivogen) for 10 days. To generate monoclonal cell lines, fluorescence-activated cell sorting was used, employing fluorescein isothiocyanate-labeled OKM-1 monoclonal antibody (mouse IgG2b) specific for human CD11b and APC-labeled anti-CD18 (Exbio, Vestec, Czech Republic). Stably transfected individual clones were then subcultured and expanded in the presence of antibiotics and antimycotics (1%, A5955, Sigma–Aldrich, St. Louis, MO, USA) in F-12 medium supplemented with heat-inactivated bovine serum (10%, A15-151, PAA Laboratories, USA).

For use in experiments, the cells were harvested from plates in 0.02% EDTA diluted in phosphate-buffered saline and washed three times with 10 ml of cold modified HBSS (140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes-Na, 50 mM glucose, pH 7.4).

CyaA binding to cells

Approximately 10^6 CHO or CHO-CD11b/CD18 cells were incubated in modified HBSS with 100, 250, or 500 ng/ml CyaA for 5 min at 37 °C, prior to removal of unbound toxin by four washes in cold modified HBSS. Cells were lysed with 0.1% Triton X-100 for determination of cell-bound adenylate cyclase enzyme activity that was measured using the procedure of White [38], as modified by Hanoune et al. [39] and described in full elsewhere [40]. The reaction was performed at 30 °C in 50 μl of a medium containing 60 mM Tris–HCl, pH 8, 7 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1% (w/w) Triton X-100, 2 mM [α-^{32}P]ATP, 1 mg/ml bovine serum albumin, 0.1 mM [2,8-^{3}H]cAMP, and 0.1 μM calmodulin. One unit of adenylate cyclase corresponds to 1 μmol of cAMP formed in 1 min at 30 °C at pH 8.

Determination of cytosolic potassium levels using the PBFI–AM fluorescent indicator method

Prior to experiments, glass coverslips (20 × 20 mm, Art. No. 8000103, Hirschmann Laborgeräte, Germany) were cut to 10 × 20 mm and washed repeatedly with ethanol, HCl, and water and heat-sterilized at 200 °C for 3 h. Cells grown on prepared glass coverslips (in six-well plates) were washed in HBSS and loaded with 9.5 μM PBFI–AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C in the presence of 0.05% (w/w) Pluronic F-127 (Sigma), in the dark. After being washed in HBSS the individual glass coverslips were promptly mounted into custom-made polypropylene holders which were placed horizontally into 10 × 10-mm quartz cuvettes filled with HBSS. The coverslip was illuminated at the angle of 40° and the emission was detected at 50°. The design of the coverslip holder allows continuous stirring with a small magnetic stirrer and addition of the toxin by a syringe during the experiment. Ratiometric measurements were performed using a FluoroMax-3 spectrofluorimeter (Jobin Yvon Horriba, France)

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