



## cAMP imaging of cells treated with pertussis toxin, cholera toxin, and anthrax edema toxin

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### ABSTRACT

The enzymatic activity of the three most studied bacterial toxins that increase the cytosolic cAMP level: pertussis toxin (PT), cholera toxin (CT), and anthrax edema toxin (ET), was imaged by fluorescence videomicroscopy. Three different cell lines were transfected with a fluorescence resonance energy transfer biosensor based on the PKA regulatory and catalytic subunits fused to CFP and YFP, respectively. Real-time imaging of cells expressing this cAMP biosensor provided time and space resolved pictures of the toxins action. The time course of the PT-induced cAMP increase suggests that its active subunit enters the cytosol more rapidly than that deduced by biochemical experiments. ET generated cAMP concentration gradients decreasing from the nucleus to the cell periphery. On the contrary, CT, which acts on the plasma membrane adenylate cyclase, did not. The potential of imaging methods in studying the mode of entry and the intracellular action of bacterial toxins is discussed.

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Many pathogenic bacteria produce toxins that elevate the intracellular cAMP concentration and this activity has a major impact on different cell types [1,2]. The finding that targeting cAMP-dependent processes allows toxins to modulate the immune response of the host can, at least partially, account for the wide distribution of this evolutionary strategy [3–5]. Further, cAMP-elevating toxins are indispensable tools in cell biology and cellular microbiology [6,7].

The most intensively studied cAMP-elevating toxins are: pertussis toxin (PT) from *Bordetella pertussis*, cholera toxin (CT) from *Vibrio cholerae*, and edema toxin (ET) from *Bacillus anthracis*. Although the common outcome of their activity is a rise of the intracellular cAMP concentration, their mechanisms of action are distinct. In fact, ET is an adenylate cyclase (AC) [8], whereas PT and CT ADP-ribosylate distinct intracellular G proteins [9]. PT ADP-ribosylates the inhibitory  $G_{i\alpha}$  protein of the plasma membrane AC and relieves its inhibition with ensuing increase of cyto-

solic cAMP [10]. However, PT also affects other G proteins which control different cell functions [3], including the dynamics of the actin cytoskeleton with changes in cell shape typically observed in PT-treated Chinese hamster ovary (CHO) cells [11,12]. Similarly, CT ADP-ribosylates the stimulatory  $G_{s\alpha}$  protein of the plasma membrane AC leading to its up-regulation [13]. The consequent rise of cAMP causes loss of water and ions through cAMP-gated ions channels in intoxicated intestinal epithelial cells.

PT, CT, and ET also differ in their modes of cell entry. CT is endocytosed and is retrogradely transported from the endosomal compartment to the Trans Golgi Network, to the Golgi and then to the endoplasmic reticulum, wherefrom it reaches the cytosol and diffuses to its targets [13]. ET is endocytosed after binding to either of its two cell surface receptors, tumor endothelial marker 8 (TEM8) or capillary morphogenesis protein 2 (CMG2) [14]. Available evidences indicate that the catalytic subunit of ET translocates into the cytosol from late endosomes and then remains attached to the endosomal membrane, where it produces cAMP [15–17]. Comparably less is known on how PT reaches the cytosol [18,19]. These distinct mechanisms of action and modes of cell entry are expected to result in different dynamics and intracellular distribution of cAMP elevation. Indeed, space and kinetics of the toxin-induced intracellular cAMP variations are the two crucial parameters of the intoxication process. On the one hand, mounting evidence indicates that signal compartmentalization is an essential mechanism to determine the specificity of signaling events [20,21].

**Abbreviations:** AC, adenylate cyclase; CMG2, capillary morphogenesis protein 2; CT, cholera toxin; Caco-2, human colonic adenocarcinoma cells; ET, anthrax edema toxin; FRET, fluorescence resonance energy transfer; HBE, human bronchial epithelial cells; PT, pertussis toxin; TEM8, tumor endothelial marker 8.

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Therefore, both for infection-oriented research and for the use of toxins as tools in cell biology, it is important to know the site of cAMP increase. On the other hand, the time taken by a toxin to reach the cytosol, corresponding here to the onset of cAMP rise, can provide information about the mode of cell entry [22]. Moreover, the kinetics of the enzymatic action of these three toxins can lead to different levels of cAMP with distinct alterations of cellular functions.

Here, we have used a recently established powerful method [23] to provide novel information on the action of cAMP-elevating toxins in different cell lines in culture. Fluorescence resonance energy transfer (FRET)-based single-cell imaging allows one to monitor the spatially- and temporally-resolved increase of intracellular cAMP levels [24]. The PT, CT, and ET action was measured in three different cell lines, to evaluate the role of the cellular environment in cAMP elevation and to obtain results of rather general validity. Human bronchial epithelial (HBE) cells were chosen because airways epithelial cells are targeted by cAMP-elevating toxins during respiratory tract infections with *B. pertussis*. Similarly, Jurkat T cells are a relevant model for anthrax toxins as well as for PT and CT [3]. Intestinal epithelial cells are directly affected by CT during infection with *V. cholerae* and by anthrax toxins in intestinal anthrax, and therefore we have used here human colonic adenocarcinoma cells (Caco-2).

## Materials and methods

**Reagents, plasmids, and proteins.** Cell culture media, fetal calf serum (FCS), L-glutamine, and antibiotics were purchased from Gibco. Fibronectin, poly-D-lysine, non-essential amino acids, forskolin, IBMX, Hepes and CT were from Sigma; bovine serum albumin (BSA) and FuGENE HD from Roche, collagen type I from BD Biosciences, and PT from List Biological Laboratories, Inc. (CA, USA). The plasmids pCDNA3-RIIRI-CFP and pCDNA3-C-YFP coding, respectively, for the regulatory subunit of PKA fused to CFP and for the catalytic subunit of PKA fused to YFP were previously described [25]. Protective antigen (PA) and edema factor (EF) were obtained as previously described [16,26].

**Cell culture.** HBE cells and CaCo-2 cells were maintained in minimum essential medium (MEM) with Earle's Salts supplemented with 15% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Further, 1% of non-essential amino acids was added to the medium for CaCo-2 cells. Jurkat JEG-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10 mM Hepes. CHO cells were kept in F12-K medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% non-essential amino acids. Flasks and glass coverslips used to grow HBE cells were treated for 2 h at 37 °C with a coating solution containing 20 µg/ml fibronectin, 100 µg/ml BSA, and 30 µg/ml collagen type I in MEM with Earle's Salts, which was removed by aspiration. Cells were maintained at 37 °C and 5% CO<sub>2</sub> in a humid environment.

**Cell transfection and FRET imaging of intracellular cAMP dynamics.** Live imaging of intracellular cAMP dynamics was performed as previously described. Briefly,  $1 \times 10^5$  HBE, and CaCo-2 cells were plated on glass coverslips and immediately transfected with 1 µg each of pCDNA3-RIIRI-CFP and pCDNA3-C-YFP in Opti-MEM, using 6 µl FuGENE HD. Jurkat cells ( $9 \times 10^5$ ) were prepared the evening before transfection and 20 µg each of pCDNA3-RIIRI-CFP and pCDNA3-C-YFP were introduced into cells kept in 400 µl of culture medium and electrically shocked at 250 V and 950 F in electroporation cuvettes with 0.4 cm gap (Bio-Rad) using a GenePulser Xcell electroporator (Bio-Rad). Cells were then plated at  $5 \times 10^5$  cells/ml

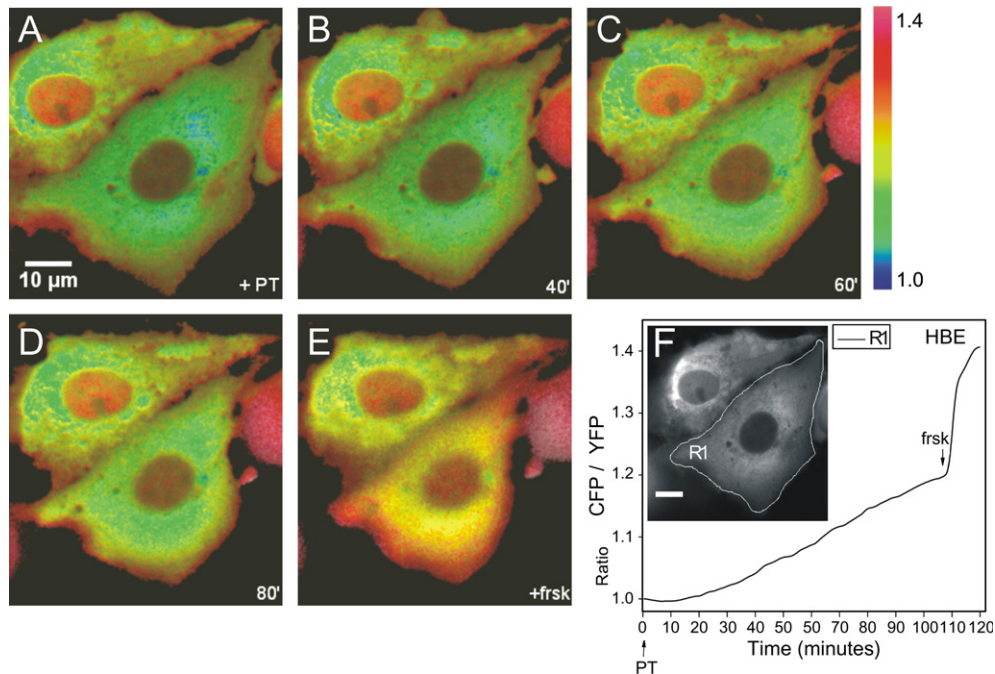
on poly-D-lysine-coated glass coverslips, and 24–48 h after transfection, were transferred to a microscope-adapted micro-incubator equipped with a temperature controller (HTC s.r.l., Italy) at 37 °C and constant 5% CO<sub>2</sub> pressure in 135 mM NaCl, 5 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 20 mM Hepes, 1.8 mM CaCl<sub>2</sub>, and 5.4 mM glucose, pH 7.4. The toxins (5 nM CT or 20 nM PT or 10 nM EF + 20 nM PA) were added, and, after 5–10 min of equilibration, images were acquired every 20 s. An oil immersion 60 × PlanApo 1.40 NA objective was used in a IX81 inverted microscope (Olympus), equipped with an MT20 illumination system with 150 W Xe arc burner (Olympus), a Dual-View beam splitter (Optical Insights, Germany) with a 505DCXR dichroic mirror and D480/30 and D535/40 band filters (Chroma Technologies Corp., USA), and an F-View II digital camera (Olympus). The acquisition software was cell^R (Olympus) and the integration time 100 ms or less. Images were processed with WCIF ImageJ v1.37 (<http://rsb.info.nih.gov/ij>). The intracellular cAMP level was determined as the ratio between the background-subtracted cyan emission (480 nm) and the background-subtracted yellow emission (540 nm) upon excitation at 430 nm (CFP/YFP ratio). Ratio images were created in pseudo-colors ranging from blue to red, which indicates increasing cAMP levels.

## Results

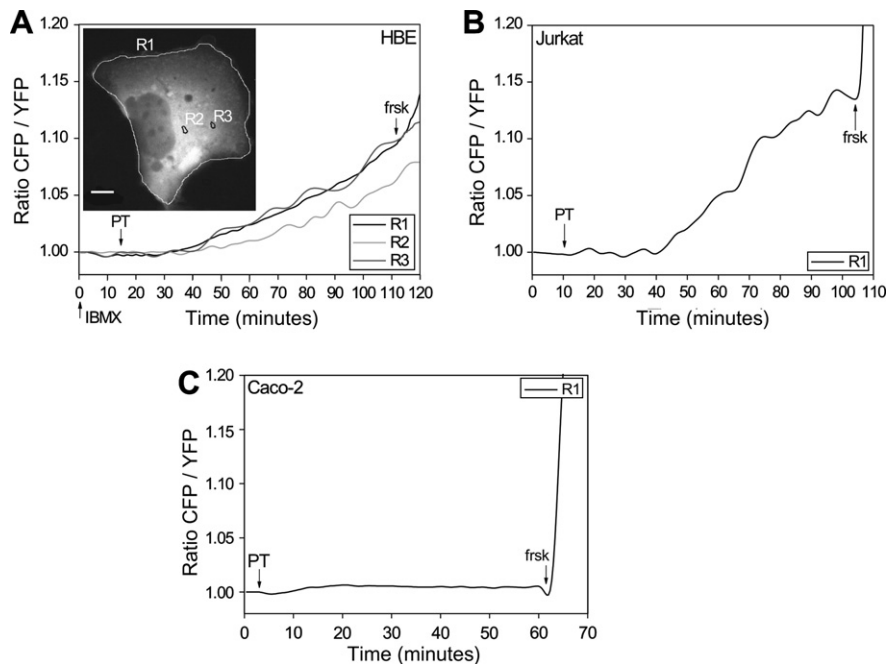
### Dynamics of intracellular cAMP rise induced by pertussis toxin

A fluorescent biosensor based on the cAMP-binding protein kinase A (PKA) which exploits the phenomenon of FRET was employed for space- and time-resolved live imaging of intoxicated cells. This probe was generated by fusing the regulatory RII-β subunit and the catalytic C-α subunit of PKA to the cyan (CFP) and the yellow (YFP) variants of the green fluorescent protein, respectively [23–25]. In quiescent cells, the R-CFP and C-YFP subunits are associated in a holotetrameric complex and FRET occurs among them, but cAMP induces their separation with loss of FRET. HBE, Caco2, and Jurkat T cells were transfected and expressed good levels of C-YFP and R-CFP. These fluorescent cells were treated with toxin doses defined in preliminary experiments not to saturate the fluorescent signal and this was verified at the end of each experiment by addition of 25 µM forskolin.

Fig. 1 shows the effect of PT on the intracellular cAMP level monitored by FRET in HBE cells, as a change of pseudo-colors in a scale going from blue (low cAMP) to red (high cAMP). The increase of cytosolic cAMP is evident after 1 h from toxin addition, though it appears to be rather low as compared with that obtained by addition of forskolin (Fig. 1E). The level of cAMP in different microdomains of the cells can be determined by selecting sub-areas of the cells where the ratiometric signal is monitored. This provides a quantitative, though relative, evaluation of cAMP which is particularly valuable when followed in time. Fig. 1F shows that about 30 min after PT addition the cytosolic cAMP level begins to rise. cAMP rises similarly in different sub-areas of the HBE cells (Fig. 2A); these profiles are representative of many cells ( $n = 10$ ) which have been transfected and imaged. Fig. 2B shows that PT induces in Jurkat T cells a sharper rise of cAMP which begins after a lag phase of 20–30 min ( $n = 7$  cells); in these cells the signal was recorded on the entire cell area owing to the small size of this lymphocytic cell. At variance, PT did not alter the basal level of cAMP in Caco2 cells, which are very reactive to forskoline (Fig. 2C). The purified B oligomer, lacking the enzymatic S1 subunit of PT, has not effect on the level of cAMP of HBE and Jurkat cells (not shown). Taken together, the imaging profiles obtained with Jurkat and HBE cells indicate that the time course of entry of the enzymatic



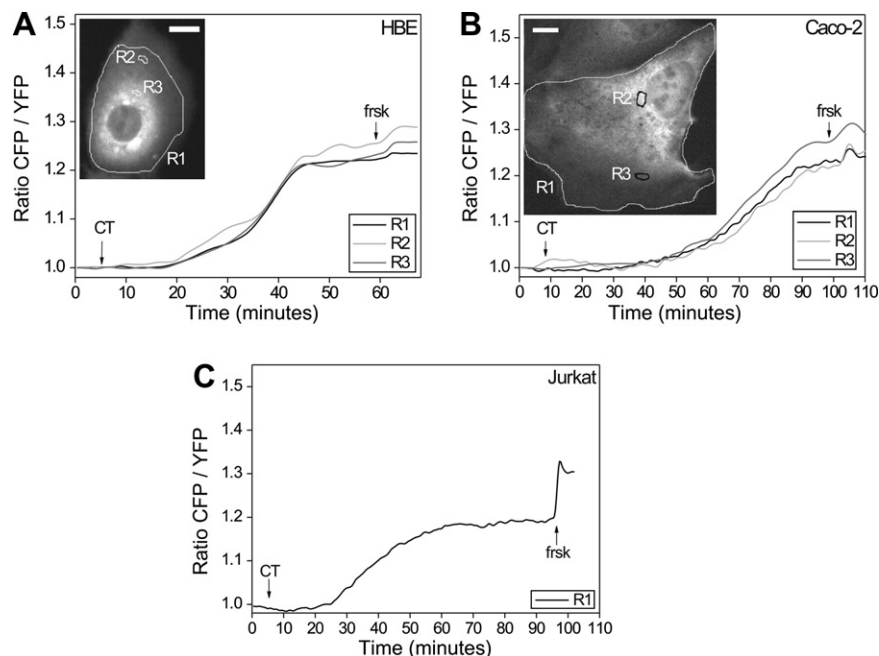
**Fig. 1.** Imaging of the rise of cAMP induced by pertussis toxin in HBE pulmonary cells transfected with PKA-based fluorescent probes. HBE cells expressing the catalytic PKA subunit-YFP and the regulatory PKA subunit-CFP in the cytosol were imaged after treatment with PT (20 nM). Cells were kept in a micro-incubator at 37 °C and constant 5% CO<sub>2</sub> pressure, under the microscope objective. (A–D) Two cells at the given time periods after toxin addition: the pseudo-colors reflect the increasing cAMP concentration from blue (low cAMP) to red (high cAMP). (E) Effect of the addition of forskolin. (F) Change of cAMP as ratioed in the encircled sub-area indicated in the inset, measured as CFP/YFP ratio. Bars: 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)



**Fig. 2.** Time courses of the rise of cAMP as imaged with PKA-based fluorescent probes in HBE, Jurkat cells and Caco-2 treated with pertussis toxin (PT). The three panels show traces of the increase of cytosolic cAMP as detected with the method outlined in Fig. 1 in the indicated cell lines treated with 20 nM PT. (B) Record obtained monitoring the entire cell area, given the reduced dimension of the cytosol of the Jurkat T cells, while three different sub-areas are monitored in the larger HBE cell. The increase in cAMP in the Jurkat T cells and in the pulmonary HBE cells is low, as compared to other toxins (see Figs. 3 and 4), but it is clearly evident after a lag phase of 30–40 min; this gives an *in vivo* indication of the time required for PT to traffic its S1 ADP-ribosyltransferase subunit into the cytosol. In contrast, Caco-2 cells do not rise their cAMP level following the action of PT, though the cells are fully responsive as one can see from the effect of the adenylate cyclase agonist forskolin (abbreviated frsk in A). Bars: 10 µm.

cally active S1 subunit of PT is more rapid than that reported recently [19]. This may be due to the fact that these estimates were based on biochemical modifications of PT subunits or of G proteins,

assayed with radioactive tracers. These methods measure the bulk of the phenomenon and can include modification of inactive targets, while the major advantage of the present imaging method



**Fig. 3.** Time courses of the rise of cAMP as imaged with PKA-based fluorescent probes in HBE, Caco-2 and Jurkat cells treated with cholera toxin (CT). The three cell lines were transfected and imaged as in Figs. 1 and 2, after the exposure to 5 nM CT. The three panels show that all the three cell lines are reactive to CT and that this toxin induces a consistent increase in cAMP cytosolic concentration which begins to rise earlier in Jurkat and HBE than in Caco-2 cells. Bars: 10  $\mu$ m.

is that it detects the actual functional signal as it is being generated.

#### Effect of cholera toxin on the dynamics of intracellular cAMP

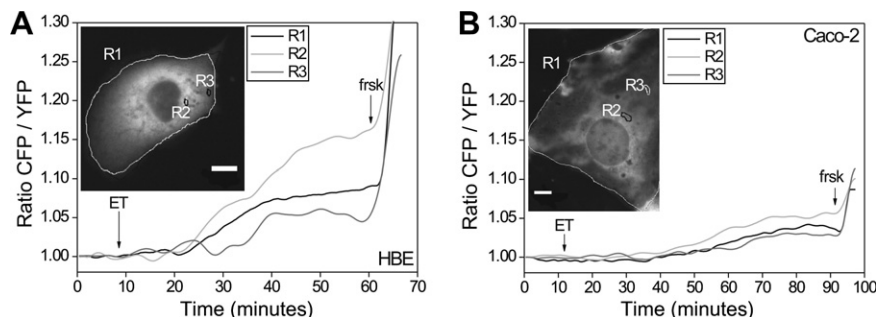
The first bacterial toxin identified to elevate cytosolic [cAMP] was CT [27]. *In vivo*, CT acts mainly within the intestinal polarized epithelial lining, but it has been used as a molecular tool in a variety of cells. Fig. 3 shows the increase of cytosolic cAMP in the different cells tested here. In all cases, CT induces a strong increase in [cAMP] which begins to rise after a variable lag phase and reaches levels which are only slightly increased by forskolin (except in Jurkat cells). Notwithstanding the fact that the CT-stimulated AC is located on the cytosolic face of the plasma membrane, there appears to be no relevant intracellular cAMP gradients, i.e. this second messenger is rather evenly distributed in the cell cytosol. The profile of the rise of cytosolic cAMP as a function of time differs in the three cell lines tested here. We have no explanation for this difference, but it is likely to be related to different time courses of the entry of the A1 ADP-ribosyltransferase subunit into cells. Though, it is well established that CT reaches the AC via the

retrograde transport pathway from the plasma membrane to the ER and the reverse translocon, the exact time course of the process has not been defined, and it may vary from cell to cell type [14,27,28]. The different profiles and time course obtained indicate that this trafficking pathways vary with cell type and this factor should be considered when using CT as a tool in the study of cell physiology.

#### Anthrax edema toxin induces intracellular concentration gradients of cAMP

At variance from CT and PT, the anthrax ET is itself an AC that reaches the cell cytosol and raises the cytosolic cAMP concentration. ET has been the first bacterial toxin whose intracellular activity has been imaged *in vivo* [16]. Here, we have extended the study to other cell lines to gather more general information on its mode of cell entry.

Fig. 4 shows that ET is poorly active in Caco2 cells, while in HBE cells it causes an increase in cAMP cytosolic concentration similar to the one recorded previously in HeLa cells [16]. Remarkably, also in HBE cells this toxin induces a cAMP concentration gradient



**Fig. 4.** cAMP imaging in HBE and Caco-2 cells transfected with PKA-based fluorescent probes and treated with the anthrax edema toxin (ET). Caco-2 cells are poorly reactive to the addition of protective antigen (PA, 20 nM) and of edema factor (EF, 10 nM). PA mediates the entry of EF which is an AC causing a modest rise of cAMP in Caco-2 cells, while it is active in HBE cells. Notice that this toxin causes the formation of a gradient of cAMP concentration, higher around the nucleus and lower toward the cell periphery. Bars, 10  $\mu$ m.

which decreases from the nucleus to the cell periphery which is consistent with the previously suggested localization of EF on late endosomes [16,17]. The low effect of ET on Caco2 cells may be due to the very low expression of both anthrax toxin receptors (TEM8 and CGM2) in these cells (see Fig. 1S). This finding documents that the present method is capable of detecting cAMP intracellular concentration gradients, and that the lack of evidence for such an occurrence in CT- and PT-treated cells is not artifactual.

## Discussion

The present study shows the potential of cAMP imaging in the study of cAMP-elevating toxins. The main finding presented here is that the ADP-ribosylating subunit of PT reaches its AC target and causes an increase of cAMP much faster than previously estimated by biochemical methods. The present study brings the value down from hours [19] to 30–40 min and compares well with the value obtained in the same cells with CT, whose pathway of retrograde entry via early endosomes-TGN-Golgi-ER-cytosol is well documented [13,22]. The present finding is therefore compatible with an S1 subunit of PT following the same pathway of entry in the cytosol as the one followed by the A1 subunit of CT.

A second relevant finding is that, notwithstanding the fact that the AC targeted by CT and PT is localized on the cytosolic face of the plasma membrane, there are no gradients of [cAMP] in the cytosol. This is at variance with what previously found for ET and the CyaA adenylate cyclase of *B. pertussis* [16]. Therefore, a given localization of a bacterial toxin within the host cell does not necessarily determine a gradient of effects.

A third point raised by the present work which is relevant to the very large use of CT and PT, and currently also ET, as tools in the study of cell physiology and function, is that it cannot be assumed *a priori* that these toxins necessarily lead to an increase in cytosolic cAMP concentration. In fact, PT and ET do not increase cAMP in Caco2 cells, where CT is very active.

A final consideration that derives from the present results is that different cells have different time courses and extents of cAMP response to the toxins and these parameters are of paramount importance when using these toxins to modulate cAMP to study its role in any given cell function or interaction. The rise of cAMP signals depends on the cellular machinery that controls this second messenger (ACs, phosphodiesterases and anchoring proteins) [20,21,24,28,29] and, therefore, it is almost impossible to make realistic assumptions *a priori* about the action of a cAMP-elevating toxin. cAMP imaging is a very appropriate way of testing the action of any bacterial toxin which induces, either directly or indirectly, a rise in cAMP. The method is applicable to almost any cell which can be transfected and observed without major changes of cell shape and focus under the fluorescence microscope.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2008.09.012](https://doi.org/10.1016/j.bbrc.2008.09.012).

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