Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase

REZA BEIGI,1 EIRY KOBATAKE,2 MASUO AIZAWA,2 AND GEORGE R. DUBYAK1

1Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4970; and 2Department of Bioengineering, Tokyo Institute of Technology, Midori-ku, Yokohama 226-8501, Japan

Beigi, Reza, Eiry Kobatake, Masuo Aizawa, and George R. Dubyak. Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am. J. Physiol. 276 (Cell Physiol. 45): C267–C278, 1999.—We have developed a method for measuring the local concentration of ATP at the extracellular surface of live cells. This method relies on the specific attachment to the cell surface of a chimeric protein that consists of the IgG-binding domain of Staphylococcus aureus protein A fused in-frame with the complete sequence for firefly luciferase (proA-luc). Expression of proA-luc in Escherichia coli and its one-step affinity purification are straightforward. Attachment to cells is demonstrated to be specific and antibody dependent using several suspended and adherent cell types. Light production by cell surface-attached luciferase is continuous, linearly related to ATP concentration, and sufficient to provide nanomolar sensitivity. The spatial resolution of this method enables the observation of strictly local changes in extracellular ATP during its secretion from activated platelets. Furthermore, the activity of cell-attached luciferase is relatively refractory to the inclusion of nucleotidases in the medium, arguing for its effectiveness in cell systems possessing potent ecto-ATPases.

purinergic signal transduction; cell surface microenvironment

The appearance of nucleotides, particularly ATP, in extracellular spaces is known to modulate many functions, including cardiovascular performance, neurotransmission, epithelial secretion, skeletal and smooth muscle contraction, endocrine/exocrine secretion, leukocyte adhesion, and immune, inflammatory, and thrombotic reactions (9). These effects are mediated by P2 nucleotide and P1 nucleoside receptors that are expressed in multiple subtypes on a wide array of cells and tissues (4, 9). It is recognized that ATP efflux from diverse cell types may be elicited by a variety of stimuli, including hypoxia, acidosis, mechanical deformation, hyposmotic shock, receptor stimulation, fluid shear stress, and membrane depolarization (9). Additionally, recent work has demonstrated mechanically induced release of UTP from human astrocytes (18).

There are three general mechanisms by which intracellular ATP can be released to extracellular spaces. The first involves direct, nonspecific cytolysis of healthy cells by physical and biological traumas. Given the presence of 3–5 mM ATP within the cytoplasm of all cells, any cell type can be considered a potential source of this nonselectively released ATP. A second mechanism involves the release of ATP compartmentalized within exocytosis-competent granules or vesicles. ATP is copackaged in the secretory granules of many neuronal, neuroendocrine, or endocrine cells and is rapidly released during synaptic neurotransmission and other types of regulated exocytosis. In some cases, nonlytic, stress-induced ATP release can be modulated by drugs known to alter the function of certain ATP-binding cassette type transport proteins (29). Thus a third potential route for ATP release is the efflux of cytosolic ATP via plasma membrane transport proteins.

Several factors complicate the straightforward analysis of physiologically relevant ATP release. The ubiquitous presence of ATP in all cell types makes it difficult to determine whether extracellular ATP is derived from particular, intact cell types via selective release mechanisms or whether this ATP is derived from the nonselective cytolysis of cells during experimental manipulations. In addition, most cells express ecto-nucleotidases that rapidly hydrolyze extracellular ATP present at the cell surface (26, 33). Released nucleotides may be spatially confined between cells or diluted by diffusion and dissipated by convection. For these reasons, the concentration of ATP in the proximity of cell surfaces is expected to be dynamic, and the measurement of ATP in bulk extracellular fluids may greatly underestimate the amount of ATP actually released at the cell surface. This may be particularly true in cells or tissues with substantial unstirred surface layers. It is therefore likely that ATP release in amounts that are sufficient to activate cells locally (via autocrine or paracrine mechanisms) cannot be adequately measured using bulk solution samples. Taken together, these factors confound the direct measurement of ATP efflux during most experimental manipulations and contribute to substantial disagreement between different groups (1, 15, 27).
Firefly luciferase is widely used as an extraordinarily sensitive bioluminescent ATP sensor protein (7, 19). Under suitable conditions, this assay system can detect ATP concentrations ranging from picomolar to micromolar levels. This method has been primarily used in offline experiments, wherein cell-free aliquots of extracellular medium are periodically sampled following stimulation of cells or tissue and then mixed with standard solutions of luciferase and d-luciferin. Luciferase-luciferin has also been used for online assays of ATP release from cell types such as platelets or adrenal chromaffin cells, which are readily studied as cell suspensions and which release large amounts of ATP by exocytosis of ATP-containing granules (8, 28). Unfortunately, such studies rely on the analysis of bulk solution samples that, as described above, do not accurately represent the dynamic ATP concentrations localized in the immediate vicinity of cell surfaces.

In principle, the sensitivity and utility of luciferase as a sensor of ATP release into localized or restricted extracellular spaces might be improved by selectively targeting the luciferase to the extracellular surface of a cell type being considered as a potential source of ATP. In this study, we report the use of a protein A-luciferase chimera (proA-luc) that can be stably adsorbed onto the surface of intact cells via interactions with primary IgG antibodies directed against native surface antigens. We show that local extracellular ATP levels can be continuously assayed with high sensitivity and excellent temporal and spatial resolution using either suspended cells or adherent cells that are coated with proA-luc. With this method, we are able to observe cell surface-localized transient ATP concentrations during secretion from activated platelets. This reagent and method should prove useful for the online detection of low-level ATP release from cells, such as epithelia, that utilize high localized nucleotide release for autocrine signaling or tissues, such as endothelia, that express very high levels of ecto-nucleotidase activity.

METHODS

Materials

Apyrase grade I and lysozyme were from Sigma. Luciferin was purchased from Molecular Probes. 1,4-Dithiothreitol (DTT), leupeptin, and aprotinin were obtained from Boehringer Mannheim. Luria-Bertani (LB) medium components were supplied by DIFCO Laboratories. Unless explicitly stated otherwise, all other reagents were from Sigma.

Transformation of Bacteria

pMALU5 plasmid DNA (120 ng) was incubated with 100 µl of competent JM109 Escherichia coli for transformation by heat shock. Transformants were selected on ampicillin plates, grown to saturation in LB medium (30) containing 50 µg/ml ampicillin, and stored as 15% glycerol stocks.

Growth of ProA-luc-Transformed Bacteria and Preparation of Bacterial Lysates

Transformed E. coli (500 µl) were added to 500 ml of LB medium in the presence of 50 µg/ml ampicillin and grown to mid-log phase at 37°C, at which time isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM. Cells were then grown to saturation by overnight incubation at 25–28°C with agitation. Cells were harvested by centrifugation (6,500 g) for 10 min at 4°C. The medium was poured off, and the cells were resuspended in STE buffer [containing (in mM) 150 NaCl, 10 Tris-HCl (pH 7.0), 5 EDTA, and 5 DTT] with 200 µg/ml lysozyme at a ratio of 8 ml buffer/g wet cells. The suspension was homogenized and allowed to incubate at room temperature for 30 min before the addition of protease inhibitors (final concentrations: 100 µM phenylmethylsulfonyl fluoride, 12 µg/ml leupeptin, and 10 µg/ml aprotinin). Cells were lysed by three cycles of sonication (VWR Scientific Branson Sonifier cell disrupter 185 using two or three 10-s bursts), quick freezing in ethanol-dry ice, and thawing at 37°C. After a 60-min, 4°C centrifugation at 20,000 g, the pellet was discarded and the supernatant was either quick frozen and stored at −80°C or processed immediately for purification of the expressed proA-luc.

Purification of Recombinant ProA-luc Protein

The soluble fraction of bacterial lysates was filtered through 0.2-µm Acrodiscs (Gelman Sciences). ProA-luc was purified at room temperature by affinity chromatography on immobilized bovine IgG-agarose beads (Sigma). Filtered samples (3 ml) were diluted 1:1 with Immunopure Gentile Ag/Ab binding buffer (Pierce) and incubated end-over-end with 1.4 ml of bovine IgG-agarose beads for 1 hr at room temperature. The suspended beads were packed into a 12-ml plastic column, allowed to settle, and then washed with 15 column volumes of binding buffer. ProA-luc was eluted with 8.5 ml of Immunopure Gentile Ag/Ab elution buffer (Pierce) and allowed to drop directly into 1 ml of 10× STE [containing 1.5 M NaCl, 1 M Tris-HCl (pH 8.5), and 50 mM EDTA] to rapidly neutralize the pH of the eluate. The eluate was applied to a HiTrap desalting column (Pharmacia) and was buffer exchanged into 1× STE [containing (in mM) 150 NaCl, 100 Tris-HCl (pH 7.8), and 5 EDTA]. Fractions from this column were tested for luciferase activity, and those containing the peaks of the activity were pooled and concentrated 25-fold using Ultrafree centrifugal filters (Biomax 10k, Millipore). After addition of DTT to 1 mM, the purified proA-luc was stored at 4°C.

Luciferase Activity Assays

The activity of proA-luc during purification was assayed by diluting samples in an optimized solution (FL-AAB, Sigma) containing MgSO4, DTT, EDTA, BSA, and tricine buffer salts. Luminometry was measured with a Berthold Lumat LB 9501 luminometer set to measure integrated light intensity in 5-s intervals. Assays were performed by mixing typically 5–20 µl of proA-luc fractions with 20 µl of 1.5 mM luciferin and FL-AAB buffer in 12 × 75-mm polystyrene tubes (Falcon 2008, Becton Dickinson) and then recording background luminometry. Twenty microliters of 10 µM ATP were added (1 µM ATP final), and the maximum light output during subsequent integration periods was recorded in arbitrary units. Final volumes were 200 µl. To measure specific luciferase activities, protein content was determined using the Bio-Rad protein assay and used to normalize light output by a given sample. Specific activities were expressed as relative light units (RLU) per mg protein per µM ATP.

For all experiments with intact cells, we designed a luminometer apparatus that permits continuous recording of light output. This instrument consists of a photomultiplier tube (Thorn EM1 9924B) operated at 800 V and surrounded by a light-tight machined stainless-steel housing and a sample holder that can accommodate either 12 × 75-mm tubes (for
suspended cells) or 35-mm tissue culture plates (for adherent cells). A shutter between the sample holder and the surface of the photomultiplier protects the tube from stray light during reagent addition. Output from the instrument was filtered at 10 Hz and recorded in analog fashion on chart paper. Light production measured with this apparatus was expressed as relative light output (RLO) to distinguish this arbitrary scale from that utilized by the Berthold luminometer (RLU).

SDS-PAGE and Western Blotting

SDS-PAGE (9% acrylamide) was performed under denaturing conditions in the presence of β-mercaptoethanol by standard methods (30). Proteins were either visualized directly by fixation and staining with Coomassie brilliant blue or electrophoretically transferred to polyvinylidene fluoride (Immobilon transfer membranes, Millipore) for Western blots at room temperature. In the latter case, membranes were blocked with 4% blotting grade nonfat dry milk (Bio-Rad) in 0.1% Tween 20, 0.9% NaCl, 1 mM EDTA, and 10 mM Tris (pH 7.4) and then probed directly with rabbit anti-bovine IgG-peroxidase (Sigma) at a dilution of 1:10,000 for 1 h. Chemiluminescent activity of the adsorbed peroxidase was assayed using SuperSignal chemiluminescent substrate (Pierce).

Cell Culture

HL-60 promyelocytes and Bac-1.2F5 murine macrophages were maintained as previously described (6, 24). HL-60 cells were used 36–48 h after passage by pelleting and resuspending to a density of 30 × 10⁶/ml in balanced salt solution (BSS) containing (in mM) 130 NaCl, 5 KCl, 25 HEPES (pH 7.5), 1.5 CaCl₂, 1 MgSO₄, 5 NaHCO₃, 1.5 K₂HPO₄, 10 dextrose, and 3.5 mg/ml BSA. Bac-1.2F5 macrophages were passed for 36–48 h before experiments and resuspended onto 35 × 10⁻⁶ sterile tissue culture dishes (Corning).

Preparation of Washed Human Platelets

All steps of the preparation were at room temperature. Blood was collected from healthy volunteers into acid-citrate-dextrose anticoagulant (100 mM trisodium citrate and 128 mM dextrose, pH 5) at a final ratio of 6:1. The collected blood was diluted 1:1 in 50-ml Falcon tubes with PBS containing (in mM) 9.1 Na₂HPO₄, 1.7 NaH₂PO₄, and 150 NaCl (pH 7.4); 25-ml aliquots were then layered onto 13 ml of Histopaque-plasma interface (Sigma) for pelleting mononuclear layer at the Histopaque-plasma interface. The Histopaque-plasma interface was collected using a sterile transfer pipette, diluted to 107 (Sigma) at a dilution of 1:10,000 for 1 h. Chemiluminescent activity of the adsorbed peroxidase was assayed using SuperSignal chemiluminescent substrate (Pierce).

Preparation of Washed Human Platelets

All steps of the preparation were at room temperature. Blood was collected from healthy volunteers into acid-citrate-dextrose anticoagulant (100 mM trisodium citrate and 128 mM dextrose, pH 5) at a final ratio of 6:1. The collected blood was diluted 1:1 in 50-ml Falcon tubes with PBS containing (in mM) 9.1 Na₂HPO₄, 1.7 NaH₂PO₄, and 150 NaCl (pH 7.4); 25-ml aliquots were then layered onto 13 ml of Histopaque-plasma interface (Sigma) for pelleting mononuclear layer at the Histopaque-plasma interface. The Histopaque-plasma interface was collected using a sterile transfer pipette, diluted to 107 (Sigma) at a dilution of 1:10,000 for 1 h. Chemiluminescent activity of the adsorbed peroxidase was assayed using SuperSignal chemiluminescent substrate (Pierce).

Preparation of Washed Human Platelets

All steps of the preparation were at room temperature. Blood was collected from healthy volunteers into acid-citrate-dextrose anticoagulant (100 mM trisodium citrate and 128 mM dextrose, pH 5) at a final ratio of 6:1. The collected blood was diluted 1:1 in 50-ml Falcon tubes with PBS containing (in mM) 9.1 Na₂HPO₄, 1.7 NaH₂PO₄, and 150 NaCl (pH 7.4); 25-ml aliquots were then layered onto 13 ml of Histopaque-plasma interface (Sigma) for pelleting mononuclear layer at the Histopaque-plasma interface. The Histopaque-plasma interface was collected using a sterile transfer pipette, diluted to 107 (Sigma) at a dilution of 1:10,000 for 1 h. Chemiluminescent activity of the adsorbed peroxidase was assayed using SuperSignal chemiluminescent substrate (Pierce).

Preparation of Washed Human Platelets

All steps of the preparation were at room temperature. Blood was collected from healthy volunteers into acid-citrate-dextrose anticoagulant (100 mM trisodium citrate and 128 mM dextrose, pH 5) at a final ratio of 6:1. The collected blood was diluted 1:1 in 50-ml Falcon tubes with PBS containing (in mM) 9.1 Na₂HPO₄, 1.7 NaH₂PO₄, and 150 NaCl (pH 7.4); 25-ml aliquots were then layered onto 13 ml of Histopaque-plasma interface (Sigma) for pelleting mononuclear layer at the Histopaque-plasma interface. The Histopaque-plasma interface was collected using a sterile transfer pipette, diluted to 107 (Sigma) at a dilution of 1:10,000 for 1 h. Chemiluminescent activity of the adsorbed peroxidase was assayed using SuperSignal chemiluminescent substrate (Pierce).
luminescence. The shutter was manually closed during addition of various reagents and then reopened after mixing (within 3–4 s) to record light output. In practice, we found that this small time delay did not introduce significant errors. Added reagents were mixed by agitation of the tubes (suspended cells) or dishes (adherent cells) before they were placed in the luminometer.

For some tests (see Fig. 8), we utilized a Chronolog 460-VS lumigaggrometer with continuous stirring at 37°C, which permitted the simultaneous measurement of activated platelet ATP release and aggregation.

Figure Preparation

Pen recordings of the photomultiplier tube output were traced onto white paper and scanned using a Scanjet 4C (Hewlett-Packard) and Adobe Photoshop 3.0. Figures were prepared using Adobe Illustrator 7.0 or Microsoft Excel 97.

RESULTS

Cell Attachment Strategy

In a cell-attached configuration, proA-luc is ideally situated to detect surface-localized ATP as it is released from cells and to compete for this ATP with ectoenzymes that catalyze its hydrolysis (Fig. 1A). Attachment is achieved by sequential incubations with primary antibodies against a particular surface epitope and then proA-luc. The protein A domain of proA-luc interacts strongly with the exposed Fc domains on individual antibody molecules, leading to its attachment. Cells are washed extensively afterwards to remove unbound luciferase.

Engineering of pMALU5

The previously constructed expression vector pMALU2 (16) lacked twelve residues corresponding to the amino-terminus of luciferase, which caused the luminescence behavior of the expressed protein to differ from that of wild-type luciferase. Therefore, a new plasmid was constructed (Fig. 1B) that contains the four IgG-binding domains of protein A (17, 23) fused in-frame with the complete coding sequence for firefly luciferase. The chimeric DNA is under the control of a tac promoter and is inducible by IPTG at room temperature (Fig. 2A). Ampicillin resistance is conferred by the inclusion of a gene for β-lactamase. The predicted molecular mass of proA-luc is 91 kDa.

Expression and Purification of ProA-luc

A significant fraction of recombinant proA-luc was found in the insoluble pellet from lysed cells (not shown). However, the relative inducible expression of proA-luc in the soluble phase was substantially increased at temperatures below 30°C (not shown; Ref. 21). Overproduction of the protein in J M 109 E. coli was confirmed by the presence of conspicuous bands in the region of its predicted molecular mass (Fig. 2A). Luciferase activity in the different lysates correlated with the relative intensities of the bands (not shown). Western blotting confirmed the presence of protein A activity (Fig. 2B), which also correlated with the relative band intensities.

Purification of proA-luc from E. coli lysates was achieved using affinity chromatography (Fig. 2) at room temperature. Eluted protein was desalted and thereby exchanged into STE buffer at pH 7.8. Luciferase activities were determined in arbitrary light units and normalized to the protein content; specific activities were thus expressed as relative light units (RLU) per mg protein per µM ATP (see Luciferase Activity Assays). Greater than 90% of the luciferase activity was retained by the beads as judged by the activity of the flow-through fraction (Table 1). A modest increase in specific activity was observed after purification of the
enzyme (Table 1). It is clear that a large loss in enzyme activity occurred during elution of the protein from the affinity column. Indeed, an estimate of the percent luciferase activity recovered relative to an equimolar amount of wild-type luciferase indicates that at least 75% of the purified molecules were inactive for light production (see DISCUSSION).

To assess the nature of the purified luciferase activity, wild-type luciferase (Sigma) was diluted to the point at which its light production in response to a given bolus of ATP was equal to that of an equal-volume sample of proA-luc. At all concentrations of ATP used, the two enzymes exhibited identical peak light production and decay characteristics (Fig. 3A), demonstrating the integrity of the luciferase domain when fused with protein A. Peak light output by the two enzymes was linear over the tested range of ATP concentrations (Fig. 3B), which extended into the subnanomolar and high-micromolar levels (not shown). The lower limit of reliable ATP measurement was dictated by the noise-to-signal ratio, which improved from 33% at 100 pM ATP to 8% at 320 pM to 3% at 1 nM and which continued to decrease with increasing ATP concentration.

Specificity and Antibody Dependence of Cell Attachment

Attachment of proA-luc to cell surfaces was studied using three different suspended and adherent cell types (Fig. 4). The choice of antibodies for cell surface attachment was made with several considerations in mind: 1) antibodies must be against extracellular epitopes, 2) epitopes should be in relatively high copy number per cell, and 3) antibodies should be of subtypes that possess relatively high affinity for protein A. Among mouse subtypes, binding strengths to protein A follow the order IgG2b > IgG2a > IgG1 (17). Therefore, we used either mouse IgG2a or IgG2b antibodies. Anti-HLA-ABC and anti-H-2K^d antibodies are directed against MHC class I antigens present on most nucleated human and murine cells, respectively, which facilitates proA-luc binding to a wide variety of cell types. Additionally, the HLA-ABC complex is expressed at high density in HL-60 cells (3). Anti-CD45.2 antibodies are directed against the leukocyte common antigen (Ly-5.2) present on all leukocytes of most mouse strains. The \( \alpha_{IIb} \beta_3 \) integrin (CD41) is unique to platelets and is present at roughly 40,000 copies/cell (22).

Table 1. Typical proA-luc purification characteristics

<table>
<thead>
<tr>
<th>Volume, ml</th>
<th>Filtered</th>
<th>Soluble</th>
<th>Flow-through</th>
<th>Purified proA-luc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.0</td>
<td>6.0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Protein concn, µg/ml</td>
<td>520</td>
<td>280</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Activity, RLU·µl⁻¹·µM ATP⁻¹</td>
<td>2.0 × 10⁶</td>
<td>1.3 × 10⁶</td>
<td>1.6 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>Specific activity, RLU·mg⁻¹·µM ATP⁻¹</td>
<td>3.8 × 10⁹</td>
<td>4.6 × 10⁸</td>
<td>8.0 × 10⁸</td>
<td></td>
</tr>
<tr>
<td>Relative total activity, % recovery</td>
<td>100</td>
<td>7</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Values are representative. RLU, relative light units. Relative luciferase activities of column fractions during a typical protein A-luciferase (proA-luc) purification. "Lysate" corresponds to lane c of Fig. 2A, after 1:1 dilution with Pierce binding buffer. "Flow-through" corresponds to fraction that flowed off column after end-over-end incubation for 1 h. "Purified" corresponds to eluted, desalted, and concentrated product. Activities and protein concentrations were determined as described in METHODS.
To measure cell-attached luciferase activity, cells were prepared as described in Cell-Attached Luciferase Assays and challenged with exogenous ATP in the presence of 150 µM luciferin. Only those cells preincubated with primary antibodies retained a significant amount of luciferase activity, demonstrating the specificity of cell attachment (Fig. 4A). The dependence of attached activity on primary antibody concentration was studied by incubating identical cell preparations with different concentrations of antibodies. In all cases, the amount of surface-attached luciferase increased monotonically with primary antibody concentration (Fig. 4, B–D); saturation of surface binding sites was observed with anti-HLA-ABC antibodies on platelets and HL-60 cells. The increased retention of proA-luc by anti-CD41-coated platelets vs. anti-HLA-ABC-coated platelets presumably reflects differences in the copy number of these respective surface antigens.

ATP Dependence of Cell-Attached Luciferase Activity

ProA-luc-coated cells were prepared and identical samples were tested for their response to different concentrations of exogenous ATP. Peak light production from coated HL-60 cells and from coated platelets was linear with respect to added ATP over the expected range of physiological concentrations from high-micromolar to mid-nanomolar levels (not shown). Light production was additionally proportional to the total proA-luc concentration, as demonstrated by using dilutions of coated cells (not shown). At high-micromolar concentrations of ATP, light output by proA-luc exhibited the well-characterized “flash” kinetics that are due to product inhibition (Fig. 3A, top). This behavior could be eliminated by performing assays in the presence of CoA (Fig. 5). With at least 100 µg/ml CoA in the sample, light output by coated platelets in response to exogenous ATP was sustained and responded linearly to ATP concentrations (not shown). Consistent with this observation is the idea that consumption of ATP by cell-attached luciferase is negligible on the time scale of these experiments.

Light Transients From Activated Platelets

Secretion from platelets can be elicited by a variety of stimuli, including thrombin, collagen, von Willebrand factor, ADP, vasopressin, platelet-activating factor, and Ca²⁺ ionophores (31). ATP is copackaged in platelet dense granules with serotonin, Ca²⁺, and ADP (31) and is therefore secreted when the cells are activated. We examined light production by soluble vs. cell-attached luciferase during platelet ATP secretion elicited by thrombin (Fig. 6A). The experiment with cell-attached luciferase produced a light transient that was uniquely sensitive to the local concentration of ATP at platelet cell surfaces during the secretion process (Fig. 6A, top trace), whereas an equivalent amount of soluble luciferase (25 ng/ml) was unable to yield this information (Fig. 6A, middle trace). Even at a much higher concentration (100 µg/ml), soluble luciferase was unable to detect the ATP transient near platelet cell surfaces (Fig. 6A, bottom trace).
in steady state, presumably after released ATP had diffused evenly throughout the sample volume, equivalent amounts of soluble or cell-attached luciferases produced equal amounts of light (Fig. 6A, top and middle traces). To control for washout of unbound proA-luc, the experiment was repeated using platelets that had been preincubated in the absence of primary antibody (Fig. 6A, bottom trace). Platelet secretion was also studied using platelets coated with primary antibodies against the HLA-ABC complex, in the absence or presence of CoA. As shown in Fig. 6B, the essential features of the light transients in these proA-luc-coated platelets were similar. This shows that local product inhibition of adsorbed proA-luc was not the cause of the light transients observed in thrombin-activated platelets (see DISCUSSION).

Many of the cell types known to participate in purinergic signaling possess potent ecto-ATPases that rapidly degrade extracellular ATP (26, 33). To study how extracellular ATPases might modulate the luciferase signals, platelet degranulation was triggered in the presence of a high concentration of soluble apyrase (10 U/ml). The magnitude of the light signal produced by cell-attached proA-luc was only moderately attenuated by apyrase addition (Fig. 7A). In contrast, the light signal produced by soluble luciferase was nearly eliminated in the presence of apyrase (Fig. 7B). Whereas one might conclude from the soluble luciferase experiment that ecto-ATPase activity can completely scavenge the secreted ATP before it could initiate signal transduction, the cell-attached observations demonstrate the existence of a significant local ATP concentration at the platelet surface, consistent with the potential for autocrine/paracrine stimulation of P2 receptors.

To assess the effects of stirring and temperature on the observed light and to measure another index of normal platelet function, we measured ATP secretion and aggregation simultaneously using a Chronolog lumiaggregometer. As shown in Fig. 8A, the essential characteristics of the light transients produced by surface-attached luciferase were qualitatively unaltered by stirring or an increase in temperature to 37°C. Furthermore, proA-luc-coated platelets remained competent for aggregation despite ligation of a fraction of their CD41 molecules (Fig. 8B). A subsequent addition of 10 µM ATP for calibration of the light signal indicated that the peak ATP concentration at the surface of stirred, coated platelets was in the 15–20 µM range.

Fig. 4. Cell-attached luciferase activity is specific and antibody (Ab) dependent. All results are representative of at least 3 independent experiments. A: light traces from washed human platelets treated with proA-luc (see METHODS) in presence (top trace) and absence (bottom trace) of preincubation with anti-human leukocyte antigen (anti-HLA)-ABC Ab at 2.5 µg/ml. Platelets (5 × 10^7) were suspended in balanced salt solution and incubated with 150 µM luciferin for at least 10 s before assay in 12 × 75-mm plastic tubes at room temperature. Light reactions were initiated by addition of 5 µM ATP. B: specific attachment to washed human platelets. Peak light output from platelets was tested as in A after platelets were preincubated in different concentrations of either anti-HLA-ABC or anti-CD41 Ab before treatment with saturating amounts of proA-luc (see METHODS). ATP was added to a final concentration of 10 µM. C: Ab dependence of proA-luc specific attachment to HL-60 promyelocytes. Cells (1 × 10^6) were coated with proA-luc as described in METHODS, using different concentrations of anti-HLA-ABC Ab during preincubation step. Light reactions were initiated by addition of ATP to 10 µM. D: Ab dependence of proA-luc specific binding to adherent Bac-1.2F5 cells. Cells were treated as described in METHODS and were challenged with 10 µM ATP in presence of 150 µM luciferin. Monoclonal Ab were directed against either CD45.2 or H-2Kd major histocompatibility complex class I antigen (PharMingen), as indicated.
DISCUSSION

The relatively recent classification of nucleotide receptor subtypes with respect to function, pharmacology, and primary structure (13) has been crucial to an understanding of nucleotide-based signaling. The study of ATP-based signaling has also been facilitated by the extremely sensitive and specific assay for released extracellular ATP in the form of the luciferase-catalyzed reaction. However, by the nature of the assay, it is restricted to the determination of ATP in the bulk solution in which cells are bathed. This is generally unsatisfactory because many factors (e.g., ecto-ATPases, unstirred layers) may cause the concentration of ATP in the bulk medium to be different from that in the local microenvironment of cell surfaces. It is therefore important to be able to measure extracellular ATP at the site of receptor stimulation.

Our observations of ATP secretion from platelets (Figs. 6, 7A, and 8A) demonstrate that cell-attached luciferase monitors the local rise of ATP as it is deposited in the extracellular space and the decay in this concentration as ATP diffuses into the bulk solution. Significantly, even much higher (4,000×) amounts of soluble luciferase in the medium were incapable of recording this type of “ATP transient” (Fig. 6A, inset). Although the signal from soluble luciferase was acutely sensitive to the presence of soluble apyrase, that from cell-attached luciferase was relatively refractory to such treatment (Fig. 7). The latter result is consistent with that of Aflalo (2), who monitored local ATP at the surface of respiring mitochondria using recombinant luciferase targeted to the cytoplasmic face of the mitochondrial outer membrane.

Surface-localized ATP may be transiently sequestered from the bulk solution by unstirred layer effects, even during rapid stirring of the suspended platelets (Fig. 8A). Alternatively, the shape change that accompanies platelet activation (31) leads to the invagination and sequestration of membrane areas from the bulk solution. This could explain the protection of surface-localized ATP from exogenous apyrase (Fig. 7), as well as the inability of soluble luciferase to detect the surface-localized transient (Fig. 6A). However, the equality of steady-state light production by soluble and cell-attached luciferases (Fig. 6A) does not support this interpretation. Furthermore, our data using coated platelets in the presence of exogenous apyrase (Fig. 7A) argue against the complete sequestration of extracellular ATP, given the decay of the cell-attached luciferase signal to baseline levels (presumably due to consumption of the released ATP by apyrase). Escolar et al. (10) demonstrated that CD41 moves from the periphery toward platelet centers during thrombin stimulation; however, complete sequestration of a fraction of the total CD41 did not become apparent until 3–5 min after activation.

Tethering of proA-luc to platelet surfaces greatly reduces the effective volume in which it is confined, ensuring that it will be responsive only to ATP that is in the same microregion. In contrast, soluble luciferase is freely diffusible throughout the entire assay volume. Under such conditions, the enzyme is unable to discriminate between surface-localized and bulk ATP. This feature is independent of the concentration of soluble luciferase, consistent with the inability of concentrated luciferase to detect the surface-localized transient (Fig. 6A, inset).

This method provides information about platelet secretion that has previously been unavailable. Under
the conditions of these experiments (Figs. 6, 7A, 8A), we were able to observe a transient peak ATP concentration on the order of 15–20 µM, which is sufficient to initiate autocrine stimulation of platelet P2X₁ receptors (5). This estimate assumes that all platelets degranulate with identical time courses. Numerical deconvolution of the light output from proA-luc-coated platelets should yield a time-dependent kinetic profile for the average platelet secretory response under various conditions, which should permit the testing of this assumption. The local concentration of secreted ADP is not directly measurable by this technique. However, the ATP-to-ADP ratio in human platelet dense granules is approximately two (32). If it is assumed that dense granule ATP and ADP undergo secretion with the same kinetics, our data suggest that the surface concentration of ADP following thrombin stimulation will transiently reach 7–10 µM. This is sufficient for maximal activation of the platelet P2Y₁ receptor (20) and consistent with feed-forward autocrine/paracrine activation of platelet responses (31).

Firefly luciferase catalyzes the production of 562-nm light with high quantum yield when incubated with its substrates, D-luciferin and MgATP, in the presence of molecular oxygen (7, 19). Light output by the enzyme in response to ATP concentrations >10⁻⁶ M shows flash kinetics that include a rapid rise (~300 ms) to a peak followed by a rapid decay (~0.5 s), presumably due to product inhibition (19). A variety of compounds are known to stimulate turnover by the enzyme, including CoA (11), which effectively removes the product inhibition and results in constant light output (even for ATP concentrations >10⁻⁴ M). We repeated platelet secretion studies using cell-attached luciferase in the presence of 250 µg/ml CoA to ensure that the observed light transient was not due to product inhibition of proA-luc (Fig. 6B). Peak heights observed during platelet secretion were reduced slightly in the presence of CoA (Fig. 6C).
Protein A is a component of the cell wall of Staphylococcus aureus that is bound to peptidoglycan at its carboxy terminus and is distinguished by the presence of four tandem IgG-binding domains in its amino-terminal portion (17, 23). It has been widely used as an immunoaffinity reagent (14) and as a fusion partner to facilitate the purification of recombinant proteins (23). Recently developed applications include the construction of novel bioluminescent immunoassay reagents (12, 16, 25). IgG-subtype specificity of protein A binding played an important role in the purification of proA-luc. In general, antibody binding strength by protein A is species specific and follows the order human > rabbit > guinea pig > mouse > cow > goat, rat, chicken, and hamster (30). Although we first attempted to purify proA-luc using rabbit IgG-agarose, we found that the buffers required to elute proA-luc from this resin caused significant loss of luciferase activity, due to irreversible denaturation. The intermediate affinity of protein A for bovine IgG-agarose was sufficient for depletion of proA-luc from bacterial lysates while facilitating its elution by relatively mild buffers. Under these conditions, we typically recovered 20% of the original luciferase activity (Table 1). However, affinity purification of proA-luc based on interactions with the protein A domain does present a few disadvantages. Proteolytic fragments of proA-luc copurify due to their possession of an active protein A domain (Fig. 2, A and B, left), which may be due to somewhat slower activation of luciferase under these conditions (Fig. 5A, right).

Fig. 7. Signal from surface-attached luciferase is resistant to soluble apyrase. Calibrations were performed as described for Fig. 6. A: 5 × 10⁷ coated platelets (prepared as in Fig. 6A) stimulated to secrete by addition of thrombin to 2.5 U/ml in absence (top trace) or presence (bottom trace) of 10 U/ml soluble apyrase. B: uncoated platelets in cosuspension with 10 µg/ml soluble wild-type luciferase stimulated by thrombin in absence (top trace) or presence (bottom trace) of 10 U/ml apyrase. These results are representative of at least 3 independent experiments using soluble luciferase at concentrations up to and including 100 µg/ml.

Fig. 8. Platelet secretion and aggregation measured simultaneously. Platelets were prepared as described for Fig. 6. Measurements were performed using a Chronolog lumiaaggregate meter with continuous stirring at 37°C. Final platelet densities were 3 × 10⁸ platelets/ml with 150 µM luciferin and 167 µg/ml CoA in total volumes of 300 µl; 1 µl of thrombin at 1,000 U/ml was added to initiate platelet activation. A: secretion from coated platelets (left) was calibrated by subsequent addition of 10 µM ATP. Uncoated platelets (right) were studied in presence of 100 ng/ml wild-type luciferase (Molecular Probes). B: aggregation of corresponding samples from A, measured as decreases in sample absorbance.
B). These species may compete for cell surface binding sites, occupying them nonproductively. Additionally, it is likely that a significant portion of purified proA-luc consists of active protein A moieties coupled to luciferase that is denatured during elution from the affinity column.

A potential complication of this method is that antibody binding to some cell surface epitopes can cause activation of the cells. In this preliminary study, we have not investigated the functional consequences of antibody attachment. However, consistent secretion profiles from activated platelets were obtained using either anti-HLA-ABC or anti-CD41 antibodies (Fig. 6). For cell types in which it may be difficult to select an appropriate membrane marker on which to tether proA-luc, transfection of cDNAs engineered to express intermembrane proteins that lack signaling function may provide a practical alternative.

It should be relatively straightforward to adapt this method to many different cell types, both suspended and adherent. Nanomolar changes in extracellular ATP can be observed (not shown) with excellent spatial and temporal resolution (Figs. 6–8). It should also be possible to study local ATP release in mixed populations of cell types by using antibodies against cell-specific surface antigens. Another potential application is to adapt the method for use with cooled charge-coupled device (CCD) cameras for observation of ATP secretion at the single-cell level. The feasibility of this application is supported by the findings of Oker-Blom and colleagues (25), who recorded cooled CCD bioluminescence images of protein A-luciferase attached to individual IgG-Sepharose beads.

We are indebted to Erin Clifford, Ulrich Hopfer, Ben Humphreys, Masao Ikeda-Saito, Emil Negescu, and Karen Parker for helpful discussions, to Richard Eckert for access to the Berthold lumimagrometer, and to Joan Fox for access to the Chronolog lumiaggregometer. Discussions, to Richard Eckert for access to the Berthold luminometer we used for continuous light recording. Sylvia Kertesy provided excellent technical assistance.

This work was supported in part by National Institutes of Health (NIH) Grant GM-36387 (to G. R. Dubyak), by NIH Cystic Fibrosis Feasibility Grant DK-27651 (to G. R. Dubyak), and by NIH Training Grant HL-07415 (to R. Beigi).

Portions of this work appeared in poster and abstract form at the 1998 Biophysical Society Meeting (Kansas City, MO).

Received 12 June 1998; accepted in final form 17 August 1998.

REFERENCES


