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Recombinant aequorin as a probe for cytosolic free Ca^{2+} in *Escherichia coli*

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We describe a novel and simple method for the measurement of bacterial cytosolic free calcium ($[Ca^{2+}]_i$) using recombinant aequorin reconstituted within live bacterial cells. Using this method we have measured the effects of external calcium, complement, phagocytosis and antibiotics on the $[Ca^{2+}]_i$ of *Escherichia coli*. In principle this method should be applicable to any genetically transformable organism and should suffer fewer problems than fluorescent dyes for subcellular calcium measurement.

Aequorin reconstitution; Coelenterazine; *E. coli*; Complement; Phagocytosis; Antibiotic; Cytoplasmic Ca^{2+}

1. INTRODUCTION

Measurement of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) has played a key role in establishing Ca^{2+} as the intracellular signal for many physiological and pathological phenomena in animal and plant cells [1]. However, we know little of its role in prokaryotes, reflecting the absence of a simple method for measuring $[Ca^{2+}]_i$ in living bacteria. One report [2] has described the use of the fluorescent dye Fura-2 for bacterial calcium measurement but considerable problems were encountered particularly with dye loading and with autofluorescence. Methods using fluorescent dyes can also experience problems with dye leakage, toxicity, $[Ca^{2+}]_i$ perturbation and dye compartmentalisation. Aequorin is recognised to be a more suitable probe for $[Ca^{2+}]_i$ measurement than fluorescent dyes. It is non-perturbing, non-toxic and leakage problems and compartmentalisation are not experienced [1]. This protoprotein emits measurable blue light when it binds Ca^{2+}. The energy for this light emission arises from a chemical reaction within the prosthetic group of the protein known as coelenterazine (a substituted dihydropyrazinimidazolone ring system) [1]. Despite the advantages of aequorin for calcium measurement, however, the requirement to microinject aequorin has constrained its use almost totally to large, single eukaryotic cells. We report here a simple solution to this problem. Recombinant aequorin can be reconstituted in living bacteria upon addition of coelenterazine and can be used as a probe to report the effects of external Ca^{2+}, serum complement, phagocytosis and antibiotics on $[Ca^{2+}]_i$ of *E. coli*. In principle this method is applicable to all genetically transformable organisms.

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2. MATERIALS AND METHODS

E. coli pop2136 (*malT*, P_R, *cI857*, *malPQ* — a kind gift from O. Raibaud, Institut Pasteur) cells were transformed with the plasmid pAEQ1.3 using standard methods [3]. pAEQ1.3 contains the full coding sequence from an apoaequorin cDNA clone fused to the lambda P_L promotor [4]. Over-expression was achieved by growing these transformed cells at 30°C in Luria broth containing ampicillin to 100 μg ml^{-1} to an optical density of 0.3 at 600 nm, then at 42°C for 3 h. Expression of apoaequorin was verified by Western blot analysis (not shown) of total *E. coli* protein using a polyclonal antibody raised against native aequorin purified from *Aequorea victorea* [5]. Aequorin was reconstituted by diluting cultures 1 : 1 with 100 mM KCl, 1 mM $MgCl_2$, Tris-HCl, pH 7.5, (buffer A [2]). For standard experiments 0.25 mM coelenterazine in methanol (a kind gift from Professor F. McCapra, University of Sussex) was added to 2.5 μM (final concentration) and the cells incubated at room temperature in darkness. Loaded cells were washed and finally resuspended in an equal volume of buffer A containing 0.5 mM EGTA. Reconstitution of aequorin in vitro was performed in a buffer containing 0.5 M NaCl, 5 mM mercaptoethanol, 5 mM EDTA, 0.1% gelatin (w/v), 10 mM Tris-HCl, pH 7.4, 2.5 μM coelenterazine.

Chemiluminescence was used to estimate both the total reconstituted aequorin and to estimate $[Ca^{2+}]_i$ in *E. coli*. Reconstituted aequorin in solution was discharged by adding an equal volume of 50 mM $CaCl_2$, and in *E. coli* cells by adding an equal volume of 50 mM $CaCl_2$, 1% nonidet P40.

Chemiluminescence measurements used a digital chemiluminometer with an EMI photomultiplier 9757 AM at 1 kV with a discriminator as described previously [6]. The output was either recorded graphically or by an 8-digit scaler.

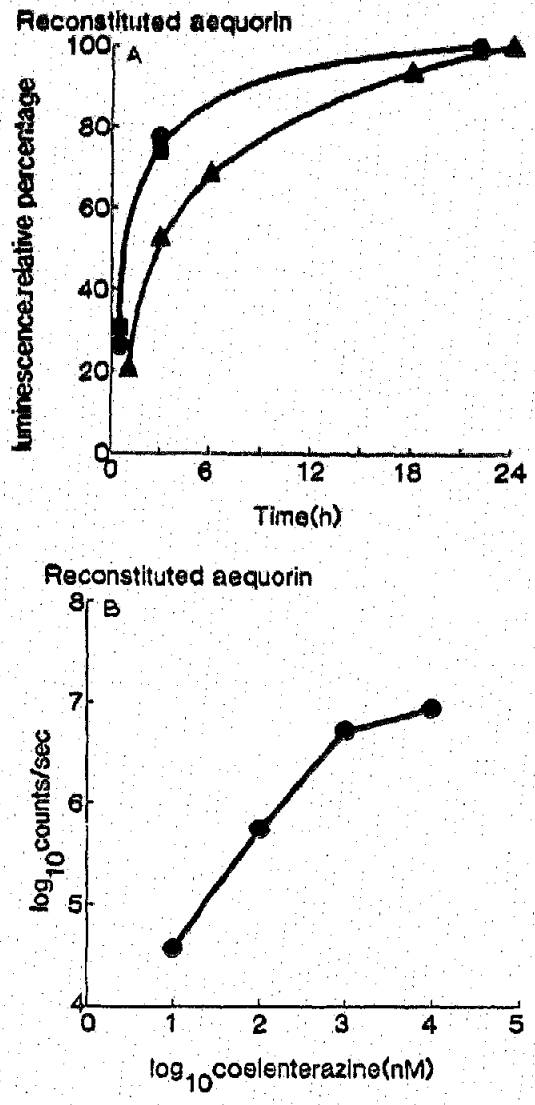

Fig. 1. (A) Kinetics of aequorin reconstitution. Plot of total amount of reconstituted aequorin against time in vitro using apoaequorin extracted from *Aequorea* (●), the single apoaequorin isoform expressed from pAEQ1.3 (■), and in vivo *E. coli* cells (▲). Luminescence values are expressed as percentages relative to the total luminescence counts at the end of the timecourse for each assay. (B) Effect of coelenterazine concentration on aequorin reconstitution in *E. coli* cells. Log_{10} plot of total reconstituted luminescence against coelenterazine concentration.

3. RESULTS AND DISCUSSION

Figure 1A shows a time course for the in vitro and in vivo reconstitution of aequorin from apoaequorin and coelenterazine. Reconstitution used in vitro a mixture of apoaequorin isoforms isolated from *Aequorea* or the single recombinant isoform expressed by pAEQ1.3 and in vivo (in *E. coli* cells), using coelenterazine at 2.5 μM. All 3 reactions show similar kinetics of reconstitution, although the in vivo reconstitution might be slightly slower initially. Routinely thereafter an overnight incubation was used to load the cells. Figure 1B shows that

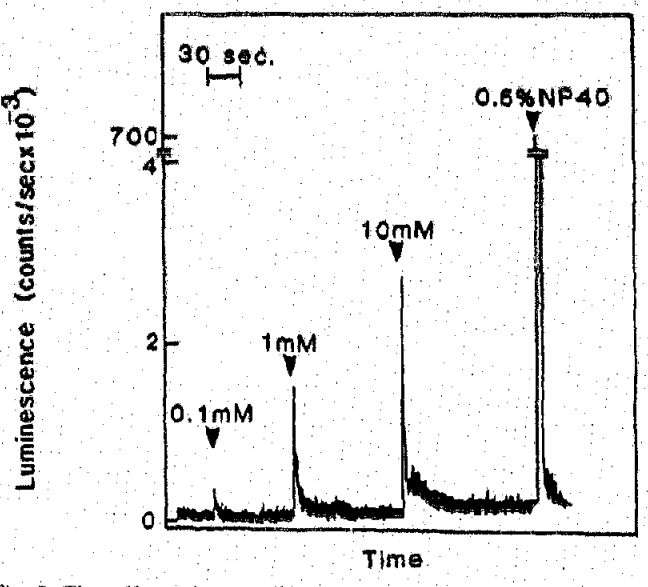

Fig. 2. The effect of extracellular calcium on *E. coli* cells. Chart recorder trace of changing luminescence from *E. coli* cells in response to 0.1 mM, 1 mM and 10 mM Ca^{2+} and 1% nonidet P40.

the reconstitution rate in *E. coli* is directly proportional to the concentration of coelenterazine up to 1 μM. Aequorin reconstituted in living *E. coli* is extremely stable with no significant loss after 8 days in a calcium-free buffer (data not shown).

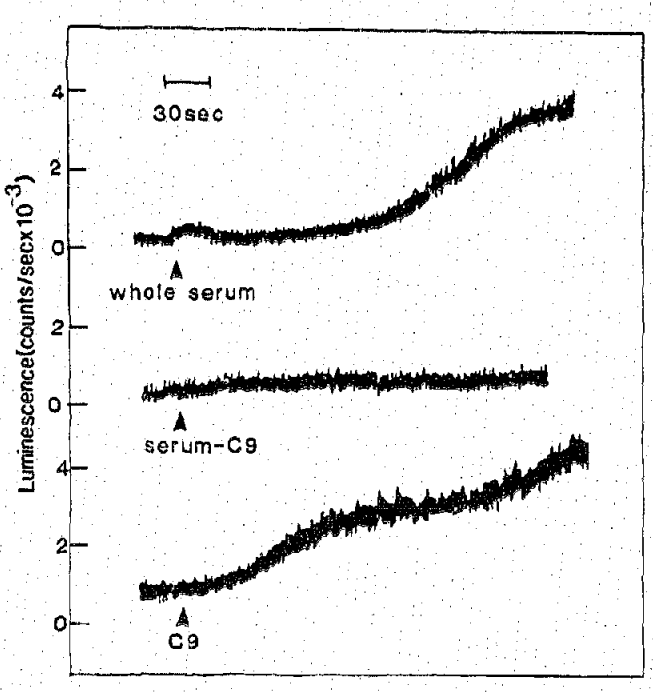

Fig. 3. The effect of complement on *E. coli* cells. Chart recorder trace of changing luminescence from *E. coli* cells in response to whole human serum, human serum deficient in C9 and human serum deficient in C9 to which purified C9 was added.

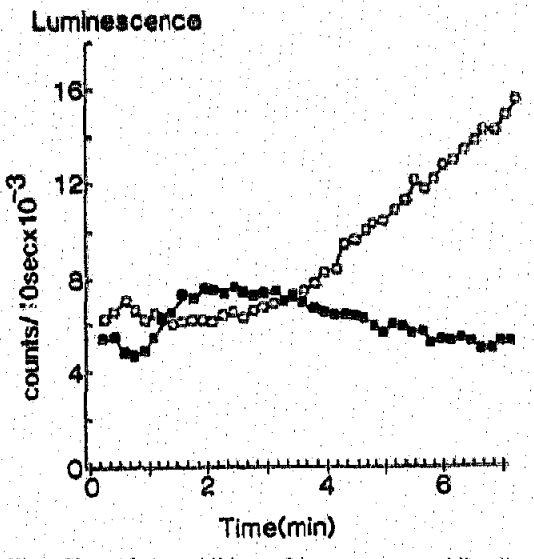

Fig. 4. The effect of the addition of human neutrophil cells on the luminescence of aequorin-containing *E. coli* cells. Luminescence counts per 10 s were measured over 7 min for *E. coli* cells incubated with (□) and without (■) human neutrophil cells.

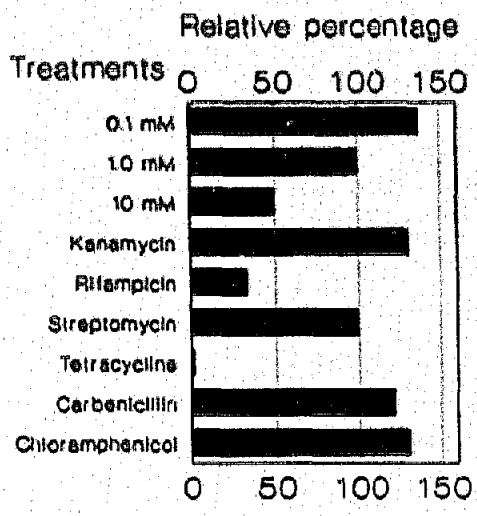

Fig. 5. The effect of antibiotics on calcium influx into *E. coli* cells. Graph shows the amount of reconstituted aequorin remaining (as a percentage relative to control cells in 1 mM Ca^{2+}; 100% = 3.8×10^6 total counts) after overnight incubation of *E. coli* cells in 0.1 mM, 1 mM and 10 mM Ca^{2+} and the antibiotics kanamycin (100 $\mu g\ ml^{-1}$), rifampicin (250 $\mu g\ ml^{-1}$), streptomycin (100 $\mu g\ ml^{-1}$), tetracycline (12.5 $\mu g\ ml^{-1}$), carbenicillin (500 $\mu g\ ml^{-1}$) and chloramphenicol (150 $\mu g\ ml^{-1}$) in 1 mM Ca^{2+}.

Figure 2 shows the response of reconstituted aequorin in *E. coli* to increasing external Ca^{2+} concentrations. The final consumption of aequorin was never greater than 8% of total in this experiment. Definite transients in luminescence were observed but low intracellular resting values were rapidly re-established. *E. coli* can therefore regulate its $[Ca^{2+}]_i$ in an apparently calcium-dependent manner. The potential for transducing signals through a change in the membrane calcium gradient is present in *E. coli*. The presence of a bacterial calcium-binding protein [7] and a prokaryotic Ca^{2+}-dependent adenylate cyclase [8] reinforce this possibility.

It has been proposed that the bacteriocidal constituent of serum, complement, acts by reversible damaging eukaryotic membrane permeability [1, 9]. However the mechanism by which it kills bacteria is unknown. Figure 3 demonstrates that reconstituted aequorin can be used as a probe to test this hypothesis. When serum was added to loaded *E. coli*, increased luminescence was observed within 3 min suggesting possibly an enhanced Ca^{2+} influx. When treated with serum from which C9 factor, the complement terminal component, had been removed by a monoclonal affinity column [10], no effect of the serum was observed until purified C9 was added back to the serum/cells incubation mixture. The final consumption of aequorin was never greater than 10% of total in this experiment. Overnight incubation in this C9-deficient serum with added C9 reduced total detectable aequorin to less than 10% of the control without added C9. These data suggest that human serum complement may cause an elevation of *E. coli* $[Ca^{2+}]_i$ similar to that seen in erythrocytes and also neutrophils [1, 9].

Figure 4 shows that reconstituted aequorin in *E. coli* can be used to probe the early events of phagocytosis. Loaded *E. coli* were mixed with active human neutrophils. Within a 4 min period a small but reproducible increase in luminescence from the loaded *E. coli* cells was observed indicating perhaps the onset of membrane permeabilisation, such as may be induced by oxygen metabolites.

The main mode of action of the antibiotics kanamycin, rifampicin, streptomycin, tetracycline, carbenicillin and chloramphenicol are all well documented. Nothing is known about the effect of these antibiotics on the integrity of bacterial cell membranes nor on their ability to provoke an influx of Ca^{2+} which is known to be cytotoxic at high concentrations [1]. Such a mechanism could be envisaged as contributing to the bacteriocidal or bacteriostatic activities of antibiotics. Transformed and loaded *E. coli* were incubated in appropriate concentrations of kanamycin, rifampicin, streptomycin, tetracycline, carbenicillin and chloramphenicol overnight and the total remaining aequorin then estimated. An overnight incubation was used as an adequately sensitive measure for the detection of small effects on Ca^{2+} flux. Figure 5 shows that of the 6 antibiotics, only rifampicin and tetracycline substantially accelerate the loss of aequorin. In addition to their well-documented effects on protein synthesis, therefore, rifampicin and tetracycline either specifically modify $[Ca^{2+}]_i$ or alter plasma membrane integrity. Colony counts showed no differences in viable cell number after the overnight incubation with the bacteriostats tetracycline, rifampicin and chloramphenicol (data not shown), thus eliminating cell death as a possible contribution to the loss of aequorin.

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