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Original Research Article

Detection of ATP Amount with Quantitative Biosensors

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Abstract

Lack of appropriate biosensors for precise local real-time measurement of extracellular ATP content in the pericellular space from individual cells under healthy and pathological settings has until now prevented a thorough understanding of the role of ATP as an extracellular signal. The relationships between neurons and glia as well as the transmission of purinergic neurotransmission to no muscular cells are discussed. Both the ontogeny and phylogeny of purinergic signaling are considered to demonstrate the ancient and pervasive nature of purinergic neurotransmission. We describe here how to immobilize firefly luciferase (Fluc) coupled with green fluorescent protein (GFP) on the plasma membrane of HEK 293 cells to create a straightforward, sensitive, and dependable dual-function biosensor for the local real-time measurement of extracellular ATP concentration in the pericellular space in livingcells.

Keywords: biosensor, ATP, purinergic, neurotransmission, signaling, real-time measurement.

Introduction

Adenosine 5'-triphosphate (ATP) was long thought of as the primary energy source in living cells, but we now know that it also serves a key physiological function as a pleiotropic extracellular messenger for cell-to-cell communication that acts at purinergic receptors on plasma membranes. Using complementing in vitro suspension-cultured cell and in planta systems, a combination of biochemical, proteomics, and bioinformatics techniques has been established to isolate, identify, and profile secreted proteins.

Extracellular ATP is often measured in cell supernatants using a soluble luciferin-luciferase assay or HPLC, which makes the process time-consuming and unreliable in most cases. We have created a chimeric luciferase that targets the plasma membrane and enables in vivo real-time imaging of extracellular ATP. As a result, of all extracellular signaling chemicals that are currently understood and that first appeared relatively early in evolution, ATP appears to be the most pervasive and ubiquitous [6].

Because of a wide variety of powerful ectonucleotides and other hydrolytic activities that break down ATP and produce ADP, AMP, and adenosine immediately after release, ATP has a half-life that is measured in seconds [7,8]. Once outside the cell, ATP uses a variety of receptors to connect to and activate in order to exert its many different effects.

We have immobilised firefly luciferase (Fluc) coupled with the green fluorescent protein (GFP on the cell plasma membrane via a glycosyl phosphate dylinositol, GPI, anchor derived from the human folate receptor 1 (FOLR1) protein in an effort to provide a straightforward and trustworthy dual-function biosensor for the local real-time measurement of extracellular ATP concentration in the pericellular space in live mammalian cells.

Material & Methods:

For the purpose of measuring ATP, the chamber is housed in a photomultiplier that is stored in a dark, frigid (4°C) box. An IBM-compatible computer equipped with a Thorn EMI photon counting board was used to detect light emission. The board let the data to be stored in the computer's memory for later study.

A Gilson peristaltic pump was utilized throughout the trials to continuously pump buffer into the thermostat compartment. To create the construct, the PCR product is transferred to the pBSK+ vector (Stratagene, La Jolla, CA), digested with the enzyme PstI, and inserted in the correct frame

between a PstI fragment encoding the GPI anchor protein (28 aa) and a PstI fragment encoding the human folate receptor (26 aa).

- **Isolation of genomic DNA, amplification and cloning**

The "primary" sequence of A, G, C, and T bases in the molecule in DNA can be directlyextracted from living, dead, and even extinct organisms. DNA may be freely read (sequenced), written (synthesized), and modified (mutated). It is crucial to distinguish

between two different types of DNA, genomic and complementary DNA (cDNA), which is created by reverse transcribed messenger RNA into DNA, before we examine how to filter, sequence, and synthesize DNA. The only information provided by c-DNA is the protein coding sequences, as mRNA lacks any intron-encoded nucleotides. In contrast, the enzyme reverse transcriptase catalyses the synthesis of complementary DNA (cDNA) from a single- stranded RNA (e.g., messenger RNA (mRNA) or microRNA) template. An enzyme called reverse transcriptase is present in retroviruses with RNA as their primary genetic material, including HIV. The TA cloning kit's reagents and transformation process were used to ligate all obtained pure DNA fragments into the pCR® II plasmid vector and then introduce them into the One Shot® TOP10 chemically competent E. coli strain for cloning (Invitrogen, Carlsbad, CA, USA).

- **Development of the cell plasma membrane-based immobilised firefly luciferase (pmeLUC)**

The pcDNA3.1 (+) vector, which had already been pre-digested with the XbaI and ApaI enzymes, was then inserted with the acquired PCR product in the correct frame. The final vector was designated as one. Light is formed when oxygen reacts with calcium, adenosine triphosphate (ATP), and the substance luciferin in the presence of the bioluminescent enzyme luciferase. The vector was pre-digested using BamHI and XhoI enzymes before the BamHI and XhoI enzymes were used to digest the vector. The resulting DNA fragment was then placed in the proper frame into that particular vector. This method does not adequatelyreflect the dynamic ATP flux at the cell membrane because it can only be used to infer the quantity of diffused ATP in bulk solutions. Hence, we created luciferase fusion proteins that could be fixed on the surface of the cell.

- **Cell transfection**

Studies of gene products and functions in eukaryotic cells are made possible by the powerful analytical tool known as transfection. The quantity, quality, and ratio of transfection reagentto DNA, as well as the origin, type, and passage of transfected cells, as well as the presence or absence of serum in the cell culture, all affect how applied 48 hours after transfection), we were also able to produce clones that could express pmeLUC in a stable manner. G418 sulphate at a concentration of 0.4 mg/ml was continuously present in stable pmeLUC-expressing clones.

- **Confocal fluorescence microscopy analysis and luciferase assay**

Since ATP is required for the firefly's Luciferin-Luciferase reaction, a highly sensitive ATP detection tool is based on this reaction. Commercially, such ATP detection kits are offered. Also extracted and capable of being expressed inside of cells is the luciferase gene. Followingthe aspiration of PBS, the cells were lysed with 20 l of the lysis solution for 5 min at room temperature before 100 l of the luciferase assay solution was added. Individual cells' responses to the same stimulation can vary in terms of gene expression, according to research using bioluminescence microscopy. The sequence of events from cellular signal transmission to gene expression, which is controlled by particular transcription factors originating from signaling cascades in distinct cells, must be observed in order to fully comprehend these phenomena. The POLARstar Omega luminometer was used to detect the bioluminescence that was created immediately (Polarstar Omega, BMG Labtech, Inc., NC 27513, USA). The total luminescence (Units) obtained for measuring luciferase activity at the emission wavelength of 560 nm was the mean of four determinations.

- **ATP measurement**

Both organic and microbial contamination is indicated by ATP. It is a straightforward way for determining the amount of light that, in direct proportion to the amount of ATP, is released when the enzyme luciferase encounters molecular ATP [19]. As it measures cleaning effectiveness, which may suggest a decreased risk of infection, ATP assessment maybe a viable method to regulate the quality of endoscope reprocessing. The ATP test is a process of rapidly measuring actively growing microorganisms through detection of adenosine triphosphate, or ATP.

The POLAR star Omega luminometer was used to detect the bioluminescence that was created immediately (Polar star Omega, BMG Labtech, Inc., NC 27513, USA). The total luminescence (Units) obtained for measuring luciferase activity at theemission wavelength of 560 nm was the mean of four determinations. The total luminescence (Units) obtained for measuring luciferase activity at the emission wavelength of 560 nm was the mean of

four determinations. The HEK 293 "ghost" was rinsed five times in succession with 200 l of PBS between the two ATP concentrations that were employed.

For the purpose of measuring ATP, 100 l of new luciferase reagent were added following the aspiration of PBS from the previous rinse. If not, unused HEK 293 "ghost" created in this manner can be kept at +40C in 1 ml of PBS.

Result:

Figure 1 depicts the schematic layout of the pmeLUC constructions utilized as biosensors to monitor the local, real-time concentration of ATP in the pericellular space in active HEK 293cells. HEK 293 cells that had not been transfected or had been transfected with the vector (9) or pmeLUC1, and pmeLuc2 underwent confocal fluorescence microscopy examination.

The pmeLUC1-expressing HEK 293 cells gave off no fluorescence (Figures 2A and 2B).

When compared to the GFP expression detected throughout transfected cells (Figure 2C), the presence of GFP expression exclusively on the surface of (clusters) (Figure 2) shows that theFluc is only expressed on the plasma membrane [10].

Hence, this would apply to cells that have been transfected with pmeLUC1, pmeLUC3, pmeLUC4, and pmeLUC5.

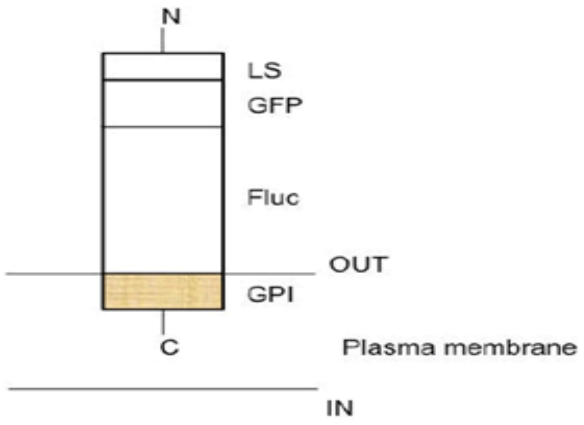

Figure 1: Schematic representation of the membrane topology of the pmeLUC constructs

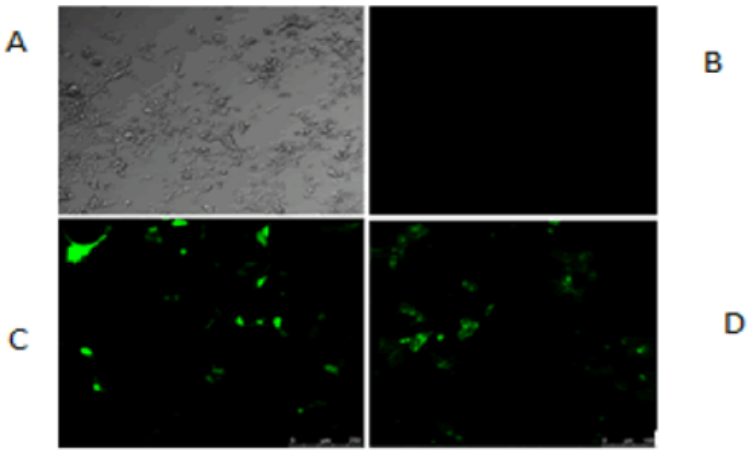

Figure 2: Confocal fluorescence microscopy analysis of HEK 293 cells

Discussion:

Many biological functions, including cell signaling and cell adhesion, depend on proteins with a GPI anchor [10]. This affects both health and sickness. Examples include the development of the scrapie form of the prion protein, the cause of Creutzfeldt-Jakob disease, and the fact that the absence of GPI on the membrane results in paroxysmal nocturnal haemoglobinuria, both of which are disorders in which GPI anchoring dysfunction isimplicated.

In conclusion, the pmeLUC probe has some benefits over currently used approaches because it was specifically targeted and expressed on the plasma membrane with the catalytic site towards the extracellular milieu: Due to this structure, the pmeLUC probe may monitor ATP increases brought on by momentary plasma membrane release, and genetic editing may make it possible to

measure ATP levels in vivo. t was therefore possible to inject our stable clones of HEK293-pmeLUC2 into living mice to quantify ATP in vivo and could as a result detect extracellular ATP levels in healthy tissues (5 M) as well as high extracellular ATP concentration at tumour sites. Our pmeLUC2 probe (without LS of FOLR1 and with GFP instead of myc tag) was structurally identical to that obtained by Pellegatti et al.

Conclusion:

For the local, real-time measurement of extracellular ATP concentration in the pericellular space in living mammalian cells, our pmeLUC2 dual-function reporter construct appears to be a suitable, sensitive, and reliable biosensor. The technique employed to build our pmeLUC2 probe may open the door for fresh approaches to logical pmeLUC design. Its application to living organisms and cells, particularly for discovering a new route for ATP secretion as a signal molecule, holds the potential to greatly increase its usefulness. To sum up, in the current study, the presence of the immobilised firefly luciferase (Fluc) on the cell plasma membrane of the pmeLUC2 transfected HEK293 cells was confirmed by (a) the presence of GFP expression, which was only visible on the surface of the pmeLUC2 transfected HEK293 cells as many dots (clusters).

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