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Novel, isotype-specific sensors for protein kinase A subunit interaction based on bioluminescence resonance energy transfer (BRET)

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Abstract

Homogeneous protein–protein interaction assays without the need of a separation step are an essential tool to unravel signal transduction events in live cells. We have established an isoform specific protein kinase A (PKA) subunit interaction assay based on bioluminescence resonance energy transfer (BRET). Tagging human R^{I}_{α}-, R^{II}_{α}-, as well as Cα-subunits of PKA with Renilla luciferase (Rluc) as the bioluminescent donor or with green fluorescent protein (GFP^2) as the energy acceptor, respectively, allows to directly probe PKA subunit interaction in living cells as well as in total cell extracts in order to study side by side PKA type I versus type II holoenzyme dynamics.

Several novel, genetically encoded cAMP sensors and–for the first time PKA type I sensors–were generated. When C- and R-subunits are assembled to the respective holoenzyme complexes inside the cell, BRET occurs with a signal up to three times above the background. An increase of endogenous cAMP levels as well as treatment with the cAMP analog 8-Br-cAMP is reflected by a dose-dependent BRET signal reduction in cells expressing wild type proteins. In contrast to type II, the dissociation of the PKA type I holoenzyme complex was never complete in cells with maximally elevated cAMP levels. Both sensors dissociated completely upon treatment with 8-Br-cAMP after cell lysis, consistent with in vitro activation assays using holoenzymes assembled from purified PKA subunits. Interestingly, incubation of cells with the PKA antagonist Rp-8-Br-cAMPS leads to a significant BRET signal increase in cells expressing PKA type I or type II isoforms, indicating a stabilization of the holoenzyme complexes in vivo. Mutant RI subunits with reduced (hRIα-R210K) or abolished (hRIα-G200E/G324E) cAMP binding capability were studied to quantify maximal signal to noise ratios for the RI-BRET sensor.

Utilizing BRET we demonstrate that PKA type II holoenzyme was rendered insensitive to β-adrenergic receptor stimulation with isoproterenol when anchoring to the plasma membrane of COS-7 cells was disrupted by either using Ht31 peptide or by depletion of membrane cholesterol.

Keywords: cAMP-dependent protein kinase; PKA; Bioluminescence resonance energy transfer; BRET; AKAP

1. Introduction

PKA, the main effector of the second messenger cAMP inside the eukaryotic cell, is composed of a regulatory (R) subunit dimer and two catalytic (C) subunits, forming the inactive holoenzyme complex (R_2C_2). The holoenzyme complex dissociates upon the cooperative binding of four molecules cAMP to two distinct binding sites on each R subunit (sites A and B), leading to the release of the catalytically active C subunits. The C subunits are then able to phosphorylate substrate proteins in the cytoplasm and in the nucleus [1–3].

In mammalian tissues two major PKA isoforms are present, type I and type II, distinguishable by their biochemical properties, expression pattern and the subcellular location of

Abbreviations: AKAP, A-kinase anchoring protein; 8-Br-cAMP, 8-bromoadenosine-3′, 5′-cyclic monophosphate; 8-Br-cAMP-AM, 8-bromoadenosine-3′, 5′-cyclic monophosphate-acetoxymethylester; BRET, bioluminescence resonance energy transfer; FCS, fetal calf serum; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; mCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; PKA, protein kinase A; Rluc, Renilla luciferase; Rp-8-Br-cAMPS, Rp-8-bromoadenosine-3′,5′-cyclic monophosphothiorate; Sp-8-Br-cAMPS, Sp-8-bromoadenosine-3′, 5′-cyclic monophosphorothioate.

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the four distinct regulatory subunit gene products (R^I_α, R^I_β, R^{II}_α, R^{II}_β) [4]. Deregulation of PKA activity has been demonstrated to contribute to a variety of diseases like diabetes, cancer and cardiovascular disease [5]. In HIV-infected T-cells, the cAMP/PKA type I pathway has been shown to be hyper-activated and in turn contributes to T-cell immune dysfunction [6–8]. Therefore, it is of high interest to selectively probe the activation of PKA isoforms in living cells, provided an experimental setup that allows for investigation and screening of potent modulators of PKA action.

Advances in fluorescence and bioluminescence technologies allow to monitor protein–protein interactions or cyclic nucleotide production in living cells contributing to the development of biological sensors for various signalling molecules [9,10]. Early PKA sensor systems, originally developed by Adams et al., were dependent on fluorescent labelling and subsequent microinjection of purified proteins into the cell [11]. In contrast, genetically encoded sensors based on variants of the green fluorescent protein (GFP) [12,13] offer easier handling and better spatial resolution. Several fluorescence resonance energy transfer (FRET)-based sensors for protein kinase activity and cAMP action have emerged and have been utilized successfully to monitor changes in intracellular cAMP concentration $[cAMP]_i$ or PKA type II activity [14,15]. However, PKA type I subunit dynamics could not be investigated in vivo so far, as the construction of a genetically encoded FRET-based reporter has not been successful. PKA type II subunit interaction was investigated using a reporter based on R subunit IIβ from rat tagged with YFP and murine Cα tagged with EGFP in live neonatal cardiac myocytes demonstrating spatial and temporal control of cAMP concentrations. Phosphodiesterase (PDE) activity restricts free diffusion of cAMP within the cell leading to the confinement of cAMP-action to discrete microdomains [15]. Besides the control of local cAMP concentrations, anchoring of PKA via the R-Subunits by A-Kinase Anchoring Proteins (AKAPs) is a widely utilized way to constrict PKA action within cellular compartments.

As an alternative to FRET-based sensors, bioluminescence resonance energy transfer (BRET) allows for the detection of direct interaction of fusion proteins without a fluorescent energy donor excited by an external light source. We established a BRET-based reporter system for human PKA type Iα as well as type IIα, by tagging human R^I_α-, R^{II}_α-, and Cα-subunits with Renilla luciferase (Rluc) as the bioluminescent donor or green fluorescent protein (GFP^2) as the energy acceptor. BRET occurs when luminescence energy, generated by Rluc catalyzed substrate oxidation, is transferred to GFP^2 in close proximity (1–10 nm). The BRET signal is determined by measuring the ratio of green (acceptor, 515 nm) over blue (donor, 410 nm) light [16,17].

Employing this technology, we are able to monitor subunit interaction for both, PKA type I and type II holoenzyme in intact cells as well as in total cell extracts, using a 96-well microplate format. We can quantify PKA subunit dynamics in response to PKA agonists and antagonists as well as to substances interfering with the binding of PKA to AKAPs, thereby affecting the local PKA activity in response to hormone stimulation in the intact cell.

2. Materials and methods

2.1. Reagents

Forskolin was obtained from Biomol, Hamburg, Germany; 3-isobutyl-1-methylxanthine (IBMX), isoproterenol (ISO), methyl-β-cyclodextrin (mCD) and cholesterol were from Sigma-Aldrich, Schnelldorf, Germany; 8-bromoadenosine-3′, 5′-cyclic monophosphate (8-Br-cAMP), 8-bromoadenosine-3′, 5′-cyclic monophosphate-acetoxymethylester (8-Br-cAMP-AM), Rp-8-bromoadenosine-3′, 5′-cyclic monophosphothiorate (Rp-8-Br-cAMPS) and Sp-8-bromoadenosine-3′, 5′-cyclic monophosphorothioate (Sp-8-Br-cAMPS) were from BioLog, Bremen, Germany.

2.2. Eucaryotic expression vectors

Plasmids carrying the full length human R^I_α, R^{II}_α and Cα1 genes were gifts of Prof. Dr. B.S. Skalhegg, Oslo University, Norway and Prof. Dr. S.S. Taylor, UC San Diego, USA, respectively.

Renilla luciferase (Rluc) fusion protein expression vectors (pRluc(h)-$N_{1\text{-}3}$ and -C_{1-3}), green fluorescent protein (GFP^2) vectors (pGFP^2-C_{1-3} and -N_{1-3}) were purchased from PerkinElmer, Rodgau, Germany. They are part of the BRET^2™ system (in the following BRET, [16]), an assay system optimized for protein expression in eucaryotic cells and for efficient resonance energy transfer. The human R^I_α and Cα1 coding sequences were amplified by using sense and antisense primers harbouring unique *Hin*dIII and *Bam*HI sites. For cloning into vectors where the fusion part was located C-terminally, the R- and C-subunits were amplified without stop codon. The fragments were subcloned in-frame into the *Hin*dIII/*Bam*HI sites of pRluc(h) and pGFP^2 vectors. The human R^{II}_α coding sequence was amplified without a stop codon using sense and antisense primers allowing for cloning with *Bam*HI and *Kpn*I. The fragments were subcloned using the pTrcHis2-Topo® TA cloning kit (Invitrogen, Karlsruhe, Germany), excised and cloned in-frame into *Bam*HI/*Kpn*I sites of pRluc-N and pGFP^2-N vectors. The mutations R210K and G200E/G324E were introduced in human R^I_α cDNA using the QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) with pRluc(h)-N_2-hR^I_α as the template. All plasmids were verified by sequencing.

2.3. Cell culture

COS-7 cells (ATCC CRL 1651) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), supplemented with 10% (v/v) fetal calf serum (FCS gold, PAA, Coelbe, Germany). HEK293T cells (ATCC CRL 11268) were grown in RPMI medium (PAA) supplemented with 10% (v/v) FCS gold (PAA). The cell lines were cultivated at 37 °C in a humidified atmosphere of 7.5% CO_2 in air. They were grown in 75-cm^2 tissue culture flasks to confluency (3–4 days), detached with Accutase™ (PAA), and counted using a coverslipped improved Neubauer chamber. Cells were seeded at a density of 2×10^6 cells per 75-cm^2 flask and used between passages 8 and 25 for all experiments.

2.4. BRET assays

COS-7 cells were seeded in a 96-well microplate (Optiplate, PerkinElmer, Rodgau, Germany) at a density of 2×10^4 cells per well. Transfections and co-transfections were carried out in the microplate 24 h later using 4 µl PolyFect (Qiagen, Hilden, Germany) per well and a total of 0.5 µg plasmid DNA per transfection, unless otherwise specified. 46–48 h post-transfection, cells were washed once with glucose-supplemented Dulbecco's PBS (D-PBS, Invitrogen), and the substrate DeepBlueC™ (PerkinElmer), a coelenterazine derivative, was added at a final concentration of 5 µM in a total volume of 50 µl D-PBS. The emission of light was detected immediately using a Fusion™ α-FP microplate reader (PerkinElmer). The light output was taken