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Nuclear β-adrenergic receptors modulate gene expression in adult rat heart

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ABSTRACT

Both β_1- and β_3-adrenergic receptors (β_1ARs and β_3ARs) are present on nuclear membranes in adult ventricular myocytes. These nuclear-localized receptors are functional with respect to ligand binding and effector activation. In isolated cardiac nuclei, the non-selective βAR agonist isoproterenol stimulated *de novo* RNA synthesis measured using assays of transcription initiation (Boivin et al., 2006 Cardiovasc Res. 71:69–78). In contrast, stimulation of endothelin receptors, another G protein-coupled receptor (GPCR) that localizes to the nuclear membrane, resulted in decreased RNA synthesis. To investigate the signalling pathway(s) involved in GPCR-mediated regulation of RNA synthesis, nuclei were isolated from intact adult rat hearts and treated with receptor agonists in the presence or absence of inhibitors of different mitogen-activated protein kinase (MAPK) and PI3K/PKB pathways. Components of p38, JNK, and ERK1/2 MAP kinase cascades as well as PKB were detected in nuclear preparations. Inhibition of PKB with triciribine, in the presence of isoproterenol, converted the activation of the βAR from stimulatory to inhibitory with regards to RNA synthesis, while ERK1/2, JNK and p38 inhibition reduced both basal and isoproterenol-stimulated activity. Analysis by qPCR indicated an increase in the expression of 18 S rRNA following isoproterenol treatment and a decrease in NFκB mRNA. Further qPCR experiments revealed that isoproterenol treatment also reduced the expression of several other genes involved in the activation of NFκB, while ERK1/2 and PKB inhibition substantially reversed this effect. Our results suggest that GPCRs on the nuclear membrane regulate nuclear functions such as gene expression and this process is modulated by activation/inhibition of downstream protein kinases within the nucleus.

1. Introduction

β-adrenergic receptors (βARs) are part of the GPCR superfamily that signal through heterotrimeric G proteins. GPCRs activate a wide range of downstream effectors and regulate diverse cellular functions in cardiomyocytes, including contractility, metabolism and gene expression. Additionally, the downstream signalling pathways activated following ligand binding can vary depending on the composition of heterotrimeric G proteins, and particularly which α subunits interact with the receptor.

In mammalian cardiomyocytes, all three known βAR subtypes have been detected. β_1ARs are the predominant subtype found in the heart, representing roughly 70% of the total βAR density [1]. Primarily involved in the regulation of cardiomyocyte contractility, β_1ARs are known to signal through $G\alpha_s$ and adenylyl cyclase (AC). β_2ARs, representing roughly 30% of the total βAR density, are also involved in the regulation of the contractility, but to a lesser extent [2]. β_1ARs and

Abbreviations: AC, adenylyl cyclase; ATP, adenosine triphosphate; βAR, β-adrenergic receptor; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DNA, deoxyribonucleic acid; DTT, dithiothreitol; ET-1, endothelin 1; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HRP, horseradish peroxide; ISO, isoproterenol; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PBS, phosphate buffered saline; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PMSF, phenylmethanesulphonylfluoride; PTX, pertussis toxin; qPCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid; RNAP, RNA polymerase; TCA, trichloroacetic acid; TX-100, Triton X-100; UTP, uridine 5′ triphosphate.

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β_2ARs signal through $G\alpha_s$ and AC with similar efficacy. However, the signals are compartmentalized differently in cardiomyocytes, possibly due to their localization in distinct membrane microdomains and/or dual coupling of the β_2AR to $G\alpha_s$ and $G\alpha_i$ [3]. β_2ARs also have a high level of spontaneous activity not detected for β_1AR [4]. During the development of heart failure, β_1ARs are internalized, their synthesis is reduced, and they begin to signal predominantly through a Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII)-dependent mechanism, while β_2ARs switch from $G\alpha_s$ to $G\alpha_i$ signalling, potentially activating cardioprotective mechanisms [2]. Additionally, expression and activity of GRK2 (βARK1), the primary GPCR kinase (GRK) in the heart, is increased during the development of heart failure, providing a molecular mechanism for βAR desensitization in heart failure [1]. While β_3ARs are also expressed in healthy cardiomyocytes, their functions remain ill-defined, and they represent a negligible part of the total βAR density [1]. In fact, as opposed to the β_1ARs and β_2ARs, β_3ARs appear to have negative inotropic effects and are actually up-regulated in response to heart failure [5]. Hence, βAR subtypes likely play non-redundant roles within the cardiomyocyte.

Recently, evidence has accrued showing that functional GPCRs are not solely localized at the plasma membrane but can also signal from different endogenous membrane compartments, including the nuclear membrane (reviewed in [6]). Recent evidence also seems to indicate that these intracellular receptors may have the capacity to regulate signalling pathways that differ from those of their plasma membrane counterparts, as was recently demonstrated for the metabotropic glutamate receptor 5 (mGluR5) [7]. In the case of the βARs, functional β_1AR and β_3AR, but not the β_2AR, have been detected at the level of the nuclear membrane in rat and mouse adult ventricular myocytes [8]. Moreover, several studies have demonstrated that a number of their normal cell surface interactors, including $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, adenylyl cyclase, and PKA, as well as other regulatory molecules known to interact with GPCRs, are also associated with the nucleus or the nuclear membrane [9,10]. In fact, literature even seems to support the existence of nuclear-localized phosphoinositide signalling pathways that can regulate nuclear PKB/Akt signalling [11]. Furthermore, βARs on the nuclear membrane have been shown to be functional with respect to both ligand binding and effector activation [8]. Indeed, in isolated nuclei, β_1ARs activate AC following treatment with isoproterenol, while β_3ARs appear to stimulate *de novo* transcription, through $G\alpha_i$ activation [8]. However, the post-receptor signalling pathways that become activated following ligand binding and lead to changes in gene transcription remain to be identified.

Given the presence of GPCRs at the level of the nuclear membrane, we wished to assess what role they might play in adult cardiomyocytes, and to determine what pathways might be activated downstream of nuclear receptor activation and lead to modulation of gene transcription. To this end we used a pharmacological approach to investigate the involvement of different MAPKs and the PI3K/PKB pathway.

2. Material and methods

2.1. Materials

Anti-PKB, anti-phospho-PKB (threonine 308 and serine 473), anti-phospho-ERK p44/42 (threonine 202/tyrosine 204), anti-MEK1/2 and anti-phospho-MEK1/2, anti-p38 and anti-JNK2 antibodies were from Cell Signaling Technology. Anti-Raf1 and Lamin B antibodies were from Santa Cruz Biotechnology. Anti-NFκB antibody was from eBioscience. Horseradish peroxide (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence (ECL) reagent Renaissance Plus was from Perkin Elmer Life Sciences (Woodbridge, Ontario). Triton X-100 (TX-100), leupeptin, PMSF and DNase I were from Roche Applied Science (Laval, Quebec). SDS-polyacrylamide gel electrophoresis reagents, Bradford Protein Assay reagent, and nitrocellulose (0.22 μm) were from Bio-Rad Laboratories (Mississauga, Ontario). Isoproterenol was from Tocris Bioscience (Ellisville, MO). Endothelin-1 (ET-1) was from Peninsula Laboratories (Torrance, CA). Pertussis toxin (PTX), microcystin LR, CGP 20712A, ICI118551 and α-amanitin were from Sigma (Mississauga, Ontario). PD98059, SB203580, SP600125, LY294002, wortmannin and triciribine were from Calbiochem. U0126 was from Upstate Cell Signaling Solutions. RNaseOut, dNTP Mix, First Strand buffer and M-MLVRT were from Invitrogen. Primers, as well as SyBR Green and ROX were also from Invitrogen. RNA extraction kits were from Qiagen. RT^2 First strand kits, SABiosciences RT^2 qPCR Master Mix and rat NFκB signalling pathway RT^2 Profiler PCR arrays were from SABiosciences. Unless otherwise stated, all reagents were of analytical grade and were purchased from VWR Canlab (Ville Mont-Royal, Quebec) or Fisher Scientific (Mississauga, Ontario). [α^{32}P]UTP (specific activity 3000 Ci/mmol) was from Perkin Elmer.

2.2. Isolation of nuclei

Rat cardiac nuclei were isolated as described previously [12]. Briefly, rat hearts were pulverized under liquid nitrogen, resuspended in cold PBS, and homogenized (Polytron, 8000 rpm; 2×10 s, Fraction 1). All subsequent steps were carried out on ice or at 5 °C. Homogenates were centrifuged for 15 min at 500 ×*g*. The resulting supernatants, referred to as Fraction 2, were diluted 1:1 with buffer A (10 mM K-HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated 10 min on ice, and centrifuged for 15 min at 2000 ×*g*. The resulting supernatant was discarded. The pellet, referred to as crude nuclei (Fraction 3) was resuspended in buffer B (0.3 M K-HEPES pH 7.9, 1.5 M KCl, 0.03 M $MgCl_2$, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4), incubated on ice for 20 min, and centrifuged for 15 min at 2000 ×*g*. The pellet, an enriched nuclear fraction (Fraction 4), was resuspended in buffer C (20 mM Na-HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 25 μg/ml leupeptin, 0.2 mM Na_3VO_4) or 1 x transcription buffer (50 mM Tris pH 7.9, 0.15 M KCl, 1 mM $MnCl_2$, 6 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 1 U/μl RNase inhibitor) and either used fresh or aliquoted, snap-frozen using liquid nitrogen, and stored at −80 °C.

2.3. Transcription initiation

Measurements of transcription initiation were performed as previously described [8]. Briefly, 10 μl of isolated nuclei (resuspended in 1 x transcription buffer) were incubated at 30 °C for 30 min in the presence of the indicated agonist/antagonist and 10 μCi [α^{32}P]UTP (3000 Ci/mmol). CTP and GTP were omitted to prevent chain elongation. Following termination of reactions by digestion with DNase I, nuclei were lysed with 10 mM Tris–HCl pH 8.0, 10 mM EDTA and 1% SDS. Duplicate 5 μl aliquots were transferred onto Whatman GF/C glass fiber filters, washed twice with 5% TCA containing 20 mM sodium pyrophosphate, and air-dried. ^{32}P incorporation was determined by liquid scintillation counting. DNA concentrations were determined spectrophotometrically and [^{32}P]UTP incorporation expressed as cpm/μg DNA. Where indicated, isolated nuclei were pre-treated with the appropriate pharmacological inhibitors. Nuclei were either pre-treated at room temperature with 10 μM PD98059 for 1 h, 20 μM U0126 for 30 min, 100 nM wortmannin for 30 min, 50 μM LY294002 for 30 min, 10 μM SB203580 for 1 h, 20 μM SP600125 for 1 h, 1 μM triciribine for 30 min, 3 μM α-amanitin (a potent inhibitor of RNA polymerase II and III) for 30 min, or vehicle (DMSO) for either 30 min or 1 h, according to which inhibitor was used.