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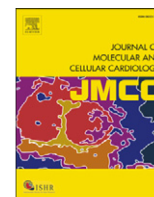

Original article

Caveolae compartmentalise β2-adrenoceptor signals by curtailing cAMP production and maintaining phosphatase activity in the sarcoplasmic reticulum of the adult ventricular myocyte

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ABSTRACT

Inotropy and lusitropy in the ventricular myocyte can be efficiently induced by activation of β1-, but not β2-, adrenoceptors (ARs). Compartmentation of β2-AR-derived cAMP-dependent signalling underlies this functional discrepancy. Here we investigate the mechanism by which caveolae (specialised sarcolemmal invaginations rich in cholesterol and caveolin-3) contribute to compartmentation in the adult rat ventricular myocyte. Selective activation of β2-ARs (with zinterol/CGP20712A) produced little contractile response in control cells but pronounced inotropic and lusitropic responses in cells treated with the cholesterol-depleting agent methyl-β-cyclodextrin (MBCD). This was not linked to modulation of L-type Ca^{2+} current, but instead to a discrete PKA-mediated phosphorylation of phospholamban at Ser^{16}. Application of a cell-permeable inhibitor of caveolin-3 scaffolding interactions mimicked the effect of MBCD on phosphorylated phospholamban (pPLB) during β2-AR stimulation, consistent with MBCD acting via caveolae. Biosensor experiments revealed β2-AR mobilisation of cAMP in PKA II signalling domains of intact cells only after MBCD treatment, providing a real-time demonstration of cAMP freed from caveolar constraint. Other proteins have roles in compartmentation, so the effects of phosphodiesterase (PDE), protein phosphatase (PP) and phosphoinositide-3-kinase (PI3K) inhibitors on pPLB and contraction were compared in control and MBCD treated cells. PP inhibition alone was conspicuous in showing robust de-compartmentation of β2-AR-derived signalling in control cells *and* a comparatively diminutive effect after cholesterol depletion. Collating all evidence, we promote the novel concept that caveolae limit β2-AR-cAMP signalling by providing a platform that not only attenuates production of cAMP but also prevents inhibitory modulation of PPs at the sarcoplasmic reticulum. This article is part of a Special Issue entitled "Local Signaling in Myocytes".

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1. Introduction

In the cardiac myocyte, many receptors signal via the second messenger cyclic AMP, but produce diverse changes in electrical, mechanical, metabolic and transcriptional activities. This occurs because different receptors produce changes in cAMP in different compartments of the cell. The idea of cAMP compartmentation is well accepted but the mechanisms responsible are not fully understood.

β1- and β2-adrenoceptors (AR), the predominant β-ARs in the heart, provide an excellent illustration of compartmentalised cAMP signalling in the adult ventricular myocyte. β1-AR stimulation promotes positive inotropic and lusitropic responses, whereas β2-AR stimulation has minimal functional effects [1,2]. These distinct β-AR functional responses can be correlated with temporal and spatial properties of cAMP generation [3], protein kinase A (PKA) activation [4] and target protein phosphorylation [5]. In essence, β1-AR signals are global, whereas β2-AR signals are localised to their site of production. For example, β1-AR stimulation promotes PKA-dependent phosphorylation of multiple targets throughout the cell — the L type Ca^{2+} channel [6], phospholamban (PLB) [7], RyR [8] and troponin I (TnI) [7]. By contrast, β2-AR signals do not access proteins of the sarcoplasmic reticulum (SR) or myofilaments [9,10].

Abbreviations: AKAP, A kinase anchoring protein; ARVM, adult rat ventricular myocyte; β-AR, beta adrenoceptor; C3SD, caveolin-3 scaffolding domain; cAMP, cyclic adenosine 3′-5′-monophosphate; Cav-3, caveolin-3; CGP, CGP20712A; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; Epac, exchange protein activated by cAMP; FRET, fluorescence resonance energy transfer; FSK, forskolin; HIV, human immunodeficiency virus; IBMX, 3-isobutyl-1-methylxanthine; $I_{Ca,L}$, L-type Ca^{2+} current; Iso, isoproterenol bitartrate; MBCD, methyl-β-cyclodextrin; PDE, phosphodiesterase; PKA, protein kinase A; PLB, phospholamban; pPLB, Ser^{16} phosphorylated PLB; PP, protein phosphatases; RyR, ryanodine receptor; TAT, trans-activating transcriptional activator; TnI, troponin I; ZNT, zinterol hydrochloride.

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We are interested in the mechanisms that restrict cAMP dependent signalling from the β2-AR.

Whilst the β1-AR couples to the stimulatory Gs protein, β2-ARs couple sequentially to Gs and the inhibitory Gi protein. The Gi pathway has been proposed as a key factor responsible for the spatial restriction of β2-AR-cAMP signals to the sarcolemmal compartment, perhaps through stimulation of protein phosphatase activity [11–13]. Another focus of interest is phosphodiesterase (PDE). Several studies have suggested that PDE 3 and 4 contribute to the creation of the distinct β2 AR cAMP signatures in the adult ventricular myocyte, because PDE3/4 inhibition increases the size and duration of the cAMP and activated PKA signals (e.g. [4,14]).

However, an additional, complementary, explanation for the diverse effects of β1- and β2-AR stimulation on cAMP is that the differential membrane distribution of signalling elements, including receptors, produces cAMP signals in different cellular compartments. The lipid bilayer is not a homogenous structure with randomly distributed proteins, but contains liquid-ordered phases enriched in cholesterol and sphingolipids known as lipid rafts. Caveolae are specialised forms of invaginated rafts characterised by the presence of caveolin and cavin proteins (see Refs. [15,16] for reviews). Caveolae have been shown to be important structures for organising G-protein coupled receptors and their downstream signalling components [17]. Within caveolae, oligomeric caveolin can assemble, and regulate, macromolecular signalling complexes [18] through its ability to bind many different proteins via its scaffolding domain [19].

In support of a role for caveolae in creating distinct β1- and β2-AR cAMP signals, differential membrane distribution of receptors has been reported in the ventricular myocyte. Whilst β1-ARs are found in caveolar and non-caveolar domains, the majority of β2-ARs are present exclusively in caveolae (in the absence of β AR stimulation) [20–23]. Other elements of the β AR signal cascade (Gαs, Gαi, adenylyl cyclase 5/6, PKA) have been shown to be present in caveolar membrane fractions [20–23], to co-immunprecipitate with caveolin 3 [22] and to be inhibited by interaction with the caveolin scaffolding domain [24–26]. The exclusive caveolar location of the β2-AR suggests a role for caveolae in its downstream signalling. Indeed we have recently shown functional evidence that caveolae compartmentalise the β2 AR response in the adult ventricular myocyte; disruption of caveolae reveals marked inotropic and lusitropic responses to β2-AR stimulation [27], associated with a globalisation of cAMP-dependent signalling indexed by phosphorylation of phospholamban [9].

The aim of the present study was to reconcile, for the first time, work showing the roles of phosphatases and phosphodiesterases in spatial control of β2-AR signalling with mechanism(s) by which caveolae compartmentalise cAMP in the adult ventricular cell. We report how disruption of caveolae impacts on β2-AR signalling at the level of cAMP, protein phosphorylation, $I_{Ca,L}$, $[Ca^{2+}]_i$ and contraction, and how this is affected by inhibition of key components linked with β2-AR compartmentation. Measuring these different indices gives insight into the mechanism of caveolar control (e.g. cAMP production/cAMP degradation/phosphatase activity) and the region of the cell (e.g. sarcolemma/SR/myofilaments) in which this control operates. We show that spatial restriction of the β2-AR cAMP signal by caveolae occurs at the level of cAMP production *and* phosphatase activity in the SR. This can be explained by the formation of specific signalling complexes in the caveolar microdomain.

2. Materials and methods

2.1. Cell isolation

Adult rat ventricular myocytes (ARVM) were enzymatically isolated from the hearts of male Wistar rats using a standard procedure outlined elsewhere [28]. Care was taken to follow the *Animals (Scientific Procedures) Act 1986*, the *Recommendation from the Declaration of Helsinki* and the *Guiding Principles in the Care and Use of Animals*. All experiments were carried out at room temperature unless otherwise stated and, except for those involving fluorescence resonance energy transfer (FRET), used ARVM on the day of isolation in an extracellular buffer containing (mM): NaCl 137, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1.0, NaH_2PO_4 0.33, glucose 5.5, HEPES 5 (pH 7.4).

2.2. Cell culture

For FRET imaging experiments only, ARVM were plated in minimum essential medium (MEM) containing insulin–transferrin–selenium complex (Invitrogen, CA, USA), bovine serum albumin (1 mg/ml), 2,3-butanedione monoxime (10 mM) and penicillin–streptomycin. After incubation for 2 h, the cells were transduced with adenoviral constructs containing protein kinase A (PKA)- or exchange protein activated by cAMP (Epac)-based cAMP biosensors, as described previously [23,29,30]. Imaging experiments were carried out 48–72 h after transduction.

2.3. β2-AR stimulation

Selective β2-AR stimulation was achieved with the β2 agonist zinterol (ZNT, 10 μM) in combination with CGP20712A (CGP, 300 nM), a specific β1-AR antagonist. For all protocols, cells were exposed to CGP for at least 5 min prior to addition of solution containing ZNT and CGP. Baseline recordings were made in the presence of CGP.

2.4. Methyl-β-cyclodextrin treatment

Freshly isolated ARVM were incubated for 1 h at 37 °C in Ca^{2+}-containing isolation solution (Ca-IS, [in mM]: NaCl 130, KCl 5.4, $MgCl_2$ 1.4, NaH_2PO_4 0.4, creatine 10, taurine 20, glucose 10, HEPES 5, (pH 7.4)) supplemented with 1–2 mM methyl-β-cyclodextrin (MBCD); cultured cells were incubated under the same conditions with MBCD dissolved in MEM-based culture medium. MBCD solution was replaced with fresh solution and treated cells used within 3 h. We have previously shown that incubation of myocytes with 1–2 mM MBCD at 37 °C for 1 h removes cholesterol and Cav-3 from buoyant caveolar membrane fractions [9] and markedly reduces caveolae number [31].

2.5. TAT-tagged peptide treatment

Peptides corresponding to the 11-residue trans-activating transcriptional activator sequence (TAT) from HIV-1, N-terminally linked via 4 glycine residues to the 20-residue caveolin scaffolding domain of Cav-3 (C3SD) or a scrambled version of this (Scram) were synthesised (Peptide 2.0, VA, USA). Complete peptide sequences were: TAT-C3SD, YGRKKRRQRRRGGGGDGVWRVSYTTFTVSKYWCYR; TAT-Scram, YGRKKRRQRRRGGGGYWTVYTKVDFCGSRYVRTSW. Lyophilised stock aliquots (0.5 mg) were reconstituted in water and diluted in Ca-IS. ARVM were incubated in 0.5 or 1 μM peptide-Ca-IS solution at 37 °C for 30 min. Peptide solutions ≥2 μM caused a marked decrease in the viability of myocyte preparations.

2.6. Cell shortening and $[Ca^{2+}]_i$ transient measurements

Unloaded shortening and $[Ca^{2+}]_i$ transients were recorded simultaneously using digital edge-detection software (IonOptix, MA, USA) and an OptoScan monochromator (Cairn Research, UK) in field-stimulated ARVM (0.5 Hz) loaded with 1.5 μM fura-2, following previously described protocols (Calaghan et al., 1998). An average of 10–12 traces under basal conditions, and at steady-state following drug application, were analysed using IonWizard 6.0 software (IonOptix).

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