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PDE4 and mAKAPβ are nodal organizers of β2-ARs nuclear PKA signaling in cardiac myocytes

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Running title: Nuclear PKA regulation by β-ARs in cardiomyocytes

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Abstract

Aims

$\beta_1$- and $\beta_2$-adrenergic receptors ($\beta$-ARs) produce different acute contractile effects on the heart partly because they impact on different cytosolic pools of cAMP-dependent protein kinase (PKA). They also exert different effects on gene expression but the underlying mechanisms remain unknown. The aim of this study was to understand the mechanisms by which $\beta_1$- and $\beta_2$-ARs regulate nuclear PKA activity in cardiomyocytes.

Methods and Results

We used cytoplasmic and nuclear targeted biosensors to examine cAMP signals and PKA activity in adult rat ventricular myocytes upon selective $\beta_1$- or $\beta_2$-ARs stimulation. Both $\beta_1$- and $\beta_2$-AR stimulation increased cAMP and activated PKA in the cytoplasm. While the two receptors also increased cAMP in the nucleus, only $\beta_1$-ARs increased nuclear PKA activity and up-regulated the PKA target gene and pro-apoptotic factor, inducible cAMP element repressor (ICER). Inhibition of PDE4, but not $G_i$, PDE3, GRK2 nor caveolae disruption disclosed nuclear PKA activation and ICER induction by $\beta_2$-ARs. Both nuclear and cytoplasmic PKI prevented nuclear PKA activation and ICER induction by $\beta_1$-ARs, indicating that PKA activation outside the nucleus is required for subsequent nuclear PKA activation and ICER mRNA expression. However, nuclear PKI decreased ICER up-regulation by $\beta_2$-AR stimulation (with concomitant PDE4 inhibition) by only 30%, indicating that other mechanisms are involved. Down-regulation of mAKAPβ partially inhibited nuclear PKA activation upon $\beta_1$-AR stimulation, and drastically decreased nuclear PKA activation upon $\beta_2$-AR stimulation in the presence of PDE4 inhibition.

Conclusions

$\beta_1$- and $\beta_2$-ARs differentially regulate nuclear PKA activity and ICER expression in cardiomyocytes. PDE4 insulates a mAKAPβ-targeted PKA pool at the nuclear envelope that prevents nuclear PKA activation upon $\beta_2$-AR stimulation.

Key words: adrenergic receptors - cAMP-dependent protein kinase - compartmentation - nucleus.
Introduction

Acute stimulation of β-adrenergic receptors (β-ARs) is essential for the adaptation of cardiac performance to physiological needs, but persistent activation exerts deleterious effects that ultimately result in heart failure (HF). The normal heart mainly expresses β₁- and β₂-AR subtypes, and although both couple to the Gₛ/cAMP/PKA cascade, their functional effects differ markedly. β₁- but not β₂-ARs elicit strong inotropic and lusitropic responses associated with concerted phosphorylation of excitation-contraction coupling (ECC) proteins¹, induce HF upon moderate expression²,³, and promote cardiomyocyte apoptosis.⁴ These diverse effects are explained by a functional compartmentation model which integrates alternative coupling of β₂-ARs to G₄,⁴,⁵ distinct location of receptors in specialized microdomains of the plasma membrane,⁶-¹⁰ differential coupling of β₁- and β₂-ARs to adenylyl cyclases (AC) type V and VI,¹¹ and differential regulation by cyclic-nucleotide phosphodiesterases (PDEs) type 3 and 4.¹² Additional differences between β₁- and β₂-ARs include receptor desensitization, with β₂-ARs being more rapidly internalized than β₁-ARs.¹³

Another critical organizer of β-AR signalling integration is constituted by A-kinase anchoring proteins (AKAPs). In cardiomyocytes, AKAPs target PKA to the plasma membrane, the sarcoplasmic reticulum and the myofilaments for local regulation of major ECC proteins.¹⁴ AKAPs also target PKA to other intracellular organelles such as the nucleus to regulate gene expression.¹⁴,¹⁵ In cardiomyocytes, the scaffold protein muscle AKAPβ (mAKAPβ) organizes a complete cAMP signalling module including PKA and PDE4D3 at the nuclear envelope. Because PDE4D3 is phosphorylated and activated by PKA¹⁶ this constitutes a negative feedback loop modulating local cAMP level and PKA activity.¹⁷ Several subsequent studies showed that mAKAPβ assemble a much larger signalosome which major function is to modulate pathological hypertrophy.¹⁸-²⁰ Upon β-AR stimulation and cAMP elevation, PKA activation also drives CREB-dependent transcriptional activation of inducible cAMP early repressor (ICER), a potent pro-apoptotic factor in cardiomyocytes.²¹,²² In addition, PKA regulates class II histone deacetylases (HDACs) 4 and 5 in the nucleus.²³-²⁵ However, thus far the mechanisms that control the dynamics of nuclear PKA activity in cardiomyocytes remain poorly understood. We and others have used genetically-encoded PKA biosensors targeted to the cytoplasm and the nucleus to show temporal segregation of PKA responses in both compartments in neonatal and adult cardiomyocytes.²⁶,²⁷ Such organization provides a mechanism by which acute β-AR stimulation may regulate contractility independently of gene expression. Our previous results also identified PDE4 as an important upstream regulator of nuclear PKA activity upon β-AR stimulation. However, how cAMP/PKA signalling
generated by β1- and β2-ARs are integrated in the nucleus of cardiomyocytes and whether this participates in functional differences observed upon stimulation of these receptors remains elusive. Here, we reveal that β1- and β2-ARs differentially regulate nuclear PKA activity and ICER expression in adult cardiomyocytes. We provide evidence that PDE4 prevents activation by β2-ARs of a mA-KAPβ-targeted PKA pool at the nuclear envelope which is required for PKA activation inside the nucleus and contributes to ICER induction.

Methods
An expanded methods section is provided in the online Data Supplement.

Experimental Animals
All procedures were performed in accordance with the European Community guiding principles in the Care and Use of Animals (2010/63/UE), the local Ethics Committee (CREEA Ile-de-France Sud) guidelines and the French decree n° 2013-118 on the protection of animals used for scientific purposes. Male Wistar rats were anesthetized by intraperitoneal injection of pentobarbital (0.1 mg/g).

FRET-Based cAMP and PKA Measurements in adult rat ventricular myocytes
After transduction of freshly isolated adult rat ventricular myocytes (ARVMs) with the appropriate adenoviruses, cells were subjected to FRET measurements as described.26

Results
β1- and β2-ARs differentially regulate nuclear PKA activity in adult cardiomyocytes
To analyse the dynamics of PKA activity in the bulk cytoplasm and in the nucleus, we used genetically encoded A-kinase activity reporters (AKAR3) targeted to these compartments by the addition of a nuclear export sequence (NES), and a nuclear localizing sequence (NLS), respectively.28 As shown previously26 and in the pseudocolour images of Fig. 1, adenoviral transfer allowed robust and compartment-specific expression of these biosensors after 24h in adult rat ventricular myocytes (ARVMs).
To determine how β1- and β2-ARs regulate cytoplasmic and nuclear PKA activities in ARVMs, we selectively stimulated these two receptors using a combination of isoprenaline (Iso) and either the β2-AR antagonist ICI 118,551 (ICI, 10 nM) or the β1-AR antagonist CGP 20712A
(CGP, 100 nM), respectively. Stimulation of cytoplasmic PKA activity by β₁- and β₂-ARs were completely abolished by 100 nM CGP and 10 nM ICI, respectively, indicating that the chosen concentrations of ICI and CGP in combination with Iso were appropriate for selective stimulation of β-AR subtypes (Supplementary Fig. 1). β₁-AR stimulation led to a fast increase in cytoplasmic PKA activity and a robust, but slower increase in nuclear PKA activity (Fig. 1A and B). In contrast, β₂-AR stimulation increased cytoplasmic PKA activity but had negligible effects on nuclear PKA activity (Fig. 1C and D). As shown in Fig. 1E, β₁-AR stimulation increased PKA activity in a concentration-dependent manner in both compartments, whereas this occurred only in the cytoplasm with β₂-AR stimulation (Fig. 1F). In Fig. 1G, nuclear PKA activation was plotted as a function of cytoplasmic PKA activation for the two receptors. The steeper slope of the linear regression further illustrates that β₁-ARs are more efficient than β₂-ARs to increase nuclear PKA activity, even when cytoplasmic PKA is activated at the same level.

**β₁- and β₂-ARs elevate cAMP in the nucleus**

Because cAMP generated by β₂-ARs was reported to be locally confined²⁹, we hypothesized that cAMP may not reach the nucleus upon β₂-AR stimulation, hence explaining the lack of PKA activation in this compartment. To test this hypothesis, we generated a nuclear-targeted version of the cytoplasmic cAMP sensor Epac-S'H₁₈⁷ by addition of 3 NLS at the C-terminus (Epac-S'H₁₈⁷-3NLS).³⁰ Adenoviral vectors allowed robust and compartment-specific expression of Epac-S'H₁₈⁷ and Epac-S'H₁₈⁷-3NLS in ARVMs at 24h (Supplementary Fig. 2A). As shown by the individual traces in Fig. 2A-D and summarized in Fig. 2E and 2F, stimulation of both β₁- and β₂-ARs increased cAMP in a concentration-dependent manner in the two compartments. Regardless of the receptor type or the Iso concentration employed, the FRET responses recorded in the nucleus were systematically higher than in the cytoplasm. This difference apparently reflected a greater sensitivity of the 3NLS version of the sensor to cAMP and not higher cAMP levels in the nucleus. When the changes in FRET induced by Iso in the cytoplasm were determined for both sensors following expression for 48h, a condition for which the Epac-S'H₁₈⁷-3NLS sensor became expressed not only in the nucleus, but also in the cytoplasm (Supplementary Fig. 2B), the cytoplasmic response of the mislocalized Epac-S'H₁₈⁷-3NLS sensor to Iso was higher than that measured with Epac-S'H₁₈⁷ (Supplementary Fig. 2C). Regardless of the difference in sensitivity of the Epac-S'H₁₈⁷ and Epac-S'H₁₈⁷-3NLS sensors, the relative response in the cytoplasmic and nuclear compartments did not differ between β₁- and β₂-ARs (Fig. 2G), in contrast to what was
observed with the PKA sensors (Fig. 1G). Thus the lack of nuclear PKA activation cannot be attributed to the absence of a global nuclear cAMP elevation upon β2-AR stimulation.

**Mechanisms that prevent nuclear PKA activation by β2-ARs**

Multiple mechanisms have been proposed to compartmentalize β2-AR activation of PKA, by acting not only on cAMP generation and propagation but also downstream. In particular, receptor coupling to Gi may not only temper cAMP synthesis, but also activate alternative signalling pathways through βγ to circumvent the concurrent PKA activation.31, 32 To test this hypothesis, we inhibited Gi with pertussis toxin (PTX, 1.5 µg/mL, 2h). As a control, we verified that PTX effectively blocked the anti-adrenergic effect of acetylcholine on the β1-AR-induced cytoplasmic PKA activation (Supplementary Fig. 3A and 3B). Under β2-AR stimulation, PTX-treated cells exhibited higher cytoplasmic PKA activity but no difference in nuclear PKA activation (Fig. 3A, 3B). Similarly, co-expression of AKAR3-NES or AKAR3-NLS with a C-terminal fragment of GRK2 (βARK-ct, supplementary Fig. 3C), that scavenges βγ subunits of heterotrimeric G proteins and prevents GRK2 phosphorylation and desensitisation of the receptor, led to a potentiation of cytoplasmic PKA activity, but was without effect on nuclear PKA activity (Fig. 3C, 3D).

Another related mechanism for β2-AR compartmentalization is receptor localization to caveolae.6, 8, 10 To address this possibility, caveolae were disrupted by treating the cells with methyl-β-cyclodextrin (MβCD, 2 mM, 1h) to deplete cholesterol, or by co-expressing a dominant negative caveolin-3 mutant (Cav3DN) together with AKAR3-NES or AKAR3-NLS.10 The efficiency of MβCD to deplete cholesterol was verified by filipin staining, whereas expression of Cav3DN was demonstrated by immunocytochemistry and western blot (Supplementary Fig. 4A-C). In both cases, caveolae-disrupted cells showed potentiation of cytoplasmic but not nuclear PKA activity upon β2-AR stimulation (Fig. 3E-H). Conversely, overexpression of wild type Cav3 induced a small decrease in β2-AR-stimulated cytoplasmic PKA activity, consistent with its previously reported effect on β2-AR-generated cytoplasmic cAMP (Supplementary Fig. 4D).10

Thus, inhibition of Gi, of GRK2, and caveolae disruption potentiated β2-AR stimulation of PKA activity in the cell cytoplasm as expected, but did not confer PKA activation in the nucleus. Because recent studies emphasised the role of PDE3 and PDE4 in controlling the activation of discrete PKA pools by β1- and β2-ARs in cardiomyocytes33, we investigated the contribution of these enzymes to the regulation of cytoplasmic and nuclear PKA. We have shown previously28 that neither the PDE3 inhibitor cilostamide (Cil, 1 µM) alone nor the PDE4
inhibitor Ro-201724 (Ro, 10 µM) alone has an effect on basal PKA activity. Under β₁-AR stimulation, PDE4 inhibition potentiated both cytoplasmic and nuclear PKA activities whereas PDE3 inhibition had no significant effect (Fig. 4A and 4B). In comparison, under β₂-AR stimulation PDE3 inhibition led to a large potentiation of cytoplasmic PKA activity but was without effect on nuclear PKA activity (Fig. 4C and 4D). Interestingly, PDE4 inhibition had a similar potentiating effect on cytoplasmic PKA activity under β₂-AR stimulation but in this case it generated a strong nuclear PKA activation (Fig. 4C and 4D). These results unveiled a critical role of PDE4 in controlling nuclear PKA activation.

β₁- and β₂-ARs differentially regulate the PKA target gene and pro-apoptotic factor, ICER

To assess the functional consequences of nuclear PKA activation by β₁- and β₂-ARs on gene expression, the expression of inducible cAMP early repressor (ICER), a member of the CREB family of transcription factors known as a critical pro-apoptotic factor in cardiomyocytes,²¹, ²², ³⁴ was studied. β₁-AR stimulation of isolated ARVMs led to a strong induction of ICER mRNA (Fig. 4E). This effect was potentiated by concomitant PDE4 inhibition, but not by PDE3 inhibition (Fig. 4E). In comparison, β₂-AR stimulation alone had no significant effect on ICER expression, but concomitant PDE4 inhibition resulted in ICER induction (Fig. 4F). In the absence of β-AR stimulation, neither PDE3 nor PDE4 inhibition had effect on ICER mRNA expression (Supplementary Fig. 5A). β₁-AR stimulation of ICER mRNA expression was not observed in cells transduced with an adenovirus encoding the selective PKA inhibitor peptide (PKI) (Supplementary Fig. 5B). To examine the specific contribution of nuclear PKA in ICER regulation, we fused the first 25 amino acids of PKI with the red fluorescent protein mCherry and appended 3 NLS at the C-terminus for nuclear targeting (Supplementary Fig. 6A). As shown in supplementary Fig. 6B, in cardiomyocytes co-expressing nuclear PKI with AKAR3-NLS, nuclear PKA activation by β₁-ARs was abolished as expected. The augmentation of ICER mRNA by β₁-AR stimulation alone or in combination with PDE4 inhibition was also suppressed by nuclear PKI expression (Supplementary Fig. 6C and 6D). Similarly, nuclear PKI completely blocked nuclear PKA activation by β₂-ARs with concomitant PDE4 inhibition (Supplementary Fig. 7A). However, ICER mRNA induction by β₂-ARs with concomitant PDE4 inhibition was only partially blocked by nuclear PKI, suggesting that alternative mechanisms contribute to this effect (Supplementary Fig. 7B).³⁵, ³⁶

Because Epac1 was localized in the perinuclear region in cardiomyocytes,¹⁸, ³⁷ similar experiments were conducted in cells pre-incubated with 10 µM of the Epac1 inhibitor,
However, as shown in supplementary Fig. 7C, Epac1 inhibition did not modify the stimulatory effect of β2-ARs + Ro on ICER mRNA expression.

**β1-AR stimulation of nuclear PKA requires an extranuclear PKA pool**

In HEK293 cells, Sample et al. showed that PDE4 controls a nuclear pool of PKA. In order to determine whether the PKA pool involved in the β1-AR-induced nuclear PKA activation is localized outside or inside the nucleus in cardiomyocytes, we fused the first 25 amino acids of PKI with the red fluorescent protein mCherry and appended a NES to the C-terminus for cytoplasmic targeting. As shown in the images and intensity profiles of Fig. 5A, the resulting constructs localized specifically in the compartment of interest in ARVMs. To verify the efficiency of PKA inhibition by cytoplasmic PKI, β-AR stimulation of Ca^{2+} transients was compared in Fura2-loaded and electrically paced ARVMs expressing cytoplasmic PKI, nuclear PKI or β-Gal. As shown in Supplementary Fig. 8, β-AR stimulation with 10 nM Iso increased Ca^{2+} transient amplitude to a similar extent in ARVMs expressing β-Gal or PKI-3NLS, whereas these effects were absent in ARVMs expressing PKI-NES. Importantly, PKI-NES completely blocked nuclear PKA activation in response to β1-ARs stimulation (Fig. 5B). Cytoplasmic PKI also blocked ICER mRNA induction by β1-ARs (Fig. 5C). These results show that upon β1-AR stimulation, PKA activation outside the nucleus is a prerequisite to enhanced nuclear PKA activity and ICER induction.

**Role of mAKAP in the activation of nuclear PKA by β1- and β2-ARs**

In cardiomyocytes, muscle A-kinase anchoring protein (mAKAPβ) has been shown to organize a cAMP-responsive network containing PKA and PDE4 at the perinuclear membrane. To study the contribution of mAKAPβ in shaping nuclear PKA responses under β1- or β2-AR stimulation, we used adenoviruses expressing a short hairpin RNA (shRNA) to reduce its expression. As shown in Fig. 6A and 6B, this led to ~50% decrease in mAKAPβ perinuclear staining 72h post-transduction. Downregulation of mAKAPβ had no effect on the bulk cytoplasmic PKA activity under either β1- or β2-AR stimulation in the presence of Ro (Supplementary Fig. 9). However, mAKAPβ silencing induced a small decrease in β1-ARs stimulation of nuclear PKA activity (Fig. 6C) which was not observed when PDE4 was inhibited (Fig. 6D). Decreasing mAKAPβ failed to unmask a nuclear PKA response to β2-AR stimulation (Fig. 6E) but induced a ~60% reduction of β2-AR+Ro stimulation (Fig. 6F). These results indicate that when PDE4 is inhibited, stimulation of β2-ARs activates a specific pool of PKA maintained by mAKAPβ at the nuclear envelope to increase PKA activity inside the
nucleus. This specific mAKAPβ-dependent PKA pool may also be mobilized upon β₁-AR stimulation, albeit contributing to total nuclear PKA activity to a much lesser extent.

**Discussion**

Numerous studies have emphasized the importance of spatiotemporal control of cAMP/PKA pools in specific subcellular compartments for physiological regulation of cardiomyocyte contractile function. By comparison, the mechanisms that control nuclear PKA activity are less well understood, despite their critical importance for modulation of gene expression and long term modification of cell growth and apoptosis by β-ARs. Here, we provide the first evidence that β₁- and β₂-ARs differentially activate nuclear PKA and gene expression in adult cardiomyocytes. As illustrated in Fig. 7, our results support a model in which PDE4 insulates a mAKAPβ-targeted PKA pool at the nuclear envelope that prevents nuclear PKA activation and ICER induction upon β₂-AR stimulation, whereas the broader β₁-AR stimulation engages additional PKA pools to enhance nuclear PKA activity and ICER expression.

In this study we found that under selective β₁-AR stimulation, PKA was activated in both the cytoplasmic and nuclear compartments, whereas β₂-AR stimulation was less efficient to activate cytoplasmic PKA and failed to activate nuclear PKA. The slower kinetics of nuclear versus cytoplasmic PKA activation observed upon β₁-AR stimulation are consistent with previous observations using isoprenaline in cardiomyocytes and suggest that the PKA holoenzyme is first activated outside the nucleus and then the catalytic subunits translocate inside the nucleus by diffusion, which is a slow process. The fact that cytoplasmic PKI abolished PKA phosphorylation of the nuclear-targeted PKA biosensor and induction of ICER upon β₁-AR stimulation strongly supports this model and argues against the contribution of a nuclear resident pool of PKA as demonstrated in HEK293 cells. These results also offer a complementary view to the local-activation/local-action of PKA signalling which prevails in other subcellular compartments of cardiomyocytes.

The robust increase in cytoplasmic PKA activity observed here for β₁-ARs compared to the smaller β₂-AR effect is consistent with previous real-time monitoring of PKA activity in mouse ventricular myocytes. However, this difference is not sufficient to explain the lack of nuclear PKA activation by β₂-ARs since for a similar increase in cytoplasmic PKA activity, β₁- but not β₂-ARs activate nuclear PKA (Fig. 1G). Thus, compartmentalization rather than intensity of
β2-AR signals must explain their inability to induce nuclear PKA activation. However, our cAMP measurements with a fourth-generation cAMP FRET sensor harboring a superior dynamic range\textsuperscript{30} clearly showed that β2-AR stimulation elevated cAMP in the nucleus. This result was not expected given a previous report that β2-AR stimulation generates locally confined cAMP signals.\textsuperscript{29} However, a recent study using the same biosensor suggests that cAMP diffusivity is equivalent upon non-selective β-AR or selective β2-AR stimulation.\textsuperscript{42} Thus, one possibility is that the cAMP generated by β2-AR stimulation is able to diffuse to the nucleus even if the receptor and organelle are located at some distance from each other. But alternatively, β2-ARs could be localized in close proximity to the nucleus in ARVMs. Indeed, β2-ARs were shown to be located in T-tubules\textsuperscript{9}, and T-tubules are known to extend from the cell surface to the nuclear envelope, where they establish close contacts with the nucleus.\textsuperscript{43, 44}

Previous studies have proposed that alternative coupling of β2-ARs to Gi and their localization to caveolae circumvent β2-AR signalling by acting not only at the level of cAMP, but also downstream, by activating phosphatases.\textsuperscript{8, 31, 32} Accordingly, inhibition of Gi or disruption of caveolae potentiated β2-AR responses in the cytoplasm. However, these manoeuvres failed to unmask an effect of β2-AR stimulation on nuclear PKA activity, making it unlikely that phosphatase activation would explain the lack of β2-AR response in this compartment. Similarly, we show that βARK-ct overexpression potentiates β2-AR-induced cytoplasmic but not nuclear PKA activity. The former is consistent with inhibition of β2-AR desensitization, and consequent increased Gs signalling, but could also be explained by a decrease in Gi signalling, since it has been shown that GRK2-mediated phosphorylation of β2-ARs is important for β2-AR coupling to Gi\textsuperscript{45, 46}. In addition, βARK-ct should also prevent the recruitment of PDE4D5 to β2-ARs\textsuperscript{47} as recently demonstrated.\textsuperscript{48} Based on our results, failure of β2-ARs to increase nuclear PKA activity was not due to coupling of β2-ARs to PDE4D5. Altogether, these data demonstrate that nuclear PKA activity can be dissociated from the bulk cytoplasmic PKA activity upon β2-AR stimulation, and that none of the above mechanisms is sufficient to explain the lack of nuclear PKA activation upon β2-AR stimulation.

It is undisputed that higher rates of local cAMP degradation by PDEs participate in curtailing cAMP signal.\textsuperscript{49} For this reason, we investigated the role of PDE3 and PDE4, the two major cAMP-hydrolyzing PDEs expressed in rat cardiomyocytes,\textsuperscript{12} in tuning cytoplasmic and nuclear PKA responses to β-AR stimulation. Our findings show that PDE3 plays a minor role in regulating β1-AR stimulation, but controls cytoplasmic PKA activity in response to β2-AR.
stimulation, whereas PDE4 acts as the main modulator of PKA activity under both β₁- and β₂-AR stimulation. These data are consistent with earlier work showing that PDE4 regulates cAMP levels under both β₁- and β₂-AR stimulation, whereas PDE3 preferentially regulates cAMP generated under β₂-AR stimulation. Strikingly, PDE4 inhibition unmasked nuclear PKA activation under β₂-AR stimulation. Although several PDE4 isoforms were shown to be associated with β₂-ARs, our cAMP measurements are not compatible with PDE4 preventing cAMP generated by β₂-AR stimulation to access the nucleus. Hence, we reasoned that PDE4 may act as a sink, isolating a discrete PKA pool from cAMP influx generated upon β₂-AR stimulation. In cardiomyocytes, a perinuclear pool of PKA is maintained together with PDE4D3 by the scaffolding protein mAKAPβ, localized at the external membrane of the nuclear envelope, a strategic position for nuclear regulation. Our results show that disrupting this PKA pool by mAKAPβ knockdown drastically reduced nuclear PKA activation by β₂-ARs with concomitant PDE4 inhibition. This identifies mAKAPβ-associated PKA as the main route for β₂-AR control of nuclear PKA. The co-localization of PDE4D3 and PKA within the mAKAP complex allows efficient activation of PDE4D3 by PKA, rapid degradation of cAMP and restoration of basal PKA activity. In line with recent studies suggesting that PKA activation within an AKAP complex does not involve dissociation of the catalytic subunits, PDE4D3 within the mAKAP complex may prevent catalytic subunit dissociation and their translocation inside the nucleus, hence explaining the lack of nuclear PKA activation by β₂-ARs. Upon PDE4 inhibition, abnormally elevated cAMP levels are reached in the complex, allowing PKA catalytic subunit dissociation and transfer to the nucleus. We have previously demonstrated that mAKAPβ selectively binds type 5 AC (AC5) in cardiomyocytes and others have shown that this AC5 is localized in T-tubules and is critical for β₂-AR enhancement of I_{Ca,L} which is revealed by application of PDE3 and PDE4 inhibitors. This parallel with our results further supports the existence of a pool of β₂-ARs localized at the interface between the T-tubular membrane and the nuclear envelope (Fig. 7). The small impact of mAKAPβ silencing on β₁-AR stimulation of nuclear PKA activity suggests that other PKA pools are involved, which may be controlled by distinct AKAPs. One possible candidate is AKAP-Lbc, which is localized in a relatively broad perinuclear region in neonatal myocytes. Indeed, AKAP-Lbc was recently shown to bind a PDE4 long isoform, the activation of which led to reduced forskolin-induced nuclear PKA activation and attenuated hypertrophic response to β-AR stimulation.

Recent studies have emphasized the critical role of PKA in the pro-apoptotic effects of β-ARs. We have shown that nuclear PKA activation by β₁-ARs is concentration-dependent (Fig. 1), requires sustained receptor stimulation, and induces ICER expression (Fig. 4E and 4F).
Supplementary Fig. 6A), which is a strong mediator of apoptosis by decreasing Bcl-2 in cardiomyocytes.\textsuperscript{21} In contrast, β2-ARs do not increase nuclear PKA activity (Fig. 1) and fail to increase ICER expression (Fig. 4F). These results are consistent with a previous study showing that cardiomyocyte fate is switched from survival to death depending on the strength of β-AR stimulation and the balance between a β2-AR/G\textsubscript{i}-mediated ERK1/2 pathway leading to Bcl-2 induction at a low concentration range of Iso, and a β1-AR-mediated PKA/CREB/ICER pathway leading to Bcl-2 repression at higher concentrations of Iso.\textsuperscript{34} Thus, segregated PKA activation between the cytoplasm and the nucleus, together with activation of surviving signaling such as ERK1/2 is likely to contribute to the differential effects of low versus high levels of β-AR stimulation as well as subtype-specific effects. However, we further show that β2-ARs can increase ICER expression when PDE4 is inhibited. At variance with β1-ARs, this effect was only partially inhibited by nuclear PKI and not modified by the Epac1 inhibitor CE3F4 (Supplemental Fig. 7), indicating that other mechanisms contribute to β2-ARs regulation of ICER when PDE4 is inhibited.

In a previous study, it was shown that in neonatal cardiomyocytes, PDE3 but not PDE4 inhibition increased CREB phosphorylation and ICER expression and thus induced Bcl-2 downregulation.\textsuperscript{22} These results contrast with the lack of effect of the PDE3 inhibitor cilostamide on basal ICER expression observed here (Supplementary Fig. 5A). This might be explained by differences in experimental conditions or the cell type used (adult versus neonatal myocytes).

In conclusion, our study unveils the molecular mechanisms by which β1- and β2-ARs differentially regulate nuclear PKA activity and ICER expression in terminally differentiated adult cardiomyocytes, and identify mAKAPβ and PDE4 as critical organizers of β2-AR nuclear signalling. Many questions remain, however, that will need to be addressed in future studies. The extent to which the findings obtained here for ICER can be generalized to the β-AR transcriptome, and the place of PKA in the control of gene expression by β-ARs versus other cAMP effectors will deserve further investigations. The contribution of intracellular β-ARs located at the nuclear envelope\textsuperscript{58, 59}, in endosomes\textsuperscript{60} and at the Golg\textsuperscript{60, 61} should be evaluated in intact adult cardiac myocytes. In a translational perspective, it will also be important to determine whether the signalling routes linking β-ARs to nuclear PKA activation identified here in rat are conserved in larger mammals and humans. Finally, in hypertrophy and heart failure, there is a profound remodelling of the β-AR/cAMP/PKA pathway. Interestingly, while the expression of β1-ARs is reduced,\textsuperscript{62} β2-ARs and PDEs are
redistributed, leading to enhanced β2-ARs signalling and loss of compartmentation.9, 33, 63
Future studies should aim to elucidate how nuclear PKA activity is controlled by β-ARs in
diseased cardiomyocytes, which may be important for identification of new therapeutic
targets in HF.

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**Conflict of interest**
None.
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Figure legends

Figure 1: Stimulation of β₁- and β₂-ARs induce differential activation of cytoplasmic and nuclear PKA activity in ARVMs.
(A-D) Representative time course of cytoplasmic and nuclear PKA activities reported by the normalized Yellow Fluorescent Protein (YFP) / Cyan Fluorescent Protein (CFP) ratio in ARVMs transduced with Ad.AKAR3-NES (A, C) or Ad.AKAR3-NLS (B, D) for 24h at a multiplicity of infection (MOI) of 1000 active viral particles/cell. β₁-AR stimulation was achieved using a combination of 10 nM isoprenaline (Iso) and 10 nM ICI 118,551 (ICI) (A, B); β₂-AR stimulation using 100 nM Iso in combination with 100 nM CGP 20712A (CGP) (C, D). Pseudo-colour images of the YFP/CFP ratio were recorded at the times indicated by the letters on the graphs. Scale bars represent 20 µm. (E, F) Mean variation (±SEM) of the YFP/CFP ratio in ARVMs expressing either AKAR3-NES or AKAR3-NLS upon β₁-AR stimulation using 1, 3 and 10 nM Iso in combination with 10 nM ICI (E) or β₂-AR stimulation using 10, 30 and 100 nM Iso in combination with 100 nM CGP (F). Number of cells/animals are indicated in brackets. Statistical significance is indicated as *** p<0.001 versus ICI+Iso 1 nM or CGP+Iso 10 nM in the cytoplasm, $$ p<0.01$$ versus ICI+Iso 1 nM in the nucleus, $$$ p<0.001$$ by nested ANOVA with Tukey’s post-hoc test. (G) Nuclear PKA activation (% increase in YFP/CFP ratio in ARVMs expressing AKAR3-NLS) is plotted as a function of cytoplasmic PKA activation (% increase in YFP/CFP ratio in ARVMs expressing AKAR3-NES) for β₁- and β₂-AR stimulations. Values (±SEM) of E and F were used for this graph.

Figure 2: Both β₁- and β₂-AR increase cytoplasmic and nuclear cAMP levels in ARVMs.
(A-D) Representative time course of the normalized CFP/YFP ratio upon selective β₁-AR stimulation with Iso (1, 3 and 10 nM) in combination with 10 nM ICI (A, B) or β₂-AR stimulation with Iso (10, 30 and 100 nM) in combination with 100 nM CGP (C, D) in ARVMs transduced with Ad.Epac-SH187(A, C) or Ad.Epac-SH187-3NLS (B, D) for 24h at a MOI of 1000 active viral particles/cell. Pseudo-color images of the CFP/YFP ratio were recorded at the times indicated by the letters on the graphs. Scale bars represent 20 µm. (E, F) Mean variation (±SEM) of the CFP/YFP ratio in ARVMs expressing either Epac-SH187 or Epac-SH187-3NLS upon β₁-AR stimulation (E) or β₂-AR stimulation (F). Number of cells/animals are indicated in brackets. Statistical significance is indicated as * p<0.05; ** p<0.01; *** p<0.001 versus ICI+Iso 1 nM or CGP+Iso 10 nM in the cytoplasm, $$ p<0.01$$, $$ p<0.001$$ versus
ICI+Iso 1 nM or CGP+Iso 10 nM in the nucleus, # p<0.05, ### p<0.001, £££ p<0.001 by nested ANOVA with Tukey’s post-hoc test. (G) Nuclear cAMP elevation (% increase in CFP/YFP ratio in ARVMs expressing Epac-SH187-3NLS) is plotted as a function of cytoplasmic cAMP elevation (% increase in CFP/YFP ratio in ARVMs expressing Epac-SH187) in response to either β₁- or β₂-AR stimulation. Values (±SEM) of E and F were used for this graph.

Figure 3: Gᵢ proteins, caveolae and GRK2 regulate cytoplasmic but not nuclear PKA activation in response to β₂-AR stimulation.

Average time course of the YFP/CFP ratio upon β₂-AR stimulation in ARVMs expressing AKAR3-NES (A, C, E, G) or AKAR3-NLS (B, D, F, H). (A, B) ARVMs treated or not with pertussis toxin (PTX, 1.5 µg/mL, 2h) were exposed to 10 nM Iso plus 100 nM CGP to stimulate β₂-ARs. In all other protocols, β₂-ARs were stimulated with 30 nM Iso plus 100 nM CGP. (C, D) ARVMs were treated or not with 2 mM methyl-β-cyclodextrin (MβCD) for 1h. (E) ARVMs were co-transduced with Ad.AKAR3-NES (MOI 200) and Ad.β-Galactosidase (β-Gal, MOI 2000) or Ad.AKAR3-NES and an adenovirus encoding a dominant-negative Cav3 mutant (Ad. Cav3DN, MOI 2000) for 48h. (F) ARVMs were co-transduced with Ad.AKAR3-NLS and Ad. β-Gal or Ad.AKAR3-NLS and Ad.Cav3DN for 48h. (G) ARVMs were co-transduced with Ad.AKAR3-NES (MOI 200) and Ad.β-Gal (MOI 1000) or Ad.AKAR3-NES (MOI 200) and Ad.βARK-ct (MOI 1000) for 48h. (H) ARVMs were co-transduced with Ad.AKAR3-NLS (MOI 200) and Ad.β-Gal (MOI 1000) or Ad.AKAR3-NLS (MOI 200) and Ad.βARK-ct (MOI 1000) for 48h. In each panel, the number of cells/animals are indicated in brackets for the different experimental conditions. Statistical significance is indicated as * p<0.05; ** p<0.01; *** p<0.001 by nested ANOVA with Tukey’s post-hoc test.

Figure 4: PDE4 is predominant for regulation of β₁- and β₂-AR induced cytoplasmic and nuclear PKA activation.

(A, B) Average variation of the YFP/CFP ratio upon β₁-AR stimulation using 1 nM Iso plus 10 nM ICI alone or in the presence of 1 µM cilostamide (Cil), a PDE3 inhibitor, or 10 µM Ro-201724 (Ro) a PDE4 inhibitor in ARVMs transduced with Ad.AKAR3-NES (A) or Ad.AKAR3-NLS (B) at MOI 1000 for 24h. (C, D) Average variation of the YFP/CFP ratio upon β₂-AR stimulation using 30 nM Iso plus 100 nM CGP alone or in the presence of 1 µM Cil or 10 µM Ro in ARVMs transduced with Ad.AKAR3-NES (C) or Ad.AKAR3-NLS (D) at MOI 1000 for 24h. Number of cells/animals are indicated in brackets. Statistical significance is indicated as * p<0.05; ** p<0.01; *** p<0.001; §§ p<0.01 versus β₁- or β₂-AR by nested ANOVA with Tukey’s post-hoc test. (E, F) ICER mRNA expression in ARVMs in primary culture for 24h
and stimulated or not by β₁-ARs (100 nM Iso plus 10 nM ICI during 2h) or β₂-ARs (100 nM Iso plus 100 nM CGP during 2h) alone or in combination with 10 µM Ro or 1 µM Cil. Number of animals is indicated in brackets. Statistical significance is indicated as *** p<0.001 versus control; $ p<0.01$, $$$ p<0.001 by Kruskal-Wallis test with Dunn’s post-hoc test (E) or one-way ANOVA with Tukey’s post-hoc test (F).

Figure 5: Cytoplasmic PKI identifies the subcellular pool of PKA involved in regulation of nuclear PKA activity and ICER expression by β₁-ARs in cardiomyocytes. (A) Top, domain scheme of cytoplasmic targeted PKA inhibitor, PKI-NES. Middle, confocal image of an ARVM transduced with Ad.PKI–NES (MOI 250, 24h). Scale bars represent 20 μm. Bottom, fluorescence intensity profile along the yellow line as indicated on the above image in arbitrary units (AU). aa, amino acids. (B) Top, confocal images of ARVMs co-transduced with Ad.PKI-NES (MOI 250) and Ad.AKAR3-NLS (MOI 1000) for 24 h. Scale bars represent 20 μm. Bottom, Average time courses of the YFP/CFP ratio upon β₁-AR stimulation using 10 nM Iso plus 10 nM ICI in ARVMs co-transduced with Ad.PKI-NES and Ad.AKAR3-NLS. ARVMs co-transduced with Ad.β-Gal (MOI 250) and Ad.AKAR3-NLS (MOI 1000) were used as control. (C) ICER mRNA expression in ARVMs transduced during 24h with Ad.β-Gal (MOI 250) alone, Ad.β-Gal with β₁-AR stimulation (100 nM Iso plus 10 nM ICI during 2h) and Ad.PKI-NES (MOI 250) with β₁-AR stimulation (100 nM Iso plus 10 nM ICI during 2h). Data of ICER mRNA expression in Ad.β-Gal-transduced cells with or without β₁-AR stimulation are the same as in Supplementary Figure 6C. Number of cells/rats (B) or rats (C) are indicated in the respective panels. Statistical significance is indicated as *** p<0.001 between β-Gal and PKI-NES by nested ANOVA with Tukey’s post-hoc test (B) or as ** p<0.01 versus β-Gal by Kruskal-Wallis test with Dunn’s post-hoc test (C).

Figure 6: The scaffold protein mAKAPβ controls β₂-AR induced nuclear PKA activity when PDE4 is inhibited. (A) Immunocytochemical detection of mAKAPβ in ARVMs 72h after sequential infection with adenoviruses encoding either a scrambled shRNA (Ad.Control shRNA MOI 2000) or a shRNA against mAKAPβ (Ad-mAKAPβ shRNA MOI 2000) for 48h followed by infection with Ad.AKAR3-NLS (MOI 1000) for 24 h. Scale bars represent 20 μm. (B) Quantification of mAKAPβ fluorescence in ARVMs co-transduced with Ad.AKAR3-NLS and Ad.Control shRNA or Ad-mAKAPβ shRNA at 72 h. (C) Mean variation of the YFP/CFP ratio upon β₁-AR stimulation with 3 nM Iso plus 10 nM ICI in ARVMs co-transduced with Ad.AKAR3-NLS and Ad.Control shRNA or Ad.AKAR3-NLS and Ad.mAKAPβ shRNA. (D) Mean variation of the
YFP/CFP ratio upon β₁-AR stimulation with 1 nM Iso plus 10 nM ICI in the presence of 10 µM Ro 201724 (Ro) to block PDE4. ARVMs were co-transduced with Ad.AKAR3-NLS and Ad.Control shRNA or with Ad.AKAR3-NLS and Ad.mAKAPβ shRNA. (E, F) Mean variation of the YFP/CFP ratio upon β₂-AR stimulation (using 30 nM Iso plus 100 nM CGP) alone (E) or in the presence of 10 µM Ro (F) in ARVMs co-transduced with Ad.AKAR3-NLS and Ad.Control shRNA or with Ad.AKAR3-NLS Ad-mAKAPβ shRNA. Number of cells/rats are indicated in brackets. Statistical significance is indicated as * p<0.05; ** p<0.01 by nested ANOVA with Tukey’s post-hoc test.

Figure 7: Proposed model for β₁- and β₂-AR regulation of cytoplasmic and nuclear PKA activity and ICER expression in adult cardiac myocytes.

Stimulation of β₁-ARs generate cAMP signals (in red) diffusing in the cytoplasm and the nucleus. Upon β₁-ARs stimulation, PDE4 (in green) regulates cAMP levels to control PKA activity in the cytoplasm. A fraction of catalytic subunits (C) of PKA dissociate from regulatory subunits (R) and translocate inside the nucleus to increase nuclear PKA activity. Elevation of nuclear PKA activity allows induction of ICER transcription, presumably through CREB phosphorylation, which may be direct or indirect (dotted arrow between C and CREB).

Stimulation of β₂-ARs also generate cAMP elevation in the cytoplasm and the nucleus resulting in activation of cytoplasmic PKA. Cytoplasmic PKA activation upon β₂-AR stimulation is restricted by caveolin3, Gᵢ, GRK2, PDE3 and PDE4. In addition, PDE4 prevents activation by β₂-ARs of a specific pool of PKA tethered by mAKAPβ at the perinuclear membrane (illustrated by the dotted line surrounding the mAKAPβ-PKA-PDE4 complex) which controls access of C subunits to the nucleus (dotted arrow) and nuclear PKA activation by β₂-ARs. When PDE4 is inhibited, nuclear PKA activation contributes to ICER up-regulation by β₂-ARs, although other PKA-independent mechanisms may be involved.
Bedioune et al Figure 2
Bedioune et al Figure 4

A. Cytoplasm

B. Nucleus

C.

D.

E.

F.
**Figure 6**

A. Composite images showing control shRNA AKAR3-NLS and mAKAP shRNA AKAR3-NLS.

B. mAKAP fluorescence (AU) comparison between control shRNA and mAKAP shRNA.

C. β₁-ARs activity showing % increase in YFP/CFP over time (min).

D. β₁-ARs + Ro activity showing % increase in YFP/CFP over time (min).

E. β₂-ARs activity showing % increase in YFP/CFP over time (min).

F. β₂-ARs + Ro activity showing % increase in YFP/CFP over time (min).

Bedioune et al
Bedioune et al. Figure 7

The diagram illustrates the interaction between various proteins and molecules in a cell. Key components include:

- **β_1** and **β_2** receptors
- **AC** (adenylyl cyclase)
- **PDE4** (phosphodiesterase 4)
- **CREB** (cAMP response element-binding protein)
- **ICER** (inhibitor of CREB)
- **PKA** (protein kinase A)
- **GRK2** (G protein-coupled receptor kinase 2)
- **Cav3** (calcium channel)
- **G_i** and **G_s** (i and s subunits of G proteins)

The diagram shows the signaling pathways involved in the regulation of ICER expression, with critical interactions in both the cytoplasm and the nucleus.
SUPPLEMENTARY MATERIAL

PDE4 and mAKAPβ are nodal organizers of β2-ARs nuclear PKA signaling in cardiac myocytes

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DETAILED METHODS

Reagents
Isoprenaline, CGP-20712A, methyl-β-cyclodextrin, filipin III and acetylcholine were from Sigma, Ro-20-1724 and Pertussis toxin were from Calbiochem, cilostamide and ICI 118,551 were from Tocris Bioscience. CE3F4 (6-Fluoro-5,7-dibromo-2-methyl-1-formyl-1,2,3,4-tetrahydroquinoline) was kindly provided by Pascal Bouyssou (Institut de Chimie Organique et Analytique, Orléans, France).

Adenoviruses
Adenoviruses (Ad.) encoding the cytoplasmic PKA biosensor AKAR3-NES and the nuclear sensor AKAR3-NLS were previously described.1 Ad. PKI encoding the rabbit muscle PKI was a kind gift from Dr. Hazel Lum (University of Chicago, Illinois).2 Ad.Cav3 and Ad.Cav3DN encoding human wild type and a dominant negative mutant of caveolin 3, respectively3 were kindly provided by Dr Sian Harding (Imperial College, London) with permission from Dr. Tsutomu Imaizumi (Kurume University, Kurume, Japan). Ad. βARK-ct, encoding for the C-terminal fragment of GRK2 was a generous gift of Dr. Walter Koch (Temple University, Philadelphia, USA). Ad. Epac-SH187, Ad. Epac-SH187-3NLS, Ad. PKI-mCherry-NES, and Ad. PKI-mCherry-3NLS were generated for this study as indicated below. The scrambled (Ad.Control shRNA) and mAKAPβ-specific shRNA (Ad-mAKAPβ shRNA) adenoviruses include hairpin sequences based upon rat mAKAP mRNA sequence (NCBI GI:5070430, base pairs 7210-7228) expressed under the control of the U6 promoter, as previously described: mAKAP siRNA:

5’-GACGAACCTTCTCTCGGATTTCAAGAGATTCGGAAGGATTCGTCATTTTTT-3’

control siRNA:

5’-GACGAACCCCTGTTCGGAATTCAAGAGATTCGGAACAGGAGGTTGCATTTTTT-3’

Isolation, culture and adenoviral transduction of adult rat ventricular myocytes
All experiments were carried out according to the European Community guiding principles in the care and use of animals (2010/63/UE, 22 september 2010), the local Ethics Committee (CREEA Ille-de-France Sud) guidelines and the French decree n° 2013-118, 1st February 2013 on the protection of animals used for scientific purposes (JORF n°0032, 7 February 2013 p2199, text n° 24). Authorizations to perform animal experiments according to this decree were obtained from the Ministère français de l'Agriculture, de l'Agroalimentaire et de la Forêt (agreement N° C 92-019-01).

Male Wistar rats (250–300 g) were anesthetized by intraperitoneal injection of pentobarbital (0.1 mg/g) and hearts were excised rapidly and transferred into a cold Ca2+-free Ringer
solution containing (in mM): NaCl 117, KCl 5.7, NaHCO₃ 4.4, KH₂PO₄ 1.5, MgCl₂ 1.7, D-glucose 11.7, Na-phosphocreatine 10, taurine 20, and HEPES 21. Hearts were then mounted on Langendorff apparatus and perfused with an oxygenated Ca²⁺-free Ringer solution for 5 minutes, followed by perfusion during 40 minutes at 37°C with the same solution containing 1 mg/mL collagenase A (Roche) plus 320 μM EGTA and CaCl₂ to reach a free Ca²⁺ concentration of 20-25 μM. The ventricles were then cut off, chopped with scissors, filtered through a sterile 500 μm mesh and the cells were allowed to settle down. The supernatant was discarded and cells resuspended two times with Ringer solution at increasing [Ca²⁺] to 300 μM, followed by two other resuspensions in a 5 mg/ml BSA-containing Ringer solution. Finally, isolated cells were resuspended in minimal essential medium (MEM) supplemented with 2.5% fetal bovine serum (FBS), 1% penicillin/streptomycin, 20 mM HEPES (pH 7.6), plated on laminin-coated (10 μg/ml) culture dishes and kept in a 5% CO₂ incubator at 37°C. After 1 to 2 h, the medium was replaced by FBS-free MEM with or without adenoviruses.

**Western Blot**

ARVMs were lysed in cold lysis buffer containing (in mM): NaCl 150, HEPES 20, EDTA 2, Glycerol 10%, Triton 0.2% supplemented with phosphatase inhibitor cocktail (Roche) and a protease inhibitor cocktail (Roche). The lysates were centrifuged for 10 minutes at 12,000 g and 4°C and proteins from the supernatant were quantified using BCA Protein Assay (Pierce). After heating the samples at 95°C for 5 minutes, proteins were subjected to SDS-PAGE then transferred onto PVDF membranes. The membranes were saturated with 3% milk for 1 hour at room temperature and incubated overnight at 4°C with anti-GRK2 antibody (sc-562, Santa Cruz), anti-Caveolin-3 (sc-5310, Santa Cruz) and anti-GAPDH (D16H11, Cell Signalling). After incubation with appropriate secondary antibodies for 1 hour, proteins were visualized by enhanced chemoluminescence. Blots were scanned and analyzed densitometrically by ImageJ (National Institute of Health).

**Subcellular PKA activity and cAMP measurements by FRET imaging**

Prior to FRET experiments, FBS-free MEM was replaced by a Ringer solution containing (in mM): NaCl 121.6, KCl 5.4, MgCl₂ 1.8, CaCl₂ 1.8, NaHCO₃ 4, NaH₂PO₄ 0.8, D-glucose 5, N pyruvate 5, HEPES 10 (pH 7.4). ARVMs were left in this solution during 2 h at room temperature for equilibrium before starting acquisition. Images were captured every 5 seconds using the 40× oil immersion objective of an inverted microscope (Nikon) connected to a software-controlled (Metafluor, Molecular Devices) cooled charge coupled (CCD) camera (Cool SNAP HQ2). CFP was excited during 300 ms by a Xenon lamp (100W, Nikon) using a 440/20BP filter and a 455LP dichroic mirror. Dual emission imaging of CFP and YFP
was performed using a Dual-View emission splitter equipped with a 510LP dichroic mirror and 480/30 nm, 535/25 nm BP filters. Average fluorescence intensity was measured in a region of interest comprising a significant portion of the cytoplasm for Epac-S\(^{H187}\) and AKAR3-NES or a region of interest inside one of the two nuclei for Epac-S\(^{H187}\)-3NLS and AKAR3-NLS. Background was subtracted and CFP bleed through in the YFP channel was corrected before calculating the YFP/CFP ratio for the AKAR3 sensors or the CFP/YFP ratio for Epac-S\(^{H187}\) sensors. Ratio images were obtained using Image J software.

**Pertussis toxin and methyl-\(\beta\)-cyclodextrin treatments**
Culture medium was replaced with fresh FBS-free MEM supplemented or not with *Pertussis toxin* (PTX; 1.5 µg/mL) and ARVMs were then incubated for 2 h in a 5% CO\(_2\) incubator at 37°C. Similar protocol was used for ARVMs treatment with methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD). ARVMs were incubated with fresh FBS-free MEM supplemented or not with M\(\beta\)CD (2 mM) for 1 h at 37 °C. Thereafter, PTX/M\(\beta\)CD solution was replaced with a Ringer solution and ARVMs were used after 2 h.

**Gene construction and adenoviral vector generation**

PKI-mCherry-NES and PKI-mCherry-3NLS fusion genes were generated by sequential subcloning. The first 25 amino acids of PKI were amplified by PCR (forward primer, 5’_AATAATAAGCTTATGACTGATGTCGAAACTACTTATGCCG; reverse primer, 5’_AATAATGGATCCATCGTGGATGGCGTTACGTC) from the full length rabbit muscle PKI\(\alpha\) (a gift of Dr. Richard A. Maurer, Oregon Health Sciences University) and ligated into the HindIII and BamHI sites of the mammalian expression vector, pcDNA3 (Invitrogen). An mCherry DNA coding sequence (a gift of Dr. Christian Poüs, University Paris-Sud, France) without the stop codon was amplified by PCR (forward primer, 5’_AATAATGGATCCATCGTGGAGCAAGGCC; reverse primer, 5’_AATAATGAATTCTTCTTGTACAGCTCGTCCATGC) and subcloned at the 3’-end of PKI 1-25 using BamHI and EcoRI sites of pcDNA3. A DNA oligonucleotide encoding the NES sequence (LPLERLTL) and a stop codon was synthesized (forward, 5’_AATTCACTGCCCCCTGGAGCGCCTGACCCTGTAAT; reverse, 5’_CTAGATTACAGGGTCAGGGCTCCAGGGGGGAGTTG) and subcloned at the 3’-end of the PKI-mCherry construct using EcoRI and XbaI sites of pcDNA3 to obtain the PKI-mCherry-NES fusion gene. The PKI-mCherry sequence was subcloned at the 5’-end of a triple NLS sequence (PKKKRKVDPKKKRKDPKKKRRK) contained into a modified pcDNA3 vector using HindIII and EcoRI restriction sites to obtain the PKI-mCherry-3NLS fusion gene.
To obtain the Epac-S187-3NLS fusion gene, a triple NLS sequence was subcloned at the 3'-end of Epac-S187 using EcoRI and StuI restriction sites. Epac-S187 and the triple NLS pcDNA3 were gifts of Dr. Kees Jalink (Netherlands Cancer Institute).

PKI-mCherry-NES, PKI-mCherry-3NLS and Epac-S187-3NLS were cloned into the pShuttle-CMV vector (Addgene plasmid # 16403) and transferred by homologous recombination into the pAdEasy-1 vector (Addgene plasmid # 16399). The resultant recombinant plasmid was digested with PacI and transfected into HEK-293 cells to generate adenoviral vectors. Viral particles were harvested and purified with a Vivapure AdenoPACK20 kit (Sartorius).

**Real-time quantitative PCR analysis of ICER mRNA induction**

ARVMs were treated with the different drugs and maintained in culture medium for 2 h in a 5% CO₂ incubator at 37 °C. Thereafter, total RNA was extracted using Trizol reagent (MRCgene). Reverse transcription of RNA samples was carried out by using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions.

Real-time PCR reactions were prepared using SYBR Green Supermix (Bio-Rad) and performed in a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad). The relative amount of ICER mRNA transcripts was quantified using the ΔCt method. The average Ct obtained in non-treated cells was used as a calibrator and RPLP2, RPL32, YWHAZ housekeeping genes were used as the reference for normalization. The following primer pairs were used:

- **ICER**: 5’_TGGCTGTAACTGGAGATGAAA, 5’_TCTGCTAGTTGCTGGGGACT.
- **RPLP2**: 5’_GCTGTGGCTGTTTCTGCTTC, 5’_ATGTCGTCATCCGACTCCTC.
- **RPL32**: 5’_GCTGCTGTAGTGCAACAAA, 5’_GGGATTGGTGACTCTGATGG.
- **YWHAZ**: 5’_AGACGGAAGGTGCTGAGAAA, 5’_GAAGCATTGGGGATCAAGAA.

**Immunocytochemistry**

Caveolin-3 and mAKAP detection in cultured cardiomyocytes was analyzed by immunocytochemistry using an anti-Caveolin-3 (sc-5310, Santa Cruz, 1:300) and anti-mAKAP (PRB-451P, BioLegend, 1:1000) respectively. After washing with PBS, ARVMs were fixed with 4% PFA in PBS for 15 minutes. Cells were then permeabilized with 0.3% Triton X-100 in PBS for 30 minutes and incubated for 15 minutes at room temperature with a blocking buffer solution containing 0.5% BSA and 1% Goat serum in PBS. Thereafter, the preparations were sequentially incubated overnight at 4°C with the anti-Caveolin-3 or 2 h at room temperature with the anti-mAKAP primary antibodies and Alexa fluorescent dye-conjugated specific-secondary antibodies (Alexa Fluor 633, Invitrogen, 1:1000) diluted in blocking buffer. Coverslips were mounted on slides using Mowiol coverslip mounting solution and imaged using a Leica TCS SP5 confocal microscope equipped with a 60× water
immersion objective. For Filipin straining, PFA-fixed cells were incubated with a 50µg/ml Filipin-containing PBS solution during 2 h followed by PBS wash. Images were then acquired.

**Deconvolution**
Confocal images were subjected to spatial deconvolution algorithm. The point spread function (PSF) generated by using 0.17 µm orange fluorescent beads (PS-Speck microscope point source kit; Molecular Probes) was used in deconvolution. The Richardson-Lucy deconvolution algorithm with 10 iterations was performed on each z-stack of images using the DeconvolutionLab plugin of ImageJ (Biomedical Imaging Group, EPFL).

**Ca²⁺ imaging**
24 h after transduction with Ad-β-Gal, Ad-PKI-NES or Ad-PKI-3NLS, isolated cardiomyocytes were loaded with 1 µM Fura-2 AM (Invitrogen) for 15 min and then washed for 15 min with Ringer solution to allow hydrolysis of the esterified groups by endogeneous esterases. Loaded myocytes were paced at 1 Hz and Fura-2 ratio (measured at 512 nm upon excitation at 340 and 380 nm) was recorded using a double excitation spectrofluorimeter coupled with a video detection system (IonOptix, Milton, MA, USA). ARVMs were perfused with normal Ringer solution for 3 min (same composition as for FRET imaging), and then the cells were stimulated with Isoproterenol. Ca²⁺ transient amplitude corresponding to the percentage of variation between the end-diastolic and the peak systolic Fura-2 ratio was calculated offline on dedicated software (IonWizard 6×, IonOptix).

**Statistics**
All results are expressed as mean ± SEM. For RT-qPCR data, normal distribution was tested by a Shapiro-Wilk normality test. For normally distributed data, differences between multiple groups were analyzed using an ordinary one-way ANOVA with Tukey’s multiple comparisons post-hoc test. A Kruskal Wallis test with Dunn’s multiple comparisons post-hoc test were used when the data did not follow a normal distribution. A nested ANOVA (which takes into account both the number of observations and the number of animals) was performed using the lme function in the nlme v3.1–131 package for R (R version 3.4.1 and RStudio version 1.0.153), followed by Tukey’s post-hoc test for all data obtained on individual cells. Differences with p values <0.05 were considered as statistically significant. The number of independent experiments performed is indicated in the figures.
Supplementary Figure 1: Specificity of β₁- and β₂-AR stimulation by a combination of isoprenaline (Iso) and ICI 118551 (ICI) or Iso and CGP 20712A (CGP). (A-C) Representative time course of the normalized YFP/CFP ratio in ARVMs transduced with Ad.AKAR-NES at MOI 1000 for 24h. (A) Specific β₁-AR stimulation using 100 nM Iso in combination with 10 nM ICI was fully blocked by subsequent addition of 100 nM CGP. (B) Specific β₂-AR stimulation with 100 nM Iso in combination with 100 nM CGP was fully blocked by subsequent addition of 10 nM ICI. (C) Combined application of 10 nM ICI and 100 nM CGP completely prevented PKA activation by Iso 100 nM. (D-F) Mean variation (±SEM) of the YFP/CFP ratio upon (D) β₁-AR stimulation before and after addition of 100 nM CGP; (E) β₂-AR stimulation before and after addition of 10 nM ICI; (F) after addition of 100 nM Iso to cells perfused with 10 nM ICI and 100 nM CGP. In D-F, numbers in brackets indicate cells/animals. Statistical significance is indicated as *** p<0.001 by nested ANOVA with Tukey’s post-hoc test.
Supplementary Figure 2 Epac-$^{H187}$-3NLS harbors increased sensitivity to isoprenaline compared to Epac-$^{H187}$ (A,B). Representative confocal images of ARVMs transduced with Ad.Epac-$^{H187}$ or Ad. Epac-$^{H187}$-3NLS at MOI 1000 for 24h (A) and 48h (B). In (B), white rectangles show the regions of interest where FRET measurements are performed. Scale bars represent 20 µm. (C) CFP/YFP ratio variation in the cytoplasmic compartment of ARVMs expressing either Epac-$^{H187}$ or Epac-$^{H187}$-3NLS after stimulation with the indicated concentrations of isoprenaline. Numbers in brackets indicate cells/animals. Statistical significance is indicated as *** p<0.001 by nested ANOVA with Tukey’s post-hoc test.
Supplementary Figure 3: Validation of Pertussis toxin as an efficient way to inhibit Gi-mediated signaling and of adenoviral infection to overexpress β-ARK-ct in ARVMs. (A) Effect of 1 μM acetylcholine (ACh) on β₁-AR response to 10 nM Iso plus 10 nM ICI in ARVMs transduced with Ad.AKAR3-NES at MOI 1000, 24h and treated or not with 1.5 μg/mL Pertussis toxin (PTX). (B) Mean variation (±SEM) of the YFP/CFP ratio in ARVMs expressing AKAR3-NES upon ACh application on top of β₁-AR stimulation using 10 nM Iso plus 10 nM ICI in ARVMs treated or not with PTX. Number of cells/animals is indicated in brackets. Statistical significance is indicated as *** p<0.001 by nested ANOVA with Tukey’s post-hoc test. (C) βARK-ct expression in ARVMs 48h after co-transduction with Ad.AKAR3-NES (MOI 200) and Ad.β-Gal (MOI 1000) or Ad.βARK-ct (MOI 1000). GRK2 was used as loading control.
Supplementary Figure 4: Validation of methyl-β-cyclodextrin treatment to deplete cholesterol, of adenoviral transduction efficiency to overexpress wild type and dominant negative caveolin-3 (Cav3) mutant, and effects of Cav3 overexpression on PKA responses to β2-ARs stimulation in ARVMs. (A) Effect of MβCD treatment (2 mM, 1h) on cholesterol content in ARVMs, as assessed by filipin (50 µg/mL) staining. Top, representative epifluorescence images of control (non-treated) and MβCD-treated ARVMs stained with filipin. Scale bars represent 20 µm. Bottom, average filipin fluorescence intensity in control and MβCD-treated ARVMs (number of cells/animals is indicated in brackets). AU, arbitrary units. *** p<0.001 by nested ANOVA with Tukey’s post-hoc test. (B) Immunocytochemical detection of Cav3 in ARVMs co-transduced with Ad.AKAR3-NES (MOI 200) and Ad. β-Gal (MOI 2000) or Ad.Cav3 (MOI 2000) or Ad.Cav3DN (MOI 2000) after 48h. (C) Western blot analysis of Cav3 and Cav3DN protein expression in ARVMs co-transduced with Ad.AKAR3-NES and Ad.β-Gal or Ad.Cav3 or Ad.Cav3DN (same MOI and duration as in B). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Number of animals is indicated in brackets. (D, E) Mean variation (±SEM) of the YFP/CFP ratio upon β2-AR stimulation using 30 nM Iso plus 100 nM CGP in ARVMs co-transduced with Ad.AKAR3-NES (D) or Ad.AKAR3-NLS (E) (both at MOI 200 for 48h) and Ad.β-galactosidase (β-Gal, MOI 2000) or Ad.Caveolin-3 (Cav3, MOI 2000). Numbers in brackets indicate cells/animals.
Supplementary Figure 5: Regulation of ICER mRNA expression by β₁-ARs, PDE3 and PDE4 in ARVMs. (A) Effects of PDE4 inhibition with 10 µM Ro or of PDE3 inhibition with 1 µM Cil during 2h on ICER mRNA expression in ARVMs in primary culture for 24h. (B) ICER mRNA expression in ARVMs transduced with Ad.β-Gal (MOI 1000) or Ad.PKI (PKA inhibitor, MOI 1000) for 48 h and stimulated or not with 100 nM Iso plus 10 nM ICI during 2h. Number of rats used is indicated in brackets. Statistical significance is indicated as *** p<0.001 versus β-Gal; $ p<0.05 by Kruskal-Wallis test with Dunn’s post-hoc test.
Supplementary Figure 6: Nuclear PKA activation is required for ICER mRNA induction by β₁-ARs stimulation alone and with concomitant PDE4 inhibition. (A) Top, domain scheme of the nuclear-targeted PKA inhibitor, PKI-3NLS. Middle, confocal image of an ARVM transduced with Ad.PKI–3NLS (MOI 250, 24h). Scale bar represents 20 μm. Bottom, fluorescence intensity profile along the yellow line as indicated on the above image in arbitrary units (AU). aa, amino acids. (B) Top, confocal images of ARVMs co-transduced with Ad.PKI–3NLs (MOI 250) and Ad.AKAR3-NLS (MOI 1000) for 24h. Scale bars represent 20 μm. Bottom, Average time course of the YFP/CFP ratio upon β₁-AR stimulation using 10 nM Iso plus 10 nM ICI in ARVMs co-transduced with Ad.AKAR3-NLS and Ad.PKI-3NLS. ARVMs co-transduced with Ad.AKAR3-NLS (MOI 1000) and Ad.β-Gal (MOI 250) were used as control. (C) ICER mRNA expression in ARVMs transduced during 24h with Ad.β-Gal alone (MOI 250), Ad.β-Gal with β₁-AR stimulation (100 nM Iso plus 10 nM ICI during 2h) and Ad.PKI-3NLS (MOI 250) with β₁-AR stimulation (100 nM Iso plus 10 nM ICI during 2h). Data of ICER mRNA expression in Ad.β-Gal-transduced cells with or without β₁-AR stimulation are the same as in Figure 5C. (D) ICER mRNA expression in ARVMs transduced as in C and stimulated or not by β₁-AR in the presence of 10 μM Ro. Numbers of cells/rats (B) or rats (C, D) are indicated in brackets in each panel. Statistical significance is indicated as *** p<0.001 by nested ANOVA with Tukey’s post-hoc test in (B) and ** p<0.01; *** p<0.001 versus β-Gal; $$$ p<0.001 by Kruskal-Wallis test with Dunn’s post-hoc test in (C, D).
Supplementary Figure 7: Nuclear PKA, but not Epac1, contributes to ICER mRNA induction by β₂-AR stimulation with concomitant PDE4 inhibition. (A) Average time course of the YFP/CFP ratio upon β₂-AR stimulation using 30 nM Iso plus 100 nM CGP in the presence of 10 μM Ro 201724 (Ro) to block PDE4 in ARVMs co-transduced with Ad.AKAR3-NLS (MOI 1000) and Ad.PKI-3NLS (MOI 250). ARVMs co-transduced with Ad.AKAR3-NLS (MOI 1000) and Ad.β-Gal (MOI 250) were used as control. Number of cells/rats are indicated in brackets. (B) Effects of β₂-AR stimulation (100 nM Iso plus 100 nM CGP during 2 h) with concomitant PDE4 inhibition (Ro, 10 μM) on ICER mRNA expression in ARVMs transduced during 24 h with Ad.β-Gal (MOI 250) or Ad.PKI-3NLS (MOI 250). (C) Effects of β₂-AR stimulation (100 nM Iso plus 100 nM CGP during 2 h) with concomitant PDE4 inhibition (Ro, 10 μM) on ICER mRNA expression in ARVMs transduced during 24 h with Ad.β-Gal (MOI 250) and pre-treated or not with 10 μM CE3F4 during 15 min at 37°C. In (B) and (C), number of rats are indicated in brackets. Statistical significance is indicated as *** p<0.001 by nested ANOVA with Tukey’s post-hoc test in (A) and *** p<0.001 versus β-Gal; $$$ p<0.001 by one-way ANOVA with Tukey’s post-hoc test in (B, C).
Supplementary Figure 8: PKI-NES but not PKI-3NLS blocks β-AR stimulation of Ca$^{2+}$ transients in ARVMs. (A-C) Representative traces of Ca$^{2+}$ transients in Fura-2-loaded ARVMs paced at 1 Hz, and transduced for 24h with Ad.β-Gal at MOI 250 (A), Ad.PKI-3NLS at MOI 250 (B) or Ad.PKI-NES at MOI 250 (C), under basal condition or 10 nM Iso stimulation. (D) Mean amplitude (±SEM) of Ca$^{2+}$ transients before and after Iso stimulation in β-Gal, PKI-3NLS and PKI-NES expressing ARVMs. Number of cells/rats are indicated in brackets. Statistical significance is indicated as *** p<0.001 by nested ANOVA with Tukey's post-hoc test.
Supplementary Figure 9: Knockdown of mAKAPβ does not modify regulation of cytoplasmic PKA activity by β1- or β2-ARs with concomitant PDE4 inhibition. ARVMs were transduced with adenoviruses encoding either a scrambled shRNA (Ad.Control shRNA, MOI 2000) or a shRNA against mAKAPβ (Ad-mAKAPβ shRNA, MOI 2000) for 48h, followed by infection with Ad.AKAR3-NES (MOI 1000) for 24h. FRET imaging was performed at 72h. β1-ARs were stimulated with 3 nM Iso + 10 nM ICI, whereas β2-ARs were stimulated with 100 nM Iso + 100 nM CGP in the presence of the PDE4 inhibitor Ro 201724 (Ro, 10 µM). Numbers in brackets indicate number of cells/rats.
SUPPLEMENTAL REFERENCES


