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## **Etude de la signalisation $\beta$ -adrénergique cardiaque nucléaire à l'aide de bio-senseurs FRET**

### **Investigating cardiac $\beta$ -adrenergic nuclear signaling with FRET-based biosensors**

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#### **Résumé en français**

En activant les récepteurs  $\beta$ -adrénergiques ( $\beta$ -ARs) membranaires, la noradrénaline et l'adrénaline constituent les stimulateurs les plus puissants de la fonction cardiaque. Les  $\beta$ -ARs sont couplés positivement à la synthèse d'AMPC, qui active la protéine kinase dépendante de l'AMPC (PKA). La PKA régule les protéines clés du couplage excitation-contraction mais également l'expression génique. Alors qu'une activation aiguë de la voie AMPC/PKA permet l'adaptation du débit cardiaque à l'effort, son activation chronique est délétère en favorisant le remodelage pathologique du cœur. L'utilisation de sondes basées sur le transfert d'énergie de fluorescence par résonance (FRET) et localisées spécifiquement au niveau du cytoplasme ou du noyau permet de mettre en évidence des mécanismes différentiels par lesquels les  $\beta$ -ARs contrôlent l'activation de la PKA dans ces deux compartiments. La caractérisation de ces mécanismes est importante afin de mieux comprendre les effets délétères d'une activation chronique de la voie  $\beta$ -adrénergique dans le cœur.

**Mots clés:** cardiomyocyte, récepteurs  $\beta$ -adrénergiques, protéine kinase AMPC-dépendante, noyau, compartimentation.

## **Résumé en anglais**

By activating membrane  $\beta$ -adrenergic receptors ( $\beta$ -AR), noradrenaline and adrenaline are the most powerful stimulators of cardiac function.  $\beta$ -ARs are coupled to the synthesis of cAMP, which activates the cAMP-dependent protein kinase (PKA). PKA regulates the key proteins of excitation-contraction coupling but also gene expression. While an acute activation of the cAMP/PKA pathway allows adaptation of cardiac output to exercise, its chronic activation is deleterious by promoting pathological remodeling of the heart. The use of probes based on fluorescence resonance energy transfer (FRET) and located specifically at the level of the cytoplasm or the nucleus make it possible to highlight the differential mechanisms by which  $\beta$ -ARs control PKA activation in these two compartments. The characterization of these mechanisms is important in order to better understand the deleterious effects of chronic activation of the  $\beta$ -adrenergic pathway in the heart.

**Keywords** : cardiomyocyte,  $\beta$ -adrenergic receptors, cAMP-dependent protein kinase, nucleus, compartmentation.

## Introduction

Noradrenaline released by the cardiac sympathetic nerves and adrenaline secreted by the adrenal glands increase cardiac contractility by activating  $\beta$ -adrenergic receptors ( $\beta$ -ARs) and the canonical cAMP/cAMP-dependent protein kinase (PKA) pathway. In the absence of cAMP, the PKA holoenzyme is a heterotetramer composed of a dimer of regulatory (R) subunits that bind and inhibit two catalytic subunits (C). When cAMP increases, it binds to the R subunits, causing the release and activation of the C subunits. PKA in turn phosphorylates specific proteins that control cardiac excitation-contraction coupling: Rad, that controls sarcolemmal L-type  $\text{Ca}^{2+}$  channels (LTCC)<sup>1</sup>, the ryanodine receptors (RyR2), mediating  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum (SR), phospholamban that controls the SR  $\text{Ca}^{2+}$  ATPase (SERCA2) responsible for  $\text{Ca}^{2+}$  re-uptake into the SR, and the sarcomeric proteins, troponine I and myosin-binding protein C.<sup>2</sup> Although PKA is the primary target of cAMP in the heart, cAMP also controls cardiac function by binding to Hyperpolarization-activated cation channels (HCN)<sup>3</sup>, exchange protein directly activated by cAMP (Epac)<sup>4</sup> and Popeye domain containing proteins (Popdc)<sup>5</sup>.

The velocity and specificity of  $\beta$ -AR regulation seems to be determined by the localization of the PKA holoenzyme to its targets by A-kinase anchoring proteins (AKAPs)<sup>6</sup> and by the spatiotemporal pattern of cAMP signaling, which results from the interplay between cAMP production by adenylyl cyclases and cAMP degradation by cyclic nucleotide phosphodiesterases (PDEs).<sup>7</sup> The study of subcellular cAMP signaling has been fostered by the development of biosensors that detect changes in both cAMP and PKA activity in real time in intact living cells with very high spatial and temporal resolution. These sensors are based on the principle of Fluorescence Resonance Energy Transfer (FRET). In the case of cAMP detection, most sensors use the cAMP binding domains present in Epac inserted between optimized variants of the FRET pair CFP and YFP. The binding of cAMP to these modules results in a conformational change and a modification of the FRET signal, which can be easily quantified.<sup>8</sup> A-kinase activity reporters (AKAR) use the same principle to measure PKA activity, except that the cAMP-binding domain is replaced by a phosphoamino acid binding domain and a PKA

consensus sequence. Phosphorylation by PKA triggers intramolecular binding of the phosphoamino acid binding domain and an increase in FRET between CFP and YFP.<sup>9</sup> In the following, we will briefly present how these approaches allowed to study nuclear cAMP/PKA signaling in intact cardiomyocytes.

### **Nuclear cAMP signaling in cardiac myocytes**

While an acute activation of the  $\beta$ -AR/cAMP/PKA pathway allows adaptation of cardiac output to exercise, its chronic activation is deleterious by promoting pathological remodeling of the heart.<sup>10</sup> Long term modification of cardiac function by PKA involve modifications of gene expression *via* phosphorylation of nuclear proteins such as the cAMP-response element binding protein (CREB) and class II histone deacetylases<sup>11-13</sup> However, how cAMP/PKA signalling generated by  $\beta$ -ARs is integrated in the nucleus of cardiomyocytes is not well understood. In order to study the spatiotemporal dynamics of cytoplasmic and nuclear cAMP and PKA activity in living cells, Epac-based reporters for cAMP and AKAR sensors can be targeted to these compartments by using specific signal sequences.<sup>9</sup> Using these probes in adult rat ventricular myocytes, we observed that stimulation of  $\beta$ -ARs with isoprenaline (Iso) led to fast cAMP elevation in both compartments, whereas PKA activity was fast in the cytoplasm but markedly slower in the nucleus. This slow activation of PKA in the nucleus was confirmed by measuring phosphorylation of CREB, which required prolonged  $\beta$ -AR stimulation, in contrast to phosphorylation of the myofilament protein MyBP-C, which occurred within 15 seconds of  $\beta$ -AR stimulation.<sup>14</sup> Similar slow increase in nuclear PKA activity in response to  $\beta$ -AR stimulation was reported in neonatal cardiomyocytes.<sup>15</sup> In these cells, treatment with wheat germ agglutinin, a putative blocker of the nuclear pores, largely attenuated nuclear PKA activation while leaving cytoplasmic PKA stimulation intact.<sup>15</sup> These results are compatible with PKA dissociation occurring outside the nucleus, and C subunits crossing the nuclear envelope by passive diffusion.<sup>16</sup> We next sought to characterize the mechanisms that counteract nuclear  $\beta$ -AR signalling. Because PDE3 and PDE4 account for the majority of the cAMP-hydrolyzing activity in ARVMs<sup>7</sup>, the contribution of these

two PDE families was tested using specific inhibitors. Whereas PDE3 inhibition had little effect, PDE4 inhibition resulted in a large potentiation of both cytoplasmic and nuclear PKA activity induced by a transient  $\beta$ -AR stimulation. In cardiac myocytes, PP1 and PP2A are the major PPs that counterbalance PKA activity.<sup>17</sup> Accordingly, concomitant inhibition of these two PPs markedly slowed down AKAR3 dephosphorylation in the cytoplasm, and greatly increased PKA response in the nucleus. However, preferential PP2A inhibition slowed down the dephosphorylation of AKAR3 in the cytoplasm but had no effect on the nuclear PKA response, and PP2B (calcineurin) inhibition had no effect. Together, these results suggest that both PP1 and PP2A oppose PKA activation in the cytoplasm whereas PP1 plays a major role in the nucleus.<sup>14</sup> In addition to PPs, Yang et al. provided evidence that protein kinase inhibitor  $\alpha$  (PKI $\alpha$ ) is important to oppose nuclear PKA activity.<sup>15</sup> In neonatal cardiomyocytes, overexpression of a cytoplasmic-targeted C subunit increased  $\text{Ca}^{2+}$  transients but not cellular hypertrophy, whereas nuclear-targeted C subunit increased hypertrophy without affecting  $\text{Ca}^{2+}$  transients.<sup>15</sup> These results suggest that cytoplasmic-nuclear PKA compartmentation allows differential regulation of cardiac contractility and gene expression and contributes to cardiomyocyte hypertrophic growth.

Cardiac  $\beta$ -ARs are predominantly of the  $\beta_1$ - and  $\beta_2$ -subtypes. These receptors produce different acute contractile effects partly because they impact on different cytosolic pools of cAMP and PKA. They also exert different long term effects on hypertrophic growth and apoptosis, but the underlying mechanisms are unclear. In a subsequent study, we compared  $\beta_1$ - and  $\beta_2$ -ARs regulation of cytoplasmic and nuclear cAMP/PKA activity in cardiomyocytes using targeted biosensors.<sup>18</sup> 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). Multiple mechanisms have been proposed to confine  $\beta_2$ -AR signalling in cardiomyocytes, in particular  $\beta_2$ -AR coupling to  $G_i$ , desensitization by GRK2, and localization in caveolae.<sup>19</sup> However, disruption of these

mechanisms failed to reveal nuclear PKA activation by  $\beta_2$ -ARs. In contrast, PDE4 inhibition disclosed nuclear PKA activation and ICER induction upon stimulation of these receptors. Although several PDE4 isoforms were shown to be associated with  $\beta_2$ -ARs<sup>19</sup>, our cAMP measurements in the nucleus were not compatible with PDE4 preventing cAMP generated by  $\beta_2$ -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  $\beta_2$ -AR stimulation. In cardiomyocytes, a perinuclear pool of PKA is maintained together with the specific PDE4 isoform PDE4D3 by the scaffolding protein mAKAP $\beta$ , localized at the external membrane of the nuclear envelope.<sup>20</sup> Our results showed that disrupting this PKA pool by mAKAP $\beta$  knockdown drastically reduced nuclear PKA activation by  $\beta_2$ -ARs with concomitant PDE4 inhibition, thus identifying mAKAP $\beta$ -associated PKA as the main route for  $\beta_2$ -AR control of nuclear PKA.<sup>18</sup>

## **Conclusion**

These studies show that in cardiomyocytes,  $\beta$ -AR stimulation of nuclear PKA activity is temporally dissociated from that of cytoplasmic PKA activation and identify PDE4, PP1 and PKI $\alpha$  as important modulators of nuclear PKA activity. This may explain in part why short term  $\beta$ -AR stimulation exerts a salutary increase in cardiac contractility, whereas sustained  $\beta$ -AR stimulation has detrimental effects by modifying gene expression. They further show that  $\beta_1$ -ARs and  $\beta_2$ -ARs differentially regulate nuclear PKA activity and the pro-apoptotic factor ICER, indicating that PKA compartmentation contributes to subtype-specific effects. Future studies should aim to elucidate how nuclear PKA activity is controlled by  $\beta$ -ARs in diseased cardiomyocytes, which may be important for identification of new therapeutic targets in heart failure.

Disclosure: None.

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