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### Functional localization of cAMP signalling in cardiac myocytes

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running title : cAMP compartmentation in cardiac cells

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#### Abstract—

The cAMP pathway is of cardinal importance for heart physiology and pathology. The spatial organization of the various components of the cAMP pathway is thought to allow the segregation of functional responses triggered by the different neuromediators and hormones that use this pathway. Phosphodiesterases (PDEs) hydrolyse cAMP (and cGMP) and play a major role in this process by preventing cAMP diffusion to the whole cytosol and inadequate target activation. The development of olfactory cyclic nucleotide-gated channels to directly monitor cAMP beneath the plasma membrane in real time allows to gain new insights into the molecular mechanisms responsible for cAMP homeostasis and hormonal specificity in cardiac cells. This review summarizes the recent results we obtained using this approach in adult rat ventricular myocytes. In particular, the role of phosphodiesterases in the maintenance of specific cAMP signals generated by  $\beta$ -adrenergic receptors and other G<sub>s</sub>-coupled receptors will be discussed.

Key words: cAMP, heart, CNG channels, G protein-coupled receptor, phosphodiesterase

### The cAMP pathway in cardiomyocytes

Cyclic AMP is the main second messenger for signals derived from the sympathetic and parasympathetic systems and for a large number of cardioactive hormones and drugs. The sympathetic mediator noradrenaline increases cardiac contractility mainly through stimulation of  $\beta_1$ -adrenergic receptors ( $\beta_1$ -AR), which are coupled to G<sub>s</sub> proteins and adenylyl cyclases. The consequent increase in cAMP in turn may activate three effectors in cardiac cells, HCN channels [1], the exchange factor for Rap1 Epac [2] and the cAMP-dependent protein kinase (PKA). PKA phosphorylates a myriad of proteins including metabolic enzymes [3], transcription factors of the CREB family [4], and key components of cardiac excitation-contraction coupling (L-type Ca<sup>2+</sup> channels [5], ryanodine receptors [6], phospholamban [7], troponin I [8], myosin binding protein C [9] and protein phosphatase inhibitor-1 [10]). The concerted regulation of calcium-handling proteins by PKA leads to increased Ca<sup>2+</sup> transient and increased contractility in response to sympathetic activation.

The cAMP pathway can be counteracted at several levels. The parasympathetic mediator acetylcholine suppresses the effect of noradrenaline on contractility by activating G<sub>i</sub> proteins and thus inhibiting cAMP synthesis [11-14]. At the other end of the cascade, cAMP actions are counterbalanced by phosphatases which dephosphorylate PKA substrates [5,10]. In between, cAMP signals can be obliterated by phosphodiesterases (PDEs), that catalyse the hydrolysis of the phosphodiester bond in cyclic nucleotides cAMP or cGMP. PDEs constitute a highly diversified class of enzymes, subdivided in 11 families [15,16] among which at least four are expressed in the heart: PDE1, which is activated by Ca<sup>2+</sup>/calmodulin; PDE2, which is stimulated by cGMP; PDE3, which is inhibited by cGMP; and PDE4. While PDE1 and PDE2 can hydrolyze both cAMP and cGMP, PDE3 preferentially hydrolyzes cAMP and PDE4 is specific for cAMP. PDE1 seems to be mainly present in other cells but cardiomyocytes [17],

and PDE2 represents a minor part of cAMP hydrolysis in cardiomyocytes [18]. Thus, in normal conditions, cAMP hydrolysis in cardiac myocytes is mainly accounted for by PDE3 and PDE4. As we will see below, PDEs play a critical role in cAMP signal termination and in establishing compartmentalized responses.

## Differences in Gs protein-coupled receptors (GsPCRs) actions and the compartmentation hypothesis

In addition to  $\beta_1$ -AR, several other GsPCRs increase cAMP in cardiac myocytes but do not reproduce the effects of  $\beta_1$ -AR stimulation. For instance, in adult rat ventricular myocytes (ARVMs)  $\beta_2$ -adrenergic receptors ( $\beta_2$ -AR), glucagon receptors (Glu-R), prostaglandin E1 receptors (PGE1-R) and glucagon-like peptide-1 receptors (GLP1-R) are present.  $\beta_2$ -AR stimulation increases contractile force but does not activate glycogen phosphorylase [19] and does not accelerate relaxation [20,21] (but see [19]). Glu-R stimulation activates phosphorylase and exerts positive inotropic and lusitropic effects, but the contractile effects fade with time [22]. PGE-1 has no effect on contractile activity or glycogen metabolism [23]. Finally, GLP-1R stimulation exerts a modest negative inotropic effect despite an increase in total cAMP comparable to that elicited by a  $\beta$ -adrenergic stimulation [24].

Early biochemical studies [25-30] showed that cAMP accumulation and PKA-dependent phosphorylation of proteins in the membranous fraction were associated with inotropic and metabolic actions. Such correlations led to the proposal that activation of different G<sub>s</sub>PCRs results in the accumulation of cAMP and phosphorylation of hormone target proteins in distinct compartments [31]. The discovery of A-kinase anchoring proteins (AKAPs), responsible for the subcellular distribution of particulate PKA [32,33] and the demonstration that  $\beta$ -adrenergic receptors regulate nearby but not remote cardiac Ca<sup>2+</sup> channels [34,35] have provided additional support for this concept.

### CNG channels: new indicators to track cAMP in single living cells

Cyclic nucleotide-gated channels are normally found in photoreceptors and olfactory sensory neurons, where they translate changes in intracellular cyclic nucleotide concentration triggered by light or odorants into variations of the membrane potential. Native CNG channels consist of  $\alpha$  and  $\beta$  subunits, with the  $\alpha$  subunit forming a non specific cationic pore gated by cAMP or cGMP [36]. Upon binding of the cyclic nucleotide, CNG channel allow Na<sup>+</sup>, K<sup>+</sup> and  $Ca^{2+}$  to enter the cell (Figure 1A) and this activity is easily detected using either patch-clamp or Ca<sup>2+</sup>-fluorescence measurements [37]. CNG channels display several characteristics that make them valuable cyclic nucleotide sensors: their gating is fast; they do not desensitize; they are virtually voltage insensitive; and they display an excellent dynamic range [38]. The rat olfactory a subunit (CNGA2) make a valuable cGMP sensor that was used recently to show cGMP compartmentation in cardiac myocytes [39]. In order to study selectively cAMP dynamics, Rich et al. (2001) have performed punctual mutations in CNGA2 to decrease the sensitivity to cGMP and increase the sensitivity to cAMP [40]. In particular, the E583M mutation confers to the channel an EC<sub>50</sub> for cAMP of  $\approx 10 \mu$ M, and the additional C460W mutation further increases the sensitivity, to about 1 µM. These CNGA2 mutants were incorporated into adenovirus constructs to allow efficient expression in various cell types.

# $\beta$ -adrenergic cAMP signals are compartmentalized by PKA-mediated activation of PDE3 and/or PDE4

We recently used the E583M and the E583M/C460W mutants to study the role of phosphodiesterases in cAMP compartmentation in cardiac myocytes. For this, ARVMs were infected with the adenoviral constructs and after 24h the whole cell CNG current ( $I_{CNG}$ ) was monitored by the patch-clamp technique as an index of subsarcolemmal [cAMP]. Using the low affinity E583M mutant, we observed that stimulation of  $\beta$ -adrenergic receptors with

isoprenaline (ISO) culminated at about 20% of the effect of a non-specific stimulation of adenylyl cyclase by the hydrosoluble forskolin analogue L-858051 (L-85). When using the high affinity E583M/C460W mutant, the effect of L-85 was augmented as predicted from the difference in sensitivity of the two channels. However, with ISO the expected shift in sensitivity did not occur and the potentiation observed was significantly less than with L-85. From these results, we concluded that only a limited number of CNG channels were activated nearly maximally on a  $\beta$ -adrenergic stimulation, which implies that cAMP reaches high concentrations in spatially-confined microdomains [41]. We next investigated the mechanisms that prevent cAMP diffusion out of these microdomains. We found that selective inhibition of PDE3 by cilostamide augmented the effect of ISO ≈3-folds whereas selective inhibition of PDE4 by Ro-201724 increased the effect of ISO ≈8-folds. Interestingly, inhibition of PKA (which does not regulate CNG channels) by H89 enhanced the effect of ISO similarly to PDE4 inhibition, but this effect was not observed in the presence of the non specific PDE inhibitor isobutyl methyl xanthine (IBMX). PDE assays showed that ISO increases the total PDE3 and total PDE4 activities in ARVM. From these results, we concluded that subsarcolemmal cAMP levels are negatively tuned by PKA-mediated activation of PDE3/4 [41]. Although PDE3 can be phosphorylated and activated by PKA [42-44], several lines of evidence would favour the implication of a PDE4 isoform. PDE4 long isoforms are phosphorylated at Ser54 and activated by PKA in vitro [45,46]. Second, PDE4D variants have been shown to co-immunoprecipitate with several AKAPs expressed in heart [47]. Third, recent data in neonatal cardiomyocytes reported an important role for PDE4B2 in controlling norepinephrine-induced cAMP increase [48].

### Distinct GsPCRs are differentially coupled to PDEs in cardiac myocytes

According to the cAMP compartmentation hypothesis, cAMP signals triggered by different GsPCRs differ in their spatio-temporal characteristics, and PDEs play a determinant role in this process. In order to test this hypothesis, we used CNG channels to compare subsarcolemmal cAMP signals elicited by different GsPCRs and L-type Ca<sup>2+</sup> current (I<sub>Ca,L</sub>) to evaluate the consequences on a downstream cAMP target [18]. As mentioned above,  $\beta_1$ -AR,  $\beta_2$ -AR, Glu-R and PGE<sub>1</sub>-R are co-expressed in ARVMs, and are all positively coupled to cAMP, but have different functional effects. When monitoring subsarcolemmal cAMP with the high affinity E583M/C460W channel, we found that activation of  $\beta_1$ -AR and Glu-R elicited readily detectable cAMP at the membrane, while  $\beta_2$ -AR and PGE<sub>1</sub>-R did not. Failure to detect cAMP elicited by  $\beta_2$ -AR and PGE<sub>1</sub>-R could be due to localization of these receptors in caveolae [31] from which CNG channels seem mostly excluded [49].

When examining PDE regulation of GsPCRs cAMP signals, we found that cAMP elicited by  $\beta_1$ -AR was regulated by PDE3 and by PDE4 (Figure 2A). In contrast, the cAMP signal generated by glucagon was specifically regulated by PDE4. In the case of  $\beta_2$ -AR and PGE<sub>1</sub>-R, concomitant inhibition of PDE3 and PDE4 was necessary to detect cAMP beneath the membrane. In the case of PGE<sub>1</sub>, inhibition of PDE1 through 4 by IBMX had a greater effect than concomitant inhibition of PDE3 and PDE4, indicating that other PDEs, most likely PDE1 or PDE2, also regulate PGE<sub>1</sub>-R-mediated intracellular cAMP signals. These results indicate that these GsPCRs generate different cAMP signals due to a specific contribution of individual PDE families (Figure 2B).

For all receptors but PGE<sub>1</sub>-R, the nature of this functional coupling determined the regulation of a major downstream cAMP target, the L-type  $Ca^{2+}$  channels. Indeed, PDE3 and PDE4 were found to regulate  $\beta_1$ -AR and  $\beta_2$ -AR regulation of I<sub>Ca,L</sub>, while the effect of glucagon on this

parameter exclusively involved PDE4. In contrast, single inhibition of PDE3 or PDE4 failed to reveal any effect of PGE<sub>1</sub> on  $I_{Ca,L}$ , suggesting that PGE<sub>1</sub>-R lie in a distinct compartment from L-type Ca<sup>2+</sup> channels. These results are consistent with that of Brunton's group that showed a lack of PGE<sub>1</sub> effect on cardiac inotropism [31].

### Future directions: identification of specific roles for PDE isoforms

These results underline the importance of PDEs for the definition of receptor-specific cAMP signals and thus hormonal specificity in cardiac cells. Given the impressive number of PDE isoenzymes expressed in cardiac myocytes [17], understanding the role of each isoform in the regulation of cAMP homeostasis represents a huge task. However, collecting this information might not satisfy only academic curiosity, as PDEs might also play a previously underestimated role in cardiac hypertrophy and failure [50].

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### **Figure legends**

### Figure 1: CNG channels as cAMP biosensors

A The  $\alpha$  subunit of olfactory rat cyclic nucleotide-gated channels (CNGA2) forms a tetrameric channel which is closed in the absence of cAMP. When cAMP is increased in the cell, the channel opens and allows a non specific cationic current to flow through the membrane. **B** Expression of CNGA2 as revealed by confocal microscopy in native and Ad-CNG-infected adult rat ventricular myocytes cultured for 24h. CNGA2 mutant was detected using a mouse monoclonal anti-CNGA2 kindly provided by Drs. F. Mueller and B. Kaupp. No CNG channels were detected in non infected cells whereas Ad-CNG infected cells demonstrated a rather ubiquituous pattern of expression with accentuation of the staining at the Z-lines.

### Figure 2: Functional coupling of GsPCRs to individual PDE families

**A** An example of cAMP monitoring with E583M/C460W in ARVMs. The amplitude of the CNG current ( $I_{CNG}$ ) recorded by the patch-clamp technique 24h after adenoviral infection is depicted as a function of time. Activation of  $\beta_1$ -AR with Iso (5 µM) in the presence of the  $\beta_2$ -AR blocker ICI 118551 (1 µM) increases  $I_{CNG}$  and selective inhibition of PDE3 with cilostamide (Cil, 1 µM) or Ro 201724 (Ro, 10 µM) strongly potentiate this response. Non selective inhibition of all PDEs with IBMX upon  $\beta_1$ -AR stimulation also increase **B** Shematic representation of PDE coupling to the different GsPCRs.  $\beta_1$ -AR cAMP is readily detected and augmented by selective inhibition of either PDE3 or PDE4, while  $\beta_2$ -AR cAMP is only detected and regulated by PDE4 only. PGE<sub>1</sub>-R cAMP is not detected unless PDE3 and PDE4 are inhibited simultaneously but other PDEs, possibly PDE1 or PDE2, prevent diffusion of cAMP along the membrane.





B



Vandecasteele et al., Figure 1



B



Vandecasteele et al., Figure 2