Functional localization of cAMP signalling in cardiac myocytes

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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. PDEs (phosphodiesterases) 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 us to gain new insights into the molecular mechanisms responsible for cAMP homoeostasis and hormonal specificity in cardiac cells. The present review summarizes the recent results we obtained using this approach in adult rat ventricular myocytes. In particular, the role of PDEs in the maintenance of specific cAMP signals generated by β-adrenergic receptors and other G_s-coupled receptors will be discussed.

The cAMP pathway in cardiomyocytes

cAMP 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 β_1-ARs (β_1-adrenergic receptors), which are coupled with G_s-proteins and adenylate cyclases. The consequent increase in cAMP in turn may activate three effectors in cardiac cells, HCN channels (hyperpolarization-activated cyclic nucleotide-gated channels) [1], the exchange factor for Rap1 Epac (exchange protein directly activated by cAMP) [2] and PKA (cAMP-dependent protein kinase or protein kinase A). PKA phosphorylates a myriad of proteins including metabolic enzymes [3], transcription factors of the CREB (cAMP-response-element-binding protein) family [4], and key components of cardiac excitation–contraction coupling (L-type Ca^{2+} 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^{2+} 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_i-proteins and thus inhibiting cAMP synthesis [11–14]. At the other end of the cascade, cAMP actions are counterbalanced by phosphatases that dephosphorylate PKA substrates [5,10]. In between, cAMP signals can be obliterated by PDEs (phosphodiesterases), which catalyse the hydrolysis of the phosphodiester bond in cyclic nucleotides cAMP or cGMP. PDEs constitute a highly diversified class of enzymes, subdivided into 11 families [15,16] among which at least four are expressed in the heart: PDE1, which is activated by Ca^{2+}/calmodulin; PDE2, which is stimulated by cGMP; PDE3, which is inhibited by cGMP; and PDE4. While PDE1 and PDE2 can hydrolyse both cAMP and cGMP, PDE3 preferentially hydrolyses cAMP and PDE4 is specific for cAMP. PDE1 seems to be mainly present in cells other than cardiomyocytes [17], and PDE2 represents a minor part of cAMP hydrolysis in cardiomyocytes [18]. Thus, under 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 G_sPCRs (G_s-protein-coupled receptors) actions and the compartmentation hypothesis

In addition to β_1-AR, several other G_sPCRs increase cAMP in cardiac myocytes but do not reproduce the effects of β_1-AR stimulation. For instance, in ARVMs (adult rat ventricular myocytes), β_2-ARs, Glu-Rs (glucagon receptors), PGE_1-Rs (prostaglandin E_1 receptors) and GLP1-Rs (glucagon-like peptide-1 receptors) are present. β_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

Key words: adrenergic receptor, cAMP, cardiac myocyte, cyclic nucleotide-gated channel (CNG channel), G-protein-coupled receptor, phosphodiesterase (PDE).

Abbreviations used: AKAP, A-kinase-anchoring protein; β-AR, β-adrenergic receptor; ARVM, adult rat ventricular myocyte; CNG channel, cyclic nucleotide-gated channel; GLP1-R, glucagon-like peptide-1 receptor; Glu-R, glucagon receptor; G_sPCR, G_s-protein-coupled receptor; IBMX, isobutylmethylxanthine; ISO, isoprenaline; PDE, phosphodiesterase; PGE_1-R, prostaglandin E_1 receptor; PKA, cAMP-dependent protein kinase.

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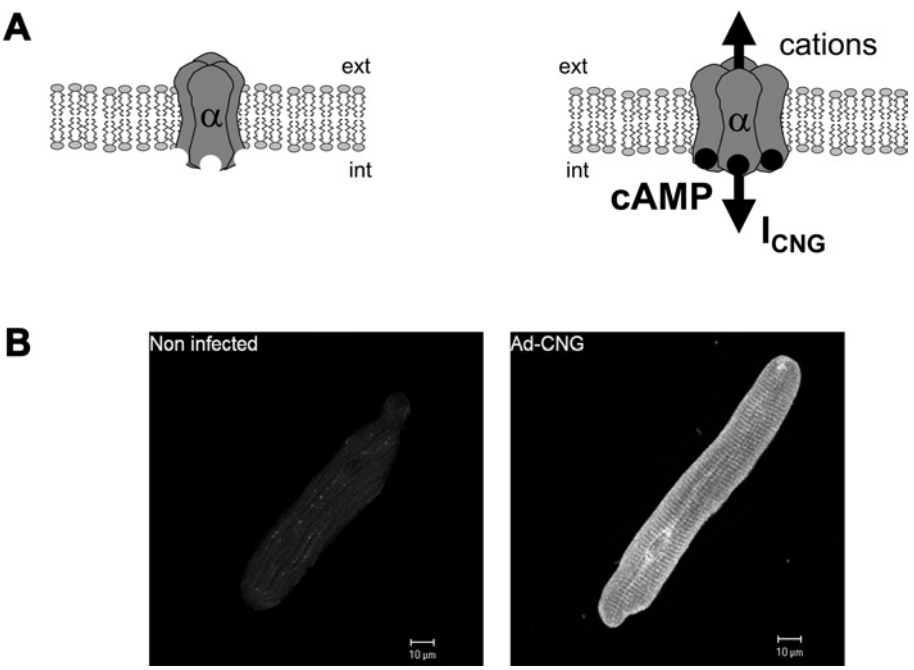
Figure 1 | **CNG channels as cAMP biosensors**

(**A**) The α subunit of olfactory rat CNG channels (CNGA2) forms a tetrameric channel that 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 (adenovirus carrying CNG)-infected ARVMs cultured for 24 h. CNGA2 mutant was detected using a mouse monoclonal anti-CNGA2 kindly provided by Dr F. Müller and Professor U.B. Kaupp (Institute for Biological Information Processing, Research Centre Jülich, Jülich, Germany). No CNG channels were detected in non-infected cells, whereas Ad-CNG-infected cells demonstrated a rather ubiquitous pattern of expression with accentuation of the staining at the Z-lines.



contractile activity or glycogen metabolism [23]. Finally, GLP-1R stimulation exerts a modest negative inotropic effect despite an increase in total cAMP comparable with that elicited by a β-adrenergic stimulation [24].

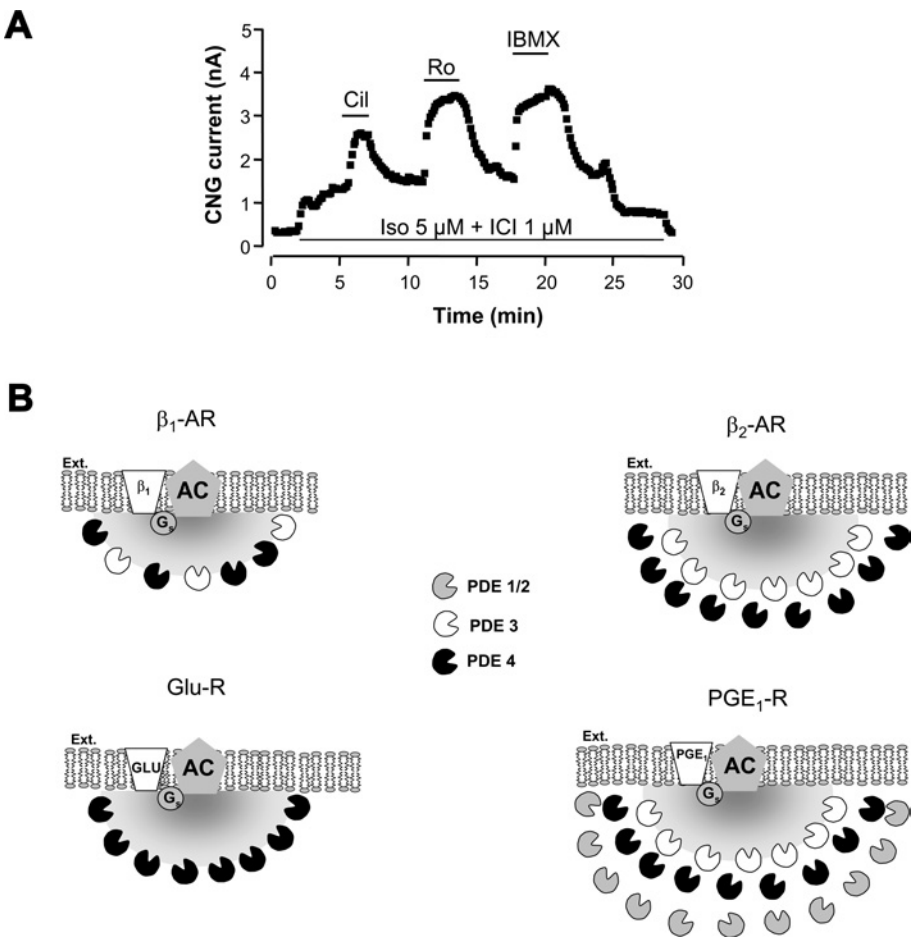
Early biochemical studies [25–30] showed that cAMP accumulation and PKA-dependent phosphorylation of proteins in the membranous fraction were associated with inotropic and metabolic actions. Such correlations led to the proposal that activation of different G_sPCRs results in the accumulation of cAMP and phosphorylation of hormone target proteins in distinct compartments [31]. The discovery of AKAPs (A-kinase-anchoring proteins), responsible for the subcellular distribution of particulate PKA [32,33], and the demonstration that β-ARs regulate nearby but not remote cardiac Ca^{2+} channels [34,35], have provided additional support for this concept.

CNG channels (cyclic nucleotide-gated channels): new indicators to track cAMP in single living cells

CNG 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 α- and β-subunits, with the α-subunit forming a non-specific cationic pore gated by cAMP or cGMP [36]. Upon binding of the cyclic nucleotide, CNG channels allow Na^+, K^+ and Ca^{2+} to enter the cell (Figure 1A) and this activity is easily detected using either patch-clamp or Ca^{2+}-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 α subunit (CNGA2) makes 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. [40] have made point mutations in CNGA2 to decrease the sensitivity to cGMP and increase the sensitivity to cAMP. In particular, the E583M mutation confers on the channel an EC_{50} for cAMP of approx. 10 μM, and the additional C460W mutation further increases the sensitivity, to approx. 1 μM. These CNGA2 mutants were incorporated into adenovirus constructs to allow efficient expression in various cell types.

Figure 2 | Functional coupling of G_sPCRs with 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 24 h after adenoviral infection is depicted as a function of time. Activation of β_1-AR with ISO (5 μM) in the presence of the β_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 potentiates this response. Non-selective inhibition of all PDEs with IBMX upon β_1-AR stimulation also increases I_{CNG}. (**B**) Schematic representation of PDE coupling with the different G_sPCRs. β_1-AR cAMP is readily detected and augmented by selective inhibition of either PDE3 or PDE4, whereas β_2-AR cAMP is only detected when PDE3 and PDE4 are inhibited. Glu-R cAMP is readily detected and regulated by PDE4 only. PGE_1-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. Ext., exterior.



β-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 PDEs in cAMP compartmentation in cardiac myocytes [41]. For this, ARVMs were infected with the adenoviral constructs and, after 24 h, 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 β-ARs with ISO (isoprenaline) culminated at approx. 20% of the effect of a non-specific stimulation of adenylate 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 upon β-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 approx. 3-fold, whereas selective inhibition of PDE4 by Ro-201724 increased the effect of ISO approx. 8-fold. 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 IBMX (isobutylmethylxanthine). PDE assays showed that ISO increases the total PDE3 and total PDE4 activities in ARVMs. From these results, we concluded that subsarcolemmal cAMP levels are negatively tuned by PKA-mediated activation of PDE3/PDE4 [41]. Although PDE3 can be phosphorylated and activated by PKA [42–44], several lines of evidence would favour the implication of a PDE4 isoform. First, PDE4 long isoforms are phosphorylated at Ser^{54} and activated by PKA *in vitro* [45,46]. Secondly, PDE4D variants have been shown to co-immunoprecipitate with several AKAPs expressed in heart [47]. Thirdly, recent results in neonatal cardiomyocytes reported an important role for PDE4B2 in controlling noradrenaline-induced cAMP increase [48].

Distinct G_sPCRs are differentially coupled with PDEs in cardiac myocytes

According to the cAMP compartmentation hypothesis, cAMP signals triggered by different G_sPCRs differ in their spatiotemporal 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 G_sPCRs and L-type Ca^{2+} current ($I_{Ca,L}$) to evaluate the consequences on a downstream cAMP target [18]. As mentioned above, β_1-AR, β_2-AR, Glu-R and PGE_1-R are co-expressed in ARVMs, and are all positively coupled with cAMP, but have different functional effects. When monitoring subsarcolemmal cAMP with the high-affinity E583M/C460W channel, we found that activation of β_1-AR and Glu-R elicited readily detectable cAMP at the membrane, while β_2-AR and PGE_1-R did not. Failure to detect cAMP elicited by β_2-AR and PGE_1-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 G_sPCRs cAMP signals, we found that cAMP elicited by β_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 β_2-AR and PGE_1-R, concomitant inhibition of PDE3 and PDE4 was necessary to detect cAMP beneath the membrane. In the case of PGE_1, inhibition of PDE1–PDE4 by IBMX had a greater effect than concomitant inhibition of PDE3 and PDE4, indicating that other PDEs, probably PDE1 or PDE2, also regulate PGE_1-R-mediated intracellular cAMP signals. These results indicate that these G_sPCRs generate different cAMP signals due to a specific contribution of individual PDE families (Figure 2B).

For all receptors except PGE_1-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 β_1-AR and β_2-AR regulation of $I_{Ca,L}$, whereas 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_1 on $I_{Ca,L}$, suggesting that PGE_1-R lies in a distinct compartment from L-type Ca^{2+} channels. These results are consistent with those of Steinberg and Brunton [31] that showed a lack of PGE_1 effect on cardiac inotropism.

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 homoeostasis 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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