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THE OLD AND NEW FACES OF CYCLIC AMP

Lessons from cAMP compartmentation in isolated cardiac myocytes

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Since the discovery of cyclic AMP by Earl Sutherland and Ted Rall in the late fifties, an amazing number of studies have appeared on how this second messenger is synthesized or degraded, on what makes its level go up or down, on what it does to target effectors by either covalent (phosphorylation) or non-covalent (direct binding to proteins, such as ion channels or guanine-nucleotide-exchange factors) mechanisms, and on how it affects a countless number of cellular functions (reviewed in: Beavo & Brunton, 2002). In certain tissues and organs, the cAMP pathway has been so fully explored over the years that one can wonder what else is there to be found. This is the case in the heart, where cAMP plays a key role in the sympathetic nerve/β-adrenergic receptor/adenylyl cyclase/protein kinase A (PKA) axis that serves to stimulate cardiac rhythm (chronotropy) as well as contractile force (inotropy) and relaxation (lusitropy) (reviewed in: Bers 2002). Yet, there is a question about cAMP signalling which we all keep in the back of our mind as a leitmotiv but which has only lately received the attention it deserves: does cAMP freely diffuse inside the cell?

Indeed, for sake of simplicity, one has often considered the cell as a homogenous system where cAMP and its effectors have uniform distributions and concentrations. While such a model may be sufficient when studying the effect of a given cAMP activator on a given cellular function, it becomes totally insufficient when comparing different cAMP activators which produce different functional effects. For instance, if we concentrate on the heart, there are many old and new studies showing that the β-adrenergic agonist isoprenaline (ISO), prostaglandin E_1 (PGE_1) and glucagon-like peptide 1 (GLP-1) elevate intracardiac cAMP levels with different consequences on contractility: while ISO augments the force of contraction, PGE_1 does not and GLP-1 exerts a negative inotropic effect (Brunton *et al.*, 1979; Vila-Petroff *et al.*, 2001) . In other words, depending on the initial stimulus used, not all

cAMP gains always access to all its possible effectors. There is a word to summarize such a postulate: *compartmentation*.

Although subcellular compartmentation of cAMP in the heart was proposed 25 years ago (Keely 1977; Brunton *et al.*, 1979; reviewed in: Steinberg & Brunton, 2001), it is only recently that multimolecular signalling complexes between cell surface receptors and intracellular targets were identified as being essential for the rapidity and specificity of signal transduction events (Davare *et al.*, 2001; Marx *et al.*, 2002; reviewed in: Tasken & Aandahl, 2004). However, how such modules allow to maintain a specificity when small diffusible molecules are generated during the signalling cascade is complex to dissect. Localized cAMP signals may be generated by the interplay between discrete production sites and restricted diffusion within the cytoplasm. In addition to specialized membrane structures that may circumvent cAMP spreading (Steinberg & Brunton, 2001; Rich *et al.*, 2000), degradation of cAMP into 5'-AMP by cyclic nucleotide phosphodiesterases (PDEs) appears critical for the formation of dynamic microdomains (Jurevičius & Fischmeister, 1996; Rich *et al.*, 2001; Bers & Ziolo, 2001; Zaccolo & Pozzan, 2002).

A deeper understanding of the mechanisms involved in cAMP homeostasis requires appropriate methods for the direct and continuous measurement of the second messenger in intact cells. Several years ago, we developed a double microperfusion technique using the L-type Ca^{2+} channel current ($I_{Ca,L}$) as a probe for cAMP/PKA activity (Jurevičius & Fischmeister, 1996). We examined cAMP compartmentation in intact frog ventricular myocytes by testing the effect of a cAMP activator applied on only one portion of a myocyte on the activity of Ca^{2+} channels in the other portion. This indirect approach led us to conclude that the cAMP signal generated in response to the β-adrenergic agonist ISO was not homogenously distributed, but remained restricted to the side of the cell where it was generated. Other more direct approaches to compartmentation are based on the use of

fluorescent PKA as a biosensor of cAMP (Goaillard *et al.*, 2001; Zaccolo *et al.*, 2000). However, careful evaluation of PKA-based indicators reveals a number of drawbacks which complicate the interpretation of the results. Among these, the kinase activity inherent to the probe and the poor dynamic range may be regarded as the most limiting (Rich & Karpen, 2002).

An alternative approach, initially developed by Rich *et al.* (2000), uses genetically modified α subunits of rat olfactory cyclic nucleotide-gated channel (CNG) as cAMP sensors. The wild type α subunit (CNGA2) on its own forms a cationic channel directly opened by cyclic nucleotides with fast kinetics, wide dynamic range and lack of desensitisation. Mutants of CNGA2 with increased cAMP sensitivity and selectivity have proven to be valuable tools for monitoring subsarcolemmal cAMP in model cells (Rich & Karpen, 2002).

In a very recent study (Rochais *et al.*, 2004), we reported the first real-time measurement of cAMP in adult rat cardiac myocytes using mutant CNGA2 channels encoded into adenoviruses. CNGA2 was not found in native myocytes but was strongly expressed in infected cells. We used the whole cell patch-clamp technique to record currents in response to ISO or forskolin, a direct activator of adenylyl cyclase. Forskolin elicited a non-selective, Mg^{2+}-sensitive current observed only in infected cells, a typical feature allowing us to identify the current measured as the CNG current (I_{CNG}). ISO also activated I_{CNG}, but its maximal efficiency was ≈5 times lower than with forskolin. However, ISO and forskolin exerted a similar maximal increase of $I_{Ca,L}$. We also used another CNGA2 mutant with a 10-fold higher sensitivity for cAMP, and found that the difference between ISO and forskolin on I_{CNG} was actually due to the activation of a localized fraction of CNG channels by ISO, while forskolin activated all channels. In other words, β-adrenergic cAMP signal was found to be compartmentalized in rat cardiomyocytes. We also tested the effect of PKA in this compartmentation, using H89 or PKI as PKA inhibitors, or PDE, using IBMX as a non-

selective inhibitor. PKA or PDE inhibition dramatically potentiated ISO- and forskolin-stimulated I_{CNG}. A similar potentiation of β-adrenergic stimulation occurred when only PDE4 (a cAMP-specific PDE) was blocked using the selective inhibitor Ro 20-1724. However, when PDE3 (a cGMP-inhibited PDE) was blocked using the selective inhibitor cilostamide, a 2-fold smaller effect than with IBMX was observed. This indicates that PDE4 plays a more important role than PDE3 in the cAMP compartmentation. In the presence of IBMX, H89 had no effect on ISO stimulation of I_{CNG}, which indicated that the effect of PKA was due to an increase in PDE activity.

Our results obtained with CNGA2 channels in rat cardiomyocytes confirmed and extended the suggestions made in our previous study in frog using the double microperfusion technique (Jurevičius & Fischmeister, 1996; Jurevičius *et al.*, 2003), and emphasized the important role of PDEs in the control of cAMP compartments in heart. In addition, we have established the importance of PKA in controlling plasma membrane PDE activity, acting as a negative tune of cAMP increases triggered by β-adrenergic receptors. This negative feedback controls global cAMP homeostasis beneath the membrane and contributes to the maintenance of restricted hormonal cAMP signals.

In conclusion, almost 50 years after its discovery, cAMP has still many hidden faces that need to be discovered. It will be one of the main challenges for cellular physiologists in the 21st century to decipher the complex spatio-temporal organisation that any given cell type has concocted to allow itself to discriminate among different external stimuli acting via a common signalling pathway.

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