PDE4 Control on cAMP/PKA Compartmentation Revealed by Biosensor Imaging in Neurons

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Abstract

Electrophysiological recordings (using the slow-AHP potassium current) together with novel biosensor imaging methods (with AKAR and Epac sensors) were used in preparations of rodent brain slices to record PKA activation in real time and in individual neurons. The experiments revealed the propagation of the PKA signal from the membrane to the cytosol and eventually to the nucleus. The experiments show how the geometry of the neurons combined with phosphodiesterase activities (mostly rolipram-sensitive PDE4) contributes to a functional compartmentation of the cAMP in subcellular domains.

Introduction

The signaling pathway of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) is involved in virtually all physiological processes. In the nervous system, the cAMP/PKA cascade plays an important role in several integrated brain functions such as neuronal survival, axonal regeneration, memory, and cognitive functions. At the cellular level, the cAMP/PKA cascade is responsible for the modulation of various processes including some specific forms of synaptic plasticity, control of excitability, regulation of nuclear factors, and imprinting of long-term changes, etc. One important target of the cAMP signal is the cAMP-dependent protein kinase (PKA), which phosphorylates various proteins including receptors and ion channels. cAMP also activates the exchange proteins directly activated by cAMP (Epac1 and Epac2) and directly modulates some ion channels like cyclic-nucleotide gated channels and I_h.

The cAMP/PKA signal takes place in the complex multi-dimensional space of the living cell, and the temporal and spatial dimensions are critical in the integration of this signal. In the temporal dimension, the signal is initiated at a precise time by the release of neuromodulators, starting a series of activating reactions, closely followed by feedback mechanisms, which stop these reactions and eventually return the cell to "basal" state. Among these negative controls, phosphodiesterases counteract the activity of adenylyl cyclases by degrading cAMP into AMP. In the spatial dimension, protein-protein interactions such as those mediated by AKAPs maintain partners of the signaling cascade in proximity in the so-called signaling microdomains [1–4]. Among these signaling enzymes, type 4 phosphodiesterase (PDE4) has been shown to play an important role in subcellular compartmentation: this enzyme is encoded by 4 different genes, each one having diverse splice variants, which determine the sub-cellular localization of the enzyme and thereby contribute to the compartmentation of cAMP. In addition to protein-protein interactions, purely geometric effects also directly impact signal integration [5]. Temporal and spatial measurements are of critical importance in the understanding of intracellular signaling and direct imaging of the cAMP/PKA signaling cascade therefore seems a very needed approach. The recent development of genetically-encoded sensors now allow time-lapse imaging in live preparations, monitoring changes in cAMP concentration using Epac-based sensors or changes in PKA activity level using AKAR sensors, providing real-time access to the spatial and temporal characteristics of intracellular signaling.

Experimental Context

Our goal was to gain a better understanding of the cellular events involved in the integration of neuromodulatory signals in the everyday work of

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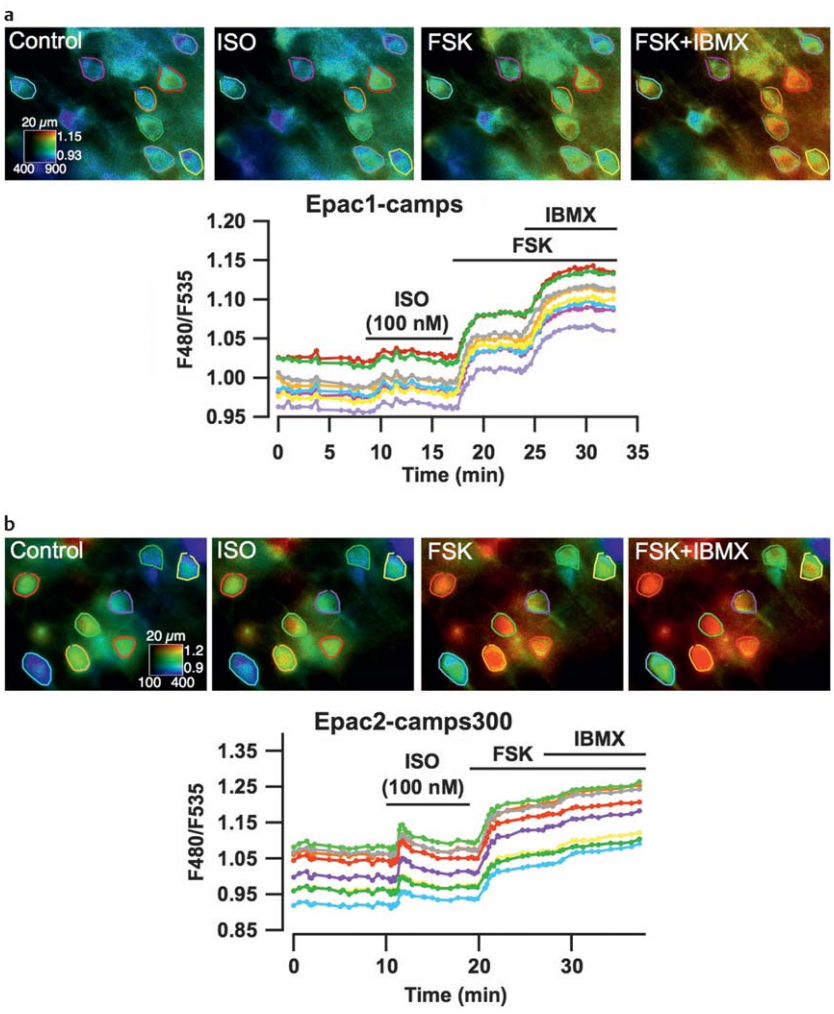

Fig. 1 Wide-field imaging of cortical brain slices expressing the Epac1-camps **a** and Epac2-camps300 **b** biosensors. Epac1-camps reported no significant cAMP signal in response to β-AR stimulation with Isoproterenol (ISO), whereas the more sensitive Epac2-camps300 did. Pseudocolor images represent the fluorescence emission ratios indicative of changes in cAMP concentration. Each trace indicates the F480/F535 emission ratio measured in the regions indicated by the color contour drawn on the pseudocolor images. ISO (100 nM Isoproterenol), FSK (13 µM Forskolin) and IBMX (200 µM) were applied in the bath for the period of time indicated by the bars. Reproduced with permission from reference [14].

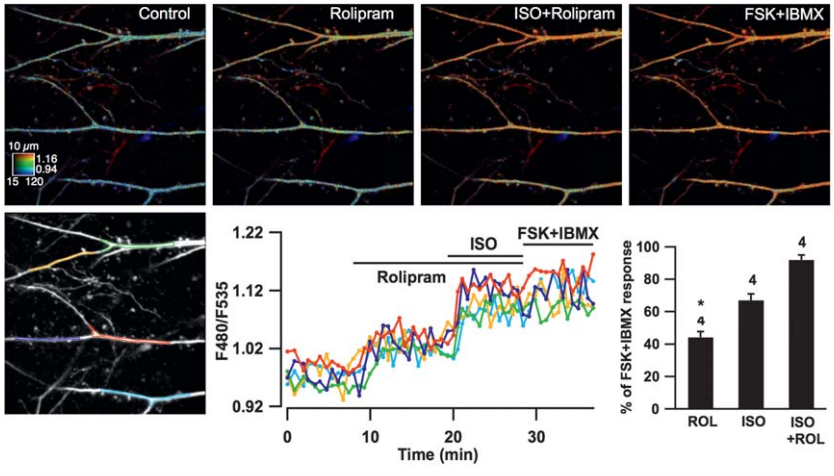

Fig. 2 PDE4 inhibition with rolipram increases PKA activity and cAMP levels in thin apical dendrites in the parietal cortex. Pseudocolor images obtained with 2-photon microscopy in a brain slice, showing Epac2-camps300-Cit emission ratio in different experimental conditions. Each trace indicates the F480/F535 emission ratio, measured on the regions indicated by the color contour on the grayscale image. Rolipram (100 nM), ISO (100 nM), FSK (13 µM), and IBMX (200 µM) were applied in the bath for the period of time indicated by the bars. The mean ratio change of Epac2-camps300-Cit is expressed as a percentage of the maximal response obtained with FSK and FSK+IBMX, respectively. The asterisk indicates a statistically significant difference. Reproduced with permission from reference [14].

an adult brain. Brain slices from neonate (and even adult) rodents have long been used for electrophysiological studies and provide an experimental access to living neurons with a preserved morphology.

First, we used an ionic current to report PKA activation at the membrane. In neurons, a calcium-activated potassium current (whose identity still remains elusive) produces the so-called "slow Afterhyperpolarization Potential" which stops bursts of action potentials. This potassium current (I_{sAHP}) is suppressed by a number of neuromodulators, leading to global membrane depolarization and longer-lasting burst of action potentials. The closure of this channel depends mainly on PKA-mediated phosphorylation. For both its physiological importance and strong sensitivity to PKA, we chose this current as an indicator of PKA-

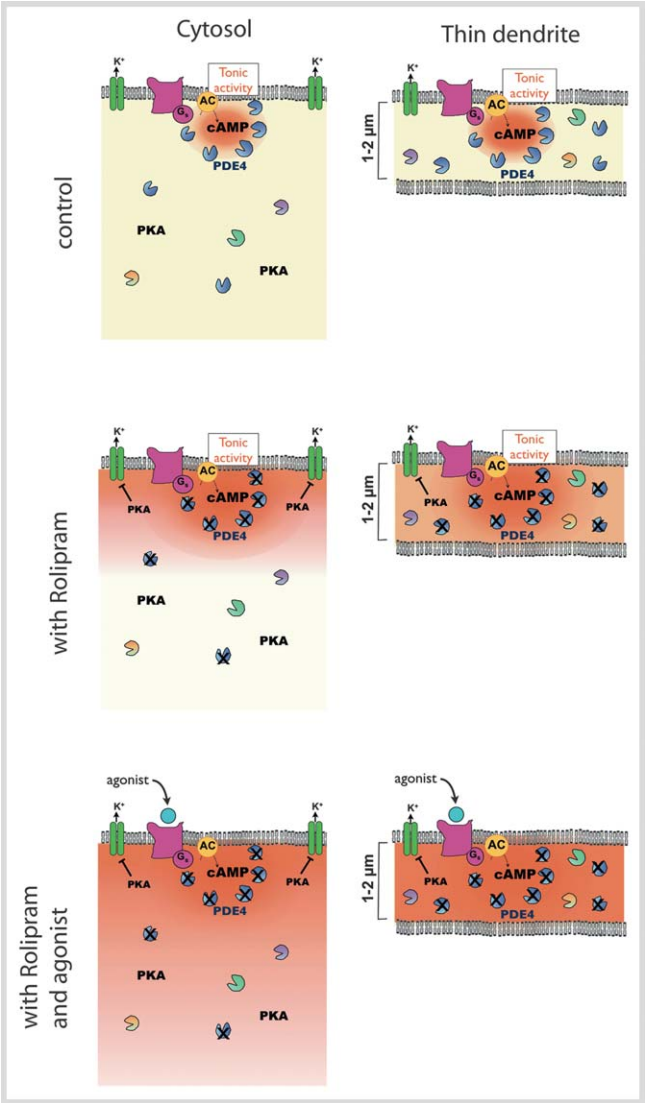

Fig. 3 PDE4 activity and sub-cellular compartmentation of the cAMP signal. In control condition (upper row), a tonic adenylyl cyclase activity maintains a low PKA activity which exerts a moderate inhibitory effect on potassium channels. This tonic cAMP production is counteracted by PDE4 since rolipram application (middle row) immediately produces a stronger inhibition of the potassium current and an increase in cAMP concentration, detectable with biosensors in small dendrites. Since there are alternative cAMP degradation routes, the global cytosolic cAMP concentration does not increase. Activation of Gs-coupled receptors (lower row) further increases adenylyl cyclase activity, which eventually overcomes the cAMP degradation processes and leads to a detectable cAMP signal in the bulk cytosol.

mediated phosphorylation events at the membrane level. This current was recorded using the classical patch-clamp technique and reported PKA activation at the membrane with a temporal resolution of less than a second.

Fluorescent biosensors have provided a novel approach for real-time monitoring of specific molecular events taking place within living cells. These sensors are commonly constituted of a domain sensitive to the biological signal of interest sandwiched between a pair of fluorophores, usually CFP and YFP or their close derivatives [6]. The signal triggers a conformational change of the biosensor, modifying the fluorescence resonance energy transfer (FRET) from CFP to YFP, changing the CFP and YFP fluorescence in opposite directions. Among these biosensors, we used the Epac1-camps [7] with a moderate (2 µM) sensitivity to cAMP, and Epac2-camps 300 [8] with a high (300 nM) sensitivity to cAMP ◉ **Fig. 1**. PKA sensors of the AKAR (A-Kinase Activity Reporter) family are recombinant protein composed of a phosphoamino acid binding domain and PKA-specific substrate fused between CFP and YFP. When phosphorylated by PKA, a conformational reorganization leads to an increase in FRET between CFP and YFP. We first used AKAR2.2 [9], and later AKAR3 [10], which provided a larger signal. In addition, the spatial dimension of the intracellular signal was accessed using a targeting domains to address the probe towards a specific sub-cellular compartment. AKAR2.2 fused with a nuclear localization signal at its C-terminal was thus used to monitor the kinetics of PKA activation inside the nucleus of neurons in brain slices.

In parallel with electrophysiology, we thus used fluorescent biosensors to directly "image" changes in the cAMP/PKA cascade in the same preparation. The brain slice preparation was optimized to keep the slices alive in vitro long enough for the neurons to express the biosensor, which was vectored by the sindbis virus [11]. Slices were imaged within less than 20 h in wide-field microscopy, or 2-photon microscopy when high spatial resolution was needed.

Signal Propagation from the Membrane Towards the Cytosol and the Nucleus

Electrophysiology and biosensor imaging were used to monitor PKA activation at the membrane, in the cytosol, and in the nucleus after the activation of serotonin 5-HT_7 receptors in intralaminar thalamic neurons. Kinetics analysis showed that the PKA signal first activated at the membrane in half a minute and reached a maximal activation level, whereas the PKA signal rose much slower in the cytosol, in ~2.5 min and only reached partial activation. This suggests that the brief release of neuromodulators can quickly and strongly modulate neuronal excitability, whereas longer presence of neuromodulators would be required for gradual and partial phosphorylation of PKA targets in the cytosol. Translocation of the PKA signal into the nucleus took even longer (more than 10 min) suggesting that the modulation of gene expression would only occur after long and sustained neuromodulatory stimuli [12].

We then wanted to understand what cellular mechanisms were involved in the dampening of the cAMP/PKA signal, from its strong and powerful effect observed at the membrane to the slower and gradual signal seen in the cytosol. We focused on phosphodiesterase (PDE) activities as these enzyme quickly degrade cAMP into AMP, and type 4 PDE (PDE4) has been reported to regulate the amplitude of the β-adrenergic response in the cortex. Moreover, the specific PDE4 inhibitor rolipram had shown interesting antidepressant and procognitive activities [13], although the cellular basis of its effect remained unclear in the brain.

Rolipram applied at a low concentration (100 nM) immediately decreased the PKA-sensitive I_{sAHP}, an effect associated with increased neuronal excitability. Since the inhibition of cAMP degradation increases PKA activity, there must be a tonic production of cAMP whereupon PDE4 activity produces a continuous repressing effect, suggesting that the removal of this brake immediately leads to an increase in cAMP concentration and PKA activation. This was indeed observed in the smallest dendritic branches of pyramidal cortical neurons, where 2-photon

microscopy revealed **Fig. 2** positive cAMP and PKA responses during rolipram application [14]. However, the effect of rolipram on cAMP concentration was not sufficient to be seen with biosensors in the bulk cytosol and the PKA sensor was only marginally activated **Fig. 1**. This result is consistent with the tonic cAMP production being too weak to fill the entire neuronal space, thus being restricted to a sub-membrane domain [5] **Fig. 3**.

We also evaluated the importance of PDE4 in the regulation of neuromodulator-evoked cAMP/PKA signal. Pyramidal cortical neurons exhibit a strong and reliable β_1-adrenergic response and the amplitude of this response, measured in the cytosol at the level of cAMP concentration or PKA activity, was larger when PDE4 was inhibited by rolipram [14]. This shows that PDE4 also controls the amplitude of the signal produced by neuromodulators during its travel from the membrane towards the cytosol **Fig. 3**.

PDE4 inhibition thus has the combined effect of depolarizing the neuron and increasing its responsiveness to neuromodulators. We propose that these 2 effects may account for the overall procognitive and antidepressant effects of rolipram, by compensating for pathologically low levels of neuromodulation and deficits in neuronal excitability. Although the nausea and emesis produced by rolipram precluded further use in clinics, PDE4 nevertheless remains a promising target to treat neuropyschiatric disorders [15], particularly with the recently uncovered implications of PDE4 in schizophrenia [16, 17]. In this context, biosensor imaging of the cAMP/PKA signaling cascade in mature central neurons will provide invaluable data about the cellular effects of PDE4 inhibitors in specific brain regions.

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