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Imaging second messenger dynamics in developing neural circuits

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Abstract

A characteristic feature of developing neural circuits is that they are spontaneously active. There are several examples, including the retina, spinal cord and hippocampus, where spontaneous activity is highly correlated amongst neighboring cells, with large depolarizing events occurring with a periodicity on the order of minutes. One likely mechanism by which neurons can “decode” these slow oscillations is through activation of second messengers cascades that either influence transcriptional activity or drive posttranslational modifications. Here we describe recent experiments where imaging has been used to characterize slow oscillations in the cAMP/PKA second messenger cascade in retinal neurons. We review the latest techniques in imaging this specific second messenger cascade, its intimate relationship with changes in intracellular calcium concentration, and several hypotheses regarding its role in neurodevelopment.

Introduction

Spontaneous neuronal activity is a common feature in the developing central nervous system, occurring in many regions including the retina (Wong et al., 1993; Feller et al., 1996), spinal cord (Gorbunova and Spitzer, 2002; Hanson and Landmesser, 2003), hippocampus (Ben-Ari et al., 1989; Garaschuk et al., 1998; Colin-Le Brun et al., 2004; Khalilov et al., 2005), neocortex (Schwartz et al., 1998; Aguilo et al., 1999; Adelsberger et al., 2005), and hindbrain (Gust et al., 2003). Although spontaneous activity is generated by circuits with vastly different synaptic connectivity, each circuit exhibits episodic events that are correlated across large populations of cells with a periodicity on the order of minutes (Feller, 1999; O'Donovan, 1999). These events are for the most part generated by depolarizations of neurons, and cause significant increases in intracellular calcium concentration.

A model system for understanding how periodic activity translates into long-term changes in neural circuits is the developing visual system. Spontaneous activity in the retina is critical for the retinotopic and eye-specific refinement of retinal projections to the thalamus and superior colliculus prior to vision (for review, see Wong, 1999; Torborg and Feller, 2005). Retinal waves are spatially correlated bursts of activity that propagate laterally across the retina prior to the development of light responses. This activity is also periodic, occurring in a given retinal ganglion cell approximately once per minute. Most hypotheses regarding how retinal waves drive the refinement of retinal projections have been based on the assumption that spontaneous activity drives synaptic competition that in turn leads to axonal refinement (Katz and Shatz, 1996; Huberman, 2006; Butts et al., 2007). There is also some indication that cell autonomous processes are dependent on patterned activity (Ruthazer and Cline, 2004). For example, rhythmic activity in retinal ganglion cells significantly enhances the rate of axonal outgrowth (Goldberg et al., 2002; Rodger et al., 2005). In addition, neuronal activity determines the polarity of responsiveness to chemorepulsive or chemoattractive signals (Wang and Zheng, 1998; Ming et al., 2001; Nicol et al., 2007).

Little is known about the specific mechanisms by which patterned activity is read out by the cell to produce changes in protein expression or regulation of existing proteins. Though it is likely that increases in intracellular calcium concentration and downstream calmodulin-dependent kinases are responsible for some of the resulting developmental changes, other second messenger cascades, such as the cAMP pathway, may be involved. Calcium and cAMP pathways are known to be closely interrelated. Calcium activates some adenylyl cyclases (ACs), the enzymes that convert ATP to cAMP to directly increase cAMP concentration (Xia and Storm, 1997). Indeed, mutant mice lacking AC1 show defects in projections to both the superior colliculus and lateral geniculate nucleus, implying that calcium dependent cAMP concentration is important for map refinement (Ravary et al., 2003; Plas et al., 2004). In some cells, increases in calcium concentration lead to decreases in cAMP levels either by inhibiting some classes of ACs (Wayman et al., 1995; Wang and Storm, 2003), or by activating a distinct class of phosphodiesterases that are responsible for the breakdown of cAMP (Sonnenburg et al., 1998).

Cyclic AMP is a ubiquitous second messenger that is involved in myriad cellular processes. Downstream targets of cyclic AMP include cAMP dependent protein kinase (PKA), a guanine nucleotide exchange factor called Exchange protein activated by cAMP (Epac) and cyclic nucleotide gated ion channels. PKA in turn phosphorylates a wide variety of targets, including many receptors and ion channels that govern the excitability of neurons. In addition, cAMP also influences actin dynamics (Meberg et al., 1998) and is critical for growth cone motility (Lohof et al., 1992; Kim and Wu, 1996; Song and Poo, 1999; Munck et al., 2004).

To determine the various roles of second messenger cascades in development, it is critical to characterize the effects of neural activity on second messengers dynamics. However, tools for assaying activity of second messenger pathways other than calcium have been sparse until recently. Particularly, the cAMP/PKA pathway has seen the advent of several indicators suitable for use in both mammalian systems and model organisms. Here, we review advances in the field of fluorescence-based assays of the cAMP/PKA pathway and the application of these techniques to developing neurons.

Indicators for dynamic imaging of cAMP/PKA cascade

Although biochemical techniques to quantify cAMP concentration and PKA activity have existed for many years, these techniques are not appropriate for live imaging since they require the lysing of cells to obtain the measurements. The first assay with the spatiotemporal sensitivity to do dynamic imaging was the protein FICRhR, a tetrameric holoenzyme consisting of the catalytic and regulatory subunits of PKA fused to fluorescein and rhodamine, respectively (Adams et al., 1991). FICRhR was used to measure evoked changes of cAMP levels by forskolin or GPCR agonists in invertebrate neurons (Bacskai et al., 1993; Hempel et al., 1996) and mammalian hippocampal neurons, (Vincent and Brusciano, 2001; Goaillard and Vincent, 2002), as well as to image spontaneous cAMP transients in spinal cord neurons (Gorbunova and Spitzer, 2002). However, the use of chemically modified recombinant proteins implies it needs to be microinjected into cells, which has more limited use in mammalian neurons where whole cell recordings dialyze the intracellular contents that may modulate second messenger signaling.

A solution to this problem is to use genetically encoded indicators. One approach is to express genetically encoded cAMP-gated cation channels (Rich et al., 2001). Though this works well in kidney cells, the introduction of novel calcium conductances may alter the excitability of neurons. The first genetically encoded indicator of cAMP was based on FICRhR, replacing fluorescein and rhodamine with GFP and BFP (Zaccolo et al., 2000). This indicator was soon updated to use CFP/YFP fluorophores, which are more suitable for FRET (Zaccolo and Pozzan,

2002) and the linker range was modified to optimize FRET efficiency (Lissandron et al., 2005). The PKA-based cAMP sensor has four binding sites for cAMP – two on each regulatory subunit – each with different affinities for cAMP due to cooperative binding. The PKA-based cAMP sensor relies on the dissociation of the regulatory and catalytic subunits to decrease FRET efficiency. The indicator is expressed throughout the cell, but since it utilizes the RII subunit of PKA, it can also localize to A-Kinase Anchoring Proteins (AKAPs). AKAPs are thought to be involved in organizing microdomains (for review, Beene and Scott, 2007), and therefore this indicator is likely to be useful in studying very localized changes in cAMP concentration. Indeed, the PKA-based cAMP sensor has been used to detect microdomains of cAMP signaling in cardiac myocytes (Zaccolo and Pozzan, 2002; Lissandron and Zaccolo, 2006). A similar indicator has been recently developed that uses a truncated form of the CFP-RII subunit targeted to the membrane. Evanescent wave microscopy is then used to detect the translocation of the YFP-catalytic subunit away from the membrane when cAMP levels increase (Dyachok et al., 2006)

Recently, novel indicators of cAMP have been developed using proteins that have a single cAMP binding site. In particular, three groups independently developed FRET sensors based on the Exchange protein activated by cAMP (Epac) (DiPilato et al., 2004; Nikolaev et al., 2004; Ponsioen et al., 2004). Epac is a guanine nucleotide exchange factor with a single cAMP binding site, whose activity links cAMP to the Ras-ERK pathway (for review Holz et al., 2006). Upon binding of cAMP to the indicators, there is a decrease in FRET efficiency. There has yet to be a rigorous comparison of the various Epac indicators, which are truncated differently but appear to have similar dynamic ranges of FRET ratios. Indicators with a single cAMP binding site provide an advantage over those that rely upon cooperative binding of cAMP in that their response times are more likely to directly reflect changes in changes in cAMP levels. FRET-based indicators have also been developed from the cAMP binding sites of the PKA regulatory subunit (Nikolaev et al., 2004) and the cAMP gated channel HCN2 (Nikolaev et al., 2006).

A third type of indicator was developed to report on PKA activity. This indicator was designed as an archetype for reporting kinase activity via FRET. AKARs consist of a fusion of CFP, a PKA target substrate, a phosphobinding region, and the YFP variant citrine (Zhang et al., 2001). Efforts to further improve AKAR have focused on increasing the off-kinetics (Zhang et al., 2005) and the dynamic range of the indicator by optimizing the orientation of YFP with respect to CFP using circular permutations of the YFP variant, Venus (Nagai et al., 2004; Allen and Zhang, 2006). The AKAR FRET paradigm has also been used to study the activity of other kinases, including PKB/Akt (Aktus, Sasaki et al., 2003), PKC (CKAR, Violin et al., 2003), PKD (DKAR, Kunkel et al., 2007), and tyrosine kinases (Ting et al., 2001).

One advantage of genetically encoded indicators is that they can be localized to specific populations of cells using specific promoters. In addition, they can be mutated to allow targeting to different subcellular compartments. For example, short peptide tags have been used to localize the indicators to nuclear, mitochondrial, cytosolic, and membrane regions in hippocampal neurons (DiPilato et al., 2004; Allen and Zhang, 2006; Gervasi et al., 2007). This ability to obtain subcellular localization is likely to be critical for distinguishing amongst the variety of targets of the cAMP/PKA pathways in neurons.

Spontaneous cAMP/PKA transients in retinal ganglion cells

We recently used two FRET indicators, ICUE2 and AKAR2.2 to assay the spontaneous activation of the cAMP/PKA pathway in retinal ganglion cells during retinal waves (Dunn, Wang, et al. 2006). Calcium influx due to retinal waves occurs approximately once per minute, and calcium dependent adenylyl cyclases are present in retinal ganglion cells suggesting

cAMP/PKA might be activated by calcium influx. However, it was unknown whether this pattern of calcium transients would cause transient or sustained activation of the cAMP/PKA pathway. Briefly, retinal explants were isolated from P0-P4 rats, electroporated with either ICUE2 or AKAR2.2, and cultured overnight to allow expression of the indicator. We found that some retinal ganglion cells exhibit spontaneous transient increases in PKA activity and cAMP concentration across the cell soma (Figure 2). Blockade of retinal waves eliminated the PKA transients. We also found that PKA activity increased within 5 seconds following a retinal wave. This is consistent with the hypothesis that retinal waves drive activation of the cAMP/PKA pathway through a calcium mediated mechanism. The implications for a role of these second messenger transients in neurodevelopment are explored below. Here we use these data as an example for illustrating various properties of cAMP and PKA indicators.

While these indicators provide significant opportunities to advance our understanding about the dynamics of the cAMP/PKA pathway, the results must be interpreted with caution. First, there may be a limitation to the sensitivity of the reporters. For example, during waves cAMP and PKA activity transients were observed in only a small subset of RGCs although all RGCs have spontaneous depolarizations. We found that depolarizations lasting less than one second did not reliably induce a detectable change in the FRET ratio of AKAR2.2 while depolarizations greater than two seconds did. We cannot determine from these experiments whether depolarizations less than one second induced changes in PKA activity that were below the sensitivity of our imaging system or whether activation of the cAMP/PKA pathway requires a threshold influx of calcium.

Second, the timecourse of the response is a convolution of the kinetics of the indicators and the second messenger cascade. For example, we found that the PKA-based cAMP sensor had a slower rise time to bath application of forskolin and IBMX than ICUE2. This may be due to the fact that a change in FRET ratio for the PKA-based cAMP sensor requires the binding of four cAMP molecules while ICUE2 requires the binding of a single cAMP molecule. Other indicators featuring a single cAMP binding site had comparable kinetics to ICUE2 (Nikolaev et al., 2004; Nikolaev et al., 2006). The timecourse of AKAR response is determined by the rate at which AKAR is determined by three components: cAMP accumulation, PKA phosphorylation rate, and the intrinsic kinetics of the indicator. Uncaging cAMP in cardiac myocytes led to an immediate increase in FRET ratio change of AKAR2, suggesting that the delay due to phosphorylation was less than five seconds (Saucerman et al., 2006). Therefore the predominant component of AKAR's time course is cAMP accumulation. However, since AKAR reports PKA activity, and a single PKA catalytic subunit can phosphorylate many target substrates, AKAR may have faster response kinetics than the indicators relying upon cAMP binding sites. Since AKAR rapidly responds to changes in cAMP concentration, the time to peak has been used as measure of the timecourse of the propagation of cAMP/PKA signals across the cytosol. In response to bath application of forskolin, the AKAR2.2 FRET ratio in the soma of hippocampal neurons increases immediately upon application of the drug, and reaches to 90% of its maximum in 2.5- min (Gervasi et al., 2007). In response to short depolarizations and short applications of forskolin the AKAR2.2 FRET ratio in the soma of retinal ganglion cells reaches to its maximum in 20 s. This difference with hippocampal neurons is likely due to the shorter stimulus time, as cAMP will continue to accumulate during constant exposure to forskolin. It is important to note that the temporal response of AKAR to phosphorylation by PKA, whether it is the onset of the response, the rate at which the ratio change increases and decreases, or the maximum FRET ratio change, may be different than the endogenous targets of PKA.

Third, overexpression of the various FRET indicators may disrupt the cAMP/PKA pathway in neurons through a variety of ways. 1) Indicators that contain high affinity binding sites for cAMP, may buffer cAMP and therefore interfere with the endogenous signaling and feedback

on the pathway. 2) Indicators, particularly the PKA-based and full length Epac-based indicators, may provide diffusion barriers that spatially sequester the cAMP more than usual. It has been suggested that the limited diffusibility of PKA allows it to serve as a barrier to cAMP diffusion in the same way that some calcium binding proteins limit the diffusion of calcium, thereby overemphasizing the presence of microdomains (Saucerman et al., 2006). 3) Catalytically active enzymes may alter PKA signaling in basal conditions, as was reported for FICRHR (Goaillard et al., 2001). However, in hippocampal neurons, AKAR overexpression did not block PKA-dependent phosphorylation of an endogenous ion channel indicating endogenous PKA substrates were not affected (Gervasi et al., 2007).

Fourth, it has been reported that FRET ratios decrease in response to nonspecific biochemical interactions with ATP (Willemse et al., 2007). Hence, it is critical to determine whether changes in FRET ratio are a result of activation of cAMP as opposed a result of changes in ATP. For this reason, it is advantageous to use pairs of indicators that have opposite FRET ratio changes in response to increases in the cAMP/PKA pathway, for example AKARs increase FRET ratio with increased PKA activity, while PKA- and Epac-based FRET sensors decrease FRET ratio with increased cAMP concentration.

Imaging the interplay of intracellular cAMP and calcium concentrations

The close interplay of cAMP and calcium pathways and the importance for both in guiding cellular responses to various inputs make combined imaging of the two an enticing prospect (DeBernardi and Brooker, 1996). For example in developing spinal cord neurons, certain patterns of calcium influx lead to transient increases in cAMP, and, reciprocally, cAMP levels regulated the frequency of calcium transients (Gorbunova and Spitzer, 2002) (Figure 3). This has led to the idea that the frequency of calcium transients is tuned to drive cAMP transients (Zaccolo and Pozzan, 2003; Borodinsky and Spitzer, 2006; Willoughby and Cooper, 2006). Simultaneous imaging of Epac based FRET reporters and the calcium indicator, fura-2, have led to detailed description of the interactions between calcium and cAMP oscillations in various non-neuronal cell types (Landa et al., 2005; Harbeck et al., 2006; Willoughby and Cooper, 2006). Applying these quantitative methods to spontaneously active neurons will yield tremendous insight into the function of periodic calcium and cAMP transients.

Hypotheses regarding the role of periodic activation of second messengers in neurodevelopment

There are several examples throughout the nervous system that indicate that infrequent activation of neurons is critical for driving specific developmental programs. These examples can be broken into two categories – regulation of transcription and post-translational modification. Using transcriptional assays, several groups have found that certain transcription factors are preferentially activated by rhythmic stimuli, not continuous stimuli (Itoh et al., 1995; Fields et al., 1997; Itoh et al., 1997; Dolmetsch et al., 1998; Li et al., 1998). In addition, altering the pattern of rhythmic activity in immature spinal cord neurons alters neurotransmitter expression, likely through transcriptional regulation (Borodinsky et al., 2004). Similarly, Hanson and Landmesser found that the frequency of rhythmic bursting in spinal motoneurons was necessary for expression of ephrinA4 (Hanson and Landmesser, 2004).

There is also evidence that periodic activation of networks alters post-translational modifications of existing proteins. Post-translation modifications such as phosphorylation are typically thought of as rapid onset, transient alterations of proteins. The repetitive nature of spontaneous activity offers a mechanism by which post-translational modifications might be maintained over long periods (Wu et al., 2001). For example, decreasing the frequency of periodic bursting in spinal cord neurons leads to a lack of polysialic acid on NCAM in spinal

motoneurons which leads to axon pathfinding errors, probably by way of failed defasciculation (Hanson and Landmesser, 2004). Whether this represents a general function of spontaneous activity in developing neurons remains to be determined.

Implications for activity-dependent developmental processes in visual system

Do slow oscillations in cAMP levels play a role in refinement of retinal ganglion cell projections? The cAMP/PKA pathway has been implicated by findings that show mice that lack adenylyl cyclase 1 (AC1) have both reduced eye-specific segregation of retinogeniculate axons within the dLGN (Ravary et al., 2003) and reduced retinotopic refinement in the SC (Ravary et al., 2003; Plas et al., 2004). AC1 is found in the retina, dLGN, and SC (Ravary et al., 2003; Plas et al., 2004; Nicol et al., 2005). Recently, Nicol et al. (Nicol et al., 2006) used a co-culture system to demonstrate that retinal explants from WT mice refine normally in the presence of explants from an AC1^{-/-} superior colliculus, while retinal explants from AC1^{-/-} mice failed to refine in the presence of SC explants from WT mice. Based on these studies, it was proposed that the failure in segregation is due to a defect of AC1 in the retina.

How does AC1 in the retina regulate activity-dependent refinement? First, it may be functioning cell autonomously in retinal ganglion cells. Recently, it has been reported that AC1 is necessary in retinal ganglion cells for repulsive responses to exogenous ephrin (Nicol et al., 2006). In addition, it was demonstrated spontaneous retinal activity is necessary for repulsive responses to ephrin (Nicol et al., 2007). This lack of responsiveness is rescued by uncaging cAMP in a periodic manner. These results lead to the intriguing hypothesis that retinal waves drive periodic activation of the cAMP/PKA pathway that is required for ephrin mediated repulsion. Second, AC1 modulates synaptic transmission at retinocollicular synapses (Shah et al., 2007), and therefore may be that the level of reading out the activity-dependent competition that leads to refinement (Katz and Shatz, 1996; Crair, 1999; Butts, 2002; Butts et al., 2007). Third, mice lacking normal level of phosphorylated CREB, a target of PKA activity have reduced map refinement, indicating a role of postsynaptic cAMP/PKA signaling in LGN neurons (Pham et al., 2001). By using imaging to determine the spatial and temporal properties of cAMP/PKA signaling, we will be able to gain insights in the specific developmental mechanisms by which activity influences several aspects of neural development.

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Figure 1. Schematics of genetically encoded, FRET based cAMP level and PKA activity sensors
 A. LEFT: Schematic of the PKA-based cAMP sensors. CFP and YFP are C-terminal fusions to the regulatory and catalytic subunits of PKA (Lissandron, 2005). MIDDLE: Fluorescence image of a dissociated retinal neuron expressing PKA based cAMP sensor. RIGHT: Time course of F_{CFP} (blue), F_{YFP} (yellow), and the FRET ratio (red) of F_{YFP}/F_{CFP} for the PKA-based cAMP sensor during application of both the adenylyl cyclase activator forskolin ($10\mu M$) and phosphodiesterase inhibitor IBMX ($100\mu M$). Bar represents time of drug applications. All ratios are corrected for CFP bleedthrough into YFP channel and differential bleaching of the two fluorophores. Elevation of cAMP leads to decreases in FRET ratio. Ratio trace is inverted to show increases in cAMP as upward deflections.

B. LEFT: Schematics of EPAC-based sensors: Different truncations of the exchange protein activated by cAMP (Epac) are sandwiched between a CFP/YFP FRET pair. The different variations have different binding affinities for cAMP (Ponsioen, 2004). MIDDLE: Fluorescence image of a dissociated retinal neuron expressing ICUE2. RIGHT: Time course of F_{CFP} (blue), F_{YFP} (yellow), and the FRET ratio (red) of $F_{\text{YFP}}/F_{\text{CFP}}$ for ICUE2 where elevation of cAMP leads to decreases in FRET ratio. Ratio trace is inverted to show increases in cAMP as upward deflections.

C. LEFT: Schematic of the PKA activity reporter, AKAR3. Modified from Zhang, 2001. A-kinase-activity reporter (AKAR) is a fusion of CFP and YFP to either a FHA1 or 14-3-3 phosphobinding region and PKA target substrate. Different variations have varying dynamic range and dephosphorylation rates. MIDDLE: Fluorescence image of a dissociated retinal neuron expressing AKAR2.2. RIGHT: Time course of F_{CFP} (blue), F_{YFP} (yellow), and the FRET ratio (red) of $F_{\text{YFP}}/F_{\text{CFP}}$ for the AKAR2.2 where elevation of PKA activity leads to increases in FRET ratio.

MIDDLE and RIGHT, Modified from Dunn, Wang et al. 2006

Fig. 2. Spontaneous changes in cAMP levels and PKA activity in retinal ganglion cells during retinal waves

A. Time course of F_{CFP} (blue), F_{YFP} (yellow) recorded simultaneously and averaged over the cell body of a RGC expressing ICUE2. The ICUE2 FRET ratio is computed as F_{YFP}/F_{CFP} , inverted to show cAMP increases as upward deflections, corrected for CFP bleedthrough into YFP channel, and corrected for differential bleaching of CFP and YFP.

B. Time course of F_{CFP} (blue), F_{YFP} (yellow) recorded simultaneously and averaged over the cell body of a RGC expressing AKAR2.2. The ratio is computed as F_{YFP}/F_{CFP} , corrected for CFP bleedthrough into YFP channel and corrected for differential bleaching. From Dunn, Wang et al., 2006.

Figure 3. Spontaneous oscillations in cAMP are driven by certain patterns of calcium spikes in spinal neurons

A. Surface plot images show a spontaneous, transient increase in cAMP concentration in an immature spinal neuron loaded with the ratiometric dye, FICRhR. Color scale represents the 520 nm/580 nm fluorescence ratio. (s) soma, (a) axon, (gc) growth cone. Scale bar, 10 μ m. B-D. Fluorescence ratio traces of F_{520}/F_{580} in a spinal neuron loaded with FICRhR show cAMP response to induced calcium influx. Arrows indicate stimulated Ca^{2+} spikes. (A) single spikes, (B) three spikes, (C) five spikes. From Gorbunova and Spitzer, 2002.