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ORIGINAL ARTICLE

Visualization of the dynamics of PSD-95 and Kir2.1 interaction by fluorescence lifetime-based resonance energy transfer imaging

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Summary Many cellular processes are orchestrated by protein—protein interactions that allow the formation of protein networks involved in subcellular compartmentalization, communication, and signalling. Postsynaptic density protein 95 (PSD-95), a member of the membrane-associated guanylate kinase protein (MAGuK) family, is a central scaffold protein of the postsynaptic density (PSD) of excitatory synapses in the mammalian central nervous system. PSD-95 serves as a matrix for targeting and accumulation of yet other PSD proteins including ion channels and receptors via its three N-terminal PDZ (PSD-95, discs large, zonula occludens-1) domains. However, the stoichiometry of PSD-95 and its binding partners in such complexes and the dynamic regulation of their interactions remain elusive. Here, we have investigated the protein—protein interaction between PSD-95 and the inward rectifier potassium channel Kir2.1, which we consider as a model for other PDZ domain based interactions at synapses. By using Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM), we show that PSD-95 and Kir2.1 directly interact within clusters which are formed at the plasma membrane of culture cells. Our in vivo FRET data indicate that Kir2.1 binds to more than one PDZ domain of PSD-95, suggesting a structural model of synergistic target binding by the first

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two PDZ domains of the scaffold protein. We show that the cluster formation is induced by the channel whereas PSD-95 alone does not form clusters. The interaction of PSD-95 and Kir2.1 is dynamically regulated by protein kinase A (PKA) mediated phosphorylation, which is directly visualized and quantified in living cells and in real time.

Introduction

Membrane-associated guanylate kinase proteins (MAGuKs) represent a family of scaffold proteins that organize intracellular trafficking, cellular adhesion, and localization of signalling complexes at cellular junctions (Funke et al., 2005). They are composed of multiple protein–protein interaction domains, which allow distinct target binding. The postsynaptic density protein 95 (PSD-95) is one of the best studied MAGuK members (Kim and Sheng, 2004; Gardoni et al., 2009). It is highly enriched in the postsynaptic density (PSD) of excitatory synapses, an electron-dense organelle opposed to the active zone of the presynapse. PSD-95 contains three PDZ (PSD-95, discs large, zonula occludens-1) domains, which mediate interaction with target molecules including certain receptors, ion channels, and cell adhesion molecules. PSD-95 binds to target proteins by recognizing special carboxy-terminal peptide motifs. For instance, PSD-95 interacts with NMDA receptor subunits and a number of potassium channels, that bear a C-terminal PDZ binding motif of the consensus sequence -X-S/T-X-V (Kornau et al., 1997; Sheng and Sala, 2001). Furthermore, PDZ domains of PSD-95 were reported for binding internal peptide sequences, which raises the possibility of protein–protein interactions (Brenman et al., 1996; Hillier et al., 1999; Tochio et al., 2000; van Ham and Hendriks, 2003).

Inwardly rectifying potassium (Kir) channels are homo- or heterotetrameric assemblies of pore-lining α-subunits, each containing two transmembrane segments. They have been categorized into seven subfamilies based on sequence homology and biophysical properties (Kubo et al., 1993; Doupnik et al., 1995; Fakler and Ruppersberg, 1996; Isomoto et al., 1997; Nichols and Lopatin, 1997; Bichet et al., 2003; Kubo et al., 2005). Kir2 subfamily members are well expressed in excitable tissue including brain, heart, and skeletal muscle (Nichols and Lopatin, 1997; Hibino et al., 2010; Lujan, 2010). They are characterized by strong inward rectification due to a voltage-dependent channel pore block by intracellular Mg^{2+} and polyamines (Lopatin et al., 1994; Fakler et al., 1995). This conductance property enables the channel to maintain the resting membrane potential and prevents shunting of action potential firing (Nichols and Lopatin, 1997; Lu, 2004).

In a previous study, we have used the Kir2.1 channel as a model target for synaptic PDZ interactions with PSD-95 to gain insight into the MAGuK-mediated clustering and immobilization of ion channels in general (Biskup et al., 2004). The Kir2.1 channel contains a C-terminal sequence motif (-RRESEI), by which it can bind to the first two PDZ domains of PSD-95, referred to as PDZ1 and PDZ2, which are arranged in a tandem like structure with an overall length of 6 nm (McCann et al., 2012). Residues upstream of the PDZ ligand motif add to PDZ recognition as they seem to contribute to binding specificity as indicated by NMR spectroscopy (Pegan et al., 2007). Up to twelve residues are involved in PDZ based interaction of PSD-95 and Kir2.1 ion channels. By exploiting Förster resonance energy transfer (FRET), we have shown that PSD-95 and Kir2.1 do not only colocalize within clusters at the plasma membrane, but directly interact with each other as they localize within close molecular vicinity to each other (<10 nm) (Biskup et al., 2004). However, our study did not reveal the stoichiometry of such PSD-95/ion channel interaction, their stability, and to what extent this interaction might be dynamically regulated. It is known that posttranslational modifications including phosphorylation may regulate protein–protein interactions. Thus, it has been shown that phosphorylation of residues within the PDZ ligand motif can abolish the interaction with PDZ domains (Pawson and Scott, 1997). For example, protein kinase A (PKA) mediated phosphorylation of Ser-440 of Kir2.3 uncouples the channel from the second PDZ domain of PSD-95 (Cohen et al., 1996; Jonas and Kaczmarek, 1996). The same consensus site for PKA phosphorylation is contained within the very C-terminus of Kir2.1.

For investigating the regulation of protein–protein interactions by phosphorylation, protein biochemical methods including co-immunoprecipitation and immobilized metal-ion affinity chromatography are often used. These techniques, however, suffer from major drawbacks, mainly a limited sensitivity and the risk of non-specific entrapment of other proteins or contaminants. Moreover, dynamic interactions and their regulation by the often short-lived events of phosphorylation and dephosphorylation cannot be followed up (Paradela and Albar, 2008).

In the present study, we employed FRET to monitor the dynamics of the PSD-95 and Kir2.1 interaction in real time. Fluorescence lifetime imaging microscopy (FLIM) was used to quantify the extent of FRET. Our approach allows for visualizing the protein–protein interaction and its regulation with high spatial and temporal resolution inside living cells. The resulting lifetime data were used to estimate the stoichiometry of the interacting proteins and the underlying structural arrangement of interaction domains. Our in vivo data indicate that Kir2.1 binds to more than one PDZ domain of PSD-95 and support former in vitro findings that suggested synergistic target binding by PDZ1 and PDZ2 (Long et al., 2003; Wang et al., 2009).

Materials and methods

Molecular biology

Fusion constructs of PSD-95 and Kir2.1 with fluorescent proteins were generated by subcloning respective cDNAs into pECFP-C1, pECFP-N1, pEYFP-C1, pEYFP-N1 vector plasmids