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The molecular architecture of neuronal kinase/phosphatase signalling complexes

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Introduction

The transduction of extracellular signals across the postsynaptic membrane to appropriate subneuronal targets is a tightly regulated process that underlies diverse physiological functions [1]. As many signalling enzymes are widely distributed and often have rather broad substrate specificities, a critical element in signal transduction is the control of specificity. How does an individual neurotransmitter direct the correct pool of second messengers to particular downstream enzymes, and in turn, how does each enzyme find its appropriate set of protein substrates? In some cases, specificity is achieved by enzymes that have limited or precise substrate recognition. However, recent evidence suggests that subcellular targeting through association with scaffold adapter and anchoring proteins is an important mechanism by which neurons localize protein kinases and phosphatases at sites where they can be accessed optimally by activators and also effect particular substrates [2]. In this article, we will focus on recent advances in our understanding of the molecular mechanisms that govern the cellular compartmentalization of kinases and phosphatases through the use of A kinase anchoring proteins (AKAPs). In addition, we examine how anchored signalling complexes consisting of both enzyme classes contribute to the modulation of neuronal ion channels.

General properties of AKAPs

The first AKAP to be identified was the microtubule-associated protein, MAP2, which was shown to copurify and interact directly with the cAMP-dependent protein kinase from brain extracts [3]. In the years since then, numerous AKAPs have been identified by gel overlays and expression library screening and over 25 AKAP cDNAs have been isolated [4,5]. Commonly these proteins are identified on the basis of their ability

Abbreviations used: AKAP, A kinase anchoring proteins; CFTR, cystic fibrosis transmembrane regulator; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C; PP1, protein phosphatase 1.

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to bind the type II regulatory subunit of the cAMP-dependent protein kinase *in vitro* although a more appropriate mode of classification is copurification with the protein kinase A (PKA) holoenzyme from tissue extracts. Collectively, these data support the notion that the AKAPs are a group of functionally related proteins, which all contain a structurally common RII binding site. However, their specificity is defined by a unique targeting domain that directs the PKA/AKAP complex to discrete subcellular compartments.

AKAP targeting interactions

A unique property of each AKAP is a targeting sequence that determines the location of the protein in the cell. A combination of subcellular fractionation and immunohistochemical studies have identified AKAPs in association with a variety of organelles including centrosomes, endoplasmic reticulum, Golgi body, mitochondria, nuclear matrix, peroxisomes, plasma membrane, and secretory granules. Although the subcellular location of many AKAPs has been determined, the precise mechanism of targeting has been determined for only a few molecules. Distinct splice variants of the dual specificity D-AKAP family, which bind RI or RII subunits, contain a consensus mitochondrial signal sequence, whereas isoforms lacking this sequence are targeted to the endoplasmic reticulum [6,7]. The small molecular mass anchoring protein AKAP15/18 is targeted to the plasma membrane through lipid modification of Gly1, Cys4 and Cys5, which are myristoylated and dually palmitoylated, respectively [8,9]. Recent evidence suggests that organelle targeting of AKAPs may be more sophisticated than originally believed. For example, two AKAPs, AKAP79 and yotiao, are located at the neuronal postsynaptic membrane [10]. Three polybasic targeting regions in AKAP79 participate in electrostatic interactions with membrane phospholipids, thereby directing the kinase in proximity to substrates on the inner face of the postsynaptic membrane.

Multienzyme signalling complexes

Although most AKAPs have been defined on the basis of their interaction with PKA, perhaps their most important property is their ability to bind additional signalling enzymes [11]. By simultaneously binding enzymes with opposing actions, such as kinases and phosphatases, these multivalent anchoring proteins may target entire signalling cascades to specific substrates. For example, AKAP79 binds PKA, protein kinase C (PKC) and the protein phosphatase calcineurin [12]. Each enzyme is inhibited when bound to the anchoring protein, providing an additional level of regulation. Because distinct activation signals are necessary to release and activate each enzyme, AKAP79 may provide a point of convergence for multiple second messenger signals, such as cAMP, calcium and phospholipids. Gravin targets both PKA and PKC to the cell periphery and filopodia of macrophage-like cells [13]. AKAP220, originally identified in association with peroxisomes, has been shown recently to bind protein phosphatase 1 (PP1) in addition to PKA. When bound, PP1 is inhibited, suggesting that AKAP220 may regulate phosphatase activity [14]. Other AKAPs have binding sites for other structural proteins. For example, the cytoskeletal component erzin, an RII binding protein, also binds EBP50/NHERF, a protein involved in regulation of sodium hydrogen transport in the apical membrane of epithelial cells. A pair of recent studies demonstrate that EBP50/NHERF binds the cystic fibrosis transmembrane regulator (CFTR) channel [15,16]. As cAMP-mediated signalling has been implicated in regulation of CFTR, these data implicate a potential role for anchored PKA via an erzin/EBP50 complex.

The consequences of PKA anchoring on ion channel function

The biological relevance of anchoring is underscored by recent functional studies using AKAPs as vectors to manipulate the subcellular distribution of PKA. To date, two approaches have been exploited: cellular disruption of PKA anchoring using inhibitor peptides and plasmids derived from Ht31; and the expression of compartment-specific AKAPs that redistribute the kinase to defined subcellular sites. Many of these studies have focused on rapid cAMP-responsive events such as modulation of ion channels. Initial studies demonstrated that perfusion of cultured hippocampal neurons with Ht31 peptides, displacing anchored PKA, caused a time-dependent rundown in AMPA-type glutamate receptor currents [17]. Using a similar approach, Ht31-mediated disruption of PKA anchoring has demonstrated a role for anchored PKA in the regulation of cardiac and skeletal muscle L-type calcium channels, brain sodium channels and calcium-activated potassium channels [18,19]. Heterologous expression of AKAP79 targets PKA to the periphery of cells in proximity to transmembrane proteins. This

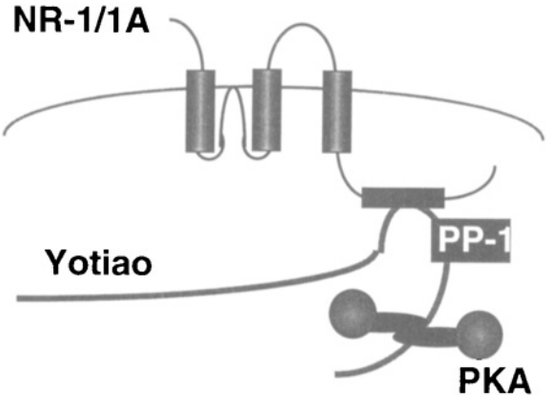
approach has been used successfully to demonstrate increased cAMP-dependent modulation of L-type calcium channels and ROM K potassium channels [18,20]. Likewise, transient transfection of AKAP18 into HEK293 cells expressing the cardiac L-type Ca^{2+} channel promoted a $34 \pm 9\%$ increase in cAMP-responsive Ca^{2+} currents. In contrast, a targeting deficient mutant of AKAP18 lacking the myristoylation and dual palmitoylation signals had no effect on Ca^{2+} currents in response to the application of a cAMP analogue. Further studies demonstrate that AKAP18 facilitates GLP-1-mediated insulin secretion in a pancreatic beta cell line (RINm5F), suggesting that membrane anchoring of the kinase participates in physiologically relevant cAMP-responsive events that may involve ion channel activation [8]. In the remainder of this article we will focus on a novel AKAP, yotiao, that associates directly with the *N*-methyl-D-aspartate (NMDA)-responsive glutamate receptor ion channels.

Yotiao

Clustering and immobilization of neurotransmitter receptors and ion channels is maintained by an intricate system of protein–protein interactions. For example, NMDA receptors are clustered and coupled to the cytoskeleton through association with PDZ proteins, α-actinin and neurofilaments. Many signalling pathways converge on the NMDA receptor allowing the regulation of channel activity in response to the generation of second messengers such as Ca^{2+} and cAMP [21,22]. Accordingly, PKA and PP1 activity have been demonstrated to modulate NMDA receptor function and appear to act in opposition to each other.

Figure 1

Model for the regulation of NMDA receptor currents by the yotiao signalling complex



Recently, a two-hybrid screen for proteins that bind the NMDA receptor subunit NR1A identified a protein called yotiao that interacts with the C-terminal C1 exon cassette of the ion-channel [23]. Independently, we isolated cDNAs encoding fragments of yotiao by an interaction cloning strategy to identify AKAPs.

Immunoprecipatations with yotiao antiserum from brain extracts isolated an RII-binding protein and were enriched several-fold for PKA catalytic subunit activity. Additional support for a yotiao/PKA complex inside cells was provided by immunocytochemistry as both proteins exhibited distinct but overlapping staining patterns in the soma and dendrites of cultured hippocampal neurons. Further mapping studies demonstrated that residues 1440–1457 of yotiao appear to include the principal determinants for RII interaction as a synthetic peptide encompassing this region blocked RII binding (see Figure 1). In sum, these findings suggest that yotiao functions to anchor PKA to NMDA receptors in neurons.

On the basis of evidence that PP1 contributes to the regulation of NMDA receptors, we performed experiments to address whether PP1 associated with yotiao. Immunoprecipitation of yotiao from brain extracts resulted in the co-purification of PP1 and the phosphatase bound to recombinant fragments encompassing residues 1171–1229 of yotiao *in vitro*. Additional experiments demonstrated that the PP1 targeting inhibitor peptide (Gm) blocked solid-phase interaction with the phosphatase and that yotiao binding had no effect on PP1 activity towards a NR1A receptor fragment as a substrate. These later results demonstrate that yotiao is not an inhibitor of PP1 activity, suggesting that yotiao targets active PP1 to the NMDA receptor.

Our biochemical studies demonstrate that yotiao anchors PKA and PP1 to the NR1A subunit of the NMDA receptor, suggesting that yotiao enables the modulation of receptor function by these enzymes. Whole-cell recordings of NMDA receptors expressed in HEK293 cells were performed to test this hypothesis. Application of cAMP rapidly and reversibly enhanced NMDA currents in yotiao-expressing cells. Additional control experiments demonstrated that the cAMP effect was blocked by whole-cell dialysis of the PKA inhibitor peptide PKI 5–24 or the RII anchoring inhibitor peptide Ht31 indicating that an anchored pool of PKA was responsible for augmentation of the current. In order to test for a role of yotiao-dependent PP1 anchoring in the

modulation of NMDA receptor activity, the PP1 targeting inhibitor peptide was dialysed into cells via the patch pipette. A time-dependent increase in NMDA receptor currents was observed in yotiao-expressing cells that plateaued within 5–10 min of establishing the whole-cell configuration. These results indicate that tonic PP1 activity associated with yotiao negatively regulates NMDA receptors.

As yotiao binds to NR1 splice variants containing the C1 exon, we propose the following model for modulation of heteromultimers in which this subunit is present (Figure 1). Under resting conditions targeting of a constitively active phosphatase favours dephosphorylation of the channel or a closely associated protein. However, upon elevation of cAMP, PKA is released from anchored sites shifting the equilibrium in favour of phosphorylation and results in enhancement of NMDA receptor activity. Although other kinase/phosphatase scaffold proteins have been identified, yotiao represents an additional level of molecular organization because it facilitates the dynamic regulation of an individual phosphoprotein by assembling a phosphatase/kinase signalling complex that is attached to the substrate. In fact, the anchoring of enzymes that work in functional opposition to each other makes yotiao distinct from other channel-modulating proteins such as InaD or AKAP15/18, that localize only kinases, and AKAP79, which anchors PKA, PKC and PP2B in an inactive state [24]. As a number of ion channels appear to be modulated by closely associated kinases and phosphatases it is reasonable to assume that other structural elements similar to yotiao exist.

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