Control of protein phosphatase I in the dendrite

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Communication between nerve cells is mediated by both electrical and chemical signals. Chemical neurotransmission can be further categorized into fast and slow components. Fast acting neurotransmitters directly influence neuronal electrical excitability by binding to cell surface receptors which serve as ligand-gated ion channels, thereby directly modulating membrane potential and cell firing. Neurotransmitter receptors may otherwise modulate neuronal excitability indirectly, by coupling to intracellular signalling pathways that impact on the functional activity of ligand- and voltage-gated ion channels, ion pumps, and the machinery for chemical neurotransmission. These indirect actions are relatively slow, and often involve cascades of protein phosphorylation which serve to alter the biochemical activities of substrate proteins, and hence cellular physiology.

Recent work has indicated that protein phosphatase 1 (PP1) is an important component of the signalling machinery that controls synaptic transmission. In the rat hippocampus, pharmacological inhibition of protein phosphatase activity prevents the induction of long-term depression (LTD) [1], a use-dependent decrease in synaptic efficacy that may contribute to the process of information storage in the brain [2]. Further work used the PP1 regulatory protein, inhibitor 1 (I1), to show that PP1 was specifically responsible for this effect. I1 is converted from an inactive enzyme into a potent PP1 inhibitor following phosphorylation by cAMP-dependent protein kinase A (PKA). When phospho-I1 peptides were infused into CA1 pyramidal cells, LTD induction was blocked [3]. Complementary experiments have addressed the role of PP1 in the induction of long-term potentiation (LTP). Pharmacological inhibition of phosphatases removed the requirement for PKA activation in the induction of LTP in CA1 pyramidal cells [4]. Infusion of phospho-I1 peptides substituted for the requirement for PKA activity in LTP induction. Thus, in contrast with LTD, which requires PP1 activity, LTP is promoted by PP1 inhibition. The physiological role played by I1 at the Schaffer collateral/CA1 synapse has recently been questioned following the examination of I1 knockout mice, which display normal synaptic plasticity at this synapse [5]. This suggests that additional neuronal PP1 regulatory elements may exist.

PP1 has been shown to be present at synaptic junctions [6] and is specifically enriched in dendritic spines [7], postsynaptic specializations that receive the vast majority of excitatory input in the brain. Several candidate substrates for PP1 exist at the synapse. The phosphorylation state and activity of calcium/calmodulin-dependent protein kinase II (CaMKII) has been shown to be regulated by PP1 specifically within the postsynaptic density compartment [8,9]. CaMKII is an attractive substrate molecule through which PP1 might mediate its effects on excitatory synaptic transmission. CaMKII has itself been shown to be enriched in dendritic spines and exerts a strong influence on synaptic efficacy [10–12]. Additional potential PP1 substrate molecules that govern synaptic plasticity include the *N*-methyl-D-aspartate (NMDA) receptor [13], protein kinase C [14], and in the nucleus, the cAMP response element-binding (CREB) protein [15]. Thus, PP1 plays a diverse role in the regulation of neuronal substrates. In fact, PP1 is involved in many different cellular processes, including regulation of gene expression, cell division cycle, glycogen metabolism, and muscle contraction [16]. How is the activity of the enzyme directed specifically to the substrates involved in these diverse cellular processes? The answer to this appears to be that PP1 is regulated by a family of proteins that are termed 'targeting subunits' [17]. These regulatory proteins are distinct from proteins in the I1 class, which are small and soluble; targeting subunits are typically large and display characteristics of cytoskeletal proteins, often being relatively insoluble in cell extracts and showing distinct sub-

Abbreviations used: AMPA, α-amino-3-hydroxy-S-methyl-4-isoxazolepropionic acid; CaMKII, calcium/calmodulin-dependent protein kinase II; CREB, cAMP response element-binding protein; DARPP32, dopamine and cAMP-regulated phosphoprotein; I1, inhibitor 1; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; PNUTS, phosphatase 1 nuclear targeting subunit; PP1, protein phosphatase 1.

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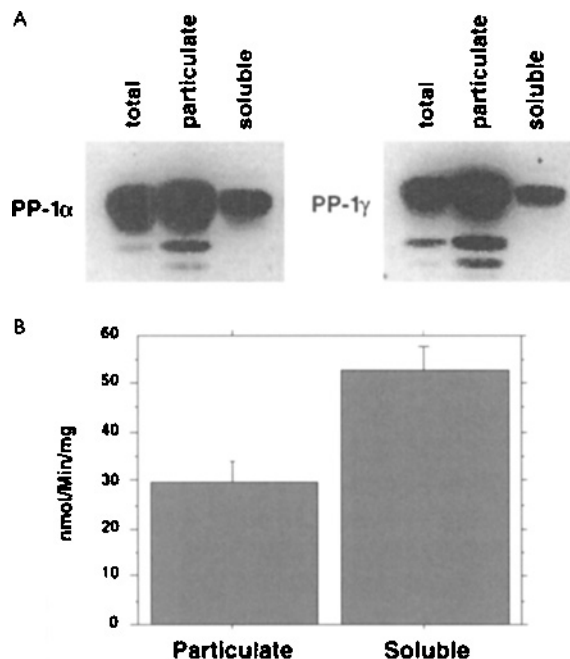
Figure 1

Distribution and activity of PP1

(**A**) Immunoblots showing the relative distribution of the α and γ isoforms of PP1 in rat brain homogenate fractions. Loading was normalized for protein content (50 μg/lane). (**B**) Catalytic activity of PP1 towards phosphorylase *a* measured in rat brain homogenate fractions normalized for total protein content. Rat brain cortex was lysed by sonication in 50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 20 μg/ml leupeptin/antipain and 5 μg/ml pepstatin/chymostatin. Homogenate was centrifuged at 19000 **g** for 20 min. Soluble and particulate suspensions were assayed for PP1 activity as described previously.



cellular localizations. Targeting subunits generally bind to PP1 with high affinity and as a result dictate the subcellular localization of PP1, thereby placing the enzyme in proximity to the substrates that are responsible for controlling a given cellular process. In addition to dictating localization, targeting subunits may also modulate PP1 catalytic activity toward selected substrates in response to specific intracellular signals [17].

Preliminary evidence for the existence of PP1 targeting proteins in the brain came from cellular fractionation studies. Particulate and soluble fractions of cortical homogenate were separated by high-speed centrifugation. The majority of the PP1 catalytic subunit was found in the particulate fraction. However, when the catalytic activity of PP1 in these two fractions was measured, using phosphorylase *a* as substrate, the majority of the activity was found in the soluble fraction (Figure 1). Purified PP1 is highly soluble. This implies that much of the PP1 in cortical homogenate is associated with proteins in the insoluble fraction, and that this association inhibits the enzyme.

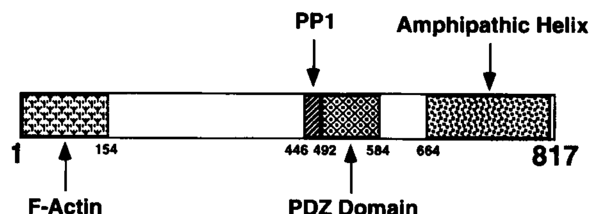
Isolation and characterization of novel PP1-interacting proteins

Using the α isoform of the catalytic subunit of PP1 as a bait, a rat brain cDNA library was screened using the yeast two-hybrid expression cloning system [18]. Several different clones were identified based on restriction enzyme digestion and sequence analysis. These included cDNAs for the PP1-binding proteins spinophilin [19], phosphatase 1 nuclear targeting subunit (PNUTS) [20] and p53BP2 [21]. Of these proteins, spinophilin has been most thoroughly characterized: it is found in a complex with PP1 in rat brain lysates, and is a potent inhibitor of PP1 activity *in vitro*. The name spinophilin is based on the specific localization of the protein in the dendritic spine compartment, a subcellular organelle in which PP1 is also specifically enriched [7]. This localization may be highly significant with respect to the role played by PP1 in the regulation of synaptic plasticity; dendritic spines are thought to contain the signal transduction machinery responsible for the regulation of glutamatergic synaptic transmission. Spinophilin contains a single PDZ domain (Figure 2); this domain is present in many cytoskeletal proteins and often serves to mediate binding to the terminus of transmembrane proteins. In the brain, the PSD-95 family of PDZ domain-containing proteins has been shown to bind to NMDA receptors and Shaker-type K^+ channels and to promote the clustering of these receptors in heterologous cells [22–24]. A second class of PDZ domain-containing proteins, the GRIP/ABP family, binds to the GluR2 glutamate

Figure 2

Domain organization of spinophilin

The N-terminus of the molecule binds to, and can bundle, F-actin. The PP1-binding domain lies immediately upstream of the PDZ domain. The amphipathic helix is thought to mediate the formation of a coiled-coil spinophilin homodimer.



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receptor subunit [24]. This implies that spinophilin might also be associated with a transmembrane protein, possibly an ion channel or neurotransmitter receptor that resides in the spine. The binding site for PP1 has been mapped to a position immediately adjacent to the PDZ domain [25]. Thus, spinophilin may place PP1 in an opportune position to exert its control over substrates that govern synaptic strength.

Spinophilin also contains a region predicted to form a coiled-coil. Recent two-hybrid analysis showed that this region of spinophilin can bind to itself, indicating that the molecule may exist as a homomultimer *in vivo*. The amino terminus of spinophilin binds to, and will bundle, F-actin [26], suggesting that spinophilin is an integral part of the dense actin filament network that exists in dendritic spines. Scanning of the spinophilin sequence for consensus motifs for phosphorylation reveals the presence of many potential sites that are clustered in the N-terminal actin-binding region. Phosphorylation at these sites has the potential to alter functional interactions with the actin cytoskeleton. In addition, PP1 has been implicated in the control of the actin–myosin cytoskeleton in non-neuronal cells [27]. This suggests that the spinophilin/PP1 complex may play a role in regulating actin dynamics in the spine and thereby control spine morphology and turnover. In developing rat cortex, spinophilin expression peaks at a time when spines are being formed [19,28] and spine structure is thought to influence synaptic efficacy [28]; therefore, modulation of the cytoskeleton provides a potential mechanism for the spinophilin/PP1-mediated regulation of synaptic transmission.

An additional contribution to the synaptic targeting of PP1 is probably provided by a protein that is related in structure to spinophilin. This protein, identified based on its ability to bind to F-actin, was named neurabin (neuronal actin binding protein) [29]. Neurabin was also found to bind to PP1 [26]. Recently, p70 ribosomal S6 kinase was shown to bind to the PDZ domain of neurabin [30]. This kinase also binds to the PDZ domain of spinophilin (P. E. Burnett and P. B. Allen, unpublished work). These results raise the possibility that a p70 ribosomal S6 kinase/spinophilin/neurabin/PP1 complex may serve to regulate local protein synthesis at the synapse. New protein synthesis is detected in response to synaptic stimulation and may be required for certain forms of long term plasticity [31,32].

Fine mapping of the PP1 binding site in spinophilin revealed that PP1 binds to a motif that is found in other PP1 regulatory proteins, namely, R/K-R/K-Hydrophobic-X-F/W [25,33]. Mutation of the phenylalanine residue to alanine abolished the binding of PP1 to spinophilin. A short peptide encompassing this site does not inhibit PP1 activity, but is still capable of binding to PP1, as shown by competition assays with full-length spinophilin [25]. When added to immunoprecipitation reactions, the spinophilin peptide antagonized the interaction with PP1. This antagonist peptide was infused into acutely dissociated striatal neurons in the whole-cell patch clamp mode to assess the physiological consequences of disrupting the PP1–spinophilin complex [34]. The paradigm examined was the time-dependent reduction of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) current response which is seen after patching on to these cells. This run-down effect can be prevented by the application of a dopamine D1 receptor agonist, which is consistent with the observation that AMPA responses are potentiated by PKA-dependent phosphorylation [35,36]. In addition, run-down was prevented by inhibiting protein phosphatases with okadaic acid, or by the infusion of a PP1 inhibitor, phospho-dopamine and cAMP-regulated phosphoprotein (DARPP32) peptide [37]. Infusion of the spinophilin antagonist peptide also prevented the AMPA current run-down, and infusion of control spinophilin peptide, incorporating the phenylalanine to alanine mutation that abolishes PP1 binding to spinophilin, had no effect on run-down. These results suggest that spinophilin-mediated targeting of PP1 to the AMPA receptor serves to maintain the channel in a dephosphorylated, less active state. The channel is stimulated by PKA-mediated phosphorylation; PKA simultaneously inhibits PP1 activity due to the phosphorylation and activation of the PP1 inhibitor, DARPP32. In binding to PP1, phospho-DARPP32 presumably also displaces PP1 from spinophilin and the AMPA channel. The results support the idea that spinophilin is a *bona fide* neuronal PP1 targeting protein.

Not all PP1 binding proteins found in the nervous system are restricted to dendrites. For instance, one of the PP1 interacting proteins identified in the yeast two-hybrid screen was localized discretely to the cell nucleus, earning this protein the acronym PNUTS (phosphatase 1 nuclear targeting subunit) [20]. PNUTS has a very high affinity for PP1 and inhibits catalytic

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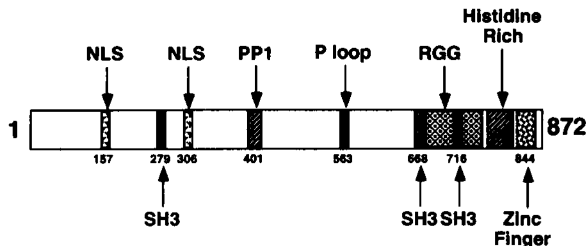
Figure 3

Domain organization of PNUTS

NLS, nuclear localization signal consensus motif; SH3, consensus for SH3 domain binding; PP1, PP1-binding site; P loop, nucleotide binding consensus motif; RGG, Arg/Gly/Gly repeat boxes implicated in RNA binding; histidine rich, region containing multiple imperfect direct repeats.



activity with an IC_{50} in the picomolar range. Several motifs can be identified from the predicted amino acid sequence that give some clue as to possible function (Figure 3). These include the expected nuclear localization signal, RGG boxes (which are implicated in RNA binding) and a putative zinc finger. Given the localization and structure of PNUTS it seems possible that it may be involved in controlling the established role that PP1 plays in regulating gene expression [38]. In this way PNUTS–PP1 may contribute to the regulation of long-term changes in neuronal activity.

In the future, it will be of interest to identify additional PP1 substrates that are involved in neuronal function and to characterize further the role played by PP1 regulatory subunits in the integration of signals that control the postsynaptic response.

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Received 1 January 1999

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