Molecular Neurobiology

ISSN0893-7648/92/5/2-3-4/229–246/$3.60

The Regulation and Function of Protein Phosphatases in the Brain

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Contents

Abstract

Data emerging from a number of different systems indicate that protein phosphatases are highly regulated and potentially responsive to changes in the levels of intracellular second messengers produced by extracellular stimulation. They may therefore be involved in the regulation of many cell functions. The protein phosphatases in the nervous system have not been well studied. However, a number of neuronal-specific regulators (such as DARPP-32 and G-substrate) exist, and brain protein phosphatases appear to have particularly low specific activity, suggesting that neuronal protein phosphatases possess considerable and unique potential for regula-

tion. Several early events following depolarization or receptor activation appear to involve specific dephosphorylations, indicating that regulation of protein phosphatase activity is important for the control of many neuronal functions. This article reviews the current literature concerning the identification, regulation, and function of serine/threonine protein phosphatases in the brain, with particular emphasis on the regulation of the major protein phosphatases, PP1 and PP2A, and their potential roles in modulating neurotransmitter release and postsynaptic responses.

Index Entries: Protein phosphatase; phosphoprotein phosphatase; dephosphorylation; brain; nervous system; depolarization; neurotransmitter; exocytosis; receptor activation.

Introduction

Protein phosphorylation is a dynamic process involving the action and regulation of both protein kinases and protein phosphatases. Although studies of protein kinases have been and continue to be extensive, protein phosphatases have been less well studied. This is particularly true in the nervous system, where protein phosphorylation is associated with the regulation of a variety of physiological processes, and where protein kinases and phosphatases are highly concentrated. Despite the availability of considerable information concerning neuronal protein kinases and their substrates, precise roles for protein phosphorylation remain unknown, and protein dephosphorylation has not been investigated in detail. It is clear that dephosphorylation will be equally important in cellular events involving protein phosphorylation, and regulation of protein phosphatases may prove to be a key event in the regulation of some of these processes. This article will provide an overview of the current status of knowledge concerning the nature and regulation of protein phosphatases, together with a detailed account of the protein phosphatases in the brain as a basis for future investigation into the role and regulation of these enzymes in neuronal function. Particular attention will be given to protein phosphatases types 1 and 2A, which are the major serine/threonine protein phosphatases in all cells, and emphasis will be placed on the mechanisms by which extracellular signals might regulate their activities. Several excellent reviews exist on the tyrosine phosphatases (Tonks et al., 1989; Hunter, 1989) and the calcium-dependent protein phosphatase, calcineurin (Klee et al., 1988; Klee and Cohen, 1988), as well as protein phosphatases in general (Cohen, 1989, Cohen and Cohen, 1989; Shenolikar and Nairn, 1990; Stemmer and Klee., 1991), to which readers are referred for details.

Classification and Regulation of Protein Phosphatases

The serine/threonine protein phosphatases comprise four main classes of enzymes, each of which represents a family of multiple isozymes, and can be identified based on its substrate specificities and sensitivity to specific inhibitors (Cohen, 1989) (Table 1). Protein phosphatase 1 (PP1) is selective for the b-subunit of phosphorylase kinase and is inhibited by two heat-stable inhibitor proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2). Type 2 protein phosphatases are selective for the α-subunit of phosphorylase kinase and are insensitive to I-1 and I-2. Type 2 protein phosphatases are subdivided into three distinct classes based on their cationic requirements. PP2A is active in the absence of divalent cations, whereas PP2B and PP2C are completely dependent on calcium and magnesium, respectively. These four activities may also be distinguished by their sensitivity to the marine toxin, okadaic acid (Haystead et al., 1989; Cohen et al., 1989). At a concentration of 1 n*M* okadaic acid, PP2A is completely inhibited, whereas significantly higher concentrations are required to inhibit PP1 and PP2B (PP2B being least sensitive). PP2C is insensitive to okadaic acid. An alternative classifica-

Table 1
Classification of Protein Phosphatases

Phosphatase class	Phosphorylase kinase substrate	I-1/I-2 sensitive	Okadaic acid sensitivity	Cation
PP1	β	Yes	1 μM	None
PP2A	α	No	1 μM	None
PP2B	α	No	>5 μM	Ca^{2+}
PP2C	α	No	None	Mg^{2+}

tion regime identifies these phosphatases as the AMD-phosphatase (ATP- and magnesium-dependent; PP1), PCS (polycation-stimulated; PP2A), calcium-dependent (PP2B; calcineurin), and magnesium-dependent (PP2C)(Merlevede, 1985). Four separate, highly conserved protein phosphatase catalytic subunits have been identified that account for these four different catalytic activities. PP1 is the most highly conserved enzyme known and shares approximately 40% homology with PP2A (Berndt et al.,1987), suggesting that they belong to the same gene family. PP2B shares some homology with PP2A (Cohen and Cohen, 1989), but PP2C is derived from a distinct gene family (Tamura et al., 1989). At least two isozymes, arising from separate genes of each phosphatase catalytic subunit, have been detected (Ohkura et al., 1989; Stone et al., 1987; Kincaid et al., 1988; McGowan and Cohen, 1987). Although present in all species and tissues examined, these isozymes are expressed in different amounts (Cohen and Cohen, 1989), and may therefore have different functions or substrate specificities.

In addition to these four enzymes, cDNA cloning has identified, in various tissues, five novel protein phosphatase genes termed PPV, PPX, PPY, and PPZ, and PP2Bw. PPX from liver shows 49% identity to PP1 and 69% identity to PP2A (da Cruz e Silva et al., 1988) as does PPV (Cohen et al., 1990). PPY from *Drosophila* also has homology that is intermediate between PP1 and PP2A, although it is more closely related to PP1 than PP2A (Dombradi et al., 1989). PPZ was found in mammalian brain, and has 68 and 44% homology with PP1 and PP2A, respectively (Cohen et al., 1990). PP2Bw is a novel calcium- and calmodulin-dependent phosphatase that is only 62% homologous to PP2B (Cohen et al., 1990). It has recently been demonstrated that both PPZ and PP2Bw are in fact yeast enzymes and not brain phosphatases as previously thought (da Cruz e Silva et al., 1991).

A fundamental dilemma in the study of protein phosphatases has been explaining how four families of enzyme with overlapping and/or broad substrate specificities can have specific actions on selected substrates. Evidence is now accumulating to suggest that protein phosphatases may be regulated in a variety of ways in response to extracellular stimulation (Cohen and Cohen, 1989) and may therefore be involved in the regulation of many cell functions.

Protein Phosphatase 1

The regulation of PP1 has been the most fully investigated, and several important regulatory mechanisms have been identified in non-neuronal tissues. Figures 1–3 summarize these findings. The heat-stable protein, I-1, is a highly selective, potent inhibitor of PP1 when phosphorylated by PKA and is also an excellent substrate for the calcium-dependent phosphatase PP2B (Nimmo and Cohen, 1978). Dephosphorylation of I-1 inactivates it, releasing the active catalytic subunit of PP1 (PP1 c) (Fig. 1). Thus, signals that generate two major intracellular second messengers (cAMP and calcium) which do not directly affect PP1, may modulate its activity through phosphorylation and dephosphorylation of I-1 (Hemmings et al., 1984).

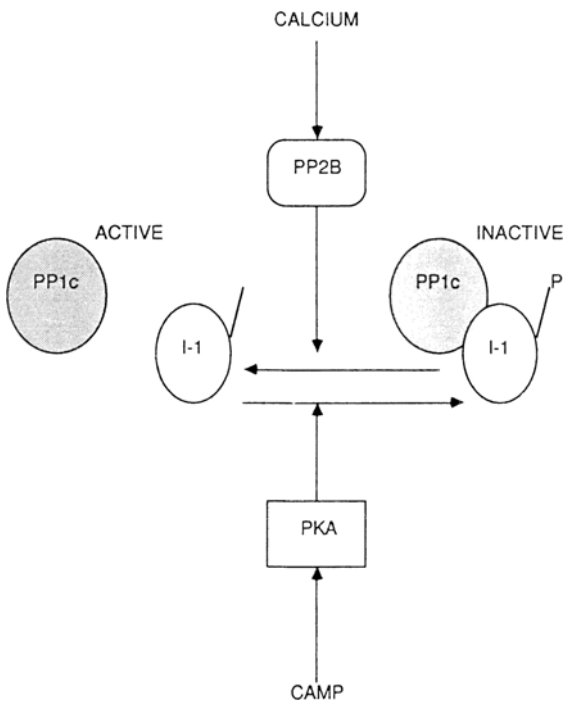

Fig. 1. Regulation of protein phosphatase-1 by inhibitor-1. PP1c = catalytic subunit of PP1; I-1 = inhibitor-1; PKA = cyclic AMP-dependent protein kinase; PP2B = calcium/calmodulin dependent protein phosphatase (Calcineurin).

PP1 is also inhibited by I-2 (Fig. 2), and can be isolated as an inactive complex (termed PP1-I) composed of I-2 and the catalytic subunit of PP1 (Hemmings et al., 1982). This form of PP1 is only transiently activated upon phosphorylation of threonine-72 (Cohen, 1989) on I-2 by an activating factor (FA) now known to be glycogen synthase kinase 3 (GSK3). I-2 is also phosphorylated on at least three serine residues by caseine kinase II (CKII) (Holmes et al., 1986), which serves to promote the phosphorylation of threonine-72 (DePaoli-Roach, 1984). The mechanisms governing the regulation of GSK3 and that of CKII are unknown, but GSK3 has been proposed to be a transmembrane signal for insulin and epidermal growth factor (Yang et al., 1989),

The substrate specificity of PP1 may be controlled by a number of different regulatory subunits both in different tissues and within the same

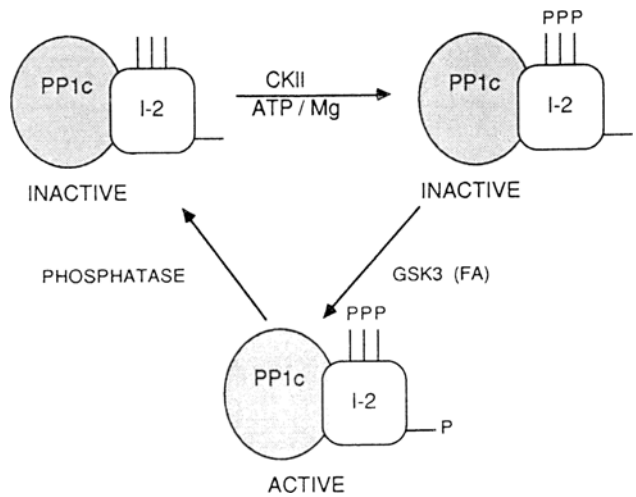

Fig. 2. Regulation of the inactive cytosolic form of protein phosphatase-1 (PP1-I). I-2 = PP1-inhibitor-2; CKII = caseine kinase II; GSK3 (FA) = the activating kinase, glycogen synthase kinase 3.

tissue. These regulatory subunits direct PP1 activity toward specific subcellular localizations (Fig. 3), and therefore toward specific substrates, through modulation of the phosphorylation state of a number of different regulatory subunits. In skeletal muscle, the glycogen-bound subunit of PP1 (G), when dephosphorylated, binds the catalytic subunit of PP1 to glycogen particles and enhances the dephosphorylation of glycogen-bound substrates (Hubbard and Cohen, 1989b). Like I-1, this subunit is phosphorylated by PKA and is an excellent substrate for PP2B (Hubbard and Cohen, 1989a). Thus, the phosphorylation/dephosphorylation of specific substrates may also be regulated by signals that generate cAMP and calcium. The glycogen-binding regulatory subunit also targets the catalytic subunit of PP1 to sarcoplasmic reticulum (Hubbard et al., 1990), but a different subunit directs it toward myosin (Chisholm and Cohen, 1988). The glycogen-bound regulatory subunit in liver is also distinct from that in skeletal muscle (Kikuchi et al., 1990).

Protein Phosphatase 2A

Mechanisms for the regulation of PP2A are largely unknown. However, the existence of sev-

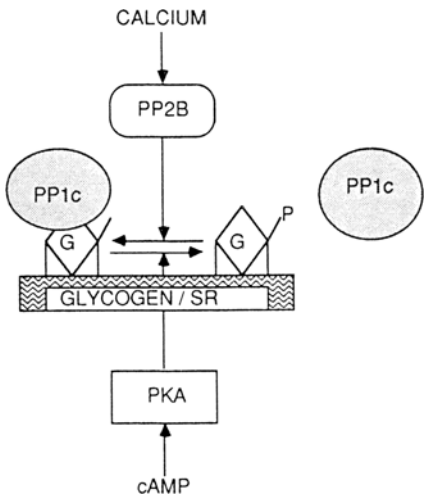

Fig. 3. Regulation of the glycogen-bound form of protein phosphatase-1 in rabbit skeletal muscle. Note that both bound and free forms of PP1 are active, but glycogen binding modifies activity toward colocalized substrates. G = glycogen-binding regulatory subunit of protein phosphatase-1; SR = sarcoplasmic reticulum.

eral potential regulatory mechanisms for PP1 that serve to limit its otherwise broad substrate specificity suggests that mechanisms should also exist to regulate the broad substrate specificity of PP2A. Indeed, it is now becoming clear that PP1 and PP2A regulate distinct processes in many cells (Kinoshita et al., 1990), and mechanisms must exist for the control of both phosphatases. PP2A may exist in a number of different holoenzyme forms, four of which have been purified (Cohen, 1989). Each enzyme contains the same catalytic subunit associated with various combinations of regulatory subunits of 65 kDa (A subunit), 55 kDa (B subunit), and 72 kDa. A 54-kDa form of the B subunit, termed B', has also been identified (Tung et al.,1985). Although little is known about the function and regulation of these multimeric forms of PP2A, they do appear to have different tissue distribution and substrate specificities (Usui et al., 1988; Waelkens et al., 1987; Chen et al., 1989). The regulatory subunits in general appear to reduce the activity of the catalytic subunit, but may also serve to modulate or target activity towards certain substrates. A potential phosphorylation site on the 65-kDa subunit has recently been found that may regulate its association with the catalytic subunit (Hemmings et al., 1990). There is also evidence to suggest that PP2A activity toward acetyl Co A carboxylase in rat liver is inhibited by PKA (Haystead et al., 1990), although the mechanism is not known. PP2A is also activated by basic proteins and polyamines, such as protamine and spermine, the extent of which varies depending on the holoenzyme being investigated (Waelkens et al., 1987). Although the normal cellular concentration of spermine is within the range at which it can regulate PP2A (Cornwell et al., 1987), it is not known if these substances are physiological regulators of the phosphatase.

Recently it has been shown that application of TPA to mouse skin stimulates a type 2A phosphatase (Gschwendt et al., 1989). Although it has not been shown if this is a direct or PKC-mediated effect, the ability of TPA to activate a phosphatase is intriguing and may have important physiological implications. These findings together, raise the possibility that, like PP1, PP2A activity can be modulated by different second messengers, either directly or through various cascade mechanisms involving PKA and PKC and, therefore, other protein phosphatases.

Protein Phosphatase 2B (Calcineurin)

PP2B has been comprehensively reviewed elsewhere (Klee et al., 1988; Klee and Cohen, 1988; Stemmer and Klee, 1991) and will only be considered briefly here. PP2B as a mediator of the effects of calcium on exocytosis and neurotransmitter release is also considered in another article of this volume (Robinson, 1992). Despite being highly concentrated in brain, specific functions for PP2B remain unknown. PP2B has a much narrower substrate specificity than the other main phosphatases, the majority of its most effective substrates being regulators of other protein kinases or phosphatases (Klee and Cohen, 1988). Thus, the major role of PP2B may be to mediate the

effect of extracellular signals that elevate calcium, on other components of second messenger systems not directly affected by calcium.

PP2B is a heterodimer composed of equal amounts of A (61 kDa) and B (19 kDa) subunits. The A subunit contains the catalytic and calmodulin binding domains, whereas the B subunit binds calcium and is highly homologous to calmodulin (Klee et al., 1988). In addition to regulation by calcium, the activity of PP2B may be modulated by myristoylation (Aitken et al.,1982), a property that may target PP2B to membrane structures. PP2B can be phosphorylated by calmodulin-dependent protein kinase II (CMKII) and protein kinase C at the same site (Tung, 1986; Hashimoto and Soderling, 1989), but although this does appear to inhibit the enzyme by increasing the K_m for the substrate, these post-translational modifications have not been shown to occur in vivo. Proteolysis of the A subunit renders PP2B fully independent of calmodulin and only partially dependent on calcium (Hubbard and Klee, 1989), but the activation is irreversible and therefore unlikely to be an important physiological regulatory mechanism. An autoinhibitory domain has also been identified that interacts with the active site (Hashimoto et al., 1990). This interaction may account for the low basal activity of PP2B in the absence of calcium/calmodulin.

Protein Phosphatase 2C

Protein phosphatase 2C (PP2C) is a monomeric protein of approximately 43 kDa, and at least two isoenzymes have been isolated (McGowan and Cohen, 1987). Beyond its dependence on magnesium, which is unlikely to regulate the phosphatase in vivo, nothing is known about the regulation of this phosphatase and specific substrates, or functional roles have not been identified. The overall contribution of PP2C to cellular dephosphorylation is relatively minor, although specific roles in regions where the contributions of PP1 and PP2A may be limited cannot be ruled out.

Identity and Localization of Neuronal Protein Phosphatases

Several studies have demonstrated the presence of protein phosphatase activity corresponding to PP1, PP2A, PP2B, and PP2C in brain extracts with the highest levels being in synaptoplasmic fractions as opposed to non-neuronal tissue (Ingebritsen et al., 1983; Shields et al., 1985; Yang, 1985).

Protein Phosphatase 1

Subcellular fractionation studies have demonstrated PP1 activity in cytosolic, synaptosolic, synaptic plasma membrane and synaptic junction fractions (Shields et al., 1985; Dokas et al.,1990), consistent with a ubiquitous distribution of this phosphatase in brain. The major mRNA species encoding either isozyme in brain are the same as in other tissues (Berndt et al., 1987; Cohen, 1988), but the particulate form of the enzyme has not been characterized. Although the particulate form is likely to contain the same catalytic subunit as the cytosolic form, the regulatory subunits that bind it to various neuronal structures have not been described. Interestingly, an inositol phosphate-stimulated protein phosphatase, which appears to be a type 1 phosphatase, has been isolated from bovine brain membranes (Zwiller et al., 1989). However, in contrast to PP1, this phosphatase is insensitive to heparin when phosphorylase is used as a substrate, has intermediate sensitivity between PP1 and PP2A to okadaic acid, and is phosphorylated by PKC (Zwiller et al., 1990), suggesting that it may be a novel brain-specific protein phosphatase. Furthermore, the effect of IP3 appears to be directly on the catalytic subunit, and the holoenzyme structure has not been investigated. It is possible that this phosphatase is related to the recently purified phosphatase (termed PP3) believed to be a novel neuron-specific phosphatase (Honkanen et al., 1991). The amount of active

PP1 present in brain extracts is considerably lower than in extracts of skeletal muscle (Ingebritsen et al., 1983; Yang, 1985), and this may in part be owing to the considerably higher levels of the inactive cytosolic form (PP1-I) that are present in the brain (Yang and Fong, 1984). Although the activating kinase (GSK3) of this form of PP1 is considered to be a cytosolic enzyme, it is also present in brain in equal or higher amounts in particulate fractions (Yang et al., 1987; Tung and Reed, 1989). The particulate form of GSK3 appears to be identical to the cytosolic form, and consists of two subunits of 52 kDa and 46 kDa (Tung and Reed, 1989). It has been suggested that GSK3 is a specific membrane protein in brain and that the cytosolic form is an artifact of homogenization (Yang et al., 1987).

Protein Phosphatase 2A

The amount of PP2A activity in brain extracts is the highest of all tissues investigated (Ingebritsen et al., 1983), and there is approximately three times as much PP2A as PP1. This is consistent with the elevated levels of PP2A mRNA detected in brain (daCruz e Silva et al., 1987; Khew-Goodall and Hemmings, 1988; Kitagawa et al., 1988) and with immunoblot studies using monoclonal antibodies specific for the catalytic subunit of PP2A, which showed highest immunoreactivity in brain (Mumby et al., 1985). Studies with polyclonal antibodies to the catalytic subunit of PP2A showed that PP2A was largely in cytosolic and synaptosolic fractions (Saitoh et al., 1989), and measurement of PP2A in subcellular fractions of brain showed that there was fivefold less PP2A than PP1 in synaptic plasma membrane and synaptic junction fractions (Shields et al., 1985), suggesting that PP2A is predominately a cytosolic enzyme in brain. However, preliminary studies in our own laboratory have found significant amounts of PP2A in particulate fractions of rat and chicken brain (Sim et al., unpublished), and the possibility that some fractionation procedures remove PP2A from membrane fractions cannot be ignored.

Immunohistochemical studies showed highest PP2A immunoreactivity in neurons and a wide regional distribution (Saitoh et al., 1989). However, the holoenzyme forms of PP2A in brain have not been fully investigated, and their regional distribution is not known. Potential classifications for the forms of PP2A that have been purified from brain are shown in Table 2. Patterson and Flavin (1986) purified PP2A from bovine brain and showed it to be similar to the trimeric holoenzymes, PP2A1 or PP2AO, although it had different substrate specificity. Another form of PP2A purified from bovine brain contained regulatory subunits of 65 and 42 kDa (Nishiwaki et al., 1990). The 65-kDa subunit is likely to be the A-regulatory subunit, but the relationship of the 42-kDA subunit to other known regulatory subunits was not established. However, using microcystin-affinity chromatography, the same group showed that PP2A purified from mouse brain contained regulatory subunits of 67 and 58 kDa, and a catalytic subunit of 42 kDa (Nishiwaki et al., 1991). PP2A purified from porcine brain was similar to PP2A1 (Yang et al., 1986). Whether these differences are related to the species differences or consequences of different purification procedures requires investigation. There is a preliminary report that a major protein phosphatase in rat hippocampus is similar to PP2AO (Barnes et al., 1989,1990), a trimeric form containing the 65 and 54 kDa subunits, although the regulatory subunits were reported to be 63 and 55 kDa, which is more characteristic of the PP2A1 form than the PP2AO form (Cohen, 1989). Isolation of tau-factor-specific phosphatases by DEAE-cellulose chromatography identified two peaks of activity (Yamamoto et al., 1990). Each peak was identified as a PP2A phosphatase, but the regulatory subunits were not characterized. PP2A has also been identified as the major component of a protein phosphatase preparation isolated from bovine brain (Yamamoto et al., 1988). Termed protein phosphatase C, this preparation is not a novel enzyme, but appears to consist of a mixture of the catalytic subunits of PP1 and PP2A.

Table 2
Identity of Neuronal PP2A Holoenzyme Forms

Study	Regulatory subunits	Catalytic subunit	Potential classification
Patterson and Flavin (1986)	63 kDa, 51 kDa	36 kDa	PP2A1/PP2AO
Nishiwaki et al. (1990)	65 kDa, 42 kDa	37 kDa	?
Nishiwaki et al. (1991)	67 kDa, 58 kDa	41 kDa	PP2A1
Barnes et al. (1989,1990)	63 kDa, 55 kDa	38 kDa	PP2A1
Yang et al. (1986)	69 kDa, 55 kDa	34 kDa	PP2A1
Mayer et al. (1991)	54 kDa β	?	Neuronal specific?

The B-subunit (55 kDa) of PP2A is encoded by two genes, α and β. Northern blot analysis showed that the β isoform (which is distinct from the B' subunit) was highly expressed in a neuronal cell line and may represent a neuron-specific isoform (Mayer et al., 1991). It remains to be seen if this isoform leads to a neuronal-specific holoenzyme of PP2A with specific functions or regulatory properties.

Protein Phosphatase 2B

The regional and subcellular localization of calcineurin has been extensively studied (reviewed in Klee et al., 1988), and PP2B exists in both cytosolic and particulate fractions (Anthony et al., 1988). However, it should be noted that fractionation procedures used can lead to selective loss of PP2B activity from membrane fractions (Shields et al., 1985) such that the precise subcellular localizations cannot always be ascertained. It appears that the isoforms of this phosphatase have different tissue and regional distributions, suggesting that they have different neuronal functions (Takaishi et al., 1991; Kincaid et al., 1990), although it is not clear if the cytosolic and particulate enzymes represent the different isoforms.

Protein Phosphatase 2C

Magnesium-dependent phosphatase activity is present in brain extracts at levels fourfold higher than skeletal muscle (Ingebritsen et al., 1983). PP2C activity is greatest in cytosolic and synaptosolic fractions, there being four-to eightfold less PP2C activity in synaptic plasma membrane and synaptic junction fractions (Shields et al., 1985). Unlike other phosphatases, PP2C was not concentrated in synaptosolic fractions when compared with whole brain cytosolic fractions (Shields et al., 1985) and is therefore not specifically concentrated in nerve terminals. The regional distribution of PP2C in the brain has not been established.

Regulation of Neuronal Protein Phosphatases

Although little is known about the regulation of neuronal protein phosphatases, several lines of evidence suggest that protein phosphatases in the brain have considerable and unique potential for regulation. A number of neuron-specific inhibitors of protein phosphatases have been identified (*see below*), and it appears that protein phosphatases in the brain are relatively inactive under basal conditions. Isolated nerve terminals contain high levels of basal phosphorylation, and although this may reflect elevated protein kinase activity, depolarization leads to considerable activation of protein kinase A and CMK II

(Dunkley and Robinson, 1986), suggesting that, at least under resting conditions, the activity of these kinases is not unusually high. Treatment of synaptosomes with okadaic acid under resting conditions only leads to a 60% increase in basal phosphorylation (in comparison to the two-to threefold increases observed in other systems) and supports the hypothesis that high basal phosphorylation in synaptosomes reflects low basal protein phosphatase activity (Sim et al., 1991). Although tissue extracts of the brain contain high levels of protein phosphatase 2A, 2B, and 2C activity/mg protein (Ingebritsen et al., 1983), the amount of these protein phosphatases present per mg protein, as determined by immunoblot and Northern blot analysis, are considerably higher than other tissues (Mumby et al., 1985; Kincaid et al., 1990). Furthermore, the brain and skeletal muscle contain the same levels of PP1 mRNA (Stemmer and Klee, 1991), but brain contains approximately half as much PP1 activity (Ingebritsen et al., 1983). Thus, protein phosphatases are likely to have a low specific activity in brain. This situation may be more critical in specific neuronal regions, for example, the nerve terminal, which is enriched in PP2B, 0.32% of synaptosomal protein being this phosphatase (Anthony et al., 1988).

Protein Phosphatase 1

Although mammalian brain contains the PP1 inhibitor I-1, a neuronal isoform, DARPP-32, also exists in selected regions of the brain (Hemmings et al., 1984). This may point to specific functions for PP1 in these regions. DARPP-32 is concentrated in dopaminoceptive neurons and like I-1, is a substrate for protein kinase A, and is dephosphorylated by PP2B (King et al., 1984). Although highly homologous to I-1, DARPP-32 differs in that it can be phosphorylated on serines 45 and 102 by CKII, in addition to phosphorylation on threonine-34, by protein kinase A. Phosphorylation by CKII does not directly affect the potency of DARPP-32 as a PP1-inhibitor, but promotes the phosphorylation of threonine-34 (Girault et al., 1989). DARPP-32 isolated from intact brain slices is phosphorylated on these serine residues (Girault et al., 1989), but the physiological significance of the mechanisms regulating this phosphorylation are unknown. However, since DARPP-32 is concentrated in dopaminoceptive neurons, it is likely that the activity of PP1 can be altered in vivo by agents (such as dopamine) that elevate cAMP (Fig. 4). Indeed, treatment of brain slices with forskolin leads to the phosphorylation of DARPP-32 on threonine-34, and this is antagonized by stimulation of the NMDA-specific glutamate receptor, which is linked to calcium channels (Halpain et al., 1990). This antagonistic effect is presumed to be owing to activation of PP2B, which then leads to activation of PP1 (Fig. 4). However, this has not been shown directly, and only one other phosphoprotein (MAP2) was shown to be dephosphorylated in response to NMDA (Halpain and Greengard, 1990). It will be important to determine if PP1 is inactive in the absence of the influx of calcium and that activation of the NMDA receptor does cause changes in the activity of PP1 in this region of the brain.

Although the localization of DARPP-32 is highly restricted, I-1 is more widely distributed (Nairn et al., 1988; MacDougall et al., 1989), suggesting that I-1 might also mediate the effects of cAMP and calcium on PP1 in other regions of the brain. Evidence to suggest that this occurs in isolated nerve terminals has been gained using okadaic acid, the cell-permeable inhibitor of PP1 and PP2A (Sim et al., 1991). When synaptosomes were incubated with ^{32}Pi, the level of phosphorylation reached by most proteins was modulated by the external calcium concentration (Robinson and Dunkley, 1985). In the presence of okadaic acid at concentrations that do not inhibit PP2B, these calcium-dependent effects were abolished (Fig. 5). Furthermore, depolarization-dependent dephosphorylation of most synaptosomal proteins was blocked by the same concentration of okadaic acid (Fig. 6). Thus, it appears that PP1 and/or PP2A is(are) the major protein phosphatase(s) in synaptosomes and is (are) responsible for mediating the effects of calcium on

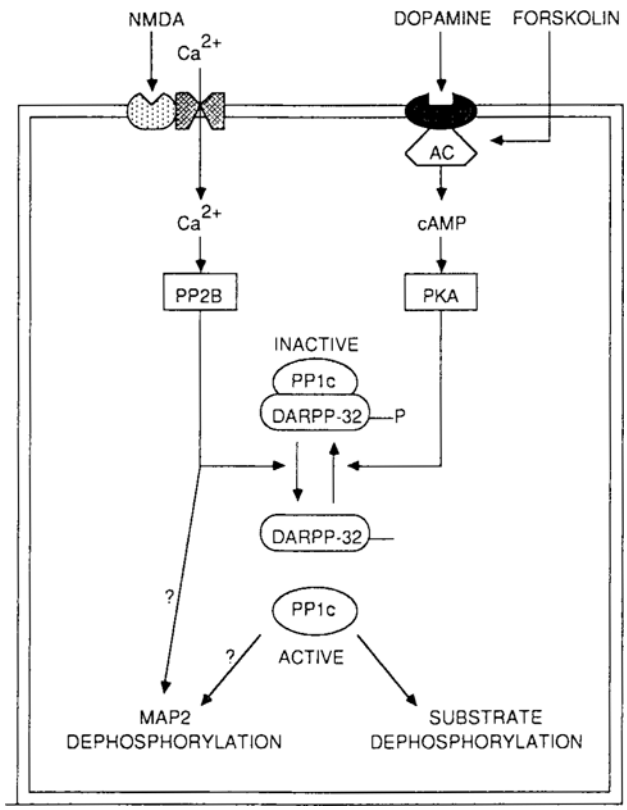

Fig. 4. Regulation of protein phosphatase-1 by DARPP-32. AC = adenylate cyclase; MAP2 = microtubule-associated protein 2.

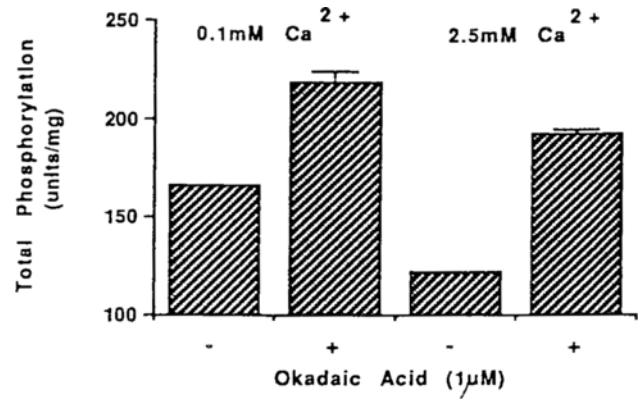

Fig. 5. Effect of okadaic acid and external calcium concentration on basal phosphorylation in synaptosomes (reproduced from Sim et al., 1991). Synaptosomes were incubated with $^{32}P_i$ for 40 min followed by 1 μ*M* okadaic acid for 5 min and reactions stopped by the addition of SDS-stop buffer. Phosphoproteins were separated by SDS-PAGE, and phosphorylation quantitated by autoradiography and densitometry scanning. Results represent the mean of three experiments, and error bars indicate the SEM. Statistical analysis showed that the difference in basal phosphorylation was significant in the absence of okadaic acid, but not significant in its presence.

synaptosomal dephosphorylation. The most likely mechanisms for this are the activation of PP2B, dephosphorylation of I-1, and activation of PP1 (Fig. 1). It will be important to investigate the phosphorylation of I-1 in synaptosomes under these conditions. Since this effect is observed in synaptosolic and particulate fractions, it is also possible that calcium modulates PP1 through a regulatory subunit analogous to the glycogen-bound form in skeletal muscle.

Substantial amounts of the inactive form of PP1 (PP1-I) have been identified in pig brain, and it was suggested that this may be the predominant type 1 phosphatase in the brain (Yang and Fong, 1984). However, this is based on the lack of PP1 activity found in this study, a finding that is not borne out by other investigations (Ingebritsen et al., 1983; Shields et al., 1985; Yang, 1985). Nevertheless, the high levels of PP1-I in brain suggest that it may have important functions. The finding that the activating kinase (GSK3) is predominantly membrane bound, but that PP1-I is absent from membrane fractions of brain (Yang et al., 1990) may suggest that regulation of this form of PP1 only occurs in cytosolic compartments that approximate membrane structures containing GSK3, and may have specific substrates and functions. GSK3 is likely to phosphorylate proteins other than PP1-I, and therefore, appears to have a dual function in promoting both phosphorylation (of PP1-I and other proteins) and dephosphorylation (via PP1). Membrane-bound GSK3 exists in two forms: a spontaneously active/trypsin-labile form and an inactive/trypsin-resistant form (Yang et al., 1990). Reconstitution studies showed that the activity of each form could be differentially modified by acidic or neutral phospholipids (Yang et al., 1990), suggesting that membrane phospholipids may play a role in the

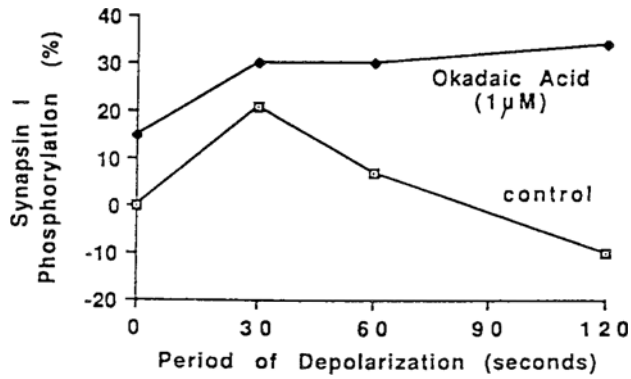

Fig. 6. Effect of okadaic acid on depolarization-stimulated dephosphorylation of synapsin I (reproduced from Sim et al., 1991). Experiments were carried out as described in Fig. 5. Prior to stopping the reactions, depolarization was induced by the addition of KCl to a final concentration of 41 μ*M*. The effect seen with synapsin I was the same for most of the synaptosomal proteins whose phosphorylation was normally increased after depolarization.

regulation of this enzyme. It would be interesting to see if the interconversion between two different forms is a physiological mechanism relevant to regulation of PP1 or the phosphorylation of other substrates.

Protein Phosphatase 2A

There are no known regulatory mechanisms governing the activity of PP2A, but five intriguing possibilities should be considered. The inhibition of calcium dependent dephosphorylation in synaptosomes by okadaic acid (Sim et al., 1991) (Figs. 5 and 6) may equally be owing to inhibition of PP2A, and mechanisms for modulating PP2A activity in response to extracellular stimulation should be investigated.

First, it has been reported that depolarization of rat hippocampal slices leads to redistribution of PP2A from cytosol to plasma membranes (Barnes et al., 1989). Although it is not clear how this occurs, it may be speculated that PP2A is targeted toward specific subcellular localizations via calcium-dependent modulation of a regulatory subunit or protein, in an analogous fashion to PP1.

Second, a 23-kDa protein (G-substrate) that is phosphorylated by cGMP-dependent protein kinase and is enriched in cerebellar Purkinje cells (Schlichter et al., 1978) is highly homologous to DARPP-32, but appears to function as an inhibitor of PP2A (Girault et al., 1989). Thus, PP2A activity may be modulated by signals that raise the levels of intracellular second messengers.

Third, it has been shown that a holoenzyme form (PP2A2) could be stimulated by tubulin and inhibited by MAP2 (Jessus et al., 1989). This raises the possibility that changes in the cytoskeletal structure (through polymerization and microtubule assembly) can influence protein phosphatase activity. It will be important to investigate if this form of PP2A exists in brain, and if these antagonistic effects of tubulin and MAP2 influence PP2A activity toward neuronal substrates.

Fourth, it has been found that stimulation of presynaptic NMDA receptors leads to increased ornithine decarboxylase activity and transient elevation of polyamines (Koenig et al., 1991). If the change in level of polyamines can be shown to modify the activity of PP2A holoenzymes, this represents a potential mechanism for the regulation of PP2A in response to signals that elevate the level of intracellular calcium, and it warrants further investigation.

Last, consistent with the hypothesis that brain protein phosphatases exist in low-activity states, studies from my own laboratory have shown that, despite containing low PP2A activity, particulate fractions of rat brain, including synaptic junctions, contain significant amounts of PP2A detected by monospecific antibodies (Sim et al., unpublished). This suggests that membrane-associated PP2A exists in a particularly inactive state, and these structures may contain high levels of a specific inhibitor of this phosphatase.

Protein Dephosphorylation and Neuronal Function

Although protein phosphorylation is involved in a variety of processes in the brain, precise molecular mechanisms involved in these processes

are not known (Hemmings et al., 1989). In particular, the molecular events involved in neurotransmitter release, which follows the rapid influx of calcium, are not known. This is despite the phosphorylation of some key proteins being strongly implicated as part of the mechanism (Llinas et al., 1985; Dunkley and Robinson, 1986; Robinson et al., 1987; Dekker et al., 1989; Nichols et al., 1990; Robinson, 1992). Depolarization of synaptosomes leads to the rapid influx of calcium through voltage-sensitive calcium channels and increases phosphorylation of several proteins (Dunkley and Robinson, 1986). However, depolarization also leads to the rapid dephosphorylation of at least three proteins of 65, 96, and 139 kDa (Robinson and Dunkley, 1985; Gomez-Puertas et al., 1991). The 65-kDa protein has many characteristics similar to the 65-kDA protein (Parafusin) whose dephosphorylation has been linked with exocytosis in *Paramecium* (Zieseness and Plattner, 1985), and similar proteins have been found in other exocytotic systems (Satir et al., 1989).

The dephosphorylation of the 96-kDa protein, now termed dephosphin, has received considerable attention and may function as the trigger for release (Robinson, 1992). Interestingly, the dephosphorylation of dephosphin is only partially inhibited by 1 μ*M* okadaic acid and requires 5 μ*M* okadaic acid for total inhibition, suggesting that it may be a substrate for PP2B (Sim et al., 1991). Since the best known substrates for PP2B are regulators of other phosphatases or kinases, it is tempting to speculate that dephosphin also has this function. The dephosphorylation of these proteins represents the earliest phosphorylation/dephosphorylation events that can be measured following depolarization, supporting the notion that dephosphorylation events are important for release. However, evidence that protein phosphatases are necessary for the series of events leading to neurotransmitter release is limited and controversial. In permeabilized chromaffin cells, thiophosphorylation (which is resistant to phosphatase activity), induced by incubation with ATPγS, inhibited the release of catecholamines, suggesting that protein phosphatase activity is necessary for release (Brooks and Brooks, 1985). This is consistent with studies using okadaic acid, which inhibited carbachol-evoked secretion from these cells (Yanagihara et al., 1991) and inhibited calcium-dependent glutamate release from guinea pig hippocampal synaptosomes (McMahon and Nicholls, personal communication). Also, microinjection of PP2B into *Paramecium* triggers exocytosis, an effect that is reversed by the PP2B antibody (Momayezi et al., 1987). However, this effect was also produced by microinjection of alkaline phosphatase, so the relevant phosphatase is not known. Conversely, in PC12 cells, ATPγS caused an increase in calcium-independent secretion of noradrenaline, as did treating the cells with protein phosphatase inhibitors (Wagner and Vu, 1990). Addition of the purified catalytic subunit of PP2A to these permeabilized cells caused a 70% decrease in secretion of noradrenaline (Wagner and Vu, 1990). In Aplysia, FMRFamide inhibition of serotonin-induced presynaptic facilitation is associated with a decrease in protein phosphorylation (Sweatt et al., 1989), which may be the result of activation of protein phosphatases. Okadaic acid also enhances the basal and evoked release of neurotransmitter at frog and lobster neuromuscular junctions, as measured by quantal analysis (Abdul-Ghani et al., 1991). Data from our laboratory show that inhibition of PP1 and PP2A with okadaic acid increases the basal release of endogenous excitatory amino acids glutamate, aspartate, and GABA (Sim et al., in preparation). It is possible that protein phosphatases govern the sensitivity of the release mechanism to stimulation, such that the ability of a given signal to initiate release is determined by the prevailing activity of protein phosphatases. It will be important to determine the effects of okadaic acid at submaximal concentrations of secretory signals in these systems. However, since PP1 and PP2A account for the majority of synaptosomal dephosphorylation (Sim et al., 1991), okadaic acid is likely to affect a number of processes, such as ion channel function, microtubule assembly, and cat-

echolamine synthesis, such that it will be difficult to separate the individual events regulated by individual phosphatases. It will be necessary to develop specific probes and conditions specific for each phosphatase to define precise roles of protein phosphatases in release.

Many postsynaptic events also involve protein phosphorylation, but, as in the presynaptic nerve terminal, precise molecular mechanisms and roles have not been established. Glutamate is the major excitatory neurotransmitter in the central nervous system, and the regulation of its receptor and the consequences of its activation have been the subject of intense study. Of particular interest has been the NMDA subtype, the activation of which is linked to the influx of calcium. Interestingly, this rise in intracellular calcium in hippocampal slices does not lead to increased phosphorylation of proteins, or to increases in CMKII or PKA activity (Halpain and Greengard, 1990). On the contrary, the only demonstrable effect of NMDA receptor activation was dephosphorylation of DARPP-32 and MAP2 (Fig. 4). Although both these effects were postulated to be the result of activation of PP2B, it is not clear whether PP2B directly dephosphorylates MAP2 or whether this is a consequence of PP1 activation through inhibition of DARPP-32. Nevertheless, the fact that the only phosphorylation/dephosphorylation events that appear to occur in response to NMDA receptor activation are dephosphorylations provides intriguing evidence for the regulatory roles of protein phosphatases in neuronal function.

Summary and Perspectives

Data emerging from a number of different systems indicate that protein phosphatases are highly regulated and potentially responsive to changes in the levels of intracellular second messengers that occur in response to extracellular stimulation. Because of their high levels, but low specific activity, and the presence of neuronal specific regulators (such as DARPP-32 and G-substrate), the protein phosphatases in the nervous system possess considerable and unique potential for regulation. That several early events following presynaptic depolarization or receptor activation of postsynaptic responses appear to involve specific dephosphorylations suggests that regulation of protein phosphatase activity is important for the control of many neuronal functions. It will therefore be important to characterize fully the protein phosphatases present in brain, and determine the native forms of the enzymes present, their regional and subcellular localization, and their neuronal substrates. The regulatory subunits of PP1 in brain should be investigated in order to determine the mechanisms whereby the particulate forms of PP1 are regulated. Elucidating the phosphorylation state of I-1 in response to different stimuli will provide important information concerning the general regulation of cytosolic PP1 in addition to its specific regulation by DARPP-32. Regional and subcellular mapping of I-2 also will be important in determining if indeed the inactive form of PP1 approximates membrane structures where its activating kinase is concentrated and, therefore, has specific substrates and functions in these regions. The potential regulation of PP2A by intracellular second messengers is an important emerging concept. Whether this occurs through different regulatory subunits or other molecules can only be established when the holoenzyme forms of PP2A in the brain are fully characterized and their regional and subcellular localization determined.

Despite a number of studies, precise roles for protein dephosphorylation in the nervous system are not known. This is primarily owing to the ubiquitous nature of protein phosphorylation as a regulatory mechanism, such that the use of general phosphatase probes (such as okadaic acid) is likely to have a myriad of effects. Although the development of specific inhibitors for individual classes of phosphatase will facilitate a more refined approach toward studying the roles of dephosphorylation, detailed information will only be forthcoming as the specific endogenous

substrates for each class of phosphatase are determined. Although many neuronal proteins are substrates for several phosphatases in vitro, it is likely that regulatory mechanisms governing the activity and localization of the protein phosphatases will serve to limit this broad substrate specificity and, consequently, specific substrates for each phosphatase will exist in vivo. As these specific substrates are identified and the mechanisms regulating their dephosphorylation are elucidated, they may then be used to probe the functional roles of particular protein phosphatases in the brain.

Acknowledgments

I would like to thank Associate Professors Peter Dunkley and John Rostas and Dr. Stephen Bunn for critically reading this manuscript, as well as for their many helpful suggestions.

Financial assistance from the National Health and Medical Research Council of Australia, the Australian Research Council, and the University of Newcastle is gratefully acknowledged.

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