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PHYSIOLOGIC IMPORTANCE OF PROTEIN PHOSPHATASE INHIBITORS

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1. ABSTRACT

Reversible protein phosphorylation is an important mode of regulation of cellular processes. While earlier studies focused on protein kinases, it is now apparent that protein phosphatases play an equally integral role in the control of cellular phosphoproteins. This review examines the role played by endogenous inhibitors of three major protein serine/threonine phosphatases, PP1, PP2A and PP2B in the control of cell physiology. The discussion highlights novel paradigms for signal transduction by protein phosphatase inhibitors that provide important avenues for signal amplification, the timing of physiological responses and cross-talk between distinct signal transduction pathways. New evidence also points to genetic abnormalities or altered expression of phosphatase inhibitors as potential mechanisms for human disease.

Together, the data emphasize the physiological importance of protein phosphatase inhibitors and establish phosphatase regulation as a key feature of hormone signaling.

2. INTRODUCTION

Reversible protein phosphorylation controls many physiological processes in both plant and animal cells with the phosphorylation of cellular proteins being defined by the protein kinases and phosphatases that act on them. Discovery of protein kinases directly activated by second messengers fostered the early view that kinases, and not phosphatases, were highly regulated in eukaryotic cells. However, analysis of cellular phosphatases soon made this concept untenable. For instance, the specific activity of non-receptor protein tyrosine phosphatases was found

Table 1. Cellular phosphatase inhibitors

PP1 Inhibitors	IC50 (nM)	Mr (kDa)	Phosphoproteins	Ref.
PP1 Inhibitors				
Inhibitor-1 (I-1)	1.6	28	yes	24
DARPP-32	1	32	yes	80
Inhibitor-2 (I-2)	3.1	23	yes	24
NIPP-1	0.01	38.5	yes	63
RIPP-1	20	23	unknown	68
CPI17	0.18	17	yes	69
PP2A Inhibitors				
Inhibitor-1 (I1PP2A)	30	30	yes	17
Inhibitor-2 (I2PP2A)	25	39	yes	17
PP2B Inhibitors				
Cain	440	240	unknown	21

to far exceed that of the protein kinases which they opposed. This required that growth factors which initiate protein tyrosine phosphorylation to elicit a physiological response, must first overcome the barrier to signaling created by the phosphatases. This strongly hinted at the presence of cellular mechanisms to suppress phosphatase activity during times of kinase activation. These mechanisms would also provide for tremendous amplification of the physiological signals. Concomitant control of kinases and phosphatases provides the cell with the capacity to rapidly switch proteins from their phosphorylated to dephosphorylated state to meet differing physiological demands. This is perhaps best illustrated during the eukaryotic cell division cycle where decisions to proceed through different stages are made by the timely phosphorylation and dephosphorylation of specific cell cycle regulators. Thus, phosphorylation-dephosphorylation events act as switches or checkpoints that ensure that a cell has fulfilled the requirements to proceed to the next cell cycle stage. Errors in checkpoint control form the most prevalent basis for aberrant cell growth seen in human cancers and can carry dire developmental consequences for an organism.

A number of mechanisms have been analyzed with an eye on their potential for coordinating kinases and phosphatases. These include changes in the expression of protein phosphatases, their subcellular localization, phosphorylation of phosphatase catalytic and regulatory subunits and regulation by endogenous phosphatase inhibitors. Of these, hormonally-regulated protein inhibitors represent the best understood mechanism for regulating the major cellular protein serine/threonine phosphatases and for which there is also extensive physiological evidence. While endogenous inhibitors of protein tyrosine phosphatases have been reported, they remain largely uncharacterized (1). Therefore, in this review, we will limit our discussion to cellular proteins that regulate the major protein serine/threonine phosphatases in response to physiological stimuli. Analysis of these proteins has introduced us to several novel paradigms for cell signaling and has largely dismissed the notion of unregulated protein phosphatase activity.

One way in which the cell coordinates the activation of kinases with the inhibition of phosphatases is via endogenous phosphatase inhibitors which are activated by second messenger-regulated protein kinases. This is indeed the case for many proteins which function as endogenous phosphatase inhibitors only after they themselves have been phosphorylated. These phosphatase inhibitors provide a direct link between hormone-induced changes in second messenger levels and alterations in phosphatase activity that may themselves account for some aspects of the physiological response. By the activation of phosphatase inhibitors, the second messengers bolster or amplify the function of protein kinases, enhancing protein phosphorylation events. The coordination of kinases and phosphatases in this manner provides for a rapid onset of the physiological response and may also increase the size of the cellular response elicited by hormones.

Yet another mode of phosphatase regulation involves inhibitors that are active only in their dephosphorylated state. These proteins represent important mechanisms for maintaining latent pools of phosphatases that can be activated in response to certain hormones. In this regard, insulin and peptide hormones activate protein phosphatases that promote the dephosphorylation of enzymes involved in cell metabolism. Alternately, phosphatase inhibitors that are inactivated by phosphorylation may function as timers of the cellular response. In this scenario, hormones would trigger the activation of protein kinases that phosphorylate multiple proteins including the phosphatase inhibitors. The inactivation of the phosphatase inhibitors may lead to a delayed activation of phosphatases that terminate the cellular response. The presence of these phosphatase inhibitors and their phophorylation would then control the duration of a cellular response to hormones.

As most protein phosphatases are not dedicated to reverse the actions of specific protein kinases, changes in phosphatase activity likely have a broad impact on dephosphorylation and turnover of phosphoproteins which are substrates for many different kinases. Thus, hormones via the modulation of phosphatase inhibitors may control many different pathways, and phosphatase inhibitors become conduits for crosstalk between multiple signaling pathways or important devices for integrating and orchestrating the physiological response.

All the inhibitor proteins, discussed in this review (table 1), show remarkable specificity towards their target phosphatases. Being themselves phosphoproteins, it has been proposed that they function as psueodsubstrate inhibitors of the phosphatases. This then raises an intriguing issue of the structural features encoded within these phosphoproteins that make them highly selective and potent inhibitors, yet not substrates of their target phosphatases. In most cases, phosphorylation of these inhibitors is reversed by phosphatases other than the ones that they inhibit. This yields a novel regulatory cascade where one phosphatase, via the dephosphorylation of an inhibitor, modulates the function of another phosphatase. With the discovery of numerous phosphatase inhibitors and regulatory subunits that undergo reversible protein

phosphorylation, there will undoubtedly be an increasing number of such protein phosphatase cascades. Finally, there is growing evidence of an intricate interplay between the inhibitors and other phosphatase regulatory subunits that controls enzyme activity so that the days of considering protein phosphatases as constitutive or unregulated enzymes are clearly over.

2.1. Introduction to protein serine/threonine phosphatases

Given the focus of this review on protein serine/threonine phosphatase inhibitors, we need to briefly discuss the enzymes targeted by these proteins. For a complete description of these enzymes, the reader is referred to a number of excellent reviews (2, 3, 4). Prior to the advent of molecular cloning, protein serine/threonine phosphatases were classified by their biochemical properties (protein composition, *in vitro* substrate specificity, metal requirement and regulation by endogenous inhibitors) into two broad groups termed type 1 and type 2 phosphatases. The type-2 phosphatases were further subdivided into three groups, PP2A, PP2B and PP2C. Molecular cloning has now identified many protein serine/threonine phosphatases. With the exception of PP2C, the primary structures of these serine/threonine phosphatases show stretches of conserved amino acids that are the hallmarks of this enzyme family. PP1 (or type 1 phosphatase) and PP2A make up more than 90 % of the serine/threonine phosphatase activity in mammalian cells and are therefore the primary focus of this review.

2.1.1. Protein phosphatase-1

Protein Phosphatase 1 (PP1) regulates many biological processes including synaptic plasticity, cell cycle, gene transcription, and carbohydrate and lipid metabolism. Four mammalian isoforms of the PP1 catalytic subunit are generated from three genes. With the exception of PP1γ2, which is predominantly expressed in testes, the other isoforms, PP1α, β and γ1, are widely expressed in mammalian tissues. The functional importance of PP1 isoforms remains unclear. It has been speculated that they may associate with distinct regulatory subunits and thereby serve unique physiological functions but so far there has been little evidence to support this idea. PP1 activity is regulated by many hormones and growth factors. As the levels of the PP1 catalytic subunit do not change in response to physiological stimuli, hormonal regulation is thought to occur primarily through endogenous inhibitors and in some cases, through regulatory subunits (5). The largest number of phosphatase inhibitors thus far identified target PP1. These include Inhibitor-1 (I-1), Inhibitor-2 (I-2), dopmine- and cAMP-regulated phosphoprotein of Mr 32,000 (DARPP-32), nuclear inhibitor of PP1 (NIPP-1), C-kinase activated phosphatase inhibitor of Mr 17,000 (CPI17), and ribosomal inhibitor of PP1 (RIPP-1). In addition, a large number of PP1-binding proteins have been shown to inhibit the phosphorylase *a* phosphatase activity of PP1 *in vitro*. The precise role of these latter proteins in controlling PP1 activity in intact cells remains unknown, but some of these PP1-binding proteins may also turn out to be phosphatase inhibitors. Regardless, PP1 inhibitors are among the best understood phosphatase regulators and have set many of the prevailing paradigms for phosphatase regulation.

One of the paradigms for PP1 regulation operates during the control of the cAMP-response binding protein, CREB, which must be phosphorylated to be an active transcription factor. In many cells, this occurs in response to activation of PKA which directly phosphorylates CREB on serine$_{133}$ (6). In other cells, such as neurons, elevation of intracellular calcium, either alone or in conjunction with phosphoinositide turnover, promotes CREB phosphorylation on serine$_{133}$ (7). Experiments in cultured cells show that despite chronic activation of PKA by cell-permeable cAMP analogs (8) or treatment of cells with the calcium ionophore, ionomycin, and the PKC activator, phorbol ester (9), only transient phosphorylation of CREB and transcription of CRE-regulated genes occurs. This suggests that the CREB phosphatase, identified as a nuclear PP1 (8), is a dominant regulator of CREB function. This was confirmed by the pharmacological treatment of cells with the phosphatase inhibitor, okadaic acid, which leads to a robust and prolonged phosphorylation of CREB and may in part account for the cytotoxic effects of this toxin. Similarly, the expression of a constitutively active form of Inhibitor-1 (I-1), a PP1-specific inhibitor, promoted CREB phosphorylation and gene transcription, in the absence of elevated second messenger levels (10). These data point to the tremendous potential for signaling through PP1 to control CRE-mediated gene transcription and raises an intriguing possibility that a PP1 regulator, particularly one that inhibits PP1 activity, may be an important mediator of signal transduction in the nucleus.

The role of PP1 in the cell cycle also emphasizes the need for endogenous regulators. It has been established that the retinoblastoma gene product (RB) must be dephosphorylated as cells exit mitosis. This dephosphorylation activates RB's function as a growth suppressor that blocks cells in the G1 phase of the cell cycle (for review see 11). Ludlow, *et. al*. identified the RB phosphatase in mitotic extracts as PP1 (12). Because RB must be rephosphorylated for cells to initiate S phase, PP1 acts as a brake to prevent DNA synthesis (13, 14). This suggests that PP1 must be inhibited to facilitate G_1/S transition and reactivated during mitosis. This was precisely what was shown by the studies of Berndt, *et. al*. (14). In these experiments, a potentially unregulated form of PP1 catalytic subunit by mutating a proposed phosphorylation site near the C-terminus was expressed in human cancer cells and blocked their entry into S phase. As PP1 levels do not change during the cell cycle, one presumes that cell cycle-dependent changes in PP1 activity must be mediated by either the covalent modification of PP1 catalytic subunit or by endogenous PP1 regulators. In this regard, a nuclear PP1-binding protein has been recently identified that may modulate PP1's activity against some cell cycle substrates (15).

In summary, considerable effort has been focused in recent years on the identification of endogenous PP1 regulators in an attempt to understand the physiological control of this protein phosphatase.

2.1.2. Protein phosphatase 2A

PP2A is also a major protein phosphatase in all eukaryotic cells and has a wide range of biological functions. These include the control of cell cycle, organization of cytoskeleton, transcription of immediate early genes, cholesterol and protein biosynthesis. PP2A is a heterotrimeric enzyme made up of a catalytic or C subunit,

and two regulatory subunits, termed A and B. To date, cDNAs have been identified for two A subunits, two C subunits, and over twenty B subunits. This suggests the existence of numerous PP2A complexes in mammalian cells. It has been speculated that each PP2A complex serves distinct functions, although at this time, there is very little direct evidence to support this notion. PP2A regulation is further complicated by recent reports that A, B and C subunits all exist as phosphoproteins (3), but the physiological relevance of these findings remains to be established. The $PP2A_C$ subunit is also the major carboxymethylated protein in mammalian cells. This modification occurs at the C-terminal $leucine_{309}$ and has been credited with various functions, including enzyme activation, assembly and localization to membranes (16). The identification of additional regulators, specifically PP2A inhibitors, is very much in its infancy. However, two PP2A inhibitors have recently been identified. Termed I_1^{PP2A} and I_2^{PP2A}, these proteins have been implicated in both physiological and pathophysiological regulation of PP2A (17).

2.1.3. Protein phosphatase 2B

PP2B, also termed calcineurin, is a calcium/calmodulin-activated protein serine/threonine phosphatase consisting of a catalytic A-subunit and a regulatory or calcium-binding B-subunit which makes this the only phosphatase directly regulated by second messengers (4). PP2B has a much narrower *in vitro* substrate specificity than either PP1 or PP2A. This is consistent with its specialized functions in the nervous system, T lymphocytes and other cells. Interestingly, the best known *in vitro* and *in vivo* substrates of PP2B are the PP1 inhibitors, I-1 and DARPP-32. Thus, PP2B controls PP1 activity and the two together form the first documented phosphatase cascade (18). PP2B has drawn much attention as the target of two clinically important immunosuppressive drugs, cyclosporin and FK506 (19). The complex of each drug with its cognate intracellular receptor, known as an immunophilin, binds to and inhibits the PP2B heterodimer. It has been speculated that the immunophilins may be physiological regulators of PP2B and the immunosuppressive drugs simply stabilize the formation of immunophilin/PP2B complexes to potentiate phosphatase inhibition (20). A more convincing candidate for an endogenous PP2B inhibitor is the newly discovered cain. This 240 kDa protein associates directly with the A or catalytic subunit and suppresses PP2B activity at micromolar concentrations (21). Several other PP2B binding proteins have been identified. Some of these like the A-kinase anchoring protein, AKAP-79, may also inhibit PP2B activity (22). PP2B associates with its substrate, the transcription factor NFAT (nuclear factor of activated T-cells). Recent studies show that the PP2B-binding domain of NFAT inhibits PP2B activity *in vitro* and in intact T-cells (23). Whether native NFAT functions as a substrate and a regulator of PP2B in mammalan cells remains to be determined.

2.1.4. Other protein serine/threonine phosphatases

A number of protein phosphatases have been identified by their homology to PP1 and PP2A. Some of these enzymes, such as PPX (or PP4) and PPV(or PP6), show intriguing and highly restricted subcellular localizations, pointing to specialized functions in cells. However, the study of these enzymes has lagged behind that of PP1, PP2A, and PP2B in large part due to problems with their expression and subsequent biochemical characterization. Thus, we know very little about the physiological functions and regulation of these enzymes in eukaryotic cells. The nomenclature for these newly discovered enzymes is also undergoing review and as such, they will not be discussed further in this review.

3. PROTEIN PHOSPHATASE INHIBITORS

3.1. PP1 inhibitors

3.1.1. Inhibitor-1

Inhibitor-1 (I-1) was first identified in rabbit skeletal muscle (24), but is widely expressed in mammalian tissues. This thermostable protein (Mr 18,700) is a potent and specific PP1 inhibitor, K_i = 1.6nM, when it is phosphorylated by PKA (25, 26). Structure-function studies show that it is closely related to DARPP-32, a predominantly neuronal PP1 inhibitor. Studies of I-1 also indicate a complex array of interactions with the PP1 catalytic subunit that together result in enzyme inhibition (27). The essential elements in I-1 that mediate PP1 inhibition include the phosphorylation of $threonine_{35}$ and a tetrapeptide consensus motif found in many PP1-binding proteins (28). I-1 is also phosphorylated *in vivo* on $serine_{67}$ by an unknown protein kinase (29). However, the functional role of this phosphorylation remains unknown.

The role of I-1 in regulating PP1 function has been investigated in many different physiological settings. These include the hormonal control of glycogen metabolism, synaptic plasticity controlled by neurotransmitters, growth of pituitary tumor cells and the control of muscle contraction. Some but not all hormones that elevate intracellular cAMP activate I-1. The precise reasons for this remain unclear but may in itself account for some of the differences in physiological effects of these hormones. In any case, hormones that activate I-1 induce much larger and more rapid changes in signal transduction pathways transduced by PP1 substrates.

3.1.1. 1. I-1 and Glycogen Metabolism

Hormones, like adrenalin, which activate PKA result in the increased phosphorylation and inactivation of glycogen synthase in mammalian skeletal muscle. This activation is not due to the direct action of PKA on glycogen synthase. Although PKA can phosphorylate and inactivate glycogen synthase *in vitro*, adrenalin promotes the phosphorylation of glycogen synthase at serines that are not PKA targets (30). Moreover, the protein kinase, GSK3, that phosphorylates these serines is itself not activated by PKA. Consequently, it was determined that the mechanism for enhanced glycogen synthase phosphorylation was the PKA-mediated activation of I-1 and the resultant inhibition of PP1 (31). This revealed a novel mechanism for hormone action in that PP1 modulation via I-1 mediated cAMP regulation of proteins that are substrates for kinases other than PKA.

Glycogen synthase is regulated by a pool of PP1 that is bound to the glycogen particle. This association is mediated through the glycogen-targeting subunit(s) of PP1. PKA-mediated phosphorylation of the skeletal muscle G-subunit is thought to cause PP1 dissociation from the G subunit and reduce glycogen synthase phosphatase activity. Thus, cAMP may coordinate PP1 translocation from

glycogen with the activation of the cytosolic inhibitor, I-1, to achieve rapid phosphatase inhibition. This would also accommodate the *in vitro* finding that glycogen-bound PP1 was less sensitive to inhibition by I-1 than free PP1 catalytic subunit. Presence of a common PP1-binding motif in I-1 and the G-subunit also predicted that interaction of these proteins with PP1 may be mutually exclusive. Recently, unpublished studies have begun to question the notion of hormone-induced translocation of PP1 from glycogen to cytosol. The full impact of these findings on the hormonal regulation of glycogen-bound PP1 has not been determined.

Other hormones, such as insulin, result in the dephosphorylation and activation of glycogen synthase. Numerous studies have reported that insulin activates PP1 in insulin-sensitive cells (32). In skeletal muscle, a number of mechanisms may combine to activate the glycogen-bound PP1 in response to insulin. For instance, insulin activates cAMP phosphodiesterase to lower cAMP levels. This would result in I-1 inactivation and in itself promote glycogen synthase dephosphorylation. Others have suggested that insulin actively promotes the dephosphorylation of I-1 by activating an I-1 phosphatase. Finally, phosphorylation of the G_M-subunit by an insulin-stimulated protein kinase, most likely PKB, also elevates glycogen synthase phosphatase activity. Combined effects of the reduced kinase and increased PP1 activity promote glycogen synthase dephosphorylation and the storage of excess blood glucose as glycogen in skeletal muscle. A number of G_M homologues have been identified in other tissues (33) and unlike the G_M-subunit, some of these are not subject to regulation by insulin (34). This potentially makes I-1's role in hormonal control of glycogen metabolism even more important in these tissues.

3.1.1. 2. I-1 and Synaptic Plasticity

Changes in synaptic transmission elicited by prior neuronal activity, have been extensively studied in the hippocampus as a potential model for learning and memory. Activity-dependent enhancement of synaptic transmission is seen as long term potentiation or LTP. Yet other stimuli depress the functions of hippocampal synapses leading to long term depression or LTD. Several protein kinases, including CaM-Kinase II, PKC (35) and fyn (36), have been implicated in LTP. Based on their electrophysiological characteristics, LTD appeared to be the converse of LTP and may therefore involve protein phosphatases. Recent studies established that two protein phosphatases, PP1 and PP2B, acted in tandem to regulate LTD (18). The PP1 inhibitor, I-1, is highly expressed in the hippocampus and functions as the link between PP2B and PP1. This demonstrated a different use of the phosphatase cascade first suppressed to control glycogen metabolism in skeletal muscle.

Interestingly, LTP and LTD are both activated by the excitatory neurotransmitter, glutamate, acting via the second messenger, calcium. So an important question has been how a single second messenger mediated such opposing physiological effects. The answer appears to lie in the different sensitivities of the calcium/calmodulin activated protein phosphatase (PP2B) and kinase (CaM-Kinase II) in neurons. It has been speculated that low calcium levels activate PP2B and induce LTD while higher concentrations of calcium activate CaMKII which is required to trigger LTP. However, LTP and LTD share many common characteristics, suggesting that they also share many signaling components. In this regard, I-1 appears to be involved in both LTP and LTD. PKA inhibitors abolish sustained postsynaptic LTP. These effects were reversed by phosphatase inhibitors, specifically those that target PP1. Subsequently, it was shown that LTP-generating stimuli also activated PKA which in turn activated the endogenous PP1 inhibitor, I-1, in hippocampal neurons (37). Thus, the current model is that LTP-generating stimuli result in the activation of both CaM-Kinase II and calcium/calmodulin-stimulated adenylyl cyclase. The role of cAMP in the LTP pathway is to activate I-1 and suppress PP1 activity, which reverses the functions of CaM-Kinase II. Hence, I-1 functions as the gatekeeper which determines whether the neuron will transition from early to intermediate or late phases of LTP. In contrast to LTP, LTD results from the small influx of calcium, which is unable to activate either CaMKII or adenylyl cyclase, and, the predominant signal transducer for LTD is PP2B or calcineurin. The subcellular organization of signaling molecules may also be an important contributor to the unique specificity of the LTP and LTD responses.

3.1.1. 3. I-1 and Cell Growth

Genetic studies in fungi implicated a critical role for PP1 in the cell cycle, particularly in the transition through mitosis. Further evidence in *Xenopus laevis* oocytes, which lack the G1 phase of the cell cycle, showed that PP1 inhibitors inhibited cell division or oocyte maturation (38). Oocytes stalled in mitosis show high levels of histone H1 phosphatase activity and are unable to activate the cyclin-dependent protein kinase, Cdc2, or MPF, the maturation promoting factor. Introduction of PP1 inhibitors not only lowered the histone phosphatase activity in these oocyte extracts but also activated MPF (39). This strongly hints at the presence of regulated PP1 inhibitors, like I-1, in the timely activation of MPF and completion of cell division.

Many growth factors activate PP1 (32), but the role for PP1 activation in cell growth remains unknown. In this regard, PP1 was required for growth of the cultured pituitary tumor cell line, GH4D1. cAMP and cyclosporin A, both antiproliferative agents, were shown to enhance the phosphorylation of I-1 in these cells. These studies provided the first evidence for I-1 as a growth regulator and suggested that chronic I-1 activity, induced by PKA activation or calcineurin (PP2B) inhibition blocked cell growth (40). These results also indicated that PP1 should not always be considered as a negative growth regulator (as discussed for RB dephosphorylation) but that it may also provide positive signals for growth by promoting other phases of the cell cycle.

3.1.1. 4. I-1 and Muscle Contraction

The β–adrenergic agonist, isoproterenol, has a positive inotropic effect in the heart. Isoprotenol increases I-1 phosphorylation and results in the inhibition of PP1 activity in the myocardium (41, 42). This decrease in PP1 activity enhances cardiac contractility by preventing the dephosphorylation of proteins such as the Na/K-ATPase, phospholamban, troponin I and voltage sensitive calcium channels which are all involved in maintaining the contractile state of heart muscle (43).

On the other hand, smooth muscle PP1 is tightly bound to myosin and is the primary myosin phosphatase (44). Dephosphorylation of myosin light chains results in the relaxation of smooth muscle. Yet, hormones, such as epinephrine, that increase intracellular cAMP and lead to I-1 phosphorylation, promote the relaxation of smooth muscle. This paradox was resolved by the finding that smooth muscle myosin light chain kinase (MLCK) is phosphorylated at an inhibitory site in response to cAMP. The dephosphorylation of MLCK at this site is also mediated by PP1. Thus, it has been proposed that hormonal activation of I-1 leads to the predicted inhibition of PP1 that acts on MLCK. Thus, MLCK is inactivated and smooth muscle relaxation ensues. Unlike CaM-kinase II which is inactivated by PP1, smooth muscle MLCK, another calmodulin-regulated enzyme, requires PP1 activity to remain in its active state. Thus, I-1 functions in opposing ways to control protein kinases in neurons and smooth muscle. This model also predicts that the myosin-bound PP1, which dephosphorylates myosin light chains to relax smooth muscle, is not regulated by I-1. This may be consistent with the presence of the common PP1-binding motif in I-1 and the 110 kDa myosin-targeting subunit of PP1. Alternately, the interplay between I-1 and the myosin and MLCK phosphatases (both PP1) may modulate the rates of protein phosphorylation and dephosphorylation that set the contractile tone of different smooth muscle beds.

3.1.2. DARPP-32 (dopamine and cAMP-regulated phosphoprotein of apparent Mr 32,000)

DARPP-32 is highly homologous to I-1 near its N-terminus and like I-1, it inhibits PP1 activity only after phosphorylation on threonine$_{34}$ by PKA (45). DARPP-32 is found mainly in the brain, especially in the basal ganglia (46), but it is also expressed in adipose tissue (47) and to a much lesser extent, in the kidney (48). Recent reports suggest that DARPP-32 is myristoylated at its N-terminus, which mediates its association with membranes. Although originally identified by its enhanced phosphorylation in response to dopamine, DARPP-32 is activated by many hormones and neurotransmitters that modulate cAMP levels (49). Interestingly, some cells in the brain and kidney express both I-1 and DARPP-32. However, DARPP-32 shows a more complex mode of regulation than I-1. For instance, DARPP-32 is phosphorylated *in vivo* by both PKA and casein kinase I. Phosphorylation by casein kinase I impairs the turnover of phosphate at the activating site, threonine$_{34}$ (50). Indeed, two different type-2 phosphatases, PP2B acting on threonine$_{34}$ and PP2C dephosphorylating the casein kinase I sites, may regulate DARPP-32 function. This led to the speculation that the two PP1 inhibitors responded differently to physiological stimuli and may be used in different ways to control PP1 activity in these cells. In brain, dopamine and glutamate have antagonistic effects on the excitability of neurons, possibly mediated by their opposing effects on DARPP-32. Dopamine acting at D1 receptors activates adenylyl cyclase, and through PKA, activates DARPP-32 (45). Glutamate, on the other hand, working through NMDA receptors, reverses DARPP-32 activity. Thus, like I-1, increases in intracellular calcium promote the dephosphorylation of DARPP-32 in the nervous system (51). Unfortunately, unlike I-1, there is only limited information on the physiological role of DARPP-32 in the brain and other tissues.

3.1.2. 1. DARPP-32 and Na/K-ATPase

In the kidney (48) as well as in the brain (45), activation of the D1 receptors by dopamine results in the blockade of Na^+/K^+-ATPase activity which in turn causes vasodilation and increased natriuresis. DARPP-32 immunoreactivity has been shown in the thick ascending limb of the loop of Henle (52). Use of DARPP-32 phosphopeptides showed that PP1 inhibition increased the phosphorylation of Na^+/K^+-ATPase. This resulted in increased Na+ excretion as the Na^+/K^+-ATPase no longer pumps Na+ from the urine back into the blood stream. Vasodilation occurs as water flows into the kidney to maintain the ion gradient (53). Thus, DARPP-32 appears to be a critical component of the control of salt balance in the mammalian kidney.

3.1.2. 2. DARPP-32, lipogenesis and lipolysis

DARPP-32, not I-1, is present in 3T3-L1 adipocytes and in these cells, it is required for adipogenesis. Active DARPP-32 maintains low PP1 activity in differentiated fat cells and this is essential for insulin to stimulate PP1 activity and facilitate triglyceride biosynthesis (34). These studies suggest that, like I-1 in other tissues, changes in DARPP-32 activity may be an important avenue for insulin signaling. cAMP-mediated activation of DARPP-32 may be critical for amplifying the phosphorylation events that lead to activation of hormone-sensitive lipase and enhanced lipolysis.

3.1.3. Inhibitor-2

Inhibitor-2 (I-2) was isolated, along with I-1, as a heat-stable protein from skeletal muscle extracts and specifically inhibits PP1 activity with a Ki = 3.1 nM (24). Unlike I-1 or DARPP-32, I-2 does not need to be phosphorylated to inhibit PP1. I-2 forms a stable and inactive complex with the PP1 catalytic subunit. This inactive complex was isolated as an ATP-Mg-dependent phosphatase which, as the name implies, was inactive until incubated with ATP-Mg. Activation of the latent complex is accompanied by the phosphorylation of I-2 on threonine$_{72}$ by GSK-3. I-2 is also phosphorylaed on three serines by casein kinase II (CK2). Phosphorylation by CK2 does not alter I-2 activity but greatly facilitates the subsequent phosphorylation by GSK-3 (54, 55). The activation cycle for the ATP-Mg-dependent phosphatase is complicated and somewhat controversial. The fundamental aspect of this cycle is that I-2 phosphorylation by GSK-3 promotes a conformational change in the PP1/I-2 complex, which does not by itself activate the enzyme. The slow autodephosphorylation of I-2 correlates best with the increase in PP1 activity. Throughout this process, I-2 remains bound to PP1 and in a longer time frame the complex relaxes back to its original inactive conformation. Recent studies show that multiple domains mediate the rapid and reversible inhibition of PP1 and the slower inactivation to the stable complex that can be reactivated by GSK-3 (56). Interestingly, the active PP1/I-2 complex can itself be inhibited by addition of exogenous I-2. Against this background of complex interactions of I-2 with PP1, its role as a PP1 inhibitor has been questioned (57).

Recent *in vitro* studies suggest that I-2 interacts with denatured PP1 to promote a rapid and effective refolding of this protein and yield an active enzyme. In support of this, recombinant PP1, which differs from native PP1 in significant ways, behaves much more like the native

enzyme after incubation with I-2 and reactivation by GSK-3 (58, 59). This has led to the proposal that I-2 is a chaperone for PP1. This hypothesis may also be consistent with preliminary reports that overexpression of I-2 in mammalian cells does not inhibit cellular PP1 activity but in fact elevates the cellular content of PP1 catalytic subunit. Studies with Glc8, the yeast homologue of I-2, came to a more complex conclusion, suggesting that under different circumstances, Glc8 was either an inhibitor or an "activator" of the yeast PP1 (60). So, despite extensive studies the physiological functions of I-2 remain elusive.

Perhaps the most intriguing property of I-2 is that its protein and mRNA levels fluctuate during the cell cycle, peaking twice, at S phase and mitosis (5). Recent experiments used I-2 fused to the green flourescent protein (GFP) to show that I-2 was cytosolic during G1 and translocated to the nucleus in S phase (61). Moreover, phosphorylation at four sites was necessary for nuclear translocation as mutations at any one serine prevented nuclear entry. These studies also identified a putative nuclear localization sequence in I-2 and showed that mutations of two lysines in this sequence abolished nuclear localization of I-2. Changes in the phosphorylation of I-2 during G1 and S phase also argued for a role of I-2 phosphorylation in its subcellular localization. As I-2 localization failed to correlate with PP1 distribution in cells, this has also raised questions about I-2's role as a PP1 regulator, but other functions for I-2 have not been identified.

3.1.3. 1. I-2 and Sperm Motility

The only physiological role proposed for I-2 is in the control of sperm motility. The testis-specific isoform, PP1γ2, forms an inactive complex with I-2. The increased PP1 activity seen in nonmotile immature sperm was accredited to an elevation in GSK-3 activity which activates the PP1/I-2 complex. Incubation of immature sperm with the phosphatase inhibitors, okadaic acid and calyculin A, induced motility (62) suggesting that I-2 inhibits PP1 activity in mature mammalian sperm to facilitate their motility.

3.1.4. NIPP-1 (nuclear inhibitor of PP1)

NIPP-1 was originally identified as two PP1 inhibitory polypeptides of 16 and 18 kDa in the particulate fraction of bovine thymus nuclei (63). Subsequent studies showed that the full length NIPP-1 cDNA encoded a protein of 38.5 kDa that was extremely sensitive to proteolysis. NIPP-1 is perhaps the most potent PP1 inhibitor thus far identified with a Ki in the picomolar range. The protein is largely nuclear in its localization and is ubiquitously expressed in mammalian tissues (64). Although NIPP-1, like I-1 and DARPP-32, possesses the consensus PP1-binding motif, it is more like I-2 in its action, being a more potent phosphatase inhibitor in the dephosphorylated state. Moreover, NIPP-1, like I-2, forms a stable, inactive complex with PP1. Following the phosphorylation of NIPP-1 by PKA on a site within the PP1-binding motif, it's activity as a PP1 inhibitor is dramatically reduced.

NIPP-1 is phosphorylated at serine-178 and serine–199, by PKA and on threonine-161 and serine-204 by CK2 (65). Both kinases reduce the affinity of NIPP-1 for PP1 and increase phosphatase activity. The effects of the two kinases in activating the NIPP-1/PP1 complex are synergistic, but do not cause dissociation of the complex. It has been speculated that NIPP-1 phosphorylation prevents its reassociation with PP1 (64).

3.1.4.1. NIPP-1 and RNA Metabolism

No clear physiological role for NIPP-1 has been identified but one clue comes from the observation that NIPP-1 contains a RNA-binding motif also found in the bacterial RNA processing enzyme, Ard-1. The extensive sequence homology between mammalian Ard1 and the carboxy-terminus of NIPP-1 suggests that the two proteins are derived from the same gene through alternate splicing (64). Pursuant to this, recombinant NIPP-1 was shown to bind to RNA and its C-terminal fragment was shown to possess endonuclease activity. While the RNA-binding was unaffected by the presence of PP1, the full-length NIPP-1 did not degrade RNA (66). Whether NIPP-1 mediates PP1's association with RNA is not certain, but other PP1-binding proteins (67) have been implicated in PP1's function to control spliceosome assembly and RNA splicing. At this time, the physiological role of NIPP-1 as a PP1 inhibitor has not been established.

3.1.5. RIPP-1 (ribosomal inhibitor of PP1)

RIPP-1 is a 23 kDa basic polypeptide that is complexed with PP1 in rat liver ribosomes. *In vitro*, RIPP-1 is a potent inhibitor of PP1 (Ki = 20 nM) with some substrates, phosphorylase a and myelin basic protein, but a much poorer inhibitor (Ki = 400nM) with other substrates, histone IIA and casein. In addition, RIPP-1 seems to be able to inactivate PP1. Incubation of PP1 with RIPP-1 for 45 minutes at 25°C converted PP1 into a less active enzyme that could not be reactivated by proteolytic degradation of RIPP-1. This inactivation of PP1 is reminiscent of I-2 except that following phosphorylation by GSK-3, I-2's effects are reversed. At this time, no mechanism for reversal of PP1 inactivation by RIPP-1 has been demonstrated. Few experiments have been done thus far to elucidate RIPP-1's physiologic role. However, RIPP-1 inhibits PP1-mediated dephosphorylation of ribosomal S6, a component of the 40 S ribosomal subunit and suggests a role in the control of protein synthesis (68).

3.1.6. CPI17 (C-kinase activated PP1 inhibitor, apparent Mr 17,000)

CPI17 is the newest member of the PP1 inhibitor family. It was isolated from porcine aorta smooth muscle as a 17 kDa PKC substrate that potently inhibits PP1 activity (69). The mRNA for CPI17 is expressed exclusively in smooth muscle, such as aorta and bladder, but not in skeletal muscle or non-muscle tissues. When phosphorylated by PKC, CPI17 shows a Ki = 0.18 nM for PP1 and a Ki = 1.3 μM in its dephosphorylated state. An unusual aspect of CPI17 is that it inhibits the myosin-bound PP1 complex as well as the free PP1 catalytic subunit (70). This implies that it functions differently from I-1 and DARPP-32 and suggests an important role for CPI17 in the control of smooth muscle contractility.

3.2. PP2A inhibitors

3.2.1. I_1^{PP2A}

I_1^{PP2A} was purified from bovine kidney as a 30 kDa heat stable protein that specifically inhibited PP2A with a Ki = 30 nM (17). Subsequent cloning of this protein showed it to be the bovine homologue of the human

putative histocompatibility leukocyte antigen class II associated protein-1 (PHAP-1) (71). PHAP-1, a protein of unknown function, was thought to be involved in the immune response as it is bound to the C-terminal region of the DR2α chain of MHC class II receptors (72). I_1^{PP2A} is highly acidic at its C-terminus and it has been hypothesized that this region mediates PP2A inhibition as the other PP2A inhibitor, I_2^{PP2A}, also has a highly acidic tail (71, 73). Little else is known about I_1^{PP2A} in terms of PP2A regulation, but it is interesting to note that I_1^{PP2A} has recently been identified as a target for granzyme A. T-lymphocytes trigger apoptotic death in target cells by releasing cytotoxic granules, containing perforin, a pore-forming protein, and several granzymes, which are serine proteases. A putative substrate trapping mutant of granzyme A identified I_1^{PP2A} and I_2^{PP2A} as binding proteins (74). Thus, it has been inferred that degradation of PP2A inhibitors may be an important signal for apoptotic cell death.

3.2.2. I_2^{PP2A}

I_2^{PP2A} was also identified as a heat stable PP2A inhibitor from bovine kidney (17). I_2^{PP2A} is a 39 kDa protein that is a homologue of the human SETα protein, also called PHAP-II. I_2^{PP2A} inhibits PP2A with a Ki = 2 nM (73). Preliminary studies show that I_2^{PP2A} is phosphorylated *in vivo* at two serines near its N-terminus, but the functional consequences of these phosphorylations remain unknown. Subcellular localization shows that I_2^{PP2A} is largely nuclear. A chromosomal translocation that leads to the fusion of a nuclear porin, NUP214, with I_2^{PP2A} has been linked to a variety of leukemias and may provide new clues to the physiological functions of I_2^{PP2A} .

3.3. Endogenous PP2B Inhibitors

PP2B is the recognized target of the two immunosuppressive drugs, cyclosporin and FK506, commonly used in organ transplantation. While cyclosporin only inhibits PP2B in conjunction with a variety of cyclophilins, recent studies suggest that PP2B associates with cyclophilin in the absence of drug (20). This has led to the speculation that cyclophilins and other immunophilins may be natural regulators of PP2B. Recently, a direct endogenous inhibitor of PP2B has been identified. Cain is a 240 kDa protein which inhibits PP2B with a Ki = 0.44 uM, making it the most potent endogenous inhibitor of PP2B so far. Co-localization studies show that both calcineurin and cain are found in the brain exclusively in neurons. In addition, it has been shown that cain binds to PP2B at a site distinct from that of FK506/FKBP12 binding, thus, identifying a novel regulatory site on PP2B (21). PP2B also associates with the PKA-binding protein, AKAP79 (22) and this association reduces the phosphatase activity. Whether this qualifies AKAP79 as a phosphatase inhibitor is not yet clear, but fragments of AKAP79 that mediate PP2B binding have been shown to suppress PP2B functions in intact cells. Finally, PP2B associates constitutively with its T-cell substrate, NFAT. It is now well established that PP2B dephosphorylates NFAT and thereby facilitates its entry into the nucleus to initiate gene transcription. Introduction of the PP2B-binding region of NFAT into cells inhibited the transcription of cytokine genes (23). Thus, one scenario is that NFAT association restricts PP2B functions solely to regulate NFAT or, alternately, additional signals are required to activate the NFAT-bound PP2B and promote rapid dephosphorylation of the transcription factor. Future studies will shed more light on the role of PP2B-binding proteins in the function of this phosphatase.

4. PHOSPHATASE INHIBITORS AND HUMAN DISEASE

The physiological relevance for phosphatase inhibitors can often be inferred from their involvement in the pathogenesis of disease. Because of its role in dopaminergic pathways in the brain, DARPP-32 has been implicated in Parkinson's disease (75). A decrease in dopamine signaling, a hallmark of Parkinson's, may at least in part be mediated by changes in DARPP-32 activity. Indeed, preliminary studies show that loss of DARPP-32 function in mice elicits locomotor defects similar to Parkinson's.

There appears to be a strong link between PP2A inhibitors and cancer. I_1^{PP2A} is highly expressed in some cancer cells (Z. Damuni, personal communication). In many acute undifferentiated leukemias, a chromosomal translocation on chromosome 9 results in the fusion of SETα (I_2^{PP2A}) to the C-terminus of NUP214, a nuclear porin. NUP214 is normally localized to the cytoplasmic side of the nucleus. In contrast, the fusion protein is localized to the interior of the nucleus (76, 77, 78). Moreover, the I_2^{PP2A}-NUP214 fusion protein is a more potent PP2A inhibitor than I_2^{PP2A} (Z. Damuni, personal communication), suggesting that the more effective inhibition of nuclear PP2A accounts for unregulated growth of leukemic cells.

Deregulation of protein phosphatases has also been implicated in insulin resistance, associated with diabetes mellitus and other metabolic disorders. To what extent the failure of hormones to control protein phosphatase activity in this setting is due to errors in the function of endogenous inhibitors is not certain. Future studies that define the physiological role of protein phosphatases and their regulators should provide new insights into the molecular basis for insulin resistance and other human disorders.

It has long been known that protein serine/threonine phosphatases are the targets for over 50 environmental toxins. While these have been useful tools for studying the phosphatases, they also reveal the essential nature of protein serine/threonine phosphatases to cell physiology. Many of these toxins are also tumor promotors, suggesting the importance of serine/threonine phosphatase inhibition to unregulated cell growth. Additional evidence for this idea comes from the fact that several viral proteins have also been identified which target the inhibition of protein phosphatases to help take control of the cell. Perhaps the best examples of this is the polyoma middle T and SV40 small T antigen which selectively inhibit PP2A. More recently, a viral protein, A238L, from the african swine fever virus has been found to inhibit PP2B (79). The existence of toxins and viral proteins that target serine/thrreonine phosphatases reveals the importance of reversible regulation of endogenous phosphatase inhibitors for proper functioning of phosphatases in cells and suggests the possibility of the involvement of disregulation of inhibitors in the pathogenesis of cancers.

5. PERSPECTIVE

This review has focused on the known inhibitor proteins for the major protein serine/threonine phosphatases, PP1 and PP2A, while recognizing that the family of protein phosphatases has continued to expand over the last few years. At this time, there may be more than 200 functionally different serine/threonine phosphatase complexes in mammalian cells. In addition, 100 or more protein tyrosine phosphatases have also been identified. Complete understanding of mechanisms that regulate these enzymes, in many cases, awaits the identification of suitable physiological targets. In this respect, PP1 and PP2A represent the founder members of the protein phosphatase family and have provided us with valuable models for enzyme regulation that could be applied to other phosphatases. Our discussion has attempted to highlight the manner in which phosphatase inhibitors provide for amplification of hormone signals, cross-talk between distinct signal transduction pathways, control the size and duration of a physiological response and act as gatekeepers or decision makers in cell signaling. The presence of many different inhibitors of an individual phosphatase also emphasizes the range of options available to a cell to communicate physiological signals through the protein phosphatase. With the recent expansion in the number of phosphatase catalytic and regulatory subunits, the days of considering protein phosphatases as unregulated or constitutive enzyme activities are finally over.

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