multimedia

REVIEW ARTICLE

The role of serine/threonine protein phosphatases in exocytosis

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Modulation of exocytosis is integral to the regulation of cellular signalling, and a variety of disorders (such as epilepsy, hypertension, diabetes and asthma) are closely associated with pathological modulation of exocytosis. Emerging evidence points to protein phosphatases as key regulators of exocytosis in many cells and, therefore, as potential targets for the design of novel therapies to treat these diseases. Diverse yet exquisite regulatory mechanisms have evolved to direct the specificity of these enzymes in controlling particular cell processes, and functionally driven studies have demonstrated differential regulation of exocytosis by individual protein phosphatases. This Review discusses the evidence for the regulation of exocytosis by protein phosphatases in three major secretory systems, (1) mast cells, in which the regulation of exocytosis of inflammatory mediators plays a major role in the respiratory response to antigens, (2) insulin-secreting cells in which regulation of exocytosis is essential for metabolic control, and (3) neurons, in which regulation of exocytosis is perhaps the most complex and is essential for effective neurotransmission.

Key words: β-cell, exocytosis, mast cell, neuron, phosphatase, signal transduction.

INTRODUCTION

The incorporation of membrane vesicles into the plasma membrane by exocytosis is a ubiquitously expressed process that mediates cell–cell communication. Although the demand for exocytosis varies considerably between cell types and calls for specialized adaptations of the basic process to suit the demands of different cell types, many components of the exocytotic machinery are strongly conserved from yeast to neurons [1–4].

Several groups of protein that are involved in the fundamental exocytotic machinery are found in all eukaryotic cells [3]. SNARE [soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor] proteins are present on both vesicular and plasma membranes and form stable fusion ('core') complexes [5]. NSF is a chaperone-like protein that forms ATP-dependent interactions, via SNAP (soluble *N*-ethylmaleimide-sensitive adaptor protein), with all members of the SNARE core complex [6]. NSF has ATPase activity that is believed to promote disassembly of SNARE complexes during core complex recycling. SM (Sec1Munc18-like) proteins, which also form interactions with SNARE proteins (predominantly syntaxins) are required for all intracellular vesicular-traffic steps [7]. The neuronal SM protein Munc18 is essential for regulated exocytosis and functions in a late stage in the fusion process, where its dissociation from syntaxin determines the kinetics of post-fusion events [8]. Lastly, a large family of GTP-binding proteins, namely Rab proteins, are also involved in multiple aspects of exocytotic pathways, but their individual roles are incompletely understood [4].

Although specific roles for individual members of these groups of proteins in the exocytotic process continue to be elucidated, investigation of the molecular mechanisms underlying exocytosis has established that protein phosphorylation is an essential regulatory component, and members of each of these fundamental groups of proteins are regulated by phosphorylation [8–12]. Signals that lead to exocytosis result in the activation of protein kinases and protein phosphatases with concomitant changes to the phosphorylation levels of many proteins involved in the exocytotic machinery. The phosphorylation of a given protein is determined by the co-ordinate control of both protein kinases and protein phosphatases, and alteration in either enzyme can lead to changes in exocytosis. Compared with protein kinases, the mechanisms underlying regulation of protein phosphatase activity in response to cellular signalling are poorly understood. Nevertheless, evidence is beginning to emerge for specific regulatory roles for protein phosphatases in exocytosis. Whereas there is growing evidence for the involvement of protein tyrosine phosphatases in exocytosis [13–16], the focus of this Review will be the serine/threonine phosphatases. The Review summarizes the major regulatory features of serine/threonine protein phosphatases and provides an analysis of the evidence that supports a role for serine/threonine protein phosphatases in regulating exocytosis in three major cellular systems. Each system illustrates the diversity of roles for protein phosphatases within the exocytotic process

Abbreviations used: SNARE, soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor; SNAP, soluble *N*-ethylmaleimide-sensitive adaptor protein; SM, Sec1Munc18-like; PP1 etc., protein phosphatase 1 etc.; PPP, phosphoprotein phosphatase; PPM, protein phosphatase M (Mg^{2+}-dependent phosphatases); PKA, protein kinase A; AKAP, A kinase anchoring protein; CG-NAP, centrosome-and-Golgi-localized protein kinase N-associated protein; NMDA, *N*-methyl-D-aspartate; LTP, long-term potentiation; PKC, protein kinase C; MLCK, myosin light chain kinase; WD repeat, tryptophan-aspartic acid repeat; SV40, simian virus 40; CaNB, calcineurin regulatory subunit B; DSCR1, Down's syndrome candidate region; JNK, c-Jun N-terminal kinase; TPR, tetratricopeptide repeat; ILK1, integrin-linked kinase 1; ILKAP, ILK1-associated phosphatase; MKK, mitogen-activated protein (MAP) kinase kinase; MKKK, MAP kinase kinase kinase; TGF-β, transforming growth factor-β; I_1, inhibitor-1; I_2, inhibitor-2; PHAP, putative histocompatibility leucocyte antigens class II-associated protein; CsA, cyclosporin A; KHC, kinesin heavy chain; 4AP, 4-aminopyridine.

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and identifies a number of potential loci of action of particular protein phosphatases that may not be immediately recognized through investigation of a single system.

STRUCTURE AND REGULATION OF SERINE/THREONINE PROTEIN PHOSPHATASES

The human genome encodes over 300 serine/threonine protein kinases, but less than 40 serine/threonine protein phosphatases [17]. Thus, although protein kinases have diversified through variation of the catalytic subunit, a relatively small number of protein phosphatases is responsible for dephosphorylating a vast array of proteins. This concept has historically promoted the view of protein phosphatases as relatively uncontrolled, but it is now clear that specific changes in substrate phosphorylation levels are regulated at both kinase and phosphatase levels, with protein phosphatases providing the fine control by regulating the rate and duration of key steps involved in cellular processes [18]. To exert this specificity, protein phosphatases have evolved exquisite control mechanisms that target distinct pools of enzyme to specific subcellular locations and modulate substrate specificity through dynamic binding to relevant protein partners. Thus, whilst *in vitro* studies might indicate a degree of selectivity for substrates by individual phosphatases, the relevant phosphatase *in vivo* is determined by the relative location and concentration of each phosphatase relative to each particular substrate. A number of recent reviews that provide comprehensive analysis of the structure and regulation of protein phosphatases already exist [17,19–29]. The following is a summary of the key features, and readers are referred to these excellent and more extensive reviews for further detail.

Classification of serine/threonine protein phosphatases

In general, serine/threonine protein phosphatases can be classified into two families on the basis of similarity in the primary amino acid sequence between the different catalytic subunits. The largest of these families is the PPP (phosphoprotein phosphatase) family, which includes the abundant PP1, PP2A and PP2B enzymes, as well as the more recently identified PP4 (formerly PPX), PP5, PP6 and PP7 enzymes. Figure 1 shows a schematic diagram of the basic native forms of the three best-characterized PPP enzymes. Early attempts to classify protein phosphatases utilized their different capacities to dephosphorylate certain substrates and responses to heat-stable inhibitor proteins [30]. Thus PP1 was inhibited by the heat-stable proteins inhibitor-1 (I_1) and inhibitor-2 (I_2), whereas type 2 phosphatases (PP2A, PP2B and PP2C) were not. PP1 also preferentially dephosphorylated the β-subunit of phosphorylase kinase, whereas type 2 phosphatases preferentially dephosphorylated the α-subunit of phosphorylase kinase. Of the type 2 phosphatases, PP2B is Ca^{2+}-activated, whereas PP2C is Mg^{2+}-dependent. PP2A is active in the absence of bivalent cations. Although this classification holds remarkably true today, the advent of molecular genetics has enabled a more comprehensive classification based on sequence similarity of the catalytic subunit. It is now clear that PP2C belongs to a distinct [PPM (protein phosphatase M/Mg^{2+}-dependent phosphatases)] gene family, whereas the remaining type 2 and type 1 phosphatases belong to the same (PPP) gene family [27]. In comparison with the PPP family, the PPM family has a limited number of members [27].

PP1

The catalytic subunit of PP1 (PP1c) has four mammalian isoforms (α, β/δ, γ^1 and γ^2). The γ isoform has two alternatively spliced variants, and there is also evidence for two alternatively spliced variants of the α isoforms [17,20]. Differential expression and subcellular translocation of these isoforms may account for a degree of specificity in phosphatase action [31]. For example, in brain, the α and γ^1 isoforms are highly expressed in the striatum, whereas the β isoform is not [32]. In the cell nucleus the α isoform localizes to the nuclear matrix, but translocates to the centrosome during mitosis. Similarly, the β isoform moves from non-nuclear chromatin to the chromosomes, and the γ^1 from nucleoli to microtubules, during mitosis [33]. Dynamic relocalization of PP1 isoforms has been demonstrated in a number of systems [34], but these isoforms are approx. 90% homologous, diverging only in their N- and C-termini, and have broad and overlapping substrate specificity. Differential expression and subcellular translocation of catalytic subunit isoforms is therefore insufficient to fully account for control of PP1 specificity, and it is now established that interaction with additional regulatory subunits is the primary means of control.

The concept of regulatory subunits which govern PP1 specificity was first presented in 1993 from an analysis of glycogen-associated PP1 [35]. This enzyme comprised the catalytic subunit complexed to a glycogen-bound protein, G_M, and was

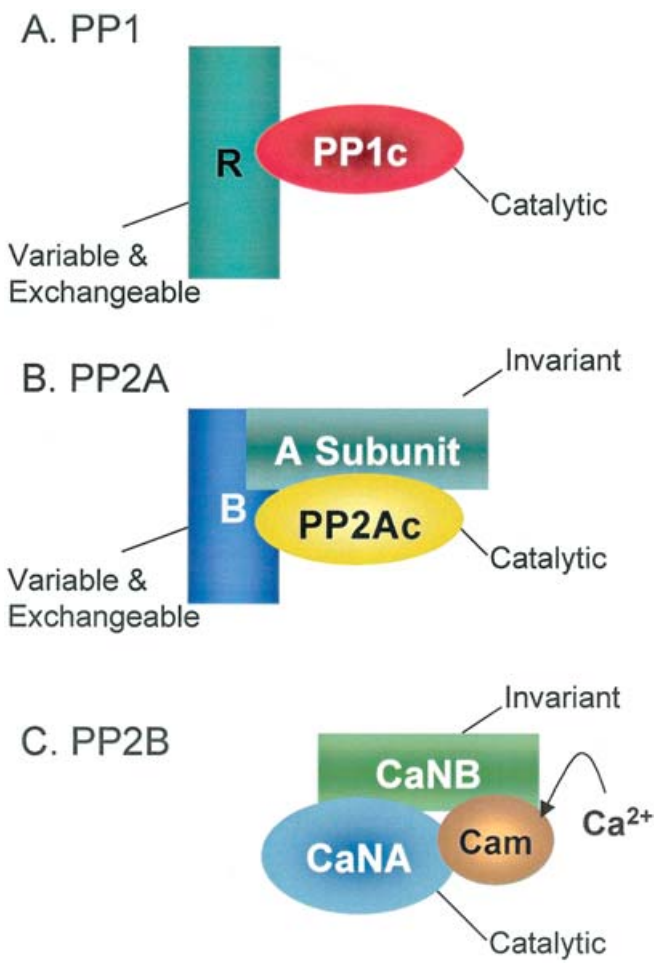

Figure 1 Schematic diagram of the core configurations of type 1, 2A and 2B PPP phosphatases

(**a**) PP1: R, regulatory subunit. (**b**) PP2A: B, subunit B; PP2Ac, catalytic subunit of PP2A. (**c**) PP2B: CaNA, calcineurin regulatory subunit A; Cam, calmodulin.

eight times more active than the free PP1c. In this way, targeting PP1 to glycogen selectively enhanced substrate dephosphorylation of glycogen-associated substrates. In addition, G_M was regulated by phosphorylation at two sites, one which enhanced activity and the other which led to dissociation of PP1c, providing a means to dynamically control PP1 localization. Currently approx. 50 different PP1 regulatory subunits have been described, many of which have distinct tissue or subcellular distribution and which display one or more of the characteristics of G_M regulation [17,36]. A useful systematic nomenclature has been developed for all PP1 regulatory proteins [17,36], with each protein given the designation PPP1R followed by a specific identifier such as 1A (e.g. I_1 is PPP1RA). Despite having little or no significant sequence similarity, the binding of most PP1 regulatory subunits is mutually exclusive, suggesting a common site of interaction. This and other lines of evidence led to the discovery of a conserved RVXF one-letter code; 'X' is any amino acid) motif on most PP1 regulatory subunits which interacts with a hydrophobic groove close to the C-terminus of PP1c [37]. Analysis of PP1 regulatory proteins which do not strictly conform to this motif resulted in a recent revision of this motif to (R/K)X1(V/I)X2(F/W), in which X1 can be absent or any amino acid, with the exception of those with large hydrophobic side chains, and in which X2 can be any amino acid, with the same exceptions as X1 or phosphoserine [17]. The introduction into intact cells of disrupting peptides with this same sequence or constructs of proteins mutated in this sequence has demonstrated a functional role for this motif [38,39]. However, this sequence is found in over 10% of all proteins encoded in the human genome, strongly suggesting that not all proteins containing this motif are likely to be legitimate PP1 regulatory subunits and that the accessibility of this motif to the PP1 binding site is critical for functional interaction. Thus a range of other determinants of PP1 specificity, which regulate exposure of the RVXF motif to PP1c, is beginning to emerge. For example, the presence of ankyrin repeats found in the p53 binding protein, P53BP2 appears critical for interaction with PP1c [40]. Also, proteins which interact with PP1c at additional sites are emerging, such as the anchoring protein AKAP220 [41], GADD34 (growth-arrest and DNA-damage-inducible protein 34) [42], NIPP1 (nuclear inhibitor of protein phosphatase-1) [28] and inhibitors-1 and -2 [17,43].

The mutual exclusivity of most PP1 regulatory subunit binding suggests that the native form of PP1c is a dimer comprising the catalytic subunit and one regulatory subunit. However, additional proteins may interact with the PP1 heterodimer through these regulatory subunits, such that PP1 often exists as part of larger multi-protein complexes. For example PP1 is linked to protein kinase A (PKA) at a number of different subcellular locations through interaction with A kinase anchoring proteins (AKAPs), all of which bind PKA, but which also possess unique subcellular targeting domains [44,45]. Thus AKAP149 targets PP1 to nuclear-membrane lamina [46,47], AKAP220 targets PP1 to peroxisomes [48], AKAP450 [CG-NAP (centrosome-and-Golgi-localized PKN-associated protein)] targets PP1 to centrosomes [49] and Yotiao (named after a popular Chinese breakfast food consisting of long strands of fried dough by Lin et al. [49a]) targets PP1 to *N*-methyl-D-aspartate (NMDA) receptors in the post-synaptic density of neurons [38]. In addition, many of these anchoring proteins also bind a number of other proteins, including substrates and signalling enzymes, to co-ordinate specific cellular events. This raises the question as to how PP1 activity is regulated in these complexes to ensure appropriate responses to extracellular signals.

Allosteric regulation through interaction with its regulatory subunits or substrates provides a variety of influences over PP1 activity to provide specific control of signalling events. This has been most elegantly demonstrated in the regulation of PP1 activity in glycogen metabolism. The glycogen-associated form of PP1 dephosphorylates both glycogen synthase (resulting in enzyme activation) and glycogen phosphorylase (resulting in inactivation) to provide insulin-mediated co-ordination of glycogen metabolism. However, by an allosteric interaction, the phosphorylated form of glycogen phosphorylase inhibits the capacity of PP1 to dephosphorylate glycogen synthase to ensure that, during periods of high energy demand, glycogen synthesis is inhibited [50,51]. The binding of PP1 to AKAPs also modulates its activity in different ways. Thus PP1 is inhibited by interaction with AKAP220 [41], but interaction with Yotiao does not changes its capacity to dephosphorylate co-localized substrates [38]. The role of AKAP220 is therefore to limit PP1 activity towards co-localized substrates, whereas interaction with Yotiao provides tonic dephosphorylation of the NMDA receptor.

A second mechanism of regulating native forms of PP1 is by phosphorylation of its regulatory subunits. Although there are limited examples of this to date, the power of the control and response to extracellular signals seen in these examples suggests that phosphorylation is likely to be a major mechanism of control of many regulatory subunits. PKA leads to changes in PP1 location by phosphorylation of NIPP1 [19], neurabin I [52] and G_M [35]. In each of these proteins, phosphorylation occurs at sites close to the RVXF motif and diminishes their capacity to bind PP1. However, PP1 association with G_M promotes activity towards glycogen-bound substrates and phosphorylation has a negative influence on dephosphorylation of these substrates, whereas phosphorylation of NIPP1 and neurabin I decreases their capacity to inhibit PP1 and therefore promotes substrate dephosphorylation. PKA also inhibits PP1 activity through increasing the phosphorylation of I_1 and its homologue DARPP-32 (dopamine- and cAMP- regulated phosphoprotein of 32 kDa), which is enriched in dopaminergic neurons [53–56]. These proteins are also substrates for the Ca^{2+}-dependent phosphatase 2B, such that the potential for modulation and co-ordination of PP1 activity by two major second-messenger systems is considerable. Importantly, this control has been shown to have a significant role to play *in vivo*. Mice lacking DARPP-32 have an altered response to dopaminergic stimulation [57], whilst mice lacking I_1 have deficient long-term potentiation (LTP) [58], a model of synaptic plasticity. Interestingly, the decrease in LTP was not found in all synapses tested, suggesting that additional PP1 control mechanisms are involved in regulating synaptic efficacy. In addition to PKA, a number of other protein kinases, including protein kinase C (PKC), myosin light-chain kinase (MLCK), CKII (casein kinase II) and cdk5 (cyclin-dependent kinase 5) regulate PP1 by phosphorylation of associated proteins [17,19,56,59].

More recently it has been shown that the level of expression of some PP1 regulatory subunits can be dynamically altered. For example levels of G_L (glycogen-binding subunit of PP1) and PTG (protein targeting to glycogen) are substantially decreased in diabetic rats and normal levels can be restored by insulin therapy [60].

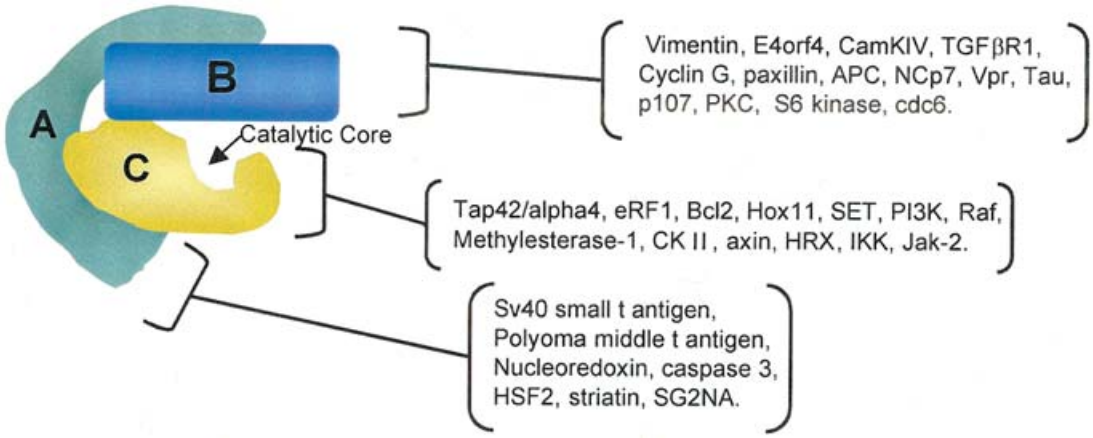

Figure 2 Schematic diagram of the structure of the native trimeric form of PP2A

A range of interacting proteins is shown with an indication of their site of interaction. Further abbreviations: E4orf4, adenovirus E4orf4 protein; CamKIV, calmodulin-dependent kinase IV; APC, adenomatous polyposis coli; NCp7, HIV-1 nucleocapsid protein 7; Vpr, viral protein R; tau, microtubule-associated protein Tau; p107, retinoblastoma family protein p107; cdc6, cell-division cycle 6 protein; Tap42, a regulator of type 2A-related protein phosphatases; eRF1, protein release factor eRF1; SET, putative class II human histocompatibility leucocyte-associated protein II (PHAP-II); PI3K, phosphoinositide 3-kinase; Raf, Ras-activated factor; HRX, human trithorax; IKK, IκB (inhibitory κB) α kinase; Jak-2, Janus kinase-2.

PP2A

The catalytic subunit of PP2A has two mammalian isoforms (α and β), which share 97 % homology, and both isoforms are ubiquitously expressed and highly conserved [21,61]. However, the α isoform is 10 times more abundant than the β isoform ([62], but see [62a]). Knockout mice lacking the α isoform are embryonic-lethal, despite there being a compensatory increase in the β isoform [63]. This suggests that the isoforms are not completely redundant and isoform-specific functions may exist. Cellular expression of PP2A is tightly controlled at the translational level, and it is virtually impossible to overexpress PP2A in mammalian cells. This indicates the extreme sensitivity of mammalian cells to altered levels of PP2A and suggests active roles for PP2A in cellular regulation and the presence of carefully regulated control mechanisms.

PP2A, originally classified as a cytosolic enzyme, is broadly distributed throughout the cell, with particularly high levels in neuronal membranes [64]. Moreover, dynamic targeting of PP2A between different subcellular locations has been demonstrated in mast [65,66], kidney [67] and retinal cells [68]. The native forms of PP2A exist as heterotrimers comprising the catalytic subunit (C), a conserved scaffold subunit (A) and a variable B subunit (see Figure 1). The A subunit has two isoforms (α and β), which are approx. 86 % homologous and are widely expressed [69]. However, differential expression and interaction with B subunits has been reported [70]. Although the AC dimer has decreased activity compared with that of the free catalytic subunit, the principle role of the A subunit appears to be the recruitment of additional proteins into the PP2A complex [71]. Crystal structure analysis of the A subunit indicates that it has a hook-shaped structure which facilitates binding both C and B subunits via a hydrophobic surface created by loops between 15 imperfect, 39-amino-acid repeats ('HEAT repeats'). This arrangement leaves an external face capable of interacting with other proteins, including caspase 3, HSF2 (heat-shock factor 2), striatin, nucleoredoxin and a number of tumour antigens [72] (see Figure 2).

At least three families of mammalian B subunit (B, B′, B″) have been identified as part of the native PP2A heterotrimer. These arise from distinct gene families and have no apparent sequence homology. However, recent sequence alignment of the two A subunit binding domains from the B′ subunit revealed significant homology with all other B subunits [73]. Indeed database searching using only these domains successfully identified 95 % of all known B subunits. Importantly, no new B subunits were identified by this alignment search, suggesting that other A-subunit-binding proteins are not classic B subunits and contain separate binding determinants. In general, B subunit association with the AC dimer diminishes (but does not block) activity. Subcellular targeting is considered the predominant role for the B subunits, and a minimal reduction in activity *in vitro* may actually have limited consequences *in vivo*, where a particular form of PP2A becomes highly concentrated close to its substrates. Each family of B subunits gives rise to a number of isoforms, and consequently the number of potential different trimeric configurations is quite large (approx. 75).

The B (B55, PR55 and cdc55) subunit family in mammals comprises four gene products (Bα, Bβ, Bγ and Bδ), which show differential patterns of tissue expression and subcellular distribution. The differential distribution has been most extensively investigated in brain, where Bβ and Bγ are most highly expressed [74]. In brain, Bα and Bβ are predominantly cytosolic, whereas Bγ is enriched in the cytoskeleton. The relative levels of B family subunits are also under developmental control, with a significant decrease in the Bβ/Bγ ratio after birth [74]. Similar changes do not occur with either the C or A subunit, suggesting that the B subunits are primarily responsible for regulating PP2A specificity. The B family also contains five WD (tryptophan-aspartic acid) repeats that are characteristically involved in mediating protein–protein interactions [75]. However, the other B subunit families do not have these repeats, indicating that other determinants of PP2A targeting are likely to exist.

The B′ (PR61, B56 and RTS1) family comprises five gene families of which there are also a number of splice variants, bringing the number of different proteins to ten [21,76–78]. All B′ subunits share 80 % homology, the variable regions at the C- and N-termini being considered responsible for specific subcellular targeting or altering substrate specificity. The majority of B′ subunits are phosphorylated *in vivo* [79], but data to support a central role for phosphorylation of B′ subunit family members in regulating PP2A function has yet to emerge.

The B″ (PR72/130) family comprise alternatively spliced variants or variants arising from alternative start sites for transcription of the same gene [80]. Whereas PR130 is present in all tissues, PR72 expression is restricted to heart and skeletal muscle. Yeast two-hybrid analysis has recently identified two homologues of PR72 [81,82]. PR59 shares 56 % identity with PR72, whereas PR48 is 68 % homologous with PR59. PR59 is widely expressed except in muscle, and regulates the cell cycle through interaction with p107 [81]. PR48 is found in the nucleus, where it appears to target PP2A to its substrate cdc6 (cell-division cycle 6) for regulation of DNA replication [82].

Two other proteins that share homology with B′ subunits have been suggested to represent a fourth family of B subunit. Striatin and SG2NA (S, G_2-phase nuclear antigen) are WD-repeat proteins that also bind calmodulin in the presence of Ca^{2+} [83]. This raises the possibility of regulation of PP2A by Ca^{2+}. Striatin is concentrated at the post-synaptic density of neurons (a specialization of the neuronal plasma membrane in which neurotransmitter receptors are highly concentrated), whereas SG2NA is a nuclear protein. Although both proteins clearly bind PP2A through the A subunit, they differ from the other B-subunit families in that they do not contain the conserved A-subunit-binding domains and they do not have additional contacts with the catalytic subunit of PP2A [83]. Classification as B subunits is therefore still controversial.

Like striatin and SG2NA, a vast array of other proteins has now been shown to form stable complexes with PP2A (Figure 2). Interactions mediated by contact with C, A and B subunits have all been demonstrated and so PP2A has the potential to be part of extremely large multiprotein complexes and therefore form pools localized to distinct regions within the cell. Evidence suggests that the formation and localization of these different pools of PP2A can be dynamically altered. The simian-virus-40 (SV40) small T-antigen induces viral transformation by displacing some forms of B subunit and thereby inhibiting PP2A [84]. Moreover, B′ can displace SV40 small T-antigen and reverse its cellular effects [29], suggesting that dynamic exchange of B subunits can have dramatic effects on cell function.

Although subcellular targeting of the AC dimer, or ACB trimer, is the principle mechanism of regulating PP2A, the C-terminus of the C subunit is a focal point for regulation of PP2A complexes. Here, PP2A is subject to tyrosine phosphorylation on Tyr^{307} [85] and methylation on Leu^{309} [86–89] and undergoes stable interaction with the B subunits [90]. Despite the close proximity of these residues, they confer quite different properties on the enzyme and, since tyrosine phosphorylation blocks methylation, their effects on PP2A appear to be mutually exclusive. Tyrosine phosphorylation, by $PP60^{src}$ (phosphoprotein of 60 kDa encoded by the *src* oncogene), $PP56^{lck}$ and EGF (epidermal-growth-factor) and insulin-receptor kinases, potently inhibits enzyme activity [85,91], whereas methylation, by a specific methyltransferase [86,92], modestly increases activity. Tyrosine phosphorylation abolishes B-subunit binding [90] and modulates the ability of the AC core to bind other proteins, whilst methylation is essential for B subunit binding, but not necessarily for binding other proteins [93,94]. PP2A can also be inactivated by phosphorylation on (unidentified) threonine residues by an autophosphorylation-activated protein kinase [95,96]. The contribution of phosphorylation and methylation to PP2A function *in vivo* remains to be determined, but they are both clearly dynamic. Tyrosine phosphorylation of PP2A mediated by activation of cells with a number of growth factors is transient [97], whereas the level of PP2A methylation changes during the cell cycle [98]. The tyrosine phosphatase responsible for PP2A dephosphorylation remains to be identified, but relatively low concentrations of okadaic acid significantly increases PP2A tyrosine phosphorylation levels, suggesting that PP2A can function as its own phosphatase. The capacity of PP2A to function as a tyrosine phosphatase for other substrates has also been demonstrated, and it can be stimulated by association with two proteins, tubulin [99] and PTPA (phosphotyrosyl phosphatase activator) [100,101]. PP2A methylation has been reported to be stimulated by cAMP [102], raising the possibility of regulation by extracellular signals and a phosphatase methylesterase (PME-1) that specifically demethylates PP2A has been cloned [92].

Lastly, specific forms of PP2A might mediate the cellular effects of ceramide. A trimeric form of PP2A containing the B subunit was isolated as a major ceramide-activated protein phosphatase [92,103,104], but ceramide can also activate dimeric and monomeric forms of PP2A. Ceramide also activates PP1 and a number of protein kinases [105]. Thus the specific role of PP2A in mediating signals through ceramide remains to be determined.

PP2B

PP2B (calcineurin) is a Ca^{2+}- and calmodulin-activated enzyme with a restricted number of substrates when compared with PP1 and PP2A. Nevertheless, PP2B substrates are involved in a wide range of cellular functions, such that regulation of PP2B by Ca^{2+} is a significant mechanism for regulating cell function through extracellular signals [24–26]. The 58–64 kDa catalytic subunit of PP2B has three highly conserved mammalian isoforms (α, β and γ), which are products of separate genes. Only the α and β isoforms are ubiquitously expressed, with the γ isoform being restricted to expression in the testes [106]. Calcineurin was first discovered in brain, where it is particularly concentrated and where the α isoform is the predominant one. The isoforms are 89 % homologous for 90 % of their primary amino acid sequence, but have variable N- and C-termini. The role of these variable regions is unknown. The conserved core of the catalytic subunit also contains autoinhibitory and calmodulin-binding domains, as well as a binding domain for a 19 kDa regulatory subunit (calcineurin regulatory subunit B; CaNB) [107–109]. CaNB has two highly homologous isoforms (α, β), which are also highly conserved [23,25,110]. Expression of the β isoform appears to be restricted to the testis. CaNB has four binding sites for Ca^{2+}, three of which have affinities in the micromolar range and one that has an affinity in the submicromolar range. In the presence of submicromolar Ca^{2+}, CaNB remains bound to the catalytic subunit and there is little change in enzyme activity. Basal activity can be stimulated up to 20-fold by equimolar concentrations of calmodulin, which displaces the autoinhibitory domain on the catalytic subunit ([111,112], but see [112a]). There is also a strong co-operativity between Ca^{2+} and calmodulin in activating PP2B, the concentrations of Ca^{2+} needed for activation decreasing with increasing calmodulin concentrations and vice versa ([111,112], but see [112A]). This co-operativity allows PP2B to respond to small changes in Ca^{2+} during cellular stimulation.

The catalytic subunit of PP2B also contains a myristoylated N-terminus that is believed to mediate targeting of PP2B to membranes. This is consistent with the finding that, in brain, up to 70 % of PP2B is bound to either membranes or the cytoskeleton. However, although myristoylation of PP2B has been shown to be necessary for binding to phosphatidylserine [113,114], some mutation studies show that it is not necessary for membrane association or enzyme activity [115]. PP2B has also

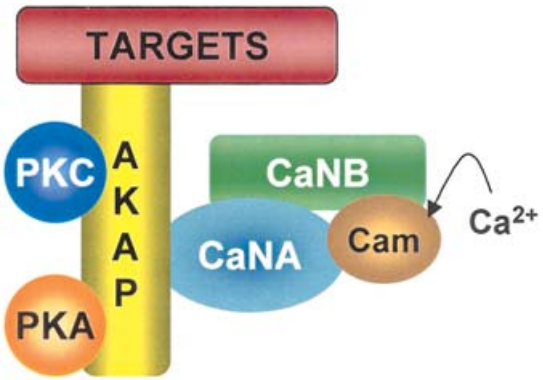

Figure 3 Schematic diagram of multienzyme signalling complex co-ordinated by AKAPs

Further abbreviations: CaNA, calcineurin regulatory subunit A; Cam, calmodulin.

been found in the nucleus of T-lymphocytes, where it regulates the NFAT (nuclear factor of activated T-cells) transcription complex [24]. In addition to CaNB, a number of other proteins that regulate enzyme activity and/or subcellular location bind PP2B. The first of these to be discovered was a member of the AKAP family. This suggests that PP2B is regulated through the formation of multienzyme signalling complexes (Figure 3). In neurons, PP2B is targeted to postsynaptic GluR1 glutamate receptors through binding AKAP79, which also targets PKA and PKC to the same region [44,45,116]. Membrane-permeable peptides that disrupt the interaction between AKAP79 and PP2B in cells cause rundown of GluR1-mediated Ca^{2+} currents, illustrating the functional importance of co-ordinated targeting of both kinases and phosphatases [117,118]. The immunophilin FKBP12 (immunophilin-ligand-FK506-binding protein 12) targets PP2B to ryanodine [119] and InsP_3 receptors [120,121], presumably to mediate regulation of Ca^{2+} release from intracellular stores. Such targeting is disrupted by FK506 and cyclosporin and leads to inhibition of PP2B activity [120,121]. Two other PP2B binding proteins (calcineurin inhibitor CAIN/Cabin1 and calcipressin) were identified using the yeast two-hybrid system. The interaction of CAIN with PP2B inhibits enzyme activity and inhibits synaptic-vesicle endocytosis [122,123]. Calcipressin is the mammalian homologue of a highly conserved family of proteins [also known as DSCR1 (Down's syndrome candidate region 1), DSCR1L2 or ZAKI-4 in humans and Rcn1p in yeast] that also inhibits PP2B through interaction with the catalytic subunit and which appear to protect the cell against acute Ca^{2+}-mediated stress damage [124]. In humans, DSCR1 is encoded on chromosome 21 within the DSCR, suggesting a role for PP2B in this disease [125]. Lastly a protein encoded by the African-swine-fever-virus genome (A238L) is a competitive inhibitor of PP2B activity through binding at a site that is shared with the transcription factor NFAT [126].

Other PPP family serine/threonine phosphatases

Although PP1, PP2A and PP2B are the best-characterized serine/threonine phosphatases to date, several other phosphatases have been identified which may play fundamental roles in cellular regulation. PP4, PP5 and PP6 are widely distributed in mammalian tissues, but their *in vivo* targets remain largely unknown [20].

PP4 (formerly PPX) is highly conserved and is particularly concentrated in centrosomes and the nucleus [20]. Association with centrosomes is dynamically altered during the cell cycle, and it is required for centrosome maturation and sperm meiosis [20,127]. Stimulation with TNFα (tumour necrosis factor-α) results in time-dependent activation of endogenous PP4 which leads to activation of c-Jun N-terminal kinase (JNK) [128]. A number of regulatory subunits for PP4 have been identified that appear to regulate PP4 in a similar manner to the regulatory subunits of PP2A. $PP4_{R1}$ is a scaffold subunit similar to the A subunit of PP2A, whereas $PP4_{R2}$ may function as a B-type regulatory subunit to target PP4 to centrosomes [130]. A homologue of $PP4_{R1}$, Rmeg, is up-regulated in the kidney of patients with glomerulonephritis [130]. Another PP4 regulatory subunit, α4, is also a binding protein for PP2A and PP6 [131].

PP5 is present in all eukaryotic cells, where it is predominantly found in the nucleus and the cytoplasm [132]. The structure of PP5 is unique in that catalytic, regulatory and targeting domains are present in a single polypeptide chain. PP5 has a characteristic tetratricopeptide repeat (TPR), which mediates protein–protein interactions [133–135]. In particular PP5 binds hsp90 (heat-shock protein 90) to specifically regulate glucocorticoid receptor function [136,137]. PP5 also binds the atrial-natriuretic-peptide (ANP) receptor [138] and potentially regulates Ca^{2+}-activated potassium (BK) channel activity [132]. Two members of the anaphase-promoting complex, CDC16 and CDC27, also bind PP5 through the TPR domain to regulate the cell cycle [139]. The TPR domain also causes auto-inhibition of PP5, which can be relieved *in vitro* by proteolysis. *In vivo*, the basal activity of PP5 is low, but it can be activated by arachidonic acid and other unsaturated fatty acids, but not by saturated fatty acids [140]. This is also facilitated by binding the TPR domain and may relieve auto-inhibition. PP5 expression is inducible by oestrogen [141] and renders breast-cancer cells insensitive to oestrogen. Two proteins, CRY1 and CRY2, that are expressed in retina and other tissues and are involved in regulating circadian rhythms, also interact with PP5 through the TPR domain, but lead to inhibition of catalytic activity [142,143].

PP6 (also referred to as PPV 6A, Sit4 and Ppe1) is highly conserved between species, but there is limited information regarding the mammalian enzymes [20]. Functional roles for PP6 are implicated from its yeast and *Drosophila* homologues, where a role in regulating the cell cycle is implicated. No regulatory mechanisms or interacting proteins for PP6 have been identified, but the N-terminal region of PPV 6A, which is not involved in modulating catalytic activity, is essential for cell-cycle functions of PP6 in yeast [144]. This region might therefore mediate protein–protein interactions and subcellular targeting of PP6.

PP7 is another Ca^{2+}-activated phosphatase, which shares 35 % similarity with PP1 and PP2A. Ca^{2+} activation is by way of five EF-hand (-like) domains [145]. Two highly homologous genes encode the human form of PP7, expression of which appears restricted to developing brain, retina and primary sensory neurons [145]. Functional roles for PP7 or other regulatory features remain unknown.

PP2C

PP2C represents a diverse family of enzymes, which to date comprises six gene products (α, β, γ, δ, Wip1, and Ca^{2+}/calmodulin-dependent protein kinase phosphatase) in mammalian cells. So far, two human isoforms of PP2Cα (α-1 and α-2) and five isoforms of the mouse PP2Cβ (β-1, β-2, β-3, β-4 and β-5), which are products of alternative splicing, have been found [147–156]. Diversity between isoforms accounts for a

degree of substrate specificity, but there is also a considerable degree of overlapping specificity, and the mechanisms of regulation of PP2C remain unclear. The native form of PP2C appears to be a monomer, with no evidence for conserved families of regulatory subunits [27]. However, a number of PP2C-like homologues have also been found as binding partners for various proteins in 'ligand fishing' studies, suggesting that, like the PPP family, association with numerous proteins regulates the PPM family. For example, ILKAP [integrin-linked kinase 1 (ILK1)-associated phosphatase, a human homologue of rat PP2C] was originally identified as an ILK1 binding partner [157]. The interaction is unique to ILKAP in specifically regulating growth-factor-stimulated ILK activity. Interestingly, whereas the majority of PP2C enzymes are Mg^{2+}-dependent, rat PP2C and ILKAP are inhibited by Mg^{2+}. Two other PP2C-like enzymes (POPX1 and POPX2) were isolated as binding partners for a guanine-nucleotide exchange factor PIX [158]. This interaction promotes the dephosphorylation of PAK (p21-activated kinase) and inhibits actin stress-fibre breakdown.

Whereas PP2C has been implicated in the regulation of many cell processes, a consistent theme in regulating stress-activated signalling pathways has emerged. Overexpression of the α-1, α-2 and β-1 isoforms inhibits p38 and JNK pathways, but not the MKK1/ERK1 [mitogen-activated protein (MAP) kinase kinase-1/extracellular-signal-regulated kinase] pathway [159]. Moreover the β-1 isoform, but not the α isoform, binds to TAK1 [TGF-β (transforming growth factor-β)-activated kinase 1] and MKKK (MAP kinase kinase kinase) in TGF-β signal-transduction pathways [156]. Thus unique interactions of binding proteins with individual isoforms may contribute to the specificity of action of PP2C.

A long-standing dilemma in understanding the role and regulation of PP2C has been the considerable overlap with PP2A in substrate specificity. Furthermore, PP2C is activated by physiological concentrations of Mg^{2+} and would therefore, without other means of regulation, be constitutively active inside cells. For example, tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, is dephosphorylated on Ser^{40} by both PP2A and PP2C, with PP2A considered to be the major phosphatase. However, kinetic analysis showed that the relative contribution of each phosphatase varies according to substrate concentration, with high concentrations favouring PP2C [160]. Thus PP2C may be more important than PP2A in regions where substrates are concentrated by targeting.

The Mg^{2+}-dependency of PP2C is also reduced by unsaturated fatty acids [161] and could therefore be regulated by signals operating through phospholipase A. PP2C is also inactivated by high concentrations of Ca^{2+} [160]. The contribution of these findings to PP2C function *in vivo* remains to be defined but they do indicate that PP2C has the potential for dynamic regulation by a range of physiological stimuli.

Protein phosphatase inhibitors

Understanding the functional roles of protein phosphatases has been heavily dependent upon studies that have utilized a number of naturally occurring inhibitors. The majority of these inhibitors have a degree of specificity for different members of the PPP family [162,163]. However, in order to evoke cellular effects, concentrations of inhibitors far in excess of the IC_{50} for most enzymes must be used, and so interpretation of results is difficult. Furthermore, difficulties arise from differences in subcellular targeting, which may mean that the local concentrations of individual phosphatases in distinct regions of the cell are quite different from others. Differential accessibility of the inhibitors to different regions of the cell also make interpretation of *in vivo* effects difficult [164]. The use of a range of inhibitors and/or alternative approaches to confidently predict roles for specific phosphatases is therefore essential.

Okadaic acid was the first such inhibitor to be discovered, and has been widely used to implicate the PPP family of phosphatases in regulating cellular function [164–166]. Derived from marine dinoflagellates, this cell-permeant inhibitor affects all members of the PPP family with a degree of selectivity for PP2A and the closely related PP4. PPM phosphatases are unaffected by okadaic acid or any other natural inhibitor. A similar degree of selectivity for the PPP family is achieved with the microcystin family of cyclic peptides, which are derived from cyanobacteria [167,168]. However, these are not cell-permeant inhibitors and have limited use in intact cell studies. Calyculin A is a potent cell-permeant inhibitor of the PPP family, but, in contrast with okadaic acid, shows little specificity for individual family members [162,163,169]. This inhibitor is used widely to manipulate general PPP activity inside cells and is particularly stable to acid or enzyme hydrolysis. Tautomycin, originally isolated from *Streptomyces*, is a unique inhibitor of the PPP family in that it has a higher affinity for PP1 over other family members and may therefore be useful in conjunction with other inhibitors to identify roles for PP1 inside cells [170]. Fostriecin is unique among inhibitors in that it was originally discovered as a novel anti-tumour agent. Fostriecin has strong selectivity for PP2A and PP4 over other PPP family members and is therefore a particularly useful tool in delineating functional roles for these phosphatases [171–174]. Fostriecin also inhibits topoisomerase II such that its use to investigate roles for PP2A/PP4 in growth arrest is limited [175]. Although such pharmacological inhibitors are widely used and have selectivity for particular protein phosphatases, it is recognized that they will broadly affect the phosphorylation states of many proteins, and indirect actions may account for the functional outcomes being measured. For example, okadaic acid stimulates lipolysis [176], and the resultant changes to the cellular lipid environment might be expected to influence cellular function. Thus studies that rely upon individual inhibitors alone are clearly indicative only, and additional approaches are required to validate the findings and to identify specific sites of action. It is likely that emerging technologies such as RNA interference will bring a greater degree of specificity into this field, but published studies are as yet limited in the serine/threonine phosphatase field [177–179].

Several endogenous heat-stable inhibitors of PP1 and PP2A can be used *in vitro* to distinguish between the activities of individual phosphatases. I_1 (and its homologue DARPP-32) specifically inhibits PP1 when phosphorylated by PKA. A phosphorylated synthetic peptide encompassing residues 9–35 of the protein retains inhibitor activity. I_2 also inhibits PP1 specifically, but does not require phosphorylation to do so. However, the full-length protein is needed [17]. I_1^{PP2A} and I_2^{PP2A} were originally isolated from kidney and potently inhibit all holoenzyme forms of PP2A [180]. I_1^{PP2A} was originally isolated as the protein PHAP-1 (putative histocompatibility leucocyte antigens class II-associated protein 1) and I_2^{PP2A} as PHAP-11 (also known as SET), proteins that are involved in leukaemogenesis, suggesting a role in modulating cell growth [181,182].

The identification of PP2B as the target for the immuno-suppressant drug cyclosporin A (CsA) not only identified a major role for this phosphatase in the immune response, but also provided the research community with a highly selective cell-permeant inhibitor with which to investigate functional roles for this phosphatase [183]. The mechanism of action of CsA

is indirect, requiring the formation of a CsA–immunophilin (cyclophilin) complex. Another similar inhibitor, FK506, requires the presence of a different immunophilin (FKBP12) to be effective. CsA and FK506 are therefore only effective in cells expressing the appropriate binding protein, and negative outcomes need to be confirmed by other means, such as PP2B activity measurements or Western blotting for the relevant binding protein. Furthermore, cyclosporin has been shown to inhibit the mitochondrial permeability transition pore apparently by direct binding to the matrix isomerase cyclophilin D component of the pore and is therefore acting independent of PP2B [184]. Thus, as with all uses of pharmacological inhibitors, any role of PP2B that is implied from studies with CsA needs to be confirmed by other means.

MULTIPLE MODES OF EXOCYTOSIS

All cells have the capacity to secrete materials into the extracellular space by exocytosis [4]. This process can be both constitutive and highly regulated, the latter being the fundamental process used by neurons, endocrine and exocrine cells to release various neurotransmitters, hormones and digestive enzymes [3]. During constitutive exocytosis, materials are released from the cell continuously, whereas in regulated exocytosis, release occurs only in response to an appropriate stimulus [185]. Although both approaches involve incorporation of membrane vesicles into the plasma membrane, regulated exocytosis involves the prior storage of neurotransmitters and hormones in secretory vesicles and the process of incorporation of these vesicles into the plasma membrane is restrained until the appropriate signal is generated, which usually involves a rise in intracellular Ca^{2+}. Several steps in regulated exocytosis are common to all cells, and regulation by protein phosphorylation might have a similar role to play in each cell type. Thus all cells require synthesis of material to be released, packaging into secretory vesicles or granules, trafficking of vesicles to the plasma membrane, docking with the plasma membrane, priming the vesicle to a state of readiness, fusion with the plasma membrane, release of soluble contents and subsequent vesicle or granule endocytosis. Each of these key steps consists of multiple molecular interactions, many of which are regulated by phosphorylation. In most cell types, vesicles or granules are returned to the Golgi for reprocessing and refilling for subsequent exocytosis, but in some cells, such as neurons, secretory vesicles can be refilled locally without returning to the Golgi. This local recycling of vesicles is largely restricted to vesicles containing low-molecular-mass signalling molecules, such as amino acids and catecholamines, whereas peptide-derived signals rely mainly upon Golgi-mediated recycling [3,4]. By the nature of the different recycling processes, these different forms of regulated exocytosis have distinct characteristics. Thus exocytosis that requires Golgi-dependent recycling is slow and sustained, occurs over a broad section of the plasma membrane, and secretory materials are contained within relatively large secretory granules (> 200 nm diameter), of which only a small number are docked prior to stimulus [186]. In contrast, exocytosis that involves local recycling is relatively fast, occurs within a restricted region of the plasma membrane, secretory materials are stored in small vesicles (< 50 nm diameter) and vesicles are organized into a number of pre-docked and reserve pools [187]. In both types of exocytosis, Ca^{2+} is the trigger for exocytosis, but in slower, Golgi-dependent exocytosis, Ca^{2+} also maintains exocytosis by influencing granule mobilization and priming for fusion. In some cell types, such as neurons, both Golgi-dependent and local recycling mechanisms exist for exocytosis of peptides and low-molecular-mass neurotransmitters respectively. Given that protein phosphorylation appears to be involved in most steps of exocytosis and endocytosis, these differences in process alone could potentially lead to different functional outcomes from experimental manipulation of kinase or phosphatase activity. Identifying specific roles for individual protein phosphatases in exocytosis is therefore difficult. Moreover, exocytosis in neurons and neuroendocrine cells has now been shown to occur through two distinct modes [1,188–192]. In addition to full incorporation of vesicles into the plasma membrane ('full fusion'), followed by clathrin-mediated endocytosis, exocytosis can occur by a mechanism in which the vesicle rapidly pinches off without full integration into the plasma membrane. Retrieved vesicles are rapidly refilled with cargo or those that have not dispensed their full cargo are immediately available for further exocytosis.

This so-called 'kiss-and-run' mode of exocytosis is generally favoured by strong stimulation and high Ca^{2+} conditions and provides a mechanism for very fast vesicle recycling in response to intense signalling [193,194]. However, it is now clear that a spectrum of kiss-and-run-like mechanisms exist for dense core vesicles in different cell types and, in many cases, kiss-and-run-like mechanisms prevail across a range of Ca^{2+} concentrations, and exocytosis involving complete integration of vesicle and plasma membranes rarely occurs [195]. For example, in PC12 cells, secretory granules are recycled virtually intact after exocytosis [196], yet in chromaffin cells, high Ca^{2+} levels are required to switch the mode to kiss-and-run [193]. Moreover, in insulin-secreting cells, kiss-and-run is also the major form of exocytosis, but, as a result of fusion pore size and time of opening, the cell can selectively release different types and amounts of vesicle cargo [197]. This latter finding led to the description of three forms of kiss-and-run: 'pure', which releases only very small molecules, 'mixed', where the fusion pore size is greater to allow exocytosis of larger molecules, and 'full', where the release of all vesicle cargo is permitted [197].

It is not yet clear if kiss-and-run represents a different form of endocytosis (clathrin-independent) or a failure to complete full-fusion exocytosis [192,193]. Furthermore, the molecular mechanisms that determine whether a particular nerve ending utilizes kiss-and-run or full-fusion modes of exocytosis and the relative contribution of each mode in normal and pathological conditions remain unknown. Although the evidence that these two modes can co-exist and operate on different timescales supports the notion that the two modes represent distinct mechanisms [198,199], the most recent popular view is that the molecular machinery is the same for each mode, the choice between kiss-and-run or full fusion being determined by the capacity of the vesicle to be rapidly retrieved from the plasma membrane [192,193]. Given the central role of protein phosphorylation in exocytosis, it is conceivable that differential expression of protein phosphatase enzymes, subunits or targets play a role in defining the particular mode of exocytosis in a given cell type in response to particular signals.

The remainder of this Review presents the evidence for a functional role for a number of protein phosphatases in regulated exocytosis in three major cell systems which have been extensively studied and in which major roles for protein phosphatases have been identified. It is recognized that the processes of exocytosis and endocytosis are integrally linked, and changes to endocytosis will have consequences for subsequent exocytotic events. Although protein phosphatases, in particular PP2B, have been shown to have modulatory roles in endocytosis [200], the intention of this Review is to focus specifically on recent developments in regulation of exocytosis by protein phosphatases.

It is, however, important that experiments designed to investigate their role in the exocytotic process are interpreted within the context of possible effects on endocytosis.

The first cell system to be discussed is the mast cell, in which the exocytosis of inflammatory mediators is slow, and sustained and granule recycling is Golgi-dependent. Secondly, insulin-secreting cells will be discussed, in which the exocytosis of insulin is also Golgi-dependent, but in which some organization of secretory granules occurs prior to stimulus. Lastly, neurons, in which both Golgi-dependent and Golgi-independent mechanisms of recycling exist, and in which both full-fusion and kiss-and-run modes of exocytosis occur, will be reviewed. Identification of the targets for phosphatase action in each system has the potential for developing new tools to specifically manipulate exocytosis in that system. However, few data exist to link specific phosphatase substrates as the targets that mediate their specific action on exocytosis.

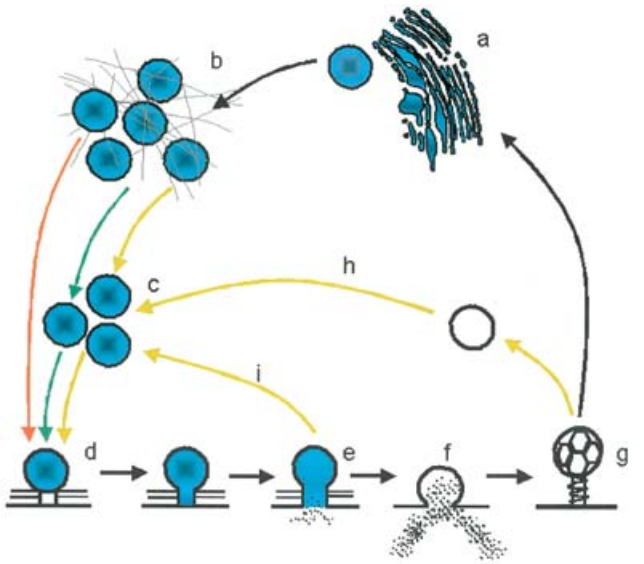

Figure 4 Schematic diagram of the different modes of exocytosis in mast cells, insulin-secreting cells and neurons

Black lines indicate aspects that are common to all cell systems. The orange arrow indicates the specific processes that occur in mast cells in which there is no organization of granules into distinct pools. The green arrows indicate the trafficking of granules from reserve to readily releasable pools in insulin-secreting cells. Although trafficking between different pools also occurs in insulin-secreting cells, the mechanisms of this may or may not be similar to that which occurs in the neuron. Therefore separate arrows (olive green) are used to indicate those processes that occur in neurons. Key aspects of the exocytotic process in which protein phosphatases may play a role are indicated: a, Golgi processing; b, cytoskeletal attachment of vesicles; c, mobilization to release ready pools; d, docking with the plasma membrane; e, fusion-pore expansion; f, full fusion of the vesicle with the plasma membrane; g, clathrin-mediated endocytosis; h, local recycling of vesicles; i, kiss-and-run exocytosis. An animated version of this Figure is available at: http://www.BiochemJ.org/bj/373/bj3730641add.htm.

Mast-cell exocytosis

Exocytosis of inflammatory mediators from mast cells in response to a wide range of stimuli represents the primary cellular response in allergic disease. The majority of antigens trigger mast-cell exocytosis through cross-linked high-affinity IgE receptors on their surface, but a range of other agents that by-pass the IgE receptor, such as opioids and physical stimuli such as cold and light, can also activate mast cells to release these mediators [201]. When antigens, such as pollen or house-dust-mite particles, bind to and cross-link specific IgE on mast cells, they initiate a well-characterized sequence of initial intracellular signals. In general terms, the early events that follow receptor aggregation are increased tyrosine kinase activity, rapid hydrolysis of inositol phospholipids and elevation of intracellular Ca^{2+}, translocation of PKC to the plasma membrane, and phosphorylation of a diverse range of proteins [201]. The culmination of these signals, which occurs within seconds to minutes, is the fusion of the granule and plasma membranes and the release of intragranular inflammatory mediators such as histamine, chymases and tryptases, and chemotactic factors for neutrophils and eosinophils. These mediators recruit and activate other cells in the inflammatory cascade that leads to chronic asthma [203].

Unlike insulin-secreting cells and neurons, where secretory granules and vesicles are organized into defined reserve and ready-releasable pools, trafficking of mast-cell granules is only organized subsequent to cell stimulation (see Figure 4). Prior to cell stimulation, granules are kept apart from their fusion sites by a cytoskeletal barrier composed of predominantly actin and myosin, and remodelling of this barrier has been shown to be essential for regulated exocytosis [204]. Thus, in response to stimulation, there is a dramatic reorganization of the cytoskeleton, and distinct morphological changes in the cell occur that are necessary for exocytosis to take place. Subsequently, granules dock and fuse with the plasma membrane to allow release of granule contents over a protracted period of time, and vesicles are finally recycled by endocytosis [65]. Though delineating the role(s) of protein phosphatases by interpreting the results from inhibitor studies is particularly complex in these cells, the protracted time course of exocytosis makes these cells particularly amenable to biochemical and molecular analysis of these roles.

There is general agreement that okadaic acid produces a time- and concentration-dependent inhibition of granule-bound inflammatory mediator exocytosis from mast cells [65,66,205–211]. Similar effects are found with calyculin A [210,211], but, presumably due to greater cell permeability of this inhibitor relative to okadaic acid, cells treated with calyculin A rapidly round up and detach from culture supports, making subsequent analysis difficult. Mast cells also release granule contents in response to PMA and the Ca^{2+} ionophore A23187 [212,213], and these effects are also blocked by okadaic acid [211]. Thus the major effects of PP1/PP2A are likely to be downstream of the IgE receptor and Ca^{2+} influx. Consistent with this latter position, okadaic acid was shown to have no effect on intracellular Ca^{2+} fluxes in rat peritoneal mast cells [209].

Despite the consistent observation of inhibition by okadaic acid in a range of mast-cell types (human lung, skin, basophils, peritoneal and pleural as well as the basophilic leukemic RBL-2H3 cell line), the relatively high concentration of PP1 and PP2A in cells makes it difficult to identify which particular phosphatases are involved. A comparative study using a number of inhibitors, with different degrees of selectivity, suggested that PP1 was the predominant phosphatase involved [205]. Thus tautomycin, which has a degree of selectivity for PP1, was a more effective inhibitor than fostriecin, which is highly selective for PP2A. However, these data must be interpreted with caution because of the relative instability of these inhibitors and because of the results from other approaches, which indicate a major role for PP2A in mast-cell exocytosis.

Measurement of PP1 and PP2A activity in RBL-2H3 cells following okadaic acid treatment at concentrations that inhibit exocytosis showed that only PP2A was inhibited [66]. This phenomenon is consistent with the relative selectivity of okadaic acid for PP2A over PP1 and has been demonstrated in a number of cell lines [214]. Importantly the inhibitory effect of okadaic

acid on exocytosis correlated with the degree of inhibition of membrane-bound PP2A activity [66]. Subsequent studies showed that in response to antigen stimulation, PP2A, but not PP1, was transiently translocated to the cell membrane from the cytosol in a time course that matched the peak rate of secretion [66]. Similar changes in PP2A in response to extracellular signalling have now been reported in other systems. For example, in the retina, light and dark stimuli induce dynamic exchange between subcellular compartments to facilitate changes in rhodopsin and phosducin activity [215]. Similarly, in kidney cells, PP2A translocates to the apical membrane, where it negatively regulates tight-junction proteins to increase paracellular permeability [216]. Translocation of PP2A in mast cells was also observed following PMA and A23187 treatment, which again matched the (decreased) rate of secretion [66]. It is noteworthy that PMA alone (which does not induce exocytosis) also caused translocation of PP2A to the plasma membrane, but, without an elevation in intracellular Ca^{2+}, the translocated PP2A was inactive. The mechanism of translocation and Ca^{2+} activation of PP2A remains to be identified, and the targets for PP2A that mediate its regulation of exocytosis are not known. Given that PMA also induces translocation of PKC [217,218], co-translocation mediated by a common anchoring protein may be involved to co-ordinate the phosphorylation/dephosphorylation of common substrates. Consistent with this, localization of PKC and PP2A during PKC signalling has been described in COS cells [219] and demonstrated in mast cells (R. I. Ludowyke, J. Holst and A. T. R. Sim, unpublished work). Down-regulation of PKC itself by PP2A has also been described [220] and PP2A-mediated dephosphorylation of Thr^{500} and Ser^{660} on PKCβII is required to activate PKC autophosphorylation [221]. Other studies have shown that PKC can be dephosphorylated by a PP2A trimer and a membrane-bound form of PP2A [222]. Inhibition of PP2A with okadaic acid also prevents PKC down-regulation [223] and leads to the apparent un-regulation of PKC activity, as there is increased phosphorylation of the myosin heavy and light chains at PKC-specific sites.

Okadaic acid treatment of mast cells also inhibits the unique morphological changes that occur in mast cells to facilitate granule trafficking and fusion with the plasma membrane [65]. This suggests that, in addition to a role at the cell membrane, a major site of action of PP2A (and/or PP1) could be the cytoskeleton. The actomyosin cortical web is dynamic, being disassembled and assembled in response to extracellular signals. These dynamic changes are regulated by phosphorylation of myosin at specific sites on the heavy and light chains.

In mast cells, myosin IIA is the only conventional myosin isoform present [224] and is phosphoryated in unstimulated cells on light chains, by MLCK, and on heavy chains at a number of sites, by unknown kinases [225,226]. Importantly, activation of cells with antigen or Ca^{2+} ionophore does not alter phosphorylation at these sites, but leads to the phosphorylation of both light and heavy chains by PKC at distinct sites [227,228]. Although there is good evidence that the protein phosphatase affecting myosin phosphorylation in muscle is PP1, equally strong evidence indicates that, in mast cells, the phosphatase affecting the stimulus-dependent dephosphorylation of PKC sites on myosin is PP2A [65]. This shows that the protein phosphatase responsible for the specificity of the dephosphorylation of particular substrates can differ between cell types, presumably due to cell specific differences in the expression of targeting proteins. Moreover, in response to mast-cell stimulation with antigen or PMA, there is increased association between myosin, actin and PP2A, observed by fluorescence microscopy and immunoprecipitation studies [65]. The increased association is transient and matches the time course of secretion. Inhibition of PP2A with okadaic acid inhibited the association with myosin and the stimulus-dependent cytoskeletal rearrangements. In these studies there was also increased association of PP1 with myosin in response to stimulation, and this, too, was disrupted by okadaic acid. Given that PP1 activity is not altered by okadaic acid in these cells [66], an interaction between PP1 and PP2A was suggested. Changes in myosin phosphorylation also accompany the massive morphological changes in platelets that are required for activation and secretion [229]. In these cells, increased association of both PP1 and PP2A with the cytoskeleton was also observed, with a greater association of PP2A with the cytoskeleton after stimulation than there was with PP1 [229].

Although the specific regulatory mechanisms underlying this control of mast-cell exocytosis by PP2A are not known, a recent study in mast cells (A. T. R. Sim, J. Holst and R. I. Ludowyke, unpublished work) suggests PP2A methylation is involved. Stable transfection of a PP2A mutant that is unable to be methylated produces a cell line with significantly decreased capacity for secretion. This finding is consistent with the notion that PP2A methylation is required for exocytosis and warrants further investigation.

In contrast with those on PP1 and PP2A, there are few studies that indicate a role for PP2B in regulating mast-cell exocytosis. Treatment of a number of mast-cell lines with FK506 resulted in inhibition of antigen-mediated histamine exocytosis [231]. Furthermore, transfection of RBL-2H3 cells with a catalytically inactive mutant of the A subunit (catalytic) of PP2B increased the sensitivity of release to FK506 presumably by diverting the B subunit of PP2B away from interaction with the endogenous catalytic subunit [231].

Pancreatic-islet exocytosis

Elevation of blood glucose leads to increased intracellular Ca^{2+} levels and a biphasic exocytosis of insulin from what has been referred to as ready releasable and storage pools of secretory granules in β-cells of the pancreas (Figure 4). Although the precise mechanisms of glucose sensing are still not fully understood, it is clear that the exocytosis is mediated by glucose oxidation and the generation of intracellular signalling molecules [232–234]. Elevation of intracellular ATP from glucose oxidation leads to closure of ATP-sensitive K^+ channels (K_{ATP}), membrane depolarization, and the resultant influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels initiates the first phase of exocytosis. The second phase appears to involve additional mechanisms which are less well defined. Candidate mediators include mitochondrial glutamate, PKA and PKC, cAMP phosphodiesterase, the AMP-activated protein kinase and insulin-stimulated protein kinases such as protein kinase B and S6 kinase [234]. Clearly, multiple signalling pathways can modulate insulin exocytosis, and, in each case, the molecular mechanisms may be differentially affected by phosphorylation. A number of studies have indicated roles for okadaic acid and cyclosporin/FK506-sensitive phosphatases in regulating exocytosis from insulin-secreting cells. However, both inhibitory and stimulatory roles have been demonstrated over a 10-year period, and the precise role(s) *in vivo* remain unclear. In isolated islet β-cells, most studies which utilize glucose to induce insulin exocytosis, show inhibition by okadaic acid at concentrations that would be expected to inhibit PP1 and/or PP2A [235–237]. Although okadaic acid has been shown to decrease Ca^{2+} currents in insulin-secreting cells [238], the degree of inhibition is insufficient to explain the large

decreases in exocytosis, and a number of other studies clearly indicate effects of okadaic acid downstream of Ca^{2+} entry [235,239,240].

In contrast with effects on glucose-mediated exocytosis, studies of insulin exocytosis from β-cells induced by other secretagogues show an increase in exocytosis in response to inhibition of PP1/PP2A [237,239,241], suggesting that the targets of PP1/PP2A are different from those for glucose-mediated exocytosis and are likely to be upstream of the common vesicle-docking and fusion events. In one study the effects of okadaic acid on glucose and cAMP-mediated secretion were directly compared and clearly showed inhibition of the former and stimulation of the latter [237]. In insulin-secreting RINm5F insulinoma cells, all studies indicate increased exocytosis in response to inhibition of PP1/PP2A [238,242–244]. Since the response to glucose in RINm5F cells is not robust, most of these investigations did not use glucose to stimulate release. A recent study, however, showed increased insulin release in response to phosphatase inhibition at physiological glucose concentrations, but no effect on KCl-stimulated exocytosis [243]. Interestingly, this effect was achieved with microcystin, a non-cell-permeant inhibitor of PP1/PP2A and a major increase in intracellular Ca^{2+} was also osberved. The mechanism of action remains unknown, but was suggested to be via an extracellular phosphatase activity. However, no evidence to support either conclusion was presented.

It would therefore appear that different molecular events are regulated by PP1/PP2A in glucose- and non-glucose-mediated exocytosis of insulin. Although the targets of PP1/PP2A remain unknown in this system, potential targets that are common to both mechanisms of exocytosis may be masked by effects on differential targets that are unique to glucose and non-glucose stimulated mechanisms. Furthermore, the mechanisms governing regulation of PP1/PP2A in these cells are not known. Whilst these may be different in each cell system, diversity may also be derived from the differential utilization of similar mechanisms by each system. Two studies in RIN5mF cells show inhibition of PP2A associated with stimulation of insulin secretion [243,244], indicating a responsiveness of PP2A to extracellular stimulation. Furthermore, inhibition of PP2A demethylation using ebelactone, an inhibitor of methylesterases, inhibited insulin secretion from isolated islets and INS-1 cell lines [245]. Given that the methylation state of PP2A governs its interaction with B subunits, this finding suggests that dynamic alteration of PP2A complexes can modify exocytosis, in insulin-secreting cells as well as in mast cells.

Reports on the effects of PP2B in insulin exocytosis are also contradictory with inhibition and stimulation being measured in response to PP2B inhibition [235,236,246–253]. Treatment of rats with CsA for 21 days decreased insulin secretion, but this was likely to be due to diminished insulin synthesis [249]. A similar study showed no acute effect of FK506 treatment of cells, but there was a reversible and time-dependent decrease in secretion, accompanied by a decrease in cellular insulin content and insulin mRNA levels [254]. The most recent study demonstrating inhibition of exocytosis by inhibiting PP2B suggests that the effect is limited to a second microtuble-dependent phase of exocytosis in which dephosphorylation of kinesin heavy chain (KHC) is required for replenishment of the readily releasable pool of secretory granules [246]. Increased exocytosis from PP2B inhibition has been demonstrated in several cell types, but not in isolated β-cells. Treatment of MIN6 glucose-responsive cells with CsA resulted in a dose-dependent increase in insulin secretion, an affect that was attributed to inhibition of dephosphorylation of a synapsin-I like protein in these cells [253]. Given that the role of synapsin-1 in neurons is to tether synaptic vesicles to the cytoskeleton, it is possible that the stimulatory effect observed here with CsA is due to disruption of the ready releasable pool and may be distinct from inhibitory effects on the second phase that involve KHC. Consistent with these findings, transient inhibition of PP2B with FK506 increased cAMP-dependent insulin secretion from RINm5f cells [251]. Interestingly, in these cells, forskolin treatment (which elevates cAMP) increased PP2B activity and subsequent dephosphorylation of the synapsin I-like protein, both of which were inhibited by FK506. These apparently co-ordinated effects of PKA and PP2B were shown to be mediated by co-localization with AKAP79/150, since disruption of PKA/AKAP79 binding disrupted forskolin activation of PP2B and secretion [251]. Importantly, in these studies, no effect on insulin content was observed during the 30 min incubation with FK506. It is therefore likely that the predominant acute effect of PP2B is to negatively regulate insulin exocytosis.

Neuronal exocytosis

In contrast with mast and pancreatic β-cells, exocytosis in neurons involves both large-granule, Golgi-dependent exocytosis and small-synaptic-vesicle exocytosis involving local vesicle recycling [3]. However, within the context of neurotransmission, synaptic vesicles are considerably more abundant and more quantitatively important than the large granules. For synaptic-vesicle exocytosis, the cytoskeletal architecture governing pools of synaptic vesicles is already in place under basal conditions in neurons. Synaptic vesicles are highly organized into pre-docked and docked pools in order to respond rapidly to changes in intracellular Ca^{2+} concentration [255]. Neuronal cells have particularly high levels of PP2A associated with membranes, suggesting that any translocation associated with cytoskeletal reorganization and vesicle trafficking, such as seen in mast cells and pancreatic cells, has already taken place [256]. It is therefore unlikely that any changes in PP2A subcellular location similar to that which is seen in mast cells will be observed in neurons. However, since membrane-bound PP2A appears to respond to changes in intracellular Ca^{2+}, at least in mast cells [66], it may therefore participate in regulation of other aspects of the exocytotic processes, in addition to the cytoskeletal arrangements.

The effects of phosphatase inhibition on neuronal exocytosis have been broadly investigated, with a range of effects described. A number of early studies suggested that okadaic acid treatment of synaptosomes, neuromuscular junctions or cultured cells resulted in increased release of neurotransmitters including glutamate, GABA (γ-aminobutyric acid) and acetylcholine [257–261]. However, the most recent studies indicate inhibition of neurotransmitter release from synaptosomes or cells treated with either okadaic acid or calyculin A ([262,263], but see [263a]; [264–266]). Because of the relatively high concentrations of PP1 and PP2A in neuronal tissue, concentrations of okadaic acid much greater than *in vitro* IC_{50} concentrations are typically required to induce a response. At these concentrations a range of PPP family phosphatases may be inhibited, and so interpretation of these collective results in isolation from other studies is difficult. Interestingly, although okadaic acid increases neurotransmitter release from chromaffin cells, this has been shown to be due to a significant increase in the concentration of catecholamine in secretory granules, but is in fact accompanied by an inhibition of degranulation [267]. More recent studies have utilized different inhibitors to show that inhibitory effects of okadaic acid on neurotransmitter release in synaptosomes are mediated by inhibition of PP2A. Thus fostriecin, which has selectivity for PP2A, produces inhibition of KCl-depolarization-mediated release of glutamate [266]. Fostriecin also inhibits

KCl-depolarization-mediated release of the styryl dye FM2-10, indicating that regulation by PP2A applies to the exocytosis of most neurotransmitters.

In addition to inhibition of KCl-mediated exocytosis, fostriecin and okadaic acid also inhibit exocytosis mediated by the Na^+-channel blocker 4-aminopyridine (4AP). This effect occurs only at low concentrations of 4AP, with no effect at higher concentrations [266]. At higher concentrations of 4AP, isolated synaptosomes have been shown to switch the dominant mode of exocytosis from full fusion to kiss-and-run [189]. At these concentrations, neither okadaic acid nor fostriecin produce any effect on glutamate or FM2-10 exocytosis, suggesting that the effect of PP2A is selective for full-fusion exocytosis and has little role to play in the kiss-and-run mode of exocytosis.

The loci of PP2A action in inhibiting neuronal exocytosis remain unknown, but, given the broad substrate specificity of PP2A, a number of protein targets are likely. A number of studies have shown modulation of specific Ca^{2+} channels by okadaic acid ([257,266]; [268], but see [268a]; [269]), but this is unlikely to explain the effects seen in experiments using populations of synaptosomes, where no changes in Ca^{2+} levels are observed in okadaic acid- or fostriecin-treated synaptosomes [266]. Furthermore, since kiss-and-run is induced by high Ca^{2+} concentrations, effects of PP2A mediated predominantly through inhibition of Ca^{2+} channels might be expected to be observed for both full fusion and kiss-and-run.

A number of studies using neuromuscular junctions or cultured spinal-cord neurons have described the effects of okadaic acid on synaptic vesicle organization and clustering [270–272]. Imaging of synaptic-vesicle movements within the nerve terminal using FM1-43 steryl dyes shows a disruption of synaptic-vesicle clustering and a lateral spreading of vesicles in the presence of okadaic acid [272]. The vesicle spreading does not occur through diffusion of, now, untethered vesicles, but rather there appears to be a PP2A-sensitive mechanism that translocates vesicles bidirectionally in nerve terminals. Exactly how these vesicle movements contribute to modulation of exocytosis remains to be determined. In cultured spinal-cord neurons, okadaic acid caused a dispersal of synaptic-vesicle clusters, but actin localization at contact sites was not disturbed [271]. This is in contrast with effects seen in mast cells, where okadaic acid prevents cytoskeletal rearrangements [65]. The difference may, however, reside in the roles played by the cytoskeleton in moving secretory granules to the plasma membrane in response to stimulation in mast cells, compared with the role played by the cytoskeleton in maintaining synaptic-vesicle organization in pools near to sites of exocytosis in neurons. It is also noteworthy that okadaic acid causes dispersal of synaptic vesicles and inhibition of exocytosis not only in neurons that have formed contacts with the synaptic targets, but also in ‘naïve’ neurons that have not contacted synaptic targets [271]. This suggests that, prior to synapse formation, synaptic vesicles are already organized into pools capable of exocytosis and vesicle recycling by a PP2A-dependent mechanism.

Synapsin I is involved in tethering synaptic vesicles to the cytoskeleton, and is a known substrate for PP2A [273,274]. However, regulation of synapsin I phosphorylation by PP2A cannot account for the effects of PP2A inhibitors on exocytosis, because phosphorylation of PP2A-sensitive sites on synapsin I increases following depolarization, and phosphorylated synapsin I promotes dissociation of vesicles from the cytoskeleton and promotes exocytosis [274,275].

Evidence from yeast suggests a role for PP2A in SNARE-mediated membrane fusion events. PP2A-mediated dephosphorylation of Sso t-SNARE (target-membrane SNARE) results in enhanced ability to form SNARE complexes and increased secretion [276]. This indicates that PP2A dephosphorylation of SNARE proteins overcomes kinase-mediated restraints on SNARE complexes and therefore exocytosis. Whilst the role of SNARE phosphorylation/dephosphorylation in mammalian cells remains to be determined, these findings in yeast suggest SNARE complexes as potential targets for PP2A in regulating exocytosis. However, the differential effects of PP2A on full fusion and kiss-and-run [266] suggest that, for inhibition of SNARE dephosphorylation to explain the inhibition of mammalian exocytosis by PP2A inhibitors, this must be at a site proximal to full membrane fusion but distal to fusion-pore formation. If this were not the case, any effect of PP2A on steps prior to fusion-pore formation would result in similar effects of inhibitors on both full fusion and kiss-and-run, unless each mode uses different molecular machinery for the delivery of synaptic vesicles to the plasma membrane.

PP1 or PP2A may also be involved in regulating the capacity of pre-synaptic glutamate receptors to influence glutamate release. In synaptosomes, an initial activation of metabotropic glutamate receptors with the agonist DHPG (dihydroxyphenylglycine) facilitates evoked glutamate release, but with a second activation of the receptor evoked release is inhibited [277]. This switch from facilitation to inhibition is lost with extended intervals between stimuli, except in the presence of okadaic acid or calyculin A. Thus inhibition of PP1 or PP2A prevents the recovery and maintains the receptor coupled to inhibition of release. This inhibition is coupled to N-type Ca^{2+} channels, but the target(s) of PP1/PP2A, and the physiological relevance of this switch, are not known [277].

A role for PP2B in regulating endocytosis through dephosphorylation of dynamin and other proteins is well established [200,279–281]. However, evidence also points to a distinct role in exocytosis, although the targets remain unknown. Inhibition of PP2B either potentiates or has little effect on neuronal exocytosis, depending upon the stimulus used [264,266,269,282–288]. Exocytosis mediated by KCl depolarization is generally unaffected by inhibition of PP2B [266,269]. In contrast, exocytosis mediated by other stimuli is increased by inhibition of PP2B. In a number of studies, inhibition of PP2B with CsA or FK506 resulted in increased exocytosis [264,269,283–285,286,288], but this was only observed at high concentrations of 4AP, which typically induce a switch to the kiss-and-run mode of exocytosis. Exocytosis mediated by lower concentrations of 4AP, which use the full-fusion mode of exocytosis, are unaffected by CsA or FK506 [266]. This suggests that PP2B is a negative regulator of the kiss-and-run mode of exocytosis.

PP2B is known to regulate Ca^{2+}-channel activity [21]. However, like PP2A, the most important regulatory point for PP2B’s effect on exocytosis appears to lie downstream of Ca^{2+}-channel activation, because full fusion is unaffected by CsA or FK506, and there is no change in Ca^{2+} levels in synaptosomes following treatment with PP2B inhibitors [266,269]. Moreover, CsA and FK506 also increase exocytosis induced by ionomycin, consistent with a role downstream of Ca^{2+} entry [285]. Since PP2B appears to only affect kiss-and-run and not full fusion, these studies would also suggest that ionomycin induces the kiss-and-run mode of exocytosis. Moreover, CsA, which is used therapeutically as an immunosuppressant, has been shown to evoke acute renal hypertension in transplant patients as a result of increased neurotransmitter release from renal afferents [289]. Given that CsA inhibits endocytosis (which would result in depletion of synaptic vesicles during chronic exposure to CsA) and increases kiss-and-run exocytosis, it would therefore appear likely that CsA-induced renal hypertension is mediated by kiss-and-run in these neurons.

The targets of PP2B action remain unknown, but a role for dynamin in kiss-and-run, in addition to clathrin-mediated endocytosis, has been proposed. In one study, a population of docked secretory granules in the neuroendocrine PC12 cell line, which were observed to fuse with the plasma membrane and were retrieved at the same position (distinct from clathrin-mediated endocytosis, which occurs at sites distinct from the site of fusion), were preferentially associated with dynamin and retrieval was blocked by dynamin inhibitors [290]. However, other studies suggest dynamin-independent mechanisms underlying kiss-and-run [291].

Careful analysis of synapsin I phosphorylation sites *in vivo* show that, in response to high 4AP, there is not only rapid phosphorylation of sites 1–3 on synapsin I, but also concomitant rapid dephosphorylation of other PP2B-specific sites that are believed to provide a constraint for exocytosis [285]. This indicates a potential complex interplay between mechanisms that regulate different phosphorylation sites on the same protein and which may be involved in different aspects of the exocytotic process. It remains to be determined if synapsin I has additional roles beyond tethering synaptic vesicles prior to docking with the plasma membrane. Changes to synapsin I phosphorylation mediated by PP2B may also be of clinical significance, since, in Huntington's disease, which is characterized by defective neurotransmission, there is particular hyperphosphorylation of PP2B sites on synapsin I that is associated with decreased expression of CaNB in these patients [292].

SUMMARY AND FUTURE PERSPECTIVES

It is now well established that protein phosphatases are a complex, highly regulated, group of enzymes that are actively involved in regulating many aspects of cellular signalling. Although the core components of their native enzymes are highly conserved, protein phosphatases have evolved diverse regulatory mechanisms, each of which may be unique to a given cellular process. In order to understand the role of protein phosphatases in a given cell process, it is therefore critical to use a functionally driven approach that identifies the regulatory features specific to that process. Although much remains to be discovered in relation to the mechanisms of phosphatase regulation, functionally based analysis of exocytosis has so far identified a number of potential loci (Figure 4) for phosphatase action within the exocytotic machinery, many of which are clearly downstream of channel activity. Comparison of different exocytotic systems also indicates that phosphatases may be involved in several key aspects of the exocytotic granule or vesicle life cycle. Differential response to phosphatase inhibition in insulin-secreting cells, depending upon the stimulus used, suggest specific roles for phosphatases in individual signalling pathways within these, and perhaps other, cells. Studies of insulin-secreting cells suggest a particular role for PP1/PP2A at a second phase that requires mobilization of vesicles from either reserve or release ready pools (loci b and c of Figure 4). Evidence from mast cells indicates particular involvement of PP1 and PP2A with the cytoskeleton for trafficking of secretory granules to the plasma membrane (Figure 4, locus b) and has provided the first evidence for regulation of PP2A subcellular location in response to extracellular stimulation. In more complex systems, such as neurons, additional roles for PP2A and PP2B in regulating exocytosis downstream of vesicle trafficking are suggested (Figure 4, loci e, f and i). The differential regulation of the full fusion (Figure 4, locus f) and kiss-and-run (Figure 4, locus *e*) modes of exocytosis by PP2A and PP2B respectively suggests specific roles at the membrane fusion and retrieval level. Modulation of exocytosis is integral to the regulation of cellular signalling and a variety of disorders (such as epilepsy, hypertension, diabetes and asthma) are closely associated with pathological modulation of exocytosis. In order to develop effective and specific therapies for such disorders, it is critical to identify and characterize the key molecules involved in modulating exocytosis and to define the molecular events specific to each condition. Identifying the targets of protein phosphatase action and the specific proteins involved in directing phosphatases to these substrates represents the next major challenge in understanding the particular roles of protein phosphatases in exocytosis.

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