Effects of Serine/Threonine Protein Phosphatases on Ion Channels in Excitable Membranes

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Herzig, Stefan, and Joachim Neumann. Effects of Serine/Threonine Protein Phosphatases on Ion Channels in Excitable Membranes. Physiol. Rev. 80: 173–210, 2000.–This review deals with the influence of serine/threonine-specific protein phosphatases on the function of ion channels in the plasma membrane of excitable tissues. Particular focus is given to developments of the past decade. Most of the electrophysiological experiments have been performed with protein phosphatase inhibitors. Therefore, a synopsis is required incorporating issues from biochemistry, pharmacology, and electrophysiology. First, we summarize the structural and biochemical properties of protein phosphatase (types 1, 2A, 2B, 2C, and 3–7) catalytic subunits and their regulatory subunits. Then the available pharmacological tools (protein inhibitors, nonprotein inhibitors, and activators) are introduced. The use of these inhibitors is discussed based on their biochemical selectivity and a number of methodological caveats. The next section reviews the effects of these tools on various classes of ion channels (i.e., voltage-gated Ca\textsuperscript{2+} and Na\textsuperscript{+} channels, various K\textsuperscript{-} channels, ligand-gated channels, and anion channels). We delineate in which cases a direct interaction between a protein phosphatase and a given channel has been proven and where a more complex regulation is likely involved. Finally, we present ideas for future research and possible pathophysiological implications.

I. INTRODUCTION

Nearly all aspects of cell function involve phosphorylation of the amino acids serine/threonine. It has been estimated that one-third of cellular proteins are reversibly phosphorylated (95). Phosphorylation can regulate proteins that induce very short-term or very long-term effects like ion channels and transcription factors. Specifically, cell division, cell differentiation, neuronal activity, muscle

Tu ne quaesieris, scire nefas, quem mihi, quem tibi finem
di dederint, Leuconoe, nec Babylonios temptaris numeros.
Ut melius, quidquid erit, pati! Seu pluris hienes seu tribuit
Iuppiter ultimam, quae nunc oppositis debilitat pumicibus
mare Tyrrhenum. Sapias, vina liques, et spatio brevi spem
longam reseces. Dum loquimur, fugerit invida aetas: carpe
diem quam minimum credula postero.

Q. Horatius Flaccus: Carmen 1.11.
II. STRUCTURE AND NOMENCLATURE OF SERINE/THREONINE PROTEIN PHOSPHATASES

A. Biochemical Classification of Protein Phosphatases

Philipp Cohen (77, 79) suggested to divide phosphatases solely on an enzymatic basis into protein phosphatase (PP) 1 and PP2. This classification holds true for mammalian phosphatases that are the subject matter here. In contrast, bacterial phosphatases do not neatly follow this scheme (81). Mammalian type 2 PP were subdivided into PP2A, PP2B, and PP2C (77). Type 1 PP are characterized by their inhibition by protein inhibitors 1 and 2. Type 1 PP dephosphorylates preferentially the β-subunit of phosphorylase kinase. Type 2 PP are not inhibited by inhibitors 1 and 2 (of PP1) and dephosphorylate mainly the α-subunit of phosphorylase kinase. PP2B is characterized by its requirement for Ca$^{2+}$ and calmodulin, whereas PP2C requires Mg$^{2+}$ for activity (375). Both PP1 and PP2A do not require divalent cations for their enzymatic activity. More recently, new serine/threonine phosphatases have been cloned and sequenced (78). New mammalian phosphatases are PP4 (or PPX), PP5, PP6 (or Sit4), and PP7 (see sects. II B4 and II B5). In contrast to the other PP, PP7, when expressed in vitro, is inactive against the widely used substrate phosphorylase a (it is unknown whether this is also true in vivo); however, phosphorylated histone can be used as substrate. PP7 is dependent on Mg$^{2+}$ but not calmodulin and is activated by Ca$^{2+}$ (208).

B. Primary Structure of the Catalytic Subunits of Protein Phosphatase and Subcellular Localization

The catalytic subunits of phosphatases that dephosphorylate serine and threonine are encoded by the PPP and PPM gene families (78). These families are defined by distinct amino acid sequences and crystal structures. The PPP family includes the prototypical types 1, 2A, and 2B phosphatases. It also comprises novel phosphatases like PP4, PP5, and PP6 (78). The PPM family includes the prototypical types 1, 2A, and 2B phosphatases. The multimeric forms consist of the catalytic subunit and one or two accessory proteins. These accessory proteins are.

contraction, and metabolic functions are regulated by phosphorylation. Here, we are mostly concerned with phosphatases in mammalian cells that possess excitable membranes. At present, it seems that many important dephosphorylation reactions in these cells are catalyzed by a limited number of catalytic subunit isoforms. However, the important concept emerges that the substrate specificity and function of phosphatases are mainly regulated by ancillary proteins. Therefore, we address their putative function where required.

The past decade witnessed rapid progress on the physiological role of phosphatases. The advent and widespread experimental use of new inhibitors as pharmacological tools hastened this process. Ion channels are ideal candidates for studying the dynamics of phosphorylation and dephosphorylation of proteins, because their molecular properties can be measured on-line in single-channel experiments. Based on these considerations, we try to address the structural and biochemical properties of phosphatases and their regulatory subunits first. The tools available for physiological experiments are then described, and a paragraph is devoted to methodological problems regarding the use of such compounds. The present electrophysiological knowledge about regulation of ion channels is also summarized. We are aware of the vast amount of data in this field but have chosen to restrict our presentation to those types or families of ion channels studied in more detail. Emphasis is placed on experiments where the molecular target of the phosphatase is probably the channel itself, or where a more complex but interesting signaling cascade is involved. It should be noted here that electrophysiological experiments will usually fail to prove the exact molecular nature of the dephosphorylated protein or even the exact amino acid. Up to now, most studies have employed native cells. Space limitations necessitate us to focus the present review along several dimensions. Preference is given to cite more recent work, mainly covering the 1990s (until 4/99). For in depth discussion of earlier literature, excellent reviews are available (375). The role of phosphatases in cell division is covered by another review in this journal (319). This review is concerned with serine/threonine-specific phosphatases. However, additional phosphatases (mitogen-activated protein kinase phosphatases) exist that dephosphorylate both threonine and tyrosine residues within the same substrate protein (432). Tyrosine phosphatases, which may also be relevant for ion channel regulation, are also not covered here. Even within the remaining area of ion channel regulation by serine/threonine phosphatases, we have to skip a considerable number of valuable papers. We apologize for any omission that will have to be considered a regrettable flaw.
proteins can confer substrate specificity, can regulate enzyme activity, and can control the subcellular localization of the holoenzyme (127).

### 1. PP1

**A) CATALYTIC SUBUNITS.** Cloning revealed that the catalytic subunit of type 1 phosphatases is differentiated into types 1α, 1β, and 1γ derived from three different genes (364). Protein phosphatase 1α codes for a protein of 330 amino acids (e.g., in rat and rabbit, Refs. 19, 31, 364). The catalytic subunit of PP1β is comprised of 327 amino acids (rat, Ref. 364). The catalytic subunit of PP1γ has two splice variants called PP1γ1 and PP1γ2 that code for 323 and 337 amino acids, respectively (364). Sequences are highly conserved between species. For instance, human PP1γ is 93% nucleotide sequence similar and identical in protein sequence to the rat homolog (334).

Table 2 includes the tissue expression of the catalytic subunits of PP1. However, even within one organ, the cellular or subcellular distribution of PP1 is not necessarily uniform. For example, in rat salivary glands, only some cell types reacted with antibodies specific for PP1γ (380). In the heart, where PP1α, PP1δ, and PP1γ are immunologically detectable in whole tissue homogenates, the myofibrillar fractions contain mainly PP1α (70). This should be considered when comparing biochemical or (electro)physiological data from whole tissues and cells.

**B) ACCESSORY OR REGULATORY SUBUNITS.** There are proteins that associate in equimolar concentrations with PP1 catalytic subunits leading to dimers (for brevity, unless stated otherwise, in the remainder PP1 stands for the catalytic subunit).
catalytic subunit of PP1). These accessory protein can change (that is, inhibit or augment) the activity, substrate selectivity, or subcellular localization of the catalytic subunit. They are discussed below and in section III A. Their tissue distribution and their functional effects are summarized in Tables 3 and 4, respectively.

Many regulatory proteins of type 1 PP contain a common sequence. It was noted that the motif R (or K), V (or I), X (variable amino acid), and F is common to all PP1-binding proteins, e.g., RGL, G, M110, p53BP, inhibitor 1, DARPP-32, and NIPP1 (see below). The different regulatory subunits are mutually exclusive (114, 232). The first regulators to be identified were I1 and I2 (206, 207).

A) I1, I2, and DARPP-32. I2 (inhibitor 2 of PP1) forms a complex with the catalytic subunit of PP1 to generate the ATP-Mg-dependent PP (95, 354). I2 inhibits PP1. I1 (inhibitor 1 of PP1) and DARPP-32 only inhibit PP1 after their phosphorylation by the cAMP-dependent protein kinase or cGMP-dependent protein kinase (181). Table 3 presents their tissue distribution. These inhibitors are discussed further in section III A because they are very useful tools for electrophysiological experimentation.

B) RGL. Another regulatory subunit is the G subunit (also called RGL). It was detected as the protein that attached PP1 to glycogen in rabbit skeletal muscle (394). It could also bind the catalytic subunit of PP1 to the sarcoplasmic reticulum (SR). It could be phosphorylated by protein kinase A (PKA), and this phosphorylation was accompanied by the release of PP1 from the SR preparation or the glycogen particles (215). RGL is comprised of 1,109 amino acids (405). It is highly expressed in skeletal muscle (see Table 3 for tissue distribution). The G subunit increases the activity of PP1 toward its substrates like phosphorylase a or glycogen synthase (95). Phosphorylation of the G subunit by PKA on amino acids serine-48 (site 1) and serine-67 (site 2) after treatment of intact skeletal muscle preparations with epinephrine reduced the affinity for G subunit for PP1 (94, 295, 324). The G subunit remains attached to glycogen under these conditions, but PP1 dissociates from the glycogen particle (213). Dissociated PP1 is less active toward glycogen synthase and phosphorylase a (214). In this way, epinephrine can decrease phosphatase activity, and this leads to inactivation of glycogen synthase (213). Insulin has the opposite functional effect (in skeletal muscle) via a somewhat similar mechanism. Insulin stimulates mitogen-activated protein kinase-activated protein (MAPKAP) kinase-1. This kinase phosphorylates site 1 on the G subunit. This phosphorylation increases PP1 activity against glycogen synthase. Thus site-specific phosphorylation of RGL and subsequently altered phosphatase activity can explain both the stimulatory effect of insulin and

### Table 3. Tissue distribution of accessory proteins for PP1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DARPP-32</th>
<th>I1</th>
<th>I2</th>
<th>Gb</th>
<th>RGL</th>
<th>PPP1E5</th>
<th>NIPP1</th>
<th>CPI17</th>
<th>GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>+ + (178)</td>
<td>+ (294)</td>
<td>+ (294, 354)</td>
<td>- (104)</td>
<td>- (405)</td>
<td>+ (105)</td>
<td>+ + (423)</td>
<td>+ (126)</td>
<td>+ (338)</td>
</tr>
<tr>
<td>Heart</td>
<td>+ (294)</td>
<td>+ (294, 354)</td>
<td>- (104)</td>
<td>+ (405)</td>
<td>+ (105)</td>
<td>+ + (423)</td>
<td>- (126)</td>
<td>- (338)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>+ (294)*</td>
<td>+ + (104)</td>
<td>+ + (104)</td>
<td>+ (105)</td>
<td>+ (405)</td>
<td>+ (105)</td>
<td>+ + (423)</td>
<td>- (126)</td>
<td>- (338)</td>
</tr>
<tr>
<td>Intestine</td>
<td>- (104)</td>
<td>+ (105)</td>
<td>br + + (126)*</td>
<td>bladder</td>
<td>+ + (126)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>+ (313)</td>
<td>+ + (313)</td>
<td>- (104)</td>
<td>- (405)</td>
<td>+ (105)</td>
<td>+ (423)</td>
<td>- (126)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
<td>- (104)</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>+++ (294)</td>
<td>++ (294, 354)</td>
<td>- (104)</td>
<td>+ + (405)</td>
<td>+ + (405)</td>
<td>+ + (405)</td>
<td>- (126)</td>
<td>- (338)</td>
<td></td>
</tr>
<tr>
<td>Retina</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
<td>+ (105)</td>
</tr>
</tbody>
</table>

--, Below detection limit. See text for details. * Species dependent. **+ + + in aorta (126). Reference numbers are given in parentheses.

### Table 4. Effect of accessory/regulatory subunits on PP1 activity

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Stimulation</th>
<th>Inhibition: K, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor-1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>DARPP-32</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inhibitor-2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>NIPP-1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>RIPP-1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CPI17</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>p53BP2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>sds22</td>
<td>25,000</td>
<td></td>
</tr>
<tr>
<td>PPP1E5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Gb</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PSF (102)</td>
<td>1,000</td>
<td></td>
</tr>
<tr>
<td>L5 (193)</td>
<td>Phosphorylase a</td>
<td></td>
</tr>
<tr>
<td>Gb (68)</td>
<td>Phosphorylated myosin</td>
<td></td>
</tr>
<tr>
<td>RGL (199)</td>
<td>Epinephrine (295)</td>
<td></td>
</tr>
</tbody>
</table>

Inhibition is quantified by inhibition constant (K) values. Stimulation is mentioned for appropriate substrates or pharmacological stimuli. See text for further details.
the inhibitory effect of epinephrine on glycogen synthesis in skeletal muscle (309).

C) \(G_L\). There is a specific protein that mediates the binding of PP1 to glycogen in the liver, called \(G_L\). \(G_L\) is comprised of 284 amino acids. In contrast to \(R_{GL}\), \(G_L\) is not phosphorylated significantly by PKA. \(G_L\) inhibited dephosphorylation of phosphorylase \(a\) by PP1. It is only present in liver (see Table 3).

D) \(G_M\). A protein that attached PP1 to myosin was called M subunit or \(G_M\) (68). \(G_M\) stimulated the activity of the catalytic subunit of PP1 toward phosphorylated native myosin but not toward phosphorylase \(a\), indicating that it conveys substrate specificity. PP1 attached to \(G_M\) is only present in myofilaments and cannot be purified from cytosol (68). Two proteins were identified and termed M130/ M133 based on their apparent molecular weight. Fittingly, two clones were isolated coding for 963 and 1,004 amino acids. They differ in the 41 intervening additional amino acids in the higher molecular weight form and are probably coded by distinct genes (63, 379). Differences in function are at present unknown (217, 218). Immunologically, the M130 was detectable in many chicken tissues including aorta, stomach, and heart but absent in liver and skeletal muscle (338, see Table 3). The M130 subunit in strips from rabbit portal veins is phosphorylated by an unknown endogenous kinase, and at the same time smooth muscle phosphatase activity is attenuated (218, 416). Hence, the function of \(G_M\) might be regulated by phosphorylation.

E) NIPP1. Nuclear proteins (isolated from bovine thymus nuclei) called NIPP1a (18 kDa) and NIPP1b (16 kDa) bind to and inactivate PP1 (33, 227, 229; see Table 4). This inhibition is accompanied by phosphorylation and reversed by PP2A or inhibitors of PKA or casein kinase II (422). These heat-stable proteins are prototytically framed of a precursor protein (39–41 kDa) that has been cloned and sequenced and called NIPP1. Its cDNA predicts 351 residues, and it is ubiquitously expressed (see Table 3). There is evidence for receptor-mediated hepatic phosphorylation of NIPP1 in intact animals (227).

F) Others. A ribosomal protein called RIPP, ribosomal inhibitor of PP1, of 23 kDa was isolated from rat liver ribosomes. This protein preferentially bound and inhibited PP1 in a noncompetitive manner (32). RIPP might play a role in the regulation of protein synthesis. On the other hand, PP1 activity is stimulated by another ribosomal protein from rat liver called L5 (193). A cytosolic protein that belongs to the heat shock protein family binds to PP1\(\gamma_2\), an isoform of PP1 enriched in testis. The functional role of this association is unknown (71). PP1 can bind to the retinoblastoma gene product. This may contribute to the function of PP1 in cell division (112). However, binding has not yet been shown in vivo. With the use of the yeast two-hybrid system it was revealed that PP1\(\gamma\) binds to p53BP2. The latter is a protein that binds to the p53 protein that acts as a tumor repressor. p53BP2 also inhibits potently PP1 activity against phosphorylase \(a\) (176). PP1 also binds to a splicing factor. The factor was identified as poly(pyrimidine) tract-binding protein-associated splicing factor (PSF). PP1 was inhibited by recombinant PSF (see Table 4). It is conceivable that the inhibitory action of PSF is potentiated by phosphorylation of PSF. The interaction may play a role in the physiological regulation of splicing in the spliceosome (192). In porcine aorta, PP1 could be inhibited by a protein of \(\sim 20\) kDa. The inhibition was proportional to its phosphorylation by an unknown endogenous kinase or exogenous protein kinase C (PKC). The IC\(_{50}\) was within the physiological concentration of the inhibitory protein of 20 kDa and might thus be functionally relevant (125). This protein has been sequenced and was called CPI17 (C-kinase activated PP inhibitor; apparent molecular mass 17 kDa; Ref. 126). It is expressed in smooth muscle like aorta or bladder but not in skeletal muscle or nonmuscle tissue (Table 3; Ref. 126). A new 36-kDa protein was identified that binds and inhibits PP1 activity. It was termed PPPIR5 (105). This protein is related to \(G_L\), but it is, in contrast to \(G_L\) ubiquitously expressed (Table 3; Ref. 105).

The inhibitory or stimulatory actions of the accessory subunits of PP1 are compared in Table 4.

c) DIRECT ALTERATION OF PP1 ACTIVITY. A more direct way to modulate phosphatase 1 activity is by posttranslational modification of their catalytic subunits. Phosphorylation and methylation have been reported. All mammalian PP1 share a \(\text{TPPR}\) sequence (364), which is a recognition site for phosphorylation by cyclin-dependent protein kinases. Indeed, PP1\(\alpha\) and PP1\(\gamma_1\) are phosphorylated on threonine-320 and threonine-311, respectively, by cell cycle-dependent protein kinases (cyclin-dependent protein kinases), and this phosphorylation inhibits their phosphatase phosphatase activity (103). The phosphorylation led to the incorporation of 0.5 mol phosphate/mol protein within 30 min. At this time point, phosphatase activity decreased to 50% of the initial value (103). This phosphorylation was first observed in vitro but later also in a cell cycle-dependent manner in intact cells and was accompanied by changes in cell cycle-dependent phosphatase activity in cytosol and nucleus (264).

2. PP2A

A) CATALYTIC SUBUNIT. The catalytic subunits of PP2A (see also Tables 1 and 2 for synopsis) exist in two isoforms called PP2A\(\alpha\) and PP2A\(\beta\). They have been cloned from many species (15, 84, 85, 161, 182, 248, 249, 392, 393). Rat liver PP2A\(\alpha\) cDNA codes for a 309-amino acid protein. PP2A\(\beta\) was different from PP2A\(\alpha\) in 8 amino acids, but the cDNA coded also for 309 amino acids and is coded by a different gene. The expression on protein and mRNA is higher for PP2A\(\alpha\) than PP2A\(\beta\). For instance, in porcine heart,
the ratio is 8:1 (392). PP2A is apparently mainly cytosolic. However, some PP2A was also detectable in the nucleus (418).

**b) Accessory/regulatory subunits.** Several subunits of PP2A are known. Trimeric PP2A is formed by the A (structural component, also called PR65), B (phosphatase regulatory subunit, PR), and C (catalytic subunit) subunits. It is controversial whether the native enzyme is a trimer. Dimers (formed of equimolar A and C) might be native forms or isolation artifacts (438).

**A) A subunit.** Two isoforms of the A subunits of PP2A have been cloned (177, 430). These A subunits were also called putative regulatory subunits and have an apparent molecular mass of ~65 kDa. This gave rise to the alternative nomenclature of PR65 for the A subunit (177). The Aα and Aβ forms encode proteins of 589 and 602 amino acids, respectively. The effect of the A subunit on PP2A activity is substrate dependent. Recombinant Aα inhibited the activity (using phosphorylase a or myosin light chains as substrate) of the C subunit from the bovine heart with high potency (IC₅₀ = 0.59 nM (235)). In contrast, the B subunit (PR55) stimulated the dephosphorylation of cdk1-phosphorylated histone H (3).

**c) Direct regulation of PP2A activity.** Covalent modification of the catalytic subunit can increase or decrease the activity of PP2A as discussed above for PP1. The COOH-terminal leucine (..TPDYFL) of the catalytic subunit of PP2A can be reversibly methylated by specific enzymes (273, 275). This leads to a modest increase in PP activity (129, 275, 449). This methylation is stimulated by cAMP and inhibited by okadaic acid (136). Agonist-induced reversible methylation of PP2A has been noted in intact cells (259) and is cell cycle dependent (418). In contrast, tyrosine phosphorylation reduces PP2A activity. The COOH-terminal tyrosine-307 is probably phosphorylated (..TPDYFL). This phosphorylation is catalyzed by tyrosine kinases like Src or Lck (55).

In addition, an autophosphorylation-activated protein kinase phosphorylates a threonine residue on PP2A and inhibits the activity of PP2A (165, 166). This may lead to enhanced phosphorylation and thus stimulation of mitogen-activated protein (MAP) kinase and MAP kinase kinase (306, 395). In contrast, casein kinase 2a binds to autophosphorylated PP2A, leading to an increase in phosphatase activity against MEK 1 (184).

**3. PP2B**

PP2B, alternatively called calcineurin, is a Ca²⁺/calmodulin-dependent protein phosphatase. The enzyme consists of two subunits, the catalytic A subunit (see Table 1) of ~60 kDa (CNA) and the regulatory B subunit (CNB) of ~19 kDa. Calcineurin is present in nearly all mammalian cells studied. However, it is most highly expressed in the brain (see Table 2 for tissue distribution).

**A) Catalytic subunit (A subunit).** Cloning from rat brain indicated a length of 521 amino acids for the A subunit.
There are three mammalian genes for the A subunit, giving rise to the CNA\(\alpha\), CNA\(\beta\), and CNA\(\gamma\) isoforms. CNA\(\alpha\) and CNA\(\beta\) are highly expressed in brain, whereas CNA\(\gamma\) is testis specific. Differential splicing of CNA\(\alpha\) generates two transcripts (\(\alpha_1\) and \(\alpha_2\)). The CNA\(\beta\) gene is alternatively spliced to three transcripts CNA\(\beta_1\), CNA\(\beta_2\), and CNA\(\beta_3\). CNA\(\alpha\) and CNA\(\beta_2\) are highly expressed in neuronal tissue, whereas CNA\(\gamma\) is specific for the testis (419). The catalytic subunit (A subunit) of PP2B shows autoinhibition that is relieved by interaction with the B subunit. PP2B is quite different from PP1 and PP2A. It is the only PP clearly regulated by a second messenger, namely, Ca\(^{2+}\). The inhibition of B on A is relieved if B binds Ca\(^{2+}\). This explains why the enzyme is dependent on Ca\(^{2+}\) for activity. Using proteolysis of the autoinhibitory COOH terminus of the A subunit generates a Ca\(^{2+}\)-independent isoform. This can be used experimentally to understand the function of PP2B.

B) REGULATORY SUBUNIT (B SUBUNIT). The B subunit was sequenced at the protein level and found to comprise 168 amino acids. It shows sequence similarity to calmodulin. Like calmodulin, it binds 4 mol Ca\(^{2+}\)/mol (5). Two different B subunit genes are known that are called CNB\(\alpha\) and CNB\(\beta\). CNB\(\alpha\) gives rise to one isoform expressed in many tissues named CNB\(\alpha_1\) (170 amino acids) and, by means of a different promoter, leads to another testis-specific isoform called CNB\(\alpha_2\) (216 amino acids). Similarly, CNB\(\beta\) (179 amino acids) is only expressed in the testis (52, 419).

The substrate specificity of PP2B is quite high. Well-investigated substrates include a subunit of phosphorylase kinase, inhibitor 1 (of PP1), DARPP-32, the type II regulatory subunit of the cAMP-dependent protein kinase, and the site 2 of the glycogen binding subunit of PP1 (RGL; Ref. 213). Data with inhibitors for PP2B (see also sects. wB, 7 and 8) indicate that the activity of the PP2B is substrate dependent. Although these inhibitors decreased the activity toward a 19-amino acid peptide, they caused a stimulation of activity toward p-nitrophenylphosphate (287). PP2B might play a role in signal transduction especially in the brain, where its expression is very high.

c) ACCESSORY PROTEINS. A) AKAP 79. A protein kinase A anchoring protein (AKAP 79) was able to bind PP2B. AKAP 79 inhibited in a noncompetitive manner PP2B activity with an IC\(_{50}\) of ~4 \(\mu\)M. It did not inhibit PP1 or PP2A. The interaction was studied in bovine brain (75).

B) CAIN. Another protein called calcineurin inhibitor (cain) was more recently studied (266). Cloning predicted a sequence of 2,182 amino acids. The IC\(_{50}\) was ~0.4–0.5 \(\mu\)M. Hence, cain is more potent than AKAP 79. Cain was highly expressed on RNA and protein level in brain, kidney, and testis. It was least detectable in heart, spleen, lung, liver, and skeletal muscle. It is mainly cytosolic. It was speculated that cain may target inactivated PP2B to specific intracellular regions where its release would provide Ca\(^{2+}\)-regulated phosphatase activity to specific signaling pathways (266).

4. PP2C

PP2C is monomeric. In mammalian cells, PP2Ca and PP2Cb are known (404, 437). PP2Ca is comprised of 382 amino acids. Several isoforms of PP2Ca, namely, PP2Ca1, PP2Ca2, and PP2Ca3, have been reported (302). PP2Ca is the most abundant isoform. Alternative splicing seems to generate the isoforms PP2Ca1 and PP2Ca2. They were cloned from a mouse library and show differences in COOH termini and the 3’-untranslated region. PP2Ca1 is expressed in all mouse tissues studied, whereas PP2Ca2 is confined to heart and brain where they might serve special functions (408). PP2Ca1 and PP2Ca2 code for 390 and 389 amino acids, respectively. Tissue distribution is included in Table 2. PP2C was originally assumed to be exclusively cytosolic (375). More recent work identified PP2C also in the nucleus of mammalian cells (87).

5. PP3

A PP3 has been suggested to exist (203). It was described as a particulate protein in the bovine brain that was inhibited by okadaic acid but stimulated by inhibitor 2 and inositol phosphates (471). Because there has not been any recent report on PP3, it seems likely that this enzyme may have been artifactual.

6. PP4

PP4, also called PPX (44), is expressed highly in testis; however, it was also detectable in all other tissues investigated (see Table 2). Its structure, like that of PP6, is reminiscent of the paradigmatic PP2A. PP4 is comprised of 307 amino acids (rabbit); PP4 is mainly localized in the nucleus, although smaller amounts are also present in the cytosol (44). Regulatory subunits of PP4 are thought to exist but have not been clearly identified (78).

7. PP5

PP5 is ubiquitously expressed in human tissues (see Table 2). The calculated molecular mass of the protein is ~58 kDa (the 5’-end of the sequence was incomplete in the initial report, Ref. 58). PP5 contains an autoinhibitory domain. Polyunsaturated fatty acids can relieve this inhibition (57). PP5 was detectable mainly in the nucleus, although some immunoreactivity was also present in the cytosol (56, 58, 67). PP5 interacts with the atrial natriuretic peptide receptor and was isolated in complex with a glucocorticoid receptor (56). These associations might indicate some kind of regulatory interaction.
8. PP6

PP6 is structurally related to PP2A. PP6 (303) has so far been identified in all mammalian tissues examined (see Table 2). Sit 4, the \textit{Saccharomyces} homolog of PP6, has regulatory subunits (130). Therefore, regulatory subunits of PP6 are thought to exist but have not been clearly identified (78).

9. PP7

PP7 is comprised of 653 amino acids. With a comparison of RNA from various human tissues, PP7 was only detectable in the retina and not, for instance, in the heart (see Table 2).

III. MODULATORS OF PROTEIN PHOSPHATASE ACTIVITY

In contrast to some tyrosine phosphatases, serine/threonine phosphatases are probably not located transmembranic but subplasmalemmal. This is no problem when using cell-free extracts. In more intact systems, inhibitors or activators have to pass the membrane. This is easiest if the compounds are freely permeable. Otherwise, the membrane has to be broken. This can be done mechanically (injection through pipette), electrically (gene pulser), chemically (transfection reagents), or by viral gene transfer. Hence, if information is available on cell permeability, it will be provided for the compounds listed in this section.

A. Protein Inhibitors

All these inhibitors are comprised of amino acids and are thus not expected to pass cell membranes easily.

1. Inhibitor 1 PP1

Inhibitors 1 and 2 of PP1 were identified by Huang and Glinsmann (206, 207). They share unusual physical properties. Both are heat stable and are not precipitated by 1% trichloroacetic acid, in contrast to most other proteins. The sequence of I1 has been reported first by direct protein sequencing from rabbit skeletal muscle and then from a rat skeletal muscle library and a human brain library by cloning and results in a predicted protein of 171 amino acids (4, 117, 119). The NH2-terminal region is highly conserved. Only in the COOH termini were differences between rabbit, rat, and human noted (119).

I1 from rabbit skeletal muscle and human brain has a calculated molecular mass of 18.7 and 19.2 kDa, respectively (4, 119). The apparent molecular mass is ~26 kDa on SDS-PAGE. This discrepancy has been explained by a low degree of order in the protein. I1 binds to and inhibits PP1 only after being phosphorylated on threonine-35 by cAMP-dependent protein kinase or cGMP-dependent protein kinase (181). It is very selective for PP1. For instance, phosphorylated recombinant human I1 inhibited PP1 and PP2A with IC50 values of 1.1 and 21,000 nM, respectively (119). Phosphorylation of I1 occurs in vivo in skeletal muscle, heart, and cardiomyocytes after \(\beta\)-adrenergic stimulation (76, 168, 329). In vitro-phosphorylated I1 has been used as a tool to study in permeabilized preparations whether a process involves PP1. For instance, I1 phosphorylated by cGMP-dependent protein kinase induced force generation in permeabilized smooth muscle cells (413). As expected, mutagenesis of threonine-35 to alanine yielded a mutant I1 that could not be phosphorylated by I1 and that did not inhibit PP1. Mutation of threonine-35 to aspartic acid, which is intended to mimic phosphorylation, led to a mutant form of I1 that inhibited both PP1 and PP2A with IC50 values of 24 and 25 \(\mu\)M, respectively (119). Immobilized I1 binds \(\sim\)10 times better to PP1 than to PP2A, independent of its phosphorylation state. Amino acids 9–12 KIQF are conserved in rat, rabbit, and human, and they seem to be crucial for binding and inhibition of PP1 (114). If this sequence is deleted, phosphorylation of I1 is unable to inhibit PP1 activity (119). Interestingly, I1 is present in the liver of rabbits, guinea pigs, and sheep but absent from mouse and rat liver (210, 294). I1 is present, for example, in skeletal muscle, heart, kidney, uterus, and adipose tissue (117, 294). I1 is a cytosolic protein.

2. DARPP-32

DARPP-32 is similar to I1 in function but derived from a different gene, and it is mainly expressed in the brain (178). It has been sequenced on protein and cDNA level from bovine brain (262, 444) and rat brain (115). DARPP-32 from bovine brain has a predicted molecular mass of 22.6 kDa and, like I1, a higher apparent molecular mass of 32 kDa on SDS-PAGE. The same threonine residue on DARPP-32 is phosphorylated by cAMP-dependent protein kinase but also by cGMP-dependent protein kinase. Phosphorylation of DARPP-32 changes its IC50 for PP1 from 1 \(\mu\)M to 2 nM (96, 97), underscoring its high selectivity. Under unphysiological conditions (mM Mn\(^{2+}\) in the assay), I1 and DARPP-32 are dephosphorylated and inactivated by PP1. Both I1 and DARPP-32 are dephosphorylated by PP2A and even better by PP2B (98, 178, 180). The dephosphorylation by PP2B is dependent on the presence of Ca\(^{2+}\). Hence, it was suggested that this might be a way for Ca\(^{2+}\) levels to control protein phosphorylation (214). DARPP-32 is a cytosolic protein.

Thiophosphorylated I1 or DARPP-32 is not (easily) dephosphorylated and has been successfully used to...
study the physiological role of PP1-mediated phosphorylation in muscle contraction.

3. Inhibitor 2 PP1

I₂ from rabbit skeletal muscle is comprised of 204 amino acid and has a calculated molecular mass of 22.9 kDa (198). Similarly to I₁, its apparent molecular mass on SDS-PAGE is larger and amounts to ~31 kDa. It is unrelated in sequence to I₁. Its tissue distribution has been studied (294, 354). It binds to and inhibits PP1 regardless of phosphorylation. Therefore, it has been used to study the effect of PP1 in intact cells. It forms complexes with PP1 and is therefore discussed in depth above. A caveat is noteworthy. I₂ inhibits the free catalytic subunit of PP1 in the nanomolar range. However, the glycogen-associated PP1 is poorly inhibited, and the smooth muscle myosin-PP1-associated PP is not inhibited at all (7, 213, 214). However, it is known that glycogen-associated PP and the myosin-associated PP contain the catalytic subunit of PP1 (95). Hence, inability to block a process by addition of I₂ does not prove that a PP1 is not involved but requires additional experimentation. The binding site for I₂ is probably close to that of okadaic acid (460). Mutational analysis suggests that I₂ inhibits via interaction with the amino acid tyrosine-272 on PP1 because its IC₅₀ is changed from 13 to 180 ng/ml in the mutant Y272K (458).

4. Inhibitor 1 PP2

Inhibitor 1 PP2 has been isolated from bovine kidney. It is thermostable and not inactivated by 1% trichloroacetic acid. Its apparent molecular mass was 30 kDa. (277). Later, it was identified as putative class II human histocompatibility leukocyte-associated protein (PHAP) I (279). PHAP I had been cloned and sequenced before by Shibata et al. (376) noted that OA increased tone in smooth muscle preparations. Erroneously, this was interpreted as opening of Ca²⁺ channels and activation of a Ca²⁺-dependent kinase and phosphorylation of regulatory proteins. Moreover, OA was studied as a skin tumor promoter in mice (396). However, Takai et al. (400) were the first to

5. Inhibitor 2 PP2

This inhibitor has been isolated from bovine kidney. It is also thermostable and not inactivated by 1% trichloroacetic acid. Its apparent molecular mass was initially reported as 20 kDa (277). Protein sequencing revealed that the protein had been described before as SET (278, 426, 427), PHAP II (421), and template activating factor-1β (322). SET has a predicted molecular mass of 32,100 Da and an observed molecular mass of ~39 kDa and is largely located in the nucleus (138). Thus proteolysis should account for the lower molecular mass reported initially. Ubiquitous expression of SET was reported (2). Interestingly, SET was phosphorylated on serine in intact cells. Whether this alters phosphatase inhibitory function remains to be established (2). These inhibitors might be useful tools to study the physiological function of PP2A. It has been speculated that I₁ and I₂ of PP2A might be involved in signal transduction. Specifically, they were suggested to mediate the effects of insulin on PP2A (277).

6. Simian virus 40 small tumor antigen

Simian virus 40 (SV40) is a member of the papova family of small DNA tumor viruses. Its lytic cycle takes place in permissive monkey cells. SV40 infection leads to the production of proteins that are immunogenic and that were called tumor antigens. One such antigen is the SV40 small tumor antigen. It can inhibit PP2A activity with an IC₅₀ of 10–15 nM. This has been reported for substrates such as myosin light chains, phosphorylated by myosin light-chain kinase (453). As mentioned above, PP2A can occur as a monomeric, dimeric, or trimeric species: C (catalytic subunit), AC, or ABC. SV40 inhibits PP2A activation after forming a complex with the AC species (453). This occurs with brain PP2A or in CV-1 cells that all contain the Ba form of the the B subunit of PP2A. Here, SV40 small tumor antigen displaces the B subunit from the PP2A holoenzyme (386, 453). In cells the interaction of small tumor antigen with PP2A leads to deinhibition and thus activation of MAP kinase and MEK which induces cell proliferation (386). SV40 small tumor antigen can conceivably be used in cell extracts to quantify the amount of PP2A activity. The protein is not expected to pass through intact cell membranes. However, it can be injected into cells or alternatively might be delivered to cells as their coding DNA by various means of transfection including virus vectors (386, 387).

B. Nonprotein Inhibitors

1. Okadaic acid and derivatives

The history of okadaic acid (OA) is paradigmatic (80). Okadaic acid is a polyether compound with a C-38 structure, isolated from the black sponge Halichondria okadai named in honor of Yaichiro Okada (146). Shibata et al. (376) noted that OA increased tone in smooth muscle preparations.Erroneously, this was interpreted as opening of Ca²⁺ channels and activation of a Ca²⁺-dependent kinase and phosphorylation of regulatory proteins. Moreover, OA was studied as a skin tumor promoter in mice (396). However, Takai et al. (400) were the first to
report that OA is a potent phosphatase inhibitor in smooth muscle preparations (34). Okadaic acid inhibited PP1, PP2A, and PP2B with IC\textsubscript{50} values of 272, 1.6, and 3,600 nM, respectively (35). PP2C, phosphotyrosyl phosphatase, acid phosphatase, and alkaline phosphatase were not inhibited by up to 10 µM OA. Inhibition was noncompetitive, mixed competitive, and reversible (35). Hescheler et al. (190) noted that OA inhibited PP activity in skeletal muscle preparations and increased currents through cardiac L-type Ca\textsuperscript{2+} channels. It is currently thought that tumor promotion by OA and related compounds like calyculin A and microcystin LR is due to inhibition of PP1 and PP2A (for review, see Ref. 146). The OA binding site of PP is not the substrate binding site because OA inhibits PP1 and PP2A noncompetitively (35). However, a caveat is warranted. Okadaic acid in concentrations <2 nM inhibits also PP4, PP5, and PP6 that are present in mammalian cells (78).

At least 16 derivatives of OA are known, and potent inhibitors include, in addition to OA, dinophysistoxin-1 and acanthifolicin (146). Dinophysistoxin-1 was first isolated from the hepatopancreas of the mussel Mytilus edulis. It caused shellfish poisoning in Japan. Its name is derived from its source in the dinoflagellate Dinophysis fortii (146). Chemically, it is 35-methylokadaic acid (199). Acanthifolicin is an episulfide derivative of okadaic acid. It occurs naturally in the sponge Pandaros acanthifolium. Treatment of acanthifolicin with diazomethane led to acanthifolicin-methyl ester (146). Of importance, some derivatives are inactive and can be used as negative controls. These include OA methyl ester, nor-okadacon, acanthifolicin methyl ester. Interestingly, chemical degradation products of OA, namely, OA spiroketal I and II, were still able to inhibit type PP2A, indicating that it may be possible to design simpler but still active OA derivatives (146). Okadaic acid increases the phosphorylation state of a number of proteins. Some have been identified; these include vimentin and the 27-kDa heat shock protein (hsp27) (53). The permeation of OA through cells seems to be rather poor. It has been estimated that OA penetrates the cell membrane 100-fold less readily compared with calyculin A (128). Peroral application of radioactive OA led only to 1% absorption. Intraperitoneal application of radioactive OA indicated that OA is excreted through hepatobiliary circulation (146). However, OA freely permeated through the lipid membrane of multilayer vesicles in a liquid-crystalline state, indicating that OA gains access to receptors in the cytosol (325). Okadaic acid (458, 460) binds probably to YRCG (amino acids 267–270) or the vicinity. Mutational analysis suggests that OA inhibits via interaction with amino acid tyrosine-272 on PP1 because its IC\textsubscript{50} is changed from 200 to 50,000 nM in the mutant Y272S (458).

2. Cantharidin and analogs

Cantharidin, cantharidic acid, palasonin, and endo- thall are structural analogs (268). Cantharidin (CA) is the vesicant in blister beetles (beetle in Greek is κανθαρός) and present in Spanish flies, palasonin is an anthelmintic in seeds of a medicinal tree, and endo- thall is a synthetic herbicide. The toxic action of cantharidin is thought to be due to phosphatase inhibition. Initially CA was reported to bind to PP2A in mouse liver (281). However, CA inhibits PP1 and PP2A with IC\textsubscript{50} values of ~500 and 40 nM (200, 282, 330). Palasonin and cantharidic acid exhibited similar inhibitory activity (282). Endo- thall inhibited both PP1 and PP2A with IC\textsubscript{50} values of ~5,000 and 1,000 nM (282). Neither compound inhibited PP2B (>30,000 nM) or PP2C (>1 nM). All compounds are herbicides. However, endo- thall is more potent, possibly due to the expression of other PP in plant or to permeability differences (282). Cantharidin is a terpenoid. It is cell membrane permeable. It caused phosphorylation of regulatory proteins in rhabdomyocytes and leiomocytes (251, 330). This phosphorylation was accompanied by contraction of papillary muscles and coronary arterial preparations (250, 286, 330). It is less potent and selective than okadaic acid but is inexpensive. Mutational analysis indicates that OA and CA might act on different amino acids on PP1 (460). In mutational analysis of amino acids 274–277 of PP1, no change in the IC\textsubscript{50} for cantharidic acid was noted, in contrast to OA, which was much more active when GEFT was changed to the mutant YRCG (460). It has been claimed that CA and endo- thall are not readily permeable through cell membranes but are taken up by hepatocytes (122, 124). However, others reported that endo- thall is membrane permeable. Okadaic acid displaced cantharidin from PP2A (281).

3. Calyculin A

Calyculin A (CyA) was isolated from another marine sponge, Discodermia calyx. It is an octamethylpolyhy-
Microcystins

Whereas OA and CyA are fatty acid derivatives, microcystin and nodularin are peptide toxins. Microcystins are of toxicological relevance. They cause death in cattle and humans exposed to water contaminated by certain algae. These include colonial and filamentous algae and cyanobacteria such as *Microcystis aeruginosa* (48, 49, 146). Algae and prokaryotes seem not to contain PP1 or PP2A; therefore, they can survive these toxins in contrast to other phyla. Microcystins are cyclic heptapeptides containing five constant (some of which are unique) and two variable amino acids. The variable amino acids in microcystins are given in the one-letter code. Hence, their short-hand notation is microcystin-LR, -YR, and -RR. More than 40 additional microcystins have been identified (146). Microcystin-LR inhibits PP1 and PP2A with IC<sub>50</sub> values of 0.1 nM each (298) or 1.7 and 0.04 nM, respectively (202). It inhibits PP2B with an IC<sub>50</sub> of 0.2 μM and does not inhibit PP2C up to 4 μM (298). Okadaic acid prevents the interaction of microcystin with PP2A, implying a similar site of action. Moreover, binding of inhibitor 2 to PP1 prevented the binding of microcystin-LR to PP1. It is important to keep in mind that the newer mammalian PP4 and PP5 are also inhibited by <2 nM microcystin. Hence, it is possible that some effects thought to result from PP1 or PP2A inhibition actually result from PP4 or PP5 inhibition (44, 58, 303). Moreover, inhibition of PP6 by microcystin has not been tested, and other phosphatases will likely be cloned in the future. Microcystin is the most potent (and toxic) PP inhibitor. As expected for a peptide, cell permeation is a problem. In fibroblasts, microcystin-LR did not increase phosphorylation. However, it led to hyperphosphorylation in hepatocytes (123). This is consistent with the clinical observation that intoxications with microcystin-contaminated water led to hepatic necrosis and subsequent death, as shown recently by an epidemic caused by microcystin-contaminated dialysis fluid (230). Peroral microcystin is taken up with a bile acid transport system across the ileum into hepatocytes (123). When radioactive microcystin was given intravenously to mice, label was detected mainly in liver but also in kidney, gut, and lung. No label was found in spleen and heart. Hence, no transport system for microcystin seems to exist in cardiomyocytes or splenocytes (357). Interestingly, these investigators clearly demonstrated that hepatic radioactive label persisted after single administration and that the label was covalently bound to a protein that they did not identify further but that is expected to be a PP. Permeability problems can be overcome by permeabilizing preparations with, e.g., α-toxin or β-escin. With the use of this approach, microcystin increased the tone in isolated preparations from guinea pig femoral artery, guinea pig ileum, rabbit femoral artery, and rabbit portal veins (158).

The three-dimensional structure of microcystin-LR bound to PP1 has been determined (18, 155). The modified amino acid N-methyl-dehydroalanine (Mdha) in microcystin was bound to cysteine-273 in PP1 (155). In a two-step mechanism, microcystin-LR first binds to and inactivates PP1 or PP2 within minutes. Thereafter, a covalent modification of Mdha in microcystin was formed (within hours) with PP1 or PP2A (82). Mutation of cysteine-273 in PP1 to alanine impeded covalent binding of microcystin to PP1. However, this mutation does not reduce the potency of microcystin, OA, nodularin, tautomycin, and inhibitor 2 to inhibit PP1 activity. This strongly implies a two-step mechanism and indicates that binding to PP1 and inhibition of activity are distinct processes (360). Likewise, another laboratory also identified cysteine-273 in PP1 as the amino acid that is covalently bound to microcystin. However, in their hands, mutation of cysteine-273 to alanine increased the IC<sub>50</sub> of microcystin on PP1 from 0.2 to 4.0 nM. They argued that this opposite finding could be due to different dilutions of the PP1 between laboratories (290). It was extrapolated that microcystin should covalently bind to cysteine-266 in PP2A (360). Mutational analysis suggested that microcystin and other toxins like OA, nodularin, and CyA competed for the same inhibitory site on PP1 near amino acids 273–276 (460). Mutational analysis suggests that microcystin inhibits via interaction with amino acid tyrosine-272 on PP1 because its IC<sub>50</sub> is changed from 0.3 to 14 nM in the mutant Y272K (458).

5. Nodularin

Nodularin was isolated from the toxic water cyanobacterium *Nodularia spumigena* (49). It is a cyclic pentapeptide. Like microcystin, it is toxic to the liver.
does not penetrate into fibroblasts, but it is active in hepatocytes, like microcystins (146). Nodularin R and its derivative called motuporin (nodularin V) potently inhibit PP1 as well as PP2A with IC\textsubscript{50} values of 1.6 and 0.03 nM, respectively (201). Hence, it inhibits PP1 and PP2A ∼10 times more potently than OA. It is ∼70-fold selective for PP2A. It inhibits PP2B with an IC\textsubscript{50} of 8.7 μM but does not affect PP2C (201). Hence, the IC\textsubscript{50} values are comparable to those of microcystin-LR. In contrast to microcystins, nodularin R or V does not covalently bind to PP1 or PP2A (82). Mutational analysis suggests that nodularin inhibits PP activity via interaction with amino acid tyrosine-272 on PP1 because its IC\textsubscript{50} is changed from 0.5 to 150 nM in the mutant Y272S (458). The three-dimensional solution structure of nodularin closely resembles that of microcystin-LR (13).

6. Tautomycin

Tautomycin was isolated from *Streptomyces spirorverticillatus* as an antibiotic because it is toxic to yeast and fungi. Its structure as a polyketide resembles somewhat that of OA (205, 296). It inhibits PP1 and PP2A with IC\textsubscript{50} values of 0.7 and 0.65 nM, respectively (146), or 0.16 and 0.4 nM, respectively (296). Others reported IC\textsubscript{50} values of 0.4 and 34 nM for PP1 and PP2A, respectively (401). The latter results might be interpreted as selectivity for PP1. However, p-nitrophenylphosphate was used as substrate, whereas other laboratories that reported more potent inhibition of PP2A used the more conventional and perhaps more relevant substrate phosphorylase a. Tautomycin does not inhibit PP2C, and its IC\textsubscript{50} for PP2B is 100 μM. Okadaic acid prevents the interaction of tautomycin with the catalytic subunit of PP2A. Unlike microcystin, tautomycin led to hyperphosphorylation in all cell types tested, like keratinocytes and K562 cells (146). Hence, it is apparently cell membrane permeable. Mutational analysis suggests that tautomycin inhibits via interaction with amino acid tyrosine-272 on PP1 because its IC\textsubscript{50} is changed from 1.1 to 2,600 nM in the mutant Y272K (458). Like CyA, it can be used in comparison with OA. This might indicate whether a physiological effect is mediated by PP1 or PP2.

7. Ciclosporin

Ciclosporin, or cyclosporin A (a cyclic undecapeptide), and FK-506 (a macrocyclic lactone) inhibit PP2B (342). This inhibition is not direct. First ciclosporin and FK-506 bind to cyclophilin and a FK-506-binding protein (FKBP), respectively. Thereafter, they interact with the latch region of the CNB subunit of PP2B (74, 314). Then inhibition of CNA, the catalytic subunit, occurs. The three-dimensional structure of the calcineurin-FK-506 and FKBP complex supports this notion (162, 247). Rapamy-
(see below). This property has led to a procedure to separate these phosphatases using a heparin-based affinity column chromatography. Spermine inhibits both PP1 and PP2A with similar potency (375). It was speculated that polycationic compounds might mimic the action of some unknown intracellular factor. Their use as tools to study PP function is hampered by their lack of membrane permeability.

12. Thrysiferyl 23-acetate

This compound is, like OA, a polyether fatty acid and contains a squalene carbon skeleton. It was isolated from the red alga *L. obtusa*. It is unique because it is a selective inhibitor of PP2A. Up to 1 mM it does not inhibit PP1, PP2B, PP2C, or tyrosine phosphatase activity. Its IC₅₀ for PP2A is ~4 μM. Hence, it is several orders of magnitude less potent than OA or CyA. However, it can be used in cell extracts to distinguish between type 2A and other phosphatases. It is expected to be cell membrane permeant (307).

The potency of inhibitors is usually 10–100 times less in intact tissue or cells than in enzymatic inhibition assays (54). This has been explained by reduced uptake into cells, their preferential localization within the lipid phase, or by the high intracellular concentrations of the targeted phosphatases (76, 122; see also sect. iv).

C. Activators

1. 2,3-Butanedione monoxime

2,3-Butanedione monoxime (BDM) was initially described as a chemical phosphatase. However, it was demonstrated that BDM does not directly dephosphorylate substrates like phosphorylase *a*. Instead, BDM activates the phosphatase holoenzyme of PP1 and/or PP2A (468). A caveat is in order. BDM is probably a poor tool because it is no specific phosphatase activator but exhibits various effects on additional proteins. Indeed, there are numerous examples where BDM directly blocks ion channels but does not cause dephosphorylation (9, 116, 367, 384, 457).

2. Sphingosine derivatives

Ceramide can stimulate the activity of trimeric PP2A. However, the activity of the dimer (CA) or the free catalytic subunit (C) cannot be stimulated by ceramide (101). Ceramide might be an important second messenger for cell membrane-located receptors (20, 252).

3. Other activators

PP2A is activated by polylysine, protamine, polybrene, and histone H1 (120, 343). Histone H1 is only present in the nucleus and might thus be a physiological stimulator of PP2A that is detectable in the nucleus in small amounts (see above). Protamine did not stimulate the activity of the purified catalytic subunit of PP2A, but the PP2A1 (trimeric ABC) was stimulated. Protamine could also stimulate the dimeric form of PP2A reconstituted with recombinant SV40 tumor small antigen (234). Protamine can stimulate or inhibit PP2A, depending on the substrate studied. Protamine stimulated and inhibited trimeric PP2A when myosin light chain or histone-1 were used as substrates, respectively (234). In contrast, heparin can stimulate trimeric PP2A independently of the substrate under study (234). The activation probably does not result from dissociation of trimeric PP (64). However, the subtype of B subunit is important. Heparin did not displace Bo/PR55α from the trimeric form (the main bovine brain isoform) but did displace Bβ/PR55β. In both cases, the PP2A activity was enhanced. This implies that dissociation is not necessary for stimulation of phosphatase activity by heparin (234). Heparin, protamine, and polylysine are not present within the cytosol of mammalian cells. However, they might mimic the effect of an endogenous activator. Arachidonic acid inhibits PP1 but activates PP2A (159) and PP5 (57).

IV. METHODOLOGICAL CONSIDERATIONS

The vast majority of studies cited below, which address the role of protein phosphatases in the regulation of ion channels, make use of phosphatase inhibitors in more or less intact biological systems, i.e., cells, tissue slices, isolated organs, or channels in isolated membrane patches or bilayers. It is important to point out the limitations of those results for a proper assessment of the present state of knowledge. A number of caveats apply to the above-mentioned approach, and these will be briefly outlined in this chapter. Table 6 compiles a selection of frequently used phosphatase inhibitors, together with their subtype selectivity as known from the cited biochemical studies. It may serve as a guideline for choosing the appropriate tools to investigate selected phosphatases, but “cookbook” advice is strongly discouraged. The so-called selectivity (i.e., difference between inhibition constants against various molecular targets) applies to the comparison between phosphatase isoforms. Effects on other proteins may have to be taken into account. For example, fluoride ions (forming aluminum fluoride in the absence of chelators in any physiological solution) are known to affect G proteins, and orthovanadate is a well-known inhibitor of Na⁺-K⁺-ATPase. Such risk may be reduced when using high-affinity agents such as the microcystins or okadaic acid derivatives, but not all potential candidates (protein kinases, nucleotide-binding pro-
proteins, ATPases, or unknown) for unspecific action have been thoroughly studied.

A way to minimize this problem would be to undertake the appropriate control experiments. Several examples can be given. It is reasonable to use a second or third compound from a different chemical class, but with a similar selectivity profile, as a positive control, e.g., results with OA suggesting a role for PP2A can be substantiated using cantharidin or protein inhibitors of PP2A if possible. Data with CyA can be complemented with nanomolar OA (PP2A), or with PP1 inhibitor 2. Protein preparations of inhibitor 2 of PP1 should still be active after heat inactivation of possible contaminants. PP2B inhibition can be achieved with ciclosporin, and PP2B activity should be negligible when intracellular Ca\(^{2+}\) is strongly buffered with EGTA or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Negative controls are possible if inactive derivatives are available, as with OA (i.e., norokadaone, okadaic acid methyl ester). Studies with peptide inhibitors should be accompanied by inactive peptides of the same physicochemical properties, e.g., by using a scrambled sequence.

A second point of concern relates to the comparison between biochemical inhibition constants and functional effects and their concentration dependency. First, little is known about the extent and time course of intracellular accumulation of those compounds that penetrate cell membranes. There may be considerable differences among drugs (see, for example, Ref. 128). Even if the cellular content would be known, the free concentration might be much lower than expected due to accumulation in subcellular compartments or binding to membrane or specific targets. Accordingly, biochemical inhibition constants are often far lower than concentrations necessary for functional effects (see, for example, Refs. 326, 327, 330). Another point is of importance here. When measuring the phosphorylation state of a protein or, even more indirectly, the activity of an ion channel, a steady state is observed, which may vastly differ from the (pseudo)equilibrium conditions of an enzyme activity assay. The fraction of phosphoprotein here is a result of the balance between underlying protein kinase and phosphatase activities, as illustrated by a simple model calculation. Assume a phosphoprotein phosphorylated at one site by one protein kinase, and dephosphorylated by one or two different protein phosphatases. The steady-state phosphoprotein level PrP (fraction of 1) is then defined by the law of mass action as

\[
PrP = \frac{1}{[(K_1 \times PP1 + K_2 \times PP2)/PK] + I}
\]

(1)

where PP1 and PP2 are the total activity of two phosphatases, PK is the total kinase activity (all used in arbitrary units), and \(K_1\) and \(K_2\) are the remaining fractions of phosphatase activity in the presence of a selective inhibitor I, also following simple mass action

\[
K_1 = 1 - \frac{1}{K_{D1} + I}
\]

(2)
where $K_{D1}$ is the true inhibition constant of PP1 against I (and analogous for $K_{D2}$ and PP2).

A quantitative problem arises even in the simple case where only one phosphatase is involved (PP2 = 0) (see Fig. 1, A and B). Data were calculated (thin, noisy lines) and fitted (solid lines, using 1- or 2-site models of a Langmuir isotherm, Eq. 2, $K_{D1}$ was 1 nM (log = 0), and the kinase activity was always set to PK = 1. The difference between the calculations in Figure 1, A and B, resides in the counteracting phosphatase activity (A: PP1 = 0.5, B: PP1 = 10). In both cases, the apparent half-inhibitory concentration (arrow) is higher than predicted (log = 0). This shift amounts to more than 10-fold in case B. The apparent inhibitory concentration approximates the true value I when PK >> PP1. However, then phosphoprotein levels at baseline are close to 1, and a phosphatase inhibitor would have nearly no absolute effect. The problem is even more serious if a subtype-selective inhibitor is used to assess the relative proportion of two different phosphatases in dephosphorylating a common substrate. In Figure 1, C and D, a second phosphatase is included in the model, and a pronounced subtype selectivity was chosen ($K_{D1}$ = 1 nM, log = 0, and $K_{D2}$ = 1 μM, log = 3). The half-inhibitory constants are right-shifted as above, but the proportions of the two components also depend on the relationship between kinase activity and total phosphatase activity; PK was set 1, and PP1 and PP2 were equieffective (C: PP1 = PP2 = 0.25, D: PP1 = PP2 = 10). The fraction attributed to the more sensitive PP1 is seriously underestimated, especially in Figure 1D, where baseline PrP level is low. Thus, in cases where two phosphatases act in an intact cellular system, their relative contribution cannot be easily estimated from inhibitor experiments. These aspects can account in part for the fact that functional inhibition constants are much higher than expected based on in vitro data (Table 6).

Different phosphatases can operate sequentially under physiological conditions, e.g., PP2B dephosphorylates inhibitor 1 of PP1 (see sects. II A2 and II B3). To check whether a phosphatase acts at the channel itself, and not somewhere upstream a signaling pathway, measurements of channels in bilayers or inside-out patches are often performed. However, it has to be taken into consideration that endogenous enzymes may coexist (and even copurify) with the channels, as known not only for protein kinases (see Ref. 89), but also for PP2B (371). Examples suggesting close association between phosphatases and channels are given below. Another point of interest is that biochemical data (as in Table 6) usually relate to skeletal muscle phosphatase preparations, with model phosphoprotein substrates such as phosphorylase a (see above). It is not clear whether these inhibition constants depend on the substrate, although this problem refers more likely to the high-molecular-weight protein inhibitors with (allo) steric mechanisms of inhibition, rather than active-site inhibitors such as the microcystins or OA (see above). Tissue or species variations of inhibition constants for a given catalytic subunit have only scarcely been addressed but can profoundly hamper interpretation of data (186). It is therefore recommended, at least for results that are hard to interpret, to check the biochemical activity of a phosphatase inhibitor by purifying and using the same source of enzyme as present in the functional experiments. Finally, the effects of phosphatase inhibitors and interpretation of their concentration-response curve critically depend on proper preparation and storage of stock solution or choice of solvent (16). Rather than relying on manufacturers information, solutions should be checked.
using a well-defined and robust assay, e.g., an isolated phosphatase enzyme assay.

V. EFFECTS OF PHOSPHATASES ON ION CHANNEL ELECTROPHYSIOLOGY

A. Voltage-Dependent Ca\textsuperscript{2+} Channels

1. L-type Ca\textsuperscript{2+} channels

Similar to the paradigm of cAMP-dependent phosphorylation, cardiac L-type channels served as a classical model system to study regulation of voltage-dependent ion channels by protein phosphatases and their inhibitors. PKA and PP2A reduced the isoproterenol-stimulated whole cell current in guinea pig myocytes (189). In rat heart cells (109), PP2A, but not PKA, reduced the current also under basal conditions. Inhibition of PKA and OA at micromolar concentrations elevated baseline current density and potentiated the stimulatory response to isoproterenol in the guinea pig (189, 190). At the single-channel level, OA (5 \textmu M) slowed channel rundown after patch excision (339). Wang et al. (435) measured cardiac channels in lipid bilayers. Here, OA (0.1 \textmu M) dramatically increased channel activity, suggesting that endogenous phosphatases are still associated with the channels after reconstitution in the bilayer system. Neumann and co-workers (326, 327, 330) published a series of papers on the phosphatase inhibitors OA, CyA, and CA, respectively. All compounds increased single-channel activity and enhanced the phosphorylation of other, regulatory proteins like phospholamban, troponin inhibitor, and myosin light chains in myocytes. Contractile force of multicellular preparations was also increased. Notably, the concentrations eliciting these functional effects were consistently higher than those required for inhibition of cardiac phosphatases in vitro. Incomplete cellular penetration and the more complex situation of an intact phosphorylation-dephosphorylation system (see sect. IV) may account for this discrepancy. In expression systems using cardiac \( \alpha_{1C} \)-subunits, several investigators failed to detect significant PKA-mediated increase of the Ca\textsuperscript{2+} current (344, 383, 470). On the other hand, PKA reduced the current (383) in the \textit{Xenopus} model. Forskolin as well as OA elevated the currents when previously inhibited by the PKA inhibitor H-89 (344). Furthermore, prepulse-induced facilitation could be enhanced by PKA and by OA in some (51, 372, 373) but not all studies (236). Gao et al. (152) were the first to report robust cAMP-dependent modulation in an expression system. Their data suggest that for appropriate PKA-dependent stimulation and phosphorylation of \( \alpha_{1C} \)-subunits, the protein kinase has to be anchored in the membrane by proteins like AKAP 79, both in the expression system and in native cardiac cells. The results, including site-directed mutagenesis of Ser-1928 to abolish cAMP-dependent regulation, await confirmation by other groups. There is still no firm evidence that the cardiac \( \alpha_{1C} \)-subunit itself serves as the substrate for regulatory phosphorylation and dephosphorylation, although this has been shown directly for neuronal and skeletal muscle L-type channels (175, 461).

Although L-type channels from heart are consistently stimulated by inhibitors of PP1 and PP2A (see above and Refs. 173, 253), the situation can be more complex. In intestinal smooth muscle cells, CyA was reported to stimulate (420) or depress (436) Ca\textsuperscript{2+} currents, and the direction of the effects of OA differed between intracellular dialysis and bath application (270). Later, Obara and Yabu (335) noted a biphasic response of whole cell currents to increasing concentrations of both OA and CyA. They concluded that PP2A inhibition reduces and PP1 inhibition stimulates the channels in these cells. In vascular smooth muscle cells, OA antagonized the current reduction exerted by PKC inhibitors (336). When studied at the single-channel level, preferential inhibition of PP1 using tautomycin (1–100 nM) led to a reduction in the channel availability, i.e., the probability that a voltage step will elicit channel openings and lead to an active sweep (163). On the other hand, preferential inhibition of PP2A (by 1 \mu M OA) increased open probability within active sweeps by promoting long openings (mode 2), and PP2A reduced open probability. Although these studies differ in the assignment regarding phosphatase subtype to function, they show that qualitatively different effects are mediated by PP2A and PP1 in smooth muscle L-type channels. This could indicate the presence of distinct regulatory phosphorylation sites with differential sensitivity toward phosphatases, as shown biochemically for skeletal muscle Ca\textsuperscript{2+} channels (267). In rabbit heart, Ono and Fozzard (340) noted two types of effects of OA at the single L-type channel level. Here, both availability and open probability (open times) were raised as expected (see above), but in different concentration ranges of OA (availability being more sensitive, suggesting PP2A to be involved). Allen and Chapman (8) used BDM to assess the effect of dephosphorylation on single cardiac L-type channels. Open probability was reduced by the proposed “chemical phosphatase,” mainly due to lengthening of long closures. In addition, availability was reduced, and kinetic analysis of the slow gating process revealed that the oxime reduced the lifetime of the available state (as expected for increased dephosphorylation), but also increased the lifetime of the unavailable state. The use of BDM as a tool is complicated, however, because of nonspecific inhibitory effects unrelated to channel phosphorylation (9). Wiechen et al. (443) compared the effects of OA, its inactive derivative norokadaone, and CyA (1 \mu M each) on single
channels in guinea pig cardiomyocytes. Availability was stimulated by OA and by CyA, and a more complex analysis of slow gating was compatible with a selective drug effect on the dephosphorylation rate. Okadaic acid, but not CyA, increased open probability, mode 2 gating, and prepulse-facilitated mode 2. They concluded that in these cells, like in vascular smooth muscle, PP2A controls open probability and modal gating, whereas PP1 governs availability (see also Ref. 187). In summary, several lines of evidence indicate differential modulation of L-type channels by PP1 and PP2A, although the exact single-channel mechanism may differ between species, tissues, and channel isoforms.

Relatively little is known about the effects of PP2B on cardiac L-type channels, although this phosphatase is abundant in heart and capable of dephosphorylating various sarcolemmal substrates (301). Inhibition of PP2B by peptide inhibitors (140) or by cyclosporin (186) had little or no stimulatory effect, respectively. One has to consider, however, that whole cell studies are inherently associated with some level of intracellular Ca$$^{2+}$$ buffering, which may affect basal PP2B activity. Indeed, a liposomal preparation of PP2B was reported to affect action potentials in embryonic chick ventricle (417), but this effect has not yet been confirmed at the Ca$$^{2+}$$ current level. In GH$$^3$$ cells, neuronal L-type channel inactivation was unaffected by cyclosporin and FK-506 (424), thus not supporting the “calcineurin hypothesis” (50) originally raised for neuronal Ca$$^{2+}$$ channels (see also Ref. 144). In smooth muscle cells, however, both PP2B and cyclosporin strongly affect channel activity, and PP2B seems to be one mediator of the inhibitory action of Ca$$^{2+}$$ on these channels by reducing open probability as well as availability (370).

L-type Ca$$^{2+}$$ current in pinealocytes, carried by the $\alpha_{1D}$-tore subunit, is decreased by various protein phosphatase inhibitors (66). Conversely, secretory function of pancreatic $\beta$-cells can be enhanced by OA (11), but the moderate increase found for L-type currents can only partially explain this effect, possibly more at threshold potentials for current activation (171). Okadaic acid may even decrease Ca$$^{2+}$$ current (365) and insulin secretion in isolated islets (365) or RINm5F cells (12). Interestingly, activation of PP2B can mediate the inhibitory effects of somatostatin, galanin, and an $\alpha_2$-agonist on insulin secretion. This effect is independent of changes of the Ca$$^{2+}$$ current and can be blocked by deltamethrin and a PP2B inhibitory peptide, but not by OA.

Modulation of protein phosphatase activity as a physiological mechanism of Ca$$^{2+}$$ channel regulation has been addressed in a number of studies. Herzig et al. (187) have analyzed by a discrete-time Markov analysis the slow gating process governing availability. The prolongation by isoproterenol (337) of the lifetime of the available state, thought to represent phosphorylated channels, can be prevented by OA, supporting the concept that PKA stimulation can indirectly inhibit PP1 by phosphorylation of inhibitor 1 (329). A very similar model has been proposed for the regulation of neuronal L-type, but also N- and P-type, channels by $D_1$ receptor-induced PKA activation with subsequent phosphorylation of DARPP-32 and inhibition of PP1 (397). Muscarinic receptor-mediated reduction of PKA-stimulated cardiac L-type currents, in addition to the well-known cAMP-dependent mechanism (188), may partly be due to phosphatase stimulation (186) in guinea pig ventricular myocytes, but not in frog heart cells (233). The proposed mechanism resembles the signal transduction cascade (G$$^\text{G_0}$-induced PKA stimulation) described below for some K$$^+$ channels (258, 442), but the details of possible intermediate steps are unknown. Insoluble hexakisphosphate and related mediators have been proposed to serve as second messengers to increase Ca$$^{2+}$$ currents and insulin secretion via protein phosphatase inhibition (271). Finally, the peptide chromastatin lowers Ca$$^{2+}$$ entry, presumably through L-type channels, into chromaffin cells via PP2A stimulation (151), and an $\alpha_2$ adenosine receptor agonists exerts a similar effect via phosphatase stimulation (305). There may also be developmental changes of protein phosphatases, e.g., in the heart, where a decreased sensitivity of the adult versus newborn heart cells to various phosphatase inhibitors was found (157, 289).

2. Non-L-type Ca$$^{2+}$$ channels

In central and peripheral neurons, whole cell Ca$$^{2+}$$ current represents a mixture of low- and high-voltage-activated currents. The latter can be dissected pharmacologically into L type (dihydropyridine sensitive, encoded by $\alpha_{1C}$- or $\alpha_{1V}$-subunits), N type ($\omega$-conotoxin GVIA sensitive, encoded by $\alpha_{1N}$-subunits), P or Q type (sensitive to $\omega$-agatoxin IVA or $\omega$-conotoxin MVIIIC, respectively, encoded by $\alpha_{1A}$-subunits), and R type (toxin resistant, probably encoded by $\alpha_{1E}$-subunits). With the notable exception of two studies in sympathetic neurons (42, 440), addition of phosphatase inhibitors led to an increase in neuronal Ca$$^{2+}$$ channel amplitude and/or a reduced extent of inactivation. Dolphin (106) demonstrated that cAMP-dependent stimulation of currents in rat dorsal root ganglia is mimicked by an active fragment of inhibitor 1 of PP1, suggesting that endogenous PP1 dephosphorylates a regulatory PKA site. Unfortunately, many studies employed OA in concentrations too high to discriminate between PP1 and PP2A inhibition. This holds true for snail neurons, where a PKC-mediated stimulation of currents by serotonin was amplified by 500 nM OA (191), and, in another study, the rate of inactivation was reduced by dialysis with either 50 $\mu$M OA or a peptide inhibitor of CaM kinase II (451). Both kinases were apparently not
involved in the action of the muscarinic agonist carbachol (156), as intracellular injection of OA and of microcystin-LR, but not bath application of 50 μM ciclosporin, mimicked and occluded the stimulation exerted by carbachol. In a mammalian neuronal cell line, OA (100 nM) prevented part of the inhibitory action of a dopamine D2 agonist on N-type currents (45). This part (the late or sustained component) of inhibition was not voltage dependent and likely reflects regulation of a PKA site, whereas the okadaic acid-insensitive part (on the rate of activation) represents voltage-dependent regulation by direct G protein interaction of the channels. Synaptosomes from rat hippocampus were studied with fura 2 Ca2+ imaging (23). Here, preincubation with 100 nM OA markedly potentiated the stimulation by a PKC activator, suggesting a strong basal PP1 and/or PP2A activity in this system. In cultured rat cortical neurons, Thomas et al. (411) demonstrated that postsynaptic excitatory currents are increased in amplitude by 1 μM CyA (in the bath) or microcystin (10 μM in the pipette). This effect was not mimicked by PP2B inhibitors (10 μM ciclosporin or 2 μM FK-506). These compounds had been shown earlier by the same group (425) to increase the frequency but not amplitude of postsynaptic currents, which seems to indicate a presynaptic mechanism involving PP2B.

There is strong evidence for a role of PP2B in the control of neuronal Ca2+ channels. Immunoreactivity of this phosphatase is colocalized with Ca2+ channel β-subunits in dorsal root ganglia (291). PP2B interferes with G protein inhibition of N-type channels in sympathetic neurons (467), where the response to α2-adrenergic or somatostatin receptor stimulation is inhibited by an autoinhibitory PP2B fragment or by ciclosporin, but not by OA. High-voltage-activated currents in lactotrophs from rat pituitary were stimulated by PKC activation, and ciclosporin mimicked this effect in the absence of a PKC inhibitor (137). In rat cortical synaptosomes, ciclosporin and FK-506 increased Ca2+ influx and glutamate release (374), in line with the electrophysiological results of Victor et al. (425). In the NG 108–15 cell line, overexpression of PP2B reduced N-type currents, and this reduction could be overcome by FK-506 (292). Calcium-dependent dephosphorylation of neuronal channels may also be involved in several other studies using OA (59, 256, 315, 466), where channel stimulation was mimicked by calmodulin antagonists or impeded by Ca2+, respectively. Because the OA concentrations used here were in the micromolar range, it is impossible to tell whether a PP2B has been nonselectively inhibited here by the toxin or whether PP1 was involved indirectly (e.g., through PP2B-mediated dephosphorylation of endogenous PP1 inhibitors).

In summary, both PP1/2A and PP2B mechanisms control neuronal high-voltage-activated Ca2+ currents. In most cases, current is raised directly or made resistant against inhibitory modulation by inhibition of these phosphatases. The work reviewed, however, does not sufficiently address the exact molecular substrate for phosphorylation. Given the prominent role for N-type and P/Q-type channels in neurotransmitter release, it is important to further investigate this question in reductionistic systems (single-channel recording, reconstituted channels, and expression systems allowing for site-directed mutagenesis and biochemical measurements).

B. Voltage-Dependent Na+ Channels

Voltage-dependent Na+ channels are phosphorylated by PKA and PKC. The functional consequences seem to be different among the respective channel subtypes from brain, heart, and skeletal muscle (26, 347, 385). The role of protein phosphatases has been studied in more detail for rat brain Na+ channels. Here, PP2B and PP2A are active, but with different selectivities for the various serine residues phosphorylated by PKA (321). These results are in line with the presence of five different soluble Na+ channel dephosphorylating enzymes in rat brain, which have been pharmacologically and immunologically identified as either 2A-like or 2B-like (62). There may be an exception in striatal neurons, where Na+ currents are reduced by the PP1-specific inhibitor phosphorylated DARPP-32 (366). Interestingly, a convergence of PKC- and PKA-mediated phosphorylation (280) of Na+ channels could be due to an inhibition of dephosphorylation of PKA sites by PP2A or PP2B in the presence of PKC (254).

C. K+ Channels

1. Inward rectifier K+ channels

Inward rectifier channels may be activated or inhibited by phosphorylation, depending on the particular type and system under study. In nucleus basalis neurons, substance P leads to a PKC-mediated suppression of currents, an effect which can be potentiated and rendered irreversible by 100 nM OA (402). This suggests a dephosphorylation of the PKC site by PP2A or PP1. In the pituitary GH3 cell line, thyrotropin inhibits inward rectifiers by a Ca2+-dependent mechanism, and this effect can be reverted by the catalytic subunit of PP2A, but not of PP1 (21). The cardiac ventricular inward rectifier current is inhibited by stimulation of β-adrenergic stimulation, an effect mimicked by forskolin, cAMP derivatives, or the catalytic subunit of PKA (257). This inhibition can be reversed by muscarinic stimulation using acetylcholine. The muscarinic effect can be abolished by 1 μM OA, both at the whole cell level and in the single-channel configuration (258). Importantly,
the effect of the phosphatase inhibitor was still obtained when channels were studied in the inside-out configuration [with guanosine 5’-O-(3-thiotriphosphate) or G, used to antagonize PKA-induced inhibition]. This suggests a pathway leading from muscarinic receptors via pertussis toxin-sensitive G proteins to a protein phosphatase that is closely associated with the channel and dephosphorylates a PKA site. Unfortunately, the concentration of OA (1 µM) does not allow one to conclude whether PP2A or PP1 is involved, but the authors checked the phosphatase specificity of their approach using the inactive derivative norokadaone.

β-Adrenergic regulation is very different for the cardiac acetylcholine-gated K+ channel, which also shows some extent of inward rectification. Here, the directly G protein-mediated activation due to muscarinic receptors is inhibited by β-adrenergic stimulation or PKA (239), suggesting that the phosphorylated channel has a lower open-state probability. Alkaline phosphatase reverts this effect. Dephosphorylation also seems to account for spontaneous desensitization of the activated native (241) and recombinant (GIRK1/GIRK4 heteromultimeric) channels (244). The endogenously present phosphatase seems to be located in the cytosol (241), requires Ca2+, and is inhibited by 3 mM orthovanadate, but not by OA (241). This is somewhat puzzling because PP2B would be a good candidate for Ca2+-dependent dephosphorylation, but this enzyme is relatively insensitive to orthovanadate (375). In guard cells from plants, a PP2B is likely responsible for dephosphorylation and inhibition of inward rectifiers (290). In contrast, dephosphorylation (of a site phosphorylated by unknown kinases) and inhibition of inward rectifier currents in guinea pig chromaffin cells is probably catalyzed by PP2A or PP1, because the effect is blocked by micromolar CyA or OA concentrations (223, 224). Rundown of recombinant Kir 2.1 channels is also prevented by inhibition of PP2A and PP1 by microcystin, but this effect is linked to a PKA site in the Xenopus expression system used (361). In view of the functional and structural diversity of inward rectifiers, it is not astonishing that a common pattern of phosphorylation- or dephosphorylation-linked events does not emerge from the literature.

2. Transient outward K+ channels

Only a few studies have addressed the role of dephosphorylation with respect to transient outward currents. Recombinant neuronal channels have been postulated (196) to be dephosphorylated by PP2B, which resulted in reduction of currents. As shown in a later study, the rate of inactivation was markedly accelerated by PP2B and slowed by calmodulin kinase II (358). In rat heart, BDM was used to infer a role of phosphatases in the control of cardiac transient outward currents. Indeed, the BDM-induced reduction of current was reversed by cAMP-dependent stimulation (448), but a nonspecific effect of BDM was not excluded, and the subtype of the putative endogenous phosphatase is not known. FK-506 reduced I_K in rat cardiomyocytes, but this effect appears unrelated to PP2B inhibition (108). In pituitary GH3 cells, a subpopulation of slowly inactivating channels was characterized at the single-channel level, which was depressed by a membrane-permeant cAMP derivative. Recovery was observed after somatostatin (72), which stimulates Ca2+-dependent K+ channels in these cells via PP2A activation (442), but it remains to be elucidated whether this mechanism is also involved in the control of transient inward currents.

3. Delayed rectifier K+ channels

In the classical studies on cardiac Ca2+ currents, Hescheler and co-workers (189, 190) also noted some role of phosphatases on the cardiac delayed rectifier current, i_K. Although OA (5 µM, bath applied) amplified the increase of this current after isoproterenol (190), cell dialysis with PP1 by itself did not affect the current but prevented isoproterenol-induced stimulation (189). In frog cardiac myocytes, intracellular dialysis with micromolar OA or microcystin concentrations reduced a slowly activating delayed rectifier current (140), an effect opposite to that of isoproterenol and similar to that of ATP depletion. The authors concluded that delayed rectifier currents are regulated by at least two phosphorylation sites, one which has to be phosphorylated for channel activity, and another that mediates inhibition by phosphatase inhibitors. The apparent discrepancy between the studies may be due to species differences or to the heterogeneity of i_K (362). Unfortunately, none of these experiments reveals which phosphatase subtype mediates channel dephosphorylation under physiological conditions. Duchatelle-Gourdon et al. (110) suggested a role of a Mg2+-stimulated (i.e., PP2C) phosphatase to revert catecholamine stimulation of cardiac delayed rectifiers, but direct biochemical or pharmacological evidence was not given. Regrettably, no specific tools are available to inhibit PP2C.

In an attempt to characterize phosphatase regulation of an expressed delayed rectifier potassium channel, Lopatin and Nichols (288) studied effects of BDM on Kv2.1 channels expressed in Xenopus oocytes. Unfortunately, they found reversible inhibition by the drug, regardless of the phosphorylation conditions, and had to conclude that direct block rather than chemical phosphatase activity was the most likely mechanism. Kang et al. (237) investigated in detail the mechanism of angiotensin II-induced increase in delayed rectifier current of rat hy-
porthalamic neurons. This effect was pertussis toxin sensitive and inhibited by nanomolar OA. Involvement of PP2A was further substantiated by the effect of antibodies directed against PP2A, which blocked the angiotensin II effect. This is yet another example of a signaling chain leading from receptors via PTX-sensitive G proteins to PP2A (212).

4. **ATP-dependent K⁺ channels**

In rabbit heart cells, ATP-dependent K⁺ currents are modulated by PKC phosphorylation (284, 285). This effect is stabilized by nanomolar OA and reverted or prevented by addition of PP2A to inside-out patches. This finding suggests that the PKC site involved is dephosphorylated by a PP2A located in close vicinity of the channel. The direction of PKC effects apparently depends on the ambient ATP concentration. At low ATP, where channels are basally active, PKC inhibits the current, whereas at higher ATP, where channels usually are silent, PKC activates the current. This is consistent with a less steep ATP concentration dependence of phosphorylated channels (284). These data (at low ATP concentrations) are somewhat in contrast to those of Kwak et al. (263) obtained in rat cardiomyocytes. They demonstrated that 100 nM OA slowed spontaneous rundown of channel activity, whereas orthovanadate accelerated the decrease in current observed in inside-out patches in the absence of ATP. The opposite effects were exerted by PP2A and a tyrosine phosphatase, and exact nature of the serine/threonine kinase involved was not investigated. In the absence of PKC activity, a PKA site might thus be responsible for long-term stability of channel activity. Along similar lines, in smooth muscle cells of the gallbladder, glibenclamide-sensitive currents are enhanced by PKA activation, and this effect is stabilized by 5 μM OA (457). In this preparation, an increase of channel activity (under physiological conditions) is caused by PKA phosphorylation, and the dephosphorylation may be catalyzed by PP2A or PP1. In renal tubule cells, the ATP-dependent K⁺ currents run down after excision of the patches in low-ATP solution. Rundown is blocked by Mg²⁺ withdrawal, orthovanadate, and 1 μM OA (260). Because PP2A, but not Ca²⁺, PP1, or PP2B reduced channel activity, the authors concluded that rundown is caused by a membrane-associated PP2A. Interestingly, McNicholas et al. (310), using the ROMK1 (195) channel expressed in *Xenopus* oocytes, reproduced the prevention of rundown by Mg²⁺ withdrawal and orthovanadate, but 1 μM OA or CyA was not equally effective in their hands. They concluded that rundown is mediated by a Mg²⁺-dependent dephosphorylation (by PP2C?!) of a PKA site. However, it seems conceivable that the differences between their results and those of Kubokawa et al. (260) are due to lack of a native channel-associated 2A phosphatase in the expression system. Alternatively, the rundown was so rapid (>50% in 30 s) that the time of exposure to the organic phosphatase inhibitor may have been insufficient for steady-state effects. The interpretation of the orthovanadate effects is certainly even more difficult, due to the indiscriminate character of its effects (for instance, inhibition of protein kinases, Na⁺-K⁺-ATPases, or tyrosine phosphatases; Ref. 263).

5. **Ca²⁺-activated K⁺ channels**

There are a considerable number of studies on dephosphorylation of Ca²⁺-activated K⁺ channels, probably due to their vast abundance and important physiological role for regulation of the activity of neuronal, endocrine, and smooth muscle function. No unifying picture can be drawn based on this information. This is not so much related to the gross structural heterogeneity of these channels, classically signified by their conductance (small-conductance SKCa channels, intermediate-conductance IKCa channels, and large-conductance, “maxi” or “big” BKCa channels). Rather, the latter group of BKCa channels has been examined in the majority of studies, with results differing between tissues or cell types, protein kinases, and phosphatase subtypes. This may of course be related to the respective regulatory scenario of the cell and/or the particular channel isoform (one of the many possible splice variants), but this idea has yet to be confirmed at the level of an in vitro expression system. Up to now, most experiments have been done with native channels, and kinase regulation is not easily reconstituted in channel expression systems (464).

As a starting point, the study of Reinhart et al. (352) is worthwhile of detailed consideration. They found that BKCa channels prepared from rat brain and reconstituted in lipid bilayers showed two different biophysical and regulatory phenotypes: so-called type 1 channels (fast gating, high sensitivity to charybdotoxin, activated by low Ca²⁺) are stimulated by PKA in the majority of cases, and this effect was reversed by PP2A, but not by PP1. Type 2 channels (slower open and closed times, less sensitive to charybdotoxin and Ca²⁺) are downregulated by PKA, an effect also specifically reverted by PP2A. The same group (73) showed that these type 2 channels can also be stimulated by phosphorylation, mediated by an endogenous kinase present in the bilayer system, because Mg²⁺ plus ATP and adenosine 5’-O-(3-thiotriphosphate) were sufficient to cause stimulation. This ATP effect could be reversed by PP1 (10–40 nM), but not PP2A (60 nM), also suggesting a different site involved compared with the site that mediates suppression of channel activity. In another study, exogenous PKC could mimic and triphosphorylated inhibitor 1 of PP1 could stabilize this type of upregulation (353). The conclusions from these systematic ex-
Peripherals are manifold. 1) Within the same experimental system, different channel subtypes reveal qualitatively distinct responses (stimulation vs. inhibition of type 1 vs. type 2 channels by PKA). 2) The same type (here, the so-called type 2 BK\textsubscript{c}\textsuperscript{a} channels) is bidirectionally modulated by PKA and PKC, and 3) the backward reactions are also catalyzed in a phosphatase subtype-specific manner (PP2A vs. PP1). Finally, both kinases and phosphatases must exist in close association with the channel molecule, because their influences are still present after channel reconstitution in the bilayer. Based on kinetic studies of BK\textsubscript{c}\textsuperscript{a} channels in excised patches from the pituitary, Bielefeldt and Jackson (36) argued that a kinase (involved here in maintaining channel activity) is in very close association with the channel ("intramolecular model"), more so than the corresponding phosphatase (diffusion-limited "intemolecular model"). It is therefore tempting to speculate that slow, spontaneous oscillations in the open probability of BK\textsubscript{c}\textsuperscript{a} channels, already described by Reinhart et al. (352), and termed "Wanderlust" kinetics by Silberberg et al. (382) may reflect phosphorylation-dephosphorylation reactions catalyzed by endogenous, channel-associated enzymes.

In the papers mentioned subsequently, dephosphorylation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (or associated regulatory proteins) leads to an increase in activity. In the rat pituitary tumor cell line GH\textsubscript{1}C\textsubscript{1}, Armstrong's group (441) showed that atrial natriuretic peptide stimulates BK\textsubscript{c}\textsuperscript{a} channels through an elevation of cGMP, stimulation of cGMP-dependent protein kinase (PKG), and activation of a phosphatase sensitive to 10 nM OA (PP2A). This final mechanism resembles, but the initial cascade differs from the effect of somatostatin in the same cells (442). Somatostatin does not induce a rise in cGMP, and it requires a pertussis toxin-sensitive G protein, but ultimately also leads to a channel stimulation inhibited by OA. Later studies provided evidence that the signaling cascade initiated by the activation of somatostatin receptor leads to accumulation of lipoxygenase metabolites of arachidonic acid (111). A biochemical link between eicosanoids and the activation of PP2A (159) has already been mentioned, and stimulation of this phosphatase may be of broader physiological importance. Zhou et al. (463) demonstrated that BK\textsubscript{c}\textsuperscript{a} channels from bovine tracheal smooth muscle are stimulated by PP2A in excised patches and that an indirect stimulation exerted by an active fragment of PKG is blocked by the appropriate concentrations of phosphatase inhibitors. Interestingly, a similar type of regulation was shown in the same paper for intermediate-conductance K\textsubscript{c}\textsuperscript{a} channels in Chinese hamster ovary cells expressing recombinant PKG Ia. In both cases, PP1 was ineffective at equimolar concentrations (350–700 nM), and PP2A was detected in the membranes by Western blot analysis. Activation of PKG, PP2A, and finally BK\textsubscript{c}\textsuperscript{a} channels was again proposed as the common pathway of cGMP-dependent regulation in these systems. Similar mechanisms may operate in some neurons. In hippocampal neurons, secreted \beta-amyloid precursor protein causes hyperpolarization via BK\textsubscript{c}\textsuperscript{a} channels (148). The authors proposed that cGMP, PKG, and a protein phosphatase (sensitive to 10 nM OA) are involved. BK\textsubscript{c}\textsuperscript{a} channels in a clonal pituitary cell line have similar sensitivity toward OA. Interestingly, PP2A regulation here seems to get targeted to the channel by glucocorticoid treatment (412). Holm et al. (197) investigated BK\textsubscript{c}\textsuperscript{a} channels at the whole cell level in mouse cortical neurons. Neurtrophin-3 and nerve growth factor activated the current, and this effect was blocked by 30 nM OA, which leaves some uncertainty about the phosphatase subtype involved. The nature of the upstream mechanisms also remained somewhat unclear but likely involved a tyrosine kinase and a phospholipase C. In summary, stimulation of BK\textsubscript{c}\textsuperscript{a} channels by dephosphorylation through PP2A seems to be a common mechanism to reduce cellular excitability, present in various tissues and initiated by a number of signaling cascades, most notably by cGMP and PKG.

On the other hand, protein dephosphorylation may also reduce BK\textsubscript{c}\textsuperscript{a} channel activity, as already mentioned above. A number of such examples come from studies in above smooth muscle cells. Archer et al. (14), using rat pulmonary artery, showed that nitric oxide increased whole cell BK\textsubscript{c}\textsuperscript{a} currents. Based on pharmacological evidence (inhibition by methylene blue, mimicking by Sp-guanosine 3',5'-cyclic monophosphothioate and by 2 \mu M OA), they proposed that this effect is mediated by phosphorylation of the channels. Along similar lines, Stockand and Sansom (389) and Sansom et al. (363) described a cGMP-dependent stimulation of BK\textsubscript{c}\textsuperscript{a} channels from human mesangial smooth muscle cells (see Ref. 390 for review). The effect was exerted by PKG and by cell-permeant cGMP derivatives. The activation was biphasic, and the second declining phase of activity was abolished by low concentrations of OA and cantharidin, but not by CyA. Conversely, PP2A but not PP1 inhibited channel activity in excised patches. These results are most easily explained by a PKG site that must be phosphorylated to support channel activity. This site also seems to be specifically dephosphorylated by PP2A. Note that arachidonic acid activates BK\textsubscript{c}\textsuperscript{a} channels in this system, but in a manner independent of phosphorylation (391). Phosphorylation and stimulation by PKA and cAMP derivatives have been addressed by Schubert et al. (369) in myocytes from rat tail artery. Astonishingly, the presence of 1 \mu M OA in the whole cell pipette did not modify the response to iloprost, a prostacyclin analog shown to act via PKA activation. It is feasible that longer incubation times or larger pipette diameters would have been required to achieve sufficient intracellular dialysis of the phosphatase inhibitor. In gastrointestinal smooth muscle cells, phosphatase inhibitors led to an increase in channel activity.
These effects were observed in excised patches (47), where PKA also had stimulatory effects. The phosphatase subtype could not be identified unequivocally, since CyA appeared to have a lower potency and/or efficacy than OA. In neuroendocrine and neuronal cells, there are some examples (36, 228, 274) of BK$_{ca}$ channel stimulation by phosphorylation and inhibition by phosphatases, but in none of these studies have the respective enzyme isoforms been precisely identified. FK-506 increases single-channel activity in cultured hippocampal neurons, but again (see sect. vC2), this effect is unrelated to PP2B inhibition (409). In collecting duct cells of the rat nephron (194), both small- and intermediate-conductance K$_{ca}$ channels are activated by phosphorylation, evidently via PKG. The phosphatase inhibitors CyA (10 nM) and OA (1 μM) were tested and effective at concentrations that do not firmly discriminate between PP2A and PP1.

In summary, native Ca$^{2+}$-activated K$^{+}$ channels, notably BK$_{ca}$ channels, display a variety of regulatory pathways, including phosphorylation by PKG and PKA and dephosphorylation by PP2A and PP1. These enzymes are, both functionally and anatomically, in tight association with the channels, and a number of physiologically important regulatory mechanisms converge at this target. However, it is desirable to address the diversity of the responses in various systems at the molecular level. In particular, information about the particular sites of phosphorylation would be required from future biochemical studies or from electrophysiological studies after site-directed mutagenesis of the cloned channels.

D. Ligand-Gated Cation Channels

1. N-methyl-D-aspartate receptor channels

Glutamate receptors of the NMDA type are nonselective cation channels critical for neuronal excitability and particularly for Ca$^{2+}$-dependent modulation of synaptic plasticity. They are regulated by phosphorylation and dephosphorylation. Evidence for a more direct interaction between protein phosphatases and these channels can be derived from single-channel studies. In cultured hippocampal neurons, both exogenous PP1 and PP2A depressed open probability, and CyA and OA exerted opposing effects (434). Calyculin A (20 nM) also increased the whole cell current, with effects on kinetics depending on the glycine concentrations. In the mouse nucleus accumbens, DARPP-32 is essential for the dopamine-induced phosphorylation of a NMDA receptor subunit (131). These findings hint that endogenous PP1 or PP2A regulate channel phosphorylation, activity, and kinetics. There is ample literature, on the other hand, on NMDA receptor regulation by PP2B. Lieberman and Mody (283) tested the effect of 10 μM OA on single-channel currents of neurons from dentate gyrus. The phosphatase inhibitor increased open times and open probability. These effects were mimicked by FK-506 but not by lower concentrations of OA. Stimulation of channels by phosphatase inhibition depended on Ca$^{2+}$ instead of Ba$^{2+}$ as the charge carrier. Calcineurin by itself had an inhibitory effect. At the whole cell level, inhibitors of both PP1/2A and PP2B prevented either long-term rundown (311) or short-term desensitization/inactivation (414) or had no effects at all on these phenomena (276, 359, 429). This leaves some uncertainty on the important question whether or under which conditions Ca$^{2+}$ entering through the channel are regulating the channel itself by activating Ca$^{2+}$-dependent phosphatases. This question may crucially depend on the type of neuron studied, since PP2B expression and PP2B inhibitor effects are both absent in hippocampal interneurons (381). Convincing data favoring the idea that PP2B mediates NMDA receptor desensitization were obtained by measurements of excitatory postsynaptic currents (EPSC) in hippocampal cell cultures. Here, desensitization of NMDA receptors was gauged by the change in EPSC amplitude after a train of repetitive agonist applications in the absence or presence of an NMDA receptor blocker. Strong Ca$^{2+}$ chelation as well as various PP2B inhibitors, but not CyA, completely abolished desensitization (415). The kinase involved in this system is a tonically active PKA, which can be modulated by β-adrenoceptor activation (349), providing a physiological pathway between noradrenergic input and synaptic strength at a glutamatergic synapse. Phosphatases may also participate in physiological long-term changes in NMDA receptor function such as long-term depression (447) or long-term potentiation (37), but the signaling pathway as well as the phosphatase subtype involved are not known. In summary, for a better understanding of the multitude of functional data, it would be important to define the susceptibility of the phosphorylation sites of the cloned NMDA receptor subunits toward protein phosphatases.

2. Other nonselective cation channels

As described for NMDA and AMPA (446) receptors, various other ligand-gated cation currents are stimulated by phosphatase inhibitors. Examples include 5-HT$_{3}$ receptors (38), capsaicin receptors (102), and P$_{2x}$ purine receptors (246), all of which apparently desensitize (i.e., current decreased in the continuous presence of agonist) via PP2B activity. In contrast, P$_{1}$ purine receptors are less active in the presence of PP1/2A or PP2B inhibitors (345). Okadaic acid induced phosphorylation of the recombinant nicotinic acetylcholine receptor δ-subunit, but the functional consequences have not yet been studied (255). Cyclic nucleotide-gated nonselective cation channels may be regulated by protein phosphatases, as demon-
regulated for the cGMP-gated channel in the retina (160). Here, an endogenous phosphatase seems to sensitize the channel toward cGMP, an effect mimicked by exogenous PP1 but counteracted by PP2A.

The hyperpolarization-activated nonselective cation current \( i_\text{fc} \) in heart tissues is regulated by (de)phosphorylation, as shown by an increase in current amplitude after application of CyA. In sinus node cells, this effect can be clearly discriminated from the well-known direct regulation by cAMP: here, the CyA response was not accompanied by a shift in the activation curve along the voltage axis (1), although such a shift was reported earlier (455) for ventricular myocytes. A hyperpolarization-induced cation current in peripheral neurons, which is also regulated by cAMP, does not respond to conditions altering protein kinase or phosphatase activity (221).

Other nonselective cation currents have been reported to be stimulated by OA (43, 403), by microcystin-LR (445), or by low Mg\(^{2+}\) and vanadate (222, 223), suggesting regulation by PP1/2A or, perhaps, PP2C, respectively.

In summary, phosphorylation sites coupled to increased channel activity and agonist sensitivity apparently exist in a vast number of nonselective cation channels. Physiological regulation pathways involving the phosphatases encountered here remain to be resolved, except for the obvious role of Ca\(^{2+}\)-dependent PP2B activity in some cases.

E. Anion Channels

1. Cystic fibrosis transmembrane conductance regulator channels

The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes a cAMP-dependent Cl\(^-\) channel present in heart, epithelia, and other tissues. Its regulation has been recently reviewed in detail (150). The channel is activated by PKA and phosphorylated on at least nine different consensus sites (142, 149). However, other kinases like type II PKG (142) and certain PKC isoforms (30) have also been shown to phosphorylate and activate the channel. PP2A, but not PP1 or PP2B, inactivated and dephosphorylated the channel (30). Exogenous PP2A and PP2C exerted different kinetic effects on single CFTR channels expressed in baby hamster kidney cells (293). Even at nanomolar concentrations (350), OA accelerated activation (323) and slowed down or prevented inactivation when conditions were chosen to minimize de novo phosphorylation (216, 348). PP2B inhibitors increased CFTR currents when stably expressed in fibroblasts, but not the currents endogenous to two epithelial cell lines (135), suggesting that this mechanism has no physiological significance.

A role of another phosphatase has early been postulated by Hwang et al. (216); in the presence of high OA concentrations, only part of the cAMP-dependent current was preserved. A proposed candidate would be alkaline phosphatase, since blockers like levamisole, bromotetramisole (but see Ref. 269), or methylxanthines can activate CFTR currents (27–29, 219). Notably, an antibody against alkaline phosphatase increased channel activity (27), and the enzyme as well as its inhibitors affected \(^{32}\)P incorporation into CFTR protein (28). However, it has been argued that the effect of alkaline phosphatase was nonspecific, because channel activity was restored upon application of fresh ATP solution (30), and this enzyme should not have access to the phosphorylation sites in intact cells (150).

Genistein, a known inhibitor of tyrosine kinases, stimulated CFTR currents (65, 141, 351, 452), and it increases the sensitivity of the channel toward PKA-dependent activation (204). The mechanism involved in these effects is controversial. Reduced tyrosine phosphorylation would be expected to increase PP2A activity (55). Indeed, genistein has been proposed to inhibit a phosphatase distinct from PP2A (65, 351), possibly PP2C (452). However, the results of French et al. (141) and Wang et al. (433) argue against a dephosphorylation mechanism (150). Importantly, the pattern of activation by CyA and genistein differs at the single-channel level (452), and the two compounds act in an additive manner (351).

The report of Zhou et al. (462) demonstrates stimulation of cardiac CFTR currents by the Cl\(^-\) channel blocker anthracene-9-carboxylate via phosphatase inhibition. Their data suggest that yet another unknown phosphatase, pharmacologically distinct from PP2A, PP2C, or alkaline phosphatase, might be of some physiological importance for CFTR regulation. Of note, in addition to appropriate substrate specificity, intimate colocalization of phosphatases with their substrate may be an important determinant of physiological function (e.g., Refs. 27, 134).

2. GABA\(_A\) receptor Cl\(^-\) channels

GABA\(_A\) channel currents are downregulated by PKA, and an endogenous PKA phosphorylates these channels (406). It is likely, however, that another protein kinase (e.g., tyrosine kinase, Ref. 211) is responsible for phosphorylation of a site associated with increased channel activity. Several groups have shown that Ca\(^{2+}\), entering through activation of NMDA channels diminishes GABA\(_A\) currents. A likely mechanism is Ca\(^{2+}\)-dependent activation of PP2B, since the pathway was blocked by nanomolar fenvalerate (211), cypemethrin (388), or delthametin (10, 355), by calcineurin inhibitory peptide (60), and by Ca\(^{2+}\) buffering with BAPTA (60, 317). The latter authors also found an effect of micromolar microcystin-LR or OA concentrations but could not exclude indirect mecha-
nisms (317). An additional pathway of such inhibitory cross-talk between NMDA receptors and GABA_A channels may be the nitric oxide system (355).

3. Other Cl⁻ channels

Okadaic acid has been shown to activate a variety of Cl⁻ channels, including a 50-pS channel from shark rectal gland epithelia (265), a Ca²⁺-activated Cl⁻ channel from osteoclasts (147), and a PKA-activated channel from Necturus gallbladder epithelia (132, 133). In the latter case, low concentrations of OA were sufficient, and PP2A, but not PP1, had an opposite effect on channel activity after reconstitution in bilayers (133).

On the contrary, Cl⁻ conductance in skeletal muscle was slightly diminished by 0.5 μM OA, and a stimulatory response to insulin-like growth factor I was blocked by the phosphatase inhibitor (90) and mimicked by a ceramide (91), suggesting a role of PP2A (see sect. mC2).

In a renal cell line, the open probability and, remarkably, the single-channel conductance of an outwardly rectifying Cl⁻ channel were increased by insulin. These effects were mimicked by PP2B and by a tyrosine phosphatase, and they were blocked by cyclosporin, but not by OA. The authors (304) propose that insulin action in this case should be mediated by calmodulin-dependent activation of PP2B, which in turn would be able to dephosphorylate a regulatory tyrosine residue.

An outwardly rectifying Cl⁻ channel from a human intestinal cell line is stimulated by calmodulin-dependent protein kinase II. This activation is spontaneously reverted by OA and microcystin-sensitive phosphatase. Interestingly, inositol 3,4,5,6-tetrakisphosphate exerted inhibitory effects, which depended on uninhibited phosphatase activity, but were not mediated directly by phosphatases, suggesting a complex regulatory scheme (450).

A voltage-dependent Cl⁻ channel present in the same cells was increased in activity by low osmolarity. This regulation was abolished by OA (1 μM) or CyA (50 nM) (143). Hypotonicity induced a Cl⁻ current in chick cardiac myocytes under whole cell but not perforated-patch recording conditions (172). It became insensitive to osmotic challenge even under whole cell dialysis when intracellular cAMP was raised or endogenous phosphatases were blocked by 100 nM OA. Okadaic acid and 20 nM CyA also rendered expressed CLC-3 channels insensitive to hypotonicity (107). When a critical serine residue (position 51) was mutated to alanine, the channel was tonically active, not further stimulated by hypotonicity, and no longer inhibited by PKC stimulation (107). These results indicate a direct relationship between volume regulation by this Cl⁻ channel and its (de)phosphorylation.

VI. SUMMARY AND PERSPECTIVES

As exemplified in the preceding sections, ion channel regulation by phosphatases takes place through numerous more or less complex pathways, as sketched in Figure 2.

Some limitations of studies leading to this picture are obvious. If, for instance, 10 μM OA is applied in intact cells and an altered current through an ion channel is noted, this does not really prove direct modulation of the channel protein by phosphatase type 1 or 2. First, other phosphatases are also blocked by OA (see Table 6), and these might thus be involved (see Fig. 1). Moreover, it is conceivable that a protein kinase is stimulated, a kinase that is activated by phosphorylation, thus the effect of a change of kinase activity on channel function is actually measured. Thus a naive direct correlation of phosphatase inhibition and channel function by channel phosphorylation might lead to wrong conclusions. This indicates that an integrated approach based on biochemical, biophysical, and physiological methods will be important for progress in the field.

Clearly, first all channels mentioned above as putatively regulated by phosphatases have to be identified unambiguously. As mentioned in section 1, the efforts should continue to express cloned channel proteins in vitro to use highly controlled systems to study their biophysical function. This approach has its pitfalls. Crucial posttranslational modifications might be missed in the expression system. Expression systems possess their own signal transduction machinery, which may fail to work at the expressed target protein, or lead to regulatory effects that have no physiological significance. Because expression systems have become very popular, some of the very recent studies illustrate these problems.

Ideally, the phosphorylation site of the channel protein should be identified by direct sequencing on the protein level. Here, the problem might arise that the physiological kinase is unknown. Hence, an unphysiological phosphorylation site might be identified. Alternatively, putative phosphorylation sites can be identified from the predicted protein sequence. Then it would be possible to use site-directed mutagenesis to remove these phosphorylation sites and look whether the channel activity is now altered. One could also consider a biophysical approach. It would be possible to measure the protein-protein interaction of channels and phosphatases in bilayers. Ultimately, and possibly in the near future, a three-dimensional picture from cocrystallization of a channel and a phosphatase should yield clear-cut answers where they actually interact. However, the same interactions are likely but not necessarily realized in the intact cell.

Information can be gained by dialysis or injection of phosphatases or antibodies into intact cells, studying alteration in channel activity. This approach has already
been used in the past. This method can be complicated if the site of injection, or appropriate targeting, is crucial for the function of the protein. Moreover, sufficient amounts of the protein of interest might not be readily available. Alternatively, expression vectors, namely, adenovirus constructs, could be useful to transfer the channel or phosphatase of interest into cells. These versatile methods would be helpful to establish by mutational analysis which parts of phosphatases are actually crucial for their function. However, most phosphatases do not function alone but in concert with their regulatory or targeting protein. It might be important to cotransfect these auxiliary proteins to get meaningful results. Regrettably, in expression systems is it is difficult to control the amount of overexpressed protein.

On the basis of transfection of isolated cells, there will be the development of transgenic mice that overexpress phosphatases, channels, regulators, and the combination thereof. Indeed, recently, PP1 (332) and PP2B (316) have been overexpressed. Both transgenic models show altered physiological function. Soon, data will become available whether channels are altered in these transgenic lines. In addition, several groups are currently working on knock-out mice for phosphatases or their regulators. Even here a word of caution is warranted. Some knockouts are likely to be lethal, for instance, of PP2Aa. Moreover, it is expected from precedence that overexpression and knockout leads to compensatory changes of other regulatory proteins (69). Hence, the results may be difficult to interpret. One possible route that will be used is the conditional, or tissue-specific, overexpression and knockout of phosphatases in animals.

In addition to their importance in fostering our understanding of nature, research on phosphatases and their action on channels is likely to have additional benefits in medicine. There is a growing body of evidence that phos-
phatase are altered in, for example, cardiovascular disease. Pathological states like hypertrophy (40), cardiac failure (328), and postischemic contractile dysfunction (17, 209) have been accompanied by alterations in phosphorylation of proteins and/or phosphatase function (22, 169), including channel regulation by phosphatases (368). As mentioned above, overexpression of phosphatases can lead to cardiac hypertrophy, strengthening the connection (316, 332). In addition to these pathophysiologial changes, phosphatases, in part by altering channel activity, can change the contractility of muscle (heart or vasculature, Refs. 250, 251, 286). Hence, phosphatases might be favorably altered by application of phosphatase activators and inhibitors or by directly altering cellular protein phosphatase expression, using gene therapy.

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