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Regulation of Sodium Channel Activity by Phosphorylation

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Abstract

Voltage-gated sodium channels carry the major inward current responsible for action potential depolarization in excitable cells as well as providing additional inward current that modulates overall excitability. Both their expression and function is under tight control of protein phosphorylation by specific kinases and phosphatases and this control is particular to each type of sodium channel. This article examines the impact and mechanism of phosphorylation for isoforms where it has been studied in detail in an attempt to delineate common features as well as differences.

Keywords

sodium channels; phosphorylation; kinase; neurotransmitter; phosphatase; ion channels

1. Introduction

Voltage-gated sodium channels are responsible for the initial depolarization of the action potential in most excitable cells. In addition, non-inactivating persistent sodium current (I_{Na}) supports maintained depolarization during and between action potentials. Finally, a component of I_{Na} termed resurgent is triggered upon repolarization and supports repetitive firing in some types of neurons. In the 30 years since sodium channels were first isolated, it has become recognized that they are regulated in a variety of ways including by phosphorylation. Here we review current information on effects of phosphorylation and the specific molecular events that are responsible. Voltage-gated sodium channels include an α subunit that contains the ion conducting pore and the gating machinery (Fig. 1) [1]. Each α subunit consists of 4 homologous domains arranged pseudosymmetrically around the central axis. Each domain consists of a pore forming module that contributes to the ion conducting pore and a voltage gating module. Sodium channels also contain one or two β subunits, a $\beta 2$ or $\beta 4$ subunits that is disulfide linked to the α subunit as well as a $\beta 1$ or $\beta 3$ subunit. There are 9 α subunits in the genome that have been functionally expressed. The transmembrane portions of different isoforms are extremely similar to each other. Instead, the N and distal C-termini as well as the first two intracellular loops connecting the homologous domains are divergent. The intracellular loop connecting homologous domains I and II (loop I-II) contains many of the functionally relevant phosphorylation sites in most isoforms (Fig. 1, red). Other intracellular regions contain additional targets for isoform-specific phosphorylation and protein-protein interactions [2].

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This review highlights phosphorylation events that are reflected in sodium channel function or expression and for which specific phosphorylation sites have been identified. Although the α subunits are structurally similar, regulation is different and thus each is considered separately.

2. Modulation of $\text{Na}_V1.1$, $\text{Na}_V1.2$, $\text{Na}_V1.3$ α subunits by phosphorylation

$\text{Na}_V1.1$, $\text{Na}_V1.2$ and $\text{Na}_V1.3$ sodium channel α subunits are highly expressed in the brain and may be similarly regulated by a variety of kinases. $\text{Na}_V1.2$ has been studied in the greatest detail and is emphasized here. Physiological regulation of brain sodium channels by protein kinase A (PKA, cAMP-dependent protein kinase) in a variety of brain neurons is well-established. In rat striatal neurons the dopamine D1 receptor agonist, SKF 81297, raises threshold for firing and this change is associated with a 35% decrease in the peak amplitude of I_{Na} [3, 4]. These effects required activation of PKA as they were blocked by application of the peptide PKA inhibitor, PKI [4]. Similar effects of D1-like receptor activation were observed in hippocampal neurons and cortico-striatal pyramidal neurons [5, 6]. Since $\text{Na}_V1.6$ is poorly modulated by PKA [6, 7], it is likely that this modulation is largely due to regulation of $\text{Na}_V1.1$, $\text{Na}_V1.2$ and $\text{Na}_V1.3$.

Purified brain sodium channels were phosphorylated by PKA in biochemical studies. The major sites of serine phosphorylation of brain sodium channels were isolated to loop I-II of $\text{Na}_V1.2$. Additional threonine phosphorylation sites found in the whole channel were not accounted for in this region [8, 9]. Five potential sites of PKA phosphorylation were identified in loop I-II [9, 10].

Activation of PKA reduced current due to expression of $\text{Na}_V1.2$ in *Xenopus* oocytes [11] or mammalian cells [12]. A similar reduction was observed for $\text{Na}_V1.1$ [13]. Sites in loop I-II were shown to be physiologically relevant since replacement of loop I-II with that of skeletal muscle $\text{Na}_V1.4$, which lacks these sites, prevented modulation by PKA. Analysis of specific sites by site-directed mutagenesis showed activation of PKA had no effect when all 5 potential sites had been eliminated. Of these, mutation of S573 to alanine blocked all modulation; substitution of an aspartate to mimic phosphorylation reduced the current and occluded modulation [5, 14, 15]. Substitution of S610 and S623 with alanine also reduced the effect of phosphorylation [15]. Reductions in I_{Na} due to PKA stimulation were greater at depolarized holding potentials [16] due to PKA phosphorylation stabilizing sodium channel slow inactivation [17, 18].

PKA is intimately associated with sodium channels. When sodium channels are purified from rat brain preparations, PKA activity and PKA catalytic and regulatory subunits copurify with them [19]. A kinase anchoring protein (AKAP) 15 which binds PKA to subcellular substrates was associated with the sodium channel [19]. In hippocampal neurons, the PKA-dependent dopamine D1 receptor mediated decrease of I_{Na} was abolished if local anchoring of PKA was disrupted with competing peptide HT31, which prevents PKA binding to AKAPs [20]. A binding site for AKAP15 was identified in the N-terminal half of loop I-II that would localize PKA near its phosphorylation sites in this loop [21].

$\text{Na}_V1.2$ is also modulated by activation of protein kinase C (PKC). PKC activation reduced I_{Na} and slowed its inactivation [22]. Both effects were prevented by inhibitors of PKC or by mutation of a conserved consensus site for PKC phosphorylation (S1506 in $\text{rNa}_V1.2\text{a}$) in loop III-IV, the inactivation gate [22]. Activation of M1-like muscarinic receptors with carbachol in isolated hippocampal neurons also reduced I_{Na} in a PKC-dependent manner [23]. The PKC isoform responsible for I_{Na} modulation in hippocampal neurons was PKC ϵ [24].

Interestingly, effects of PKA and PKC interact molecularly and physiologically. Mutation S1506 (phosphorylated by PKC but not PKA) to alanine that blocked modulation by PKC, also blocked the reduction in current due to activation of PKA, suggesting that S1506 must be phosphorylated for effective phosphorylation by PKA [25]. Thus, modulation by PKA and PKC interact molecularly. They are synergistic physiologically as well. In the absence of modulation by PKC, modulation by PKA requires depolarization. Activation of PKC allows modulation by PKA without depolarization and potentiates effects of phosphorylation by PKA at all potentials [16]. These processes are regulated by a complex interaction of phosphorylation sites on the I-II linker [26]. Two sites in that linker are phosphorylated by activation of PKC, S576 and S610. Four sites, S523, S610, S623 and S687, are phosphorylated by PKA. S554, S573 and S1506 are required for reduction of peak current by PKC. Conversely, S573 is required for reduction of peak current by PKA. Finally, S576 and S687 are required for the PKC-dependent increase in PKA efficacy [26]. Both PKA and PKC act by enhancing slow inactivation and their effects are greatly reduced by mutations that prevent slow inactivation [17, 18]. The identification of slow inactivation as an effector and the concentration of relevant phosphorylation sites in loop I-II suggests that phosphorylation drives conformational changes in loop I-II which interact with the transmembrane portion of the channel to favor slow inactivation.

Consistent with phosphorylation by PKA and PKC reducing I_{Na} , activation of phosphatases increased I_{Na} in a variety of preparations [11]. The details of dephosphorylation are also intricate. Biochemical studies showed that specific sites differed in their susceptibility to protein phosphatases. All sites were dephosphorylated by calcineurin but S623 was particularly susceptible. Instead, PP2A specifically dephosphorylated S610 [27]. Interestingly, depolarization of cells causes increased phosphorylation of each of the sites phosphorylated by PKA, but not due to increased PKA activity. Instead, Ca^{2+} influx and PKC activation were required. PKC phosphorylation of the channel resulted in reduced dephosphorylation of PKA sites by calcineurin or PP2A. This provides a partial biochemical explanation for the crosstalk between PKA and PKC activation [28]. An additional level of regulation is provided by PKA activation of DARPP32 which in turn inhibits PP1 and thus enhances sodium channel phosphorylation [29, 30].

Recently mass spectrometry studies have begun to provide new information about phosphorylated sites. Analysis of phosphorylation in $Na_v1.2$ identified 12 new phosphorylation sites of unknown function; 3 novel sites were identified in $Na_v1.1$ [31]. Further analysis of this type will greatly expand the universe of known phosphorylation sites.

$Na_v1.2$ α subunits are also regulated by tyrosine phosphorylation. Activation of growth factor receptors that couple via Src family tyrosine kinases, enhance inactivation of I_{Na} [32, 33]. Rat brain sodium channels interact with Fyn, 1 of 4 Src family tyrosine kinases expressed in brain. Sodium channels and Fyn colocalize within cells. $Na_v1.2$ sodium channels coimmunoprecipitate with Fyn from cotransfected tsA-201 cells. Coexpression of Fyn with $Na_v1.2$ increases the rate of inactivation and causes a negative shift in the voltage dependence of inactivation [33]. Fyn kinase binds to a Src homology 3 (SH3) domain in the second half of loop I-II. Mutation of that site prevents binding of Fyn and the effect of Fyn on inactivation. Y66 and Y1893 are consensus sites for binding of Fyn to Src homology 2 (SH2) domains after phosphorylation and these residues are required for effects of Fyn. Y730 in loop I-II and Y1497 and Y1498 in the inactivation gate are also required. Phosphorylation of the latter sites likely promotes fast inactivation. $Na_v1.1$ is not regulated by Fyn and lacks an SH3 domain in loop I-II [34].

Nav1.2 sodium channels are also regulated by tyrosine phosphatases. Tyrosine phosphatase inhibitors result in a negative shift in the voltage-dependence of inactivation, as does tyrosine phosphorylation. Sodium channels specifically associate with receptor tyrosine phosphatase β (RPTP β). Cotransfection with RPTP β caused dephosphorylation of the channel, a depolarizing shift of inactivation and slowed inactivation kinetics [35].

3. Nav1.4 α subunit

Skeletal muscle sodium channels were phosphorylated by PKA despite lacking the large loop I-II that is the target of PKA in other sodium channels [36]. Nav1.4 expressed in HEK293 cells is phosphorylated by activation of PKA or PKC pathways on overlapping and/or interacting sites [37]. However, activation of PKA had little effect on the current [14, 38]. Activation of PKC caused a large decrease in current that was blocked by inhibitors of PKC [39, 40]. This effect was not blocked by mutation of the conserved phosphorylation site in loop III-IV [40].

The activity of Nav1.4 sodium channels is also increased by myotonic dystrophy kinase. This kinase creates a gain of function in sodium channel gating kinetics. In mice lacking the kinase, I_{Na} is reduced [41].

4. Nav1.5 α subunit

Nav1.5 is the major sodium channel expressed in the heart. In cardiac myocytes activation of the β adrenergic system using isoproterenol produced variable effects on I_{Na} , some of which were attributed to activation of PKA while others were attributed to direct actions of G proteins on the channel. Most reports suggested an increase in current, at least partially due to a hyperpolarizing shift in the voltage dependence of activation and inactivation [42, 43].

Activation of PKA increased I_{Na} due to Nav1.5 sodium channel α subunits expressed in *Xenopus* oocytes. However, mutation of 5 consensus PKA sites in loop I-II failed to block these effects [44] but sequences in loop I-II were required since its replacement with that of Nav1.4 prevented modulation [38]. In general, these effects of PKA activation are quite slow with the current increasing for many minutes without evidence of saturation.

Activation of PKA also increased a persistent (non-inactivating) component of I_{Na} that is enhanced in particular disease-related mutant channels of Nav1.5 (D1760G). Such persistent current is particularly important physiologically as it strongly influences the shape and duration of the action potential. Enhancement was blocked if both serine 36 in the N-terminus and serine 525 in loop I-II were mutated to alanine but single mutations were ineffective [45].

The slower effects of PKA stimulation were attributed to insertion of new Nav1.5 channels in the cell membrane. Chloroquine or monensin, which disrupt cell membrane trafficking, prevented the increase. These effects of PKA were also not supported by loop I-II of Nav1.4 [46]. Biochemical experiments had shown that rNav1.5 was phosphorylated on 2 non-canonical PKA sites in loop I-II, S526 and S529 [47]. While mutation of other serines in PKA consensus sites did not prevent increased Nav1.5 current in response to PKA stimulation, when both of the analogous biochemically identified serines (S525 and S528) were mutated to alanine in human Nav1.5, the slow increase in current due to PKA stimulation was prevented. However, replacement of these amino acids with glutamate to mimic constitutive phosphorylation, current increased normally in response to PKA, indicating that their phosphorylation was necessary but not sufficient for the increase. An additional unidentified phosphorylation event appears also to be required [48]. In addition to

phosphorylation, endoplasmic reticulum (ER) retention sequences in loop I-II were also required for these effects of PKA. Mutation of either of 2 RXR ER retention motifs in loop I-II, (an RRR motif), blocked the increase in current due to PKA [48].

In contrast to PKA, activation of PKC reduces $I_{NaV1.5}$ current in myocytes and expressed in tissue culture cells [49]. In addition to its effect on peak current, persistent I_{Na} that is increased by several disease-causing mutations is also reduced by activation of PKC. These effects are blocked by mutation S1505A in the inactivation gate [50, 51]. This effect has been attributed to specific activation of the PKC ϵ isoform for $NaV1.5$ expressed in *Xenopus* oocytes and natively in rat ventricular myocytes [52]. Recently, the protein encoded by GPDL1 which has been associated with Brugada syndrome and sudden infant death syndrome (SIDS), was shown to act through PKC to reduce I_{Na} [53].

Reports of effects of CaMKII on I_{Na} in the heart are varied. Overexpression of CaMKII δ in rabbit ventricular myocytes shifted the voltage-dependence of availability toward more negative potentials, retarded slow inactivation with intermediate kinetics, caused slowed recovery from inactivation, and increase a persistent component of I_{Na} . These effects were blocked by inhibitors of CaMKII. In addition, $NaV1.5$ channel protein was associated with CaMKII and phosphorylated by the kinase [54]. Na-channel-associated and activated endogenous CaMKII also has been reported to have similar effects on peak I_{Na} in rat ventricular myocytes [55].

$NaV1.5$ is also modulated by the tyrosine phosphorylation [56]. For $NaV1.5$ expressed in HEK 293 cells, insulin caused a depolarizing shift in steady-state inactivation of I_{Na} which was prevented by the inhibitor of SRC family tyrosine kinases, PP2. Coexpression with catalytically active Fyn caused a 10 mV depolarizing shift in steady-state inactivation whereas catalytically inactive Fyn did not. Effects of catalytically active Fyn were blocked when Y1495 in loop III-IV was mutated to phenylalanine. Consistent with this, Y1495 is a good substrate for tyrosine phosphorylation and tyrosine-phosphorylated sodium channel was immunoprecipitated from rat cardiac membranes [56]. EGF receptor-mediated tyrosine phosphorylation also enhanced I_{Na} and increased tyrosine phosphorylation of $NaV1.5$, acting through EGF receptor kinase [57].

Evidence for specific tyrosine phosphatase systems has also been found. A screen for proteins that interacted with the last 66 amino acids of the C-terminus of $NaV1.5$ identified protein tyrosine phosphatase 1 (PTPH1). Coexpression of PTPH1 with $NaV1.5$ shifted inactivation toward more negative potentials [58], an effect that is opposite that of expressing activated Fyn [56]. Thus, activation of $NaV1.5$ appears to also be bidirectionally modulated by tyrosine kinase/phosphatase systems.

5. $NaV1.6$ α subunit

Current due to $NaV1.6$ is reduced by activation of PKA or PKC [7]. However, the degree of reduction is far less in neurons [6] and in tissue culture cells [7] relative to modulation of $NaV1.2$ after analogous stimulation. Conversely, $NaV1.6$ is potently modulated by p38 MAP kinase [59]. This kinase is activated after a variety of insults including injury and hypoxia. P38 MAP kinase colocalizes and coimmunoprecipitates with $NaV1.6$, and kinase activation strongly decreases I_{Na} . Only loop I-II of $NaV1.6$ is phosphorylated by p38 MAP kinase in vitro. Within that loop, S553 is phosphorylated and substitution S553A prevents the physiological reduction of $NaV1.6$ current in response to kinase activation [59]. S553 is the serine of a Pro-Gly-Ser-Pro motif. Phosphorylated Pro-Gly-Ser-Pro is the sequence to which Nedd4 ubiquitin ligase binds. Binding leads to endocytosis. Pharmacologic block of endocytosis completely prevented I_{Na} reduction. Interestingly, Nedd4 also interacts with Pro-Ser-Tyr1945 in the C terminus of the $NaV1.6$ α subunit channel and reduces current.

The reduction in sodium channel activity required both loop I-II and C terminal motifs. Activating p38 MAP Kinase when binding to the C-terminal tail Pro-Ser-Tyr1945 had been prevented caused activation of p38 MAP kinase to increase I_{Na} [60]. The mechanism for this increase and whether it is activated by physiological stimuli is unknown.

6. Nav1.7 α subunit

Effects of PKA regulation of Nav1.7 depend on expression system and whether long (11L) or short (11S) splice variants affecting loop I-II are expressed. PKA decreased currents due to Nav1.7 11L expressed in *Xenopus* oocytes [61] but had no effect after expression in mammalian cells. PKA caused currents due to Nav1.7 11S expressed in mammalian cells to activate at more negative potentials [62]. Conversely, PKC causes a large depolarizing shift in activation of currents due to Nav1.7 11L expressed in *Xenopus* oocytes [61]. Peptide inhibitors of specific receptors for activated C kinase (RACKs) [63, 64] indicated that this effect was due to PKC ϵ and PKC β II isoforms.

Various signals including neurotrophic factors, cytokines and a variety of pathologic signals result in the activation of MAP kinases. Current due to Nav1.7 increases in diabetic neuropathy due to activation of PKC acting through p38 MAP kinase [65]. Similarly, Nav1.7 protein in adrenal chromaffin cells increases in response to serum deprivation, but this increase requires activation of ERK1 and ERK2 MAP kinases but not p38 MAP kinase [66].

More acute actions of MAP kinase signaling are also seen. Neurotrophic factors activate ERK1/2 MAPK in DRG neurons. Activated phosphorylated ERK1/2 (pERK1/2) depolarizes resting membrane potential and increases firing of DRG neurons. At least a portion of these effects is due to activated pERK causing a 7 mV negative shift in activation and inactivation of sodium channels; inhibition of pERK causes a depolarizing shift in activation and inactivation. Fusion protein assays showed that pERK phosphorylates 4 potential sites on loop I-II of Nav1.7. Likewise, mutation of no single site produced a significant reduction in the pERK effect on sodium channel properties whereas mutation of combinations of multiple loop I-II sites prevents effect of pERK1/2 on the channel [67].

7. Nav1.8 α subunit

Hyperalgesia mediated by DRG neurons due to adenosine, PGE2 and serotonin effects have been linked to activation of PKA [68-70]. PGE2 increases the excitability of neonatal dorsal root ganglion (DRG) neurons acting at least partially through Nav1.8 (SNS, PN3) TTX-resistant sodium channels. TTX-resistant I_{Na} was increased by shifting the voltage-dependence of activation and inactivation to more negative potentials. This effect is mimicked by activation of PKA and blocked by a peptide inhibitor of PKA [70, 71].

Nav1.8 contains multiple consensus sites for serine/threonine protein kinases in loop I-II [72, 73]. Alanine substitution of 5 sites of PKA phosphorylation in loop I-II of Nav1.8 largely eliminated phosphorylation and shifted the voltage dependence of activation and inactivation to more positive potentials for Nav1.8 expressed in COS7 cells. The increase in current normally observed for WT Nav1.8 in response to PKA activation was blocked [74]. Thus, PKA phosphorylation of Nav1.8 at 5 sites in the I-II linker, produces an increase in I_{Na} that is at least partially due to a negative shift in the voltage dependence of activation [74].

Membrane trafficking has been implicated in the PKA-dependent increase in Nav1.8 current. PKA-induced increases in current and/or cell surface expression were blocked by inhibitors of protein transport to the plasma membrane for Nav1.8 expressed in *Xenopus*

oocytes [61], and in HEK293 cells, or for the native channel expressed in DRG neurons [75]. Three arginines residues between phosphorylation sites 2 and 3 in loop I-II had previously been shown to be an ER retention signal that could be masked by expression of the $\beta 3$ subunit to increase surface expression [76]. Mutation of the same 3 arginine residues to alanine also caused loss of regulation of channel number in response to forskolin or PGE2. Mutation of the nearby second, but not the third, PKA phosphorylation site prevented regulation by forskolin. Interestingly, the RRR/AAA mutation did not affect the acute effects of PKA activation on channel voltage dependence [75].

Effects of activating PKC are less well-defined. Both decreases and increases in current have been reported. Similarly, interactions with PKA in effects of PGE2 are controversial [71, 75].

Nerve inflammation and injury of peripheral tissues elevate p38 MAP kinase and results in hyperexcitability of peripheral nerves. The cytokine TNF- α increases TTX-resistant I_{Na} in cultured DRG neurons and this increase requires activation of p38 MAP kinase [77]. Nav1.8 is phosphorylated by activation of p38 and sites of phosphorylation were isolated to intracellular loop I-II. Of four potential MAP kinase phosphorylation sites in this loop, two, S551 and S556, were responsible for the biochemical phosphorylation. Mutation of either of these key serines to alanine prevented p38 MAP kinase effects on sodium channel function [78]. This contrasts with the reduction in Nav1.6 current in response to phosphorylation by p38 MAP kinase described above.

8. Na channel β subunits

Sodium channel $\beta 1$ subunits are substrates for tyrosine phosphorylation which inhibits interaction of the intracellular C-terminal tail of $\beta 1$ with ankyrin [79]. In cardiac myocytes, tyrosine phosphorylated $\beta 1$ subunits were localized to intercalated disks whereas unphosphorylated $\beta 1$ subunits were found in the transverse tubules [80]. Thus, $\beta 1$ subunit phosphorylation controls its interactions and localization in a variety of cells.

9. Conclusions[55]

Phosphorylation acts to link a broad range physiologic stimuli to an equally broad range of sodium channel responses. Phosphorylation results in changes in gating, acute increases and decreases in current as well as longer term changes in current that are mediated by channel trafficking onto and off of the membrane. A surprisingly common theme is the involvement of loop I-II in the majority of these diverse effects, regardless of sodium channel isoform. Future work will certainly add new dimensions to the effects of adding phosphate to this intracellular loop about which we know little.

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Abbreviations

I_{Na}	sodium current
PKA	protein kinase A
PKC	protein kinase C
loop I-II	intracellular loop connecting homologous domains I and II
loop III-IV	intracellular loop connecting homologous domains III and IV
p38 MAPK	p38 mitogen activated protein kinase

Fig. 1. Folding model of the voltage-gated sodium channel showing α , $\beta 1$ and $\beta 2$ subunits. The pore-forming S5 and S6 segments of each homologous domain are shown in green. The S1-S3 transmembrane segments of the voltage-sensing domain are shown in white with the charged S4 segment shown in yellow. Loop I-II (red) is shown containing PKA (circles, P) and PKC (diamonds, P) phosphorylation sites identified in $\text{Na}_v1.2$. The highly conserved inactivation gate formed by loop III-IV (blue) contains critical residues for inactivation (h) and a key PKC phosphorylation site.