Introduction

Voltage-dependent Na⁺ channels are responsible for initial action potential depolarization in many excitable cells. Na⁺ channels open or activate in response to membrane depolarization and then, within a few milliseconds, inactivate. The molecule responsible for these activities must contain a highly selective ion pore as well as appropriate machinery for gating the flow of ions through the channel. Biochemical studies have shown that rat brain Na⁺ channels are heterotrimeric proteins consisting of a 260 kDa α subunit, a 36 kDa β1 subunit and a disulphide-linked 33 kDa β2 subunit [1]. Each of these subunits is subject to N-linked glycosylation. Na⁺ channel α-subunit isoforms have been cloned and sequenced from a variety of tissues, and expression studies indicate that the α subunit alone is capable of most aspects of Na⁺-channel function. The α subunit contains four predicted transmembrane homologous domains (I–IV) (Figure 1). Each homologous domain comprises six predicted transmembrane α helices, termed S1–S6. A segment between transmembrane helices S5 and S6 contains the binding site for tetrodotoxin, which is thought to act by ‘plugging’ the ion pore [2]. Of particular interest for the process of activation is the S4 segment of each homologous domain, which contains a positively charged residue at every third amino acid position [3]. The Na⁺ channel must respond to voltage in order to be activated and this region has been proposed to function as a voltage-sensor for

Abbreviations used: cAMP, cyclic AMP; cA-PK, cAMP-dependent protein kinase.
opening of the channel [3]. This idea is supported by charge neutralization and reversal mutations that reduce the voltage-dependence of channel opening [4]. A highly conserved intracellular loop between homologous domains III and IV (L_{III/IV}) is important for channel inactivation. Site-specific antipeptide antibodies directed against sequences in this loop block inactivation whereas antibodies directed against other intracellular portions of the channel have no effect [5,6]. Likewise, Na⁺ channels that are discontinuous in this region have altered inactivation [4]. Finally, mutation of each residue in a triplet of hydrophobic amino acids in this loop, Ile^{1468}–Phe^{1489}–Met^{1490}, each to glutamine blocks inactivation. Mutations of individual amino acids indicate that the phenylalanine is the critical residue, as changing it to glutamine almost completely removes inactivation whereas mutation of the isoleucine or methionine have only modest effects [7].

The α subunit is a substrate for phosphorylation by cyclic AMP (cAMP)-dependent protein kinase (cA-PK) [8] and protein kinase C (PKC) [9]. The remainder of this article reports progress in two major areas: cloning and functional effects of the rat brain Na⁺-channel β1 subunit, and functional effects of Na⁺-channel phosphorylation by cA-PK and PKC.

Structure and functional effects of the β1 subunit

Multiple β1 subunit subtypes are expressed in rat brain, skeletal muscle, sciatic nerve and heart, as detected by subunit-specific antibodies [10]. The recent cloning and expression of the β1 subunit gave the first evidence concerning the role of this subunit in Na⁺-channel biology [11]. The sequence predicts a protein with a molecular mass of 23 kDa, which matches the experimentally observed molecular mass of the deglycosylated mature β1-subunit protein [12]. Sequence analysis suggests that this subunit has a single transmembrane α helix with an extracellular N-terminus containing four sites for N-linked glycosylation and an intracellular C-terminus [11] (Figure 1). Northern blot analysis of total RNA from various tissues using this β1 subunit as a probe detected strong expression in rat brain and spinal cord with weaker expression in rat heart and skeletal muscle. No specific hybridization was observed in rat liver [11]. Although most aspects of Na⁺-channel function are reproduced when the α subunit is expressed in isolation by injection of mRNA encoding it into X. laevis oocytes, the inactivation of expressed currents is abnormal in three ways. First, inactivation is unusually slow, requiring several milliseconds rather than being complete in under a millisecond. Second, the steady-state voltage-dependence of channel inactivation, which determines the availability of channels for activation from a given holding potential, is shifted to more positive potentials. Finally, the recovery from inactivation after returning to the holding potential is slow. During repetitive depolarizations, this results in a pulsewise reduction in current as inactivation develops during each depolarization but is not reversed completely at the holding potential between depolarizations. Thus, high frequency activity is inhibited. Coexpression of the β1 subunit with the α subunit corrects these defects and Na⁺-channel function is relatively normal [11]. An additional effect of the β1 subunit is to increase channel expression. If equivalent amounts of α subunit mRNA are injected into X. laevis oocytes, expression is approx. twice as great when it is coexpressed with β1 [11].

Na⁺ channel phosphorylation

Rat brain Na⁺ channels are good substrates for phosphorylation in vivo and in vitro by cA-PK [8,13,14] and by PKC [9,15]. The sites on the Na⁺ channel phosphorylated by cA-PK have been identified using a combination of protease digestion, two-dimensional phosphopeptide mapping of the resulting peptides, immunoprecipitation of the phosphopeptides with site-directed antibodies and phosphopeptide microsequencing [16]. Four sites of in vitro cA-PK phosphorylation are clustered in the intracellular loop between homologous domains I and II (L_{I/II}) (Figure 1).

The biochemical evidence for rat brain Na⁺-channel phosphorylation suggested the likelihood of physiological effects. Modulation of rat brain Na⁺ channels by cA-PK [17] was studied by adding the purified cA-PK catalytic subunit in the presence of ATP to the cytoplasmic surface of excised membrane patches. Phosphorylation of excised patches from cultured rat brain neurons and from Chinese hamster ovary cells expressing the rat brain type IIA α subunit (CNaIIA-1 cells) reduced Na⁺ current by 40–50%. No change in the Na⁺-current time course or its voltage-dependence of activation or inactivation were observed. When either ATP or cA-PK was applied alone, no reduction occurred. To test whether Na⁺ channels in these cells were tonically modulated at the unstimulated level of cA-PK activity, the CNaIIA-1 cells expressing Na⁺ channels were stably transfected with a dominant-negative mutant form of the regulatory subunit of Ca-PK [18] yielding CNaIIA/Revβ, cells. The
mutant regulatory subunit has two mutant cAMP-binding sites, making it incapable of binding cAMP and releasing active catalytic subunit. Resting cA-PK activity was < 10% of the resting cA-PK activity in the parent CNaIIA-1, and stimulation of cA-PK by cAMP was blocked in these cells [17]. CNaIIA/Revab cells thus provided an excellent system for studying the effects of phosphorylation by cA-PK.

Na+ current was increased in CNaIIA/Revab cells and this increase in current came from two sources. First, the number of Na+ channels was increased by 54% in CNaIIA/Revab cells without any change in Kd, as measured by binding of [3H]saxitoxin. Measured Na+ currents were increased 2.6-fold in whole cells and 3.4-fold in cell-attached patches compared with the parent CNaIIA cells. The number of saxitoxin-binding sites per cell increased 1.9-fold as did the number of saxitoxin-binding sites per μm². Thus the increase in Na+ current could be partially attributed to a second mechanism, an increase in the current per channel, presumably caused by prevention of tonic channel modulation by cA-PK phosphorylation in these cells with minimal basal cA-PK activity [17].

As expected from these results, Na+ current in excised patches from CNaIIA/Revab cells was reduced by 54% when cA-PK and ATP were added to the cytoplasmic surface of excised inside-out patches but not when either was added alone. Na+ current was not reduced when cA-PK and ATP were added in combination with a peptide inhibitor of cA-PK, PKI, 5-24 (obtained from Peninsula Labs) [19]. Finally, after Na+ currents had been reduced in response to cA-PK and ATP, washing the patch with intracellular solution containing a mixture of phosphatases I and IIA substantially reversed the reduction in current [17]. These results confirm that current through rat brain Na+ channels is modulated by cA-PK.

Na+ current through rat brain Na+ channels is also modulated by PKC. Activation of PKC in rat brain neurons or in CNaIIA-1 cells by treatment with diacylglycerols causes two effects: the peak current is reduced and macroscopic inactivation is slowed [20]. Both of these effects can also be observed by treating the cytoplasmic surface of excised inside-out membrane patches with purified PKC. If cells are pre-microinjected with a peptide inhibitor corresponding to the pseudosubstrate site of PKC [21], both effects are blocked, but they occur normally when cells are injected with the peptide inhibitor of cA-PK [19]. Similar effects have been observed in cells expressing the rat muscle Na+ channel [22].

The intracellular loop between homologous domains III and IV (LIII/IV) has been implicated in Na+ channel inactivation, as described above. The residues surrounding Ser1506 in this loop in the rat brain type IIA Na+ channel correspond to a consensus site for PKC phosphorylation. Mutation of this serine residue to alanine (mutant S1506A) blocks both the slowing of inactivation and a reduction of current by PKC activation [23]. Thus, phosphorylation of this site is required for both modulatory effects of PKC.

Other evidence suggests that the slowing of inactivation and the reduction of peak current by PKC activation may actually be two independent effects [24]. When low concentrations of diacylglycerol or PKC are used to phosphorylate the channel, slowing of inactivation is observed without reduction in peak current. Only at relatively high concentrations are both effects observed. This suggests that phosphorylation at two sites may actually be required for reduction of peak Na+ current. To test this idea, Na+ channels with mutations in other PKC consensus sequences were tested for modulation by PKC. Mutation of a serine in a PKC consensus sequence near the amino terminal of LIII/IV to an alanine prevented the reduction of peak Na+ current but had little effect on the slowing of macroscopic inactivation [24]. This result in combination with the effects of the mutation at Ser1506 suggests that phosphorylation of this residue in LIII/IV is necessary for the reduction of peak Na+ current but reduction is only seen if Ser1506 is also phosphorylated.

The finding that reduction of current by PKC requires phosphorylation at a site in LIII/IV raises the possibility that reduction of current by cA-PK at site(s) in this loop also requires phosphorylation of Ser1506 in LIII/IV. In fact, in mutant S1506A, which can not be phosphorylated at this site in LIII/IV, no reduction in current is observed when cA-PK at high concentration is applied to the cytoplasmic surface of excised inside-out patches [25]. Purified Na+ channels and peptides corresponding to this site can be phosphorylated in vitro by PKC but not by cA-PK. Thus, phosphorylation of Ser1506 by PKC is required before current can be reduced by cA-PK phosphorylation in LIII/IV.

This idea suggests that activating PKC and, thus, phosphorylating Ser1506 will greatly potentiate the effects of cA-PK. Na+ current in cell-attached patches is normally not reduced when cA-PK is activated using membrane-permeant cAMP analogs by themselves. If, however, one applies concentrations of diacylglycerol that are sufficient to slow
Na⁺ current inactivation but do not reduce the current, then application of the membrane-permeant cAMP analog 8-bromo cAMP reduces Na⁺ current [25].

A second line of evidence involves altering the sequence at Ser₁⁵⁰⁰ to make it phosphorylatable by cA-PK [26]. The two charged residues following the phosphorylated serine inhibit cA-PK phosphorylation but this negative effect can be removed by mutating them to alanine. Na⁺ current in cells expressing this mutant Na⁺ channel are reduced by 8-bromo cAMP in the absence of PKC activation. Likewise, far lower concentrations of cA-PK were expected to produce greater effects than when neuronal signals mediated through these parallel pathways. The phosphorylation of the rat brain Na⁺ channel by PKC and cA-PK. Activation of these kinases in concert is necessary before phosphorylation but this negative effect can be removed by mutating them to alanine. Na⁺ current in inactivated but do not reduce the current, then application of the membrane-permeant cAMP analog 8-bromo cAMP reduces Na⁺ current [25].

These results demonstrate a convergent regulation of the rat brain Na⁺ channel by PKC and cA-PK. Activation of these kinases in concert is expected to produce greater effects than when either kinase is activated alone. This mechanism of regulation by two kinases may serve to integrate neuronal signals mediated through these parallel signalling pathways.


Received 20 December 1993