Effects of PKC and PKA Inhibitors on the cAMP-Stimulant-Induced Enhancement of Tetrodotoxin-Resistant Na^+ (Nav1.8) Currents

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Abstract: The protein kinase C (PKC) inhibitor bisindolymaleimide Ro-31-8425 (Ro-31-8425) decreases the peak tetrodotoxin-resistant (TTX-R) Na^+ (Nav1.8) current in nodose ganglion (NG) neurons, and this decrease is not altered by simultaneous application of 8-bromo-cAMP (8-Br-cAMP), phorbol 12-myristate 13-acetate (PMA, a PKC activator) or forskolin (a cAMP analogue). Intracellular application of the endogenous protein kinase A (PKA) inhibitor, protein kinase inhibitor (PKI) abolishes the increase in the peak Nav1.8 current that occurs in response to the applications of 8-Br-cAMP, PMA, forskolin, or prostaglandin E_2 (PGE_2, an adenyl cyclase activator). At a higher concentration (0.5 mM) compared with a sufficient concentration (0.01 mM) to block the cAMP-stimulant Nav1.8 current, PKI still attenuated the Ro-31-8425-induced decrease in peak Nav1.8 current. When we considered these results together, cAMP-stimulant-induced modification of Nav1.8 currents is mediated by the activation of both PKA and PKC, and PKC may be located upstream of PKA.

INTRODUCTION

In electrophysiological studies of the Na^+ current of sensory neurons, two distinct types of the current have been identified : tetrodotoxin-sensitive (TTX-S) Na^+ currents, which are blocked by nanomolar concentrations of TTX, and TTX-resistant (TTX-R) Na^+ currents, which are not blocked or affected by micromolar concentrations of TTX [1, 2]. The TTX-R Na^+ (Nav1.8) current is found more often in capsaicin- and prostaglandin-sensitive neurons [3, 4], and the TTX-S Na^+ current is more common in capsaicin-insensitive neurons [5, 6]. More specifically, Nav1.8 currents are expressed in a population of small dorsal root ganglion (DRG) neurons consisting of the capsaicin-sensitive Aδ- and C-type sensory neurons [5, 6]. The Nav1.8 current is modificated by hyperalgesic inflammatory mediators, which induce enhanced nociceptive excitability [4, 7, 8]. Nav1.8 channels are present in the DRG cell body *in vivo* conditions [9], and it has been speculated that these channels are present on the central [10, 11] and peripheral terminals of primary nociceptive afferents [12, 13]. The expression of Nav1.8 channels is increased in the rat digital nerve and DRG after intraplanter administration of complete Freund's adjuvant or carrageenan, respectively [14, 15]. Furthermore, the spinal nerve ligation at the L_5-L_6 region is known to elicit immunohistochemical and electrophysiological changes in Nav1.8 channels [16]. These observations lead us to suggest the Nav1.8 channel plays a potential role in the sensation of neuropathic pain [16]. Indeed, it has been demonstrated that human patients with chronic neurogenic pain or chronic local hyperalgesia show increased Nav1.8 channel expression proximal to a peripheral injury site [17-19].

Nav1.8 channels are cloned from rat DRG neurons and the expression of these channels is sensitive to capsaicin [20]. Knockdown of Nav1.8 channel expression produces anti-nociception of neuropathic pain [21]. In view of these observations, taken together, it is possible to speculate the idea that the Nav1.8 channel is an effective target for treatment of neuropathic pain. The persistent current that is produced by Nav1.9 in mouse DRG neurons [22] is not observed in neonatal rat TTX-R nodose ganglion (NG) neurons but the production of Nav1.9 channel protein is found in these neurons [23]. Indeed, Nav1.9 current characteristics have not been demonstrated in neonatal NG neurons [23, 24]. The slow onset of Nav1.9 channel opening probably implies that this type of Na^+ channels makes a minor contribution to the action potential amplitude [25]. The majority of voltage-gated sodium channels (VGSCs) are inhibited by either protein kinase C (PKC) or protein kinase A (PKA) [26-32]. The VGSC into the skeletal and cardiac muscles is not significantly affected by the activation of PKA [33, 34]. However, there are increasing evidences that the enhancement of Nav1.8 currents is seen after the application of hyperalgesic inflammation mediators, such as PGE_2 and serotonin, in sensory neurons [7, 35-37]. In fact, PGE_2 application can enhance the responsiveness of primary nociceptive neurons to bradykinin and/or capsaicin [38]. Additionally, in neurons expressing sensory nociceptive neurospecific Nav1.8 channels, PGE_2 shigts the activation curve of the Na^+ current to more negative potentials and potentiates the amplitude of the current, and such effects are mimicked by the application of drugs, which regulate cAMP-dependent PKA phosphorylation of the channel [7, 24, 36]. The PKA phosphorylation of Nav1.8 channels is therefore to be an important mechanism underlying the hyperalgesic responses in sensory neurons. This is further confirmed by evidence that five serines located with the intracellular I-II loop of sensory neuron-specific Nav1.8 channels are identified as the major sites of PKA modification [39]. In contrast, based on evidence that the inhibition of PKC greatly inhibited PGE_2-induced enhancement of the Nav1.8 current from adult rat DRG neurons insentive to TTX and that PKA inhibitors have little or no effect on the PKC activator-induced increase in the Nav1.8 current, Gold *et al.* [36] concluded that PKC activity may play an important role in regulating subsequent PKA-mediated modulation of Nav1.8 currents, This conclusion is in agree-

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ment with the observation demonstrating that PKC-induced phosphorylation of the channel protein at serine 1506 in III-IV loop of the brain type II_A Na^+ channels is required to enable PKA-induced phosphorylation of other sites on the channel protein [40]. In NG neurons insensitive to TTX, both PKC and PKA signal transduction mechanisms are involved in the modification of Nav1.8 currents seen after the application of cAMP-stimulants [23, 24]. Therefore, it is obvious that the activation of thses two protein kinases, PKC and PKA, is closely related to the modulation of Nav1.8 currents obtained from neonatal rat NG neurons, particularly when the level of cAMP is increased after the drug application. These findings and other studies describing about Nav1.8 currents are discussed in this article. We focus on the relationships between Nav1.8 current and PKC or PKA activity, as well as their combined effects.

MODULATION OF NAV1.8 CURRENTS BY PKC

Many phsophorylation sites have been found on the Na^+ channels of excitable cells [41-45], and one of the phosphorylation sites of the rat brain type II_A α-subunits- at serine 1506 on the cytoplasmic loop between domains III and IV- has been identified as a functional PKC modulation site for Na^+ channel activity [40]. This is in agreement with evidence suggesting that PKC plays an important role in regulating the subsequent PKA-mediated modification of Nav1.8 currents [36]. Indeed, the basal Nav1.8 current obtained in neonatal rat NG neurons insensitive to TTX may be regulated by the level of PKC activity [46]. There is evidence that PKC inhibitors alone decreased the baseline Nav1.8 current density, but that neither extracellular nor intracellular application of either of two PKA inhibitors (WIPTIDE and R_P-cAMPs) had marked effects on the baseline Nav1.8 current [36]. This has been further confirmed in mammalian brain neurons by evidence that a reduction in the peak Na^+ current by cAMP-dependent protein kinase (CA-PK) mammalian brain neurons is required for concurrent activation of PKC and is abolished by blocking of phosphorylation of serine 1506, a channel inactivation site of the channels at which phosphorylation is mediated by PKC but not by CA-PK [40]. Furthermore, activation of the PKC activity by treatment with an intermediate concentration (25 μM) of oleoylacetylglycerol (OAG) slows the inactivation but does not reduce the peak Na^+ current [47]. Under these conditions, subsequent application of 8-Br-cAMP in the presence of OAG reduces the peak Na^+ current, indicating that PKC–mediated phosphorylation is needed to obtain the effect of phosphorylation by CA-PK [40]. In fact, when the cells were pretreated with a PKC inhibitor (Ro-31-8425) at concentrations ranging from 0.001 to 1.0 μM, a concentration of 0.5 μM Ro-31-8425 application produced the greatest decrease in the baseline peak Nav1.8 current amplitude, accompanied by a hyperpolarizing shift in the conduction-voltage (G-V) curve, and it also attenuated an increase in the Nav1.8 current in response to application of the PKC activator, phorbol 12-myristate 13-acetate (PMA), at 0.1 μM [23]. Ro-31-8425 at 0.5 μM inhibited TTX-R Na^+ channel activity completely; increasing its concentration up to 1 μM did not cause a further decrease in the Nav1.8 current compared with that seen after 0.5 μM Ro-31-8425 application [23], indicating that the PKC inhibitor Ro-31-8425 is not a non-specific inhibitor over the full range of either PKC activation. Similarly, we found that application of 0.5 μM Ro-31-8425 application blocked an increase in Nav1.8 currents induced by either 8-Br-cAMP or the cAMP analogue forskolin [23]. This was supported by the fact that application of a PKC inhibitor (staurosporine or PKC_{19-36}) caused a marked inhibition of the forskolin-induced increase in the $G_{V1/2}$ base (i.e. the percentage change in G at baseline $V_{1/2}$) [24, 36, 46]. The PKC activator and inhibitor can modulate the peak amplitude of Nav1.8 current without any shift of voltage-dependent activation kinetics in the DRG neurons, and phorbol 12, 13-dibutyrate (PDBu)-induced changes in the Nav1.8 current are associated with an increase in the rates of activation and inactivation of the current as compared to the other PKC activator PMA [36]. The latter effect probably reflects the difference between PMA and PDBu on the Nav1.8 properties. Staurosporine is known to block other protein kinases, such as PKA, protein kinase G (PKG), calmodulin-dependent protein kinase and myosin light chain kinase [48]. Considering these results together, it is possible that the increase in Nav1.8 currents induced by applications of cAMP analogues and activators is involved in the activation of PKC.

MODULATION OF NAV1.8 CURRENTS BY PKA

PGE_2, an agent producing hyperalgesia, activates adenyl cyclase. As a result, it stimulates PKA, which ultimately phosphorylates serine residuces on the sensory- neuron-specific Nav 1.8 channels [49]. Indeed, in sensory neurons insensitive to TTX, the effect of PGE_2 on Nav1.8 currents is mimicked by the drugs that upregulate cAMP-dependent PKA phosphorylation of the channel [7, 23, 24]. The membrane-permeable dibutyl cAMP (db-cAMP) is considered to be less efficient than direct adenyl cyclase activators, such as forskolin or PGE_2, because this substrate is hydrolyzed by a variety of phosphodiesterases in intact cells [36], but a similarity between the effects of forskolin or PGE_2 and db-cAMP on the Nav1.8 current has been found in neonatal rat DRG neurons [7]. By using site-directed mutagenesis, five serines located within the intracellular I-II loop of sensory neuron-specific Nav1.8 channels were identified as the major sites of modification by PKA [49]. A number of studies have demonstrated that PKA activation is responsible for the underlying mechanisms of inflammatory mediator-induced hyperalgesia [50, 51] and nociceptor sensitization [38, 52]. This is based on the fact that PKA inhibitors attenuate the PGE_2-induced modification of the Nav1.8 currents in adult rat DRG [36] and neonatal NG neurons [24], as well as the 8-Br-cAMP-induced enhancement of the Nav1.8 current [23]. We used fluorescence retrograde tracing and perforated patch-clamp techniques in an investigation of how PGE_2 affects the excitability of trigeminal ganglion (TG) neurons projecting onto the superficial layer of the cervical dorsal horn (C_1 level); after PGE_2 application, we observed increased peak Nav1.8 current amplitude and a hyperpolarizing shift in the activation curve, as well as an increased number of action potentials [53]. Recent evidence has clearly demonstrated that even when the experiments are performed in using the technique represented by a perforated patch-clamp, changes in Nav1.8 current properties following PGE_2 application [54] are quite similar to those as reported by those in the studies of England *et al.* [7] and Gold *et al.* [36]. We used the same technique of intracellular application described in previous studies [55-57] to examine the modulation of the endogenous PKA inhibitor PKI in neonatal TTX-R NG neurons; we found that intracellular application of

PKI abolished the excitability of Nav1.8 currents after applications of PGE_2 and 8-Br-cAMP [23, 24].

INTERACTION BETWEEN PKC AND PKA ON NAV 1.8 CURRENTS

The PKC-induced phosphorylation of the TTX-R Na^+ channel protein at serine 1506 is required to enable PKA-induced phosphorylation of the other sites (I-II loop) on the channel protein [40]. The application of the PKC inhibitor PKC_{19-36} significantly suppresses the forskolin-induced increase in the Nav1.8 current but the PKA inhibitor WIPTIDE has no significant effect on the PKC activator phorbol12, 13-dibutyrate (PDBu)-induced increase in the current [36]. When considering these results together, it is possible that PKC-induced phosphorylation of the channel protein at serine 1506 is required to enable PKA-induced phosphorylation of other sites on the channel protein suggested by Li *et al.* [40] in a relationship between PKC and PKA on the convergent regulation of Na^+ channels. However, we found that larger inhibitory effects of 0.5 mM PKI application in a full range from 0.01 to 0.5 mM on the peak Nav1.8 current amplitude, as well as values for the slope factor in both activation and inactivation curves, were observed in the continuing presence of 0.5 μM Ro-31-8425, which maximally inhibited Nav1.8 currents [23]. This probably implies that part of the PKA signaling is still active in the full range of inhibition of the PKC activity. Accordingly, the cAMP-analogue-induced modification of the Nav1.8 current is mediated by a common pathway involving the activation of both PKA and PKC, and that its modification of PKC appears to be located upstream of PKA. If the concentration of cAMP analogues and/or adenyl cyclase activators used is higher than those that cause maximal increases in Nav1.8 currents, the concentration-response curve is "bell-shaped" [36]. In fact, we found the addition of 8-Br-cAMP in the presence of PMA did not show the enhancement of increased Nav1.8 current, indicating the limiting effect [23].

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