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UP-REGULATION OF LOW-THRESHOLD TETRODOTOXIN-RESISTANT NA^+ CURRENT VIA ACTIVATION OF A CYCLIC AMP/PROTEIN KINASE A PATHWAY IN NOCICEPTOR-LIKE RAT DORSAL ROOT GANGLION CELLS

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Abstract—The effects of forskolin on low-threshold tetrodotoxin-resistant (TTX-r) Na^+ currents was studied in small diameter (average ≈ 25 μm) dorsal root ganglion (DRG) cells. All DRG cells included in the study were categorized as type-2 or non-type-2 based on the expression of a low-threshold A-current. In all type-2 and some non-type-2 DRG cells held at −80 mV, the adenylyl cyclase (AC) activator forskolin (10 μM) up-regulated TTX-r Na^+ currents evoked with steps to −55 mV through −35 mV (low-threshold current). Up-regulation of low-threshold current by forskolin was mimicked by the protein kinase A (PKA) agonist Sp-cAMPs and the inflammatory mediator serotonin, and blocked by the PKA antagonist Rp-cAMPs. Forskolin-induced up-regulation of low-threshold current evoked from a holding potential of −60 mV was blocked by 40 ms steps to 0 mV, which presumably induced a long lasting inactivation of the low-threshold channels. Reducing to 3 ms the duration of steps to 0 mV, significantly increased the number of DRG cells where low-threshold current was up-regulated by forskolin, presumably by reducing the long-lasting inactivation of the low-threshold channels. In the same cells, high-threshold current, evoked by 40 ms or 3 ms steps to 0 mV, was consistently up-regulated by forskolin. The selective $Na_V1.8$ channel blocker A-803467 markedly blocked high-threshold current but not low-threshold current. The different voltage protocols observed to activate and inactivate the low- and high-threshold currents, and the observation that A-803467 blocked high- but not low-threshold current suggests that the two currents were mediated by different channels, possibly $Na_V1.8$ and $Na_V1.9$, respectively. Inflammatory mediators may simultaneously up-regulate $Na_V1.8$ and $Na_V1.9$ channels in the same nociceptor via a AC/PKA signaling pathway, increasing nociceptor signaling strength, and lowering nociceptor threshold, respectively.

Key words: $Na_V1.9$, $Na_V1.8$, A-803467, sensory neuron, hyperalgesia, inflammation.

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Abbreviations: AC, adenylyl cyclase; A-D/D-A, analog to digital/digital to analog; cAMP, cyclic AMP; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; GTP, guanosine triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HP, holding potential; MΩ, megaohm; PKA, protein kinase A; PKC, protein kinase C; TEA, tetraethylammonium chloride; TEA-OH, tetraethylammonium hydroxide; TP, test potential; TTX, tetrodotoxin.

$Na_V1.9$ channels are expressed by a large proportion of nociceptors. In addition to their presence in nociceptor somata, there is evidence that $Na_V1.9$ channels are distributed to nociceptor axons, peripheral receptors, and central terminals (Amaya et al., 2000; Black and Waxman, 2002; Coggeshall et al., 2004; Keh et al., 2008; Padilla et al., 2007; Wells et al., 2007). They are hypothesized to be involved in the regulation of nociceptor excitability and action potential firing patterns (Baker et al., 2003, Baker 2005; Fang et al., 2002; Herzog et al., 2001; Maingret et al., 2008; Ostman et al., 2008). $Na_V1.9$ channels mediate a "low-threshold" tetrodotoxin-resistant (TTX-r) Na^+ current, which begins to activate at significantly more negative voltages than the TTX-r $Na_V1.8$ channels that mediate action potentials in nociceptor somata.

Available evidence suggests that $Na_V1.9$ channels play an important role in the transmission of nociceptive information. Studies with $Na_V1.9$ knockout mice suggest that $Na_V1.9$ channel expression is required for the development of inflammatory pain associated with injection of carrageenan or complete Freund's adjuvant into the rat hindpaw (Priest et al., 2005; Amaya et al., 2006). Also, $Na_V1.9$ channels were shown to be up-regulated in the somata of afferents serving rat knee joint following local inflammation induced by injection of carrageenan or complete Freund's adjuvant, and in inflamed human tooth pulp (Strickland et al., 2008; Warren et al., 2008; Wells et al., 2007).

However, little is known regarding the inflammatory mediators and signaling pathways involved in the modulation of $Na_V1.9$ channels in different types of nociceptors. A previous report demonstrated that $Na_V1.9$ channels in a subpopulation of rat nociceptors were up-regulated by serotonin (Scroggs, 2010). Other previous reports provide evidence that up-regulation of $Na_V1.9$ channels involves G-proteins and protein kinase C (PKC) (Baker et al., 2003; Baker 2005; Rush and Waxman, 2004; Ostman et al., 2008). The present work extends the author's previous report that serotonin modulates $Na_V1.9$ channels by exploring the possible role of cyclic AMP (cAMP) and protein kinase A (PKA) signaling pathways in the modulation of $Na_V1.9$.

EXPERIMENTAL PROCEDURES

Preparation of DRG cells

All procedures involving animals were approved the University of Tennessee Health Science Center Animal Use and Care Committee. Male rats (50–100 g), Sprague–Dawley (purchased from Harlan Laboratories, Indianapolis, IN, USA) were rendered uncon-

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scious with isoflurane (inhalation, 0.5 ml in a 1 L covered Telflon bowl), decapitated, and dorsal root ganglion (DRG) from lumbar and thoracic regions dissected out. The ganglia were incubated at 34 °C for 1/2 h in Tyrode's solution (composition below) containing 2 mg/ml collagenase type 1A (Sigma-Aldrich, St. Louis, MO, USA) and 2 mg/ml protease, (Sigma-Aldrich). After the enzymes were washed away from the DRG, individual DRG cell bodies were dispersed by trituration. A suspension of isolated DRG cells was immediately transferred to a plastic 35-mm petri dish where many adhered to the bottom within 5 min. The cells were superfused with Tyrode's solution containing (in mM): 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4 with NaOH.

Whole cell patch clamp recording

Whole-cell recordings were carried out using 35-mm petri dishes as baths. Experiments were restricted to relatively small diameter DRG cells (average diameter ≈ 25 μm), which were classified as type-2 or non-type-2 based on the expression of a low-threshold A-current (Fig. 1B, C). The categorization of small diameter DRG cells into types 1, 2, 3, and 4 based on their expression of various ion currents was proposed by Cardenas et al. (1995). Subsequently, it was discovered that DRG cells in the type-2 category expressed high-threshold and low-threshold TTX-r Na^+ currents that were up-regulated by serotonin (Cardenas et al., 1997; Scroggs, 2010). Thus, it was felt that categorization of DRG cells into type-2 and non-type-2 might be helpful in the present study.

Data acquisition and analysis were accomplished with P-Clamp 8.2, using an Axopatch 200A/B amplifier coupled with a Digidata analog to digital/digital to analog converter (Molecular Devices, Sunnyvale, CA, USA). Data were leak subtracted using the P/4 method. Patch electrodes were coated with Sylgard to about 200 μm from the tip, and fire polished to a final resistance of 0.8–2.0 M. The electrodes were filled with a solution containing (in mM): 120 CsCl, 5 2Na-ATP, 0.4 2Li-GTP, 5 $MgCl_2$, 5 EGTA, 1.86 $CaCl_2$, 20 HEPES, adjusted to pH 7.4 with CsOH. (Total $[Ca^{2+}]_i$ is calculated to be 100 nM). A-current was assayed for at the beginning of experiments in Tyrode's solution and TTX-resistant Na^+ currents were recorded in an external solution containing (in mM): 140 NaCl, 10 TEA, 2 $CaCl_2$, 5 $MgCl_2$, 10 HEPES, 0.1 $CdCl_2$, 0.0005 tetrodotoxin (TTX), pH 7.4 with TEA-OH (final concentration of TEA≈14 mM). Solutions were changed around the cell under study by means of a small glass capillary tube placed near the cell in the bath, as described earlier (Cardenas et al., 1997). Chemicals and salts used to make up the external and pipette solutions were obtained from Sigma-Aldrich. The patch clamp amplifier was tuned to null the whole-cell capacity transients, prediction was typically set at 80–85%, and series resistance was compensated by 80–85%.

Statistical analysis

All statistical analyses were preformed in SYSTAT (SPSS, Inc.). The significance of differences in means of paired data or independent data were determined using the paired *t*-test or the Student's *t*-test respectively. The Fisher's exact test was used determine probability of differences between groups of DRG cells regarding the proportion of DRG cells where up-regulation of Na^+ current was observed. Finally, an analysis of variance with repeated measures was used to determine the significance between independent groups regarding changes in current amplitude over time.

The amplitude of low-threshold TTX-r Na^+ currents was determined by averaging the current over the last 20 ms of the 40 ms records. Since the amplitude of low-threshold currents varied significantly in some DRG cells under control conditions, the mean value of 3–5 successive current amplitudes was used to represent a given DRG cell for control and/or treatment conditions. However, the same number of current amplitudes was always averaged to generate data points for control and treatment conditions for a given comparison. The data are expressed as the mean ± standard error of the mean.

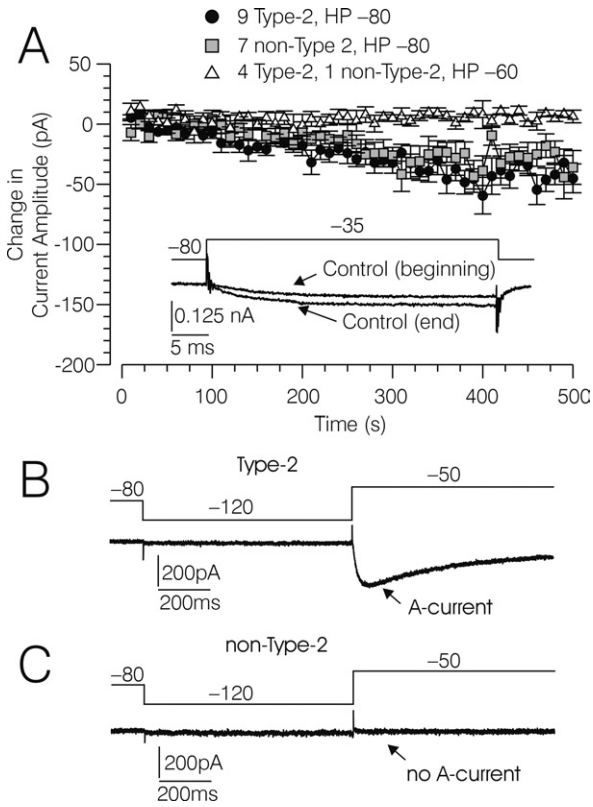

Fig. 1. Effects of DRG cell type and holding potential on run-up of low-threshold TTX-r Na^+ current. (A) Plot of change over time of TTX-r Na^+ current evoked by steps to −35 mV from a holding potential of −80 mV in nine type-2 DRG cells (circles), seven non-type-2 DRG cells (squares), and from a holding potential of −60 mV in a group containing four type-2 DRG cells and one non-type-2 DRG cell. The change in current versus time did not vary between the nine type-2 and seven non-type-2 DRG cells held at −80 mV (P=0.5, ANOVA, repeated measures). The inset in (A) shows the averaged sweeps for both type-2 and non-type 2 DRG cells corresponding to the beginning and end of the ≈ 8 min recording period. When all DRG cells held at −80 mV were grouped together, TTX-r Na^+ current increased significantly more over time compared to DRG cells held at −60 mV (P<0.05, ANOVA, repeated measures). (B) Example of the current signature of a type-2 DRG cell. The A-current (arrow) is inward because the pipette solution lacks K^+ and the external solution contains 4 mM K^+. (C) Example of the current signature of a typical non-type-2 DRG cell. Note the lack of the A-current.

RESULTS

Effects of modulators on low-threshold TTX-r Na^+ currents evoked from HP −80 mV

An initial series of experiments was carried out to test the effects of forskolin, forskolin+Rp-cAMPs, Sp-cAMPs, and 5-HT on TTX-r Na^+ currents (low-threshold currents) evoked by a step to −35 mV from a holding potential (HP) of −80 mV (Fig. 2A–K). To account for the gradual run-up of low-threshold current under these conditions, each treatment was compared to a set of 16 control experiments