

Characterization of Voltage-Gated Na⁺ Channels (VGSCs) in Primary Neuronal Cultures using Membrane Potential- and Calcium-Sensitive Dyes and the FLIPR[®].

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VGSC inhibitors that are currently available have limited therapeutic utility due to a combination of their dose-limiting side effects and general lack of potency. The development of potent, subtype-selective VGSC inhibitors may offer a therapeutic advantage in disease states caused by altered VGSC function, including persistent pain following damage to sensory neurons (Wood, *et al.*, 2002). Unfortunately, the identification of subtype-selective inhibitors has been limited by a lack of robust and predictive *in vitro* functional assays for screening a large number of compounds at multiple VGSC subtypes (Gonzalez *et al.*, 1999; Mattheakis & Savchenko, 2001; Xu *et al.*, 2001). The present study describes three novel FLIPR[®] assays designed to characterize the pharmacological properties of tetrodotoxin (TTX)-sensitive (TTX-S) and TTX-resistant (TTX-R) VGSCs in primary neuronal cultures of neonatal rat cerebellar granule neurons (CGN) and dorsal root ganglion (DRG) neurons, respectively. The assays were developed using both a membrane potential (FMP), and a calcium-sensitive (fluo-4/AM) dye.

Briefly, primary neuronal cultures of CGN at 4-6 days *in vitro* (DIV) and DRG neurons at 1 DIV were loaded with FMP or fluo-4/AM. The alkaloid toxin veratridine (40 μ M) evoked membrane depolarization in FMP dye-loaded CGN, and an increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i) in fluo-4/AM-loaded CGN cells. TTX produced a concentration-dependent inhibition of the veratridine-evoked increase in membrane depolarization and [Ca²⁺]_i with pIC₅₀ values (mean \pm S.D.; n = 3-7) of 7.57 \pm 0.35 and 7.92 \pm 0.28, respectively. There was good correlation between the pIC₅₀ values for a range of Na⁺ and Ca²⁺ channel blockers for inhibition of veratridine-evoked membrane depolarization, and calcium response (r² = 0.97, slope = 0.96). Similarly, veratridine (30 μ M) evoked membrane depolarization in FMP dye-loaded CHO-K1 cells stably-transfected with rat Na_v1.2a (rNa_v1.2a-CHO-K1). There was also good correlation between the pIC₅₀ values for inhibition of veratridine-evoked membrane depolarization in CGN cells and rNa_v1.2a-CHO-K1 cells (r² = 0.80, slope = 0.73). Furthermore, the potency of compounds for rNa_v1.2a determined using the FMP dye and FLIPR[®] or a [¹⁴C]-guanidium influx assay were very similar. Veratridine (42 μ M) also evoked membrane depolarization in FMP dye-loaded DRG neurons. The response to veratridine was inhibited completely by TTX (125 nM). In contrast, in the presence of the type II pyrethroid deltamethrin (4.2 μ M), but not the type I pyrethroid tetramethrin (4.2 μ M), TTX (125 nM) inhibited the veratridine-evoked response by only 59%. The TTX concentration response curve appeared biphasic, with a high affinity site and a low affinity site (pIC₅₀ values 8.3 and 4.9, respectively), likely representing inhibition of endogenous TTX-S and TTX-R VGSCs in DRG neurons.

These novel FLIPR[®] assays represent useful tools for the determination of the relative potencies of compounds at native TTX-S and TTX-R VGSCs.

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