TRPV1-MEDIATED PRESYNAPTIC SIGNALING IN SENSORY NEURONS

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TRPV1 activation induces prolonged presynaptic $[Ca^{2+}]_i$ elevation and synaptic activity

TRPV1 is a critical molecular detector of noxious signals in primary sensory neurons. A broad range of pain-producing stimuli either directly activate or modulate TRPV1. This includes noxious heat, protons, lipid-derived endovanilloids and inflammatory mediators (Caterina and Julius, 2001, Szallasi et al., 2006). Behavioral studies indicate that TRPV1 plays a crucial role in the development of chronic pain states, including those associated with cancer and arthritis. Accordingly, TRPV1 presents an attractive target for analgesics, and several TRPV1 antagonists are currently under clinical trials (Szallasi et al., 2006).

TRPV1 is a non-selective cation channel with a high Ca^{2+} permeability (P_{Ca}/P_{Na} = 9.6), and robust Ca^{2+} entry into the cell is a hallmark of receptor activation. At the peripheral terminals of primary nociceptors, TRPV1-mediated Ca^{2+} influx triggers the release of neuropeptides, which initiates neurogenic inflammation and contributes to peripheral sensitization (Caterina and Julius, 2001). TRPV1 is also found in the central processes of primary afferent neurons (Guo et al., 1999) suggesting involvement of the receptor into gating nociceptive transmission at the first sensory synapse in the dorsal horn of the spinal cord. Notably, intrathecal administration of selective TRPV1 antagonists produces analgesia in various pain models, highlighting the important role of TRPV1 in the spinal cord (Christoph et al., 2006; Cui et al., 2006).

Despite growing evidence to a significant role of TRPV1 in pain transduction in the spinal cord, mechanisms contributing to this process remain unclear. Here, we used digital imaging of cytosolic and mitochondrial Ca^{2+} in the axonal boutons of dorsal root ganglion (DRG) neurons in conjunction with postsynaptic patch-clamp recording in spinal cord (SC) neurons to examine TRPV1-mediated signaling at sensory synapses between DRG and SC neurons in culture (Usachev et al., 2002, Medvedeva et al., 2008).

The TRPV1 agonist capsaicin (1 μM, 30 s) elicited a robust elevation of presynaptic $[Ca^{2+}]_i$ from 0.11 ± 0.02 to 4.05 ± 0.31 μM in 72% of the small-to-medium sized DRG neurons (n = 47 boutons/14 cells; Fig. 1, *a*). After removal of capsaicin, $[Ca^{2+}]_i$ rapidly recovered to a new steady-state level ranging between 0.5 and 1.5 μM. This delayed phase of Ca^{2+} response ($[Ca^{2+}]_i$ plateau) typically lasted more than 10-15 min. In parallel experiments, glutamate release was monitored by recording spontaneous EPSCs (sEPSCs) in SC neurons in the presence of the $GABA_A$ receptor selective antagonist bicuculline (10 μM) and the glycine receptor antagonist strychnine (2 μM).

Stimulation with capsaicin (1 μM, 30 s) induced a more than 150-fold increase in the sEPSC frequency (0.27 ± 0.07 Hz at rest and 48.01 ± 9.90 Hz during the first 15 s of stimulation, n = 7) (Fig. 1, *b*). Capsaicin induced no response in

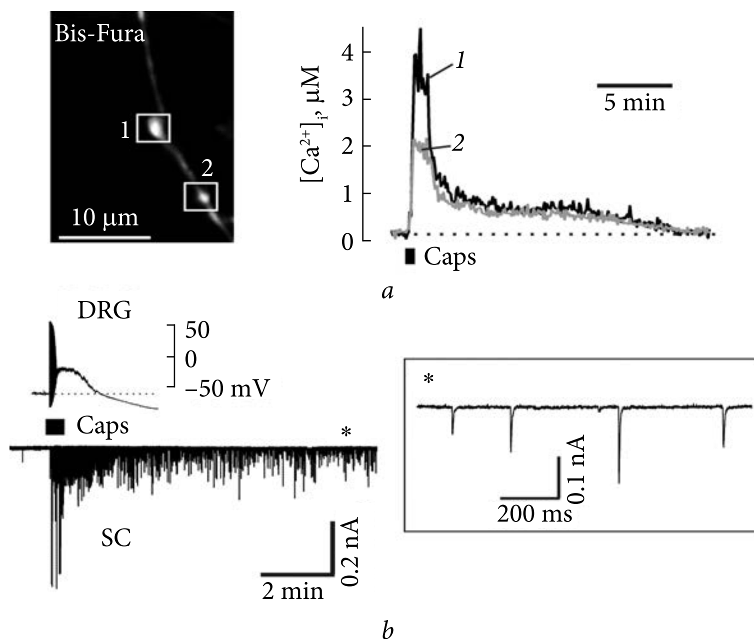

Fig. 1. TRPV1 activation leads to a robust and prolonged synaptic response at the first sensory synapse studied in DRG/SC coculture: *a* — Capsaicin application (1 μM, 30 s) induced long-lasting $[Ca^{2+}]_i$ elevation in two presynaptic boutons (white boxes, *left*) of a DRG neuron loaded with Bis-Fura. The dotted line shows resting $[Ca^{2+}]_i$; *b* — Capsaicin (1 μM, 30 s) induced a transient depolarization and action potential firing in DRG neurons (*upper left*) and a robust and long-lasting increase of excitatory synaptic activity, as measured in SC neurons (*lower left*). The inset (*right*) shows sEPSC recordings on an expanded time scale for the time frame indicated by the asterisk (*)

the cultures containing only SC neurons (n = 4; not shown). Similar to presynaptic $[Ca^{2+}]_i$ elevation, enhanced release of glutamate was observed long after the capsaicin washout, and the sEPSC frequencies were 1.68 ± 0.40 Hz and 1.00 ± 0.33 Hz at 5 and 10 min after stimulation, respectively (n = 7). In contrast, in DRG neurons, capsaicin-induced action potential firing and depolarization rapidly diminished upon washout of the drug (Fig. 1, *b*). Like capsaicin, the endogenous TRPV1 agonist N-arachidonoyl-dopamine (NADA) evoked a robust elevation of presynaptic $[Ca^{2+}]_i$ and a long-lasting enhancement of synaptic activity (Medvedeva et al., 2008). Both the $[Ca^{2+}]_i$ and the synaptic responses were blocked by the TRPV1 selective antagonists capsazepine (10 μM) and SB-366791 (10 μM). The antagonist of glutamate AMPA receptors CNQX (10 μM) completely and reversibly inhibited sEPSCs, suggesting that the synaptic response was mediated by non-NMDA glutamate (most likely AMPA) receptors. Thus, vanilloids induce long-lasting presynaptic $[Ca^{2+}]_i$ elevation and prolonged facilitation of glutamate release in DRG neurons.

Vanilloid-induced presynaptic Ca^{2+} entry is mediated by TRPV1

Vanilloids can potentially induce Ca^{2+} entry in peripheral and central terminals of primary nociceptors directly via TRPV1 and indirectly, by triggering depolarization and activation of voltage-gated Ca^{2+} channels (VGCCs). To examine this important unresolved *question*, we compared presynaptic $[Ca^{2+}]_i$ elevations elicited by two sequential capsaicin applications (1 μM, 10 s, 20 min apart) in control (no additional treatment; Fig. 2, *a*) and after blockade of VGCCs by 200 μM Cd^{2+} dur-

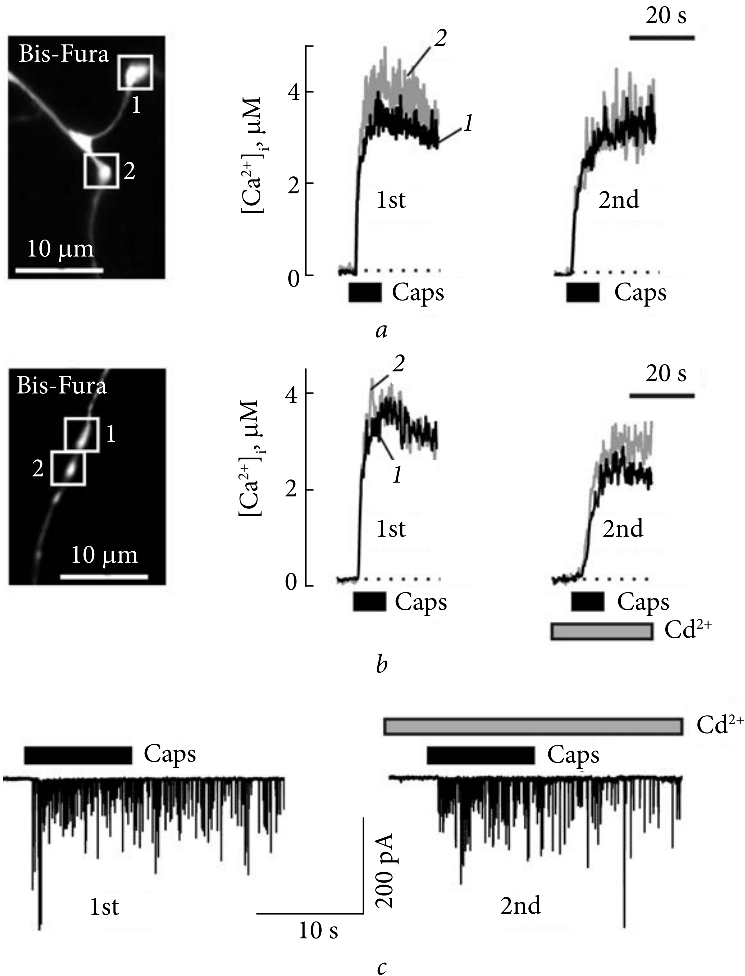

Fig. 2. Vanilloid-induced presynaptic Ca^{2+} entry is mediated by TRPV1: *a, b* — Presynaptic $[Ca^{2+}]_i$ responses in DRG neurons were studied during two sequential applications of capsaicin (1 μM, 10 s, 20 min apart) in control (*a*) or in the presence of 200 μM Cd^{2+} during the second application. *c* — sEPSCs were recorded from a SC neuron voltage-clamped at –60 mV during two sequential applications of capsaicin (1 μM 10 s, 20 min apart), either with no additional treatment (*left*) or in the presence of 200 μM Cd^{2+} (*right*)

ing the second capsaicin application (Fig. 2, *b*). In control experiments, the amplitude of the second Ca^{2+} response (A_2) was slightly smaller than that of the first one (A_1), with an A_2/A_1 ratio of 86 ± 9 % (n=14 boutons/4 cells). This effect was possibly due to incomplete recovery of TRPV1 from desensitization. A comparable decrease in the amplitude of $[Ca^{2+}]_i$ elevation was observed when cells were treated with Cd^{2+} (200 μM) during the second capsaicin application (A_2/A_1 = 81 ± ± 7 %; n = 12 boutons/5 cells; P = 0.68, Student's *t*-test), indicating that VGCCs play a negligible role in capsaicin-induced Ca^{2+} entry. Accordingly, treatment with Cd^{2+} did not prevent a pronounced increase in synaptic activity induced by capsaicin (Fig. 2, *c*). In an additional series of experiments, capsaicin-induced propagation of electrical activity and activation of VGCCs were prevented by blocking neuronal excitation upon treating cells with 1 μM tetrodotoxin (TTX) and by reducing external Na^+ concentration to 35 mM (equimolar replacement by choline). Under these conditions, action potentials were completely blocked, and only a negligible depolarization was developed in response to capsaicin (3 ± 1 mV, n = 7). However the suppression of electrical activity and depolarization by incubation in lowNa^+/TTX did not prevent a rapid capsaicin-induced presynaptic $[Ca^{2+}]_i$ rise and enhancement of synaptic activity (Medvedeva et al., 2008). Together with the Cd^{2+} experiments, these findings suggest that

Ca^{2+} influx via TRPV1 is the major route for presynaptic Ca^{2+} entry following stimulation with vanilloids.

Mitochondria control presynaptic $[Ca^{2+}]_i$ plateau and sustained synaptic activity

The initial vanilloid-induced rapid $[Ca^{2+}]_i$ elevation was followed by a sustained $[Ca^{2+}]_i$ plateau and concomitant synaptic activity (Fig. 1). We further explored the involvement of presynaptic mitochondria in this phenomenon. In order to prevent the spread of electrical excitation induced by vanilloids and, thus, to minimize the influence of extrasynaptic TRPV1, all of the following experiments were performed using a lowNa^+/TTX solution, unless indicated otherwise.

Mitochondria buffer and release Ca^{2+} via distinct mechanisms causing Ca^{2+} to cycle across the mitochondrial membranes (Kostiuk and Verkhratsky, 1995; Shishkin et al., 2002). Ca^{2+} uptake is mediated by the mitochondrial uniporter and is driven by the mitochondrial membrane potential $\Delta\Psi_{mt}$ (−150÷ −180 mV). We blocked Ca^{2+} uptake by depolarizing mitochondria with the electron chain inhibitor antimycin A1, which was added in the presence of F1Fo ATP-synthase inhibitor, oligomycin B, to prevent ATP depletion via the reversed mode of the ATP synthase (Nicholls and Budd, 2000). Combined treatment with 0.3 μM antimycin and 1 μM oligomycin (Ant + Ol) increased the peak of capsaicin-induced presynaptic $[Ca^{2+}]_i$ elevation and virtually eliminated the presynaptic $[Ca^{2+}]_i$ plateau (Fig. 3; n = 17 boutons/4 cells). Impor-

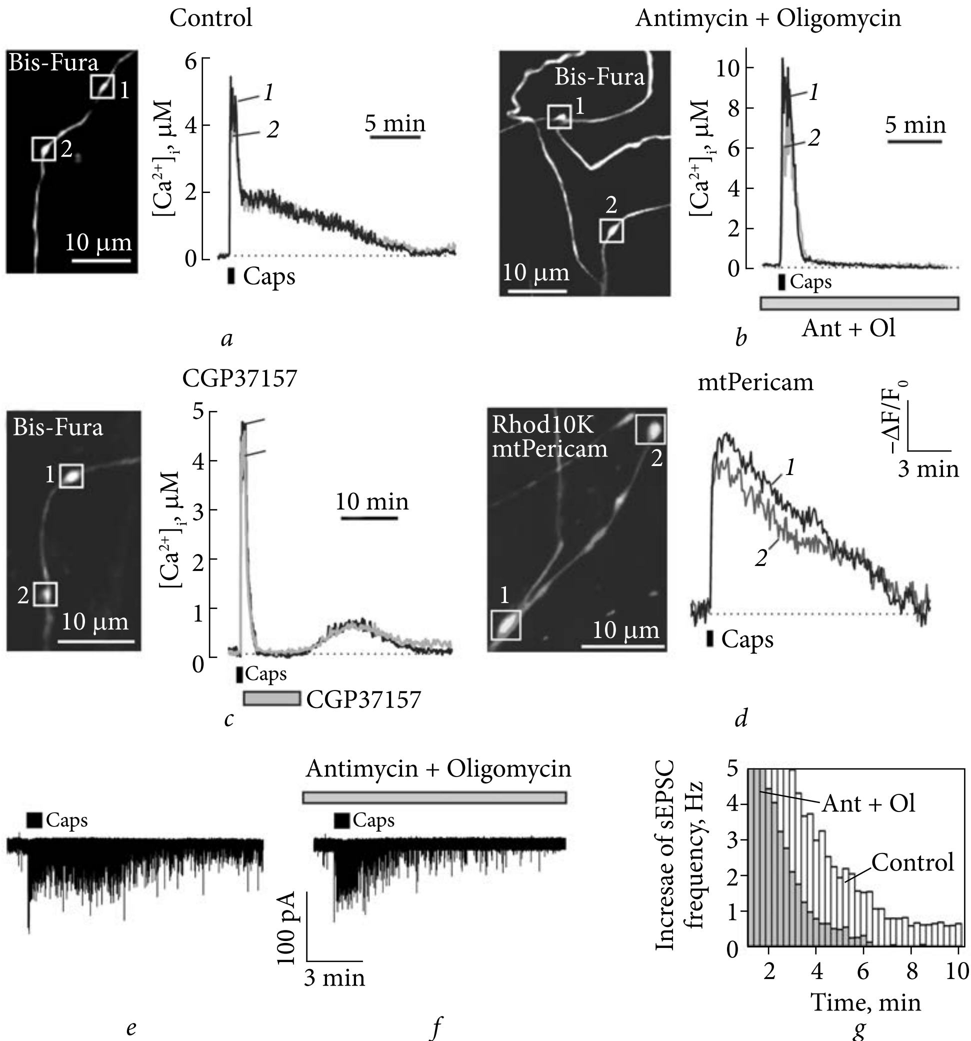

Fig. 3. Mitochondria regulate TRPV1-mediated synaptic transmission at the first sensory synapse: *a-c* — Presynaptic $[Ca^{2+}]_i$ changes induced by capsaicin application (1 μM,\ 30 s) in control cells (*a*) and in cells treated with either 0.3 μM antimycin A1 and 1 μM oligomycin (*b*) or with 10 μM CGP37157 (*c*). *d* — DRG neurons transfected with mtPericam (green) were subsequently loaded with Rhod10K (red) via a patch pipette to visualize presynaptic boutons. The plot shows capsaicin-induced (1 μM, 30 s) changes of mitochondrial Ca^{2+} in two presynaptic boutons. All of the recordings were done in lowNa$^+$/TTX buffer. *e, f* — Representative postsynaptic responses to capsaicin (1 μM, 30 s) in the absence (*e*) or presence of 0.3 μM antimycin and 1 μM oligomycin (*f*) were recorded from SC neurons voltage-clamped at -60 mV in lowNa$^+$/TTX buffer. *g* — Summary of the antimycin and olygomycin (Ant+Ol) effects on the sEPSC frequency obtained from experiments like those described in (*e*) and (*f*). The Y-axis was truncated to emphasize the effects of Ant + Ol on the long-lasting enhancement of synaptic transmission

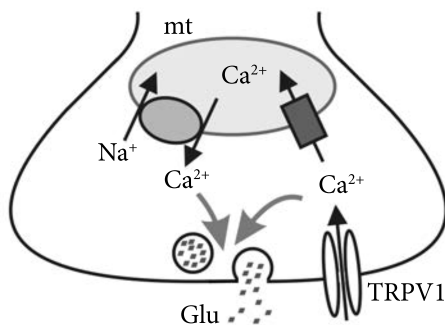

Fig. 4. A model of pre-synaptic $[Ca^{2+}]_i$ signaling mediated by TRPV1 and mitochondria in DRG neurons

tantly, the Ant + Ol treatment markedly shortened capsaicin-induced synaptic activity (Fig. 3, *e-g*). In complementary series of experiments, Ca^{2+} release from mitochondria was blocked by the selective inhibitor of mitochondrial Na^+/Ca^{2+} exchanger CGP37157 (10 μM). This treatment blocked the vanilloid-induced $[Ca^{2+}]_i$ plateau in presynaptic boutons (Fig. 3, *c*).

Interestingly, the washout of CGP37157 was accompanied by a secondary presynaptic $[Ca^{2+}]_i$ elevation, which likely resulted from the release of Ca^{2+} previously trapped within mitochondria by CGP37157. Furthermore, direct imaging of Ca^{2+} within mitochondria using the mitochondria-targeted Ca^{2+} indicator mt-Pericam showed a robust and long-lasting Ca^{2+} accumulation within presynaptic mitochondria in response to TRPV1 activation (Fig. 3, *d*).

The data described above suggest that stimulation of presynaptic TRPV1 induces a biphasic response. During the initial phase, Ca^{2+} entry mediated primarily by TRPV1 triggers presynaptic $[Ca^{2+}]_i$ elevation and glutamate release (Fig. 4). A significant portion of Ca^{2+} entering the cell is taken up by presynaptic mitochondria. Subsequently, this Ca^{2+} is gradually transported back to the cytosol, providing a long-lasting supply of presynaptic Ca^{2+} for glutamate release during the sustained phase of the response, until Ca^{2+} is eventually extruded through the plasma membrane (Fig. 4). Given that the rate of Ca^{2+} efflux from mitochondria is largely constant and independent of Ca^{2+} load, this model predicts that the duration of the synaptic response is determined by the stimulus strength and amount of Ca^{2+} initially captured by mitochondria. Indeed, we found that the duration of presynaptic $[Ca^{2+}]_i$ elevation, enhanced glutamate release, and the resulting electrical activity of postsynaptic neurons significantly increased with increasing the capsaicin concentration from 0.1 to 1 μM (Medvedeva et al., 2008).

We conclude that mitochondria control vanilloid-induced neurotransmission by translating the strength of presynaptic TRPV1 stimulation into duration of postsynaptic response at the first sensory synapse.

Acknowledgements. This work was supported by grants from the NIH and American Heart Association.

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