ELSEVIER

Neuroscience Letters 288 (2000) 57–60

Neuroscience Letters

www.elsevier.com/locate/neulet

The cAMP-dependent kinase pathway does not sensitize the cloned vanilloid receptor type 1 expressed in *Xenopus* oocytes or *Aplysia* neurons

Yong-Seok Lee[a,1], Jin-A Lee[a,1], Jooyoung Jung[b], Uhtaek Oh[b,2], Bong-Kiun Kaang[a,*]

[a]*Molecular Neurobiology Laboratory, Institute of Molecular Biology and Genetics, School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea*
[b]*Sensory Research Group, National Creative Research Initiatives, College of Pharmacy, Seoul National University, Seoul 151-742, South Korea*

Received 13 April 2000; received in revised form 18 May 2000; accepted 19 May 2000

Abstract

Capsaicin-activated channels present in sensory neurons are ligand-gated cation channels that largely account for mediating some types of pain. The cAMP-dependent protein kinase (PKA) signal pathway was suggested to mediate the prostaglandin-induced enhancement of capsaicin-evoked inward current (I_{CAP}) in rat sensory neurons. It is not clear, however, whether PKA acts directly on the capsaicin-sensitive channel that is responsible for I_{CAP}. To address this issue, we overexpressed the cloned capsaicin receptor, VR1, in heterologous expression systems such as *Xenopus* oocytes or *Aplysia* R2 neuron and stimulated PKA pathways. As a result, activation of PKA by applying either 8-bromo-cAMP or forskolin with 3-isobutyl-1-methylxanthine or through activation of β_2 adrenergic receptors failed to enhance I_{CAP} in oocytes or R2 neurons expressing VR1. Our results raise two possibilities. (1) Direct phosphorylation of VR1 by PKA may not be responsible for the sensitization; instead, phosphorylation of regulatory proteins associated with VR1 would account for the sensitization of I_{CAP} evoked by prostaglandin E_2 in dorsal root ganglion (DRG) neurons. (2) DRG neurons may have a different PKA signaling mechanism that is not replicable in *Xenopus* oocytes or *Aplysia* R2 neurons.

Keywords: Capsaicin; Protein Kinase A; *Xenopus* oocytes; *Aplysia* neuron; Vanilloid receptor 1; β_2 Adrenergic receptor; Cystic fibrosis transmembrane regulator

Capsaicin, the main pungent chemical in hot peppers, causes a pain sensation by depolarizing nociceptive sensory neurons [2,15,16]. Excitation of sensory neurons by capsaicin is now known to be mediated by a cation current that is activated by capsaicin [13,18]. A cDNA encoding the capsaicin receptor was cloned from rat sensory neurons [4]. The cloned receptor, also known as the vanilloid receptor type 1 (VR1), is thought to be a major channel-forming subunit because homomeric expression in oocytes or human embryonic kidney (HEK) cells exhibits capsaicin-sensitive currents that resemble in many respects the native capsaicin channels in sensory neurons. Although the kinetic and pharmacological properties of the capsaicin receptors are well characterized, little is known about the modulation of the receptors by the specific signal pathways. A recent study indicates that capsaicin-activated current (I_{CAP}) is sensitized by protein kinase (PKA) when the dorsal root ganglion (DRG) neurons are treated with prostaglandin E_2 [11]. It is not clear, however, whether the sensitization by PKA results from a direct modulation of VR1 or occurs indirectly through the modulation of regulatory proteins associated with VR1. To address this issue, we determined whether the PKA pathway directly sensitizes VR1 in heterologous expression systems such as *Xenopus* oocytes or *Aplysia* neurons.

We expressed VR1 channel in oocytes by microinjecting in vitro-transcribed VR1 cRNA. VR1 current from the injected oocytes was measured as described elsewhere [10] by two-electrode voltage clamp technique with holding

* Corresponding author. Tel.: +82-2-880-7525; fax: +82-2-874-1206.

E-mail addresses: kaang@snu.ac.kr (B.-K. Kaang), utoh@plaza.snu.ac.kr (U. Oh).

[1] These authors contributed equally to this work.

[2] Corresponding co-author: Tel.: +82-2-880-7854; fax: +82-2-872-0596.

0304-3940/00/$ - see front matter
PII: S0304-3940(00)01208-8

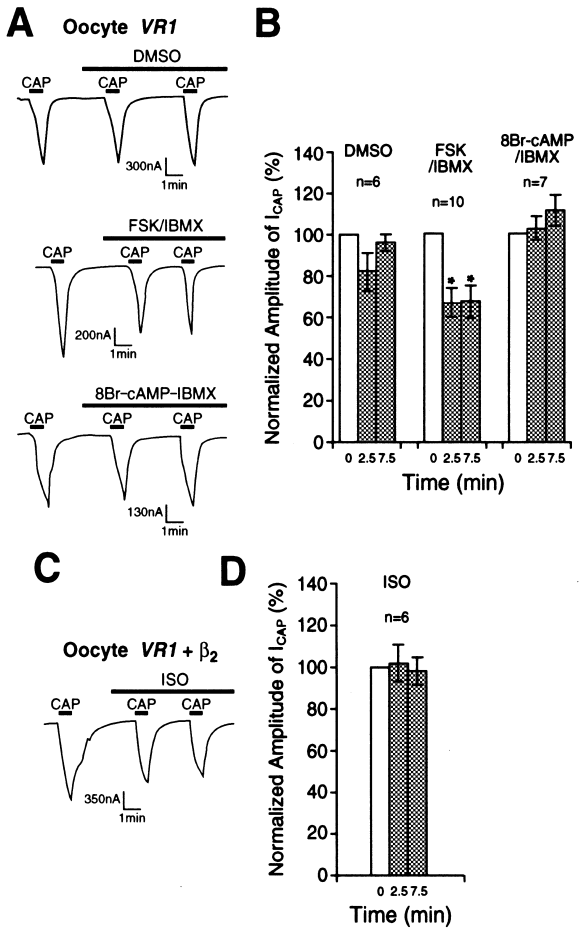

Fig. 1. Capsaicin-evoked inward current (I_{CAP}) from *Xenopus* oocytes expressing VR1. Stage V–VI *Xenopus laevis* oocytes were prepared and injected with VR1 cRNA (2.5 ng) alone (A,B) or with β_2 adrenergic receptor cRNA (250 pg) along with VR1 cRNA (2.5 ng) (C,D). cRNAs were synthesized with SP6 RNA polymerase in vitro as described elsewhere [10]. After injection, oocytes were incubated for 2–3 days at 18°C. An injected oocyte was placed in a chamber (bath volume, 700 μl). Oocyte was perfused continuously with Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) at a flow rate of 4 ml/min. For electrophysiological measurements the oocytes were impaled with two electrodes (resistance 0.5–1.5 MΩ) filled with 3 M KCl. Two-electrode voltage clamp was performed with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) at room temperature. The membrane potential was held at −70 mV. All drugs were dissolved in Ca^{2+}-free ND96 solution and applied to the bath through the perfusion lines. Concentration of drugs in the bath: Capsaicin (CAP), 1 μM; 8-Br-cAMP, 10 μM; Forskolin (FSK), 10 μM; IBMX, 1 mM; DMSO, 0.25%; isoproterenol (ISO), 1 μM; all these drugs were from Sigma Chemical Co. (St. Louis, USA). DMSO was used to dissolve forskolin, 8-Br-cAMP, and IBMX. Capsaicin was dissolved in ethanol. (A,C) Representative recording traces of I_{CAP}. Bars indicate the presence of drugs in the bath solution. (B,D) Group data showing the effect of drugs that activate the PKA pathway. The second and third peak amplitudes (filled bars) were normalized in percentage to the first peak amplitude (blank bar). Group data of normalized values are shown as the mean ± SEM. Time indicates min after the drugs were applied. n, the number of oocytes recorded. *$P < 0.01$, two-tail Wilcoxon signed rank test.

potential of –70 mV. As shown in Fig. 1A, a brief (30 s) application of capsaicin (1 μM) (Sigma Chemical Co.) evoked a great inward current (I_{CAP}) in the oocytes that were injected with VR1 cRNA. Repeated application of capsaicin in 5-min intervals exhibited a stable magnitude of I_{CAP} without an apparent desensitization in the Ca^{2+}-free bath solution. We then applied chemicals that specifically stimulate the PKA pathway to examine whether PKA activation leads to a change in VR1-mediated inward current. We used 8-bromo-cAMP (8-Br-cAMP, a membrane-permeable PKA activator, Sigma Chemical Co.), 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor, Sigma Chemical Co.), and forskolin (an adenylyl cyclase activator, Sigma Chemical Co.) to specifically activate the PKA pathway. As shown in Fig. 1A,B, application of neither 8-Br-cAMP (10 μM) with IBMX (1 mM) nor forskolin (10 μM) with IBMX (1 mM) augmented the capsaicin-evoked inward current. Instead, forskolin with IBMX reduced I_{CAP} significantly ($P < 0.01$) (Fig. 1B).

As an alternative way to activate PKA, we expressed β_2 adrenergic receptor in oocytes by microinjecting in vitro-transcribed cRNA for the receptor as described elsewhere [5]. This receptor is known to be coupled to Gs protein, thus leading to the activation of adenylyl cyclase [9]. In oocytes co-expressing VR1 and β_2 adrenergic receptor, application of a β_2 adrenergic receptor agonist, isoproterenol (1 μM) (Sigma Chemical Co.), failed to augment I_{CAP} (Fig. 1C,D). Furthermore, the failure of I_{CAP} augmentation by PKA was also observed even in the presence of Ca^{2+} in the bath that is required for desensitization of I_{CAP} [19] (data not shown).

For control experiments to test whether our protocols of the chemical application activate the PKA pathway effectively in oocytes, we co-expressed β_2 adrenergic receptor with cystic fibrosis transmembrane regulator (CFTR). The CFTR is a chloride channel that is known to be activated by PKA-mediated phosphorylation rather than through direct gating by cAMP [1,3,17]. Therefore, the heterologous expression of CFTR has been widely used as a marker for PKA activation in *Xenopus* oocytes [5,8,12,17]. In oocytes expressing β_2 adrenergic receptor and CFTR, isoproterenol (1 μM) invariably activated the channel and evoked inward current (Fig. 2A). We also applied 8-Br-cAMP (10 μM) or forskolin (10 μM) with IBMX (1 mM) to see if CFTR was activated. Applications of 8-Br-cAMP or forskolin also evoked the CFTR current, indicative of the PKA activation by these manipulations (Fig. 2A). IBMX potentiated the CFTR current that was induced by 8-Br cAMP or forskolin (Fig. 2A). None of the drugs tested elicited any response in uninjected oocytes (Fig. 2B). Taken together, these results indicate that the activation of PKA fails to augment the capsaicin-evoked VR1 current even when its activation effectively opens phosphorylation-dependent channel such as CFTR.

Lack of augmentation of VR1 by the PKA pathway was also observed in other heterologous system, an *Aplysia* giant neuron R2 expressing VR1 and β_2 adrenergic receptors.

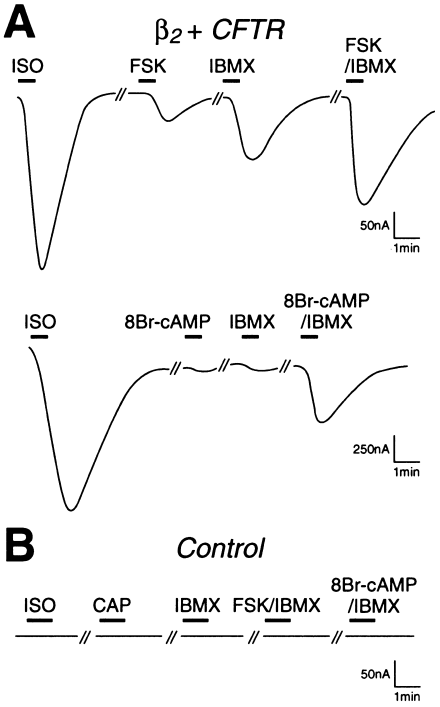

Fig. 2. CFTR current produced by PKA activation. (A) Two representative recording traces from oocytes that were injected with CFTR cRNA (2.5 ng) and β_2 adrenergic receptor cRNA (250 pg). (B) A control recording trace from an oocyte not injected with cRNA. Voltage clamping and drug application were done as described in Fig. 1. Drug was washed out for 5 min before application of another drug.

Heterologous expression in R2 neurons was achieved by microinjection of the expression plasmids pNEXδ-VR1 and pNEXδR-β_2 as described elsewhere [6,7]. The R2 neuron is normally electrically silent. But, in neurons expressing VR1, rapid depolarization with a brief volley of action potentials was observed after capsaicin (1 μM) was applied (Fig. 3A, left). In these neurons, capsaicin (1 μM) exhibited an inward current (Fig. 3B, left). In contrast, uninjected R2 neurons did not respond to capsaicin (1 μM) at all (Fig. 3A,C, right). To activate PKA pathways, isoproterenol (1 μM) was applied to *Aplysia* R2 neurons expressing both VR1 and β_2 adrenergic receptor. In these neurons, treatment with isoproterenol failed to augment but reduced I_{CAP} (Fig. 3B). The reduction of I_{CAP} after the repeated applications of CAP in 5-min intervals seemed to result from high concentration of Ca^{2+} present in artificial seawater. This reduction or desensitization of I_{CAP} also occurred to a similar degree even without activation of β_2 adrenergic receptor by isoproterenol (data not shown). For control experiments testing the efficiency of the expressed β_2 adrenergic receptor in activating PKA, CFTR was co-expressed with β_2 adrenergic receptor by microinjecting into R2 neurons the plasmids pNEXδ-CFTR and pNEXδR-β_2. In these neurons, isoproterenol (1 μM) invariably evoked large inward CFTR currents, indicating the specific activation of PKA by activation of β_2 adrenergic receptor (Fig. 3C, left).

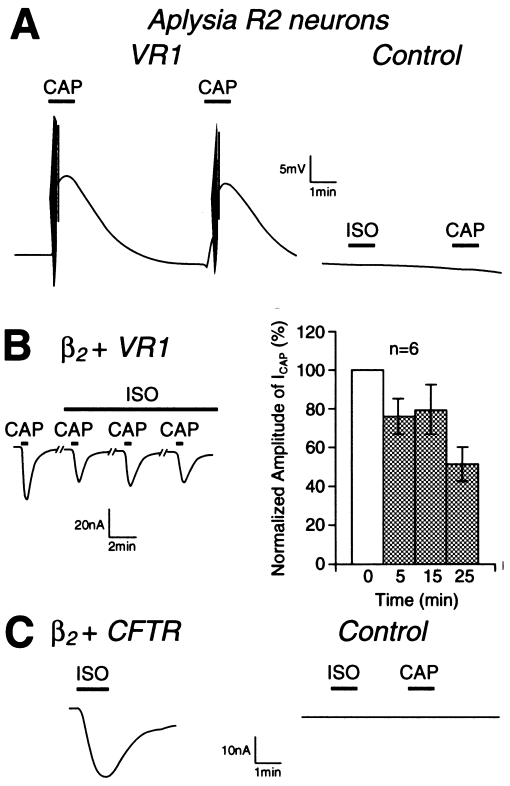

Fig. 3. VR1 expression in *Aplysia* R2 neuron. Full-length cDNAs for VR1, β_2 adrenergic receptor, and CFTR were subcloned into restriction sites, HindIII/KpnI, NcoI/XhoI, and AvaI/SacI of the *Aplysia* expression vector pNEXδ [7] to generate pNEXδ-VR1, pNEXδR-β_2, and pNEXδ-CFTR, respectively. DNA microinjection into *Aplysia* neurons and preparation of the abdominal ganglion are described elsewhere [6,7]. pNEXδ-GFP (4 mg/ml) was included in DNA injection solution as a marker of gene transfer. Only green fluorescent neurons were selected for electrophysiological recording. (A) Electrical response to 1 μM capsaicin (*CAP*) of R2 neuron expressing VR1 (Left) and uninjected control R2 neuron (Right). In VR1-expressing neuron, a transient volley of spikes was generated during early phase of capsaicin-elicited membrane depolarization (Left). Neither capsaicin (1 μM) nor isoproterenol (1 μM) (ISO) produced any change in the membrane potential (Right). Recording was done in normal artificial seawater (ASW) (in mM: 460 NaCl, 10 KCl, 55 $MgCl_2$, 11 $CaCl_2$, 10 HEPES, pH 7.6). Concentration of pNEXδ-VR1 in DNA injection solution was 3 mg/ml. (B) Capsaicin-evoked VR1 current in R2 neuron expressing VR1 and β_2 adrenergic receptor. Two-electrode voltage-clamp was done as described in Fig. 1 except that bath solution was Na^+-free ASW (in mM: 460 Tris–Cl, 10 KCl, 55 $MgCl_2$, 11 $CaCl_2$, 10 HEPES, pH 7.6) containing a K^+ channel blocker, 10 mM tetraethylammonium (Eastman Kodak) (left). Holding potential was −70 mV. Right panel shows group data. The second, third, and fourth peak amplitudes (filled bars) were normalized in percentage to the first peak amplitude (blank bar). Normalized values are shown as mean ± SEM. Time indicates min after the drugs were applied. The concentration of pNEXδ-VR1 and pNEXδR-β_2 was 3 mg/ml each. (C) CFTR current evoked by activation of coexpressed β_2 adrenergic receptor in R2 neuron (Left). Two-electrode voltage-clamp was done as described in Fig. 1 except that bath solution was normal ASW. Neither isoproterenol (ISO) nor capsaicin (CAP) produced any current in uninjected control R2 neuron in a voltage-clamp mode (Right). The concentration of pNEXδR-β_2 and pNEXδ-CFTR was 5 mg/ml and 1 mg/ml, respectively. pNEXδR is a pNEXδ derivative that contains NcoI and XhoI sites in multiple cloning sites.

Isoproterenol (1 μM) alone did not evoke inward currents in uninjected R2 neurons (Fig. 3A,C, right). Taken together, these results further suggest that the PKA pathway does not either sensitize capsaicin-induced VR1 current or recover VR1 from desensitization.

Prostaglandins are known to sensitize capsaicin-evoked current in the DRG neurons [11,14]. Sensitization of the capsaicin receptor is mediated possibly via PKA pathway because block of PKA reduced the sensitizing effect of prostaglandin [11]. Furthermore, forskolin and cAMP analogs like prostaglandin E_2 produced 2- to 4-fold transient increases in the amplitude of I_{CAP} with a time to peak of approximately 10 min [11]. Pitchford and Levine [14] also illustrated that treatment of rat sensory cells with prostaglandins or the cAMP analogs resulted in an increase in whole cell currents of I_{CAP}. Single-channel current analysis done by Lopshire and Nicol [11] suggested that phosphorylation by PKA increased the sensitivity of the capsaicin receptor or the open channel probability of the channel. Our data, however, did not support the augmentation of I_{CAP} by PKA because the activation of PKA by various mechanisms did not augment or sensitize I_{CAP} through VR1 in two different heterologous expression systems such as *Xenopus* oocytes and *Aplysia* neuron R2. Currently, it seems uncertain why there is a discrepancy between results of the present experiments and those of Lopshire and Nicol [11]. We do not rule out a possibility that nociceptive DRG neurons may have a PKA signaling mechanism that is different from those in *Xenopus* oocytes or *Aplysia* neurons. Another possible explanation is that prostaglandin E_2 may sensitize I_{CAP} by PKA pathway acting on regulatory proteins associated with VR1 rather than VR1 itself. It would be interesting to identify such regulatory proteins whose phosphorylation by PKA leads to sensitization of VR1.

We thank Dr David Julius (VR1), Dr Lap-Chee Tsui (CFTR), Dr Robert Lefkowitz (β_2-adrenergic receptor), and Dr Douglas Melton (pSP64T) for their generous gifts of the plasmids used in this study. This research was supported by the Korean Ministry of Health & Welfare (HMP-96-D-1-1020) and partly by the Korean Ministry of Science and Technology under the Brain Science Research Program. J.-A.L is supported by BK21 Research Fellowship from the Korean Ministry of Education.

[1] Bear, C.E., Duguay, F., Naismith, A.L., Kartner, N., Hanrahan, J.W. and Riordan, J.R., Cl-channel activity in *Xenopus* oocytes expressing the cystic fibrosis gene, J. Biol. Chem., 266 (1991) 19142–19145.

[2] Bevan, S. and Szolcsanyi, J., Sensory neuron-specific actions of capsaicin: mechanisms and applications, Trends Pharmacol. Sci., 11 (1990) 330–333.

[3] Birnbaum, A.K., Wotta, D.R., Law, P.Y. and Wilcox, G.L., Functional expression of adrenergic and opioid receptors in *Xenopus* oocytes: interaction between alpha 2- and beta 2-adrenergic receptors, Mol. Brain. Res., 28 (1995) 72–80.

[4] Caterina, M.J., Shumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway, Nature, 389 (1997) 816–824.

[5] Chang, D.J., Li, X.-C., Lee, Y.S., Kim, H.K., Kim, U.S., Cho, N.J., Lo, X., Weiss, K.R., Kandel, E.R. and Kaang, B.-K., Activation of a heterologously expressed octopamine receptor coupled only to adenylyl cyclase produces all the features of presynaptic facilitation in *Aplysia* sensory neurons, Proc. Natl. Acad. Sci. USA, 97 (2000) 1829–1834.

[6] Kaang, B.-K., Parameters influencing ectopic gene expression in *Aplysia* neurons, Neurosci. Lett., 221 (1996) 29–32.

[7] Kaang, B.-K., Kandel, E.R. and Grant, S.G.N., Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in *Aplysia* sensory neurons, Neuron, 10 (1993) 427–435.

[8] Kaneko, S., Nakamura, S., Adachi, K., Akaike, A. and Satoh, M., Mobilization of intracellular Ca^{2+} and stimulation of cyclic AMP production by kappa opioid receptors expressed in *Xenopus* oocytes, Mol. Brain. Res., 27 (1994) 258–264.

[9] Kobilka, B.K., MacGregor, C., Daniel, K., Kobila, T.S., Caron, M.G. and Lefkowitz, R.J., Functional activity and regulation of human β-adrenergic receptors expressed in *Xenopus* oocytes, J. Biol. Chem., 262 (1987) 15796–15802.

[10] Lee, Y.-S., Park, Y.-S., Chang, D.J., Hwang, J.M., Min, C.K., Kaang, B.-K. and Cho, N.J., Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorabditis elegans*, J. Neurochem., 72 (1999) 58–65.

[11] Lopshire, J.C. and Nicol, G.D., The cAMP transduction cascade mediates the prostaglandin E_2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies, J. Neurosci., 18 (1998) 6081–6092.

[12] Luebke, A.E., Dahl, G.P., Roos, B.A. and Dickerson, I.M., Identification of a protein that confers calcitonin gene-related peptide responsiveness to oocytes by using a cystic fibrosis transmembrane conductance regulator assay, Proc. Natl. Acad. Sci. USA, 93 (1996) 3455–3460.

[13] Oh, U., Hwang, S.W. and Kim, D., Capsaicin activates a nonselective cation channel in cultured neonatal rat dorsal root ganglion neurons, J. Neurosci., 16 (1996) 1659–1667.

[14] Pitchford, S. and Levine, J.D., Prostaglandins sensitize nociceptors in cell culture, Neurosci. Lett., 132 (1991) 105–108.

[15] Szallasi, A., The vanilloid (capsaicin) receptor: receptor types and species specificity, Gen. Pharmacol., 25 (1994) 223–243.

[16] Szolcsanyi, J., Actions of capsaicin on sensory neurons. In J. Wood (Ed.), Capsaicin in the Study of Pain, Academic Press, London, 1993, pp. 1–26.

[17] Uezono, Y., Bradley, J., Min, C., McCarty, N.A., Quick, M., Riordan, J.R., Chavkin, C., Zinn, K., Lester, H.A. and Davidson, N., Receptors that couple to 2 classes of G proteins increase cAMP and activate CFTR expressed in *Xenopus* oocytes, Receptors Channels, 1 (1993) 233–241.

[18] Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeats, J. and Bevan, S., Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture, J. Neurosci., 8 (1988) 3208–3220.

[19] Yeats, J.C., Docherty, R.J. and Bevan, S., Calcium-dependent and –independent desensitization of capsaicin-evoked responses in voltage-clamped adult rat dorsal root ganglion (DRG) neurones in culture, J. Physiol. (Lond.), 446 (1992) 390 p.