Biochemical Pharmacology, Vol. 59, pp. 1403–1406, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00260-4

SHORT COMMUNICATION

Capsaicin-Stimulated Release of Substance P from Cultured Dorsal Root Ganglion Neurons: Involvement of Two Distinct Mechanisms

John Purkiss, Mary Welch, Sarah Doward and Keith Foster*
CAMR (Centre for Applied Microbiology and Research), Porton Down, Salisbury, Wiltshire SP4 OJG, U.K.

ABSTRACT. Capsaicin, the pungent component of "hot" chilli peppers, selectively activates a distinct population of primary sensory neurons responsive to noxious stimuli. Many of these fibres express neuropeptides including the tachykinin, substance P. Using cultured dorsal root ganglion neurons, we found that capsaicin (10 μM) stimulated a 2-fold increase in release of substance P in the absence of extracellular Ca^{2+}. Elevated potassium (75 mM) was unable to induce release under these conditions. The introduction of Ca^{2+} enhanced capsaicin-induced release and brought about a robust response to potassium. Preincubation of cells with botulinum neurotoxin A (100 nM) completely blocked potassium-induced release but the capsaicin response, in the absence of Ca^{2+}, was unaffected. However, toxin treatment dramatically reduced capsaicin-stimulated release in the presence of Ca^{2+}. It is concluded that capsaicin induces release of substance P from dorsal root ganglion neurons via two mechanisms, one requiring extracellular Ca^{2+} and the intact synaptosomal-associated protein 25 kDa (SNAP-25), and the other independent of extracellular Ca^{2+} and not involving SNAP-25. BIOCHEM PHARMACOL **59**;11:1403–1406, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. capsaicin; botulinum neurotoxin A; dorsal root ganglion neurons; neurotransmitter release; synaptosomal-associated protein of 25 kDa (SNAP-25); substance P

Capsaicin (8-methyl *N*-vanillyl-6-noneamide) is the pungent component of plants of the capsicum family. The sensation of burning pain it elicits is due to the selective activation of unmyelinated C-fibres and thinly myelinated Aδ primary sensory neurons [1]. These fibres are polymodal nociceptors whose cell bodies are located in the dorsal root ganglion. Transmission to the CNS occurs in the dorsal horn of the spinal cord where glutamate is most likely to be the fast transmitter [2]. However, expression and release of neuropeptides, including substance P, is also known to occur [3].

Activation of capsaicin-sensitive neurons is dependent on the presence of a recently cloned receptor which is a non-selective cation channel [4]. The channel is insensitive to blocking agents such as dihydropyridines, ω-conotoxin, and tetrodotoxin [5], and capsaicin-induced neurotransmitter release is unaffected by these conventional channel blockers [6]. Ca^{2+} entering through the activated capsaicin receptor/channel therefore appears able to directly stimulate the release mechanism.

Elevation of intracellular Ca^{2+} is widely accepted as the signal for neurotransmitter release, though the source of Ca^{2+} may vary according to the stimulus [7]. In accordance with this, substance P release in the spinal cord in response to both depolarising and capsaicin stimuli has been reported to be dependent on the presence of extracellular Ca^{2+} [8, 9]. However, using cultured eDRG† neurons, we describe, in this report, a component of the capsaicin-stimulated substance P release that occurs in the absence of extracellular Ca^{2+}. Further, we describe different dependencies of the Ca^{2+}-dependent and -independent mechanisms of release on SNAP-25, which is considered a key component in vesicle docking and fusion [10]. This we have shown using the SNAP-25-specific clostridial Zn^{2+}-dependent endopeptidase, BoNT/A.

MATERIALS AND METHODS

eDRG Neuron Cultures

Rat eDRG neurons were prepared from 14–16-day gestation embryos dissected under low-power magnification. Ganglia were treated with trypsin/EDTA solution (Sigma) for 40 min and fully dissociated in growth medium (Dulbecco's modified Eagle's medium, 5% inactivated foetal bovine serum, 5 mM glutamine, B27 supplement [GIBCO],

* Corresponding author: Dr. John Purkiss, CAMR (Centre for Applied Microbiology and Research), Porton Down, Salisbury, Wiltshire SP4 0JG, U.K. Tel. 44 (0) 1980 612733; FAX 44 (0) 1980 612731; E-mail: john.purkiss@CAMR.org.uk
Received 5 May 1999; accepted 25 November 1999.

† *Abbreviations:* BoNT/A, botulinum neurotoxin A; BSS, balanced salt solution; eDRG, embryonic dorsal root ganglion, and SNAP-25, synaptosomal-associated protein of 25 kDa.

100 ng/mL of nerve growth factor [Becton Dickinson], 0.6% dextrose) by trituration through fire-polished glass Pasteur pipettes. Cells were plated onto Matrigel-coated (Becton Dickinson) 12-well tissue culture plates (Falcon) at a density equivalent to 5 embryos per plate. After two days, cultures were treated with 10 μM cytosine arabinoside (Sigma) to control growth of dividing cells. Medium was changed three times per week and cells were used two weeks after preparation.

Substance P Release Assay

Cells were washed twice with 1 mL BSS with no added Ca^{2+} (137 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 5 mM glucose, 20 mM HEPES, pH 7.4). Basal release was established with 0.5 mL Ca^{2+}-free BSS applied for 4.5 min. Cells were then stimulated for 4.5 min with capsaicin or 75 mM KCl (67 mM NaCl) in BSS with no added Ca^{2+} and 0.5 mM EGTA. Finally, cells were stimulated for 4.5 min with capsaicin or 75 mM KCl in BSS with 2 mM $CaCl_2$. Superfusates were transferred to ice and then centrifuged. Samples of supernatant were treated with 2 M acetic acid, 0.1% trifluoroacetic acid (final concentrations) and dehydrated in a spinvac centrifuge. Total substance P was determined in 2 M acetic acid, 0.1% trifluoroacetic acid cell extracts, following dehydration. Substance P immunoreactivity was measured with an enzyme immunoassay kit (Cayman Chemical Co.) using the supplier's protocol.

Western Blot Analysis of SNAP-25

SNAP-25 was analysed by Western blot of the hydrophobic proteins extracted from cells treated for 18–20 hr with BoNT/A. Proteins were extracted by the method of Bordier [11] and separated by SDS–PAGE on 4–20% Tris–glycine gels (Novex). Proteins were transferred to nitrocellulose and blots probed with a rabbit primary antibody specific for intact SNAP-25 (raised against a synthetic peptide of SNAP-25, residues 195–206.). The secondary antibody (donkey anti-rabbit horseradish peroxidase conjugate, Jackson ImmunoResearch Laboratories Inc.) was detected using Amersham Enhanced Chemiluminescence reagent and Hyperfilm (Amersham) according to the manufacturer's instructions.

RESULTS

Stimulation of cultured eDRG neurons with 75 mM KCl did not induce release of substance P above basal level when Ca^{2+} was absent from the medium (Fig. 1). When Ca^{2+} was introduced into the medium, a 5-fold increase in substance P release was seen (Fig. 1). By contrast, 10 μM capsaicin induced a 2-fold increase in release in the absence of extracellular Ca^{2+}. This stimulation rose to six times the basal level when Ca^{2+} was included (Fig. 1). The concentration of capsaicin used (10 μM) was not cytotoxic, as determined by trypan blue exclusion, over the time of these experiments (data not shown).

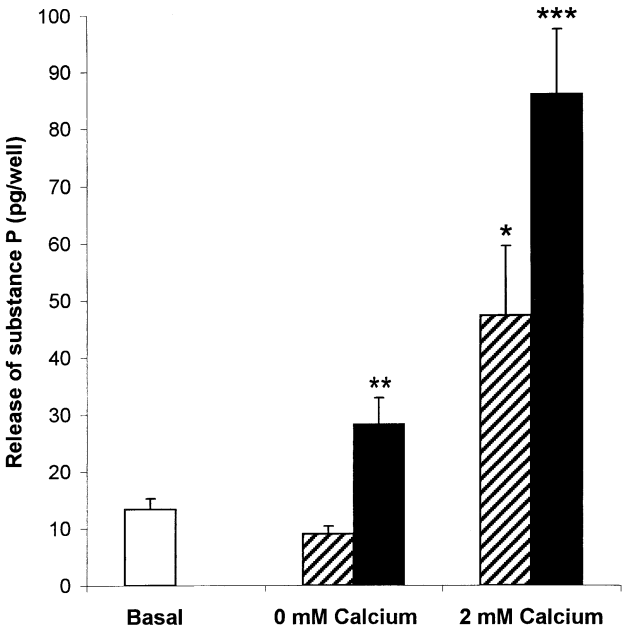

FIG. 1. Calcium dependence of KCl- and capsaicin-stimulated substance P release. Release is expressed ± SE in pg substance P/well. As an example, basal release (open bar) when expressed as a percentage of the total cellular content was 0.43 ± 0.07% (N = 8). Cells were stimulated with KCl (75 mM, N = 4, hatched bars) or capsaicin (10 μM, N = 4, filled bars) for 4.5 min in the absence (0 mM calcium) or presence (2 mM calcium) of Ca^{2+} (2 mM). The data were collected from two separate experiments. Release significantly higher than basal denoted by *($P < 0.05$), **($P < 0.005$), and *($P < 0.00005$).**

The dose-dependence of capsaicin-induced release in the absence of Ca^{2+} was found initially to be a very shallow curve. Release was only significantly enhanced above basal at 10 μM ($P < 0.05$) and 30 μM ($P < 0.0001$) capsaicin. The curve was not fully described at 30 μM (Fig. 2). In the presence of Ca^{2+}, the curve was composed of two rising phases separated by a plateau. All concentrations of capsaicin tested caused significant enhancement of release ($P < 0.05$). The second rising phase corresponded to the curve generated in the absence of Ca^{2+}. The first rise of the curve was complete at 0.3 μM capsaicin.

Treatment of eDRGs with a range of concentrations of BoNT/A gave a dose-dependent reduction in SNAP-25, as determined by Western blot, between 0.01 and 100 nM (Fig. 3). Complete SNAP-25 cleavage was consistently produced by 100 nM BoNT/A. Following this treatment, cells were stimulated with 75 mM KCl or 10 μM capsaicin. The KCl-induced release of substance P was completely abolished by neurotoxin, whereas the Ca^{2+}-independent, capsaicin-induced release was unaffected by toxin (Fig. 4 compared to Fig. 1). However, the Ca^{2+}-dependent component of capsaicin-induced release was markedly reduced by BoNT/A treatment (Fig. 4 compared to Fig. 1). This is shown by a reduction in the increase induced by the

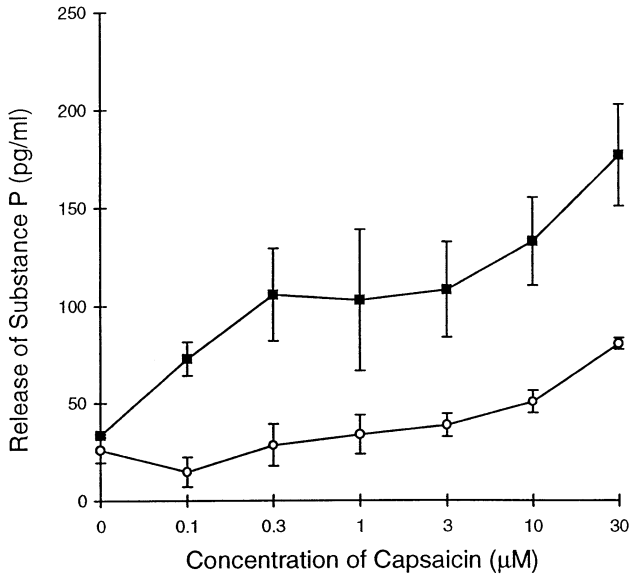

FIG. 2. Dose dependence of capsaicin-induced substance P release in the presence (■) and absence (○) of Ca^{2+}. Release is expressed as pg/well of substance P. Cells were stimulated with capsaicin in the absence of Ca^{2+} for 4.5 min and then in the presence of Ca^{2+} for 4.5 min. Results are the means ± SE of three experiments each determined in duplicate.

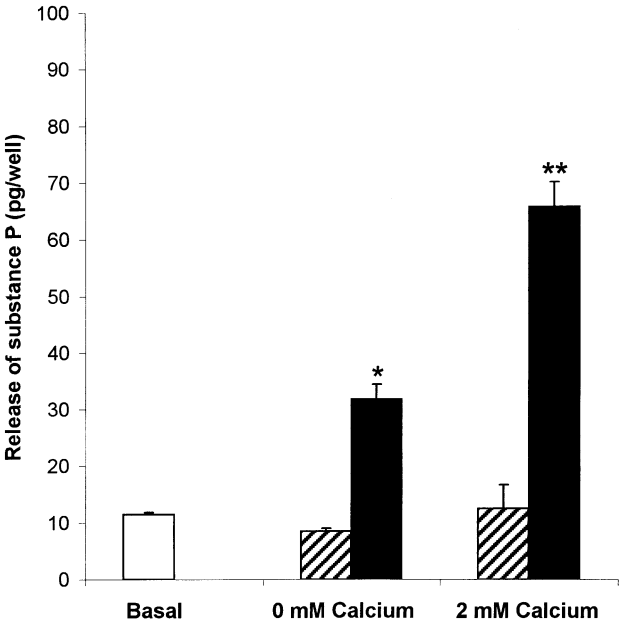

FIG. 4. KCl- and capsaicin-stimulated substance P release from BoNT/A-treated cells. Release is expressed ± SE in pg substance P/well. As an example, basal release (open bar) when expressed as a percentage of the total cellular content was 0.38 ± 0.06% (N = 8). Following 18–20 hr BoNT/A treatment (100 nM), cells were stimulated with KCl (75 mM, N = 4, hatched bars) or capsaicin (10 μM, N = 4, filled bars) for 4.5 min in the absence (0 mM calcium) or presence (2 mM calcium) of Ca^{2+} (2 mM). The data were collected from two separate experiments. Release significantly higher than basal denoted by *($P < 0.001$) and **($P < 0.00005$).

introduction of Ca^{2+} such that, without BoNT/A treatment, Ca^{2+} increased capsaicin-induced release 3-fold, although after BoNT/A treatment this increase was only 2-fold.

DISCUSSION

Capsaicin-induced neurotransmitter release from sensitive neurons has previously been described as entirely dependent upon extracellular Ca^{2+} [6, 8, 9]. However, here we describe stimulation of substance P release from cultured eDRG neurons when Ca^{2+} was absent from the superfusing medium. Re-instatement of Ca^{2+} enhanced the stimulated release. Further, we have shown that the Ca^{2+}-independent component of the release is insensitive to the action of BoNT/A. It would therefore appear that in these cultured cells capsaicin induces substance P release by two mechanisms: the first is Ca^{2+}-dependent, maximally stimulated by 0.3 μM capsaicin, and requires intact SNAP-25; and the second is Ca^{2+}-independent, becomes activated at 3–10 μM capsaicin, and is insensitive to BoNT/A. The latter observation indicates that release is through a mechanism that does not have SNAP-25 as an essential component.

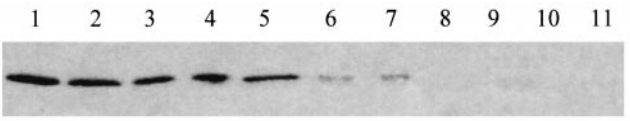

FIG. 3. Western blot analysis of SNAP-25 in eDRG neurons following BoNT/A treatment. Hydrophobic proteins of eDRG neurons, treated with BoNT/A for 18 hr, were probed with antibody specific for intact SNAP-25. Lane 1, control; lanes 2 and 3, 0.01 nM; lanes 4 and 5, 0.1 nM; lanes 6 and 7, 1 nM; lanes 8 and 9, 10 nM; and lanes 10 and 11, 100 nM BoNT/A. The blot shown is from one experiment representative of three.

The Ca^{2+}-independent stimulation of substance P release by capsaicin would appear to be a previously undocumented phenomenon. Previous studies that have described total dependence on extracellular Ca^{2+} have differed from ours in several ways, the most significant difference being that capsaicin was used at lower concentrations (1 μM [8] and 3.3 μM [9]). At this level of stimulation, the Ca^{2+}-independent component, in the present study, was only just observable. Also, the present study concerned embryonic rat neurons in culture, whereas the other two studies employed tissue slices from 4-day-old and adult rats ([8] and [9], respectively).

The mechanism of Ca^{2+}-independent capsaicin-stimulated release still remains to be established. The cytotoxic action of capsaicin is dependent on Ca^{2+}-influx, so that cell death and lysis cannot be responsible for the observed release in the absence of extracellular Ca^{2+} [12]. Though inositol phosphate turnover, arachidonic acid release, and cGMP accumulation have all been shown to occur with capsaicin stimulation in the presence of Ca^{2+}, the removal of Ca^{2+} abolishes all the alterations in these second messenger levels [13]. Ca^{2+}-independent BoNT-insensitive neurotransmitter release is, however, not unprecedented.

Black widow spider neurotoxin, α-latrotoxin, stimulates noradrenaline release from BoNT/C and D pretreated synaptosomes in the absence of Ca^{2+}. α-Latrotoxin is known to interact with a specific receptor, but the Ca^{2+}-independent BoNT-insensitive release mechanism is believed to be non-vesicular and via membrane pores [14]. A similar mechanism is unlikely to be responsible for release of a neuropeptide. The lack of dependence of Ca^{2+}-independent capsaicin-stimulated release on SNAP-25 therefore remains suggestive of a novel mechanism. However, if present, the non-neuronal homologue of SNAP-25 (SNAP-23) could have facilitated release from intoxicated cells, since it is resistant to BoNT/A [15]. We do not as yet know if cultured eDRG neurons express SNAP-23.

Multiple capsaicin receptors have been proposed previously [16]. Though the cloned receptor is clearly a receptor/channel [4], other receptors may exist with alternative coupling mechanisms to the release process. Thus, the two different release mechanisms described here may be operated by separate receptor populations with differing capsaicin sensitivities.

References

1. Szolcsányi J, Actions of capsaicin on sensory neurones. In: *Capsaicin in the Study of Pain* (Ed. Wood JN), pp. 1–26. Academic Press, London, 1993.
2. Salt TE and Hill RG, Neurotransmitter candidates of somatosensory primary afferent fibres. *Neuroscience* **10:** 1083–1103, 1983.
3. Urban L, Thompson SW and Dray A, Modulation of spinal excitability: Co-operation between neurokinin and excitatory amino acid transmitters. *Trends Neurosci* **17:** 432–438, 1994.
4. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD and Julius D, The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* **389:** 816–824, 1997.
5. Wood JN, Winter J, James IF, Rang HP, Yeats J and Bevan S, Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci* **8:** 3208–3220, 1988.
6. Del Bianco E, Santicioli P, Tramontana M, Maggi CA, Cecconi R and Geppetti P, Different pathways by which extracellular Ca^{2+} promotes calcitonin gene-related peptide release from terminals of capsaicin-sensitive afferents of guinea pigs: Effect of capsaicin, high K^+ and low pH media. *Brain Res* **566:** 46–53, 1991.
7. Purkiss JR, Nahorski SR and Willars GB, Mobilisation of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores supports bradykinin- and muscarinic-evoked release of [^{3}H]noradrenaline from SH-SY5Y cells. *J Neurochem* **64:** 1175–1183, 1995.
8. Theriault E, Otsuka M and Jessell T, Capsaicin-evoked release of substance P from primary sensory neurons. *Brain Res* **170:** 209–213, 1979.
9. Gamse R, Molnar A and Lembeck F, Substance P release from spinal cord slices by capsaicin. *Life Sci* **25:** 629–636, 1979.
10. Sudhöf TC, The synaptic vesicle cycle: A cascade of protein–protein interactions. *Nature* **375:** 645–653, 1995.
11. Bordier C, Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* **256:** 1604–1607, 1981.
12. Chard PS, Bleakman D, Savidge JR and Miller RJ, Capsaicin-induced neurotoxicity in cultured dorsal root ganglion neurons: Involvement of calcium-activated proteases. *Neuroscience* **65:** 1099–1108, 1995.
13. James IF, Ninkina NN and Wood JN, The capsaicin receptor. In: *Capsaicin in the Study of Pain* (Ed. Wood JN), pp. 1–26. Academic Press, London, 1993.
14. Davletov BA, Meunier FA, Ashton AC, Matsushita H, Hirst WA, Lelianova VG, Wilkin GP, Dolly JO and Ushkaryov YA, Vesicle exocytosis stimulated by α-latrotoxin is mediated by latrophilin and requires both external and stored Ca^{2+}. *EMBO J* **17:** 3909–3920, 1998.
15. Chen F, Foran P, Shone CC, Foster KA, Melling J and Dolly JO, Botulinum neurotoxin B inhibits insulin-stimulated glucose uptake into 3T3–L1 adipocytes and cleaves cellubrevin unlike type A toxin which failed to proteolyze the SNAP-23 present. *Biochemistry* **36:** 5719–5728, 1997.
16. Szallasi A, The vanilloid (capsaicin) receptor: Receptor subtypes and species differences. *Gen Pharmacol* **25:** 223–243, 1994.