

Venom From the Platypus, *Ornithorhynchus anatinus*, Induces a Calcium-Dependent Current in Cultured Dorsal Root Ganglion Cells

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de Plater, G. M., P. J. Milburn, and R. L. Martin. Venom from the platypus, *Ornithorhynchus anatinus*, induces a calcium-dependent current in cultured dorsal root ganglion cells. *J Neurophysiol* 85: 1340–1345, 2001. The platypus (*Ornithorhynchus anatinus*), a uniquely Australian species, is one of the few living venomous mammals. Although envenomation of humans by many vertebrate and invertebrate species results in pain, this is often not the principal symptom of envenomation. However, platypus envenomation results in an immediate excruciating pain that develops into a very long-lasting hyperalgesia. We have previously shown that the venom contains a C-type natriuretic peptide that causes mast cell degranulation, and this probably contributes to the development of the painful response. Now we demonstrate that platypus venom has a potent action on putative nociceptors. Application of the venom to small to medium diameter dorsal root ganglion cells for 10 s resulted in an inward current lasting several minutes when the venom was diluted in buffer at pH 6.1 but not at pH 7.4. The venom itself has a pH of 6.3. The venom activated a current with a linear current-voltage relationship between -100 and -25 mV and with a reversal potential of -11 mV. Ion substitution experiments indicate that the current is a non-specific cationic current. The response to the venom was blocked by the membrane-permeant Ca^{2+} -ATPase inhibitor, thapsigargin, and by the tyrosine- and serine-kinase inhibitor, k252a. Thus the response appears to be dependent on calcium release from intracellular stores. The identity of the venom component(s) that is responsible for the responses we have described is yet to be determined but is probably not the C-type natriuretic peptide or the defensin-like peptides that are present in the venom.

INTRODUCTION

Many invertebrate and vertebrate species produce venoms that are predominantly used either for food procurement or in self-defense. In keeping with these functions, such venoms contain peptides and other molecules that can induce immobilization and death. By contrast, the venom of one of the few living venomous mammals, the platypus (*Ornithorhynchus anatinus*), is believed to be used in defense of breeding territory (Grant and Temple-Smith 1998). Venom is produced in the crural glands of the male during the breeding season and is aggressively inflicted through a calcaneous spur on each hindlimb (Grant 1995). Because the venom appears to have a different function from venoms produced by nonmammalian species, it may contain peptides or molecules whose principal

effects are non-life threatening but nevertheless may seriously impair the victim.

That this could be the case is evident from the symptoms of platypus envenomation. In the human the most remarkable symptom is an immediate and excruciating pain (Fenner et al. 1992; Martin and Tidswell 1895; Spicer 1876; Tonkin and Negrine 1994). Edema rapidly develops around the wound and gradually spreads throughout the affected limb (Fenner et al. 1992; Martin and Tidswell 1895; Spicer 1876; Tonkin and Negrine 1994). Information obtained from case histories (Fenner et al. 1992; Tonkin and Negrine 1994) and anecdotal evidence indicates that the pain develops into a long-lasting hyperalgesia that persists for days or even months. Morphine analgesia partially alleviates the pain, but, in the one detailed case history, wrist block was necessary to effect complete pain relief (Fenner et al. 1992). These symptoms differ markedly from those associated with envenomation by snakes, for example, which can include systemic effects such as paralysis, myolysis, defibrination coagulopathy, and renal failure.

Recently we commenced a physiological and biochemical characterization of *Ornithorhynchus anatinus* venom (*OaV*). Like other venoms, it contains an hyaluronidase, which probably facilitates the spread of the venom through tissues (de Plater et al. 1995). It also contains a C-type natriuretic peptide, *Ov*-CNP (de Plater et al. 1995), that results in substantial edema when subcutaneously injected (de Plater et al. 1998b). This probably reflects *Ov*-CNP-induced mast cell degranulation (de Plater et al. 1998b) and is consistent with reduction, in the rat, of the edema by the 5-HT₂ receptor antagonist, ketanserin (de Plater et al. 1995).

The venom-induced mast cell degranulation suggests that some of the pain results from release of inflammatory mediators. However, the venom may also act on nociceptors themselves. To test this hypothesis, we applied *OaV* to small-medium diameter (20–40 μm) cultured dorsal root ganglion neurons (DRGs). These neurons are believed to exhibit properties that reflect those of peripheral nociceptor terminals in vivo (Gold et al. 1996; Senba and Kashiba 1996).

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METHODS

Cell preparation

Procedures used were very similar to those described by Gold et al. (1996), in which the properties of DRG neurons in vitro have been characterized. Thoracic and lumbar dorsal root ganglia were obtained from 5- to 8-wk-old Wistar rats that were decapitated under halothane anesthesia (4% in O₂). All experimental procedures were approved by The Animal Experimentation Ethics Committee at the Australian National University. Ganglia were dissociated at 36°C for 45 min in H-16 Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin G and 0.1 mg/ml streptomycin, and containing 2.5 U/ml collagenase (type I). After washing, the ganglia were incubated in 5 mg/ml dispase at 36°C for 30 min, then resuspended in DMEM supplemented with 10% fetal calf serum and the cell bodies dissociated by trituration. Cells were distributed in tissue culture plates containing coverslips coated with collagen and poly-D-lysine and incubated in CO₂ at 37°C until used. For electrophysiological recording a coverslip was placed in the bath on the stage of an inverted microscope, and the cells were perfused at 1–2 ml/min with an HEPES-buffered solution of the following composition (in mM): 130 NaCl, 3 KCl, 1.2 NaHCO₃, 0.6 MgCl₂, 2.5 CaCl₂, 10 HEPES, and 10 glucose, titrated to pH 7.4 with NaOH and osmolarity adjusted to 320 mOsm with sorbitol.

Electrophysiology

Whole cell recordings were made at room temperature from 20–40 μ m diameter DRGs between 6 and 24 h after plating; only cells that had smooth membranes under phase contrast microscopy were selected for study. The recording pipettes contained (in mM) 100 KCl, 2 2Na-ATP, 0.5 2Na-GTP, 11 EGTA, 1 CaCl₂, 2 MgCl₂, and 10 HEPES, titrated to pH 7.2 with KOH to give a final [K⁺] of 147 mM, and adjusted to 305 mOsm with sorbitol. Currents were measured using an Axopatch 1D (Axon Instruments) and data acquired on an IBM-PC compatible computer using pClamp software (20-kHz digitization rate). Slow records of the experimental measurements were made using a MacLab (AD Instruments), MacIntosh computer, and a digitization rate of 2 Hz. Electrode resistance ranged from 1.5 to 3 M Ω , series compensation from 80–90%, and corrections were applied for liquid junction potentials (JPCalc, Prof. P. H. Barry, UNSW, Australia). Resting membrane potential was measured prior to *OaV* or drug application, and only cells with corrected resting membrane potentials more negative than –50 mV were studied.

Materials

Whole venom from *Ornithorhynchus anatinus* was diluted at a concentration of 1 mg/ml in either MES-buffered solution at pH 6.1 (composition as for the HEPES-buffered solution but pH adjusted to 6.1 with HCl). The osmolarity of *OaV*-containing solutions was adjusted to 320 mOsm using sorbitol. The venom was applied to individual neurons by pressure ejection (5–10 psi) through flow pipes attached to fine plastic tubing that was back-filled with 100–300 μ l of test solution. Other drugs used were capsaicin (Fluka Chemical), freshly prepared from stock to a final concentration of 1 μ M in 0.01% ethanol, and thapsigargin (Research Biochemicals International) and k252a (Alomone Labs), each prepared from stock to give a final solution that contained 0.02% DMSO.

RESULTS

Of 96 DRGs subjected to a 10-s application of 1 mg/ml of *OaV* diluted in either MES or HEPES at pH 6.1, 69 responded with a long-lasting (duration 246.7 ± 36.5 s, mean \pm SE) inward current at –60 mV whose average latency to onset was

23.3 ± 3.0 s. The current was characterized by multiple transient events (Fig. 1A) and had an estimated mean peak amplitude of -4.65 ± 0.34 nA. Neurons that failed to respond to *OaV* ($n = 27$) usually responded to either buffer at pH 6.1 or to 1 μ M capsaicin with a single, transient inward current (Fig. 1B). Prior to venom application the resting membrane potential averaged -59.8 ± 1.7 mV ($n = 59$) for responsive neurons and -60.9 ± 1.5 mV ($n = 24$) for the unresponsive neurons. When *OaV* was buffered at pH 7.4, its application to DRGs produced little or no response ($n = 5$, Fig. 1C). There was no correlation between sensitivity to capsaicin and *OaV* ($n = 10$; resting membrane potential prior to *OaV* application -60.4 ± 2.5): of six capsaicin-sensitive neurons, four responded to *OaV* and of four capsaicin-insensitive neurons, three responded to *OaV*.

To determine the ionic basis of the *OaV*-induced current, voltage ramps from –110 to 60 mV were applied at a rate of 226 mV/s (Fig. 2, A–D). Subtraction of the curve obtained before *OaV* application from that obtained during its application yielded a linear inward current-voltage (*I*-*V*) relationship in the –100- to –50 mV range, which suggests activation of a voltage-independent current. At values more positive than –50 mV, another inward current was observed; but when K⁺ channels were blocked (see below), this current was not observed. Thus it is not likely to be a venom-activated current and probably reflects changes in the quality in the voltage-clamp between voltage ramps due to the large current flowing across an incompletely compensated access resistance. From this point on, we concentrated on characterizing the voltage-independent current. The linear range ($r = 0.97$ – 0.99) of the *I*-*V*

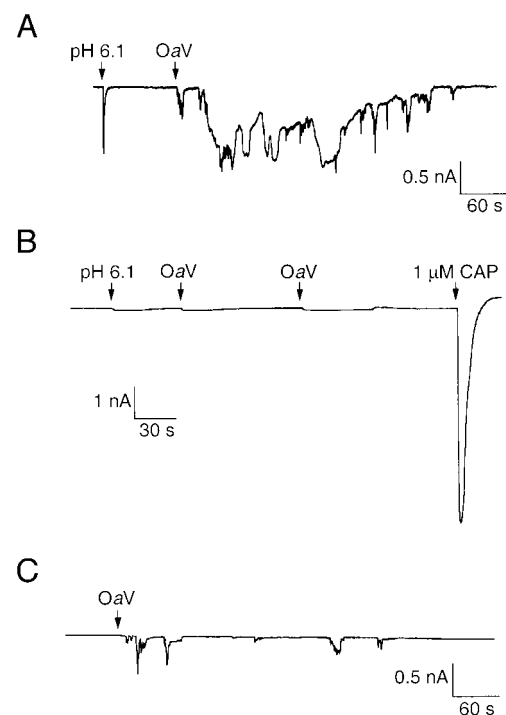


FIG. 1. Responses of cultured dorsal root ganglion (DRG) neurons to *Ornithorhynchus anatinus* venom (*OaV*). A: inward current measured at –60 mV when either MES at pH 6.1 or *OaV* buffered at pH 6.1 was applied to an acutely cultured DRG neuron. B: a DRG neuron that failed to respond to either pH 6.1 or to *OaV*, was responsive to 1 μ M capsaicin. C: at pH 7.4 the response to *OaV* was significantly reduced.

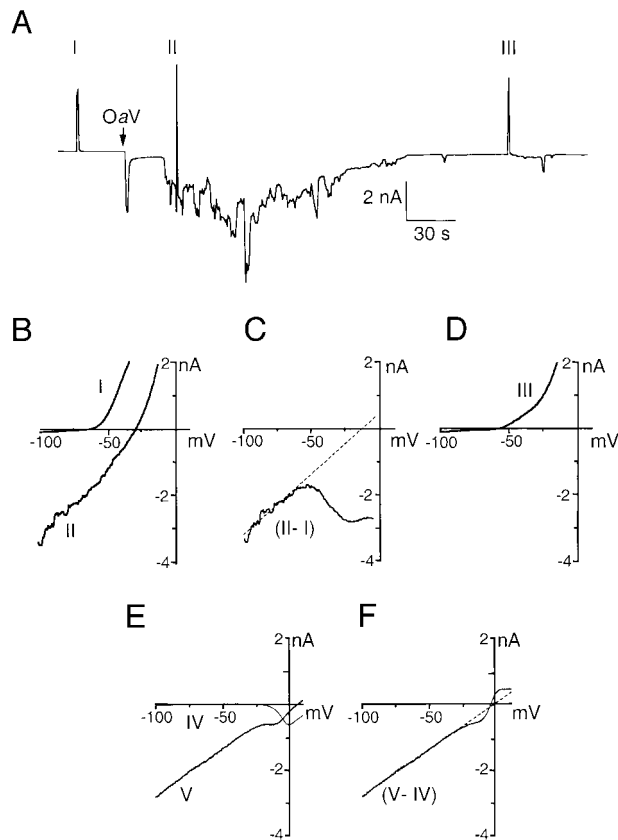


FIG. 2. Current-voltage relationships for the response to *OaV*. *A*: another example of the current induced by *OaV*; voltage ramps from -100 to 60 mV, at a rate of 226 mV/s, were applied prior to (I), during (II), and after (III) application of *OaV*. *B*: current-voltage relationships before (I) and during (II) the *OaV*-induced current. To more clearly illustrate the principal effects of the venom, only responses to part of the voltage-ramp are plotted. *C*: difference current-voltage relationship obtained by subtracting curve II from curve I. The linear portion of the relationship was extrapolated to give an estimate of the reversal potential of the current. *D*: current-voltage relationship obtained after the *OaV*-induced current had abated (III). *E-F*: current-voltage relationship when K^+ in the pipette was replaced with TEA. In *E* the responses before (IV) and after (V) venom application are shown, and in *F*, the difference current (V-IV) as well as the linear fit between -100 and -50 mV. The reversal potential is similar to that shown in *C*.

relationship was extrapolated to give a mean reversal potential for the *OaV*-activated current of -11.4 ± 2.0 mV ($n = 22$; Fig. 2*C*). This is close to the calculated E_{Cl} of -6.9 mV and to E_{rev} for the nonselective cationic current that is known to occur in DRGs (Crawford et al. 1997; Currie and Scott 1992), thus suggesting the involvement of either Cl^- and/or nonselective cationic currents. Replacement of extracellular (perfusate) Cl^- with gluconate caused a significant shift in E_{rev} to 5.2 ± 1.9 mV ($n = 4$; $P < 0.01$), but this value fell far short of the calculated E_{Cl} of 61.6 mV. Replacement of recording pipette Cl^- with gluconate was without major effect ($n = 5$; E_{rev} of -1.1 ± 3.5 mV compared with calculated E_{Cl} of -70.7 mV). These data suggest that the *OaV*-induced current is dominantly carried by cations rather than by Cl^- . We also studied the linear current when pipette K^+ was replaced with TEA to block a variety of K^+ currents (Hille 1994). The large outward current at membrane potentials more positive than -60 mV was abolished, and inward currents, probably voltage-gated Na^+ or Ca^{2+} currents, were revealed (Fig. 2*E*). However, E_{rev}

for the linear current only shifted to -1.8 ± 2.7 mV ($n = 6$; Fig. 2*F*).

Ca^{2+} -dependent nonselective cationic currents with linear *I-V* relationships have been demonstrated in DRGs, and these are activated by Ca^{2+} release from internal stores (Currie and Scott 1992). Therefore we tested the effects of *OaV* in the presence of thapsigargin, a membrane-permeant Ca^{2+} -ATPase inhibitor that prevents the refilling of intracellular Ca^{2+} stores (Verkhatsky and Shmigol 1996). *OaV* was applied twice to DRGs (~ 7 -min interval between applications), and the ratio of the second to the first response was calculated (average response = $1/T \int I dt$, where T is the duration of the response). In controls the ratio of the second to the first response was 2.69 ± 0.46 ($n = 9$; Fig. 3*A*). These data were compared with the ratio obtained from successive applications of *OaV* when $1 \mu M$ thapsigargin was applied extracellularly (Fig. 3*B*). In the presence of thapsigargin the inward current in response to the first *OaV* application was similar to controls, presumably because the intracellular stores still contained some Ca^{2+} . However, the second was very small, and as a result the ratio of the second to the first response fell to 0.17 ± 0.09 ($n = 5$; Fig. 3*B*). Thapsigargin did not wash out as demonstrated by the lack of response to a third application of *OaV* (Fig. 3*B*). The inward current induced by *OaV* was also blocked by 200 nM k252a, a tyrosine and serine-threonine kinase inhibitor at this concentration (ratio of 2nd to the 1st response = 0.44 ± 0.16 , $n = 4$; Fig. 3*C*). The response to *OaV* was restored after wash out of k252a, which is consistent with the known reversibility of some actions of this drug (Knight et al. 1997). Comparison of

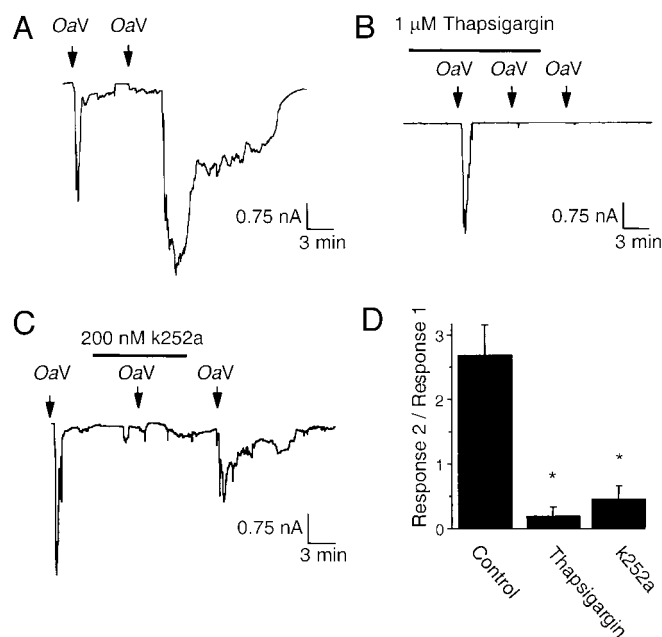


FIG. 3. Effects of thapsigargin and k252a on the response to *OaV*. *A*: repeated applications of *OaV* generally gave rise to a larger inward current on the 2nd application compared with the 1st. *B*: application of $1 \mu M$ thapsigargin beginning 5 min prior to application of *OaV* did not prevent a response to the 1st application of *OaV* but severely attenuated the 2nd response; the response to *OaV* was not restored by wash out of thapsigargin. *C*: in the presence of 200 nM k252a, the *OaV*-induced inward current was significantly attenuated, but this effect reversed on wash out of k252a. *D*: the ratio of the 2nd response to *OaV* relative to the 1st response in controls, thapsigargin, and k252a. The asterisks mark significant differences from controls ($P = 0.025$, Dunnett's test).

the ratio responses in controls, thapsigargin- and k252a-treated cells (Fig. 3D) revealed a highly significant effect of treatment (1-way ANOVA, $P < 0.001$) and significant differences between controls and both other treatment groups (Dunnett's test, $P = 0.025$). Although the concentration of thapsigargin used may block high-voltage-activated and, to a lesser extent, low-voltage-activated Ca^{2+} currents in DRGs (Rossier et al. 1993; Shmigol et al. 1995), this is unlikely to be its route of action because only the response to a second application of *OaV* was reduced.

DISCUSSION

This study is the first to report an action of an animal venom on isolated sensory neurons. We believe that a high proportion of the cells from which we recorded were probably nociceptors. To prepare cultured DRG neurons from adult rats, we used a procedure very similar to that described by Gold et al. (1996). These authors reported that about 60% of neurons responded to capsaicin, and we report a similar figure. A further 16% of all DRGs express a structural homologue of VR1 that has a high-threshold for noxious heat but is capsaicin insensitive (Caterina et al. 1999). Thus we can reasonably expect that around 80% of our recordings were from cell bodies of nociceptive neurons, and the figure may be higher because we tried to select smaller diameter cells, which are those associated with nociception in vivo (Harper and Lawson 1995).

We chose to use cultured DRGs as model nociceptors to minimize the amount of venom required for each experiment. Lack of nerve growth factor in culture medium is known to lead to the progressive loss of proton and capsaicin sensitivities over several days (Bevan 1996). Therefore we used the cultured DRGs within 6–24 h of plating, and, accordingly, the proton and capsaicin sensitivities remained robust.

A very short application of *OaV* to putative nociceptors produced a long-lasting inward current. The possibility that this current reflects cell damage (due, for example, to protease activity in the venom) can be excluded for two reasons. First, when the current eventually ceased, the holding current was usually similar to that prior to application of *OaV*, and, second, when *OaV* was applied twice with a short interval between applications, the response to the second test was bigger than that to the first. It is also unlikely that the observed responses arise from venom-induced metabolic inhibition because they are completely different from those reported in DRGs under such circumstances (Duchen 1990).

If the inward current occurred in vivo, it would be expected to lead to firing and the perception of pain. This correlates well with reports of immediate and intense pain after platypus envenomation of humans (Fenner et al. 1992). The pH sensitivity of the response is notable given that an interaction between various pain-producing molecules and pH has previously been reported (Kress et al. 1997; Petersen and Lamotte 1993; Tominaga et al. 1998) and that inflammatory exudates are acidic in nature. We have measured the pH of platypus venom and found it to be 6.3. Although this suggests that the venom component, or components, activating nociceptors in vitro, could probably do so in vivo, interstitial fluids may buffer the venom at a higher pH.

Our data indicate that *OaV* principally activates a nonselec-

tive cationic current that is probably dependent on Ca^{2+} release from intracellular stores. It is somewhat surprising that such a current could be activated with 11 mM EGTA in the recording pipette. However, when using a pipette solution of similar composition to that used here, and additionally containing IP_3 , Li and Zhao (1998) reported induction of a long-lasting current in DRGs, characterized by transient events and a very long latency to onset (2–4 min). Clearly, Ca^{2+} released from intracellular stores was not completely buffered by the high concentration of EGTA. The long latencies to onset (about 23 s) of the *OaV*- or IP_3 -induced responses do not appear to be the result of Ca^{2+} buffering either because responses to the metabotropic glutamate receptor agonist, 1-amino-1,3-cyclopentanedicarboxylic acid [(1S,3R)-ACPD], develop with latencies of 2–4 min when only 0.1 or 1 mM EGTA is used in the pipette solution (Crawford et al. 1997). Responses to ryanodine and caffeine also develop with very long latencies (Ayar and Scott 1999).

Our conclusion that the *OaV*-induced response is Ca^{2+} dependent arises largely from its block by 200 nM thapsigargin. This conclusion is consistent with the fact that in DRG neurons transient events imposed on transmembrane currents are only associated with agonists known to release Ca^{2+} from intracellular stores, such as 1S,3R-ACPD, ryanodine, caffeine, and cGMP, and not with capsaicin or low pH. It is also important to note that Ca^{2+} -dependent nonspecific cationic currents have been described in a wide variety of tissues and they have linear *I-V* relationships between -100 and -60 mV (Partridge et al. 1994). Thapsigargin at 200 nM concentration is not detrimental to sensory neurons, although it does depress high-voltage-activated Ca^{2+} currents by about 40% (Shmigol et al. 1995). The block of the *OaV*-induced current could not be attributed to an effect on the latter since the first response to *OaV* in the presence of thapsigargin was similar to that in its absence.

The results presented suggest that the venom-induced release of Ca^{2+} from intracellular stores involves activation of tyrosine or serine-threonine kinases. The tyrosine kinase, TrkA, binds nerve growth factor (NGF) with high affinity, and NGF evokes increases in cytosolic Ca^{2+} in both TrkA-expressing C6–2B glioma cells (De Bernardi et al. 1996) and in 3T3 cells (Jiang et al. 1999). Interestingly, we have found that *OaV* contains an NGF (de Plater et al. 1998a) and NGF forms a substantial component of snake venoms (Kostiza and Meier 1996). The response to subcutaneous injection of recombinant NGF in humans has not been measured any earlier than 3 h postinjection, but at this time it caused pressure allodynia and lowered the heat-pain threshold in a significant percentage of subjects (Dyck et al. 1997). Eventually, most subjects experienced allodynia and hyperalgesia that lasted 21–27 days (Dyck et al. 1997; Petty et al. 1994). The nature and time course of these symptoms bear a striking similarity to those reported after platypus envenomation (Fenner et al. 1992).

However, the acute activation of an inward current in DRGs by *OaV* is not consistent with the known actions of NGF and may therefore depend on activation of a serine kinase that induces intracellular Ca^{2+} release, for example, protein kinase C (PKC). PKC-epsilon has been implicated in the sensitization of the noxious heat response by bradykinin (Cesare et al. 1999) and PKC-gamma in neuropathic pain (Malmberg et al. 1997).

Further studies are required to elucidate the signal transduction mechanisms involved in the response we have reported.

Platypus venom contains many constituents (de Plater et al. 1995), and we have commenced studies to identify the component or components responsible for activating an inward current in DRG neurons. We can exclude involvement of the C-type natriuretic peptide (*OaV-CNP*) present in the venom (de Plater et al. 1995) because neither CNP purified from whole venom nor synthetic CNP induced a current in DRG neurons (data not shown), even though the synthetic form is capable of forming channels in artificial membranes (Kourie 1999). The venom has some protease activity that is associated with high molecular weight venom components (de Plater et al. 1995). In preliminary studies we have tested the activity of gel filtration/HPLC fractions on DRG neurons and have found that the fractions capable of inducing the inward current are of much lower molecular weight. Furthermore, if the *OaV*-induced current resulted from irreversible proteolytic cell damage, then recovery of the current to baseline, as observed in 56 of 69 neurons, or repeated responses from the same cell would be unlikely. The venom is known to contain four defensin-like peptides (Torres et al. 1999, 2000), and these have some structural similarity with the sodium neurotoxin peptide, ShI (Torres et al. 1999). Defensins are also capable of forming channels in membranes (reviewed by Kourie and Shorthouse 2000). However, Torres et al. (1999) reported that defensin-like peptide 1 had no effect on dorsal root ganglion sodium currents, and none of the platypus defensin-like peptides have antimicrobial, myotoxic, or cell growth-promoting activities (Torres et al. 2000). Another possible explanation for the responses we have observed in DRG neurons is that the venom contains free glutamate. While we have not established whether this is the case, the HPLC elution profile of active fractions suggests that glutamate is not responsible for the activity.

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