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Algogen-specific pain processing in mouse spinal cord: differential involvement of voltage-dependent Ca^{2+} channels in synaptic transmission

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1 The effects of intrathecal (i.t.) administration of N-, P/Q- or L-type voltage-dependent Ca^{2+}-channel blockers were tested in two pain models involving bradykinin (BK)- and α,β-methylene ATP (α,βmeATP)-induced activation of primary afferent neurons in mice.

2 The nociceptive response (amount of time spent licking and biting the hindpaw) induced by intraplantar injection of BK (500 pmol mouse^{-1}) was significantly attenuated by both ω-conotoxin GVIA (N-type blocker) and calciseptine (L-type) but not by ω-agatoxin IVA (P/Q-type).

3 The nociceptive response induced in a similar way by α,βmeATP (100 nmol) was significantly inhibited by both the above N- and P/Q-type Ca^{2+}-channel blockers but not by the L-type blocker.

4 The nociceptive responses elicited by BK and α,βmeATP were dose-dependently inhibited by a tachykinin-NK1-receptor antagonist (L-703,606) and an N-methyl-D-aspartate (NMDA)-receptor antagonist (D-AP5), respectively.

5 Intrathecal administration of substance P (SP) (1.8 nmol) or NMDA (350 pmol) elicited algesic responses, such as licking, biting and scratching of the hindquarters. The SP-induced algesic behaviour was significantly inhibited by the L-type blocker but not by the N-type. The NMDA-induced response was not affected by either the N- or the P/Q-type blocker.

6 These findings suggest that BK and ATP most likely excite different types of sensory neurons in the periphery and that within the spinal cord the former stimulates peptidergic transmission regulated by presynaptic N- and postsynaptic L-type Ca^{2+} channels, while the latter stimulates glutamatergic transmission regulated by presynaptic N- and P/Q-type channels.
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Abbreviations: α,βmeATP, α,β-methylene ATP; ACSF, artificial cerebrospinal fluid; BK, bradykinin; CAL, calciseptine; CGRP, calcitonin-gene-related peptide; DRG, dorsal root ganglion; i.pl., intraplantar; i.t., intrathecal; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; VDCCs, voltage-dependent Ca^{2+} channels; ω-AgTX, ω-agatoxin IVA; ω-CgTX, ω-conotoxin GVIA

Introduction

High-threshold voltage-dependent Ca^{2+} channels (VDCCs) mediate Ca^{2+} influx into neurons upon depolarization and thereafter influence synaptic mediator/receptor systems, membrane excitability levels and gene expression. So far, five distinct types of high-threshold VDCCs have been identified: these are designated L, N, P, Q and R (Bean, 1989; Llinas *et al*., 1989; Hess, 1990; Zhang *et al*., 1993; Randall & Tsien, 1995). Immunohistochemical studies have demonstrated that although all five types are present in the dorsal horn, the N- and P/Q-types are the predominant ones associated with primary afferent fibres in the superficial layers of the spinal cord, with the N-type being present in much greater numbers (Westenbroek *et al*., 1998). The perineural administration of antagonists of neuronal N-type VDCCs has been shown to relieve heat-hyperalgesia and mechano-allodynia in an experimental model of painful peripheral neuropathy (Xiao & Bennett, 1995). In a formalin model of inflammation, N-type and P/Q-type VDCCs, but not the L-type, have been shown to be involved in the inflammation-evoked hyperexcitability of dorsal horn neurons (Malmberg & Yaksh, 1994, 1995; Diaz & Dickenson, 1997). In the latter study, an N-type Ca^{2+}-channel blocker reduced the first and second phases of the formalin response to equal extents, whereas a P/Q-type blocker inhibited only or predominantly the second phase. As for spinal L-type channels, the estimations of their contribution to nociceptive processing have varied from study to study (Coderre & Melzack, 1992; Malmberg & Yaksh, 1994; Dogrul & Yesilyurt, 1998). Collectively, the above studies implicate VDCCs, especially the N-type, as important contributors to the processing of nociceptive information at the spinal-cord level. However, the possibility remains that each type of VDCC could preferentially regulate or be coupled to a particular type of synaptic transmission within the spinal cord.

Pain models, such as the formalin test and the writhing test, are often used for studies of spinal Ca^{2+} channels

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(Malmberg & Yaksh, 1994, 1995; Diaz & Dickenson, 1997; Dogrul & Yesilyurt, 1998). However, the mechanisms underlying the nociceptive responses in these models remain unclear and are probably complicated. In addition to exogenous algogens, like formalin, various endogenous inflammatory mediators are involved in pain sensation at peripheral sites (Shibata *et al*., 1989) and these may elicit different patterns of excitation of phenotypically heterogeneous sensory neurons. Consequently, when we use the aforementioned pain models to try to elucidate the role played by each type of VDCC in the spinal processing of nociceptive information, the data we obtain can be quite perplexing. Indeed, despite the accumulating evidence implicating VDCCs in nociceptive neurotransmission, a full understanding of their role remains elusive.

For the present purposes, in order to simplify pain processing as much as possible we used specific pain-producing substances (namely, α,βmeATP (an ATP analogue) and BK) as peripheral stimuli and avoided complicated pain models like the formalin test. In fact, BK and ATP are known to be endogenous algesic substances that elicit pain through direct activation of specific receptors at peripheral sites; B2 and P2X, respectively, being the receptors involved (Bathon & Proud, 1991; Dray *et al*., 1988; Bland-Ward & Humphrey, 1999; Hamilton *et al*., 1999; Tsuda *et al*., 2000). Using these two algogens, we investigated the contributions made by N-, P/Q- and L-type Ca^{2+} channels to the synaptic transmission of pain at the spinal-cord level. Our observations suggest that BK- and α,βmeATP-induced nociceptive responses are regulated within the dorsal horn by different populations of VDCCs.

Methods

Animals

Male ddY mice weighing 18 to 20 g were used. They were purchased from the Seac Yoshitomi Ltd. (Fukuoka, Japan) and housed at a temperature of $22\pm1^{\circ}C$ with a 12 h light-dark cycle. Food and water were available *ad libitum*. This study was reviewed and approved by the review board of Fukuoka Dental College for the regulation of animal experimentation and it was conducted in accordance with ICLA guidelines.

BK- and ATP-induced nociceptive responses

The observation chamber was a 20-cm-diameter glass cylinder set on a transparent acrylic-plate floor. Beneath the floor, a large mirror was mounted at a 45° angle to allow clear observation of the animal's paws. Each mouse was habituated to the chamber for 5 min before injection of BK (500 pmol mouse^{-1}) or α,βmeATP (100 nmol mouse^{-1}), 5 μl of one of these algogens being administered into the plantar side (i.pl.) of the left hindpaw. Then, the animal was immediately returned to the chamber and its nociceptive response was observed for 5 min. Within this period, the total time (in seconds) spent licking and biting the injected paw was taken as an indicator of nociception. In preliminary experiments, we established which doses of these substances would produce roughly similar nociceptive responses.

Algesic behavioural responses elicited by i.t. administration of SP and NMDA

Substance P (SP) (1.8 nmol/5 μl) or N-methyl-D-aspartate (NMDA) (350 pmol/5 μl) was administered i.t.; then, the algesic response (such as licking, biting and scratching of the hindquarters) was observed for 5 min. The total time (in seconds) spent in these behaviours was measured within this period. In preliminary experiments, we ascertained that the algesic responses elicited by SP and NMDA at these doses were roughly equivalent.

Intrathecal injection of drug solutions

Blocking drugs were dissolved in a volume of 5 μl, and an intrathecal injection (i.t.) was given according to the method of Hylden & Wilcox (1980). All i.t. injections were performed *via* a disposable 30-gauge 1/2-inch needle (Hamilton, Rino, NV, U.S.A.) mated to a 10-μl luer-tip syringe (Hamilton, Rino, NV, U.S.A.). The site of injection in this experiment was the subarachnoid space between L5 and L6. Each blocking-drug administration was made 5 min before either the i.pl. injection of BK or ATP, or the i.t. injection of SP or NMDA. The effect of each blocker was tested against its own vehicle control.

Incline plane test

Motor coordination was assessed using incline plane performance according to the method of Surber *et al*. (1959). Mice were placed on the incline screen (45°) 5 min after i.t. injection of a blocking drug. The time for which a mouse could remain on the screen was recorded, with a cut off at 5 min.

Materials

Bradykinin (BK) (Peptide Institute Inc., Osaka, Japan) and α,β-methylene ATP (α,βmeATP) (Sigma, St. Louis, MO, U.S.A.) were dissolved in phosphate-buffered saline (PBS; pH 7.4). ω-Conotoxin GVIA (ω-CgTX), calciseptine (CAL), ω-agatoxin IVA (ω-AgTX) and substance P (SP) (Peptide Institute Inc., Osaka, Japan), and D(−)-AP-5 and N-methyl-D-aspartate (NMDA) (Sigma, St. Louis, MO, U.S.A.) were dissolved in artificial cerebrospinal fluid (ACSF; composition in (mM): NaCl, 138.6; KCl, 3.35; $CaCl_2{\cdot}2H_2O$, 1.26; $MgCl_2{\cdot}6H_2O$, 1.16; $NaH_2PO_4{\cdot}2H_2O$, 0.58; $NaHCO_3$, 21.0; glucose, 10.0; pH 7.4). *cis*-2-(Diphenylmethyl)-N-[(2-indophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate (L-703,606) (RBI, Natick, MA, U.S.A.) was dissolved in ACSF containing 2-hydroxypropyl-β-cyclodextrin (H-107) (RBI, Natick, MA, U.S.A.).

Statistical analysis

Data were examined by ANOVA followed by a *post hoc* Scheffé's test for multiple comparisons *versus* vehicle control.

Results

In control mice (given 5 μl of ACSF intrathecally), intraplantar injection of BK caused relatively short-lasting

nociceptive responses. At a dose of 500 pmol paw^{-1}, the licking and biting response began within 10 s and lasted less than 5 min in all animals, with the most behavioural response being observed no later than 2 min after the injection. This response was not different from that observed in normal mice (without i.t. ACSF). A potent and selective N-type VDCC blocker, ω-CgTX (1–10 pmol mouse^{-1} i.t.), produced a dose-dependent inhibition of the nociceptive response induced by BK, as did a selective L-type blocker, CAL (1–10 pmol i.t.) (Figure 1). On the other hand, ω-AgTX (1–10 pmol), a P/Q-type blocker, had no effect on this response. At these doses, the mice showed no motor deficits after any of the Ca^{2+}-channel blockers, as judged by incline plane performance; all animals remained on the incline screen over a cut-off time.

Short-lasting nociceptive responses, similar to those evoked by BK, were evoked by α,βmeATP (100 nmol paw^{-1}), a relatively stable ATP agonist, in control mice. In almost all animals (47/55) the response had ended within 5 min after the intraplantar injection of α,βmeATP. As shown in Figure 2, ω-CgTX (1–10 pmol mouse^{-1} i.t.) dose-dependently inhibited the α,βmeATP-induced nociceptive response. By contrast, CAL (1–10 pmol mouse^{-1} i.t.) failed to affect it; even at a dose of 100 pmol there was no significant inhibition (data not shown). Although ω-AgTX was completely ineffective against BK-induced responses, it strongly inhibited the α,βmeATP-induced responses at 3 and 10 pmol mouse^{-1}.

Thus, the pain responses induced by BK and α,βmeATP would seem to require different populations of VDCCs. To try

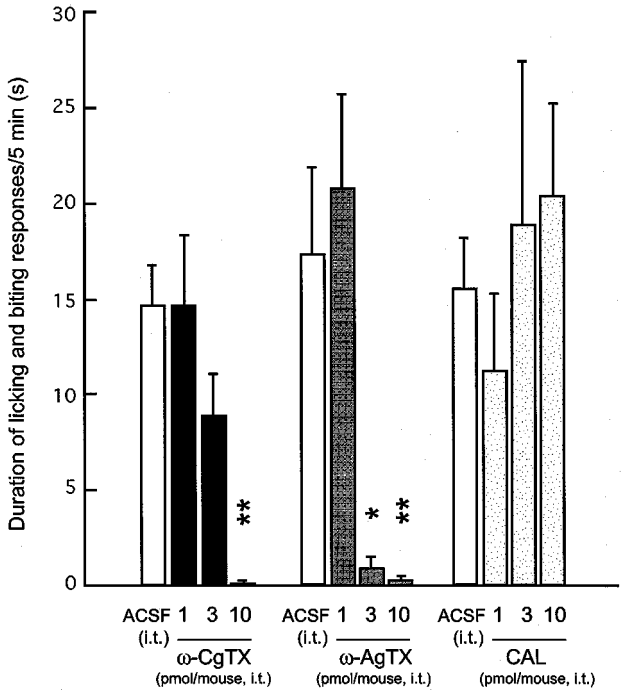

Figure 2 Effects of N-, P/Q- and L-type Ca^{2+}-channel blockers on α,βmeATP-induced nociceptive responses. Each Ca^{2+}-channel blocker was administered i.t. 5 min before an i.pl. injection of α,βmeATP (100 nmol). Amount of time spent licking and biting the injected paw was observed in the 5 min after α,βmeATP injection. Each column represents the mean$\pm$s.e. ($n=5-8$). $^*P<0.05$ and $^{**}P<0.01$ *vs* corresponding ACSF-injected control group.

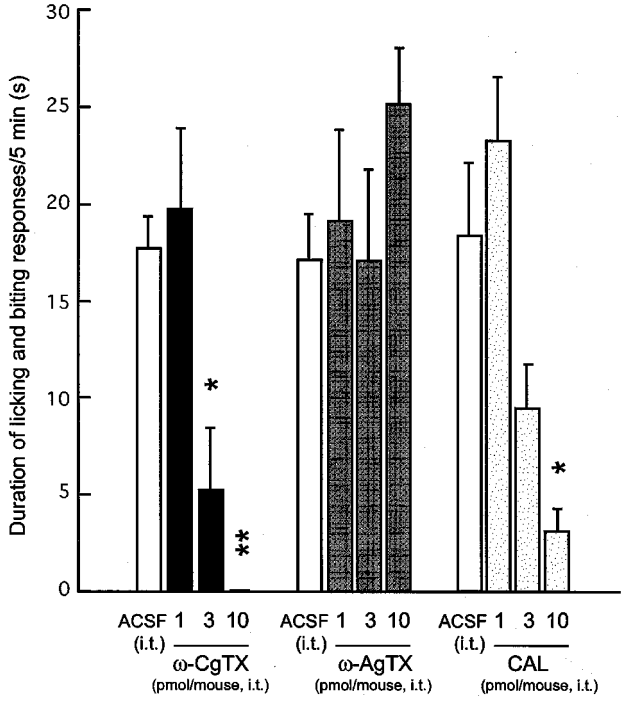

Figure 1 Effects of N-, P/Q- and L-type Ca^{2+}-channel blockers on BK-induced nociceptive responses. Each Ca^{2+}-channel blocker was administered i.t. 5 min before an i.pl. injection of BK (500 pmol). Amount of time spent licking and biting the injected paw was observed in the 5 min after BK injection. Each column represents the mean$\pm$s.e. ($n=6-11$). $^*P<0.05$ and $^{**}P<0.01$ *vs* corresponding ACSF-injected control group.

to clarify the underlying mechanisms, we investigated the neurotransmitters involved at the spinal level. A tachykinin-NK1-receptor antagonist, L-703,606, was administered intrathecally 5 min before the BK i.pl. injection. At 500 pmol, this antagonist significantly inhibited the licking and biting responses induced by BK (Figure 3). An N-methyl-D-aspartate (NMDA)-receptor antagonist, D(−)-AP-5, had no effect on the BK-induced responses even at a dose of 1500 pmol (i.t.) but it dose-dependently inhibited the α,βmeATP-induced nociceptive response (Figure 4). By contrast, L-703,606 had no significant effect on the α,βmeATP response.

We next examined the N- and L-type channel blockers that had been effective against the BK-induced nociceptive response to see if they would affect the SP-mediated response, since the contrasting results between BK and α,βmeATP seemed to indicate that the former excites peptidergic neurons and the latter glutamatergic ones. Similarly, we examined the N- and P/Q-type blockers that had reduced the α,βmeATP-induced response to see if they would affect the NMDA-mediated response. In these tests, i.t. administration of SP (1.8 nmol mouse^{-1}) elicited an algesic response (involving licking, biting and scratching of the hindquarters). This response was adequately inhibited by the NK1 receptor antagonist L-703,606 at the same dose as that needed to produce an inhibition of the BK-response. ω-CgTX (1–10 pmol mouse^{-1} i.t.) had no effect on the SP-induced algesic response, while CAL significantly inhibited it at 10 pmol (Figure 5). On the other hand, in the case of NMDA (350 pmol mouse^{-1} i.t.), which produced a similar nociceptive response to SP, antagonism was seen only with the

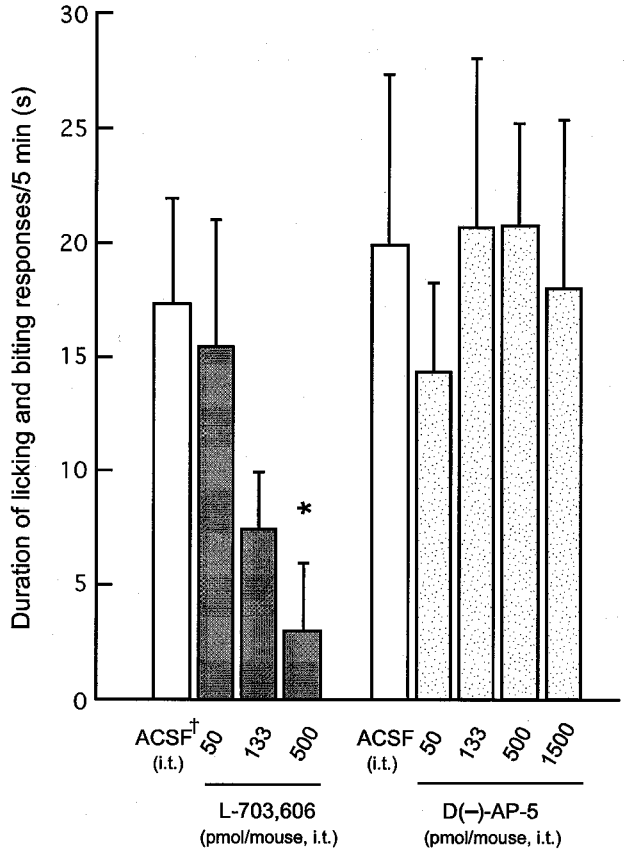

Figure 3 Effects of tachykinin NK1- and glutamate NMDA-receptor antagonists on BK-induced nociceptive responses. Each antagonist was administered i.t. 5 min before an i.pl. injection of BK (500 pmol). Amount of time spent licking and biting the injected paw was observed in the 5 min after BK injection. Each column represents the mean ± s.e. ($n = 5-8$). ACSF†: ACSF containing 2-hydroxypropyl-β-cyclodextrin (H-107). *$P < 0.05$ *vs* corresponding control group (ACSF- or ACSF†-injected).

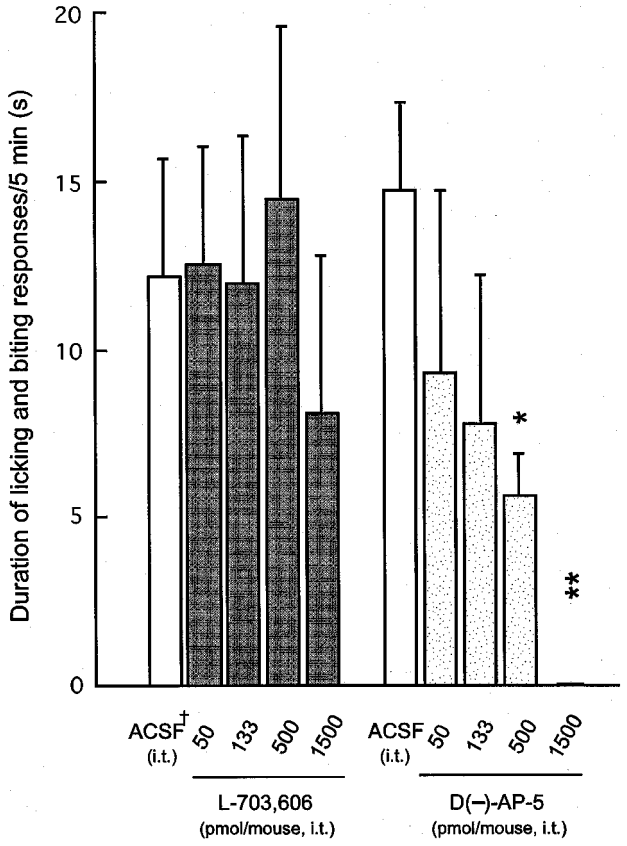

Figure 4 Effects of tachykinin NK1- and glutamate NMDA-receptor antagonists on α,βmeATP-induced nociceptive responses. Each antagonist was administered i.t. 5 min before an i.pl. injection of α,βmeATP (100 nmol). Amount of time spent licking and biting the injected paw was observed in the 5 min after α,βmeATP injection. Each column represents the mean ± s.e. ($n = 5-12$). ACSF†: ACSF containing 2-hydroxypropyl-β-cyclodextrin (H-107). *$P < 0.05$ and **$P < 0.01$ *vs* corresponding control group (ACSF- or ACSF†-injected).

NMDA-receptor antagonist D(−)-AP-5 (used at the same dose as that needed to inhibit the α,βmeATP-induced response). Both ω-CgTX (1–10 pmol i.t.) and ω-AgTX (1–10 pmol i.t.) failed to affect the NMDA-induced algesic response (Figure 6).

Discussion

Published evidence suggests that BK and ATP may act *via* relatively simple mechanisms, in contrast to other nociceptive tests: probably, they selectively activate small-diameter neurons *via* specific receptors (Inoue *et al*., 1998; Bland-Ward & Humphrey, 1997). In our study, the nociceptive responses elicited by small doses of BK and α,βmeATP were short lasting (less than 5 min), and no visible swelling or redness was observed during the 5-min measurement period. We observed that the BK-induced nociceptive response was inhibited by an NK1-receptor antagonist, L-703,606, but not by an NMDA-receptor antagonist, D(−)-AP-5. By contrast, the α,βmeATP-induced response was reduced by the NMDA-receptor antagonist but not by the NK1-receptor antagonist. As a result of studies carried out using three markers – the heavy neurofilament protein NF200, the neuropeptide calcitonin gene-related peptide (CGRP) and the purinoceptor P2X3 – nearly all neurons in dorsal root ganglia (DRG) have been assigned to three subclasses: namely, large-diameter myelinated neurons and small-diameter unmyelinated peptidergic and non-peptidergic neurons (Bennett *et al*., 1998; Averill *et al*., 1995; Bradbury *et al*., 1998). Primary afferent C fibres release excitatory amino acids (such as glutamate) and peptides (such as substance P and CGRP) that serve to induce excitation of secondary neurons *via* various receptors, including the NMDA and NK1 receptors. Release of substance P and CGRP from sensory neurons is known to follow stimulation by BK (Kano *et al*., 1994; Yonehara *et al*., 1995) and it has been reported that activation of P2X receptors on DRG neurons elicits glutamate release from sensory-neuron synapses (Gu & Macdermott, 1997). Similarly, it has been suggested that intrathecal administration of α,βmeATP may evoke spinal glutamate release (Tsuda *et al*., 1999). Furthermore, it has recently been reported that capsaicin-sensitive polymodal C fibres can be divided into at least two types (Ueda *et al*., 2000): the first are stimulated by many algesic substances (including BK) and use substance P as a primary afferent neurotransmitter, while the others are stimulated by ATP through the P2X3 receptor and use

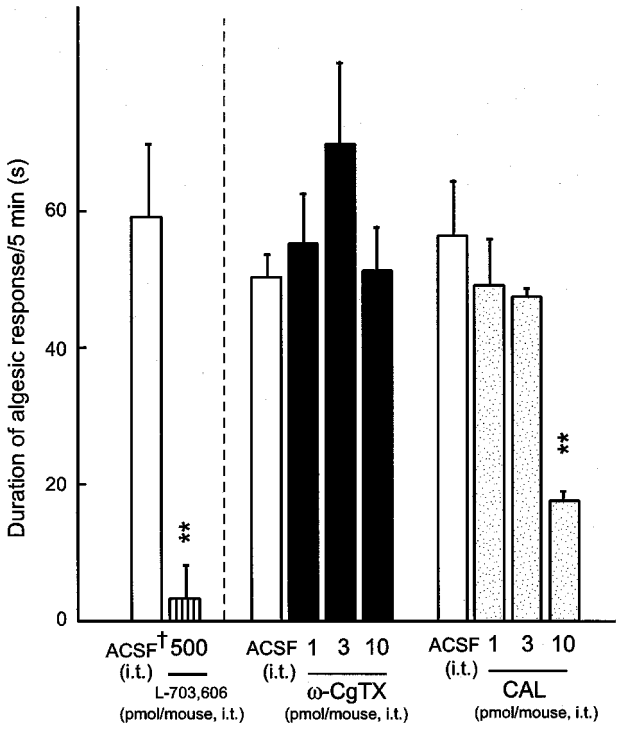

Figure 5 Effects of N- and L-type Ca^{2+}-channel blockers on the algesic response induced by i.t. injection of SP. One of the Ca^{2+}-channel blockers or L-703,606 was administered i.t. 5 min before i.t. injection of SP (1.8 nmol). Amount of time spent licking, biting and scratching the hindquarters was observed in the 5 min after SP injection. Each column represents the mean ± s.e. ($n = 5-8$). ACSF†: ACSF containing 2-hydroxypropyl-β-cyclodextrin (H-107). $^{**}P < 0.01$ *vs* corresponding control group (ACSF- or ACSF†-injected).

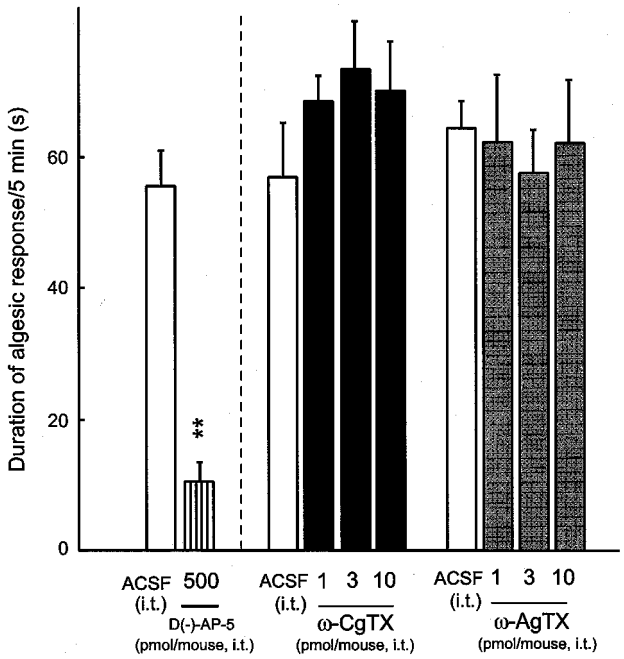

Figure 6 Effects of N- and P/Q-type Ca^{2+}-channel blockers on the algesic response induced by i.t. injection of NMDA. One of the Ca^{2+}-channel blockers or D-AP-5 was administered i.t. 5 min before i.t. injection of NMDA (350 pmol). Amount of time spent licking, biting and scratching the hindquarters was observed in the 5 min after NMDA injection. Each column represents the mean ± s.e. ($n = 5-8$). $^{**}P < 0.01$ *vs* corresponding ACSF-injected control group.

glutamate as a neurotransmitter. Our findings are consistent with these reports insofar as they suggest that BK and ATP are likely to elicit excitation of different types of sensory neurons at peripheral sites and to evoke release of substance P and glutamate respectively, within the spinal cord.

In the present study, a selective N-type Ca^{2+} channel blocker, ω-CgTX, inhibited both the BK- and α,βmeATP-induced nociceptive responses. Increased Ca^{2+} influx mediated through the opening of VDCCs leads to the release of primary afferent neurotransmitters like substance P and glutamate. In co-cultures of rat DRG and spinal neurons, excitatory transmission between DRG and spinal-cord neurons was found to be substantially reduced by ω-CgTX; by contrast, excitatory transmission between spinal neurons was not altered by this blocker (Yu *et al*., 1992). Furthermore, N-type-channel immunoreactivity has been found with a higher density in nerve terminals forming synapses on the cell bodies than in the cell–surface membrane within the superficial laminae of the spinal cord (Westenbroek *et al*., 1998). Thus, N-type Ca^{2+} channels seem to be present mainly on the presynaptic membrane and thus may modulate neurotransmitter release within the spinal cord. Indeed, N-type antagonists have been shown to block release of substance P from primary sensory neurons in culture (Holz *et al*., 1988) and N-type channels have been reported to play a dominant role in glutamatergic sensory neurotransmission via a presynaptic mechanism (Gruner & Silva, 1994). Actually, in the present study ω-CgTX had no effect on SP- or NMDA-induced algesic responses. Thus, the analgesia induced by N-type channel blockade in the present study may have been exerted mainly *via* an effect at the primary afferent nerve terminals – by inhibition of substance P release in the case of the BK-response and of glutamate release in the case of the α,βmeATP-response – with little or no effect being exerted on the second-order neuron itself. The above circumstantial evidence and our results, taken together, suggest that N-type-channel activity is involved in the presynaptic regulation of pain transmission, possibly between unmyelinated C fibres and their targets within the spinal cord, whether transmission is peptidergic or non-peptidergic.

Turning now to the P/Q type of Ca^{2+} channels, our results show that ω-AgTX reduced the α,βmeATP-induced nociceptive response but not the BK-induced response. The available evidence suggests that N-type channels are the predominant VDCCs associated with substance-P-containing primary afferent fibres within the superficial layers of the cord, whereas P/Q-type channels also exist at nerve terminals but are rarely co-localized with substance P (Westenbroek *et al*., 1998). Rather, the latter channels have been reported to be involved in glutamatergic transmission in the lamprey spinal cord, together with the N-type (Krieger *et al*., 1999). Our results are consistent with these studies inasmuch as the α,βmeATP-induced nociceptive response (thought to be transmitted *via* glutamate release) was reduced by ω-AgTX. Furthermore, in our study ω-AgTX had no effect on the NMDA-induced algesic response. Hence, taken together the above findings strongly suggest that the P/Q-type channel is localized in the presynaptic membrane and is mainly involved in glutamatergic neurotransmission, not in peptidergic transmission.

L-type Ca^{2+} channels are found throughout the dorsal horn, localized mainly in the cell bodies (Westenbroek *et al*., 1998). In hippocampal neurons, a similar pattern is seen, with clusters of L-type channels in the postsynaptic membrane (Hell *et al*., 1993, 1996). Furthermore, blockade of L-type VDCC does not inhibit the electrically-evoked release of CGRP from rat spinal-cord slices (Santicioli *et al*., 1992) or of noradrenaline from rat hypothalamic slices (Troger *et al*., 1994), whereas blockade of the N-type does. In addition, while the N-type plays an important role in both the bradykinin-induced release of CGRP from guinea-pig atria and the NMDA-stimulated release of noradrenaline from rat hippocampal and cortical slices, L-type channels have not been found to play a part in these processes (Geppetti *et al*., 1990; Keith *et al*., 1989). In the present study, an L-type blocker, CAL, failed to inhibit the α,βmeATP-induced nociceptive response. On the other hand, this L-type blocker did inhibit the BK-induced response, which is thought to be transmitted *via* SP release, and it also reduced the algesic response elicited by exogenous SP. In view of these results, and the evidence of a preferential distribution of L-type channels on soma and the apparent non-involvement of these channels in neurotransmitter release, the inhibitory effect of CAL on BK-induced responses may be mediated through modulation of post-synaptic neurons in the dorsal horn. The second-order neuron synaptically connected to the primary afferent C fibre stimulated by ATP may be different from the target involved in the BK-response (which seems to be rich in L-type channels). Such divergency would account for the ineffectiveness of CAL against the α,βmeATP-induced response.

It has recently been reported that ATP produces thermal hyperalgesia and mechanical allodynia as well as algesia (Hamilton *et al*., 1999; Tsuda *et al*., 2000) and BK, the most potent algesic substance known, has a well-established involvement in a variety of pathophysiological conditions. Hense, we should expect our pain models to be capable of providing a wealth of information about pain transmission. The present data not only suggest that distinct populations of VDCCs may be involved in BK-evoked peptidergic and ATP-evoked glutamatergic sensory transmission at the spinal-cord level, but may also provide a molecular basis for the future development of clinically effective Ca^{2+}-channel blockers for the control of pain.

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