British Journal of Pharmacology (2003) 140, 558–566
www.nature.com/bjp

Voltage-dependent calcium channels are involved in neurogenic dural vasodilatation *via* a presynaptic transmitter release mechanism

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1 A missense mutation of the *CACNA1A* gene that encodes the α_{1A} subunit of the voltage-dependent P/Q-type calcium channel has been discovered in patients suffering from familial hemiplegic migraine. This suggested that calcium channelopathies may be involved in migraine more broadly, and established the importance of genetic mechanisms in migraine.

2 Channelopathies share many clinical characteristics with migraine, and thus exploring calcium channel functions in the trigeminovascular system may give insights into migraine pathophysiology. It is also known that drugs blocking the P/Q- and N-type calcium channels have been successful in other animal models of trigeminovascular activation and head pain.

3 In the present study, we used intravital microscopy to examine the effects of specific calcium channel blockers on neurogenic dural vasodilatation and calcitonin gene-related peptide (CGRP)-induced dilation.

4 The L-type voltage-dependent calcium channel blocker calciseptine significantly attenuated ($20\,\mu g\,kg^{-1}$, $n=7$) the dilation brought about by electrical stimulation, but did not effect CGRP-induced dural dilation.

5 The P/Q-type voltage-dependent calcium channel blocker ω-agatoxin-IVA ($20\,\mu g\,kg^{-1}$, $n=7$) significantly attenuated the dilation brought about by electrical stimulation, but did not effect CGRP-induced dural dilation.

6 The N-type voltage-dependent calcium channel blocker ω-conotoxin-GVIA ($20\,\mu g\,kg^{-1}$, $n=8$ and $40\,\mu g\,kg^{-1}$, $n=7$) significantly attenuated the dilation brought about by electrical stimulation, but did not effect CGRP-induced dural dilation.

7 It is thought that the P/Q-, N- and L-type calcium channels all exist presynaptically on trigeminovascular neurons, and blockade of these channels prevents CGRP release, and, therefore, dural blood vessel dilation. These data suggest that the P/Q-, N- and L-type calcium channels may be involved in trigeminovascular nociception.
British Journal of Pharmacology (2003) **140,** 558–566. doi:10.1038/sj.bjp.0705456

Keywords: Calcium channels; missense mutations; CGRP; intravital microscopy; trigeminovascular; presynaptic

Abbreviations: ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; KCL, potassium chloride; s.e.m., standard error of the mean

Introduction

Migraine pathophysiology is beginning to be understood, and it is likely to involve the activation of trigeminal afferents (Goadsby *et al*., 2002). Trigeminal sensory nerve fibres that innervate the cranial vasculature contain calcitonin gene-related peptide (CGRP), substance P and neurokinin A (Uddman *et al*., 1999). Trigeminal ganglion stimulation, which recruits both Aδ- and C-fibres (Lee *et al*., 1985; O'Connor & van der Kooy, 1988), results in the release of CGRP and substance P from perivascular trigeminal sensory nerves in humans (Goadsby *et al*., 1988). In acute migraine, only CGRP and neurokinin A (Goadsby *et al*., 1990; Gallai *et al*., 1995), but not substance P (May & Goadsby, 2001) levels, are increased. Moreover, CGRP and not substance P is elevated in acute cluster headache (Goadsby & Edvinsson, 1994). Taken together, these data suggest that CGRP has a pivotal role in trigeminovascular nociception.

Calcium influx into cells is believed to be responsible for neurotransmitter release *via* the voltage-dependent calcium channels (Katz & Miledi, 1967; Dunlap *et al*., 1995). It has recently been established that a rare form of migraine, familial hemiplegic migraine, is due to either a missense mutation of the *CACNA1A* gene that encodes the α_{1A} subunit of the voltage-dependent P/Q-type calcium channel (Ophoff *et al*., 1996), or due to mutations in *ATP1A2* that encode the α_2 subunit of the Na^+/K^+ pump (De Fusco *et al*., 2003). These findings, and the clinical similarities between other channelopathies (Griggs & Nutt, 1995) and migraine (Ferrari, 1998), have suggested that migraine may involve a genetic mutation to one or more of these calcium channels.

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Advance online publication: 26 August 2003

The P/Q-type calcium channels are located neuronally, and have been found to be involved in presynaptic calcium influx into cells and neurotransmitter release in the mammalian brain (Llinas *et al*., 1992; Dunlap *et al*., 1995). The P/Q-type calcium channel is present in the rat trigeminal ganglion (Westenbroek *et al*., 1995) and in a small number of cells in the rat spinal trigeminal nucleus (Westenbroek *et al*., 1995; Craig *et al*., 1998), a key area that is believed to be involved in the pain of acute migraine. The N-type calcium channel, so called for its neuronal localisation, is also involved in the presynaptic regulation of neurotransmitter release in the rat and guinea-pig (Maggi & Giuliani, 1991; Olivera *et al*., 1994; Wheeler *et al*., 1994). A mutation of the *CACNA1B* gene encoding the α_{1B} subunit of the N-type calcium channel has been identified (Ino *et al*., 2001; Miller, 2001), although the phenotype of the mutation is unclear. There is evidence of α_{1B} immunoreactivity in the trigeminal nucleus and trigeminal ganglion in the rat brain (Kim & Chung, 1999; Chung *et al*., 2000).

A third type of voltage-dependent calcium channel is the 'long-lasting' L-type calcium channel. The L-type calcium channel is sensitive to drugs, such as verapamil and diltiazem, and has also been found to be involved in transmitter release in the skin and knee joint (Just *et al*., 2001; Kress *et al*., 2001). The L-type calcium channel has been found to be present on smooth muscle, more specifically on mammalian cardiac and brain vascular smooth muscle (Nakazawa *et al*., 1992; Dunlap *et al*., 1995; Matsuoka *et al*., 1997; Seisenberger *et al*., 2000). L-type calcium channels have also been found in rat trigeminal ganglion neurons, and are involved in long-term synaptic changes in the sensory nucleus of the rat trigeminal nerve after trigeminal ganglion stimulation (Kim & Chung, 1999; Guido *et al*., 2001).

In this study, we used the model of intravital microscopy (Williamson *et al*., 1997a, b) that allows continuous measurement of dural blood vessel diameter, while inducing dilation both prejunctionally with electrical stimulation, and postjunctionally with intravenous CGRP injection. It has been shown that electrical stimulation of a cranial window results in dural vessel dilation inhibited by the CGRP antagonist $CGRP_{8-37}$ (Williamson *et al*., 1997a). Antagonists at both the neurokinin 1 and 2 receptors were not able to produce any inhibition of neurogenic dural vasodilatation (Williamson *et al*., 1997a). Several antimigraine agents, sumatriptan, naratriptan, rizatriptan (Ferrari *et al*., 2002) and dihydroergotamine (Ferrari, 1998), have been shown to inhibit the dural dilation brought about by electrical stimulation (Williamson *et al*., 1997a–c). Exogenous CGRP also causes dural vasodilatation, presumably by activating CGRP receptors on the smooth muscle of dural arteries (Williamson *et al*., 1997a). We investigated the role of P/Q-, N- and L-type voltage-dependent calcium channel blockers with both neurogenic dural vasodilatation and CGRP-induced dural dilation, to characterise the pharmacology of these channels in the trigeminovascular innervation of the dura mater.

Methods

Surgical preparation

All experiments were conducted under the U.K. Home Office (Scientific Procedures) Act (1986). Male Sprague–Dawley rats (220–385 g) were anaesthetised throughout the experiments with sodium pentobarbitone (60 mg kg^{-1} i.p. initially and then 18 mg kg^{-1} h i.v. infusion). The left femoral artery and vein were cannulated for blood pressure recording, and intravenous infusion of anaesthetic and test compounds, respectively.

Temperature was maintained throughout, using a homeothermic blanket system. The rats were placed in a stereotaxic frame and ventilated with oxygen-enriched air, 3–5 ml, 60–80 strokes min^{-1} (Small Rodent Ventilator—Model 683, Harvard Instruments, U.K.). End-tidal CO_2 was monitored (Capstar-100, CWE Inc., U.S.A.) and kept between 3.5 and 4.5%, and blood pressure was monitored continually. The skull was exposed and the right or left parietal bone thinned by drilling with a saline-cooled drill until the blood vessels of the dura were clearly visible through the intact skull.

Intravital microscopy

The cranial window was covered with mineral oil (37°C) and a branch of the middle meningeal artery viewed using an intravital microscope (Microvision MV2100, U.K.), and the image displayed on a television monitor. Dural blood vessel diameter was continuously measured using a video dimension analyser (Living Systems Instrumentation, U.S.A.) and displayed with blood pressure on a data analysis system (Spike4 v2, Cambridge Electronic Design, Cambridge, U.K.).

Experimental protocols

Defining electrical stimulation parameters Electrical stimulation was used to evoke dilation of the dural blood vessels with a bipolar stimulating electrode (NE 200X, Clark Electromedical) that was placed on the surface of the cranial window approximately 200 μm from the vessel of interest. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 s (Grass Stimulator S88, Grass Instrumentation, MA, U.S.A.) with increasing voltage, until maximal dilation was observed. Subsequent electrically induced responses in the same animal were then evoked using the same voltage (Williamson *et al*., 1997a; Akerman *et al*., 2002).

Effects of calcium channel blockers on electrical stimulation and CGRP-induced dilation

The effects of the L-type calcium channel blocker calciseptine were studied. Calciseptine (7 μg kg^{-1}) was administered intravenously at least 10 min after a control response to electrical stimulation or bolus CGRP (1 μg kg^{-1}; it has previously been defined that this dose provides maximum dilation of dural blood vessels when given intravenously (Williamson *et al*., 1997a)); the electrical stimulation or CGRP bolus was repeated after 5–10 min. Then, at least a further 10 min later, an increased dosage of calciseptine (10 μg kg^{-1}) was administered, followed by electrical stimulation or CGRP bolus after 10 min. A final dose of calciseptine (20 μg kg^{-1}) was administered after another 10 min, and a final repeat electrical stimulation or CGRP bolus given after a further 10 min. The effects of an N-type (ω-conotoxin-GVIA – 10, 20 and 40 μg kg^{-1}) and a P/Q-type (ω-agatoxin-IVA – 3, 10 and 20 μg kg^{-1}) calcium channel blockers were also studied using the same protocol. A P/Q-type (ω-agatoxin-TK – 3, 10 and 20 μg kg^{-1}) calcium channel blocker used the same protocol, but was only tested

against electrical stimulation. Up to three doses of each test substance was given during a single experiment; not all animals had every dose.

Data analysis

The peak effects of electrical stimulation and CGRP infusion on dural vessel diameter were calculated as a percentage increase from the prestimulation baseline diameter. The nature of the experimental set-up, where the magnification of the dural vessel selected for study was different in each set-up, made it impractical to standardise the dural vessel measurement; therefore, the dural vessel diameter was measured in arbitrary units, and all calculations related to the premanipulation baseline. All data are expressed as mean ± s.e.m. Statistical analysis was performed using an ANOVA for repeated measures and *post hoc* comparison was made with a Student's paired *t*-test, to examine the level of dilation after administration of test compounds at specific doses compared to the control dilation, if repeated measures proved significant (SPSS v10.0). The reproducibility of both the neurogenic vasodilator and CGRP responses has been tested previously using four consecutive saline-controlled stimuli (Akerman *et al*., 2002) in the same experimental set-up. For the control studies, the consecutive determinations of the neurogenic vasodilator response were compared to each other, and also the baseline dilations obtained for the studies where there was pharmacological intervention. This was done using an ANOVA for repeated measures. Significance was assessed at the $P<0.05$ levels.

Drugs

The infusion of anaesthetic, CGRP and calcium channel blocker was *via* the same catheter, after flushing with saline for several minutes between each compound. CGRP, calciseptine (IC_{50} 27 nM), which is isolated from the black mamba (Yasuda *et al*., 1994), ω-agatoxin-IVA and ω-agatoxin-TK, which are equipotent (IC50 20 nM) (Mintz *et al*., 1992), which are both isolated in the funnel web spider (all Sigma-Aldrich, U.K.), and ω-conotoxin-GVIA (IC_{50} 3 nM) (Grantham *et al*., 1994), which is isolated in the marine snail (Peptide Institute, U.K.), were all dissolved in deoxygenated water, aliquotted and frozen until required. They were then redissolved in 0.9% saline, to make up to a volume of 0.2 ml before intravenous administration. The doses that were given for each calcium channel blocker were determined by the fact that a 10 $\mu g\,kg^{-1}$ dose is effective in inhibiting the N-type calcium channel *in vivo* (Pruneau & Belichard, 1992), without being toxic, and the IC_{50} values for each blocker are similarly in the nM region.

Results

Control groups

The results for the series of control dilations using electrical stimulation and CGRP-induced dilation with saline intervention have been published, and showed no difference across the cohort (Akerman *et al*., 2002). These control effects were compared using an ANOVA to the control dilations used in the pharmacological studies, and there were no significant differences.

Effect of the L-type calcium channel blocker on electrical stimulation and CGRP-induced dilation

In rats treated with calciseptine (7 and 10 $\mu g\,kg^{-1}$, $n=8$ and 20 $\mu g\,kg^{-1}$, $n=7$), the dilation brought about by electrical stimulation was significantly reduced with the 20 $\mu g\,kg^{-1}$ dose of calciseptine from 122 ± 14 to 85 ± 19% ($P<0.05$, $t_6=5.0$). Increases in dural blood vessel diameter evoked by CGRP (1 $\mu g\,kg^{-1}$, i.v.) showed no significant difference between the control CGRP-induced dilation and the dilation produced after calciseptine (7, 10 and 20 $\mu g\,kg^{-1}$, $n=7$; $F_{4,20}=0.384$, $P=0.82$; Figure 1). Calciseptine injections caused a slight reduction in blood pressure, which was accompanied by a slight increase in blood vessel diameter; neither were significant for each dose (Table 1). Both returned to preinjection levels by the time of the repeated vasodilator challenge.

Effect of the P/Q-type calcium channel blocker ω-agatoxin-IVA on electrical stimulation and CGRP-induced dilation

In rats treated with ω-agatoxin-IVA (3, 10 and 20 $\mu g\,kg^{-1}$, $n=7$), the dilation brought about by electrical stimulation was significantly reduced with all doses of ω-agatoxin-IVA: 111 ± 13–91 ± 10% (3 $\mu g\,kg^{-1}$, $P<0.05$, $t_6=3.9$), 111 ± 13–81 ± 12% (10 $\mu g\,kg^{-1}$, $P<0.05$, $t_6=3.6$) and 111 ± 13–63 ± 11% (20 $\mu g\,kg^{-1}$, $P<0.05$, $t_6=3.9$). Increases in dural blood vessel diameter evoked by CGRP (1 $\mu g\,kg^{-1}$, i.v.) showed no significant difference between the control CGRP-induced dilation and the dilation produced after ω-agatoxin-IVA (3, 10 and 20 $\mu g\,kg^{-1}$, $n=6$, $F_{4,20}=0.05$, $P=0.46$, Figure 2). ω-agatoxin-IVA injections had no significant effect on blood pressure or blood vessel diameter (Table 1).

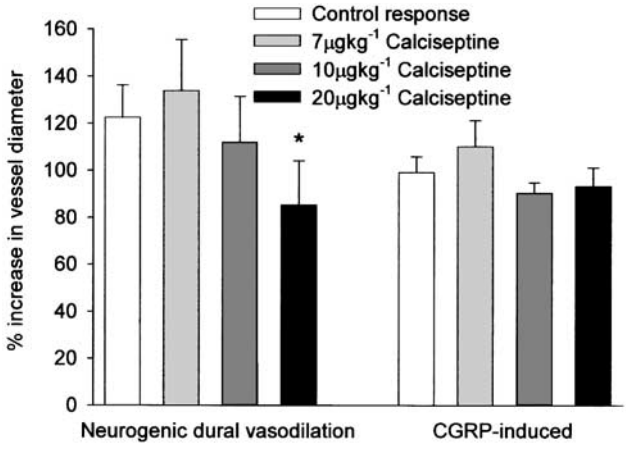

Figure 1 The effects of repeated electrical stimulation or CGRP injection (1 μgkg^{-1}) with calciseptine treatment on dural blood vessel diameter. Following control responses, rats were injected with calciseptine (7, 10 and 20 $\mu g\,kg^{-1}$) and electrical stimulation or CGRP injection repeated. *$P<0.05$ significance compared to the control response used in the calciseptine series of experiments.

Table 1 Summary of blood pressure and dural blood vessel diameter changes under the different drug regimens

Drug	*Dosage* (n)	*Change in blood pressure* (mmHg)	*Change in dural blood vessel diameter* (%)
Calciseptine	7 $\mu g\,kg^{-1}$ ($n=10$)	-1 ± 1	$+6\pm4$
Calciseptine	10 $\mu g\,kg^{-1}$ ($n=10$)	-4 ± 2	$+4\pm6$
Calciseptine	20 $\mu g\,kg^{-1}$ ($n=10$)	-8 ± 4	$+3\pm2$
ω-Agatoxin-TK	3 $\mu g\,kg^{-1}$ ($n=7$)	-2 ± 2	-4 ± 2
ω-Agatoxin-TK	10 $\mu g\,kg^{-1}$ ($n=7$)	-3 ± 2	-7 ± 1*
ω-Agatoxin-TK	20 $\mu g\,kg^{-1}$ ($n=7$)	-2 ± 2	-8 ± 2
ω-Agatoxin-IVA	3 $\mu g\,kg^{-1}$ ($n=13$)	-2 ± 1	-2 ± 1
ω-Agatoxin-IVA	10 $\mu g\,kg^{-1}$ ($n=13$)	-0.3 ± 1	$+1\pm2$
ω-Agatoxin-IVA	20 $\mu g\,kg^{-1}$ ($n=13$)	-2 ± 1	$+3\pm3$
ω-Conotoxin-GVIA	10 $\mu g\,kg^{-1}$ ($n=14$)	-44 ± 2#	$+20\pm6$*
ω-Conotoxin-GVIA	20 $\mu g\,kg^{-1}$ ($n=14$)	-7 ± 3	$+9\pm6$
ω-Conotoxin-GVIA	40 $\mu g\,kg^{-1}$ ($n=14$)	-6 ± 3	$+23\pm12$

Effects of calcium channel blockers on blood pressure and dural blood vessel diameter. *$P<0.05$ significance compared to blood vessel diameter prior to calcium channel blocker administration. #$P<0.05$ significance compared to blood pressure prior to calcium channel blocker injection.

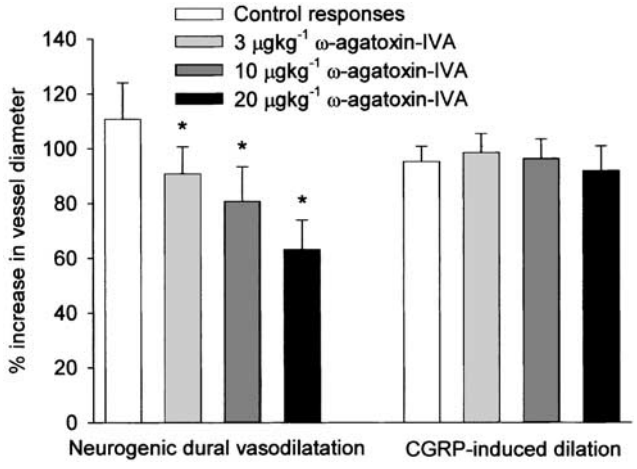

Figure 2 The effects of repeated electrical stimulation or CGRP injection (1 $\mu g\,kg^{-1}$) with ω-agatoxin-IVA treatment on dural blood vessel diameter. Following control responses, rats were injected with ω-agatoxin-IVA (3, 10 and 20 $\mu g\,kg^{-1}$) and electrical stimulation or CGRP injection repeated. *$P<0.05$ significance compared to the control response used in the ω-agatoxin-IVA series of experiments.

Effect of the P/Q-type calcium channel blocker ω-agatoxin-TK on electrical stimulation

In rats treated with ω-agatoxin-TK (3, 10 and 20 $\mu g\,kg^{-1}$, $n=8$) the dilation brought about by electrical stimulation was significantly reduced with the 10 and 20 $\mu g\,kg^{-1}$ doses of ω-agatoxin-TK: 157 ± 9–$112\pm23\%$ ($P<0.05$, $t_7=2.4$) and 157 ± 9–$92\pm18\%$, respectively ($P<0.05$, $t_7=3.6$) (see Figure 3). ω-agatoxin-TK injections caused a slight decrease in blood pressure, which was accompanied by a small increase in blood vessel diameter (Table 1); this was only significant for the 10 $\mu g\,kg^{-1}$ dose. Both returned to preinjection levels by the time of the repeated vasodilator challenge.

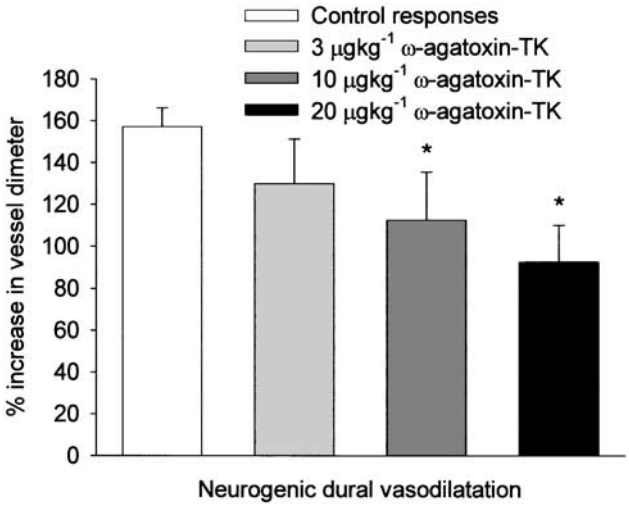

Figure 3 The effects of repeated electrical stimulation with ω-agatoxin-TK treatment on dural blood vessel diameter. Following control responses, rats were injected with ω-agatoxin-TK (3, 10 and 20 $\mu g\,kg^{-1}$) and electrical stimulation repeated. *$P<0.05$ significance compared to the control response used in the ω-agatoxin-TK series of experiments.

Effect of the N-type calcium channel blocker ω-conotoxin-GVIA on electrical stimulation and CGRP-induced dilation

In rats treated with ω-conotoxin-GVIA (10 and 20 $\mu g\,kg^{-1}$, $n=8$ and 40 $\mu g\,kg^{-1}$, $n=7$), the dilation brought about by electrical stimulation was significantly reduced with all doses: 102 ± 13–$83\pm17\%$ (10 $\mu g\,kg^{-1}$, $P<0.05$, $t_7=3.0$), 102 ± 13–$82\pm17\%$ (20 $\mu g\,kg^{-1}$, $P<0.05$, $t_7=3.0$) and 91 ± 7–$74\pm9\%$ (40 $\mu g\,kg^{-1}$, $P<0.05$, $t_6=3.8$). Increases in dural blood vessel diameter evoked by CGRP (1 $\mu g\,kg^{-1}$, i.v.) showed no significant difference between the control CGRP-induced dilation and the dilation produced after ω-conotoxin-GVIA (Figure 4; 10, 20 and 40 $\mu g\,kg^{-1}$, $n=6$, $F_{4,20}=0.27$, $P=0.9$). The initial (10 $\mu g\,kg^{-1}$) ω-conotoxin-GVIA injection caused a significant decrease in blood pressure of 44 ± 2 mmHg, accompanied by a $20\pm6\%$ ($n=14$, $P<0.01$, $t_{13}=20.65$) increase in vessel diameter (Table 1). The increase in vessel diameter returned to its preinjection level by the time of the repeated vasodilator challenge. The blood pressure drop did not return completely to its preinjection level, remaining 31 ± 3 mmHg below the initial level. There was no significant change in either the blood pressure or vessel diameter with the second and third doses of ω-conotoxin-GVIA.

Effect of calcium channel blockers on the hypotensive effect of CGRP injections

The four consecutive CGRP injections (as described in Akerman *et al*., 2002) and the control group of the Results section produced decreases in mean blood pressure of 41 ± 7, 38 ± 5, 37 ± 7 and 33 ± 3 mmHg, respectively (Table 2). There was no difference across the cohort ($F_{3,12}=1.492$, $P=0.283$). We compared these effects with an ANOVA for repeated measures to the control dilations used in the pharmacological studies, and there were no significant differences. Changes in blood pressure caused by CGRP (1 $\mu g\,kg^{-1}$, i.v.) showed no significant difference between the control CGRP change and

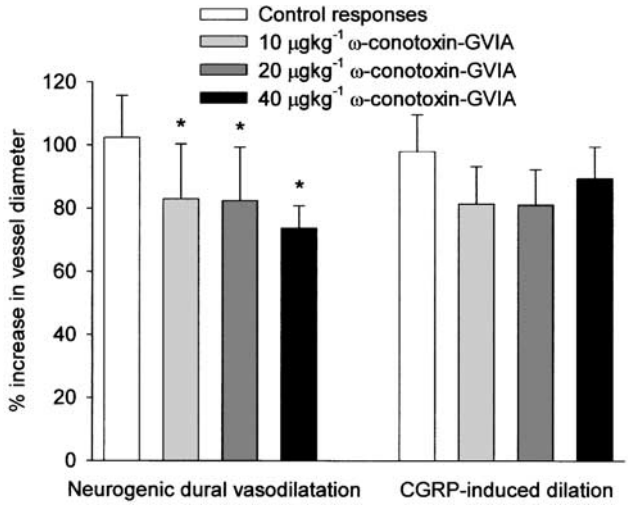

Figure 4 The effects of repeated electrical stimulation or CGRP injection ($1\,\mu g\,kg^{-1}$) with ω-conotoxin-GVIA treatment on dural blood vessel diameter. Following control responses, rats were injected with ω-conotoxin-GVIA (10, 20 and $40\,\mu g\,kg^{-1}$) and electrical stimulation and CGRP injection repeated. $^*P<0.05$ significance compared to the control response used in the ω-conotoxin-GVIA series of experiments.

the change produced after calciseptine (7, 10 and $20\,\mu g\,kg^{-1}$, $n=5$, $F_{3,12}=2.34$, $P=0.17$) or ω-agatoxin-IVA (3, 10 and $20\,\mu g\,kg^{-1}$, $n=7$, $F_{3,18}=2.91$, $P=0.11$). The changes in blood pressure with CGRP injection after ω-conotoxin-GVIA ($n=14$) were significantly reduced for each dose from 40 ± 6 to 16 ± 2 mmHg ($10\,\mu g\,kg^{-1}$, $P<0.05$, $t_5=3.15$), 22 ± 3 mmHg ($20\,\mu g\,kg^{-1}$, $P<0.05$, $t_5=2.6$) and 17 ± 3 mmHg ($40\,\mu g\,kg^{-1}$, $P<0.05$, $t_5=4.1$). There was no difference in the blood pressure drop with CGRP between the given different doses of ω-conotoxin-GVIA.

Discussion

The data suggest that each of the P/Q-, N- and L-type voltage-dependent calcium channels are involved in neurogenic trigeminovascular dural vasodilatation. The specific employed blockers were all able to attenuate dural vasodilatation, but none had effects on CGRP-induced dural vasodilatation. This is likely to be due to presynaptic inhibition of calcium influx preventing the release of CGRP. It is thus likely that these calcium channels are not present postsynaptically and, therefore, were unable to affect exogenous CGRP.

P/Q-type calcium channel blockers and neurogenic dural extravasation

The P/Q-type calcium channel blockers ω-agatoxin-TK and ω-agatoxin-IVA were able to attenuate neurogenic dural vasodilatation in the rat, brought about by electrical stimulation. It seems likely that blockade of the P/Q-type voltage-dependent calcium channels prevents CGRP release presynaptically from perivascular trigeminal sensory nerve fibres, since we saw no effect on the dural dilation elicited by exogenously administered CGRP. This effect is analogous to that seen with triptans, serotonin 5-$HT_{1B/1D}$ agonists (Williamson *et al*., 1997b, c) and the opioid receptor agonists (Williamson *et al*., 2001). The P/Q-type calcium channel blocker α-eudesmol inhibits plasma protein extravasation in the rat dura, and attenuated the vasodilatation in the facial skin, as monitored by laser Doppler flowmetry after trigeminal ganglion stimulation, and is thought to use the same mechanism, blocking the presynaptic release of neuropeptides from perivascular trigeminal terminals (Asakura *et al*., 2000).

N-type calcium channel blockers and neurogenic dural extravasation

The N-type calcium channel blocker, ω-conotoxin-GVIA was able to attenuate neurogenic dural vasodilatation in the rat. It is likely that this attenuation is *via* a similar mechanism to the P/Q-type calcium channel inhibition. The N-type calcium channel blocker is, therefore, preventing CGRP release from perivascular trigeminal sensory nerve fibres and inhibiting the dilation of the dural blood vessels. Indeed, ω-conotoxin-GVIA, the recognised N-type calcium channel blocker (Olivera *et al*., 1985), has been shown to inhibit presynaptic neuropeptide release, including CGRP, after electrical stimulation of the ureter in the guinea-pig and KCl-evoked CGRP release in the rat spinal cord (Maggi *et al*., 1990a, b; Maggi & Giuliani, 1991). Also, recently, blockade of the N-type calcium channel by bathing the brain stem area with ω-conotoxin-GVIA (Richter *et al*., 2002) inhibited the central sensitisation of trigeminal nucleus caudalis neurons after dilation of the meningeal blood vessels with CGRP (Cumberbatch *et al*., 1999; 2001). Calcium influx into nerve terminals contributes to the release of neurotransmitters; therefore, it seems likely that ω-conotoxin-GVIA is preventing the release of CGRP by inhibiting calcium influx into nerve terminals, and thus preventing exocytosis and the mechanism for transmitter release (Dunlap *et al*., 1995).

Table 2 Summary of blood pressure changes with CGRP injection under different drug regimens.

CGRP injection ($1\,\mu g\,kg^{-1}$)	*Saline (0.2 ml) drop in BP* (mmHg)	*Calciseptine (dosage and drop in BP)*	*ω-Agatoxin-IVA (dosage and drop in BP)*	*ω-Conotoxin-GVIA (dosage and drop in BP)*
1	41 ± 7	(Control) 32 ± 2 mmHg	(Control) 41 ± 3 mmHg	(Control) 40 ± 6 mmHg
2	38 ± 5	($7\,\mu g\,kg^{-1}$) 31 ± 4 mmHg	($3\,\mu g\,kg^{-1}$) 42 ± 3 mmHg	($10\,\mu g\,kg^{-1}$) 16 ± 2 mmHg*
3	37 ± 7	($10\,\mu g\,kg^{-1}$) 22 ± 2 mmHg	($10\,\mu g\,kg^{-1}$) 36 ± 5 mmHg	($20\,\mu g\,kg^{-1}$) 22 ± 3 mmHg*
4	33 ± 3	($20\,\mu g\,kg^{-1}$) 27 ± 2 mmHg	($20\,\mu g\,kg^{-1}$) 31 ± 2 mmHg	($40\,\mu g\,kg^{-1}$) 17 ± 3 mmHg*

BP = blood pressure. Control represents with initial CGRP injection prior to any drug intervention. There was no difference in the change in BP across all CGRP injections in the saline group. There was no difference when the controls used for each drug were compared using an ANOVA for repeated measures to the changes in the saline group. $^*P<0.05$ significance compared to the control blood pressure drop.

L-type calcium channel blockade and neurogenic dural extravasation

The L-type calcium channel blocker calciseptine was also able to attenuate the neurogenic dural vasodilatation that occurs after electrical stimulation of the cranial window. We could not see evidence in the literature that L-type calcium channels contribute to transmitter release specifically on trigeminal neurons, although there is evidence that they are involved in dopaminergic mechanisms in the striatum and caudate putamen in the brain, to promote neurotransmitter release from presynaptic nerve endings (Galarraga *et al*., 1997; Okita *et al*., 2000; Baufreton *et al*., 2003), and they are also involved in transmitter release in the periphery (Just *et al*., 2001; Kress *et al*., 2001). There are no data for calciseptine acting on any other sites in the brain or periphery at the doses used in these experiments, although we cannot discount an action at another site at this concentration. From the evidence available, it seems that calciseptine is acting on a receptor or channel site presynaptically, attenuating the release of CGRP, and therefore the subsequent dilation. Without direct evidence that the L-type calcium channel is involved in presynaptic neurotransmitter release in the trigeminovascular region, or whether calciseptine may be acting on another non-L-type calcium channel site, we cannot be sure if this is the mechanism involved. It is worth noting that the dose required to produce any inhibition with the L-type calcium channel blocker is higher than the lowest dose required with the P/Q- and N-type calcium channel blockers, despite the fact that all the compounds have IC_{50} values in the nm region. This discrepancy may reflect a lower distribution of L-type calcium channels on trigeminal nerves.

Although each calcium channel blocker was able to attenuate to some degree the vasodilatation caused by electrical stimulation, none of them were able to totally block the effect. Earlier studies using this model have shown that only the CGRP receptor blocker $CGRP_{8-37}$ was able to fully block the neurogenic dural vasodilatation, while the triptans were never able to fully inhibit the response (Williamson *et al*., 1997b, c). The triptans were, however, more effective than the calcium channel blockers used in this study. This would seem to imply that there is not a single channel that governs the release of CGRP from sensory trigeminal nerve fibres. It is more likely that two or more contribute to the transmitter release in this region, which would make any attempted therapeutic intervention complex. It may be useful to apply a nonspecific calcium channel blocker in the model, or even the individual channel blockers simultaneously to see if this is more effective at fully blocking the neurogenic dural vasodilatation.

The mechanism by which the calcium channel blockers attenuate neurogenic dural vasodilatation may be by preventing presynaptic release of CGRP from trigeminal sensory terminals, although our data cannot confirm this. Electrical stimulation of dural sites provokes presynaptic CGRP release from trigeminal sensory nerve fibres that innervate the dura mater. This CGRP release causes vasodilatation by acting on postsynaptic CGRP receptors on the smooth muscle of dural arteries. If the dilation is blocked or attenuated by a mechanism that is not postsynaptic, then it seems likely to be *via* a presynaptic mechanism. It is necessary to study this point more thoughtfully, so that one can be certain that the mechanism involved is due to the blockade of presynaptic CGRP release rather than some other mechanism. This argument also stands for the data produced by the Williamson group.

One possibility would be to look at the peptide content of blood samples taken from the external jugular vein after electrical stimulation of the cranial window, before and after calcium channel blocker treatment, mirroring the studies done by Goadsby *et al*. (1988; 1990) on both migraine patients and experimental animals after trigeminal ganglion stimulation. It would also be useful to repeat the trigeminal ganglion stimulation studies using calcium channel blockers, to see if the different channels are preferentially situated on neurons that release either CGRP, substance P or neurokinin A, and also looking at the response of the different calcium channel blockers on extravasation, compared to vasodilatation, as the response of α-eudesmol on extravasation was more profound than on the vasodilatation of facial skin (Asakura *et al*., 2000).

Effect of calcium channel blockers on CGRP-induced dilation

Intravenous CGRP induces dural vessel dilation in the rat, and CGRP blockers inhibit this by acting postjunctionally at CGRP receptors in the smooth muscle (Williamson *et al*., 1997a). The P/Q-, N- and L-type calcium channel inhibitors were all unable to block the CGRP-induced dilation, when given intravenously. It is unlikely then that the P/Q-, N- and L-type calcium channel blockers are situated postsynaptically, as they are unable to prevent the dural vessel dilation caused by exogenous CGRP.

Blood pressure effects of calcium channel blockers

Neither calciseptine nor ω-agatoxin had any profound effects on blood pressure *per se*, and they had no significant effect on dural blood vessel diameter, except the $10\,\mu g\,kg^{-1}$ dose of ω-agatoxin-TK. This seems a rather erroneous result, as the higher dose had no great effect on blood vessel diameter, and the three doses of ω-agatoxin-IVA had no great effect either, and the two compounds are equipotent as P/Q-type calcium channel blockers. CGRP, as well as being a potent vasodilator, is also known to cause a dose-dependent drop in blood pressure (Brain *et al*., 1993; Williamson *et al*., 1997a). We have shown previously that this vasodilatation is reproducible (Akerman *et al*., 2002), and, in this study, that the blood pressure response is also reproducible. Both calciseptine and ω-agatoxin-IVA were unable to alter the effects of CGRP on blood pressure.

ω-Conotoxin-GVIA had a profound effect on blood pressure; this was only the case on its first injection where it produced a significant drop in blood pressure and a corresponding significant increase in vessel diameter. It would seem that the blood pressure effect was irreversible as the blood pressure only slightly increased after this, and subsequent injections did not further affect the blood pressure. The blood vessel diameter was restored to its original level after 5 min postinjection; therefore, this response was not irreversible. The vasodilator response would seem to be linked to the blood pressure change, as subsequent injections of ω-conotoxin-GVIA did not affect either blood vessel diameter

or blood pressure. This may reflect the fact that N-type calcium channels appear to be essential for the proper functioning of the sympathetic nervous in circulatory regulation (Pruneau & Belichard, 1992; Ino *et al*., 2001). On first inspection of the effect of ω-conotoxin on the CGRP-induced blood pressure drop, it would seem that all doses of the peptide had a significant effect on this response. In reality, it is more likely that the drop in blood pressure caused by the initial ω-conotoxin-GVIA, which was maintained throughout the remainder of the experiment, meant that CGRP was unable to drop the blood pressure that much more. It is important to note that the CGRP was still able to cause a consistent dural vasodilatation despite the limited blood pressure response. This indicates that it is the response of CGRP on the smooth muscle of meningeal vessels that cause the vasodilatation, and not a response to the blood pressure change.

There is considerable evidence for the involvement of the P/Q-type voltage-gated calcium channel in more common forms of migraine (May *et al*., 1995; Nyholt *et al*., 1998), in addition to clear evidence for its involvement in familial hemiplegic migraine (Ophoff *et al*., 1996). It is likely that the phenotype of these P/Q-type calcium channel mutations takes the form of opening the presynaptic calcium channels of sensory nerve terminals, although the functional consequences are variable (Hans *et al*., 1999). Several mutations of the gene that encodes the α_{1B} subunit of the N-type calcium channel have been identified (Ino *et al*., 2001; Miller, 2001), although none are specifically related to migraine. Interpreting the data for the L-type calcium channel is slightly more difficult, as there is no evidence of their existence causing transmitter release in the CNS, although they are present in the trigeminal system (Kim & Chung, 1999; Guido *et al*., 2001). The fact that they are situated in the trigeminal ganglion means that it is possible that a mutation of the genes that encode subunits that make up the L-type calcium channels may cause the channels to remain open and evoke exocytosis and transmitter release. This may influence responses to cortical spreading depression (Choudhuri *et al*., 2002), which is believed to represent the aura phase of migraine, where L-type channel expression is altered.

The therapeutic possibilities of using specific calcium channel blockers in the acute treatment of migraine are limited as their effects in this model, while being significant, did not completely block the neurogenic dural vasodilatation, and were less effective than the triptans. However, there may be possibilities as a combination therapy, if it is found that the use of two or more calcium channel blockers is better able to inhibit the neurogenic dural vasodilatation. The limiting factor may be the effect they have on blood pressure, especially the N-type calcium channel blocker. Most calcium channel blockers that are used in the clinic are used to prevent angina and hypertension; therefore, an inevitable side effect, or consequence, of their use is the reduction of blood pressure. If one can establish an ideal combination that limits the impact on blood pressure, calcium channel blockers may yet be of use in the treatment of acute migraine. It is also important to note that all the studies mentioned above were done in either mice, rats or guinea-pig. The affinity and efficacy of the compounds used may differ in the human and, therefore, this issue will need to be addressed if a combination therapy were to be of any use.

In summary, we have shown the involvement of P/Q-, N- and L-type voltage-gated calcium channels in neurogenic trigeminovascular dural vasodilatation, which is not directly related to the activation of postsynaptic CGRP receptors. We can suggest that each of the P/Q-, N- and L-type calcium channels may represent novel therapeutic targets in migraine. Further pharmacological and human genetic studies in migraine may elucidate a role for these channels in primary headache.

We thank Thorsten Bartsch, Kevin Shields, Yolande Knight, James Storer and Paul Hammond of the Headache Group at the Institute of Neurology for both assistance and technical support during these experiments. This work has been supported by the Wellcome Trust. PJG is a Wellcome Senior Research Fellow.

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(Received April 15, 2003
Revised July 8, 2003
Accepted July 16, 2003)