Partial reversal of conduction slowing during repetitive stimulation of single sympathetic efferents in human skin

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Abstract

Aims: To describe and identify the function of a class of human C fibre with an unusual response to repetitive electrical stimulation. Other C fibres slow progressively at 2 Hz (type 1), reach a latency plateau (type 2) or hardly slow at all (type 3).

Methods: C fibres innervating hairy skin were recorded by microneurography in the superficial peroneal nerves of 19 healthy volunteers. Baseline electrical stimulation of the skin was at 0.25 Hz, and activity-dependent slowing recorded during stimulation at 2 Hz for 3 min and after a 3-min pause in stimulation.

Results: In 41 units, there was a partial recovery of latency during repetitive stimulation. These were classified as 'type-4' units, and identified as sympathetic efferents, since they exhibited spontaneously activity, which was enhanced by manoeuvres that increase sympathetic outflow (15 of 16 cases) and/or suppressed by a proximal anaesthetic block (eight of eight cases). The peak slowing during 2 Hz trains averaged 6.47 ± 2.06% (mean ± SD, n = 41), but after 3 min the slowing had reduced to 4.90 ± 2.20%, which was less than in all type 1 (nociceptor) fibres but similar to that in type 2 (cold) fibres. Compared with cold fibres, type-4 sympathetic fibres slowed more after the first 10 impulses at 2 Hz (2.57 ± 0.45%) and also after a pause in stimulation (1.66 ± 0.51%).

Conclusions: The distinctive activity-dependent slowing profiles of these type-4 sympathetic C units may help identification *in vitro*, and suggest that hyperpolarization-activated channels have a particularly prominent role in the axonal membrane.

Keywords conduction velocity, human, nerve fibres, skin, sympathetic, unmyelinated.

An action potential propagated in an unmyelinated axon causes both short-lasting (~1 s) and long-lasting (up to a few minutes) changes in conduction velocity. The short-lasting changes may be in the direction of speeding up (supernormality) or slowing down (subnormality) and probably depend on whether the balance of charge movement during the action potential (primarily due to an influx of Na^+ and Ca^{2+} ions, and an efflux of K^+ ions) leave the membrane depolarized or hyperpolarized at the end of the action potential (Bostock *et al.* 2003). The long-lasting changes are normally in the direction of slowing, and are caused in part by hyperpolarization by the electrogenic sodium pump that reverses the movements of Na^+ and K^+ ions (Rang & Ritchie 1968). These long-lasting changes accumulate during trains of action potentials, in a way

that depends on the complex interaction of membrane ion channels and pumps, and can be used to identify different functional types of afferent and efferent C fibres innervating skin, both in animals (Thalhammer *et al.* 1994, Gee *et al.* 1996) and humans (Serra *et al.* 1999, Weidner *et al.* 1999).

We first reported that the profile of activity-dependent slowing during 3-min trains at 2 Hz differentiates three types of human C fibre: type 1, which slow progressively during the 3 min, were identified as nociceptor afferents; type 2, which reach a plateau of slowing at a latency increase of about 5%, were specific cold afferents; a rarer type 3, which hardly slow at all, were unidentified (Serra *et al.* 1999, Campero *et al.* 2001). Following Weidner *et al.*'s (1999) report that slowing at very low rates of stimulation differentiates mechano-insensitive from mechano-responsive C nociceptors, we subdivided type 1 into those that slow by less than 2% after a 3-min pause in stimulation at 0.25 Hz (type 1A, mechano-responsive) and those that slow by more than 2% after a pause (type 1B, mechano-insensitive) (Serra *et al.* 2004). Using the same protocol of baseline stimulation at 0.25 Hz, interrupted by 3-min periods at 0 Hz and 2 Hz, we now report a further, very distinctive profile of activity-dependent slowing, in which the slowing quickly reaches a peak and is followed by a phase of relative acceleration. These 'type 4' C fibres are sympathetic efferents, and the ability to distinguish them from afferent C fibres by means of activity-dependent velocity changes alone may prove useful in studies of C fibres *in vitro*, when telltale efferent activity is absent.

Methods

Subjects and microneurographic recordings

Microneurographic recordings were obtained from 19 healthy volunteers (10 males, nine females, aged 16–52 years). The subjects gave written informed consent and the local ethics committee approved the test protocol. They sat relaxed in a recliner with the legs supported in a padded platform. Room temperature was maintained at 21 °C. Microneurographic recordings were obtained from the superficial peroneal nerve at ankle level. A disposable lacquer-insulated tungsten microelectrode, 200 μm in diameter (FHC, Bowdoinham, ME, USA) was inserted manually through the skin into the nerve. A subcutaneous reference electrode was inserted 1–2 cm away. The nerve signals were filtered, amplified, monitored by oscilloscope and loudspeaker, and digitized by personal computer, using QTRAC (© Institute of Neurology, London, UK) or AXOSCOPE® (Axon Instruments, Whipple City, CA, USA) software (Fig. 1).

Measurements of latency and activity-dependent slowing of C fibres

Search for action potentials with C-fibre latency was performed by electrical stimulation of the skin by a pair of needle electrodes. When an action potential was found, the needles were slightly inserted in the skin and stimulation at 0.25 Hz begun. Square wave electrical pulses (0.25 ms duration, 10–150 V) were delivered using a Grass S48 stimulator with stimulus isolation unit (SIU5).

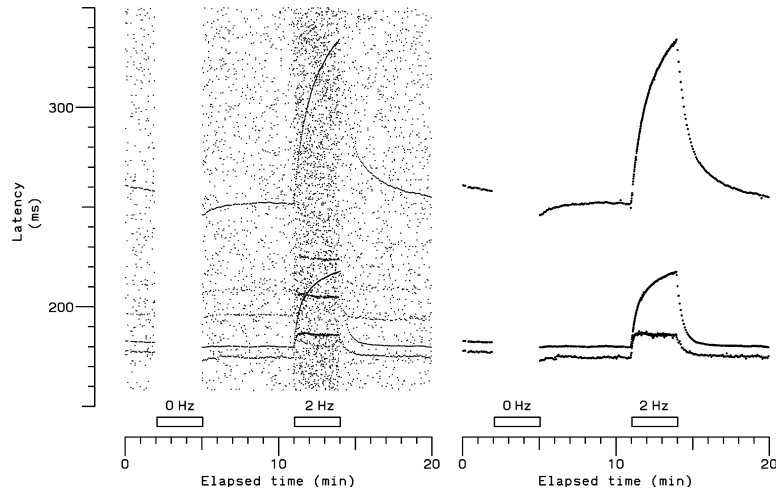

Figure 1 C-fibre raster plots. Electrical stimulation at 0.25 Hz was interrupted by a 3-min period without stimulation and by a 3-min period of stimulation at 2 Hz, to reveal patterns of activity-dependent slowing of different C fibres. Left: detailed raster plot (showing 18 largest peaks in neurogram at each time point) reveals presence of at least five units. Right: remeasured latencies of three largest units, identified from slowing profiles as type 1B (mechano-insensitive nociceptor) at 252 ms, type 1A (mechano-responsive nociceptor) at 180 ms, and new type-4 unit at 175 ms baseline latency. Two further type-4 units can be discerned in the detailed raster plot at latencies of 196 and 209 ms.

The latencies of all units excited were displayed in a raster plot [equivalent to the compressed latency display of Torebjörk and Hallin (1974)] using Qtrac© (Fig. 1). Activity-dependent slowing was assessed at 0.25 and 2 Hz. To assess slowing at the baseline frequency of 0.25 Hz, stimulation was interrupted by a pause of 3 min. After a 6-min period of stimulation at 0.25 Hz, the rate was increased to 2 Hz for 3 min, followed by another recovery period of 6 min at 0.25 Hz. In a few experiments, slowing was also assessed at 1 and 4 Hz. Conduction velocity was estimated from the latency at the baseline frequency and the distance between stimulating and recording electrodes.

Identification of cutaneous sympathetic efferents

Sympathetic units were identified on the basis of spontaneous activity which (a) was increased by 'sympathetic manoeuvres', and/or (b) was blocked by an injection of lidocaine (3–6 mL, 2%) close to the nerve, proximal to the recording site. Spontaneous activity was recognized by fluctuations in baseline latency, as previously described for cold-specific C fibres (Campero *et al.* 2001). In some cases, a sympathetic fibre was so spontaneously active, that the baseline latency was only measurable after lidocaine block (e.g. Fig. 5, below). 'Sympathetic manoeuvres', known to increase cutaneous sympathetic activity, included taking a deep breath, the Valsalva manoeuvre (at onset), startle by an unexpected shout, and stress caused by mental arithmetic (Delius *et al.* 1972). In this study, no attempts were made at cooling or warming the whole body, which could have discriminated vasoconstrictor from sudomotor subclasses of sympathetic fibres (Macefield & Wallin 1999).

Results

'Type 4' profile of activity dependent slowing and acceleration

Figure 2 illustrates four fibres with a distinctive profile of latency changes on stimulation for 3 min at 2 Hz on a background of stimulation at 0.25 Hz. The new 'type-4' units on the left are compared with type-2 (cold) units on the right, which have the most similar latency profile among the previously described types of human C fibre (Serra *et al.* 1999). Nociceptor fibres behave very differently, slowing progressively for 3 min at 2 Hz, and with a much greater degree of slowing achieved by

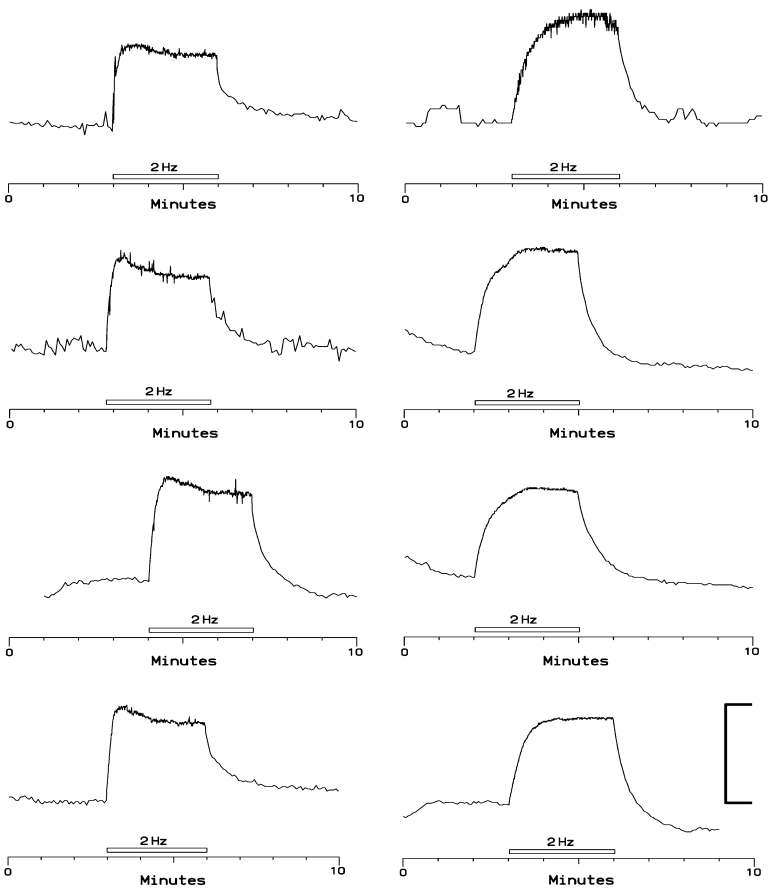

Figure 2 Comparison of type-4 and -2 slowing profiles at 2 Hz. Top row: type 4 (left) and type 2 (right) profiles of activity-dependent slowing recorded from sympathetic and cold fibres respectively in the same nerve at the same time. Vertical scale for all traces is % slowing, calibration bar 5%. Type-4 profiles characterized by faster initial rise to peak and subsequent partial recovery during train, Rows 2–4: further examples of type 4 and 2 slowing profiles from different subjects.

Table 1 Axonal properties distinguishing the type 4 (accelerating) units from type 2 (plateau) units

	Type 2 [mean ± SD (*n*)]	Type 4 [mean ± SD (*n*)]	*P*-value*
Conduction velocity (ms^{-1})	0.98 ± 0.36 (34)	0.63 ± 0.19 (41)	2.9×10^{-6}
Slowing after 5 s at 2 Hz (%)	1.03 ± 0.37 (18)	2.57 ± 0.45 (14)	9.5×10^{-10}
Peak slowing within the first min at 2 Hz (%)	4.65 ± 1.27 (27)	6.47 ± 2.06 (41)	1.6×10^{-4}
Slowing after 1 min at 2 Hz (%)	4.65 ± 1.27 (27)	5.65 ± 1.94 (30)	2.5×10^{-2}
Slowing after 3 min at 2 Hz (%)	4.88 ± 1.42 (36)	4.90 ± 2.20 (41)	0.92
Slowing at 0.25 Hz after pause (%)	0.93 ± 0.39 (9)	1.66 ± 0.51 (17)	1.1×10^{-3}

*Values in this column are probabilities that type-2 and -4 units are drawn from populations with the same mean (Student's *t*-test).

3 min, averaging 21% for mechano-responsive (type 1A) and 34% for mechano-insensitive (type 1B) nociceptors (Serra *et al.* 2004). Type-2 and -4 units have several features in common, which may cause them to be confused: both types reach a limit of slowing of about 5%, much less than in nociceptor fibres, both display spontaneous activity at normal skin temperatures, and both types are insensitive to mechanical probing of the skin with von Frey hairs.

Conduction velocities and parameters of slowing of type-2 and -4 units are compared in Table 1. Some type 2 (cold) fibres have conduction velocities in the Aδ range (>2 ms^{-1}) (Campero *et al.* 2001). Such fibres are not included in Table 1. By definition, the type-4 units are differentiated by a lesser degree of slowing after 3 min at 2 Hz than the peak slowing during the first minute. The slowing after 3 min at 2 Hz was not significantly different between the two types of unit, but the peak slowing during the first minute was on average significantly greater for type-4 units. The single variable that discriminated best between the two classes of units, when it could be measured accurately, was the initial rate of slowing, measured over the first 5 s of stimulation at 2 Hz. We found no overlap of this parameter between type-2 units (0.38–1.70%) and type-4 units (1.91–3.50%). On average, type-4 units also conducted more slowly than type-2 units, and they were more sensitive to low rates of stimulation, as indicated by the slowing at 0.25 Hz after a 3-min pause in stimulation. In this respect, type-2 and -4 units both come between the values for type 1A (mechano-responsive nociceptors) and type 1B (mechano-insensitive nociceptors) which slow by 0.30 ± 0.4% and 4.5 ± 1.4%, respectively after a pause (Serra *et al.* 2004).

The phase of acceleration in the response of type-4 units to repetitive stimulation was dependent on stimulation rate. Figure 3 illustrates a fibre stimulated for 3-min periods at 1, 2 and 4 Hz. At 1 Hz the latency reached a plateau but did not recover during the train. Comparison with the previously published data for this protocol for type-1 and -2 fibres indicates a further

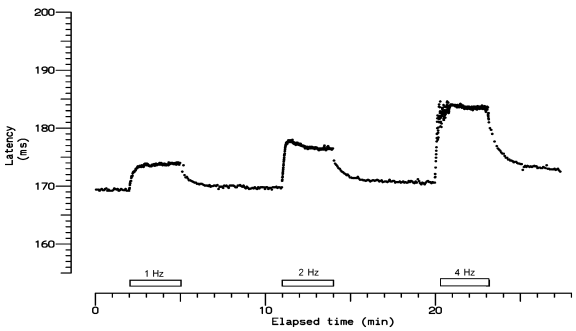

Figure 3 Activity dependent slowing as a function of impulse rate. A single sympathetic fibre was stimulated at 0.25 Hz, and this rate was increased for 3-min periods to frequencies of 1, 2 and 4 Hz. At each frequency there was a fast initial slowing, but only at 2 and 4 Hz was there a phase of relative acceleration.

distinguishing feature of type-4 units. Whereas plateau height increased more than linearly with stimulation rate for type 1, and close to linearly for type 2, it increases less than linearly for type-4 units. The mean ratio of slowing at 2 Hz (i.e. extra slowing after 3 min at 2 Hz, above baseline latency at 0.25 Hz) to that at 1 Hz was 1.34 ± 0.14 (mean ± SD) for the fibre in Figure 3 and three others, whereas for the type-1 and -2 fibres measured previously (Serra *et al.* 1999) the mean ratios were 2.64 and 2.00, respectively.

Identification of 'type-4' units as sympathetic efferents

Of the 34 type-4 units recorded with adequate signal-to-noise to identify electrically evoked spikes unambiguously, all showed evidence of spontaneous activity in the form of fluctuations in baseline latency. These fluctuations were so marked that some units with less favourable signal-to-noise were not visible in the raster plots until stimulated at 2 Hz, which stopped the random fluctuations. Blocking of the spontaneous activity by a centripetal impulse train suggests that the spontaneous activity is efferent, and the only C fibre efferents innervating human skin are sympathetic fibres,

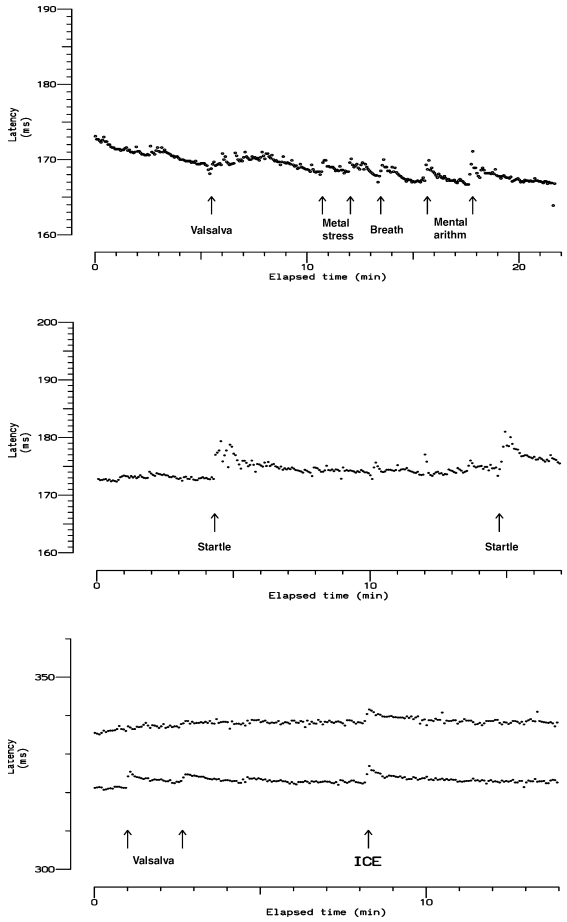

Figure 4 Baseline spontaneous activity of type-4 units is enhanced by manoeuvres that normally increase sympathetic tone. Top: ongoing activity is transiently increased by Valsalva manoeuvres, mental stress and by a deep breath. Middle: another unit displaying a significant amount of baseline activity as shown by latency fluctuations. This fibre was strongly activated by startling the subject with a shout. Bottom: recording of two type-4 units illustrating the more selective activation of the short latency unit by Valsalva manoeuvre, while both units were activated by ice applied in the cervical region.

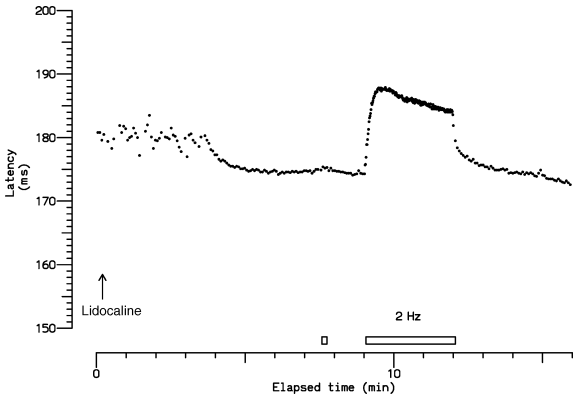

Figure 5 Block of efferent activity in type-4 unit by local anaesthetic. Latency fluctuations due to spontaneous activity in a type-4 unit were blocked 3.5 min after injecting lidocaine close to the nerve, proximal to the recording site.

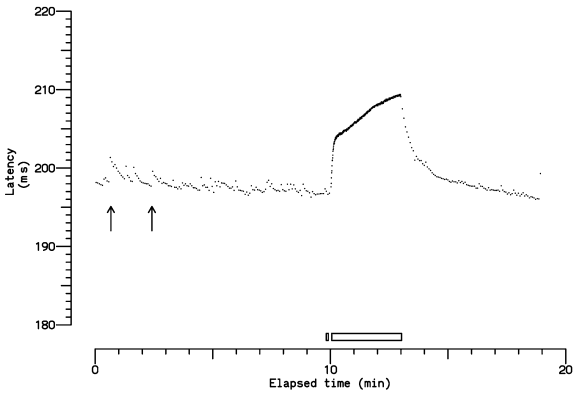

Figure 6 Sympathetic unit with different profile of activity-dependent slowing. Spontaneous unit, conducting at 0.58 ms^{-1}, which was activated by cold air and ice on the back of the neck (arrows), and therefore presumed to be a sympathetic efferent, but showed a different pattern from the type-4 units when stimulated at 2 Hz for 3 min (bar).

so we tested the effects of manoeuvres known to activate sympathetic discharges on the spontaneous activity. In 15 of 16 cases tested, clear evidence of increased activity was found (Fig. 4), confirming that those units were sympathetic. Additionally, a proximal anaesthetic block with lidocaine (e.g. Fig. 5) was effective in blocking spontaneous activity (as evidenced by latency fluctuations) in every case (eight of eight units tested), confirming that the activity was efferent, and therefore most likely sympathetic.

Other sympathetic fibres

Although the first clearly identified sympathetic units that we studied with repetitive stimulation all exhibited the phase of acceleration that identifies them as type-4 units, we have subsequently recorded some sympathetic units (identified by spontaneous activity that was either increased by sympathetic manoeuvres or blocked by proximal lidocaine) without an acceleration phase. These units are less unambiguously distinguished from cold units by their profile of activity-dependent slowing than the 'type-4' units. They share the relatively rapid slowing during the first 5 s at 2 Hz, but this may be followed by a plateau or a further slow increase in latency. An example is illustrated in Figure 6. It is not yet clear whether these represent a different functional subclass of sympathetic fibre (e.g. pilo-erector or sudomotor as against vasomotor) or variation within sympathetics of the same class(es) (see Discussion).

Discussion

This study has shown that some sympathetic cutaneous efferents in humans display a highly distinctive pattern of activity-dependent slowing, which enables them to be distinguished from other C fibres. This observation enhances the potential utility of activity-dependent slowing measurements for determining C-fibre function. However, we have evidence that not all sympathetic fibres exhibit the 'type 4' pattern of activity-dependent slowing described here. This study also raises the question as to what peculiar membrane properties of the type-4 sympathetic fibres can account for their unique property of speeding up while being stimulated repetitively.

What subtype(s) of sympathetic efferent exhibit activity-dependent acceleration?

The sympathetic manoeuvres used in this study do not permit separation of vasomotor from sudomotor subtypes. This is best achieved by warming or cooling the whole body (Bini *et al.* 1980, Macefield & Wallin 1999). A possible clue to the nature of the type-4 sympathetic fibres is their mean conduction velocity of 0.63 ms^{-1}, which is closer to Fagius & Wallin's (1980) estimate of 0.77 ms^{-1} for the peroneal fibres mediating reflex vasoconstriction, than their estimate of 1.27 ms^{-1} for fibres mediating the sudomotor reflex. However, the correspondence between reflex latency and the mean velocities measured over the distal few centimetres may not be close, so this comparison must be treated with caution. Also, in a microneurographic study in which whole-body warming and cooling was used to help identify sympathetic fibre type, Schmelz *et al.* (1998) tentatively classified one unit conducting at 0.78 ms^{-1} as vasoconstrictor, and one conducting at 0.68 ms^{-1} as sudomotor. The sympathetic units which did not exhibit a phase of acceleration at 2 Hz did not have significantly different velocities (0.59 ± 0.14 ms^{-1}, mean $\pm$ SD, $n = 20$). Further experiments will be required to determine whether activity-dependent acceleration is associated with a particular subtype of sympathetic fibre.

Mechanism of acceleration during repetitive stimulation

One clue to the mechanism of acceleration is the non-linear increase in latency with stimulation rate. For cold fibres, the increase in steady-state latency with stimulation rate was approximately linear (i.e. extra slowing above baseline at 2 Hz was twice that at 1 Hz), which may be simply explained by the effects of an outward, electrogenic pump current, proportional to the increase in intracellular sodium concentration, on a constant membrane resistance. For sympathetic fibres, the less than linear increase in latency plateau with stimulation rate suggests that either (a) the sodium influx per impulse gets smaller, or (b) pump activation reaches saturation, or (c) that the electrogenic hyperpolarization is limited by inward rectification. The first two possibilities are unlikely, since (a) hyperpolarization should increase sodium current, and a reduction in sodium current would result in more slowing, not less, and (b) if the pump was failing to remove sodium ions at the higher rates, the recovery after the train would take very much longer, which does not occur. On the other hand, it is likely that the electrogenic hyperpolarization is limited by the inward rectifier I_H, since this has been demonstrated to limit slowing during repetitive stimulation at low rates in human C fibres *in vitro* (Grafe *et al.* 1997).

If I_H is responsible for the non-linear increase in slowing with stimulation rate, could it also be responsible for the acceleration phase of type-4 fibres? I_H is a slowly activated current, so that in current clamp a steady hyperpolarizing current applied to axons produces a hyperpolarization which 'sags' over a few hundred millisecond (Grafe *et al.* 1997). However, it seems unlikely that I_H activation could be slow enough to account for the acceleration phase of type-4 fibres, which may last for at least 2 min (Fig. 2). Possibly the build up of extracellular potassium around these fibres during the 2 Hz train results in an increase in I_H current (which is carried by Na^+ and K^+ ions), which would result in reduced hyperpolarization and relative acceleration.

Whatever the detailed mechanism of the acceleration of type-4 sympathetic units, our results suggest that I_H is particularly important in limiting electrogenic hyperpolarization in these fibres. The type-4 latency profile may provide a useful positive identification of these fibres in *in vitro* (skin–nerve and nerve bath) preparations, in which efferent activity is absent. Other sympathetic fibres, which do not exhibit the phase of acceleration at 2 Hz, may also be unambiguously distinguishable from cold fibres by their axonal properties, since we have recently found that the latency recovery cycles of sympathetic units (both refractory period and duration of supernormality) are much longer than those of cold fibres (Bostock *et al.* 2003).

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References

Bini, G., Hagbarth, K.E., Hynninen, P. & Wallin, B.G. 1980. Thermoregulatory and rhythm-generating mechanisms governing the sudomotor and vasoconstrictor outflow in human cutaneous nerves. *J Physiol* **306**, 537–552.

Bostock, H., Campero, M., Serra, J. & Ochoa, J. 2003. Velocity recovery cycles of C fibres innervating human skin. *J Physiol* **553**, 649–663.

Campero, M., Serra, J., Bostock, H. & Ochoa, J.L. 2001. Slowly conducting afferents activated by innocuous low temperature in human skin. *J Physiol* **535**, 855–865.

Delius, W., Hagbarth, K.E., Hongell, A. & Wallin, B.G. 1972. Manoeuvres affecting sympathetic outflow in human skin nerves. *Acta Physiol Scand* **84**, 177–186.

Fagius, J. & Wallin, B.G. 1980. Sympathetic reflex latencies and conduction velocities in normal man. *J Neurol Sci* **47**, 433–448.

Gee, M.D., Lynn, B. & Cotsell, B. 1996. Activity-dependent slowing of conduction velocity provides a method for identifying different functional classes of C-fibre in the rat saphenous nerve. *Neuroscience* **73**, 667–675.

Grafe, P., Quasthoff, S., Grosskreutz, J. & Alzheimer, C. 1997. Function of the hyperpolarization-activated inward rectification in nonmyelinated peripheral rat and human axons. *J Neurophysiol* **77**, 421–426.

Macefield, V.G. & Wallin, B.G. 1999. Respiratory and cardiac modulation of single sympathetic vasoconstrictor and sudomotor neurones to human skin. *J Physiol* **516**, 303–314.

Rang, H.P. & Ritchie, J.M. 1968. On the electrogenic sodium pump in mammalian non-myelinated nerve fibres and its activation by various external cations. *J Physiol* **196**, 715–730.

Schmelz, M., Schmidt, R., Bickel, A., Torebjork, H.E. & Handwerker, H.O. 1998. Innervation territories of single sympathetic C fibers in human skin. *J Neurophysiol* **79**, 1653–1660.

Serra, J., Campero, M., Ochoa, J. & Bostock, H. 1999. Activity-dependent slowing of conduction differentiates functional subtypes of C fibres innervating human skin. *J Physiol* **515**, 799–811.

Serra, J., Campero, M., Bostock, H. & Ochoa, J. 2004. Two types of C nociceptors in human skin and their behaviour in areas of capsaicin-induced secondary hyperalgesia. *J Neurophysiol* **91**, 2770–2781.

Thalhammer, J.G., Raymond, S.A., Popitz-Bergez, F.A. & Strichartz, G.R. 1994. Modality-dependent modulation of conduction by impulse activity in functionally characterized single cutaneous afferents in the rat. *Somatosens Mot Res* **11**, 243–257.

Torebjörk, H.E. & Hallin, R.G. 1974. Responses in human A and C fibres to repeated electrical intradermal stimulation. *J Neurol Neurosurg Psychiatry* **37**, 653–664.

Weidner, C., Schmelz, M., Schmidt, R., Hansson, B., Handwerker, H.O. & Torebjörk, H.E. 1999. Functional attributes discriminating mechano-insensitive and mechano-responsive C nociceptors in human skin. *J Neurosci* **19**, 10184–10190.