Activity-dependent excitability changes suggest Na\(^+\)/K\(^+\) pump dysfunction in diabetic neuropathy

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The present study was undertaken to evaluate the role of Na\(^+\)/K\(^+\) pump dysfunction in the development of diabetic neuropathy (DN). Nerve excitability techniques, which provide information about membrane potential and axonal ion channel function, were undertaken in 15 patients with established DN and in 10 patients with diabetes who had no evidence of neuropathy (DWN). Excitability parameters were recorded at baseline, and then before and after 1 min of maximal voluntary contraction (MVC) of abductor pollicis brevis. Compared to controls, CMAP amplitude was significantly decreased in DN patients with associated reductions in strength-duration time constant and refractoriness, consistent with a reduction in nodal Na\(^+\) conductances. Following MVC for 1 min, there was an increase in normalized threshold in all diabetic patients and controls, consistent with axonal hyperpolarization. When compared to control values, the increase in threshold following MVC was significantly less in DN patients (DN group 13.1 \(\pm\) 2.2%; controls 20.4 \(\pm\) 1.9%; \(P\) \(<\) 0.05) and the rate of recovery was slower (\(P\) \(<\) 0.01). In DWN patients, CMAP amplitude was preserved, and excitability values following MVC were not significantly different to control values. The reduced threshold change and slower recovery in DN patients following MVC are likely to be secondary to Na\(^+\)/K\(^+\) pump dysfunction. Alteration in Na\(^+\)/K\(^+\) pump function, coupled with reductions in nodal Na\(^+\) currents, may be sufficient to trigger conduction failure in DN patients and are likely to contribute to the clinical symptoms of weakness and fatigue.

Keywords: diabetic neuropathy; Na\(^+\)/K\(^+\) pump; nerve excitability; sodium channel

Abbreviations: ADH = activity-dependent hyperpolarization; CMAP = compound muscle action potential; DN = diabetic neuropathy; DWN = diabetes without neuropathy; HbA\(_1\)C = glycosylated haemoglobin; MVC = maximal voluntary contraction; NSS = neuropathy symptom score; T-NSS = total neuropathy symptom score; TE = threshold electrotonus; TED = depolarizing threshold electrotonus; TEh = hyperpolarizing threshold electrotonus; VDT = vibration detection threshold


Introduction

Diabetes may be complicated by the development of a symmetrical length-dependent polyneuropathy presenting initially with sensory symptoms of paraesthesia and pain (Lozeron et al., 2002). The incidence of diabetic neuropathy (DN) increases with duration of disease (DCCT Research Group, 1988), affecting up to 50% of diabetic patients after 25 years of disease (Pirart, 1978).

The pathophysiology of DN has not been established although pathological studies have suggested an ischaemic basis, possibly related to microangiopathy (Sima, 1996). More recently, a number of studies have uncovered biophysical abnormalities in DN. Of relevance, excitability studies have established that refractoriness and the duration of the relative refractory period are decreased, consistent with an overall reduction of nodal Na\(^+\) conductances (Mackel and Brink, 2003; Misawa et al., 2004; Krishnan and Kiernan, 2005), a finding which has also been highlighted by previous in vitro studies (Brismar and Sima, 1981; Brismar et al., 1987; Brismar, 1993).

Considerable attention has also been focused on the role of Na\(^+\)/K\(^+\) pump dysfunction in the development of DN (Greene and Lattimer, 1984; Greene, 1986), which has in turn been related to C-peptide deficiency (Wahren et al., 2000) and the biochemical consequences of high glucose levels (Greene et al., 1988; Stevens et al., 1995; Sima, 1996). A previous study...
that applied transient limb ischaemia in DN demonstrated paradoxically reduced threshold changes during the ischaemic period in diabetic nerves (ischaemic resistance), a finding attributed to biochemical alterations, including increased substrate stores for anaerobic metabolism, rather than Na⁺/K⁺ pump dysfunction (Strupp et al., 1990).

Nerve excitability techniques may provide a means of detecting changes in membrane potential caused by activation of the Na⁺/K⁺ pump (Bostock and Grafe, 1985; Bostock and Bergmans, 1994; Kuwabara et al., 2004). Vagg and colleagues (1998) showed that the activity-dependent hyperpolarization (ADH) of Kiernan Bostock and Bergmans, 1994; Kuwabara et al., 2004). Separate studies were also undertaken in 10 diabetic patients (three male, seven female, age range 46–78, mean 60.8 years), who had no clinical or electrophysiological evidence of neuropathy.

Baseline clinical data for DN patients are detailed in Table 1. A clinical history and neurological examination were undertaken in all patients and symptoms were staged using subsets 1B, 2A and 2B of the Neuropath Symptom Score (Dyck et al., 1980, 1985; Dyck, 1988). The number of symptoms present in each subset was added to give a Total Neuropathy Symptom Score (T-NSS). All patients underwent automated vibration detection threshold (VDT) testing. A CASE IV™ quantitative sensory testing system (WR Electronics Co., Stillwater, USA) was used for automated vibration detection threshold (VDT) testing. The vibration stimulus was placed over the left big toe in all patients and VDT was estimated using a 4-2-1 stepping algorithm (Dyck et al., 1993). After an estimated VDT was established, the automated protocol was used to determine the just noticeable vibration threshold using the forced-choice testing method. The severity of neuropathy was staged as follows Stage 0: no neuropathy (T-NSS <2 with normal VDT); Stage 1:- asymptomatic neuropathy (T-NSS = 0 with abnormal VDT); Stage 2: symptomatic neuropathy (T-NSS ≥ 2 with normal VDT or T-NSS ≥ 1 with abnormal VDT. Neuropathic symptoms non-disabling); Stage 3: disabling neuropathy (T-NSS ≥ 2 with normal VDT or T-NSS ≥ 1 with abnormal VDT. Neuropathic symptoms reported to be disabling). Serum electrolytes including bicarbonate levels (HCO₃⁻) were assayed due to the potential effect of acid-base abnormalities on excitability parameters, particularly strength-duration time constant (Baker and Bostock, 1999), as was glycosylated haemoglobin (HbA1C), an indicator of diabetic control over the previous 3 months (Gabbay et al., 1977).

Excitability recordings utilized an automated threshold tracking system, in which the amplitude of the test stimulus was automatically increased or decreased by a percentage step after each response, depending on the difference between the recorded response and the target response. For measurements of multiple excitability parameters, an automated protocol (QTRAC version 5.2a, Institute of Neurology, Queen Square, London, with multiple

Methods

Studies were undertaken in 15 diabetic patients (10 male, five female, age range 44–63 years, mean 53.9 years) referred for neuropathy screening. All patients had type 2 diabetes and gave informed consent to the procedures, which had been approved by the South East Sydney Area Health Service Human Research Ethics Committee (Eastern Section) and the Committee on Experimental Procedures Involving Human Subjects of the University of New South Wales. Patients with concurrent neuropathy-related comorbidities were excluded from the study. Patients with type 1 diabetes were not studied due to the findings of previous studies, which demonstrate a pattern of progressive degeneration of the paranodal ion channel barrier, which is specific to type 1 diabetes (Sima et al., 1986, 2004). None of the patients had symptoms of carpal tunnel syndrome. Separate studies were also undertaken in 10 diabetic patients (three male, seven female, age range 46–78, mean 60.8 years), who had no clinical or electrophysiological evidence of neuropathy.

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**Table 1** Demographic and clinical data

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Mean ± SEM 53.9 ± 1.3 97 ± 1.3 76 ± 0.4 21.8 ± 0.8 9.1 ± 3.1 2.6 ± 0.2 2.3 ± 0.1

Neuropathy was staged according to a modified form a previously devised 3-stage system (see Methodology) and was based on the results of the Neuropathy Symptom Score (NSS) and vibration detection threshold (VDT) testing. VDT abnormalities are defined as a percentile ≥ 95% for age, sex and stimulus site and appear in bold text. Normal range for glycosylated haemoglobin (HbA1C) is 4.4–6.4%.
An excitability protocol TRONDXM2 was used that contained a proportional tracking system, in which the change in the stimulus was proportional to the difference between actual and target responses (Kiernan et al., 2000). The median nerve was stimulated at the wrist and compound muscle action potentials (CMAPs) were recorded from abductor pollicis brevis (APB; Fig. 1). Stimulus-response curves were generated using test current impulses of 0.2 ms and 1 ms. The peak 1-ms response was used to calculate the target response (40% of the supramaximal CMAP response). The ratio between the stimulus-response curves for the two different stimulus durations was used to calculate rheobase, which refers to the threshold current for the target response when the stimulus is infinitely long, and strength-duration time constant. Calculations of $r_{50}$ were based on the threshold currents for stimuli of 0.2 ms and 1.0 ms duration and were performed offline using formulae derived from Weiss’ Law (Weiss, 1901).

The threshold changes that occur in response to subthreshold depolarizing and hyperpolarizing pulses, referred to as threshold electrotonus (Bostock and Baker, 1988; Kiernan and Bostock, 2000), were measured by altering nerve excitability using prolonged subthreshold polarizing currents of 100 ms duration, set to 40% of the control threshold current. A current-threshold relationship was obtained by tracking the changes in threshold of 1-ms test pulses that occurred following subthreshold polarizing currents of 200 ms duration. The conditioning currents were altered in a stepwise fashion from +50% (depolarizing) to −100% (hyperpolarizing) of the 1-ms control threshold in steps of 10%. This part of the protocol provided an assessment of the rectifying properties of the internodal membrane. Finally, the recovery cycle of excitability of motor axons was assessed by tracking the changes in threshold that occurred following a supramaximal-conditioning stimulus of 1-ms duration. Test stimuli were delivered at 18 different conditioning-test intervals, decreasing from 200 to 2 ms. At each conditioning-test interval, three stimulus combinations were delivered: (i) unconditioned test stimulus of 1 ms duration; (ii) supramaximal conditioning stimulus alone; and (iii) conditioning and test stimulus, with the test response in this situation measured after online subtraction of the response measured in the second condition.

Excitability was also assessed before and after maximal voluntary contraction (MVC) of APB against resistance for 60 s. Stimuli were delivered at 0.8 s intervals and rotated sequentially through channels (Fig. 1). On channel 1, a 1-ms stimulus was delivered and proportional tracking achieved the target response, set to 40% of the pre-contraction maximal CMAP. On channel 2, a hyperpolarizing conditioning stimulus of 60-ms duration, set to 40% of threshold, preceded the test stimulus. On channel 3, a fixed supramaximal stimulus was delivered to produce a CMAP of maximal amplitude. A stimulus 20% greater than that on channel 3 was delivered on channel 4, ensuring that the CMAP on channel 3 was truly maximal.

Fig. 1 (A) Configuration of stimulus patterns for excitability studies incorporating voluntary contraction. Vertical arrows indicate threshold tracking of test potential (set to 40% of the maximal potential). Stimuli were delivered at 0.8-s intervals and were rotated through the four channels sequentially. On channel 1, a 1-ms stimulus was delivered and proportional tracking achieved the target response, set to 40% of the pre-contraction maximal CMAP. On channel 2, a hyperpolarizing conditioning stimulus of 60-ms duration, set to 40% of threshold, preceded the test stimulus. On channel 3, a fixed supramaximal stimulus was delivered to produce a CMAP of maximal amplitude. A stimulus 20% greater than that on channel 3 was delivered on channel 4, ensuring that the CMAP on channel 3 was truly maximal. (B) Configuration of stimulating electrodes (double circles) and recording electrodes (squares) for excitability studies. Black circle represents skin temperature probes.


maximal amplitude. A stimulus 20% greater than that on channel 3 was delivered on channel 4, ensuring that the CMAP on channel 3 was truly maximal. Surface EMG was recorded in all patients during the period of contraction to ensure adequate effort and all studies in DN patients were performed by the same investigator. While DN generally presents as a length-dependent process, with greater lower limb than upper limb involvement, studies of activity-dependent excitability changes were performed on upper limb nerves in the present study. The major reason for this relates to the fact that assessment of activity-dependent changes in lower limb nerves is fraught with difficulty due to electrode movement during the period of contraction and problems with stabilization of the limb.

Baseline excitability values were compared to previously established normative values (Kiernan et al., 2000). Values for MVC were compared to data obtained from 15 control subjects (age range 28–66 years, mean 48.5 years). Datasets were tested for skew prior to the application of statistical tests. Single comparisons in excitability parameters were analysed using Student’s unpaired t-tests. A P-value of <0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean unless otherwise indicated.

Results

All 15 DN patients had symptoms of neuropathy with a mean of two symptoms per patient (2.06 ± 0.2). Glycosylated haemoglobin (HbA1C), used as a measure of diabetic control, was elevated in 75% of DN patients (7.6 ± 0.4%; Table 1). Mean serum bicarbonate was within normal limits (mean 25.8 ± 0.6 mmol/l, range 21–30 mmol/l, normal: 22–32 mmol/l). Abnormalities of VDT, defined as a VDT >95% percentile for age, sex and stimulus site, were noted in 60% of DN patients (Table 1). In terms of neuropathy severity, 10 patients had stage 2 neuropathy and five patients had stage 3 neuropathy.

Baseline excitability parameters demonstrated changes similar to those noted in previous studies in DN patients (Mackel and Brink, 2003; Kitano et al., 2004). CMAP amplitude was reduced in DN patients compared to controls (DN group 4.9 ± 0.8 mV; controls 9.0 ± 0.6 mV; P < 0.0005). Strength-duration time constant (Fig. 2A), a voltage-dependent property of the nodal membrane that provides an indirect measure of a nodal persistent Na⁺ conductance (Mogyoros et al., 1996; Bostock and Rothwell, 1997), was lower in the DN patients (DN group 0.30 ± 0.06 ms; normal controls 0.43 ± 0.02 ms; P < 0.05) and rheobase was increased (DN group 7.3 ± 0.6 mA; normal controls 3.2 ± 0.2 mA; P < 0.0005). Alterations were also noted in threshold electrotonus parameters, with reductions in depolarizing and hyperpolarizing threshold electrotonus, leading to a ‘fanned-in’ appearance (Kiernan and Bostock, 2000; Kaji, 2003). In particular, depolarizing threshold electrotonus at 90–100 ms (TEd 90–100), previously established as a sensitive indicator of alteration in membrane potential (Kiernan and Bostock, 2000), was lower in DN patients (DN group 38.7 ± 1.3%; controls 45.6 ± 0.8%; P < 0.0005). The changes in threshold electrotonus were accompanied by alteration in recovery cycle measures (Fig. 2B), with reductions in refractoriness (DN group 22.5 ± 3.3%; controls 33.3 ± 3.1%; P < 0.05), superexcitability (DN group –23.1 ± 1.2%; controls –25.3 ± 1.0%) and late subexcitability (DN group 11.1 ± 1.4%; controls 14.8 ± 0.7%), findings that have been noted in previous studies of DN patients (Kitano et al., 2004; Misawa et al., 2004).

Excitability changes induced by MVC

Following MVC for 1 min, there was an increase in normalized threshold in all diabetic patients and controls, as illustrated for a single representative patient in Fig. 3. This was accompanied by an increase in hyperpolarizing threshold electrotonus at 60 ms, consistent with axonal hyperpolarization. When compared to control values, the increase in threshold for a stimulus of 1-ms duration (Fig. 4) was significantly less in DN patients (DN group 13.1 ± 2.2%; controls 20.4 ± 1.9%; P < 0.05).

Following MVC, there was reduction in maximal CMAP amplitude in DN patients as illustrated for the single patient in Fig. 3 (maximal reduction 33.8%) and for the group as a whole (mean reduction 4.5 ± 3.3%; Fig. 4) although this difference was not statistically significant. Reductions in maximal CMAP amplitude were not noted in any of the normal controls. Moreover, the degree of reduction in maximal CMAP amplitude noted in DN patients correlated inversely with baseline refractoriness (r = −0.65, P < 0.05), such that the greatest reductions in
maximal CMAP amplitude following MVC occurred in those patients with the least refractoriness.

In view of the activity-dependent abnormalities demonstrated in DN patients, a further series of studies was conducted in a cohort of 10 diabetic patients who had no clinical or electrophysiological evidence of neuropathy (i.e. NSS = 0, normal VDT/NCS). This was done in order to explore whether activity-dependent changes in DN patients were a manifestation of hyperglycaemia per se or whether they were restricted to patients who had evidence of clinical neuropathy. Excitability parameters recorded in DWN patients before and after 1 min of MVC demonstrated a mean post-contraction threshold increase that was within the range of values obtained from normal controls (DWN patients 24.6 ± 5.5%; controls 20.4 ± 1.9%; Fig. 5). Moreover, none of the patients in the DWN group manifested activity-dependent reduction in maximal CMAP amplitude.

Correlations were performed to explore possible links between hyperglycaemic control, the severity of neuropathy and excitability changes in DN patients and demonstrated greater abnormalities of superexcitability in patients with stage 3 neuropathy (stage 2 neuropathy −20.2 ± 1.0%; stage 3 neuropathy −14.1 ± 1.5%, P < 0.01), as demonstrated previously (Krishnan and Kiernan, 2005). Similarly, more significant reductions in TEd 90–100 ms were noted in stage 3 neuropathy patients (stage 2 neuropathy 40.6 ± 1.1%; stage 3 neuropathy 37.0 ± 1.2%, P < 0.05). Correlations were also noted between VDT and the degree of superexcitability, such that patients with higher VDT
manifested greater abnormalities of superexcitability ($r = 0.68$, $P < 0.05$). In terms of post-contraction excitability changes, there was no correlation between these changes and glycosylated haemoglobin, neuropathic symptoms or VDT abnormalities. When assessed according to neuropathy stage, mean post-contraction unconditioned threshold was less in stage 3 patients (stage 2 neuropathy $1.14 \pm 0.03\%$, stage 3 neuropathy $1.12 \pm 0.03\%$) as was threshold electrotonus (stage 2 neuropathy $1.22 \pm 0.03\%$, stage 3 neuropathy $1.19 \pm 0.03\%$) although the differences did not reach statistical significance. There was no significant correlation between excitability values and serum electrolyte levels, including serum bicarbonate.

To explore the possibility that the lesser changes in threshold following activity in DN patients (Fig. 4A) may reflect alterations in axonal Na\(^+/\)K\(^+\) pump function, recovery of threshold in the early post-contraction period was compared between DN patients and controls (Fig. 6). When expressed as a percentage of the maximal change in threshold and then normalized, the slope of the recovery in threshold (Fig. 6) was flatter in DN patients compared to controls and repeated measures ANOVA demonstrated significant differences in thresholds during the recovery period ($P < 0.01$). The findings are consistent with a slower recovery of threshold in DN patients and suggest reduced Na\(^+/\)K\(^+\) pump function in DN.

**Discussion**

The present study has investigated changes in axonal excitability that accompany natural activity in patients with DN, as a means of assessing axonal membrane potential and specifically Na\(^+/\)K\(^+\) pump function. All DN patients had type 2 diabetes with symptomatic neuropathy, with 10 patients diagnosed with stage 2 neuropathy and five patients with stage 3 neuropathy. Baseline excitability parameters demonstrated ‘fanning-in’ of threshold electrotonus curves and reduced refractoriness in DN patients.

When compared to controls and to DWN patients, MVC induced smaller changes in threshold for DN patients. These changes were accompanied by minor reduction in maximal CMAP amplitude. The possible biophysical changes that may underlie these abnormalities, including changes in Na\(^+/\)K\(^+\) pump function and nodal Na\(^+\) channel concentration, will form the focus of discussion.

In the present study, there was an increase in threshold following MVC in DN patients, DWN patients and controls, consistent with ADH due to a post-MVC increase in Na\(^+/\)K\(^+\) pump activity (Vagg et al., 1998). The magnitude of ADH was however less in DN patients compared to either DWN patients or controls. The reduced threshold changes in DN patients are unlikely to be secondary to neurogenic weakness alone which may be expected to induce a greater post-contraction threshold increase than that noted in control subjects, due to higher firing frequencies of surviving motor units inducing greater activity-dependent changes (Vucic et al., 2007). Furthermore, studies of excitability parameters at baseline and following natural activity in DWN patients demonstrated no significant difference from values obtained in normal controls. Patients in the DWN group had activity-dependent threshold changes similar to those noted in the control group and had no reduction in maximal CMAP amplitude in the post-contraction period, suggesting that hyperglycaemia alone is insufficient to induce these activity-dependent abnormalities.

What then causes the lower ADH noted in DN patients? Given that ADH is a consequence of increased Na\(^+/\)K\(^+\) pump activity, the possibility of Na\(^+/\)K\(^+\) pump dysfunction as a cause of the lesser changes in DN patients needs to be considered. Previous studies of Na\(^+/\)K\(^+\) pump function in other neuropathies have failed to demonstrate any differences between patients and controls (Krishnan and Kiernan, 2006; Krishnan et al., 2006). In particular, return of threshold to baseline values in the post-contraction period,
when expressed as a function of the maximal change, has not been prolonged in those patient groups. However, the same form of analysis, when applied to DN patients in the present study, has revealed a slower return of threshold to pre-contraction values (Fig. 6). Given the prominent role of the Na\(^+/\)K\(^+\) pump in the restoration of membrane potential following activity, such a finding provides further supportive evidence for Na\(^+/\)K\(^+\) pump dysfunction in DN, a finding that has been demonstrated in animal models of DN (Greene and Lattimer, 1984; Greene, 1986; Greene et al., 1987). Such Na\(^+/\)K\(^+\) pump dysfunction may be reversed with insulino-mimetic C-peptide therapy (Zhang et al., 2001).

**Clinical implications: fatigue in DN**

Previous studies which have investigated peripheral changes underlying fatigue in diabetes have postulated that alterations in contractile speed and tension production in muscle may underlie the development of fatigue, while a possible contribution of peripheral nerve abnormalities has largely been overlooked (Cotter et al., 1989; Cameron et al., 1992). The present study has demonstrated activity-dependent conduction failure in DN patients, with a post-contraction reduction in maximal CMAP amplitude in excess of 30% in some patients, a finding that was not observed in any of the healthy controls. Moreover, there was significant correlation between refractoriness and CMAP amplitude reduction, such that DN patients who manifested greater baseline reductions in Na\(^+\) channel parameters also had greater reduction in CMAP amplitude following natural activity.

Reduction in axonal Na\(^+\) currents has been demonstrated in both animal and human studies of DN (Brismar and Sima, 1981; Brismar et al., 1987; Brismar, 1993; Mackel and Brink, 2003; Misawa et al., 2005). Previous studies have not however assessed the possible connection between alteration in Na\(^+\) conductances and changes in Na\(^+/\)K\(^+\) pump in DN. During the period of voluntary contraction, an influx of Na\(^+\) leads to membrane depolarization, which if left unchecked could lead to Na\(^+\) channel inactivation and a reduction in CMAP amplitude. Normally, this sequence of events is prevented by an increase in Na\(^+/\)K\(^+\) pump activity which restores ionic balance and prevents conduction failure (Burke et al., 2001). It is however plausible that in situations of Na\(^+/\)K\(^+\) pump dysfunction this restorative mechanism may be impaired, leading to Na\(^+\) channel inactivation and a reduction in CMAP amplitude as was noted in the present study. Furthermore, a reduction in the inward Na\(^+\) current would further lower the safety margin for conduction (Burke, 2006), which in the setting of post-contraction membrane hyperpolarization, may be sufficient to trigger conduction failure. The findings of the present study therefore suggest a link between Na\(^+/\)K\(^+\) pump dysfunction and reduction in nodal Na\(^+\) conductances and raise the possibility that these biophysical abnormalities may contribute to weakness and fatigue, common symptoms in DN patients.

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### References


