Voltage-dependent calcium currents are enhanced in dorsal root ganglion neurones from the Bio Bred/Worchester diabetic rat

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1. Whole-cell, high-threshold, voltage-dependent calcium currents (I_{Ca}) were enhanced in acutely dissociated, capsaicin-sensitive dorsal root ganglion neurones from diabetic Bio Bred/Worchester (BB/W) rats, compared with those from age-matched, non-diabetic controls. The magnitude of the enhancement increased with the duration of diabetes, and reached significance at diabetic durations of 6 months (diabetic: 6·3 ± 0·4 nA; current density (CD), 157 ± 12 pA pF^{-1}; means ± S.E.M., $n = 9$, $P < 0·01$; control: 3·9 ± 0·6 nA; CD, 116 ± 11 pA pF^{-1}; $n = 18$) and 8 months (diabetic: 7·6 ± 0·4 nA; CD, 177 ± 25 pA pF^{-1}; $n = 11$, $P < 0·005$; control: 5·1 ± 0·5 nA; CD, 111 ± 26 pA pF^{-1}; $n = 15$). Low-threshold, voltage-dependent I_{Ca} were also enhanced in neurones from animals diabetic for 8 months (diabetic: 2·5 ± 0·7 nA, $n = 4$, $P < 0·05$; control: 0·7 ± 0·5 nA, $n = 6$).

2. The I_{Ca} enhancement was prevented by long-term treatment of diabetic animals with an aldose reductase inhibitor (ARI; peak I_{Ca} at 6 months: 4·41 ± 0·48 nA, $n = 2$; at 8 months: 4·32 ± 0·60 nA, $n = 9$).

3. The I_{Ca} enhancement was not due to a shift in the voltage dependence of either the current–voltage relationship or steady-state inactivation.

4. The L channel antagonist nifedipine and preferential N channel antagonist ω-conotoxin GVIA (ω-CgTX) caused a greater inhibition of high-threshold I_{Ca} in diabetic neurones compared with controls (nifedipine: control: 25 ± 3%, $n = 26$; diabetic: 36 ± 7%, $n = 11$; ω-CgTX: control: 40 ± 4%, $n = 21$; diabetic: 50 ± 7%, $n = 7$). Diabetic neurones also demonstrated a significantly greater residual current (2·44 ± 0·34 nA, $n = 7$) in the presence of both antagonists *vs.* controls (1·28 ± 0·30 nA, $n = 8$, $P < 0·05$), suggesting that N-, L- and additional non-N-, non-L-type high-threshold I_{Ca} were enhanced.

Diabetic neuropathy is the most common form of peripheral neuropathy in the Western world, with metabolic, functional and morphological changes in peripheral nerves documented in both human and animal models of diabetes mellitus (Behse, Buchtal & Carlsen, 1977; Brismar, 1983; Sima *et al.* 1988). Despite this, there is relatively little data on the effect of diabetes on neuronal function at the cellular level.

Abnormalities described in peripheral nerves of animal and human diabetics include decreased conduction velocity, morphological changes such as axonal swelling, axo-glial dysfunction, atrophy and nerve fibre loss (Sima & Brismar, 1985), and metabolic alterations such as decreased *myo*-inositol tissue levels, decreased Na^+,K^+-ATPase activity, and mitochondrial membrane dysfunction linked to the production of sorbitol by the polyol pathway in response to hyperglycaemia (Greene, Lattimer & Sima, 1988). Decreased Na^+,K^+-ATPase activity has also been reported to occur in both superior cervical and dorsal root ganglia (DRG) (Green, King, Thomas & Baron, 1986), suggesting that similar pathophysiological mechanisms may be operative in autonomic neuropathy. Decreased *myo*-inositol levels have been associated with decreased production of intracellular second messengers such as inositol trisphosphate and diacylglycerol (Greene *et al.* 1988). In both human and animal studies, treatment with oral aldose reductase inhibitors or dietary *myo*-inositol supplementation have been shown to reverse some of these metabolic changes, and improve the nerve conduction defect (Greene *et al.* 1988; Sima *et al.* 1988; Sima, Prashar, Zhang, Chakrabarti & Greene, 1990).

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Considerable evidence suggests that metabolic overstimulation by cytosolic calcium ($[Ca^{2+}]_i$) may contribute to neuronal cell injury (Nicotera, Bellomo & Orrenius, 1992). An important contribution to $[Ca^{2+}]_i$ in excitable tissues is made by the entry of external Ca^{2+} through voltage-dependent calcium channels (Thayer & Miller, 1990). Indirect evidence suggests that calcium signalling is abnormal in cardiac myocytes (Nobe, Aomine, Arita, Ito & Takaki, 1990), vascular smooth muscle (Kamata, Miyata & Kasuya, 1988), and other tissues (Levy, Gavin & Sowers, 1994) from diabetic animals. Therefore, the goal of the present study was to examine directly whether calcium influx via voltage-dependent calcium channels was altered in primary afferent sensory neurones in diabetes mellitus, and to evaluate the effect of an aldose reductase inhibitor on any observed alteration in calcium influx. We utilized the Bio Bred/Worchester (BB/W) rat, which developes an immunologically mediated type of diabetes that has morphological and metabolic characteristics similar to human type I diabetes mellitus (Brismar, 1983).

These studies have been presented in preliminary form (Hall, Mann & Wiley, 1993*a*).

METHODS

Animal model

Prior approval for these experiments was obtained from the University of Michigan Committee on Use and Care of Animals (Authorization no. 3593A(2)), according to NIH guidelines. Three groups of male BB/W rats were used: (i) adult non-diabetic; (ii) age-matched diabetic treated with daily insulin injection; and (iii) diabetic treated with daily insulin injection and the aldose reductase inhibitor (ARI) WAY 121,509 (courtesy of Wyeth-Ayerst Research, Princeton, NJ, USA; 3 mg (kg body weight)$^{-1}$ day^{-1} by oral gavage starting one week after onset of diabetes). Prediabetic and non-diabetes-prone animals were obtained from the NIH-sponsored colony at the University of Massachusetts (Worchester, MA, USA) and maintained at the Michigan Diabetes Research and Training Center. After the onset of diabetes, as determined by the onset of glucosuria during daily urine glucose monitoring, ultralente insulin (0·4–3·0 i.u. day^{-1} subcutaneous injection; Novo Nordisk, Princeton, NJ, USA) was administered to maintain hyperglycaemic blood glucose levels between 16 and 25 mmol l^{-1} (300–450 mg dl^{-1}) and prevent ketoacidosis. Body weight, urinary glucose and ketone bodies were monitored daily and the insulin dose titrated as described previously (Sima, 1983). Blood glucose levels were monitored daily until the desired plasma glucose levels were achieved, and monthly thereafter. Sciatic nerve conduction velocity was measured on the day before animals were killed (Sima *et al.* 1990).

Preparation of acutely dissociated DRG neurones

Isolated, acutely dissociated DRG neurones were aseptically prepared from the three groups of rats outlined above using a technique similar to that described previously (Gross, Wiley, Ryan-Jastrow & Macdonald, 1990). Rats were killed by inhalation of 100% CO_2, the spinal column was removed, and the thoracic and lumbar DRGs identified and extracted. The DRGs were trimmed, minced, incubated with 2 ml 0·3% collagenase Type II (Sigma) and 0·1% trypsin Type I (bovine; Sigma), then triturated and centrifuged. Enzymes were prepared in minimal essential medium (MEM; Gibco) supplemented with 16 mM $NaHCO_3$ and 28 mM glucose (320 mosmol kg^{-1}). The isolated DRGs were resuspended in supplemented MEM containing 10% horse serum (Gibco) without nerve growth factor, and plated onto poly-L-lysine-coated glass coverslips. The coverslips were incubated in 93% air–7% CO_2 at 37 °C, and recordings performed at room temperature between 1·5 to 7 h after plating.

Whole-cell voltage-clamp recordings

Voltage-clamp recordings using the whole-cell variant of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were made at room temperature with glass recording patch pipettes (Fisher microhematocrit tubes; Fisher Scientific, Pittsburgh, PA, USA). Electrode resistances were 1–2 MΩ, and seal resistances were greater than 1 GΩ. The external bath solution contained (mM): 5 $CaCl_2$, 67 choline chloride, 100 tetraethylammonium chloride, 5·6 glucose, 5·3 KCl, 10 Hepes and 0·8 $MgCl_2$ (pH 7·3–7·4, 320–330 mosmol kg^{-1}). Recording electrodes were filled with (mM): 140 CsCl, 10 Hepes, 10 EGTA, 5 MgATP and 0·1 LiGTP (all reagents from Sigma). The pH was adjusted to 7·2–7·3 with 1 M CsOH after addition of ATP and GTP, and the final osmolality (280–290 mosmol kg^{-1}) was adjusted to 10–15% below that of the external recording solution using distilled water. The recording solution was kept on ice throughout the experiment.

Drug preparation

ω-Conotoxin GVIA (ω-CgTX; 1 mM stock solution; Sigma) was prepared with filtered distilled water, lyophilized in 10 μl aliquots, and stored at −20 °C. On the recording day, 1–10 μM ω-CgTX was prepared using external recording solution. Nifedipine (1–10 μM) in external solution was prepared on the recording day and kept covered with aluminum foil to exclude light. Capsaicin stock solution (10 mM; Sigma) in 95% ethanol was stored at 4 °C and diluted to 0·1–1 μM on the recording day. All drug solutions were kept on ice throughout the experiment.

Experimental protocol

Phase-bright cells, 23–38 μm in diameter, were selected using a micrometer in the microscope objective lens and depolarized with voltage steps generated by the program CLAMPEX (pCLAMP; Axon Instruments, Foster City, CA, USA). Currents were recorded using an Axopatch 1B patch-clamp amplifier (Axon Instruments) with an input resistance of 1–3 MΩ, filtered with a Bessel filter at 10 kHz (−3 dB), sampled at 20 kHz and stored on hard disk as binary data files. Drugs prepared as described above were applied to the isolated cells using pressure ejection (compressed air; 0·5 psi) from glass micropipettes with 10–40 μm openings positioned approximately 50 mm from the cell. The pipettes were lowered into the bath immediately prior to a 2–5 s application of drug. Two seconds after drug application, currents were evoked, and the pipette removed from the bath. In neurones in which currents could still be elicited following administration of ω-CgTX and nifedipine, capsaicin (0·1–1·0 μM) was applied to assess capsaicin sensitivity (>10% reduction in maximum inward current).

Analysis of current components

Several calcium current (I_{Ca}) components (T, N, L, P) have been described in primary afferent (nodose and DRG) neurones (Fox, Nowycky & Tsien, 1987; Llinas, Sugimori, Lin & Cherksey, 1989). The low-threshold T current (if present) was elicited by

Table 1. Body weight, blood glucose values and nerve conduction velocities in control, diabetic and ARI-treated diabetic BB/W rats

	n	Body weight (g)	Blood glucose (mmol l^{-1})	Nerve conduction velocity (m s^{-1})
4 months				
Control	8	355·6 ± 4·3	7·07 ± 0·19	54·1 ± 0·4
Diabetic	8	298·9 ± 5·9**	22·47 ± 0·38**	45·2 ± 0·9**
6 months				
Control	18	468·1 ± 3·4	5·74 ± 0·18	55·2 ± 0·5
Diabetic	9	369·7 ± 6·7**	21·21 ± 0·73**	42·8 ± 0·7**
ARI-treated diabetic	2	358·7 ± 9·2*	22·04 ± 0·40**	52·3 ± 1·0
8 months				
Control	15	489·2 ± 2·8	5·9 ± 0·19	58·6 ± 0·7
Diabetic	11	382·7 ± 6·8**	22·29 ± 0·53**	41·9 ± 0·9**
ARI-treated diabetic	9	378·9 ± 7·3**	21·15 ± 0·19**	53·2 ± 0·9

*$P < 0·005$, ** $P < 0·001$ by ANOVA.

depolarizing the cell from holding potentials (V_h) of −80 or −90 mV to clamp potentials (V_c) of −60 to −30 mV. High-threshold currents were evoked by 100 ms depolarizations to a V_c of +10 mV at 1 min intervals with the cell alternately held at a V_h of −80 mV and −40 mV. Current records were analysed with the program CLAMPAN (pCLAMP; Axon Instruments). Leak currents assessed by hyperpolarizing commands of equal value to those used to depolarize the cell were digitally subtracted from I_{Ca}. To control for the possibility that alterations in I_{Ca} between the control and diabetic groups occurred simply on the basis of variations in cell size, the current density (CD; in picoamps per picofarad) was determined. Small depolarizing calibration voltage pulses ($V_c = +5$ mV for 12 ms, or −10 mV for 100 ms; $V_h = -80$ mV) were performed immediately after patch rupture. Whole-cell capacitance (proportional to surface area) was calculated using the following formula:

$$C = \frac{A}{V},$$

where C is capacitance (in picofarads), A (in picofarads per millivolt) is the area under the capacitance current curve from the peak inward current to the point at which I_{Ca} is 0, and V is calibration voltage step (in millivolts). Peak inward currents were divided by the cell capacitance, and the normalized currents expressed in units of picoamps per picofarad.

Current–voltage curve normalization

Current–voltage (I–V) curves were generated by depolarizing neurones from a V_h of −80 mV to a V_c of −110 to +90 mV in 10 mV steps (100 ms duration). The peak inward current for each depolarization step was plotted as a function of V_c. The baseline was determined by a least-squares fit of the line connecting the points from −110 to −60 mV, and this line subtracted from the data points at each voltage step to create a baseline-subtracted I–V curve. The curves were averaged to produce mean ± S.E.M. values at each V_c for control, diabetic and ARI-treated diabetic neurones.

Assessment of voltage dependence of steady-state inactivation

Cells were depolarized to a V_c of +10 mV for 100 ms from progressively more positive V_h (−90 to 0 mV by 10 mV steps). The cell was held at each V_h for 15 s prior to depolarization. Peak current amplitude at each V_h was plotted as a function of V_h, and fitted with a Boltzman curve generated with the following formula:

$$\frac{I_{step}}{I_{max}} = \frac{1}{1 + \exp((V_m - V_h)/k)},$$

where I_{step} is peak inward current at holding potential V_h, I_{max} is the maximum peak inward current elicited, V_h is holding potential in millivolts, V_m is the voltage at which half-maximal inactivation occurred, and k is the slope.

Statistical analysis

Significance was determined using analysis of variance and Student's two-tailed t test, and defined as a P value less than 0·05 (Mendenhall, 1975). Results are given as means ± S.E.M.

RESULTS

BB/W rat body weights, hyperglycaemia and nerve conduction velocities

Prediabetic BB/W rats developed diabetes at a mean age of 74 ± 3 days. Body weight gain was significantly decreased in diabetic rats (non-treated and ARI-treated) at all time points compared with that of non-diabetic control rats (Table 1).

Blood glucose values were elevated above 20 mmol l^{-1} in all diabetic rats, and ARI treatment had no effect on blood glucose levels (Table 1). Compared with control animals, diabetic rats developed significant functional neuropathy as reflected by slowing of sciatic nerve conduction velocity, which was ameliorated following ARI treatment (Table 1).

Calcium currents in DRGs from diabetic and control animals

Whole-cell voltage-dependent I_{Ca} were elicited in DRGs between 23 to 38 μm in diameter (control: 29 ± 3 μm, $n = 25$; diabetic: 30 ± 4 μm, $n = 17$), by depolarization to a V_c of +10 mV from a V_h of −80 alternating with −40 mV at 1 min intervals (Fig. 1).

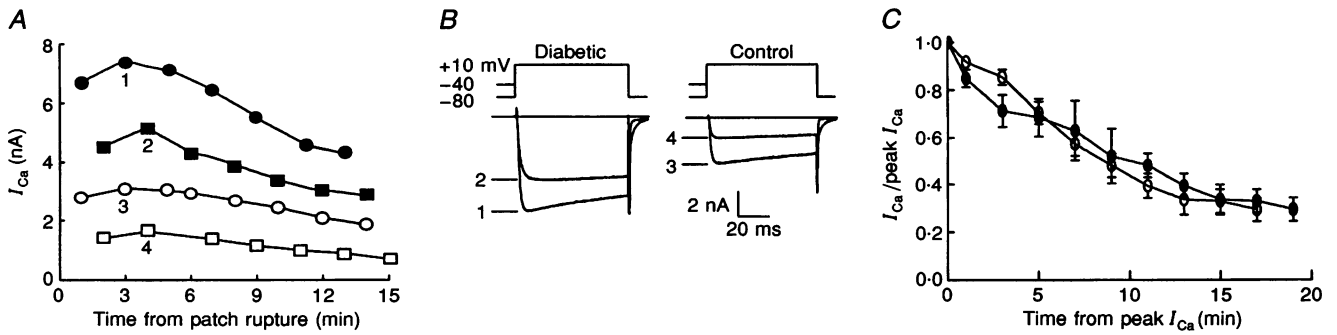

Figure 1. Diabetes mellitus increased high-threshold calcium currents in BB/W rat DRGs

A, the maximum amplitude of the inward current elicited by depolarization to +10 mV from a V_h of −80 mV (circles) and −40 mV (squares) plotted *vs.* time from patch rupture in minutes for a diabetic neuron (filled symbols) and a control neuron (open symbols). *B*, current tracings corresponding to the numbered points in *A*. *C*, run-down of current in control (○) and diabetic (●) neurons is shown as the mean ± S.E.M. of I_{Ca}/peak I_{Ca} plotted *vs.* time from peak I_{Ca}.

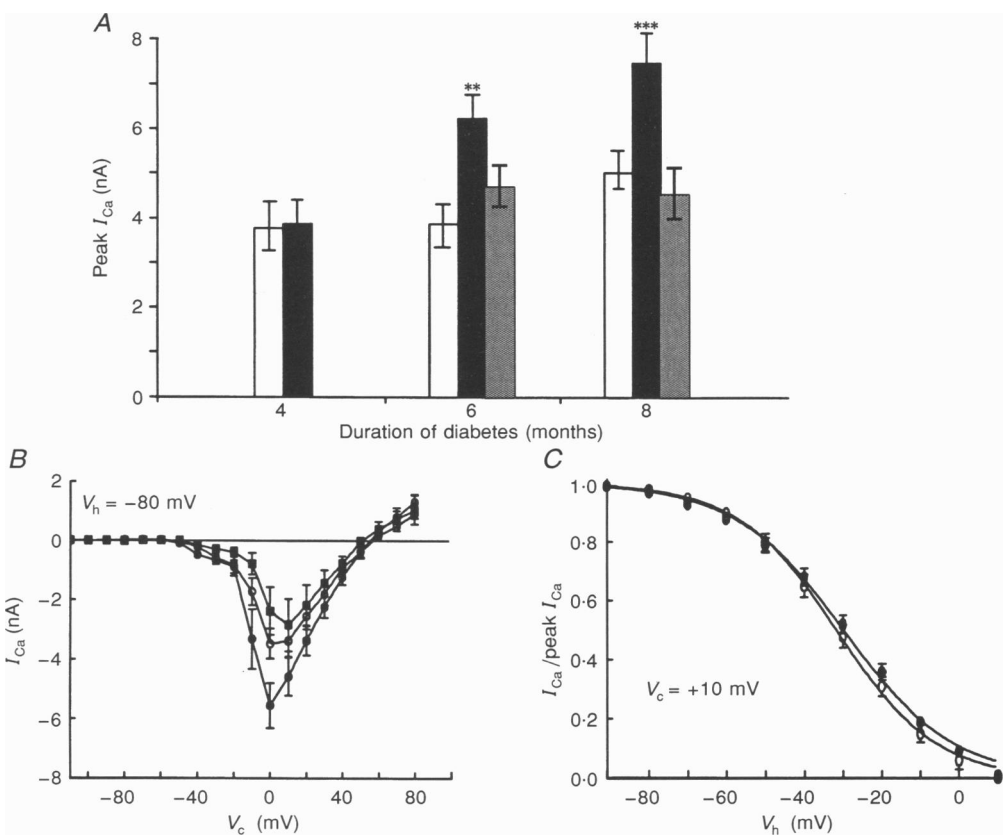

Figure 2. Enhancement of calcium currents in diabetes was prevented by an aldose reductase inhibitor

A, means ± S.E.M. of currents elicited by depolarization to +10 mV from a V_h of −80 mV in neurones from control (□), diabetic (■) and ARI-treated diabetic (▨) animals (diabetic duration 4 months: 8 control, 8 diabetic neurones; diabetic 6 months: 18 control, 9 diabetic, 2 ARI-treated diabetic neurones; diabetic 8 months: 15 control, 11 diabetic, 9 ARI-treated diabetic neurones; *** $P < 0{\cdot}005$, ** $P < 0{\cdot}01$ by ANOVA). *B*, *I–V* curves and *C*, steady-state inactivation curves generated as described in Methods for 10 control (○), 10 diabetic (●), and 11 ARI-treated (■, *B* only) neurones.

Table 2. Diabetes mellitus associated with enhanced calcium current density (CD) in rat DRGs

				$V_h = -80$ mV		$V_h = -40$ mV	
	n	Diameter (μm)	Capacitance (pF)	Peak I_{Ca} (nA)	CD (pA pF^{-1})	Peak I_{Ca} (nA)	CD (pA pF^{-1})
4 months							
Control	8	28 ± 2	24·9 ± 3·1	2·7 ± 0·8	98 ± 12	1·6 ± 0·4	85 ± 24
Diabetic	7	27 ± 3	32·4 ± 0·8	3·2 ± 0·7	108 ± 14	1·9 ± 0·8	85 ± 19
6 months							
Control	17	26 ± 2	22·4 ± 2·5	2·4 ± 0·3	116 ± 11	1·8 ± 0·3	110 ± 10
Diabetic	9	25 ± 2	19·7 ± 3·1	4·9 ± 0·4**	157 ± 12*	3·1 ± 0·3*	101 ± 14
8 months							
Control	14	34 ± 4	39·1 ± 4·2	4·3 ± 0·3	111 ± 26	3·1 ± 0·4	75 ± 12
Diabetic	10	36 ± 3	43·8 ± 4·6	6·2 ± 0·3***	177 ± 25**	4·8 ± 0·4**	115 ± 12*

*** $P < 0·005$, ** $P < 0·01$, * $P < 0·05$ by ANOVA.

High-threshold voltage-dependent currents (N-, L-type) were observed in all cells; however, low-threshold voltage-dependent currents (T-type) were detected in only 8% of neurones. Following patch rupture, the maximum amplitude of high-threshold I_{Ca} increased during the initial 2–5 min, after which run-down of the current was observed in both diabetic and control neurones (Fig. 1*A*). In Fig. 1*B* the current traces corresponding to the numbered points in Fig. 1*A* are shown. There was no significant difference in the leak conductance measured in control *vs.* diabetic neurones (control, 68 ± 44 pA; diabetic, 87 ± 32 pA). The time course of current run-down was similar in diabetic and control neurones (Fig. 1*C*). Approximately 80% of both control and diabetic neurones in the size range evaluated were responsive to capsaicin, as defined by a >10% decrease in current amplitude to capsaicin. Application of capsaicin (0·1 μM) decreased currents by 58 ± 9% in control neurones ($n = 12$) and 44 ± 13% in diabetic neurones ($n = 11$, no significant difference). Data from both capsaicin-sensitive and -insensitive neurones were combined for subsequent analysis.

Enhanced calcium currents observed in diabetes

Diabetes mellitus was associated with an increase in the peak of the voltage-dependent I_{Ca} evoked from a V_h of −80 mV at a V_c of +10 mV (Fig. 2*A*).

The magnitude of the enhancement increased with the duration of diabetes, and reached significance at diabetic durations of 6 months (control: 3·9 ± 0·6 nA, $n = 18$; diabetic: 6·33 ± 0·41 nA, $n = 9$, $P < 0·01$) and 8 months (control: 5·1 ± 0·5 nA, $n = 15$; diabetic: 7·59 ± 0·38 nA, $n = 11$, $P < 0·005$). To control for cell size, the CD for each cell was calculated by dividing I_{Ca} elicited by depolarization to a V_c of +10 mV by the whole-cell capacitance as described in Methods.

Enhancement of CD was observed at 6 months of diabetes in neurones held at a V_h of −80 mV, and at both V_h values of − 40 and −80 mV in neurones from animals diabetic for 8 months (Table 2).

Enhancement of voltage-dependent calcium currents in diabetes mellitus was prevented by long-term treatment with an aldose reductase inhibitor

Diabetic rats treated with oral WAY 121,509 (3 mg kg^{-1}) as described in Methods did not exhibit enhancement of high-threshold DRG I_{Ca} compared with age-matched, non-diabetic controls (Fig. 2*A*). Peak high-threshold I_{Ca} elicited by depolarization to +10 mV from a V_h of −80 mV at 6 months of diabetes was 4·41 ± 0·48 nA ($n = 2$), and at 8 months was 4·32 ± 0·60 nA ($n = 9$), values that were not significantly different from I_{Ca} recorded from age-matched, non-diabetic control DRGs. The degree of hyperglycaemia in the present study was comparable in the untreated diabetic and ARI-treated diabetic groups (Table 1). The mean cell diameters of ARI-treated neurones at 6 and 8 months were 25 ± 2 and 34 ± 3 μm, respectively; values not significantly different from those in control and diabetic neurones in the same age ranges (Table 2).

Diabetes mellitus was not associated with a shift in the current–voltage relationship

Diabetic neurones did not exibit any shift in the I–V relationship compared with control neurones (Fig. 2*B*). The peak of the I–V curve was shifted +10 mV more positive in ARI-treated neurones compared with both control and diabetic cells; however, this shift was by the same amount as the test interval. To determine whether a true shift was present, a subset of cells had I–V curves generated by depolarization from −20 to +20 mV at 5 mV intervals (6 control, 5 diabetic, 4 ARI-treated). At +5 mV intervals, the peak current in control, diabetic and ARI-treated cells occurred at a V_c of +10 mV, and therefore the 10 mV shift between the curves was deemed to be an artifact of the sampling interval.

Diabetes did not change the voltage dependence of steady-state inactivation

Steady-state inactivation curves were generated as described in Methods and averaged for control and diabetic neurones (Fig. 2*C*). The average curves were fitted with a Boltzman equation as described in Methods, with values for V_m and k of −30·07 and 14·29 mV for the control curve, and −29·18 and 13·94 mV for the diabetic curve, respectively. There was no significant shift in the voltage dependence of steady-state inactivation in diabetic neurones compared with controls.

Diabetes mellitus was associated with enhancement of N, L, and non-N, non-L calcium currents

We hypothesized that if diabetes preferentially enhanced only the N-type current, then the ratio of the peak current amplitudes elicited by depolarizing diabetic DRGs to +10 mV from a V_h of −40 mV *vs.* −80 mV (−40 : −80 ratio) would be less than that observed in controls, due to inactivation of the N-type, high-threshold I_{Ca} at more positive V_h (Fox *et al.* 1987). However, comparison of the −40 : −80 ratio at increasing duration of diabetes did not demonstrate any significant difference between control, diabetic and ARI-treated diabetic neurones, and therefore the observed enhancement in whole-cell I_{Ca} in 6 and 8 month diabetic neurones was unlikely to be due to an enhancement in N-type current alone. To elucidate the contribution of N- and L-type currents to the observed increase in I_{Ca}, we evaluated the effect of the L-type channel antagonist nifedipine (3 μM) and the N-type channel antagonist ω-CgTX (10 μM) on I_{Ca} in diabetic and control DRGs by applying these antagonists during the current run-down phase. Antagonists were applied for 2 s by pressure ejection from micropipettes placed close to the neurone (see Methods). Application of diluent alone had no effect on the I_{Ca} amplitude. The agonist concentrations used were close to, or at the maximum level of, the published dose–response (Fox *et al.* 1987), and our previous studies (Hall *et al.* 1993*a*) suggested that the concentrations of nifedipine and ω-CgTX employed in these studies produced near-maximal antagonism of the L- and N-type I_{Ca}, respectively. Prior to application of nifedipine, neurones were held at −40 mV to enhance voltage-dependent inactivation of N-type channels and thereby increase the relative contribution from L-type channels to the total evoked current. Nifedipine reduced I_{Ca} elicited by depolarization to +10 mV for 100 ms from a V_h of −40 mV by 25 ± 3% in control DRGs ($n = 25$) and 36 ± 7% in diabetic DRGs ($n = 11$). ω-CgTX reduced currents evoked by depolarization to +10 mV from a V_h of −80 mV by 40 ± 4% in control DRGs ($n = 21$) and by 50 ± 7% in diabetic DRGs ($n = 7$). In the presence of both antagonists, a residual current could be elicited by depolarization to a V_c of +10 mV from a V_h of −80 mV, which was significantly larger in diabetic neurones (2·44 ± 0·34 nA, $n = 7$) than controls (1·28 ± 0·30 nA, $n = 8$, $P < 0·05$). The percentage reduction in current due to either antagonist was greater in diabetic neurones, suggesting that the enhancement observed in diabetic DRG I_{Ca} involved both N- and L-type currents. In addition, the significantly larger residual current observed in diabetic neurones when N- and L-type antagonists were administered concurrently suggested that diabetes was associated with enhancement of additional non-N-, L-type, high-threshold, voltage-dependent I_{Ca} described in various neuronal tissues (Llinas *et al.* 1989).

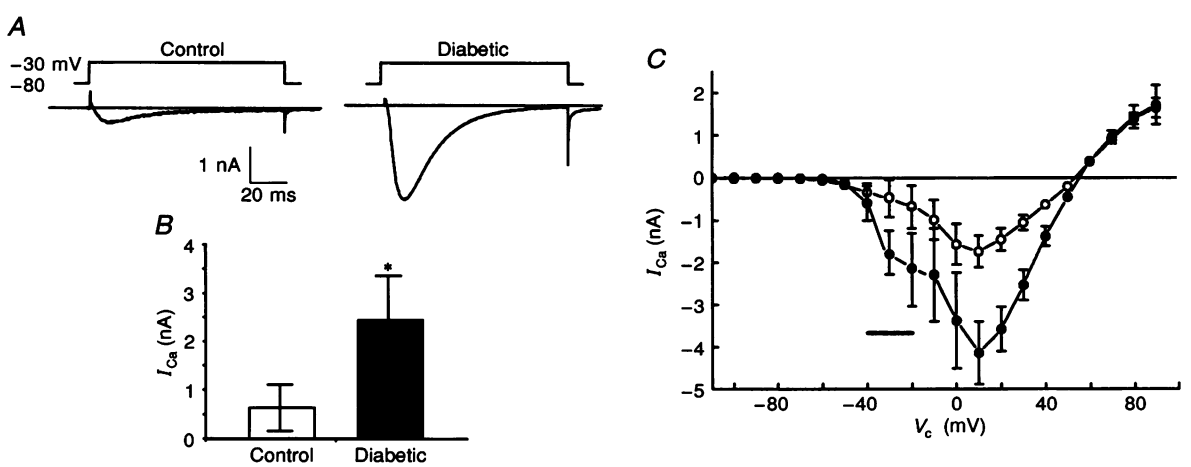

Figure 3. Diabetes-enhanced low-threshold (T-type) calcium current

A, representative transient (T-type) I_{Ca} elicited by depolarization to −30 mV from a V_h of −80 mV in a control and diabetic neurone. *B*, peak T currents (means ± S.E.M.) in DRGs from 4 diabetic and 6 control animals aged 8–10 months (*$P < 0·05$). *C*, *I–V* relationship of neurones that expressed T currents. *I–V* curves were generated as described in Methods for neurones demonstrating T currents (location of peak T currents denoted by horizontal bar).

Effect of diabetes on low-threshold (T-type) calcium currents

Approximately 8% of neurones demonstrated transient low-threshold I_{Ca} that activated and inactivated rapidly when elicited by depolarization to −30 mV from a V_h of −80 mV (Fig. 3*A*). These currents were inactivated when the neuron was held at −40 mV, and correspond to the T-type current described previously (Fox *et al.* 1987).

The average magnitude of the peak T current (Fig. 3*B*) was significantly greater in diabetic neurones (2·5 ± 0·7 nA, $n = 4$) compared with controls (0·7 ± 0·5 nA, $n = 6$). Analysis of the *I–V* relationship of cells that expressed T currents (Fig. 3*C*) did not demonstrate any shift in the voltage dependence of the peak low-threshold I_{Ca} (location denoted by horizontal bar) in diabetic neurones compared with controls.

DISCUSSION

High-threshold, voltage-dependent calcium currents were enhanced in acutely dissociated nociceptive DRGs from diabetic rats

We hypothesized that small- to medium-sized (20–38 μm), capsaicin-sensitive DRGs would show abnormalities early in diabetes, as there is evidence for early and preferential loss of small, unmyelinated, nociceptive nerve fibres and their corresponding cell bodies in diabetes (Sima & Brismar, 1985). In our study, approximately 80% of DRG neurones, 23–38 μm in diameter, were responsive to capsaicin 0·1 μM (>10% decrease in peak I_{Ca}), and thus the majority of neurones recorded were probably of nociceptive origin. Isolated, acutely dissociated DRG neurones in this size range demonstrated characteristic low- and high-threshold voltage-dependent I_{Ca} (Fox *et al.* 1987; Llinas *et al.* 1989), which were enhanced in DRGs from diabetic BB/W rats compared with age-matched controls. The magnitude of enhancement increased with the duration of diabetes, and was not observed in diabetic animals receiving long-term treatment with the aldose reductase inhibitor WAY 121,509. The increase in I_{Ca} was not due to a bias towards recording from larger neurones in the diabetic group, since the mean cell diameter of non-diabetic and diabetic neurones was not significantly different. Current density measurements, which correct for cell size by dividing the whole-cell current by the cell capacitance (proportional to cell surface area), were also significantly higher in neurones from diabetic animals at 6 and 8 months.

The voltage dependence of the current–voltage relationship and steady-state inactivation were not altered in diabetes

Alterations in current amplitude in response to noradrenaline, opiates and protein kinase A (PKA) have been correlated with shifts in the voltage dependence of activation (Bean, 1989; Doupnik & Pun, 1992) and inactivation (Gross & Macdonald, 1989), and therefore possible explanations for the enhanced amplitude of currents in diabetic neurones could be shifts in either the voltage dependence of the peak current towards more negative potentials, or inactivation towards more positive potentials. However, there was no significant shift in the voltage dependence of either the *I–V* relationship, or steady-state inactivation of currents in diabetic neurones compared with controls. Increased expression of functional calcium channels could underlie the observed enhancement, as could other potential mechanisms operating at the single-channel level, such as increased conductance, opening frequency or duration.

Multiple high-threshold calcium currents were enhanced in diabetes mellitus

Several results in this study suggest that multiple high-threshold I_{Ca} were enhanced in neurones from diabetic animals. Currents elicited at more positive holding potentials ($V_h = -40$ mV) contain proportionately less rapidly inactivating N-type I_{Ca}, and proportionately more slowly inactivating L-type current (Fox *et al.* 1987) than currents elicited from more negative potentials ($V_h = -80$ mV). If the N- or L-type current were preferentially enhanced by diabetes, the ratio of the high-threshold currents elicited by depolarization from V_h of −40 : −80 would probably be altered with longer durations of diabetes. Although there was a trend towards a decreased ratio at 8 months in the diabetic group (suggesting either increased L- or decreased N-type current), this was not significant. Therefore, more than one type of current appeared to be affected. The CD at 6 months of diabetes, was significantly larger only at a V_h of −80 mV, but by 8 months, current densities elicited by depolarization from V_h values of both −80 and −40 mV were significantly enhanced in diabetic neurones. This result could indicate that: (i) diabetes increased the more rapidly inactivating (N-type) current, and that the significant current enhancement observed by 8 months at a V_h of −40 mV could have been due to a component of a large residual N-type current that was not completely inactivated at this V_h, or (ii) initially only the N-type current was enhanced at 6 months, and with increasing diabetic duration to 8 months, N-type and other high-threshold currents were enhanced. However, using voltage criteria alone, it is not possible to unequivocally separate the current components; therefore, we employed pharmacological antagonists. The N-type channel antagonist ω-CgTX and the L-type antagonist nifedipine both decreased currents by a greater percentage in DRGs from 6 and 8 month diabetic animals. Despite the larger percentage reduction of current, the absolute magnitude of the remaining current after antagonist application was significantly larger in diabetic

neurons, indicating that diabetes enhanced at least N- and L-type I_{Ca}. Additionally, in the presence of both antagonists, the residual current was significantly larger in diabetic DRGs, suggesting that enhancement of additional high-threshold currents (Llinas *et al.* 1989) may occur in diabetes mellitus. Low-threshold rapidly inactivating (T-type) currents were also significantly larger in diabetic cells. The observation that multiple I_{Ca} types were affected, suggests that diabetes mellitus may alter an intracellular pathway that regulates multiple calcium channels.

Treatment with aldose reductase inhibitor prevented calcium current enhancement in diabetes

In previous human and animal studies, ARI treatment has been shown to improve nerve conduction defects, and normalize *myo*-inositol and Na^+,K^+-ATPase levels in diabetes (Sima *et al.* 1990). In this study, chronic oral treatment with the aldose reductase inhibitor WAY 121,509 initiated 1 week after onset of diabetes prevented the I_{Ca} enhancement observed in neurones from untreated diabetic animals. This effect was observed in neurones from animals with moderate to long duration of diabetes, suggesting that long-term ARI treatment may be of benefit in disease of long-standing duration. The decreased currents in ARI-treated animals were not due to decreased availability of glucose, as the level of hyperglycaemia was comparable in the diabetic and ARI-treated groups. In the hyperglycaemic state, aldose reductase converts a significant amount of glucose into sorbitol (Greene *et al.* 1988). Accumulation of sorbitol produced by the polyol pathway in response to hyperglycaemia has been implicated as a cause of the decreased *myo*-inositol and Na^+,K^+-ATPase levels described previously in sciatic nerves from diabetic rats (Das, Bray, Aguayo & Rasminsky, 1976; Greene *et al.* 1988). Decreased *myo*-inositol uptake in diabetes has been linked to decreased production of inositol trisphosphate (Green *et al.* 1988), diacylglycerol (Zhu & Eichberg, 1990), and protein kinase C (PKC; Kim, Rushovich, Thomas, Uedat, Agranoff & Greene, 1991).

Putative mechanisms underlying enhanced calcium currents in diabetes

One mechanism that could underly the increase in I_{Ca} observed in this study is altered phosphorylation of voltage-dependent calcium channels by protein kinases such as PKC or PKA. Phorbol esters, which activate endogenous PKC, have been reported both to decrease and enhance high-threshold I_{Ca} (Gross & Macdonald, 1989; Yang & Tsien, 1993). This discrepancy may be due to differences in endogenous PKC levels and/or activity between tissue types. Alternatively, phorbol esters may have non-specific effects independent of their action to activate PKC (Hockberger, Toselli, Swandulla & Lux, 1989). Direct application of constitutively active PKC causes enhancement of I_{Ca} in DRGs (Hall, Tan, Browning & Macdonald, 1993*b*) and invertebrate tissues (DeRiemer, Strong, Albert, Greengard & Kaczmarek, 1985). Decreased cytosolic PKC levels have been described in diabetic neurones (Kim *et al.* 1991); however, in other tissues, increased PKC activity has been documented (Tanaka *et al.* 1991). One possible explanation for these discrepancies in PKC activity may lie in the recent demonstration of selective activation of PKC isoforms in diabetes (Inoguchi, Battan, Handler, Spotsman, Heath & King, 1992; Tang, Parker, Beattie & Houslay, 1993). Alternatively, alterations in other components of signal transduction that affect calcium channel function, such as guanine nucleotide-binding (G) proteins, may be present in diabetes. Houslay and colleagues (Gawler, Milligan, Spiegel, Unson & Houslay, 1987; Bushfield *et al.* 1990) have described decreased levels and functional activity of inhibitory G proteins in hepatocytes from diabetic rats. Of interest, enhancement of I_{Ca} in pancreatic and pituitary cell lines by exposure to serum from diabetic patients has been reported recently (Juntti-Berggren *et al.* 1993). These authors concluded that a protein in the IgM fraction of serum was responsible for the observed enhancement. Therefore, circulating factors in diabetic serum may also contribute to increased calcium influx in diabetes.

In conclusion, we observed an enhancement in multiple high-threshold, voltage-dependent I_{Ca} in capsaicin-sensitive DRG neurones in diabetic BB/W rats compared with age-matched, non-diabetic controls, suggesting that alteration of a common pathway, involving either the expression of functional calcium channels or their regulation, occurs in diabetes mellitus. As altered calcium homeostasis resulting in elevation of $[Ca^{2+}]_i$ may contribute to neuronal cell injury and death (Nicotera *et al.* 1992), enhanced calcium influx may contribute to impairment of neuronal function in diabetes mellitus. Finally, a recent study demonstrated a beneficial effect of the L-type calcium channel antagonist nimodipine on streptozotocin-induced diabetic neuropathy in the rat (Kappelle, Biessels, Bravenboer, van Buren, Traber, de Wildt & Gispen, 1994), suggesting that abnormalities in cellular calcium influx in diabetes mellitus may provide possible new targets for treatment.

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