Diabetes-induced changes in calcium homeostasis and the effects of calcium channel blockers in rat and mice nociceptive neurons

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Abstract

Aims/hypothesis. Distal neuropathy is the most common complication of diabetes mellitus, making it important to reveal the cellular mechanisms leading to its development, one of which might be the alteration in intracellular calcium homeostasis in primary and secondary nociceptive neurons. We aimed to investigate these possible changes.

Methods. Control and streptozotocin-treated diabetic rats and mice were used. Changes in intracellular free calcium concentrations ($[Ca^{2+}]_i$) were measured fluorometrically in primary nociceptive neurons from dorsal root ganglia and in secondary nociceptive neurons from substantia gelatinosa of spinal dorsal horn slices.

Results. Measurements of $[Ca^{2+}]_i$ increases induced in dorsal root ganglion and dorsal horn neurons by membrane depolarization did not show any substantial difference in their peak amplitudes in control and diabetic animals. However, a definite prolonga-

tion of the decay phase of the transients was observed under diabetic conditions. Caffeine application to dorsal root ganglion and dorsal horn neurons induced a transient elevation of $[Ca^{2+}]_i$ which was less prominent in cells from diabetic animals. Short-term application of a calcium channel blocker nifedipine showed a substantial amplification of its action in diabetic neurons. However, chronic administration of nimodipine induced a clear increase in the peak values of transients in dorsal root ganglion neurons of diabetic animals compared with those of untreated animals.

Conclusion/interpretation. The described changes of calcium signalling in nociceptive neurons could be the reason for the development of distal polyneuropathy and its symptoms in the early stages of diabetes mellitus. [Diabetologia (2001) 44: 1302–1309]

Keywords Diabetes mellitus, streptozotocin, calcium channels, dihydropyridines, spinal cord, neurons,ryanodine receptor calcium release channel.

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Abbreviations: DRG, Dorsal root ganglion; GABA, gamma-aminobutyric acid; DHP, dihydropyridines; STZ, streptozotocin; SG, substantia gelatinosa; DMSO, dimethyl sulfoxide; DH, dorsal horn; TTX, tetrodotoxin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IP₃, inositol 1,4,5-trisphosphate; CICR, calcium-induced calcium release; VOCC, voltage-operated calcium channel; SERCA, sarco(endo)plasmatic reticulum calcium ATPase; HVA, high-voltage-activated channels

Understanding the origin of and discovering a treatment for neurological complications in diabetes mellitus is one of the most important challenges facing both experimental investigation and clinical practice because of their severity and the large numbers of patients affected. Nevertheless, the mechanisms of the development of distal diabetic neuropathy, in particular the incidence of hyperalgesia and allodynia, pain syndromes or, the opposite, loss of sensitivity are not well understood. In particular, data about the changes of intracellular metabolism in nociceptive neurons under diabetic conditions are scarce, especially about changes in intracellular calcium signalling which could be quite important for

alterations of synaptic transmission of nociceptive impulses.

The synaptic connections between primary (in the dorsal root ganglia) and secondary (in the spinal cord dorsal horn) nociceptive neurons are clearly important for changes in transmission of nociceptive messages in neuropathic complications of diabetes [1]. Therefore, a detailed study of possible changes in the signalling mechanisms of these neurons during experimentally-induced diabetes will yield important results. In initial experiments performed in our laboratory on mice with streptozotocin (STZ)-induced diabetes, a definite potentiation of synaptic transmission from primary to secondary nociceptive neurons correlating with the degree of hyperglycaemia has been shown by recording changes in the negative potential wave from the dorsal spinal cord surface [2]. These changes could be reversed by the administration of insulin whereas streptozotocin by itself did not show any effect before inducing hyperglycaemia [3, 4]. Parallel recordings of depolarization-induced calcium signals from primary sensory neurons showed a substantial prolongation of their time course which also depended on the degree of hyperglycaemia [5]. At the same time, no substantial changes in calcium signals could be shown in non-nociceptive sensory neurons. These findings raised an important question as to possible cellular mechanisms responsible for the observed alterations in intracellular signalling which might be one of the reasons for pathological potentiation of transmission of nociceptive impulses to secondary neurons. A direct recording of possible changes in the signalling mechanisms in secondary nociceptive neurons of the spinal dorsal horn could also be an important direction of studies clarifying the course of diabetic neuropathy. Because preparation of tissue slices from mice spinal cord is technically difficult, it would be more reliable to conduct a similar series of experiments on rats with experimentally-induced diabetes. A detailed comparison of changes in intracellular signalling in primary and secondary nociceptive neurons of diabetic mice and rats would be quite important for evaluation of their true importance, taking into account the differences in incidence and time course of this pathology in different types of experimental animals. Preliminary experimental results of experiments on rat spinal dorsal horn neurons have actually shown substantial changes in Ca^{2+} signalling during STZ-induced diabetes [4, 6].

Materials and methods

Induction of experimental diabetes. In our experiments we used either male Wistar rats (21 days old) or male AC57Black mice (30 days old). Diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (STZ, Sigma-Aldrich Chemie, Deisenhofen, Bayern, Germany) dissolved in 0.9% NaCl solution

80 mg/kg (single) and 40 mg/kg (5 times) for rats and mice, respectively. STZ-injected and control animals were kept on the same diet. Some of the animals were used for experiments in 2–3 days after injection of STZ and before inducing hyperglycaemia (STZ-control group). Others were used for experiments in 4–5 weeks after STZ injection. The glucose concentrations in blood samples obtained from tail vein were measured by a strip-operated blood glucose sensor (Roche Diagnostics, Mannheim, Baden-Württemberg, Germany). The glucose concentration in blood serum was in the range of 5–9 mmol/l, and 12–28 mmol/l for normal and diabetic animals, respectively. Animal experiments were approved by the Bogomoletz Institute of Physiology Clinic of Animal Care and Use.

Spinal cord slice preparation. Experiments were done on spinal cord slices acutely isolated from normal and diabetic rats. The experimental technique for acute preparation of thin spinal cord slices has been described elsewhere [7]. Briefly, rats were anaesthetised with isoflurane and the spinal cord was excised. Slices, 300 μm -thick, were cut from the L5-L7 segments with the use of a vibratome (Campden Instruments, Loughborough, UK) and transferred to the experimental chamber, mounted on the stage of an upright microscope (Axioscope, Zeiss, Oberkochen, Baden-Württemberg, Germany) and continuously superfused with a recording solution. The solution was bubbled with 5% CO_2 + 95% O_2 (pH 7.4) and maintained at 32°C. Cells were visualised through a long-distance water-immersion objective (60 $\times$, NA 0.9). All neurons tested were chosen from the substantia gelatinosa (SG) of the dorsal horn. To distinguish between neurons and glial cells, we recorded cell responses to the application of high potassium solution. All experiments were done at 32°C.

DRG neurons preparation. The experimental technique for acute preparation of isolated DRG neurons was similar to that used for mice and rats. Ganglia were enzymatically treated in a Tyrode medium supplemented by 1 mg/ml protease (Sigma, Type XIV). The tissue was incubated in isolation medium at 35°C for 18–20 min. After enzymatic treatment the cells were isolated by gentle pipetting in Tyrode solution. The cell suspension was plated on sterile glass coverslips. All neurons were divided into two groups depending on their size: 35–50 μm for large and 25–30 μm for small neurons. An additional fast application system allowed a rapid (< 100 ms) change of the solution surrounding chosen neurons.

Measurement of intracellular calcium in spinal cord neurons. Dorsal horn neurons were loaded with a calcium indicator, fura-2, by incubating spinal cord slices with fura-2 acetoxymethyl ester (fura-2/AM). For such bulk loading, spinal cord slices were incubated in a solution supplemented with fura-2/AM (10 $\mu\text{mol/l}$, diluted in DMSO) and pluronic F-127 detergent (0.02%) for 50 min at 35°C. For the period of dye-loading, slices were kept under controlled air environment (5% CO_2 + 95% O_2). Subsequently, slices were incubated in physiological solution for an additional 40 min to ensure fura-2/AM de-esterification.

For fura-2 excitation, cells were alternately illuminated at wavelengths of 360 ± 5 nm and 390 ± 5 nm. Excitation filters were mounted in a filter wheel set at 5 rev/s. The emitted light was collected at 530 ± 10 nm by a photomultiplier. The filter wheel and photomultiplier output were controlled by the Fura-2 system (Luigs and Neumann, Rattingen, Germany). Signals corresponding to both excitation wavelengths were fed to an IBM compatible PC using a TIDA interface (Batelle, Germany). $[\text{Ca}^{2+}]_i$ values were calculated offline.

To reduce the background fluorescence and select the region of interest, the UV illumination was attenuated by an adjustable diaphragm installed in the light path. Furthermore, a 1 mm pinhole was inserted in front of the photomultiplier housing so that fluorescence collection was equivalent to a 40 μm spot at the object plane. Dye-loaded dorsal horn neurons were positioned in such a way that the fluorescent signal was collected from their soma.

The actual $[\text{Ca}^{2+}]_i$ was calculated from the ratio (R) of fluorescence recorded at 360 and 390 excitation wavelengths using the equation [8]:

$$[\text{Ca}^{2+}]_i = K_d B (R - R_{\min}) / (R_{\max} - R),$$

where $R_{\min}$ is the fluorescence ratio of Ca^{2+} -free fura-2 and $R_{\max}$ is the ratio of Ca^{2+} -bound fura-2; the constant K_d was taken from [8]; the constant B was determined empirically. The system was calibrated in vitro [8]. The parameters $K_d B$, $R_{\min}$ and $R_{\max}$ characterising the system were 3225 nmol/l, 0.9 and 10, respectively. Residual $[\text{Ca}^{2+}]_i$ was calculated using the following equation:

$$R = (\Delta[\text{Ca}^{2+}]_{60} / \Delta[\text{Ca}^{2+}]_{\text{peak}}) \cdot 100\%;$$

where $\Delta[\text{Ca}^{2+}]_{60} - [\text{Ca}^{2+}]_i$ at 60 s after the termination of the application minus resting $[\text{Ca}^{2+}]_i$, and $\Delta[\text{Ca}^{2+}]_{\text{peak}} - \text{peak } [\text{Ca}^{2+}]_i$ minus resting $[\text{Ca}^{2+}]_i$.

Measurement of intracellular calcium in DRG neurons. Cytosolic free Ca^{2+} concentration was measured with a Ca^{2+} sensitive fluorescent dye indo-1. Neurons were loaded with the indicator by incubation with Tyrode solution supplemented with 7 $\mu\text{mol/l}$ indo-1/AM (diluted with DMSO) and 0.02 % Pluronic F-127 detergent for 30 min followed by the washout in normal Tyrode solution for the same period at 22 °C. For $[\text{Ca}^{2+}]_i$ measurements glass coverslips with dye-loaded cells were placed in the experimental chamber mounted on the stage of an inverted fluorescent microscope equipped with epifluorescence. Indo-1 fluorescence was induced with a single excitation wavelength and dual emission was measured to determine $[\text{Ca}^{2+}]_i$. Cells were illuminated at 360 nm through the objective lens (40 $\times$ Zeiss) with a 120 W mercury arc lamp. The emitted light was passed through an aperture centred on the cell body, split by dichroic mirrors and collected by a pair of photomultiplier tubes in bands between 395–415 nm and 490–510 nm. After filtering at 20 Hz, an analogue divider delivered the fluorescence ratio on line. The actual $[\text{Ca}^{2+}]_i$ values were calculated offline on the basis of the Grynkiewicz equation [8]. Residual $[\text{Ca}^{2+}]_i$ was calculated in the same way as for DH neurons.

Solutions. The physiological salt solution used for the preparation procedure contained (in mmol/l): NaCl – 125; KCl – 5; CaCl_2 – 2.5; MgSO_4 – 1.5; NaHCO_3 – 25; KH_2PO_4 – 1.6; glucose – 10 and recording solution: NaCl – 128; KCl – 2; CaCl_2 – 2.5; MgSO_4 – 1.5; NaHCO_3 – 25; KH_2PO_4 – 1.6; glucose – 10; pH – 7.4 when continuously gassed with 5 % CO_2 + 95 % O_2 .

In order to prevent the involvement of synaptic transduction in generation of $[\text{Ca}^{2+}]_i$ transients, all slice experiments were performed in the presence of 1 $\mu\text{mol/l}$ TTX in bath solution.

Tyrode solution was composed of (in mmol/l): NaCl – 140; KCl – 4; CaCl_2 – 2; MgCl_2 – 2; HEPES/NaOH – 5; glucose – 10; pH – 7.4. For producing high-potassium extracellular solution 50 mmol/l Na^+ in Tyrode solution was replaced isoosmotically with 50 mmol/l K^+ . Nifedipine concentration used for application to spinal cord slices and DRG neurons was 50 $\mu\text{mol/l}$, caffeine 30 mmol/l for DH neurons and caffeine 20 mmol/l for DRG ones.

Fura-2/AM and indo-1/AM were obtained from Molecular Probes (Eugene, Ore., USA). TTX was obtained from Alomone Labs (Jerusalem, Israel) and all other chemicals were from Sigma-Aldrich Chemie, Deisenhofen, Germany.

For studying the long-term dihydropyridine (DHP) action nimodipine was administered per os (40 mg/kg daily during 3 weeks) in mice with STZ-modulated diabetes. This part of experiments was done on mice rather than on rats because of technical problems involving the isolation of DRG neurons from rats aged over 12 weeks.

Data analysis. Calcium recording traces were analysed using Tida software (Batelle, Germany). Data are expressed as Means $\pm$ SEM. The number of cells used for data analysis is indicated in round brackets after the mean values throughout the text. Statistical significance was calculated using two-tailed two-sample equal variance Student's *t* test. A *p* value of less than 0.05 was considered statistically significant.

Results

A total of 30 control, 6 STZ-control and 39 diabetic rats were used in the experiments. Animals for the STZ-control group were used 2–3 days after STZ injection. We did not observe any difference in calcium signalling between control and STZ-control groups, indicating that STZ itself does not affect calcium homeostasis in neurons. Diabetic rats and mice were used 4–5 weeks after STZ injection. On the average, the glucose concentration in blood serum was 7.5 ± 1.0 mmol/l ($n = 30$) and 20.6 ± 1.3 mmol/l ($n = 39$) for control and diabetic animals, respectively.

The basal concentration of cytoplasmic free calcium concentration ($[\text{Ca}^{2+}]_i$) was measured in dorsal root ganglion (DRG) neurons from control and diabetic rats. We did not observe a considerable difference in basal $[\text{Ca}^{2+}]_i$ in neurons of the two groups. On the average, in large DRG neurons basal $[\text{Ca}^{2+}]_i$ was 113 ± 4 nmol/l ($n = 31$) in control cells and 101 ± 3 nmol/l ($n = 39$) in diabetic neurons. In small DRG neurons from the STZ-induced diabetic rats the basal $[\text{Ca}^{2+}]_i$ (103 ± 4 nmol/l, $n = 31$) was also not substantially different from that in control animals (94 ± 6 nmol/l, $n = 19$).

The most important characteristics of functional changes in neuronal calcium homeostasis are the changes in intracellular calcium concentration during cell activation by membrane depolarization (depolarization-induced calcium transient). To elicit such transients, we applied high-potassium (50 mmol/l) solution externally.

No substantial differences were observed in the rise time and the amplitude of transients in DRG neurons of STZ-treated animals compared with control ones (Fig. 1). The main difference in the characteristics of $[\text{Ca}^{2+}]_i$ transients in DRG neurons between control and diabetic animals was the time course of their decay after termination of membrane

Fig. 1. Depolarization-induced $[Ca^{2+}]_i$ transients recorded from DRG (A) and DH (B) neurons from control (upper trace) and diabetic (lower trace) rats. C Residual $[Ca^{2+}]_i$ concentration at 60 s after the termination of 50 mmol/l KCl depolarization presented as a percentage of the transient peak; * = $p < 0.05$, *** = $p < 0.001$

depolarization. The residual intracellular $[Ca^{2+}]_i$ measured 60 s after termination of depolarization and presented as a percentage of the transient peak was significantly higher in small DRG neurons from diabetic animals: $8.1 \pm 0.4\%$ ($n = 23$) in control and $11.0 \pm 1.0\%$ ($n = 11$) in the case of STZ-induced diabetes, $p < 0.05$ (Fig. 1). Interestingly, in large DRG neurons there were no substantial changes in the residual $[Ca^{2+}]_i$: $8.0 \pm 1.0\%$ ($n = 29$) and $7.6 \pm 1.0\%$ ($n = 39$) in neurons from control and diabetic rats, respectively. (Fig. 1). This finding indicates the presence of significant changes in the functioning of intracellular Ca^{2+} handling mechanisms in DRG neurons from STZ-induced diabetic rats.

As has been shown [6], the same parameters in the spinal dorsal horn (DH) neurons revealed similar changes in residual $[Ca^{2+}]_i$ measured 60 s after termination of membrane depolarization: $2.8 \pm 0.5\%$

($n = 23$) in control and $14.1 \pm 1.2\%$ ($n = 19$) in the case of STZ-induced diabetes, $p < 0.001$ (Fig. 1). These results indicate that under diabetic conditions similar changes in calcium signalling occur both in primary and secondary nociceptive neurons.

We also checked the changes in depolarization-induced transients in DRG neurons from control and diabetic mice. Some decrease in the transient amplitudes was observed at short time (0.5 s) depolarization (311 ± 36 nmol/l and 269 ± 32 nmol/l for control and diabetic mice correspondingly). This decrease disappeared with the lengthening of depolarization duration. Thus, at 5 s depolarization the mean amplitudes were 468 ± 63 nmol/l and 451 ± 52 nmol/l for neurons from control and diabetic animals, respectively. The changes in other parameters of $[Ca^{2+}]_i$ transient, such as basal concentration and residual calcium were similar to those obtained from rats. Re-

Fig. 2. Amplitudes of caffeine- and ionomycin-induced $[Ca^{2+}]_i$ transients in DH and DRG neurons of control and diabetic rats. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

sidual $[Ca^{2+}]_i$ increases measured 60 s after termination of depolarization increased to $17.0 \pm 2.9\%$ of the peak amplitude as compared to $6.8 \pm 1.3\%$ in control ($p < 0.05$).

Although several studies have reported alterations in the functioning of the IP_3 -sensitive Ca^{2+} stores of nerve cells under diabetic conditions [9], very little information is available about changes in the activity of ryanodine-sensitive Ca^{2+} stores in secondary nociceptive neurons. These stores release Ca^{2+} in response to increases in $[Ca^{2+}]_i$. This calcium-induced Ca^{2+} release (CICR) can be activated by the application of caffeine which reduces the receptor sensitivity threshold to calcium ions.

Application of 20 mmol/l caffeine in Ca^{2+} -free external solution to DRG neurons induced a transient elevation of $[Ca^{2+}]_i$ both in neurons of control and STZ-treated animals (Fig. 2). However, the amplitude of these transients was considerably smaller in neurons of STZ-induced diabetic rats compared with control. On the average, these amplitudes were 466 ± 48 nmol/l, $n = 14$ for control and 343 ± 36 nmol/l, $n = 16$ for diabetic animals, $p < 0.01$. In DRG neurons we checked caffeine-induced $[Ca^{2+}]_i$ increases only in large cells, since small DRG neurons lack caffeine-releasable calcium stores [10]. The difference in response to 30 mmol/l caffeine application in Ca^{2+} -free solution to DH neurons was more prominent than that observed when applied to DRG neurons (268 ± 29 nmol/l, $n = 13$ for control and 41 ± 9 nmol/l, $n = 17$ diabetic animals, $p < 0.001$). These results led us to conclude that Ca^{2+} in both DRG and DH neurons of diabetic rats are stored and/or released from ryanodine-sensitive calcium stores less effectively than in neurons from control animals. This conclusion was further supported by experiments with ionomycin. Application of 1 μ mol/l ion-

Fig. 3. Increased block of depolarization-induced $[Ca^{2+}]_i$ transients by 50 μ mol/l of nifedipine in diabetic DRG and DH neurons. Data are expressed as % of $[Ca^{2+}]_i$ transient under nifedipine action relative to control transient. The first two bars represent data of small DRG neurons; the second pair – large neurons; the third pair – DH neurons. * = $p < 0.05$, ** = $p < 0.01$

omycin in Ca^{2+} -free solution induced a smaller $[Ca^{2+}]_i$ increase both in DRG and DH neurons from diabetic rats compared with those from control animals. The amplitudes of such an increase in DRG neurons were 159 ± 22 nmol/l, $n = 27$ for control and 80 ± 16 nmol/l, $n = 10$ for diabetic animals, $p < 0.05$. The amplitudes of such elevation in DH neurons were 188 ± 25 nmol/l, $n = 8$ for control and 85 ± 13 nmol/l, $n = 6$ for diabetic animals, $p < 0.05$.

Thus, in STZ-induced diabetes, even at early stages, definite alterations in calcium signalling of DRG and DH neurons occur as substantial prolongation of the time course of depolarization-induced $[Ca^{2+}]_i$ transients. Such prolongation alters the transmission of nociceptive signals through the corresponding neurons, at least partly through changes in the functioning of the intracellular Ca^{2+} -regulating mechanisms.

For further clarification of possible changes in the function of cellular structures involved in the above-described alterations of intracellular calcium homeostasis, we also investigated the effect of different calcium channel blockers on depolarization-triggered calcium transients.

The application of an L-type calcium channel blocker nifedipine (50 μ mol/l) to DRG and DH neurons induced a substantial suppression of the amplitude of depolarization-induced $[Ca^{2+}]_i$ transients. As shown in Figure 3, this suppression was considerably stronger in neurons of STZ-treated rats compared with control animals. In small DRG neurons the amplitudes of depolarization-induced $[Ca^{2+}]_i$ transients under nifedipine treatment were reduced to $64 \pm 5\%$ ($n = 11$) in neurons of control animals and to $46 \pm 5\%$ ($n = 9$) in those from diabetic rats with respect to control depolarization-induced transient

without the drug. The difference was statistically significant ($p < 0.05$). In large neurons these amplitudes decreased to $57 \pm 6\%$ ($n = 16$) and $34 \pm 6\%$ ($n = 8$), $p < 0.05$ for control and diabetic animals, respectively. A similar phenomenon was observed in DH neurons (Fig. 3). Depolarization-induced peak amplitudes of calcium transients under nifedipine treatment were lowered to $69 \pm 5\%$ ($n = 8$) in control animals and to $35 \pm 3\%$, ($n = 13$) in diabetic rats with respect to control depolarization-induced transient. The difference was $p < 0.01$. Nifedipine had no effect on increased residual calcium in neurons of diabetic animals.

Our data indicate that although the amplitudes of $[Ca^{2+}]_i$ transients induced by activation of voltage-operated calcium channels do not alter considerably under diabetic conditions, definite changes do occur in the activity or expression of L-type channel. Such alterations in the case of short-term application of L-type calcium channel blockers fit the observation of other investigators on different tissues under diabetic conditions [11]. Such increased efficacy of high-voltage calcium channel blockers in diabetes could result either from a shift in relative contribution of different channel types to the total Ca^{2+} influx into the neurons of diabetic animals, or to a changed pharmacological sensitivity of corresponding channels.

These results prompted us to investigate not only the immediate effects of calcium channel blockers on depolarization-induced calcium transients but also their possible effects during prolonged administration. For this purpose, we used a per os administration of nimodipine (L-type calcium channel blocker from a class of dihydropyridines) over a period of 3 weeks to STZ-induced diabetic and control mice. The amplitudes of calcium transients increased by $12.8 \pm 3\%$ ($n = 7$) in small-sized DRG neurons from healthy nimodipine-treated mice compared with those obtained from untreated animals ($p < 0.05$). An even greater increase was observed in neurons of STZ-induced diabetic animals which chronically received nimodipine compared to untreated diabetic mice.

The amplitudes of calcium transients increased by $39 \pm 2\%$ ($n = 15$, $p < 0.01$). The concentration of residual calcium measured 60 s after termination of depolarization reached $13.0 \pm 3\%$ in control nimodipine-treated animals compared with $6.8 \pm 0.8\%$ in control untreated animals ($p < 0.01$); these values were $19.2 \pm 2.3\%$ in diabetic animals which received nimodipine and $17.7 \pm 2.9\%$ in diabetic mice not treated with nimodipine (the difference is not significant, $p > 0.1$) (Fig. 4). Thus, the concentration of residual calcium became greater at long-term nimodipine administration even in healthy animals (the concentration of residual calcium is given according to 5 s depolarization). This result suggests that not only diabetes but also long-term treatment with L-type chan-

Fig. 4. Residual $[Ca^{2+}]_i$ at 60 s after the termination of 50 mmol/l KCl depolarization of DRG neurons presented as a percentage of the transient peak. The first two bars represent residual $[Ca^{2+}]_i$ in neurons from nimodipine-treated and non-treated control animals. The second pair represent residual $[Ca^{2+}]_i$ in neurons from nimodipine-treated and non-treated diabetic animals. ** = $p < 0.01$

nel blockers by itself could alter the function of corresponding channels. Such an alteration could be due to some common mechanisms which are modulated by the long-lasting application of L-type calcium channel blockers and amplified in the case of diabetes mellitus.

Discussion

Our data show that substantial changes in calcium signalling in nociceptive neurons occur even at early stages of experimentally-induced diabetes mellitus. These changes were practically identical in diabetic mice and rats, indicating their general importance for the development of diabetic neuropathy. Of note, such changes were basically identical in primary and secondary nociceptive cells, although the mechanisms responsible for the termination of calcium signals and elimination of excessive Ca^{2+} ions from the cytosol are quite different in both cases. As has been shown previously, in small-sized (nociceptive) DRG neurons of mice the endoplasmic reticulum does not participate in this process; no Ca^{2+} release could be evoked from it either by the application of caffeine or metabotropic receptor agonists [10, 12]. For this reason, changes in Ca^{2+} accumulation and its subsequent release by mitochondria are of major importance for the formation of calcium signals in this type of cell [13, 14]. Similar findings were obtained for small-sized DRG neurons of rats. In contrast, in all tested rat dorsal horn neurons, transient increases in cytosolic Ca^{2+} could be evoked by the application of caffeine; superfusion of slices with Ca^{2+} -free solu-

tion did not affect the transients, showing that they are produced by Ca^{2+} release from intracellular stores [6]. Thus, Ca^{2+} uptake by endoplasmic reticulum in these neurons can be an important component in the mechanisms which are shaping the time course of depolarization-induced calcium transients. Depression of such uptake under diabetic conditions could be an important factor responsible for the prolongation of calcium signals (in combination with possible changes in the calcium-regulating function of mitochondria).

A general conclusion to be drawn from our experiments is that diabetic pathology is related with a reduced capacity of nociceptive neurons to eliminate Ca^{2+} from the cytosol. The participation of more rapidly operating mechanisms (Ca^{2+} uptake by mitochondria and endoplasmic reticulum) is definitely predominant in this elimination. Obviously, Ca^{2+} extrusion to the extracellular space by plasmalemmal Ca^{2+} -ATPase can also be altered, but it might be a less important due to its slowness.

There are several metabolic pathways which influence the Ca^{2+} -accumulating function of mitochondria and endoplasmic reticulum that can be altered under diabetic conditions. The excess of glucose is converted into sorbitol and fructose, an increase which is linked to lower concentrations of myoinositol, disturbances in phospholipid metabolism and generation of diacylglycerol [15]. High glucose concentrations advance the formation of advanced glycation end-products [16]. Finally, toxic effects of glucose can be mediated by an imbalance in the generation and scavenging of reactive oxygen species and formation of lipid peroxidation by-products [17].

We have shown that in neurons from diabetic rats the amount of Ca^{2+} released from caffeine-sensitive stores is much smaller than in cells from control animals. Similar observations were made on aortic smooth muscle [18] and cardiomyocytes [19]. Therefore, we can assume that Ca^{2+} accumulation by the endoplasmic reticulum during the incidence of Ca^{2+} transients is reduced in DH and DRG neurons under diabetic conditions, probably because of a decrease in the activity of corresponding SERCA-pumps. As a result, the restoration of $[\text{Ca}^{2+}]_i$ to the basal concentration after termination of membrane depolarization will also be slowed down. Other mechanisms could also be involved in the decrease of caffeine-induced $[\text{Ca}^{2+}]_i$ transients under diabetic conditions: changes in the sensitivity of ryanodine-receptors; a reduction in the Ca^{2+} capacity of the endoplasmic reticulum; increases in the Ca^{2+} -permeability ("the Ca^{2+} leak pathway") of the endoplasmic reticulum. In any case, our results show that ryanodine-sensitive calcium stores of endoplasmic reticulum play a major role in the prolongation of calcium transients in nociceptive DRG and DH neurons under diabetic conditions and, together with mitochondria, they can participate in the modulation of calcium transients [20].

Our data also indicate that voltage-operated calcium channels involved in the generation of depolarization-induced $[\text{Ca}^{2+}]_i$ transients are clearly altered under diabetic conditions. The L-channel antagonist nifedipine caused a greater inhibition of $[\text{Ca}^{2+}]_i$ transients in neurons from diabetic animals than from control animals. These findings imply that a relative contribution of L-type high-threshold Ca^{2+} currents to Ca^{2+} signalling became enhanced.

The findings are quite controversial. For instance, an increase of calcium transient peaks in cardiomyocytes from diabetic animals has been shown [19]. Calcium currents in capsaicin-sensitive DRG neurons under diabetic conditions increased too [21]. However, other authors did not observe any definite differences in calcium transients in peripheral neurons at normal and high glucose concentrations [22].

It is known that HVA channels can transit into short and long open states. The interrelation between the two states of calcium channels could be changed under diabetic conditions. This change could result from alterations in the processes of channel phosphorylation due to changes in the PKC activity [21] or in membrane structure [23]. According to some authors, such changes can directly influence the structural orientation of phospholipids, membrane structure and, as a result, the activity of incorporated ion channels [24]. The same effects were found with cardiomyocytes [25].

Potentiation of the action of HVA calcium channel blockers was found in DH and DRG neurons, with predominant enhancement of the inhibitory action of dihydropyridines (DHP) only with a short-term application. Such an influence of calcium channel blockers (especially nifedipine) has been already shown for microvessel cells and cardiomyocytes [26, 27]. Some authors showed that in skeletal muscle cells the effectiveness of DHP under short-term application is regulated by Ca^{2+} and different second messengers [11]. Others reported the changes in pharmacological sensitivity of HVA calcium channels to DHP [28].

In the case of long-term DHPs treatment, different mechanisms seem to be involved than in the short-term application. In contrast to neurons from untreated animals, the neurons from animals which were treated with nimodipine for 3 weeks showed an increase in the amplitude of calcium transient. An increase in the amplitudes of calcium transients under chronic treatment by nifedipine was also observed by other authors in cardiomyocytes of healthy animals [27]. The authors also showed that $[\text{Ca}^{2+}]_i$ transient amplitude was significantly higher in ventricular myocytes obtained from nifedipine-treated rats than in myocytes isolated from untreated animals. Investigators have also shown increased calcium currents under a long-term blockade of L-type calcium channels with nifedipine and an increase in the number

of binding sites for nifedipine in skeletal muscle of healthy animals [11]. The mechanism by which the long-term treatment of DHP can induce such effects is not known. It could be due to an increase in the number of receptors or to a decrease in the rate of degradation of the receptors. A higher rate of calcium channel expression could also be a reason for the effects of DHP. Some authors have speculated that the cause of such action of nifedipine could be the activation of protein kinase C or endogenous phosphatases [27]. Exactly which mechanisms act in case of nociceptive neurons needs to be clarified.

In conclusion, the problem of changes in calcium homeostasis in nociceptive neurons during diabetes mellitus is of major importance for the understanding of the mechanisms of neurological complications in diabetes and definitely needs further clarification.

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