Potassium channel dysfunction in hypothalamic glucose-receptive neurones of obese Zucker rats

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1. We have shown, using intracellular and cell-attached recordings, that glucose-receptive (GR) neurones of obese Zucker rats exhibit abnormal electrophysiological responses to changes in extracellular glucose concentration, whereas GR neurones of lean Zucker and control rats respond normally.

2. In inside-out recordings from obese rat GR neurones it was shown that the 150 pS ATP-sensitive K^+ (K_{ATP}) and the 160 pS calcium-activated K^+ (K_{Ca}) channels were absent, whereas both were present in GR neurones of lean Zucker and control rats.

3. The potassium channel most frequently observed in inside-out patches from obese GR neurones was characterized by a conductance of 213 pS, was activated by raising internal calcium and inhibited by application of internal ATP. This channel (which we have termed K_{fa}) was not observed in lean or control rat GR neurones.

4. Tolbutamide (100 μM) was found to induce no effect or to elicit a small depolarization of obese rat GR neurones in the absence of glucose, in contrast to its clear excitatory actions on control or lean Zucker GR neurones.

5. Intracellular, cell-attached and inside-out recordings from obese rat non-GR neurones showed that there was no alteration in their membrane properties or firing characteristics or in the characteristics of the large-conductance calcium-activated K^+ channel (K_{Ca}) present in these neurones as compared with lean and control rats.

6. It is concluded that the K_{fa} channel is specific to GR neurones of obese Zucker rats and that the presence of this channel coupled with the absence of K_{ATP} and K_{Ca} channels results in the abnormal glucose-sensing response of these neurones.

Obesity increases the risk of non-insulin-dependent diabetes mellitus (NIDDM), hyperlipidaemia and hypertension in humans and experimental animals and involves a genetic component (Bray & York, 1979). Recent studies indicate that one form of genetic obesity in rodents is due to a mutation in the *obese* (*ob*) gene (Zhang, Proenca, Maffei, Barone, Leopold & Friedman, 1994). This gene encodes an adipose tissue-derived signalling molecule (leptin) which is considered to act as an obesity regulating factor or hormone. The absence of functional leptin results in profound obesity (*ob/ob* mice) which can be corrected in this strain by exogenous administration of recombinant ob protein (Pelleymounter *et al.* 1995; Halaas *et al.* 1995; Campfield, Smith, Guisez, Devos & Burn, 1995). Recent evidence suggests that the gene (*db*) underlying the recessive obesity mutation in mice (*db/db*) encodes the receptor for the *ob* gene product, leptin. The recent cloning of this receptor indicates that it is a single membrane-spanning receptor of the class 1 cytokine receptor family (Tartaglia *et al.* 1995). The receptor has alternatively spliced forms and one of these is abnormally spliced in *db/db* mice giving rise to a mutant with the C-terminal cytoplasmic region missing (Lee *et al.* 1996), which may well result in aberrant signal transduction.

The autosomal recessive *fa* gene produces obesity in Zucker and Wistar Kyoto rats when homozygous (Zucker & Zucker, 1962; Ikeda, Shino, Matsuo, Iwatsuka & Suzuoki, 1981). Zucker (*fa/fa*) obese rats are hyperinsulinaemic, hyperphagic and relatively normoglycaemic but exhibit increased central and peripheral insulin resistance (Ikeda *et al.* 1986; Zarjevski, Doyle & Jeanrenaud, 1992). The transfer of the *fa* gene into Wistar Kyoto rats brings about overt diabetic

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symptoms as well as obesity in male rats (Ikeda *et al.* 1981). Therefore the *fa* gene is of interest in the development of obesity and NIDDM. There is no abnormality associated with the *ob* gene in obese Zucker (*fa/fa*) rats but an increased ob mRNA in adipose tissue is observed in this (Murakami & Shima, 1995) and the Wistar (*fa/fa*) strain (Masuzaki *et al.* 1996). The *fa* gene is considered to map to a region syntenous with the mouse *db* locus (Truett, Bahary, Friedman & Leibel, 1991) and thus the defect resulting in an obese phenotype may also be present at the level of the *ob* receptor in these animals. The *ob* receptor splice variant associated with the *db* gene is expressed at a high level in the hypothalamus (Lee *et al.* 1996) and has led to the suggestion that leptin interacts with this receptor in the hypothalamus, resulting in weight loss.

The hypothalamus has a profound influence on metabolism through its effects on appetite and satiety as part of the long-term regulation of body mass, and its effects on pancreatic, adrenal and liver function for short-term control (Morley & Levine, 1985; Niijima, 1989). The hypothalamic areas of particular importance in the regulation of glucose metabolism and feeding are the lateral hypothalamic area and the ventromedial hypothalamic nucleus (VMHN) (Niijima, 1989). Lesion experiments have shown that disruption of the VMHN results in hyperphagia and obesity (Bray & York, 1979) and also gives rise to an enhanced ob mRNA expression (Maffei *et al.* 1995; Funahashi *et al.* 1995). Glucose-receptive (GR) neurones make up approximately 30% of the neurones in the VMHN and are thought to mediate in the central control of glucose metabolism (Oomura, Ono, Ooyama & Wayner, 1969; Ono, Nishino, Fukuda, Sasaki, Muramoto & Oomura, 1982). The GR neurones depolarize and increase firing of action potentials in response to a rise in extracellular glucose concentration (Ono *et al.* 1982; Minami, Oomura & Sugimori, 1986). We have demonstrated previously that closure of ATP-sensitive K^+ (K_{ATP}) channels by glucose metabolism increases the excitability of rat GR neurones (Ashford, Boden & Treherne, 1990*a*) in a manner similar to that seen for pancreatic β-cells (Ashcroft, 1988). We were therefore interested to determine whether the Zucker (*fa/fa*) rat displayed changes in GR neurone physiology and have consequently investigated the glucose responsiveness and properties of K_{ATP} and calcium-activated potassium channels of hypothalamic GR neurones of Zucker rats.

METHODS

Drugs and solutions

All solutions were made using glass-distilled water and Analar grade chemicals. The artificial cerebrospinal fluid (ACSF) for rat hypothalamic slices contained (mM): NaCl, 128·0; KCl, 5·0; NaH_2PO_4, 1·2; $CaCl_2$, 2·4; $MgCl_2$, 1·3; $NaHCO_3$, 26·0; D-glucose, 10·0; pH 7·4. Before single channel recording, neurones were washed thoroughly with normal external saline which consisted of (mM): NaCl, 135·0; KCl, 5·0; $CaCl_2$, 1·0; $MgCl_2$, 1·0; Hepes, 10·0. The pH was adjusted to 7·2 with NaOH. For cell-attached recording the patch pipette was filled with the following solution (mM): KCl, 140·0; $CaCl_2$, 1·0; $MgCl_2$, 1·0 (or $CaCl_2$, 5·0; $MgCl_2$, 5·0); Hepes, 10·0 (pH adjusted to 7·2 with KOH) and the bathing solution was the normal external saline. The solution in the recording pipette used for inside-out patch studies either contained the same solution as for cell-attached recording (i.e. symmetrical 140 mM KCl) or normal external saline (i.e. approximately physiological ionic gradients). The bathing solution for inside-out patch recordings contained (mM): KCl, 140·0; $MgCl_2$, 1·0; $CaCl_2$, 0·9; EGTA, 1·0; Hepes, 10·0. The pH was adjusted to 7·2 with KOH and the free Ca^{2+} concentration was 0·8 μM. In experiments to ascertain the Ca^{2+} sensitivity of potassium channels the concentrations of Ca^{2+} and Mg^{2+} were varied in order to produce the required change in the concentration of Ca^{2+} whilst keeping the concentration of Mg^{2+} constant. ATP (Na^+ and K^+ salts, vanadium free) and tolbutamide were purchased from Sigma.

Electrical recordings and data analysis

Male rats fed *ad libitum* were stunned and decapitated and the hypothalamus dissected out. Coronal slices (350 μm thick) of hypothalamus were cut from brains of Zucker, Wistar Kyoto or Sprague–Dawley rats with a Vibratome (Oxford Instruments). The slices were maintained at room temperature in ACSF. For intracellular recording, the slices were transferred to a recording chamber where they were superfused with ACSF at 37 °C. Electrodes were filled with 3 M potassium acetate and had DC resistances of 100–150 MΩ when measured in physiological saline. A period of 30 min was allowed for equilibration following impalement. Input resistances were derived from the slope of the current–voltage plot obtained by measuring the electrotonic potential during current injection. Pulses, of greater than 100 ms duration, were applied in order to ensure complete capacitance saturation of the membrane. In the case of Zucker rats, naivety of neurones was ensured by making only one recording from each slice. These recording procedures have previously been described in full (Boden & Hill, 1988). Acutely dissociated neurones were prepared as described previously (Ashford *et al.* 1990*a*). In brief, male rats (Zucker, Wistar or Sprague–Dawley strains, 100–300 g) fed *ad libitum* were stunned and decapitated and the hypothalamus dissected out. Ventromedial nuclei were isolated from 350 μm coronal slices of rat hypothalamus. The isolated hypothalamic sections were incubated for 1 h in collagenase (0·5 mg ml^{-1}) and trypsin (1 mg ml^{-1}) in ACSF and dispersed by trituration. For simplicity we have combined the data from Wistar Kyoto and Sprague–Dawley rats as control animals throughout as no detectable differences were observed.

Single channel currents were recorded from cell-attached and inside-out membrane patches. Recording pipettes, when filled with electrolyte, had resistances between 8 and 15 MΩ. Current recordings were obtained using a patch-clamp amplifier (EPC-7, List Electronic, Germany or Axopatch-2D, Axon Instruments, USA) and recorded onto magnetic tape (Racal Store 4D or 4DS, Southampton, UK). The tape was later replayed through an 8-pole Bessel filter (−3 dB at 1 kHz), amplified and digitized at 5 kHz with a 12-bit analog-to-digital converter (Data Translation 2801A, Marlborough, MA, USA) and analysed on either an Elonex 386 or an Apricot Xen-i 286/45 computer using PAT 6.2 software (Patch-clamp analysis program 6.2, J. Dempster, University of Strathclyde, UK). The data were analysed for current amplitudes and open-state probability (P_{open}) as described previously (Dempster, 1988; Kozlowski & Ashford, 1990). In multichannel patches (usually confined to those with two channels), channel activity was determined by integration of the current signal over 1–2 min at a constant voltage and the result expressed as the function $N_f P_o$, where N_f is the number of functional channels and P_o is the open-

state probability (Kozlowski & Ashford, 1990). Single channel records used for illustrative purposes were replayed into a chart recorder (Gould, 2200) which had a nominal frequency response of 140 Hz. The potential across the membrane patch is described following the usual sign convention for membrane potential (i.e. inside negative). All patch-clamp experiments were carried out at room temperature (20–25 °C). All data in the text and tables are presented as mean values ± S.E.M.

RESULTS

The mean weight for male lean heterozygous (+/*fa*) and homozygous (+/+) Zucker rats at 8 weeks was 190·3 ± 5·5 g ($n = 6$), and for age-matched obese homozygous (*fa*/*fa*) Zucker rats was 278·7 ± 7·5 g ($n = 6$; $P < 0·005$). We made no attempt to distinguish the lean phenotypes as identical data were obtained within this group (22 animals were used and the corresponding theoretical ratios were 2:1; +/*fa*:+/+) The obese rats exhibited marked changes in their distribution of body fat consistent with previous observations (Zucker & Zucker, 1961).

Intracellular recordings from brain slices

Intracellular recordings were made from neurones in hypothalamic slices taken from lean and obese male Zucker and age-matched Wistar rats (2–6 months old). A total of forty neurones from thirty-one slices were used in this study. Experiments were performed on sixteen neurones from the VMHN of obese (*fa*/*fa*) Zucker rats and nine from lean (+/*fa* or +/+) Zucker rats; fifteen neurones from Wistar Kyoto rats were used for control data. In lean Zucker rats, GR neurones (identified as neurones which responded to acute changes in external glucose concentration and/or were excited upon the application of tolbutamide; see Ashford *et al.* 1990*a*; Ashford, Boden & Treherne, 1990*b*) were found

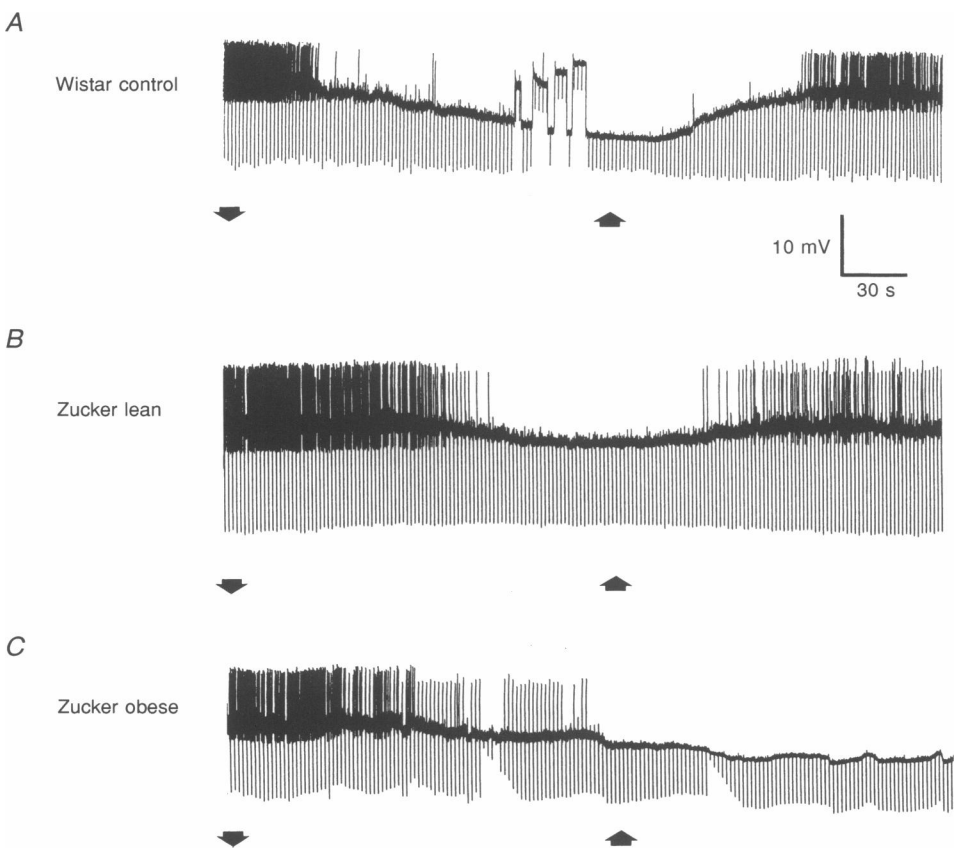

Figure 1. Intracellular recordings from hypothalamic GR neurones in rat brain slices

In all 3 recordings the extracellular glucose concentration in the ACSF superfusing the slice was reduced from 10 mM to 0 at the downward arrow, and 10 mM glucose was reintroduced at the upward-pointing arrow. *A*, record obtained from a control rat GR neurone. The removal of glucose induced membrane hyperpolarization and cessation of spontaneous action potentials. Associated with these changes was a decrease in input resistance which was not voltage induced (as determined by constant current injection to offset hyperpolarization). The hyperpolarization and input resistance change were reversed upon readministration of 10 mM glucose, and action potential activity returned. *B*, a similar response was observed on removal of and readmission of 10 mM extracellular glucose in a GR neurone from a lean Zucker rat. *C*, the response of GR neurones from obese Zucker rats to the removal of glucose was markedly different. Membrane hyperpolarization was observed but was slow in onset and was not reversed by readministration of 10 mM glucose. Furthermore, oscillations in the resting membrane potential of the GR neurone were observed following removal and reapplication of 10 mM glucose.

Table 1. Resting membrane parameters of VMHN neurones

Rat type	Control		Lean		Obese	
Neurone type	GR	Non-GR	GR	Non-GR	GR	Non-GR
n/total	6/15	9/15	4/9	5/9	9/16	7/16
Membrane potential (mV)	−58·8 ± 1·7	−57·1 ± 1·6	−58·5 ± 5·7	−64·0 ± 4·3	−57·6 ± 1·9	−60·3 ± 3·3
Input resistance (MΩ)	199 ± 29	231 ± 17	194 ± 24	206 ± 18	211 ± 20	238 ± 30
MHP in 0 glucose (mV)	4·5 ± 1·1	None	6·1 ± 1·3	None	4·5 ± 0·6	None
Input resistance change in 0 glucose (%)	38·3 ± 8·9	None	35·3 ± 4·3	None	18·6 ± 2·5*	None

Data from all 3 types of rat used in the study are presented. Forty neurones were used from 31 slice preparations (25 Zucker and 6 control). * Significantly different from control or lean ($P = 0{\cdot}0024$, Student's unpaired t test). Data in column 1, rows 4 and 5 are not different from those reported previously (Ashford *et al.* 1990*a*). MHP, membrane hyperpolarization; *n*, number of cells.

to make up approximately 44% of the population of VMHN neurones tested electrophysiologically and had a mean resting membrane potential of −58·5 ± 5·7 mV and input resistance of 194 ± 24 MΩ ($n = 9$). These neurones responded to changes in extracellular glucose concentration in a manner indistinguishable from GR neurones in control rats (Fig. 1*A* and *B*, Table 1; see also Ashford *et al.* 1990*a*, *b*), with the removal of extracellular glucose leading to membrane hyperpolarization and a fall in input resistance. The changes in membrane potential and input resistance were completely reversed in all cases upon reapplication of ACSF containing 10 mM extracellular glucose.

In obese (*fa/fa*) rats, GR neurones (identified by their response to tolbutamide in the presence of glucose or by their response to the removal of glucose; other neighbouring neurones do not respond to these challenges) made up approximately 56% of the population of the VMHN ($n = 16$), had a mean resting membrane potential of −57·6 ± 1·9 mV and mean input resistance of 211 ± 20 MΩ. However, distinct differences were seen between neuronal responses to glucose removal in obese (*fa/fa*) rats when compared with normal Wistar and lean Zucker rats. In the obese group, the removal of glucose resulted in membrane hyperpolarization and decreased input resistance of GR neurones but this effect was slower in onset than for the other two groups studied (Fig. 1*C*) and the decrease in resistance was significantly less than for the lean or control groups (Table 1). Furthermore, readmission of glucose (10 mM) did not reverse the action of the glucose-free ACSF with respect to resting membrane potential or input resistance. Recordings were continued for up to 2 h but the GR neurones of obese rats never recovered from the glucose-free challenge and both in the absence of glucose and following reintroduction of 10 mM glucose, membrane potential oscillations were apparent. The non glucose-receptive neurones in the VMHN, which neither depolarized nor hyperpolarized on removal of extracellular glucose, made up 55 and 44% of the population of lean and obese rat nuclei, respectively. These neurones displayed normal electrophysiological characteristics when compared with age-matched control rats (Table 1), although lean Zucker rats did have membrane potentials slightly more hyperpolarized than Wistar controls ($P > 0{\cdot}05$). These findings suggested to us that there were changes in the ion channels underlying glucose-dependent excitability of GR neurones in obese rats.

Patch-clamp recordings from isolated VMHN GR neurones

Cell-attached and inside-out patch-clamp recordings were obtained from freshly dissociated VMHN neurones isolated from hypothalamic slices from male lean (+/+ and +/*fa*) and obese (*fa/fa*) Zucker rats and age-matched controls (2–6 months old). In cell-attached recordings 32% of the neurones from the VMHN of lean Zucker rats ($n = 19$) and 35% of those from control rats ($n = 20$) were found to be glucose receptive. These GR neurones were identified by their response to raising extracellular glucose concentration in the bath saline from 0 mM to between 5 and 10 mM. Under these conditions, the high level of channel activity observed in the absence of extracellular glucose was markedly reduced upon glucose application (Fig. 2*A* and *B*). The glucose-induced reduction in channel open probability (P_{open}) was identical in both lean Zucker and control rats, with a typical reduction in P_{open} from approximately 0·7 to 0·01 (Fig. 2*A* and *B*). Similar data were obtained in a further two cell-attached recordings.

In experiments where glucose concentrations were not manipulated, we identified GR neurones in cell-attached recordings as those which, in the absence of glucose, were clearly not of the non-GR type (characterized by infrequent channel activity and a continuous firing of action potentials). Under symmetrical 140 mM KCl recording conditions, inside-out patches from GR neurones were demonstrated to contain a K^+ channel with a mean single channel conductance of 148·6 ± 7·0 pS ($n = 5$) and 147·0 ± 5·0 pS ($n = 5$) for lean Zucker and control animals, respectively. The P_{open} of this channel was insensitive to changes in either intracellular Ca^{2+} ion concentration (5 nM to 1 mM) or membrane potential (−60 to +60 mV) but was inhibited by the application of 1 mM ATP to the cytoplasmic surface of the

membrane (Fig. 3A), with a mean reduction in P_{open} of $63 \pm 12\%$ and $66 \pm 14\%$ for lean ($n = 3$) and control ($n = 3$) animals, respectively. Therefore this channel was identified as the K_{ATP} channel, previously characterized in GR neurones of control rats (Ashford *et al.* 1990*a*, *b*).

Inside-out patches from GR neurones of lean Zucker rats also displayed (in approximately 40% of patches, a similar proportion to that observed in control animals; Treherne & Ashford, 1991) a large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel which had a mean conductance of 160 ± 13 pS ($n = 3$) in symmetrical 140 mM KCl. The P_{open} of this channel was increased upon depolarization of the membrane or when the Ca^{2+} ion concentration at the inner surface of the membrane was increased (from 0·8 to 100 μM), but was unaffected by the application ($n = 6$) of 1 mM ATP (Fig. 3B), as previously described (Treherne & Ashford, 1991). Similar data were obtained from inside-out recordings from GR neurones of control animals ($n = 6$).

Therefore, the K_{ATP} and K_{Ca} channels described in GR neurones from lean Zucker and control rats are identical to those previously described in GR neurones of Wistar Kyoto and Sprague–Dawley rats (Ashford *et al.* 1990*a*, *b*; Treherne & Ashford, 1991).

The intracellular recordings from GR neurones in obese Zucker rats demonstrate that they respond abnormally to changes in extracellular glucose concentration (Fig. 1C), while non-GR neurones appeared identical in their electrophysiological responses to non-GR neurones of lean and control animals. Furthermore, the responses of obese Zucker rat GR neurones in cell-attached recordings were also abnormal compared with GR neurones from lean and control rats. Potassium channel activity in obese rat GR neurones

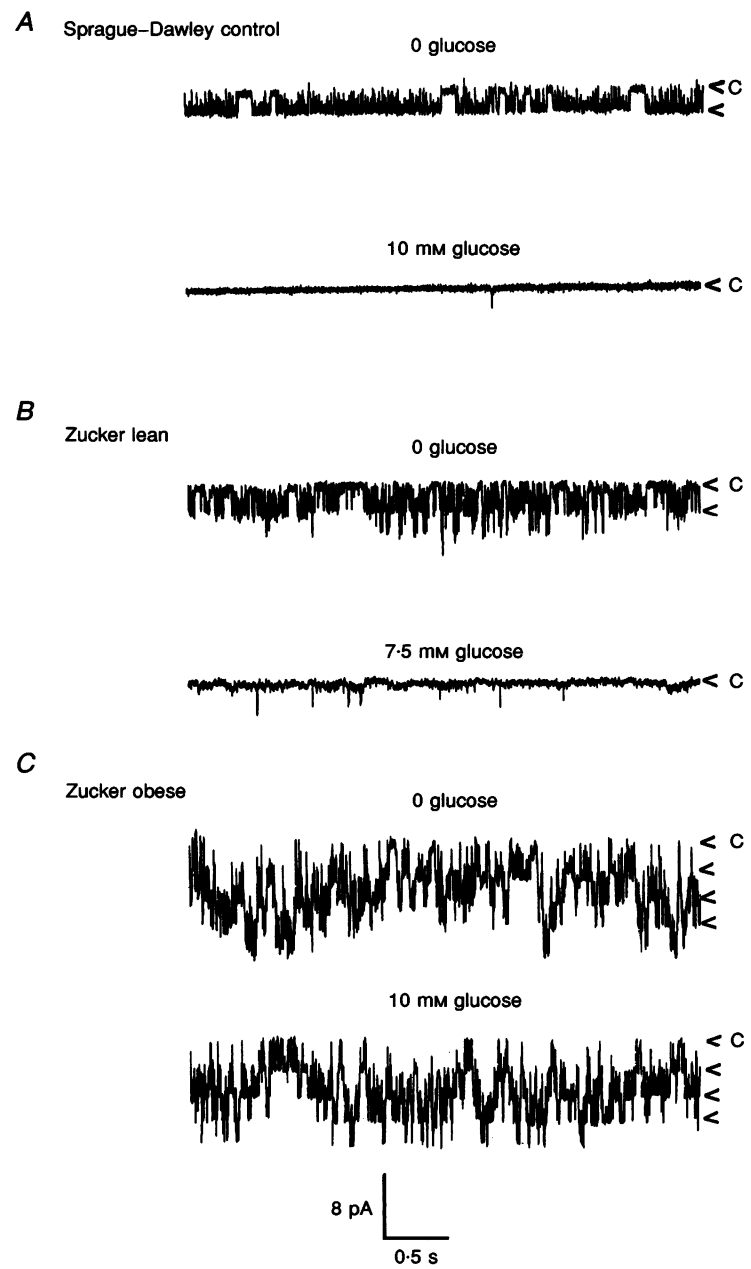

Figure 2. Cell-attached recordings from isolated hypothalamic GR neurones

All recordings were made in normal saline using pipettes containing 140 mM KCl and a holding potential of 0 mV. *A*, control GR neurone. In the absence of glucose, channel activity was high ($P_{open} = 0{\cdot}72$) and was substantially reduced on addition of 10 mM extracellular glucose ($P_{open} = 0{\cdot}01$). *B*, a similar response was observed on replacement of the 0 glucose solution with one containing 7·5 mM glucose in a GR neurone obtained from a lean Zucker rat. The value of P_{open} was reduced from 0·67 to 0·008 following exposure to glucose. *C*, a typical example of the response observed for GR neurones from obese Zucker rats. In the absence of extracellular glucose there is a high level of channel activity ($P_{open} = 0{\cdot}62$). However, on application of 10 mM extracellular glucose no significant inhibition of channel activity occurred. In this and subsequent figures, the C on the right-hand side indicates the closed state.

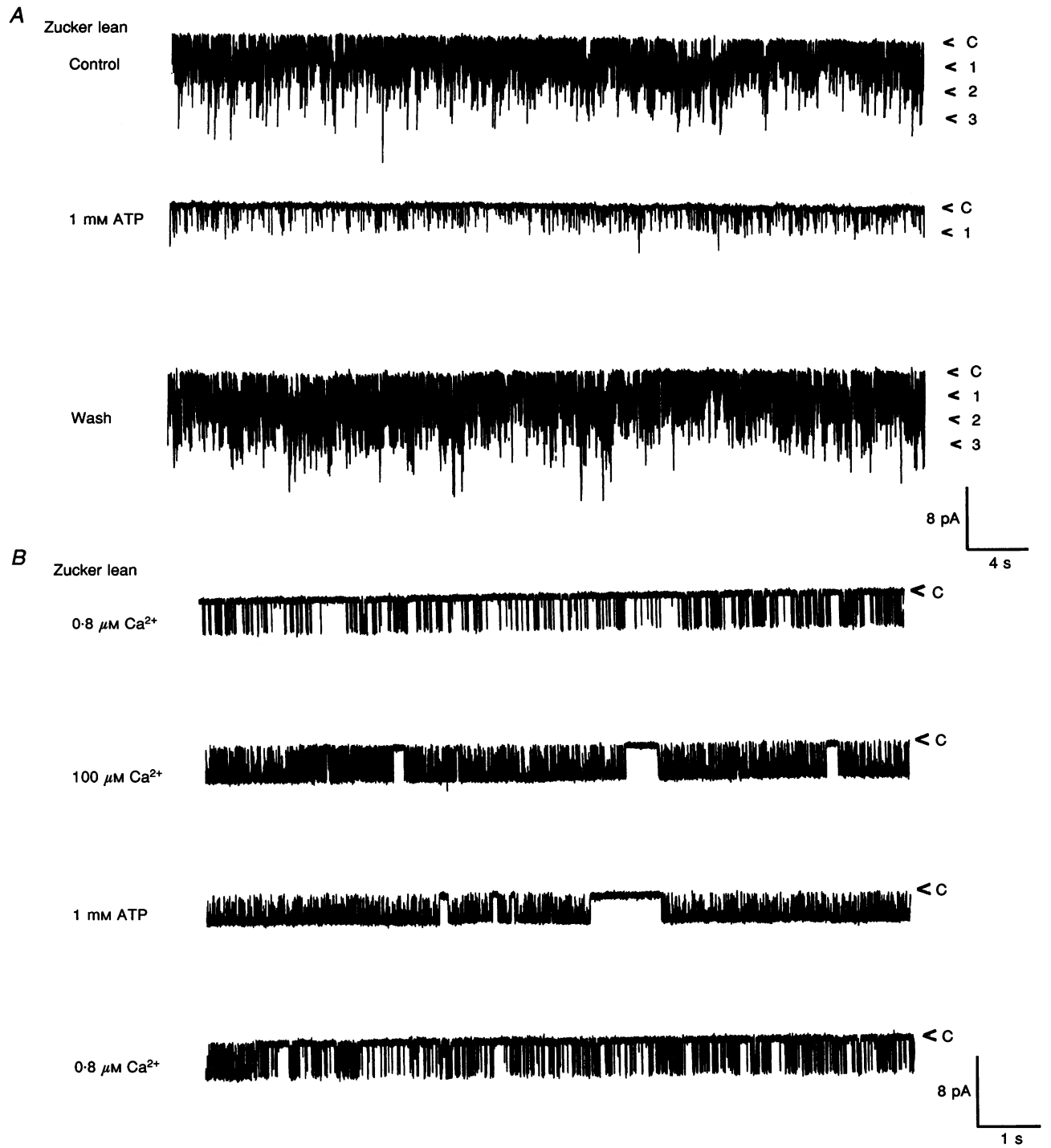

Figure 3. GR neurones of lean Zucker rats have K_{ATP} and K_{Ca} channels

Inside-out single channel current recordings of the 150 pS K_{ATP} channel (*A*) and the 160 pS K_{Ca} channel (*B*) from GR neurones obtained from lean Zucker rats. The recordings were made in symmetrical 140 mM KCl at a membrane potential of −30 mV. *A*, K_{ATP} channels were initially very active ($N_fP_o = 0{\cdot}89$; upper trace) although following application of 1 mM ATP to the bathing solution, channel activity was reduced markedly ($N_fP_o = 0{\cdot}02$; middle trace). Channel activity returned to its previously high level when ATP was removed upon washout ($N_fP_o = 1{\cdot}13$; lower trace). *B*, the upper trace shows channel activity in the presence of 0·8 μM free Ca^{2+} ($P_{open} = 0{\cdot}18$). Channel activity increased on addition of 100 μM Ca^{2+} ($P_{open} = 0{\cdot}61$; second trace). The addition of 1 mM ATP in the presence of 100 μM Ca^{2+} did not alter P_{open} (0·64; third trace). The Ca^{2+} sensitivity of the channel was unchanged following addition of ATP. The reintroduction of 0·8 μM Ca^{2+} reduced P_{open} again (0·24; bottom trace).

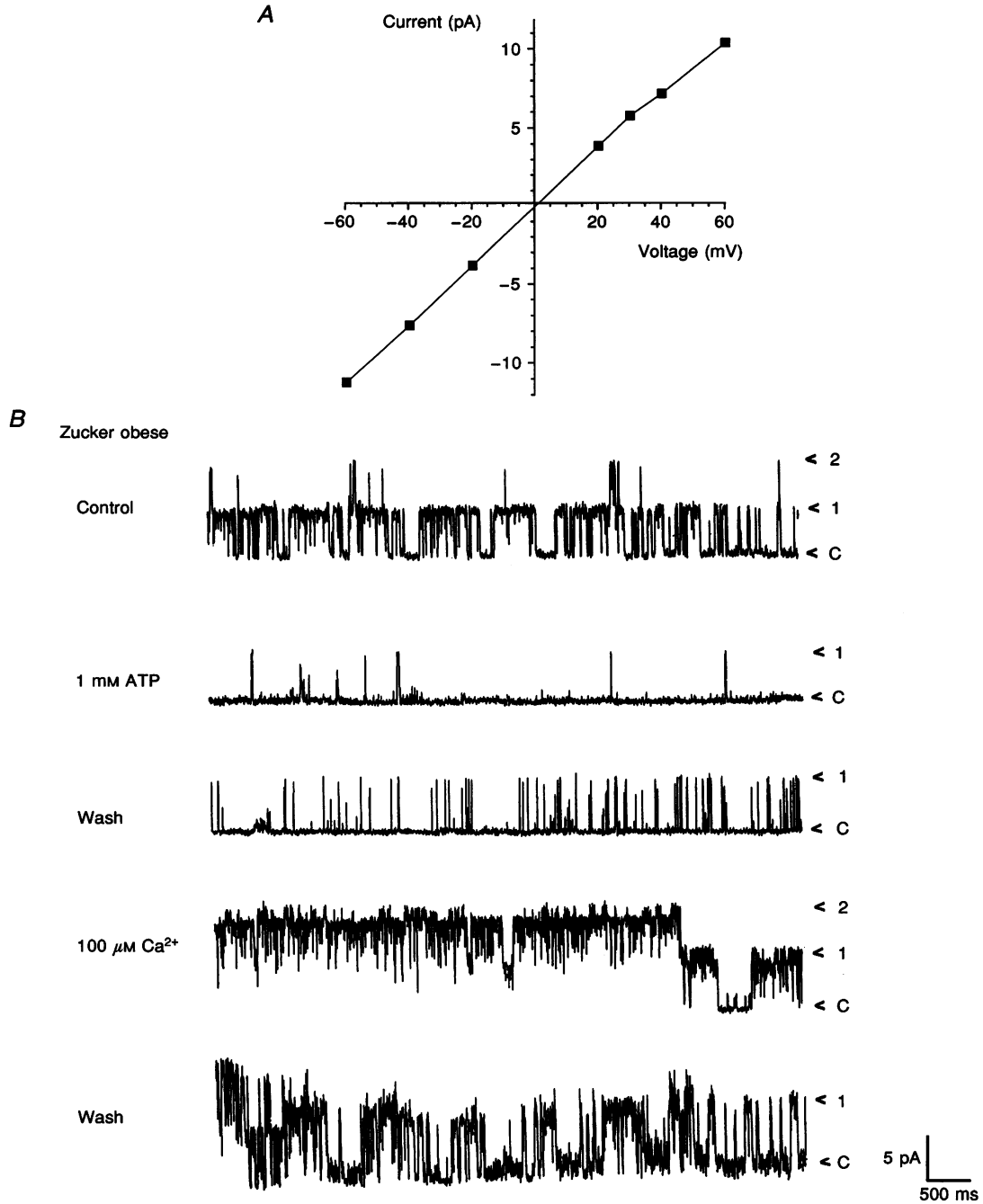

Figure 4. GR neurones of obese Zucker rats contain a novel K^+ channel

A, single channel current–voltage relationship for the K_{fa} channel recorded from an inside-out membrane patch excised from an obese Zucker rat GR neurone in symmetrical 140 mM KCl with a free calcium concentration of 100 μM in the bath solution. The conductance of this channel determined from the slope of the line fitted to the data by linear regression was 196 pS. *B*, single channel activity obtained from an inside-out patch from an obese Zucker rat GR neurone in symmetrical 140 mM KCl. The membrane potential was +30 mV in all traces. The upper trace shows channel activity recorded in the presence of 0·8 μM free Ca^{2+} ($P_{open} = 0·39$). The application of 1 mM ATP (second trace) produced a significant reduction in channel activity ($P_{open} = 0·01$). This inhibition was not reversed (third trace) when the ATP was washed out ($P_{open} = 0·08$). However, inhibition was reversed by the addition (fourth trace) of 100 μM free Ca^{2+} to the bath ($P_{open} = 0·74$) and when the Ca^{2+} concentration was finally returned to 0·8 μM (bottom trace), channel activity reverted to control levels ($P_{open} = 0·38$). Note that following the increase in Ca^{2+} concentration in the fourth trace a second type of channel characterized by small conductance (30–40 pS) and short openings was observed. The activity of this channel was not reduced on reduction of calcium to 0·8 μM (bottom trace).

was characterized by a high open-state probability in the absence of extracellular glucose, as seen in lean and control animals, but in contrast, channel activity was not significantly reduced ($n = 3$) on application of up to 10 mM extracellular glucose (Fig. 2*C*). Subsequent analysis of the single channel currents recorded from inside-out patches did not match any of the K^+ channels seen in GR or non-GR neurones in lean Zucker and control rats. This K^+ channel had an almost linear current–voltage relation (Fig. 4*A*) with a mean single channel conductance of 213 ± 15 pS ($n = 5$) in symmetrical KCl. The channel P_{open} increased upon depolarization or when the intracellular Ca^{2+} ion concentration was raised (e.g. from 0·8 to 100 μM). The voltage dependence of channel P_{open} was similar to that observed for other K_{Ca} channels, but P_{open} was less Ca^{2+} sensitive with $V_{0.5}$ (the potential at which P_{open} was 0·5) values of +30 mV and −8 mV in 0·8 μM and 100 μM Ca^{2+}, respectively. In addition, channel P_{open} was reduced markedly by the application of 1 mM ATP (Fig. 4*B*) to the inner surface of the membrane, by $77{\cdot}0 \pm 14{\cdot}0\%$ ($n = 5$). This inhibition was difficult to reverse even following prolonged washout. However raising the Ca^{2+} ion concentration from 0·8 to 100 μM in the bath solution could overcome the ATP inhibition (Fig. 4*B*).

These biophysical features were particular to this channel and have not been observed in K_{ATP} or K_{Ca} channels obtained from GR neurones of lean Zucker and control rats. Thus far we have not observed any 150 pS K_{ATP} channels in

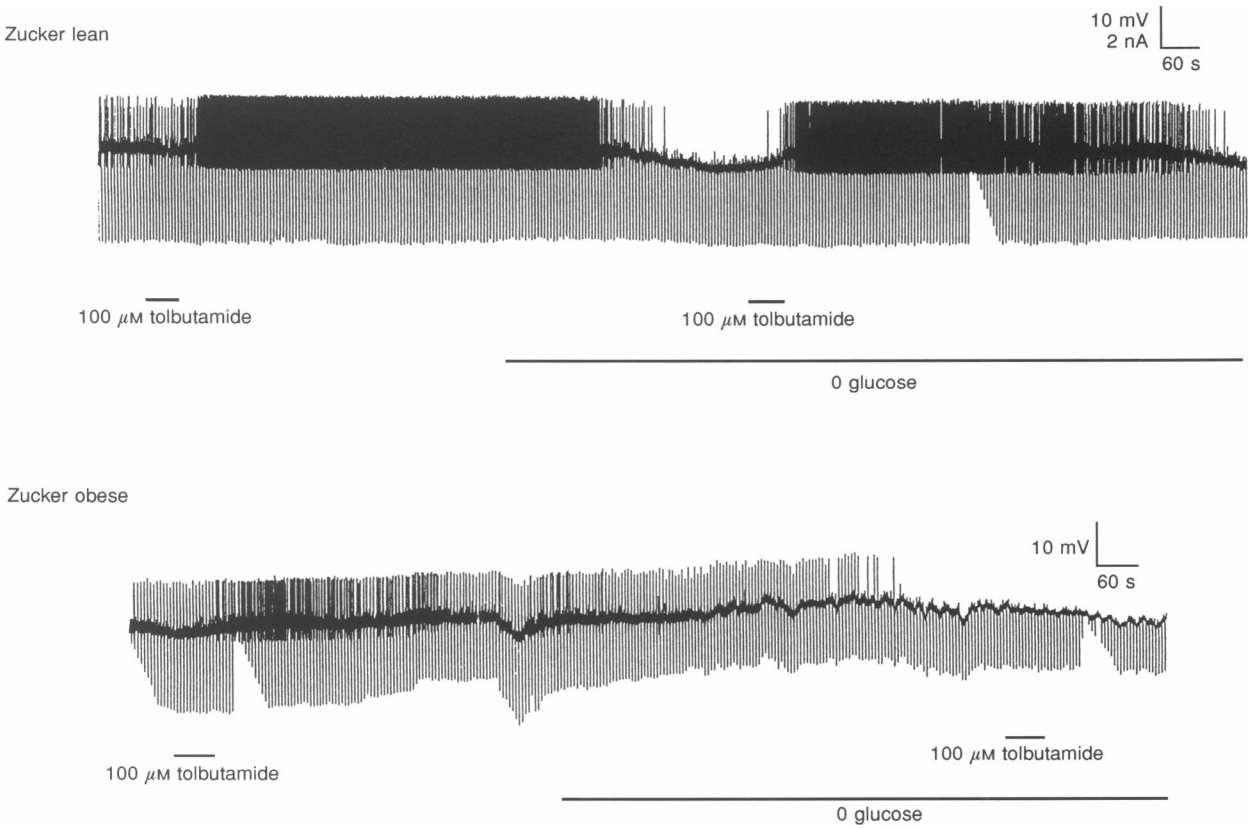

Figure 5. Differential sensitivity of control and obese Zucker GR neurones to tolbutamide
Intracellular recordings, made under current clamp conditions, from single GR neurones in a lean Zucker (upper trace) and obese Zucker (lower trace) rat hypothalamic slice. In both recordings the ACSF initially contained 10 mM glucose. Application of tolbutamide (100 μM) for 1 min caused a long-lasting depolarization and increase in the frequency of action potentials in the lean Zucker GR neurone recording (upper trace) but induced a much-diminished and briefer response in the obese Zucker GR neurone recording (lower trace). Nine minutes after tolbutamide application and washout, removal of extracellular glucose resulted in membrane hyperpolarization accompanied by a fall in apparent input resistance in the lean Zucker GR neurone (upper trace) but a much slower and less marked response from the obese Zucker GR neurone (lower trace). The response of the obese Zucker GR neurone to the application of 0 glucose was similar to that shown in Fig. 1*C*, with oscillations in membrane potential evident and no recovery following reapplication of 10 mM glucose (not shown). Reapplication of tolbutamide (100 μM) during the period of membrane hyperpolarization caused a clear excitatory response in the lean Zucker GR neurone but had no effect on the obese Zucker GR neurone. The action of tolbutamide was shown to be recoverable following washout and reapplication of a glucose-free ACSF in the lean Zucker GR neurone (upper trace).

dissociated GR neurones obtained from obese Zucker rats ($n = 18$ patches from 13 rats) though on one occasion a 160 pS K_{Ca} channel similar to that of GR neurones was observed. A further difference between GR neurones of obese Zucker and lean Zucker or control rats is their sensitivity to the sulphonylurea, tolbutamide (Fig. 5). Intracellular recordings from obese Zucker GR neurones demonstrate that in the presence of 10 mM glucose, tolbutamide (100 μM) induced a mild increase in action potential frequency in three out of four neurones tested and, following removal of glucose, had no effect in five out of seven experiments; in the other two it induced only a small depolarization. This contrasts markedly with the clear excitatory actions of this concentration of tolbutamide on control and lean Zucker GR neurones (Fig. 5 and Ashford *et al.* 1990*b*).

Patch-clamp recordings from isolated VMHN non-GR neurones

Cell-attached recordings from other VMHN neurones isolated from obese, lean or control animals typically displayed continuous firing of action potentials and occasional channel activity associated particularly with the repolarization phase of action currents (Fig. 6). These neurones were unaffected by an increase in extracellular glucose from 0 to 10 mM. Subsequent inside-out patch recordings indicated that the most common potassium channel observed was a large-conductance calcium-activated (K_{Ca}) channel. These K_{Ca} channels were characterized by a linear current–voltage relationship with a single channel conductance in symmetrical KCl of 244·3 ± 3·0 pS ($n = 10$), 244·2 ± 3·7 pS ($n = 12$) and 247·8 ± 4·1 pS ($n = 5$) for lean, obese

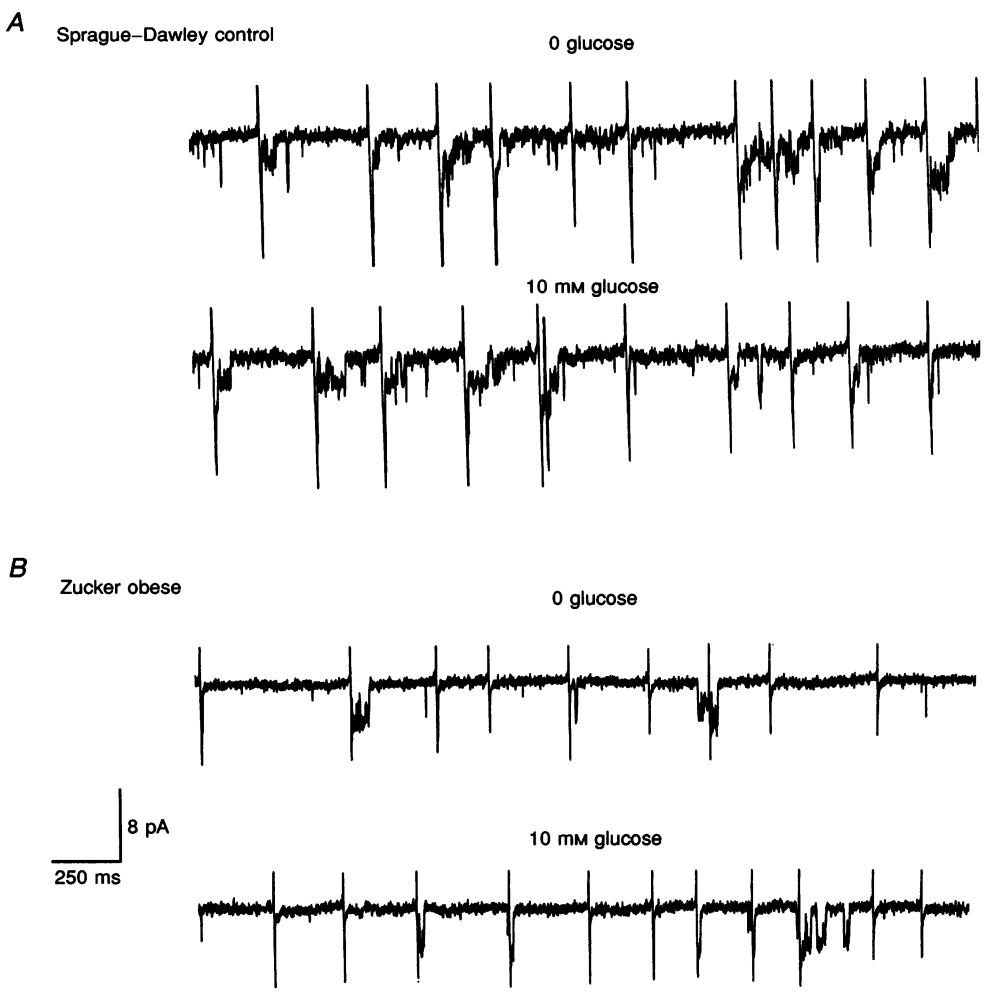

Figure 6. Cell-attached recording from non-GR neurones from obese and control rats

Cell-attached recordings from a non-GR neurone of a control rat (*A*) and obese Zucker rat (*B*). The cells were bathed in normal saline, the patch pipette contained 140 mM KCl and the membrane was held at 0 mV in both recordings. The upper traces in both *A* and *B* show the activity recorded in the absence of extracellular glucose. The neurones displayed continuous firing of action currents with single channel currents associated predominantly with the repolarization phase of the action currents. The firing frequency and characteristics of the single channel currents were unaffected by the application of 10 mM glucose to the superfusing saline solution (lower traces in *A* and *B*). Similar recordings in the presence and absence of glucose were observed in non-GR neurones of lean Zucker rats (not shown).

and control animals, respectively. The K_{Ca} channel P_{open} increased with membrane depolarization and with intracellular Ca^{2+} ion concentration (Fig. 7) and in all three groups of rats the channel displayed a similar voltage dependence (15–20 mV per e-fold change in P_{open}). The voltage dependence of the channel was not affected by the Ca^{2+} ion concentration but $V_{0.5}$ was shifted to more positive values when the Ca^{2+} ion concentration was reduced. For

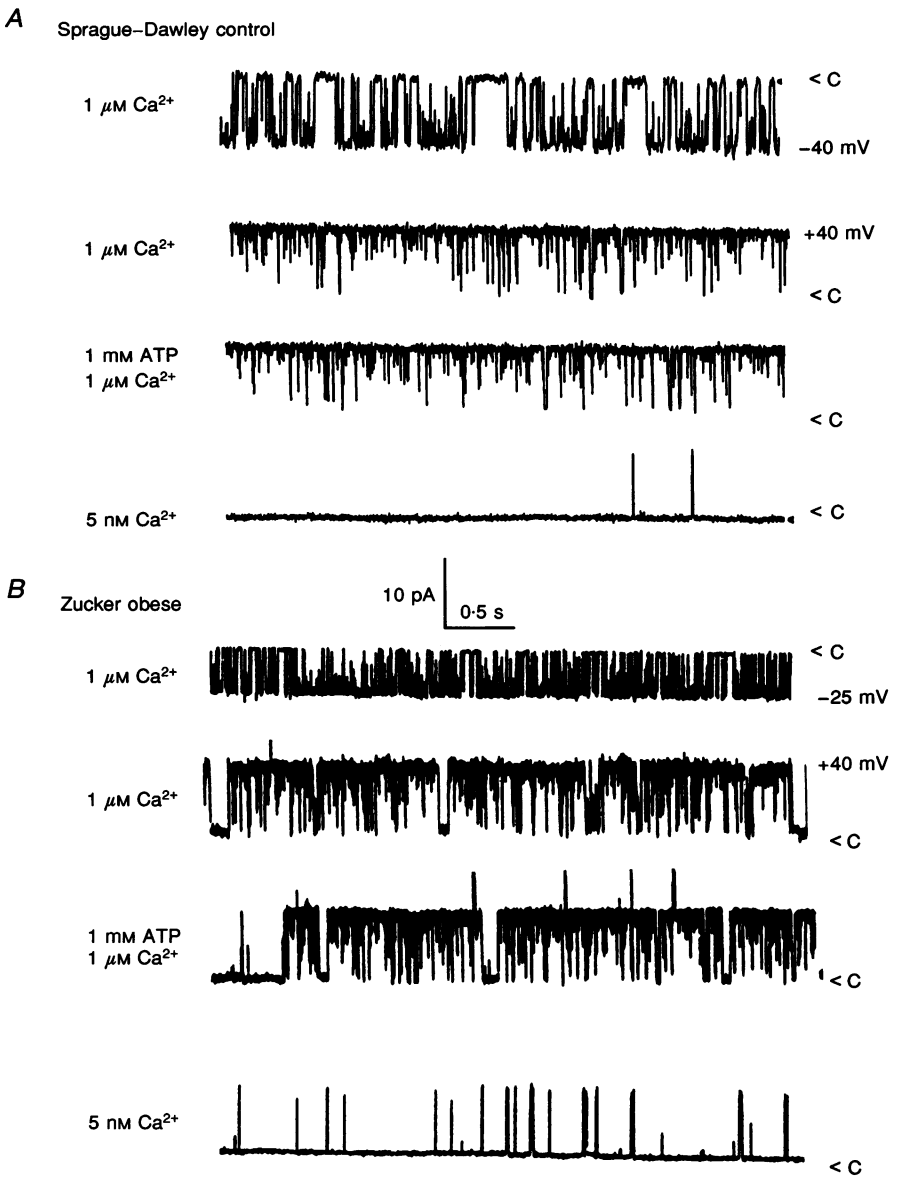

Figure 7. Non-GR neurones of control and Zucker rats contain 250 pS K_{Ca} channels

Inside-out patch recordings from dissociated non-GR neurones from control (*A*) and obese Zucker rats (*B*). Recordings were made under symmetrical 140 mM KCl solutions with a free calcium concentration of 0·8 μM in the bath solution. Under these conditions the predominant channel observed in non-GR neurones from both control (*A*) and obese (*B*) rats was a large-conductance (~250 pS) K_{Ca} channel. *A*, single channel activity was voltage dependent with $P_{open} = 0{\cdot}37$ at −40 mV (upper trace) and $P_{open} = 0{\cdot}89$ at +40 mV (upper middle trace). Addition of 1 mM ATP (lower middle trace) at a membrane potential of +40 mV and in the presence of a Ca^{2+} concentration of 0·8 μM had no effect on channel activity ($P_{open} = 0{\cdot}87$). The channel activity remained at this level after washout of the ATP (not shown). On reduction of the free Ca^{2+} concentration to approximately 5 nM (bottom trace), channel activity at +40 mV membrane potential was markedly reduced ($P_{open} = 0{\cdot}008$), demonstrating the channel to be Ca^{2+} sensitive. *B*, essentially identical data were obtained from an inside-out patch from a non-GR neurone of an obese Zucker rat. The top trace shows channel activity at a membrane potential of −25 mV ($P_{open} = 0{\cdot}46$) and the upper middle trace at a potential of +40 mV ($P_{open} = 0{\cdot}71$). Addition of 1 mM ATP (lower middle trace) at a membrane potential of +40 mV did not affect channel activity ($P_{open} = 0{\cdot}66$) although following washout of the ATP and lowering of free Ca^{2+} to approximately 5 nM (bottom trace), channel activity was severely reduced ($P_{open} = 0{\cdot}008$).

example, when the Ca^{2+} ion concentration was reduced from 100 to 0·8 μM, $V_{0.5}$ was shifted from −40 to +10 mV and from −34 to +8 mV for obese and control rat neurones, respectively. Thus the K_{Ca} channel of non-GR neurones in control and lean Zucker rats is more sensitive to Ca^{2+} than the K_{fa} channel of obese Zucker rat GR neurones. However, in contrast to a previous study (Treherne & Ashford, 1991), we found that in control animals and lean Zucker rats this K_{Ca} channel was not consistently sensitive to the application of cytoplasmic ATP. Inhibition was only observed in two out of eight patches from non-GR neurones from lean Zucker rats and three out of nine from control rats (not shown), and channel sensitivity to ATP was much less than for the K_{ATP} channel from GR neurones. The application of 1 mM ATP produced a 20% inhibition of K_{Ca} channel activity ($n = 5$; lean and control data combined) in symmetrical KCl as opposed to a 77% inhibition of the K_{ATP} channel ($n = 5$) under the same conditions.

DISCUSSION

GR neurones from lean Zucker rats exhibited values for resting membrane potential, input resistance and responses to changes in extracellular glucose concentration indistinguishable from those obtained from Wistar or Sprague–Dawley rats, in both the present (Table 1) and previous studies (Ono *et al.* 1982; Ashford *et al.* 1990*a*). In the presence of 10 mM glucose the mean resting membrane potential and input resistance values of GR neurones of obese (*fa/fa*) Zucker rats were not statistically different from the corresponding mean values recorded from GR neurones of lean and control rats (Table 1). However, on removal of extracellular glucose the GR neurones of obese Zucker rats displayed an abnormal response compared with lean Zucker and control animals. Although the magnitude of the hyperpolarization was identical to that of lean and control animals, the time course of response was clearly altered, being much slower in GR neurones of obese rats. Other differences were also apparent, the decrease in input resistance was significantly depressed, the hyperpolarization was not reversed on reapplication of 10 mM glucose and the membrane potential displayed oscillations in the absence or presence of glucose. In contrast, no differences were apparent in the steady-state electrophysiological characteristics of non-GR neurones, in the presence or absence of glucose, between the obese, lean or control groups. Thus these data strongly implicate a malfunction in the mechanism by which Zucker obese GR neurones sense glucose. Previous studies (Ashford *et al.* 1990*a*,*b*; Treherne & Ashford, 1991) and our control data in this paper indicate that GR neurones from the VMHN of Sprague–Dawley and Wistar Kyoto rats contain both 150 pS K_{ATP} and 160 pS K_{Ca} channels. In this study we show that GR neurones of lean Zucker rats have essentially identical channels. For example, in cell-attached recordings the potassium channel active in the absence of extracellular glucose is inhibited on application of glucose in an identical manner to that of GR neurones from control rats. Inside-out single channel studies show that the channel underlying this glucose sensitivity is characterized by a single channel conductance of 150 pS, is insensitive to membrane voltage and changes in intracellular calcium concentration but is inhibited by application of millimolar concentrations of cytoplasmic ATP. These data are indistinguishable from the characteristics of the 150 pS K_{ATP} channel previously described in GR neurones of control rats (Ashford *et al.* 1990*a*, *b*). Thus it is reasonable to believe that the K_{ATP} channel observed in lean Zucker rats is identical to its counterpart in Sprague–Dawley and Wistar rats, and therefore fufills the same glucose-sensing function.

The 160 pS K_{Ca} channel observed in inside-out recordings from GR neurones of lean Zucker rats was also identical to that seen in control animals and was shown to be insensitive to cytoplasmic ATP. The channel P_{open} increased upon depolarization and with the raising of intracellular Ca^{2+} ion concentration in agreement with previous observations and the suggestion that the channel contributes to both action potential repolarization and the after-hyperpolarization (Treherne & Ashford, 1991). Recordings from inside-out patches from neurones of obese and lean Zucker rats identified as non-glucose receptive in cell-attached experiments indicate that the predominant K^+ channel observed was a 250 pS K_{Ca} channel. The channel is voltage- and Ca^{2+}-sensitive and is similar to the K_{Ca} channel described in an earlier study of potassium channel activity in VMHN neurones of control rats (Treherne & Ashford, 1991). Therefore we conclude that non-GR neurones from the VMHN of obese and lean Zucker rats and control rats contain essentially identical 250 pS K_{Ca} channels. The ATP sensitivity observed in a proportion of recordings may be due the presence of a protein kinase associated with the channel protein, able to modulate channel activity by phosphorylation in a manner similar to that reported for rat brain K_{Ca} channels (Chung, Reinhart, Martin, Brautigan & Levitan, 1991; Reinhart, Chung, Martin, Brautigan & Levitan, 1991; Lee, Rowe & Ashford, 1995). The lack of ATP sensitivity displayed by most of the K_{Ca} channels may simply be due to the absence or inhibition of the appropriate kinase in the majority of excised patches. The functional significance of this modulation is unclear but requires further investigation.

Cell-attached recordings from GR neurones of obese rats clearly show that their response to the addition of glucose following a period in glucose-free saline is abnormal compared with that of the GR neurones of lean and control animals and supports the data obtained from intracellular recordings. The most striking feature observed in inside-out patches from GR neurones of obese Zucker rats is the absence of normal K_{ATP} (150 pS) and K_{Ca} (160 pS) channels. Instead a K^+ channel is observed with mean conductance (213 pS) and voltage sensitivity closer to that of the 250 pS K_{Ca} channel seen in non-GR neurones, but with ATP sensitivity similar to the 150 pS K_{ATP} channel recorded

from normal GR neurones. The Ca^{2+} sensitivity of this channel also differed from that observed for K_{Ca} channels of non-GR neurones. It is unlikely that this K^+ channel, which we have called K_{fa}, was recorded from non-GR neurones as we made strenuous efforts to identify GR and non-GR neurones of obese rats and recordings from the non-GR neurones revealed the presence of a 250 pS K_{Ca} channel, which was identical to the large K_{Ca} channel recorded from non-GR neurones of lean Zucker and control rats. Therefore we suggest that the K_{fa} channel is specific to GR neurones within the VMHN of obese animals and that non-GR neurones are unaffected and can be considered normal in relation to their counterparts in lean and control animals.

This study provides further strong evidence for the notion that a K_{ATP} channel mediates glucose sensing of hypothalamic GR neurones. In obese Zucker rats the GR neurones displayed abnormal responses to changes in extracellular glucose concentration and when their K^+ channel activity was examined, normal K_{ATP} activity was absent. In lean Zucker rats and control animals the K_{ATP} channel was present and the GR neurones displayed normal responses to changes in extracellular glucose concentration. Therefore intact 150 pS K_{ATP} channels are likely to be required for normal glucose sensing in GR neurones. The results obtained from inside-out patch recordings from GR neurones of obese animals may help to explain the abnormal responses of these neurones to glucose in intact cells. For example, the lack of immediate reversibility of ATP action on channel activity may contribute to the slow hyperpolarization of GR neurones in brain slice recordings in response to removal of glucose. In addition the lack of response of GR neurones from obese Zucker rats to readministration of glucose after glucose-induced hyperpolarization in intracellular recordings was also matched by the high level of K^+ channel activity observed in cell-attached patches from isolated GR neurones in the absence and presence of 10 mM glucose. This latter observation and the oscillation of membrane potential seen in intracellular recordings from these cells may be associated with the K_{fa} channel sensitivity to both ATP and calcium. It is also feasible that the K_{fa} channel is under the influence of other intracellular signals which do not normally mediate in the glucose response involving K_{ATP} channels. Clearly the regulation of the K_{fa} channel requires further investigation.

The conserved synteny between the *fa* and *db* genes in terms of chromosomal location suggests that the phenotype of the resultant mutation should be identical. The mouse *db/db* mutation results in aberrant splicing of the leptin receptor splice variant (Ob-Rb) which is highly expressed in the hypothalamus, resulting in a truncated form with most of the cytoplasmic C-terminal region missing (Lee *et al.* 1995). Recent studies (Phillips *et al.* 1996) indicate that the rat *fa/fa* obese phenotype is also associated with a mutation in the Ob-Rb isoform of the leptin receptor in the hypothalamus. No aberrant splicing of the transcript occurs but instead there is a mis-sense mutation of the *fa* gene resulting in a Gln269Pro substitution. It has been suggested that the *db* mutation may give rise to leptin receptors defective in signal transduction (Lee *et al.* 1996) and the *fa* mutation to receptors perhaps unable to undergo dimerization (Phillips *et al.* 1996). We do not yet know whether the changes we see in the potassium channels of the GR neurones are directly causally linked to the *fa* gene mutation. However one interpretation of the data is that the expression of the *fa* gene (i.e. a malfunctioning leptin receptor, Ob-Rb, in the hypothalamus) leads in some, as yet undefined, way to altered expression or combination of certain potassium channel subunits or to a change in the functional control of homo/heteromeric potassium channel complexes (e.g. through phosphorylation or association/dissociation with regulatory subunits) in hypothalamic GR neurones. Clearly, further experiments are required to examine the potassium channels of hypothalamic GR neurones of other animal models of genetic obesity, in particular the *db/db* and *ob/ob* mouse strains. Thus alterations to potassium channel characteristics in GR neurones associated with genetic obesity may present a novel potential therapeutic site for pharmacological intervention.

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