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Glucose effects on gastric motility and tone evoked from the rat dorsal vagal complex

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1. To examine the effects of glucose on the central components of the vago-vagal reflex control of gastric function, we performed both *in vivo* and *in vitro* experiments on neurones in the medial nucleus of the tractus solitarius (mNTS) and in the dorsal motor nucleus of the vagus (DMV).
2. In the *in vivo* anaesthetized rat preparation, unilateral microinjection of D-glucose (10 or 50 mM (60 nl)$^{-1}$) in mNTS produced inhibition of gastric motility and an increase in intragastric pressure. D-glucose had no effect in the DMV.
3. In the *in vitro* rat brainstem slice preparation, whole-cell recordings of DMV neurones showed that increasing the glucose concentration of the perfusion solution from 5 mM to 15 or 30 mM produced outward currents of 35 ± 5 pA ($n = 7$) and 51 ± 10 pA ($n = 11$), respectively. These were blocked by tetrodotoxin and picrotoxin, indicating that glucose was acting indirectly to cause the release of GABA. Decreasing the glucose concentration of the perfusing solution by one-half produced an inward current of 36 ± 5 pA ($n = 7$).
4. Stimulation of the NTS evoked inhibitory postsynaptic currents (IPSCs) in DMV neurones. The amplitude of the evoked IPSCs was positively correlated with glucose concentration. Perfusion with the ATP-sensitive K^+ (K_{ATP}) channel opener diazoxide mimicked the effect of reduced glucose, while perfusion with the K_{ATP} channel blocker glibenclamide mimicked the effects of increased glucose.
5. Our data indicate that glucose had no direct excitatory effect on DMV neurones, but DMV neurones appear to be affected by an action of glucose on cell bodies of mNTS neurones via effects on an ATP-sensitive potassium channel.

Diabetes mellitus has been reported to be associated with a significant incidence of gastrointestinal (GI) tract symptoms, including constipation, nausea, abdominal pain and diarrhoea (Enck *et al.* 1994). Studies carried out in tertiary care centres have shown GI symptoms in 20–60 % of diabetic patients (Feldman & Schiller, 1983; Clouse & Lustman, 1989), though the prevalence of GI symptoms in diabetic patients selected from the general population is much less common (Janatuinen *et al.* 1993; Malecki *et al.* 2000). However, data from several studies show a weak but significant correlation between GI tract symptoms and delayed gastric emptying in diabetic patients (Horowitz *et al.* 1986, 1991; Wegener *et al.* 1990). Although the cause of delayed gastric emptying is not completely understood, Yamano & colleagues (1997) surmised that this may be caused by changes in blood glucose levels, in addition to the autonomic neuropathic changes known to occur with long-standing diabetes mellitus (Sampson *et al.* 1990; Vinik & Suwanwalaikorn, 1997). Indeed, there is evidence from both human and animal studies indicating that blood glucose level can act as a modulator of gastric motility (Sakaguchi & Shimojo, 1984; Barnett & Owyang, 1988; Bjornsson *et al.* 1994).

Animal studies to date have implicated vago-vagal reflexes in the action of glucose to alter gastric function (Sakaguchi & Shimojo, 1984; Sakaguchi *et al.* 1994). These reflexes consist of three components, the first of which is a sensory limb comprising chemosensory and mechano-sensory elements linked to vagal afferent nerves (Rogers *et al.* 1995). Data received by these sensory elements are funnelled via a glutamatergic synapse into the brainstem at the level of the nucleus of the tractus solitarius (NTS) (Rogers *et al.* 1995; Sykes *et al.* 1997). Many of the peripheral vagal afferents that synapse in the NTS do so at the level of the medial subnucleus of the tractus solitarius (mNTS) (Altschuler *et al.* 1989). The NTS, in turn, sends projections to the efferent vagal neurones in the dorsal motor nucleus of the vagus (DMV), which

project to the parasympathetic ganglia and the enteric ganglia innervating the digestive tract (Rogers *et al.* 1995). Most of the projections from the NTS to the DMV appear to be inhibitory (McCann & Rogers, 1994) and, although the neurotransmitter released is unknown, indirect evidence suggests that it is GABA (Feng *et al.* 1990; Travagli *et al.* 1991; Washaban *et al.* 1995; Sivarao *et al.* 1998; Browning & Travagli, 1999).

Glucose exerts pronounced effects both on vagal sensory nerves and on central components of the reflexes. The hepatic portal area appears to have glucose sensors linked to hepatic vagal afferent nerves (Sakaguchi & Shimojo, 1984; Sakaguchi *et al.* 1994). In fact, glucose administered into the hepatic portal vein has been reported to decrease hepatic vagal afferent discharge rate (Niijima, 1969; Niijima & Mequid, 1994). Neurones in both NTS and DMV have also been shown to be affected by glucose. Glucose injected into the DMV of anaesthetized rats has been shown to decrease gastric motility and intragastric pressure (Sakaguchi *et al.* 1985, 1994). Conversely, gastric motility or pressure did not seem to be affected when glucose was injected into the NTS, although additional studies indicated that glucose injected into the NTS could reduce gastric acid secretion (Sakaguchi & Sato, 1987).

Electrophysiological studies of these two brainstem nuclei indicate the presence of both glucoresponsive (i.e. gluco-excitatory) and glucosensitive (i.e. gluco-inhibitory) neurones (Mizuno & Oomura, 1984; Adachi *et al.* 1995), and, in the case of the NTS, the majority of the gluco-responsive neurones were shown to be linked to the K_{ATP} channel (Dallaporta *et al.* 2000).

The purpose of the present study was to evaluate the potential role of each of these brainstem nuclei in the effects of glucose to modulate vagal control of gastric motility and tone. This was accomplished using two complementary approaches, namely, microinjection of glucose into the mNTS and the DMV of the anaesthetized rat while monitoring intragastric pressure and motility, and patch-clamp analysis of electrical activity of DMV neurones exposed to different concentrations of glucose. A preliminary account of this paper has been presented previously (29th Annual Meeting of the Society for Neuroscience, 1999).

METHODS

In vivo rat studies

Animal preparation. Experiments were performed on male Sprague-Dawley rats ($n = 59$) weighing 250–350 g (Taconic, Germantown, NY, USA). Prior to each experiment, food was withheld overnight but water was provided *ad libitum.* Animals were anaesthetized with an intraperitoneal injection of a cocktail (3 ml kg^{-1}) containing urethane (800 mg kg^{-1}) and α-chloralose (60 mg kg^{-1}) dissolved in 3 ml of 0.9% saline. Body temperature was monitored by a rectal thermometer and maintained at 37 $\pm$ 1 °C with an infrared heating lamp. Before surgery, all animals were pretreated with dexamethasone (0.8 mg, subcutaneously) to minimize brain swelling. Animal care and experimental procedures were performed in accordance with the NIH guidelines and with the approval of the Animal Care and Utilization Committee of Georgetown University, Washington, DC, USA.

Surgery. Rats were intubated to maintain an open airway and for instituting artificial respiration when necessary. The carotid artery was cannulated with polyethylene tubing (PE 50) to monitor blood pressure. Blood pressure was recorded using a bridge amplifier connected to a MacLab (ADInstruments, Milford, MA, USA) data acquisition system. Data were stored on computer (Apple Macintosh G3 connected to MacLab) for analysis at a later time. In some animals, ligatures were placed around the vagus nerves and were tied, to be avulsed at a later time. For each experiment an intra-gastric balloon, made from the little finger of a small latex glove, was tied around polyethylene tubing (PE 160) and was inserted into the stomach via the fundus. The tubing was connected to a pressure transducer, which was connected to a bridge amplifier (MacLab). Data were saved for analysis at a later time. The stomach was inflated by introducing warm saline (2–3 ml) into the balloon, to achieve a baseline pressure of 6–15 mmHg. The animals were then positioned in a stereotaxic apparatus (David Kopf, Tujunga, CA, USA). A partial dorsal craniotomy was performed to expose the fourth ventricle. The cerebellum was retracted slightly while using a 26 gauge needle to cut the subarachnoid covering. Calamus scriptorius (cs) was viewed from the dorsal aspect and used as a point of reference (see below).

Microinjection technique. D-Glucose was dissolved in 0.9% saline. A histological marker (Fast Green dye) for locating injection sites was added to drug solutions in a 1–2 mg ml^{-1} concentration. The pH of all drug solutions was brought to 7.0–7.2. Double-barrelled pipettes with a tip diameter of between 30 and 60 μm were used. All micro-injections were given unilaterally. Injections were given in volumes of 60 nl and administered by hand-controlled pressure. Micro-injections were given within 5 s. Calamus scriptorius was used as a zero reference point. Stereotaxic co-ordinates were originally chosen based on histology in Paxinos & Watson (1986). Co-ordinates for the DMV ranged from 0.3–0.5 mm rostral to cs, medial–lateral 0.3–0.5 mm lateral from the midline, and dorsal–ventral 0.5–0.7 mm from the dorsal surface of the medulla. Co-ordinates for the mNTS ranged from 0.3–0.5 mm rostral to cs, 0.5–0.7 mm lateral from the midline, and from 0.4–0.6 mm from the dorsal surface of the medulla. These co-ordinates were chosen based on co-ordinates developed in our earlier published study of nicotine (Ferreira *et al.* 2000).

Histologic verification. At the end of the experiment all rats were killed with an overdose of pentobarbital. Brains were removed and fixed in a mixture of 4% paraformaldehyde and 20% sucrose for at least 24 h. The brain was cut into 50 μm thick coronal sections and stained with Neutral Red. The location of nuclear groups was studied in relation to microinjection sites using the atlas of Paxinos & Watson (1986).

Data analysis. Data were analysed using the Chart Software for data analysis made for MacLab (ADInstruments). Before micro-injections were performed, the lowest points of the intragastric pressure (IGP) trace were obtained over a 5 min control period, and a single value was calculated as the mean of all of these points and used as an index of gastric tone. After microinjections into the mNTS, the maximum value in the trace was taken as the largest increase in gastric tone. The percentage change from baseline in IGP was then calculated. Data for IGP are reported as percentage change from baseline since baseline IGP varied between animals. It should be noted that all data that are shown to be statistically significant are significant when analysed both as raw data and as percentage change from baseline. Data appear as means (percentage change from baseline for IGP) $\pm$S.E.M. To calculate the area under the curve for the

phasic contractions appearing in the IGP traces, the MacLab data analysis package was utilized. The program calculates the area under the curve as an integral that is represented by:

$$\sum(y - y\text{Baseline}(t)) \times \Delta t,$$

where $y - y$Baseline denotes the first and last data point in the selection and Δt is the sample interval (5 min in our study). Student's paired t tests were performed in all cases as animals served as their own controls. Differences were considered significant at $P < 0.05$.

In vitro brain slice studies

Gastric-projecting DMV neurones were labelled as described previously (Browning *et al.* 1999). Briefly, 12-day-old rat pups of either sex were anaesthetized deeply (indicated by abolition of the foot-pinch withdrawal reflex) with a 6% solution of 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane) with air (400–600 ml min^{-1}) before an abdominal laparotomy was performed. During surgery anaesthesia was maintained by placing the head of the rat in a custom-made anaesthetic chamber through which the halothane mixture was perfused. Crystals of the retrograde tracer DiI were applied to the serosal surface of the gastric fundus, corpus or the antrum/pylorus. The application site was embedded in a fast-hardening epoxy resin that was allowed to dry for several minutes before the entire surgical area was washed with warm saline. The wound was closed with 5/0 silk suture and the animal allowed to recover for 10–15 days.

The brainstems were removed as described previously (Travagli *et al.* 1991; Browning *et al.* 1999). Briefly, the rats were placed in an anaesthetic chamber and anaesthetized with halothane before being killed by severing the major blood vessels in the chest. The brainstem was removed and placed in oxygenated Krebs solution at 4 °C (see below for composition). Using a vibratome, six to eight coronal sections (200 μm thick) containing the dorsal vagal complex were cut and stored in oxygenated Krebs solution at 30 °C for at least 1 h prior to use. A single slice was transferred to a custom-made perfusion chamber (volume 500 μl) and kept in place using a nylon mesh. The chamber was maintained at 35 °C by perfusion with warmed, oxygenated Krebs solution at a rate of 2.5–3.0 ml min^{-1}.

Prior to electrophysiological recording, gastric-projecting DMV neurones were identified using a Nikon E600-FS microscope equipped with epifluorescence filters suitable for visualizing DiI. Once the identity of a labelled neurone was confirmed, whole-cell recordings were made under brightfield illumination.

Electrophysiological recording. Whole-cell recordings were made with patch pipettes (3–8 MΩ resistance) filled with a potassium gluconate solution (see below for composition) using an Axopatch ID single electrode voltage-clamp amplifier (Axon Instruments, Foster City, CA, USA). Perforated-patch recordings were made using pipettes (3–8 MΩ resistance) filled with a potassium gluconate solution containing gramicidin (see below for composition and Spruston & Johnston (1992) for detailed technique).

Recordings were made from neurones unequivocally labelled with DiI. Data were filtered at 2 kHz, digitized via a Digidata 1200C interface (Axon Instruments), acquired, stored and analysed on an IBM PC utilizing pCLAMP 8 software. Recordings were accepted only if the series resistance (i.e. pipette + access resistance) was < 15 MΩ. In addition, the neuronal membrane had to be stable at the holding potential, the action potential evoked following injection of depolarizing current had to have an amplitude of at least 60 mV and the membrane potential had to return to the baseline value following the action potential after-hyperpolarization.

Electrical stimulation. Bipolar tungsten electrodes were used to electrically stimulate the centralis and medialis subnuclei of the NTS. Paired stimuli (0.1–1.0 ms, 10–500 μA; 50–300 ms interval) were applied every 20 s to evoke submaximal excitatory (EPSCs) or inhibitory (IPSCs) postsynaptic currents.

All experiments involving evoked IPSCs were carried out in the presence of 1 mM kynurenic acid (to eliminate spontaneous and evoked EPSCs) and with 0.5 mM QX314 in the internal pipette solution (to eliminate antidromically evoked action potentials). QX314, 0.5 mM, was also used in the internal pipette in experiments involving evoked EPSCs. A minimum of six control EPSCs or IPSCs were obtained prior to each drug application. Drugs were applied to the bath via a series of manually operated valves. To assess the effects of drugs, each neurone served as its own control (i.e. the results obtained after administration of a drug were compared to those before administration using Student's paired t test). Results are expressed as means ± S.E.M. Significance was set at $P < 0.05$.

Drugs and solutions. Materials used for the *in vivo* rat studies were as follows: D-glucose, L-glucose, urethane, α-chloralose, Fast Green dye and dexamethasone. All were purchased from Sigma Chemical Co. (St Louis, MO, USA) except dexamethasone which was purchased from Elkins-Sinn (Cherry Hill, NJ, USA).

Materials used for the *in vitro* studies were as follows: extracellular solution materials (Krebs solution) (mM): NaCl, 126; $NaHCO_3$, 25; KCl, 2.5; $MgCl_2$, 1.2; $CaCl_2$, 2.4; NaH_2PO_4, 1.2; and glucose, 5; maintained at pH 7.4 by bubbling with 95% O_2–5% CO_2. Equi-molar Krebs solutions were prepared by either substituting sodium chloride for glucose (as in the case of 2.5 mM solution) or vice versa (as in the case of 11, 15 and 30 mM solutions).

Intracellular solution materials (mM): potassium gluconate, 128; KCl, 10; $CaCl_2$, 0.3; $MgCl_2$, 1; Hepes, 1; EGTA, 1; ATP, 2; and GTP, 0.25; adjusted to pH 7.35 with KOH.

Intracellular solution materials for perforated patch (mM): potassium gluconate, 128; KCl, 10; $CaCl_2$, 0.3; $MgCl_2$, 1; Hepes, 1; and EGTA, 1; with 100 μg ml^{-1} gramicidin, adjusted to pH 7.35 with KOH.

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($\text{DiIC}_{18}(3)$; DiI) was purchased from Molecular Probes (Eugene, OR, USA). 2-Bromo-2-chloro-1,1,1-trifluoroethane (Halothane) and all other materials listed for the composition of the solutions were purchased from Sigma Chemical Co.

RESULTS

In vivo studies

Gastric motility was decreased and intragastric pressure was increased by microinjection of D-glucose in the mNTS but not in the DMV

Unilateral microinjections of D-glucose (60 nl of a 10 mM solution) into the DMV were performed in eight anaesthetized rats while monitoring intragastric pressure and motility. We did not observe any effect on either intragastric pressure or gastric motility in any of the experiments (Figs 1 and 2). In three experiments, D-glucose (60 nl of a 50 mM solution) was tested by unilateral microinjection into the DMV. Again, no effect of D-glucose on gastric function was observed (Fig. 2). Finally, to provide a more sensitive endpoint (i.e. a higher level of baseline gastric motility) for detecting an inhibitory effect of D-glucose microinjected into the DMV, we administered insulin (5 units) subcutaneously in three animals prior to unilateral microinjection of 60 nl of

a 10 mM solution of D-glucose into the DMV. Despite the increase in gastric motility produced by insulin-induced hypoglycaemia, unilateral microinjection of D-glucose into the DMV had no effect on gastric motility (data not shown). The sites in the dorsomedial medulla where D-glucose was microinjected in the experiments described above are depicted in Fig. 3. As can be noted, all sites were located within the confines of the DMV.

Contrary to the lack of effect of microinjections of D-glucose into the DMV, unilateral microinjection of 60 nl of a 10 mM solution of D-glucose into the mNTS produced inhibition of gastric motility with an immediate onset, but a relatively slow developing increase in intragastric pressure ($n = 5$; Figs 2 and 4). These effects dissipated over a period of 15–30 min but could be reproduced in the same experiment by repeated microinjection of 60 nl of a 10 mM D-glucose solution (data not shown). When 60 nl of a 50 mM solution of D-glucose was microinjected unilaterally into the mNTS ($n = 5$), the effect on motility and intragastric pressure appeared to be intensified (Fig. 2). Three types of control experiments were performed. First, D-glucose (60 nl of a 10 mM solution) was microinjected into sites outside the mNTS (Fig. 3). These microinjections did not produce an effect on motility or on intragastric pressure (data not shown). The other two types of controls were microinjection of either 60 nl of 0.9% saline solution or 60 nl of a 10 mM L-glucose solution into the mNTS or DMV (Fig. 3). When 10 mM L-glucose was microinjected into the mNTS, a $2.7 \pm 1.5\%$ change in intragastric pressure ($n = 5$; $P > 0.05$) and a -52 ± 49 mmHg s^{-1} change in the area under the curve ($n = 4$; $P > 0.05$) occurred. When 10 mM L-glucose was microinjected into the DMV, a $1.2 \pm 0.7\%$ change in intragastric pressure ($n = 3$; $P > 0.05$) and a -36 ± 24 mmHg s^{-1} change in the area under the curve ($n = 3$; $P > 0.05$) occurred. There was no significant difference between the responses elicited by saline or L-glucose microinjected into these nuclei, ruling out the possible role of injectate osmolarity in the response of D-glucose. In no case did these control microinjections affect motility or intragastric pressure (Fig. 2).

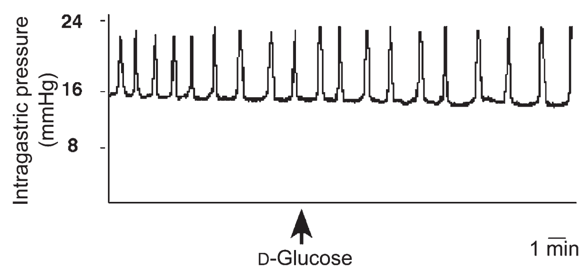

Figure 1

Effect of unilateral microinjection of D-glucose (60 nl of 10 mM solution) into the DMV on intragastric pressure and gastric motility (see text for details).

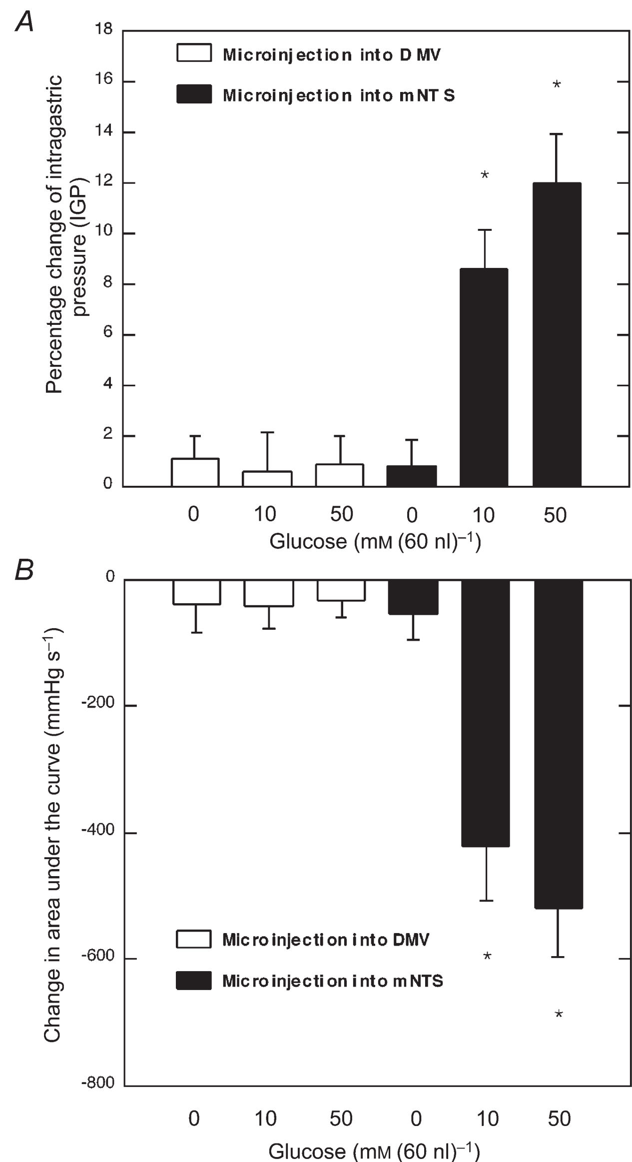

Figure 2. Effects of D-glucose microinjected into the DMV and mNTS on intragastric pressure (IGP) and the area under the curve (i.e. area of phasic antral contractions)

A, percentage change of IGP due to microinjection of saline (designated as 0 glucose), 10 and 50 mM (in 60 nl) D-glucose into the DMV (□), and the response due to microinjection of saline (designated as 0 glucose), 10 and 50 mM (in 60 nl) D-glucose into the mNTS (■). *B*, change of the area under the curve (mmHg s^{-1}) in response to microinjection of saline (designated as 0 glucose), 10 and 50 mM (in 60 nl) D-glucose into the DMV (□), and microinjection of saline (designated as 0 glucose), 10 and 50 mM (in 60 nl) D-glucose into the mNTS (■). IGP data are represented as the means ± S.E.M. of 3–6 microinjection sequences. Change in the area under the curve data are represented as the means ± S.E.M. of 3 or 4 microinjection sequences. * $P < 0.05$ using Student's group t test.

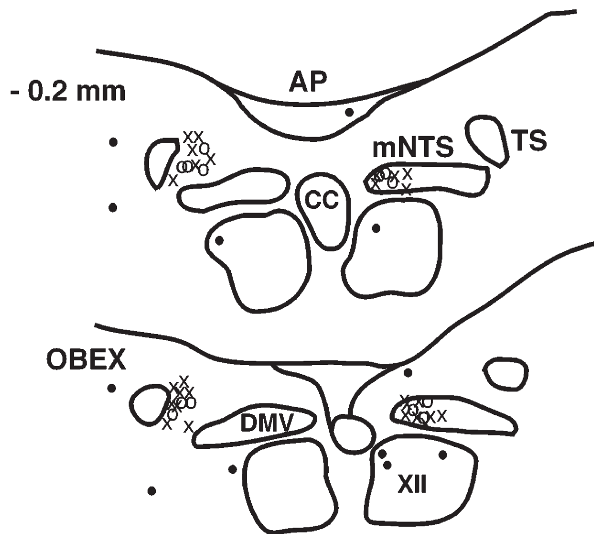

Figure 3. Microinjection sites for D-glucose and control studies

Two camera lucida drawings of coronal sections of the medulla are depicted to illustrate the location of the pipette tips indicating the microinjection sites of D-glucose (×), D-glucose controls (●) and L-glucose or saline (○). Microinjection sites (tip of micropipette) were located in these two rostral–caudal areas of the medulla, namely at obex, and slightly caudal to obex (i.e. 0.2 mm caudal to obex). For clarity, all mNTS injection sites are shown on the left and DMV sites are shown on the right. mNTS, medial nucleus of the tractus solitarius; TS, tractus solitarius; CC, central canal; DMV, dorsal motor nucleus of the vagus; XII, hypoglossal; AP, area postrema.

Ipsilateral vagotomy had no effect on either the inhibition of gastric motility or the increase in intragastric pressure induced by D-glucose ($n = 4$; Table 1). Bilateral cervical vagotomy, however, prevented D-glucose from producing an increase in IGP ($n = 4$; Table 1). We were not able to evaluate bilateral cervical vagotomy on phasic contractions because sectioning both cervical vagus nerves *per se* abolished all phasic contractions (Table 1). The lack of effect of ipsilateral vagotomy and the requirement for bilateral vagotomy for abolishing responses evoked from the mNTS was noted before (Ferreira *et al.* 2000), and is due to the fact that neurones of the mNTS connect to both the right and left DMVs (Blessing *et al.* 1991). Finally, the sites in the dorsomedial medulla where D-glucose was microinjected in the experiments described above are depicted in Fig. 3. As can be noted, all sites were located within the confines of the mNTS.

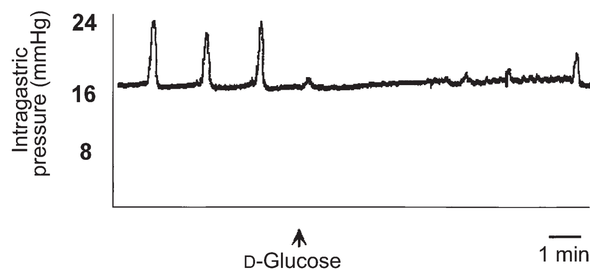

Figure 4

Effect of unilateral microinjection of D-glucose (60 nl of 10 mM solution) into the mNTS on intragastric pressure and gastric motility (see text for details).

In vitro studies

Postsynaptic effects: increasing the extracellular glucose concentration induced an outward current in DMV neurones

Whole-cell recordings. Whole-cell patch clamp recordings were made from 77 identified gastric-projecting neurones (35 fundus, 22 corpus and 20 antrum/pylorus-projecting neurones). In 75 of these neurones, the glucose concentration was raised from 5 to 11 mM ($n = 8$), 15 mM ($n = 28$) or 30 mM ($n = 49$) (note: some neurones were exposed to more than one concentration of glucose). Twenty-nine neurones (38 %) responded with an outward current, 1 neurone (1 %) responded with an inward current with the remaining 47 neurones (61 %) showing no effect.

The magnitude of the outward current was 12 ± 1.5 pA ($n = 4$), 36 ± 4.6 pA ($n = 7$) and 51 ± 9.7 pA ($n = 11$)

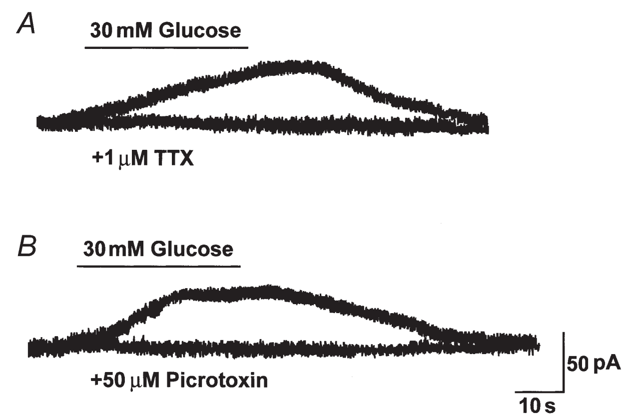

Figure 5. Superfusion with 30 mM glucose induced an outward current that was sensitive to tetrodotoxin and picrotoxin

Representative traces of gastric-projecting DMV neurones voltage clamped at −50 mV showing that elevating the glucose concentration of the superfusing Krebs solution from 5 to 30 mM induced an outward current (current allowed to plateau, then wash was performed so that the current could return to baseline levels, typically after 10 min) that was inhibited by the synaptic blocker tetrodotoxin (incubation of 10 min; 1 μM, *A*) as well as by the $GABA_A$ receptor channel-selective antagonist picrotoxin (50 μM, *B*).

Table 1. Effects of vagotomy on glucose-induced (10 mM (60 nl)$^{-1}$) changes in intragastric pressure and phasic activity evoked from the mNTS

	Pre-Vx	Post-ipsilateral Vx	Bilateral Vx
IGP	+7.9 ± 0.4	+8.2 ± 1.1	+0.9 ± 0.3*
Phasic	−439 ± 78.0	−397 ± 64.0	Phasic activity was absent and no response to D-glucose was observed

Values represent means ± S.E.M. ($n = 4$). Intragastric pressure (IGP) is expressed as percentage change from baseline and phasic activity is in mmHg s^{-1}. Vx, vagotomy. *$P < 0.05$ using ANOVA followed by Duncan's test. Baseline values: IGP, 12.4 ± 3.6 mmHg and Phasic, 470 ± 90 mmHg s^{-1}.

when glucose was raised from 5 mM to 11, 15 and 30 mM, respectively (Fig. 5). Concentrations above 30 mM glucose Krebs solution were not tested. In 19 neurones, the effects of a decrease in glucose concentration (from 5 to 2.5 mM) were assessed. Seven of these neurones (37 %) responded with an inward current of 35 ± 5 pA amplitude with the remaining 12 neurones (63 %) displaying no effect.

The glucose-induced outward current in DMV neurones was due to an indirect effect of glucose. As in the case of the *in vivo* studies, the *in vitro* brain slice studies also indicated no evidence for a direct effect of D-glucose on DMV neurones. In fact, in the presence of the synaptic transmission blocker tetrodotoxin (TTX, 1 μM), the outward current induced by increasing the

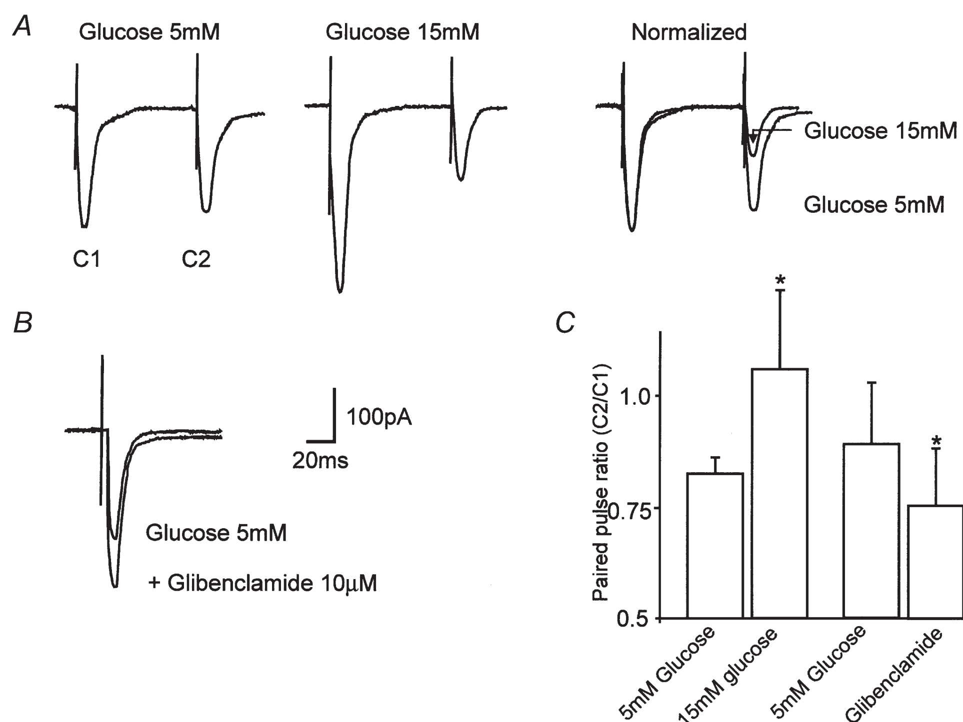

Figure 6. Elevated glucose concentration increased evoked excitatory synaptic current (EPSC) amplitude via actions at a presynaptic site

A, representative traces showing that application of paired stimuli (85 ms apart) to the NTS induced EPSCs in the recorded gastric-projecting DMV neurone. Increasing the concentration of glucose in the superfusing Krebs solution from 5 to 15 mM increased the amplitude of the evoked EPSCs. Normalization of the amplitudes of the first EPSCs (C1) revealed that the ratio of the paired currents (C2:C1) was altered. *B*, the presynaptic excitatory action of an increase in glucose concentration was mimicked by the ATP-sensitive potassium channel blocker glibenclamide (10 μM). *C*, summary histogram illustrating the alteration in the ratio of the paired EPSCs (C2:C1) following an increase in the glucose concentration of the superfusing Krebs solution or application of glibenclamide, indicating that such effects occurred presynaptically. *$P < 0.05$.

glucose concentration to 30 mM was decreased from 61 ± 17.0 pA in control to 8 ± 1.6 pA in the presence of TTX (i.e. an 84 ± 6 % reduction; $n = 6$, $P < 0.05$; Fig. 5). Similarly, in the presence of picrotoxin (50 μM), a selective blocker of the $GABA_A$-gated chloride channel, the outward current induced by 30 mM glucose was reduced from 38 ± 3.1 pA in control to 4 ± 2.0 pA in the presence of picrotoxin (i.e. an 89 ± 5 % reduction; $n = 5$, $P < 0.05$; Fig. 5).

Perforated-patch recordings. To circumvent the need to include ATP within the patch pipette solution and avoid any potential problems of unwittingly closing ATP-sensitive potassium channels, recordings were also made from gastric-projecting DMV neurones using the perforated-patch technique. Gramicidin (100 μg ml^{-1}) perforated-patch recordings were made from 17 gastric-projecting neurones. Of these, four (24 %) responded to an elevation of glucose concentration from 5 to 30 mM with an outward current of 47 ± 5 pA that did not differ significantly from the outward current induced by the same increase in glucose concentration in cells recorded with the whole-cell patch clamp method ($P > 0.05$). In addition, as with the whole-cell patch clamp technique, the outward currents observed with the perforated-patch technique were reduced by 72.4 % following exposure to TTX (1 μM; $n = 2$) and by 91 % following superfusion with picrotoxin (50 μM; $n = 2$).

Presynaptic effects: increasing the extracellular glucose concentration increased the amplitude of electrically evoked currents

In 34 neurones, the effects of altered extracellular glucose concentrations were assessed on the amplitudes of IPSCs and EPSCs evoked by stimulation of the NTS. In five neurones, increasing extracellular glucose levels from 5 to 11 mM increased the evoked IPSC amplitude from 165 ± 23 pA to 207 ± 21 pA (i.e. 130 ± 14 % of control; $P < 0.05$). Similarly, increasing extracellular glucose levels from 5 to 15 mM or to 30 mM also increased the evoked IPSC amplitudes from 254 ± 57 pA to 313 ± 66 pA and from 218 ± 51 pA to 276 ± 64 pA, respectively ($n = 5$ for both, $P < 0.05$). In each case, the ratio of the peak amplitude of the evoked currents (paired-pulse ratio) was altered suggesting a presynaptic site of action (Trombley & Westbrook, 1990; Travagli &

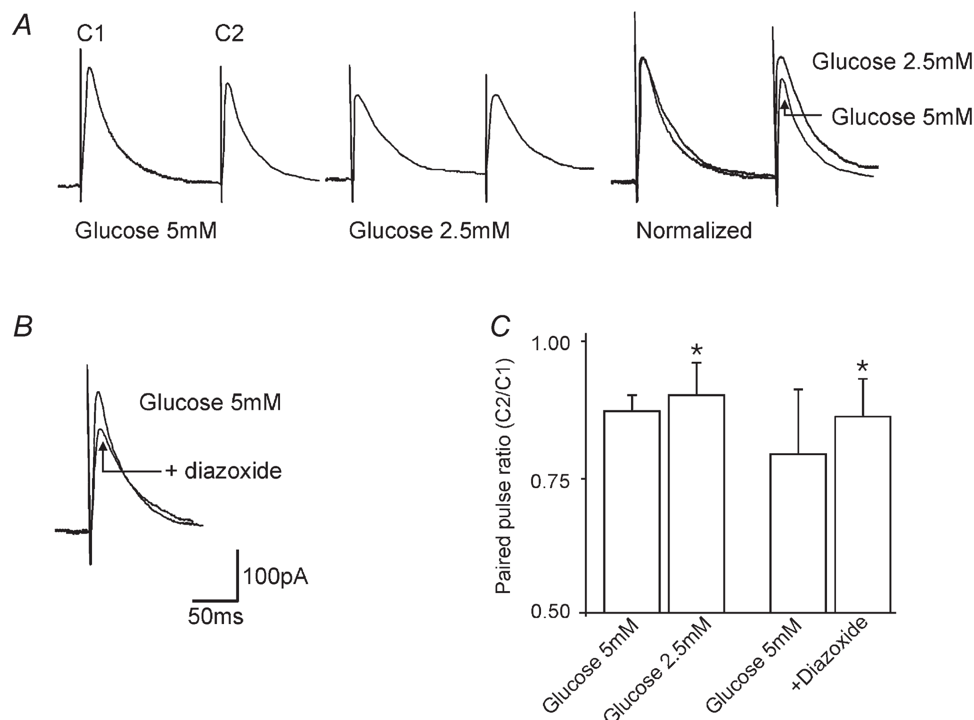

Figure 7. Reduced glucose concentration decreased evoked inhibitory synaptic current (IPSC) amplitude via actions at a presynaptic site

A, representative traces showing that application of paired stimuli (150 ms apart) to the NTS induced IPSCs in the recorded gastric-projecting DMV neurone. Decreasing the concentration of the superfusing Krebs solution from 5 to 2.5 mM reduced the amplitude of the evoked IPSCs. Normalization of the amplitudes of the first IPSCs (C1) revealed that the ratio of the paired currents (C2:C1) was altered. *B*, the presynaptic inhibitory action of a decrease in glucose concentration was mimicked by the ATP-sensitive potassium channel opener diazoxide (300 μM). *C*, summary histogram illustrating the alteration in the ratio of the paired IPSCs (C2:C1) following a decrease in the glucose concentration of the superfusing Krebs solution or application of diazoxide. $^*P < 0.05$.

Williams, 1996; Bertolino *et al.* 1997; Browning & Travagli, 1999). For example, elevating glucose levels from 5 to 15 mM increased the paired-pulse ratio from 0.76 ± 0.13 to 0.83 ± 0.14 ($P < 0.05$).

This presynaptic site of action was confirmed by use of the $GABA_A$-receptor agonist muscimol (100 μM), applied by pressure ejection, to evoke an outward current in the recorded neurone. The amplitude of the muscimol-evoked outward current was not affected by increasing the extracellular glucose concentration from 5 to 15 mM: the muscimol-induced current was 443 ± 77 pA in 5 mM glucose and 452 ± 71 pA in 15 mM glucose ($n = 5$; $P > 0.05$).

Elevated extracellular glucose concentrations had similar effects on evoked EPSC amplitudes. Specifically, in five neurones, increasing the extracellular glucose concentration from 5 to 11 mM increased the amplitude of evoked EPSCs from 241 ± 34 to 287 ± 26 pA (i.e. 123 ± 12% of control; $P < 0.05$). Elevating the glucose concentration from 5 to 15 mM increased evoked EPSC amplitude from 134 ± 18 to 170 ± 22 pA (i.e. 128% of control; $n = 5$, $P < 0.05$; Fig. 6). Similarly, elevating the extracellular glucose concentration from 5 to 30 mM increased evoked EPSC amplitude from 224 ± 59 to 288 ± 61 pA (i.e. 136% of control; $n = 5$, $P < 0.05$). In each instance, the ratio of the paired current amplitudes was altered, suggesting a presynaptic site of action. For example, increasing glucose concentration from 5 to 15 mM increased the paired-pulse ratio from 0.82 ± 0.04 to 1.06 ± 0.18 ($P < 0.05$; Fig. 6).

The ATP-sensitive potassium channel blocker glibenclamide (10 μM) mimicked the actions of elevated glucose concentrations on synaptic transmission. Glibenclamide increased the amplitude of evoked EPSCs from 219 ± 61 to 268 ± 65 pA ($n = 6$; $P < 0.05$; Fig. 6), while in a further five neurones, glibenclamide increased the amplitude of evoked IPSCs from 188 ± 19 to 221 ± 24 pA ($n = 5$; $P < 0.05$). In both instances, glibenclamide altered the paired-pulse ratio again suggesting a presynaptic site of action. Specifically, glibenclamide increased the paired-pulse ratio of evoked IPSCs from 0.54 ± 0.17 to 0.60 ± 0.16 ($P < 0.05$) while the paired-pulse ratio of evoked EPSCs was decreased from 0.89 ± 0.14 to 0.75 ± 0.13 ($P < 0.05$; Fig. 6).

Presynaptic effects: decreasing the extracellular glucose concentration decreased the amplitude of electrically evoked currents

In five neurones, lowering the glucose concentration from 5 to 2.5 mM reduced the amplitude of evoked IPSCs to 77 ± 2% of control (from 245 ± 43 to 189 ± 31 pA; $P < 0.05$; Fig. 7). The ratio of the amplitude of the paired evoked currents was increased from 0.87 ± 0.03 to 0.90 ± 0.06 ($P < 0.05$; Fig. 7) indicating that the effect was presynaptic in nature.

This presynaptic site of action was confirmed by use of the $GABA_A$ receptor agonist muscimol (100 μM), applied by pressure ejection, to evoke an outward current in the recorded neurone. The amplitude of the muscimol-evoked outward current was not affected by decreasing the extracellular glucose concentration from 5 to 2.5 mM. The muscimol-induced current was 539 ± 85 pA in 5 mM glucose and 540 ± 75 pA in 2.5 mM glucose ($n = 4$; $P > 0.05$).

The action of lowered glucose concentration to reduce the amplitude of evoked IPSCs was mimicked by the ATP-sensitive potassium channel opener diazoxide (300 μM). Specifically, in five neurones, superfusion with diazoxide reduced the amplitude of evoked IPSCs from 231 ± 50 to 198 ± 50 pA (i.e. to 84 ± 5% of control; $P < 0.05$; Fig. 7). Diazoxide also increased the paired-pulse ratio from 0.79 ± 0.12 to 0.86 ± 0.07 ($P < 0.05$; Fig. 7) suggesting a presynaptic site of action.

Similarly, in five neurones, lowering the glucose concentration from 5 to 2.5 mM reduced the amplitude of evoked EPSCs from 222 ± 78 to 183 ± 70 pA (i.e. 79 ± 4% of control; $P < 0.05$). The ratio of the amplitude of the paired evoked currents was increased from 0.88 ± 0.14 to 1.06 ± 0.17 ($P < 0.05$) indicating that the effect was presynaptic in nature. The actions of lowered glucose concentration to reduce the amplitude of evoked EPSCs was mimicked by diazoxide (300 μM). Specifically, in five neurones, superfusion with diazoxide reduced the amplitude of evoked EPSCs from 261 ± 75 to 211 ± 76 pA (i.e. 76 ± 1% of control; $P < 0.05$). Diazoxide also increased the paired-pulse ratio from 0.98 ± 0.15 to 1.19 ± 0.19 ($P < 0.05$), suggesting a presynaptic site of action.

DISCUSSION

The results of the present study indicate that acute hyperglycaemia affects the vagal brainstem neurocircuitry controlling gastric function, primarily via actions on the synaptic connections between the mNTS and the DMV. A summary of our evidence is as follows: (i) microinjection of D-glucose into the mNTS inhibited gastric motility and increased gastric tone; (ii) microinjection of the same doses of D-glucose into the DMV of anaesthetized rats had no effect on either gastric motility or intragastric pressure; (iii) whole-cell recordings in identified gastric-projecting DMV neurones revealed no direct effect of acute hyperglycemia; and (iv) increasing the glucose concentration increased the amplitude of synaptic currents evoked from the NTS.

Although we do not have sufficient evidence to conclude that all the effects of hyperglycaemia were due to glucose actions on mNTS neurones, our *in vivo* data obtained following microinjection of glucose into the mNTS suggest that the mNTS is a strong candidate for the site

where glucose-evoked changes are occurring. The data obtained from the *in vitro* experiments further support this suggestion (see below).

In vivo experiments

The starting point in these studies was to assess whether microinjection of D-glucose into the DMV of anaesthetized rats would decrease intragastric pressure and inhibit gastric motility. Our rationale for pursuing these experiments was based on the findings of Sakaguchi & colleagues, who reported that microinjection of D-glucose into the DMV can depress gastric motility of rats (Sakaguchi *et al.* 1985, 1994).

Our data confirm that acute hyperglycaemia produces robust effects to decrease gastric motility and increase gastric tone, but in addition indicate, for the first time, that these effects are due to exposure of mNTS and not DMV neurones to elevated glucose concentrations.

Our data are in contrast with previous reports that suggested that the DMV is a brainstem site at which glucose acts to inhibit gastric function (Sakaguchi *et al.* 1985). Their evidence was obtained using much higher concentrations of glucose than those tested in the present study (e.g. 10 and 50 mM in the current study as compared to 138, 277 and 555 mM in their study). Furthermore, Sakaguchi *et al.* (1985) microinjected volumes of glucose ranging from 500 to 1000 nl, as compared to the 60 nl volume used in the present study. Volumes this large microinjected into the DMV, in our opinion, are very likely to affect nearby structures such as the NTS. Finally, the gastric response noted by Sakaguchi *et al.* (1985) was a glucose-induced *decrease* in intragastric pressure. We observed the opposite response with glucose microinjected into the mNTS, namely, an *increase* in intragastric pressure. In a subsequent study by this group, Sakaguchi *et al.* (1994) reported that 500 nl of 4 mM glucose injected into the DMV of rats reduced gastric motility as reflected by a downward shift in the power spectrum for motility but no effect of this dose of glucose was noted following injection into the NTS. Similarly, 500 nl of 10 mM glucose injected into the DMV of rats decreased gastric pressure. Finally, Sakaguchi *et al.* (1994) showed that injection of 500 nl of a 7.5 and a 10 mM glucose solution into the DMV of rats decreased the intensity area of motility. This latter endpoint of gastric function is analogous to our measurement of gastric motility. In our study, 60 nl of neither a 10 mM nor a 50 mM glucose solution microinjected into the DMV had any effect on gastric motility. The reason(s) for the observed differences between the results of Sakaguchi *et al.* (1994) and our own findings is unclear, though we surmise that our microinjection technique may have allowed more precise localization of glucose effects to the mNTS and the DMV by virtue of the much smaller injection volumes. In support of this, in a recent report we showed that microinjections of drugs in the same volume as that used in the present study provided functional responses that could, in fact, be attributed to discrete activation of *either* mNTS *or* DMV (Ferreira *et al.* 2000; Ferreira, 2000).

In addition, the involvement of GABAergic neurones that project from the NTS to DMV in the control of gastric functions has been well established. Microinjection of the $GABA_A$ antagonist bicuculline into the DMV has been reported to produce a pronounced effect on gastric mechanical function (Sivarao *et al.* 1998; Ferreira, 2000), and in the present study we have shown that GABA-mediated currents occur in DMV neurones upon exposure to elevated glucose levels. That NTS neurones are excited by elevated glucose levels has also been shown by several investigators using *in vitro* preparations (Mizuno & Oomura, 1984; Adachi *et al.* 1995; Yettefti *et al.* 1995; Dellaporta *et al.* 1999, 2000). Based on the accumulated evidence for the involvement of GABAergic neurones projecting from the NTS to the DMV, we postulate that GABA-mediated effects on DMV neurones lead to reduced excitatory input to the stomach and this is reflected by inhibition of phasic activity in the antrum.

The explanation for the observed increase in gastric tone, on the other hand, is unclear. In an attempt to elucidate the mechanism, a few experiments were performed *in vitro* wherein glucose was tested on DMV neurones projecting to the antrum and to the fundus (retrograde tracer was applied to these gastric sites several days prior to performing patch clamp recordings). Our purpose was to determine whether glucose would exert differing effects on antral-projecting *vs.* fundus-projecting neurones. In these preliminary studies no difference in the effect of glucose on antral- and fundus-projecting neurones was noted. Another possible explanation for why gastric tone increased is that a withdrawal of the gastric relaxation provided by an increased inhibition of non-adrenergic non-cholinergic vagal pathways (Krowicki & Hornby, 1996) had occurred, as a result of an increased GABAergic tone from the mNTS to the DMV.

In vitro experiments

In the *in vitro* experiments, hyperglycaemia produced an outward current in DMV neurones that was antagonized by the synaptic blocker tetrodotoxin and by the GABA receptor channel blocker picrotoxin. Thus, we concluded that glucose may be acting on an afferent input to the DMV to release GABA. Indeed, hyperglycaemia increased the amplitude of synaptic currents evoked by electrical stimulation of the NTS, and our data suggest that this effect was presynaptic. Thus, the response of gastric-projecting DMV cells to direct application of the $GABA_A$-selective agonist muscimol was unaffected by variations of the extracellular glucose concentration; and the paired-pulse ratio was altered following hyperglycaemia.

In addition, acute hypoglycaemia also produced significant effects on the central component of the vago-vagal reflex. The *in vitro* whole-cell recordings indicated the occurrence of an inward current in a percentage of neurones similar to the percentage responding to hyperglycaemia with an outward current. Acute hypoglycaemia also altered the amplitude of electrically evoked currents such that the paired-pulse ratio was altered, while the amplitude of the current induced by local application of muscimol was not. Overall these data suggest that hypoglycaemia induced at the level of the DMV membrane also acts via a presynaptic site of action. These *in vitro* observations therefore support the *in vivo* data that suggest that NTS neurones tonically inhibit the activity of DMV neurones (Sivarao *et al.* 1998; Ferreira, 2000).

Even though these studies cannot determine the exact site of action where glucose affects DMV neurones, our data with picrotoxin suggest that a major part of the effect of glucose is to excite a neuronal projection, maybe from the NTS, to the DMV, that releases GABA (McCann & Rogers, 1994). Similar effects of changes in glucose concentration in the brain to release GABA have been demonstrated by several investigators. For example, Amoroso *et al.* (1990) reported that exposure of substantia nigra slices to glucose increased GABA release. Similarly, During *et al.* (1995) reported that glucose perfusion of the substantia nigra *in vivo* increased GABA release.

The mechanism responsible for glucose-induced GABA release has been hypothesized to be through ATP-dependent potassium channels (K_{ATP} channels) (Amoroso *et al.* 1990; During *et al.* 1995; Levin *et al.* 1999). Glucose is proposed to act as a signalling molecule to close the K_{ATP} channels resulting in a slight depolarization which, in turn, leads to the activation of Ca^{2+} and Na^{+} channels, followed by full depolarization of the neurone. Evidence that K_{ATP} channels were involved in glucose-evoked responses in the present study is twofold. First, the perfusion of the slice with the K_{ATP} channel blocker glibenclamide (Amoroso *et al.* 1990) mimicked the actions of glucose on evoked currents, as well as on the paired-pulse response ratio. Second, perfusion of the slice with the K_{ATP} channel opener diazoxide had the opposite effect to glucose (and glibenclamide) on evoked currents. Indeed, the effect of diazoxide on evoked currents was similar to that observed by decreasing the normal glucose concentration.

Our data therefore imply that the responses obtained with glucose in the brain slice preparation are presumably due to actions of glucose on K_{ATP} channels of mNTS neurones. In further support of this conclusion, no differences were observed in the current amplitude between experiments conducted in whole-cell recording or in the perforated-patch recording configuration. ATP is routinely included in the patch-pipette solution during whole-cell recordings (to avoid run down of second-messenger-mediated responses), but its addition is not necessary in the perforated-patch configuration (where there is no exchange of large intracellular components between the cell and the recording pipette). Since ATP closes K_{ATP} channels, the fact that no differences were observed in these two configurations argues in favour of an effect of glucose on cells *other* than the ones on which the recording is made.

Since K_{ATP} channels have been hypothesized to be the underlying mechanism responsible for the behaviour of 'glucose-responsive' (GR) neurones (Levin *et al.* 1999), we suggest that the glucose-induced inhibition of gastric motility noted in our study is due to increased firing of GR GABAergic neurones in the mNTS that, in turn, inhibit DMV neurones projecting to the phasically contracting antrum. Furthermore, K_{ATP} channels appear to be responsible for the excitatory effects of elevated glucose on NTS neurones (Dallaporta *et al.* 2000), the majority of which were also activated by the sulphonylurea drugs tolbutamide and glibenclamide, and were inhibited by the K_{ATP} channel opener pinacidil.

In terms of extrapolating our data obtained with wide ranging concentrations of glucose to animals that are euglycaemic, hypoglycaemic and hyperglycaemic, euglycaemia in the rat is considered to be approximately 5.9 mM (Borg *et al.* 1994), and the extremes of plasma glucose in this species range from approximately 2.5 mM (Borg *et al.* 1994) to approximately 22.2 mM (Thomson *et al.* 2001). Our brain slice studies were conducted using glucose concentrations of 2.5 mM (hypoglycaemia), 5 mM (euglycaemia), and 11, 15 and 30 mM (hyperglycaemia). Except for the 30 mM concentration of glucose, our experimental conditions parallel the conditions tolerated by the rat in studies of the physiological effects of hypo- and hyperglycaemia. In our *in vivo* study, we micro-injected 60 nl of a solution of either 10 or 50 mM of glucose into brainstem nuclei. The experimental conditions using 10 mM glucose agree with concentrations used by other investigators to study the effects of high blood-glucose levels on brain function (e.g. Silver & Erecinska, 1994). Since drugs applied locally to brain tissue diffuse and equilibrate, the tissue concentration is actually severalfold lower than the amount initially administered (Fu *et al.* 1999). Hence, although 60 nl of 50 mM glucose was microinjected, the actual tissue concentration of glucose was severalfold less, and was probably in the range compatible with an extreme hyperglycaemic state.

Conclusions

In conclusion, we have shown that acute hyperglycaemia affects the central components of vago-vagal reflexes by inducing a decrease in gastric motility and an increase in intragastric pressure. The combined results of our *in vivo* and *in vitro* studies suggest that these effects may be mediated by an increase of GABA release from the mNTS to gastric-projecting neurones of the DMV. In contrast to previous studies in this area, our results implicate the

mNTS, rather than the DMV, as the major glucose-responsive area of the dorsal vagal complex. Further studies will be necessary to confirm this conclusion, and to identify the specific GR NTS cells responsible for these effects, including the possibility that separate populations of NTS cells may be responsible for the decrease in motility *versus* the increase in intragastric pressure.

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