The role of the sensory peptide calcitonin-gene-related peptide(s) in skeletal muscle carbohydrate metabolism: effects of capsaicin and resiniferatoxin

Brendan LEIGHTON* and Elizabeth A. FOOT
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

1. The content of calcitonin-gene-related-peptide-like immunoreactivity (CGRP-LI) in various rat muscles was measured. Starvation for 24 h did not affect the content of CGRP-LI in these muscles, except for a decreased level in the starved-rat diaphragm. Higher contents of CGRP-LI were observed in well-vascularized muscles. 2. Capsaicin (at 1, 10 and 100 μM) inhibited insulin-stimulated rates of glycogen synthesis in isolated stripped incubated soleus muscle preparations by a mechanism independent of catecholamine release, since the effects of capsaicin were not altered by the β-adrenoreceptor antagonist DL-propranolol. 3. Resiniferatoxin (10 nM), which is a potent capsaicin agonist, also significantly inhibited the insulin-stimulated rate of glycogen synthesis. Furthermore, the concentration of resiniferatoxin required to inhibit glycogen synthesis was 100 times less than the concentration of capsaicin needed for the same effect. 4. Capsaicin (10 μM) decreased the content of CGRP-LI in isolated stripped incubated soleus muscle preparations by about 40%. 5. Neonatal treatment of rats with capsaicin, which causes de-afferentation of some sensory nerves such, we hypothesize, that CGRP can no longer be released to counteract the effects of insulin *in vivo*, caused increased rates of glycogen synthesis and increased glycogen content in stripped soleus muscle preparations *in vitro* when muscles were isolated from the adult rats. 6. These findings support the hypothesis that capsaicin and resiniferatoxin elicit an excitatory response on sensory nerves in skeletal muscle *in vitro* to cause the efferent release of CGRP. Consequently, CGRP is delivered to skeletal muscle fibres to inhibit insulin-stimulated glycogen synthesis. The role of CGRP in recovery of blood glucose levels during hypoglycaemia is discussed.

INTRODUCTION

Calcitonin-gene-related peptides (CGRPs) are 37-amino-acid peptides [1]. CGRPs share about 45% and 10–15% amino acid sequence identity with the 37-amino-acid pancreatic hormone amylin [2] and the 52-amino-acid peptide adrenomedullin [3] respectively. Amylin is primarily synthesized, stored and secreted by pancreatic β-cells [4–6]. One important target organ for amylin action is skeletal muscle [7,8]. In this tissue amylin is known to diminish the effects of insulin on intracellular glucose metabolism (mainly glycogen synthesis) [7–12]. Indeed, acute intravenous administration of amylin increases blood glucose concentrations [10]. These findings are consistent with the hypothesis that amylin is a counter-regulatory hormone, working against the effects of insulin to raise blood glucose levels [7].

CGRP is present in motor-end plates [13–15] and in sensory nerves in skeletal muscle [16]. Specific binding sites for CGRP have been identified on muscle sarcolemmal membranes [17] and on isolated stripped muscle membranes [18]. CGRP is a potent inhibitor of insulin-mediated glycogen synthesis both *in vitro* [7,19] and *in vivo* [9,20,21]; however, low concentrations of CGRP (i.e. < 20 nM) do not increase the rate of lactate formation in rat soleus muscle preparations *in vitro* [21]. The importance of CGRP as a counter-regulatory agent to insulin's effects in skeletal muscle needs to be clarified. For example, it is not clear if there is sufficient CGRP in skeletal muscle to affect insulin-mediated glucose metabolism. Consequently amylin, delivered by the bloodstream, is anticipated to be the primary mediator of insulin's effects on glucose metabolism in skeletal muscle. There may be a role, possibly in partnership with amylin, for CGRP, if it is released from skeletal muscle nerves *in vivo*, to act as an agent which diminishes the effects of insulin.

Capsaicin, at 1 μM, causes a significant release of CGRP-LI (CGRP-like immunoreactivity) from superfused rat soleus muscle preparations [22–24]. Furthermore, this capsaicin-induced release of CGRP-LI can be inhibited by the competitive antagonist capsazepine at the capsaicin receptor [22]. A low pH (pH 5.0) or a high KCl concentration (80 mM) also causes an increased rate of release of CGRP-LI from the superfused rat soleus muscle [22].

The source of CGRP released from electrically stimulated skeletal muscle is largely the sensory nerves [25]. We investigated whether CGRP is released from these nerves to modulate insulin-mediated glucose metabolism in isolated stripped incubated skeletal muscle preparations. Firstly, we measured the content of CGRP in a number of muscles. Then we used agents which exert potent excitor effects on peripheral sensory neurons (i.e. unmyelinated afferent nerve endings of the C-fibre type [26]). For this purpose we used two agents: capsaicin, which has maximal effects in other biological systems at low levels (1–10 μM), and the potent capsaicin mimetic agent, resiniferatoxin (10–100 nM) [27]. The effect of capsaicin on the content of CGRP in incubated soleus muscle and the effects of capsaicin and resiniferatoxin on insulin-stimulated glucose metabolism in soleus muscle preparations *in vitro* were measured.

Neonatal capsaicin treatment of rat pups causes de-afferentation of functional small-diameter unmyelinated sensory C-fibres that are capsaicin-sensitive, which also leads to a decrease in the content of CGRP [28–30]. Effectively, this results in little release of these sensory peptides from de-afferentated fibres. In a

Abbreviations used: CGRP, calcitonin-gene-related peptide; CGRP-LI, CGRP-like immunoreactivity.
* To whom correspondence should be addressed.

preliminary examination of the role of sensory nerves in modulating the response of glucose metabolism to insulin in skeletal muscle *in vivo* we treated a group of neonatal rats with capsaicin. Thus a chronic depletion of sensory peptides in nerves in skeletal muscle may lead to an enhanced response of glucose metabolism to insulin, assuming that a sensory peptide (e.g. CGRP) does counteract the effects of insulin *in vivo*. Soleus muscles were isolated from adult control and neonatal-capsaicin-treated rats and the response of glucose metabolism to insulin was measured *in vitro*.

Preliminary reports of some of these results have been presented in abstract form [31–33].

MATERIALS AND METHODS

Animals

Male Wistar rats (140–160 g; Harlan-Olac, Bicester, Oxon., U.K.) were kept in the animal house of the Department of Biochemistry, University of Oxford, in controlled conditions (23±1 °C; 12 h-light/12 h-dark cycle) and received standard laboratory chow and water *ad libitum*. The treatment of animals adhered to the guidelines laid down by the Animals Scientific Procedures Act, U.K. (1986). Food was withdrawn for the periods indicated in the legends to the Tables. Water was always provided *ad libitum* to all rats.

Neonatal capsaicin treatment

Male albino rats were bred in the animal house of the Department of Biochemistry, University of Oxford. Newborn rats (2–3 days old) were injected subcutaneously with capsaicin (50 mg/kg) suspended in a 0.9% (w/v) sterile saline solution containing 10% (v/v) ethanol and 10% (w/v) Tween 80. After this procedure the pups were kept warm under a tungsten lamp before being returned to their mothers. Rats were weaned after about 1 month and were killed 7–9 weeks after capsaicin treatment (weight 140–190 g). Results from a preliminary experiment showed that the response and sensitivity of glucose metabolism to insulin in incubated soleus muscle preparations were the same in muscles isolated from rats either bred in the Department of Biochemistry, University of Oxford, or obtained from a commercial source (results not shown). Neonatal control rats were treated with the appropriate volume of saline vehicle containing 10% (v/v) ethanol and 10% (w/v) Tween 80.

Materials

Enzymes, biochemicals and radiochemicals were purchased from sources previously given [7,17,34], except for capsaicin, substance P, galanin, cholecystokinin (Sigma Chemical Co., Poole, Dorset, U.K.), resiniferatoxin (Marketing Scientific Limited, Barnet, Herts., U.K.), [2-^{125}I-His^{10}]CGRP (human) (Amersham International), and the anti-CGRP-II serum RAS 6012, goat anti-rabbit antiserum and rabbit serum (Peninsula Laboratories).

Incubation procedures

Rats were killed by cervical dislocation and stripped solei were prepared as described previously [35]. The tendons of the muscles were ligated, and the muscles were rapidly weighed, tied at the resting length *in situ* on stainless steel clips and placed in 25 ml Erlenmeyer flasks containing 3.5 ml of oxygenated Krebs–Ringer bicarbonate buffer plus 7 mM Hepes, pH 7.4, 5.5 mM glucose, and 1.5% (w/v) de-fatted BSA. Flasks were sealed and aerated continuously with O_2/CO_2 (19:1). After pre-incubation of muscles in this medium for 30 min at 37 °C in an oscillating water bath (100 rev./min), the muscle strips were transferred to similar vials containing identical medium with added [U-^{14}C]glucose (0.5 μCi/ml) and insulin (100 μunits/ml). The flasks were sealed, and re-gassed for the initial 15 min period in a 1 h incubation. At the end of the incubation period, muscles were blotted and rapidly frozen in liquid nitrogen. The concentration of lactate in the incubation medium was determined spectrophotometrically [36] and [U-^{14}C]glucose incorporated into glycogen (glycogen synthesis) was measured [37]. Rates of glycogen synthesis were measured in terms of μmol of glucosyl units/h per g wet wt. Rates of glucose oxidation were measured as previously described [35]. Basal rates of lactate formation were measured in the presence of either 1 or 10 μunits of insulin/ml, since previous experiments have demonstrated that the rates of both processes are not different when stripped soleus muscle preparations are incubated in the presence of basal concentrations (1 or 10 μunits/ml) or in the complete absence of insulin [37]. The rates of lactate release and glycogen synthesis in the absence of insulin were (all values μmol/h per g) 7.25±0.56 ($n = 6$) and 1.37±0.10 ($n = 6$) respectively in the present study (see Table 3), and 8.10±0.75 ($n = 12$) and 1.10±0.17 ($n = 12$) respectively in a previous study [37]. To gauge the effect of insulin the basal rates for both processes are given in each table reporting insulin-stimulated rates of lactate release and glycogen synthesis.

Capsaicin and resiniferatoxin incubations

Capsaicin and resiniferatoxin were dissolved and diluted in absolute ethanol. Similar volumes (35 μl) of absolute ethanol were added to control flasks. Stripped soleus muscle preparations were incubated with capsaicin (1–10 μM) for 1 h. After the incubation, the immunoreactive CGRP content in frozen soleus muscles was measured with rabbit antiserum directed against this peptide (the cross-reactivity with amidated amylin was < 0.01%). [2-^{125}I-His^{10}]CGRP (human) was used as tracer. The incubation was terminated and the muscles were frozen in liquid N_2. For measurement of CGRP-LI, muscles were powdered under liquid N_2 at −20 °C and the powder was weighed before homogenization in 0.1 M HCl (two 15 s bursts at setting 5; Polytron homogenizer). Tissue was extracted at 90 °C for 10 min before cell debris was removed by centrifugation at 4 °C. The supernatant was lyophilized and the residue was reconstituted in assay buffer [0.1 M phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl, 0.1% (w/v) BSA, 0.01% (w/v) NaN_3] with brief homogenization with a Polytron homogenizer. Standard rat CGRP-1 or samples (100 μl) were incubated with 100 μl of rabbit anti-(human CGRP-II) serum for 24 h at 4 °C. Human [^{125}I-His^{10}]CGRP (100 μl) was added and incubated for a further 24 h. After addition of 100 μl of goat anti-rabbit antiserum (1:2000 dilution) and 100 μl of rabbit serum (1:2000 dilution) the samples were incubated at 20 °C for 2 h. Assay buffer (0.5 ml) was added to the sample buffers before centrifugation at 3000 ***g*** for 20 min. The coefficient of variance was less than 10% for values between 25 and 250 pM. Inter- and intra-assay coefficients of variation were 9% and 6% respectively. The cross-reactivity of the antiserum was 100% for rat CGRP-1 and CGRP-2 and human CGRP.

RESULTS AND DISCUSSION

There is now abundant evidence that, at least in some tissues, the release of CGRP from sensory nerves is the mechanism underlying the effects of the acute administration of capsaicin [38–40]. For example, capsaicin is a powerful stimulator of the con-

Table 1 CGRP-LI content in various tissues isolated from fed and fasted rats

Results are presented as means ± S.E.M. ($n = 5$). Significant differences (non-paired Student's t test) between fed and fasted values are denoted by $^{*}P < 0.05$.

Tissue	Content of CGRP-LI (fmol/g wet wt.)	
	Fed	Fasted
Heart	1416 ± 159	1514 ± 303
Diaphragm	2733 ± 369	1864 ± 129*
Soleus	1012 ± 150	900 ± 50
Extensor digitorum longus	752 ± 51	700 ± 36
Epitrochlearis	301 ± 130	267 ± 86
Gastrocnemius	644 ± 98	752 ± 241

tractility of the spontaneously beating guinea-pig atrium *in vitro* [41–43]. These effects of capsaicin are mimicked by CGRP [39,40]. The capsaicin-induced stimulation of atrial contractility becomes tolerant to the effects of CGRP (i.e. exhibits tachyphylaxis), suggesting an involvement of the neuropeptide in this response [39,40]. Capsaicin, at 1 μM, causes a significant release of CGRP-LI from superfused rat soleus muscle preparations [28–30]. Also, the major source of CGRP released from electrically stimulated skeletal muscle is sensory nerves [22].

The effects of starvation for 24 h on the content of CGRP-LI in the heart and various skeletal muscles are given on Table 1. Starvation significantly decreased the CGRP-LI content only in the diaphragm, which had the highest CGRP-LI content (Table 1). The rank order of CGRP-LI content in the various skeletal muscle preparations was as follows: diaphragm > heart > soleus > gastrocnemius = extensor digitorum longus > epitrochlearis. The greater content of CGRP-LI in well-vascularized skeletal muscle may reflect greater sensory nerve innervation around the smooth muscle of the blood vessels and capillaries [44,45].

Capsaicin significantly inhibited insulin-stimulated (100 μunits/ml) rates of glycogen synthesis at concentrations of 1, 10 and 100 μM; no effect was observed at concentrations less than 1 μM (Table 2). Capsaicin at all concentrations except 100 μM did not affect the rate of lactate formation. Clearly the magnitude of the effects of capsaicin at 100 μM on the rates of lactate release and glycogen synthesis are significantly greater than at lower capsaicin concentrations. In other biological systems the activation of sensory nerves by capsaicin is maximal at a concentration of 10 μM [46,47]. Consequently, it is a possibility that the effects of capsaicin at 100 μM were not entirely due to the activation of sensory nerves and subsequent release of sensory peptides. Because of the latter possibility we wanted to use only concentrations of capsaicin (i.e. 1 and 10 μM) or resiniferatoxin (i.e. 10 or 100 nM; see below) which are known to work via a mechanism that only involves activation of sensory nerves [46,47].

We could not rule out the possibility that capsaicin at low concentrations (1 to 10 μM) acted directly on skeletal muscle cells to affect glycogen synthesis, rather than acting indirectly via the release of CGRP from sensory nerves. Therefore we conducted similar experiments with the potent capsaicin mimetic agent resiniferatoxin, which has been shown to act on the same neurons by the same mechanism as capsaicin. Resiniferatoxin is a phorbol-related diterpene and has a different chemical structure from that of capsaicin, but the threshold concentration for activation of sensory neurons by resiniferatoxin is about 10 nM (the threshold concentration for capsaicin is > 1 μM) [48]. Thus resiniferatoxin can cause effects in biological systems at concentrations 100 times lower than the concentration of capsaicin required to elicit the same effect [48]. Resiniferatoxin at 10, 100 and 1000 nM significantly inhibited the insulin-stimulated rates of glycogen synthesis in isolated stripped incubated soleus muscle preparations, with the inhibition being significant at 100 and 1000 nM (however, we did routinely obtain significant effects with 10 nM resiniferatoxin; see Table 3). This demonstrated that the concentration of resiniferatoxin required as an inhibitor was 100 times less than the concentration of capsaicin required for the same effect (Table 2). Therefore both resiniferatoxin and capsaicin inhibited insulin-mediated glycogen synthesis (Table 2) at concentrations (10 nM and 1 μM respectively) of both compounds that are used to activate sensory nerves and cause release of sensory peptides in other biological systems [25–27,46–48].

In a large number of experiments in the present study we incubated stripped soleus muscle preparations with a concentration of insulin (100 μunits/ml) that caused half-maximal activation of the rates of lactate formation and glycogen synthesis [8]. However, in some experiments there was significant variation in the rates of glycogen synthesis or lactate formation in the control muscles stimulated by 100 μunits of insulin/ml (see Table 2). Our experiments were conducted over a long period of time, and we consider that the sensitivity of skeletal muscle to insulin may vary depending on when the experiment is conducted in the annual cycle. Nevertheless, the variation in the control values

Table 2 Concentration-dependent effects of capsaicin and resiniferatoxin on the rates of glycogen synthesis and lactate formation in isolated stripped soleus muscle preparations incubated with insulin

All muscles were incubated in the presence of 100 μunits of insulin/ml and in the absence or presence of various concentrations of capsaicin or resiniferatoxin. Values (which contain a basal rate) are presented as means ± S.E.M. ($n = 4$). The rates of lactate release and glycogen synthesis in the absence of insulin were 7.25 ± 0.56 ($n = 6$) and 1.37 ± 0.10 ($n = 6$) μmol/h per g respectively. The statistical significance of differences between muscles incubated in the absence and the presence of capsaicin or resiniferatoxin are denoted by $^{*}P < 0.05$.

Capsaicin concn. (μM)...	0	0.001	0.1	1.0	10	100
Rate of glycogen synthesis (μmol/h per g)	3.68 ± 0.22	3.70 ± 0.18	3.28 ± 0.22	2.76 ± 0.13*	2.67 ± 0.23*	0.47 ± 0.11*
Rate of lactate formation (μmol/h per g)	12.8 ± 0.9	13.1 ± 0.8	12.5 ± 0.6	13.2 ± 0.4	14.4 ± 1.3	22.09 ± 0.85*
Resiniferatoxin concn. (μM)...	0	0.0001	0.001	0.01	0.1	1.0
Rate of glycogen synthesis (μmol/h per g)	4.52 ± 0.30	5.34 ± 0.43	4.14 ± 0.55	3.65 ± 0.46	3.22 ± 0.24*	3.45 ± 0.34*
Rate of lactate formation (μmol/h per g)	14.5 ± 1.2	16.6 ± 1.1	16.3 ± 1.2	14.8 ± 0.6	12.7 ± 0.7	12.6 ± 1.3

Table 3 Effects of capsaicin (10 μM) and resiniferatoxin (100 nM) on insulin-mediated rates of lactate formation and glycogen synthesis in isolated stripped soleus muscle of the rat

Values (which contain a basal rate) are presented as means $\pm$ S.E.M. for at least six separate experiments. Significant differences (non-paired Student's t test) between control (insulin alone) and treated are denoted by $^*P < 0.05$.

Insulin concn. (μunits/ml)	Control	Capsaicin (10 μM)	Control	Resiniferatoxin (0.1 μM)
Rate of glycogen synthesis (μmol/h per g)				
0	1.37 ± 0.10	1.17 ± 0.24	1.03 ± 0.12	1.07 ± 0.09
10	1.67 ± 0.15	1.37 ± 0.21	1.83 ± 0.31	1.13 ± 0.10
100	3.65 ± 0.28	2.74 ± 0.36*	3.86 ± 0.40	2.04 ± 0.26*
1000	4.93 ± 0.26	4.43 ± 0.52	4.49 ± 0.51	3.87 ± 0.23
Rate of lactate formation (μmol/h per g)				
0	7.25 ± 0.56	8.62 ± 0.65	8.64 ± 2.04	11.96 ± 1.12
10	8.37 ± 0.46	10.0 ± 0.46*	11.07 ± 1.51	13.27 ± 1.10
100	12.61 ± 0.78	13.50 ± 0.43	15.39 ± 2.15	16.55 ± 2.42
1000	13.46 ± 0.60	14.95 ± 0.78	16.03 ± 1.72	17.75 ± 1.72

Table 4 Effect of combination of resiniferatoxin and capsaicin on insulin-stimulated rates of glycogen synthesis in isolated incubated stripped rat soleus muscle preparations

All muscles were incubated in the presence of insulin (100 μunits/ml), in the absence or presence of capsaicin (10 μM), resiniferatoxin (10 nM), or both capsaicin (10 μM) and resiniferatoxin (10 nM). Values (which consist of a basal and insulin-stimulated rate) are presented as means $\pm$ S.E.M. ($n = 4$). The rate of glycogen synthesis in the absence of insulin was 1.37 $\pm$ 0.10 μmol/h per g (6). The statistical significance (non-paired Student's t test) of differences from control values is denoted by $^*P < 0.05$.

	Glycogen synthesis (μmol/h per g)
Control	4.14 ± 0.11
Capsaicin	2.91 ± 0.35*
Resiniferatoxin	3.38 ± 0.30*
Capsaicin + resiniferatoxin	2.73 ± 0.30*

demonstrates the necessity to conduct control muscle incubations with this biological incubated preparation, which has always been our practice [6,7,19,31–35,37].

We considered it possible that capsaicin might release other sensory peptides which could influence insulin's effects in muscle. Indeed, other sensory peptides (i.e. substance P, dynorphin, galanin and cholecystokinin) have been located in sensory nerves in skeletal muscle [49,50]. Addition of these peptides to the incubation medium at high concentrations had no effect on the insulin-stimulated rates of glycogen synthesis (results not shown). Also, none of these sensory peptides (substance P, dynorphin, galanin and cholecystokinin) affected the CGRP-mediated inhibition of insulin-stimulated rates of glycogen synthesis in isolated stripped incubated soleus muscle preparations (results not shown). When stripped soleus muscle preparations were incubated in the absence or presence of capsaicin (10 μM) for 1 h there was a significant decrease (40 %) in the content of CGRP-LI (control, 1451 $\pm$ 134; capsaicin-treated, 929 $\pm$ 43 fmol/g wet wt; means $\pm$ S.E.M.; $n = 5$; $P < 0.05$). If this amount of CGRP is released locally into the extracellular space of the muscle then the approximate local concentration would be at least 2 nM (the volume of water in the extracellular space is taken to be 0.27 ml per g wet wt.), which is a sufficient concentration of CGRP to inhibit significantly the insulin-mediated rate of glycogen synthesis [8,18].

Another possibility was that capsaicin acted on adrenergic fibres to release catecholamines which might inhibit insulin-stimulated (100 μunits/ml) glycogen synthesis via a β-adrenergic mechanism. However, the β-adrenoceptor antagonist DL-propranolol (10 μM; 2.39 $\pm$ 0.21 μmol/h per g; $n = 3$) did not affect capsaicin-mediated inhibition (10 μM; 2.11 $\pm$ 0.27 μmol/h per g; $n = 3$) of insulin-stimulated glycogen synthesis (100 μM; 3.54 $\pm$ 0.39 μmol/h per g; $n = 3$) in isolated incubated soleus muscle preparations. The rates of lactate formation were not affected by either propranolol or capsaicin alone or in combination (results not shown). In general, no toxic effects of capsaicin on either sympathetic or parasympathetic neurons have been noted [51–53]. Nevertheless, we established that the inhibition of glycogen synthesis by capsaicin occurs in the presence of the β-adrenoceptor antagonist DL-propranolol. In other tissues the effects of CGRP are also exhibited in the presence of a β-adrenoceptor antagonist [9]. In summary, the effects of capsaicin cannot be accounted for by the release of other modulatory sensory peptides (at least by the peptides that we tested, i.e. substance P, dynorphin, galanin and cholecystokinin) or a catecholamine molecule. We believe that the decreased content of CGRP-LI is the result of the increased rate of release of CGRP from sensory nerves caused by capsaicin. The released CGRP then binds to specific high-affinity receptors on skeletal muscle sarcolemma to affect insulin-stimulated carbohydrate metabolism.

Rat soleus muscle preparations were isolated and incubated in the absence or presence of insulin and capsaicin (10 μM) or resiniferatoxin (100 nM) and the rates of glucose metabolism were measured. Insulin significantly stimulated the rates of both lactate formation and glycogen synthesis (Table 3). In the complete absence of insulin neither capsaicin nor resiniferatoxin affected the basal rates of lactate formation or glycogen synthesis (Table 3). Therefore, these agents *per se* did not affect basal glucose metabolism. However, only the sub-maximal (i.e. at 100 μunits/ml) insulin-stimulated rate of glycogen synthesis was significantly inhibited by capsaicin and resiniferatoxin (Table 3). Thus acute *in vitro* treatment of soleus muscle preparations may be associated with an alteration in the sensitivity of glycogen synthesis to insulin.

We investigated whether capsaicin and resiniferatoxin were operating via a similar mechanism by measuring the effects of capsaicin (10 μM) or resiniferatoxin (10 nM) alone or in combination on insulin-stimulated rates of glycogen synthesis (Table 4). Both resiniferatoxin (10 nM) and capsaicin (10 μM) significantly decreased the rate of glycogen synthesis (but had no effect on the rate of lactate formation; results not shown). The addition of both agents to the incubation medium did not lead to a significantly different decrease in the rate of insulin-stimulated glycogen synthesis (Table 4). The lack of an additive effect suggests that a common mechanism is employed by capsaicin and resiniferatoxin to affect glycogen synthesis in soleus muscle.

Neonatal capsaicin treatment of rat pups at the dose used in the present study (i.e. 50 mg/kg) causes de-afferentation of some small-diameter unmyelinated sensory fibres (C-fibres) that are capsaicin-sensitive; this also leads to a decrease in the content of CGRP [23–25]. Incubated stripped soleus muscle preparations were isolated from adult rats that had been treated as neonates with capsaicin, and it was found that the rate of glycogen synthesis was increased at a low concentration of insulin (i.e. 1 μunit/ml) and at sub-maximal (100 μunits/ml) and maximal

Table 5 Effects of neonatal capsaicin treatment of rats on the response of lactate formation, muscle glycogen content and glucose oxidation to insulin in incubated stripped soleus muscle preparations isolated from adult male Wistar rats

Results (which consist of a basal and insulin-stimulated rate) are presented as means ± S.E.M. for the numbers of separate experiments given in parentheses. The rates of lactate release and glycogen synthesis in the absence of insulin were 7.25 ± 0.56 (6) and 1.37 ± 0.10 (6) μmol/h per g respectively. Significant differences between control and neonatal-capsaicin-treated rats are denoted by * $P < 0.05$.

Insulin concn. (μunits/ml)	Control	Neonatal capsaicin
Rate of lactate formation (μmol/h per g)		
1	8.53 ± 0.44 (7)	7.89 ± 0.31 (7)
10	11.48 ± 1.59 (7)	9.34 ± 0.89 (7)
100	13.68 ± 1.96 (7)	10.87 ± 0.64 (7)
1000	12.92 ± 0.88 (7)	12.48 ± 0.54 (7)
Glycogen content (μmol/g)		
1	15.63 ± 0.92 (7)	19.20 ± 1.10* (7)
10	16.57 ± 0.74 (7)	19.22 ± 1.06* (7)
100	20.33 ± 1.23 (7)	25.17 ± 1.96* (7)
1000	24.13 ± 0.66 (7)	28.13 ± 1.40* (7)
Rate of $^{14}CO_2$ formation (μmol/h per g)		
1	0.30 ± 0.01 (4)	0.49 ± 0.05* (4)
10	0.46 ± 0.05 (4)	0.45 ± 0.03 (4)
100	0.42 ± 0.06 (4)	0.39 ± 0.04 (4)
1000	0.46 ± 0.08 (4)	0.49 ± 0.04 (4)
Rate of glycogen synthesis (μmol/h per g)		
1	1.82 ± 0.24 (7)	2.86 ± 0.23* (7)
10	2.04 ± 0.30 (7)	2.67 ± 0.08* (7)
100	4.85 ± 0.22 (7)	6.15 ± 0.42* (7)
1000	7.42 ± 0.27 (7)	10.6 ± 0.40* (7)

(1000 μunits/ml) insulin concentrations (Table 5). The content of glycogen in the incubated soleus muscle preparations isolated from neonatal-capsaicin-treated rats was also significantly increased at all concentrations of insulin (Table 5). Generally, the responses of neither lactate formation nor glucose oxidation to insulin were affected by treatment of the rats as pups with capsaicin (Table 5). These preliminary experiments suggest that sensory nerves may play a role in modulating the response of glucose metabolism in skeletal muscle in rats *in vivo*. Interestingly, neonatal capsaicin treatment of rats affects the sensitivity of glucose metabolism to insulin in the rat when measured by the euglycaemic clamp technique (S. Koopmans, personal communication). Clearly, further *in vivo* experiments need to be carried out in order to characterize fully the release *in vivo* of CGRP from sensory nerves and its role as a modulator of insulin-mediated responses in skeletal muscle. This may be possible with the advent of specific CGRP receptor antagonists [54] and a capsaicin receptor antagonist [25]. Capsaicin, at 10 μM (Table 6), did not affect insulin-mediated glucose metabolism in soleus muscle preparations isolated from neonatal-capsaicin-treated rats, although capsaicin inhibited insulin-stimulated glycogen synthesis in stripped soleus preparations isolated from untreated rats (both experiments were carried out together on the same day). The lack of effect of capsaicin on insulin-mediated rates of glycogen synthesis in muscles from neonatal rats (Table 6) suggests that the sensory nerves in skeletal muscle were deafferenated and, perhaps, that CGRP could not be released to inhibit the rate of insulin-mediated glycogen synthesis.

An important issue raised by the present study is the involvement of sensory nerves, and their efferent release of CGRP, in counter-regulatory mechanisms against the effects of insulin *in vivo*. We, and others, have demonstrated that CGRP is a potent inhibitor of insulin-stimulated glycogen synthesis in skeletal muscle *in vitro* and *in vivo* [7,9,17,19]. The present study suggests that activation of sensory nerves with capsaicin or resiniferatoxin is associated with impaired activation of glycogen synthesis by insulin, at least *in vitro*. Capsaicin also caused a significant decrease in the content of CGRP in isolated incubated stripped soleus muscle. Therefore it might be anticipated that sensory nerve activation in skeletal muscle might occur *in vivo* in some conditions where there is a rapid decline in the blood glucose levels. One study provides evidence for the involvement of capsaicin-sensitive sensory nerves in glucose homeostasis. Zhou et al. [55] treated neonatal rats with capsaicin to cause the selective de-afferentation of unmyelinated C-fibres (sensory nerves). Both control and neonatal capsaicin-treated rats were subjected to co-infusion of insulin (1 unit/kg, which causes acute hypoglycaemia) and somatostatin administration (to suppress glucagon secretion). The recovery of blood glucose to normal levels was monitored. In vehicle-treated control rats, blood glucose values return to normal values within 90 min, perhaps in response to counter-regulatory responses (which did not involve glucagon). However, there was very little recovery of blood glucose levels in conscious adult rats treated as neonates with capsaicin. Therefore we suggest that, during severe hypoglycaemia, release of CGRP from sensory nerves in skeletal muscle *in vivo* becomes an important mechanism to accelerate the recovery of blood glucose levels to normal.

Table 6 Effects of capsaicin on insulin-stimulated glucose metabolism in isolated incubated stripped soleus muscle preparations isolated from vehicle-treated control rats and neonatal-capsaicin-treated rats

Results (which consist of a basal and insulin-stimulated rate) are presented as means ± S.E.M. for at least three separate experiments. The rates of lactate release and glycogen synthesis in the absence of insulin were 7.25 ± 0.56 (6) and 1.37 ± 0.10 (6) μmol/h per g respectively. All muscles were incubated in the presence of 100 μunits of insulin/ml. Significant differences between control and neonatal-capsaicin-treated rats are denoted by * $P < 0.05$.

	Control		Neonatal capsaicin	
Capsaicin concn. (μM)...	0	10	0	10
Rate of lactate formation (μmol/h per g)	13.03 ± 0.70	13.62 ± 0.23	14.97 ± 1.29	15.10 ± 0.86
Glycogen content (μmol/g)	21.65 ± 0.94	17.37 ± 1.45*	27.57 ± 2.72	22.94 ± 2.66
Rate of glycogen synthesis (μmol/h per g)	4.14 ± 0.34	2.16 ± 0.20*	5.62 ± 0.50	4.32 ± 0.53

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REFERENCES

1 Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. and Evans, R. M. (1982) Nature (London) **298**, 240–244
2 Cooper, G. J. S., Willis, A. C., Turner, R. C., Sim, R. B. and Reid, K. B. M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8628–8632
3 Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuto, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. **192**, 553–560
4 Leffert, J. D., Newgard, C. B., Okamoto, H., Milburn, J. L. and Luskey, K. L. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3127–3130
5 Cooper, G. J. S., Day, A. J., Willis, A. C., Roberts, A. N., Reid, K. B. M. and Leighton, B. (1989) Biochim. Biophys. Acta **1014**, 247–258

6 Leighton, B. and Cooper, G. J. S. (1990) Trends Biochem. Sci. **15**, 295–299
7 Leighton, B. and Cooper, G. J. S. (1988) Nature (London) **335**, 632–635
8 Leighton, B. and Foot, E. A. (1990) Biochem. J. **269**, 19–23
9 Molina, J. M., Cooper, G. J. S., Leighton, B. and Olefsky, J. M. (1990) Diabetes **39**, 260–265
10 Young, D. A., Deems, R. O., Deacon, R. W., McIntosh, R. A. and Foley, J. A. (1990) Am. J. Physiol. **259**, E457–E461
11 Koopmans, S. J., van Mansfeld, A. D. M., Hansz, H. S., Krans, H. M. J. K., Radder, J. K., Frolich, M., de Boer, S. F., Kreutter, D. K., Andrews, G. C. and Maassen, J. A. (1991) Diabetologia **34**, 218–224
12 Frontoni, S., Choi, S. B., Banduch, D. and Rossetti, L. (1991) Diabetes **40**, 568–573
13 Ohlen, A., Lindbom, L., Staines, W., Hokfelt, T., Cuello, A. C., Fischer, J. A. and Hedqvist, P. (1977) Naunyn-Schmiedeberg's Arch. Physiol. **336**, 87–93
14 Forgren, S., Bergh, A., Carlsson, E. and Thornell, K. E. (1993) Cell Tissue Res. **274**, 439–446
15 Piehl, F., Avidsson, U., Hokfelt, T. and Cullheim, S. (1993) Exp. Brain Res. **96**, 291–303
16 Rodrigo, J., Polak, J. M., Ferandez, L., Ghatei, M. A., Mulderry, P. and Bloom, S. R. (1985) Gasterenterology **88**, 444–451
17 Popper, P. and Micevych, P. E. (1989) Brain Res. **496**, 180–186
18 Chantry, A., Leighton, B. and Day, A. (1991) Biochem. J. **277**, 139–143
19 Leighton, B., Foot, E. A., Cooper, G. J. S. and King, J. M. (1989) FEBS Lett. **249**, 357–361
20 Yamaguchi, A., Chiba, T., Morishita, T., Nakamura, A., Inui, T., Yamatani, T., Kadowaki, S., Chihara, K., Fukasee, M. and Fujita, T. (1990) Diabetes **39**, 168–174
21 Choi, S. B., Frontoni, S. and Rossetti, L. (1991) Am. J. Physiol. **260**, E321–E325
22 Santiciol., P., Del Bianco, E., Figini, M., Beran, S. and Maggi, A. (1993) Br. J. Pharmacol. **110**, 609–612
23 Santicioli, P., Del Bianco, E., Geppetti, P. and Maggi, C. A. (1992) Neurosci. Lett. **143**, 19–22
24 Geppetti, P., Del Bianco, E., Patacchini, R., Santicioli, P., Maggi, C. A. and Tramontana, M. (1991) Neuroscience **41**, 295–301
25 Sakaguuchi, M., Inaishi, Y., Kashihara, Y. and Kuno, M. (1991) J. Physiol. (London) **434**, 257–270
26 Holtzer, P. (1988) Neuroscience **24**, 739–768
27 Szallai, A. and Blumberg, P. M. (1989) Neuroscience **30**, 515–520
28 Jansco, G. E., Kiraly, E. and Jancso-Gabor, A. (1977) Nature (London) **270**, 741–743
29 Sterni, C. and Brecha, N. (1985) Gastroenterology **88**, 1600–1605
30 Sterni, C. and Brecha, N. (1986) Gastroenterology **90**, 1155–1163
31 Leighton, B. and Foot, E. A. (1991) Diabetologia **34**, A42
32 Leighton, B. and Foot, E. A. (1989) Biochem. Soc. Trans. **18**, 477–478
33 Leighton, B. and Foot, E. A. (1992) Ann. N.Y. Acad. Sci. **657**, 549–551
34 Leighton, B., Dimitriadis, G. D., Parry-Billings, M., Lozeman, F. J. and Newsholme, E. A. (1989) Biochem. J. **261**, 383–387
35 Leighton, B., Kowalchuk, J. M., Challiss, R. A. J. and Newsholme, E. A. (1988) Am. J. Physiol. **255**, E41–E45
36 Engel, R. C. and Jones, J. B. (1978) Anal. Biochem. **88**, 475–484
37 Challiss, R. A. J., Espinal, J. and Newsholme, E. A. (1983) Biosci. Rep. **3**, 675–679
38 Ju, G., Hokfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J. A., Frey, P., Elde, R. P. and Brown, J. C. (1987) Cell Tissue Res. **247**, 417–431
39 Maggi, C. A., Santicioli, P., Theodorsson-Norheim, E. and Meli, A. (1987) Neurosci. Lett. **78**, 683–688
40 Miyauchi, T., Ishikawa, T., Sugishita, Y., Saito, A. and Goto, K. (1987) J. Cardiovasc. Pharmacol. **10**, 675–682
41 Franco-Cereceda, A. and Lundberg, J. M. (1985) Naunyn-Schmiedeberg's Arch. Pharmacol. **331**, 146–151
42 Franco-Cereceda, A. and Lundberg, J. M. (1988) Naunyn-Schmeideberg's Arch. Pharmacol. **337**, 649–655
43 Fukada, F. and Fumjiwara, M. (1969) J. Pharm. Pharmacol. **21**, 622–624
44 Gibbons, M. N., Wattchow, D. and Coventry, B. (1987) Brain Res. **414**, 143–148
45 Fleming, B. P., Gibbins, U. I. L., Morris, J. L. and Gannon, B. J. (1989) Microvasc. Res. **38**, 255–268
46 Franco-Cereda, A., Henke, H., Lundberg, J. M., Peterman, J. B., Hökfelt, T. and Fischer, J. A. (1987) Peptides **8**, 399–410
47 Renzi, D., Santicioli, P., Maggi, C. A., Surrenti, C., Pradelles, P. and Meli, A. (1988) Neurosci. Lett. **92**, 254–258
48 Winter, J., Dray, A., Wood, J. N., Yeats, J. C. and Bevan, S. (1990) Brain Res. **520**, 131–140
49 Ohlen, A., Thureson-Klein, A., Raud, J., Hokfeld, T. and Hedqvist, P. (1988) Acta Physiol. Scand. **134**, 579–583
50 Gibbins, I. L., Furness, J. B. and Costa, M. (1987) Cell Tissue Res. **248**, 417–431
51 Molnar, J., Gyorgy, L., Unyi, G. and Kenyeres, J. (1969) Acta Physiol. Acad. Sci. Hung. **35**, 369–374
52 Lunberg, J. M., Hua, X.-Y. and Fredholm, B. (1984) Naunyn-Schmeideberg's Arch. Pharmacol. **325**, 176–182
53 Gamse, R. A., Molnar, A. and Lembeck, F. (1979) Life Sci. **25**, 629–636
54 Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., Fukase, M., Noda, T. and Fujita, T. (1989) Am. J. Physiol. **256**, E331–E335
55 Zhou, X.-F., Jhamandas, K. H. and Livett, B. G. (1990) Am. J. Physiol. **258**, E212–E219

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