ELSEVIER

European Journal of Pharmacology 285 (1995) 189–197

ejp

Different effects of two aldose reductase inhibitors on nociception and prostaglandin E

Nigel A. Calcutt [a,*], Li Li [a], Tony L. Yaksh [b], Annika B. Malmberg [b]

[a] *Department of Pathology, University of California at San Diego, La Jolla, CA, USA*
[b] *Department of Anesthesiology, University of California at San Diego, La Jolla, CA, USA*

Received 1 May 1995; revised 23 June 1995; accepted 30 June 1995

Abstract

This study examined the effect of two structurally dissimilar aldose reductase inhibitors, *N*-[[5-(trifluoromethyl)-6-methoxy-1-napthalenyl]thioxomethyl]-*N*-methlyglycine (tolrestat) and 4-amino-2,6-dimethylphenyl-sulphonyl nitromethane (ICI 222155), on formalin-evoked behavioural responses in control and diabetic rats and on capsaicin-evoked release of prostaglandin E from spinal cord slices in vitro. Both compounds, given orally for 4 weeks, prevented hyperalgesia in diabetic rats 5–20 min after hindpaw formalin injection. ICI 222155 also prevented hyperalgesia in diabetic rats 21–60 min after formalin, whereas tolrestat suppressed activity in diabetic rats below controls and also suppressed activity in controls when given orally or intrathecally. Capsaicin-evoked release of prostaglandin E from spinal cord slices of control rats was significantly reduced by tolrestat, but not ICI 222155. These data suggest that hyperalgesia in diabetic rats is related to glucose metabolism by aldose reductase, whereas tolrestat has specific effects on formalin-evoked nociception associated with an ability to reduce spinal prostaglandin release.

Keywords: Diabetes; Formalin test; Aldose reductase; Polyol pathway; Pain; Diabetic neuropathy

1. Introduction

Sensory dysfunction in diabetic patients may range from inappropriate pain sensations to anaesthesia associated with fibre loss. The pathogenesis of painful diabetic sensory neuropathy is unknown and treatment strategies are currently restricted to alleviation of symptoms rather than any rational preventative therapy. Experimental diabetes may provide insight into mechanisms underlying altered sensory function that allows development of therapeutic agents. There have been a number of studies in rodents investigating the effect of diabetes on the hot plate and tail flick tests (Chu et al., 1986; Bansinath et al., 1989; Lee and McCarty, 1990, 1992; Courteix et al., 1993; Calcutt et al., 1994a) which measure behavioural responses to acute high threshold nociceptive stimuli and are analogous to measures of thermal pain thresholds in humans. However, the possibility that experimental hyperglycaemia alters behavioural responses in experimental paradigms that utilize prolonged nociceptive stimuli, such as the formalin test (Dubisson and Dennis, 1977) has only recently been addressed. Courteix et al. (1993) reported hyperalgesia in diabetic rats during the first 15 min after formalin injection, and in a detailed examination of the time course of this effect we noted that hyperglycaemia in rats causes enhanced activity during the normally inactive period (Q phase) 5–20 min after injection of 5% formalin, with no modification of the earlier (phase 1) or later (phase 2) periods of pain-associated behaviour (Malmberg et al., 1993; Calcutt et al., 1994a).

The mechanism by which hyperglycaemia causes hyperalgesia in the formalin test is not known. Treatment with AGF 44, an ester derivative of ganglioside GM1, reduced the exaggerated flinching of diabetic rats during the Q phase but also suppressed activity during phases 1 and 2 in both normal and diabetic rats, suggesting a generalized antinociceptive effect that is unrelated to any specific pathogenic mechanism arising from hyperglycaemia (Malmberg et al., 1993). Treating diabetic rats with *N*-[[5-(trifluoromethyl)-6-methoxy-1-

* Corresponding author. Department of Pathology, University of California at San Diego, La Jolla, CA 92093-0612, USA. Tel. 619 534 5331, fax 619 534 1886, email ncalcutt@ucsd.edu.

SSDI 0014-2999(95)00402-5

napthalenyl]thioxomethyl]-*N*-methlyglycine (tolrestat), an inhibitor of the polyol pathway enzyme aldose reductase (EC 1.1.1.2.1) also reduces flinching during the Q phase, and does so without discernable effect on phase 1 or 2 behaviour or on any aspect of the formalin test in control rats injected with 5% formalin (Calcutt et al., 1994a). This suggests that exaggerated flux through aldose reductase may be involved in the pathogenesis of the disorder and is of interest given reports that aldose reductase inhibitors can improve qualitative sensory assessments in diabetic patients (Masson and Boulton, 1990).

It is becoming apparent that tolrestat has physiological and biochemical effects not shared by other aldose reductase inhibitors, including inhibition of prostaglandin F_1 synthase and receptor-mediated macrophage chemotaxis (Carper et al., 1989; Calcutt et al., 1994b,c). The present study was initiated to confirm that the effect of tolrestat on the Q phase of diabetic rats was related to the capacity to inhibit aldose reductase, by comparing it with a potent and structurally dissimilar aldose reductase inhibitor, 4-amino-2,6-dimethylphenyl-sulphonyl nitromethane (ICI 222155). Moreover, formalin concentration-response curves (Wheeler-Aceto and Cowan, 1993) have suggested that formalin tests using 5% solutions, as used in our previous studies, provide a supramaximal stimulus which evokes maximal responses and may preclude measurement of hyperalgesia. We therefore repeated our experimental paradigm involving diabetic rats and both of the aldose reductase inhibitors after determining a sub-maximal dose of formalin. The surprising and specific antinociceptive effects of tolrestat on both control and diabetic rats led us to investigate a potential mechanism of action by measuring the release of E-series prostaglandins from spinal cord slices in vitro, based on recent reports that inhibition of spinal prostaglandin production also produces antinociception in the formalin test (Malmberg and Yaksh, 1992, 1995).

2. Materials and methods

2.1. Experimental design and treatments

All studies used adult female Sprague-Dawley rats (Harlan Industries, San Diego, USA). In experiments involving hyperglycaemia, insulin-deficient diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg body weight) dissolved in sterile 0.9% saline. Hyperglycaemia was confirmed two days later by measurement of tail vein blood glucose concentration using a glucose oxidase-impregnated test strip (Ames Glucostix, Myles Elkhart, USA) and only animals with a blood glucose concentration of above 15 mmol/l were considered diabetic. All animals were allowed free access to food and water and maintained under approved standard conditions. Aldose reductase inhibitor-treated rats received either tolrestat (*N*-[[5-(trifluoromethyl)-6-methoxy-1-napthalenyl]thioxomethyl]-*N*-methlyglycine, Wyeth-Ayerst, Princeton, USA) or ICI 222155 (4-amino-2,6-dimethylphenyl-sulphonyl nitromethane, Zeneca Pharmaceuticals, Macclesfield, UK) suspended in water and Tween 80, once daily by oral gavage. The dose for each aldose reductase inhibitor was based upon the manufacturers recommendations as being suitable to achieve complete blockade of the accumulation of polyol pathway metabolites in peripheral nerve and was 50 mg/kg per day for tolrestat and 15 mg/kg per day for ICI 222155. Treatment began on the day that hyperglycaemia was confirmed and continued for the duration of the experiment (4 weeks). Control rats received equivalent volumes of vehicle alone by daily gavage to preclude potential effects of handling and gavaging per se on behavioural measurements.

2.2. Formalin test and intrathecal drug delivery

Rats were lightly anaesthetized with halothane (2% in O_2) to subdue voluntary movements and 50 μl of a freshly prepared formalin solution (0.2–5.0%) was injected subcutaneously into the dorsal surface of the right hind paw. Animals were then transferred to an observation chamber constructed to allow continuous visualization of the paws. Animals rapidly recovered normal motor function (< 30 s after injection) and the number of flinches during 1 min periods were counted at 5 min intervals for the next 60 min by an observer who was unaware of the treatment group of each animal. Based upon the time-response curves of normal rats (Fig. 1), phase 1 was defined as the initial measurement of flinching (1–2 min post injection) the Q (quiescent) phase as the measurements made at 5–6, 10–11, 15–16 and 20–21 min and phase 2 as all subsequent measurements post injection. Comparisons of activity during each phase were made by summing the flinches recorded at measurement points within the phase.

For examination of the effect of intrathecal aldose reductase inhibitors on the formalin test, rats were implanted with lumbar intrathecal catheters under halothane anaesthesia (Yaksh and Rudy, 1976). Each polyethylene (PE-10) catheter extended from the cisterna to the rostral edge of the lumbar enlargement. Rats that showed neurological deficits after recovering from anaesthesia were immediately euthanized. Studies were performed in conscious, unrestrained animals, 5–7 days after catheter placement. The aldose reductase inhibitors were dissolved in sterile 0.9% saline to concentrations that allowed all doses to be delivered over a period of 40–60 s in a total volume of 10 μl

followed by 10 μl of saline to flush the catheter. Formalin testing was performed exactly as described above, 10 min or 6 h after delivery of the aldose reductase inhibitor or saline vehicle.

2.3. Biochemical assays

After completion of behavioural testing, animals were weighed, decapitated and a blood sample collected for determination of plasma glucose concentration by spectrophotometric assay (glucose assay kit, Sigma, St. Louis, USA). Portions of sciatic nerve and spinal cord were removed and stored at −70°C until determination of sugar and polyol content to establish the efficacy of the aldose reductase inhibitors in restricting accumulation of polyol pathway metabolites. Tissue sugars and polyols were measured by assay of their trimethylsilyl derivatives using a Hewlett Packard 5890 gas chromatograph fitted with a 25 m × 0.2 mm Hewlett Packard Ultra 1 capillary column and flame ionization detector and with α-methyl mannoside as an internal standard.

2.4. In vitro studies

In vitro release studies were performed as previously described (Malmberg and Yaksh, 1994). Briefly, spinal cords were removed from decapitated rats by hydraulic extrusion, placed into ice-cold Krebs buffer and dissected on a glass plate placed on crushed ice. A 2 cm segment of the lumbar enlargement was divided into dorsal and ventral portions. The dorsal portion was further cut into pieces of 0.5 mm cross-section and five of these were dispersed inside a perfusion chamber (25 mm internal diameter filter holder; Millipore Corporation, Bedford, USA) modified to have an internal dead volume of approximately 0.5 ml. The chambers were submerged in a water bath maintained at 37°C and perfused at a rate of 0.2 ml/min with modified Krebs buffer solution containing 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 118.3 mM NaCl, 2.5 mM $CaCl_2$ and 3.3 mM dextrose. The perfusate was maintained at 37°C and pH was adjusted to 7.4 by bubbling with 5% CO_2 in 95% O_2. Tolrestat or ICI 222155 were added to the perfusate from the onset of the experiment. After an initial wash-out period of 45 min, a baseline sample was collected over a 10 min period (approximately 2 ml) into polypropylene tubes placed on ice. The tissue was then challenged by perfusion for 10 min with buffer as described above with the addition of 10 μM capsaicin (8-methyl-*N*-vanillyl-6-noneaneamide; Sigma Chemical Co., St. Louis, USA). Immediately after collection, the samples were frozen on dry ice, lyophilized, and reconstituted in 200 μl Krebs solution for assay of prostaglandin E by radioimmunoassay using commercially available antisera with less than 1.0% cross-reactivity with prostaglandins $F_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$ and prostaglandins of the A, B or D series (Advanced Magnetics, Cambridge, USA). This assay kit detects prostaglandin E_2 with a 50% cross-reactivity to prostaglandin E_1, although the former is far more abundant in nervous tissue. The assay sensitivity was 4.1 pg/ml according to the manufacturer. At the conclusion of the experiment, the five pieces of tissue were recovered from the perfusion chamber and protein content measured using a commercial assay kit (Coomassie Blue G-250; Pierce Chemical Co., Rockford, USA). Release was calculated as the prostaglandin E level during 10 min of capsaicin challenge minus levels over the preceding 10 min.

2.5. Data analysis

All statistical comparisons, unless otherwise noted, were made using 1 way analysis of variance, and where

Table 1
Sciatic nerve and cord sugars and polyols after 4 weeks of diabetes in rats injected with 5% formalin for behaviour studies. Data are means ± S.E.M. (nmol/mg dry wt). Statistical comparison by analysis of variance with Newman-Keuls post-hoc test. nd = not detected

	Body weight (g)	Plasma glucose (mmol/l)	Nerve				Cord			
			Glucose	Sorbitol	Fructose	myo-Inositol	Glucose	Sorbitol	Fructose	myo-Inositol
Control	239 ± 4^a	6.3 ± 0.5^a	9.7 ± 2.2^a	nd	0.9 ± 0.3^a	10.0 ± 0.7^a	1.5 ± 0.2^a	nd	0.7 ± 0.3^a	25.7 ± 2.7
Diabetic	154 ± 10^b	40.1 ± 3.4^b	54.0 ± 5.2^b	3.8 ± 0.6	19.5 ± 1.3^b	7.0 ± 0.3^b	12.4 ± 2.1^b	1.5 ± 0.2	7.9 ± 1.0^b	22.9 ± 1.4
Diabetic + tolrestat	147 ± 9^b	47.2 ± 2.9^b	67.3 ± 4.5^b	nd	1.4 ± 0.7^a	10.2 ± 1.2^a	16.8 ± 4.0^b	nd	2.6 ± 0.6^c	21.3 ± 2.7
Diabetic + ICI222155	131 ± 6^b	49.9 ± 3.9^b	66.6 ± 6.3^b	nd	1.2 ± 0.3^a	9.4 ± 0.4^a	13.2 ± 1.5^b	nd	3.2 ± 0.3^c	22.7 ± 1.5
	^{a}vs.^{b} $P < 0.001$	^{a}vs.^{b} $P < 0.001$	^{a}vs.^{b} $P < 0.001$		^{a}vs.^{b} $P < 0.001$	^{a}vs.^{b} $P < 0.01$	^{a}vs.^{b} $P < 0.001$		^{a,c}vs.^{b} $P < 0.001$ ^{a}vs.^{c} $P < 0.05$	

Table 2
Sciatic nerve and cord sugars and polyols after 4 weeks of diabetes in rats injected with 0.5% formalin for behaviour studies. Data are mean ± S.E.M. (nmol/mg dry weight). Statistical comparisons by analysis of variance with Newman-Keuls post-hoc test. nd = not detected

	Body weight (g)	Plasma glucose (mmol/l)	Nerve				Cord			
			Glucose	Sorbitol	Fructose	myo-Inositol	Glucose	Sorbitol	Fructose	myo-Inositol
Control	251 ± 7^a	5.5 ± 0.7^a	6.4 ± 1.0^a	nd	2.4 ± 0.4^a	12.5 ± 1.0^a	1.3 ± 0.2^a	nd	0.8 ± 0.1^a	26.7 ± 1.9
Diabetic	190 ± 10^b	29.2 ± 4.0^b	40.0 ± 5.1^b	8.4 ± 1.3	29.7 ± 2.1^b	8.5 ± 0.8^b	7.9 ± 1.9^b	0.8 ± 0.1	3.8 ± 0.5^b	23.8 ± 2.9
Diabetic + tolrestat	187 ± 5^b	30.6 ± 1.6^b	52.9 ± 4.0^c	nd	2.1 ± 0.3^a	12.6 ± 0.7^a	7.6 ± 1.7^b	nd	1.2 ± 0.2^a	23.4 ± 1.7
Diabetic + ICI222155	205 ± 7^b	31.9 ± 2.0^b	56.9 ± 3.5^c	nd	3.0 ± 0.6^a	11.9 ± 0.8^a	7.4 ± 0.9^b	nd	1.7 ± 0.4^a	28.2 ± 3.6
	^{a}vs.^{b} $P < 0.001$	^{a}vs.^{b} $P < 0.001$	^{a}vs.^{b,c} $P < 0.001$ ^{b}vs.^{c} $P < 0.05$		^{a}vs.^{b} $P < 0.001$	^{a}vs.^{b} $P < 0.05$	^{a}vs.^{b} $P < 0.05$		^{a}vs.^{b} $P < 0.001$	

$P < 0.05$, between-group differences were identified by the Newman-Keuls post-hoc test.

3. Results

4 weeks of streptozotocin-induced diabetes was associated with a reduction in body weight and elevation of plasma nerve and cord glucose levels (Tables 1 and 2). In untreated diabetic rats there was accumulation of sorbitol and fructose in the nerve and cord, which in the sciatic nerve was associated with reduced myo-inositol levels. Both tolrestat (50 mg/kg per day) and ICI 222155 (15 mg/kg per day) completely prevented the accumulation of sorbitol in nerve and cord, attenuated the accumulation of fructose in nerve and cord, and restored nerve myo-inositol levels.

In control rats, formalin injection induced a concentration-dependent increase in flinching behaviour (Fig. 1). Untreated diabetic rats injected with 5% formalin exhibited responses similar to controls during both phases 1 and 2, but also showed exaggerated flinch activity during the intervening Q phase ($P < 0.01$ vs controls; Table 3). This increased activity was significantly reduced by concurrent treatment with tolrestat or ICI 222155 (both $P < 0.05$ vs. untreated diabetics), while activity during phases 1 and 2 was not affected by either compound.

Injection of 0.5% formalin, for sub-maximal stimulation of behavioural responses, evoked normal behavioural responses in diabetic rats during phase 1 compared to untreated controls (Table 4), but exaggerated flinch activity during the Q phase ($P < 0.001$) and phase 2 ($P < 0.05$). Treatment of diabetic rats with

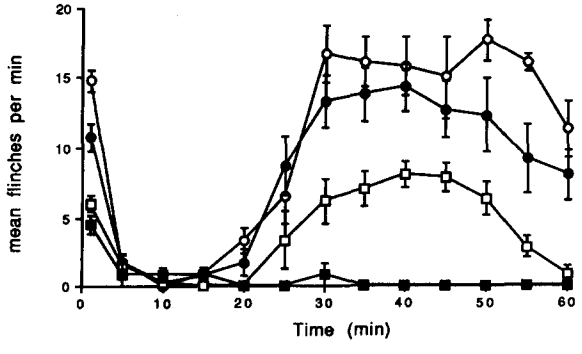

Fig. 1. Time-response curves of flinching behaviour after the injection of 50 μl of 0.2% (solid squares), 0.5% (open squares), 1% (solid circles) or 5% (open circles) formalin into the hindpaw of non-diabetic rats. Data are means ± S.E.M. $n = 4$–6 rats per group.

Table 3
Flinch behaviour evoked by 5% formalin. Data are means ± S.E.M. (sum flinches). Statistical comparisons by analysis of variance with Newman-Keuls post hoc test

	n	Phase 1	Phase Q	Phase 2
Control	7	13 ± 1	3 ± 1^a	108 ± 8
Diabetic	7	12 ± 1	25 ± 7^b	96 ± 8
Diabetic + tolrestat	7	11 ± 1	10 ± 2^c	90 ± 8
Diabetic + ICI 222155	6	11 ± 1	12 ± 3^c	85 ± 12
			^{a}vs.^{b} $P < 0.01$ ^{b}vs.^{c} $P < 0.05$	

Table 4
Flinch behaviour evoked by 0.5% formalin. Data are means ± S.E.M. (sum flinches). Statistical comparisons by analysis of variance with Newman-Keuls post hoc test

	n	Phase 1	Phase Q	Phase 2
Control	7	9 ± 1	4 ± 1^a	81 ± 6^a
Diabetic	6	9 ± 1	21 ± 7^b	106 ± 8^b
Diabetic + tolrestat	6	6 ± 1	8 ± 2^c	54 ± 7^c
Diabetic + ICI 222155	6	8 ± 1	10 ± 3^c	83 ± 8^a
			^{a}vs.^{b} $P < 0.001$ ^{b}vs.^{c} $P < 0.01$	^{a}vs.^{b,c} $P < 0.05$ ^{b}vs.^{c} $P < 0.001$

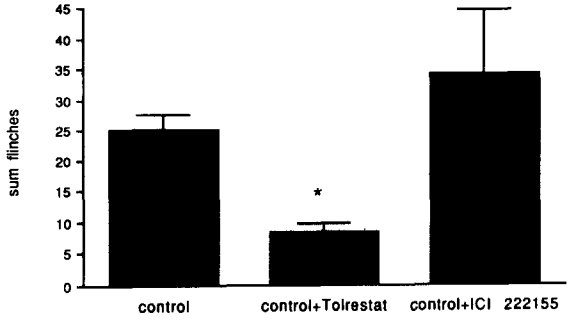

Fig. 2. The phase 2 response to 50 μl of 0.5% formalin in non-diabetic control (vehicle-treated) rats, and rats treated with tolrestat or ICI 222155 (both 50 mg/kg per day) for 1 month. Data are means ± S.E.M. $^{*}P < 0.05$ vs. control or ICI 222155-treated rats. Statistical comparisons by 1 way analysis of variance followed by Newman-Keuls post-hoc test. $n = 6$ rats per group.

tolrestat (50 mg/kg per day) or ICI 222155 (15 mg/kg per day) for the duration of hyperglycaemia reduced the exaggerated activity of diabetic rats during the Q phase (both $P < 0.01$ vs. untreated diabetics). ICI 222155 restored phase 2 to control levels ($P < 0.05$ vs. untreated diabetics), whereas tolrestat depressed activity during phase 2 compared to all other groups ($P < 0.05$ vs. controls or ICI 222155-treated diabetics and $P < 0.001$ vs. untreated diabetics). In a separate study, daily treatment of non-diabetic rats with tolrestat (50 mg/kg per day) for 1 month caused a significant ($P < 0.05$) reduction in activity during phase 2 of the response to 0.5% formalin compared to vehicle or ICI 222155-treated (50 mg/kg per day) non-diabetic rats (Fig. 2), without notably affecting the other phases. Treating non-diabetic rats with tolrestat (50 mg/kg) or ICI 222155 (50 mg/kg) on the day prior to testing and then 1 h before testing did not significantly alter phase 2 activity in response to 0.5% formalin (vehicle-treated = 89 ± 23; tolrestat-treated = 74 ± 13; ICI 222155-treated = 70 ± 17 mean sum flinches during phase 2 ± S.E.M., $n = 4–5$ per group).

Intrathecal delivery of 10 μg tolrestat to non-diabetic rats, 10 min before injection of 5% formalin into the hindpaw, caused a significant ($P < 0.05$) suppression of phase 2 flinch activity (69 ± 17: mean sum flinches during phase 2 ± S.E.M.) compared to animals that received an equivalent volume of 0.9% saline vehicle alone (127 ± 6; Fig. 3A) and this effect was partially maintained when this dose of tolrestat was given 6 h prior to formalin injection (100 ± 14). A similar effect was noted after delivery of 100 μg of tolrestat 10 min before testing (tolrestat-treated = 75 ± 14 vs. controls = 120 ± 10; $P < 0.05$) whereas 100 μg of ICI 222155 (133 ± 8), was without effect ($P < 0.05$ vs. tolrestat-treated rats; Fig. 3B).

Spinal cord slices from non-diabetic rats were exposed to concentrations of tolrestat or ICI 222155 before determination of capsaicin-evoked prostaglandin

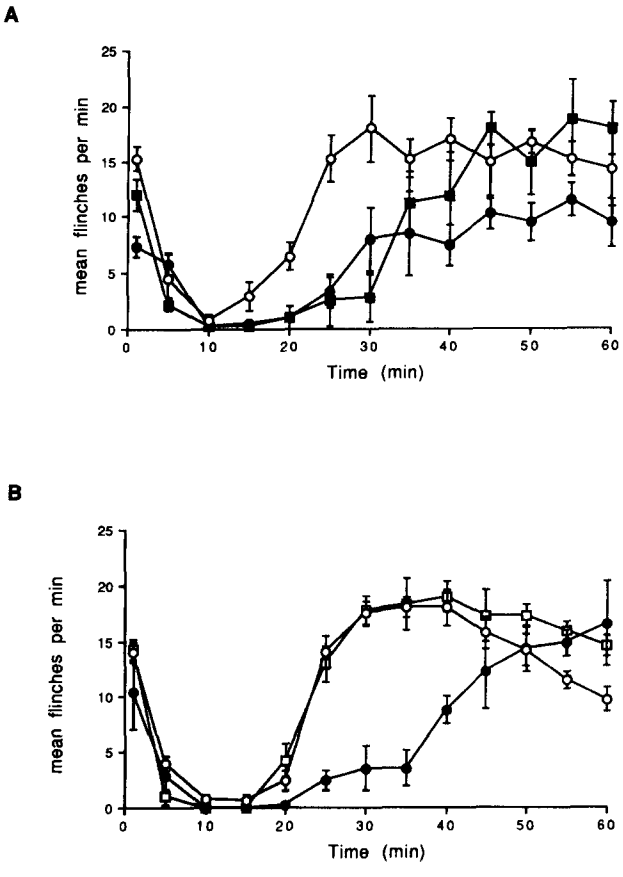

Fig. 3. Time-response curves of flinching behaviour in non-diabetic rats in response to 50 μl of 5% formalin after the intrathecal delivery of (A) saline (open circles, for delivery 10 min before testing), 10 μg tolrestat (closed circles for delivery 10 min before testing; closed squares for delivery 6 h before testing) or (B) controls (open circles), 100 μg tolrestat (closed circles for delivery 10 min before testing) and 100 μg ICI 222155 (open squares for delivery 10 min before testing). Data are means ± S.E.M. $n = 4–6$ rats per group.

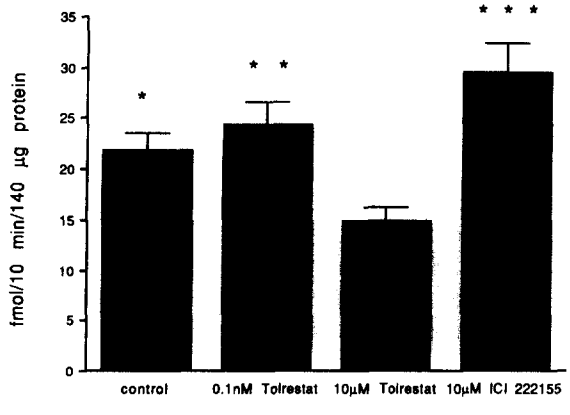

Fig. 4. Release of prostaglandin E from in vitro spinal cord slice preparations of non-diabetic rats during a 10 min challenge with 10 μM capsaicin, with or without the presence of tolrestat or ICI 222155. Data are means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. 10 μM tolrestat group. Statistical comparisons by 1 way analysis of variance followed by Newman-Keuls post-hoc test. $n = 4$ experiments per group.

E release. The presence of 10 μM ICI 222155 did not significantly alter capsaicin-evoked prostaglandin E release (29.4 ± 3.0 fmol/140 μg protein) compared to slices where aldose reductase inhibitors were not included in the perfusate (21.8 ± 1.7; Fig. 4). Tolrestat, at 10 μM (14.7 ± 1.6), but not 0.1 nM (24.3 ± 2.3) significantly ($P < 0.05$) reduced capsaicin-evoked prostaglandin E release compared to slices exposed to aldose reductase inhibitor-free perfusate.

4. Discussion

The initial aim of this study was to provide further evidence for a role of flux through aldose reductase in the hyperalgesic behaviours of diabetic rats, by excluding the possibility that the previously reported effect of tolrestat (Calcutt et al., 1994a) was unrelated to its capacity to inhibit aldose reductase. We therefore compared the effect of tolrestat with that of ICI 222155, a potent aldose reductase inhibitor of a different structural series, being napthaglycine and sulphonylnitromethane derivatives, respectively. Both aldose reductase inhibitors, given at the manufacturers recommended doses, achieved complete block of the accumulation of sorbitol in the nerve and spinal cord of diabetic rats, and a near complete block of fructose, suggesting a largely effective inhibition of aldose reductase in these tissues. In agreement with our previous study with tolrestat, the hyperalgesic responses of diabetic rats during the Q phase were inhibited by both aldose reductase inhibitors (Calcutt et al., 1994a). This supports the suggestion that it is the common ability to inhibit the exaggerated flux through aldose reductase that attenuates the hyperalgesia induced by diabetes in the formalin test model. The presence of a similar increase in Q phase activity in galactose-fed rats (Calcutt et al., 1994a), which display hexose sugar hyperglycaemia in the absence of insulin deficiency, is also consistent with an association between flux through aldose reductase and hyperalgesia. However, it is not yet clear where the primary lesion lies. In the sciatic nerve, aldose reductase is localized to the cytoplasm of myelinating Schwann cells (Powell et al., 1991), while distribution in the central nervous system is not well documented. Furthermore, the physiologic and pharmacologic features of the Q phase of the formalin test are ill defined in normal rats and it is not clear whether it represents passive inactivity of primary afferents and dorsal horn neurons or an active suppression. The latter possibility is supported by reports that behavioural activity can be induced during the Q phase of control rats by systemic treatment with opioid (Wheeler-Aceto and Cowan, 1993) or γ-aminobutyric acid (Franklin and Abbott, 1993) antagonists.

We repeated our studies of the effects of diabetes and aldose reductase inhibition on the formalin test using a lower concentration of formalin because of concerns that 5% formalin evokes maximal responses, preventing detection of any potential hyperalgesia during active phases of the test. A concentration of 0.5% formalin was chosen based on concentration-response studies indicating the induction of discernable, but sub-maximal, behavioural activity. While exaggerated activity during the Q phase in diabetic rats was detected and aldose reductase inhibitor-preventable using either 5% or 0.5% formalin, untreated diabetic rats injected with the lower concentration also showed hyperalgesia during phase 2, suggesting that 5% formalin obscures subtle physiologic abnormalities by providing a supramaximal stimulus.

Consideration of the mechanisms that underlie formalin-induced nociceptive behaviour may provide some clues to the pathogenesis of hyperalgesia in diabetic rats. It is believed that phase 1 behaviour in the formalin test is directly related to primary afferent input from the site of injection. In contrast, pharmacologic studies have suggested that, while the response remains dependent on primary afferent activity (Dickenson and Sullivan, 1987; Coderre et al., 1990b), phase 2 involves amplification of the nociceptive input at the spinal level associated with glutaminergic *N*-methyl-D-aspartate (NMDA) and peptidergic neurokinin (NK_1) receptor activation (Yamamoto and Yaksh, 1991, 1992), cyclooxygenase-mediated prostaglandin E release (Malmberg and Yaksh, 1992, 1995) and nitric oxide production (Malmberg and Yaksh, 1993). Any one, or a combination of these systems may be altered by diabetes. For example, prostaglandin E production is elevated in glomeruli from diabetic rats and this is attenuated by aldose reductase inhibitor treatment (Chang et al., 1991; Ramsammy et al., 1993). A similar situation in the spinal cord of diabetic rats could produce hyperalgesia, as occurs after intrathecal prostaglandin E_2 injection (Taiwo and Levine, 1986, 1988), and would be consistent with our observation that the aldose reductase inhibitor ICI 222155 restored phase 2 behaviour to control levels. However, in other tissues prostaglandin E release is reduced by hyperglycaemia (Kunisaki et al., 1991; Rothe et al., 1994) and it is clear that measurements of stimulus-evoked spinal prostaglandin E release are necessary to provide insight into this potential mechanism of hyperalgesia.

It has been reported that the spinal cord of diabetic rats shows increased substance P binding sites (Kamei et al., 1990). As substance P enhances nociceptive processing in the spinal cord and peptidergic NK_1 receptor antagonists inhibit phase 2 of the formalin test (Yamamoto and Yaksh, 1991) this also provides a potential mechanism for hyperalgesia in diabetic rats. An increased number of substance P receptors may occur to counter the deficit in peripheral nerve content

and axonal transport of substance P (Robinson et al., 1987; Tomlinson et al., 1988; Calcutt et al., 1990), should this lead to impaired release of the peptide. Should hyperalgesia be the consequence of reduced nerve substance P levels, treatments that maintain nerve substance P levels should be accompanied by prevention of hyperalgesia. Interestingly, aldose reductase inhibition prevents both nerve substance P depletion (Robinson et al., 1987; Diemel et al., 1992) and hyperalgesia during phase 2 in diabetic rats. At present no studies have reported the effects of diabetes and aldose reductase inhibition on spinal release of nociceptive neurotransmitters and modulators or receptor numbers and function.

Our finding of hyperalgesia during phase 2 of the response to 0.5% formalin in diabetic rats contrasts with a report that diabetic mice show an absence of phase 2 after injection of 0.5% formalin (Kamei et al., 1993). It is plausible that there are species differences in the effect of diabetes on the formalin test. In a preliminary study, we found that severe diabetes attenuated phase 2 of the formalin test in rats. This was not affected by aldose reductase inhibition but coincided with animals being obviously sick and unresponsive to stimuli. We have subsequently become cautious in interpreting reduced behavioural responses to stimuli in sick rats, but are confident of our present findings in which behavioural activity was enhanced by diabetes.

An unexpected finding was that, when using 0.5% formalin as the stimulus, tolrestat but not ICI 222155, reduced phase 2 below control values in diabetic rats and also suppressed phase 2 in control rats. Suppression of phase 2 was present after delivery by oral gavage after 4 weeks, but not 24 h, of treatment. This may reflect the pharmacokinetics of oral delivery and maintenance of effective levels of tolrestat in nervous tissue (Dvornik et al., 1994), as a rapid effect was noted when tolrestat was delivered by intrathecal catheter 10 min before injection of formalin and therefore 30 min before the onset of phase 2. Suppression of phase 2 by tolrestat is probably not related to the capacity to inhibit aldose reductase, as equal oral and intrathecal doses of ICI 222155 were without effect on control rats, despite the latter being a more potent inhibitor of aldose reductase. Other compounds with a similar ability to suppress phase 2 behaviour after spinal delivery include inhibitors of glutamatergic NMDA (Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992) and tachykinin NK_1 receptors (Yamamoto and Yaksh, 1991) and inhibitors of enzymes involved in the synthesis of prostanoids and nitric oxide (Malmberg and Yaksh, 1992, 1993). It is not yet known whether tolrestat interferes with any of these signalling pathways. This is not the first description of properties of tolrestat that are not shared by other aldose reductase inhibitors. Tolrestat, but not sorbinil or statil, inhibits prostaglandin F_1 synthase activity (Carper et al., 1989) and also the receptor-mediated chemotaxis of macrophages (Calcutt et al., 1994b).

To investigate a potential mechanism by which tolrestat inhibits phase 2 of the formalin test, we measured the capsaicin-evoked release of E-series prostaglandins from spinal cord slices. There are binding sites for prostaglandin E_2 in laminae I and II of the dorsal horn (Matsumura at al., 1992) and prostaglandin E_2 is released in the spinal cord after noxious thermal and formalin stimulation (Coderre et al., 1990a; Malmberg and Yaksh, 1995) and from cord-derived astrocytes by substance P (Marriott et al., 1991). The production of prostanoids in the spinal cord has been implicated in sustaining phase 2 of the formalin test by studies that have shown inhibition of this phase by assorted non-steroidal anti-inflammatory agents with a potency order that agrees with their capacity to inhibit cyclooxygenase (Malmberg and Yaksh, 1992) while spinal delivery of prostaglandin E_2 antagonists also suppresses phase 2 activity (Malmberg et al., 1994). Moreover, intrathecal delivery of prostaglandins produce pain behaviour and thermal hyperalgesia in rats (Minami et al., 1994), further supporting the association between spinal prostanoids and maintenance of hyperalgesia. The finding that tolrestat, but not ICI 222155, reduced prostaglandin E release from spinal cord slices at a concentration of 10 μM is consistent with the possibility that the effect of tolrestat on phase 2 of the formalin test is related to reduction of spinal prostanoid production. It is not yet known whether tolrestat possesses the capacity to inhibit cyclooxygenase or other enzymes involved in the formation of prostaglandin E. Alternately, the effect may involve disruption of capsaicin-evoked signal transduction pathways, as we have previously reported that tolrestat but not other aldose reductase inhibitors, prevents receptor-mediated macrophage chemotaxis without interfering with chemotaxis towards agents that act in a non-receptor-mediated manner (Calcutt et al., 1994b). These possibilities merit further investigation.

Acknowledgements

This study was supported by NIH grants NS 14162 and NS 16541. We thank Dr. T. Hohman of Wyeth-Ayerst and Dr. D. Mirrlees of Zeneca Pharmaceuticals for supplies of Tolrestat and ICI 222155, respectively.

References

Bansinath, M., K. Ramabadran, H. Turndorf and M.M. Puig, 1989, Effect of yohimbine on nociceptive threshold in normoglycemic and streptozotocin-treated hyperglycemic mice, Pharmacol. Biochem. Behav. 33, 459.

Calcutt, N.A. A.B Malmberg, T. Yamamoto and T.L. Yaksh, 1994a, Tolrestat treatment prevents modification of the formalin test model of prolonged pain in hyperglycemic rats, Pain 58, 413.

Calcutt, N.A., H.F. McMurray, D.F. Moorhouse, M. Bache, S. Parthasarathy, H.C. Powell and A.P. Mizisin, 1994b, Inhibition of macrophage chemotaxis and peripheral nerve regeneration in normal and hyperglycemic rats by the aldose reductase inhibitor Tolrestat, Exp. Neurol. 128, 226.

Calcutt, N.A., A.P. Mizisin and M.W. Kalichman, 1994c, Aldose reductase inhibition, Doppler flux and conduction in diabetic rat nerve, Eur. J. Pharmacol. 251, 27.

Calcutt, N.A., D.R. Tomlinson, G.B. Willars and P. Keen, 1990, Axonal transport of substance P-like immunoreactivity in ganglioside-treated diabetic rats, J. Neurol. Sci. 96, 283.

Carper, D.A., G. Wistow, C. Nishimura, C. Graham, K. Watanabe, Y. Fujii, H. Hayashi and O. Hayaishi, 1989, A superfamily of NADPH-dependent reductases in eukaryotes and prokaryotes, Exp. Eye Res. 49, 377.

Chang, W.P., E. Dimitriadis, T. Allen, M.E. Dunlop, M. Cooper and R.G. Larkins, 1991, The effect of aldose reductase inhibitors on glomerular prostaglandin production and urinary albumin excretion in experimental diabetes mellitus, Diabetologia 34, 225.

Chu, P.C., M.T. Lin, L.R. Shian and S.Y. Leu, 1986, Alterations in physiologic functions and in brain monoamine content in streptozotocin-diabetic rats, Diabetes 35, 481.

Coderre, T.J. and R. Melzack, 1992, The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury, J. Neurosci. 12, 3671.

Coderre, T.J., R. Gonzales, M.E. Goldyne, J. West and J.D. Levine, 1990a, Noxious stimulus-induced increase in spinal prostaglandin E_2 is noradrenergic terminal-dependent, Neurosci. Lett. 115, 253.

Coderre, T.J., A.L. Vaccarino and R. Melzack, 1990b, Central nervous system plasticity in the tonic pain response to subcutaneous formalin injection, Brain Res. 535, 155.

Courteix, C., A. Eschalier and J. Lavarenne, 1993, Streptozocin-induced diabetic rats: behavioral evidence for a model of chronic pain, Pain 53, 81.

Dickenson, A.H. and A.F. Sullivan, 1987, Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre and post formalin, Pain 30, 349.

Diemel, L.T., E. Stevens, G.B. Willars and D.R. Tomlinson, 1992, Depletion of substance P and calcitonin gene-related peptide in sciatic nerve of rats with experimental diabetes; effects of insulin and aldose reductase inhibition, Neurosci. Lett. 137, 253.

Dubuisson, D. and S.G. Dennis, 1977, The formalin test: a quantitative study of the analgesic effects of morphine, meperidine and brain stem stimulation in rats and cats, Pain 4, 161.

Dvornik, D., J. Millen, D.R. Hicks and M. Kraml, 1994, Tolrestat pharmacokinetics in rat peripheral nerve, J. Diabetes Comp. 8, 18.

Franklin, K.B.J. and F.V. Abbott, 1993, Pentobarbital, diazepam and ethanol abolish the interphase diminution of pain in the formalin test: evidence for pain modulation by $GABA_A$ receptors, Pharmacol. Biochem. Behav. 46, 661.

Kamei, J., H. Hitosugi and Y. Kasuya, 1993, Formalin-induced nociceptive responses in diabetic mice, Neurosci. Lett. 149, 161.

Kamei, J., M. Ogawa and Y. Kasuya, 1990, Development of supersensitivity to substance P in the spinal cord of the streptozotocin-induced diabetic rat, Pharmacol. Biochem. Behav. 35, 473.

Kunisaki, M., F. Umeda, T. Inoguchi and H. Nawata, 1991, Vitamin E restores reduced prostacyclin synthesis in aortic endothelial cells cultured with a high concentration of glucose, Metabolism 41, 613.

Lee, J.H. and R. McCarty, 1990, Glycemic control of pain threshold in diabetic and control rats, Physiol. Behav. 47, 225.

Lee, J.H. and R. McCarty, 1992, Pain threshold in diabetic rats: effects of good versus poor diabetic control, Pain 50, 231.

Malmberg, A.B. and T.L. Yaksh, 1992, Antinociceptive actions of spinal non-steroidal anti-inflammatory agents on the formalin test in the rat, J. Pharmacol. Exp. Ther. 263, 136.

Malmberg, A.B. and T.L. Yaksh, 1993, Spinal nitric oxide synthesis inhibition blocks NMDA induced thermal hyperalgesia and produces antinociception in the formalin test in rats, Pain 54, 291.

Malmberg, A.B. and T.L. Yaksh, 1994, Capsaicin-evoked prostaglandin E_2 release in spinal cord slices: relative effect of cyclooxygenase inhibitors, Eur. J. Pharmacol. 271, 293.

Malmberg, A.B. and T.L. Yaksh TL, 1995, Cyclooxygenase inhibition and the spinal release of prostaglandin E_2 and amino acids evoked by paw formalin injection: a microdialysis study in unanesthetized rats, J. Neurosci. 15, 2768.

Malmberg, A.B., T.L. Yaksh and N.A. Calcutt, 1993, Anti-nociceptive effects of the GM1 ganglioside derivative AGF 44 on the formalin test in normal and streptozotocin-diabetic rats, Neurosci. Lett. 161, 45.

Malmberg, A.B., M.F. Rafferty and T.L. Yaksh, 1994, Antinociceptive effect of spinally delivered prostaglandin E receptor antagonists in the formalin test on the rat, Neurosci. Lett. 173, 193.

Marriott, D.R., G.P. Wilkin and J.N. Wood, 1991, Substance P-induced release of prostaglandins from astrocytes: regional specialisation and correlation with phosphoinositol metabolism, J. Neurochem. 56, 259.

Masson. E.A. and A.J.M. Boulton, 1990, Aldose reductase inhibitors in the treatment of diabetic neuropathy: a review of the rationale and clinical evidence, Drugs 39, 190.

Matsumura, K., Y. Watanabe, K. Imai-Matsumura, M. Connolly, Y. Koyama, H. Onoe and Y. Watanabe, 1992, Mapping of prostaglandin E2 binding sites in rat brain using quantitative autoradiography, Brain Res. 581, 292.

Minami, T., R. Uda, S. Horiguchi, S. Ito, M. Hyodo and O. Hayaishi, 1994, Allodynia evoked by intrathecal administation of prostaglandin E_2 to conscious mice, Pain 57, 217.

Powell, H.C., R.S. Garrett, P.F. Kador and A.P. Mizisin, 1991, Fine structural localization of aldose reductase and ouabain-sensitive, K^+-dependent p-nitro-phenylphosphatase in rat peripheral nerve, Acta Neuropathol. 81, 529.

Ramsammy, L.S., B. Haynes, C. Josepovitz and G.J. Kaloyanides, 1993, Mechanism of decreased arachidonic acid in the renal cortex of rats with diabetes mellitus, Lipids 28, 433.

Robinson, J.P., G.B. Willars, D.R. Tomlinson and P. Keen, 1987, Axonal transport and tissue contents of substance P in rats with long-term streptozotocin-diabetes. Effects of the aldose reductase inhibitor "statil", Brain Res. 426, 339.

Rothe, H., C. Ongoren S. Martin, P. Rosen and H. Kolb, 1994, Abnormal TNF-alpha production in diabetes-prone BB rats: enhanced TNF-alpha expression and defective PGE_2 feedback inhibition, Immunology 81, 407.

Taiwo, Y.O. and J.D. Levine, 1986, Indomethacin blocks central nociceptive effects of PGF_2 alpha, Brain Res. 373, 81.

Taiwo, Y.O. and J.D. Levine, 1988, Prostaglandins inhibit endogenous pain control mechanisms by blocking transmission at spinal noradrenergic synapses, J. Neurosci. 8, 1346.

Tomlinson, D.R., J.P. Robinson, G.B. Willars and P. Keen, 1988, Deficient axonal transport of substance P in streptozotocin-induced diabetic rats. Effects of Sorbinil and insulin, Diabetes 37, 488.

Wheeler-Aceto, H. and A. Cowan, 1993, Naloxone causes apparent antinociception and pronociception simultaneously in the rat paw formalin test, Eur. J. Pharmacol. 236, 193.

Yaksh, T.L. and T.A. Rudy, 1976, Chronic catheterization of the spinal sub-arachnoid space, Physiol. Behav. 17, 1031.

Yamamoto, T. and T.L. Yaksh, 1991, Stereospecific effects of a nonpeptide NK1-selective antagonist, CP 96–345: antinociception in the absence of motor dysfunction, Life Sci. 49, 1955.

Yamamoto, T. and T.L. Yaksh, 1992, Comparison of the antinociceptive effects of pre- and post-treatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat, Anesthesiology 77, 757.