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Muscarinic receptor density is reduced in diabetic rat atria, an effect prevented by the aldose reductase inhibitor, Statil

A. KOFO-ABAYOMI, P. D. LUCAS*, *Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire LE11 3TU, UK*

Binding assays using [^{3}H]quinuclidinyl benzilate (QNB) as a muscarinic receptor ligand were carried out on atrial tissue from 6 control and 11 six-week streptozocin-diabetic rats. Five of the latter had received the aldose reductase inhibitor, Statil (25 mg kg^{-1} day^{-1} orally). Dissociation constants for specific (atropine displaceable QNB) binding were not changed either by diabetes or Statil treatment. Muscarinic receptor numbers, as estimated by B_{max} values, were, however, significantly depressed in tissues from the untreated diabetic group, compared with tissues from either the controls or the Statil-treated diabetic group. These results may explain the reduced sensitivity to muscarinic agonists previously observed in atria from similarly diabetic rats and indicate the involvement of the sorbitol pathway in the change. Possible mechanisms are discussed.

Autonomic neuropathy is a major secondary complication of diabetes (Clarke et al 1979). Defective parasympathetic control of the heart is one of the early changes which may be detected as a persistent tachycardia or loss of normal variation in beat-to-beat time interval during deep breathing (Wheeler & Watkins 1973; Lloyd-Mostyn & Watkins 1975; Feldman 1981). Abnormalities in vagal control of the heart have been observed in experimentally diabetic rats. An increased sensitivity to carbachol has been found in perfused hearts from 6, 8 and 12-month alloxan- or strepotozocin-diabetic rats (Vadlamundi & McNeill 1983). This change may represent denervation supersensitivity since a loss of cholinergic innervation accompanied by raised sensitivity to acetylcholine has been reported to occur in atria from 8-month alloxan-diabetic rats (Tomlinson & Yusof 1983). In contrast, cardiac sensitivity to cholinergic agents appears to be reduced in shorter term diabetic rats (Foy & Lucas 1976; Vadlamundi & McNeill 1983). Increased transmitter release has been suggested as being the cause of this change (Vadlamundi & McNeill 1983), although whether the change involves alterations in receptors or receptor–effector mechanisms has not been defined. The purpose of the present study was to determine whether muscarinic receptor changes occur in atria from short-term diabetic rats and, if they do, whether they are modified by a proposed treatment of diabetic neuropathy, aldose reductase inhibition. [^{3}H]Quinuclindinyl benzilate ([^{3}H]QNB) is a specific muscarinic receptor ligand (Birdsall & Hulme 1976) and was therefore used in this study.

* Correspondence.

Materials and methods

Experimental animals. Diabetes was induced in 11 male Wistar rats (200–250 g) by a single injection of 1 mL kg^{-1} of a freshly prepared solution of streptozocin (40 mg mL^{-1}, pH 4·5 citrate buffer) via a tail vein. Six controls received buffer alone. Three days after injection, glucose concentrations were assessed in a drop of blood, obtained by puncturing a tail vein with a sterile needle, using test strips (BM-test-BG; Boehringer Mannheim). All rats which had received streptozocin gave blood glucose readings of 22 m mol L^{-1} or above. From day four, 5 of the diabetic animals received by mouth 25 mg kg^{-1} Statil (2·5 mL kg^{-1} of a 10 mg mL^{-1} solution in Tween 80) daily. The other 6 diabetic and 6 control rats received oral Tween 80 only. Statil (ICI 128436; 3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazin-1-yl acetic acid) was a gift from Imperial Chemical Industries Pharmaceuticals Division.

Receptor assay. Six weeks after the tail vein injection the rats were weighed, anaesthetized with pentobarbitone (60 mg kg^{-1} i.p.) and the hearts removed and placed in ice-cold physiological saline. When contractions had ceased the atria were carefully trimmed free of the ventricles and other non-atrial tissue and frozen by immersion in liquid N_2. The tissues were subsequently stored at −20 °C. A 0·1 mL sample of blood was taken for blood glucose assay according to the method of Asatoor & King (1954).

Atria were homogenized in 30 volumes of ice-cold 50 mM Tris-HCl buffer, pH 8·0. Sufficient 4 M KCl was added to produce a 0·5 M KCl solution and, after cooling on ice for 10 min, the mixture was centrifuged at 49 000*g* for 10 min. The supernatant was discarded and the pellet resuspended in the same buffer, centrifuged and the supernatant again removed. The pellet was finally resuspended in 30 volumes of 50 mM Tris-HCl buffer, pH 8. Aliquots of this suspension were incubated at 37 °C for 60 min with increasing concentrations of [^{3}H]QNB (30 Ci mmol^{-1} solution Amersham, UK and diluted with 5 mM HCl) with or without 1 mol L^{-1} atropine sulphate. At the end of the incubation period the samples were rapidly filtered under vacuum using Whatman GF/C filter papers. These were then washed twice with 3 mL of the ice-cold 5 mM Tris-HCl buffer and transferred to scintillation vials containing 5 mL of scintillation fluid. The vials were shaken for 1 h and the radioactivity counted (LKB, Rackbeta, liquid scintilla-

tion counter) at an efficiency of approximately 45%. Specific binding was calculated for each [^{3}H]QNB concentration from the difference in radioactivity between those tubes containing atropine and those that did not. B_{max} and K_D values were obtained by standard Scatchard analysis. B_{max} values were expressed as fmol (mg protein)$^{-1}$. Protein concentrations of the tissue suspensions used in the binding assay were measured by the method of Lowry et al (1951) using bovine serum albumin as the standard.

One-way analysis of variance was carried out on data from the three groups. Where P values of 0·05 or less were obtained then differences between individual means were assessed by Duncan's multiple comparisons.

Results

These are presented in Table 1. Diabetes was confirmed in all animals that had received streptozocin as they had raised blood glucose concentrations and low (negative in most cases) growth rates compared with controls. Statil had no effect on these measurements. [^{3}H]QNB binding studies revealed reduced B_{max} values in atrial tissue from the untreated diabetic animals compared with those obtained in tissue from either control or Statil-treated diabetic animals. K_D values were similar from tissue from all three groups. Weights also appeared to be unchanged by either diabetes or Statil treatment.

Table 1. Experimental data from control and six-week streptozocin-diabetic rats with or without Statil treatment.

	Controls (6)	Diabetic (6)	Diabetic + Statil (5)
Blood glucose concentration (mmol L^{-1})	4·34 ± 1·84^c	28·36 ± 5·95	27·05 ± 5·48
Weight gain over 6 weeks (g)	78·30 ± 35·54^c	−4·33 ± 12·10	−14·33 ± 28·42
[^{3}H]QNB binding:			
B_{max}, (fmol (mg protein)$^{-1}$)	318·3 ± 70·14^b	214·3 ± 36·0	287·5 ± 14·4^a
K_D (pmol)	34·47 ± 4·70	30·81 ± 6·27	33·47 ± 9·21

Data are means ± standard deviation, with numbers per group in parentheses. Significances of differences from the untreated diabetic group (Duncan's multiple range tests) are indicated by: ^{a} $P < 0·05$, ^{b} $P < 0·01$, ^{c} $P < 0·001$.

Discussion

The present study demonstrated a reduction in atrial muscarinic receptor density, as defined by [^{3}H]QNB binding, in six-week untreated streptozocin-diabetic rats. K_D values for the receptor–ligand interaction appeared not to be changed. These results may explain previous reports of reduced cardiac sensitivity to muscarinic agents in short-term alloxan- and streptozocin-induced diabetes in rats (Foy & Lucas 1976; Vadlamundi & McNeill 1983). Baroreceptor reflexes have been reported to be more active in 12-week streptozocin-diabetic rats by Chang & Lund (1986). Vagal nerve stimulation has been found by Stuesse et al (1982) to result in a more pronounced bradycardia in 7- to 9-week alloxan-diabetic rats. These two reports suggest that there may be excessive exposure of atrial tissue to acetylcholine in the earlier stages of diabetes. Acute (3 h) exposure of chicken cultured heart cells to high concentrations of muscarinic agonists has been shown to decrease muscarinic receptor number as measured by [^{3}H]QNB binding (Galper & Smith 1980). A compensatory decrease in atrial muscarinic receptor number in response to increased exposure to acetylcholine may, thus, have occurred in the untreated diabetic rats used in the present study. The aldose reductase inhibitor, Statil, appeared to prevent this change. A metabolic effect of diabetes which is prevented by aldose reductase inhibition, is a depression of neuronal Na^+,K^+-ATPase, possibly via a reduction in intracellular myo-inositol concentrations (Green & Lattimer 1985).

Acute inhibition of Na^+,K^+-ATPase has been shown to increase the turnover of both acetylcholine and noradrenaline (Vizi et al 1982). The reduced atrial muscarinic receptor density observed in the present study and the reduced cardiac sensitivity to cholinergic agents observed previously in short-term diabetic rats may, thus, be due to an increased release of acetylcholine by vagal nerve endings. The increased vagal activity may, in turn, be a consequence of Na^+,K^+-ATPase depression and be reversible by aldose reductase inhibition.

The importance of the above to longer term vagal neuropathy requires further study.

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Lymphoid tissue responses to a novel perfluorochemical emulsion in rats*

A. D. BOLLANDS, K. C. LOWE†, S. K. SHARMA‡, S. S. DAVIS‡, *Departments of Zoology and ‡Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, UK*

The effects of a novel perfluorochemical emulsion on rat lymphoid tissues and antibody production against sheep red blood cells (SRBC) have been studied. The responses were compared with those following injection of identical doses of the proprietary emulsion, Fluosol-DA 20% (F-DA). Liver weight was increased up to 15% at 8 days following intravenous (i.v.) or intraperitoneal (i.p.) injection of the novel emulsion but was unaffected by F-DA injection. Spleen weight also increased by a maximum of 20% in response to i.p. injection of the novel emulsion but this was less than increases of up to 44% which occurred in F-DA-injected rats. Thymus weight decreased ($P < 0.05$) following i.p. injection of the novel emulsion whereas mesenteric lymph node (MLN) weight remained unchanged. However, MLN weight was increased in response to i.v. injected F-DA, while thymus weight showed a small increase following i.p. F-DA injection. Mean plasma antibody titres to SRBC were significantly ($P < 0.01$) increased at 7 days after immunization in rats pretreated with i.p. injections of either the novel emulsion or F-DA; titres in animals pretreated with i.v. injections of either emulsion were similar to control.

Emulsified perfluorochemicals (PFCs) have properties which make them attractive as physiological oxygen-transport fluids. Such properties include: the ability to dissolve substantial volumes of oxygen and other respiratory gases; and small particle sizes (<0·25 μm) which enable them to pass readily through capillary beds. In addition, due to the strength of the carbon–fluorine bond (ca 116 kcal mol^{-1} kJ mol^{-1}), PFC molecules are generally regarded as being both chemically and biologically inert (Riess & Le Blanc 1982). Some of the physiological effects of emulsified PFCs have been studied in several species, and also in experiments using cells in culture (Lowe & Bollands 1985; Lowe 1986, 1987).

A commercial emulsion, Fluosol-DA 20% (F-DA; Green Cross, Japan), which contains perfluorodecalin (FDC) and perfluorotripropylamine (FTPA) emulsified with the poloxamer surfactant, Pluronic F-68, has been tested in human trials in several countries (Mitsuno et al 1982; Tremper et al 1982; Waxman et al 1984; Stefaniszyn et al 1985; Gould et al 1986). This emulsion is also being evaluated for possible therapeutic uses related to tumour oxygenation (Lustig & McIntosh 1986) and perfusion of ischaemic tissues (Forman et al 1985). However, one inherent problem with F-DA is its tendency for destabilization on storage, resulting in increased particle size (Riess & Le Blanc 1982). This effect is minimized by storage of the stem emulsion component of F-DA in a frozen state, as recommended by the manufacturers (Naito & Yokoyama 1978).

The major cause of droplet growth in emulsions is coalescence, but this can normally be retarded using emulsifying agents which form electrostatic and mechanical barriers at the oil-water interface. However, a more subtle means of instability can occur by a process of molecular diffusion known as Ostwald ripening and this can occur even if particles have excellent barriers to coalescence (Davis et al 1981).

We have recently reported the development and preliminary physicochemical assessment of novel compositions of emulsified PFCs for possible biological uses related to oxygen transport (Davis et al 1986; Sharma et al 1986). The new emulsions were based on FDC and contained small quantities of polycyclic, perfluorinated, higher boiling point oil (HBPO) additives to stabilize against instability caused by Ostwald ripening. Since there have been no biocompatibility studies using these preparations, the present experiments were undertaken to investigate the effects of one such emulsion on lymphoid tissues and antibody production against an antigenic 'challenge' in the form of sheep red blood cells (SRBC) in rats. A particular objective was to compare the responses with those following injection of identical doses of F-DA. A preliminary report of some of these results has already been published (Bollands et al 1986).

Materials and methods

Care of animals and experimental procedures. Female Wistar rats (140–160 g, n = 37) were used. They were maintained in the laboratory animal house under controlled conditions (13 h light, 11 h dark; temperature 24 ± 1 °C) and had free access to a standard food

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† Correspondence.