Streptozotocin-induced diabetes elicits the phosphorylation of hepatocyte $G_{i2}\alpha$ at the protein kinase C site but not at the protein kinase A-controlled site

Nicholas J. MORRIS*, Mark BUSHFIELD† and Miles D. HOUSLAY‡

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Davidson Building, Institute of Life and Biomedical Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

Streptozotocin-induced diabetes caused a profound increase in the steady-state level of phosphorylation of the α -subunit of the adenylate cyclase inhibitory protein G_{i2} in hepatocytes. Unlike hepatocytes from control animals, those from streptozotocin-diabetic animals showed no increase in the phosphorylation of $G_{i2}\alpha$ in response to a challenge with the protein kinase C activator phorbol myristate acetate. However, a stimulatory effect of 8-bromo-cAMP on $G_{i2}\alpha$ phosphorylation was evident in hepatocytes from diabetic animals but this was severely reduced compared with that observed in hepatocytes from normal animals. Two-dimensional tryptic phosphopeptide mapping showed that $G_{i2}\alpha$ in resting hepatocytes from diabetic animals was phosphorylated exclusively at the protein kinase C site (C-site) but no labelling was evident at the protein kinase A-regulated site (AN-site). Treatment of hepatocytes from diabetic animals with phorbol myristate acetate did not change this

pattern of labelling. In contrast, challenge of hepatocytes from diabetic animals with 8-bromo-cAMP led to the appearance of a new labelled phosphopeptide that was consistent with labelling at the AN-site. Analysis of the C-site and AN-site phosphopeptides from hepatocytes of diabetic animals treated with 8-bromo-cAMP showed that the increase in labelling of $G_{i2}\alpha$ caused by this ligand could be attributed almost entirely to labelling at the AN-site. Thus streptozotocin diabetes appears to cause enhanced labelling of hepatocyte $G_{i2}\alpha$ by exclusively increasing phosphorylation at the C-site. It is suggested that the increased labelling at the C-site reflects an augmentation of the protein kinase C signalling system in hepatocytes from streptozotocin-induced diabetic animals. This may have widespread functional consequences for these cells and may result either from an increased protein kinase C activity and/or a reduction in protein phosphatase 1 and/or 2A activity.

INTRODUCTION

Insulin and glucagon exert antagonist effects on liver glucose metabolism [1,2]. This is thought to be mediated by the opposing effects they exert on levels of the second messenger cAMP [1,2]. Glucagon elevates cAMP levels by stimulating adenylate cyclase activity [1–4], whereas insulin decreases levels by inhibiting adenylate cyclase and activating cAMP-specific phosphodiesterase activities (see refs. [5,6] for reviews).

Adenylate cyclase is under the dual control of the stimulatory G-protein G_s and the inhibitory G-protein G_i in hepatocytes [7]. Activation of protein kinase C causes the phosphorylation of the α -subunit of this G-protein [8,9] and this is thought to occur on Ser¹⁴⁴ (C-site) [10]. Phosphorylation of $G_{i2}\alpha$ in hepatocytes [7–11] and other cells [12–16] appears to cause loss of GTP-dependent inhibition of adenylate cyclase. This effect, however, is not observed in all cell types and may be a cell-specific phenomenon. It has been suggested that hepatocyte G_{i2} is controlled by a futile cycle of phosphorylation and dephosphorylation involving protein kinase C and protein phosphatase activity [7,17]. Thus either stimulation of protein kinase C or inhibition of protein phosphatase activity may inhibit the functioning of G_{i2} . Phosphorylation of $G_{i2}\alpha$ also occurs at a

second site, suggested to be Ser²⁰⁷ (AN-site), which results upon elevation of cAMP levels [10].

Streptozotocin treatment induces type-I insulin-dependent diabetes in rats (see ref. [18] for a review). This changes the functioning of a plethora of signalling systems, including hepatocyte adenylate cyclase activity (see, e.g. refs. [19,20]), G-protein functioning [21–25] and protein kinase C expression [26]. Streptozotocin-induced diabetes causes a reduction in the level of G_{i2} in hepatocytes [27] but not in whole liver [27,28] because of compensatory changes occurring in the non-parenchymal cells. Such chemically induced diabetes also causes the abolition of GTP-mediated G_i functioning and an increase in protein kinase C activity [26]. We show here that, in streptozotocin-diabetic animals, the C-site of the α -subunit of G_{i2} becomes fully phosphorylated without any concomitant phosphorylation at the AN-site.

MATERIALS AND METHODS

Materials

[³²P]P_i was obtained from Amersham International. Phorbol 12-myristate 13-acetate (PMA) was from Cambridge Bioscience. Hormones and Protein A-agarose were from Sigma. Cellulose

Abbreviations used: G_i , inhibitory G-protein controlling adenylate cyclase activity; G_s , stimulatory G-protein controlling adenylate cyclase activity; PMA, phorbol 12-myristate 13-acetate; DNP, dinitrophenyl.

* Present address: Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756, U.S.A.

† Present address: Pfizer Central Research, Sandwich, Kent, U.K.

‡ To whom correspondence should be addressed.

TLC plates were from Eastman Kodak Company, Rochester, NY, U.S.A. Tos-Phe-CH₂Cl ('TPCK')-treated trypsin, α -chymotrypsin and *Staphylococcus aureus* V8 protease were obtained from Lorne Laboratories Ltd., Reading, Berks., U.K. Okadaic acid was from Mona Bioproducts, Honolulu, Hawaii, U.S.A. All other biochemicals were from Boehringer, and all other chemicals were of AR grade from BDH.

Hepatocyte preparation and labelling conditions

Hepatocytes were prepared as previously described [10] using 220–250 g fed male Sprague–Dawley rats. Cells (10^6 – 10^7 cells/ml) were preincubated for 65 min at 37 °C in Krebs–Henseleit buffer [10] supplemented with 2.5% (w/v) BSA, 2.5 mM CaCl₂ and 10 mM glucose and containing 50 μ M potassium phosphate and 1 mCi of [³²P]P_i. Cells were gassed with O₂/CO₂ (19:1, v/v) for 30 s every 10 min. Ligands were added in less than 1% of the total incubation volume and, after an appropriate time, the reactions were stopped by the addition of 10 vol. of ice-cold Krebs–Henseleit buffer [10]. The cells were harvested by centrifugation (100 g; 2 min).

Immunoprecipitation of G_i2 α

This was performed as described previously in some detail [10]. Briefly, a pellet of cells (10^6 /ml) was extracted by the addition of 1 ml of a buffer containing 1% (v/v) Triton X-100, 10 mM EDTA, 100 mM NaH₂PO₄, 100 μ M Na₃VO₄, 2 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 nM okadaic acid and 50 mM Hepes, pH 7.2. After 1 h at 4 °C, unsolubilized material was removed by centrifugation (14000 g; 10 min; 4 °C). Labelled G_i2 α was immunoprecipitated using antiserum 1867, the specificity of which for G_i2 α has been discussed in detail elsewhere [10]. Immunoprecipitation of G_i2 α was detected by subsequent immunoblotting for the presence of an approx. 40 kDa phosphorylated species. Gel electrophoresis and autoradiography and the detection, excision and analysis of labelled bands were carried out as described in detail previously by us [10]. Protein analyses were done by the method of Laemmli [29].

Elution of ³²P-labelled G_i2 α from polyacrylamide gels and the separation of ³²P-labelled enzymic cleavage products

Tryptic phosphopeptide analyses were performed exactly as described in Morris et al. [10]. In essence, G_i2 α was immunoprecipitated and then subjected to SDS/PAGE with subsequent autoradiography to identify the 40 kDa band of G_i2 α . The autorad was then used to locate G_i2 α on the polyacrylamide gel slab before its elution as described previously [10]. The eluted protein was then treated with trypsin, digestion being carried out under one of two conditions [10]. Briefly, the 'higher-temperature' condition involved digestion for 8 h at 37 °C before the addition of a further 10 μ l of protease and incubation continued for 16 h. The 'lower-temperature' digestion with trypsin was performed at 30 °C for periods of 6 h and 16 h. A sequence of lyophilization and resuspension was then carried out to remove residual NH₄HCO₃. The product of the final lyophilization was then subjected to electrophoresis at pH 1.9 followed by ascending chromatography in butanol/pyridine/acetic acid/water (15:10:3:12, by vol.). This was performed in the presence of marker dyes ϵ -dinitrophenyl (DNP)-lysine (positive charge), DNP-DL-glutamic acid (neutral charge) and xylene cyanol FF (negative charge). The labelled peptides were identified by autoradiography [10].

RESULTS

As described previously [10], we were able to immunoprecipitate phosphorylated G_i2 α from hepatocytes of streptozotocin-induced diabetic rats (results not shown here) and confirm that the level of labelling of G_i2 α was not increased by challenging these cells with the phorbol ester, PMA (Table 1). This was in contrast with the marked increase observed in hepatocytes from normal animals [10]. In addition, we noted here that treatment of hepatocytes from diabetic animals with the protein phosphatase 1/2A inhibitor, okadaic acid [30], did not increase the labelling of G_i2 α (Table 1), but did elicit a profound increase in hepatocytes from normal animals [17]. A challenge with 8-bromo-cAMP did, however, increase the labelling of G_i2 α (Table 1), although this effect was considerably smaller than that observed using hepatocytes from normal animals (Table 1).

Hepatocyte G_i2 α is much more highly phosphorylated in diabetic animals than in normal animals [27]. In order to understand the basis of this we undertook a two-dimensional tryptic phosphopeptide analysis using lower- and higher-temperature trypsin digestion strategies [10]. These allow identification of the single protein kinase C site [10] as two closely migrating phosphopeptides (C1, C2) in the lower-temperature digestion and three peptides (C1, C2, C3) in the more exhaustive higher-temperature digestion [10]. The protein kinase A-controlled phosphorylation site is recognized as a single phosphopeptide, called the AN-peptide [10] using both of these digestion strategies.

In [³²P]P_i-loaded hepatocytes from streptozotocin-diabetic animals incubated under basal conditions, two-dimensional tryptic phosphopeptide analysis of immunoprecipitated G_i2 α showed that only the labelling of the C group of peptides occurred (Figures 1a and 1b). This was apparent using either the lower- or higher-temperature digestion strategy. In no instance did we obtain evidence indicating labelled AN-peptide. Thus the increase in labelling of G_i2 α in diabetic animals is due exclusively to phosphorylation at the C-site.

The pattern of labelling seen using hepatocytes from diabetic animals, incubated under basal conditions, was not altered by challenging the cells with the protein kinase activator PMA (Figures 1c and 1d). Furthermore, little, if any (< 8%; $n = 4$), change in the degree of labelling of the C group of peptides occurred.

Treatment of hepatocytes of diabetic animals with 8-bromo-cAMP did, however, lead to the appearance of labelled AN-peptide (Figures 1e and 1f) but did not alter (< 7%; $n = 4$) the

Table 1 Labelling of G_i2 α in hepatocytes from streptozotocin-induced diabetic rats

Hepatocytes from normal and streptozotocin-induced diabetic rats were used. Untreated cells were analysed (none), as were cells that had been challenged for 15 min with 10 ng/ml PMA, those treated for 15 min with 300 μ M 8-bromo-cAMP and those treated for 20 min with 100 nM okadaic acid. Results are means $\pm$ S.D. for four separate experiments. The amount of G_i2 α in samples were determined by quantitative immunoblotting as described previously [10,17].

Ligand	Labelling (mol of ³² P/mol of G _i 2 α)	
	Normal	Diabetic
None	0.41 $\pm$ 0.08	0.98 $\pm$ 0.07
PMA	0.59 $\pm$ 0.04	1.04 $\pm$ 0.09
8-Bromo-cAMP	0.97 $\pm$ 0.10	1.36 $\pm$ 0.11
Okadaic acid	1.1 $\pm$ 0.08	1.05 $\pm$ 0.09

Figure 1 Two-dimensional tryptic phosphopeptide maps of hepatocyte G_i2 α

Hepatocytes from streptozotocin-induced diabetic rats were incubated to isotopic equilibrium with [³²P]P_i. Where indicated they were then challenged with ligands. They were then harvested and solubilized for the immunoprecipitation of G_i2 α with subsequent SDS/PAGE to localize G_i2 α and then trypsin digestion under the lower- or higher-temperature conditions to allow for the two-dimensional resolution of labelled phosphopeptides by autoradiography (see the Materials and methods section and ref. [10]). The results of such a final autoradiography are shown for typical experiments using the higher- (a, c, e) and lower- (b, d, f) temperature strategies. Shown is the origin for sample application, the solvent front (SF), direction of electrophoresis (+/-) and the DNP-lysine marker. All experiments show data for animals made diabetic with streptozotocin. Untreated cells (a, b) were analysed, as were cells that had been challenged for 15 min with 10 ng/ml PMA (c, d) or for 15 min with 300 μ M 8-bromo-cAMP (e, f).

degree of labelling of the C group of peptides. Thus the increase in labelling of hepatocyte G_i2 α from diabetic animals on challenge with 8-bromo-cAMP (Table 1) must be due, almost exclusively, to the novel labelling of the AN-site.

DISCUSSION

Streptozotocin-induced diabetes causes profound changes in signalling systems. In the case of the inhibitory G-protein G_i2, both protein [21,27] and transcript [27] levels fall with residual G_i2 α showing an increased level of phosphorylation compared with that seen in hepatocytes of normal animals [27]. The functional consequences of such changes in hepatocyte G_i2 α are an abolition of GTP-elicited G_i inhibition of adenylate cyclase and a corresponding increase in glucagon-stimulated adenylate cyclase activity [21,27].

We have shown that G_i2 α can be phosphorylated at two sites [10]. Phosphorylation at the C-site is elicited through protein kinase C action and that at the AN-site occurs concomitantly with elevation of cAMP levels [10]. In order to define the site of the increased labelling of G_i2 α seen in the hepatocytes of streptozotocin-diabetic animals, we undertook a two-dimensional tryptic phosphopeptide analysis of G_i2 α . This analysis identified (Figure 1) a phosphopeptide pattern that was identical with that found in the hepatocytes of both normal animals and those treated with agents that activated protein kinase C [10], i.e. the only labelled species observed were the G-group of peptides (Figure 1). This was apparent irrespective of whether the higher- or lower-temperature tryptic phosphopeptide analytical procedures were employed (Figure 1). The only

difference between hepatocyte G_i2 α from the normal and streptozotocin-diabetic animals was the amount of label incorporated at the C-site [12]. This indicates that streptozotocin-induced diabetes perturbs exclusively labelling at the C-site of G_i2 α . We have suggested that phosphorylation of the C-site of G_i2 α may be modulated by a futile phosphorylation-dephosphorylation cycle in hepatocytes [7,17]. Thus increased labelling may be due to either enhanced protein kinase C activity or decreased protein phosphatase activity. In this regard we have noted profound changes in expression of protein kinase C isoforms in hepatocytes from streptozotocin-diabetic rats [26]. This may reflect a more general perturbation, as others have noted diabetes-induced changes in protein kinase C expression in other organs and tissues (see, e.g., refs. [31–34]). In addition, reduced levels of protein phosphatase 1 have been noted in the livers of diabetic mice [35] and in the muscle of diabetic humans [36]. Thus, if diabetes results in lowered protein phosphatase levels in the hepatocytes of streptozotocin-diabetic animals, this might also be expected to increase the level of phosphorylated G_i2 α in a similar fashion to that elicited by the protein phosphatase inhibitor okadaic acid [17]. The selective action of protein phosphatase inhibition in modulating labelling at the C-site, but not the AN-site [17], appears to be because the basal activity of protein kinase A is too low to sustain cycling of phosphatase at this site [7]. That the increase in labelling was restricted to the C-site would be consistent with our earlier studies [27] which indicated a stoichiometry of labelling of approx. 1.2 for G_i2 α from streptozotocin-diabetic hepatocytes compared with approx. 0.33 for normal animals, implying modification of the C-site in the entire G_i2 α pool of hepatocytes from diabetic animals.

Streptozotocin-induced diabetes did not elicit $G_{i2\alpha}$ labelling of the protein kinase A-regulated AN-site but neither did it prevent labelling occurring in hepatocytes challenged with 8-bromo-cAMP. However, challenging hepatocytes from diabetic animals with 8-bromo-cAMP led to an increase in $G_{i2\alpha}$ labelling which was much smaller (Table 1) than that found using hepatocytes from normal animals. This was because, unlike in normal animals where 8-bromo-cAMP increased labelling at both the C- and AN-sites [10], the increase in labelling of hepatocytes from diabetic animals was due entirely to modification at the AN-site. Such observations are consistent with streptozotocin-induced diabetes having selectively caused phosphorylation of the C-site of $G_{i2\alpha}$.

Our results suggest that a perturbation of the phosphorylation of protein kinase C substrates may characterize streptozotocin-induced diabetes in hepatocytes. As there is much evidence to suggest that protein kinase C can modify the functioning of a plethora of signal-transduction systems (see, e.g., ref. [37]), this is likely to lead to pleiotropic effects. Such changes may have relevance to our understanding of the molecular pathology associated with diabetes and the clinical complications associated with this condition.

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