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Functional alterations of G-proteins in diabetic rat retina: a possible explanation for the early visual abnormalities in diabetes mellitus

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Summary. We examined changes in guanosine triphosphate-dependent signal transduction mechanisms in the retina from the early stages of the streptozotocin-diabetic rat, a model for Type 1 (insulin-dependent) diabetes mellitus. Guanosine triphosphate binding, guanosine triphosphatase activity, and binding of (azido) guanosine triphosphate decreased significantly in the retina as early as 2 weeks after the induction of diabetes. The ability of guanosine triphosphate to inhibit forskolin-stimulatable adenyl cyclase was also abolished. These data suggest functional deterioration of G-proteins, especially Gi, in diabetic retina. Further studies using retinal rod outer segments revealed deterioration in light-sensitive, guanosine triphosphate-dependent functions of transducin in diabetic rats. Pertussis toxin-catalysed ADP ribosylation of the α subunit of transducin, a heterotrimeric G-protein of rod outer segments, was also reduced in diabetes. No functional effects were seen in purified subunits of transducin subjected to non-enzymatic glycation in vitro. On the other hand, incubation of non-diabetic rod outer segments with (12-0-tetradeconyl) phorbol-13-acetate, a protein kinase C agonist, in the presence of magnesium and adenosine triphosphate resulted in the reduction of guanosine triphosphate-binding and hydrolysis, thus indicating that protein kinase C may be involved in the regulation of these activities. The significance of these observations in the early visual abnormalities associated with diabetes is discussed.

Key words: Diabetes mellitus, retina, rod outer segments, GTP-binding proteins, visual abnormalities.

A growing body of experimental evidence indicates that guanosine triphosphate (GTP)-dependent signal transduction mechanisms play a crucial role in biological processes [1, 2], including insulin secretion [3] and vision [4, 5]. The GTP-dependent signal transduction cascade is comprised of three functional components: receptor for signal acquisition, G-protein for signal amplification, and effector for signal expression. Acquisition of signal by the receptor catalyses the exchange of GTP for bound guanosine diphosphate (GDP) on the α subunit, and the complex of GTP and α subunit complexed with GTP activates the effector system [1, 2, 6, 7]. Although there is growing evidence to suggest the existence of other G-proteins, such as the small molecular weight and large molecular weight G-proteins [8, 9], only the heterotrimeric G-proteins have been extensively studied so far. Despite recent progress in understanding the role of G-proteins in signal transduction phenomena, their metabolism in pathological states such as diabetes mellitus is relatively not well understood [10]. Gawler et al. [11] were the first to document functional impairment of G-proteins in hepatocytes from a diabetic rat. More recently, Green and Johnson [12] described a similar defect in diabetic adipocytes and documented reversal of this lesion by treating diabetic animals with insulin, suggesting that insulinoprevia or hyperglycaemia or both may cause such a lesion.

It is well established that diabetic retinopathy is the leading cause of blindness in human diabetic patients. The phenomenon of diabetic retinopathy involves the interplay of various biochemical, endocrine, and haemodynamic factors, with capillary damage being the most prominent pathological lesion [13]. However, several visual abnormalities have been reported in diabetic humans even before capillary damage takes place [14–17].

The present study was undertaken to quantitate early changes in GTP-dependent signal transduction mechanism(s) in retina and rod outer segments (ROS) from the streptozotocin-diabetic rat, a model for Type 1 (insulin-dependent) diabetes. In addition, evidence is also presented to explain the possible mechanism(s) by which hyperglycaemia may change the G-protein metabolism of the retina in the early stages of diabetes.

Materials and methods

Guanosine-5-0-(3-thiotriphosphate) (GTPγS) [^{35}S] (1000 Ci/nmol), cGMP [^{3}H] (20 Ci/nmol), ATP [^{32}P] (3000 Ci/nmol), GTP [^{32}P] (50 Ci/mmol), NAD [^{32}P] (800 mCi/mmol), and glucose [^{14}C] (11.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, Mass., USA), and 8-N3 [^{32}P] GTP (5.8 mCi/mmol) was from ICN Radiochemicals (Costa Mesa, Calif., USA). Forskolin and TPA (12-0-tetradeconyl phorbol-13-acetate), and adenosine-5′-(β-γ-imido)triphosphate (App(NH)p) were purchased from Calbiochem (San Diego, Calif., USA). Glycogel-B was from Pierce (Rockford, Ill., USA), and pertussis toxin was obtained from List Biological Labs (Campbell, Calif., USA). All other reagents employed in the study were of analytical grade.

Animals and induction of diabetes

Male Sprague-Dawley rats (150–170 g) were fasted overnight, and streptozotocin was administered intraperitoneally (60 mg/kg body weight) as described previously [18, 19]. The animals were allowed free access to water and chow. They were housed in a light-cycled room and cared for according to National Institutes of Health and Institutional Animal Care and Use Committee guidelines. The degree of hyperglycaemia was monitored by quantitating the plasma glucose using o-toluidine reagent [18]. Insulin was administered subcutaneously (2 IU/day) to a group of diabetic animals ($n = 6$) to study the preventive effects of insulin therapy in the development of diabetes-associated lesions in GTP-dependent functions. Insulin treatment of diabetic rats significantly reduced the plasma glucose; almost to those of non-diabetic rats ($\leq$ 5 mmol/l in non-diabetic and insulin-treated diabetic rats vs $\leq$ 16.7 mmol/l in diabetic rats).

Isolation of retina and ROS

Animals were dark-adapted for 15 h before being killed by decapitation; retinae were removed using a magnifying glass under a dim red light. Retinal homogenates were prepared in a glass homogenizer containing 100 mmol/l Tris-HCl, pH 7.4, 5 mmol/l dithiothreitol (DTT), and 5 μmol/l each of leupeptin and pepstatin. These retinal preparations were found to be free from erythrocytes, originating from retinal capillaries, as confirmed by haemoglobin measurements in these preparations [19]. ROS were prepared by sucrose density gradient centrifugation as described by O'Brien et al. [20]. They were homogenized in 50 mmol/l Tris-HCl, pH 7.4, containing 40% (weight/volume, w/v) sucrose, 65 mmol/l NaCl, 2 mmol/l $MgCl_2$, 0.1 mmol/l phenyl methyl sulphonyl fluoride and 5 μmol/l each of leupeptin and pepstatin.

Biochemical assays in retina and ROS

GTPγS binding. GTPγS binding was measured according to a procedure described by Yamazaki et al. [4, 5]. The assays were carried out in a final volume of 100 μl containing 50 mmol/l Tris-HCl, pH 7.4, 5 mmol/l $MgSO_4$, 1 mmol/l EGTA, and retinal or ROS membranes. Reaction was initiated by the addition of 1 μmol/l GTPγS [^{35}S]. After incubation at 4°C for 30 min, 80 μl of the reaction mixture was applied to a Millipore filter (HA, pore size 0.45 μm; Millipore, Boston, Mass., USA) pre-soaked in washing buffer (10 mmol/l Tris-HCl, pH 7.4, containing 1 mmol/l EGTA and 5 mmol/l $MgSO_4$). Filters were washed four times with (4 ml each) of washing buffer, and the radioactivity on the filters was determined by scintillation spectrometry using Formula 963 (New England Nuclear, Boston, Mass., USA). Non-specific binding was determined in the presence of a 100-fold excess of unlabelled GTPγS. Activity was expressed as pmols of GTPγS bound per mg protein.

GTPase activity. GTPase activity was measured as described by Yamazaki et al. [4, 5] with minor modifications. Enzyme reaction was carried out in a final volume of 100 μl containing 10 mmol/l Tris-HCl, pH 7.5, 1 mmol/l DTT, 5 mmol/l $MgCl_2$, and 110 mmol/l NaCl. The reaction was initiated by the addition of 2 μmol/l [^{32}P]GTP and was carried out at 33°C for 10 min. Optimization experiments showed that the activity was linear up to 10 μg protein and for a 20-min incubation period. ^{32}Pi released was quantitated after precipitating the protein with trichloroacetic acid (TCA) and adsorbing the unhydrolysed nucleotide onto charcoal. Activity was expressed as pmols of ^{32}Pi released per mg protein per min.

Adenylyl cyclase activity. Adenyl cyclase activity was measured in a total volume of 100 μl containing 50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 5 mmol/l $MnCl_2$, 2.5 mmol/l isobutyl methyl xanthine, 15 mmol/l creatinine phosphate, 50 μg/ml creatinine kinase, 0.8 mmol/l [^{32}P]ATP, and 1 mmol/l cAMP. [^{3}H]cAMP (0.1 mCi) was added as an internal standard. Reaction was carried out for 15 min at 37°C at the end of which 20 μl of 1 mol/l HCl was added. cAMP was separated on neutral alumina and AG1X2 (BioRad, Richmond, Calif., USA) as described by Birnbaumer et al. [21]. The recovery of cAMP was about 80% by this method.

To study the effects of light on GTP-dependent functions in ROS, the assays were carried out under a dim red light or in ambient room light.

Photoaffinity labelling. Photoaffinity labelling was carried out as described by Yada et al. [22]. In brief, the reaction mixture consisted of 20 mmol/l 3-(N-morpholino) propane sulphonic acid (MOPS) buffer, pH 7.5, 1 mmol/l EDTA, 100 mmol/l NaCl, 25 mmol/l $MgCl_2$, 1 mmol/l App(NH)p (non-hydrolysable analogue of ATP), 10 μmol/l 8-N3 [^{32}P]GTP and 20–30 μg non-diabetic and diabetic retinal protein. The reaction was carried out in the presence or absence of 50 μmol/l GTP to study the specificity of the reaction. The mixture was incubated in the dark for 10 min at 4°C and then irradiated for 10 min with an ultraviolet lamp (Mineral light; 254 nm) from a distance of 1 cm. The labelled proteins were separated on 6.5–16.5% SDS-PAGE and identified by autoradiography as we described previously [23, 24].

ADP ribosylation of transducin. Pertussis toxin-mediated ADP ribosylation of α subunit of transducin, a retinal ROS G-protein, was carried out as described by Yamazaki et al. [4, 5]. In brief, the reaction mixture in a total volume of 50 μl consisted 8 mmol/l MOPS buffer, pH 7.4, 35 mmol/l NaCl, 50 mmol/l KCl, 2 mmol/l DTT, 25 μg/ml pertussis toxin (preactivated in the presence of ATP and DTT) and 20–30 μg ROS proteins from non-diabetic and diabetic rats. The reaction was initiated by the addition of 10 μmol/l [^{32}P]NAD, and carried out at 33°C for 30 min. It was terminated by the addition of Laemmli buffer [25], and the contents of the tubes were heated at 80°C for 5 min. The labelled proteins were separated on SDS-PAGE, and the magnitude of radiolabelling was quantitated by autoradiography. As a control, ROS proteins were incubated with [^{32}P]NAD in the absence of pertussis toxin to determine the specificity of the reaction.

Non-enzymatic glycation (NEG) of retinal proteins in diabetes: effects of in vitro NEG of purified subunits of transducin on its function

NEG of retinal proteins. The degree of NEG of retinal proteins from non-diabetic and diabetic rats was quantitated by glycogel-B affinity chromatography as we described earlier [18]. Retinal proteins were applied to the glycogel-B column and the glycated (bound) form of the proteins was eluted from the column using a buffer containing sorbitol. Details of the method utilized in this study have been described elsewhere [18]. The amounts of proteins in the bound and unbound fractions were measured according to Bradford [26].

NEG of purified subunits of transducin. Subunits of transducin (α/β-γ), a heterotrimeric G-protein of ROS were purified from frog ROS as described by Yamazaki et al. [27]. NEG was carried out under sterile conditions as we previously described [28, 29]. In brief, the

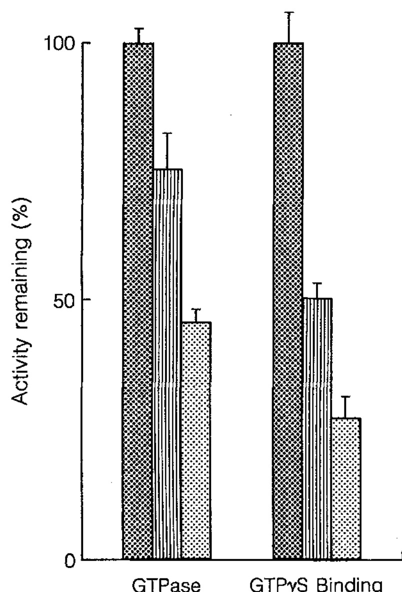

Fig. 1. Guanosine-5-0-(3-thiotriphosphate) (GTPγS) binding and guanosine triphosphate (GTP)ase activities in non-diabetic and diabetic retinal homogenates. GTPγS binding and GTPase activities were measured in retinal homogenates from rats with diabetes of 2 and 7 weeks duration. Data were compared with the values obtained from non-diabetic animals taken as 100%. The basal activities for these parameters were: 240 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein for GTPase activity and 410 pmol/mg protein for GTPγS binding. Data are mean ± SEM obtained from three different rats in each group, [▩] control, [▥] 2 weeks diabetes, and [▨] 7 weeks of diabetes

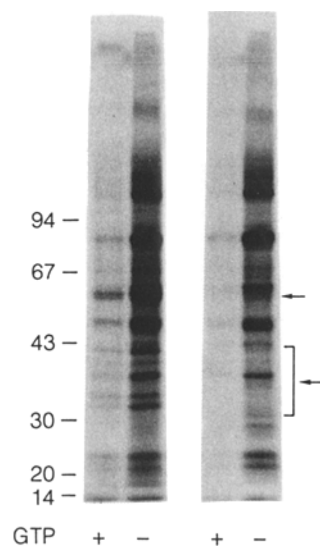

Fig. 2. Photoaffinity labeling of guanosine triphosphate (GTP)-binding proteins in non-diabetic and diabetic retina. Photoaffinity labelling of GTP-binding proteins was carried out in non-diabetic (left panel) and diabetic (right panel) retinae as described in the Methods section. Labelling was carried out in the presence (+) and absence (−) of unlabelled GTP (50 μmol/l). Arrows indicate the regions where marked reductions in the binding were observed in diabetic retinae

purified subunits were incubated in phosphate buffered saline containing 200 mmol/l [^{14}C]glucose. Sodium azide (0.02%) was also added to the medium to prevent bacterial growth. Parallel incubations were carried out in the presence of unlabelled glucose for measurements of the effects of NEG on GTP binding and hydrolytic functions of transducin. Incubations were also carried out with fatty acid-free human serum albumin (Sigma, St. Louis, Mo., USA) to compare the degree of NEG between human serum albumin and transducin subunits. In order to prevent the thermal denaturation of purified transducin subunits, NEG was carried out at 4°C instead of 22°C. These steps were essential since our pilot results indicated a significant reduction in GTP-dependent functions of purified transducin subunits when incubated at room temperature for prolonged time periods. In order to compare the rates of NEG at 4°C and at 22°C, human serum albumin was used as a test protein. Incubations were carried out at specified temperatures for 21 days. The degree of incorporation of glucose into pure proteins was measured after separating on SDS-PAGE followed by scintillation counting of the label in individual bands. GTPase activity and GTPγS binding in the glycated and unmodified transducin subunits were measured in a reconstituted system using urea-washed ROS membranes as a source of rhodopsin [4, 5].

GTP-dependent functions in non-diabetic and diabetic ROS under phosphorylating conditions

Non-diabetic retinal homogenates (10–15 μg) were incubated in the presence or absence of protein kinase C modulator, TPA, along with 50 μmol/l ATP or App(NH)p and 5 mmol/l $MgSO_4$ for 10 min at 33°C. Following this pre-incubation, GTPase activity and GTPγS binding were quantitated in these mixtures as described above.

Statistical analysis

Statistical significance of differences between control and experimental groups was evaluated by Student's *t* test using a computer program Statistics (Blackwell Scientific Software, Oxford, 1990). A p value < 0.05 was considered statistically significant.

Results

GTPase and GTPγS binding in retinal homogenates: effects of diabetes

Retinal homogenates from non-diabetic and diabetic (2-weeks duration) rats were used in these studies. The GTPase activity was 25% lower in retinal homogenates from diabetic rats than in retinal homogenates from non-diabetic rats (Fig. 1). After 7 weeks, activity in retinal homogenates from diabetic rats was only 50% of the control activity (Fig. 1). In retinal homogenates from non-diabetic rats, GTPγS binding was: (a) saturable with respect to GTPγS concentration, (b) linear with protein concentrations up to 100 μg, and (c) saturable by 30 min of incubation at 30°C.

Under these optimal conditions of assay, GTPγS binding was significantly lower in retinal homogenates from diabetic rats (Fig. 1). After 2 weeks of diabetes, GTPγS binding had decreased by 50% and after 7 weeks of diabetes, the binding had decreased to 25% of the control animals. These data in Figure 1 show that the GTP-dependent functions continue to decline with the duration of diabetes. However, we observed no major differences in these activities in control animals between the starting and the ending stages used in our studies (i. e. 4 to 6 months of age), suggesting that these differences are not associated with the age of the rat but with diabetic state of the animal.

Photoaffinity labelling of GTP-binding proteins in non-diabetic and diabetic retinal homogenates

In order to confirm the data described above, retinal homogenates from non-diabetic and diabetic rats were incubated in the presence of azido [^{32}P]GTP, and the degree of

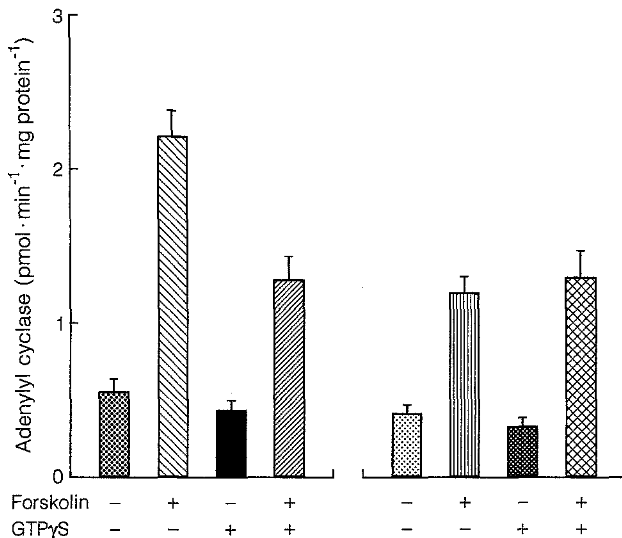

Fig. 3. Loss of inhibitory G-protein function in diabetic retina. Adenylyl cyclase activity was measured in non-diabetic (left panel) and diabetic (right panel) retinal homogenates with and without forskolin (100 µmol/l) and guanosine-5-0-(3-thiotriphosphate) GTPγS) (50 nmol/l). Data are mean ± SEM from three experiments done in triplicate. GTPγS inhibited forskolin-stimulatable adenylyl cyclase activity in retinal homogenates from non-diabetic rats ($p < 0.05$) compared to no inhibition in retinal homogenates from diabetic rats

labelling was monitored. Data in Figure 2 show that at least 6–8 proteins were labelled in non-diabetic retinal homogenates. Inclusion of unlabelled GTP (50 µmol/l) in the incubation medium decreased the labelling of these proteins, suggesting that the labelling was specific for GTP binding proteins. In diabetic retinal homogenates, the intensity of protein labelling (indicated by arrows) was relatively low as compared with non-diabetic retinal homogenates. These data, thus, confirm the above findings and suggest a decreased expression of G-proteins in diabetic retinae.

Effect of diabetes on G-protein function in the retina

It has been shown that forskolin, a diterpene, stimulates adenylyl cyclase activity in several cell types [30, 31]. Low concentrations of GTP inhibit forskolin-stimulated adenylyl cyclase activity involving inhibitory G-protein. Therefore, we measured this activity and its inhibition by GTP in non-diabetic and diabetic retinal homogenates. The data given in Figure 3 (left panel) show that forskolin (100 µmol/l) stimulated the adenylyl cyclase activity in non-diabetic retinal homogenates by nearly five-fold. GTPγS (50 nmol/l) inhibited this stimulation by 44 %, suggesting the functionality of inhibitory G-protein in retinal homogenates.

When similar quantitations were carried out in diabetic retinal homogenates (Fig. 3, right panel), we observed that the basal activity of adenylyl cyclase was reduced significantly in diabetic retinal homogenates as compared with non-diabetic retinal homogenates. Also, forskolin stimulated this activity to a lesser extent (approximately three-fold) in diabetic retinal homogenates. More interestingly, the ability of GTP to inhibit forskolin-stimulated adenylyl cyclase was abolished in diabetic retina. These data provide evidence for the functional inactivation of inhibitory G-protein in diabetic retina.

Light-sensitive GTPγS binding and GTPase activities in ROS from non-diabetic and diabetic rats

We extended the above-described studies to ROS preparations, since light-sensitive GTP-dependent signal transduction mechanisms have been extensively studied in this layer of retina [4, 5]. GTPγS binding and GTPase activities were quantitated in non-diabetic and diabetic ROS both in dark (under a dim red light) and in ambient room light. Data given in Table 1 show that both of these activities decreased significantly in diabetic ROS compared with non-diabetic ROS. However, the ratio of GTPase activity in light to dark remained the same in non-diabetic and diabetic ROS. These data suggest functional inactivation or decreased expression of transducin in diabetic ROS or both. Moreover, we also observed that treatment of diabetic rats with insulin for 10 days normalized the diabetes-associated reductions in GTPγS binding and GTPase activities in ROS, suggesting that insulinoprevia or hyperglycaemia or both may cause this lesion.

Effects of diabetes on pertussis toxin-mediated ADP ribosylation of transducin

Pertussis toxin catalyses the incorporation of ADP-ribose moiety into the α subunit of transducin when incubated in the presence of NAD [4, 5]. Non-diabetic and diabetic ROS were incubated with [^{32}P]NAD in the presence or absence of pertussis toxin and the degree of labelling of the α subunit of transducin was monitored by autoradiography after SDS-PAGE. The autoradiogram shows a significant reduction in the labelling of the α subunit in diabetic ROS as compared with non-diabetic ROS (Fig. 4). Incubation of ROS with NAD in the absence of pertussis toxin did not result in the labelling of α subunit, suggesting the specificity of the reaction. These data may imply a weak interaction among the subunits of transducin, since the ADP ribosylation of transducin has been shown to require both α and β, γ subunits. However, these data do not rule out the possibility of either a decreased expression of, or defective subunits of, transducin in diabetic ROS or both.

Functional consequences of transducin NEG in vitro

We extended the above-studies to determine the molecular and cellular mechanisms underlying the reduction of G-protein function in diabetes. It has been well documented that NEG of proteins affects their function [32]. Some of these proteins include: calmodulin [29], carbonic anhydrase [33], haemoglobin [34] and superoxide dismutase [35]. Therefore, initial studies were directed toward quantitation of NEG in non-diabetic and diabetic retinal

Table 1. Guanosine-5-0-(3-thiotriphosphate) (GTPγS) binding and guanosine triphosphate (GTP)ase activities in non-diabetic, diabetic, and diabetic rats treated with insulin: effects of light

Rod outer segment	GTPγS binding (pmols/mg)		GTPase activity (pmols · mg^{-1} · min^{-1})	
	Dark	Light	Dark	Light
Non-diabetic	176 ± 10	399 ± 21	266 ± 20	532 ± 41
Diabetic	142 ± 6	226 ± 14	187 ± 11	387 ± 24
Diabetic + insulin	168 ± 10	361 ± 11	248 ± 13	492 ± 24

GTPγS binding and GTPase activities were measured in rod outer segments from non-diabetic, 2-week diabetic, and 7-week diabetic rats treated with insulin as described in the Methods section. Data are mean ± SEM from six rats in each group. p values are < 0.05 in all conditions between non-diabetic and diabetic rod outer segments. There were no significant differences between non-diabetic and insulin-treated diabetic rod outer segments

Table 2. Non-enzymatic glycation (NEG) of retinal proteins in non-diabetic and diabetic rats

Type of animal	NEG (%)
Non-diabetic	2.4 ± 0.05
Diabetic	4.6 ± 1.3[a]

[a] $p < 0.05$ compared with non-diabetic animals
NEG of retinal proteins from non-diabetic and diabetic animals (n = 4, each) was measured as described in the text

homogenates. Data in Table 2 show a significant increase in the NEG of retinal proteins in diabetes. However, these data do not prove that the retinal G-proteins are glycated in vivo and that NEG of G-proteins increases in diabetes. Therefore, we extended these studies by glycating the purified transducin subunits in vitro and studying their function upon incubation with high glucose. Table 3 shows that all three subunits of transducin underwent NEG and the rates of glucose incorporation were comparable. However, the rates of glycation of transducin subunits were significantly lower compared with those of human serum albumin incubated under identical conditions. As expected, the amount of glucose incorporation was much higher at 22 °C compared to 4 °C. The data suggest that the purified subunits of transducin undergo NEG and that it may be higher under physiological conditions.

We quantitated the GTP binding and hydrolytic functions of transducin glycated in vitro and compared them with the unmodified form (incubated under similar conditions without glucose). GTPγS binding and GTPase activities of unmodified and glycated α subunit of transducin were measured in the presence of β/γ subunits of transducin and urea-washed membranes (as a source of rhodopsin). The data are given in Figure 5 and suggested no functional impairment in the α subunit of transducin as a consequence of NEG. We obtained similar results with glycated or unmodified β/γ subunits of Gt preparations or both. These data suggest that under present experimental conditions, NEG of transducin subunits α or β/γ does not result in any significant changes in its ability to bind (GTPγS binding) and hydrolyse (GTPase activity) GTP (Fig. 6). It is still conceivable that the effects of NEG on transducin function may be too small to measure under the present experimental conditions.

Regulation of G-protein function by phosphorylation: effects of diabetes

It has been shown recently that G-proteins undergo phosphorylation [36–39]. Available information suggests that the α-subunit of transducin is phosphorylated by insulin receptor kinase and protein kinase C and Lee et al. [40] have demonstrated significant translocation of protein kinase C activity into the membrane fraction of the retina within one week of diabetes. It is suggested that activation of protein kinase C is related to the translocation of enzyme to the membrane fractions.

We undertook a study to evaluate the effects of phosphorylating conditions on the GTP-dependent functions in non-diabetic ROS. The data given in Table 4 suggest that GTPγS binding and GTPase activities may be regulated by phosphorylation of ROS. Incubation of ROS with ATP and Mg^{++} in the presence of TPA resulted in 74% inhibition of GTPase activity and a 32% inhibition in the GTPγS binding. Incubation with App(NH)p, under similar conditions did not cause any inhibition, suggesting hydrolysis of ATP is involved in this step. Therefore, it is likely that protein kinase C is involved in regulation of these activities and that transducin may be in a more phosphorylated state in diabetic ROS.

Discussion

Our results provide biochemical evidence for impaired GTP-dependent functions in the retina in very early stages of streptozotocin diabetes. Alterations in the G-protein metabolism in diabetes have also been reported in hepatocytes [11], adipocytes [12, 41], and platelets [42]. A systemic nature of the effects of diabetes on reduction of GTP-dependent functions is questionable since we recently observed that GTPγS binding remained the same in non-diabetic and diabetic erythrocytes, while the GTPase activity was significantly increased [43]. The present studies also demonstrate that the observed early changes can be reversed by treatment of diabetic rats with insulin.

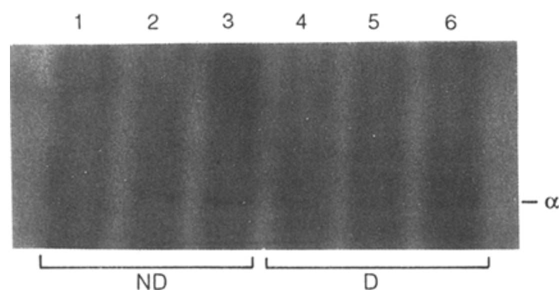

Fig. 4. Pertussis toxin-mediated ADP ribosylation of transducin (T)α from non-diabetic and diabetic rod outer segments (ROS). Non-diabetic and diabetic ROS were incubated with [^{32}P]NAD in the presence or absence of pertussis toxin. Incorporation of the label into Tα was monitored by autoradiography after SDS-PAGE. Lanes 1–3 and 4–6 represent non-diabetic (ND) and diabetic (D) ROS, respectively. Lanes 1 and 4 were ROS incubated with NAD in the presence of pertussis toxin. Lanes 1, 2, 4, and 5 had 5 µg ROS protein, while lanes 3 and 6 had 10 µg ROS protein

Table 3. Non-enzymatic glycation (NEG) of purified transducin (T) subunits from frog rod outer segments: a comparison with NEG rates of human serum albumin

Protein	Incubation temperature (°C)	Degree of glycation (mol glucose/mol protein)
HSA	22	2.84
HSA	4	0.90
Tα	4	0.33
Tβ	4	0.36
Tγ	4	0.42

Human serum albumin (HSA), Tα, and Tβ/γ(10–15 μg) were incubated under sterile conditions in phosphate buffered saline containing 200 mmol/l [^{14}C]glucose. Incubations were carried out at 4°C and 22°C for 21 days. The reaction was terminated by addition of Laemmli buffer [25] and the contents were heated for 5 min. The proteins were separated on SDS polyacrylamide gels (6.5–16% gradient gels) and stained with Coomassie blue. Individual proteins were sliced and the radioactivity determined by scintillation spectrometry. Degree of incorporation of glucose into individual proteins was calculated from the specific activity of glucose and the protein concentration in the incubation medium

In a recent communication, Hadjiconstanau et al. [44] reported an apparent loss in inhibitory G-protein activity in diabetic retina in very early stages of diabetes. Our studies reach a similar conclusion, but by a different experimental approach. We also observed that the basal adenylyl cyclase activity, as well as its stimulated activity by forskolin, were decreased in diabetic retina. This suggests that diabetes may induce alterations in the catalytic component of adenylyl cyclase in the membrane through which forskolin exerts its effects [31]. Indeed, we [45] and others [46] have demonstrated that diabetes causes significant alterations in the membrane phospholipid asymmetry. It may be added that similar diabetes-associated reductions in the basal activity of the adenylyl cyclase have been reported in adipocytes [41] and hepatocytes [11].

By photoaffinity labelling technique, we observed an overall reduction in the labelling of G-proteins in diabetic retina. This may suggest that the content of G-proteins is decreased in diabetes. This evidence, in addition to the data on the reduction in GTPγS binding in diabetic retina, may suggest that the expression of G-proteins is reduced in diabetic retina. Recent studies by Griffith and Houslay have indeed described reduced mRNA levels of Gα subunits in different tissues from diabetic rats [47].

We observed that subunits of transducin can be glycated in vitro; however, under the experimental conditions described herein, it does not undergo impaired function because of NEG. The observed deterioration in transducin function in diabetic ROS may not result from NEG since these reactions take several days and are not subjected to short-term regulation, such as reversal by insulin treatment. On the contrary, phosphorylation of protein components in the retina seems to regulate such functions.

In recent years, numerous studies have reported on phosphorylation of G-proteins in several cell types, including adipocytes [38] and myocytes [39]. It has been shown that the phosphorylation of Gα subunits results in decreased ADP ribosylation of the phosphorylated subunit in cardiac myocytes [39]. We also observed decreased ADP ribosylation of α subunits of transducin in diabetic ROS. It is likely that in diabetic ROS the α subunit of transducin is in a more phosphorylated state, thus resulting in the decreased interaction among the transducin subunits, as evidenced by decreased ADP ribosylation. It may also be possible that other membrane components, responsible for the optimal interactions of transducin, are undergoing these modifications in diabetic ROS. Support for our finding on the regulation of transducin function by protein kinase C comes from recent reports on the localization of protein kinase C activity in retina [48], as well as ROS [49, 50], and the ability of protein kinase C to phosphorylate transducin in vitro [37]. In addition, the present data indicate that phosphorylation may be a regulatory mechanism because of its short-term nature. Indeed, we

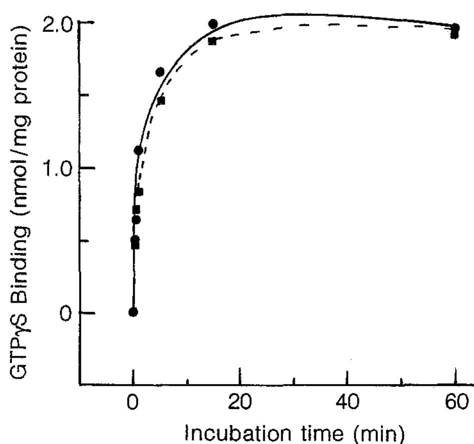

Fig. 5. Effect of non-enzymatic glycation (NEG) on guanosine-5-0-(3-thiotriphosphate) (GTPγS) binding in purified transducin (T)α from frog rod outer segments. NEG of purified Tα was carried out as described in the text. As a control, Tα was incubated under similar conditions without glucose (unmodified form). GTPγS binding was assayed as described in the Methods section in [●] unmodified and [■] glycated Tα. Each data point represents the mean of triplicate measurements

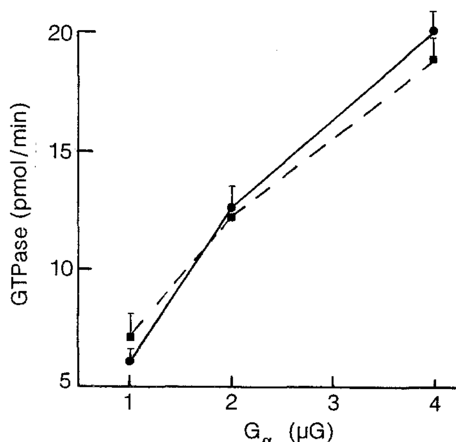

Fig. 6. Effect of non-enzymatic glycation (NEG) on guanosine triphosphate (GTP)ase activity in purified transducin (T)α from frog rod outer segments (ROS). NEG and measurement of GTPase activity of purfied Tα were carried out as described in the text. The data on GTPase activity in the unmodified [●] and glycated [■] Tα were the mean of triplicate measurements

Table 4. Effect of phosphorylating conditions on guanosine-5-0-(3-thiotriphosphate) (GTPγS) binding and guanosine triphosphate (GTP)ase activity in non-diabetic rod outer segments (ROS)

Condition	GTPγS binding (pmols/mg)	GTPase activity (pmols · min^{-1} · mg^{-1})
None	397 ± 21	562 ± 41
TPA (2 µmol/l)	382 ± 18[a]	588 ± 22[a]
TPA (2 µmol/l) + ATP (50 µmol/l)	260 ± 7[b]	133 ± 6[b]

[a] Not significant compared with the activities in the absence of TPA.
[b] $p < 0.05$ compared with activities in the absence of ATP
Non-diabetic ROS were incubated with (12-0-tetradeconyl) phorbol-13 acetate (TPA) in the presence or absence of ATP for 10 min at 33 °C, and GTPγS binding and GTPase activities were measured. Data are mean ± SEM of two experiments done in triplicate. Inclusion of adenosine-5′-(β-γ-imido) triphosphate (App(NH)p) (50 µmol/l) instead of ATP did not result in inhibition of any of these activities

have recently documented that diabetes affects the phosphorylation state and thereby, the function of erythrocyte sodium-potassium ATPase activity [19]. It is premature to exclude that alterations in phosphatase levels cause the observed changes in diabetic retina since recent studies have suggested that the interplay of kinase-phosphatase system is critical for the optimal function of various processes in photoreceptors [51].

Recent studies also described visual abnormalities in human diabetic patients even before capillary damage take place. These include: abnormalities in colour vision [14], dark adaptation [15], prolonged photostress recovery time [16], and decreased photopigment bleaching [17]. The rate at which these changes take place in human diabetic patients may not be the same since there is a certain degree of metabolic control in human diabetic patients which is not possible in the "uncontrolled diabetic" animal model. A clear understanding of these changes would be useful in preventing these lesions by pharmacological intervention. Indeed, we present evidence that early changes are reversible by insulin treatment of diabetic rats. Thus, they may define a "point of return" from these progressive changes in diabetic retina.

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