

Diabetic neuropathy: inhibitory G protein dysfunction involves PKC-dependent phosphorylation of G_{oα}

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Abstract

We examined the hypothesis that decreased inhibitory G protein function in diabetic neuropathy is associated with increased protein kinase C (PKC)-dependent phosphorylation of the G_{oα} subunit. Streptozotocin-induced diabetic rats were studied between 4 and 8 weeks after onset of diabetes and compared with aged-matched healthy animals as controls. Opioid-mediated inhibition of forskolin-stimulated cyclic AMP was significantly less in dorsal root ganglia (DRGs) from diabetic rats compared with controls. Activation of PKC in DRGs from control rats was associated with a significant decrease in opioid-mediated inhibition of forskolin-stimulated cyclic AMP that was similar to the decrease in inhibition observed in DRGs from diabetic rats. Both basal and PKC-mediated labeling of G_{oα} with ³²P_i was significantly less in DRGs from

diabetic rats, supporting increased endogenous PKC-dependent phosphorylation of G_{oα}. Probing of immunoprecipitated G_{oα} with an anti-phospho-serine/threonine specific antibody revealed a significant increase in baseline phosphorylation in diabetic DRGs. Activation of PKC produced a significant increase in phosphorylation in control DRGs but no significant increase in G_{oα} in diabetic DRGs. Phosphorylation of PKC-α was increased, PKC-β₁₁ was unchanged and PKC-δ decreased in diabetic DRGs. These results suggest that diminished inhibitory G protein function observed in DRGs neurons from diabetic rats involves an isoform-specific PKC-dependent pathway.

Keywords: diabetes mellitus, diabetic neuropathy, G protein, PKC.

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Diabetic neuropathy is the most common peripheral neuropathy in the Western hemisphere (Greene *et al.* 1992). Functional, morphological and metabolic changes in peripheral nerves appear to be similar in human and animal models of type 1 and type 2 diabetes mellitus (Bischoff 1973). Hypotheses that have been proposed to link hyperglycemia with development of diabetic neuropathy include: (i) formation of reactive oxygen species; (ii) formation of advanced glycation end products; (iii) increased activity of aldose reductase; (iv) activation of protein kinase C (PKC); and (v) impaired regulation of calcium homeostasis (Levy *et al.* 1994; Hall *et al.* 1995; Biessels and Gispen 1996; Biessels *et al.* 1999; Cameron *et al.* 1999; Nishikawa *et al.* 2000; Voitenko *et al.* 2000).

Our previous studies indicated that diabetic neuropathy was associated with diminished inhibitory guanine nucleotide-binding (G_{i/o}) protein function in dorsal root ganglion (DRG) neurons from diabetic rats using direct measurements of opioid receptor-mediated activation of GTPase activity (Hall *et al.* 2001). Opioid receptors are also coupled to inhibition of adenylate cyclase in a variety of tissues,

including neurons (Xie and Lewis 1997; Allouche *et al.* 1999). Inhibition of adenylate cyclase is mediated by G_{oα} subunits of the G_{i/o} family (Simonds 1999). It is unknown whether this pathway is affected in diabetic peripheral neuropathy.

The underlying mechanism(s) responsible for impaired inhibitory G protein function in diabetic neuropathy may involve phosphorylation of G proteins by intracellular signaling molecules, such as protein kinase C (PKC) or protein kinase A (PKA) (Katada *et al.* 1985). Activation of PKC and phosphorylation of the alpha subunit of G_{i2} was

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Abbreviations used: DAMGO, [D-Ala(2), N-MePhe(4), Gly(5)-d]-enkephalin; DRG, dorsal root ganglion; IP, immunoprecipitation; PDBu, phorbol 12, 13-dibutyrate; PKA, protein kinase A; PKC, protein kinase C; STZ, streptozotocin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

associated with impaired G_{i2} -mediated inhibition of adenylate cyclase in hepatocytes from streptozotocin (STZ)-induced diabetic rats (Morris *et al.* 1996). The existence of similar abnormalities in diabetic neuropathy has not been explored, although previous studies suggest that activation of PKC occurs in diabetic neuropathy and that impaired inhibitory G protein function is associated with increased calcium influx in acutely dissociated DRG neurons obtained from diabetic rats (Hall *et al.* 1996, 2001; Cameron *et al.* 1999).

The goals of the current study were: (i) to confirm that diabetic neuropathy is associated with diminished inhibitory G protein function. We evaluated whether opioid-mediated inhibition of forskolin-stimulated cyclic AMP production was decreased in DRGs from diabetic rats compared with controls in the absence and presence of PKC activation; (ii) to assess whether DRGs from diabetic rats demonstrated increased phosphorylation of the $G_{\alpha x}$ subunit via a pathway that involved activation of PKC; and (iii) to examine whether diabetic neuropathy was associated with differential phosphorylation of PKC isoforms in DRG neurons obtained from diabetic rats.

Materials and methods

Animal model

The studies described in this manuscript were approved by the University Committee on Use and Care of Animals. We employed the streptozotocin (STZ)-induced diabetic rat model in these studies. Many of the abnormalities observed in diabetic peripheral neuropathy in humans are noted in this animal model, including decreased axonal transport, reduced nerve conduction velocity and impaired axon regeneration (Stevens *et al.* 1994; Biessels *et al.* 1999). Male Sprague-Dawley rats (Charles River) aged 4–5 months were rendered diabetic by injection with 45 mg/kg STZ (Sigma, St. Louis, MO, USA) as described previously (Stevens *et al.* 1994; Srinivasan *et al.* 2000). Age-matched animals injected with vehicle served as controls. Animals injected with STZ were given 10% sucrose in water for 48 h after injection to prevent hypoglycemia and subsequently maintained on an *ad lib.* diet. Blood glucose levels were monitored daily until induction of diabetes, which usually occurred within 1–3 days, then bi-weekly thereafter. Animals were studied between 4 and 8 weeks after induction of diabetes. Weight and blood glucose were measured on the day of euthanasia.

DRG preparation

Isolated thoracic and lumbar DRG neurons were aseptically removed using techniques described previously (Hall *et al.* 1995). All studies were performed on the same day that the DRGs were removed from the animals.

Cyclic AMP assay

Measurement of cyclic AMP activity was performed using a cyclic AMP [^3H] assay kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA; code TRK432) (Tovey *et al.* 1974). Control and diabetic rat DRGs were treated *in vitro* with the opioid agonist, [D-Ala(2),

N-MePhe(4), Gly(5)-ol]-enkephalin (DAMGO), 1 μM for 5 min, followed by 3 μM forskolin + DAMGO for 25 min in the presence or absence of phorbol esters, 12-O-tetradecanoylphorbol-13-acetate (TPA), 500 nM pre-treatment for 60 min. In some studies, the inactive analog, 4- α phorbol, was used as a control. DRGs were extracted by homogenization in buffer containing 4 mM EDTA. Samples were mixed with assay reagents and placed in scintillation vials for counting. Blank cpm were determined, as were cpm of samples bound in the absence of unlabeled cAMP (Co). After subtracting the blank cpm from Co to give the blank-corrected cpm (Cx), the ratio of Co/Cx was calculated and plotted against pM of cAMP/tube. The latter value was obtained by comparing values against a standard curve generated using serial dilutions of known concentrations of cAMP.

Phosphorylation studies with $^{32}\text{P}_i$

These studies were patterned after Morris *et al.* (1996). Minced thoracic and lumbar DRGs from one animal (usually 20–24 ganglia) were pre-incubated in a 1.5 mL tube for 60 min at 37°C in 200 μL minimal essential medium (MEM) supplemented with 2 mCi of [$^{32}\text{P}_i$] (NEN LifeScience, Boston, MA, USA), 2.5% bovine serum albumin (BSA), 2.5 mM CaCl_2 , 10 mM glucose supplemented with the phosphatase inhibitor beta-glycerophosphate (10 mM; Sigma). In some studies, phorbol ester, phorbol 12, 13-dibutyrate (PDBu, 500 nM for 60 min) \pm the membrane-permeable PKC inhibitor NPC-15437 (10 μM) or 8-bromo-cyclic AMP (2 mM for 60 min), were added to the buffer during the incubation period. Some samples were treated with the inactive phorbol, 4 α -phorbol (500 nM for 60 min). The DRGs were harvested by centrifugation (12 000 g for 10 s). Lysis buffer [one tablet of proteinase inhibitor-cocktail (Roche, Mannheim, Germany) in 50 mL 20 mM HEPES/100 mM NaCl buffer, plus 0.1% Triton X 100] was added, and the tissue was homogenized to release the proteins.

Immunoprecipitation, gel electrophoresis and autoradiography of $G_{\alpha x}$

Homogenized DRG tissue was briefly centrifuged to eliminate the cell debris, and the supernatant fluid was treated with anti- $G_{\alpha x}$ polyclonal antibody [Santa Cruz Biotechnology, Santa Cruz, CA, USA; it has no cross-reactivity with other members of the inhibitory G protein (G_i) family], mixed with 10% protein G agarose (Sigma) for purified the $G_{\alpha x}$. The mixture was incubated for 60 min at room temperature or overnight at 4°C. Immune complexes were collected as protein G agarose pellets by centrifugation (12 000 g for 5 s); the pellets were washed three times in a 20 mM HEPES, 100 mM NaCl buffer (pH 7.2), and were finally re-suspended in the buffer and placed in a boiling water bath for 5 min. Samples were then centrifuged (12 000 g for 5 s) and the supernatant fluids taken for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE). This was performed at 50 mA for 2 h in 10% acrylamide gels. After electrophoresis, gels were dried and subjected to autoradiography. Gels were scanned and analyzed quantitatively using a Molecular Dynamic, ImageQuant System (Bio-Rad Laboratories, Hercules, CA, USA). In some experiments, $G_{i\alpha}$ was immunoprecipitated using a pan anti- $G_{i\alpha}$ antibody at a dilution of 1 : 1000 (Santa Cruz Biotechnology). Immunoprecipitated samples were processed as described above. While validating the immunoprecipitation methodology, we tested ($n = 6$) for the presence of

$G_{\alpha x}$ in the supernatant fluid after centrifugation of the protein G pellets and observed negligible levels, confirming that $G_{\alpha x}$ was reproducibly and reliably bound to the pellets.

Western immunoblot analysis

Western analysis was performed using methods similar to those described previously (Hall *et al.* 2001). Whole DRGs were homogenized and the protein content was determined using a Lowry assay system (Bio-Rad). Protein (25 μ g) was added to each lane. Samples were separated using 10% (for $G_{\alpha x}$ studies) or 7.5% (for PKC studies) polyacrylamide Ready Gel (Bio-Rad) and electrotransferred to a polyvinylidene fluoride (PVDF) microporous membrane. Isoform-specific antibodies to $G_{\alpha x}$ or PKC in 5% non-fat Carnation milk at a dilution factor of 1 : 1000 (unless otherwise stated) were added to the membranes. Secondary antibody conjugated to horseradish peroxidase enhanced chemiluminescence (ECL + Plus, western blotting detection reagents, Amersham Pharmacia Biotech) was used to counterstain the bands. Protein bands were then analyzed using ImageQuant software (Molecular Dynamics, Bio-Rad). For experiments determining the expression of $G_{\alpha x}$ in diabetic and control tissue, purified $G_{\alpha x}$ was used as a standard and results normalized to staining produced by 0.5 μ g $G_{\alpha x}$.

Assessment of phosphorylation of $G_{\alpha x}$ using an anti-phospho-serine/threonine specific antibody

To examine directly whether the alpha subunit of G_o undergoes increased phosphorylation in diabetic neuropathy, we probed immunoprecipitated $G_{\alpha x}$ from control and diabetic rats with an anti-phospho-serine/threonine antibody (ChemCon, Temecula, CA, USA; 1 : 2000 dilution). In some experiments, the DRGs were treated with phorbol ester (PDBu, 500 nM, 60 min) to assess the effect of PKC activation on the level of phosphorylation in DRGs from control and diabetic rats. Some tissue samples were treated with the inactive analog, 4- α phorbol, as a control.

Assessment of PKC- α , PKC- β_{II} and PKC- δ phosphorylation in DRGs using anti-phospho-site specific antibodies

These studies were performed using membrane preparations reported previously (Hall *et al.* 2001). Comparative studies were also performed to examine the level of phosphorylation with immunoprecipitated proteins using isoform specific antibodies and, subsequently, probing the level of phosphorylation with anti-phospho-site specific antibodies. Whole cell proteins (20 μ g/sample) were incubated with anti-PKC- α , anti-PKC- β_{II} (Santa Cruz Biotechnology) or anti-PKC- δ (Upstate Biotechnology, Lake Placid, NY, USA), plus protein G beads for immunoprecipitation (IP) (4°C overnight). Purified proteins were loaded on 10% gel after IP, then processed for gel transferring and milk blocking (Carnation). Membranes were exposed to anti-phospho-PKC- α (Ser 657) antibody (1 : 1000), anti-phospho-PKC- β_{II} (Ser 660) antibody (1 : 1000) (Santa Cruz Biotechnology) or anti-phospho-PKC- δ (Thr 505) antibody (1 : 1000, Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. Samples were subsequently treated with secondary antibody at room temperature for 1 h.

Statistical analysis

Data were analyzed using an unpaired *t*-test. Significance was accepted at the $p < 0.05$ level. Results are presented as mean \pm SE.

Results

Animals were studied at 4–6 months of age, 4–8 weeks following induction of diabetes with STZ. The diabetic rats used in the current studies demonstrated similar elevations in blood glucose and reduction in body weight as our previous studies that documented diminished nerve conduction velocity reversed by treatment with insulin (Srinivasan *et al.* 2000). Blood glucose ranged from 300 to 550 mg/dL in STZ-injected animals. We observed no significant difference in results obtained from rats with diabetes for 4 weeks versus 8 weeks.

Opioid (DAMGO)-mediated inhibition of forskolin-stimulated cyclic AMP production was significantly reduced in DRGs from diabetic rats compared with controls. Treatment with phorbol-ester reversed DAMGO-mediated inhibition of forskolin-stimulated cyclic AMP in control DRGs

Our previous studies suggested that inhibitory G protein (G_i/G_o) function was reduced in diabetic neuropathy (Hall *et al.* 2001). One objective of the current studies was to generate additional evidence that inhibitory G protein function is impaired in diabetic peripheral neuropathy by measuring opiate-mediated inhibition of forskolin-stimulated production of cyclic AMP. It is relevant that the expression of opioid receptors was not altered in DRGs from diabetic rats compared with controls (Hall *et al.* 2001). Application of the preferential mu-opioid receptor agonist DAMGO (1 μ M) inhibited forskolin (3 μ M)-induced cyclic AMP production that was significantly decreased in DRGs from diabetic rats compared with controls (Fig. 1a). Basal levels of cyclic AMP were 1.2 ± 0.3 (control) and 1.5 ± 0.5 (diabetic) pM/tube. Treatment with forskolin increased the cyclic AMP levels to 4.8 ± 0.6 (control; $n = 8$) and 5.1 ± 0.7 (diabetic; $n = 8$) pM/tube (Fig. 1b). Pre-treatment with the opioid agonist DAMGO decreased forskolin-stimulated cyclic AMP levels by $20 \pm 3\%$ in control DRGs (Fig. 1a; $n = 8$, $p < 0.05$) but had no significant effect in diabetic DRGs [$4 \pm 1.8\%$ decrease; not significant (NS); $n = 8$; Fig. 1b].

Pre-treatment with phorbol ester (TPA, 500 nM for 60 min) reversed opioid-mediated inhibition of forskolin-induced cyclic AMP levels in DRGs from control rats. (Fig. 1a). Treatment with the inactive phorbol ester analog, 4- α phorbol (500 nM for 60 min), had no significant effect on opioid-mediated inhibition of forskolin-stimulated cyclic AMP levels.

Protein levels of $G_{\alpha x}$ in DRG soma from control and diabetic rats were similar

There was no significant difference in the expression of $G_{\alpha x}$ in DRGs from control and diabetic rats when measured by semi-quantitative western blot analysis (Fig. 2a). This indicates that alterations in phosphorylation and function of G_o are unlikely to be due to altered levels of the α subunit in diabetic DRGs.

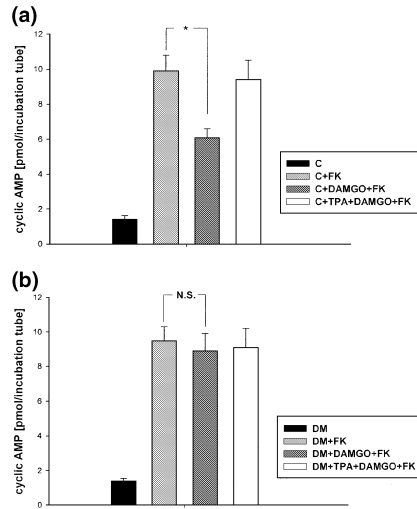


Fig. 1 Cyclic AMP production in DRGs from control rats was increased by treatment with forskolin (FK: $3 \mu\text{M} \times 25 \text{ min}$) (a) Treatment with forskolin and the mu-opioid receptor agonist DAMGO (DAMGO + FK: DAMGO ($1 \mu\text{M}$) pre-treatment for 5 min prior to (FK) significantly decreased the response to forskolin ($*p < 0.05$; $n = 8$). (b) A similar increase in cyclic AMP production was observed in response to forskolin treatment in DRGs from diabetic rats, but DAMGO demonstrated no significant inhibitory effect on forskolin-mediated cAMP production ($n = 8$, NS). Pre-treatment with phorbol ester (TPA, 500 nM for 60 min) reversed opioid-mediated inhibition of forskolin-stimulated cAMP production in DRGs from control rats (a) ($n = 5$, $*p < 0.05$).

Baseline endogenous phosphorylation of $G_{\alpha\alpha}$ was significantly greater in DRGs from diabetic rats

Endogenous phosphorylation of $G_{\alpha\alpha}$ was assessed by incubating acutely harvested DRGs from control and diabetic rats with $^{32}\text{P}_i$, and subsequently immunoprecipitating $G_{\alpha\alpha}$ with a specific antibody (Fig. 2b). Since exogenously-administered $^{32}\text{P}_i$ labels phosphorylation sites on $G_{\alpha\alpha}$ not occupied by unlabeled P_i , we interpreted a decrease in labeling with $^{32}\text{P}_i$ as indicating that endogenous unlabeled phosphate groups already occupied more sites. In experiments run in parallel with identical amounts of $^{32}\text{P}_i$ label and protein concentrations, immunoprecipitated $G_{\alpha\alpha}$ in DRGs from diabetic rats demonstrated significantly less labeling with $^{32}\text{P}_i$ (decreased by $40 \pm 8\%$) when compared with non-diabetic controls ($n = 5$, $p < 0.05$). Since there was no change in $G_{\alpha\alpha}$ protein levels, this result suggested that the level of endogenous phosphorylation of $G_{\alpha\alpha}$ was significantly increased in diabetes.

Endogenous PKC-dependent phosphorylation of $G_{\alpha\alpha}$ was significantly increased in DRGs from diabetic rats

We next assessed PKC-dependent phosphorylation in DRGs from control and diabetic rats (Fig. 3). Freshly-harvested DRGs from control and diabetic rats were incubated with $^{32}\text{P}_i$ with and without PDBu (500 nM for 60 min). Counts were

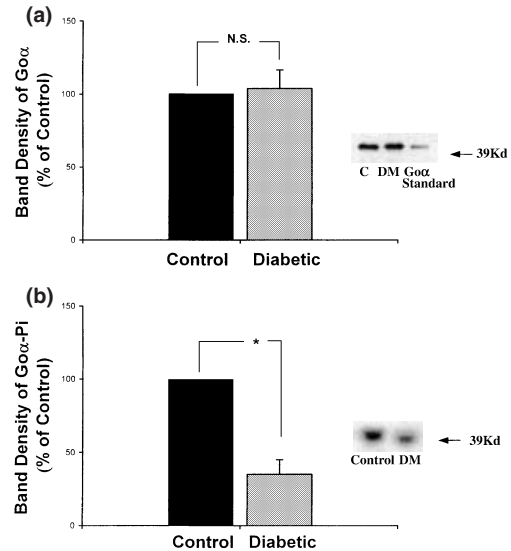


Fig. 2 Expression of $G_{\alpha\alpha}$ protein measured using semi-quantitative western blot was similar in control (C) and diabetic (DM) DRGs ($n = 6$ animals for each group). Protein concentrations were $25 \mu\text{g}$ in C and DM lanes. Bands were normalized to pixel density of $0.5 \mu\text{g}$ of $G_{\alpha\alpha}$ standard run in parallel. (a) $^{32}\text{P}_i$ -mediated phosphorylation of immunoprecipitated $G_{\alpha\alpha}$ from diabetic and control DRGs. (b) Compared with control tissue, diabetic DRGs incorporated significantly less $^{32}\text{P}_i$ label ($*p < 0.05$; $n = 5$), indicating fewer un-occupied phosphorylation sites on $G_{\alpha\alpha}$ in DRGs from diabetic animals.

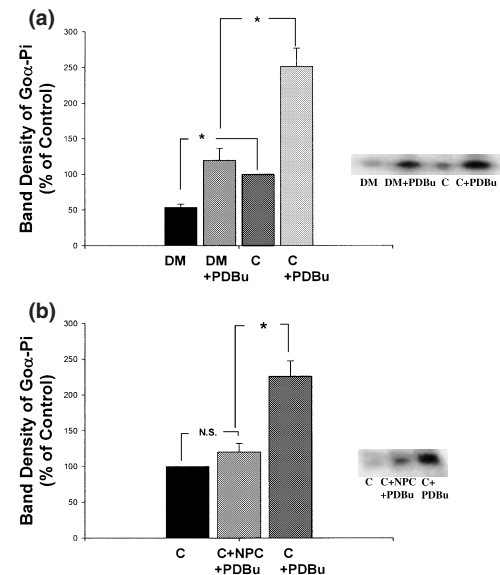


Fig. 3 Unstimulated (baseline) phosphorylation [$^{32}\text{P}_i$] of $G_{\alpha\alpha}$ was significantly greater in DRGs from control (C) compared with diabetic (DM) rats. (a) DRGs from control animals also demonstrated significantly greater phosphorylation in response to treatment with phorbol ester PDBu ($500 \text{ nM} \times 60 \text{ min}$) compared with DRGs from diabetic animals ($n = 5$; $p < 0.05$). (b) Pre-treatment with the PKC inhibitor NPC-15437 (NPC: $10 \mu\text{M}$ for 20 min) significantly decreased phosphorylation of $G_{\alpha\alpha}$ by PDBu ($n = 5$; $*p < 0.05$).

expressed as a percentage of the untreated controls. Phorbol ester-mediated phosphorylation ($^{32}\text{P}_i$) of $G_{\alpha x}$ in DRGs from control rats was significantly greater than that observed in DRGs from diabetic rats ($n = 5$, $p < 0.05$) (Fig. 3a), suggesting that fewer endogenous phosphorylation sites were occupied in control tissue. Treatment with the inactive phorbol analog, 4- α phorbol, had no significant effect. Pre-treatment of DRGs with the membrane-permeable PKC inhibitor, NPC-15437 (10 μM), caused a substantial reduction in PDBu-mediated phosphorylation ($^{32}\text{P}_i$) of $G_{\alpha x}$ (Fig. 3b, $p < 0.05$), confirming that PDBu-mediated phosphorylation of $G_{\alpha x}$ involved activation of PKC.

Increased phosphorylation of $G_{\alpha x}$ in DRGs from diabetic rats was confirmed using an anti-phospho-serine/threonine specific antibody. Treatment with phorbol ester increased phosphorylation in control DRGs but did not significantly alter phosphorylation in diabetic DRGs

To examine directly whether $G_{\alpha x}$ undergoes increased phosphorylation in DRGs from diabetic rats, we probed the immunoprecipitated alpha subunit of G_o from control and diabetic rats using an anti-phospho-serine/threonine specific antibody. The level of phosphorylation of $G_{\alpha x}$ was significantly greater ($45 \pm 8\%$, $p > 0.05$, $n = 4$) in DRGs from diabetic rats compared with controls (Fig. 4). Treatment with phorbol ester (PDBu, 500 nM for 60 min) caused a significant increase in phosphorylation ($35 \pm 7\%$, $p > 0.05$, $n = 4$)

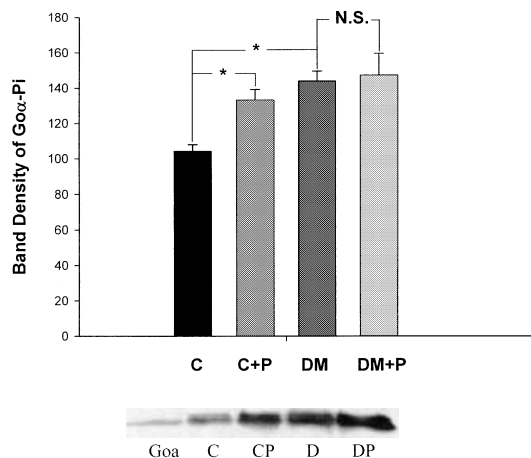


Fig. 4 Increased phosphorylation of $G_{\alpha x}$ in DRGs from diabetic rats was confirmed using an anti-phosphorylation site-specific antibody. Immunoprecipitated $G_{\alpha x}$ was probed with anti-phospho-serine/threonine specific antibody. The $G_{\alpha x}$ standard is depicted in the left band. Baseline phosphorylation was significantly increased in DRGs from diabetic rats ($*p < 0.05$, $n = 4$). Treatment with phorbol ester (PDBu, 500 nM for 60 min) was associated with a significant increase in phosphorylation in DRGs from healthy controls ($*p < 0.05$, $n = 4$) but did not have a significant effect on DRGs from diabetic rats.

in control DRGs but did not significantly change the level of phosphorylation in diabetic DRGs. These results provide direct evidence that $G_{\alpha x}$ undergoes increased phosphorylation in diabetic peripheral neuropathy, and that the pathway may involve activation of PKC. Treatment with the inactive phorbol ester analog, 4- α phorbol, had no significant effect on the level of phosphorylation.

Activation of cyclic AMP-dependent protein kinase A (PKA) did not increase phosphorylation of $G_{\alpha x}$ in DRG

It is possible that the increased level of endogenous phosphorylation in diabetic DRGs might be due to other kinases, such as cyclic AMP-dependent PKA. To test whether PKA-mediated phosphorylation was involved, freshly-harvested DRGs from control and diabetic rats were incubated with 8-bromo-cAMP (2 mM for 60 min) in the presence of $^{32}\text{P}_i$ (Fig. 5a). $G_{\alpha x}$ was immunoprecipitated and subjected to electrophoresis. There was no significant change in the level of phosphorylation of immunoprecipitated $G_{\alpha x}$ in DRGs from control or diabetic rats ($n = 5$). To confirm that PKA was activated in our assay, we tested 8-bromo-cAMP-mediated phosphorylation ($^{32}\text{P}_i$) of $G_{i\alpha}$ in excised rat liver under similar conditions (Fig. 5b). Previous reports indicate that PKA phosphorylates the inhibitory G protein, $G_{i\alpha}$, in rat hepatocytes (Bushfield *et al.* 1990). Treatment with 8-bromo-cAMP resulted in a significant ($p < 0.05$) increase in phosphorylation of $G_{i\alpha}$ ($32 \pm 3\%$) over basal levels ($n = 4$), indicating that the 8-bromo-cAMP treatment

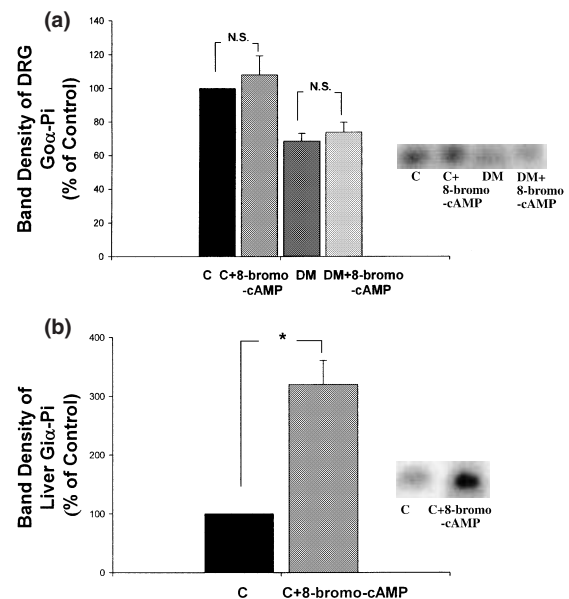
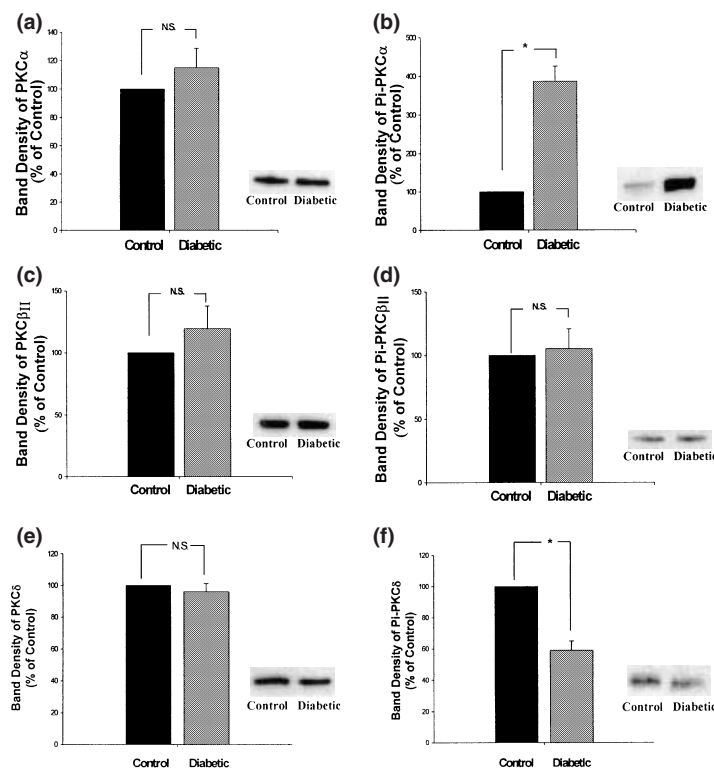


Fig. 5 (a) Phosphorylation [$^{32}\text{P}_i$] of $G_{\alpha x}$ was not affected by treatment with 8-bromo-cAMP (2 mM for 60 min) in control (C) and diabetic (DM) DRGs (NS: not significant). (b) Phosphorylation [$^{32}\text{P}_i$] of $G_{i\alpha}$ was significantly increased after treatment with 8-bromo-cAMP (2 mM for 60 min) in control (C) hepatic tissue ($n = 4$; $*p < 0.05$).

Fig. 6 (a) Protein levels of PKC- α measured by western blot analysis in DRGs from control and diabetic animals were unchanged. (b) Analysis of PKC- α phosphorylation using anti-phospho-site-specific PKC- α antibody demonstrated significantly increased phosphorylation of residue Ser 657 in DRGs from diabetic animals ($n = 5$ animals each group; $*p < 0.05$). (c) Protein levels of PKC- β_{II} measured by western blot analysis in DRGs from five control and five diabetic animals were unchanged. (d) PKC- β_{II} phosphorylation using anti-phospho-site-specific PKC- β_{II} antibody did not show any difference in either the expression or level of phosphorylation at residue Ser 660 ($n = 5$ animals each group; $*p < 0.05$). (e) Western blot analysis of PKC- δ demonstrated similar levels in DRGs from control and diabetic rats. (f) Phosphorylation of PKC- δ at Thr505 was significantly decreased in DRGs from diabetic rats compared with controls ($n = 5$ animals in each group, $*p < 0.05$).



conditions used in our assay were sufficient to activate PKA-dependent phosphorylation in liver tissue.

Endogenous phosphorylation of PKC- α was significantly increased in DRGs from diabetic rats

Western blot analysis revealed that protein levels of PKC- α were not significantly different in DRGs from control and diabetic rats (Fig. 6a). However, DRGs from diabetic rats demonstrated a significant fourfold increase in phosphorylation of PKC- α at Ser 657, as measured by a phospho-specific antibody (Fig. 6b).

Endogenous phosphorylation of PKC- β_{II} was not increased in DRGs from diabetic rats

Western blot analysis indicated that protein levels of PKC- β_{II} were not significantly different in DRGs from control and diabetic rats (Fig. 6c). In contrast to PKC- α , phosphorylation of PKC- β_{II} at Ser 660 was not significantly different in DRGs from control and diabetic rats (Fig. 6e).

Endogenous phosphorylation of PKC- δ was decreased in DRGs from diabetic rats

Western blot analysis revealed that protein levels of PKC- δ were not significantly different in DRGs from control and diabetic rats (Fig. 6e). However, DRGs from diabetic rats demonstrated a significant decrease ($41 \pm 7\%$) in phosphorylation of PKC- δ (Thr 505), as measured by a phospho-specific antibody (Fig. 6f).

Discussion

We demonstrated previously that opioid-mediated activation of inhibitory G protein function was significantly impaired in DRGs from diabetic rats (Hall *et al.* 2001). The diminished efficacy of opioids in diabetes was not likely due to a decrease in opioid receptor number because [3 H]-naloxone binding was not affected by diabetes. In this manuscript we have added the following complementary observations regarding inhibitory G protein function in DRGs from control and diabetic rats: (i) opioid-mediated inhibition of forskolin-stimulated cyclic AMP levels in diabetic DRGs was significantly decreased compared with controls; and (ii) activation of PKC resulted in decreased opioid-mediated inhibition of forskolin-stimulated cyclic AMP levels in control DRGs but did not significantly change the attenuated effect of opioid application in diabetic DRGs. These observations provide additional evidence that diabetes is associated with impaired inhibitory G protein function, and that activation of PKC reproduces in control DRGs the abnormalities in opioid function observed in diabetic DRGs.

We hypothesized that the decreased inhibitory G protein function in diabetic peripheral neuropathy involved increased phosphorylation of $G_{o\alpha}$ via a PKC-dependent pathway. The following observations support this hypothesis.

(I) Protein levels of $G_{o\alpha}$ were similar in DRGs from control and diabetic rats.

(II) Baseline labeling of G_{α} in the presence of $^{32}P_i$ was significantly less in DRGs from diabetic rats compared with controls, implying that more sites were occupied by endogenous phosphorylation in diabetic rats. As the level of G_{α} was unchanged, it is unlikely that this result was due simply to altered expression of G_{α} .

(III) Phorbol ester-mediated phosphorylation of G_{α} in the presence of $^{32}P_i$ was significantly less in DRGs from diabetic rats compared with controls. Phorbol ester-mediated phosphorylation of G_{α} was prevented by the membrane-permeable PKC inhibitor, NPC-15437, supporting the specific involvement of a PKC-dependent pathway.

(IV) Direct assessment of phosphorylation of immunoprecipitated G_{α} using an anti-phospho-serine/threonine specific antibody revealed that the baseline phosphorylation was significantly increased in diabetic DRGs compared with controls. Treatment with phorbol ester increased the level of phosphorylation of G_{α} in control DRGs but did not have a significant effect in DRGs from diabetic rats.

(V) Increased phosphorylation was not likely the result of PKA-mediated effects, as treatment of DRGs with 8-bromo-cyclic AMP was not associated with a significant increase in phosphorylation of G_{α} in the presence of $^{32}P_i$, whereas phosphorylation of hepatocyte $G_{i\alpha}$ was increased.

(VI) DRGs from diabetic rats demonstrated increased phosphorylation of PKC- α , no change in phosphorylation of PKC- β_{II} and decreased phosphorylation of PKC- δ . The protein levels of these three isoforms were unchanged in DRGs from diabetic rats compared with controls. This last observation suggests that diabetic peripheral neuropathy is associated with differential activation of PKC isoforms.

Our results suggest that diabetes mellitus may have region-specific effects on neuronal function. For example, opioid-mediated G-protein activation in the pons and medulla were unaltered in STZ-induced diabetic mice and the non-obese diabetic mouse model, despite an attenuation of opioid effect on tail flick response to pain (Ohsawa *et al.* 1999; Pieper *et al.* 2000). This discrepancy could be explained by differences between rats and mice in metabolic pathways affected by diabetes, or the relatively modest changes occurring in CNS function in early diabetes mellitus when compared with peripheral nerve function (Biessels *et al.* 1999).

Heterotrimeric G proteins are an integral component of post-receptor signal transduction (Birbaumer *et al.* 1990). The specific functions of the heterogeneous α and β/γ subunits of G_i/G_o are an active area of investigation. Inhibitory G protein α subunits combine with effector sites on the intracytoplasmic domain of specific receptor populations that are coupled to G_i/G_o (Iiri *et al.* 1998). The G_{α} subunits also regulate activation of adenylate cyclase (Simonds 1999). Inhibitory β/γ subunits modulate calcium channel function by combining with an effector site on the 1–11 intra-cytoplasmic loop of the voltage-gated calcium

channel (De Waard *et al.* 1997; Garcia *et al.* 1998). G proteins are themselves targets for modulation of function by intracellular proteins that regulate G protein stimulation (RGS) under physiological and pathophysiological conditions (Huailing and Neubig 2001). Our results suggest that inhibitory G protein function in DRGs can also be regulated by activation of PKC.

Reports of the direction and magnitude of changes in total PKC in diabetic neuropathy have been conflicting, with some investigators reporting increased levels of expression and/or activation, and other investigators reporting opposite results (Cameron *et al.* 1999; Ways and Sheetz 2001). Much of the controversy can be explained by either tissue-specific changes in the levels of PKC, and/or the likelihood that diabetes differentially affects the expression and/or activity of PKC isoforms. A previous report suggested that the STZ-induced diabetic rat demonstrated enhanced phosphorylation of hepatocyte $G_{i2\alpha}$ exclusively at PKC-dependent sites (Morris *et al.* 1996). This could result from increased PKC activity, a decrease in phosphatase activity, or a combination of both. We have not identified the specific amino acids on the α subunit of G_o in DRGs that undergo increased phosphorylation in diabetic neuropathy. Phorbol ester-mediated activation of protein kinase C was associated with phosphorylation at Ser 144 on $G_{i2\alpha}$ in hepatocytes from diabetic rats that correlated with a decrease in GTP-dependent inhibition of adenylate cyclase (Bushfield *et al.* 1990; Morris *et al.* 1996).

Our observation that diabetes had differential effects on PKC isoforms has support from other reports. Differential expression of PKC isoforms in sciatic nerve and spinal cord of diabetic rats has been reported (Roberts and Mclean 1997). PKC- α isoform translocated from cytosolic to particulate fraction, while levels of PKC- β_{II} isoform were reduced in the cytosolic fraction, and the β_I and γ -isoforms were unaffected. Increased PKC activity has been implicated in the pathogenesis of other diabetic complications, including retinopathy and nephropathy (Ways and Sheetz 2001). In contrast, Mathew *et al.* (1997) observed that the level of PKC- α in sciatic nerve from the Zucker diabetic fatty rat (a model of type 11 diabetes) was significantly reduced. We detected no changes in the protein levels of immunoreactive PKC- α , - β_{II} or - δ in DRGs from diabetic rats using semi-quantitative western blot analysis. However, we did observe a marked increase in phosphorylation of immunoprecipitated PKC- α , no change in PKC- β_{II} and a decrease in PKC- δ using anti-phospho-site specific antibodies. These observations provide support that PKC isoforms are differentially activated in diabetic peripheral neuropathy. The anti-phospho-specific PKC- α antibody we employed recognizes phosphorylated Ser 657, the PKC- β_{II} antibody recognizes phosphorylated Ser 660 and the PKC- δ antibody recognizes Thr 505. Phosphorylation of PKC- α at Ser 657 appears to control

accumulation of active enzyme and contributes to its phosphatase-resistant state (Bornancin and Parker 1996). Phosphorylation of PKC- β_{II} at Ser 660, part of the conserved carboxyl-terminal hydrophobic motif, regulates the catalytic and regulatory domains of PKC that are associated with higher affinity binding of ATP and Ca^{2+} (Edwards and Newton 1997). Phosphorylation of PKC- δ at Thr 505 (part of the activation loop) does not appear to be a requirement for basal or activator-dependent enzymatic activity but may contribute to enzyme activation along with phosphorylation of the adjacent residue Tyr 512 (Konishi *et al.* 2001). Our results with PKC- δ are similar to those of Sakaue *et al.* (2003) who observed that the level of phosphorylated PKC- δ , but not total PKC- δ , decreased in DRGs from diabetic rats. Additional studies will be required to clarify whether other known isoforms of PKC are differentially expressed and activated in this model of diabetic neuropathy. For example, Cesare *et al.* (1999) observed specific involvement of PKC- ϵ in sensitization of the neuronal response to painful heat. It is noteworthy that this group did not observe expression of PKC- α in DRGs obtained from 3- to 5-day-old neonatal rats using an antibody from Life Technologies (Gibco). In contrast, Khasar *et al.* (1999) did identify PKC- α in mouse DRGs using the same antibody as the one we employed from Santa Cruz Biotechnology.

Our results suggest that the pathway mediating impaired inhibitory G protein function in diabetic peripheral neuropathy involves activation of PKC and phosphorylation of the alpha subunit of G_o . The PKC-dependent pathway may involve direct or indirect phosphorylation of $G_{o\alpha}$. Previous studies support phorbol ester-mediated phosphorylation of $G_{i2\alpha}$ in intact acutely-harvested hepatocytes from diabetic STZ rats (Morris *et al.* 1996). However, this study did not examine whether PKC was acting directly or indirectly to phosphorylate $G_{i2\alpha}$. Kozasa and Gilman (1996) observed that catalytic PKC directly phosphorylates $G_{12\alpha}$ and $G_{2\alpha}$ *in vitro*, but neither $G_{o\alpha}$ nor $G_{i2\alpha}$ were targets for phosphorylation by PKC. It is possible that additional pathways may be involved in PKC-dependent phosphorylation of $G_{o\alpha}$. Exposure to phorbol esters activates the mitogen-activated phosphorylation (MAP) kinase pathway and extracellular signal-regulated kinases in several tissues, including neurons (Stariha and Kim 2001). It is not known whether either selective activation of MAP kinase is associated with phosphorylation of $G_{o\alpha}$ or blockade of this pathway affects PKC-dependent phosphorylation of $G_{o\alpha}$. It is noteworthy that using the Scansite consensus searching method with high stringency, neither G protein alpha o subunit (rGNAO) nor G protein alpha i1 subunit (GNAI1) demonstrate consensus PKA or PKC sites. This suggests that PKC may be acting indirectly to phosphophorylate $G_{o\alpha}$.

In summary, we have presented a body of data in support of the interpretation that impaired inhibitory G protein function in diabetic neuropathy involves a pathway that

includes activation of PKC and phosphorylation of the $G_{o\alpha}$ subunit. These observations may provide a mechanistic basis for the increase in calcium influx observed in diabetic peripheral neuropathy and suggest potential targets for therapeutic interventions (Ahlgren and Levine 1994; Nakamura *et al.* 1999; Carter 2000).

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