Rapid Publications

Protein Kinase C Agonists Acutely Normalize Decreased Ouabain-inhibitable Respiration in Diabetic Rabbit Nerve

Implications for (Na,K)-ATPase Regulation and Diabetic Complications

DOUGLAS A. GREENE AND SARAH A. LATTIMER

SUMMARY

Diminished (Na,K)-ATPase activity in diabetic peripheral nerve is attributed to an underlying depletion of free *myo*-inositol, but no biochemical mechanism linking *myo*-inositol metabolism and (Na,K)-ATPase has emerged. Since inositol phospholipid turnover releases inositol-(1,4,5)-tris-phosphate and diacylglycerol, two putative "second messengers" that modulate protein kinase C, the effect of protein kinase C agonists on (Na,K)-ATPase activity was examined in diabetic nerve. Phorbol myristate acetate or the diacylglycerol *sn*-1,2-dioctanoylglycerol acutely normalized depressed ouabain-inhibitable respiration [a measure of (Na,K)-ATPase activity], suggesting that *myo*-inositol metabolism modulates (Na,K)-ATPase activity via protein kinase C, and that reduced *myo*-inositol impairs (Na,K)-ATPase activity in diabetic nerve by this mechanism.

DIABETES 1986; 35:242–45.

Tissues susceptible to diabetic complications (peripheral nerve, renal glomerulus, retina, and arterial smooth muscle) exhibit a characteristic decline in their intracellular free *myo*-inositol (MI) levels following exposure to elevated ambient glucose concentrations: Competitive inhibition by glucose of Na-dependent cellular MI uptake and increased "sorbitol pathway" activity have been invoked to explain this phenomenon.[1–7] Diabetes also diminishes (Na,K)-ATPase activity in these tissues, an effect blocked in nerve and glomerulus by either MI supplementation or aldose reductase inhibition, both of which prevent depletion of tissue MI.[6–10] Hence MI and the (Na,K)-ATPase appear linked in a complex, potentially self-reinforcing, metabolic defect initiated by elevated ambient glucose concentrations in tissues susceptible to diabetic complications (Figure 1). Because MI is primarily incorporated into phospholipid, defects in phosphoinositide metabolism[11–16] have been invoked in this postulated cycle in diabetic peripheral nerve (Figure 1).

Membrane phosphoinositide turnover releases inositol-(1,4,5)-tris-phosphate (IP_3) and diacylglycerol (DG),[17] two metabolites that respectively mobilize intracellular Ca^{2+} (although Ca^{2+}-independent protein kinase activation has recently been proposed for IP_3[18] and activate protein kinase C (by lowering its Ca^{2+} requirement to within the physiologic intracellular concentration range.[19] Hence, these two "second messengers" may act independently or synergistically in translating exogenously stimulated phosphoinositide turnover into intracellular messages for metabolic regulation.[17,19] (Na,K)-ATPase activity in erythroleukemic cells is regulated by cyclic-AMP-independent membrane-bound protein kinase activity;[20] similar regulation in peripheral nerve by phosphoinositide-mediated protein kinase C activation could explain the MI-related (Na,K)-ATPase defect in diabetic nerve. If diminished (Na,K)-ATPase activity in diabetic nerve reflects a reduction in protein kinase C activity, then agonists that stimulate protein kinase C, such as DG and phorbol esters, should accordingly normalize (Na,K)-ATPase in diabetic peripheral nerve.[21] The present studies explore the ability of two protein kinase C agonists, phorbol myristate acetate (PMA) and *sn*-1,2-dioctanoylglycerol (diC_8),[22] to acutely normalize the reduced ouabain-inhibitable oxygen consumption in diabetic endoneurial preparations that reflects the reduced (Na,K)-ATPase activity.[23–25]

MATERIALS AND METHODS

Diabetes (fasting plasma glucose >300 mg/dl) was induced in 1.5–2.0-kg male New Zealand white rabbits by i.v. alloxan monohydrate (90 mg/kg).[24] Two weeks later, alloxan-injected and untreated control rabbits were fasted overnight (mean ± SEM fasting plasma glucose in diabetics of 477 ± 16 mg/dl, N = 42) and anesthetized, after which

This work was presented in part at the annual meeting of the American Society for Clinical Investigation, Washington, D.C., May 1985.
From the Diabetes Research Laboratories of the Department of Medicine, School of Medicine, University of Pittsburgh, Presbyterian University Hospital, Room 3304, 230 Lothrop Street, Pittsburgh, Pennsylvania 15261.
Address reprint requests to D. A. Greene, M. D., at the above address.
Received for publication 30 October 1985 and in revised form 15 November 1985.

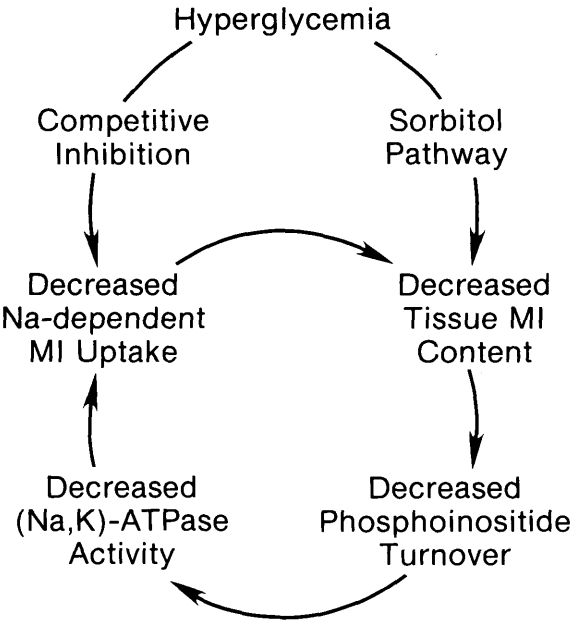

FIGURE 1. Proposed scheme linking hyperglycemia via decreased Na^+-dependent MI uptake and/or increased sorbitol pathway activity to abnormalities in MI metabolism and (Na,K)-ATPase activity in tissues with diabetic complications.[1–10] The sorbitol pathway is implicated in this sequence by the ability of aldose reductase inhibitors to normalize tissue MI and (Na,K)-ATPase in several diabetic tissues.[2,5–7,28] The nature of the biochemical link between sorbitol pathway and the MI-phosphoinositide-(Na,K)-ATPase cycle is presently obscure.

paired endoneurial preparations were derived from their left and right sciatic nerve using controlled collagenase (Type I) digestion and microdissection, as previously described in detail.[3,23,24] Endoneurial preparations were equilibrated for 10 min and incubated for 1 h in 3 ml of 4.5% dialyzed defatted bovine serum albumin (fraction V powder) and 5 mM glucose in Krebs-Ringer bicarbonate buffer (pH 7.4) equilibrated with 5% CO_2/95% O_2 in 10-ml Erlenmeyer flasks in a Dubnoff metabolic shaker at 88 cycles/min and 37°C. Stocks of PMA, 4-alpha phorbol (4a-P) (both 300 μg/ml), and diC_8 (50 mM) were prepared in dimethylsulfoxide (DMSO), and 10 μL was added to 3 ml of incubation medium for final phorbol concentrations of 2 μM for PMA and 167 μM for diC_8 during the 1-h incubation period; 10 μl of DMSO was added to control flasks for a DMSO concentration of 0.33% in all incubation flasks. Ouabain (2 mM) or amiloride (Merck Sharp and Dohme, West Point, Pennsylvania) (0.2–1 mM) was added in selected experiments for the last 10 or 30 min, respectively, and was present during the measurement of oxygen consumption. Linear oxygen consumption was recorded for at least 15 min in a Model 53 biological oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio) containing 3 ml of fresh medium. In selected experiments, incubated endoneurial preparations were snap frozen and homogenized for enzymatic determination of ouabain-inhibitable respiration, as previously described in detail for rat nerve[8] only here using a higher saturating concentration of ouabain (2 mM). Data are shown as mean ± SEM. Differences between groups were analyzed by Student's two-tailed *t*-test, and between pairs by the *t*-test for paired comparisons. All reagents were purchased from Sigma Chemical Co., St. Louis, Missouri, and were of the highest available purity unless otherwise specified.

RESULTS

PMA had no acute effect on resting oxygen consumption in endoneurial preparations from nondiabetic animals (Figure 2, column 1). Resting oxygen consumption was reduced by approximately 40% in endoneurial preparations derived from 2-wk-old alloxan-diabetic animals, but PMA acutely corrected this abnormality in vitro (Figure 2, column 2). Ouabain completely eradicated the effect of PMA (Figure 2, column 3), indicating that PMA acted on the ouabain-inhibitable component of respiration, which mirrors (Na,K)-ATPase activity.[24,25] In human leukemic cells, phorbol esters stimulate (Na,K)-ATPase activity indirectly by enhancing (Na^+,H^+)exchange, an effect originally thought to be blocked by 0.2 mM amiloride,[26] but later shown to require fivefold higher concentrations of amiloride that also appeared to directly inhibit protein kinase C.[27] In order to determine if the action of protein kinase C agonists on (Na,K)-ATPase in diabetic nerve was similarly mediated by (Na^+,H^+)exchange, PMA was tested on paired diabetic endoneurial samples exposed to 0.2 or 1 mM amiloride for 30 min [recognizing that an effect at the higher concentration could reflect either mediation by the (Na^+,H^+)exchanger or direct inhibition of protein kinase C]. At both concentrations, the full stimulatory effect of PMA on respiration was preserved (Figure 2, columns 4 and 5), ex-

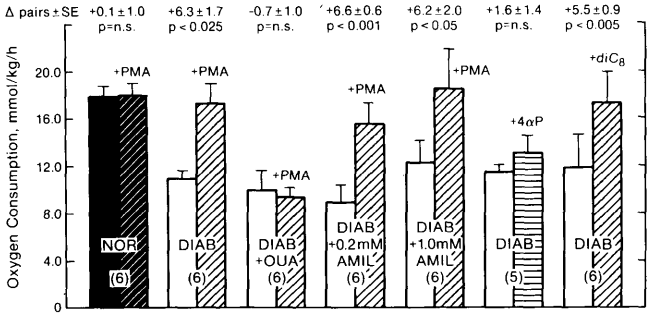

FIGURE 2. Effect of protein kinase C agonists on nondiabetic and diabetic endoneurial respiration. Oxygen consumption, expressed as mmol/kg/h, was measured in pairs of endoneurial preparations derived from normal (NOR, solid bars) and 2-wk-old alloxan-diabetic (DIAB, open bars) rabbits. One member of each pair of endoneurial preparations was studied after in vitro exposure to the active protein kinase C agonists, phorbol myristate acetate or *sn*-1,2-dioctanoylglycerol (+PMA or +diC_8, diagonally hatched bars), or the inactive phorbol analogue, 4-alpha phorbol (+4aP, horizontally hatched bar). The other member of each pair was exposed to dimethylsulfoxide, the vehicle for phorbol and diacylglycerol additions. In one set of experiments, 2 mM ouabain (OUA) was present in incubation medium for 10 min before and during the measurement of oxygen consumption to inhibit the (Na,K)-ATPase. In two sets of experiments, amiloride (AMIL) in 0.2 or 1.0 mM concentrations was present for 30 min before and during the measurement of oxygen consumption in order to inhibit (Na^+,H^+)exchange. Numbers of paired samples are shown in parentheses. Error bars = SEM. Mean differences of paired samples ± SEM are given numerically above each bar, and were used to calculate P-values by the *t*-test for paired comparisons. Endoneurial oxygen consumption was lower than previously reported,[24] this difference being entirely attributable to the effect of dimethylsulfoxide (DMSO): in separate studies 0.33% DMSO reduced resting oxygen consumption in normal endoneurial preparations from 22.2 ± 1.5 to 17.4 ± 0.7 mmol/kg/h, mean difference of pairs −4.9 ± 0.9 mmol/kg/h, N = 6, P < 0.01. This effect of DMSO is restricted to the non-ouabain-inhibitable component of respiration, since respiration in diabetic endoneurial preparations with 2 mM ouabain was equally depressed by DMSO compared with previous observations in the absence of DMSO.[24]

cluding a significant role for amiloride-sensitive (Na^+,H^+)exchange in this phenomenon. To establish that the effect of PMA on ouabain-inhibitable respiration was mediated by an alteration in (Na,K)-ATPase activity, ouabain-inhibitable ATPase activity was measured in crude homogenates of paired diabetic endoneurial preparations exposed to PMA or DMSO alone. PMA significantly increased ouabain-inhibitable ATPase activity by approximately 90% from 14.7 ± 2.2 to 27.8 ± 2.4 μmol/g/h (mean difference of paired samples, 13.1 ± 2.5 μmol/g/h, $N = 7$, $P < 0.005$). To examine the specificity of the action of PMA on diabetic nerve (Na,K)-ATPase, a protein-kinase C-inactive phorbol analogue (4a-P) and a nonphorbol protein-kinase–stimulating DG were tested. 4a-P had no effect on diabetic endoneurial respiration (Figure 2, column 6), but diC_8, a short-chain symmetrical DG known to stimulate protein kinase C in human platelets,[22] reproduced the effect of PMA (Figure 2, column 7). Thus, the reduction in ouabain-inhibitable respiration in endoneurial preparations from diabetic animals was acutely, specifically, and completely corrected by agonists that stimulate protein kinase C activity. This effect was not mediated by amiloride-sensitive (Na^+,H^+)exchange, or changes in water-soluble cofactors or substrates for the (Na,K)-ATPase, since the effect persisted in broken cell preparations.[8]

DISCUSSION

Decreased steady-state respiration in endoneurial preparations from diabetic rabbits is attributed to a reduction in (Na,K)-ATPase activity; endoneurial MI is also depressed by alloxan diabetes in the rabbit.[24] An analogous (Na,K)-ATPase defect in the streptozocin-diabetic rat measured enzymatically in crude nerve homogenates is preventable by either dietary MI supplementation[8] or aldose reductase inhibitors[28] that normalize the decreased nerve MI content. In view of the action of PMA and diC_8 on ouabain-inhibitable respiration in diabetic endoneurial preparations, and the rapidly emerging relationship between inositol phospholipid metabolism and protein kinase C modulation,[17,19] it is reasonable to infer that this reduction in (Na,K)-ATPase activity in diabetic nerve most likely mirrors decreased protein kinase C activity. These data are compatible with a direct phosphorylation of (Na,K)-ATPase or an associated regulatory membrane protein by protein kinase C. [A less likely possibility is that lipophilic agents that activate protein kinase C interact with a homologous lipid domain of the (Na,K)-ATPase, thereby directly modulating its activity as well.] In any case, the expression of the (Na,K)-ATPase defect in the broken cell preparations of diabetic rat nerve localizes the biochemical lesion entirely within the (Na,K)-ATPase–membrane complex.

Poorly understood consequences of hyperglycemia on nerve, retina, renal glomerulus, and arterial wall metabolism are thought to heavily influence the development of diabetic complications. These tissues share in common a reduction in cellular free MI and (Na,K)-ATPase in response to elevated ambient glucose concentration;[1-7] by extension of the present observations in nerve, a common defect in protein kinase C activity may be inferred in these other tissues. Although its endogenous substrates have not yet been fully defined, protein kinase C phosphorylates a broad range of proteins.[19] In nerve, this MI-related protein kinase C defect appears to explain the rapidly reversible slowing of conduction velocity in diabetic rats via its effect on (Na,K)-ATPase activity.[29] A reduction of protein kinase C activity in nerve, retina, renal glomerulus, and arterial wall might also explain other complex and poorly understood responses of these tissues to the diabetic state. Thus, protein kinase C may become a major focus of research into the complications of diabetes, and diabetes might now serve as a model in which to explore the physiologic role of protein kinase C and its possible modulation of (Na,K)-ATPase.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of Lisa Tamres; the editorial assistance of Drs. Jan Ulbrecht, Patricia Carroll, and Ann Marie Mackway; and the helpful suggestions of Drs. Frederick DeRubertis and Klaus Hofmann who reviewed the draft manuscript. The diC_8 was a generous gift from Dr. Robert Basford.

These studies were supported in part by USPHS Research Grant R01 AM29892 and the Harry Soffer Memorial Research Fund of the University of Pittsburgh.

REFERENCES

[1] Greene, D. A., DeJesus, P. V., Jr., and Winegrad, A. I.: Effects of insulin and dietary myoinositol on impaired peripheral motor nerve conduction velocity in acute streptozotocin diabetes. J. Clin. Invest. 1975; 55:1326–36.

[2] Finegold, D., Lattimer, S. A., Nolle, S., Bernstein, M., and Greene, D. A.: Polyol pathway activity and *myo*-inositol metabolism. Diabetes 1983; 32:988–92.

[3] Greene, D. A., and Lattimer, S. A.: Sodium and energy-dependent uptake of *myo*-inositol by rabbit peripheral nerve: competitive inhibition by glucose and lack of an insulin effect. J. Clin. Invest. 1982; 70:1009–18.

[4] Simmons, D. A., Winegrad, A. I., and Martin, D. B.: Significance of tissue *myo*-inositol concentrations in metabolic regulation in nerve. Science 1982; 219:848–51.

[5] Beyer-Mears, A., Ku, L. and Cohen, M. P.: Glomerular polyol accumulation in diabetes and its prevention by oral sorbinil. Diabetes 1984; 33:604–607.

[6] MacGregor, L. C., Rosecan, L. R., Laties, A. M., and Matchinsky, F. M.: Altered retinal metabolism in diabetes. I. Microanalysis of lipid, glucose, sorbitol and *myo*-inositol in the choroid and in the individual layers of the rabbit retina. In press. J. Biol. Chem. 1985.

[7] Morrison, A. D.: Aortic smooth muscle metabolism: effect of polyol pathway inhibition. Clin. Res. 1984; 32:851A.

[8] Greene, D. A., and Lattimer, S. A.: Impaired rat sciatic nerve sodium-potassium ATPase in acute streptozocin diabetes and its correction by dietary *myo*-inositol supplementation. J. Clin. Invest. 1983; 72:1058–63.

[9] Cohen, M. P.: Reduced glomerular sodium-potassium adenosine triphosphatase activity in acute streptozotocin diabetes and its prevention by oral sorbinil. Diabetes 1985; 34:1071–74.

[10] Simmons, D. A., Winegrad, A. I., and Martin, D. B.: Phosphatidylinositol turnover regulates arterial Na^+-K^+-ATPase activity. Clin. Res. 1983; 31:546A.

[11] Clements, R. S., Jr., and Stockard, G. R.: Abnormal sciatic nerve *myo*-inositol metabolism in the streptozotocin-diabetic rat: effects of insulin treatment. Diabetes 1980; 29:227–35.

[12] Eichberg, J., Bell, M. E., and Peterson, R. G.: Metabolism of polyphosphoinositides and other phospholipids in peripheral nerve of normal and streptozotocin-diabetic rats. *In* Phospholipids in the Nervous System. Vol. 1, Horrocks, L., Ed. New York, Raven Press, 1982:271–81.

[13] Natarajan, V., Dyck, P. J., and Schmid, H. O.: Alterations of inositol lipid metabolism of rat sciatic nerve in streptozotocin-induced diabetes. J. Neurochem. 1981; 36:413–19.

[14] Hothersall, J. S., and McLean, P.: Effect of experimental diabetes and insulin on phosphatidylinositol synthesis in rat sciatic nerve. Biochem. Biophys. Res. Commun. 1979; 88:477–84.

[15] Palmano, K. P., Whiting, P. H., and Hawthorne, J. N.: Free and lipid *myo*-inositol in tissues from rats with acute and less severe streptozotocin-induced diabetes. Biochem. J. 1977; 167:229–35.

[16] Mayhew, J. A., Gillon, K. R. W., and Hawthorne, J. N.: Free and lipid inositol, sorbitol and sugars in sciatic nerve obtained post-mortem from diabetic patients and control subjects. Diabetologia 1983; 24:13–15.

[17] Berridge, M. J.: Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 1984; 220:345–60.

[18] Whitman, M. R., Epstein, J., and Cantley, L.: Inositol 1,4,5-trisphosphate stimulates phosphorylation of a 62,000-dalton protein in monkey fibroblast and bovine brain cell lysates. J. Biol. Chem. 1984; 259:13652–55.

[19] Kikkawa, U., and Nishizuka, Y.: Protein kinase C. *In* The Enzymes. In press. Krebs, E., Ed. New York, Academic Press.

[20] Ling, L., and Cantley, L.: The (Na,K)-ATPase of Friend erythroleukemia cells is phosphorylated near the ATP hydrolysis by an endogenous membrane-bound kinase. J. Biol. Chem. 1984; 259:4089–95.

[21] Blumberg, P. M., Jaken, S., Konig, S., Sharkey, N. A., Leach, K. L., Jeng, A. Y., and Yeh, E.: Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. Biochem. Pharmacol. 1984; 33:933–40.

[22] Lapetina, E. G., Reep, B., Ganong, B. R., and Bell, R. M.: Exogenous sn-1,2-diacylglycerols containing saturated fatty acids function as bioregulators of protein kinase C in human platelets. J. Biol. Chem. 1985; 260:1358–61.

[23] Greene, D. A., and Winegrad, A. I.: In vitro studies of the substrates for energy production and the effects of insulin on glucose utilization in the neural components of peripheral nerve. Diabetes 1979; 28:878–87.

[24] Greene, D. A., and Lattimer, S. A.: Impaired energy utilization and (Na,K)-ATPase in diabetic peripheral nerve. Am. J. Physiol. 1984; 246:E311–18.

[25] Ritchie, J. M.: The oxygen consumption of mammalian non-myelinated nerve fibers at rest and during activity. J. Physiol. 1967; 188:309–29.

[26] Besterman, J. M., and Cuatrecasas, P.: Phorbol ester rapidly stimulate amiloride-sensitive Na^+/H^+ exchange in human leukemic cell line. J. Cell Biol. 1984; 99:340–43.

[27] Besterman, J. M., and Cuatrecasas, P.: Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C. 1985. J. Biol. Chem. 1985; 260:1155–59.

[28] Greene, D. A., and Lattimer, S. A.: Action of sorbinil in diabetic peripheral nerve: relationship of polyol pathway inhibition to a *myo*-inositol-mediated defect in sodium-potassium ATPase activity. Diabetes 1984; 33:712–16.

[29] Greene, D. A., Yagahashi, S., Lattimer, S. A., and Sima, A. A. F.: Nerve Na^+-K^+-ATPase, conduction, and *myo*-inositol in the insulin-deficient BB rat. Am. J. Physiol. 1984; 247:E534–39.