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A *myo*-inositol pool utilized for phosphatidylinositol synthesis is depleted in sciatic nerve from rats with streptozotocin-induced diabetes

(propranolol/phosphatidyl CMP/diabetic neuropathy)

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ABSTRACT **Peripheral nerve from experimentally diabetic rats exhibits lowered levels of *myo*-inositol (MI) and decreased incorporation of [^{3}H]MI into phosphatidylinositol (PI). There are indications that diminished PI turnover may be causally related to reduced Na^+,K^+-ATPase activity in diabetic nerve. We have investigated whether a metabolic compartment of MI that is essential for PI synthesis is decreased in this tissue. Sciatic nerve segments from streptozotocin-induced diabetic and age-matched normal rats were incubated *in vitro* with either $^{32}P_i$ or [^{3}H]cytidine in the presence of propranolol. This cationic amphiphilic agent redirected nerve phospholipid metabolism to produce enhanced ^{32}P incorporation into PI and decreased labeling of phosphatidylcholine and phosphatidylethanolamine. The accumulation of phosphatidyl CMP (CMP-PA) was also demonstrated by chromatographic and enzymatic means. The incorporation of [^{3}H]cytidine into CMP-PA in normal nerve increased up to 15-fold when 0.6 mM propranolol was present. In diabetic nerve, the liponucleotide incorporated 2- to 3-fold more isotope and was more readily labeled at lower drug concentrations as compared to normal nerve. The buildup of [^{3}H]CMP-PA was reduced in a dose-dependent manner in the presence of MI in the incubation medium at concentrations up to 3 mM. However, if MI was added after liponucleotide accumulation, preformed CMP-PA could not be utilized for PI synthesis. The difference in liponucleotide labeling between normal and diabetic nerve was nearly abolished at 0.3 mM medium MI, a concentration much less than the level of cyclitol in the tissue. These results strongly suggest the presence in nerve of a pool of MI that is not in equilibrium with the bulk of nerve MI and that is preferentially used for PI synthesis. This metabolic compartment is depleted in diabetic nerve but can be readily replenished by exogenous MI and may correspond to the MI pool that has been proposed to be required for the turnover of a portion of tissue PI involved in maintenance of normal Na^+,K^+-ATPase activity.**

myo-Inositol (MI) has been suggested to play an important role in the development of experimental diabetic neuropathy (1–3). Mammalian tissues maintain millimolar concentrations of MI, which in peripheral nerve is at least 40-fold higher than in plasma (4, 5). It has long been known that MI content is reduced substantially in peripheral nerve from diabetic animals (1, 4, 6). Evidence from both diabetic nerve preparations and neural cell cultures grown in the presence of elevated medium glucose concentrations indicates that the depletion of cellular MI levels is associated with increased intracellular sorbitol, alterations of inositol phospholipid metabolism, and decreased Na^+,K^+-ATPase activity (2, 7, 8), which is critical for the maintenance of resting membrane potential. Impairment of this enzyme has been linked to reduced nerve conduction velocity that is characteristic of both human and experimental diabetes (2). Furthermore, supplementing the diet of diabetic animals with MI tends to restore the cyclitol level, as well as Na^+,K^+-ATPase activity and nerve conduction velocity, to normal (1, 9, 10).

There is evidence that both Na^+,K^+-ATPase activity and a component of phosphatidylinositol (PI) turnover in normal tissues are inhibited by incubation in MI-free medium (11, 12). Consequently, reduced MI content in diabetic nerve could reflect depletion of the MI supply that specifically serves as substrate for PI metabolism, which in turn is integral to regulation of Na^+,K^+-ATPase activity.

To investigate this possibility, we have used propranolol, which like several other cationic amphiphilic drugs markedly enhances accumulation of phosphatidyl CMP (CMP-PA) in a number of tissues including iris muscle, pineal gland, and pancreatic islets (13–15). This occurs because propranolol redirects the metabolism of phospholipids, primarily by inhibiting PA phosphatase (16, 17), thereby decreasing the formation of phosphatidylcholine and phosphatidylethanolamine and enhancing the synthesis of PI and phosphatidylglycerol. In the presence of propranolol, the accumulation of CMP-PA, the immediate precursor of the latter two phospholipids, will be determined by its rate of biosynthesis relative to its rate of utilization. The consumption of the liponucleotide is governed by the availability of endogenous MI, since its appearance is prevented by inclusion of sufficient MI in the medium (15, 16). Thus, measurement of CMP-PA formation can serve as an index of tissue MI available for the synthesis of PI. In the present study, this approach was used to evaluate the possibility that a specific pool of MI required for PI synthesis is depleted in diabetic nerve.

MATERIAL AND METHODS

[5-^{3}H]Cytidine, streptozotocin, DL-propranolol, MI, and a diagnostic kit (no. 510) for analysis of blood glucose concentration were purchased from Sigma. $^{32}P_i$ in aqueous solution (HCl free) was obtained from DuPont/NEN. Silica gel K6 plates (10 × 20 cm; 250 μm thick) for thin-layer chromatography were products of Whatman. Authentic CMP-PA and other phospholipid standards were obtained from Serdary Research Laboratories (London, ON, Canada).

Diabetes was induced in male Sprague-Dawley rats by intraperitoneal injection of streptozotocin (60 mg per kg of body weight) as described (18). Diabetic rats and age-matched normal rats were sacrificed by decapitation between 15 and 25 weeks after the induction of disease. Blood samples were collected for glucose determinations. In agreement with

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Abbreviations: MI, *myo*-inositol; PI, phosphatidylinositol; CMP-PA, phosphatidyl CMP; PA, phosphatidic acid.

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previous reports, diabetic rats had developed cataracts, were markedly hyperglycemic [540 ± 18 for diabetics; 109 ± 8 for controls (mg/dl ± SEM)], and had undergone a large weight loss [243 ± 16 for diabetics; 439 ± 19 for controls (g ± SEM)].

The sciatic nerves were dissected and epineuria were removed as described (18). Nerves were cut into 36 segments ≈1.5 mm thick. Nine segments were placed in an incubation medium composed of 0.3 ml of Krebs–Ringer bicarbonate buffer, pH 7.4/5.5 mM glucose together with 5 μM cytidine and various concentrations of DL-propranolol and MI as desired. The nerve segments were preincubated at 37°C in 95% O_2/5% CO_2 for 1 hr. Then another 0.3 ml of medium of the same composition and containing either [^{3}H]cytidine (20 μCi/ml; 1 Ci = 37 GBq) or $^{32}P_i$ (40–50 μCi/ml) was added and the incubation was continued for 2 hr.

Incubations were terminated by cooling to 0°C and medium aliquots were taken for radioactivity determination. The nerve segments were washed with ice-cold saline and further divided into three groups, which were treated as triplicate samples thereafter. Lipids were extracted according to Bell *et al.* (7) and dried under N_2. The residue was resuspended in 0.3 ml of chloroform/methanol/water (75:25:2; vol/vol). Aliquots of each extract were used for total lipid phosphorus measurement as described by Chen *et al.* (19). When [^{3}H]cytidine was the precursor, additional aliquots of each lipid extract were transferred to scintillation vials, air dried, and assayed. [^{32}P]Phospholipids were separated by TLC on silica gel either in one dimension using chloroform/methanol/20% aqueous methylamine (60:36:10; vol/vol) or in two dimensions using chloroform/methanol/water/glacial acetic acid (50:43:3:1; vol/vol) as the first and chloroform/methanol/20% aqueous methylamine (60:36:10; vol/vol) as the second solvent system. Individual radioactive phospholipid classes were located by autoradiography and identified by comparison with authentic standard phospholipids. The appropriate areas of silica gel were scraped off for radioassay.

Calculation of isotope incorporation was based on the specific activity of radioactive precursor in the incubation medium. Statistical significance was evaluated by Student's *t* test.

RESULTS

Propranolol-Mediated CMP-PA Accumulation in Normal and Diabetic Nerves. The effects of propranolol on ^{32}P incorporation into phospholipids of peripheral nerve were similar to those of the drug in other tissues. Fig. 1 shows that in normal rat sciatic nerve incubated in the presence of a series of propranolol concentrations, the incorporation of isotope was increased into phosphatidylinositol 4-phosphate, PI, and PA but was decreased into phosphatidylcholine. The maximal effect was achieved at 0.6–1.0 mM propranolol. Other experiments indicated that 3 mM propranolol diminished ^{32}P incorporation into most phospholipids and that the inhibitory effect was greatest for phosphatidylcholine and phosphatidylethanolamine (96% and 90% inhibition, respectively, relative to controls).

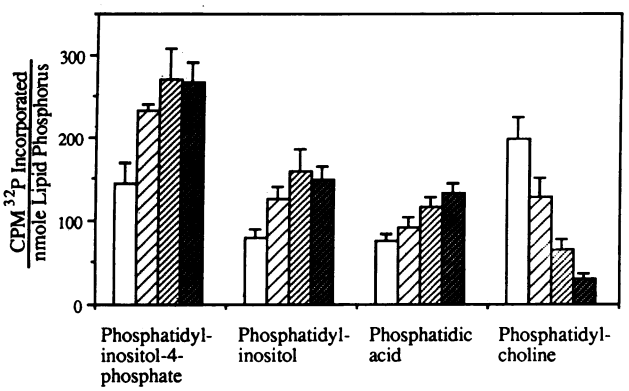

FIG. 1. Incorporation of ^{32}P into normal nerve phospholipids in the presence of various concentrations of propranolol (□, 0 mM; ▨, 0.3 mM; ▩, 0.6 mM; ■, 1.0 mM). Nerve segments were incubated and radioactive phospholipids were separated as described in the text. The results are averages ± SEM for three groups of nerve segments.

Two-dimensional thin-layer chromatography of [^{32}P]phospholipids followed by autoradiography (Fig. 2) revealed that [^{32}P]CMP-PA could not be detected in extracts from either normal or diabetic nerve incubated in the absence of propranolol (Fig. 2 *a* and *c*). However, a radioactive spot that comigrated with authentic CMP-PA was visible on autoradiograms obtained for lipids of normal nerve that had been incubated in the presence of 0.6 mM propranolol. When an equal amount of phospholipid from diabetic nerve was chromatographed, the labeling of [^{32}P]CMP-PA was observed to be substantially increased (Fig. 2 *b* and *d*).

Normal nerves incubated with [^{3}H]cytidine and 0.6–1.0 mM propranolol exhibited maximal isotope incorporation into nerve lipids, which was 10–15 times that for nerves labeled in the absence of drug (Fig. 3). Enhanced labeling was abolished in the presence of 2 mM propranolol. The rate of [^{3}H]cytidine incorporation with time into nerve lipids in the presence of 0.6 mM propranolol was linear for up to at least 3 hr (Fig. 4*a*).

Two-dimensional chromatographic separation and autoradiography of [^{3}H]cytidine-labeled lipids demonstrated the presence of a single radioactive spot, which accounted for ≈95% of the total radioactivity in the nerve lipid extract and which comigrated with authentic CMP-PA. To further identify the lipid product, the area on the thin-layer plate corresponding to [^{3}H]CMP-PA was eluted (14). Upon incubation of the contents of the eluate with rat brain microsomes

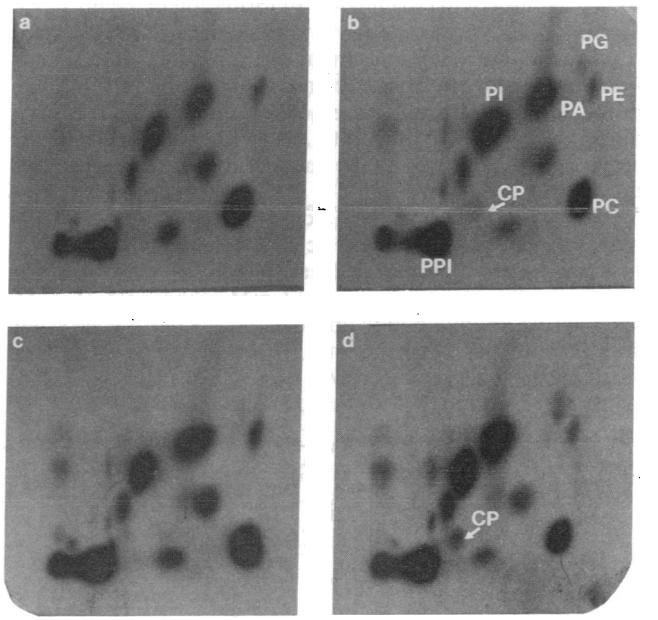

FIG. 2. Autoradiogram of two-dimensional thin-layer chromatographic separation of ^{32}P-labeled nerve phospholipids. After nerve incubation in either the presence or absence of propranolol and lipid extraction, samples containing equal quantities of lipid phosphorus were applied to the plates, which were developed as described in the text. (*a*) Normal nerve, no propranolol. (*b*) Normal nerve, 0.6 mM propranolol. (*c*) Diabetic nerve, no propranolol. (*d*) Diabetic nerve, 0.6 mM propranolol. CP, CMP-PA; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PPI, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate.

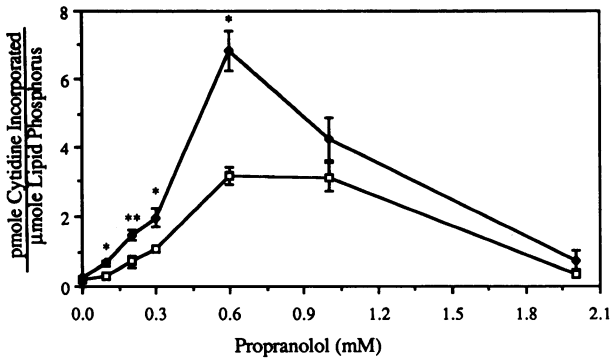

FIG. 3. Incorporation of [^{3}H]cytidine into nerve lipids from normal (□) and diabetic (●) animals in the presence of various concentrations of propranolol. Nerves were incubated and lipids were extracted as described in the text. The results are averages ± SEM for 12 groups of nerve segments pooled from four separate experiments. *, $P < 0.01$; **, $P < 0.05$ (different from normal).

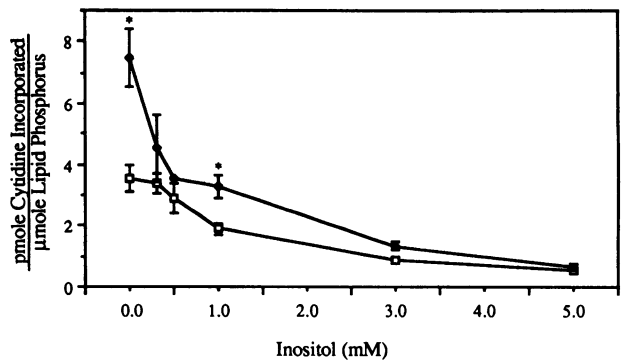

FIG. 5. Effect of MI on propranolol-stimulated CMP-PA accumulation in normal (□) and diabetic (●) nerves. Nerves were incubated with various concentrations of MI in the presence of [^{3}H]cytidine and 0.6 mM propranolol. The results are averages ± SEM for nine groups of nerve segments pooled from three separate experiments. *, $P < 0.01$ (different from normal).

according to Benjamins and Agranoff (20), there was a substantial MI-dependent release of water-soluble radioactivity, consistent with the synthesis of PI and concomitant liberation of [^{3}H]CMP.

Incubation of nerves from diabetic rats with the same range of drug concentrations caused significantly more CMP-PA to accumulate as compared to normal nerve in the presence of as little as 0.1 mM propranolol (Fig. 3). The effect was maximal at 0.6 mM propranolol when more than twice the amount of [^{3}H]liponucleotide was present in diabetic compared to normal nerve. At higher drug concentrations, as incorporation of radioactivity into the liponucleotide declined, the difference between normal and diabetic nerve disappeared.

Regulation of PI Formation and CMP-PA Accumulation by MI. The ability of MI to enhance PI formation in the presence of propranolol was examined by adding the cyclitol (10 mM) to an incubation medium containing $^{32}P_i$ and 1.0 mM propranolol. Under these conditions, the labeling of [^{32}P]PI in normal nerve segments increased 25% above the level obtained in the absence of cyclitol, as would be expected if the supply of MI was limiting the conversion of CMP-PA to this inositol phospholipid.

The inclusion of increasing concentrations of MI in the incubation medium during [^{3}H]cytidine labeling resulted in a dose-dependent progressive decrease in [^{3}H]CMP-PA, which accumulated in the presence of 0.6 mM propranolol (Fig. 5). Radioactive liponucleotide approached the basal level when the concentration of exogenous MI reached or exceeded the presumptive endogenous level of cyclitol (2–3 mM). The ability of medium MI to utilize previously accumulated CMP-PA was examined by comparing the levels of radioactive liponucleotide in nerve samples to which 3 mM MI was added either simultaneously with [^{3}H]cytidine or later. If MI was added together with the radioactive precursor, much less CMP-PA accumulated than if no MI was present (Fig. 4 *a* and *b*). In contrast, the addition of MI after either 1 or 2 hr of isotope incorporation almost completely prevented further accumulation of [^{3}H]CMP-PA but did not cause the disappearance of any previously formed liponucleotide (Fig. 4 *c* and *d*).

The presence of 0.3 mM MI in the incubation medium, a concentration far below the reported concentration of nerve cyclitol in the diabetic state, did not affect the amount of [^{3}H]CMP-PA that accumulated in normal nerve, but it nearly eliminated the increment of liponucleotide present in diabetic nerve over that in normal tissue (Fig. 5). Successively higher concentrations of MI caused a progressive and parallel decline in the residual labeled liponucleotide in both normal and diabetic nerve.

DISCUSSION

Propranolol altered normal nerve metabolism in a manner similar to that observed in other tissue preparations such that the incorporation of both [^{32}P]- and [^{3}H]cytidine into CMP-PA was greatly enhanced and the labeling of [^{32}P]PI and [^{32}P]PA was also elevated concomitant with sharply reduced entry of isotope into [^{32}P]phosphatidylcholine. In addition to inhibiting PA phosphatase, thereby increasing the availability of PA for CMP-PA formation, this agent has also been reported to affect the activities of several other enzymes that

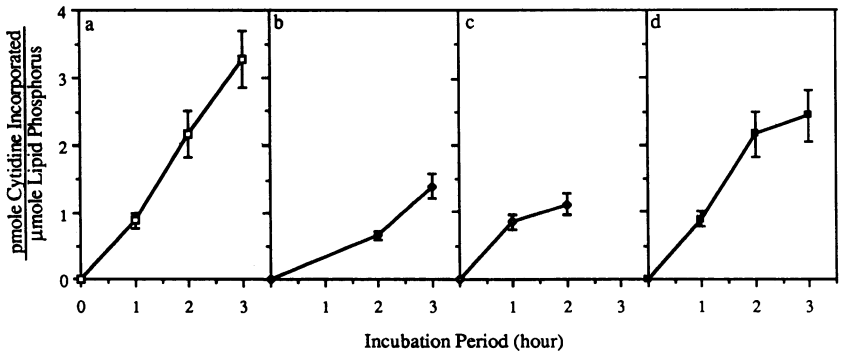

FIG. 4. Effect on CMP-PA formation of MI addition to incubation medium at various times during normal nerve incubations. Nerves were incubated with [^{3}H]cytidine in the presence of 0.6 mM propranolol for 3 hr. (*a*) No MI added. (*b*) MI (3 mM) added simultaneously with [^{3}H]cytidine. (*c*) MI (3 mM) added 1 hr after [^{3}H]cytidine. (*d*) MI (3 mM) added 2 hr after [^{3}H]cytidine. The results are averages ± SEM for six groups of nerve segments pooled from two separate experiments.

metabolize phospholipids, including PI kinase (21). This may be the explanation for the enhanced formation of phosphatidylinositol 4-phosphate elicited by the drug, a phenomenon that has also been observed in brain cortex mince (22).

Over a range of propranolol concentrations, [^{3}H]CMP-PA, the immediate precursor of PI, accumulated to a greater extent in incubated sciatic nerve from streptozotocin-induced diabetic rats than from normal rats. The formation of [^{3}H]liponucleotide was almost entirely prevented by exogenous MI at concentrations that approximate tissue levels of this cyclitol. These results provide evidence that nerve from diabetic animals is depleted of a MI pool needed for PI synthesis.

Such an interpretation is valid provided that the enhanced labeling of [^{3}H]CMP-PA in diabetic compared to normal nerve reflects an increase in liponucleotide mass and is not merely the reflection of a difference in the specific activity of its precursor, [^{3}H]CTP. The kinetics of radioactive CTP formation will be largely determined by the rates of [^{3}H]cytidine entry into the tissue and subsequent phosphorylations of the nucleoside by ATP. Preincubation of the tissue with cytidine and propranolol was carried out to preload the tissue with these compounds. Subsequently, [^{3}H]cytidine uptake into [^{3}H]CMP-PA in the two preparations displayed parallel time courses, suggesting that this factor was not appreciably affected by the diabetic state. Previously, we found that the levels and specific radioactivities of [^{32}P]ATP in normal and diabetic nerve segments were nearly the same after several hours of incubation (18), consistent with comparable potentials for cytidine phosphorylation. Moreover, the quantities of radioactivity in [^{3}H]CMP-PA were not significantly different either when propranolol was absent or a drug concentrations (1–2 mM) not far above the levels at which differences in isotope uptake were seen. Taken together, these considerations make it most unlikely that altered CTP specific activity can account for the elevated [^{3}H]CMP-PA accumulation in diabetic nerve in the range of effective propranolol concentrations.

One surprising observation was the inability of nerve to utilize CMP-PA for PI synthesis unless MI was present at the time liponucleotide formation occurs in the tissue and not if the cyclitol is added later. This finding suggests that CMP-PA once synthesized rapidly enters and accumulates in a tissue compartment where it becomes inaccessible for PI formation. The location of this putative sequestered CMP-PA pool remains to be established.

The blockade of CMP-PA accumulation by MI strongly supports the conclusion that availability of the cyclitol plays a major role in regulating the consumption of the liponucleotide under conditions induced by propranolol in which increased formation of PI is favored. Additional experiments in this laboratory (unpublished results) as well as by others (23–25) indicate that Li^+, an ion that would be expected to decrease tissue MI by inhibition of inositol monophosphatase (26), also enhances the formation of CMP-PA in nerve, brain, and parotid gland.

In view of the well-documented finding that MI content is reduced appreciably in nerves from experimentally diabetic animals, it was of particular interest that an exogenous MI concentration much less than these endogenous levels was sufficient to prevent most of the excess CMP-PA accumulation that occurred in diabetic relative to normal nerve. This result is consistent with the presence of a discrete pool of cyclitol in nerve that is not in equilibrium with the bulk of tissue MI and that is utilized for PI synthesis. This compartment of MI is both readily depleted and replenished in diabetic nerve. One possible cause for loss of MI from such a critical pool might be greater leakage of cyclitol from nerve segments due to structural damage produced by chronic diabetes (27). A second might be reduced synthesis of endogenous MI from glucose in the tissue preparation. While inositol-1-phosphate synthase activity has been detected in cell-free nerve preparations (28), the capacity of this biosynthetic pathway in nerve to supply MI has not been rigorously evaluated. A third factor could be impairment of Na^+-dependent MI uptake, which is known to be competitively inhibited by glucose (29). The inhibition is reversed in the presence of an aldose reductase inhibitor, which also normalizes tissue sorbitol levels. These findings suggest a fourth explanation—namely, that efflux of MI might be enhanced in the presence of elevated polyol (30).

PI synthase activity has been reported to be diminished in cell-free preparations from diabetic compared to normal nerve (28, 31, 32). However, since the level of exogenous MI that nearly abolished the increment in liponucleotide accumulation in the diabetic state was well below the measured K_m (1.5–4.5 mM) for the enzyme in nervous tissue (20, 33, 34), it is unlikely that CMP-PA buildup is due to reduced activity of this enzyme in nerve segments. While PI synthase is usually considered to be localized in the endoplasmic reticulum, a recent report described a PI synthase activity exhibiting a markedly lower K_m in the plasma membrane of a pituitary tumor cell line (35). If such a component of this enzyme activity were present in axolemma or Schwann cell plasma membrane, it is conceivable that the turnover of some cellular PI could be regulated by variations in micromolar concentrations of exogenous MI.

The present findings are in accord with earlier reports that incorporation of [^{3}H]MI into phospholipids is reduced in diabetic compared to normal nerve (36, 37). Moreover, they extend and lend support to the results of Winegrad and coworkers (11, 12), who have reported that incubation of normal sciatic nerve endoneurium and aortic intima–media preparations in the absence of medium MI abolished a portion of the Na^+,K^+-ATPase activity concomitant with disappearance of a component of PI turnover. These authors postulated that these alterations are due to the depletion of a discrete intracellular MI pool, since these deficits did not occur in the presence of low levels of exogenous MI, which they proposed is essential for rapid turnover of a PI compartment necessary for maintenance of normal levels of Na^+,K^+-ATPase activity.

Since protein kinase C activators have been reported to restore the missing component of Na^+,K^+-ATPase activity in diabetic nerve, while having no effect on the enzyme from normal nerve (38), the link between PI turnover and maintenance of the enzyme may be continual production of an adequate supply of 1,2-diacylglycerol. This concept has recently received support from the finding that the content of 1,2-diacylglycerol is reduced in diabetic compared to normal nerve (39). Furthermore, on the basis of molecular species analysis, this natural activator of protein kinase C appears to be derived in large part from the breakdown of inositol phospholipids and, in diabetic tissue, the bulk of the decrease in 1,2-diacylglycerol is due to a loss of the stearoyl, arachidonoyl molecular species, which is characteristic of these phospholipids.

The onset of reduced Na^+,K^+-ATPase activity in several tissues from diabetic animals is generally explained by glucose-induced stimulation of the polyol pathway, which causes decreased MI uptake, reduces cyclitol content, and thereby perturbs inositol phospholipid metabolism. However, in renal glomeruli from diabetic animals, it has been reported that decreased Na^+,K^+-ATPase activity precedes a detectable reduction in MI levels (40). Moreover, alterations in MI concentrations could not be measured in nerve biopsy samples from chronically diabetic human patients (41). These apparently disparate observations can be reconciled by the concept that, whereas reduction of total tissue MI is not a sensitive indicator of biochemical abnormalities, depletion of

a small, metabolically discrete MI pool in nerve and possibly other tissues subject to diabetic complications will disrupt proper regulation of PI metabolism and Na^+,K^+-ATPase activity.

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