Sustained Recovery of Na^+-K^+-ATPase Activity in Sciatic Nerve of Diabetic Mice by Administration of H7 or Calphostin C, Inhibitors of PKC

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We have previously shown that intraperitoneal injection of H-7, an inhibitor of PKC, restores completely the activity of Na^+-K^+-ATPase in sciatic nerve of diabetic mice; however, the effect was transient, with a half-life of ~1 h under the conditions used. This work assessed whether calphostin C, a new more potent and specific inhibitor of PKC, is also able to restore the activity of Na^+-K^+-ATPase in sciatic nerve of ALX-induced diabetic mice and also assessed if continuous administration of H-7 or calphostin C can afford sustained recovery of the ATPase. Small amounts of calphostin C (i.e., 2 μg/kg) restore entirely the activity of the enzyme. Larger doses (e.g., 30 μg/kg) can be administered with equal results. The ED_{50} was ~0.5 μg/kg. This indicates that calphostin C is ~20,000 times more potent than H-7 in restoring the ATPase activity in diabetic mice. A single intraperitoneal injection of 1 or 10 μg/kg of calphostin C maintains the enzyme for 4 and 8 h, respectively. Administration of H-7 by continuous delivery from micro-osmotic pumps implanted in the back of the mice maintains the Na^+-K^+-ATPase for 24 h, although the activity decreases thereafter. This is the result of instability of H-7 in solution. Continuous administration of calphostin C maintains the activity of the ATPase at nearly normal values for at least 2 wk. The results support the hypothesis that, in sciatic nerve tissue of diabetic animals, the activity of PKC is increased, leading to higher phosphorylation of Na^+-K^+-ATPase, which results in the decreased activity observed. Also, the results show that inhibitors of PKC such as calphostin C can be of therapeutic interest. *Diabetes* 42:257–62, 1993

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H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PKC, protein kinase C; ALX, alloxan; cGMP, cyclic GMP.

Diabetic neuropathy is the most common peripheral neuropathy in the Western world. The pathogenetic mechanism for diabetic neuropathy remains unclear (1), although it is thought that morphofunctional abnormalities in experimental diabetic neuropathy are attributable to decreased Na^+-K^+-ATPase activity (1,2).

Na^+-K^+-ATPase is phosphorylated by PKC (3). Furthermore, gangliosides, which inhibit PKC (4), restore the activity of Na^+-K^+-ATPase in sciatic nerve of ALX-induced diabetic rats (5,6).

The effect of gangliosides on the ATPase activity could be the result of the inhibition of PKC. Therefore, we tested if H-7, an inhibitor of PKC, is able to restore the activity of Na^+-K^+-ATPase in sciatic nerve tissue of diabetic mice. We found that H-7 restores the activity of the enzyme; however, the effect was maintained only for a short time, probably because the H-7 is rapidly degraded or eliminated (7).

Recently, calphostin C, a new more potent and specific inhibitor of PKC, has become commercially available (8,9). We assessed if calphostin C is able to restore the activity of the Na^+-K^+-ATPase in sciatic nerve of ALX-induced diabetic mice and tested if continuous administration of H-7 or calphostin C could afford sustained recovery of the ATPase.

Calphostin C restores completely the activity of the enzyme. Moreover, ATPase can be maintained at normal levels for >2 wk. These results confirm that diminished activity of Na^+-K^+-ATPase in experimental diabetes is caused by increased phosphorylation by PKC.

RESEARCH DESIGN AND METHODS

Male albino mice were used. Animals were made diabetic with a single subcutaneous injection of 200 mg ALX per kg/body wt (66 mg/ml in 0.1 M citrate-phosphate

buffer, pH 4.5). Control mice were injected with the same volume of the citrate-phosphate buffer. Blood was taken from the tail vein, and glucose was determined enzymatically as described by Kunst et al. (10). Mice identified as diabetic on the basis of blood glucose levels were divided into subgroups as required by the experiment (at least four mice per subgroup). Experiments were started 5 days after the injection of ALX when the activity of Na^+-K^+-ATPase has decreased to ~50% of the controls.

The PKC inhibitors H-7 (Sigma, St.Louis, MO) and calphostin C (Kamiya Biomedical, Thousand Oaks, CA) were used. H-7 inhibits similarly different isoenzymes of PKC (11), whereas, as far as we know, no data are available about the isoform specificity of calphostin C. H-7 and calphostin C were dissolved in dimethyl sulfoxide (100 and 1 mg/ml, respectively) and then diluted with water. The solvent alone had no effect on the Na^+-K^+-ATPase activity. For acute experiments, the inhibitors were injected intraperitoneally. For continuous delivery of the inhibitors, miniature implantable pumps from Alza (Palo Alto, CA) were used. The mini- or micro-osmotic pumps were filled with the solutions of inhibitors and implanted in the back of the mice. At least four animals per group were used.

After treatment, mice were killed by cervical dislocation, and both sciatic nerves were taken and homogenized in 20 vol of a prechilled solution of 0.25 M sucrose, 1.25 mM EGTA, 10 mM Tris-HCl, pH 7.5. Aliquots were frozen and stored at −80°C until used. Na^+-K^+-ATPase activity was determined spectrophotometrically by the coupled enzymatic method described by Penefski and Bruist (12) as the difference between total and ouabain-insensitive ATPase activity. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 2.5 mM phosphoenolpyruvate, 50 μM NADH, 2.5 mM ATP, 0.2 U of pyruvate kinase, 0.2 U of lactate dehydrogenase, and the sample (10 μg of protein). Ouabain, when added, was 3 mM. Five different assays were conducted for each animal. Incubations were conducted at 30°C, and the absorbance at 360 nm was followed for 15 min. The activities are expressed as nanomole of NADH oxidized per hour per milligram of protein.

To assess the effect of the injection of the inhibitors on the activity of PKC in sciatic nerve tissue, mice were injected with saline (controls), H-7 (30 mg/kg), or calphostin C (30 μg/kg). After 15 min, mice were killed by cervical dislocation, and both sciatic nerves were taken and homogenized in 20 vol of medium A (20 mM Tris-HCl, pH 7.5, 50 mM β-mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA, 20 μg/ml aprotinin, and 100 μg/ml leupeptin). The homogenate was centrifuged at 4°C for 30 min at 100,000 *g*. The supernatant (cytosolic fraction) was taken and kept in ice until used. The pellet was suspended by homogenization in 25% of the original volume of medium A containing 0.5% Triton X-100, stirred at 4°C for 20 min, and then diluted 10 times with medium A to bring the concentration of Triton X-100 to 0.05%. Samples were centrifuged at 4°C for 30 min at 100,000 *g*, and the supernatant was used as the membrane fraction for the assay. The incubation mixtures contained in a final volume of 75 μl of 50 mM Tris-HCl, pH 7.5; 1 mM calcium acetate; 75 μg/ml phosphatidylserine; 2 μg/ml phorbol 12-myristate 13-acetate; 75 μM peptide substrate (Amersham, Arlington Heights, IL); 2.5 mM dithiothreitol; 15 mM magnesium acetate; 50 μM [Γ-^{32}P]ATP (0.25 μCi/tube); and 15, 20, or 25 μl of the sample. For the blanks, calcium and phosphatidylserine were omitted. Samples were incubated for 15 min at 25°C, and the reaction was stopped by adding 100 μl of 150 mM ortophosphoric acid; 125 μl of the mixture were pipetted onto a square of P81 Whatman paper, which was then immersed in 75 mM orthophosphoric acid. After washing, radioactivity retained in the filter (incorporated in the peptide) was counted. Values are given as nanomoles of ^{32}P incorporated in the peptide per milligram of sample protein.

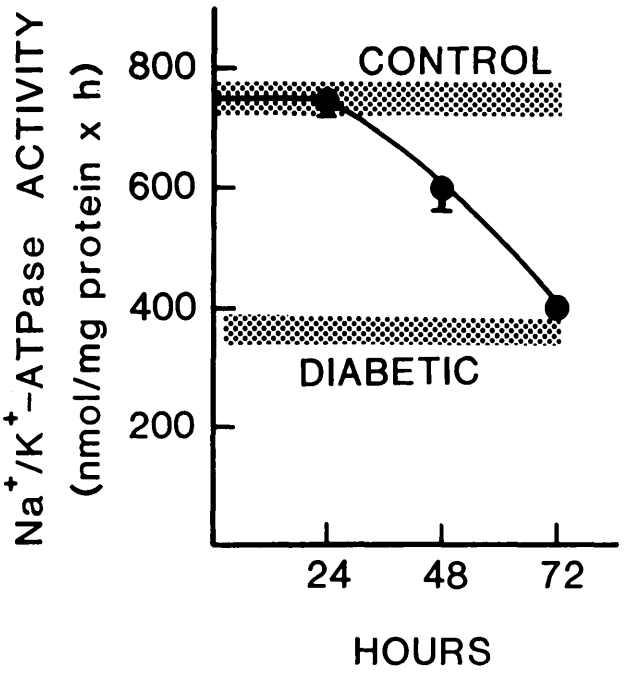

FIG. 1. Effect of continuous delivery of H-7 on the activity of Na^+-K^+-ATPase in sciatic nerve of diabetic mice. Mice were injected subcutaneously with 200 mg ALX/kg body wt. After 4 days, blood was taken from tail vein, and glucose was determined. Groups of four diabetic or control mice were used. Micro-osmotic pumps were filled with 100 μl of solution of H-7 (250 mg/ml) in 50% dimethyl sulfoxide and implanted in back of diabetic mice. These pumps released 1 μl/h. Groups of four mice were killed at indicated times, both sciatic nerves were taken, and activity of Na^+-K^+-ATPase was determined. Dotted areas are means ± SD for controls and diabetic (nontreated with H-7) mice, respectively. For points lacking SD, SD was less than width of point. Activity after 72 h of implantation of pumps was not significantly different from diabetic, nontreated mice.

RESULTS

The activity of Na^+-K^+-ATPase in sciatic nerve of ALX-induced diabetic mice decreases by ~50% after 3 days and very slightly thereafter. Intraperitoneal injection of 30 mg/kg of H-7 completely restored the activity of the enzyme; however, the effect of H-7 is transient, with a half-life of ~1 h under these conditions. Injection of H-7 to control mice did not affect significantly the activity of Na^+-K^+-ATPase (7).

The first objective of this work was to assess if continuous administration of H-7 can maintain the activity of

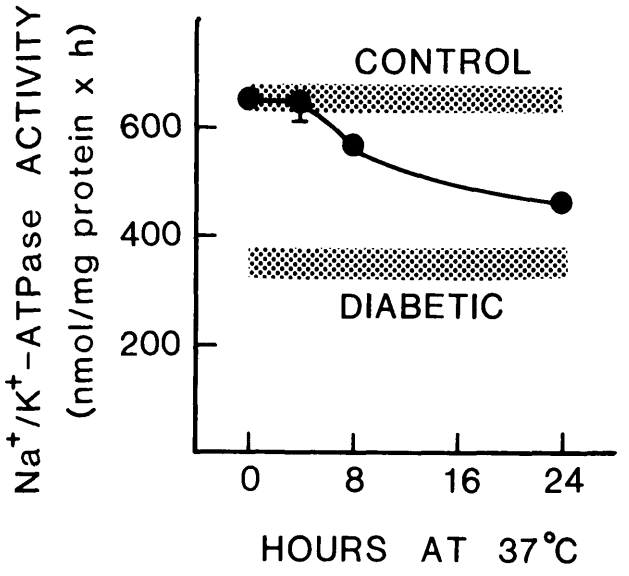

FIG. 2. Effect of heating H-7 at 37°C for different periods of time on its ability to restore Na^+-K^+-ATPase activity in sciatic nerve of diabetic mice. Mice were made diabetic as in Fig. 1. Solution of H-7 (10 mg/ml) in 10% dimethyl sulfoxide was prepared and used immediately (time 0) or after heating at 37°C for indicated times. Groups of four diabetic mice were injected intraperitoneally with 15 mg/kg of H-7 and killed 15 min later. Groups of control and diabetic, nontreated mice were also killed. Both sciatic nerves were taken, and activity of Na^+-K^+-ATPase was determined. Dotted areas are means ± SD of values for control and diabetic (nontreated with H-7) mice, respectively. For points lacking SD, SD was less than width of point. All values for experimental mice were statistically different from diabetic, nontreated mice.

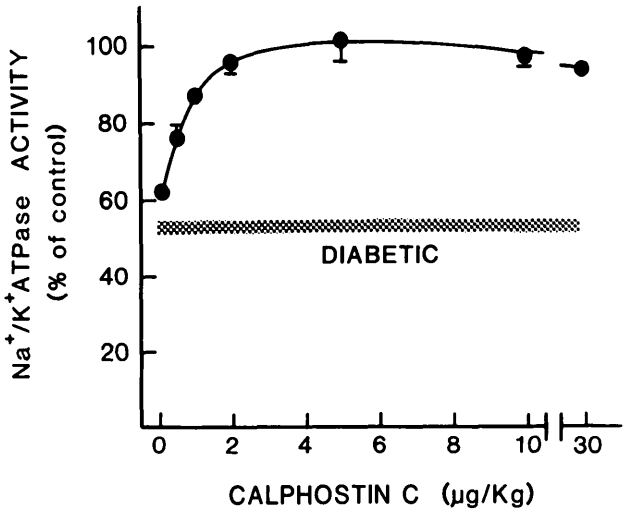

FIG. 3. Dose-dependent recovery by calphostin C of Na^+-K^+-ATPase activity in sciatic nerve tissue of diabetic mice. Mice were injected subcutaneously with 200 mg ALX/kg body wt. After 4 days, blood was taken from tail vein, and glucose was determined. On day 5, groups of 5 diabetic mice were injected intraperitoneally with the indicated doses of calphostin C and killed 15 min later. Groups of control and diabetic, nontreated mice were also killed. Both sciatic nerves were taken and Na^+-K^+-ATPase activity was determined. Values are the percentage of activity of controls. Dotted area is mean ± SD of values for diabetic, nontreated mice. For points lacking SD, SD was less than width of point. Values for all groups of mice injected with calphostin C were significantly different from diabetic, nontreated mice.

Na^+-K^+-ATPase for longer periods of time. We administered the H-7 by continuous delivery from micro-osmotic pumps implanted in the back of the mice. As shown in Fig. 1, the activity of Na^+-K^+-ATPase was restored to normal values. The activity remained as 100% of control for 24 h and decreased thereafter. After 48 h, 60% of the activity lost as a result of diabetes was recovered by H-7, whereas after 72 h no difference was observed between diabetic mice treated or not treated with H-7. No untoward effects were noted in mice bearing the osmotic pumps during this 72-h period.

These results clearly demonstrate that continuous administration of H-7 can maintain the activity of Na^+-K^+-ATPase at normal levels for 24 h but not thereafter. Because H-7 is aways delivered at the same rate during the experiment, H-7 is probably not stable under these conditions. To assess this possibility, we heated solutions of H-7 for different periods of time at 37°C and injected 15 mg/kg i.p. of the inhibitor to diabetic mice. As shown in Fig. 2, freshly prepared H-7 is able to restore completely the Na^+-K^+-ATPase activity. After heating at 37°C for 4 h, the recovery is still complete; however, only 73 and 40% of the lost activity is recovered with H-7 heated for 8 and 24 h, respectively. This indicates that H-7 is fairly unstable under these conditions and explains why the effect of H-7 is not maintained after 72 h (Fig. 1).

Calphostin C, a new more potent and specific inhibitor of PKC (8,9), has recently become available. Thus we tested if calphostin C is able to restore the activity of Na^+-K^+-ATPase in sciatic nerve tissue of diabetic mice. As is the case for H-7, injection of calphostin C to control mice did not affect the activity of the ATPase or slightly decreases it (by 5–10%). As shown in Fig. 3, intraperitoneal injection of calphostin C restores the activity of Na^+-K^+-ATPase in sciatic nerve of diabetic mice. The recovery was complete at ~2 µg/kg and is maintained at the same level with the larger doses tested, e.g., 30 µg/kg. The ED_{50} was ~0.5 µg/kg. We have previously shown that for H-7 the recovery was complete at ~20

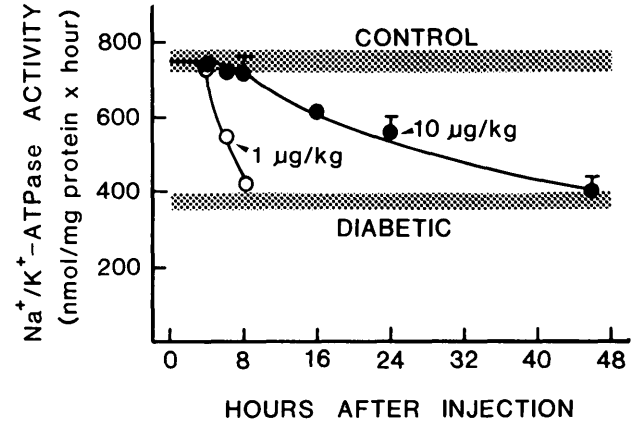

FIG. 4. Effect of a single injection of calphostin C on activity of Na^+-K^+-ATPase after different times. Mice were injected subcutaneously with 200 mg ALX/kg body wt. After 4 days, blood was taken from tail vein, and glucose was determined. On day 5, groups of 5 mice were injected intraperitoneally with 1 (○) or 10 (●) µg/kg of calphostin C and killed at indicated times. Groups of control and diabetic nontreated mice were also killed. Both sciatic nerves were removed, and Na^+-K^+-ATPase activity was determined. Dotted areas are means ± SD of values for controls and diabetic nontreated mice, respectively. For points lacking SD, SD was less than width of point.

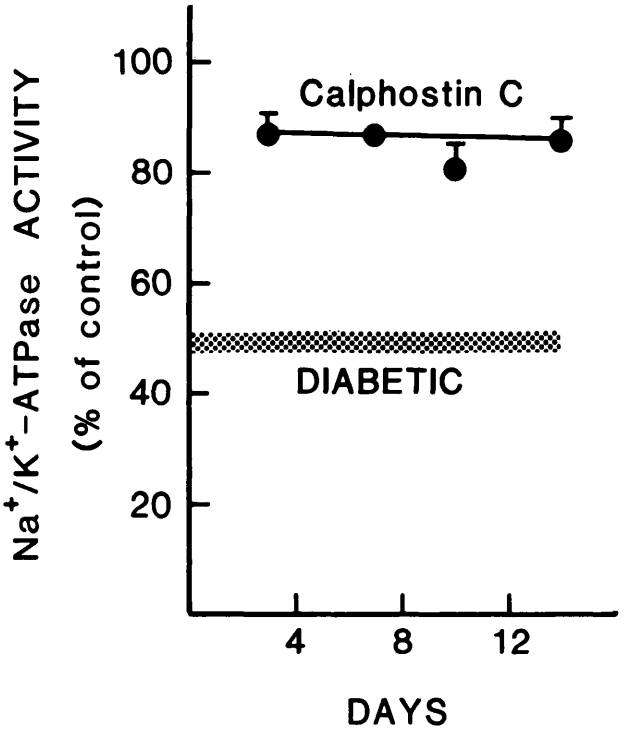

FIG. 5. Effect of continuous delivery of calphostin C on Na^+-K^+-ATPase activity in sciatic nerve tissue of diabetic mice. Mice were injected subcutaneously with 200 mg ALX/kg body wt. After 4 days, blood was taken from tail vein, and glucose was determined. Groups of four diabetic or control mice were used. Mini-osmotic pumps were filled with a solution of 50 μg/ml of calphostin C in 10% dimethyl sulfoxide. These pumps released 0.5 μl/h. Groups of four mice were killed at indicated times, both sciatic nerves were taken, and Na^+-K^+-ATPase activity was determined. Dotted area is mean ± SD of values for diabetic, nontreated mice. For points lacking SD, SD was less than width of point. Values for calphostin C-treated mice were statistically different from diabetic mice for all times.

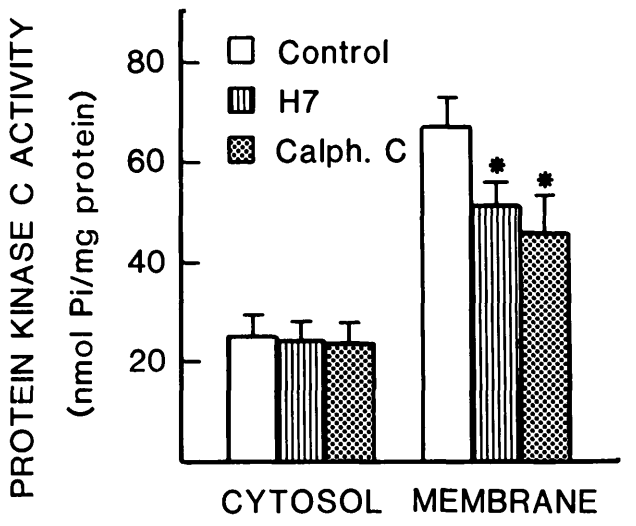

FIG. 6. Effect of intraperitoneal injection of H-7 and calphostin C on activity of PKC in sciatic nerve. Mice were injected intraperitoneally with H-7 (30 mg/kg) or calphostin C (30 μg/kg). After 15 min, mice were killed by cervical dislocation. Both sciatic nerves were taken, and cytosol and membrane fractions were isolated. For assay of PKC activity, three different amounts of sample (15, 20, and 25 μl) were used to ensure linearity of assay, which was carried out as described in Experimental Procedures. Two to four mice/group were used in each experiment. Three different experiments were conducted. Values are means ± SD of 8 mice per group. * Statistically ($P \leq 0.001$) different from controls.

mg/kg, and the ED_{50} was ~10 mg/kg. The results shown in Fig. 3 indicate, therefore, that calphostin C is ~20,000 times more potent than H-7 in restoring the ATPase activity in diabetic mice.

We then studied how long a single injection of calphostin C can actively maintain the Na^+-K^+-ATPase. As shown in Fig. 4, intaperitoneal injection of 1 μg/kg of calphostin C totally restores the ATPase activity for 4 h, then decreases rapidly. Injection of 10 μg/kg of calphostin C restores the Na^+-K^+ activity completely for 8 h. Then the activity decreases slowly. As mentioned above, H-7 restores transiently the activity of the ATPase in sciatic nerve of diabetic mice. Injection of 30 mg/kg of H-7 restores completely the activity after 15 min, but only 50% of the activity is recovered after ~1 h (7). The above results clearly show that calphostin C is much more effective.

We then tested how long the Na^+-K^+-ATPase can be maintained completely active if calphostin C is administered continuously. Miniosmotic pumps containing a solution of calphostin C (50 μg/ml) were implanted in the back of diabetic mice which were killed after 3, 7, 10, or 14 days. As shown in Fig. 5, 3 days after implantation of the pumps, the Na^+-K^+-ATPase activity in sciatic nerves was 86 ± 4% of the controls, whereas for diabetic, nontreated mice it was 48 ± 2%. Therefore, under these conditions, the recovery was nearly complete after 3 days and remained at the same level (86%) for as long as 14 days (Fig. 5), which is longer than we can test with the pumps used. The level of glucose in blood was not affected by the treatment with calphostin C. For example, the values for controls, diabetic nontreated, and diabetic mice bearing the miniosmotic pumps for 14 days were 8.6 ± 0.5, 31.9 ± 4.3, and 30.1 ± 4.1 mM, respectively, the day before implanting the pumps and 8.9 ± 0.5, 31.7 ± 5.7, and 31.5 ± 3.3 mM 14 days later.

We then assessed whether PKC activity in nerve tissue is actually reduced after injection of H-7 (30 mmol/kg) or calphostin C (30 μmol/kg). To this end we assayed the in vitro activity of PKC in cytosol and membrane fractions obtained from sciatic nerves of mice both injected and not injected with the inhibitors. As shown in Fig. 6, PKC activity in the cytosol was not affected by injection of H-7 or calphostin C, whereas the activity in the membrane fraction was significantly reduced 15 min after injection of H-7 (by 23%) and of calphostin C (by 31%).

DISCUSSION

The above results clearly show that H-7 and calphostin C, two different inhibitors of PKC, restore the activity of Na^+-K^+-ATPase in sciatic nerve tissue of diabetic mice. Also, this activity can be maintained at nearly normal values for at least 2 wk by continuous administration of calphostin C. This treatment did not produce any noticeable untoward effect nor affect the blood glucose levels.

The activity of Na^+-K^+-ATPase decreases in experimental diabetes (5,13,14). Impairment of Na^+-K^+-ATPase activity plays a pivotal role in the pathogenesis of

diabetic neuropathy (1,2). However, the reason for the decreased activity of the ATPase remains controversial. It has been suggested that, in diabetes, an altered *myo*-inositol uptake and metabolism leads to derangement of phosphoinositide turnover (1,15). This would lead to altered activity of PKC. It has been suggested that the activity of PKC is decreased in diabetes (14) and that the reduction in the activity of Na^+-K^+-ATPase probably mirrors decreased PKC activity (15,16). This suggestion was based on experiments showing that activators of PKC acutely normalized decreased ouabain-inhibitable respiration in diabetic rabbit nerve (14). However, it should be considered that the compounds used produce a transient activation of PKC followed by a rapid down-regulation. Incubation with phorbol 12-myristate 13-acetate for 1 h, as shown by Greene and Lattimer (14), actually decreases the activity of PKC in different types of cells (17–19). Therefore, the recovery of the Na^+-K^+-ATPase induced by the activators of PKC could be caused by inhibition instead of activation of PKC. Alternatively, it is possible that phorbol esters stimulate the pump by a mechanism other than PKC activation, as suggested by Tomlinson and Ettlinger (20).

We previously reported that the activity of Na^+-K^+-ATPase is restored in sciatic nerve of diabetic mice by in vivo administration of H-7 (7). We suggested that the phosphorylated form of the ATPase is less active than the unphosphorylated one and that the increased activity of PKC in diabetic animals could be responsible for the reduced activity of Na^+-K^+-ATPase (7). In fact, PKC is activated in glomeruli of diabetic rats (21).

Na^+-K^+-ATPase is phosphorylated by PKC (3). However, the effect of this phosphorylation on the activity of the ATPase is not clear. Some reports suggested that activation of PKC stimulates Na^+-K^+-ATPase (22), whereas others suggested that activators of PKC inhibit the ATPase (23). The reason for these differing results is not clear but could be the result of the use of different experimental approaches in different tissues. However, Bertorello et al. (24) clearly showed that PKC phosphorylates purified Na^+-K^+-ATPase, and that this phosphorylation was associated with an inhibition of its activity of ~40–50%. Note that, as shown here and previously (6,7), the activity of the Na^+-K^+-ATPase in sciatic nerve tissue of diabetic rats or mice is also decreased by ~50%, supporting the concept that inhibition of the ATPase is caused by increased phosphorylation by PKC. A recent preliminary report by Tomlinson and Ettlinger (20) also suggests that PKC exerts a tonic inhibition of the Na^+-K^+ pump in rat sciatic endoneurium. This agrees with the results reported herein and previously (7), showing that inhibitors of PKC restore completely the activity of Na^+-K^+-ATPase in sciatic nerve tissue of diabetic mice.

The results shown in Fig. 6 indicate that PKC is inhibited in the membrane fraction of the sciatic nerves of mice injected intraperitoneally with H-7 or calphostin C, supporting the idea that the restoration of the Na^+-K^+-ATPase activity is mediated by inhibition of PKC. The activity of PKC was inhibited in the membrane fraction but not in the cytosol. This would reflect selective retention of the inhibitors in the membrane-bound PKC during fractionation of the samples. In any case, as PKC depends on Ca^{2+} and phospholipids, it is considered to be active when bound to the membrane and inactive when in the cytosol. Therefore, the results shown in Fig. 6 indicate that intraperitoneal injection of H-7 or calphostin C actually reduces the activity of PKC in sciatic nerve tissue.

The possibility that H-7 and calphostin C could restore the ATPase activity by means other than PKC inhibition cannot be completely ruled out. However, the relative potencies of H-7 and calphostin C to restore Na^+-K^+-ATPase are similar to their relative potencies to inhibit PKC. Also, these inhibitors act on different domains of the kinase, H-7 on the catalitic domain and calphostin C on the regulatory domain. Therefore, it seems unlikely that both inhibitors act on a putative nonkinase enzyme. The possibility that H-7 and calphostin C modulate a different protein kinase cannot be excluded; H-7 is a nonspecific inhibitor of PKC, it also inhibits protein kinases dependent on cAMP and cGMP with a similar K_i. However, HA 1004, which inhibits cAMP and cGMP-dependent protein kinases with K_i values ~20- and 30-fold lower than for PKC (25), did not restore the activity of Na^+-K^+-ATPase (7), suggesting that cAMP- and cGMP-dependent protein kinases are not involved in this effect. Calphostin C is a highly specific inhibitor of PKC (8,9); however, it cannot be excluded that it could also inhibit a different, not yet identified, protein kinase. However, the most likely explanation for the results reported herein and earlier (7) seems to be that H-7 and calphostin C restoration of Na^+-K^+-ATPase is mediated by inhibition of PKC.

In conclusion, our results support the hypothesis that, in sciatic nerve tissue of diabetic animals, the activity of PKC is increased, leading to higher phosphorylation of Na^+-K^+-ATPase that results in the decreased activity observed. These alterations can be reversed for long periods of time by continuous administration of inhibitors of PKC. This provides an experimental approach to assess the role of decreased Na^+-K^+-ATPase activity in the origin of other neuropathological alterations such as decreased nerve conduction velocity.

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