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In vivo phosphorylation of the Na,K-ATPase α subunit in sciatic nerves of control and diabetic rats: Effects of protein kinase modulators

(sodium pump/protein kinase C/protein kinase A/diabetic neuropathy)

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ABSTRACT **The phosphorylation state of the Na,K-ATPase α subunit has been examined in ^{32}P-labeled sciatic nerves of control and streptozotocin-treated diabetic rats. Intact nerves were challenged with protein kinase (PK) modulators and α-subunit ^{32}P labeling was analyzed after immunoprecipitation. In control nerves, the PKC activator phorbol 12-myristate 13-acetate (PMA) had little effect on α-subunit ^{32}P labeling. In contrast, staurosporine, a PKC inhibitor, and extracellular calcium omission decreased it. In Ca^{2+}-free conditions, PMA restored the labeling to basal levels. The cAMP-raising agent forskolin reduced the ^{32}P labeling of the α subunit. The results suggest that nerve Na,K-ATPase is tonically phosphorylated by PKC in a Ca^{2+}-dependent manner and that PKA modulates the phosphorylation process. In nerves of diabetic rats, PMA increased ^{32}P labeling of the α subunit. In contrast to staurosporine or extracellular calcium omission, the decreased state of phosphorylation seen with forskolin was no longer significant in diabetic nerves. No change in the level of α-subunit isoforms (α1 or α2) was detected by Western blot analysis in such nerves. In conclusion, the altered effect of PK activators on Na,K-ATPase phosphorylation state is consistent with the view that a defect in PKC activation exists in diabetic nerves.**

Na,K-ATPase is a key enzyme in the function of all mammalian cells. In addition to maintaining membrane potential, the Na^+ gradient produced by this pump drives the transport across the cell membrane of ions as well as amino acids, glucose, and *myo*-inositol. The functional enzyme comprises distinct α and β subunits of, respectively, 100 and 40–60 kDa and possibly a third γ subunit of 12 kDa (1). The α and β subunits both exist as multiple isoforms (2, 3). The short-term regulation of the pump is still poorly understood. A phosphorylation/dephosphorylation regulatory mechanism via protein kinases and phosphatases has been suggested. In particular, it was found that the catalytic α subunit of Na,K-ATPase can serve both in subcellular fractions and in intact cells as a substrate for protein kinases (PKs). Phosphorylation of the enzyme by PKC and cAMP-dependent PKA in subcellular fractions from different cell types has been described (4–6). With either kinase, phosphorylation was associated with inhibition of enzyme activity (5). PKC activation by phorbol 12,13-dibutyrate was shown to increase ^{32}P labeling of the α subunit in kidney epithelial cells, thus supporting the notion that PKC might also phosphorylate the enzyme in intact cells (7).

In peripheral nerves, diabetes leads to a reduction of Na,K-ATPase activity as measured directly or indirectly as ouabain-inhibitable oxygen consumption (8–10). This defect is proposed to be the common denominator for altered energy metabolism, diminished nerve conduction, and paranodal swelling found in acute experimental diabetes (11). Furthermore, the impaired activity of the pump can be corrected by PK modulators, thus providing a model for the functional implication of phosphorylation of the pump. Published results based on indirect approaches are, however, conflicting. On the one hand, the acute application of PKC activators specifically corrected the impaired activity of the pump in peripheral nerves (10, 12, 13) and in erythrocytes of diabetic animals (14). On the other hand, altered Na,K-ATPase activity in sciatic nerves of diabetic mice was apparently increased by inhibitors of PKC (15, 16). PKA activation also improved Na,K-ATPase activity in nerves of diabetic rats (17).

In view of these conflicting observations, it is important to define whether PKs act directly on the Na,K-ATPase in intact nerves. This would help to elucidate both the mechanism of regulation of the pump and the defect underlying the initiating events of diabetic peripheral neuropathy. In the present study, we have examined the phosphorylation state of the Na,K-ATPase α subunit in sciatic nerves of normal and diabetic rats under basal conditions and in response to PK modulators.

MATERIALS AND METHODS

Animals and Surgery. Male Wistar rats (200–250 g) were made diabetic by injection of streptozotocin (65 mg/kg) as described (18) and were used 9–10 weeks after injection. Age-matched controls received buffer only. Diabetic rats displayed marked hyperglycemia [35.6 ± 3.1 mM glucose for diabetics; 6.6 ± 1.1 mM glucose for controls (mean ± SEM)], moderate weight gain [124 ± 21 g for diabetics; 275 ± 20 g for controls (mean ± SEM)], and cataract formation. Sciatic nerves were dissected under anesthesia and rapidly desheathed in a nominally Ca^{2+}-free Krebs–Ringer bicarbonate Hepes buffer (KRBH; 140 mM NaCl/3.8 mM KCl/0.2 mM NaH_2PO_4/0.5 mM $MgSO_4$/10 mM Hepes/2 mM $NaHCO_3$, pH 7.4) supplemented with glucose (10 mM) and *myo*-inositol (1 mM). Thereafter, random nerves were tied with surgical thread at both extremities and pooled by pairs. When indicated, *n* refers to nerve pairs.

Nerve ^{32}P Labeling and Incubation. Nerve pairs were preincubated (15 min) in cold KRBH (with or without 1.5 mM $CaCl_2$, as mentioned) supplemented with glucose (10 mM),

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Abbreviations: PK, protein kinase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, intracellular calcium concentration; PLC, phospholipase C; DAG, diacylglycerol.

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myo-inositol (1 mM), and pyruvate (5 mM) and gradually brought to 37°C. They were then transferred to the solution containing [^{32}P]orthophosphate (New England Nuclear) (1 mCi/ml in KRBH; 1 Ci = 37 GBq) (37°C) for 60 min. Thereafter, the nerves were placed in KRBH containing the stimuli for 15 min. The reaction was terminated by rinsing the nerves in ice-cold phosphate-buffered saline/0.5 mM EGTA, pH 7.4, containing NaF (30 mM), Na_3VO_4 (1 mM), and $Na_4O_7P_2$ (30 mM).

Subcellular Fractionation. Each pair of nerves was homogenized in 20 mM Tris·HCl/2 mM EGTA/2 mM EDTA/6 mM 2-mercaptoethanol/10 μg of leupeptin per ml/4 μg of aprotinin per ml/1 mM Na_3VO_4/30 mM NaF/30 mM $Na_4O_7P_2$, pH 7.4, whereupon cytosolic and membrane fractions were prepared (18). Rat brain membranes were similarly prepared. The protein content of the samples was determined by the Bradford method (19) using bovine serum albumin as standard. The recovery of Na,K-ATPase between the cytosolic and membrane fractions was evaluated by Western blot analysis. For both control and diabetic rats, the α1 and α2 isoforms of the α subunit were reproducibly detected exclusively in the membrane fraction (n = 6 for each group; data not shown).

Membrane Phosphoprotein Pattern and α-Subunit Immunoprecipitation. Phosphorylated membrane proteins (10 μg) were analyzed by SDS/PAGE and autoradiography (−70°C; 6–14 h) of dried gels with preflashed Fuji x-ray films. The α subunit was immunoprecipitated from phosphorylated membrane samples (100 μg), as described (20), by addition of anti-α, a polyclonal rabbit antiserum raised against the *Bufo marinus* enzyme (21). Its ^{32}P labeling was revealed by autoradiography (7–15 days exposure) after SDS/PAGE. Western blot analysis of the immunoprecipitate obtained from rat brain membranes, using isoform-specific antibodies defined below (data not shown), demonstrated that anti-α does not discriminate between the α1, α2, and α3 α-subunit isoforms.

Western Blotting. Western blotting was done essentially as described (18) using mouse monoclonal anti-α1 McK1 (22), rabbit polyclonal anti-α2 (Upstate Biotechnology, Lake Placid, NY), mouse monoclonal anti-α3 McB-X3 (23), and anti-α (21) as primary antibodies with horseradish peroxidase conjugates as secondary antibodies. Immunoreactivity was visualized by chemiluminescence (Amersham).

Quantitative Analysis of Autoradiograms. Quantification of both the ^{32}P labeling and the immunoreactivity of antibodies was done under conditions of linearity using the VD620 videodensitometer (Bio-Rad). To take into account differences in ^{32}P uptake between nerves, the intensity of the immunoprecipitated band was normalized to the mean value of two phosphoproteins of 160–165 (P1) and 55–60 (P2) kDa seen in the general membrane phosphoprotein pattern. These two bands (see Figs. 1 and 3) were chosen as they were not changed by the stimuli or by diabetes. With okadaic acid (GIBCO), the levels of phosphorylation of the α subunit were evaluated as -fold increase (see ref. 24). Results are expressed as means ± SEM. Values were compared by the Mann–Whitney U test.

RESULTS

***In Vivo* Phosphorylation of the Na,K-ATPase α Subunit in Control Sciatic Nerves.** Nerve pairs from normal rats labeled with [^{32}P]orthophosphate were challenged or not for 15 min with PK modulators. Since Na,K-ATPase α subunit is detected exclusively in the nerve membrane fractions (18), the latter were analyzed for phosphoproteins. Treatment of the nerves with phorbol 12-myristate 13-acetate (PMA) (10 μM), the PKC inhibitor staurosporine (100 nM), or forskolin (10 μM) did not cause major changes in the general pattern of phosphoproteins (Fig. 1*A*). At least 12 phosphoproteins of molecular mass ranging from 20 to 170 kDa were detected. Two protein bands (≈30 and ≈25 kDa) displayed the strongest ^{32}P labeling. According to their molecular mass and relative abundance (>80% of all stained proteins), they probably represent the protein P0 and a proteolysis product of P0 (24, 25). PMA application led to increased ^{32}P incorporation in an ≈80-kDa band (Fig. 1*A*, ×); conversely, staurosporine decreased it. This band probably represents the MARCKS protein (26). Since it was an easily detectable PKC substrate in rat sciatic nerves, it was used as a positive control for the effect of PMA (27). However, no resolved phosphoprotein band could be identified as the Na,K-ATPase α subunit (≈100 kDa) (Fig. 1*A*). Therefore, immunoprecipitation of the α subunit from the same membrane fractions shown in Fig. 1*A* was performed in order to examine unequivocally its ^{32}P labeling. A single radioactive band was detected in each lane (≈100 kDa; Fig. 1*B*) and was identified as the Na,K-ATPase α subunit in parallel experiments by Western analysis. In the absence of any stimulus, the α subunit showed a basal ^{32}P labeling (Fig. 1*B*), which was not affected by PMA, in contrast to staurosporine application, which reduced it significantly (Figs. 1*C* and 2; Table 1).

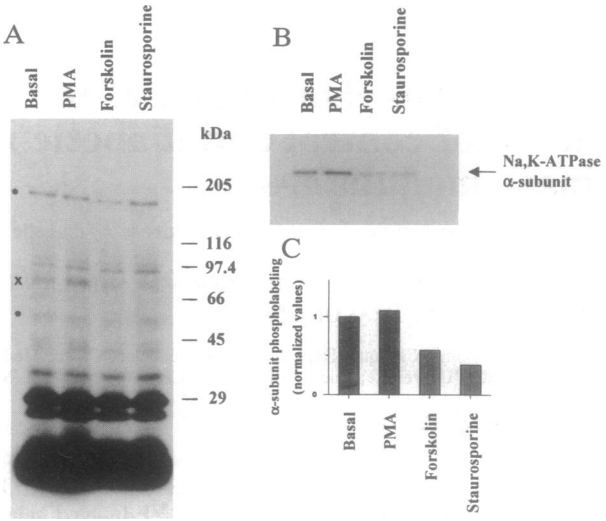

FIG. 1. Effects of PK modulators on protein and Na,K-ATPase α-subunit phosphorylation in normal rat sciatic nerve. Nerves labeled (60 min) with [^{32}P]orthophosphate were challenged (15 min) with either no test substance (basal) or with PMA (10 μM), forskolin (10 μM), or staurosporine (100 nM) in normal KRBH. (*A*) General phosphorylation pattern of membrane proteins. Membrane proteins (10 μg) were analyzed by SDS/PAGE (5–15% polyacrylamide gradient) and autoradiography. Molecular size markers (kDa) are indicated on the left. ●, P1 and P2 proteins used for normalization; ×, 80-kDa PKC substrate. (*B*) α-Subunit immunoprecipitation. Membrane proteins (100 μg) shown in *A* were immunoprecipitated with the anti-α antiserum and the precipitates were analyzed by SDS/PAGE and autoradiography. (*C*) Relative effect (%) of PMA, forskolin, and staurosporine on α-subunit ^{32}P labeling: intensities of bands shown in *B* (6.2, 7.9, 3.8, 3.5) were normalized to that of P1 (2.9, 3.8, 3.9, 5.0) and P2 (3.6, 3.8, 3.1, 4.6) shown in *A*. Values in parentheses correspond to intensities (arbitrary units) measured by densitometric scanning for basal, PMA, forskolin, and staurosporine, respectively.

To investigate the mechanisms underlying the basal α-subunit ^{32}P labeling, nerves were incubated in nominally Ca^{2+}-free buffer (Fig. 2; Table 1). This maneuver is known to decrease both intracellular calcium concentration ($[Ca^{2+}]_i$) (29) and basal inositol phospholipid turnover in peripheral nerves (30). Low calcium throughout decreased the ^{32}P labeling of the α subunit. Under these conditions, PMA (31) or readdition of extracellular Ca^{2+} (data not shown) restored basal phosphorylation levels in 15 min. These results, to-

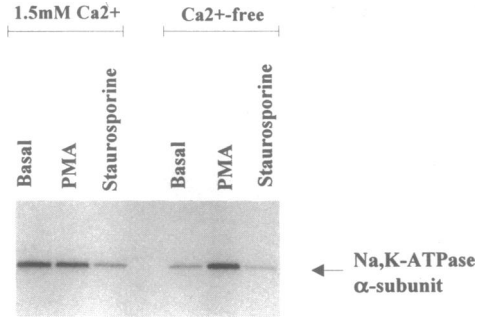

FIG. 2. Effect of PKC modulators and extracellular Ca^{2+} omission on α-subunit phosphorylation in normal sciatic nerve. After labeling (60 min) with [^{32}P]orthophosphate, nerves were challenged (15 min) or not (basal) with PMA (10 μM) or staurosporine (100 nM) in normal (first three lanes) or in nominally calcium-free (Ca^{2+}-free; last three lanes) KRBH. Experimental procedure is similar to that described in legend to Fig. 1.

gether with the inhibitory effect of staurosporine, seen only in the presence of Ca^{2+} (Table 1), suggest that Ca^{2+} is involved in the tonic Na,K-ATPase phosphorylation via the activation of PKC. To test whether PKA directly phosphorylates the Na,K-ATPase, forskolin (10 μM) was applied to the nerves. Forskolin significantly reduced the labeling of the α subunit to a similar extent as extracellular Ca^{2+} omission (Fig. 1; Table 1). It is noteworthy that forskolin was shown to stimulate $^{45}Ca^{2+}$ efflux and to decrease $[Ca^{2+}]_i$ in peripheral nerves (32). To test whether forskolin was acting indirectly through an effect on $[Ca^{2+}]_i$, the drug was added in Ca^{2+}-free buffer. Indeed, under these conditions, the inhibitory effect of forskolin was almost obliterated (Table 1). Furthermore, when PMA was added simultaneously with forskolin, the effect of forskolin was also canceled (Table 1). These results indicate that, in contrast to PKC, PKA negatively regulates phosphorylation of the α subunit.

The rapid reduction of the ^{32}P labeling caused by both staurosporine and forskolin points to the presence of a strong phosphatase activity in the vicinity of the Na^+ pump. Therefore, the effect of the protein phosphatase inhibitor okadaic acid was tested on the basal labeling of the α subunit. Okadaic acid (1 μM) treatment for 15 min enhanced the degree of phosphorylation [(2.4 ± 0.25)-fold increase; n = 4] of all phosphoprotein bands as well as that of the α subunit [(3.2 ± 0.3)-fold increase; n = 4] evaluated after immunoprecipitation. In preliminary experiments (n = 2), the simultaneous application of forskolin with okadaic acid did not further increase the labeling of the subunit. This was observed

Table 1. Effect of PK modulators on Na,K-ATPase α-subunit ^{32}P labeling in sciatic nerves from control rats

Test substance	$[Ca^{2+}]$ in buffer	^{32}P incorporation into α subunit, %	
PMA (10 μM)	1.5 mM	106 ± 11 (8)	NS
Staurosporine (100 nM)	1.5 mM	41 ± 3 (5)	**
Forskolin (10 μM)	1.5 mM	67 ± 9 (6)	**
PMA (10 μM) + forskolin (10 μM)	1.5 mM	93 (2)	ND
None	Ca^{2+}-free	69 ± 9 (6)	*
PMA (10 μM)	Ca^{2+}-free	101 ± 13 (4)	NS
Staurosporine (100 nM)	Ca^{2+}-free	61 ± 17 (3)	*
Forskolin (10 μM)	Ca^{2+}-free	57 ± 2 (3)	*

Values are means ± SEM of the number of experiments indicated in parentheses. They are expressed as percentage of the value obtained under basal conditions in control nerves; $[Ca^{2+}]$ in the Ca^{2+}-free buffer was <1 μM, as determined with the fluorescent dye Fluo3 (28). *, $P < 0.05$; **, $P < 0.01$ as compared with basal conditions. NS, not significantly different; ND, not determined.

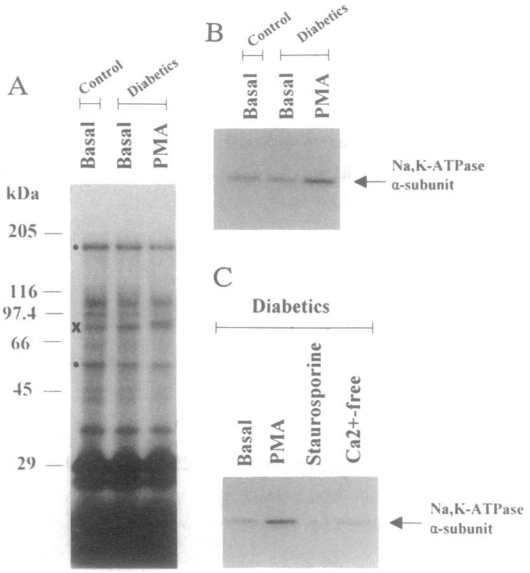

FIG. 3. Effect of PKC modulators and extracellular Ca^{2+} omission on α-subunit phosphorylation in diabetic sciatic nerve. Autoradiograms of two independent experiments showing the effect of PMA (10 μM) (*B*) or PMA (10 μM), staurosporine (100 nM), and extracellular Ca^{2+} omission (*C*). Experimental procedures were similar to those described in Figs. 1 and 2. (*A*) General phosphorylation pattern of membrane proteins used in *B*. In *A* and *B*, the effect of PMA in diabetic nerves is compared to a nonstimulated control (basal). Molecular size markers (kDa) are indicated on the left. ●, P1 and P2 proteins; ×, 80-kDa PKC substrate.

despite the synergism of okadaic acid and forskolin on the labeling of other proteins (data not shown).

***In Vivo* Phosphorylation of the Na,K-ATPase α Subunit in Diabetic Sciatic Nerves.** α-Subunit phosphorylation levels were compared in sciatic nerves from diabetic rats and age-matched controls 9 weeks after diabetes induction (Fig. 3), by which time peripheral neuropathy is known to have developed (33). The relative basal level of ^{32}P incorporation in the α subunit (Fig. 3*B*) was slightly lower in the diabetic nerves (86% ± 8% of controls; n = 8; P = 0.15). However, the α-subunit ^{32}P incorporation was markedly increased by PMA (Fig. 3 *B* and *C*; Table 2). Furthermore, in diabetic nerves, PMA application had a much greater phosphorylating effect on the α subunit (300% ± 72%; n = 3) than on the 80-kDa PKC substrate (142% ± 13%; n = 4). Indeed, the labeling effect of PMA on the 80-kDa protein was not significantly greater in diabetics compared to controls (120% ± 6%; n = 4; P = 0.06). Staurosporine and extracellular Ca^{2+} omission still caused a decrease in the phosphorylation level of the α subunit in diabetics (Fig. 3*C*). The reducing effect of forskolin, although marginal, was no longer significant (P = 0.06; Table 2).

Table 2. Effect of PK modulators on Na,K-ATPase α-subunit ^{32}P labeling in sciatic nerves from streptozotocin-induced diabetic rats

Test substance	$[Ca^{2+}]$ in buffer	^{32}P incorporation into α subunit, %	
PMA (10 μM)	1.5 mM	300 ± 72 (3)	**
Staurosporine (100 nM)	1.5 mM	58 ± 20 (4)	*
Forskolin (10 μM)	1.5 mM	73 ± 14 (4)	NS
None	Ca^{2+}-free	44 ± 19 (3)	*

Values are means ± SEM of the number of experiments indicated in parentheses. They are expressed as percentage of the value obtained under basal conditions in diabetic nerves. *, $P < 0.05$; **, $P < 0.01$ as compared with basal conditions. NS, not significantly different.

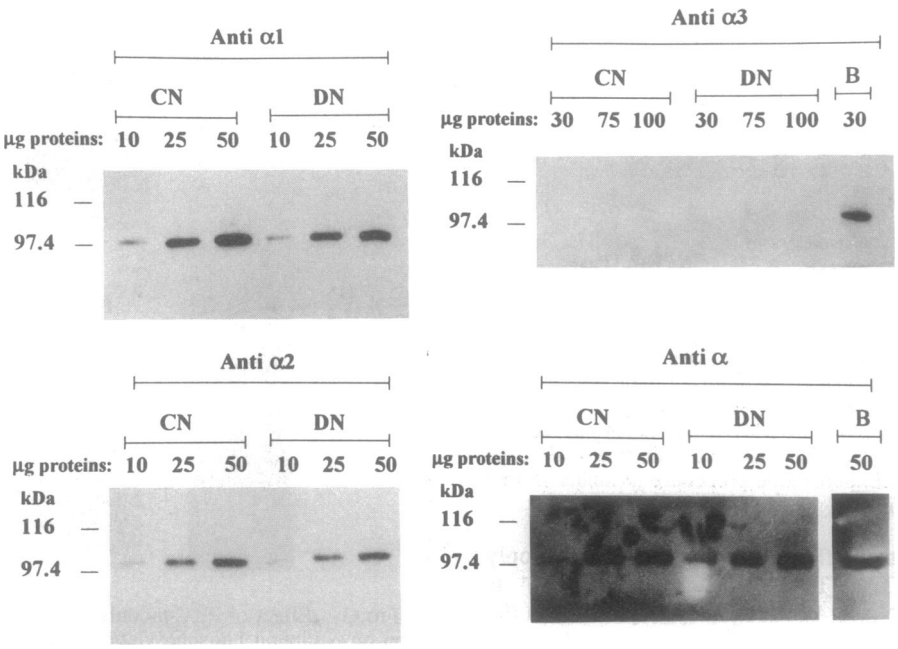

FIG. 4. Representative Western blot analysis of Na,K-ATPase α-subunit isoforms in sciatic nerves of control and diabetic rats. Membrane proteins of control (lanes CN) and diabetic (lanes DN) nerves were resolved by SDS/PAGE (8% polyacrylamide), transferred to nitrocellulose, and probed with anti-α1 (*Upper Left*), anti-α2 (*Lower Left*), anti-α3 (*Upper Right*), and anti-α (*Lower Right*) antibodies. Lanes B, rat brain membrane proteins as control. Molecular size markers (kDa) are indicated on the left.

Western Analysis of Na,K-ATPase α-Subunit Isoforms in Sciatic Nerve of Control and Diabetic Rats. To test for a change in the level of Na,K-ATPase α-subunit expression in diabetic rats, which could underlie the difference in sensitivity to PMA, Western analysis with isoform-specific antibodies was performed. The α1 and α2 isoforms were identified in sciatic nerve; in contrast to brain, the α3 isoform was not detected (Fig. 4). The nondiscriminating anti-α antibody detected a doublet both in peripheral nerve and in brain membranes, although in the former the upper band was much fainter than in the latter. The lower band in both preparations was also stained by the anti-α1-specific antibody. Although this suggests that α1 is the main isoform of the peripheral nerve, further characterization is needed to substantiate this. As evaluated by densitometric scanning, no significant difference in α-subunit immunoreactivity was observed with the anti-α (100% ± 5% vs. 96% ± 11%; $n = 4$; $P = 0.88$), with the anti-α1 (100% ± 14% vs. 87% ± 12%; $n = 6$; $P = 0.34$), and with the anti-α2 antibodies (100% ± 15% vs. 103% ± 11%; $n = 6$; $P = 0.65$) for controls and diabetics, respectively.

DISCUSSION

This report describes the *in vivo* phosphorylation state of the Na,K-ATPase α subunit in rat sciatic nerve. The results suggest a tonic regulation of this phosphorylation in the normal rat sciatic nerve. Although PMA did not enhance the α-subunit ^{32}P labeling in normal nerves, the involvement of PKC is suggested from the inhibitory effect of staurosporine. Since low extracellular Ca^{2+} significantly decreased the labeling of the α subunit that could be restored to control values by PMA, Ca^{2+} is suggested to activate PKC indirectly. This is supported by the previous demonstration that ionomycin, a Ca^{2+} ionophore, promoted the translocation of PKC to the membrane fraction of this nerve (18). Hence, by modulating the Ca^{2+}-sensitive phospholipase C (PLC) of sciatic nerve (34), Ca^{2+} may sustain diacylglycerol (DAG) formation and thus basal PKC activity underlying the tonic phosphorylation of Na,K-ATPase. In this way, Ca^{2+} would link the rapid basal turnover of specific inositol phospholipid pools, PKC, and Na,K-ATPase activity (11, 35, 36). Indeed, basal inositol monophosphate production is also inhibited by Ca^{2+}-entry blockers in peripheral nerve (30). Furthermore, the rapid reduction of ^{32}P labeling by staurosporine and, conversely, the pronounced increase in labeling by okadaic acid both indicate that a strong phosphatase activity permits a phosphorylation/dephosphorylation process with rapid turnover.

Even though Na,K-ATPase α subunit can serve as substrate for PKA *in vitro* (5, 6), the reduction by forskolin of the phosphorylation process suggests that the situation is apparently different in intact sciatic nerve. The inhibitory effect of PKA is probably secondary to an inhibition of PKC activation rather than to the direct stimulation of a dephosphorylation process. Indeed, no PKA-activated protein phosphatase has been reported so far in the literature (37). Lower PKC activity may thus result from the inhibition of DAG production by PLC consequent to either a decrease in nerve $[Ca^{2+}]_i$ caused by forskolin (32) or to an inhibitory action of PKA on PLC (38). Furthermore, the lack of phosphorylating effect of forskolin in calcium-free buffer or in the presence of okadaic acid further argues against the participation of PKA in phosphorylation of the nerve Na,K-ATPase.

In diabetic nerves, ^{32}P labeling of the Na,K-ATPase α subunit was specifically increased by PMA, while phosphorylation of the 80-kDa PKC substrate was not potentiated by diabetes. Although okadaic acid was not tested in diabetic nerves, the preserved inhibitory actions of staurosporine and extracellular calcium omission apparently exclude a defect in phosphatase activity. Rather, these findings point to a defect in PKC activation. The well-documented diminution in diabetic nerves both of specific inositol phospholipid pools (11, 36) and of arachidonyl-containing species of DAG (39), the physiological activators of PKC, may be implicated in this defect. This is strengthened by previous demonstrations (18, 40) that no alteration of PKC itself was detected in the Na,K-ATPase-containing fractions of diabetic nerves. Taken together, these findings favor the view that the decreased Na,K-ATPase activity measured in sciatic nerves of streptozotocin-induced diabetic rats may result from an alteration

of its phosphorylation state by PKC. The similar basal ^{32}P incorporation in the α subunit in controls and in diabetics contrasted with the responses obtained with PMA. Major differences in the total amount or in α-subunit isoform expression explaining this phenomenon were ruled out by Western analysis. Nevertheless, reciprocal changes in isoform expression in Schwann cells and axons as well as other diabetes-linked alterations could still be involved. The latter include (*i*) an increased number of nonphosphorylated serine and threonine sites due to decreased PKC activation and/or heightened phosphatase activity combined with an increased phosphorylation of distinct sites by other kinases; and (*ii*) modifications in [γ-^{32}P]ATP specific activity in discrete ATP pools associated with Na,K-ATPase phosphorylation (41), on the one hand masking a decreased α-subunit basal phosphorylation, and on the other hand amplifying the ^{32}P-labeling response of PMA.

It is still under debate whether the nerve Na,K-ATPase is activated (10, 12, 13) or inhibited (15, 16) after its phosphorylation, as suggested in other tissues (5, 42). Thus, a defect in Na,K-ATPase activity has been proposed to be associated with decreased adenylyl cyclase activity in diabetic nerves (17). However, while cAMP analogues apparently enhance Na,K-ATPase activity in both normal and diabetic tissues (17), phorbol esters do so only in diabetic nerve (10, 12, 13). Consequently, a positive correlation between the phosphorylation state of the α subunit described here and the reported effects of kinase modulators on the pump activity in diabetic nerves can be established only for the activation of PKC (10, 12, 13, 16, 17). Thus, stimulation of the pump by PKA activators must be exerted on other closely related targets also sustaining directly or indirectly the activity of the pump.

In summary, Na,K-ATPase phosphorylation in the normal sciatic nerve appears to be tonically controlled via regulation of PKC activity. The latter is proposed to depend on the rate of DAG production by the nerve PLC influenced by both $[Ca^{2+}]_i$ and PKA activity. The dynamic phosphorylation state of the α subunit would thus result mainly from the balance between a strong phosphatase activity and the degree of PKC activation. Although the phosphorylation process was highly sensitive to staurosporine, a participation of Ca^{2+}/calmodulin-dependent protein kinases (43) cannot be excluded. If the extent of phosphorylation caused by PMA application is considered to reflect the Na,K-ATPase-associated PKC activity, it follows that there is a deficit in PKC activation in the diabetic nerve.

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