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Defective sarcolemmal phosphorylation associated with noninsulin-dependent diabetes

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Noninsulin-dependent diabetes is associated with a decrease in the activity of sarcolemmal phosphatase 1, but no change in the activities of phosphatase 2A, 2B, or 2C. Also unaffected by diabetes were the activities of protein kinase C, cAMP-dependent protein kinase and calcium-calmodulin protein kinase. Because of the decrease in phosphatase 1 activity, ^{32}P incorporation into sarcolemmal phosphoproteins catalyzed by either intrinsic protein kinases or extrinsic cAMP-dependent protein kinase was elevated in the diabetic. Among the proteins whose phosphorylation was elevated in diabetes was the phospholamban-like protein, which has been implicated in the regulation of ATP-dependent calcium transport. The phosphate-linked increase could be prevented by exposing the membranes to a phosphatase inhibitor and either extrinsic cAMP-dependent protein kinase or alamethicin. In addition to the phosphatase-linked effects, analysis of individual sarcolemmal phosphoproteins by SDS-polyacrylamide gel electrophoresis indicated that diabetes caused a specific elevation in membrane phosphorylation of some proteins (43 kDa and 78 kDa), but a decrease in the phosphorylation state of other phosphoproteins (31 kDa and 49 kDa). The data indicate that membrane phosphorylation is dramatically altered by diabetes. The possibility that this contributes to altered myocardial function is discussed.

Introduction

One of the important complications of noninsulin-dependent diabetes (NIDD) is the development of a cardiomyopathy, a condition characterized by defects in myocardial energy metabolism, cardiac contractility, diastolic compliance and the rate of myocardial relaxation [1–3]. A similar complication develops in insulin-dependent diabetes (IDD); however, unlike the cardiomyopathy associated with NIDD, the IDD cardiomyopathy in rats shows no evidence of reduced diastolic compliance [4]. Moreover, many of the metabolic aberrations of the IDD cardiomyopathy are absent in the NIDD disease [3]. Therefore, it has been proposed that the two conditions represent distinct diseases [1].

While numerous studies have focused on various factors which may contribute to the development of the IDD cardiomyopathy, little is known about the causes of the NIDD cardiomyopathy. Studies using the neonatal rat model of NIDD reveal that defects in sarcoplasmic reticular calcium transport and tissue calcium content occur in the heart, raising the possibility that abnormalities in calcium movement may contribute to impaired mechanical performance of the NIDD heart [2]. The change in calcium homeostasis might also account for some of the alternations in energy metabolism associated with the cardiomyopathy, including reduced glucose transport and utilization [5]. Another factor which presumably contributes to the severity of the cardiomyopathy is insulin resistance. Schaffer et al. [3] have shown that the stimulation of glucose transport by insulin is dramatically attenuated in the NIDD heart. Moreover, insulin-mediated increase in myocardial mechanical function are less in the NIDD heart [6].

Another factor which has not been considered, but deserves some attention, is that the regulation of cell processes by protein phosphorylation may be altered in NIDD. This idea is attractive because protein phosphorylation serves as a major regulator of calcium transport [7–13], cardiac metabolism [5,14] and insulin resistance [15]. Moreover, changes in the phosphorylation state of some key metabolic enzymes contributes to the development of abnormal energy metabolism in the IDD heart [16,17]. For this reason, the effect of NIDD on mem-

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brane phosphorylation and the activity of key kinases and phosphatases was examined.

Materials and Methods

The noninsulin-dependent diabetic condition was produced as described previously [2,6]. Briefly, male Wistar rats were injected intraperitoneally at 2 days of age with 90 mg/kg of streptozotocin (kindly provided by Dr. Albert Chang, Upjohn, Kalamazoo, MI). These animals became progressively more glucose intolerant with age. By 12 months, their fasting and nonfasting blood glucose levels were 136 ± 6 and 185 ± 21 mg/dl, respectively, which are slightly elevated in comparison to corresponding values of 112 ± 2 and 138 ± 3 mg/dl for the age-matched controls. One hour following a glucose challenge of 2 g/kg i.p., blood glucose levels typically rose to values between 500 and 600 mg/dl in the NIDD rats, but only between 200 and 300 mg/dl in the control rats. At the same time, plasma insulin levels increased to values about 2-fold greater than controls (about 19 ng/ml for the noninsulin-dependent diabetic and 9 ng/ml for the nondiabetic). There was no difference in weight between the age-matched control and the NIDD rats (593 ± 28 g for control vs. 572 ± 10 g for NIDD group), indicating that the diabetic animals were not wasting. The glucose challenge and body weight data indicate that the rats exhibited features characteristic of noninsulin-dependent diabetes. All animals were studied after 10–12 months of age.

The sarcolemma preparation was isolated from nondiabetic and NIDD hearts using a slight modification of the method of Pitts [18], as described previously [19]. Membrane yield was similar in the two groups (0.24 ± 0.01 mg/g wet wt. for nondiabetic and 0.22 ± 0.02 mg/g wet wt. for NIDD). The method of Doyle et al. [20] was used to evaluate membrane sidedness and revealed that both preparations contained approx. 30% leaky, 35% inside-out and 35% right-side-out vesicles. Ouabain-sensitive Na^+/K^+-ATPase activity was 32.1 ± 2.5 and 18.1 ± 2.2 μmol P_i/mg per h for nondiabetic and NIDD membrane, respectively. Another sarcolemmal enzyme marker, adenylate cyclase, exhibited activity of 245 ± 29 and 250 ± 24 pmol cAMP/mg per min in nondiabetic and NIDD membrane, respectively. In both the diabetic and nondiabetic sarcolemma, the sarcolemmal markers were concentrated approx. 9–13-fold relative to the homogenate. By comparison, the purity factor for cytochrome-*c* oxidase was 0.3–0.4 for the two preparations, indicating minimal mitochondrial contamination of the sarcolemma preparation. Since both oxalate-facilitated and *p*-nitrophenyl phosphate supported calcium accumulation represented less than 10% of total calcium uptake in the two preparations, sarcoplasmic reticular contamination is also minimal.

Total membrane phosphorylation was determined using the method of Caroni and Carafoli [7]. Sarcolemma (20 μg protein) were preincubated for 5 min at 37 °C in 20 mM Mops buffer (pH 7.0) containing 160 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EGTA in the presence and absence of 150 units cAMP-dependent protein kinase catalytic subunit, 10 mM NaF plus 150 units of cAMP-dependent protein kinase catalytic subunit or 10 mM NaF plus alamethicin (1 mg alamethicin/mg protein). The phosphorylation reaction was initiated by addition of 50 μM [$\gamma - ^{32}$P]ATP and terminated at the appropriate time by addition of 3 ml of 20 mM Mops buffer (pH 7.0) containing 160 mM ATP and 40 mM K_2HPO_4 and by rapidly filtering the reaction mixture over Whatman GF/B filters. The filters were washed twice with 3 ml of the 20 mM Mops buffer and then counted for radioactivity.

A slight modification was introduced in the phosphorylation procedure when the analysis of individual sarcolemmal protein bands by gel electrophoresis was carried out. The reaction was allowed to proceed for 30 s, at which time it was terminated by the addition of 10 μl of a 0.125 M Tris (pH 6.8) stop solution containing 20% glycerol, 7 mM β-mercaptoethanol and 5% SDS followed by heating the samples for either 3 min at 80 °C or 1 min at 100 °C. The samples were then subjected to SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide slab gel. The system was programmed to run for 45 min at a voltage of 200 V and a temperature of 10 °C. Upon completion of the run, the gels were stained with Coomassie blue and then dried. The dried gels were exposed to Kodak X-AR5 X-ray film using intensifying screens and stored at −70 °C. After the proper time of exposure, the films were developed. The molecular weights of the sarcolemmal proteins were assessed based on the migration of known standards (β-galactosidase, 116 kDa; bovine serum albumin, 66 kDa; egg albumin 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa).

Standard assays were used to measure the activities of key protein kinases. After treating the membranes with 1 mg alamethicin/mg membrane protein, cAMP-dependent protein kinase was determined by the method of Corbin and Reimann [21]. The procedure of Tuana et al. [22] was used to measure calmodulin-dependent protein kinase activity, while protein kinase C was assayed by the method of Yuan and Sen [23].

To measure the activities of individual protein phosphatases in the sarcolemma, the method developed by Ingebritsen et al. [24] was used. This assay is based on the specificity of each enzyme. Protein phosphatase 2B activity is blocked by 100 μM trifluoperazine while protein phosphatase 1 is inhibitor 2 sensitive. Mag-

nesium is required for the expression of protein phosphatase 2C activity. The isozyme assayed in the absence of magnesium and in the presence of manganese and inhibitor 2 is protein phosphatase 2A. In these studies, two protein phosphatase substrates, ^{32}P-phosphorylase a and denatured ^{32}P-sarcolemmal membranes, were used. Labelled phosphorylase a was prepared according to the procedure of Shenolikar and Ingebritsen [25], while the method of Sulakhe and Drummond [26] was used to prepare denatured ^{32}P-sarcolemma.

Results

Incubation of isolated sarcolemma with [^{32}P]ATP in the presence or absence of the catalytic subunit of protein kinase led to rapid phosphorylation of both nondiabetic and NIDD sarcolemma (Fig. 1). In the absence of added protein kinase, the rate of phosphorylation appeared to be slightly greater in the diabetic (Fig. 1A). Pretreatment of the membrane with phosphorylase phosphatase failed to alter either pattern.

Inclusion of extrinsic cAMP-dependent protein kinase in the reaction medium increased both the rate and extent of phosphorylation. As expected, the initial rate of phosphorylation in the presence of the extrinsic protein kinase was identical for both diabetic and nondiabetic sarcolemma (Fig. 1A). However, the extent of phosphorylation was greater in the diabetic membrane (Fig. 1A). When both protein kinase and NaF were included in the buffer, the initial rate of the phosphorylation reaction was reduced relatively to the reaction carried out in the presence of only extrinsic protein kinase; however, the extent of phosphorylation at longer reaction times was enhanced (Fig. 1B). This observation is most easily attributed to the nonspecificity of NaF, which is a potent protein phosphatase inhibitor, but can also inhibit protein kinase activity. The combination of protein kinase and NaF also eliminated any differences between the diabetic and nondiabetic relative to rate and extent of phosphorylation (Fig. 1B). Another combination which increased the rate and extent of phosphorylation, while eliminating differences between the diabetic and nondiabetic, was NaF and alamethicin, a peptide ionophore which is known to activate several

TABLE I

Effect of noninsulin-dependent diabetes on the activity of protein phosphatases associated with cardiac sarcolemma

Sarcolemma from nondiabetic and noninsulin-dependent diabetic rats was prepared according to the method of Pitts [18]. The activities of protein phosphatases 1, 2A, 2B and 2C associated with the isolated membrane preparation were assayed according to the method of Ingebritsen et al. [24]. All four enzymes were assayed using denatured ^{32}P-sarcolemma as substrate. In addition, protein phosphatases 1 and 2A were assayed using ^{32}P-phosphorylase a as a substrate. Values shown represent the mean ± S.E. of four preparations. n.d. signifies no detectable activity while * represents significant difference between the diabetic and nondiabetic group ($P < 0.05$).

Enzyme	Activity (pmol/mg per h)	
	nondiabetic	diabetic
Protein phosphatase 1		
Sarcolemma	36.8 ± 4.0	16.4 ± 3.6*
Phosphorylase *a*	292.0 ± 29.6	165.2 ± 24.4*
Protein phosphatase 2A		
Sarcolemma	60.0 ± 12.8	48.8 ± 9.2
Phosphorylase *a*	388.0 ± 34.0	441.6 ± 30.8
Protein phosphatase 2B	n.d.	n.d.
Protein phosphatase 2C	4.8 ± 3.2	5.6 ± 2.4

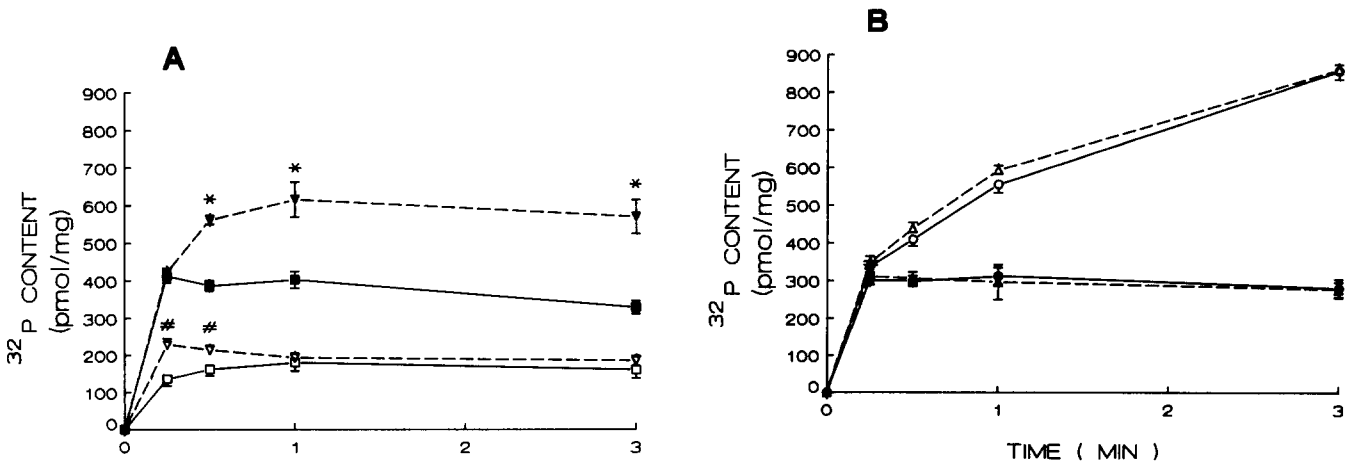

Fig. 1. Effect of noninsulin-dependent diabetes on membrane phosphorylation. Sarcolemma from nondiabetic (●, ○, ■, □) and noninsulin-dependent diabetic (▲, △, ▼, ▽) hearts was prepared according to the method of Pitts [18]. In figure (A) the rate of ^{32}P incorporation into membrane proteins was determined using sarcolemma incubated in medium containing no additions (□, ▽) or 150 units cAMP-dependent protein kinase catalytic subunit (■, ▼). In figure (B) the incubation medium contained 10 mM NaF plus 150 units cAMP-dependent protein kinase catalytic subunit (○, △) or 10 mM NaF plus 1 mg alamethicin/mg membrane protein (●, ▲). Values shown represent the means ± S.E. of four preparations. * and # denote significant difference between diabetic and control sarcolemma for each condition ($P < 0.05$).

TABLE II

Effect of noninsulin-dependent diabetes on activity of sarcolemmal-associated protein kinases

Sarcolemma from nondiabetic and diabetic rats were prepared according to the method of Pitts [18]. The activity of individual protein kinases associated with the membrane were assayed using standard procedures described in the Methods. Noninsulin-dependent diabetes had no effect on the activity of these protein kinases. Values shown represent the means ± S.E. of four preparations.

Enzyme	Activity (nmol/mg per min)	
	nondiabetic	diabetic
cAMP-dependent protein kinase	0.19 ± 0.02	0.19 ± 0.04
Protein kinase C	0.88 ± 0.08	1.10 ± 0.20
Calmodulin-dependent protein kinase	0.03 ± 0.01	0.03 ± 0.01

sarcolemmal enzymes, including intrinsic cAMP-dependent protein kinase [27].

One possible explanation for the observation that inclusion of the protein phosphatase inhibitor, NaF, and either extrinsic cAMP-dependent protein kinase or alamethicin in the incubation medium eliminated differences in sarcolemmal ^{32}P incorporation between the diabetic and nondiabetic is that diabetes specifically alters the activity of the protein phosphatases. To test this idea, the activity of each of the membrane-associated protein phosphatases was determined [24]. Using labelled, denatured cell membrane as substrate, it was found that more than 90% of the protein phosphatase activity of nondiabetic and NIDD sarcolemma was either protein phosphatase 1 or 2A (Table I); the sarcolemma contained no detectable protein phosphatase 2B and only a small amount of protein phosphatase 2C activity. Interestingly, the only enzyme affected by NIDD was protein phosphatase 1. Depending upon the substrate used, phosphatase 1 activity was reduced either 44% (phosphorylase a as substrate) or 56% (labelled membrane as substrate) in sarcolemma isolated from NIDD rats.

Recognizing that membrane phosphorylation is also dependent upon the amount of protein kinase associated with the isolated sarcolemma, the activity of several protein kinases also were examined. In agreement with previous studies [23,28], significant levels of cAMP-dependent protein kinase and protein kinase C were associated with the isolated sarcolemma. By comparison, the activity of calmodulin-dependent protein kinase in these preparations was very low (Table II). NIDD was found to have no significant effect on the activity of any of the kinases.

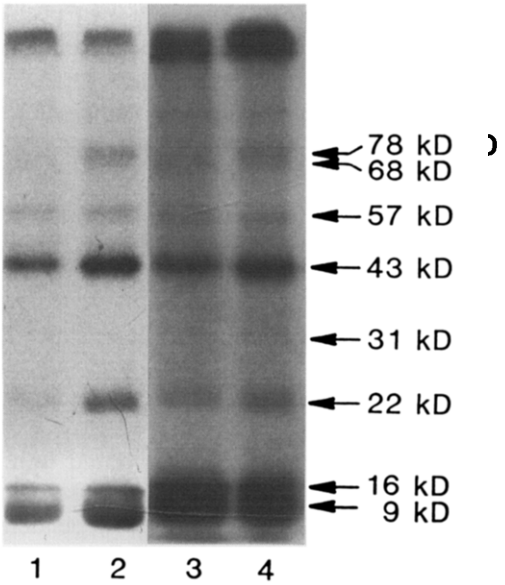

Fig. 2. Effect of noninsulin-dependent diabetes on sarcolemmal protein phosphorylation pattern. Sarcolemma was isolated from nondiabetic and noninsulin-dependent diabetic hearts using the method of Pitts [18]. The isolated membranes were phosphorylated in medium containing [^{32}P]ATP, supplemented with or without 150 units cAMP-dependent protein kinase catalytic subunit. After termination of the reaction as described in the Methods, solubilized membrane proteins were subjected to polyacrylamide gel electrophoresis, followed by autoradiography. Shown is an autoradiogram of nondiabetic (lanes 1 and 3) and diabetic (lanes 2 and 4) samples incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of cAMP-dependent protein kinase.

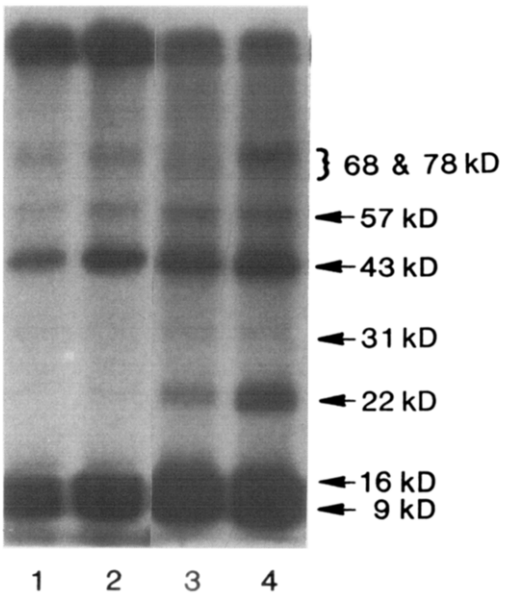

Fig. 3. Effect of phosphatase inhibition on noninsulin-dependent diabetic and nondiabetic sarcolemmal membrane phosphorylation. Sarcolemma was prepared according to the method of Pitts [18]. The isolated membrane was phosphorylated as described in Fig. 2 except 10 mM NaF was included in the incubation medium of some samples. After termination of the reaction, samples were heated for 1 minute at 100 °C and then subjected to polyacrylamide gel electrophoresis followed by autoradiography. Shown is an autoradiogram of nondiabetic (lanes 1 and 3) and diabetic (lanes 2 and 4) samples incubated with 150 units cAMP-dependent protein kinase in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 10 mM NaF.

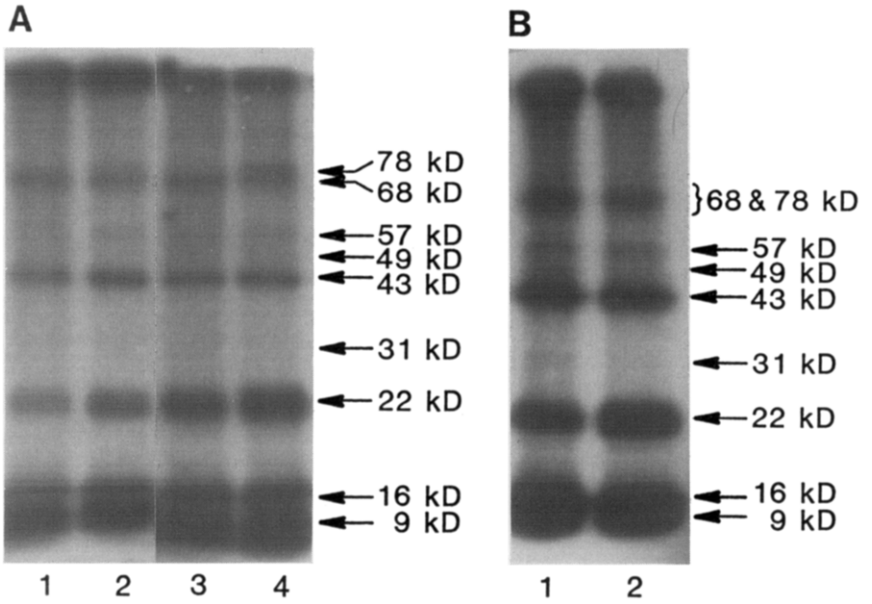

Fig. 4. Effect of noninsulin-dependent diabetes and alamethicin on membrane phosphorylation. Sarcolemma was prepared according to the method of Pitts [18]. The isolated membrane was phosphorylated as described in Fig. 2 except 10 mM NaF and 1 mg alamethicin/mg membrane protein was also included in the incubation medium. The solubilized proteins were subjected to polyacrylamide gel electrophoresis, followed by autoradiography. Figure (A) shows an autoradiogram of nondiabetic (lanes 1 and 3) and diabetic (lanes 2 and 4) sarcolemmal proteins phosphorylated in the presence (lanes 3 and 4) and absence (lanes 1 and 2) of 150 units cAMP-dependent protein kinase catalytic subunit. Lanes 3 and 4 of figure (A) represent the same run as figure (B) except the length of time the X-ray film was exposed to the dried gel was prolonged (20 h in (A) and 48 h in (B). The longer exposure was necessary to clarify differences (bands at 31 kDa and 49 kDa) between the nondiabetic (lane 1) and diabetic (lane 2) samples.

Previous studies have shown that each protein kinase of isolated sarcolemma exhibits its own peculiar phosphorylation profile [22,29–31]. To examine which phosphoproteins were affected by diabetes, sarcolemma from NIDD and nondiabetic hearts were phosphorylated under varying conditions and individual membrane phosphoproteins were then separated from each other by SDS-polyacrylamide gel electrophoresis. Fig. 2 reveals that in the absence of NaF, the intrinsic protein kinases associated with the sarcolemma were capable of phosphorylating sarcolemmal proteins with molecular weights of 9, 16, 43, 57 and 68 kDa. The intensity of each of these bands was increased by incubation of the membrane with cAMP-dependent protein kinase. In addition, proteins with molecular weights of 31, 49 and 78 kDa, which were not phosphorylated to any appreciable extent by the intrinsic protein kinases, were clearly phosphorylated by extrinsic cAMP-dependent protein kinase. In either the presence or absence of extrinsic protein kinase, the extent of phosphorylation of 43, 57 and 78 kDa proteins was clearly greater in the diabetic. Also, phosphorylated to a greater extent in the diabetic was the phospholamban-like protein, which migrated as a 9 kDa protein (Fig. 3).

In agreement with the membrane ^{32}P incorporation studies, the degree of phosphorylation of the individual bands was also affected by inclusion of NaF and extrinsic cAMP-dependent protein kinase in the phosphorylating medium (Fig. 3). As expected, many of the differences between diabetic and nondiabetic membrane disappeared in the presence of NaF. From Fig. 3 it is seen that in the presence of 10 mM NaF the only bands which clearly exhibited a higher degree of phosphorylation in diabetic membrane than nondiabetic sarcolemma were the 43 kDa and 78 kDa proteins. Similar changes occurred in the presence of 10 mM NaF and 1 mg alamethicin/mg membrane protein; not only did total membrane phosphorylation increase relative to membranes lacking both substances, but also diabetes-induced increases in the extent of phosphorylation of the 57 kDa and 68 kDa proteins disappeared (Fig. 4). Interestingly, the combination of alamethicin, NaF and extrinsic protein kinase uncovered two proteins (31 kDa and 49 kDa), which were more extensively phosphorylated in nondiabetic sarcolemma (Fig. 4).

Discussion

The two major finding of this study are that diabetes leads (1) to an increase in total sarcolemmal protein phosphorylation and (2) to changes in the pattern of phosphorylation; that is, the phosphorylation of some proteins is enhanced while others is reduced. Different mechanisms appear to account for these two phenomena. The first observation can most easily be attributed to the decrease in phosphatase 1 activity. As seen in Fig. 1A, when the phosphorylation reaction was carried out in the absence of both a phosphatase inhibitor and

extrinsic protein kinase, the initial rate of protein phosphorylation was greater in diabetic sarcolemma, presumably because of its lower phosphatase activity. Inclusion of extrinsic cAMP-dependent protein kinase in the reaction medium resulted in a rapid stimulation in the rate of membrane phosphorylation. Although the initial rate of phosphorylation was identical in the nondiabetic and diabetic membrane under those conditions, the lower phosphatase activity of the diabetic was reflected in the greater extent of phosphorylation. In keeping with the view that the phosphatase was responsible for the observed differences between the diabetic and nondiabetic membrane, it was found that inclusion of the phosphatase inhibitor, NaF, and either alamethicin or extrinsic protein kinase (to increase protein kinase activity) in the reaction medium eliminated all differences between the two groups (Fig. 1B). These data also suggested that diabetes-induced phosphorylation differences were not related to variations in intrinsic protein kinase activity of diabetic and nondiabetic membrane, a supposition that was subsequently confirmed upon assaying protein kinase activity of the isolated membrane preparations.

Assays of the individual sarcolemmal-associated phosphatases revealed that only phosphatase 1 activity was decreased in NIDD. Since the activity of rat liver phosphates 1 has been shown to decline in IDD, but restored to normal by insulin therapy, hormonal control of phosphatase 1 appears to be a general characteristic of the enzyme [32,33].

Some insight into which proteins serve as substrates for phosphatase 1 can be obtained by comparing SDS gels of diabetic and nondiabetic membrane following phosphorylation in the presence and absence of the phosphatase inhibitor NaF. The rationale for this approach is that inhibition of protein phosphatase activity, coupled with a reasonably high protein kinase activity (such as that seen by either adding alamethicin to active intrinsic protein kinase activity or adding extrinsic protein kinase) should eliminate all differences in the phosphorylation pattern caused by varying phosphatase activity. Thus, those phosphoproteins whose extent of phosphorylation in diabetic membrane was greater than nondiabetic membrane only when the phosphorylation reaction was carried out in the absence of NaF must be substrates of the phosphatase. As seen in Figs. 2–4, only a few proteins satisfied these conditions. When the phosphorylation reaction was carried out either in the presence or absence of extrinsic protein kinase, but lacking NaF, the phospholamban-like protein, as well as proteins with molecular masses of 43 kDa, 57 kDa and 78 kDa were more intensely labelled in the diabetic. By comparison, only the 78 kDa and 43 kDa proteins exhibited more intense phosphorylation bands in reactions carried out in the presence of the phosphatase inhibitor NaF and either alamethicin or extrinsic protein kinase. It therefore follows that the phospholamban-like and 57 kDa proteins are probably substrates of phosphatase 1. However, it is recognized that this analysis is inexact and that the complete substrate specificity of this important phosphatase awaits its purification and characterization.

In addition to differences in phosphorylation due to reduced phosphatase 1 activity, alterations in the membrane phosphorylation pattern of diabetic sarcolemma can also be caused by other diabetes-linked changes. These differences can be uncovered by running the reactions in the presence of NaF and either alamethicin or extrinsic protein kinase, conditions which eliminate all differences due to variation in phosphatase activity. As shown in Figs. 3 and 4, diabetes was associated with a decrease in the degree of phosphorylation of some proteins (with molecular weights of 31 kDa, 49 kDa and 68 kDa) while increasing two other proteins (43 kDa and 78 kDa).

In a related study Lamers et al. [34] reported that the extent of ^{32}P incorporation into sarcolemmal phospholamban-like protein was reduced during ischemia. They reasoned that the decrease in phosphorylation was caused by (1) changes in substrate degradation leading to reduced substrate levels, (2) solubilization of the phosphoprotein by detergent amphiphiles which accumulate in ischemia or (3) altered membrane structure. In diabetes, similar factors could theoretically also contribute to the observed alterations in membrane phosphorylation. However, analysis of the Coomassie blue staining pattern of diabetic and nondiabetic gels reveals no apparent differences. Moreover, myocardial levels of lipid amphiphiles, such as long chain acylcarnitine and fatty acids, do not increase in NIDD, ruling out this mechanism as a cause for the observed change in phosphorylation. Thus, the most logical explanation for the observed findings is that membrane structural changes, perhaps associated with altered membrane lipid composition, may affect phosphorylation of the diabetic sarcolemma.

The significance of the diabetes-induced phosphorylation changes are difficult to evaluate, primarily because the role of the individual proteins has not been definitively established. It is interesting, and perhaps significant, that all of the putative substrates for phosphatase 1 are also substrates of cAMP-dependent protein kinase. While this suggests that the phosphatase may be able to reverse some of the effects of the cAMP-dependent protein kinase, this interpretation is complicated by the fact that some of these same phosphoproteins also serve as substrates of other protein kinases [31].

While membrane phosphorylation is thought to affect both glucose metabolism [5] and insulin resistance [15], the area which has received the most attention is the stimulation of calcium transport by membrane

phosphorylation. It has been proposed that phosphorylation of the calcium channel by cAMP-dependent and calmodulin-dependent protein kinases are required for full activation of the slow calcium current [12]. In skeletal muscle, calcium channel subunits of 52 kDa and 170 kDa are phosphorylated by cAMP-dependent protein kinase [12]. However, the amount of calcium channel found in the membrane is quite low and therefore unlikely to be phosphorylated to an extent which would be detectable in the gels.

The other major calcium transporter affected by phosphorylation is the calcium pump [7–9]. Caroni et al. [7] were the first group to report that cAMP-dependent protein kinase-induced phosphorylation stimulated ATP-dependent calcium transport. Subsequently, they found that the 140 kDa Ca^{2+}-stimulated ATPase protein was not directly phosphorylated by cAMP-dependent protein kinase [8]. Questions still remain regarding the nature of the phosphoprotein modulator. Lamers [9] has proposed that a phospholamban-like protein is responsible for regulating the calcium pump. On the other hand, Presti et al. [10] claim that the phospholamban-like protein is merely a sarcoplasmic reticular contaminant which becomes associated with the sarcolemma during purification. More recent studies by Louis et al. [35] appear to support the initial finding of Lamers [9]. Although this study does not clarify this issue, it does indicate that the phospholamban-like protein probably is a substrate of sarcolemmal-associated phosphatase 1. We also found that the phospholamban-like protein was phosphorylated to a greater extent in the diabetic (Fig. 3). How this relates to the calcium pump is difficult to assess since there is some evidence that diabetes effects an uncoupling between calcium transport and ATP hydrolysis.

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