Doctoral thesis

The role of Na^+/K^+ -ATPase in the pathomechanism of diabetes mellitus

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease, which affects nearly all metabolic pathways in addition to carbohydrate metabolism. Relative or absolute insulin deficiency is the leading symptom of the disease, contributing to the development of hyperglycemia, oxidative stress and the pathological activation of the renin-angiotensin system (RAS) and the endogenous digitalis system. All of these components play a role in the development of the long-term consequences of diabetes, namely macro- and microvascular complications that significantly reduce the life expectancy of diabetes patients. Among macrovascular complications cardiovascular disease is responsible for more than 50% of deaths attributable to diabetes. Among microvascular complications, nephropathy affects 20-40% of diabetes patients and is responsible for the majority of kidney transplants. Diabetes induces pathological tissue-specific cellular hypertrophy, interstitial and perivascular fibrosis in both the heart and the kidneys, which lead to severe structural damage. In the heart the destruction of cardiomyocytes and the accumulation of connective tissue leads to dyastolic and systolic dysfunction and cardiac failure; damage to kidney tubuli and glomeruli may lead to renal failure. Na^+/K^+ -ATPase (NKA) is among the most important, membrane-bound enzymes required for the adequate functioning of both the myocardium and the renal cortex. It plays a crucial role in maintaining contractility in the myocardium, while in the kidney it is required both for excretion and for reabsorption. The enzyme is composed of two α and two β subunits. In the kidneys the tetramer is supplemented by the additional γ subunit. Several isoforms have been identified with significant differences in expression among various tissues. Myocardium is

characterized by α_1, α_2, β_1 and β_2 isoforms; α_1 and β_1 are the predominant subunit isoforms in the kidney. Diabetes is accompanied by NKA dysfunction. The function of the enzyme is significantly influenced by its location within the cell. When bound to the plasma membrane the enzyme transports Na and K ions across the membrane accompanied by the hydrolysis of ATP. In designated locations of the plasma membrane, in caveolae NKA is not involved in ion transport but initiates a variety of signaling pathways. In these locations, it primarily functions as a receptor for endogenous or exogenous digitalis. In addition, it can also enter endosomes from these locations. From endosomes it may return to the plasma membrane, undergo lysosomal degradation or affect signal transduction pathways when located intracellularly. Tissue-specific alterations in NKA activity occurring in diabetes have been reported in several studies, however the subcellular localization of the enzyme in diabetes including changes in its distribution between the intracellular membranes and the plasma membrane and the effects of insulin and angiotensin on the above mentioned processes have not been described in cardiac and renal tissue. My doctoral thesis summarizes the results of experiments carried out in rats suffering from type 1 diabetes. Our studies verify that the intracellular localization, and thus the activation of NKA changes in the diabetic myocardium and renal cortex. Our experimental results confirm that pharmacotherapeutic agents used in today's clinical practice (e.g. insulin, losartan, metformin,) may be used not only for the symptomatic treatment of carbohydrate metabolism and high blood pressure, but may actually reverse the changes in the function of NKA that we observed in diabetes. The experiments clearly show that examining basic cellular functions may shed light on pathological activity underlying the

wide range of complications associated with complex diseases, such as diabetes.

OBJECTIVES

Our aim was to investigate:

1. in the myocardium,
    a. the effect of diabetes, chronic insulin treatment and ANGII on antioxidant
       capacity and left ventricular hypertrophy, as well as
    b. the effect of acute insulin treatment on the expression of NKA isoforms, intracellular localization and pump activity in control and the streptozotocin (STZ) diabetic rat models;
2. in the renal cortex,
    a. the effect of the diabetic state on the expression and subcellular localization of NKA isoforms, and the activity of the enzyme,
    b. the effect of ANGII treatment on the expression and subcellular localization of NKA isoforms, and the activity of the enzyme,
    c. changes in the subcellular localization and activity of NKA in diabetic animals treated with insulin, angiotensin II receptor type 1 (AR1) blocker losartan and metformin.

METHODS

Experiments were performed on four-week old male Wistar rats (weighing 100±30g). All experimental protocols were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (TUKEB 99/94).

The experimental protocol consisted of the following animal groups

1) control (C),
2) STZ-induced diabetic rats (3 weeks, 4 weeks, 7 weeks) (D),
3) diabetic treated with insulin (DI),
4) control treated with angiotensin II (CA),
5) diabetic treated with angiotensin II (DA),
6) diabetic treated with losartan (DL)
7) diabetic treated with metformin (DMet)

Each experimental group contained $n \geq 5$ animals.

STZ-induced diabetes

Rats were rendered diabetic with STZ (65 mg/kg iv, Sigma Chemical Co., Budapest) dissolved in 0.1M citrate buffer (pH 4.5). Only STZ-treated animals with plasma glucose concentrations above15 mmol/L were considered diabetic and included in the study.

Acute and chronic effect of insulin treatment, ANGII administration, losartan and metformin treatment

For insulin stimulation studies rats were anaesthetized by an intraperitoneal injection of pentobarbital sodium (40 mg/kg pentobarbital, Abbott Laboratories, Budapest), than received tail vein injections of regular insulin (1.5 units/100 g body weight) or saline (0.01 mL/100 g body weight) 20 min before tissue removal. Blood and urine samples were obtained then the animals were killed by decapitation. Tissue samples were removed and stored at $^{-}80°C$.

Chronic insulin treatment consisted of two daily dose of intermediate effect insulin (5-5 IU Humulin N, Lilly) administered subcutaneously from the onset of STZ- diabetes.

Angiotensin II was administered with an osmotic minipump (pumping rate: 24h, 33μg/kg/h 7 weeks after the induction of DM), which was implanted subcutaneously under pentobarbital anaesthesia (40 mg/kg pentobarbital sodium, Abbott Laboratories).

Losartan was administered per os 4 weeks after the induction of diabetes and control animals for 3 weeks (dose: 5 mg/kg/day) Metformin was administered orally for 18 days 4 weeks after the induction of diabetes (dose: 250 mg/kg).

Measurement of general and laboratory (metabolic and renal) parameters

Renal parameters were photometrically determined with commercially available kits (Boehringer-Mannheim Diagnostic Systems) on a Hitachi-917 automated spectrophotometer. Serum glucose concentration was measured using a reagent kit from Boehringer Mannheim. Serum fructosamine concentration was measured using a kit from Roche Diagnostics Ltd.

Mean arterial pressure

Blood pressure was monitored using a radiotelemetry system. Transmitters (type: TL11M2-C50-PXT, Data Sciences International), were introduced into the abdominal aorta and the body of the transmitter was sutured to the abdominal wall. Receivers were placed under each animal's cage. The data were collected, saved and evaluated using the Dataquest IV. Software (Data Sciences).

Total scavenger capacity (TSC)

The total scavenger capacity (TSC) of the plasma was measured using a chemiluminescent method in a Lumat LB 9501 luminometer, based on the

method of Blázovics et al. The full wavelength range was analyzed. A 0.15 ml plasma sample was added to the H_2O_2-luminol-microperoxydase system. The photon output was measured for 30 seconds and expressed in RLU% (relative light unit %). Each measuring point represented five parallel data points from experiments (CV was <5%).

RT-PCR

All reagents, enzymes and isolation kits were purchased from: Quiagen GmBH, Hilden, Németország. Total RNA extraction, first-strand cDNA synthesis and PCR reaction for NKA alpha-1 and glyceraldeyde-3-phosphate dehydrogenase (GAPDH) were performed as previously described (Fekete et al., 2004). Primers: GenBank NKAα1 (NM 012504), F sequence: 5'-AGA TTT GAG CCG AGG CCT AAC ACC-3'; R sequence: 5'-TCC GCC CTT CAC CTC CAC CAG AT-3' (product length: 418 bp). As internal standard mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was isolated and the expressed amount was measured F: 5'-GGT GAA GGT CGG AGT CAA CG-3'; R: 5'-CTC ATC GCG CTT GCC AGT G-3' (product length: 496 bp). PCR products were separated on 2.5% ethidium bromide agarose gel.

Tissue homogenization and cellular protein fractionation

The kidney cortex samples were homogenized in chilled extraction buffer (60 mM HEPES, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 100 mM NaF, 0.5 mM PMSF, 0.75 mg/L leupeptin and 0.1 mM DTT), using a Potter-Elvehjem homogenizer. The total renal cortical tissue homogenate (TOT) was centrifuged at 680 g for 5 min at 4ºC and stored at -80ºC for further analysis. Triton X-100 extraction fractionates the cellular pool of NKA into an insoluble pellet (cytoskeletal-associated fraction) and a soluble supernatant by simple differential centrifugation.

Western Blot Analysis

Samples were solubilized, identical amount (5-50μg) of proteins were separated by SDS-PAGE using 10 % polyacrylamide gels and transferred onto nitrocellulose filters. Filters were blocked with Tween-20 containing Western blot buffer and then incubated with sufficiently diluted antibodies against the different subunit isoforms of NKA (UBI, Lake Placid, NY, USA- polyclonal, and Santa Cruz, Ca, USA- monoclonal). Blots were washed in a Tween-20 containing phosphate buffer and then incubated with peroxidase-conjugated secondary antibodies. Blots were developed with enhanced chemiluminescence Western blotting detection (APBiotech). Computerized densitometry of the specific bands was analyzed with Gel-Pro Analyzer 3.1 software. The values were normalized to beta-actin (Sigma Chemical Co.) as an internal standard and expressed as relative optical density.

Cell surface biotinylation

Cardiomyocytes were isolated according to the method of Abel et al (2004). Half of the cells were stimulated with 10 nM insulin with and without Wortmannin (200 nmol/L; Sigma) for 30 minutes Cardiomyocytes were then biotinylated in a solution containing PBS with sulfo-N-hydroxysuccinimide-S-S-biotin ((1,5 mg/ml, Pierce, Rockford, IL) for 60 min at 4 °C. The reaction was stopped by PBS solution containing 100 mM glycine. Cells were then washed and homogenized. 1 mg protein was then incubated overnight with 50 µl streptavidin beads (in 50% PBS) containing solution. Beads were washed and Laemmli buffer was added to each sample and heated for 56°C for 20 min. Samples were subjected to Western blot analysis.

NKA enzyme activity

NKA activity was assayed by measuring the strophantidine sensitive 3-O-methylfluoresceinphosphatase activity. The activity was determined in the presence of 19.5 mmol/L M 3-Omethylfluoresceinphosphate, 4 mmol/L MgCl2, 1 mmol/L EDTA, 80 mmol/L Tris-HCl (pH 7.6), 10 mmol/L KCl and 100 μg tissue homogenate, and pre-incubated with 0.1% Na deoxycholate (pH 7.4) for 30 min at 24°C. Inhibition percentages were calculated by comparing the activities in the presence or absence of 5 mmol/L strophanthidine. NKA activity can be calculated and given in units of micromol fluorescein/mg protein/hour.

Fluorescent immunohistochemistry

Kidney sections were immediately snap-frozen in 30% sucrose for immunohistochemical analysis. They were embedded in Shandon cryomatrix (ThermoElectron Co.), cut into 5-10μm thick slices with a cryostat and stored at ⁻80ºC until use. Slides were washed and incubated with the primary antibody and after repeated washing with the appropriate secondary antibody.. DNA was stained with Hoechst 33342 (Sigma Co.) for 10 min at room temperature. Slides were then rinsed in PBS and cover slipped with Vectashield fluorescent mounting medium (Vector Laboratories.). Appropriate controls were performed omitting the primary antibodies to ensure the same specificity with Western blot methods and to avoid auto-fluorescence. Confocal images were taken on a Zeiss Axiovert LSM510. PAS staining proved that the tissue section represented proximal tubules.

Statistical analysis

Data were analyzed on STATISTICA.6 software (StatSoft® Inc.). Data are presented as means± SD, and were tested for normal distribution with

Kolmogorov-Smirnov test. Multiple comparisons and possible interactions were evaluated by two-way ANOVA followed by Scheffe-correction as a post-hoc test. For non-parametrical data the Kruskal-Wallis ANOVA on ranks was used. Criterion for significance was $P < 0.05$ in all experiments.

SUMMARY OF RESULTS

The following observations were made with respect to changes in the myocardium of diabetic rats:

1. We have shown that TSC in the type 1 diabetic animal model was five times that of control animals (TSC: C: 0.665±0.097; D: 3,439±0.548; $p<0.00003$). No increase was detected in the left ventricular weight/total bodyweight ratio in animals three weeks after the onset of diabetes.
2. We have shown that long-term administration of insulin and ANGII reduces TSC in the plasma in animals with early diabetes (TSC D vs. CA, CIA, DA, DIA; $p<0.05$). Insulin treatment reduced TSC to a greater extent than ANGII. The two treatments have not proven to be additive. A small, but significant increase in the left ventricular weight/total bodyweight ratio (C vs. DI, DIA; $p<0.01$) was observable in diabetic rats treated with insulin. Administration of angiotensin did not increase the left ventricular weight/total bodyweight ratio.
3. We have shown that both in the control and diabetic myocardium low-affinity ouabain binding sites (α_1 subunit isoform) are mostly found bound to the plasma membrane (C: 90%, D: 90% of the total α_1 subunit is membrane-bound), while the majority of high-affinity ouabain binding sites (α_2 subunit isoform) are bound to intracellular pools (C:>70%, D:57% of the total α_2 subunit is membrane-bound).

4. In the myocardium of diabetic rats the NKA activity in the intracellular membrane fraction was 44.27% lower, while that in the plasma membrane fraction was 21.01% lower than in control animals (C vs. D PM $p<0.05$; IM $p<0.01$). The primary cause of the reduction in activity observable in diabetes is due to the significantly reduced expression of the catalytic subunit. In C and in D myocardium most of the α_1 isoform was found bound to the plasma membrane, with the quantity of the subunit decreased by 17.3% in diabetes as compared to control. The α_2 subunit isoform was found in the fraction containing the intracellular membrane in both control and diabetic myocardium. In diabetes the relative amount of this isoform is decreased by 40.2% ($p<0.01$). The amount of the regulatory β_1 isoform was decreased by 25.9% ($p<0.05$) in the plasma membrane fraction, and by 51.8% ($p<0.01$) in the intracellular membrane fraction in diabetes. The β_2 subunit isoform is also decreased in both membrane fractions (PM 21.2±2.9% $p<0.05$ and IM 23.7% $p< 0.05$) in diabetes.
5. The amount of α_2 and β_1 subunits isoforms occurring mostly in intracellular pools is decreased to a greater extent in diabetes than the amount of α_1, β_1 and β_2 subunits bound to the plasma membrane.
6. Our investigations confirm the insulin-dependent translocation of catalytic NKA α_1, α_2 and non-catalytic β_1 subunits in both control and diabetic myocardium. The translocation of the α_1 subunit into the plasma membrane induced by the effect of insulin could only be detected with the cell surface biotinylation method, where we excluded protein loss arising from the subcellular fractioning method (α_1: CI 1.37-fold and DI 1.25-fold). The translocation of α_2 was decreased in diabetes as

measured by both methods (α_2: KI 2.89-fold, DI 1.72-fold; β_1: CI 2.34-fold; DI 1.51-fold).

7. According to our results, insulin-induced translocation of NKA in the myocardium could be inhibited by the selective PI3 kinase inhibitor wortmannin.
8. In diabetic animals, the acute effect of insulin prevails only to a smaller extent probably due to the significantly decreased subunit expression. The decreased effect of insulin can primarily be explained by the significant reduction in the amount of α_2 and β_1 subunit isoforms. The α_1 subunit isoform plays only a minor part in the acute insulin response.

The following observations were made with respect to changes in the renal cortex of diabetic rats:

1. We have shown that NKA α_1 subunit isoform mRNA and protein expression in diabetic rats is increased as compared to control animals ($p<0.05$ C vs. D, CA vs. DA).
2. Administration of ANGII further increased protein levels ($p<0.05$ C vs. CA, D vs. DA). The mRNA and protein expression and enzyme activity increased in an additive manner in the kidneys of diabetic ANGII treated rats ($p<0.05$ C vs. DA).
3. Enyzme activity increased in accordance with the changes in mRNA and protein expression observable in both the STZ diabetic and the ANGII treated groups ($p<0.0001$ C vs. D, $p< 0.001$; C vs. CA, $p<0.01$ D vs. DA).
4. Similarly to the total renal cortex homogenate, the amount of the NKA α_1 subunit isoform protein in the intracellular membrane fraction was increased in diabetic rats as compared to control animals ($p<0.002$ C vs.

D; $p<0.05$ CA vs. DA). The administration of ANGII in both the C and D groups further increased protein levels ($p<0.01$, C vs. CA; D vs. DA). The amount of the α_1 subunit isoform did not change significantly in the fraction representing plasma membrane proteins in either group.

5. We have demonstrated that the increased activity observed in diabetes and following ANGII treatment does not derive from NKA activity found in the plasma membrane, but from the increased amount of pumps found in the intracellular pools ($p<0.002$ C vs. D; $p<0.05$ CA vs. DA).
6. Ser23 phosphorylation could be detected primarily in the intracellular fraction. The diabetic state increased the Ser23 phosphorylation of the α1 subunit isoform ($p<0.05$ C vs. D, CA vs. DA). Ser23 phosphorylation increased significantly following ANGII administration in both C and D rats ($p<0.05$; C vs. CA, D vs. DA). Ser23 phosphorylation presumably leads to the internalization of NKA from the basolateral membrane of the proximal convoluted tubule.
7. In diabetic rats NKA α_1 subunit isoform mRNA and protein expression as well as Ser23 phosphorylation in the intracellular membrane fraction was reduced to levels seen in the control group after the administration of losartan. Losartan treatment counteracts the above mentioned effects of diabetes and ANGII administration.
8. Metformin treatment failed to significantly reduce the increased α1 subunit isoform level in the renal cortex homogenate of diabetic rats. Metformin significantly increases the amount of the NKA α1 subunit isoform in the plasma membrane fraction ($p<0.007$), while decreasing the amount detected in the intracellular membrane fraction ($p<0.003$). This indicates α_1 subunit isoform translocation following treatment with

metformin. Acute effect of insulin resulted in no change in the subcellular localization of α_1 subunit.

DISCUSSION

The biochemical background of diabetic cardiopathy; the potential role of NKA

In diabetic cardiomyopathy the deterioration of myocardial contractility is linked to the pathological alteration of myocardial structure. Left ventricular hypertrophy is one of the macroscopic indicators of this alteration. Based on our observations plasma TSC increases by five-fold in diabetes mellitus, thus the amount of scavengers participating in the antioxidant defense system is significantly increased. We may not draw conclusions with respect to the amount of prooxidants and their activity from our studies. According to our results chronic insulin treatment leads to a reduction in TSC while the degree of left ventricular hypertrophy developing in diabetes is enhanced. Long-term insulin treatment leads to exogenous hyperinsulinemia, which consists of successive periods characterized by low and high insulin levels. Rapid changes in the concentration of insulin induce oxidative stress in the organism; deleterious consequences may include pathological tissue proliferation. Insulin treatment and the subsequent drop in blood sugar levels may reduce free radical production and thus may lead to a potential reduction in compensatory antioxidant defense capacity. Based on our experiments this explanation appears less plausible, since blood sugar levels remained quite high following insulin treatment. Exogenous hyperinsulinemia may contribute to the development of myocardial hypertrophy by influencing other signal transduction pathways.

The reduced activity of cation transport systems is a characteristic alteration in cardiomyopathy. The acute effect of insulin treatment changes the membrane potential maintained by NKA. NKA activation is likely to be responsible for the change in concentration. In our investigations however, we were unable to detect a change in NKA activity measured in homogenate following insulin treatment. At the same time, we detected an increase in both NKA activity and quantity in the plasma membrane. According to our results α1 and α2 subunit isoforms are located in different compartments within left ventricular cardiomyocytes. The α1 subunit isoform was mostly detected in the plasma membrane both in control and diabetic myocardium, while the α2 subunit isoform was mostly found in intracellular pools. The diabetic state reduces myocardial responsiveness to acute effect of insulin. This may be explained by the significant decrease in the amount of α2 és a β1 subunit isoforms in diabetes. The α1 subunit isoform plays a minor part in the acute response to insulin. In the diabetic myocardium acute insulin response leads to a reduction in intracellular NKA activity that is greater than the increase in activity observable in the plasma membrane. In diabetes the expression of β subunits is decreased to a greater degree than that of α subunits, therefore raising the possibility that the large-scale decrease in the amount of β1 subunit isoforms inhibits the formation of α2β1 heterotetramers that are capable of translocation. Our results show that the insulin induced translocation of NKA could be inhibited with the selective PI3 kinase inhibitor wortmannin. PI3 kinase plays a central role in the insulin signaling pathway, that is known to play a part in the insulin-dependent translocation of GLUT4 and NKA into the membrane. Thus the total amount of NKA is reduced in the diabetic myocardium, and the same

reduction is observable in plasma membrane-bound NKA activity that is crucial for the transport of ions. The change in NKA activity is presumably part of an adaptive mechanism, which is expected to compensate for the deteriorated myocardial contractility and pump function in diabetes.

The role of NKA in the development of diabetic nephropathy

Hyperglycemia and hypertension are obviously involved in the pathogenesis of diabetic nephropathy, speeding up the progression of the renal complication. Diabetes is characterized by an increase of local and systemic ANGII levels. Blood pressure lowering and antihyperglycemic medications (e.g. insulin, metformin) have proven to have a beneficial effect in diabetes.

Reports confirm that ANGII plays a role in the redistribution of other Na^+ channels as well. The precise mechanism of intracellular translocation and the role of NKA in the renal cortex has not been fully elucidated as of yet. We have proven in our experiments that the deteriorating effect of diabetes and ANGII treatment on renal function is additive and lead to the development of ”superimposed” nephropathy with supposedly a diabetic and RAS-activated factor.

Based on our results, ANGII plays a role in the redistribution of NKA in diabetes. The diabetic state and ANGII treatment both increase the expression of NKA and the quantity of the protein. They also enhance the internalization of NKA α_1 subunit isoform, suggested by the distribution of pumps between the plasma membrane and intracellular membrane fractions. We may not exclude the possibility however, that our experiments represent temporary states, in which pumps produced in excess due to the diabetes or treatment with ANGII have not yet reached their physiological target, the plasma membrane, and are temporarily located in the cytosol. All these data confirm that when we evaluate the increase in NKA activity measured in the

tissue homogenate we need to be aware of the fact that activity connected to the plasma membrane does not necessarily match the changes detected in the homogenate. In addition to pump protein quantity and subcellular localization, pump function may also be influenced by posttranslational covalent modifications. According to our studies, the amount of α_1 subunit isoform located in the cytosol and the degree of Ser23 phosphorylation is significantly increased in STZ diabetes and after treatment with ANGII. This is suggested by the observation that subunits moving from the plasma membrane into the intracellular membrane pool are Ser23 phosphorylated. Ser23 phosphorylation leads to the internalization of pumps, however their activity remains uninhibited, unlike that occurring during Ser18 phosphorylation. Ser23 phosphorylation could not be detected in cytoskeleton-associated plasma membrane bound NKA. Based on our observations treatment with the ANGII receptor blocker losartan reversed the increase in NKA expression; the majority of pumps were located in the basolateral membrane and the degree of detectable Ser23 phosphorylation decreased to levels similar to that seen in the control group. This finding is in accordance with clinical observation, with respect to use of ANGII receptor blockers delaying the progression of nephropathy in diabetic patients irrespective of how effective the same treatment was in controlling hypertension. We were also able to observe the normalization of pathological NKA activity developed due to diabetes in experiments carried out with the antihyperglycemic agent, metformin, suggesting that several signaling pathways may influence NKA activity. One of the main energy-sensing enzymes within the cell, AMPK is activated by metformin. Additionally, the activity of AMPK presumably deteriorates in diabetes, as energy deficiency, oxidative stress both fail to induce adequate activation.

This may not only worsen the effectivity of compensatory mechanisms in diabetes, but may also lead to increased tissue damage. In addition to the reinstatement of NKA activity, the pharmacological activation of AMPK presumably affects additional signalling pathways and research into these areas may prove to be significant in the future.

The increase in NKA activity and expression in diabetes and/or ANGII treatment (RAS activation) is presumably a part of an adaptive response on behalf of the kidney, which is meant to compensate the initial hyperfiltration and increased blood pressure seen in diabetes.

The role of pathological alterations of NKA function in tissue remodelling, and the progression of complications

Based on our results, it is presumable that the net result of changes in hormonal environment seen in diabetes, the attenuated insulin signal, the increased systemic and local ANGII level and the increased concentration of endogenous digitalis results in a change in the subcellular distribution of NKA in the myocardium and the renal cortex. The net effect of endogenous and exogenous regulatory agents presumably determines NKA activity, which also depends to a large extent on the subcellular localization of the enzyme. Change in the quantity and activity of pumps in the plasma membrane affects ion transport, while alterations of the pumps in signalosomes and intracellular membranes affect the activity of proliferative and apoptotic signaling pathways connected to NKA. MAPK cascade, PI3K and PKC are the key targets in the signaling pathways affected in diabetes, and are influenced by insulin, ANGII and endogenous digitalis. These molecules are branching points in signaling pathways that are affected by diabetes in multiple ways. Their changed activity may lead to fibrosis,

apoptosis and hypertrophy and may lead to specific dysfunction in affected tissue.

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