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A study of the activity, localization and regulation of sodium:calcium exchange in mesangial cells from Dahl/John Rapp salt-sensitive and salt-resistant rats.

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A STUDY OF THE ACTIVITY, LOCALIZATION AND REGULATION OF SODIUM:CALCIUM EXCHANGE IN MESANGIAL CELLS FROM DAHL/JOHN RAPP SALT-SENSITIVE AND SALT-RESISTANT RATS

by

ANGELA NICOLE ALFORD MASHBURN

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Physiology and Biophysics
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ABSTRACT OF DISSERTATION
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Title A Study of the Activity, Localization and Regulation of Sodium:Calcium Exchange in Mesangial Cells from Dahl/John Rapp Salt-sensitive and Salt-resistant Rats

Mesangial cells (MC) are smooth muscle-like cells found within the glomerulus that are thought to function in the regulation of glomerular filtration. These cells exhibit Na^+:Ca^{2+} exchange activity, a transport pathway that operates *in vivo* as a Ca^{2+} efflux pathway which helps to regulate intracellular calcium concentrations ($[Ca^{2+}]_i$). This transport pathway may also be involved in the altered regulation of $[Ca^{2+}]_i$ that is known to occur in certain forms of hypertension. The purpose of these studies was to determine the existence, activation, and regulation of the exchanger in cultured MC from Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats. Both functional and immunoblot assays were used to identify the exchanger in MC. Studies with the fluorescent Ca^{2+} probe fura 2 found that increases in $[Ca^{2+}]_i$ resulting from lowering of bath sodium ($[Na^+]_o$) were consistent with a reverse-mode Na^+:Ca^{2+} exchange activity that was the same between S and R MC. Using antibodies formed against the cardiac exchanger to probe MC revealed no difference in baseline expression of the exchanger in S and R MC. Other work tested the possibility that protein kinase C (PKC) is involved in Na^+:Ca^{2+} exchange regulation. S and R MC were incubated with phorbol 12-myristate 13-acetate (PMA), an activator of PKC, for 15 min then Na^+:Ca^{2+} exchange activity and protein expression assessed. Na^+:Ca^{2+} exchange activity and protein levels increased after PKC stimulation in R but not S MC indicating a defect in PKC stimulation of the exchanger in S MC. Further studies probed for PKC isozymes using both RT-PCR and

immunoblot to determine if S and R MC expression was different. Both S and R MC expressed similar amounts of α, δ, ε, η, and ζ isozymes. In conclusion, these results indicate that PKC activation of the Na^+:Ca^{2+} exchanger is defective in S MC. This defect could lead to diminished Ca^{2+} efflux and may be responsible, in part, for altered $[Ca^{2+}]_i$ that occurs in salt-sensitive hypertension.

Abstract Approved by: Committee Chairman P. Wann Bell

Program Director GR Hageman

Date 6/12/96 Dean of Graduate School John F. Lyden

DEDICATION

This dissertation is dedicated to all the people who helped make it possible. To all my friends at UAB for adding some fun to graduate school. To my parents, Richard and Bonnie, for their love and support and for providing me every opportunity to reach whatever goals I chose. And last but not least to my husband, Eric, for his love, support and encouragement throughout the last 5 years.

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LIST OF ABBREVIATIONS

%	percent
α	alpha
AA	afferent arteriole
AM	acetoxymethyl
ATP	adenosine triphosphate
β	beta
BSA	bovine serum albumin
Ca^{2+}	calcium
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
cps	counts per second
δ	delta
DDG	2 Deoxy-D-Glucose
dNTP	2'-deoxynucleoside 5'-triphosphate
ε	epsilon
EA	efferent arteriole
EGTA	ethylene glycolbis (β -aminoethyl ether) N, N, N, N tetraacetic acid
Fig.	figure
γ	gamma
g	gram
η	eta
h	hour

ι	iota
IgG	immunoglobulin G
IgM	immunoglobulin M
K^+	potassium
kDa	kilo Daltons
K_m	Michaelis constant
L	liter
λ	lambda
μ	mu
MC	mesangial cell(s)
mg	milligram
min	minute
mL	milliliter
ml	milliliter
μM	micromolar
mM	millimolar
mm	millimeter
μmol	micromole
mmol	millimole
mRNA	messenger ribonucleic acid
Na^+	sodium
Na^+:Ca^{2+} exchanger	sodium:calcium exchanger
NaCl	sodium chloride
Na+:K+ ATPase	sodium:potassium adenosine triphosphatase
Na_e	bath sodium

LIST OF ABBREVIATIONS (Continued)

ng	nanogram
nM	nanomolar
nm	nanometer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
pmole	picomole
psi	pounds per square inch
θ	theta
R	Dahl/Rapp salt-resistant rat
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
S	Dahl/Rapp salt-sensitive rat
s	seconds
SBFI	sodium-binding benzofuran isophthalate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHR	spontaneously hypertensive rat
V	volts
v/v	volume/volume concentration
V_{max}	maximum velocity
vsm	vascular smooth muscle
wk	week
WKY	Wistar-Kyoto rat

wt/vol	weight/volume concentration
ζ	zeta
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_o$	extracellular calcium concentration
$[Na^+]_i$	intracellular sodium concentration
$[Na^+]_o$	extracellular sodium concentration
°C	degrees Celsius
µg	microgram
µL	microliter
µl	microliter

INTRODUCTION

The work described in this dissertation has two main objectives. First, to demonstrate the presence and characteristics of sodium:calcium (Na^+:Ca^{2+}) exchange in cultured rat mesangial cells (MC) and second to determine possible differences in the exchanger or its regulation in salt-sensitive hypertension. Studies were performed using MC cultured from the Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats.

Mesangial cells are vascular smooth muscle-like cells found within the glomerulus that may function in the regulation of glomerular blood flow and ultrafiltration (27). Furthermore, cultured MC have been shown to demonstrate contractile activity in the presence of vasoactive agents (24, 39, 44), thereby lending evidence for their role in the regulation of glomerular filtration rate. In order for MC to contract and relax, intracellular calcium concentration ($[Ca^{2+}]_i$) must be tightly regulated. For instance, contraction is generally associated with an increase in $[Ca^{2+}]_i$ produced by Ca^{2+} influx from the extracellular fluid or release from intracellular organelles, while relaxation is associated with a decrease in $[Ca^{2+}]_i$ produced by Ca^{2+} uptake into intracellular organelles or efflux out of the cell. Regulation of $[Ca^{2+}]_i$ in contractile cells occurs at the plasma membrane through numerous influx pathways including Ca^{2+} channels and efflux pathways including Ca^{2+} ATPase and Na^+:Ca^{2+} exchange (21). The Na^+:Ca^{2+} exchanger in plasma membrane is a bidirectional antiporter that translocates 3 Na^+ ions for 1 Ca^{2+} ion (23) and may function, at least in some cells, to lower $[Ca^{2+}]_i$ after agonist-induced elevations in $[Ca^{2+}]_i$ (25, 26, 41). The exchanger was originally cloned from canine cardiac tissue and transports only Na^+ and Ca^{2+} (29). Subsequently, the Na^+:Ca^{2+} exchanger has been cloned from other tissues in

different species (1, 13, 17, 18, 20). An alternate form of the exchanger has also been cloned and is encoded by a different gene. The $Na^+:Ca^{2+}/K^+$ exchanger was cloned from rod photoreceptors and co-transports Ca^{2+} and K^+ with a stoichiometry of 4 Na^+/ 1 Ca^{2+} +1 K^+ (35). Studies described in this dissertation have focused on the $Na^+:Ca^{2+}$ form of the exchanger.

The canine cardiac form of the $Na^+:Ca^{2+}$ exchanger was cloned and sequenced and is 970 amino acids in size with 11 transmembrane regions (29). The subsequent development of both monoclonal and polyclonal antibodies by Philipson et al. (32) to the cardiac exchanger has shown the presence of 70, 120, and 160 kDa proteins from cardiac sarcolemma. The 120 kDa protein is the most likely candidate for the fully functional exchange protein, while the 70 kDa band appears to be a proteolytic fragment. In kidney, the $Na^+:Ca^{2+}$ exchanger has been localized to connecting tubule using the antibody described above (37) and proximal tubule using an antibody raised against a synthetic peptide sequence of the exchanger (13). In other work, Dominguez et al. (13) failed to identify the expression of a $Na^+:Ca^{2+}$ exchanger in whole glomeruli. However, because these studies were performed on whole glomeruli, it is possible that the antibodies were prevented from reaching MC, which reside within the glomerulus. Also, the antibodies developed by Philipson were not tested in these tissues.

There appears to be a high degree of homology (13, 18, 36) between the heart and kidney forms of the exchanger, with the kidney form having deletions in the intracellular loop region. The exchanger found in rabbit kidney is predicted to be 941 amino acids in length and the deduced amino acid sequence is 96% identical in the membrane-associated domain and 94% identical in the cytoplasmic domain compared to the canine cardiac sarcolemma exchanger (36). In rat kidney a clone has been identified which shares 89% nucleotide and 98% amino acid sequence identity with the canine cardiac exchanger (46). This homology between heart and kidney allowed us to use

antibodies formed against the cardiac exchanger to probe for the existence of the exchanger in MC.

It is generally recognized that alterations in the mechanisms responsible for the regulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ in vasoactive cells may play an important role in the development of hypertension. Blaustein and Hamlyn proposed the Natriuretic Hormone/Na^+:Ca^{2+} exchange/hypertension hypothesis (7) as a possible means of correlating $[Na^+]_i$ with $[Ca^{2+}]_i$ in hypertension. In this scheme, an increase in $[Na^+]_i$ results in a diminution of Na^+:Ca^{2+} exchange activity, which leads to less Ca^{2+} extrusion. The result is an increased $[Ca^{2+}]_i$ and elevated tension in contractile cells, potentially resulting in elevated total peripheral resistance and hypertension. However, in several different tissues from various animal models of hypertension, no consistency in Na^+:Ca^{2+} exchange activity has been found (2, 3, 22, 42, 43). Na^+:Ca^{2+} exchange activity has been reported to be enhanced, depressed or unchanged. Our studies will address whether Na^+:Ca^{2+} exchange activity is altered in a salt-sensitive form of hypertension.

Because other investigators have demonstrated functional activity of the exchanger in human MC (25, 26), we chose to study the exchanger in MC from S and R rats using fluorescent probes that measure Na^+ and Ca^{2+} concentrations. Na^+:Ca^{2+} exchange activity is influenced by the electrochemical gradients across the cell membrane for Na^+ and Ca^{2+}. Normally there is a large inwardly directed electrochemical gradient for Na^+ which is maintained by Na^+:K^+ ATPase. Forward mode operation of the exchanger (Figure 1) is, therefore, defined as the electrogenic movement of Na^+ into and Ca^{2+} out of the cell. Interestingly, the exchanger has been co-localized with Na^+:K^+ ATPase near Ca^{2+} storage sites of the sarcoplasmic reticulum (28) suggesting that there may be close interaction between these two transport pathways. One means of evaluating Na^+:Ca^{2+} exchange activity is to use reverse mode Na^+:Ca^{2+} exchange (Figure 1). This experimental approach involves a reduction in bath

Na^+ concentration ($[Na^+]_o$) which results in Ca^{2+} influx and increased $[Ca^{2+}]_i$ as measured by the fluorescent probe, fura 2. Reductions in $[Na^+]_o$ have been reported to directly mobilize Ca^{2+} in fibroblasts (40); however, several investigators (5, 8, 26) have shown convincingly that elevations in $[Ca^{2+}]_i$ in contractile cells during reductions in $[Na^+]_o$ occur through the Na^+:Ca^{2+} exchanger. Although changes in $[Ca^{2+}]_i$ during reverse mode Na^+:Ca^{2+} exchange are well documented, little information exists concerning alterations in $[Na^+]_i$ that occur during reductions in $[Na^+]_o$. This prompted us to measure both $[Na^+]_i$ and $[Ca^{2+}]_i$ using fluorescent probes and to correlate changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ during decreases in $[Na^+]_o$.

Altered $[Ca^{2+}]_i$ regulation is a well known characteristic of hypertension. Studies by several investigators have shown Ca^{2+} dependent increases in vascular tone in hypertensive models (4, 33). PKC plays an important role in second messenger-mediated transmembrane signal transduction resulting in the phosphorylation of certain proteins. Inactive PKC is a soluble cytosolic protein that binds to the plasma membrane in the presence of Ca^{2+} and phospholipid and is activated by diacylglycerol. Once activated, PKC phosphorylates serine and threonine residues on other proteins (10). Although the regulation of the Na^+:Ca^{2+} exchanger is not well understood, various investigators have suggested that exchange activity may be regulated by phosphorylation (12). Iwamoto has demonstrated PKC-dependent phosphorylation of the Na^+:Ca^{2+} exchanger in aortic smooth muscle in response to growth factors (15). Studies by Vigne suggest that PKC up-regulates Na^+:Ca^{2+} exchange in aortic smooth muscle cells (45). However, Mene demonstrated exchange inhibition in human MC upon exposure to activators of PKC (25). At present, the role of PKC in the regulation of the exchanger remains unclear. In addition, there is no information concerning a potential alteration in PKC regulation of the exchanger in hypertension. Therefore, studies were performed to examine PKC activation of the Na^+:Ca^{2+} exchanger in MC cells form S and R rats.

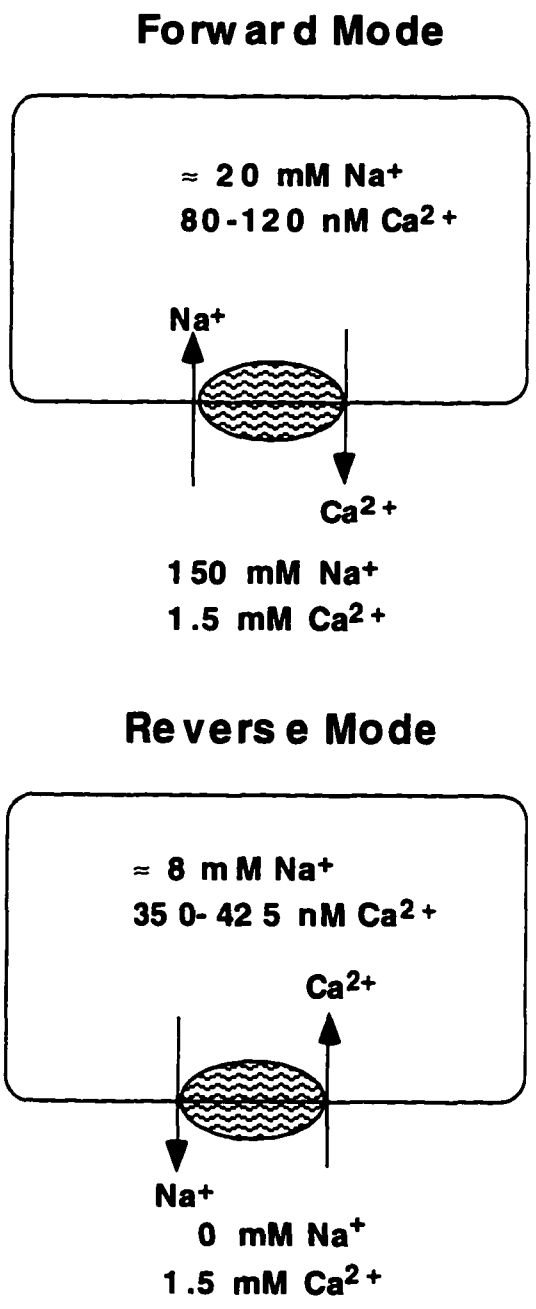

Figure 1. Schematic demonstrating forward and reverse-mode Na^+:Ca^{2+} exchange.

At present, there are 12 known isozymes of PKC that are grouped in three categories: 1) Ca^{2+} dependent and phorbol ester-responsive, α, βI, βII, and γ; 2) Ca^{2+} independent and phorbol ester-responsive, δ, ε, η, θ, and μ; and 3) Ca^{2+} independent and phorbol ester-unresponsive, ι, λ, and ζ (11). Of these, eight (α, βI, βII, γ, δ, ε, η, and ζ) are known to exist in rat (19, 30). Therefore, the role of PKC in various cells is dependent upon the number and types of PKC isozymes present. Previously, four PKC isozymes (α, δ, ε, and ζ) have been demonstrated immunologically in MC (14). Our studies establish which of the different PKC isozymes are present in MC from a hypertensive strain of rat using both reverse transcription-polymerase chain reaction (RT-PCR) to determine the presence of mRNA as well as immunoblot analysis to establish the presence of specific PKC isozyme proteins.

Dahl/Rapp salt-sensitive and salt-resistant rats, a well defined genetic model of hypertension (34), were chosen for our studies. These rats were originally developed by Dahl, who demonstrated that when a heterogeneous population of Sprague-Dawley rats were fed a high salt (8.0 % NaCl) diet some rats became markedly hypertensive while others remained normotensive (9). Dahl then selectively inbreed, for three generations, those rats that became hypertensive on high salt and designated them as salt-sensitive. In addition, he also selectively inbred, for three generations, those rats that did not become hypertensive on high salt and designated them as salt-resistant. Both strains have subsequently been inbred for more than 20 generations by Rapp and are homozygous at every locus, making them an excellent model for studying salt sensitive hypertension (34). The ability to culture MC from Dahl/Rapp S and R rats provided us with the opportunity to investigate potential differences in Na^+:Ca^{2+} exchange activity between S and R MC. Cultured MC have the advantage of removing hemodynamic and hormonal influences allowing the investigator to study the intrinsic differences in cellular function. In order to study inherent differences between S and R rats that might

lead to the development of hypertension, all MC cultured for these experiments were originally from young rats (50 g) prior to the onset of hypertension.

Na^+:Ca^{2+} exchange activity has been studied in several tissues, including vascular smooth muscle cells (45) and MC (26) by a variety of techniques. However, the localization, regulation and other characteristics of Na^+:Ca^{2+} exchange in MC remain largely unknown. We propose that there may be differences in the activity, localization, and/or regulation of the Na^+:Ca^{2+} exchanger in S and R rats, which might be involved in the pathogenesis of hypertension. For example, if MC contraction plays a role in hypertension, the following hypothesis might account for the differences demonstrated between S and R (Figure 2). In order for MC to contract there must be a signal causing an increase in $[Ca^{2+}]_i$ (e.g., a vasoactive hormone). This hormone would work through second messenger systems that would activate diacylglycerol and inositol phosphate. Inositol phosphate would, in turn, cause an increase in $[Ca^{2+}]_i$ (Figure 2A). The rise in $[Ca^{2+}]_i$ and diacylglycerol would then activate PKC, which produces a variety of potential cellular effects. One action of PKC might be to activate Na^+:Ca^{2+} exchange resulting in Ca^{2+} efflux, thus causing $[Ca^{2+}]_i$ to return to control levels (Figure 2B). In this manner, PKC activation of Na^+:Ca^{2+} exchange would operate as a regulatory mechanism that returns $[Ca^{2+}]_i$ to normal after agonist-induced stimulation. In the S rat, some part of this pathway may be defective, allowing $[Ca^{2+}]_i$ to remain elevated and the cells contracted. The above hypothesis will be addressed in three manuscripts comprising the main body of this dissertation.

The first manuscript, titled "Relationship Between $[Na^+]_i$ and $[Ca^{2+}]_i$ During Variations in $[Na^+]_o$ in Cultured Dahl/Rapp Rat Mesangial Cells," demonstrates functional characteristics consistent with Na^+:Ca^{2+} exchange activity in rat MC.

The second manuscript, titled "Immunodetection and Phorbol Ester Stimulation of the Sodium-calcium Exchanger in Cultured Mesangial Cells," demonstrates localization as well as regulation of the Na^+:Ca^{2+} exchanger in MC. In this study

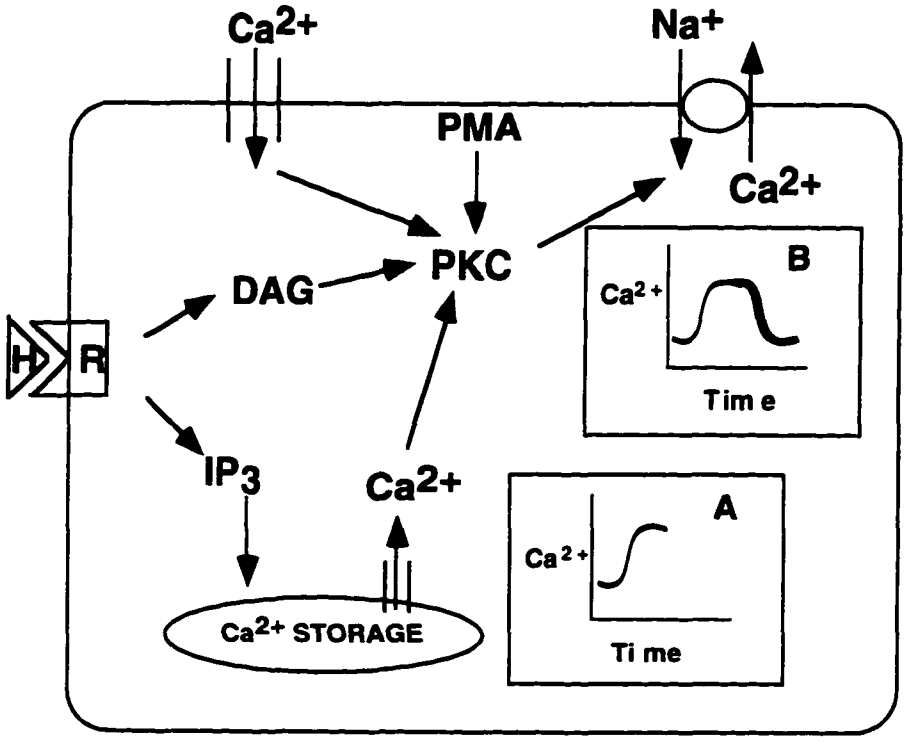

Figure 2. Schematic demonstrating a possible pathway for exchange regulation in MC. DAG = diacylglycerol, IP_3 = inositol phosphate, H = hormone, and R = receptor.

antibodies were used to immunodetect the exchanger in MC. In addition, the effects of phorbol ester on Na^+:Ca^{2+} exchange were determined using both functional as well as immunoblot methods.

The third manuscript, titled "Protein Kinase C Isozymes in Cultured Mesangial Cells and Whole Glomeruli From Salt-sensitive and Salt-resistant Rats," evaluates both the message and protein levels of five PKC isozymes that may be responsible for affecting Na^+:Ca^{2+} exchange.

Finally, the appendix of this dissertation contains two manuscripts that are related to the work presented in the three main manuscripts.

RELATIONSHIP BETWEEN $[Na^+]_i$ AND $[Ca^{2+}]_i$ DURING VARIATIONS IN $[Na^+]_o$ IN CULTURED DAHL/RAPP RAT MESANGIAL CELLS

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Running Head: Na^+:Ca^{2+} Exchange in Mesangial Cells

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ABSTRACT

Mesangial cells (MC) from Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats exhibit a sodium-calcium (Na^+:Ca^{2+}) exchange mechanism. Little is known regarding temporal changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ during activation of the exchanger by alterations in bath Na^+ concentration ($[Na^+]_o$). To address this issue, MC grown on coverslips were loaded with either fura 2 AM or SBFI AM. Baseline $[Ca^{2+}]_i$ was 122 ± 10 nM (n = 105) in S and 80 ± 5.9 nM (n = 100) ($p < 0.05$) in R, while baseline $[Na^+]_i$ was 22.4 ± 1.38 mM (n = 74) in S and 25.8 ± 1.72 mM (n = 70) in R (not significant). $[Na^+]_o$ (150 mM) was decreased to either 0, 10, 25, 50, or 75 mM Na^+ causing $[Ca^{2+}]_i$ to increase and $[Na^+]_i$ to decrease. Changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ were not different between S and R MC. The decrease in $[Na^+]_i$ was biphasic, consisting of a large, rapid decrease followed by a gradual decline in $[Na^+]_i$. The monophasic increase in $[Ca^{2+}]_i$ began after the initial decrease in $[Na^+]_i$ had occurred. The early loss of $[Na^+]_i$ appeared to be Na^+:Ca^{2+} exchange independent and may be due, in part, to Na^+ movement through the Na^+:K^+ ATPase. Thus, the majority of increase in $[Ca^{2+}]_i$ occurred during the gradual decline in $[Na^+]_i$. The magnitude and temporal pattern of changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ found in these studies are consistent with a Na^+:Ca^{2+} exchanger in MC.

Key words: Na^+:Ca^{2+} exchange, fura 2, SBFI, salt-sensitive hypertension

INTRODUCTION

Regulation of $[Ca^{2+}]_i$ in contractile cells occurs through numerous pathways including Na^+:Ca^{2+} exchange (16). This exchanger is a bidirectional antiporter that translocates 3 Na^+ ions for 1 Ca^{2+} ion (22) and may function, at least in some cells, to lower $[Ca^{2+}]_i$ after agonist-induced elevations in $[Ca^{2+}]_i$. It has been studied in a variety of cells including vascular smooth muscle cells (40) and mesangial cells (MC) (24). The Na^+:Ca^{2+} exchanger has been cloned from cardiac tissue and is 970 amino acids in length and contains 11 transmembrane regions (29). Recent work has suggested

that the exchanger derived from renal tissues has a high degree of homology with the exchanger cloned from cardiac tissues (9, 15, 31).

Na^+:Ca^{2+} exchange activity is influenced by the electrochemical gradients for Na^+ and Ca^{2+}. Normally there is a large inward electrochemical gradient for Na^+ which is maintained by Na^+:K^+ ATPase. Forward-mode operation of the exchanger is, therefore, defined as the electrogenic movement of Na^+ into and Ca^{2+} out of the cell. Interestingly, the exchanger has been co-localized with Na^+:K^+ ATPase near Ca^{2+} storage sites of the sarcoplasmic reticulum (26) suggesting that there may be close interaction between these two transport pathways. One means of evaluating Na^+:Ca^{2+} exchange activity is to use reverse-mode Na^+:Ca^{2+} exchange. This experimental approach involves a reduction in $[Na^+]_o$ that results in Ca^{2+} influx and increased $[Ca^{2+}]_i$. Although reductions in $[Na^+]_o$ have been reported to directly mobilize Ca^{2+} in fibroblasts (34), work by several investigators in contractile cells has provided substantial evidence that elevations in $[Ca^{2+}]_i$ during reductions in $[Na^+]_o$ occur through the Na^+:Ca^{2+} exchanger (3, 8, 24).

At present there is a lack of information concerning temporal changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ in response to decreased $[Na^+]_o$. This prompted us to measure $[Na^+]_i$ and $[Ca^{2+}]_i$ using fluorescent probes and to correlate changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ during decreases in $[Na^+]_o$. These studies were performed in cultured MC because previous work has demonstrated functional activity of the exchanger in these cells (23, 24).

It is generally recognized that alterations in the mechanisms responsible for the regulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ may play an important role in the development of hypertension. Blaustein et al. proposed that $[Na^+]_i$ and $[Ca^{2+}]_i$ might be linked via Na^+:Ca^{2+} exchange to hypertension (5). However, changes in Na^+:Ca^{2+} exchange activity in several different tissues from various models of hypertension have been variable (i.e., enhanced, depressed or unchanged) (1, 2, 21, 36, 38). The ability to culture MC from Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats, a well defined

genetic model of hypertension (30), provided us with the additional opportunity to investigate potential differences in Na^+:Ca^{2+} exchange activity between S and R MC.

METHODS

Isolation and culture of glomerular mesangial cells

Animals used in this study were male Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 50 g and fed standard rat chow (Prolab RMH 1000; Agway; ~170-180 meq/kg of feed of both Na^+ and K^+) and water *ad libitum*. As outlined in recent reports (17, 37), S rats from Harlan Sprague Dawley were genetically contaminated as early as March 1993. It was noted that approximately 25% of these animals failed to develop hypertension when placed on a high salt diet when the contamination was first discovered, but soon a much higher percentage of S rats were found to no longer display the salt-sensitive phenotype. Animals used in this study were obtained prior to March 1993 and, therefore, prior to the reported problems associated with the breeding colonies at Harlan Sprague Dawley. No genetic studies were performed on these rats. Also, because of the difficulty of culturing MC from older rats, MC were cultured from young rats, which prevented measurements of blood pressures on a high salt diet. For these studies, primary cultures of MC were established from 17 S and 17 R rats. The responses obtained in MC derived from the S animals appear homogeneous; therefore, we believe these rats were not contaminated.

Rat kidneys were aseptically removed and glomerular MC were isolated and cultured as previously described, with minor modifications (18, 25). In brief, renal cortical tissue was minced with a razor blade and passed through a #70 copper sieve (Fisher Scientific, Pittsburgh, PA). Tissue was then passed through progressively smaller nylon sieves (Tetko Inc., Briar Cliff Manor, NY) ranging in size from 315 μm to 75 μm in order to separate glomeruli from the remaining kidney tissue. Glomeruli were then treated with 21 units/ml collagenase (Sigma, St. Louis, MO) for 30 min and plated onto 60 mm x 15 mm petri dishes (Costar, Cambridge, MA). Cells were grown in RPMI

1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (Intergen, Purchase, NY), 240 μg/ml L-glutamine (Gibco), 82 units/ml penicillin, 82 μg/ml streptomycin (Sigma), and 2μg/ml amphotericin B (Gibco) for twenty-one days in humidified 95% air/5% CO_2 at 37°C. Media was changed twice a week. Cells were then subcultured and plated onto two 40 mm x 12 mm glass coverslips in petri dishes and were grown for an additional twenty-one days before use in experiments. With electron microscopy, the following characteristics were used to identify these cells as MC: prominent microfilaments, dense bodies, well-developed rough endoplasmic reticulum, gap junctions and attachment plaques. Staining for vimentin, which is a cytoskeletal filament indicating myogenic origins, was also positive in these cells.

Measurement of intracellular calcium concentration

$[Ca^{2+}]_i$ was measured using the acetoxymethyl ester form of the fluorescent dye fura 2 (Molecular Probes, Eugene, OR). Cells were incubated in 5 μM fura 2 AM in media for 1 h at 37°C to allow loading of the dye into cells. Fluorescence measurements were performed using a cuvette-based/dual-excitation wavelength system (Photon Technologies International, Inc., South Brunswick, NJ) in which the excitation beam and emission photometer are at a 90° angle. Coverslips containing either S or R MC were positioned diagonally in the cuvette and bathed in a 150 mM NaCl Ringer's solution. Excitation wavelengths were set at 340 nm and 380 nm, and the emission wavelength was 510 nm. The Ringer's solution contained (in mM): 4.2 KCl, 0.8 K_2HPO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 20 Hepes, 5.5 D-glucose, 5.0 L-alanine, 1.1 $CaCl_2$, 150 Cl, and 150, 75, 50, 25, 10, or 0 Na^+. N-methyl-D-glucamine was used to isosmotically replace Na^+ (all reagents from Sigma). All solutions were bubbled with O_2; pH was 7.4 and temperature maintained at 37°C.

Measurements of $[Ca^{2+}]_i$ were performed according to the following procedure. Coverslips were placed in the cuvette and allowed to equilibrate for 10 min. Ringer's

solution was continuously exchanged via a perfusion pump-suction system at a rate of 6.8 ml/min. To ensure adequate loading of the dye, fluorescence (counts per sec, cps) was measured at both 340 nm and 380 nm. Experiments were performed only if there were at least 5 x 10^5 stable cps at both excitation wavelengths. For the experiment, the baseline fura 2 fluorescence ratio (340 nm/380 nm) was monitored for at least 3 min. Then the bathing solution was changed to either the 0, 10, 25, 50, or 75 mM Na^+ Ringer's solution and the ratio was monitored for an additional 2 to 3 mins. Once the $[Ca^{2+}]_i$ response had peaked in some experiments, the solution was changed back to 150 mM NaCl Ringer's solution and baseline values for the fura 2 ratio were re-evaluated.

Intracellular calibrations were performed in order to convert fura 2 ratios into $[Ca^{2+}]_i$ values. Ionomycin (5 μM) was added to a Ringer's solution that contained either 3 mM Ca^{2+} or no Ca^{2+} and 3 mM EGTA. Cells were exposed to these solutions for a sufficient length of time (usually 2 to 3 min) to obtain stable maximum and minimum ratio values. $[Ca^{2+}]_i$ was calculated by the following equation:

$$[Ca^{2+}]_i = K_d \times (S_{f2}/S_{b2}) \times (R - R_{min}) / (R_{max} - R) \qquad (12)$$

where K_d is the dissociation constant of the dye and has a value of 224 nM (39), R is the fluorescence ratio at 340 nm/380 nm, and R_{min} and R_{max} are the ratios in the absence (3 mM EGTA) or presence of Ca^{2+} (3 mM Ca^{2+}) respectively. Fluorescence (cps) was measured at 380 nm in the absence of Ca^{2+} (S_{f2}) and in the presence of Ca^{2+} (S_{b2}).

Measurement of intracellular sodium concentration

$[Na^+]_i$ was measured using the acetoxymethyl ester form of the fluorescent dye sodium-binding benzofuran isophthalate (SBFI) (Molecular Probes, Eugene, OR). Cells were incubated in 10 μM SBFI AM in 10 ml of media plus 10 μl of 20% pluronic acid (Molecular Probes, Eugene, OR) for 2 h at 37°C. Fluorescence measurements were performed at the same wavelengths used in the fura 2 AM experiments (excitation wavelengths = 340 nm and 380 nm; emission wavelength = 510 nm). The bathing solution compositions were the same as those used for the $[Ca^{2+}]_i$ experiments. Since

quantum yield for SBFI is less than fura 2, cells were considered sufficiently loaded with dye if SBFI fluorescence averaged at least 1 x 10^5 cps at each excitation wavelength and was stable. The basic protocol with regard to baseline measurements and solution changes was the same as those described for the fura 2 experiments.

Intracellular calibrations using SBFI and 10 μM gramicidin (Sigma) in the presence of either 150 mM NaCl Ringer's or 0 mM NaCl Ringer's were used to convert ratio measurements to $[Na^+]_i$. SBFI ratio was converted to $[Na^+]_i$ using the following equation, which is the same as that used for fura 2:

$$[Na^+]_i = K_d \times (S_{f2} / S_{b2}) \times (R - R_{min}) / (R_{max} - R) \qquad (13)$$

where K_d is the dissociation constant of the dye and has a value of 16 mM (39), R is the fluorescence ratio at 340 nm/380 nm, and R_{min} and R_{max} are the ratios obtained in the absence or presence of 150 mM Na^+ respectively. Fluorescence (cps) was measured at 380 nm in the absence of Na^+ (S_{f2}) and in the presence of Na^+ (S_{b2}).

Drug studies

To determine possible pathways by which Na^+ may exit cells during reductions in Na_o, MC were loaded with SBFI AM. Cells were bathed in 150 mM NaCl Ringer's alone or with the addition of either: 1 mM ouabain or 0 mM $[K^+]_o$, for Na^+:K^+ ATPase inhibition; 3 mM EGTA, a Ca^{2+} chelator; or preincubated with 5 mM 2-deoxy-D-glucose (DDG) for 45 min to deplete ATP. The bathing solution was then switched to these same solutions except that $[Na^+]_o$ was reduced to 75 mM. The effects of a reduction in Na_o from 150 to 75 mM were assessed in the presence or absence of these various agents as previously described. All drugs were obtained from Sigma.

Statistics

Data are expressed as the mean ± standard error (SE). Statistical significance was established using paired and unpaired Student's t-test where appropriate. Comparisons within S and R groups were made using one-way ANOVA. Significance was accepted at $p < 0.05$.

RESULTS

Measurements of $[Ca^{2+}]_i$ and $[Na^+]_i$ were performed under baseline conditions ($[Na^+]_o$ = 150 mM) and after changing $[Na^+]_o$ to 0, 10, 25, 50, or 75 mM. Baseline measurements were taken only after the ratiometric fura 2 or SBFI fluorescence signals had been constant for at least 3 min. Baseline measurements for both $[Ca^{2+}]_i$ and $[Na^+]_i$ are listed in Table 1. In each group, $[Ca^{2+}]_i$ tended to be higher in S versus R MC. This difference achieved statistical significance in Groups C (25 mM $[Na^+]_o$) and E (75 mM $[Na^+]_o$). Overall, $[Ca^{2+}]_i$ averaged 122 ± 10 nM (n = 105) in S cells and 80 ± 5.9 nM (n = 100) in R cells, and this difference was statistically different ($p < 0.05$). Baseline $[Na^+]_i$ levels did not differ between or within the experimental groups.

These studies were performed in an attempt to correlate changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ when $[Na^+]_o$ was reduced. In order to perform such a correlation it was necessary to take into account the various characteristics of the response of $[Na^+]_i$ and $[Ca^{2+}]_i$ to changes in $[Na^+]_o$. These characteristics are illustrated in Figs. 1A and B, and the descriptive terms used to evaluate these responses are shown in Table 2. When $[Na^+]_o$ was altered, as shown in Figs. 1A and B, a brief lag time was observed followed by a monophasic increase in $[Ca^{2+}]_i$ and a biphasic decrease in $[Na^+]_i$. Part of this delay was due to the time required for exchange of the bathing solution in the cuvette (approximately 25 s). Interestingly, as shown in Fig. 2A, the lag time for $[Ca^{2+}]_i$ was nearly twice that of $[Na^+]_i$. This finding was consistent between S and R and over the range of $[Na^+]_o$ tested. Second, $[Na^+]_i$ responded to decreased $[Na^+]_o$ in a biphasic manner, exhibiting a large and rapid decline in $[Na^+]_i$ lasting approximately 30 s followed by a slow decrease that lasted over 150 s or until either $[Na^+]_o$ was increased or $[Na^+]_i$ reached equilibrium. The time required for the initial decrease in $[Na^+]_i$ was termed the response time while the magnitude of the decline in $[Na^+]_i$ is termed $\Delta [Na^+]_{i(initial)}$. $[Ca^{2+}]_i$ on the other hand, responded to decreased $[Na^+]_o$ in a monophasic manner that peaked at approximately 150 s. Therefore, response time for $[Ca^{2+}]_i$ was

Table 1.
Baseline intracellular calcium and sodium values

		Group A	Group B	Group C	Group D	Group E
		0 mM	10 mM	25 mM	50 mM	75 mM
		Na^+_o	Na^+_o	Na^+_o	Na^+_o	Na^+_o
S	$[Ca^{2+}]_i$	126 ± 21.6	89.0 ± 15.2	132 ± 21.7*	109 ± 28.2	139 ± 20.1†
	nM	n = 34	n = 16	n = 20	n = 13	n = 22
R	$[Ca^{2+}]_i$	82.3 ± 15.8	64.6 ± 12.8	81.9 ± 7.96*	81.7 ± 11.8	83.8 ± 10.8†
	nM	n = 30	n = 13	n = 27	n = 16	n = 14
S	$[Na^+]_i$	18.56 ± 2.27	22.47 ± 4.02	23.43 ± 2.7	24.91 ± 3.65	24.28 ± 3.45
	mM	n = 21	n = 10	n = 15	n = 15	n = 13
R	$[Na^+]_i$	24.80 ± 3.1	19.22 ± 3.04	21.64 ± 1.99	32.59 ± 5.10	29.64 ± 4.23
	mM	n = 24	n = 9	n = 12	n = 13	n = 12
				* $p < 0.05$		† $p < 0.05$

$[Ca^{2+}]_i$ and $[Na^+]_i$ in S and R mesangial cells under basal conditions. Each group denotes the treatment to be used after baseline measurements. Means were considered significantly different at $p < 0.05$ by unpaired t-test. Values are means ± SE. n = the number of coverslips used. The 179 S coverslips contained MC cultured from 17 individual S rat preparations. The 170 R coverslips contained MC cultured from 17 individual R rat preparations.

Table 2.
Term definitions

$\Delta [Ca2+]_{i(initial)}$	$[Ca^{2+}]_i$ at the end of the initial rapid decrease in $[Na^+]_i$ (i.e. 55 s) minus baseline $[Ca^{2+}]_i$.
$\Delta[Ca2+]_{i(secondary)}$	The peak value for $[Ca^{2+}]_i$ minus $[Ca^{2+}]_i$ at the end of the initial rapid decrease in $[Na^+]_i$.
$\Delta [Ca2+]_{i(total)}$	Peak $[Ca^{2+}]_i$ minus baseline $[Ca^{2+}]_i$.
Lag $[Ca^{2+}]_i$ & $[Na^+]_i$	The time required from switching solutions to the initial change in $[Ca^{2+}]_i$ or $[Na^+]_i$.
Response $[Ca^{2+}]_i$	The time required after the $[Ca^{2+}]_i$ lag time for the total increase in $[Ca^{2+}]_i$ to occur.
$\Delta [Na^+]_{i(initial)}$	Baseline $[Na^+]_i$ minus $[Na^+]_i$ after the initial rapid decrease in $[Na^+]_i$.
$\Delta [Na^+]_{i(secondary)}$	$[Na^+]_i$ after the initial rapid decrease in $[Na^+]_i$ minus $[Na^+]_i$ at peak $[Ca^{2+}]_i$ (i.e. 150 s).
$\Delta [Na^+]_{i(total)}$	Baseline $[Na^+]_i$ minus $[Na^+]_i$ at peak $[Ca^{2+}]_i$ (i.e. 150 se.
Response $[Na^+]_i$	The time required after the $[Na^+]_i$ lag time for the initial rapid decrease in $[Na^+]_i$ to occur.

Terms used in Figure 1 and throughout the text are defined in this table.

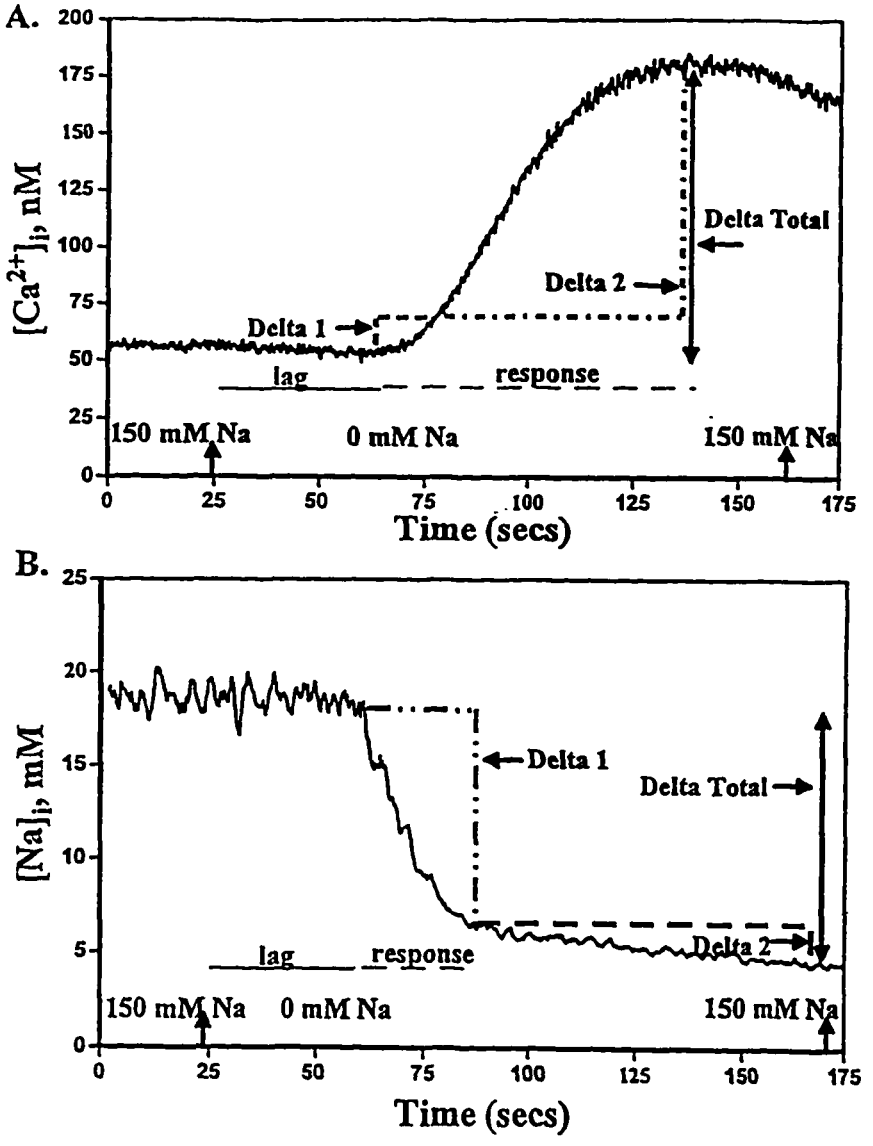

Fig. 1. Representative traces of (A) $[Ca^{2+}]_i$ (nM) and (B) $[Na^+]_i$ (mM) in S MC. $[Na^+]_o$ was changed at the arrows. Measurements, as described in Table 2, are depicted on this figure.

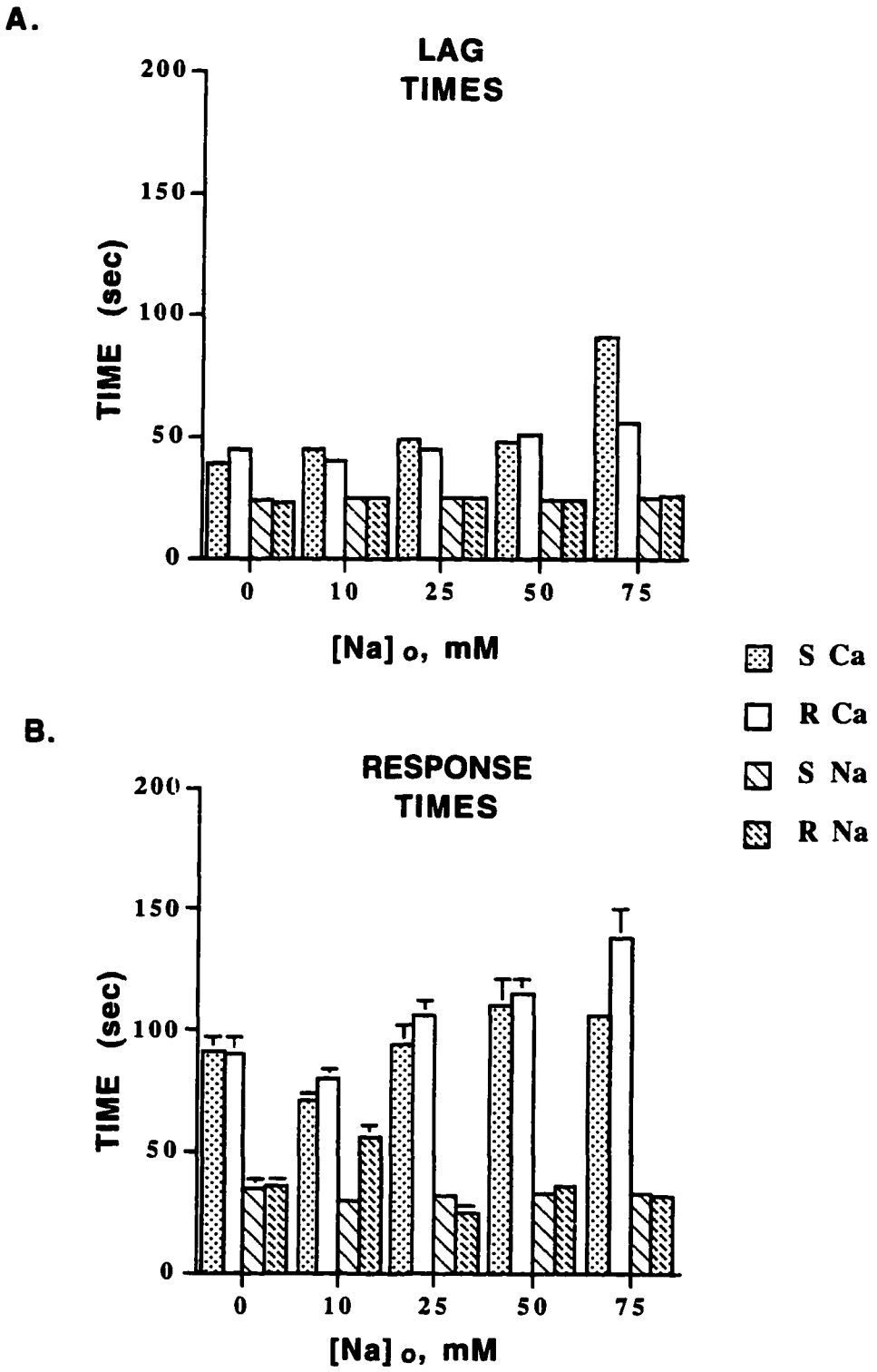

Fig. 2. Time measurements for changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ in S and R MC. A. Lag time (defined in Table 2) was not different between S and R but was significantly greater for $[Ca^{2+}]_i$ compared to $[Na^+]_i$. B. Response times (defined in Table 2) was also not different between S and R but was greater for $[Ca^{2+}]_i$ compared to $[Na^+]_i$.

was defined as the time required for Ca^{2+} to reach peak values after the lag time. Figure 2B compares response times and shows that the monophasic increase in $[Ca^{2+}]_i$ occurred over a much longer time interval than was required for the initial decline in $[Na^+]_i$. The increase in $[Ca^{2+}]_i$ was more closely associated with the small gradual decrease in $[Na^+]_i$ ($[Na^+]_{i(secondary)}$) and not the initial decrease in $[Na^+]_i$.

These observations prompted us to select specific time points at which to take measurements, and these time points are also illustrated in Figs. 1A and B and defined in Table 2. Fig. 3 illustrates changes in $[Na^+]_{i(initial)}$ and $[Ca^{2+}]_{i(initial)}$ during the initial 55 s after changing to an $[Na^+]_o$ of 75, 50, 25, 10, or 0 mM Na^+ in both S and R cells, excluding lag time. During this time period there were minimal changes in $[Ca^{2+}]_i$, while there were large decreases in $[Na^+]_i$. Fig. 4 presents changes in $[Na^+]_{i(secondary)}$ and $[Ca^{2+}]_{i(secondary)}$ in response to decreasing $[Na^+]_o$ to 0, 10, 25, 50, or 75 mM Na^+ in both S and R cells. During this time period, there were large increases in $[Ca^{2+}]_i$ and only small decreases in $[Na^+]_i$. By comparing Figs. 3 and 4 it can be seen that the majority of the decrease in $[Na^+]_i$ occurred prior to the increase in $[Ca^{2+}]_i$. Fig. 5 summarizes total change in $[Na^+]_i$ and $[Ca^{2+}]_i$ in response to decreases in $[Na^+]_o$ to 0, 10, 25, 50, or 75 mM, in both S and R cells. The responses of $[Ca^{2+}]_i$ and $[Na^+]_i$ to changes in $[Na^+]_o$ were similar between S and R MC. Interestingly, the magnitude of the changes in $[Na^+]_i$ was the same regardless of the change in $[Na^+]_o$. This was unexpected since we initially assumed that there should be graded responses of both $[Ca^{2+}]_i$ and $[Na^+]_i$ to changes in $[Na^+]_o$.

Further studies were performed to investigate why changes in $[Na^+]_i$ did not appear to correlate with changes in $[Na^+]_o$. To this end, experiments were performed to determine whether the initial large decrease in $[Na^+]_i$ occurred through pathways other than Na^+:Ca^{2+} exchange. Fig. 6 compares the effect of reducing $[Na^+]_o$ to 75 mM in the presence of 1 mM ouabain or 0 mM $[K^+]_o$ (inhibition of Na^+/K^+ ATPase), 3 mM EGTA (chelate $[Ca^{2+}]_o$) or 2 Deoxy-D-Glucose (DDG) (ATP depletion). During a reduction of

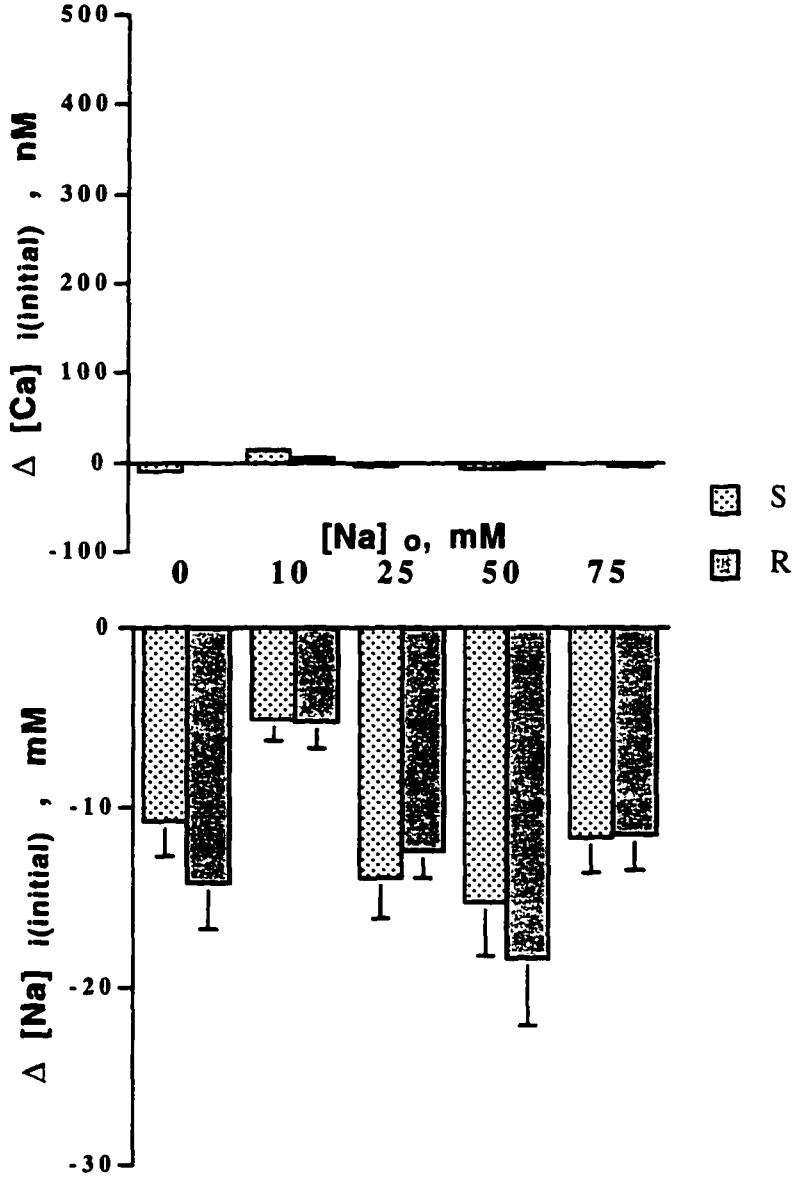

Fig. 3. The effect of reducing $[Na^+]_o$ to either 0, 10, 25, 50, or 75 mM on $[Ca^{2+}]_i$ and $[Na^+]_i$. Shown are the "initial" delta changes (defined in Table 2) in $[Ca^{2+}]_i$ (top) and $[Na^+]_i$ (bottom).

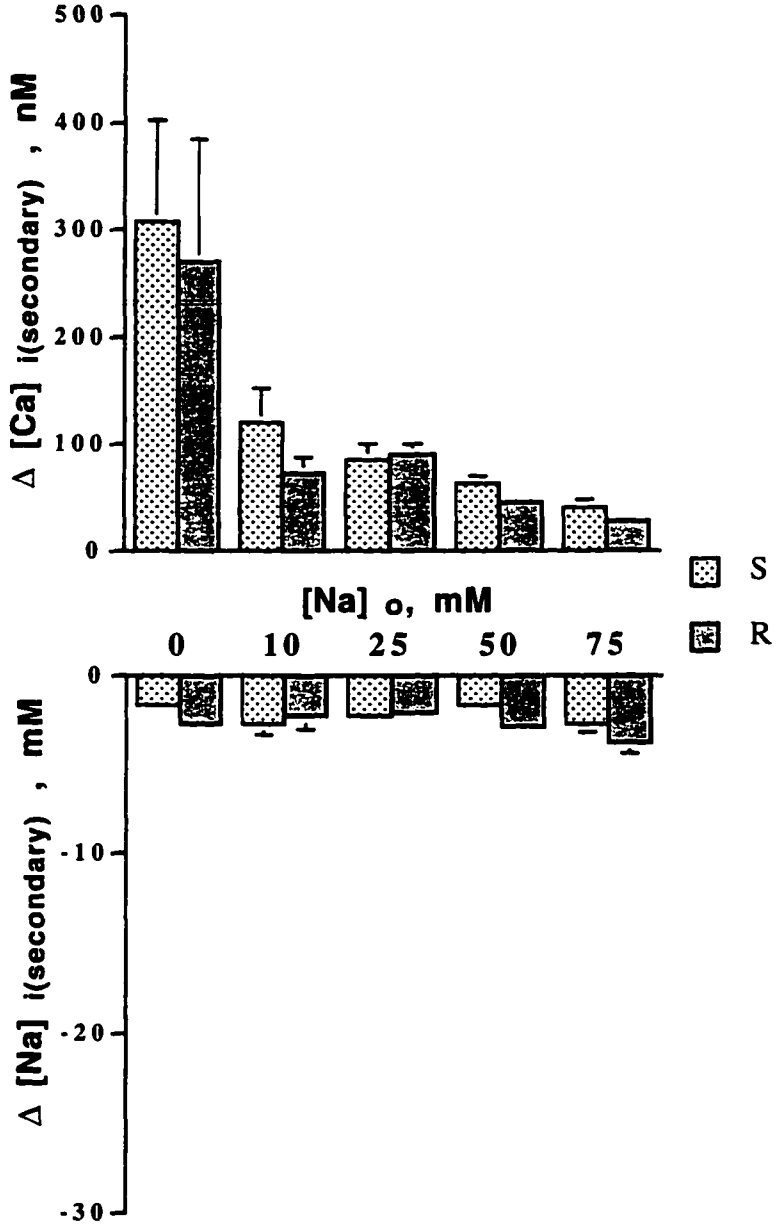

Fig. 4. The effect of reducing $[Na^+]_o$ to either 0, 10, 25, 50, or 75 mM on $[Ca^{2+}]_i$ and $[Na^+]_i$. Shown are the "secondary" delta changes, (defined in Table 2) in $[Ca^{2+}]_i$ (top) and $[Na^+]_i$ (bottom).

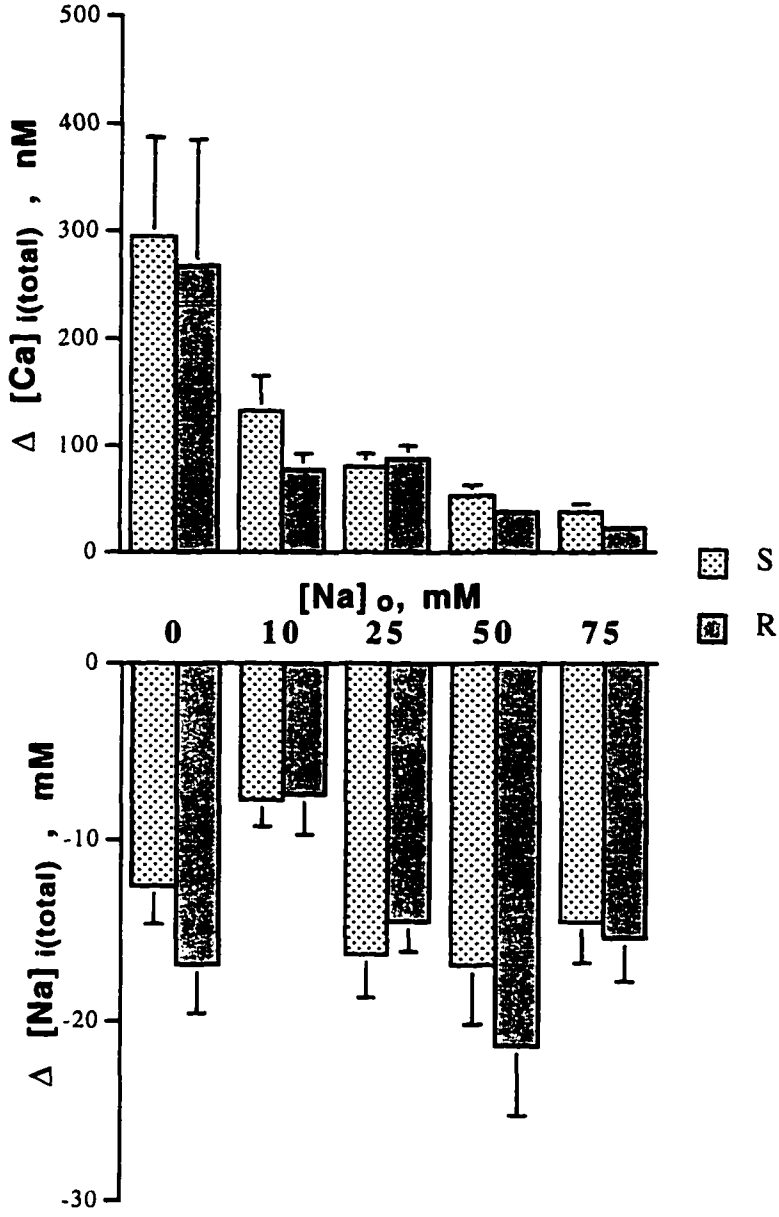

Fig. 5. The effect of reducing $[Na^+]_o$ to either 0, 10, 25, 50, or 75 mM on $[Ca^{2+}]_i$and $[Na^+]_i$. Shown are total changes (defined in Table 2) in $[Ca^{2+}]_i$ (top) and $[Na^+]_i$ (bottom).

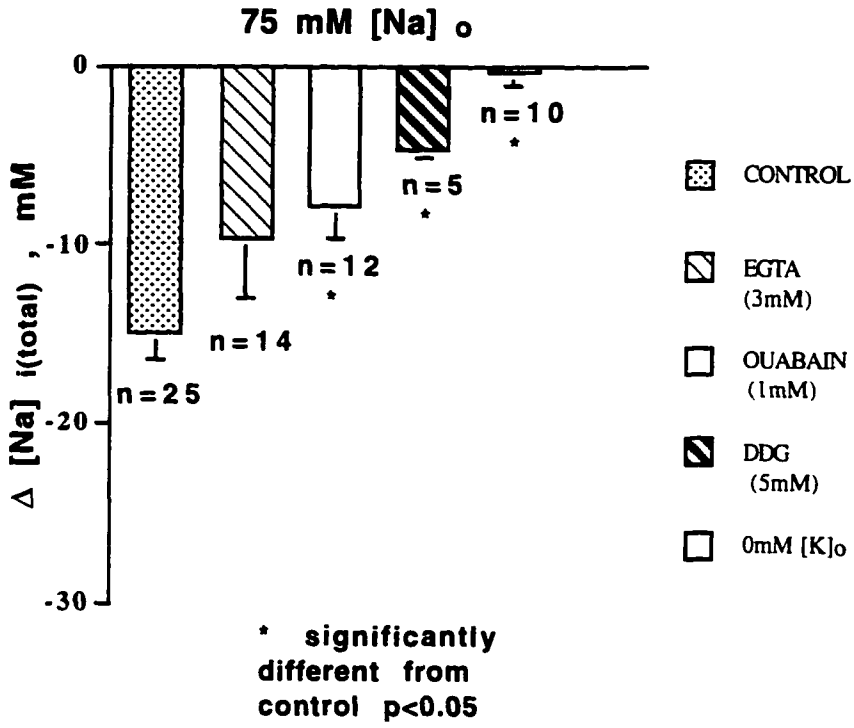

Fig. 6. The effect of reducing $[Na^+]_o$ to 75 mM in the absence or presence of EGTA, ouabain, DDG and 0 mM K^+. The reduction in $[Na^+]i$ was significantly less than control in the presence of ouabain, DDG and 0 mM K^+.

$[Na^+]_o$ from 150 to 75 mM, $[Na^+]_{i(total)}$ decreased by 14.9 ± 1.6 mM (n = 25). Elimination of $[Ca^{2+}]_o$ failed to alter the magnitude of change in $[Na^+]_i$ during reduction in $[Na^+]_o$. However, the addition of ouabain (1 mM) or elimination of $[K^+]_o$ significantly attenuated the decrease in $[Na^+]_i$ with a reduction of $[Na^+]_o$ to 75 mM. The addition of DDG, which depletes cellular ATP, also significantly attenuated the decrease in $[Na^+]_i$ during reductions in $[Na^+]_o$.

DISCUSSION

Results from the present studies and previous work in both mesangial and smooth muscle cells have provided functional evidence for the existence of a Na^+:Ca^{2+} exchanger (2, 20, 24, 27, 35). In these studies, exchanger activity was assessed by reductions in $[Na^+]_o$ which result in Na^+ exit and Ca^{2+} entry through the exchanger (3, 24). The resulting increase in $[Ca^{2+}]_i$ is dependent upon a number of factors, including the electrochemical driving forces for both Na^+ and Ca^{2+} across the plasma membrane. These studies (3, 24) as well as work in our laboratory (10, 28, 32) have found that the magnitude of increase in $[Ca^{2+}]_i$ is enhanced by prior Na^+_i loading and blocked by either removal of extracellular Ca^{2+} or addition of nonspecific inhibitors of the exchanger, such as nickel. These findings are all consistent with the presence of a Na^+:Ca^{2+} exchanger that can be examined using reverse-mode Na^+:Ca^{2+} exchange. In addition, the present studies and previous work (3, 10, 24, 28, 33) indicate that, in certain cell types, reverse-mode Na^+:Ca^{2+} exchange activity can be elicited without prior cellular Na^+ loading. Interestingly, this finding is not a general feature of all cultured MC (24) since reverse-mode Na^+:Ca^{2+} exchange activity is minimal or absent in MC cultured from the Sprague-Dawley rat (unpublished observation).

In MC, baseline $[Ca^{2+}]_i$ measurements averaged 122 nM for S and 80 nM for R cells. $[Ca^{2+}]_i$ measured in these cells agrees with fura 2 measurements taken in other cell types. $[Ca^{2+}]_i$ in cultured human MC was 72 nM (24) while it was 90 nM (27) and 119 nM (35) in cultured vascular smooth muscle cells from Sprague-Dawley rats. In

response to a decrease in $[Na^+]_o$ to zero there were comparable increases in $[Ca^{2+}]_i$ in both S and R MC. $[Ca^{2+}]_i$ increased by 295 nM in S and 268 nM in R cells. These increases in $[Ca^{2+}]_i$ compare favorably with the approximately 450 nM increase in $[Ca^{2+}]_i$ obtained in response to zero Na^+ in human MC by Mene et al. (24). In agreement with other reports (3, 24), we found that the increase in $[Ca^{2+}]_i$ was entirely monophasic. This finding is most consistent with a single mechanism (Na^+:Ca^{2+} exchange) being responsible for the increase in $[Ca^{2+}]_i$.

Baseline $[Na^+]_i$ measurements using SBFI averaged 22 mM in S and 26 mM in R MC. Other investigators using SBFI have reported $[Na^+]_i$'s of 4.4 mM in A7r5 vascular smooth muscle cells (7), 6.4 mM in guinea pig myocytes (14), and 8.6 mM in immortalized aortic myocytes (20). The higher values for $[Na^+]_i$ in the S and R MC may help explain why it was possible to demonstrate reverse-mode Na^+:Ca^{2+} exchange without Na^+-loading. In response to a reduction in $[Na^+]_o$ there was a decrease in $[Na^+]_i$ that was biphasic, consisting of a large initial decrease in $[Na^+]_i$ followed by a slow gradual decline. To our knowledge, this is the first report that there is a biphasic response of $[Na^+]_i$ to lowering of $[Na^+]_o$.

Basing our conclusions on the pattern of $[Na^+]_o$-induced changes in $[Ca^{2+}]_i$, we defined several terms (given in Table 2) that characterized the temporal changes in $[Ca^{2+}]_i$. Lag was comprised of both the time required to change solutions in the cuvette (~25 s) plus additional time for the initiation of the response. Interestingly, the lag time for changes in $[Ca^{2+}]_i$ was significantly greater than it was for $[Na^+]_i$. This supports the idea that changes in $[Ca^{2+}]_i$ were secondary to $[Na^+]_o$-induced changes in $[Na^+]_i$. In order to limit these studies to Na^+:Ca^{2+} exchange, experiments were generally terminated when $[Ca^{2+}]_i$ reached a plateau. As shown in other studies (1, 24, 28), the continued absence of $[Na^+]_o$ results in a return of $[Ca^{2+}]_i$ toward baseline values or relaxation of contractility due to extrusion by Ca^{2+}-ATPase and sequestration of Ca^{2+}. Therefore, the experimental period ended when $[Ca^{2+}]_i$ reached a plateau value, which

averaged 150 s. This period of time was then referred to as the Ca^{2+} response time. We elected to define the $[Na^+]_i$ response time as the period of time for the initial rapid fall in $[Na^+]_i$, since after this initial event there was a continued slow decline in $[Na^+]_i$ that did not generally exhibit a definitive end-point. The important point is that the time required for $[Ca^{2+}]_i$ to reach a plateau value was much longer than the time required for the initial decrease in $[Na^+]_i$.

Another means of examining this same issue was to examine the change in $[Na^+]_i$ and $[Ca^{2+}]_i$ that occurs at several defined points. Excluding the lag time, 0 to 55 s was taken to be the "initial" phase of the response and corresponded to the fast rapid decrease in $[Na^+]_i$, while 56 to 150 s was termed the "secondary" phase. We also analyzed the total change in both $[Na^+]_i$ and $[Ca^{2+}]_i$. This analysis (Figs. 3-5) revealed that during the initial decrease in $[Na^+]_i$ there was no significant change in $[Ca^{2+}]_i$, while during the secondary phase nearly all of the increase in $[Ca^{2+}]_i$ occurred while there was a slow decline in $[Na^+]_i$. This relationship between changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ can be clearly seen in Fig. 7. This example illustrates that in response to a decrease in $[Na^+]_o$, there was a rapid fall in $[Na^+]_i$ while $[Ca^{2+}]_i$ remained essentially unchanged. Subsequently, there is the large monophasic increase in $[Ca^{2+}]_i$ that was most closely associated with the slow gradual decline in $[Na^+]_i$. Overall, it should be emphasized that the approximately 2.5 mM decrease in $[Na^+]_i$ that occurred during the secondary phase is more than enough to account for the approximately 300 nM increase in $[Ca^{2+}]_i$.

Other studies by Hayashi and Satoh (14, 33) have also attempted to correlate $[Na^+]_i$ and $[Ca^{2+}]_i$. Hayashi et al. simultaneously measured $[Na^+]_i$ and $[Ca^{2+}]_i$ using two fluorescent probes with different wavelengths, SBFI and fluo 3 respectively. $[Na^+]_i$ was increased by perfusing cells with either a K^+ free solution or with strophanthidin, a cardiac glycoside. With inhibition of Na^+:K^+ ATPase, they found that Na^+ entered the

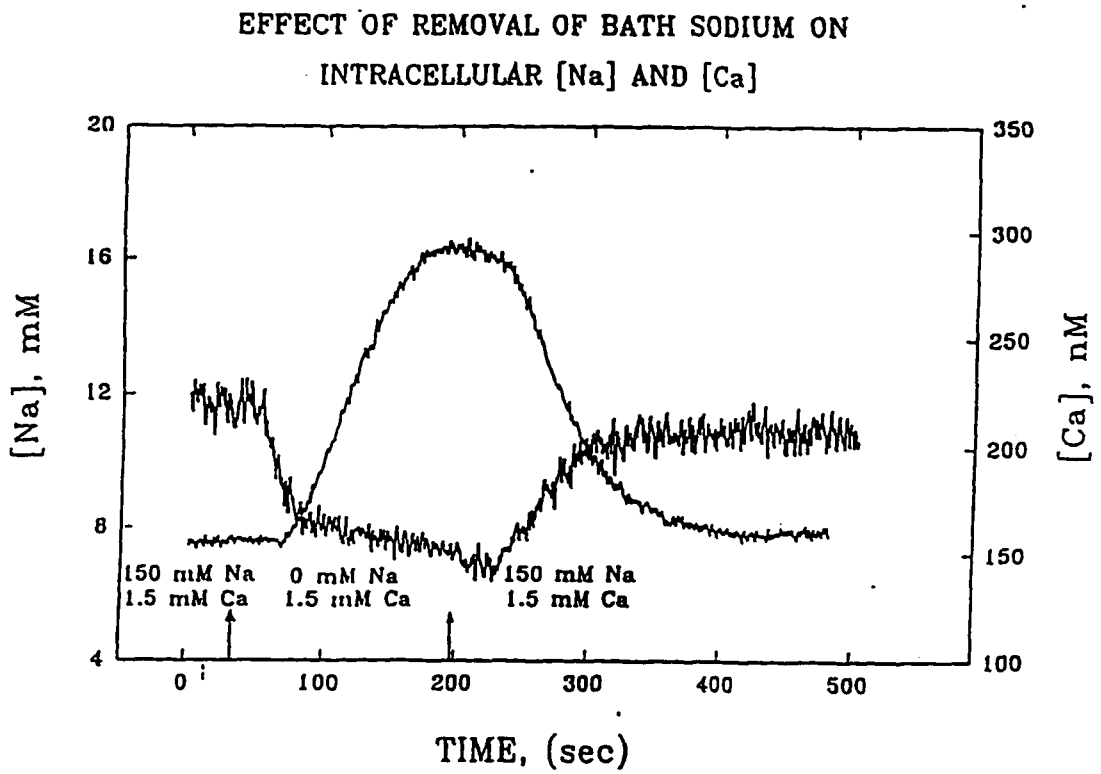

Fig. 7. Overlay traces showing the correlation between changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ upon reduction in $[Na^+]_o$ in cultured MC.

cell through the Na^+:H^+ exchanger and that the increased $[Na^+]_i$ resulted in increases in $[Ca^{2+}]_i$ via Na^+:Ca^{+2} exchange. They were able to demonstrate a correlation between increases in $[Na^+]_i$ and $[Ca^{2+}]_i$.

In our experiments, we evaluated the effects of changes in $[Na^+]_o$ from 150 mM to 75, 50, 25, 10 and 0 mM. We found that changes in $[Ca^{2+}]_i$ were dependent upon the magnitude of change in $[Na^+]_o$ and, as would be expected, the largest increase in $[Ca^{2+}]_i$ occurred when $[Na^+]_o$ was reduced to zero. This relationship between $[Na^+]_o$ and $[Ca^{2+}]_i$ has been reported in previous studies in MC (24), smooth muscle cells (3) and renal afferent arterioles (10). Changes in $[Na^+]_i$ at various $[Na^+]_o$'s produced surprising results. Instead of the expected dependency of $[Na^+]_i$ on the magnitude of change in $[Na^+]_o$, we found that the decrease in $[Na^+]_i$ was the same, regardless, if $[Na^+]_o$ was reduced to 0, 10, 25, 50 or 75 mM. It should be noted that the vast majority of this change in $[Na^+]_i$ represents the initial rapid decline in $[Na^+]_i$, since during the subsequent slow phase there were only small decreases in $[Na^+]_i$.

This finding prompted additional studies to determine the possible route(s) for the initial, large Na^+ exit. Since the decrease in $[Na^+]_i$ occurred before there was evidence for Na^+:Ca^{+2} exchange activity, we suspected that some other pathway such as the Na^+:K^+ ATPase might be responsible for the decrease in $[Na^+]_i$. However, to verify that the drop in $[Na^+]_i$ was not dependent upon $Ca^{2+}{}_o$, initial studies were performed in the absence of extracellular Ca^{2+} and in the presence of EGTA. We found no difference in the magnitude of change in $[Na^+]_i$ when $[Na^+]_o$ was decreased from 150 to 75 mM in the presence or absence of $[Ca^{2+}]_o$. Other studies were performed to determine if this decrease in $[Na^+]_i$ required energy. This was accomplished by incubation of MC in 2-dexoy-D-glucose (DDG), a non-metabolized sugar. As shown in other work, incubation in DDG results in a decrease in cellular ATP levels (19). We found that prior incubation in DDG significantly reduced the magnitude of the decrease in $[Na^+]_i$ obtained when extracellular $[Na^+]_o$ was reduced to 75 mM. To implicate a role for Na^+:K^+ ATPase as

the pathway for the initial rapid decrease in $[Na^+]_i$, MC were exposed to ouabain or K^+ was eliminated from the extracellular solution. Both of these maneuvers significantly diminished the magnitude of drop in $[Na^+]_i$ with reduced $[Na^+]_o$. Removal of extracellular K^+ was very effective in blocking the decrease in $[Na^+]_i$. It is likely that the inhibitory effect of K^+ was due to an inhibition of Na^+:K^+ ATPase. However, removal of K^+ should hyperpolarize the cell and there may be a contribution of membrane potential to the marked attenuation of the drop in $[Na^+]_i$. The inhibition of the change in $[Na^+]_i$ by ouabain further supports a role of the Na^+:K^+ ATPase. Although ouabain was not as effective as zero K^+ in blocking the decrease in $[Na^+]_i$, this is understandable because ouabain is not a very effective inhibitor of Na^+:K^+ ATPase in the rat (11). Therefore, these findings suggest that in response to a decrease in $[Na^+]_o$, there is rapid exit of Na^+ through the Na^+:K^+ ATPase, the majority of which occurs before there is any change in $[Ca^{2+}]_i$.

Blaustein and Hamlyn have proposed that membrane permeability to various ions, notably Na^+ and Ca^{2+}, is altered in hypertension (6). They proposed the Natriuretic Hormone/Na^+:Ca^{2+} exchange/hypertension hypothesis as a possible means of correlating cellular Na^+ to Ca^{2+} in hypertension. In this scheme, an increase in $[Na^+]_i$ results in a diminution of Na^+:Ca^{2+} exchange activity, which leads to less Ca^{2+} extrusion. The result is an increased $[Ca^{2+}]_i$ and elevated tension in contractile cells and hypertension. In this regard, we found that, overall, $[Ca^{2+}]_i$ was higher in S versus R MC. This is an important finding since it indicates that differences in Ca^{2+} levels can still occur in cultured cells that are removed from the internal milieu. In addition, similar increases in $[Ca^{2+}]_i$ have been observed in several different cell types and models of hypertension (4, 16). At the present time, the mechanism that is responsible for the higher baseline $[Ca^{2+}]_i$ in the S versus R MC is unknown. There was no significant difference in $[Na^+]_i$ between S and R MC, which is not consistent with the above scheme of Blaustein and Hamlyn (6). However, some caution should be noted since $[Na^+]_i$ is

measured in mM and there could be small differences in $[Na^+]_i$ not detectable by SBFI. Such small differences in $[Na^+]_i$ might be important in $Na^+:Ca^{2+}$ exchange activity because μM changes in $[Na^+]_i$ are all that are required for the nM changes in $[Ca^{2+}]_i$, which could result in the increased $[Ca^{2+}]_i$ in S MC. In addition, we did not find differences in reverse-mode $Na^+:Ca^{2+}$ exchange activity between S and R MC. These results do not rule out the possibility that there may be alterations in the regulation of the exchanger. Nonetheless, the present studies indicate that no major differences in $Na^+:Ca^{2+}$ exchange activity exist between S and R MC, at least as assessed by alterations in $[Na^+]_o$.

In summary, these studies examined $Na^+:Ca^{2+}$ exchange activity in MC derived from salt-sensitive and salt-resistant rats. Reverse-mode $Na^+:Ca^{2+}$ exchange activity was similar between S and R MC. Baseline $[Na^+]_i$ was not different between S and R cells, while S cells exhibited a higher baseline $[Ca^{2+}]_i$ compared to R cells. In response to changes in $[Na^+]_o$ there was a monophasic increase in $[Ca^{2+}]_i$ that directly correlated with the magnitude of change in $[Na^+]_o$. In contrast, the decrease in $[Na^+]_i$ with reductions in $[Na^+]_o$ was biphasic and did not correlate with the magnitude of change in $[Na^+]_o$ over the range of $[Na^+]_o$ studied (0-75 mM). We found that the initial, large decrease in $[Na^+]_i$ in response to a decrease in $[Na^+]_o$ was due to Na^+ exit through the $Na^+:K^+$ ATPase and occurred before any appreciable increase in $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ occurred during the secondary slow decline in $[Na^+]_i$, the magnitude of which was more than enough to account for the increase in Ca^{2+} flux through the $Na^+:Ca^{2+}$ exchanger.

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IMMUNODETECTION AND PHORBOL ESTER STIMULATION OF THE SODIUM-CALCIUM EXCHANGER IN CULTURED MESANGIAL CELLS

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Running Head: Na^+:Ca^{2+} exchange in mesangial cells

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ABSTRACT

The purpose of these studies was to determine if the sodium:calcium (Na^+:Ca^{2+}) exchanger protein is present in mesangial cells (MC) and to determine if protein kinase C (PKC) plays a role in Na^+:Ca^{2+} exchange regulation. Homogenates of canine heart, rat heart, and S and R rat MC were separated by SDS-PAGE and immunoblotted using monoclonal and polyclonal antibodies formed against the cardiac exchanger. Immunoblots showed 120 kDa and 70 kDa bands, which are consistent with previous reports, in dog heart, rat heart, and MC. Regulation studies were performed on MC left untreated or treated with phorbol 12-myristate 13-acetate (PMA) and loaded with fura 2 for measurement of intracellular calcium concentration ($[Ca^{2+}]_i$). The exchanger can be activated in the reverse direction by removal of extracellular sodium ($[Na^+]_o$), which results in an increase in $[Ca^{2+}]_i$. Resting $[Ca^{2+}]_i$ were not significantly different between S and R MC, nor were increases in $[Ca^{2+}]_i$ after removal of $[Na^+]_o$. R cells incubated for 15 min with 100 nM PMA showed a significant enhancement of the increase in $[Ca^{2+}]_i$ with decreases in $[Na^+]_o$ compared to untreated R MC. Conversely, S cells incubated for 15 min with PMA did not show significant enhancements in $[Ca^{2+}]_i$ responsiveness. Immunoblots of PMA treated MC revealed an increase in the Na^+:Ca^{2+} protein in R but not S MC after 15 min PMA. Therefore, PMA failed to enhance the $[Ca^{2+}]_i$ response in S compared to R MC. These data demonstrate that PKC enhances Na^+:Ca^{2+} exchange activity in MC of R but not S rats, indicating that there may be a defect in the PKC-Na^+:Ca^{2+} exchange regulation pathway in S MC.

Key words: Na^+:Ca^{2+} exchanger, protein kinase C, mesangial cells

INTRODUCTION

The sodium:calcium (Na^+:Ca^{2+}) exchanger, expressed in a variety of tissues including kidney, is thought to participate in the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$). It functions as a bidirectional antiporter transporting 3 sodium ions for 1 calcium ion (18) and is, therefore, influenced by the electrochemical gradients

for Na^+ and Ca^{2+}. Because there is normally a large inwardly directed electrochemical gradient for Na^+, the exchanger results in the electrogenic movement of Na^+ into and Ca^{2+} out of the cell. This is referred to as the "forward-mode" operation of the exchanger. Experimentally, the exchanger can function in the reverse mode by decreasing $[Na^+]_o$ thus causing Ca^{2+} to enter the cell in association with a decrease in $[Na^+]_i$ (3, 20). Previous work in our lab has established functional characteristics of Na^+:Ca^{2+} exchange via reverse-mode exchange in rat renal mesangial cells (MC) as well as afferent and efferent arterioles from rat and rabbit (6, 16, 23).

At the molecular level, cardiac and rod photoreceptor forms of the Na^+:Ca^{2+} exchanger have been cloned and found to be encoded by different genes (24, 28). The cardiac form of the exchanger is 970 amino acids in size with 11 transmembrane spanning regions. Philipson et al. (26) have developed both polyclonal and monoclonal antibodies to the Na^+:Ca^{2+} exchanger found in canine cardiac sarcolemma. These antibodies are immunoreactive with proteins 70, 120, and 160 kDa in size. The 160 kDa protein is seen under nonreducing conditions. The 120 kDa protein is thought to be the fully functional protein, while the 70 kDa protein appears to be a proteolytic fragment (26). Recent work has suggested that the canine cardiac exchanger has a high homology with exchangers derived from rat and rabbit renal tissue (5, 11, 29). Therefore, antibodies formed against the cardiac exchanger can be used to probe for the Na^+:Ca^{2+} exchanger in rat glomerular MC.

Currently, there is a lack of information concerning the regulation of Na^+:Ca^{2+} exchange and potential alterations in regulation of the exchanger that might occur in certain disease processes. In this regard, we have been particuarly interested in the role of protein kinase C (PKC) in the regulation of the exchanger. Previous work by other laboratories (19, 36) has resulted in conflicting data with PKC activation resulting in no change, stimulation or inhibition of the exchanger. In our studies, we have found that phorbol ester stimulation of PKC results in enhanced Na^+:Ca^{2+} exchange activity in

afferent arterioles (6, 23) These results are supported by recent studies showing that PKC directly phosphorylates the Na^+:Ca^{2+} exchanger (10). Therefore, phosphorlyation by PKC has been proposed as a possible mode of regulation (36).

In the current study, we sought to determine whether PKC activation alters Na^+:Ca^{2+} exchange activity and membrane protein levels in cultured MC from a genetic hypertensive model, the Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rat (27). Altered PKC regulation of the exchanger might have important consquences to the regulation of $[Ca^{2+}]_i$ in these contractile cells.

METHODS

Animals

Animals used in this study were male Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 50 g and fed standard rat chow (Prolab RMH 1000; Agway; ~170-180 meq/kg of both Na^+ and K^+) and water *ad libitum*. As outlined in recent reports (12, 32), S rats from Harlan Sprague Dawley were genetically contaminated as early as March 1993. It was noted that some of the S animals failed to develop hypertension when placed on a high salt diet. Animals used for the functional studies were obtained prior to the contamination while animals used for the immunodetection studies were obtained after the S colony had been regenerated (Summer 1994 to present). Currently, we are analyzing liver samples from all S and R rats by PCR and DNA analysis in order to ensure that experiments are performed on pure S rats (17).

Isolation and culture of glomerular mesangial cells

Rat kidneys from both S and R were aseptically removed and glomerular MC were isolated and cultured as previously described with minor modifications (13, 16, 21). In brief, renal cortical tissue was minced with a razor blade and passed through a #70 copper sieve (Fisher Scientific, Pittsburgh, PA). Tissue was then passed through progressively smaller nylon sieves (Tetko Inc., Briar Cliff Manor, NY) ranging in size

from 315 μm to 75 μm in order to separate glomeruli from the remaining kidney tissue. Glomeruli were then treated with 21 units/ml collagenase (Sigma Chemical Co., St. Louis, MO) and plated onto 60 mm x 15 mm petri dishes (Costar, Cambridge, MA). Cells were grown in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (Intergen, Purchase, NY), 240 μg/ml L-glutamine (Gibco), 82 units/ml penicillin and 82 μg/ml streptomycin (Sigma), and 2 μg/μl amphotericin B (Gibco) for twenty-one days in humidified 95% air/5% CO_2 at 37°C. Media was changed twice a week. Cells were then subcultured and plated either directly in petri dishes or on two 40 mm x 12 mm glass coverslips in petri dishes. MC were grown to confluency for an additional twenty-one days before being used in experiments. Previous studies identified these cells as MC (16, 33, 38). Electron microscopy demonstrated prominent microfilaments, dense bodies, well developed rough endoplasmic reticulum, gap junctions and attachment plaques. Vimentin, a cytoskeletal filament was also found in these cells.

Protein preparation

Cultured MC were either untreated, exposed for 15 min to 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma), a diacylglycerol analogue which stimulates PKC, in RPMI 1640, or treated for 24 h with 100 nM PMA to down-regulate PKC. MC were washed three times in phosphate buffered saline (PBS). Two milliliters of homogenizing buffer (20 mM Tris, 1 mM EGTA, pH 7.0) were then added and the cells scraped and harvested. Cells were then homogenized using a Teflon-on-glass homogenizer and centrifuged for 15 min at 12,000 rpm. This process was repeated and the supernatant discarded. The pellet, containing membranes, was resuspended and aliquoted for protein determinations and immunoblotting. Whole cell homogenates of dog heart, left ventricle, (supplied by Dr. Gilbert Hageman) or rat heart, left ventricle, were used as positive controls for immunoblotting. Protein concentrations were determined using the Lowry method (14).

Antibodies

The monoclonal antibody, C2C12, is an immunoglobulin M (IgM) that reacts with three proteins, 160, 120 and 70 kDa in canine sarcolemma. This antibody was obtained as previously described (7) from a mouse immunized with isolated reconstituted canine cardiac Na^+:Ca^{2+} exchanger. The epitope for the antibody is on the intracellular side of the plasma membrane in the region of amino acids 371-525. C2C12 was the generous gift of Dr. Kenneth D. Philipson.

The polyclonal antibody is an immunoglobulin G (IgG) that reacts with three proteins, 160, 120 and 70 kDa in canine sarcolemma. The antibody was formed in rabbit against a reconstituted, purified Na^+:Ca^{2+} exchanger from canine cardiac sarcolemma (26). This antibody was obtained from SWant, Bellinzona, Switzerland.

Immunobot

Twenty five μg of dog or rat heart protein or 50 μg of S and R MC protein samples to be immunoblotted were separated by 7.5% sodium dodecyl sulfate-polyacrylimide gel electrophoresis and transferred for 1.25 h onto nitrocellulose electrophoretically. The blots were then blocked in 10% non-fat dry milk + PBS and 0.1% Tween 20 at room temperature for 1 h then refrigerated overnight in 3% bovine serum albumin + PBS and 0.1% Tween 20. After blocking, blots were incubated in PBS with a 1:1000 dilution of either the monoclonal (IgM) or the polyclonal (IgG) antibody overnight. After washing 3 times in PBS, blots were incubated in PBS with a 1:1500 dilution of either horseradish peroxidase conjugated goat anti mouse IgM or horseradish peroxidase conjugated goat anti rabbit IgG (Hyclone, Logan, UT) at room temperature for 4 h. Blots were then washed 5 times with PBS and developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Measurement of intracellular calcium concentration

Na^+:Ca^{2+} exchange activity experiments were performed on cells that had either been incubated in RPMI 1640 media + fetal bovine serum until the time of the

experiment (serum-fed) or RPMI 1640 media alone for 24 h prior to measurements of $[Ca^{2+}]_i$ (serum-deprived). $[Ca^{2+}]_i$ was measured using the fluorescent dye fura 2 AM (Molecular Probes, Eugene, OR). Cells were incubated in media containing 5 μM fura 2 AM dissolved in dimethyl sulfoxide for 1 h at 37°C to allow loading of the dye into cells. $[Ca^{2+}]_i$ measurements were performed using dual excitation wavelength fluorescence microscopy (Photon Technologies International, Inc., South Brunswick, NJ). Coverslips containing either S or R MC were positioned in the microscope (Leitz) stage chamber and bathed in a 140 mM NaCl Ringer's solution. Single cell $[Ca^{2+}]_i$ measurments were performed using a compact Leitz photometer (6) with a variable diaphragm. Excitation wavelengths were set at 340 nm and 380 nm, and the emission wavelength was 510 nm. The Ringer's solution contained (in mM): 4.2 KCl, 0.8 K_2HPO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 20 Hepes, 5.5 D-glucose, 5.0 L-alanine, 1.1 $Ca^{2+}Cl_2$, 150 Cl. Choline or N-methyl-D-glucamine was used to isosmotically replace Na^+ (all reagents from Sigma). No differences in functional responses were noted between these two Na^+ replacements. All solutions were bubbled with O_2; pH was 7.4 and temperature maintained at 37°C. Baseline measurements were made after 15 min in the 140 mM NaCl solution. Replacement of the 140 mM NaCl solution with a zero mM Na^+ solution resulted in a transient increase in cytosolic Ca^{2+}. Activity of the reverse-mode exchanger was assessed by measurement of the difference between baseline $[Ca^{2+}]_i$ and the maximum increase in cytosolic Ca^{2+}.

To assess phorbol ester regulation of the Na^+:Ca^{2+} exchanger, cells were incubated for 15 min in 140 mM NaCl containing 100 nM PMA, prior to and during the reductions in bath Na^+. In some experiments, cells were incubated for 24 h in RPMI 1640 containing 100 nM PMA. In these experiments, 100 nM PMA was also present during the reduction in bath Na^+.

Intracellular calibrations were performed in order to convert fura 2 ratios into $[Ca^{2+}]_i$ values. Ionomycin (5 μM) was added to a Ringer's solution that contained either

3 mM Ca^{2+} or no Ca^{2+} and 3 mM EGTA. Cells were exposed to these solutions for a sufficient length of time (usually 2 to 3 min) to obtain stable maximum and minimum ratio values. $[Ca^{2+}]_i$ was calculated by the following equation:

$$[Ca^{2+}]_i = K_d \times (S_{f2} / S_{b2}) \times (R - R_{min}) / (R_{max} - R) \qquad (8)$$

where K_d is the dissociation constant of the dye and has a value of 224 nM (34), R is the fluorescence ratio at 340 nm/380 nm, and R_{min} and R_{max} are the ratios in the absence (3 mM EGTA) or presence of Ca^{2+} (3 mM Ca^{2+}) respectively. Fluorescence was measured at 380 nm in the absence of Ca^{2+} (S_{f2}) and in the presence of Ca^{2+} (S_{b2}).

Statistics

Means are expressed ± standard error (SE). Analysis was done using Student's unpaired t-test or ANOVA single factor test. Significance was taken as $p < 0.5$.

RESULTS

Immunoblot studies were performed to determine, by direct means, if the Na^+:Ca^{2+} exchanger is present in MC from S and R rats. Monoclonal antibodies formed against the canine cardiac Na^+:Ca^{2+} exchanger recognized the 120 kDa and 70 kDa bands in rat heart (Fig. 1). These bands are comparable to those found in dog heart. The 120 kDa and 70 kDa bands were also detected in R and S MC. Western blots of S and R MC probed with a polyclonal antibody to the Na^+:Ca^{2+} exchanger also showed immunodetection of bands 120 kDa in size (figure not included). Thus these results suggest that the Na^+:Ca^{2+} exchanger is present in cultured MC from S and R rats; however, the amount of exchanger protein in MC was much less than that detected in rat or canine heart.

Reports by Mene indicate that Na^+:Ca^{2+} exchange activity in MC is enhanced by serum depriving the cells for 24 h (19). We confirmed this observation in serum-fed versus serum-deprived (24 h prior to Ca^{2+} measurements) S and R MC by evaluating changes in $[Ca^{2+}]_i$ upon $[Na^+]_o$ removal. Table 1 lists the baseline $[Ca^{2+}]_i$ values for each treatment group used in this study. It can be seen that serum deprivation

Fig. 1. Western blot using the monoclonal antibody C2C12 to probe membrane preparations for the Na^+:Ca^{2+} exchanger. Lane 1 = 25 µg dog heart, lane 2 = 25 µg rat heart, lane 3 = 50 µg S MC, and lane 4 = 50 µg R MC. The arrows mark the major immunoreactive proteins. A band 120 kDa in size is found in dog heart and all rat tissues. A 70 kDa band is strongly immunodetected in both dog and rat heart and weakly in both S and R MC.

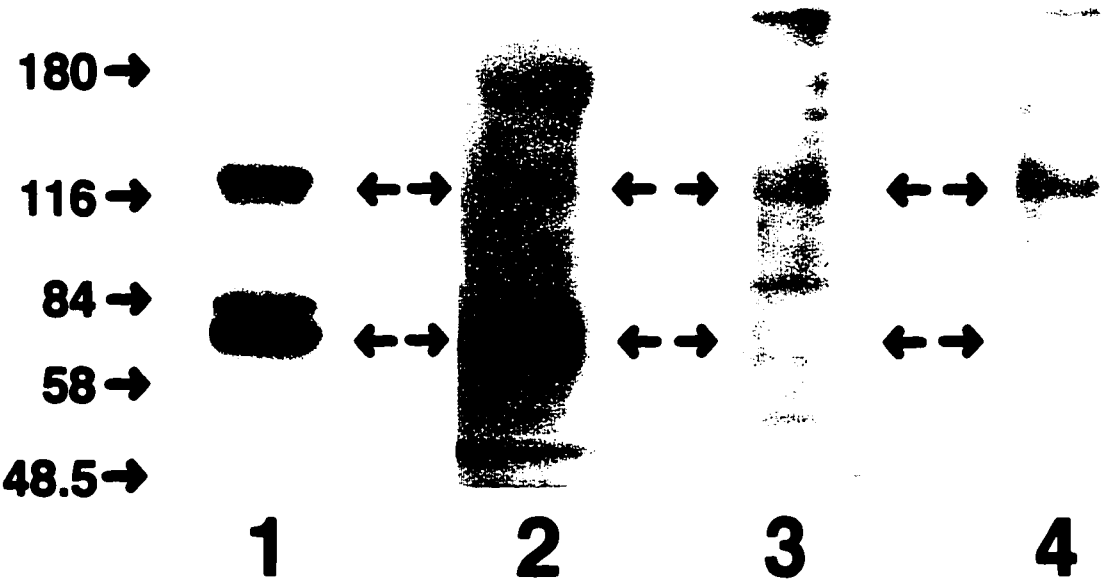


Table 1.
Baseline intracellular calcium values

Treatment	S (nM)	R (nM)
Serum Fed Control for 15 min PMA	87±14	86±6.9
	n=21	n=22
Serum Fed 15 min PMA	89±7	99±6.8
	n=30	n=24
Serum Deprived Control for 15 min PMA	139±18	135±22
	n=12	n=11
Serum Deprived 15 min PMA	126±13	118±12.8
	n=15	n=19
Serum Deprived Control for 24 hour PMA	152±13.6	141±15.8
	n=25	n=22
Serum Deprived 24 hour PMA	130±10	156±16.6
	n=19	n=17

Baseline $[Ca^{2+}]_i$ was significantly different by one-way ANOVA between serum-fed and serum-deprived groups. Baseline $[Ca^{2+}]_i$ were not different between S and R within serum-fed and serum-deprived groups. The serum-fed cells were from two S and two R MC preparations. The serum-deprived cells were from 3 S and 3 R MC preparations.

increases baseline $[Ca^{2+}]_i$ measurements in both R and S MC compared to serum fed MC. However, no differences in baseline $[Ca^{2+}]_i$ were seen between S and R within the serum-fed or serum-deprived groups. In response to a reduction in $[Na^+]_o$, increases in $[Ca^{2+}]_i$ in both S and R MC were greater in the serum-deprived versus serum-fed (compare control responses in Figs. 3 and 4).

PMA was used to determine if PKC affects Na^+:Ca^{2+} exchange activity. MC were incubated in 100 nM PMA for either 15 min (to activate PKC) or 24 h (to downregulate PKC) (25). Na^+:Ca^{2+} activity was measured as the peak change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) in response to the reduction of $[Na^+]_o$. Fig. 2 shows the $\Delta[Ca^{2+}]_i$ upon removal of $[Na^+]_o$ in control and 15 min PMA treated serum-fed S and R MC. $\Delta[Ca^{2+}]_i$ for S control MC was 167 ± 43 nM; n = 21 and was unaltered with PMA treatment in S MC (202 ± 3 nM, n = 30). $\Delta[Ca^{2+}]_i$ for R controls was 92 ± 2nM; n = 22 and increased significantly to 242 ± 62 nM; n = 30 in 15 min PMA treated R MC. Fig. 3 shows the results of 15 min PMA treatment on exchange activity in S and R MC; however, these cells have been serum-deprived for 24 h. Control $\Delta[Ca^{2+}]_i$ was 604 ± 118 nM; n = 12 and 566 ± 118 nM; n = 11 for S and R respectively. After 15 min PMA, S $\Delta[Ca^{2+}]_i$ was not statistically different compared to the control responses (840 ± 143 nM, n = 15), while R $\Delta[Ca^{2+}]_i$ increased significantly to 1187 ± 178 nM, n = 10. Thus, after 15 min incubation in 100 nM PMA, exchange activity was significantly increased in serum-fed and serum-deprived R but not S MC compared to untreated cells. Treatment of MC with 100 nM PMA for 24 h downregulates PKC activity, so this maneuver should abolish PKC stimulation in response to 15 min PMA incubation. As shown in Fig. 4, prior 24 h PMA abolished the increase in exchange activity in R MC obtained with addition of PMA for 15 min. Incubation for 24 h in PMA did not change activity from control levels in either S or R.

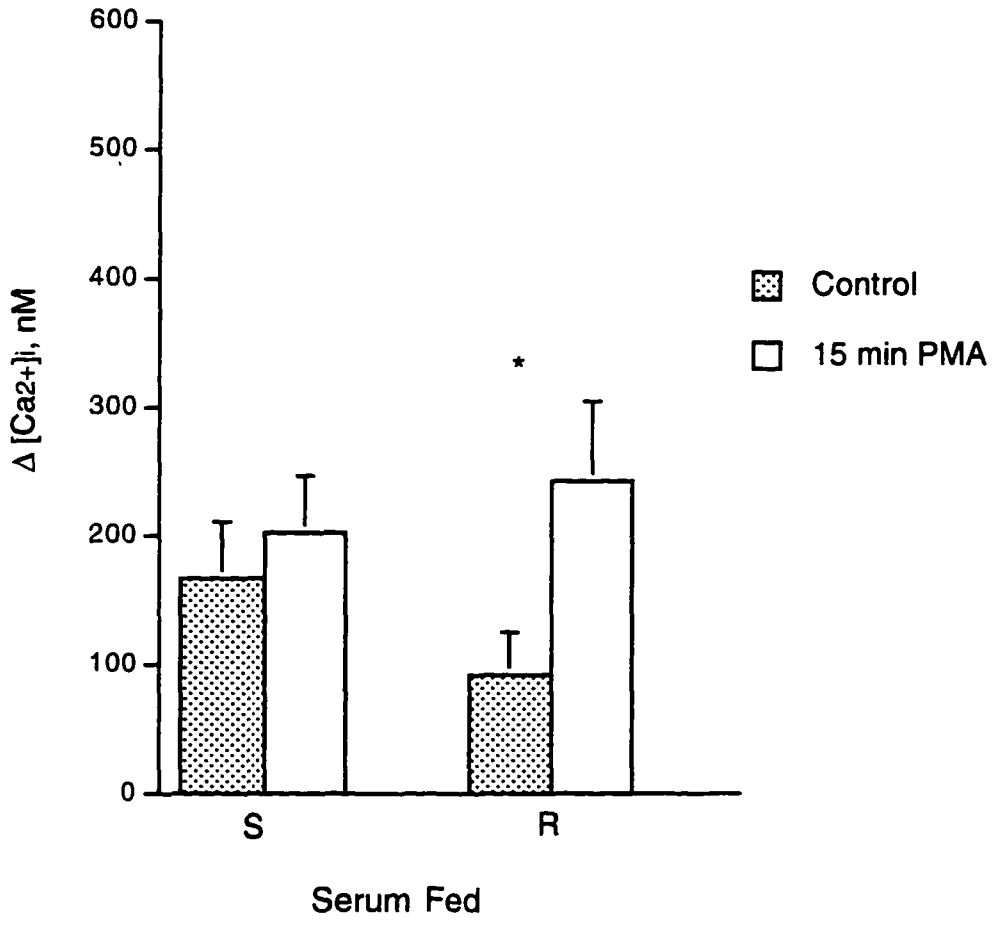


Fig. 2. Changes in $[Ca^{2+}]_i$ in response to decreased $[Na^+]_o$ in serum-fed S and R MC under control conditions and after a 15 min incubation with 100 nM PMA.
* = $p < 0.05$.

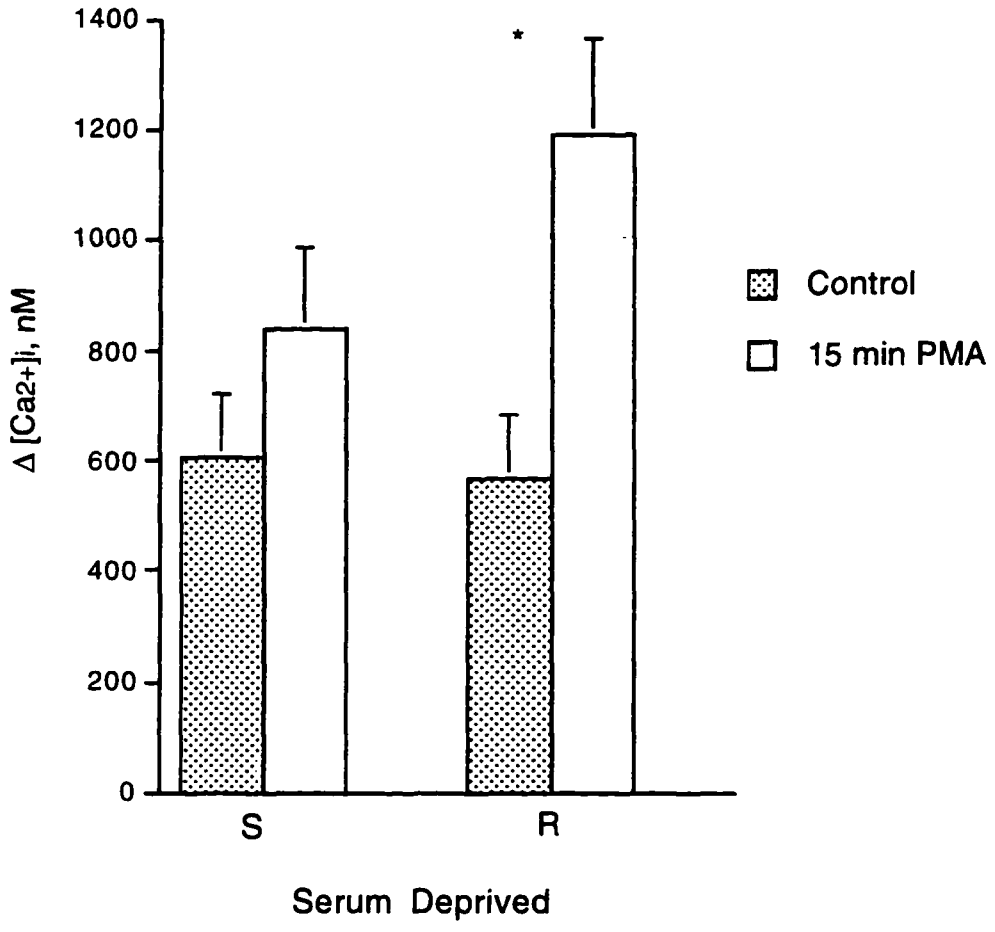


Fig. 3. Changes in $[Ca^{2+}]_i$ in response to decreased $[Na^+]_o$ in serum-deprived S and R MC under control conditions and after a 15 min incubation with 100 nM PMA.
* = $p < 0.05$.

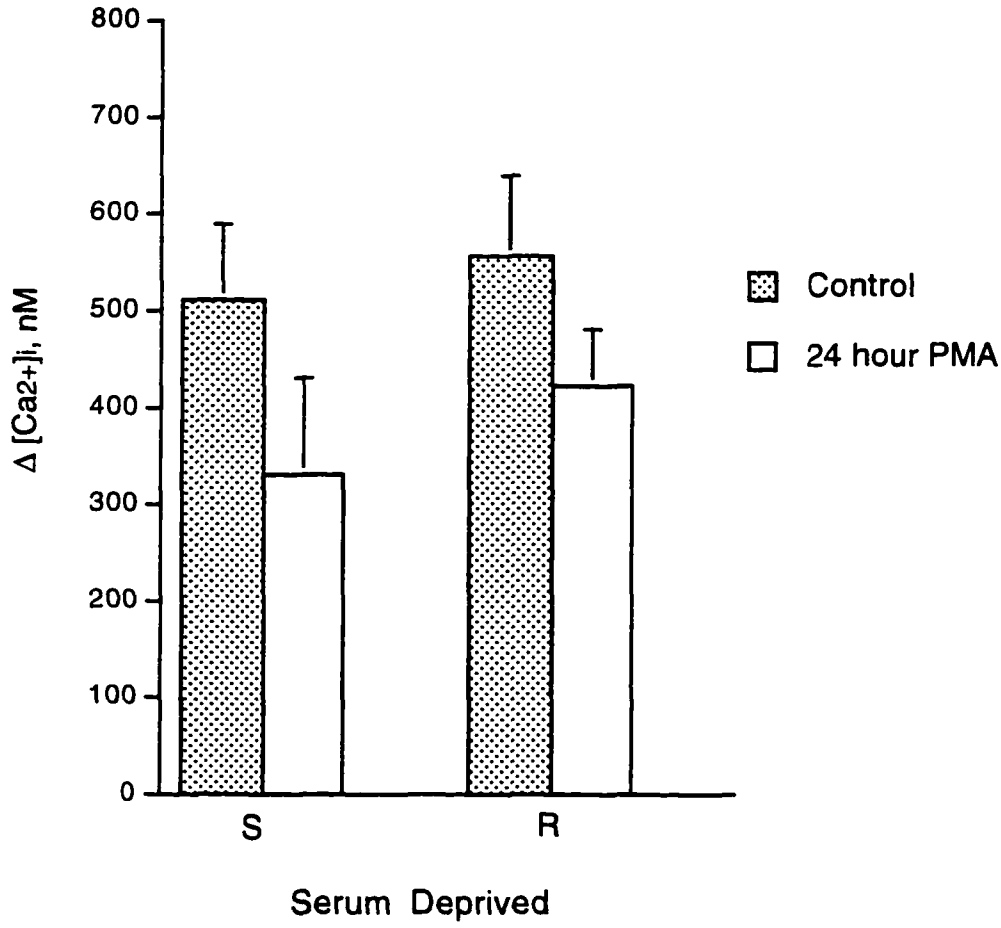


Fig. 4. Changes in $[Ca^{2+}]_i$ in response to decreased $[Na^+]_o$ in serum-deprived S and R MC under control conditions and with 24 h incubation in 100 nM PMA.

As a result of the above findings, we sought to determine if PMA affects the amount of immunodectable Na^+:Ca^{2+} exchange protein in MC incubated with 100 nM PMA for 15 min or 24 h. In R cells, 15 min PMA increased the amount of immunoreactive protein (120 kDa) while 24 h PMA decreased the amount of the 120 kDa band back toward control. This finding was the same using either the monoclonal or polyclonal antibodies (Figs. 5 and 6). In S cells the intensity of the 120 kDa band did not appear to respond to PMA. Therefore, both functional and antibody localization studies indicate differences in protein kinase C regulation of the Na^+:Ca^{2+} exchanger in cultured MC between salt-sensitive and salt-resistant rats.

DISCUSSION

The purpose of the present studies was to examine, using functional and immunological technniques, expression and regulation of the Na^+:Ca^{2+} exchanger in cultured mesangial cells. The development of monoclonal and polyclonal antibodies to the exchanger has provided a means of directly demonstrating the existence of the exchanger in a number of tissues including cardiac sarcolemma (35), proximal tubule, glomeruli (5), and connecting tubule (30). To our knowledge, the exchanger has not been directly immunolocalized in MC. In fact, Dominguez (5) reported no immunolocalization of the exchanger to whole glomeruli. In the present study, protein bands 120 and 70 kDa in size were detected in S and R MC with both a monoclonal and a polyclonal antibody directed against canine cardiac sarcolemma. The same size bands were found in control studies using homogenized dog and rat heart. Although the amount of exchanger protein was considerably less in MC cells compared to heart tissue, the clear presence of these bands serves as a positive identification of the Na^+:Ca^{2+} exchanger in MC cells. The reason that the exchanger was found in our studies and not in previous work is probably due to the fact that the MC cultures allowed for direct assess of the antibody to these cells. This may not have been the case in previous studies using whole glomeruli. In addition, there was no difference in the amount of exchanger

Fig. 5. Western blot using the monoclonal antibody and showing the effects of 100 nM PMA on the Na^+:Ca^{2+} exchange protein in S and R MC, 50 μg. C = control, 15 = 15 min 100 nM PMA, 24 = 24 h 100 nM PMA. Both the 120 and the 70 kDa bands (denoted with brackets) can be seen in all samples. However, the 120 kDa band is predominately detected by the monoclonal antibody. In the R_{15} sample both the 120 and 70 kDa bands are increased in intensity, while in the R_{24} sample both bands are reduced. Densitometric analysis indicate a 142% increase in the R_{15} 120 kDa band and a 123% increase in the 70 kDa band. There was no significant change in band intensity in response to PMA in S MC.

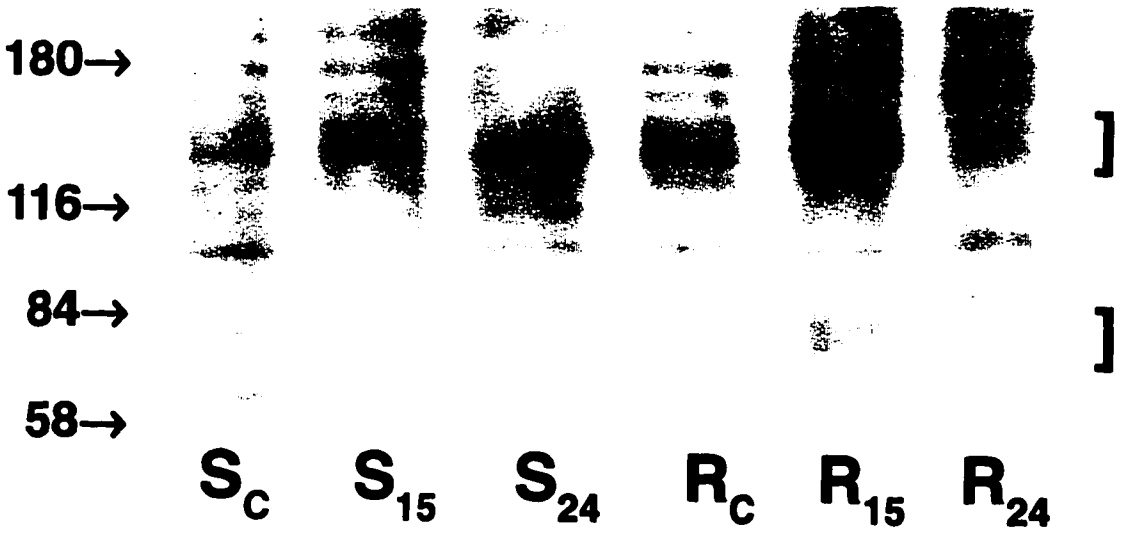
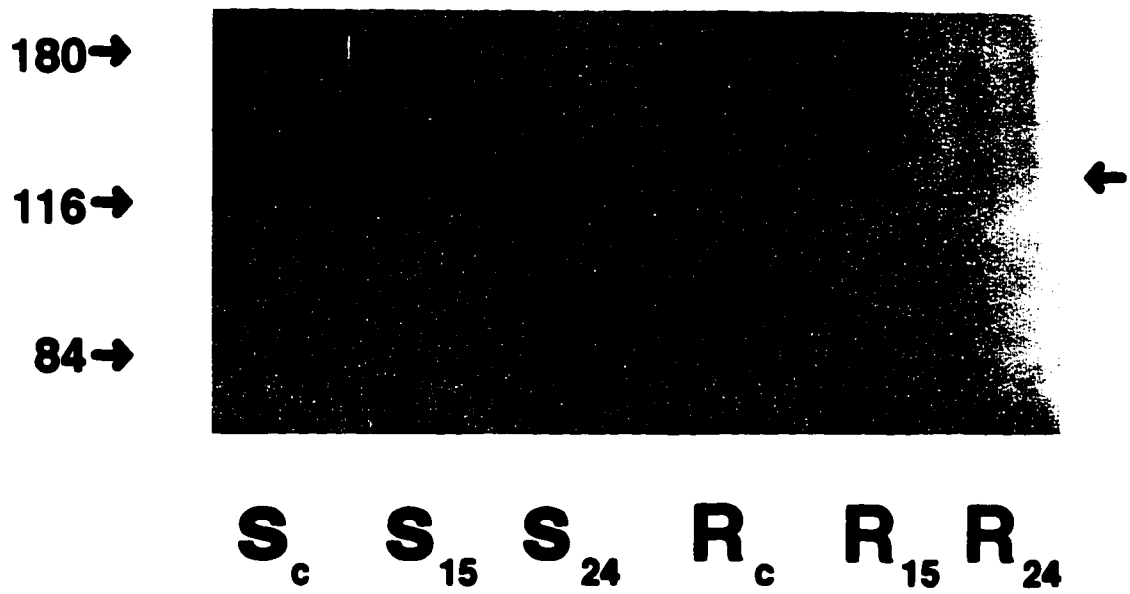


Fig. 6. Western blot using the polyclonal antibody and showing the effects of 100 nM PMA on the Na^+:Ca^{2+} exchange protein in S and R MC, 50 μg. C = control, 15 = 15 min 100 nM PMA, 24 = 24 h 100 nM PMA. The 120 kDa band is also predominately detected with the polyclonal antibody. The 120 kDa band is increased in intensity (125% by densitometric analysis) in the R_{15} sample as compared to R_C, while S_{15} does not increase compared to S_C.



protein between S and R MC. This finding is consistent with functional studies that indicate no difference in basal Na^+:Ca^{2+} exchange activity between S and R MC (16).

Previous studies have functionally demonstrated Na^+:Ca^{2+} exchange activity in several cell types including vascular smooth muscle cells and MC (2, 15, 16, 20, 22, 23, 31). Several different functional assays have been developed to study Na^+:Ca^{2+} exchange, including the measurement of tension in isolated smooth muscle rings in response to removal of bath Na^+ (1). A more direct approach has been to measure changes in $[Ca^{2+}]_i$ using Ca^{2+} sensitive fluorescent probes (16, 20). Other work as involved the use of ^{45}Ca efflux studies as a way of assessing Na^+:Ca^{2+} exchange activity (15). In the present studies, we measured changes in MC $[Ca^{2+}]_i$ during reductions in bath Na^+. Reducing bath Na^+ results in an increase in $[Ca^{2+}]_i$, which reflects the operation of the exchanger in what has been termed the reverse mode. That is, when bath Na^+ is reduced, Na^+ exits while Ca^{2+} enters the cell. In previous work (16), we have demonstrated that this increase in $[Ca^{2+}]_i$ is associated with changes in $[Na]_i$. Suggesting that the increase $[Ca^{2+}]_i$ is the direct result of a decrease in $[Na]_i$ which occurs, at least in part, through the Na^+:Ca^{2+} exchanger. In addition, recent studies have demonstrated that the increase in $[Ca^{2+}]_i$ with elimination of bath Na^+ can be blocked by pretreatment of the cell with an antisense oligonuclotide directed against the Na^+:Ca^{2+} exchanger (37). These studies clearly establish that the increase in $[Ca^{2+}]_i$ during reduction in bath Na^+ occurs through the Na^+:Ca^{2+} exchanger.

At this time, regulation of the Na^+:Ca^{2+} exchanger in MC or in any other tissues is not well understood. In order to study possible modes of regulation, we first needed to determine the optimum conditions for studying Na^+:Ca^{2+} exchange activity. As indicated, reverse-mode exchange; i.e. Ca^{2+} influx upon removal of bath Na^+, was used as a measure of Na^+:Ca^{2+} exchange activity. Mene (19) reported that depriving cells of serum prior to measuring Ca^{2+} fluxes enhanced Na^+:Ca^{2+} exchange activity, resulting in a larger influx in Ca^{2+} after bath Na^+ removal. Our results are consistent with those of

Mene. Serum-deprived cells had significantly greater baseline Ca^{2+} values and larger Ca^{2+} influx responses compared to serum fed cells. Although we do not know the mechanism for enhanced activity after serum deprivation, one explanation concerns the elevated basal $[Ca^{2+}]_i$ in the serum-deprived group. Previous studies (4) have shown that an elevated $[Ca^{2+}]_i$ directly enhances $Na^+:Ca^{2+}$ exchange activity. Although the mechanism whereby cytosolic Ca^{2+} regulates exchange activity is not known, the elevated $[Ca^{2+}]_i$ levels in the serum-deprived group may help explain the enhanced exchange activity found in this group.

Studies by Vigne (36) have shown that activation of PKC results in enhanced $Na^+:Ca^{2+}$ exchange activity in vascular smooth muscle cells. Although other work has not found this stimulatory effect of PKC (19), these studies prompted us to determine if PKC can alter exchange activity in cultured MC. We found that a 15 min incubation in PMA, which activates PKC (9) in MC, resulted in enhanced $Na^+:Ca^{2+}$ exchange activity in R but not S MC in both serum-fed and serum-deprived cells. The specificity of this effect was tested by 24 h incubation in PMA, a maneuver that down regulates PKC. Twenty four hours of PMA abolished the increase in $Na^+:Ca^{2+}$ exchange activity in R MC. Thus, PKC appears to upregulate $Na^+:Ca^{2+}$ exchanger at least in R MC. PMA stimulation of the exchanger has also been found in MC derived from the Sprague-Dawley rat (unpublished observation). Also, a similar up regulation has been shown in afferent arterioles from rabbit and R rat kidney (6, 23). These results lead us to conclude that the lack of a significant stimulation of exchange activity in S MC represents a defect in the PKC-$Na^+:Ca^{2+}$ exchange regulatory pathway. Similar conclusions have been reached in studies of afferent arterioles from the S rat (23).

Although the mechanism by which PKC enhances $Na^+:Ca^{2+}$ exchange activity is unknown, we wanted to examine the effect of PMA on protein expression of the exchanger. One possibility was that the enhanced $Na^+:Ca^{2+}$ activity seen in R MC might be due to a recruitment of exchangers at the plasma membrane. Therefore, the

final aspect of this study was to determine whether Na^+:Ca^{2+} exchange protein levels were affected by PMA stimulation.

MC from S and R rats were incubated for 15 min or 24 h with PMA, or left untreated. Immunoblots of the membrane fractions of these cells showed an increase in the amount of immunoreactive protein after 15 min PMA stimulation in the R but not S rat. Although we do not know the specific mechanism for this increase in membrane protein in the R rat, it is possible that PKC activation causes a recruitment of exchangers from a cytosolic pool to the membrane. We do not believe that the increased membrane protein in R MC is due to an activation of protein synthesis because the time period of PMA stimulation is only 15 min. In addition, because PKC can directly phosphorlyate the exchanger (10), the relationship is unclear between Na^+:Ca^{2+} exchange phosphorylation and possible exchanger recruitment to the plasma membrane. Further work in this area is needed to determine the exact mechanism of PMA-stimulated increases in Na^+:Ca^{2+} exchange protein levels.

Taken together, the functional and immunological data from S and R rat MC suggest that there is a difference in the Na^+:Ca^{2+} exchanger response to PKC activation. Incubation with PMA results in an increase in the activity of Na^+:Ca^{2+} exchange and a increase in membrane expression of the exchanger in R MC. In contrast, the S MC does not exhibit enhanced exchange or protein levels in response to PMA. These studies should lead to further work to identify which step in the PKC-Na^+:Ca^{2+} exchange pathway is defective in S MC. One consideration is to determine if there are differences in PKC isoform expression in MC from the S and R rats.

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PROTEIN KINASE C ISOZYMES IN CULTURED MESANGIAL CELLS AND WHOLE GLOMERULI FROM SALT-SENSITIVE AND SALT-RESISTANT RATS

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Running Head: PKC isozymes in mesangial cells

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ABSTRACT

Mesangial cells (MC) from Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats exhibit sodium:calcium (Na^+:Ca^{2+}) exchange activity. Previous studies have shown activation of the Na^+:Ca^{2+} exchanger by phorbol esters, thus indicating a possible role for protein kinase C (PKC) in Na^+:Ca^{2+} exchange regulation. The present studies were performed to determine which PKC isozymes are present in cultured MC from S and R rats. We also studied isozyme expression in whole glomeruli obtained from S and R rats fed 8.0% or 0.3% NaCl diets to determine if changes in blood pressure alter isozyme expression in the S rat. Reverse transcription-polymerase chain reaction (RT-PCR) indicated the presence of messenger RNA (mRNA) for specific PKC isozymes. Degenerate oligonucleotide primers as well as a zeta specific primer were used to amplify specific regions of PKC. The PCR products were subjected to restriction enzyme analysis and revealed the presence of α, δ, ε, η, and ζ isozymes in S and R whole glomeruli and MC. Western blot analysis using isozyme specific polyclonal antibodies detected the presence of five PKC isozymes, α, δ, ε, η, and ζ, in whole glomeruli and MC from S and R rats. Quantification of immunoreactive protein by densitometry showed no difference in the amount of each isozyme between S and R rats. Identification of the specific PKC isozymes in MC allows for further investigation of the role of PKC as a regulator of Na^+:Ca^{2+} exchange.

Key words: protein kinase C, isozymes, mesangial cells, glomeruli, Dahl/Rapp rats.

INTRODUCTION

Recent studies have suggested that protein kinase C (PKC) may upregulate the sodium:calcium (Na^+:Ca^{2+}) exchanger, a membrane transport protein that contributes importantly to the regulation of cytosolic calcium concentration ($[Ca^{2+}]_i$). In our studies, PKC activation with 100 nM phorbol 12-myristate 13-acetate (PMA) [1] resulted in increased Na^+:Ca^{2+} exchange activity. These results were obtained in mesangial cells (MC) cultured from either Dahl/Rapp salt-resistant rats (R) or Sprague-

Dawley rats. In addition, stimulation of the exchanger was found in afferent arterioles obtained from either rabbit or R rat kidneys. Interestingly, PKC did not stimulate exchange activity in MC cultured from Dahl/Rapp salt-sensitive rats (S) or in afferent arterioles obtained from the S rat. These results indicate a difference in PKC regulation of the Na^+:Ca^{2+} exchanger in R versus S rats. It is not known whether the difference in Na^+:Ca^{2+} exchange activity is due to intrinsic differences in the exchanger or in the PKC regulatory pathway. As an initial attempt to address this issue, we have examined PKC isozyme expression in this genetic model of hypertension.

Protein kinase C plays an important role in second messenger-mediated transmembrane signal transduction resulting in the phosphorylation of certain proteins. Inactive PKC is a soluble cytosolic protein which binds to the plasma membrane in the presence of calcium (Ca^{2+}) and phospholipid and is activated by diacylglycerol. Once activated, PKC can phosphorylate serine and threonine residues [2].

To date there are at least 12 isozymes of PKC which are divided into three categories: 1) calcium-dependent and phorbol ester-responsive, α, βI, βII and γ; 2) calcium-independent and phorbol ester-responsive, δ, ε, η, θ and μ; and 3) calcium-independent and phorbol ester-unresponsive, ι, λ, and ζ [3]. Eight of these isozymes (α, βI, βII, γ, δ, ε, η, and ζ) are known to exist in rat [4, 5]. Therefore, the role of PKC in various cells may depend on the number and types of PKC isozymes present. Studies by Huwiler using immunoblot techniques [6] have demonstrated the presence of four PKC isozymes (α, δ, ε, and ζ) in MC from rat. Our studies were undertaken to establish which of the PKC isozymes are present in S and R MC at the mRNA and protein levels, using reverse transcription-polymerase chain reaction (RT-PCR) and immunoblot analysis, respectively, and to determine if differences in isozyme type or amount exist between cultured S and R MC. In addition, we sought to determine, by the same methods, which isozymes are present in whole glomeruli from S and R rats fed either a 0.3% or 8.0% sodium chloride (NaCl) diet. Glomeruli were studied as a means of

evaluating whether isozyme expression differs *in vivo* between S and R rats and if expression changes as the S phenotype shifts from a normotensive (0.3% NaCl diet) to hypertensive (8.0% NaCl diet) state.

METHODS

Animals

Male Dahl/Rapp salt-sensitive (S) and salt-resistant (R) rats (Harlan Sprague Dawley, Indianapolis, IN) weighing approximately 50 g were used in this study. Normally, pure-bred S rats will become markedly hypertensive on an 8.0% NaCl diet, while R rats will remain normotensive. However, as outlined in recent reports [7, 8], S rats from Harlan Sprague Dawley were genetically contaminated as early as March 1993. It was noted that approximately 25% of these animals failed to develop hypertension when placed on a high salt diet when the contamination was first discovered, but soon a much higher percentage of S rats were found to no longer display the salt-sensitive phenotype. Animals used in this study were obtained after the colony had been regenerated (Summer 1994 to present) and liver samples from all S and R rats were subjected to PCR and DNA analysis in order to ensure that experiments were performed on pure S rats. In addition, blood pressure measurements were taken from rats not used for cell culture which had been fed either an 8.0% or 0.3% NaCl diet (Dyets Inc., Bethlehem, PA) for five days to ensure that the S phenotype was present.

Blood pressure measurements

Rats were divided into four groups each consisting of 4 rats maintained for 5 days on high or low salt diets: S on 8.0% NaCl diet, R on 8.0% NaCl diet, S on 0.3% NaCl diet and R on 0.3% NaCl diet. Systolic blood pressures were measured on 3 successive days via standard tail cuff plethysmography using a Grass polygraph.

Genotyping of S and R rats

PCR was performed on genomic DNA as follows. S and R rats were killed by cervical dislocation and subsequent decapitation. Genomic DNA was harvested using a

modification of the method of Blin and Stafford as follows [9]: 1 g liver was excised, frozen in liquid nitrogen and crushed into a fine powder. This was added to a digestion buffer containing: 0.1 mg/mL proteinase K, 0.5% SDS, 10 mmol/L Tris-HCl [pH 8], 25 mmol/L EDTA [pH 8], 100 mmol/L NaCl, and incubated in a shaking waterbath, 50°C, overnight. DNA was extracted three times with Tris-buffered phenol [pH 8], chloroform, and isoamyl alcohol mixture [25:24:1]. DNA was then collected by ethanol precipitation. DNA purity and yield was determined by optical density at 260 and 280 nm with a ultraviolet/visible spectrophotometer (Perkin-Elmer).

PCR primers amplifying 3 microsatellite markers as described by Lewis et al. [7] were used to test DNA from rats used in our experiments. Markers R721 and R1041 have been found to distinguish between contaminant and non contaminant S rat DNA as well as S versus R DNA. Marker R354 distinguishes between contaminant and non contaminant S rat DNA only. Genomic DNA samples from rats predating the contamination episode by several years as well as samples from genetically contaminated S rats were used as standards. PCR was performed in a 25 µL reaction volume containing 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/KCl, 1.5 mmol/l Mg, 0.001% (wt/vol) gelatin, 200 µmol/l dNTPs and 2.5 units of *Amplitaq* DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Twenty pmoles of each primer and 100 ng of rat genomic DNA were used. PCR amplification was performed with a Perkin Elmer Thermal Cycler using the following cycle protocol: 94°C, 5 min initial denaturation; 94°C, 1 min; 55°C, 1 min annealing; 72°C, 1 min 30 s for 35 cycles. PCR reaction products were size-fractionated on a 2.0% Nusieve 1:3 agarose gel (FMC Bioproducts, Rockland, MD) and stained with ethidium bromide.

Isolation of whole glomeruli and culture of glomerular mesangial cells

S and R rats fed standard rat chow (Prolab RMH 1000; Agway; ~170-180 meq/kg Na^+ and K^+) were used for culturing MC while S and R rats fed either an 8.0% or 0.3% NaCl diet were used to obtain whole glomeruli. Rat kidneys were aseptically

removed, whole glomeruli isolated, and glomerular MC cultured from individual rats as previously described with minor modifications [10-12]. MC were grown in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (Intergen, Purchase, NY), 240 μg/mL L-glutamine (Gibco Laboratories, Grand Island, NY), 82 units/mL penicillin, 82 μg/ mL streptomycin (Sigma Chemical Co., St. Louis, MO), and 2 μg/μL amphotericin B (Gibco Laboratories, Grand Island, NY) and incubated in humidified 95% air/5% CO_2 at 37°C for 21 days. The media was changed twice a week. Cells were then subcultured and grown for an additional 21 days before being used in experiments. Previous studies identified these cells as MC [11, 13, 14]. Electron microscopy demonstrated prominent microfilaments, dense bodies, well developed rough endoplasmic reticulum, gap junctions and attachment plaques. Vimentin, a cytoskeletal filament, was also found in these cells.

Protein preparation for immunoblot

Whole glomeruli from one rat or 100 mm petri dishes containing cultured MC from single rats were washed three times in 5 mL phosphate buffered saline (PBS). Two mL of buffer containing 10% sodium dodecyl sulfate (SDS) were then added and the cells scraped and harvested. The glomeruli or cells were sonnicated, boiled and aliquoted for protein determinations and immunoblotting. Protein concentrations were determined using the Lowry method [15].

MC to be used in PMA experiments were either left untreated or incubated for 30 min or 24 h in media containing 300 nM PMA (LC Laboratories, Woburn, MA). After treatment, the cells were washed and scraped in PBS, the membranes disrupted under high pressure, and spun at 100,000g to separate the cytosolic and membrane proteins. The membrane fraction was then resuspended in SDS buffer and protein concentrations determined.

Antibodies

Primary antibodies were affinity-purified rabbit polyclonal IgG antibodies raised against peptides corresponding to the following carboxy terminal PKC isozyme amino acid residues: 651-672 of rabbit alpha (α), 657-673 of rat delta (δ), 722-736 of rabbit epsilon (ε), 669-683 of mouse eta (η), and 573-592 of rat zeta (ζ). All antibodies were from Santa Cruz Biotech (Santa Cruz, California). The secondary antibody was a horseradish peroxidase conjugated goat anti-rabbit IgG (Hyclone, Logan, UT).

Immunoblot

Ten μg of protein to be immunoblotted were separated by 8.0% SDS-polyacrylamide gel electrophoresis and transferred for 1 h onto nitrocellulose electrophoretically. The blots were blocked in Tris-buffered saline with 0.2% Tween (TBST) and 5.0% BSA for 30 min. After blocking, the blots were incubated overnight in blocking buffer with a 1:2000 dilution of either the α, δ, ε, or ζ antibody or a 1:1000 dilution of η. After washing 3 times in blocking buffer, the blots were incubated in blocking buffer with a 1:3000 dilution of secondary antibody at room temperature for 4 h. The blots were washed 5 times with TBST and developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

RT-PCR studies

The method of RT-PCR used in this study is based on the method of Kohout and Rogers [4]. Total RNA extraction was performed using the acid guanidine thiocyanate method with TRIzol™ (Gibco) reagent. Briefly, 100 mm plates containing MC were washed 3 times with PBS; 4 mL of TRIzol™ were added to each plate and the cells harvested; or, glomeruli were collected as described previously and washed twice in PBS, and 250 μL of the suspended glomeruli (approximately the total glomeruli from one rat kidney) were taken for RNA extraction, spun down and resuspended in 500 μL TRIzol™. Glycogen was added to the cell or glomerular lysate and sonnication was performed to completely rupture the cells. One mL of the lysate was transferred to an

Eppendorf tube and the remainder stored at -80°C. Chloroform extraction removed DNA and protein, with the RNA remaining in the aqueous phase. RNA was recovered by precipitation with isopropyl alcohol. The RNA precipitate was then washed in 75% ethanol in DEPC-treated water, air dried and resuspended in DEPC-treated water. The RNA was treated with DNase prior to reverse transcription.

Superscript II reverse transcriptase was used to form single strand cDNA for use in subsequent PCR reactions. Reverse transcription was performed by incubating the extracted RNA in DEPC-treated water in a solution containing 50 mM KCl, 20 mM Tris-HCl [pH 8.4], 2.5 mM $MgCl_2$, 0.1 mg/mL BSA, 10 mM DTT (Gibco), 1 mM dNTP stock (Pharmacia, Piscataway, NJ), 10 ng/μL oligo-p(dt)12-18 (Pharmacia), 1 units/μL RNasin (Promega, Madison, WI) and 10 units/μL Superscript (Gibco) for 60 min at 42°C. The reverse transcriptase was inactivated by incubation at 95°C for 7 min. No genomic contamination was present in controls performed without the reverse transcriptase Superscript.

For the PCR experiments, degenerate 18-mer primers designed to recognize the conserved cysteine-rich or ATP binding regions of the PKC sequence motif were chosen to amplify the α, β, γ, δ, ε and η isoforms [4].

PKC 1 (Forward) 5'-AC(G/T) TTC TG(C/T) GA(C/T) CAC TGT-3'

PKC 2 (Reverse) 5'-CTT (G/T/C)CC AAA (G/A)CT (G/C)CC (T/C)TT-3'

Due to the unique nature of the ζ isoform, a specific primer pair 22 nucleotides in length was designed. The sense primer was selected to anneal to a cysteine-rich region that does not bind Ca^{2+}, and the anti-sense primer was selected to anneal to the ATP binding region.

Zeta 1 (Forward) 5'- CGG GAA CAT CCT GAT TAC CAG C-3'

Zeta 2 (Reverse) 5'- GCC CCA TAT CCT TTC GCT GCA C-3'

PCR was performed by adding the cDNA product to a reaction solution containing the following: 50 mM KCl, 20 mM Tris-HCl [pH 8.4], 2.5 mM $MgCl_2$, 0.1

mg/mL BSA, 0.1 mM dNTP stock, 0.5 pmol/μL of each primer and 0.02 units/μL of Taq polymerase (Fisher, Pittsburg, PA). PCR amplification was performed with a Perkin Elmer Thermal Cycler using the following cycle protocol: 94°C, 1 min denaturation; 55°C, 1 min annealing; 72°C, 1 min polymerization with 4 s extension per cycle for 45 cycles, and a final extension for 7 min at 72°C. PCR reaction products were run on an 2.0% agarose gel and stained with ethidium bromide.

The PCR amplified DNA was purified and precipitated using the following protocol. First, 0.1X volumes of 3 M Na acetate (pH 5.0) and 2.5X volumes of absolute ethanol were added to the PCR reaction mixture, vortexed and incubated at -20°C for 2 h. The mixture was then centrifuged at 12,000g for 30 min at 4°C. The supernatant was discarded and 1 mL of 75% ethanol was added. The sample was again centrifuged and the ethanol discarded. The pellet was air dried and resuspended in water. The purified DNA was run on a 2.0% agarose gel and stained with ethidium bromide.

Restriction enzyme digestion was used to analyze the purified DNA. Aliquots of the DNA were treated with various restriction enzymes according to the manufacturers protocol, and the products of the digestions were run on a 2% agarose gel and stained with ethidium bromide. The following enzymes were from Stratagene (LaJolla, CA): BamH I, Bcl I, Hind III, Kpn I, Pst I, and Stu I. Aat II and Bst XI were from New England Biolabs (Beverly, MA).

RESULTS

Mean systolic blood pressures (Figure 1) were significantly higher in S (128.5 ± 4.97 mmHg, n = 4) rats versus R (115 ± 2.61 mmHg, n = 4) rats on a 0.3% NaCl diet and significantly higher in S (162 ± 2.91 mmHg, n = 4) rats versus R (131 ± 2.12 mmHg, n = 4) rats on an 8.0% NaCl diet. Significant elevations ($p < 0.05$) in blood pressures in the S rats fed an 8.0% NaCl diet indicate an intact S phenotype.

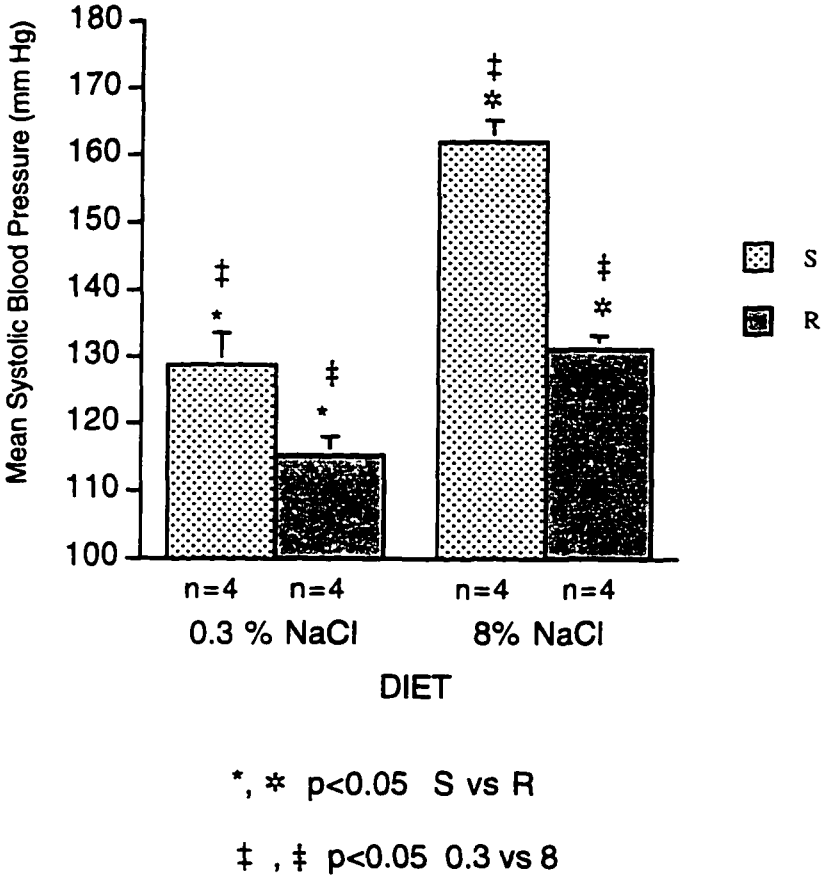


Figure 1. Mean systolic blood pressures were measured in S and R rats after 5 days on either 0.3% or 8.0% NaCl diet. Pressures were significantly different between S and R and between diets. Error bars represent standard errors.

Genotyping of S and R rats was performed using the three markers described previously. Each marker produced a distinct band for a noncontaminated S control, contaminated S control, or noncontaminated R control. As seen in Figure 2, marker R354 is amplified as a single band for noncontaminated S and R controls but as a double band for the contaminated S control. Marker R721 and R1041 (not shown) also amplifies the contaminated S control as a double band but in addition amplifies S and R noncontaminated controls as different size bands. S rats are designated noncontaminated if the banding pattern matches that of the S noncontaminated control. To date no contamination has been found from the re-derived colony.

RT-PCR was performed to detect the presence of PKC isozyme mRNA. Restriction enzyme analysis (Table 1 and Figure 3) was used to determine which isozymes were amplified. Lanes 1, 12, and 15 are size markers. Lane 2 contains the following 4 uncut bands: 719 bp, 503 bp, 356 bp, and 338 bp. Lanes 3 -10 and 14 show the digestion products. The 719 bp band was cut by BamH I and Bcl I (lanes 3 and 4), indicating the presence of α. Kpn I and Aat II, which are specific for β and γ, did not cut any bands (lanes 5 and 6). Stu I, BstXI and Pst I (lanes 8, 9, and 10) cut the 503 bp band, indicating ε is present. The 356 bp band was cut by Hind III and Stu I (lanes 7 and 8) indicating the presence of δ. BstXI (lane 9) cut the 338 band, which represents η. Lane 13 contains an uncut band at 391 bp that is cut in lane 14 by Stu I, indicating ζ is present. The results in Figure 3 were taken from the PCR products of MC from an R rat. S rat mesangial cell data were identical and are not shown.

RT-PCR analysis revealed the presence of PKC isozymes α, δ, ε, η, and ζ in both S and R cultured MC. We performed immunoblotting to determine if the mRNA found in MC was also expressed at the protein level. As can be seen in Figure 4, all 5 isozymes found by RT-PCR were also found by immunoblot. No differences were seen in the amount of protein expressed between S and R rat MC. Incubation of the PKC

Figure 2. S and R genomic DNA was isolated and tested with microsatellite markers. M = markers, Sc = noncontaminate S control, Rc = noncontaminate R control, S* = contaminate S control, S = S to be tested, and R = R to be tested. S rats used in this study were show to be noncontaminated by matching the S control banding pattern.

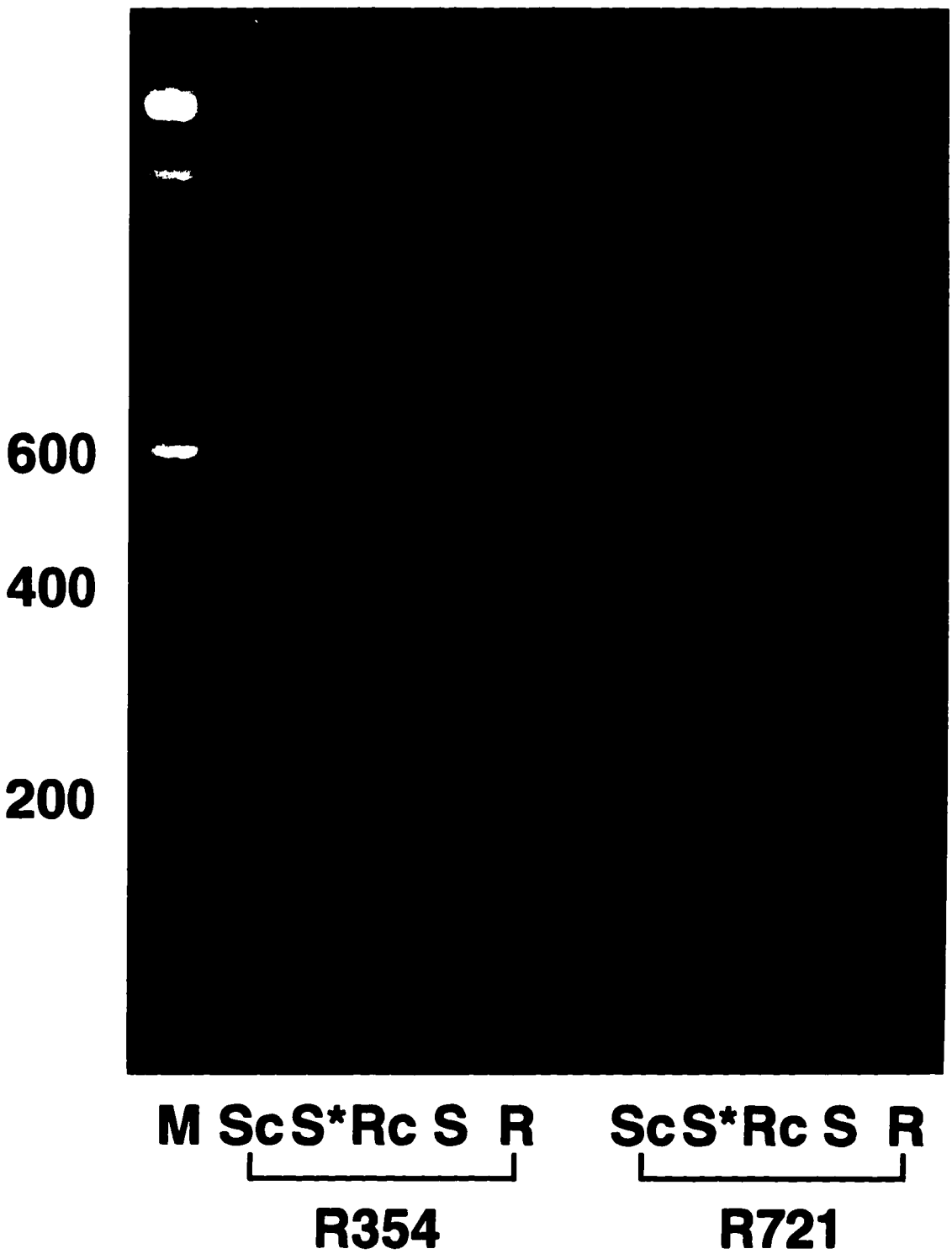


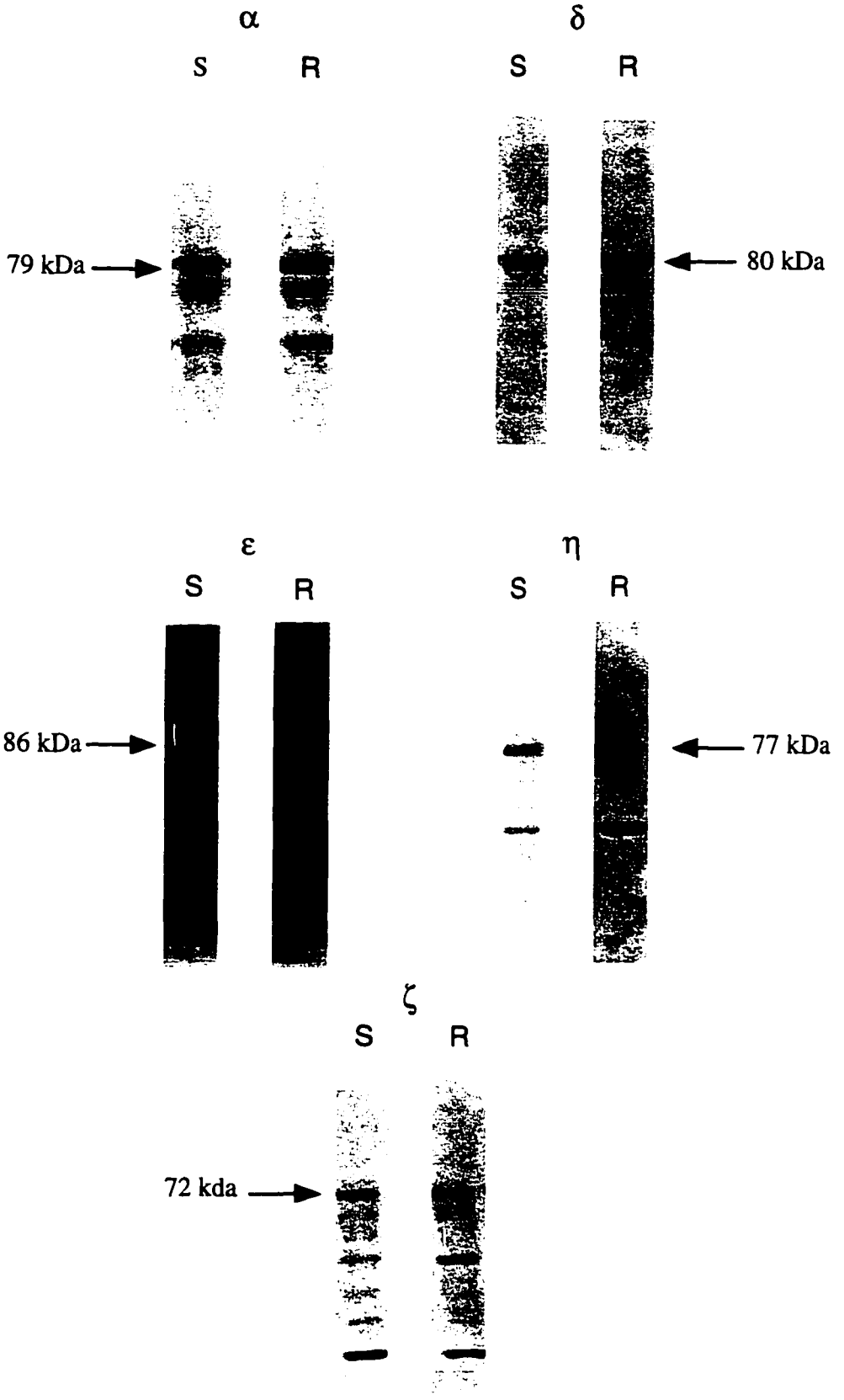
Table 1
PKC isoforms and digestion products

ISOFORM	PCR PRODUCT (BASE PAIRS)	ENZYME	FRAGMENT SIZE (BASE PAIRS)
α	719	BamH I	480, 221, 18
		Bcl I	625, 86, 8
δ	356	Hind III	190, 166
		Stu I	214, 142
ε	503	Bst XI	300, 139, 64
		Pst I	452, 51
		Stu I	336, 167
η	338	Bst XI	189, 149
ζ	391	Stu I	224, 167

Five PKC isoforms were found in S and R rat MC. The size of each isoform and digestion fragments are given as base pairs.
Information in this table was taken from Table 1 in Kohout and Rogers, where specific references regarding the development and validation of these enzymes can also be found [4].

Figure 3. Restriction enzyme analysis of PCR products from R MC. Lanes 2-11 are products of the degenerate PKC primer and distinguish between α, δ, ε, and η PKC isoforms. Lanes 13-14 = are products of Zeta primer and distinguish ζ only. The primers identify the 719 bp band as α, the 503 bp band as ε, the 356 bp band as δ, the 338 bp band as η, and the 391 bp band as ζ.

Figure 4. Western blot of S and R MC. 10 μg of S or R crude MC homogenate was loaded onto each lane. Arrows mark the major immunoreactive bands for each antibody. Approximate molecular weights of PKC α, δ, ε, η, and ζ were 79, 80, 86, 77, and 72 kDa respectively. No difference in immunoreactivity was seen between S and R.



antibodies to the peptides against which they were formed demonstrated the specificity of each antibody (data not shown).

In addition to demonstrating immunoreactivity with α, δ, ε, η, and ζ isozyme antibodies in S and R MC, we chose to test whether PMA could induce activation or membrane translocation of the phorbol ester-sensitive isozymes, α, δ, ε, and η. Figure 5 shows western blots of S and R MC treated with 300 nM PMA for 30 min or left untreated. Cells were homogenized then spun at 100,000g to separate membrane and cytosolic fractions. An increase in immunoreactive membrane protein was seen after 30 min PMA treatment in α, δ, and η in both S and R MC. No changes were detected in ε (not shown). ζ, which is phorbol ester-unresponsive, did not change after 30 min PMA. We also treated some cells for 24 h to downregulate PKC. A decrease in the α band after 24 h can be seen on the top blot in Figure 5.

Figures 3 and 4 establish that there is no difference in isozyme expression between control S and R cultured MC. Therefore, we chose to look at expression in whole glomeruli from S and R rats fed either a high or low NaCl diet. Freshly isolated whole glomeruli provide the opportunity to see if isozyme expression changes as the rat phenotype changes. RT-PCR and immunoblot were used to determine isozyme expression in freshly isolated whole glomeruli. Figure 6 shows the restriction enzyme digestion products of an RT-PCR from an S rat fed 8.0% NaCl for 5 days. Digestion analysis revealed the presence of α, δ, ε, η, and ζ PKC isozymes (Figure 3). The same 5 isozymes were detected in whole glomeruli from S and R rats fed 0.3% or 8.0% NaCl diet for 5 days (data not shown). Western blots of the high and low salt glomeruli are shown in Figure 7. All 5 PKC isozymes were shown in both S and R glomeruli fed either a 0.3% or 8.0% NaCl diet. No differences were detected in protein expression between S and R on either low or high salt diets, indicating that expression of the 5 isozymes studied does not change as the S phenotype changes from normotensive to hypertensive.

Figure 5. Western blot of S and R MC treated with PMA. 25 μg of S or R MC membrane was loaded onto each lane. Cells had been either untreated (con) or treated for 30 min or 24 h with 300 nM PMA. Arrows mark the α, δ, η and ζ immunoreactive bands.

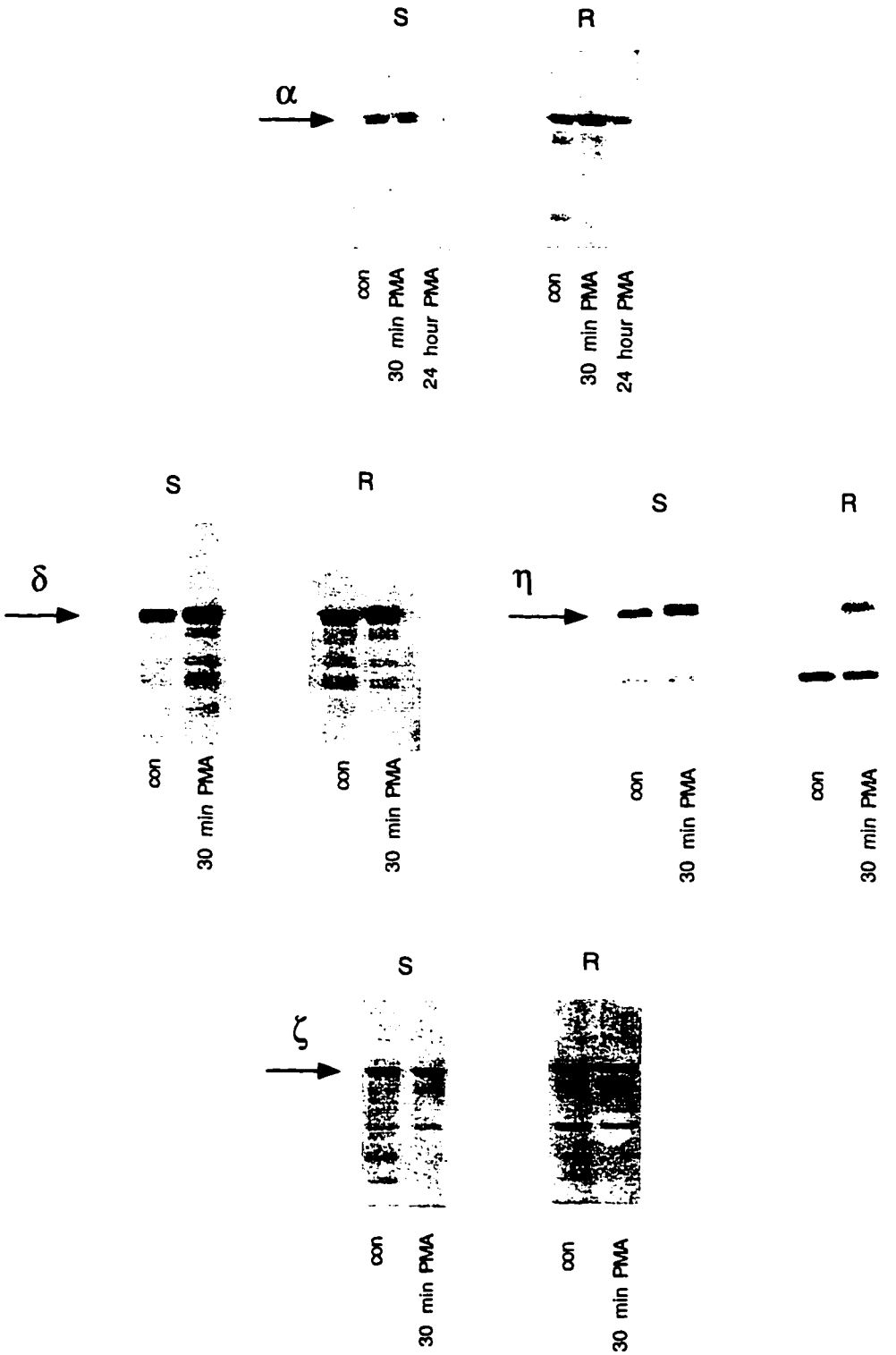
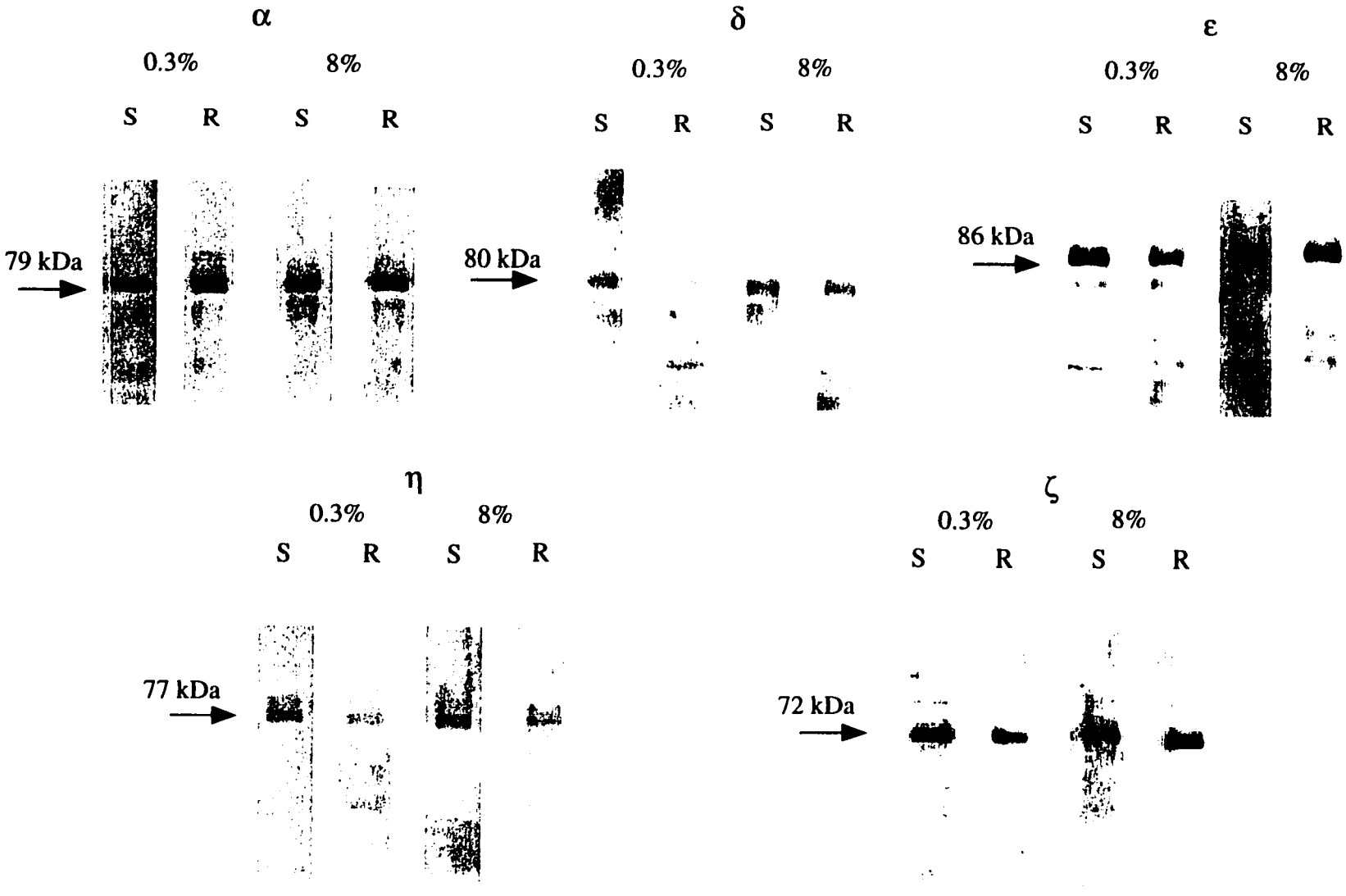


Figure 6. Restriction enzyme analysis on PCR products of whole glomeruli from S rats fed an 8.0% NaCl diet for 5 days. Lanes 2-11 are products of the degenerate PKC primer and distinguish between α, δ, ε, and η PKC isoforms. Lanes 13-14 = are products of Zeta primer and distinguish ζ only. The primers identify the 719 bp band as α, the 503 bp band as ε, the 356 bp band as δ, the 338 bp band as η, and the 391 bp band as ζ.

Figure 7. Western blot of glomeruli from S and R rats after 5 days on either a 0.3% or 8.0% NaCl diet. 10 μg of S or R crude whole glomerular homogenate was loaded onto each lane. Arrows mark the major immunoreactive bands for each antibody. No difference in immunoreactivity was seen between S and R or between 0.3% and 8.0% NaCl diet.



DISCUSSION

As the result of the recent contamination problems in the Dahl/Rapp salt-sensitive rats from Harlan Sprague Dawley, it has become essential to verify the use of genetically pure rats. The S rats used in these studies were from the re-derived S colony from Harlan Sprague Dawley. As described by Lewis et al. [7], the source of contamination within the colony was determined and corrective measures were undertaken to ensure that the re-derived colony was genetically pure. Nevertheless, we used both genotyping and blood pressure measurements in an effort to verify the use of a homogenous population of S and R rats. We chose to use the DNA markers that were originally used to describe the S contamination. Although it is not possible to determine genetic purity at every loci, we can conclude that all S rats used in the present studies did not contain the previously described contamination. Although we did not evaluate blood pressure responses of rats used for cell culture (because of their small size), in a limited series of additional experiments, S rats became hypertensive when placed on a high salt diet. Finally, Dr. Paul Sanders, in the Nephrology Research and Training Center at UAB, has found no contamination in S rats used during the same time period. Dr. Sanders has also repeated experiments done prior to the contamination and found the same results in S rats from the re-derived colony (unpublished results).

Intraglomerular mesangial cells have contractile-like properties, and it has been proposed that they play an important role in the regulation of glomerular filtration rate [16]. It is well established using *in vitro* cell culture systems that relaxation/contraction events that occur in MC are under hormonal control [17-19]. In addition, studies have been performed to elucidate second messenger pathways that mediate the effects of vasoactive agents, including angiotensin II, endothelin and thromboxanes [17, 20-25]. These studies, as well as work from our laboratory [1], have resulted in the following general concept regarding MC activation by vasoconstrictive agents. In response to a vasoactive hormone, there is an increase in MC $[Ca^{2+}]_i$ that results in a cascade of

intracellular events which ultimately lead to contraction. In addition, a rise in $[Ca^{2+}]_i$ would activate PKC, which has been shown to have a variety of cellular effects. However, one action of PKC may be to activate Na^+:Ca^{2+} exchange, resulting in Ca^{2+} efflux, thereby contributing to the return of $[Ca^{2+}]_i$ to control levels. In this manner, PKC activation of Na^+:Ca^{2+} exchange would operate as a regulatory mechanism for returning $[Ca^{2+}]_i$ levels to normal after agonist-induced stimulation. Previous work in our laboratory [1, 11], has shown that PMA stimulates Na^+:Ca^{2+} exchange in MC and afferent arterioles from R but not S rats and that baseline $[Ca^{2+}]_i$ tends to be elevated in S MC. In addition, Iwamoto [26] has demonstrated PKC-dependent phosphorylation of the exchanger in smooth muscle cells. From these results we propose that, in the hypertensive salt-sensitive rat, some part of this pathway may be defective, allowing $[Ca^{2+}]_i$ to remain elevated and cells contracted. Therefore, an important first step in unraveling this altered pathway is to determine which of the various PKC isozymes are present and their level of expression in MC from salt-sensitive and salt-resistant rats.

Using both immunoblot and RT-PCR techniques, we found that MC from S and R rats express the following 5 isozymes: α, a Ca^{2+} dependent and phorbol ester-responsive isozyme; δ, ε, and η, Ca^{2+} independent and phorbol ester-responsive isozymes; and ζ, a Ca^{2+} independent and phorbol ester-unresponsive isozyme. These results are consistent with studies by Huwiler [6], which revealed α, δ, ε, and ζ isozymes in MC, but not η because this antibody was not used to probe MC. The α, δ, ε, and ζ isozymes have been demonstrated in glomeruli, renal epithelial cells, proximal tubule and rat fibroblasts [5, 6, 27, 28]. Additionally, RT-PCR studies have demonstrated the presence of α, δ, ε, η, and ζ isozymes in cardiac myocytes [4]. Although we found expression of 5 of the PKC isozymes in S and R MC, we cannot draw any conclusions regarding which one(s) of the isozymes may be dominant. This is due to the fact that the reactivity of each of the antibodies to a PKC isozyme is different. Nevertheless, the

important point of this work was that no differences were found in either mRNA or protein levels of the 5 isozymes between S and R MC.

Although baseline expressions of the isozymes were similar, it is possible that there maybe differences between S and R MC in the activation of phorbol reactive isozymes. To evaluate this possibility, we incubated S and R MC with PMA for either 30 min or 24 h and repeated the immunoblots. Incubation for 30 min with PMA should result in membrane translocation of the phorbol ester-responsive isozymes (α, δ, ε, and η). It should be noted, however, that recent studies [29, 30] have indicated that PMA activation and downregulation (24 h of PMA) of PKC is more complex than was orginally thought. In other words, each PMA-sensitive isozyme shows a distinct time course for both activation and downregulation in response to PMA. Nonetheless, we were able to demonstrate translocation of α, δ, and η. Because the signal of ε was so low, we were unable to verify membrane translocation of this isozyme. However, the results of these studies revealed no differences in membrane translocation between S and R MC.

Although there were no differences in isozyme expression in cultured MC, there was the possibility that isozyme expression between S and R might differ in response to a high salt diet. Since this question cannot be answered using an *in vitro* cell culture system, we looked at isozyme expression in freshly isolated glomeruli from S and R rats that were on low and high salt diets. It should be acknowledged that this approach is slightly more problematic since a glomerulus is certainly not a homogenous cell population. We found using RT-PCR and immunoblot the expression of the same five isozymes that were identified in MC. Our immunoblot results are consistent with those of Huwiler [6], except we did not find expression of the β isozyme while we did find expression of the η isozyme, which was not probed in that study, in glomeruli. We do not have an explanation for the difference in β expression. In addition, there was no difference in expression of any of the isozymes between S and R on low or high salt diet.

It could be argued that perhaps the existence of other cell types in the glomeruli masked our ability to detect differences in isozyme expression in MC of freshly isolated glomeruli. However, the much more likely and most straightforward interpretation is that PKC isozyme expression is not altered by either a high salt diet or the elevation of blood pressure.

Immunoblot alone is not sufficient for determining the presence or absence of specific PKC isozymes because the protein concentrations of some isozymes may be too low for immunodetection. Thus, the combination of Western blot and the RT-PCR method of characterizing PKC isozyme expression developed by Kohout and Rogers [4] provides the optimal approach for identifying PKC isozymes present in MC. This approach allowed us to establish that both the message and protein for these 5 isozymes were expressed in both S and R MC and glomeruli.

At this time, research in the area of PKC isozymes is incomplete and the number of recognized isozymes is increasing, making it possible that other isozymes exist in MC. Therefore, it is possible that although we did not find differences in isozyme expression between S and R rats, there could still be differences in the expression of another, as yet unknown, isozyme. Also, there is a lack of information regarding the MC hormone activation pathways or target proteins for each of the known isozymes. Since PKC stimulates Na^+:Ca^{2+} exchange, it will be of particular importance to elucidate the isozyme or isozymes that target this protein upon hormone stimulation.

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SUMMARY

The goal of the work presented in this dissertation was to demonstrate the existence of the Na^+:Ca^{2+} exchanger both indirectly and directly in cultured rat MC and to begin to understand possible modes of Na^+:Ca^{2+} regulation.

The first chapter (p. 10 - 37) titled "Relationship Between $[Na^+]_i$ and $[Ca^{2+}]_i$ During Variations in $[Na^+]_o$ in Cultured Dahl/Rapp Rat Mesangial Cells," demonstrated functional evidence of Na^+:Ca^{2+} exchange in cultured rat MC. Although previous investigators have shown Na^+ dependent Ca^{2+} flux (Na^+:Ca^{2+} exchange) in MC (5, 26), our studies describe the temporal relationship between $[Na^+]_i$ and $[Ca^{2+}]_i$ during alterations in $[Na^+]_o$. Many investigators have used reverse-mode Na^+:Ca^{2+} exchange (removal of $[Na^+]_o$ resulting in Ca^{2+} influx) (5, 26) to asses exchange activity. The assumption made in these experiments is that removal of $[Na^+]_o$ results in the movement of Na^+ out of the cell down its electrochemical gradient and that this provides the driving force for Ca^{2+} to enter the cell via the exchanger. By measuring changes in both $[Na^+]_i$ and $[Ca^{2+}]_i$ upon removal of $[Na^+]_o$ with fluorescent probes, we were able to demonstrate that not all of the Na^+ that leaves the cell is through the Na^+:Ca^{2+} exchanger. Na^+ efflux was biphasic, consisting of a large rapid decrease in $[Na^+]_i$ followed by a small slow decline in $[Na^+]_i$. The large initial decrease in Na^+ efflux occurred via the Na^+:K^+ ATPase, while the smaller decrease in $[Na^+]_i$ was associated with Na^+:Ca^{2+} exchange mediated increases in $[Ca^{2+}]_i$. The small decrease in $[Na^+]_i$ was large enough to account for the increases in $[Ca^{2+}]_i$ based on a Na^+:Ca^{2+} exchange stoichiometry of 3 Na^+ ions to one Ca^{2+} ion. We also found that the magnitude of Ca^{2+} influx was dependent on $[Na^+]_o$. In other words, a larger increase in $[Ca^{2+}]_i$ was seen if

the $[Na^+]_o$ was lowered from 150 mM Na^+ to 0 mM Na^+ than when $[Na^+]_o$ was lowered from 150 mM Na to 75 mM Na^+. In addition, we found no differences in exchange activity between S and R MC. Changes in $[Ca^{2+}]_i$ and $[Na^+]_i$ after reductions in $[Na^+]_o$ were similar between the two rat strains. This finding suggests no inherent differences in the Na^+:Ca^{2+} exchanger in S and R MC.

In these studies we also looked at baseline $[Ca^{2+}]_i$ and $[Na^+]_i$ in S and R MC. Baseline $[Ca^{2+}]_i$ was higher in S compared to R MC. This is consistent with the idea that $[Ca^{2+}]_i$ levels in contractile cells may be elevated in hypertension, thereby leading to enhanced vasoconstriction. Baseline $[Na^+]_i$ was not different in S and R MC.

Our next goal was to obtain direct evidence for the Na^+:Ca^{2+} exchange protein in MC and to study the effects of PKC activation on Na^+:Ca^{2+} exchange. The results of these studies are found in the second chapter (p. 38 - 63) "Immunodetection and Phorbol Ester Stimulation of the Sodium-calcium Exchanger in Cultured Mesangial Cells." We used both monoclonal and polyclonal antibodies directed against the cardiac Na^+:Ca^{2+} exchanger to probe for the exchanger in MC. Both antibodies detected proteins of the size known to be the Na^+:Ca^{2+} exchanger. We found no differences in the size or amount of Na^+:Ca^{2+} exchange protein in S and R MC under control conditions.

Next, we assessed the regulation of the exchanger by PKC. Activation of certain PKC isozymes occurs through well known second messenger pathways. In one model, a vasoactive hormone binding its receptor activates phospholipase C. Phospholipase C, in turn, generates diacylglycerol and inositol 1,4,5-trisphosphate, which promotes Ca^{2+} release from intracellular stores. Ca^{2+} and diacylglycerol activate PKC, which then phosphorylates target proteins. It should be remembered, however, that this pathway is only for the Ca^{2+} and phorbol ester-sensitive PKC isozymes, α, β, and γ. Signals for the other isozymes are still unclear. Although the regulation of the Na^+:Ca^{2+} exchanger is not completely understood, several investigators have demonstrated that the PKC pathway may be one means of regulating exchange activity. For example, Mene (26)

has demonstrated that vasoactive hormones, such as angiotensin II, upregulate exchange activity. In addition, Karim (16) demonstrated that angiotensin II activates PKC through the inositol phosphate-Ca^{2+} pathway in proximal tubule and Pfeilschifter (31) has shown that angiotensin II activates PKC-ε via diacylglycerol stimulation in MC. Also, in squid giant axon, activation of the exchanger by a magnesium ATPase is thought to be due to protein phosphorylation (12). Finally, Iwamoto (15) has shown that the Na^+:Ca^{2+} exchanger is activated by PKC-dependent phosphorylation in vascular smooth muscle cells. Based on these and other findings, we chose to examine the effect of PKC stimulation on Na^+:Ca^{2+} exchange activity.

As in the first chapter, we assessed Na^+:Ca^{2+} exchange activity as the increase in $[Ca^{2+}]_i$ after a reduction in $[Na^+]_o$. We found that serum depriving the MC for 24 h enhanced exchange activity. These results are consistent with those of Mene (25); however, the mechanism of this enhancement is not known. Baseline $[Ca^{2+}]_i$ measurements were not different between S and R MC. This finding differs from the first chapter and may be due to the method of $[Ca^{2+}]_i$ measurement. In the first chapter, $[Ca^{2+}]_i$ measurements were obtained from a population of MC resulting in a average $[Ca^{2+}]_i$ value. Measurements in the second chapter were from single MC, which have an inherently greater degree of variance. Although $[Ca^{2+}]_i$ levels were not significantly different by nonpaired comparison in the second chapter, $[Ca^{2+}]_i$ values tended to be higher in S MC.

PMA, a diacylglycerol analogue, was used to activate PKC in MC. Stimulation of PKC by incubation in PMA for 15 min enhanced exchange activity in both serum-fed and serum-deprived R but not S MC. After incubation in PMA for 24 h, a procedure that downregulates PKC activity, exchange activity was not different between S and R or from control. In addition, we have found that PKC also enhanced Na^+:Ca^{2+} exchange activity in MC from Sprague-Dawley rats (unpublished observation). The similar findings in R and Sprague-Dawley rats, which are the parental strain of both S and R

rats, suggested that the lack of PMA stimulation of the exchanger in S MC was abnormal. In addition, since there were no differences in Na^+:Ca^{2+} exchange activity under control conditions (chapter one), this lead us to speculate that the defect in the S rat may be within the PKC pathway or in the ability of PKC to phosphorylate the exchanger.

Our next step was to determine if PKC stimulation affected the amount of plasma membrane Na^+:Ca^{2+} exchange protein. S and R MC were either left untreated (control) or treated with PMA for 15 min or 24 h. Western blots were performed on the membrane fractions. Increased amounts of immunoreactive protein, compared to control, were seen in R but not S MC after 15 min PMA treatment. Protein levels were the same as control in S and R after 24 h PMA. These initial observations suggest that PKC may cause a translocation of Na^+:Ca^{2+} exchangers from intracellular pools to the plasma membrane. Because of the short time period (15 min), it is unlikely that PKC causes increased Na^+:Ca^{2+} exchange protein synthesis. However, an increase in the number of Na^+:Ca^{2+} exchangers in the plasma membrane may account for the enhanced exchange activity found in the R MC in the presence of PMA. Likewise, the lack of exchanger stimulation in S MC with PMA correlates with the observation that PMA does not alter the amount of immunoreactive protein in S MC.

Further studies in this area are needed to define the regulation of Na^+:Ca^{2+} exchange protein expression in plasma membranes. For example, total cell membranes could be collected and separated into all the component membranes. These membranes could then be probed with Na^+:Ca^{2+} exchange antibodies to detect any shifts in Na^+:Ca^{2+} exchange protein after PMA treatment. Additionally experiments might include reagents which inhibit protein translocation to the plasma membrane. Finally, *in situ* experiments could be performed with a fluorescently labeled antibody and a

confocal microscope to track the location of the exchanger under control and stimulated conditions.

Since the abnormality in S MC may reside with the PKC pathway or in the ability of PKC to phosphorylate the exchanger, we wanted to determine which PKC isozymes are present in MC from S and R rats. These results are contained in the third chapter (p. 64 - 95) entitled "Protein kinase C Isozymes in Cultured Mesangial Cells and Whole Glomeruli From Salt-sensitive and Salt-resistant Rats." Using both RT-PCR and immunoblot, we detected 5 PKC isozymes (α, δ, ε, η, and ζ) in both S and R MC. No differences in expression level were seen between S and R rats. PMA was added to some MC to confirm the detection of the phorbol ester-dependent PKC isozymes, α, δ, ε and η. Translocation of the phorbol ester-dependent isozymes was seen in both S and R MC, and there was no difference in translocation between S and R MC. Additionally, the same 5 isozymes were present in freshly isolated glomeruli from S and R rats fed either a high or low salt diet. No differences in expression were seen between S and R rats or between diets, indicating that isozyme expression does not change with elevations in blood pressure.

The reason S rats become markedly hypertensive on a high NaCl diet remains unknown, although it appears to be a volume-expanded hypertension (38). Like most forms of hypertension, there is probably no single cause but a variety of factors which contribute to elevated blood pressure. From our studies we know that MC from S rats have an elevated baseline $[Ca^{2+}]_i$. This elevation may contribute to increased contraction of the MC, causing a decrease in glomerular filtration rate that ultimately would result in an elevated blood pressure. We propose that there is a defect in the regulation of $[Ca^{2+}]_i$ in the S rat involving the Na^+:Ca^{2+} exchanger.

The hypothesis developed by Blaustein et al. (6) states that elevations in $[Ca^{2+}]_i$ may result from decreased Na^+:Ca^{2+} exchange activity due to elevated $[Na^+]_i$ levels. However, in our studies $[Na^+]_i$ was not elevated in S MC. We suggest a modification to

the Blaustein hypothesis that involves an altered hormone-PKC-Na^+:Ca^{2+} exchange response in the S rat. A defect in this pathway would cause $[Ca^{2+}]_i$ to remain elevated instead of declining after a vasoconstrictive hormone induced elevation in $[Ca^{2+}]_i$. For example, under normal conditions, a vasoactive hormone would activate phospholipase C which would activate diacylglycerol and release of Ca^{2+} from intracellular storage sites. The combined effects of diacylglycerol and elevated $[Ca^{2+}]_i$ would activate PKC. One effect of PKC might be to cause a translocation of the exchanger from intracellular pools to plasma membrane. This would enhance Na^+:Ca^{2+} exchange activity, resulting in an increase in Ca^{2+} efflux and return of $[Ca^{2+}]_i$ to resting levels. A defect in this pathway in the S rat would lead to less Ca^{2+} extrusion after a hormone-induced increase in $[Ca^{2+}]_i$ and, therefore, a prolonged contracted state.

To summarize, we found no differences in the Na^+:Ca^{2+} exchange protein or its activity under control conditions in MC from S and R rats. Nor did we find any differences in PKC isozyme expression between the rat strains. We did find, however, that baseline $[Ca^{2+}]_i$ was higher in S compared to R MC. In addition, we found differences in both Na^+:Ca^{2+} exchange activity and protein level upon PKC stimulation with PMA. We do not know why the exchanger responds differently in S and R MC with PMA stimulation. It is possible that there is a defect in the phosphorylation site of the exchanger in the S rat, making it less responsive to PKC. Alternately, there may be differences in PKC activity between S and R. Future studies in this area need to be done in order to determine the defect. For example, there may be a mutation in the Na^+:Ca^{2+} exchanger in the S rat. Sequence analysis of the exchanger in both S and R might reveal differences in the large intracellular loop domain, which is thought to be important in regulation. In addition, the regulation of the exchanger by individual PKC isozymes could be determined using antisense oligonucleotides to the isozymes to block the activation of a particular isozyme. In conclusion, the studies described in this dissertation indicate that some portion of the PKC-Na^+:Ca^{2+} exchange pathway is

different between S and R and that this difference may play a role in the development of hypertension in the S rat.

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APPENDIX A

ALTERED Na^+:Ca^{2+} EXCHANGE IN AFFERENT ARTERIOLES OF THE SHR

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Running head: Na^+:Ca^{2+} exchange in renal arterioles of SHR

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ABSTRACT

Regulation of cytosolic calcium concentration ($[Ca^{2+}]_i$) in renal arterioles has important implications in the control of renal vascular tone, and consequently, arterial blood pressure. In this regard, Na^+:Ca^{2+} exchange has been implicated in the control of smooth muscle $[Ca^{2+}]_i$ and derangements in exchanger activity have been suggested to occur in hypertension. Therefore, studies were performed to define Na^+-dependent changes in $[Ca^{2+}]_i$ (Na^+:Ca^{2+} exchange) in afferent (AA) and efferent (EA) arterioles microdissected from kidneys of 3 and 9 week (wk) SHR and WKY rats. In addition, Na^+-independent changes in $[Ca^{2+}]_i$, representing Ca^{2+} sequestering and/or extrusion mechanisms, were evaluated. $[Ca^{2+}]_i$ was assessed in fura 2 loaded arterioles bathed in a Ringer's solution in which bath Na^+ (Na^+_e) was reduced from 150 to 2 mM and replaced with N-methyl-D-glucamine. Experiments were also conducted to determine Na^+:Ca^{2+} exchange activity in Na^+ loaded arterioles (1 mM ouabain). Baseline $[Ca^{2+}]_i$ was similar in AA of 3 and 9 wk WKY and SHR. In AA of 3 wk WKY and SHR, $[Ca^{2+}]_i$ increased similarly, 89 ± 15 and 73 ± 13 nM respectively, during decreases in Na^+_e from 150 to 2 mM Na^+ ($p > 0.05$). In 9 wk hypertensive SHR (SBP = 150 mmHg), increases in $[Ca^{2+}]_i$ were attenuated (24 ± 3 nM) relative to 3 wk WKY and SHR, and 9 wk WKY (90 ± 9 nM; $p < 0.05$). Likewise, the rate of removal of Ca^{2+} in the continued presence of 2 mM Na^+_e (Ca^{2+} sequestration and/or extrusion) was markedly reduced in AA of 9 wk SHR (-0.15 ± 0.03 nM/s) versus 3 wk SHR (-0.72 ± 0.12 nM/s) and 3 and 9 wk WKY (-0.49 ± 0.10 and -0.67 ± 0.14 nM/s). In AA pre-incubated with ouabain, Na^+-dependent increases in $[Ca^{2+}]_i$ were attenuated in 9 wk SHR, suggesting that exchanger number and/or sensitivity to the transmembrane Na^+ gradient was reduced in the SHR AA. In EA, baseline $[Ca^{2+}]_i$ was similar in 3 and 9 wk WKY and SHR. In contrast to AA, the magnitude of Na^+-dependent and Na^+-independent changes in $[Ca^{2+}]_i$ were not different in EA of 3 and 9 wk WKY and SHR. These results indicate that regulation of Na^+:Ca^{2+} exchange activity may differ between AA and EA segments.

Furthermore, diminished Na^+:Ca^{2+} exchange and Na^+-independent Ca^{2+} sequestering/extrusion mechanisms in AA from SHR might contribute to altered $[Ca^{2+}]_i$ in hypertension.

INTRODUCTION

Cytosolic calcium concentration ($[Ca^{2+}]_i$), which is the major determinant of contractility in vascular smooth muscle (vsm), may play a central role in the development of hypertension. The regulation and control of $[Ca^{2+}]_i$ occurs through several different pathways, including movement of Ca^{2+} across both plasma membrane and into and out of intracellular storage sites. At the plasma membrane Ca^{2+} transport includes, but is not limited to Ca^{2+}-ATPase and Na^+:Ca^{2+} exchange. There has been considerable interest in the Na^+:Ca^{2+} exchanger in both the regulation of $[Ca^{2+}]_i$ as well as its potential involvement in the pathogenesis of certain forms of hypertension [1-4]. The Na^+:Ca^{2+} exchanger was first alluded to in heart [5, 6] and squid giant axon [7, 8], and subsequently in a variety of tissue types including rod outer segment [9, 10], vascular smooth muscle [2, 11] and kidney [12, 13], including in the renal mesangium [14]. At the present time, two forms of the exchanger, encoded by separate genes, have been identified [15]. One gene product is found in the outer rod segment of the eye and transports Na^+, K^+ and Ca^{2+} [16, 17]; while the other gene product, the "cardiac form" of the exchanger, is expressed in a number of different tissues, including kidney [18, 19]. The cardiac form of the exchanger transports Na^+ and Ca^{2+}. In addition, recent studies suggest that various tissues may express different isoforms of the exchanger, implying that the characteristics and regulation of exchanger activity may be tissue specific [19].

Much of the previous work on Na^+:Ca^{2+} exchange in vsm has been limited to examination of the larger conductance vessels (> 200 mm), such as the mesenteric vascular bed [20, 21] and abdominal aorta [3, 4, 22]; however, to a lesser extent, studies have been performed on isolated cell membrane vesicles (extracted from mesenteric arteries) of vsm [22] and cultured aortic vsm cells [23]. Results from these studies

support the existence of a bidirectional Na^+:Ca^{2+} exchange mechanism in these cells, largely based on Ca^{2+}-uptake measurements, or indirectly via contractile measurements during either Na^+ loading or reversal of the Na^+ gradient across the plasmalemmal membrane. In the forward mode of operation, the Na^+:Ca^{2+} exchanger characteristically couples 3 inward Na^+ ions to the extrusion, against its electrochemical gradient, of 1 Ca^{2+} ion [24].

Since the initial suggestion by Blaustein that the Na^+:Ca^{2+} exchanger may be involved in hypertension [12], exchange activity has been examined in vascular smooth muscle obtained from hypertensive animals with mixed results [3, 4, 25]. In aortic rings removed from Dahl S and R rats fed a high salt (8%) diet for 4 weeks, Na^+:Ca^{2+} exchange activity, assessed by measurement of the rate of contraction elicited in a 1.2 mM Na^+ solution, was lower in aortic rings from Dahl S rats than Dahl R rats [4]. This contrasts with data obtained from aortic rings of SHR and WKY rats, in which the rate of contraction was significantly higher (2-fold) in hypertensive SHR [3]. These Na^+:Ca^{2+} exchange data from SHR aorta are consistent with results derived from isolated sarcolemmal membranes (mesenteric arteries) of SHR [25]. However, no studies to date have examined Na^+:Ca^{2+} exchange in renal resistance vessels (afferent and efferent arterioles) from SHR and WKY rats, and the importance of these blood vessels in the control of renal function and arterial blood pressure is generally accepted.

Recently, we have characterized Na^+:Ca^{2+} exchange activity in afferent and efferent arterioles from rabbit kidney using the the Ca^{2+} sensitive fluorescent probe fura 2 [26]. Reversing the transmembrane gradient with low Na^+_e increased $[Ca^{2+}]_i$ in both afferent and efferent arterioles, and these responses were inversely related, in graded fashion, to $[Na^+]_e$. In addition, arterioles Na^+ loaded with ouabain showed augmented increases in $[Ca^{2+}]_i$ during low Na^+_e. These results were consistent with the presence of a Na^+:Ca^{2+} exchanger in both afferent and efferent arterioles and suggested that Na^+:Ca^{2+} exchange might play a role in controlling $[Ca^{2+}]_i$ in these important resistance

elements. The present studies were performed to extend this work by searching for alterations in Na^+:Ca^{2+} exchange activity in afferent and efferent arterioles from the spontaneously hypertensive rat. We additionally evaluated differences in Na^+-independent regulatory mechanisms controlling $[Ca^{2+}]_i$ in arterioles from these 2 strains of rats, since other studies suggest that there may be alterations in these Ca^{2+} regulatory mechanisms in hypertension [27-31].

METHODS

Tissue preparation:

Male and female Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were obtained at 3 and 9 wks of age from Charles Rivers (Raleigh, NC) and utilized in experiments within 1 week. SHR develop arterial hypertension at 5 weeks age, while WKY rats remain normotensive; thus, pre-hypertensive and hypertensive SHR as well as separate normotensive controls were utilized. Rats were fed standard laboratory chow (Prolab animal diet) containing 0.4% sodium and 0.7% potassium and water *ad libitum*. The mean of several systolic blood pressures measurements, as well as heart rates, were determined using standard tail cuff plethysmography techniques.

For the study of isolated arterioles, rats were killed by decapitation and kidneys were removed, decapsulated and sliced in thin (1 mm) coronal sections for microdissection. Kidney slices were placed in cold Ringer's solution containing (in mM): 148 NaCl, 5 KCl, 1 $MgSO_4$, 1.6 Na_2HPO_4, 0.4 NaH_2PO_4, 5.0 Dextrose, 1.5 $CaCl_2$ and 7.0 mM fura 2 acetoxymethyl ester (fura 2-AM) or 1.6 mM sodium green, tetraacetate (Na Green). Glomeruli with attached arterioles and thick ascending limb were isolated using a Wild M5A stereomicroscope and incubated for 1 hour in 7.0 mM fura 2-AM, with additional fura 2-AM (100 mM) regularly applied in close proximity via a 1 cc syringe and 26 g needle. After 1 hour of incubation in fura 2-AM, single glomeruli with arterioles were transferred to a chamber that was mounted on a Leitz inverted microscope. Glass pipettes were used to gently hold glomerulus and vascular

structures at the floor of the bathing chamber, which prevented movement of the arteriole during the experimental protocol. Temperature of the chamber was maintained at 37°C by continuous superfusion (bath exchange) with prewarmed Ringer's solution at a rate of 1.5 ml/min. The 150 and 2 mM Na^+ Ringer's bathing solutions were maintained at 37°C, bubbled with O_2, and pH was adjusted to 7.40 with Trizma HCl or base. Osmolality of solutions was frequently verified and found to equal 296 ± 2 mOsm/kg. For precise temperature control, hot water was continuously circulated through tubing located around the perimeter of the chamber.

Criteria for identification of afferent and efferent arterioles was similar to that previously described [32]. Afferent and efferent arterioles were distiguished based on size and relative position to the thick ascending limb [33]. Also, afferent arterioles were identified by concentric rings of smooth muscle cells. After identification, an adjustable optical window was positioned over the arteriole within which photon emissions were collected and measured using a Leitz compact photometer modified to perform photon counting. Wavelengths for excitation of fura 2 were 350 and 380 nm (Xenon 75 W light source), while emitted fluorescence was passed through a 510 ± 10 nm band-pass filter. The wavelength of excitation for Na Green was 507 nm, and emitted fluorescence was measured at 530 nm. Background fluorescence was determined prior to the experimental protocol and rarely exceeded 5% of the total fluorescence. Hardware, including dual monochronometers, chopper and software were obtained from Photon Technology International (PTI Deltascan System, Princeton, NJ). Software and hardware were configured for collecting 20 datapoints/s and writing 5 points/s to file.

Experimental Protocol:

Baseline fura 2 ratios were obtained while bathing the afferent or efferent arteriole in Ringer's solution containing 150 mM Na^+_e. Experiments were performed only when the fura 2 ratio (ie, $[Ca^{2+}]_i$) remained stable for at least 100 sec. After this period of time, $[Na^+]_e$ was reduced to 2 mM, which resulted in a rapid increase in

$[Ca^{2+}]_i$ followed by a return of the ratio towards baseline values. Na^+_e was returned to 150 mM to obtain recovery measurements only after a stable plateau value had been obtained for $[Ca^{2+}]_i$ at low $[Na^+]_e$. After increasing Na^+_e to 150 mM, $[Ca^{2+}]_i$ was monitored until a stable steady-state recovery value was attained. In the experiments using ouabain (Sigma Chemical Co.), control responses, as just described, were obtained, after which the arteriole was bathed in the 150 mM Na^+_e solution containing 1 mM ouabain. After incubating arterioles for 15 mins, the protocol of reducing and readdition of Na^+_e on $[Ca^{2+}]_i$ was repeated in the continued presence of ouabain. Cytosolic $[Na^+]$ was qualitatively assessed after 30' of arteriolar incubation in a solution containing the fluorometric probe Na Green.

Analysis:

$[Ca^{2+}]_i$ was determined using the ratio of fluorescence obtained at 350 and 380 nm excitation wavelengths and calculated using the equation described by Grynkiewicz et al. [34]:

$$[Ca^{2+}]_i = K_d * [(R - V * R_{min}) / (V * R_{max} - R)] (S_{f2} / S_{b2})$$

where K_d is the effective dissociation constant of fura 2, V is the viscosity correction factor (0.85) described by Poenie [35], R_{min} and R_{max} are the maximum and minimum ratios at 350 and 380 nm in the presence and absence of Ca^{2+}, respectively, and S_{f2} and S_{b2} are the emissions at 380 nm in the presence and absence of saturating Ca^{2+}. The value of 224 nM was utilized as the K_d for fura 2 [34]. Calibration was conducted *in vitro* in the bathing chamber. Composition of the calibration solution was chosen to approximate the intracellular milieu and consisted of (in mM): 115 KCl, 20 NaCl, 10 3-(N-morpholino)propanesulfonic acid, 1.1 $MgCl_2$, 1mM fura 2 pentapotassium salt (Molecular Probes, Eugene, OR) and either 3 $CaCl_2$ or no Ca^{2+} and 3 ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). R_{min} and R_{max} values averaged 0.55 ± 0.02 (n = 9) and 3.09 ± 0.38 (n = 9), while S_{f2}/S_{b2} averaged 3.23 ±

0.32. *In vitro* calibration parameters parallel those produced *in vivo* [36] and are similar to those reported in previous studies from our laboratory [32].

In order to assess Na^+-dependent and Na^+-independent effects of low $[Na^+]_e$ on arteriolar $[Ca^{2+}]_i$, experiments were analyzed as illustrated in Fig. 1. As shown in Fig. 1: *ΔCa^{2+}*, is the difference between the peak increase in $[Ca^{2+}]_i$ and baseline $[Ca^{2+}]_i$; *dy/dt (nM/s),* is the rate of increase in $[Ca^{2+}]_i$ with decreased Na^+_e; *-dy/dt (nM/s),* is the rate of decrease in $[Ca^{2+}]_i$ in the continued presence of 2 mM Na^+_e; *%NaIRec,* is the magnitude of decrease in $[Ca^{2+}]_i$ in the continued presence of 2 mM Na^+_e divided by the total recovery of $[Ca^{2+}]_i$ after re-addition of Na^+_e to 150 mM multiplied by a factor of 100 (*i.e.*, the relative recovery as a percentage of total recovery). Dy/dt and -dy/dt were determined from analytic calculation of successive cubic data fits, as provided in the Deltascan software.

Statistical analysis:

Data are presented as the mean ± standard error (SE). Comparisons of peak responses, dy/dt, -dy/dt and NaIRec, as well as the effect of ouabain within and between groups of rats were conducted with the Student's paired and unpaired t-test. Statistical significance for all comparisons was set at $p < 0.05$.

RESULTS

At 4-5 wks of age, SHR develop hypertension, while control WKY rats remain normotensive [37-39]. In the present study, tail cuff plethysmography was utilized to document age-related changes in systolic blood pressure in 3 and 9 wk old animals, and these data are depicted in Table 1. Systolic blood pressures were similar in 3 wk WKY and SHR, while at 9 wks of age blood pressure increased in both WKY and SHR. However, the increases in blood pressure were significantly greater in 9 wk SHR compared to WKY. Also, heart rates, body weights and weight gains were comparable between SHR and WKY at 3 and 9 wks of age ($p > 0.05$).

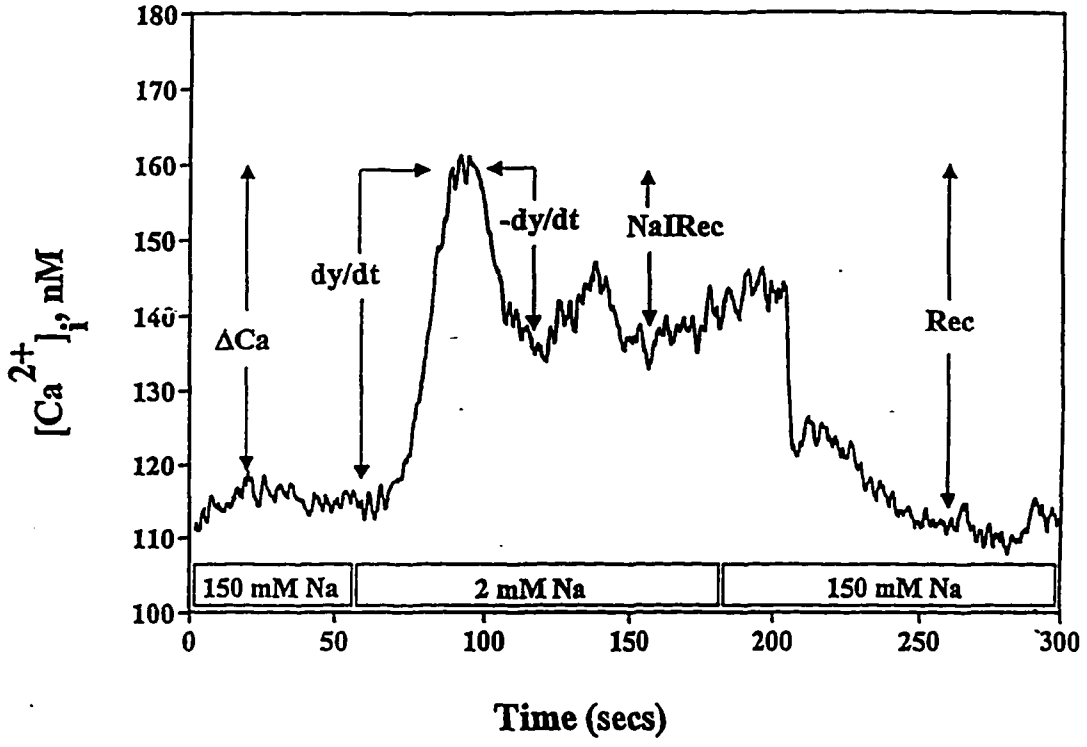

Fig. 1: Typical fura 2 trace (afferent arteriole) illustrating the experimental protocol and analysis of changes in $[Ca^{2+}]_i$. A prompt increase in $[Ca^{2+}]_i$ was observed during a reduction of Na^+_e from 150 to 2 mM. This was followed by a fall in $[Ca^{2+}]_i$ in the continued presence of 2 mM Na^+_e. After $[Ca^{2+}]_i$ had stablized, Na^+_e was returned to 150 mM. The magnitude and rate of change in $[Ca^{2+}]_i$ was determined; a more complete explanation and definitions of the terms shown in the Figure are given in the Results section.

Table 1.
Systolic blood pressure (SBP), heart rate (HR) and body weight in 3 and 9 wk WKY and SHR rats.

	SBP (mmHg)	HR (B/Min)	Body Wt. (g)
3 Wk. WKY	105	465	54.5
(n=6)	±7	±17	±3.0
9 Wk. WKY	*130	398	227.4
(n=6)	±5	±34	±6.3
3 Wk. SHR	101	486	47.4
(n=6)	±7	±16	±2.7
9 Wk. SHR	+*150	406	221.9
(n=6)	±5	±41	±4.1

*$p < 0.05$ represents significant difference between 3 and 9 wk rats; +$p < 0.05$ represents difference between 9 wk SHR and WKY.

Baseline $[Ca^{2+}]_i$ was measured in afferent and efferent arterioles bathed in 150 mM Na^+_e Ringer's solution, and the values are shown in Table 2. There were no differences in baseline $[Ca^{2+}]_i$ between SHR and WKY either at 3 or 9 wks of age under the *in vitro* conditions in which these measurements were performed. Similar results were obtained in efferent arterioles from SHR and WKY rats at 3 and 9 wks of age.

In afferent arterioles (Fig. 2A) from 3 and 9 wk WKY, reducing Na^+_e from 150 to 2 mM increased $[Ca^{2+}]_i$ similarly by 88.7 ± 14.5 and 90.0 ± 8.5 nM, respectively ($p > 0.05$). In contrast, $[Ca^{2+}]_i$ responses to a reduction in Na^+_e were markedly reduced (23.6 ± 3.4 nM) in afferent arterioles of 9 wk SHR relative to 3 wk SHR (72.9 ± 12.9 nM). While the magnitude of increase in $[Ca^{2+}]_i$ during low $[Na^+]_e$ tended to be reduced in 3 wk SHR compared with 3 wk WKY, it was not, however, statistically significant. Rates of increase in $[Ca^{2+}]_i$ (dy/dt) with a reduction in Na^+_e for afferent arterioles are shown in Table 3. Dy/dt was significantly less in 9 wk SHR compared to all three other groups. These results indicate that, in response to a reduction in Na^+_e, both the magnitude and rate of change in $[Ca^{2+}]_i$ were reduced in 9 wk SHR compared to 3 wk SHR and 3 and 9 wk WKY rats.

In the continued presence of 2 mM Na^+_e, $[Ca^{2+}]_i$ declined back towards control levels (Fig. 1). The rate of decline in $[Ca^{2+}]_i$ (-dy/dt) and the magnitude of recovery of $[Ca^{2+}]_i$ (%NaIRec) were measured in each of the four groups and the results are shown in Table 3. In afferent arterioles, both the rate of decline and magnitude of recovery of $[Ca^{2+}]_i$ in the presence of 2 mM Na^+_e were significantly less in 9 wk SHR compared to the other three groups. Thus, in afferent arterioles from hypertensive SHR, both Na^+-dependent and Na^+-independent changes in $[Ca^{2+}]_i$ were reduced when compared to 3 wk SHR and 3 and 9 wk WKY rats.

In efferent arterioles, illustrated in Fig. 2B, decreases in Na^+_e from 150 to 2 mM produced similar increases in $[Ca^{2+}]_i$ from 3 wk old (62.0 ± 14.0 nM) and 9 wk (53.2 ± 8.7 nM) WKY rats. $[Ca^{2+}]_i$ responses were also similar in 3 and 9 wk old SHR,

Table 2.
Afferent and efferent arteriolar $[Ca^{2+}]_i$ under basal conditions in 3 and 9 wk WKY and SHR rats.

	Afferent Arterioles $[Ca^{2+}]_i$, nM	Efferent Arterioles $[Ca^{2+}]_i$, nM
3 Wk WKY	155 ±16(15)	147 ±14(14)
9 Wk WKY	186 ±12(15)	166 ±12(14)
3 Wk SHR	156 ±18(15)	142 ±7(16)
9 Wk SHR	156 ±12(17)	177 ±15(16)

There were no significant differences in baseline $[Ca^{2+}]_i$ between ages and groups. Number of measurements shown in ( ).

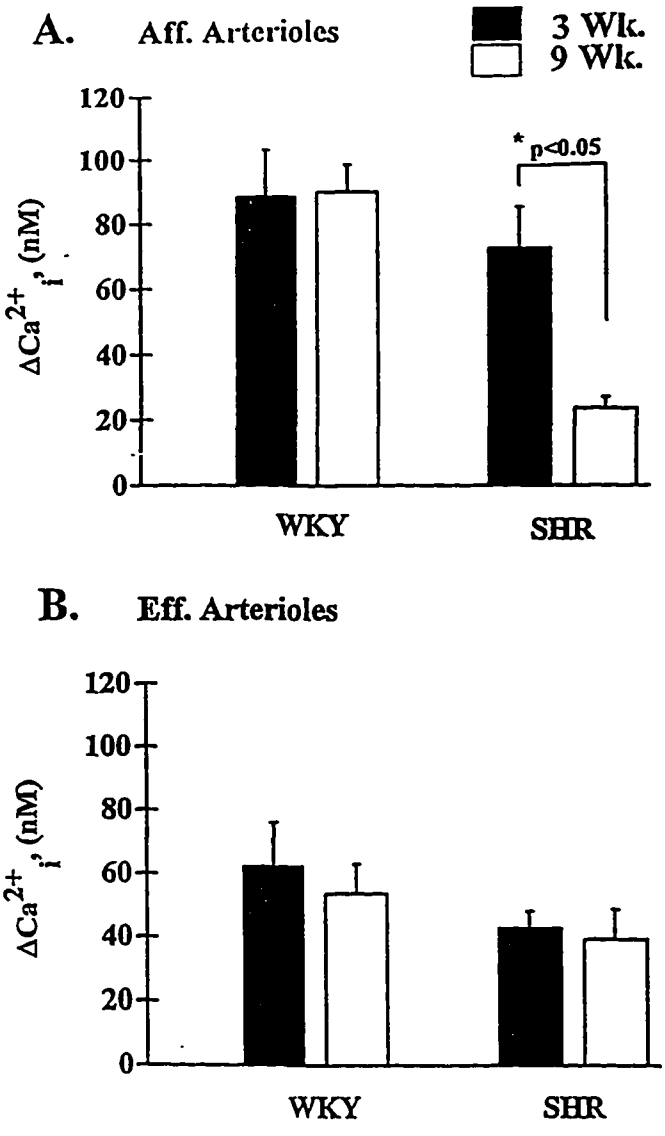

Fig. 2: Magnitude of increase in $[Ca^{2+}]_i$ during a reduction of Na^+_e from 150 to 2 mM in afferent (A) and efferent (B) arterioles from 3 (solid bars) and 9 wk (open bars) WKY and SHR. $^*p < 0.05$ represents significant difference between 3 and 9 wk SHR responses.

averaging 42.6 ± 5.4 and 39.2 ± 9.4 nM, respectively. Although changes in $[Ca^{2+}]_i$ appeared to be larger in efferent arterioles of WKY rats compared to SHR, these differences did not achieve statistical significance. As shown in Table 3, the rate of increase in $[Ca^{2+}]_i$ with decreased Na^+_e was not different in all four groups.

In the continued presence of 2 mM Na^+_e, there was a decline in $[Ca^{2+}]_i$ back toward control values. The percent recovery of $[Ca^{2+}]_i$ was not different in efferent arterioles from all four groups. The rate of decline in $[Ca^{2+}]_i$ was also similar across ages and groups, although there was a small difference that achieved statistical significance in -dy/dt between efferent arterioles from 3 and 9 wk old SHR. This may be attributed to a lower value for -dy/dt in the 3 wk old SHR rat and not to a specific difference in the 9 wk SHR compared to the other groups, as found in the afferent arteriolar studies.

In other experiments designed to decrease the transmembrane Na^+ gradient (by raising cytosolic $[Na^+]$), afferent and efferent arterioles were exposed to 1 mM ouabain for a period of 15 mins. Cytosolic $[Na^+]_i$ was qualitatively assessed before and during ouabain incubation via the fluorometric probe Na Green. A typical trace documenting the increase in $[Na^+]_i$ during 15' ouabain incubation is depicted in Fig. 3. In 4 afferent arterioles, 15' ouabain increased Na Green cps 33.7 ± 4.0% from 161024 ± 26714 to 214641 ± 34945. Similarly, in 3 efferent arterioles, ouabain increased Na Green cps 37.3 ± 1.9% from 157252 ± 33531 to 217753 ± 48185. In addition, Na Green cps decreased 9.9 ± 3.5% in afferent (n = 4) and 10.9 ± 2.9% in efferent (n = 3) arterioles bathed in 2 mM Na^+ under basal conditions. These decreases were augmented to 18.5 ±4.9% and 16.0 ± 2.3% in afferent and efferent arterioles, respectively after 15' incubation in ouabain. These results demonstrate that cytosolic Na^+ promptly and progressively increases during arteriolar incubation in ouabain. In addition, bath exchange from 150 to 2 mM Na^+ resulted in measurable decreases in intracellular Na^+,

Table 3.
Linear fit analysis of the rate of increase in $[Ca^{2+}]_i$ (dy/dt), the rate of Ca^{2+} sequestration and/or extrusion (-dy/dt), and the relative recovery of $[Ca^{2+}]_i$ (NaIRec) while Na^+_e was reduced from 150 to 2 mM.

	Afferent Arterioles			Efferent Arterioles		
	dy/dt (nM/s)	-dy/dt (nM/s)	NaIRec (% Rec)	dy/dt (nM/s)	-dy/dt (nM/s)	NaIRec (% Rec)
3 wk. WKY	2.20 ±0.39	-0.49 ±0.10	86.6 ±3.8	1.16 ±0.27	-0.40 ±0.11	64.1 ±6.5
9 wk. WKY	2.74 ±0.36	-0.67 ±0.14	76.5 ±5.5	1.19 ±0.32	-0.25 ±0.09	43.7 ±8.7
3 wk. SHR	1.89 ±0.36	-0.72 ±0.12	74.6 ±6.8	1.08 ±0.19	-0.13 ±0.04	57.4 ±8.2
9 wk. SHR	*0.63x ±0.10	*-0.15x ±0.03	56.3x ±8.1	1.39 ±0.36	*-0.40 ±0.10	66.4 ±6.5

*$p < 0.05$ represents significant differences between 3 and 9 wk old animals. x$p < 0.05$ represents differences between 9 wk SHR and WKY rats.

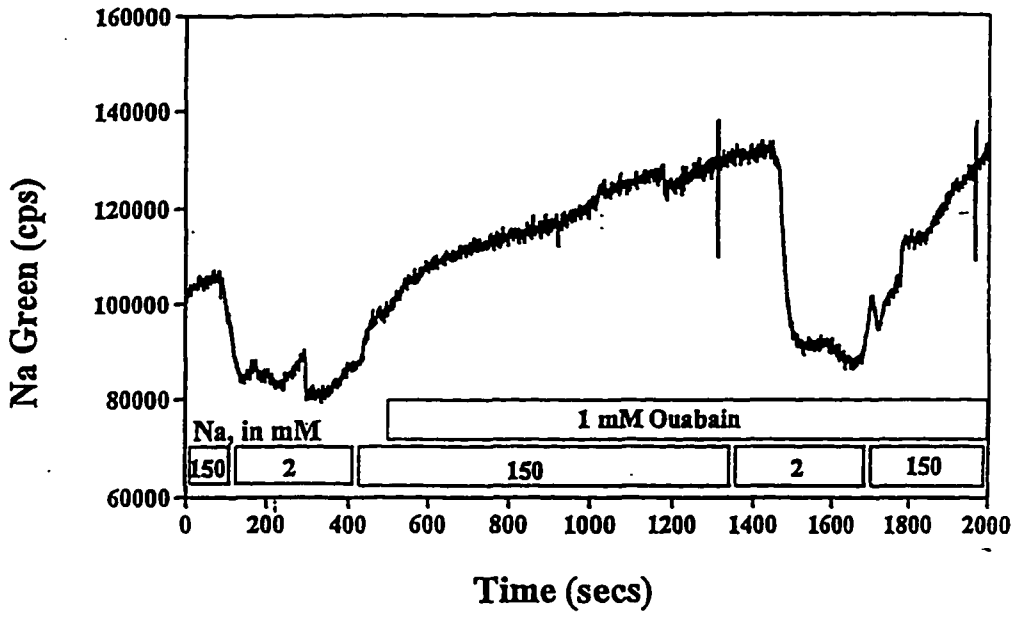

Fig. 3: Typical Na Green trace (afferent arteriole) illustrating the effect of reduction of Na^+_e from 150 to 2 mM and 15' ouabain incubation on $[Na^+]_i$. A prompt and sustained decrease in $[Na^+]_i$ was observed during a reduction of Na^+_e from 150 to 2 mM. $[Na^+]_i$ progressively increases during 1 mM incubation in ouabain.

suggesting that cytosolic Na^+ is of sufficient magnitude during basal conditions to drive reverse-mode Na^+:Ca exchange.

In afferent arterioles from 3 and 9 wk WKY rats (Fig. 4A), reduction of Na^+_e to 2 mM similarly increased $[Ca^{2+}]_i$ by 81.2 ± 18.2 (n = 9) and 96.2 ± 10.3 nM (n = 11), respectively ($p > 0.05$). Exposure to ouabain significantly increased the magnitude of changes in $[Ca^{2+}]_i$ with reductions in Na^+_e by approximately 4-fold. In response to a decrease in Na^+_e, $[Ca^{2+}]_i$ increased by 337.2 ± 111.3 and 351.7 ± 78.3 nM in 3 and 9 wk WKY, respectively. The changes in $[Ca^{2+}]_i$ were not different between 3 and 9 wk old WKY rats but were clearly different between control and ouabain treated arterioles at both 3 and 9 wks of age. In afferent arterioles from 3 and 9 wk old SHR, control increases in $[Ca^{2+}]_i$ in response to reduced Na^+_e were 77.2 ± 16.7 (n = 11) and 23.2 ± 3.7 nM (n = 15), respectively, ($p < 0.05$). These responses were similar in magnitude to those measured previously, as illustrated in Fig. 2A. After incubation in ouabain, increases in $[Ca^{2+}]_i$ at 2 mM Na^+_e were 292.3 ± 51.7 nM in 3 wk SHR and were significantly reduced in 9 wk old SHR ($[Ca^{2+}]_i$ = 129.7 ± 16.2 nM; $p < 0.05$).

In efferent arterioles obtained from 3 and 9 wk old WKY rats (Fig. 4B), increases in $[Ca^{2+}]_i$ during 2 mM Na^+_e were similar at 66.0 ± 19.2 (n = 10) and 59.7 ± 11.5 nM (n = 10), respectively ($p > 0.05$). After incubation in 1 mM ouabain, increases in $[Ca^{2+}]_i$ during 2 mM Na^+_e were augmented approximately 4-5 fold to 290.2 ± 94.8 and 276.5 ± 41.3 nM in 3 and 9 wk WKY. In efferent renal arterioles of 3 and 9 wk SHR, 2 mM Na^+_e increased $[Ca^{2+}]_i$ similarly by 33.4 ± 3.5 (n = 10) and 33.8 ± 9.7 nM (n = 13), respectively ($p > 0.05$). These responses were similar in magnitude to those measured previously, as illustrated in Fig. 2B. After ouabain, 2 mM Na^+_e increased $[Ca^{2+}]_i$ by 231.7 ± 38.4 nM in 3 wk SHR and 147.9 ± 28.6 nM in 9 wk SHR ($p < 0.05$). Thus, there appeared to be a modest, yet significant attenuation of the effect of ouabain to increase the response of $[Ca^{2+}]_i$ to reduced Na^+_e in efferent arterioles from 9 wk SHR.

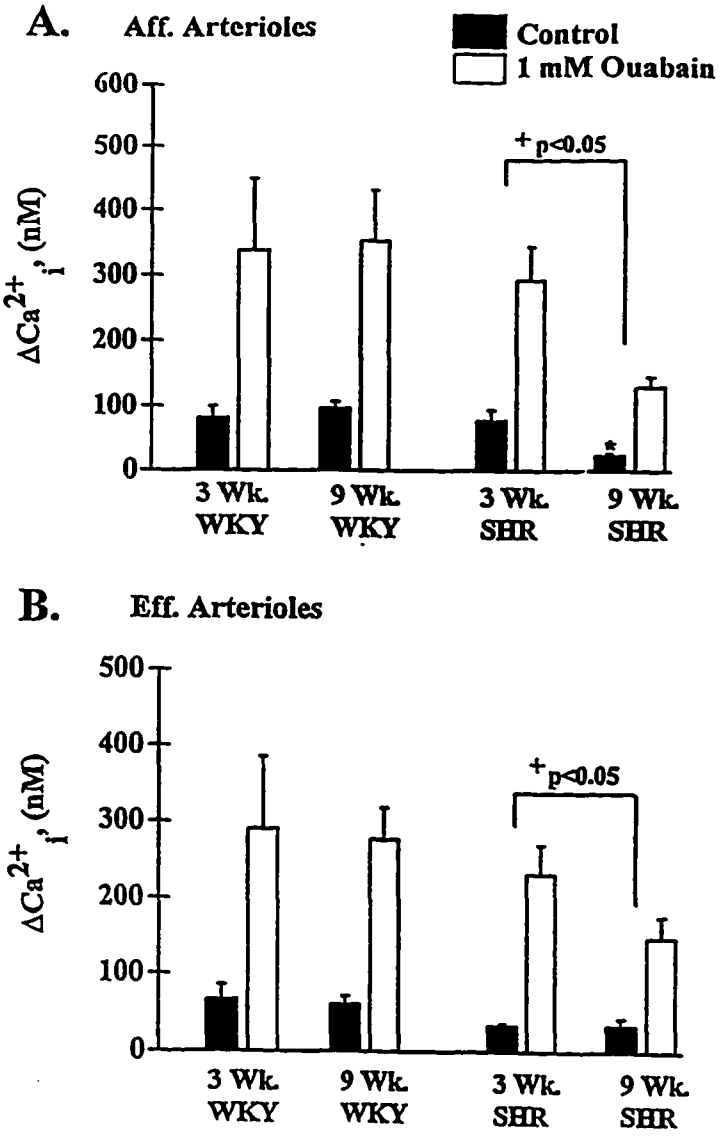

Fig. 4: The magnitude of change in $[Ca^{2+}]_i$ with a decrease in Na^+_e from 150 to 2 mM in afferent (A) and efferent (B) arterioles from 3 and 9 wk WKY and SHR before (solid bars) and after (open bars) 15 mins incubation in 1 mM ouabain. $^*p < 0.05$ represents significant difference between 3 and 9 wk SHR responses. $^+p < 0.05$ represents significant difference between control and ouabain responses in 3 and 9 wk SHR.

DISCUSSION

Elevated arterial blood pressure in spontaneously hypertensive rats (SHR) is characterized by increased peripheral and renal vascular resistance, decreased glomerular filtration rate [37-39], and renal vascular hyperreactivity to disparate agonists [40-42]. These vascular alterations may result from a common defect in the intrinsic and/or neuro-humoral regulation of $[Ca^{2+}]_i$ in vascular smooth muscle (vsm), or more than likely, result from multiple pathologies. One of the mechanisms that participates in the control of $[Ca^{2+}]_i$ in vsm, and therefore conceivably of importance in hypertension, is the Na^+:Ca^{2+} exchanger. The present studies were designed to obtain evidence for Na^+:Ca^{2+} exchange activity in renal arteriolar segments from the SHR and to compare exchanger activity between SHR and WKY rats. It should be noted that previous studies in genetic models of hypertension have examined Na^+:Ca^{2+} exchange activity in larger conductance vessels [3, 4, 20-22]. Since the characterisitics of Na^+:Ca^{2+} exchange may vary between tissues, these previous studies may not necessarily be representative or predictive of exchange activity in renal resistance vessels.

Na^+:Ca^{2+} Exchange Experiments

In the present studies, reductions in Na^+_e from 150 to 2 mM resulted in a prompt and linear increase in $[Ca^{2+}]_i$ in both afferent and efferent arterioles. This increase in $[Ca^{2+}]_i$ was similar to that found in previous studies in rabbit afferent and efferent arterioles [26]. In these studies, the most likely explanation for changes in $[Ca^{2+}]_i$ with manipulation of the transmembrane Na^+ gradient was through the Na^+:Ca^{2+} exchanger. Although there has been some evidence in human fibroblasts that removal of Na^+_e can mobilize $[Ca^{2+}]_i$ [43], similar observations have not been reported in vsm. We have found in renal arterioles that increased $[Ca^{2+}]_i$ was dependent upon the presence of extracellular Ca^{2+} and not mobilization of Ca^{2+} from intracellular storage sites. Also, nifedipine, a voltage-sensitive Ca^{2+} channel blocker, did not diminish the increased $[Ca^{2+}]_i$ obtained by lowering Na^+_e. These findings, along with additional evidence that

changes in $[Ca^{2+}]_i$ could be blocked by nickel and influenced, in predictable fashion, by changes in the transmembrane Na^+ gradient, all support the notion that Na^+:Ca^{2+} exchanger operates in renal arterioles. In other studies, elimination of Na^+_e only increased $[Ca^{2+}]_i$ by prior Na^+ loading [44]. In contrast, we found that reducing Na^+_e from 150 to 2 mM elicited increases in $[Ca^{2+}]_i$ without Na^+ loading. This finding may indicate that in renal vessels, $[Na^+]_i$ was sufficiently high enough to provide a driving force for Na^+ to exit through the exchanger in the presence of 2 mM Na^+_e (Figs 2 and 3). Presumably, in other studies, baseline $[Na^+]_i$ was lower and, therefore, it was necessary to first raise $[Na^+]_i$ in order to observe exchanger activity. Finally, in the continued presence of 2 mM Na^+_e, $[Ca^{2+}]_i$ returned toward baseline but remained significantly greater than control values. These results indicate that other Ca^{2+} regulatory mechanisms are not capable of fully restoring $[Ca^{2+}]_i$ to control levels and support the notion that the exchanger plays an important role in controlling $[Ca^{2+}]_i$ in these vascular segments.

In 3 wk old SHR and in 3 and 9 wk old WKY rats, we found significant increases in $[Ca^{2+}]_i$ at reduced Na^+_e in both afferent and efferent arterioles. However, the magnitude of the responses appeared to be greater in afferent arterioles versus efferent arterioles. These results are consistent with our recent findings in rabbit arterioles, in which Na^+-dependent changes in $[Ca^{2+}]_i$ were significantly greater in afferent compared to efferent arterioles [26]. The reason for this difference is, at the present time, unknown but could be related to differences in $[Na^+]_i$ or in the number or activity of the exchanger located in these two vascular segments. Also, recent work has shown differential $[Ca^{2+}]_i$ regulation between afferent and efferent arterioles. We have reported that afferent but not efferent arterioles functionally express voltage-gated Ca^{2+} channels [32]. The findings of the present studies may provide another example of differential $[Ca^{2+}]_i$ regulation between afferent and efferent arterioles.

In the present work, afferent arterioles dissected from hypertensive (9 wk) SHR (SBP = 150 mmHg) had a markedly attenuated increase in $[Ca^{2+}]_i$ when Na^+_e was reduced compared to 3 wk WKY and SHR and 9 wk WKY. In addition, there was no change in $[Ca^{2+}]_i$ responses obtained in the efferent arteriole of SHR and WKY at 3 and 9 wk of age, further suggesting that there are differences in Na^+:Ca^{2+} regulation between these two vascular sites. The results in afferent arterioles are consistent with studies in aortic rings removed from Dahl S and R rats fed high salt (8%) diet for 4 weeks [4]. In those studies, development of tension was utilized as an indirect assay of Na^+:Ca^{2+} exchange activity. Na^+:Ca^{2+} exchange activity, assessed by measurement of the rate of contraction elicited in a 1.2 mM Na^+ solution, was lower in aortic rings from hypertensive S rats compared to R rats [4]. However, the results of the present studies are in contrast with those found in aortic rings and isolated sarcolemmal membranes of SHR and WKY rats. In aortic rings, Ashida et al. observed a 2-fold greater rate of tension development in rings from SHR exposed to 1.2 mM bath Na^+ [3]. In another study, a slightly higher $^{45}Ca^{2+}$ uptake was noted in Na^+-loaded sarcolemmal membranes of SHR rats by Matlib and co-workers [25]. However, the level of Na^+-loading in SHR versus WKY sarcolemmal membranes (mesenteric arteries) was not determined in that study, potentially masking important differences in Na^+:Ca^{2+} exchange activity in these isolated membranes. These variable experimental findings likely reflect intrinsic differences in the regulation of Na^+:Ca^{2+} exchanger activity expressed in different blood vessels, and they underscore the importance of examining blood vessels that specifically determine vascular resistance.

To further evaluate Na^+:Ca^{2+} exchange activity in renal afferent and efferent arterioles, arterioles were Na^+ loaded by incubation in 1 mM ouabain for 15 mins. This maneuver resulted in increases in $[Na^+]_i$ and might be expected to cause an increase in $[Ca^{2+}]_i$ through the Na^+:Ca^{2+} exchanger by diminishing the transmembrane Na^+ gradient. Indeed, in studies of rabbit afferent arterioles, we found that incubation in

ouabain resulted in increases in $[Ca^{2+}]_i$ while Na^+_e was maintained at 150 mM [26]. However, in the present studies, we did not find a consistent increase in $[Ca^{2+}]_i$ during 15 mins of incubation with ouabain, despite significant increases in cytosolic Na^+. Although there were clear increases in $[Ca^{2+}]_i$ in at least some of the arterioles exposed to ouabain, these changes were not consistent within each age group or arteriolar vessel type. The reason that $[Ca^{2+}]_i$ did not consistently increase with exposure to ouabain may be related to the finding that ouabain in the rat is less effective in inhibiting Na^+,K^+-ATPase than it is in other species [45]. However, qualitative measurements of cytosolic $[Na^+]$ refute this possibility because Na Green cps increased $33.7 \pm 4.0\%$ in afferent and $37.3 \pm 1.9\%$ in efferent segments. In addition, the observation of stable $[Ca^{2+}]_i$ during ouabain suggests that voltage-activated Ca^{2+} channels are also quiescent during this maneuver. Also, in other studies, vsm or aortic rings were generally exposed to ouabain for periods of 30 min to one hour. Therefore, it is possible that we would have seen changes in $[Ca^{2+}]_i$ if the incubation time had been lengthened; and in fact, this was the case in both afferent and efferent arterioles. Ouabain exposure for 30' increased $[Ca^{2+}]_i$ in afferent segments by 43.3 ± 8.3 nM and 18.9 ± 6.0 nM in segments from efferent arterioles. However, we did find that ouabain had a large effect on Na^+:Ca^{2+} exchange activity in both SHR and WKY afferent and efferent arterioles. In both arteriolar segments exposed to ouabain, there was a 3 to 4 fold increase in the magnitude of change in $[Ca^{2+}]_i$ in response to a reduction in Na^+_e from 150 to 2 mM. These findings are consistent with other studies which found that ouabain induced contraction of vsm [46, 47]. In addition, prior incubation with ouabain enhanced ^{45}Ca uptake and increased development of wall force during reduced Na^+_e in cultured vsm cells from rat aorta [23] and isolated rat mesenteric arteries [21], respectively. The interesting finding of the present work is that the magnitude of the change in $[Ca^{2+}]_i$ with reduced Na^+_e was substantially less in afferent arterioles of hypertensive (9 wk) SHR. Ouabain also appeared to be less effective in increasing $[Ca^{2+}]_i$ at low Na^+_e in the efferent arteriole of

the 9 wk SHR. These data suggest that there was either a significant downregulation of Na^+:Ca^{2+} exchanger number or activity in the 9 wk SHR, or that ouabain was not as effective in increasing $[Na^+]_i$ in the 9 wk old SHR. This issue may be resolved by measuring $[Na^+]_i$ using fluorescent probes. However, it should be emphasized that a change of less than 1 mM $[Na^+]_i$ could account for the entire increase in $[Ca^{2+}]_i$, with reduced Na^+_e, in the presence of ouabain. Thus, it may not be possible to strictly correlate changes in $[Na^+]_i$ with changes in $[Ca^{2+}]_i$.

Nevertheless, these results demonstrate that the functional activity of the Na^+:Ca^{2+} exchanger was diminshed in the 9 wk old SHR either in the absence or presence of prior Na^+ loading. The most straightforward explanation for the differences in $[Ca^{2+}]_i$ responses is a reduction in Na^+:Ca^{2+} exchanger number or activity in this model of hypertension. In addition, this decrease in activity was more pronounced in afferent arterioles, which offers the possibility that the exchanger may be differentially regulated in these two segments. Finally, whether this alteration in Na^+:Ca^{2+} exchange activity contributes to the development of hypertension or is a consequence of the hypertensive state remains to be determined.

Na^+-Independent Experiments

A reduction in Ca^{2+}-sequestering and/or plasmalemmal Ca^{2+} extrusion capabilities could result in enhanced levels of $[Ca^{2+}]_i$ in vsm cells, leading to increased vascular resistance. In the present experiments, we examined Na^+-independent Ca^{2+} regulation by simply maintaining vessels in 2 mM Na^+_e after the peak increase in $[Ca^{2+}]_i$ had occurred (Figure 1). As mentioned previously, after the peak increase in $[Ca^{2+}]_i$ there was a decline of $[Ca^{2+}]_i$ toward baseline values. We analyzed the rate of decline in $[Ca^{2+}]_i$ and percent recovery of $[Ca^{2+}]_i$ in the continued presence of 2 mM Na^+_e and found that both the rate of recovery as well as the percent NaIRec were reduced in the afferent arterioles of 9 wk SHR. These studies, however, did not allow us

to determine if this alteration was due to decreases in Ca^{2+} sequesteration or Ca^{2+} extrusion.

Previous data concerning the function of ATP-dependent Ca^{2+} transport in genetic hypertension are limited, but in general suggest that Ca^{2+}-ATPase activity is reduced. Studies in platelets from human hypertensive subjects have shown that total Ca^{2+}-ATPase and plasma membrane Ca^{2+}-ATPase activity are inversely correlated with diastolic blood pressure [27]. With regard to vsm, mesenteric arteries obtained from SHR [28], deoxycorticosterone-salt hypertension [29], and renovascular hypertensives [30] have demonstrated a reduced ATP-dependent Ca^{2+} transport. Furthermore, aortic rings taken from Dahl S rats fed high salt (8%) showed significantly lower Ca^{2+}-ATPase activity than rings removed from R rats on high salt [4]. In these studies, the rate of ring relaxation in the continued absence of bath Na^+ (*i.e.* 1.2 mM Na^+) was taken as an index of the activity of Ca^{2+}-ATPase. In the present study, the rate of reduction in $[Ca^{2+}]_i$ (-dy/dt) in the presence of 2 mM Na^+_e was taken as suggestive of the activity of Ca^{2+}-ATPase. Further work will be required to determine whether the decrease in NaIRec is due to a reduction in Ca^{2+} sequestration and/or Ca^{2+} extrusion.

Because renal afferent arterioles demonstrated impaired Na^+-dependent and Na^+-independent $[Ca^{2+}]_i$ responses during alterations in the Na^+ transmembrane gradient, it was surprising that baseline $[Ca^{2+}]_i$ was not different in afferent and efferent arterioles from 3 and 9 wk SHR (Table 2). Previous measurements in aortic vsm cells of WKY and SHR, using lasermicrofluorospectrometry, have demonstrated elevated $[Ca^{2+}]_i$ in vsm of SHR [48]. One possible explanation concerns the fact that our studies were performed using an *in vitro* system. All arterioles were dissected free from surrounding renal parenchyma, thus removing these structures from the influence of an extraordinarily rich neural and humoral environment. Although the composition of the bathing solutions approximates that of plasma in terms of electrolytes, these solutions distinctly lack the high levels of catecholamines, angiotensin II and other ligands known

to be present in the renal interstitium *in vivo*. In addition, prior to conducting the experimental protocol, a minimum of 90 min had passed since removal of the kidney, which might tend to minimize differences in $[Ca^{2+}]_i$ in vessels from hypertensive versus normotensive animals.

Conclusions

Results from the present studies -- utilizing the transmembrane Na^+ gradient as a tool for eliciting changes in $[Ca^{2+}]_i$ -- are consistent with the operation of a Na^+:Ca^{2+} exchanger in renal afferent and efferent arteriolar segments. Exchange activity appeared to be greater in afferent versus efferent arterioles. In addition, increases in $[Ca^{2+}]_i$ during superfusion with 2 mM Na^+_e were enhanced by Na^+ loading with ouabain. Of primary significance was diminshed Na^+-dependent (Na^+:Ca^{2+} exchange activity) and Na^+-independent control of $[Ca^{2+}]_i$ observed in afferent arterioles from 9 wk old hypertensive SHR compared to 3 wk SHR and 3 and 9 wk old WKY rats. It is interesting to speculate that perhaps some common intracellular signalling pathway might be responsible for the reduction in activity of both of these Ca^{2+} regulatory pathways. Nevertheless, the reduced ability to regulate afferent arteriolar $[Ca^{2+}]_i$ *in vivo* might contribute to elevated renal vascular resistance and impairment of renal function in this model of hypertension.

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APPENDIX B

A NON-RADIOACTIVE FLUORESCENT METHOD FOR MEASURING PROTEIN KINASE C ACTIVITY

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Summary

The purpose of this study is to report the development of a non-radioactive fluorescent peptide assay for measuring protein kinase C activity (PKC). The assay is based on a glycogen synthase derived fluorescent peptide that is phosphorylated by PKC. Phosphorylation causes the peptide to migrate toward the anode while the non-phosphorylated peptide migrates toward the cathode during agarose gel electrophoresis. Quantitation of PKC activity can be accomplished by excision of the appropriate bands and measuring their relative fluorescence. Using this assay, PKC activity was measured in whole cell homogenates from cultured renal mesangial cells. The enzyme(s)-substrate system followed Michaelis-Menten kinetics under limited conditions and, therefore, Lineweaver-Burk plots were used to obtain Michaelis constant and maximum velocity values. An apparent K_M value of 40 μM was obtained for the fluorescent peptide substrate with a control V_{max} value of 300 pmol/min. Addition of phorbol 12-myristate 13-acetate increased V_{max} to 380 pmol/min.

Key words: protein kinase C, fluorescence spectroscopy, enzyme activity, phorbol esters, Michaelis-Menten kinetics, mesangial cells

Introduction

Protein kinase C (PKC) is a family of both Ca^{++} dependent and independent enzymes which appear to be involved in stimulating a wide variety of cellular processes (1). Activated PKC associates with cell membranes where it is capable of phosphorylating seryl and threonyl residues (2). Until now the most common method for assaying this enzyme(s) involved measuring the transfer of radioactive phosphate to a protein or peptide (see 7, 11, 17, 18). While this method is effective, the use of $\gamma^{32}P$ renders the assay potentially hazardous. It also suffers from the indiscriminate phosphorylation of susceptible proteins (most of which remain unidentified) resulting in increased background interference (3). The method we report, uses a fluorescent peptide (rhodamine labeled P^1-L-S-R-T-L-S^7-V-A-A-K) which changes from a net positive

charge to a net negative charge when phosphorylated by PKC. Phosphorylated and non-phosphorylated peptides can then be separated by agarose gel electrophoresis and quantified by fluorescence spectroscopy. Using this method for measuring PKC activity, Michaelis-Menten parameters were calculated from initial velocity measurements. PKC activity was quantified in cultured mesangial cells. Although this study examined PKC activity in whole cell homogenates, this method was equally effective when using other cellular fractions and should be applicable to other tissue types.

Methods

Cell Culture. Kidneys from Dahl/John Rapp SR/Jr rats were aseptically removed and glomerular mesangial cells were isolated and cultured as previously described with minor modifications (4, 5). In brief, renal cortical tissue was minced and forced through a 212 μm copper sieve (VWR, Philadelphia, PA). Tissue was then resuspended in 15 ml of a sterile modified Ringer's buffer containing 5.4 mM KCl, 0.5 mM K_2HPO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 140 mM NaCl, 1 mM $CaCl_2$, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid), 0.4 mM Na_2HPO_4, 5.0 mM dextrose, 5 mM l-alanine, 82 units/ml penicillin, 82 μg/ml streptomycin (Sigma, St. Louis, MO) and 2 μg/ml amphotericin B (Gibco BRL, Grand Island, NY). To separate glomeruli from extraneous tissue, the suspension was passed through four progressively smaller sterile nylon sieves (Tetko Inc.,Briar Cliff Manor, NY) ranging from 315 μm to 150 μm. A final 75 μm sieving collected the glomeruli. Glomeruli were then washed into a sterile 15 ml centrifuge tube, treated with 21 units/ml collagenase (Sigma) for 30 minutes, and plated onto 60 mm petri dishes (Falcon/B-D, Lincoln Park, NJ). Cells were grown in RPMI 1640 media containing 240 μg/ml l-glutamine (Gibco BRL) supplemented with 17% (v/v) fetal bovine serum (Mediatech, Herndon, VA) and antibiotics as above for 21 days in humidified 95% air / 5% CO_2 at 37°C. The media was changed twice weekly. Cells were then subcultured and plated onto 100 mm petri dishes (Costar, Cambridge, MA) and were grown in the above media for another 21 days prior to use.

Tissue Preparation. Mesangial cells from > 80% confluent 100 mm petri dishes were chilled in a shallow ice bath. Plates were then rinsed twice with ice cold buffer consisting of 100 mM HEPES, 50 mM Tris-HCl, with a pH of 8.0 (HT). Cells from several (4 or 5) plates were removed by scraping, resuspended in ice cold HT buffer, transferred to a 50 ml conical tube and centrifuged at 5000 x g for 10 minutes. Cells were resuspended in HT buffer and recentrifuged to insure removal of growth medium. A small volume (≈1 ml) of a viscous buffer, consisting of 35 mM Tris-HCl, 50 mM HEPES, 1 mM DTT, 10 mM K_3PO_4, 25% (v/v) glycerol, and a pH of 8.0 (VB), was used to resuspend the pellet after final washing with HT buffer. The suspension was twice subjected to 800 psi N_2 for 5 minutes in a bomb cell disruptor (Kontes, Vineland, NJ). Cell membranes were ruptured by quickly releasing the pressure.

Reactions. Reactions were carried out in 1.5 ml centrifuge tubes containing 5 µl of a reaction buffer (100 mM HEPES, 6.5 mM $CaCl_2$, 5 mM DTT, 50 mM $MgCl_2$, 5 mM ATP), with the addition of 5 µl of 4.2 mg/ml 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] and 5 µl of homogenate. Sufficient VB buffer was added to yield a final reaction volume of 25 µl after the reaction was initiated by addition of varying amounts of 400 ng/ml peptide substrate of sequence P^1-L-S-R-T-L-S^7-V-A-A-K (Promega, Madison, WI). Samples of homogenate were frozen for later protein content determination using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). The reaction tubes were capped, briefly vortexed, and immediately placed in a 30°C water bath for 60 seconds. The reaction was stopped by submerging the reaction tube in a dry ice/acetone bath. To ensure denaturation of all enzymes (including peptidases and proteases) frozen samples were boiled for 3 minutes. Twenty microliters of each sample were loaded onto an 0.8% agarose gel. The gel was submerged in 50 mM Tris-HCl at a pH of 8.0 and subjected to 150 V for 40 minutes at 4°C in a horizontal gel electrophoresis apparatus (Unistar Scientific, Birmingham, AL). After electrophoretic separation, the gel was photographed under UV light using Polaroid 667 film with a red filter. To ensure reproducible sample

size, bands containing the phosphorylated peptide were excised using a 1 x 0.5 cm rectangular brass punch yielding ≈0.25 cc sections of gel. Gel sections were added to 1.5 ml tubes containing 750 µl of an aqueous solution consisting of 0.01% (w/v) SDS, 5.0% (v/v) EtOH, and 15.0% (v/v) CH_3COOH. The tubes were tightly sealed in a "beaker buddy" (USA Sci., Ocala, FL) and boiled until the gel dissolved.

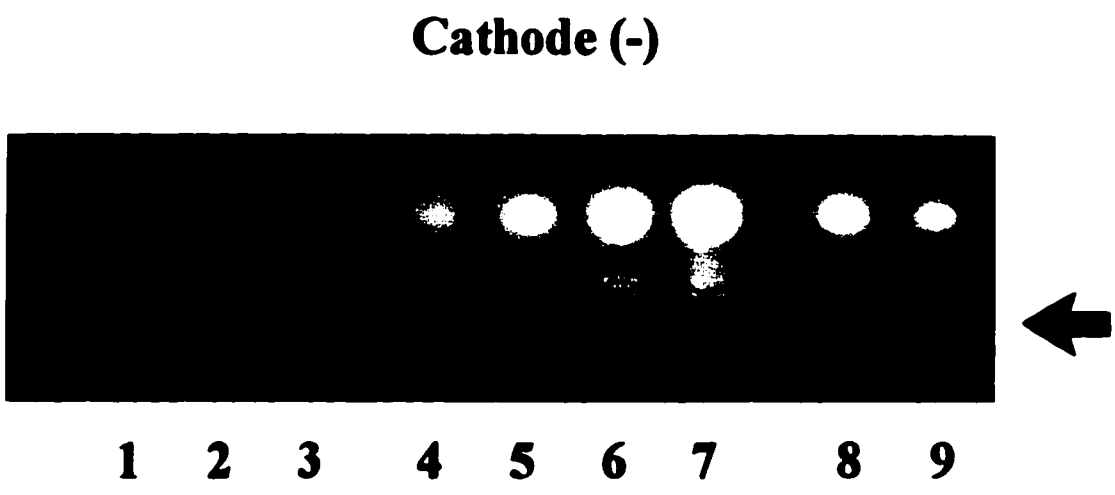
The fluorescent intensity of the phosphorylated peptide in each of the sample tubes was assayed by adding the dissolved gel to 2.0 ml of water in a UV grade 4.5 ml methacrylate cuvette (Fisher, Pittsburgh, PA). Fluorescence was measured at an excitation λ of 568 nm, an emission λ of 592 nm, and a PMT setting of 700 V using a Deltascan 1 spectrofluorometer (Photon Technologies International, South Brunswick, NJ). Absorbance values for total protein determination were measured using a micro-sample 300 spectrophotometer (Gilford, Oberlin, OH).

Results

This assay utilized a fluorescent peptide substrate to measure PKC activity. Figure 1 is a photograph of an agarose gel showing that products from a 1 minute reaction separated into three distinct bands. A neutral product was observed that stayed in the well and may be impurities in the substrate since it was observed in both the negative and positive controls where purified PKC was the only enzyme present. Substrate alone migrated toward the cathode, as shown in lane 8, while addition of purified PKC, shown in lane 9, resulted in a migration of phosphorylated substrate toward the anode. Lanes 1-7 show the separated products from preparations of equal protein concentration with increasing substrate concentration.

To acquire linear data, it was necessary to minimize peptidase and protease activity. For this reason a short reaction time was required. Plots of velocity as a function of reaction time demonstrated non-linear substrate phosphorylation for reaction times > 2 minutes

Fig. 1. This figure depicts the reaction products produced by PKC contained in mesangial cell homogenates as separated by agarose gel electrophoresis (see Methods section). The photograph clearly shows that non-phosphorylated substrate migrates toward the cathode while the phosphorylated peptide migrates toward the anode (see arrow). Wells 8 and 9 represent samples containing no PKC (negative control) and 0.25 µg purified PKC, respectively. Lanes 1-7 represent the separated products from a 1 minute incubation at 30°C of preparations having constant protein with increasing substrate concentration.



(Figure 2). To permit phosphorylation of the substrate by PKC without significant destruction of product by endogenous peptidases and proteases and to ensure sustained linear initial velocity, reaction time of 1 minute was chosen. Longer reaction times yielded multiple products of various net charges and mobility on agarose gel.

The asymptotic behavior of velocity for the enzyme/substrate system varied with reaction protein concentration which demonstrated the dependence of initial velocity on active PKC concentration.

Lineweaver-Burk plots of kinetic data, obtained for homogenized mesangial cells, yielded a V_{max} value of 300 pmol/min (Figure 6). A V_{max} value of 380 pmol/min was obtained for cells incubated in the presence of 100 nM PMA for 15 minutes prior to tissue preparation by the method described. An apparent K_M of 40 mM was obtained for the fluorescent peptide substrate.

Figure 3 illustrates a gel in which preparations of constant protein and substrate concentrations were incubated for 1, 2, 3, 4, and 5 minutes at 30°C were separated. The separation revealed four distinct bands. A fourth highly mobile negatively charged product lay farthest toward the anode. Formation of this product was proportional to reaction time and may have resulted from the destruction of phosphorylated product by proteolytic enzymes.

In order to obtain kinetic data on PKC activity, a calibration curve was constructed. Linear calibration data was observed for the non-phosphorylated substrate over a range of 0 to 60 ng/ml (Figure 4). Fluorescent intensity values from recovered phosphorylated product were compared to the calibration curve and the intensity values were corrected to reflect the dilution factor and yield reaction concentrations. Total protein concentrations were determined by the method of Bradford (6) and reflect reaction concentrations. Velocities were corrected for differences in protein concentration and expressed in units of μmol/min/(mg protein).

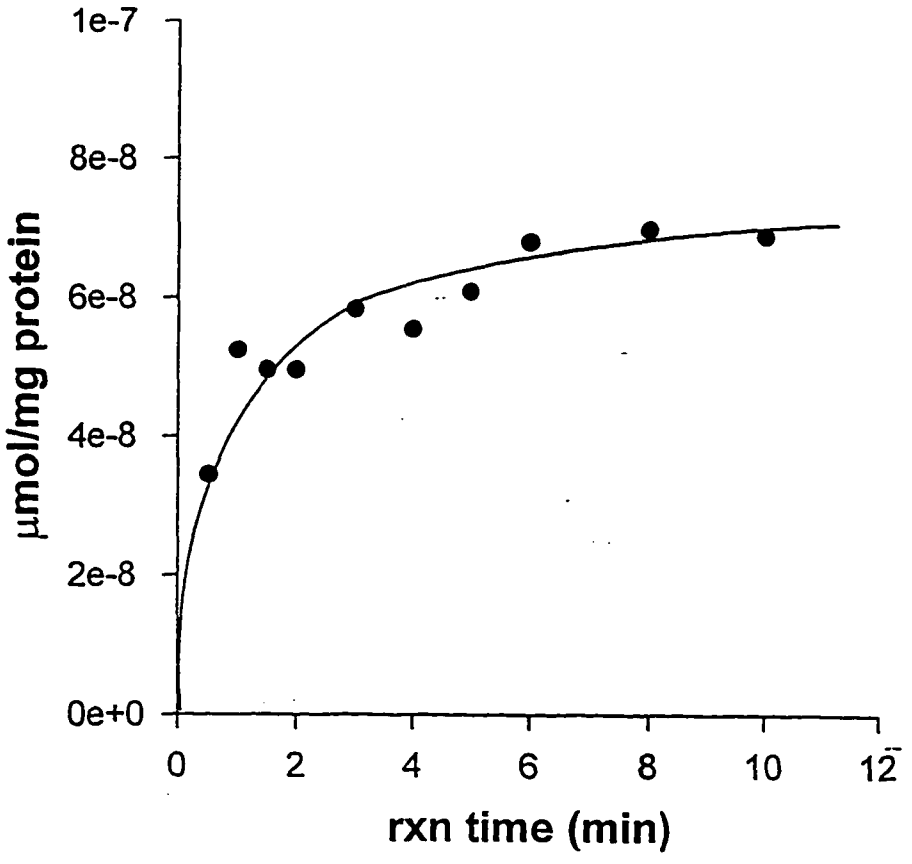
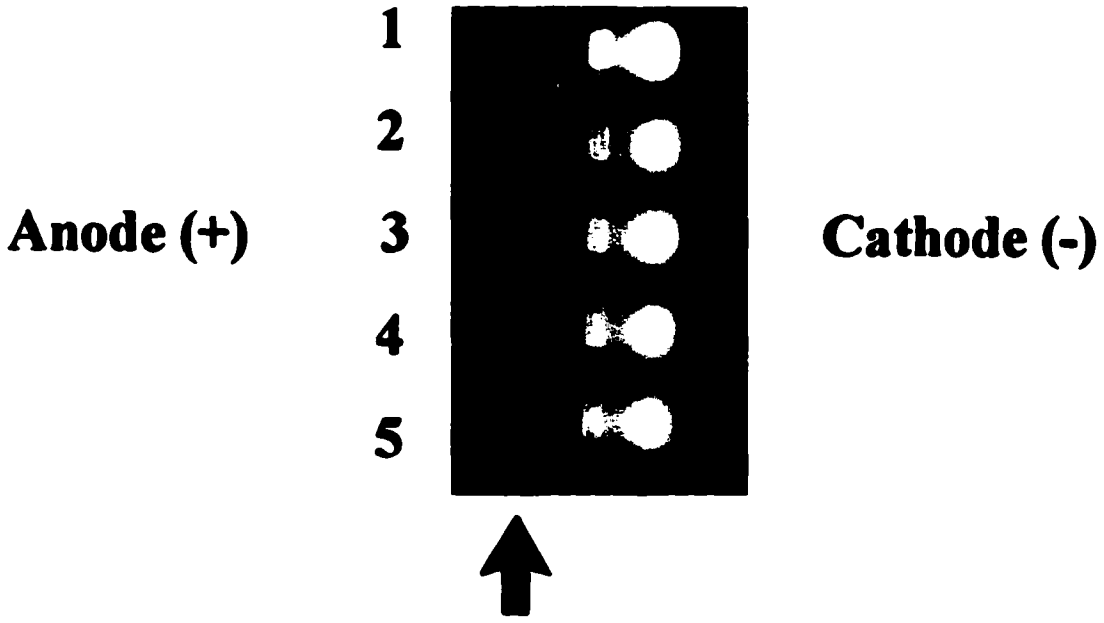


Fig. 2. A plot of velocity vs. reaction time for the products of reactions having 2.2 mg/ml protein and 32 ug/ml substrate, demonstrating a deviation from 1st order kinetics as reaction time increased. Reaction velocity began to plateau at ≈6 min due to product destruction by proteolytic enzymes. First order kinetic approximations for crude samples using this substrate require low reaction times (<2 min).

Fig. 3. Photograph of an agarose gel in which products from preparations, where reaction time was varied from 1 to 5 minutes (lanes 1-5 respectively), were separated. Reactions contained constant protein and substrate concentrations. As shown by the arrow this figure clearly indicates the formation of additional phosphorylated products with increased reaction time.



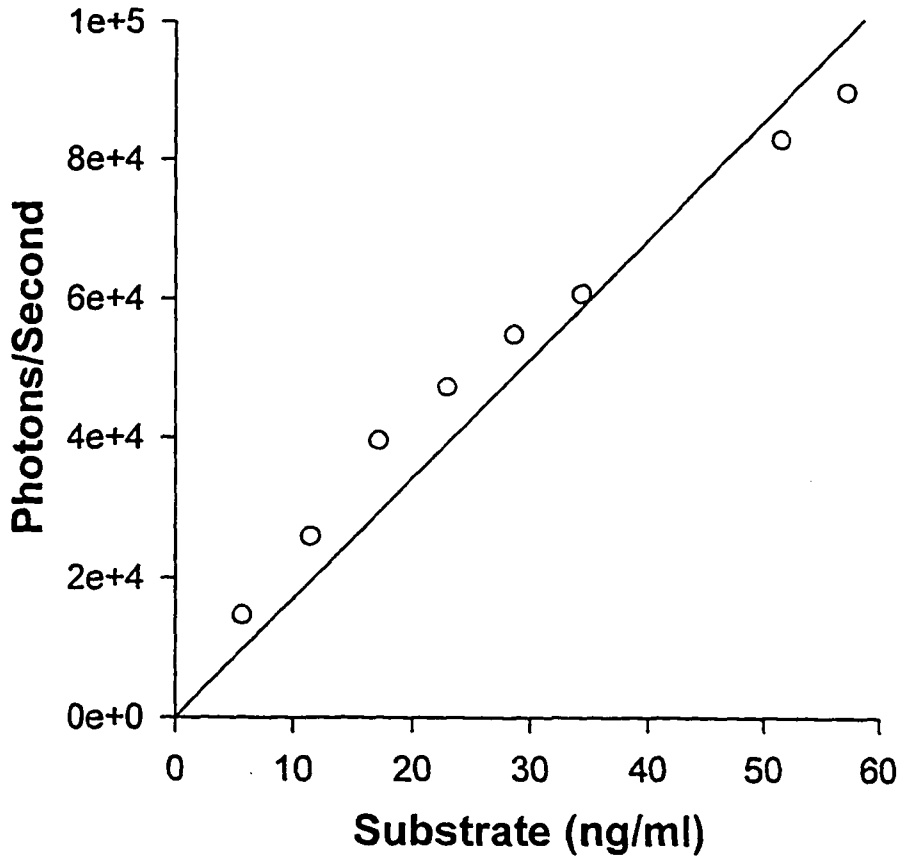


Fig. 4. Shown is a calibration curve for the fluorescent substrate over the range of 0 to 60 ng/ml. The curve fits a first order regression and yields an R value of > 0.95.

Michaelis-Menten kinetics were observed when velocity was plotted as a function of substrate concentration (i.e. the reaction reached a maximum velocity, indicating enzyme saturation (Figure 5)).

Discussion

Conventional methods for measuring PKC activity have involved the transfer of radioactive phosphate. There have been successful attempts to lower background radiation levels in methods employing $\gamma^{32}P$, but little has been achieved in eliminating side products generated by extraneous phosphorylation of endogenous proteins in biological samples (7). This extraneous phosphorylation elevates radioactive emission and subsequently misrepresents kinase activity. The method described here circumvents the pitfalls of $\gamma^{32}P$ methods since fluorescence spectroscopy does not suffer from large non-systematic background interference and by allowing the control and/or evaluation of side products. The fluorescence method also benefits from agarose gel electrophoresis which provides virtually total product separation. Side reaction products not eliminated by altering reaction conditions could be accounted for by their mobility in the agarose gel, thus eliminating the need for enzyme purification prior to analysis.

The kinetic behavior of PKC in whole cell homogenates has, until recently, been difficult to measure. In the present work we used a commercially available glycogen synthase derived fluorescent peptide substrate that is phosphorylated by PKC in a 1:1 ratio (8). The peptide substrate consisted of an eleven amino acid sequence, P^{1}-L-S-R-T-L-S^{7}-V-A-A-K, with a rhodamine moiety on the sixth amino acid from the N-terminal end, and a molecular weight of 1684. Phosphorylation of the substrate occurs at the seventh amino acid (serine) and changes the net charge on the peptide from positive to negative (9, 10). Phosphorylated and non-phosphorylated peptides can be separated by electrophoresis with the phosphorylated peptide migrating toward the anode and nonphosphorylated peptide migrating toward the cathode.

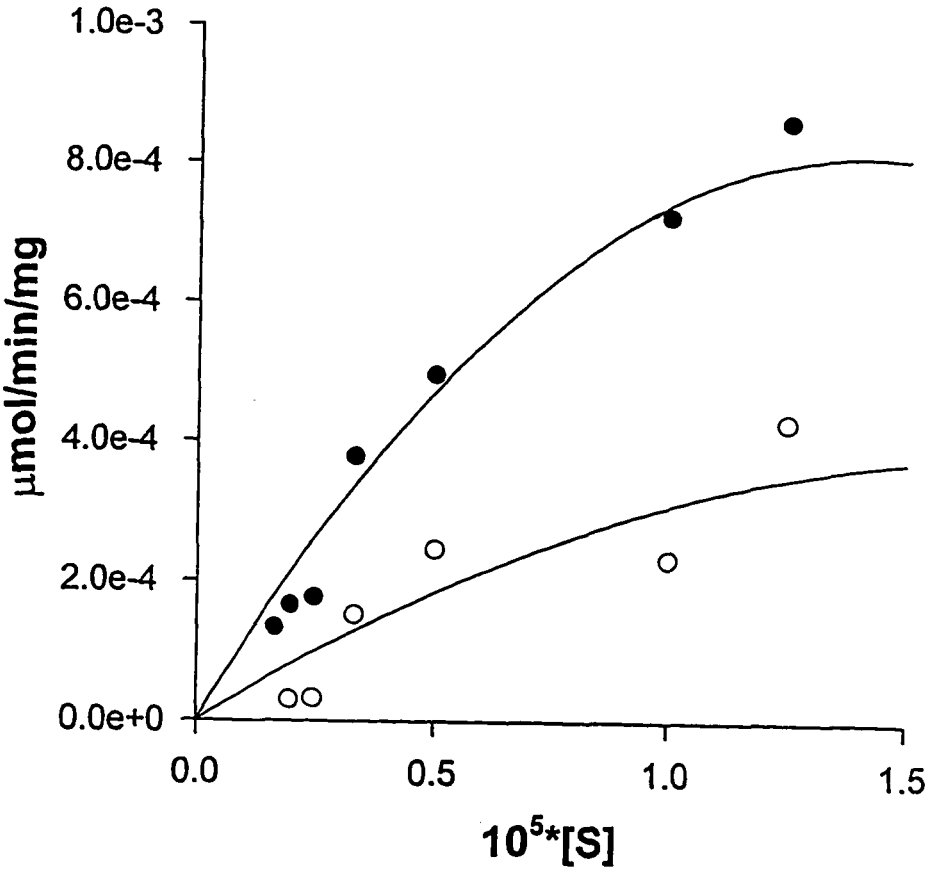


Fig. 5. A plot of velocity vs. substrate (2nd order regression, SigmaPlot 1.02) demonstrating that initial velocity was dependent on protein concentration and that the reactions approached a maximum velocity, thus proceeding by Michaelis-Menten kinetics. Closed circles represent homogenate at 0.3 mg/ml protein and open circles represent 0.15 mg/ml protein.

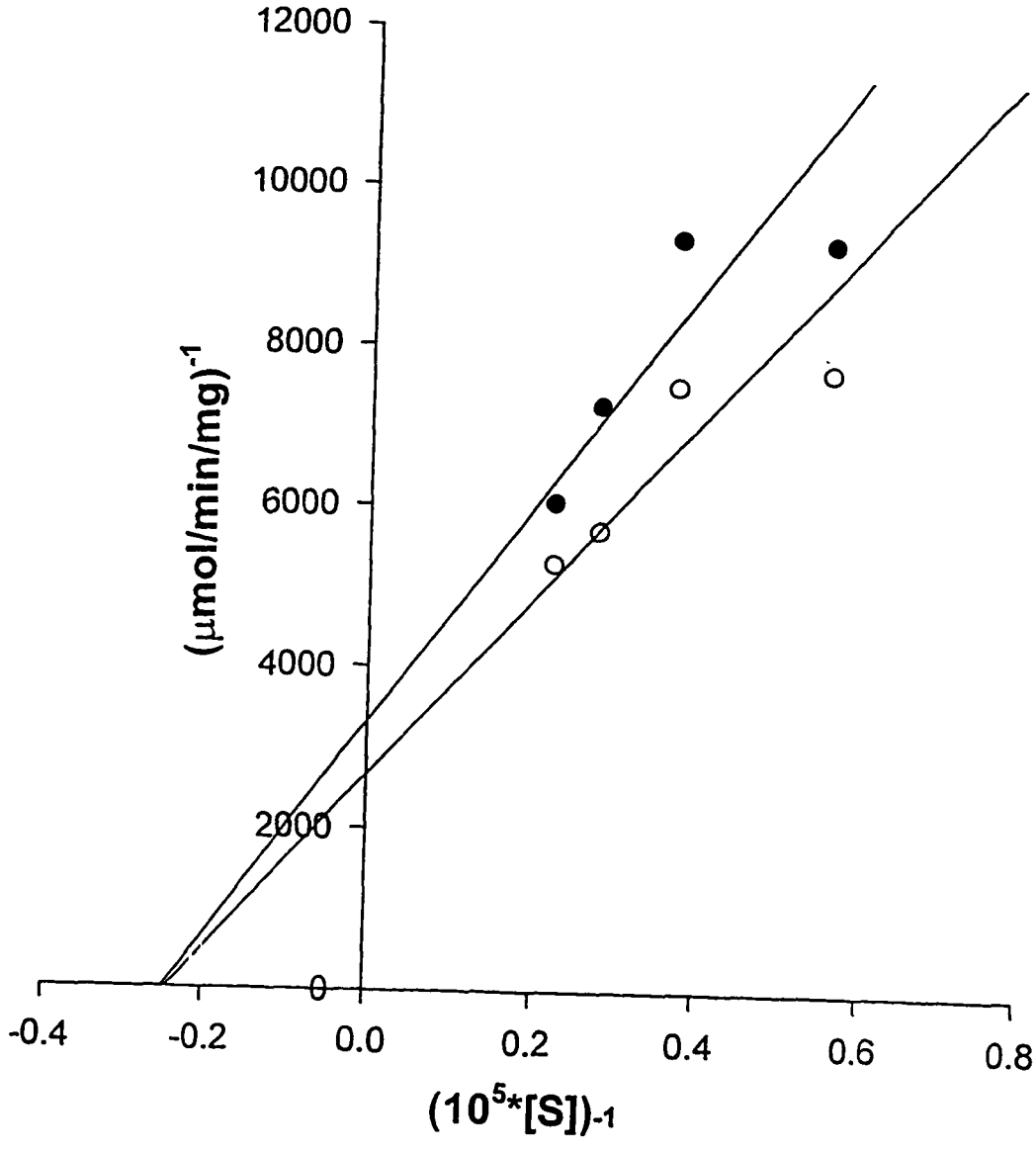


Fig. 6. Lineweaver-Burk plots for the control tissue and tissue exposed to 100 nM PMA (see Methods). These curves demonstrate phorbol ester stimulated PKC activity in mesangial cells. The apparent K_M from the data was 40 μM while the V_{max} for the control was 300 pmol/min (filled circles) and 380 pmol/min (open circles) for the stimulated tissue (n = 4).

The apparent K_M obtained by this fluorescent method agrees with a previously reported value of 33 μM obtained through a method which utilized $\gamma^{32}P$ labeled phosphate transfer to a similar glycogen synthase substrate (11). In addition to the ability of the fluorescent assay to accurately reproduce K_M values, this method has the advantage of notable sensitivity demonstrated by the detection of V_{max} differences of tens of pmol/min. The shape of the curves for the 1/V vs. 1/[S] plots (i.e. hyperbolic, see Figure 6) was not unexpected since some years ago Walter (12), Reiner (13), and Dixon and Webb (14) discussed the difficulties in analyzing a reaction mixture which contained more than one enzyme acting on the same substrate - each having different reaction rate constants. They concluded that theoretically if $K_{M_1} \neq K_{M_2} \neq K_{M_n}$ then a Lineweaver-Burk plot of such a reaction would show a hyperbolic relationship rather than the usual linear relationship. In the case of PKC, the literature reports at least 10 isoforms of this enzyme (15). Consequently, K_M values for mesangial cells can only be approximated at high substrate concentrations (16).

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