

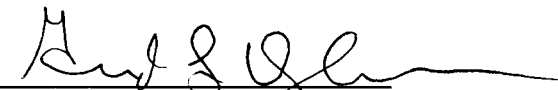
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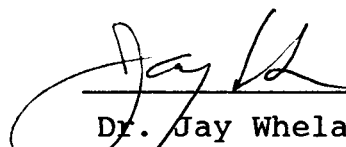
I am submitting herewith a dissertation written by Young-Cheul Kim entitled "Insulin Regulation of Vascular Smooth Muscle Calcium Metabolism and Ca^{2+} -ATPase Gene Expression". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

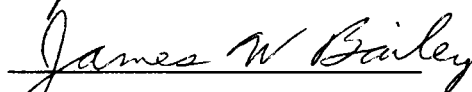


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Insulin Regulation of Vascular Smooth Muscle
Calcium Metabolism and Ca^{2+} -ATPase Gene Expression

A Dissertation
Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Young-Cheul Kim

May, 1995

DEDICATION

This dissertation is dedicated to my loving family who have given love, support, sacrifice and understanding to me. It is also dedicated to the memory of my father.

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Young-Cheul Kim

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Abstract

Insulin Regulation of Vascular Smooth Muscle Calcium Metabolism and Ca^{2+} -ATPase Gene Expression

by

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May, 1995

Adviser: Michael B. Zemel, Ph.D.

Major: Nutrition

Degree: Doctor of Philosophy

Recent evidence suggests that hypertension in insulin resistant states may result from impaired insulin regulation of vascular smooth muscle cell (VSMC) Ca^{2+} transport.

Insulin has previously been shown to attenuate vasoconstrictor responses to pressor agonists and accelerate vascular smooth muscle (VSM) relaxation and VSM Ca^{2+} efflux. To further determine the role of insulin in regulating VSMC Ca^{2+} , quiescent A7r5 cultured VSMC and human VSMC loaded with fura 2-AM were incubated with or without 10^{-7} or 10^{-8} M insulin for 1 hour; intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) responses to and rates of recovery from angiotensin II (AII) (200 nM) and arginine vasopressin (AVP) were studied fluorometrically in stirred suspension. Insulin caused an increase in the peak $[\text{Ca}^{2+}]_i$ response to AII (Peak/baseline $\times 100 = 469 \pm 96$ versus

288±74, $p<0.05$) and a decrease in the peak Ca^{2+} response to AVP (288±50 versus 389±33, $p<0.025$). However, insulin also caused a marked increase in the rate of $[\text{Ca}^{2+}]_i$ recovery to baseline after stimulation with both AII (77.3±13.8 versus 30.6±6 nM/min, $p<0.03$) and AVP ($p<0.05$), such that the cumulative exposure to elevated $[\text{Ca}^{2+}]_i$ after stimulation with either agonist (i.e., area under the $[\text{Ca}^{2+}]_i$ response curve) was reduced with insulin treatment. Insulin also caused comparable effects on Ca^{2+} recovery in the human cells but was without significant effect on peak Ca^{2+} responses to AVP. It is concluded that accelerated removal of cytosolic Ca^{2+} after agonist stimulation is likely to contribute to insulin attenuation of vasoconstrictor responses and acceleration of vascular relaxation. To evaluate the linkage between this insulin-regulation of VSMC $[\text{Ca}^{2+}]_i$ and classical actions of insulin (i.e. glucose transport and metabolism), cultured VSMCs were incubated in the presence or absence of insulin in a medium containing either pyruvate, glucose, 3-O-methylglucose (3-O-MG) or 2-deoxyglucose (2-DG). Insulin caused a 87% increase in $[\text{Ca}^{2+}]_i$ recovery rate following stimulation with AVP ($p<0.01$) and caused a marked increase in both plasmalemma and sarcoplasmic reticulum Ca^{2+} -ATPase gene expression in the presence of glucose. Comparable increases in both $[\text{Ca}^{2+}]_i$ recovery and Ca^{2+} -ATPase expression were found when glucose was replaced by 2-deoxyglucose. In contrast, no

stimulation was found in either the glucose-free or 3-O-methylglucose-containing medium. Since both glucose analogues are transported, but only 2-DG is phosphorylated, this indicates that glucose transport and metabolism to glucose-6-phosphate is essential for insulin regulation of VSMC $[Ca^{2+}]_i$, possibly via a glucose-6-phosphate-dependent carbohydrate response element in the Ca^{2+} -ATPase gene.

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ABBREVIATIONS

Angiotensin II	AngII
Arginine vasopressin	AVP
Vascular smooth muscle	VSM
Vascular smooth muscle cell	VSMC
Intracellular Ca^{2+} concentration	$[Ca^{2+}]_i$
Inositol 1,4,5-triphosphate	IP ₃
Calmodulin	CaM
Protein kinase C	PKC
Myosin light chain	MLC
Myosin light chain kinase	MLCK
Plasma membrane	PM
Sarcoplasmic reticulum	SR
Insulin dependent diabetes mellitus	IDDM
Non-insulin dependent diabetes mellitus	NIDDM
Hepes-buffered salt solution	HBSS
2-deoxyglucose	2-DG
3-O-methylglucose	3-O-MG
Dulbecco's modified eagles medium	DMEM
Fetal bovine serum	FBS
Fetal calf serum	FCS
Fura-2-acetoxymethoxy ester	Fura-2/AM
Guanidine isothiocyanate	GTC

PART I

INTRODUCTION

Part I. Introduction

A considerable body of evidence suggests that insulin resistance and/or compensatory hyperinsulinemia serves as a causative factor in the pathogenesis of hypertension (Welborn et al. 1966, Ferrannini et al. 1987, Reaven 1991, Modan 1985, DeFronzo and Ferrannini 1992, Levy et al. 1989). Presently, it is not clear whether insulin resistance or hyperinsulinemia is responsible, as insulin resistance generally causes hyperinsulinemia. Hyperinsulinemia may contribute to hypertension via a direct stimulation of sympathetic neural activity (Rowe et al. 1981, Anderson et al. 1991 & 1992) and renal sodium retention (Andres and Crabbe 1966, DeFronzo et al. 1975, Baum 1987). However, recent findings suggest that impaired cellular responses to insulin, rather than hyperinsulinemia *per se*, lead to increased vascular smooth muscle (VSM) tone characteristic of hypertension in insulin resistant states (Zemel et al. 1992b & 1993).

Insulin appears to have a direct vasodilatory effect on vascular smooth muscle, including rat aorta (Zemel et al. 1990, 1991b & 1992a, Abel and Zemel 1993), rabbit efferent renal arteriole (Juncos et al. 1992), canine femoral artery (Kahn et al. 1993), and rat mesenteric resistance arteries (Touyz and Schiffrin 1994, Tuoyz et al. 1994). Moreover, insulin has been found to attenuate *in vitro* vascular

reactivity responses to vasoconstrictor agonists in endothelial-denuded aortic strips (Rocchini 1991, Zemel et al. 1990), and to accelerate endothelium-independent relaxation after vasoconstriction, whereas these effects are blunted in aortic strips from insulin resistant (Zucker obese) rats (Zemel et al. 1991b). In addition, insulin increases forearm blood flow and decreases vascular resistance during concomitant norepinephrine infusion in humans (Nearhring et al. 1993), reduces the forearm vasoconstriction (Lembo et al. 1992), and causes a dose-dependent increase in dorsal hand-vein compliance in phenylephrine pre-constricted vessels (Feldman and Bierbrier 1993). Further, pharmacological correction with pioglitazone, ciglitazone or metformin, all of which augment peripheral insulin sensitivity, results in reduced blood pressure (Dubey et al. 1993, Pershadsingh and Kurtz 1992, Morgan et al. 1992, Kemnitz et al. 1994).

Insulin has been demonstrated to regulate intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) by affecting many transmembrane cation transport systems such as Na^+/K^+ -ATPase, Na^+/H^+ exchanger, and Ca^{2+} -ATPase (Lytton et al. 1985, Levy et al. 1990, Moore 1981, Putnam 1985, Tirupattur et al. 1993, Zemel et al. 1992, Wong et al. 1988, Aviv and Livne 1988). Since regulation of intracellular Ca^{2+} is critical to maintenance of normal vascular tone and contractility, alterations in cell Ca^{2+} regulation due to

the lack of stimulation of these pumps in insulin resistant or insulinopenic state could contribute to increased vascular smooth muscle tone and hypertension. Although vasodilatory action of insulin is not fully understood, insulin could exert its effects on vasoactive agonist-induced VSM cell (VSMC) $[Ca^{2+}]_i$ responses, including angiotensin II, arginine vasopressin, and norepinephrine (Blatter and Wier 1992, Orlov et al. 1993, Berridge 1993).

Physiological concentrations of insulin has been shown to stimulate vascular relaxation and VSM Ca^{2+} efflux in a dose-dependent manner (Zemel et al. 1992a) and attenuate $[Ca^{2+}]_i$ transient responses to angiotensin II, arginine vasopressin and norepinephrine in VSMCs isolated from rat mesenteric artery (Touyz et al. 1994). Zemel et al. (1993) recently showed that insulin stimulates both plasmalemma and sarcoplasmic reticulum Ca^{2+} -ATPase gene expression in cultured rat VSMCs, while this insulin effect is depressed in VSMCs from insulin resistant (Zucker obese) rats (Zemel et al. 1991a & 1992a).

Accordingly, it is likely that hypertension in insulin resistant states results from VSMC resistance to insulin-mediated vasodilation. Therefore, the current studies are intended to explore the role of insulin in regulating VSMC Ca^{2+} metabolism. Specifically, the overall objective of this dissertation is to :

1. Determine whether insulin attenuates $[Ca^{2+}]_i$ responses to vasoconstrictor agonists in cultured rat VSMC and human pulmonary artery VSMC preparations.
2. Determine whether insulin-regulation of VSMC $[Ca^{2+}]_i$ is linked to the "classical" actions of insulin (i.e., regulation of glucose transport and metabolism).

Literature Cited

- Abel, M.A., Zemel, M.B. (1993) Impaired recovery of vascular smooth muscle intracellular calcium following agonist stimulation in insulin resistant (Zucker obese) rats. *Am J Hypertension*. 6:500-504.
- Anderson, E.A., Hoffman, R.P., Balor, T.W., Sinkey, C.A., Mark, A.L. (1991) Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J. Clin. Invest.* 87, 2246-2252.
- Anderson, E.A., Balor, T.W., Hoffman, R.P., Sinkey, C.A., Mark, A.L. (1992) Insulin increases sympathetic activity but not blood pressure in borderline hypertensive humans. *J. Clin. Invest.* 19, 621-627.
- Andres, R., Crabbe, J. (1966) Stimulation by insulin of active sodium transport across toadskin: influence of aldosterone and vasopressin. *Arch. Int. Physiol. Biochim.* 74, 538-540.
- Aviv, A., Livne, A. (1988) The Na^+/H^+ antiport, cytosolic free Ca^{2+} and essential hypertension: A hypothesis. *Am. J. Hypertension*. 1, 410-413.
- Baum, M. (1987) Insulin stimulates volume absorption in the rabbit proximal convoluted tubule. *J. Clin. Invest.* 79, 1104-1109.
- Berridge, M.J. (1993) Inositol triphosphate and calcium signalling. *Nature* 361, 315-325.
- Blatter, L.A., Wier, W.G. (1992) Agonist-induced $[\text{Ca}^{2+}]_i$ waves and Ca^{2+} -induced Ca^{2+} release in mammalian vascular smooth muscle cells. *Am. J. Physiol.* 263, H576-H586.
- DeFronzo, R.A., Cooke, C., Andres, R., Faloona, G.R., Davis, P.J. (1975) The effect of insulin in renal handling of sodium, potassium, calcium, and phosphate in man. *J. Clin. Invest.* 55, 845-855.
- DeFronzo, R.A., Ferrannini, E. (1991) Insulin resistance: A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia and atherosclerotic cardiovascular disease. *Diabetes Care* 14, 173-194.
- Dubey, R.K., Zhang, H.Y., Boegehold, M.A., Kotchen, T.A., (1993) Pioglitazone attenuates hypertension and inhibits growth of arteriolar smooth muscle in rats. *Am. J. Physiol.* 265, R726-732.

- Feldman, R.D., Bierbrier, G.S. (1993) Insulin-mediated vasodilation: impairment with increased blood pressure and body mass. *Lancet* 342, 707-709.
- Ferrannini, E. (1992) Insulin and blood pressure : possible role of haemodynamics. *Clin. Exp. Hypertens. A.* 14, 271-285.
- Juncos, L.A., Ito, S., Carretero, O.A. (1992) Disparate effects of insulin on isolated rabbit afferent and efferent arterioles. *Hypertension* 20, 403.
- Kahn, A.M., Seidel, C.L., Allen, J.C., O'Neil, R.G. (1993) Insulin reduces contraction and intracellular calcium concentration in vascular smooth muscle. *Hypertension* 22, 735-742.
- Kemnitz, J.W., Elson, D.F., Roecker, E.B., Baum, S.T., Bergman, R.N., Meglasson, M.D. (1994) Pioglitazone increases insulin sensitivity, reduces blood glucose, insulin, and lipid levels, and lowers blood pressure in obese, insulin-resistant rhesus monkeys. *Diabetes* 43, 204-211.
- Lembo, G., Rendina, V., Lamenza, F., Iaccarino, G., Volpe, M., Trimarco, B. (1992) Insulin reduces forearm vasoconstriction evoked by reflex sympathetic activation in healthy humans. *Hypertension* 20, 409.
- Levy, J., Zemel, M.B., Sowers, J.R. (1989) Role of cellular calcium metabolism in abnormalities in glucose metabolism and diabetic hypertension. *Am. J. Med.* 87(suppl. 6A) 7-16.
- Levy, J., Sowers, J.R., Zemel, M.B. (1990) Abnormal Ca^{2+} -ATPase activity in erythrocytes of non-insulin-dependent diabetic rats. *Horm. Metab. Res.* 22, 138-142.
- Lytton, J., Lin, J.C., Guidotti, G. (1985) Identification of two molecular forms of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ in rat adipocytes: Relation to insulin stimulation of the enzyme. *J. Biol. Chem.* 260, 1177-1184.
- Modan, M., Halkin, H., Almog, S., et al. (1985) Hyperinsulinemia: a link between hypertension, obesity and glucose intolerance. *J. Clin. Invest.* 75, 809-817.
- Moore, R.D. (1981) Stimulation of $\text{Na}^+ : \text{H}^+$ exchange by insulin. *Biophys. J.* 33, 203-210.
- Morgan, D.A., Ray, C.A., Balor, T.W., Mark, A.L. (1992) Metformin increases insulin sensitivity and lowers arterial pressure in spontaneously hypertensive rats. *Hypertension* 20, 421.

- Neahring, J.M., Stepniakowski, K., Greene, A.S., Egan, B.M. (1993) Insulin does not reduce forearm alpha-vasoreactivity in obese hypertensive or lean normotensive men. *Hypertension*. 22(4), 584-590.
- Orlov, S., Resink, T.J., Bernhardt, J., Ferracin, F., Buhler, F.R. (1993) Vascular smooth muscle cell calcium fluxes: regulation by angiotensin II and lipoproteins. *Hypertension* 21, 195-203.
- Pershadsingh, H.A., Kurtz, T.W. (1992) Pharmacological reversal of hyperinsulinemia is associated with a reduction in blood pressure: A novel approach to treatment of hypertension. *Hypertension* 20, 410.
- Putnam, R.W. (1985) Effects of insulin on intracellular pH in frog skeletal muscle fibers. *Am. J. Physiol.* 248, C330-C336.
- Reaven, G.M. (1991) Insulin resistance and compensatory hyperinsulinemia: Role in hypertension, dyslipidemia, and coronary heart disease. *Am. Heart J.* 121, 1283-1288.
- Rocchini, A.P. (1991) Insulin resistance and blood pressure regulation in obese and non-obese subjects. *Hypertension* 17, 837-842.
- Rowe, J.W., Young, J.B. Minaker, K.L., Stevens, A.L., Pallotta, J., Landsberg, L. (1981) Effect of insulin and glucose infusion on sympathetic nervous system activity in normal man. *Diabetes* 30, 219-225.
- Tirupattur, P.R., Ram, J.L., Standley, P.R., Sowers, J.R. (1993) Regulation of Na⁺, K⁺-ATPase gene expression by insulin in vascular smooth muscle cells. *Am. J. Hypertension* 6, 626-629.
- Touyz, R.M., Tolloczko, B., Schiffrin, E.L. (1994) Insulin attenuates agonist-evoked calcium transients in vascular smooth muscle cells. *Hypertension* 23(Suppl.I): I-25-I-28.
- Touyz, R.M., Schiffrin, E.L. (1994) Insulin-induced Ca²⁺ transport is altered in vascular smooth muscle cells of spontaneously hypertensive rats. *Hypertension* 23(part 2): 931-935.
- Welborn, T.A., Breckenridge, A., Rubinstein, A.H., Dollery, C.T., Fraser, T.R. (1966) Serum-insulin in essential hypertension and in peripheral vascular disease. *Lancet* i,1336-1337.

- Wong, E.C., Sacks, D.B., Laurino, J.P., McDonald, J.M.
(1988) Characteristics of calmodulin phosphorylation by
the insulin receptor kinase. *Endocrinology* 123, 1830-
1836.
- Zemel, M.B., Iannucci, A., Johnson, B.A.B., Sowers, J.R.
(1991a) Reduced Ca^{2+} -ATPase expression in insulin
resistant (Zucker obese) rats. *Hypertension* 18, 442.
- Zemel, M.B., Johnson, B.A.B., Ambrozy, S.A. (1992a) Insulin-
stimulated vascular relaxation: role of Ca^{2+} -ATPase.
Am. J. Hypertension 5, 637-641.
- Zemel, M.B., Reddy, S., Shehin, S., Lockette, W., Sowers,
J.R. (1990) Vascular reactivity in Zucker obese rats.
Role of insulin resistance. *J. Vasc. Med. Biol.* 2, 81-85.
- Zemel, M.B., Reddy, S., Sowers, J.R. (1991b) Insulin
attenuation of vasoconstrictor responses to
phenylephrine in Zucker lean and obese rats. *Am. J.*
Hypertension 4, 537-539.
- Zemel, M.B., Peuler, J.D., Sowers, J.R., Simpson, L. (1992b)
Hypertension in insulin-resistant Zucker obese rats is
independent of sympathetic neural support. *Am. J.*
Physiol. 262, E368-E371.
- Zemel, M.B., Iannucci, A., Moore, J.W. (1993) Role of
insulin in regulating vascular smooth muscle Ca^{2+} -ATPase
expression. *J. Vasc. Med. Biol.* 4, 79-84.

PART II

LITERATURE REVIEW

Part II. Literature Review

A. Historical Perspectives of Intracellular Calcium

Signalling

Movement is a fundamental manifestation of animal life, and it is the diversity of muscles that makes possible such a broad spectrum of movements. In all muscle studied so far, muscle contraction appears to be basically an interaction of two proteins, actin and myosin, which is precisely controlled by the intracellular free calcium ion concentration ($[Ca^{2+}]_i$) (Ruegg 1992).

A requirement for Ca^{2+} during muscle contraction was first observed over 100 years ago, but only in the last 20 years has the role of Ca^{2+} as an important signalling molecule expanded to include nearly every eukaryotic cell. The importance of Ca^{2+} as a regulator of cell function was first recognized by Sidney Ringer (1883), who noted the requirement for Ca^{2+} in the incubation medium to maintain the contractility of the heart *in vitro*. Features of his work were later extended by others who envisaged the ion as having a regulatory role in cell metabolism (Lehninger 1950, Bygrave 1966 & 1967, Rasmussen 1970).

The concept that Ca^{2+} might serve as an intracellular messenger to couple stimulus to response in excitable tissues was developed largely in the 1960s. At the end of

that decade, the role of Ca^{2+} as a coupling factor had been established in three types of events: neurosecretion, skeletal muscle contraction, and cardiac muscle contraction (Ebashi et al. 1978, Campbell 1983). At approximately the same time, the second messenger concept of cyclic AMP (cAMP) was developed. In fact, it became evident that the molecular components of these two messenger systems were nearly universally present in all animal cells, and interacted in a variety of ways in regulating cell function (Rasmussen and Barrett 1984).

With the introduction of intracellular Ca^{2+} indicators, considerable insights have been obtained to measure dynamic, time-dependent changes in $[\text{Ca}^{2+}]_i$ (Ashley and Ridgeway 1970, Tsien et al. 1985, Cobbold and Rink 1987, Grynkiewicz et al. 1985). By their use, it was revealed that even though the rise in $[\text{Ca}^{2+}]_i$ is transient, there is a sustained alteration in the metabolism of Ca^{2+} in response to the extracellular signal: a sustained increase in the rate of Ca^{2+} influx into the cell (Mauger et al. 1985, Kojima et al. 1985). Since this sustained increase in Ca^{2+} influx rate leads neither to an increase in $[\text{Ca}^{2+}]_i$ nor in total cell calcium, it is concluded that there is an increased rate of Ca^{2+} cycling across the plasma membrane, and that this Ca^{2+} cycling has an important, spatially and temporally distinct messenger function (Rasmussen and Barrett 1984, Alkon and Rasmussen 1988). However, the

presence of appropriate plasma membrane-associated, Ca^{2+} -sensitive transducer(s) and/or response element(s) within the cell was also required in order for a change in plasma membrane Ca^{2+} cycling to alter cellular function.

Out of this line of research came the recognition that Ca^{2+} signalling was often intimately linked to the metabolism of the inositol phospholipids. (Berridge and Irvine 1984, Berridge 1985 & 1987, Nishizuka 1986, Rasmussen 1986). Accordingly, the discovery that inositol 1,4,5-triphosphate (IP_3) was the messenger linking hormone receptor interaction to intracellular Ca^{2+} release marked an important turning point in the development of this subject.

Changes in $[\text{Ca}^{2+}]_i$ brought about either by the mobilization from intracellular Ca^{2+} stores or by the influx of the ion from the extracellular medium, are the trigger of both short-term and long-term physiological responses in cells from a broad of tissues and species. Furthermore, aberrant control of Ca^{2+} fluxes appears to be associated with several pathological conditions, including both insulin resistance and hypertension.

B. Role of Ca^{2+} in Vascular Smooth Muscle Contractile Mechanism

The concentration of free Ca^{2+} in the cytosol ($[\text{Ca}^{2+}]_i$) is of crucial importance as a regulator of cell function. $[\text{Ca}^{2+}]_i$ is in dynamic equilibrium with calcium bound to the contractile and regulatory proteins (e.g. myosin, troponin, calmodulin), with extracellular calcium and with the calcium stored within cell organelles (i.e., sarcoplasmic reticulum, mitochondria). This tight regulation of $[\text{Ca}^{2+}]_i$ is fundamental to the Ca^{2+} signal transduction process whereby an extracellular stimulus is transduced into a cellular response. An elevation in $[\text{Ca}^{2+}]_i$ caused by hormonal or electrical signals can trigger responses as varied as contraction of muscles, cellular proliferation, secretion of hormones, clotting of blood, and changes in gene expression.

Activation of cell surface receptors by hormones or neurotransmitters initiates series of cellular processes leading to contraction of skeletal, cardiac and vascular smooth muscle (VSM). In smooth, skeletal and cardiac muscle, cytoplasmic calcium ions regulate contractile protein activation and tension generation. The cellular mechanisms that link surface membrane excitation with an increase in intracellular calcium and thus with the initiation of contraction are termed excitation-contraction coupling. Contractile activation in striated (skeletal and cardiac)

muscle is initiated primarily by membrane depolarization (electromechanical coupling). In smooth muscle, however, contraction can be initiated by both electrical stimulation and by binding of agonists to receptor sites on the surface membrane (pharmacomechanical coupling).

In skeletal muscle, a "sliding filament" model was proposed to account for muscle shortening (Huxley and Niedergerke 1954). According to this model, force generation is accomplished by attachment of the myosin heads (or cross-bridges) to F-actin filaments. This attachment occurs only in stimulated muscle, and it catalyzes ATP hydrolysis to generate tension. Regulation of the interaction between actin and myosin is mediated by two regulatory proteins, namely, troponin and tropomyosin. Troponin is bound to tropomyosin, which, in turn, binds actin. In a relaxed muscle, tropomyosin prevents actin-myosin interactions. However, when myoplasmic Ca^{2+} rises following stimulation of the muscle, Ca^{2+} binds troponin, allowing movement of tropomyosin to a position in which it does not interfere with cross-bridge formation between actin and myosin. Removal of Ca^{2+} results in a return of the tropomyosin to the blocking position for actin-myosin interactions and a consequent muscle relaxation.

The contractile apparatus of smooth muscle appears to also operate by means of a sliding-filament mechanism. But unlike striated muscle, the actin and myosin filaments are

not in register, and the regulation of actin-myosin interaction is also quite different. Smooth muscle contains a relatively greater content of actin and tropomyosin, and a lower content of myosin, than does skeletal muscle (Hartshorne and Gorecke 1980). Tropomyosin does not appear to be important in regulation of actin-myosin interaction (Hartshorne and Gorecke 1980); its function in smooth muscle is unclear but may involve stabilization of F-actin (Bennett and Weeds 1986). In contrast to skeletal muscle, where the major site of Ca^{2+} regulation resides on the actin filament, Ca^{2+} regulation of VSM acto-myosin resides with the myosin molecule (Hartshorne and Gorecke 1980). Myosin is the primary protein of smooth muscle thick filaments, and consists of two heavy chain subunits (approximately 200 kDa each) and two small subunits known as myosin light chains (20 and 17 kDa, respectively) (Hartshorne 1987). The head regions of light chain contain distinct sites for binding to actin, ATP hydrolysis, and association of light chain subunits.

Initial smooth muscle contraction in response to a stimulus is dependent upon a rise in intracellular Ca^{2+} from intra- and/or extracellular pools, and the calcium requirement exists early in the contraction cascade (Figure 2.1). Binding of various vasoconstrictor agonists such as norepinephrine, serotonin, angiotensin II or prostaglandin $\text{F}_{2\alpha}$ to VSM activates specific receptors and consequently

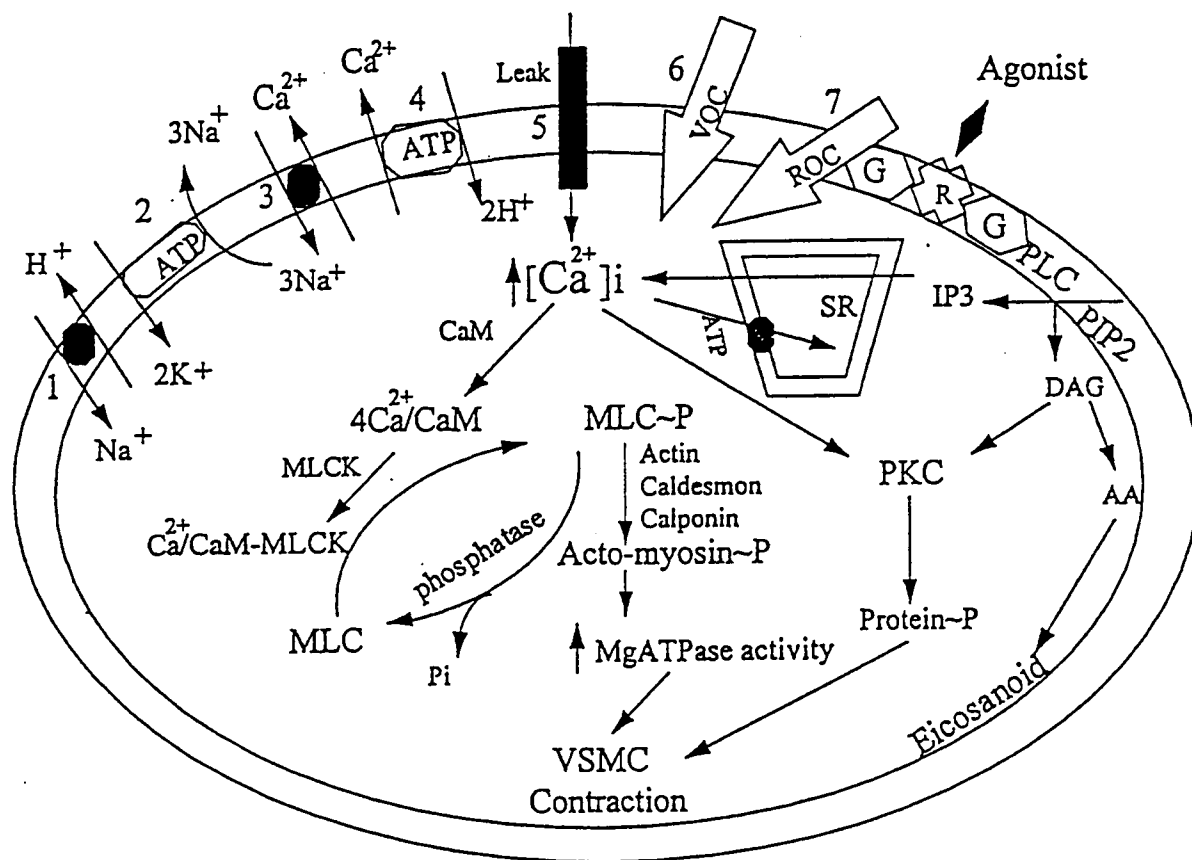


Figure 2.1 Schematic Representation of Cellular Calcium Regulation and Cellular Responses to Vasoactive agonists leading to Vascular Smooth Muscle Contraction.

1, Na^+/H^+ antiporter; 2, Na^+/K^+ -ATPase; 3, $\text{Na}^+/\text{Ca}^{2+}$ exchanger; 4, Ca^{2+} -ATPase; 5, leak channel; 6, voltage-operated channel; 7, receptor-operated channel.

Abbreviation: R, receptor; G, G-protein; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5 bisphosphate; DAG, 1,2-diacylglycerol; PKC, protein kinase C; IP₃, inositol 1,4,5-trisphosphate; SR, sarcoplasmic reticulum; CaM, calmodulin; MLC (K), myosin light chain (kinase); ~P, phosphorylation; AA, arachidonic acid; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; VSMC, vascular smooth muscle cell.

stimulates the breakdown of the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (Berridge and Irvine 1984, Rasmussen 1986, Rasmussen et al. 1987). The hydrolysis of phosphatidylinositol results in the formation of IP_3 and 1,2-diacylglycerol, and activation of protein kinase C (PKC) (Berridge and Irvine 1984, Rasmussen 1986, Rasmussen et al. 1987). The formation of these intermediate products results in the release of ionized calcium into the intracellular milieu from either intra- or extracellular stores. The water soluble IP_3 binds to a specific surface receptor of the sarcoplasmic reticulum and stimulates release of Ca^{2+} . Thus, IP_3 functions as an intracellular messenger that couples the activation of smooth muscle receptors to Ca^{2+} release from the sarcoplasmic reticulum (Somlyo 1985, Saida and van Breemen 1987).

When the $[Ca^{2+}]_i$ increases from resting concentrations of approximately 10^{-8} M to stimulated concentrations of 10^{-7} M to 10^{-5} M, Ca^{2+} bind to a 17-kDa calmodulin (CaM), a ubiquitous calcium-binding protein containing four Ca^{2+} -binding sites. CaM saturated with Ca^{2+} then binds to and modulate the activity of many cytoplasmic proteins, including Ca^{2+} /CaM-dependent myosin light chain kinase (MLCK, 130-150 kDa). Phosphorylation of the 20-kDa regulatory light chain at serine-19 by this Ca^{2+} /CaM-dependent MLCK results in conformational changes that affect

ATP hydrolysis and actin binding (Hartshorne 1987). This leads to an increase in actin-activated Mg^{2+} -ATPase with tension consequently developing from actin-myosin cross-bridge formation (Rasmussen et al. 1987).

When intracellular calcium drops below 10^{-7} M, Ca^{2+} dissociates from CaM, CaM dissociates from MLCK, and MLCK becomes inactive. Myosin light chain phosphatase activity, which is insensitive to calcium, then predominates, and myosin is dephosphorylated leading to relaxation. Although myosin light chain phosphorylation is generally agreed to play an important role in cross-bridge cycling and VSM contraction, it is not necessarily correlated with tension, and thus additional mechanisms by which calcium regulates smooth muscle contraction has been suggested.

Prolonged neuronal or hormonal stimulation of smooth muscle elicits increases in $[Ca^{2+}]_i$ and myosin light chain phosphorylation leading to contraction, which subsequently decrease to low levels during the sustained phase (Stull et al. 1991, Rembold 1992). Sustained stimulation of smooth muscle is associated with a decrease in $[Ca^{2+}]_i$ (Rembold and Murphy 1988), myosin light chain phosphorylation (Driska et al. 1981), maximal velocity of shortening and cross-bridge cycling rates (Dillon et al. 1981), and energy consumption (Krisanda and Paul 1983), whereas the developed force is still maintained. This has been termed "latch bridge", a cross-bridge which forms but either does not recycle or

recycles slowly with continuous generation of force (Murphy 1989). Although the lowering $[Ca^{2+}]_i$ might cause the "latching" of cross-bridges as suggested (Rembold and Murphy 1986, Siegman et al. 1985), it may not be the lowering Ca^{2+} concentration *per se* since myosin is dephosphorylated by a Ca^{2+} -independent protein phosphatase fairly rapidly in the absence of Ca^{2+} , while tension declines much more slowly. It seems, therefore, that dephosphorylation of attached cross-bridges at low Ca^{2+} level may be associated with latched state, since the release of ADP from myosin and cross-bridge detachment are blocked under these conditions (Butler et al. 1990). It is proposed that the dephosphorylated cross-bridge (latch bridge) detaches much slower than the phosphorylated cross-bridge and thus may be responsible for tension maintenance at low levels of myosin light chain phosphorylation (Hai and Murphy 1988). However, it is also possible that dephosphorylated bridges may also be cooperatively reattached to form a latch bridge (Somlyo and Himpens 1989).

In addition to myosin light chain phosphorylation, vertebrate smooth muscles appear to have actin thin filament-associated proteins, calponin and caldesmon, that may regulate the interaction between actin and myosin cross-bridges (Andrea and Walsh 1992, Rasmussen et al. 1987). Caldesmon is an actin- and calmodulin-binding protein of MW 93 kDa, found in both smooth muscle and nonmuscle cells,

which is probably the Ca^{2+} regulatory component of the thin filaments (Marston and Lehman 1985, Moody et al. 1990, Sobue and Sellers 1991). In the absence of Ca^{2+} , caldesmon binds to actin rather than CaM, thereby inhibiting the actin-activated Mg^{2+} -ATPase activity of phosphorylated myosin which otherwise leads to contraction. However, in the presence of Ca^{2+} , it binds to CaM instead of actin, such that its inhibitory action is reversed by Ca^{2+} /CaM (Sobue et al. 1988, Sobue and Sellers 1991, Marston 1989, Brenner et al. 1991). Moreover, the inhibition of actomyosin-ATPase activity by caldesmon is prevented by its covalent phosphorylation (Sutherland and Walsh 1989).

Recently, caldesmon from intact VSM was shown to be phosphorylated by mitogen-activated protein (MAP) kinase, suggesting that protein kinase C activation may regulate an early event in this kinase cascade, resulting in the activation of MAP kinase and the ultimate phosphorylation of caldesmon (Adam and Hathaway 1993).

Calponin is another actin- and calmodulin-binding protein of MW 34 kDa found exclusively in smooth muscle (Andrea and Walsh 1992, Winder and Walsh 1993). It binds to actin and tropomyosin and inhibits actin-activated Mg^{2+} -ATPase activity of phosphorylated smooth muscle myosin. Calponin can be phosphorylated by PKC and CaM kinase II, preventing the inhibition of Mg^{2+} -ATPase activity of myosin (Winder and Walsh 1993). Thus, when $[\text{Ca}^{2+}]_i$ increases, CaM-

dependent activation of MLCK and CaM kinase II results in myosin light chain phosphorylation and calponin phosphorylation, respectively. As a result of this, the activity of actomyosin-Mg²⁺-ATPase and cross-bridge cycling are increased, leading to smooth muscle contraction.

C. Ca^{2+} Transport Mechanisms in Vascular Smooth Muscle Cell

In the past 20 years, great advances have been made in our understanding of the mechanisms underlying the stimulus-contraction coupling in vascular smooth muscle. Smooth muscle cells, containing a multitude of receptors and electrically connected to one another via nexuses, can be activated either agonist application or membrane depolarization. As in skeletal and cardiac muscle, cytoplasmic Ca^{2+} ion concentration is the principal determinant of smooth muscle contractile activation and tone, as previously discussed. The cellular mechanisms that control $[\text{Ca}^{2+}]_i$ are complicated and not completely understood. However, there are two basic mechanisms by which extracellular stimuli activate cellular Ca^{2+} mobilization.

Depolarization of the vascular smooth muscle surface membrane, either through action potentials or graded depolarization, causes a rise in $[\text{Ca}^{2+}]_i$ and tension via a mechanism called electromechanical coupling (Johansson and Somlyo 1980). In some vascular smooth muscle, however, membrane depolarization does not appear to be a prerequisite for contraction (Somlyo, A.P. and Somlyo 1968). For instance, norepinephrine may elicit contraction of rabbit ear arteries without any change in membrane potential (Droogmans et al. 1977), and in coronary arteries

acetylcholine may elicit contraction without causing depolarization (Ito et al. 1979) in the absence of the endothelium. If the endothelium is present, however, stimulation by acetylcholine or anoxia causes relaxation without alteration of the membrane potential (Furchgott 1983). These examples of non-electric or pharmacomechanical coupling (Somlyo, A.V. and Somlyo 1968) between the cell membrane and the contractile structures demonstrate that surface membrane depolarization is not always essential in smooth muscle contraction.

1. Surface membrane Ca^{2+} channels

The roles played by surface membrane Ca^{2+} channels in the activation of contraction vary considerably with muscle types. In skeletal muscle, Ca^{2+} currents do not contribute significantly to the generation of the action potential and membrane flux of Ca^{2+} ion is not necessary for contractile protein activation. In contrast, Ca^{2+} current through L-type channels in the surface membranes of cardiac cells contributes significantly to action potential shape and calcium influx is a critical step linking membrane depolarization to muscle activation. In VSM, surface Ca^{2+} channels contribute significantly to cell excitability and act as a pathway through which extracellular Ca^{2+} can enter the cytoplasm, leading to muscle activation.

There are two distinct extracellular Ca^{2+} entry pathways across the sarcolemmal membrane; one activated by membrane depolarization (voltage-gated channels) and the other activated by the agonist interaction with its receptor (receptor-operated channels) (Khalil et al. 1990, Best et al. 1991). In addition to these two pathways, mechanical stretch of smooth muscle may also stimulate Ca^{2+} entry by opening another type of channel, referred to as a stretch-activated channel (Bevan et al. 1982). Furthermore, isotopic $^{45}\text{Ca}^{2+}$ flux studies demonstrated a transmembrane Ca^{2+} influx which existed in the absence of stimulation by depolarization, agonists, or stretch but was sensitive to blockade by lanthanum and was referred to as the leak channel (Cauvin et al. 1983). The patch-clamp technique (Hamill et al. 1981) applied to VSM studies has provided further evidence for the notion of voltage-gated channels, receptor-operated channels, and calcium leak channels. Consequently, calcium ions may cross the cell membrane through different types of calcium channels which may be distinguished by their dependence on the membrane potential and their different susceptibility to calcium channel blocking drugs, such as verapamil, nifedipine and diltiazem (Fleckenstein 1983, Sperelakis and Caulfield 1984).

2. Intracellular Ca^{2+} Regulation and Homeostasis

$[\text{Ca}^{2+}]_i$ is controlled by the interaction of cytosolic calcium buffers and three categories of transport proteins (channels, carriers, and pumps) expressed differentially on the plasma membrane and on the membranes of calcium stores.

In resting smooth muscle the $[\text{Ca}^{2+}]_i$ determined with Fura-2 (Williams et al. 1985, Pritchard and Ashley 1986) and by calcium-sensitive electrodes is in the range of approximately 50-200 nM (0.1-0.2 μM) while the extracellular free Ca^{2+} level is normally at millimolar concentrations (about 1200 μM). This is accomplished by extrusion of Ca^{2+} across the plasma membrane and by sequestration of Ca^{2+} within the cell either by pumping Ca^{2+} into intracellular components or binding of Ca^{2+} to cell structures and cytosolic proteins (Somlyo 1985) (Figure 2.1).

In most cells, extrusion of Ca^{2+} from the cell is performed primarily by two transport systems of the plasma membrane: the calcium pump (Ca^{2+} -ATPase) (Carafoli et al. 1989 & 1990, Dean 1989, Furukawa et al. 1989, Inesi and Kirtley 1990) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Sheu and Blaustein 1986, Lagnado and McNaughton 1990). There also exist intracellular compartments such as the sarcoplasmic reticulum (James et al. 1989) and mitochondria which sequester calcium via Ca^{2+} -pumping mechanisms (Gunter and Pfeiffer 1990).

Na⁺/Ca²⁺ Exchanger System

The Na⁺/Ca²⁺ exchanger, driven by the transmembrane Na⁺ concentration gradient maintained by the sodium pump (Na⁺/K⁺-ATPase), is voltage-dependent and transports Ca²⁺ out of the cell in exchange for Na⁺ influx with an apparent stoichiometry of 3Na⁺ : Ca²⁺ (Carafoli 1987, Borle 1981). The process was first demonstrated 20 years ago and was shown to occur in many cell types including vascular smooth muscle (Reuter et al. 1973). It has been described as a low-affinity, high-capacity transport system which can facilitate the efflux of large quantities of Ca²⁺ and consequently appears to be responsible for a large portion of Ca²⁺ efflux. Progress is constantly being made toward isolation, functional and molecular characterization of the plasmalemmal Na⁺/Ca²⁺ exchanger, and six distinct isoforms have been identified from rabbit brain, kidney and heart (Barzilai et al. 1984, Kofuji et al. 1994).

In vascular smooth muscle, Na⁺/Ca²⁺ exchange may contribute to Ca²⁺ extrusion, but the magnitude of its contribution is not well defined and appears to be small under physiologic conditions (Somlyo, A.P. and Somlyo 1986, Zemel et al. 1992). The significance of this process as a source of intracellular Ca²⁺ may be increased in pathologic states such as cardiovascular diseases, particularly hypertension (Blaustein and Hamlyn 1984).

Plasma Membrane Ca^{2+} -pump (PM Ca^{2+} -ATPase)

The plasmalemma Ca^{2+} -ATPase pump has been shown to have a high affinity but lower capacity for Ca^{2+} transport than the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, and it pumps Ca^{2+} out of the cell in exchange for two protons (i.e., Ca^{2+} out, 2H^+ in) (Zemel and Sowers 1990, Szasz et al. 1978). In most cells, the primary efflux pathway that increases its rate to compensate for agonist-induced changes in Ca^{2+} influx rate appears to be the Ca^{2+} -ATPase. The enzyme was discovered by Schatzmann (Schatzmann 1966) in the membrane of erythrocytes and its general reaction mechanism was related to that of the other P-type ion pumps (Katz and Blostein 1975, Knauf et al. 1974), i.e., to the formation of a phosphorylated intermediate (Pedersen and Carafoli 1987). The general, and highly simplified reaction mechanism of P-type ATPase is shown in Figure 2.2.

Basically it is assumed that these enzymes conserve the energy of ATP in a phosphorylated intermediate, and exist in two distinct conformational states, termed E1 and E2. At some step during the cycle, presumably during the $\text{E1-P} \Rightarrow \text{E2-P}$ transition, the ion is transported across the body of the ATPase to the other side of the membrane. The step of the reaction cycle during which the translocation of Ca^{2+} across the pump molecule occurs has not been fully established, although it clearly follows an aspartic acid

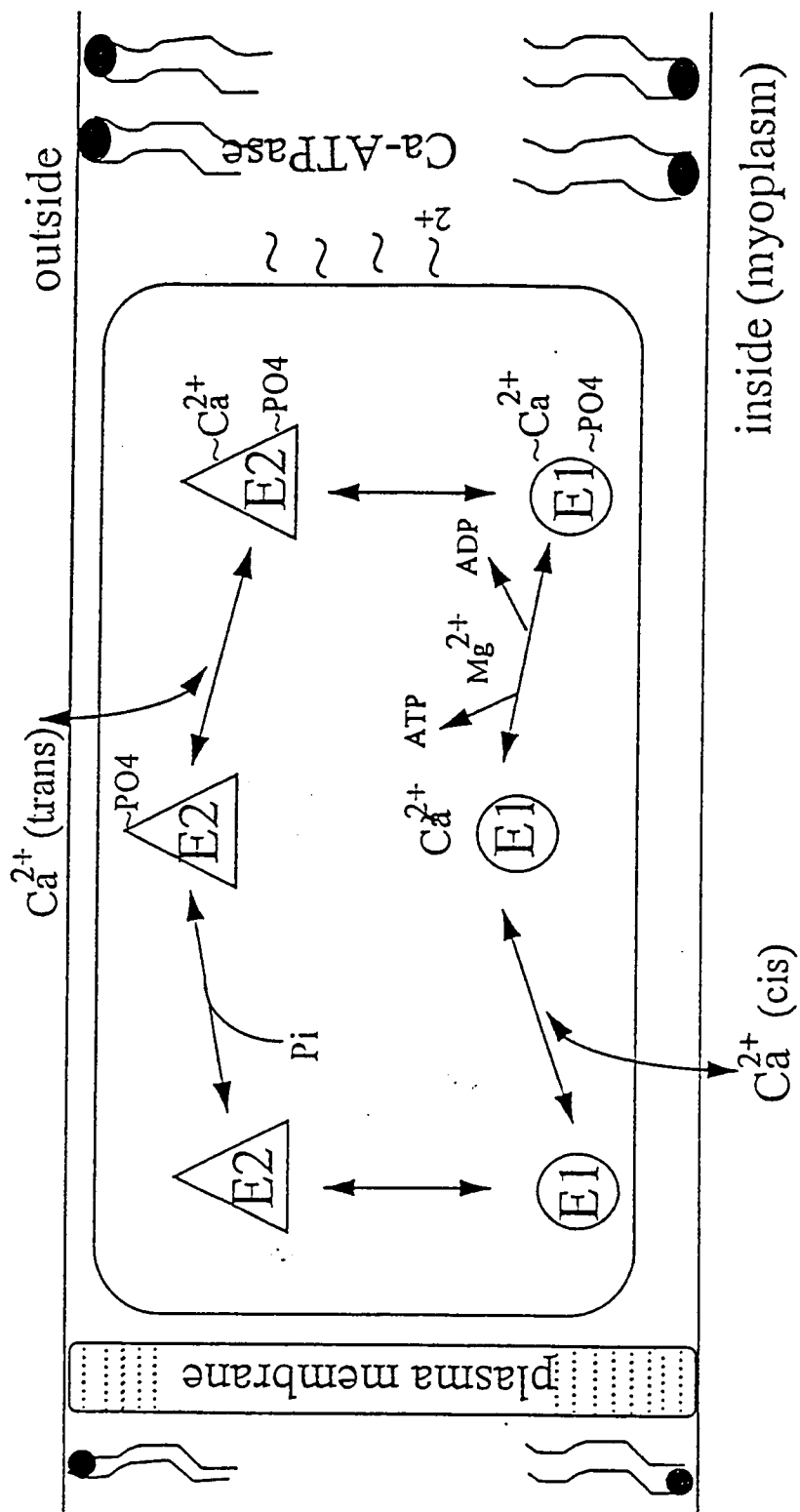


Figure 2.2 Reaction Cycle of Plasma Membrane Ca^{2+} -ATPase. Translocation process involves $\text{E1} \sim \text{PO}_4 \rightarrow \text{E2} \sim \text{PO}_4$ transition. Abbreviations: E, Ca^{2+} -ATPase; ~PO₄, phosphorylation.

phosphorylation step. Considerable evidence shows that in the E1-P state the Ca^{2+} - binding site is located on the cytoplasmic side of the membrane. It appears that the translocation of Ca^{2+} to the $\text{E1-P} \Rightarrow \text{E2-P}$ transition at the end of which the high affinity Ca^{2+} -binding site facing the interior of the cell becomes low affinity and faces the external side.

Related aspects of the reaction cycle are the effect of Mg^{2+} and the affinity for ATP. The phosphorylation of the pump is accelerated by Mg^{2+} (Rega and Garrahan 1975), which also increases the K_m of the pump for ATP (Rega and Garrahan 1975). Mg^{2+} , however, also accelerates the dephosphorylation of the phosphorylated intermediate, suggesting that Mg^{2+} and ATP act at different steps of the reaction cycle (Garrahan and Rega 1978).

In addition, calmodulin (CaM) activation of the pump was shown to be linked to the direct interaction of CaM with the ATPase, rather than to a kinase-mediated phosphorylation process (Niggli et al. 1979). The K_m of the pump for Ca^{2+} in the absence of CaM is approximately $1 \mu\text{M}$. This falls to $0.5\text{--}0.6 \mu\text{M}$ in the presence of saturating concentrations of CaM. Moreover, the V_{max} increases significantly in the presence of CaM (15- to 20-fold) (Smallwood et al. 1988). It is generally assumed that CaM increases the turnover of the pumping reaction by accelerating the rate of both the phosphorylation and the dephosphorylation of the pump

(Jeffery et al. 1981). Hence, the most immediate consequence of an increase in $[Ca^{2+}]_i$ is a Ca^{2+} -CaM-dependent activation of the Ca^{2+} -ATPase in the plasma membrane. This activation leads to a more efficient (low K_m) pump of considerably greater capacity (higher V_{max}), thus providing for a compensatory increase in the rate of calcium efflux.

The analysis of the primary structure of all plasma membrane Ca^{2+} -pump isoforms showed the general pattern and led to the identification of three important functional domains (Green 1989, Zvaritch et al. 1990, Olson et al. 1991): the domain binding fluorescein isothiocyanate, which is normally assumed to be part of the ATP-binding site; the domain surrounding the phosphorylated amino acid of the catalytic cycle, which is an aspartyl phosphate; and the calmodulin-binding domain.

Early studies have shown that smooth muscle plasmalemma Ca^{2+} pump is stimulated by CaM and inhibited by vanadate. The observation that vanadate causes maximal contraction of VSM suggests a major role of the Ca^{2+} -ATPase in the regulation of vascular tone (Rapp 1981). Similarly, certain agonists, such as oxytocin and prostaglandins have been shown to enhance their contractile activity through the inhibition of the Ca^{2+} extrusion pump (Popescu et al. 1984).

The plasmalemma Ca^{2+} -ATPase can be distinguished from other ATPases of plasmalemma and endoplasmic reticulum by its insensitivity to ouabain (distinction from Na^+/K^+ -

ATPase), high sensitivity to inhibition by vanadate (more sensitive than endoplasmic reticulum ATPase), sensitivity to K^+ (less sensitive than endoplasmic reticulum ATPase), and sensitivity to calmodulin antagonists (Popescu et al. 1984).

Defects of the plasma membrane Ca^{2+} -ATPase have been reported in a number of pathologic conditions. Several groups have described a decreased V_{max} of the pump in the sickle cell disease, but it appears that the defect is the consequence, rather than the cause, of the Ca^{2+} overload in sickled erythrocytes (Bookchin and Lew 1980, Gopinath and Vincenzi 1981, Niggli et al. 1982). The pump has also been shown to be defective in the red cells of spontaneously hypertensive rats (Vezzoli et al. 1985) where the CaM response of the pump was the major alteration. Similar alterations have also been found in platelets of human patients with essential hypertension (Resink et al. 1986) and erythrocytes of type II diabetic patients (Zemel et al. 1988 & 1990)

Sarcoplasmic Reticulum Ca^{2+} - Pump (SR Ca^{2+} -ATPase)

Since both the extracellular space and the sarcoplasmic reticulum (SR) contain Ca^{2+} at concentrations about 10,000 times higher than that in the cytoplasm, Ca^{2+} is rapidly delivered to the cytoplasm. Such a thermodynamic imbalance can be achieved only through the actions of

metabolically dependent transport processes. In most cells a variety of such processes all contribute to regulation of $[Ca^{2+}]_i$; these include the ATP- or Na^+ -dependent Ca^{2+} extrusion across the plasma membrane discussed previously, ATP- or substrate-dependent accumulation by the mitochondria, and another ATP-dependent Ca^{2+} sequestration by the sarcoplasmic reticulum (Blaustein 1985). The Ca^{2+} -ATPase of the SR is very abundant in the membranes of fast striated muscles, where it accounts for more than 50% of the total protein (Inesi 1972). The fast release and the rapid reaccumulation of Ca^{2+} by the SR trigger contraction and relaxation of the myofibrillar bundles.

Evidence for calcium sequestration by the SR came in early 1960s with the discovery of Hasselbach and Makinase (Hasselbach and Makinase 1961, 1964). They showed that preparations of vesicles inhibited myofibrillar contraction and ATPase activity by accumulating and storing calcium ions. In the presence of activating Mg^{2+} , ATP as energy source and sodium oxalate, calcium ions were actively pumped into the sarcoplasmic vesicles, where they were deposited as a calcium oxalate precipitate. In this way, the vesicles lowered the free calcium of the medium to below 10^{-8} M, a concentration at which ATP-contracted myofibrils would relax. The uptake rate of labelled calcium into these vesicles may quantitatively account for the rate of relaxation in frog muscle (Ford and Podolsky 1972). It has

been shown that by hydrolyzing one molecule of ATP two molecules of calcium are transported into the sarcoplasmic vesicles against a concentration gradient of several orders of magnitude (Meissner 1975).

As in the case of PM Ca^{2+} -pump, the SR Ca^{2+} -ATPase belongs to the family of the cation motive P-ATPases (Pedersen and Carafoli 1987) whose reaction cycle involves the formation and the cleavage of an acid-stable aspartyl phosphate intermediate. The reaction cycle of the vectorial Ca^{2+} transport from the cytosolic into the SR lumenal space is usually described according to models which contemplate a single major conformational change of the enzyme, from E2 to E1 in its nonphosphorylated form, and from the E1~P (ion) to E2~P (ion) in the phosphorylated form. Although some particular aspects differ (e.g., the stoichiometry of Ca^{2+} -transport sites, the electrogenicity, or the identity of the rate-limiting steps), the overall reaction mechanism is virtually identical to the Ca^{2+} -ATPase of plasma membrane (Carafoli 1991). The only difference between the two pumps is the prolongation of the C terminus of the PM Ca^{2+} -pump by a cytosolic portion which contains the regulatory domain. In addition, the PM Ca^{2+} -pump is regulated by direct interaction with calmodulin, while SR Ca^{2+} -pump is controlled by an accessory inhibitory (regulatory) protein phospholamban, which its hydrophilic N-terminal region shows more than 50% homology to the autoinhibitory calmodulin-

binding domain of the PM Ca^{2+} -pump. This pump is also affected by calmodulin, indirectly via its stimulation of phospholamban phosphorylation. When dephosphorylated, phospholamban interacts with the Ca^{2+} -pump and inhibits its ATPase activity (Kim et al. 1990). Moreover, a cAMP-dependent kinase has been shown to increase the rate of Ca^{2+} uptake of SR, and appears to be due to a phosphorylation of the 22 kDa protein phospholamban (Katz 1979).

Stimulation of smooth muscle by various agonists has been shown to produce a transient contractile response in the absence of extracellular Ca^{2+} or after the inhibition of Ca^{2+} influx by organic or inorganic Ca^{2+} antagonists (Cauvin et al. 1983), suggesting contribution of the intracellular Ca^{2+} stores to smooth muscle activation. Microsomal SR preparations showing energy-dependent Ca^{2+} uptake have been isolated from smooth muscle, and Ca^{2+} electrode studies of saponin-permeabilized smooth muscle have shown the operation of an ATP-dependent nonmitochondrial Ca^{2+} -pump that is blocked by vanadate (Somlyo, A.V. and Somlyo 1986).

It is now evident that SR plays the major physiologic role as the Ca^{2+} storage site and that mitochondria accumulate Ca^{2+} only when $[\text{Ca}^{2+}]_i$ is abnormally high. In skinned cultured aortic smooth muscle cells, the mitochondria do not accumulate Ca^{2+} when cytosolic Ca^{2+} is below 4 μM (van Breemen 1986). Further, the high endogenous Ca^{2+} content of mitochondria isolated from atherosclerotic

blood vessels may reflect the effects of VSM damage, and such cells containing Ca^{2+} -loaded mitochondria may become initial sites of vascular calcification (Cauvin et al. 1983). In certain pathological states, such as hypertension, the sarcoplasmic reticulum Ca^{2+} -ATPase may be altered (Resink et al. 1986, Carafoli 1991).

D. Pathophysiology of hypertension, obesity, and non-insulin dependent diabetes mellitus (NIDDM)

1. Insulin Action and Insulin Resistance

In 1889, Oskar Minkowski and Joseph von Mering first noticed that urine collected from pancreatectomized dogs attracted an inordinate number of flies due to its sweetness. This observation leads to the speculation that a substance produced by the pancreas is essential for metabolic control of blood sugar concentration (Mering and Minkowski 1980, Zeller et al. 1990). About 30 years later, the concept of an insulin-glucose axis as a key component of fuel homeostasis came into being in 1921 when Frederik Banting and his colleagues identified the active pancreatic principle as insulin, which, when absent, resulted in diabetes mellitus (McGarry 1992).

Insulin is a compact globular protein of molecular weight 6000 that is made up of two short polypeptide chains stabilized by two interchain disulfide bridges. Its characteristic secondary and tertiary structures are essential for its biological activity and are highly conserved in evolution. Insulin is released from the pancreatic β -cell and circulates free in the plasma. Insulin initiates its pleiotropic effects on cellular metabolism and growth by binding to a specific tetrameric glycoprotein

receptor located in the plasma membrane of target cells (Goldfine 1987, Rosen 1987). This receptor consists of two identical extracellular α -subunits ($M_r=130,000$ each) that contain binding sites and two identical transmembrane β -subunits ($M_r=95,000$ each) that have tyrosine kinase activity in their intracellular domains. Insulin binds to the receptor α -subunit which elicits a rapid autophosphorylation of specific tyrosine residues in the cytoplasmic domain of the β -subunit (Goldfine 1987, Yarden and Ullrich 1988). This is followed by activation of β -subunit tyrosine kinase activity which has been shown to play a key role in signal transduction by insulin (Rosen 1987, Kahn and White 1988). Among its important biological actions, insulin elicits rapid changes in the rates of membrane transport of molecules such as glucose and ions, activation or inactivation of numerous enzymes, and changes in the level of expression of many genes.

There are at least two types of diseases of this hormone response system. The first is known as type I, or insulin-dependent diabetes mellitus (IDDM) which generally appears before age 40, and results from destruction of insulin-producing β -cells (Eisenbarth 1986). The metabolic consequences follow diabetic ketoacidosis, coma, and death from insulin deficiency. The second disorder is called type II, or noninsulin-dependent diabetes mellitus (NIDDM), in which the primary defect lies at the level of the target

cells for insulin action. This is characterized not by deficiency but by the failure of the hormone to act efficiently in target tissues (insulin resistance) such as muscle, liver, and fat and by various degrees of β -cell dysfunction. In the United States, the prevalence of diabetes is estimated to be approximately 2 to 6% of the population. Of these, 10 to 25% fall into the category of IDDM while the remaining 75 to 90% of diabetic patients account for more common NIDDM (McGarry 1992). Unlike IDDM, NIDDM is often associated with obesity (Vionnet et al. 1992).

Insulin resistance is a pathological condition in which there is a shift in the dose-response curve such that the magnitude of the biological response to insulin is decreased (Kahn 1978). The insulin resistance is defined as impaired sensitivity to the effects of insulin on glucose utilization, and thus affects the non-oxidative (glycogen synthesis) and/or oxidative pathways of glucose metabolism by skeletal muscle (Golay et al 1988). In addition to obesity and NIDDM, essential hypertension (high blood pressure of unknown etiology) is commonly accompanied by insulin resistance and hyperinsulinemia (DeFronzo and Ferrannini 1991, Reaven 1991a & 1991b). Although the fundamental mechanisms underlying this insulin resistance are multiple, most cases are related to cellular abnormalities of the insulin receptor or postreceptor events

such as abnormalities in tyrosine kinase function or in the glucose transport effector system in target tissues (Garvey et al. 1991, Dohm et al. 1988).

2. Hypertension in Insulin Resistant States

Diabetes mellitus and hypertension are two of the most prevalent diseases in industrialized societies, contributing substantially to overall cardiovascular morbidity and mortality. There is clinical and epidemiological evidence for close interrelations between obesity, NIDDM, impaired glucose tolerance, hypertension and dyslipidemia (Modan et al. 1985, Reaven 1988, Zavaroni et al. 1989). Although the pathophysiological interactions among these established cardiovascular risk factors are not still completely understood, insulin resistance and compensatory hyperinsulinemia are proposed to serve as a common causative link (Modan et al. 1985, Reaven 1988, Zavaroni et al. 1989). The sensitivity and response of tissue to insulin are crucial factors in the regulation of carbohydrate metabolism, and, under certain pathophysiological conditions, affect cardiovascular complications and their etiology. Impaired sensitivity of insulin-stimulated glucose uptake leads to a rise in plasma glucose and thus to a compensatory stimulation of insulin secretion (hyperinsulinemia) to normalize blood glucose.

Insulin resistance and hyperinsulinemia are considered important risk factors contributing directly to the pathogenesis of hypertension, and there is a significant association between insulin resistance and hypertension. Moreover, this relationship persists even in non-diabetic, non-obese essential hypertensives who were not receiving antihypertensive drugs (Ferrannini et al. 1987, Modan et al. 1985, Sowers et al. 1991). Although insulin resistance and hyperinsulinemia are involved in the regulation of blood pressure, it is not clear whether this relation results from a direct manifestation of insulin resistance *per se* or hyperinsulinemia secondary to the defect in insulin action.

Substantial evidence shows that resistance to insulin-stimulated glucose uptake is a phenomenon associated with a cluster of symptoms, including impaired glucose tolerance, dyslipidemia, high blood pressure, leading to increased coronary heart disease risk. The existence of such a syndrome in which insulin resistance was the primary defect has been termed "Syndrome X" (Reaven 1988). The defect in insulin action was significantly correlated with a compensatory increase in plasma insulin concentration, enhanced hepatic VLDL-cholesterol secretion, increased plasma triglyceride concentration, decreased HDL-cholesterol concentration and hypertension (Olefsky et al. 1974, Tobey et al. 1981, Zavaroni et al. 1985, Reaven and Chen 1988).

Nevertheless, whether the linkage between insulin

resistance and hypertension is causal or consequential has been debated and still obscure. That insulin resistance develops as a result of hypertension is not tenable, since not all hypertensive patients are insulin resistant and blood pressure reduction in hypertensive patients does not uniformly improve insulin resistance (Reaven 1988). Accordingly, Reaven and Hoffman (Reaven and Hoffman 1987) proposed that hyperinsulinemia might be causally associated in the development of hypertension.

Several mechanisms have been proposed (DeFronzo and Ferrannini 1991) to explain this association. First, insulin directly activates sympathetic nervous system and releases catecholamines resulting in increased cardiac output, increased peripheral vascular resistance (Rowe et al. 1981, Rocchini 1991, Anderson et al. 1991 & 1992). Secondly, direct antinatriuretic action of insulin on the proximal and distal nephron results in reabsorption of sodium and water by the kidney, which then could contribute to volume-dependent hypertension (Andres and Crabbe 1966, DeFronzo et al. 1975, Baum 1987). Third, insulin may also stimulate Na^+ transport pathways such as the Na^+/H^+ antiporter systems, leading to intracellular alkalization and accumulation of Na^+ , thereby sensitizing the vasculature to various vasoactive agonists (DeFronzo and Ferrannini 1991). Finally, insulin has several direct effects on arterial tissue, causing structural changes in vasculature that could lead to

consequent increases in peripheral vascular resistance and hypertension (Stout 1990, Reaven 1991a & 1991b).

However, despite the potential role of hyperinsulinemia to increase peripheral vascular resistance, this mechanism has been challenged. Although hypertension in insulin resistant states has been generally attributed to hyperinsulinemia resulting in increased sympathetic neural outflow and renal Na⁺ retention, recent lines of evidence suggest a direct vasodilatory role of insulin which may be impaired in insulin resistance. Accordingly, it has been suggested that an impaired cellular response to insulin, rather than hyperinsulinemia is responsible for the increased vascular tone and reactivity which lead to hypertension. Consistent with this notion, Hall and colleagues (Hall et al. 1989 & 1991) found that hyperinsulinemia induced by chronic insulin infusion did not elevate blood pressure and peripheral vascular resistance despite observed sodium retention in normal dogs, hypertensive dogs and obese insulin resistant dogs. Moreover, in humans, chronic hyperinsulinemia and the resulting mild insulin resistance in patients with insulinoma is not associated with hypertension, and correction of hyperinsulinemia by removal of the insulinoma did not modify blood pressure (Sawicki et al. 1992). Recently, Zemel et al. (1992b) also reported that hypertension and exaggerated *in vivo* pressor sensitivity in

Zucker obese rats are independent of increased sympathetic neural support, as both persisted during ganglionic blockade. Similarly, acute or subacute insulin infusion have been found to reduce peripheral vascular resistance and blood pressure in normal and borderline hypertensive humans, respectively, in spite of the increased sympathetic neural activity (Anderson et al. 1991 & 1992), and insulin administration resulted in significant hypotension when the compensatory elevation in sympathetic nerve activity did not occur (Mathias et al. 1987). Further, insulin has been shown to attenuate *in vitro* vascular reactivity responses to vasoconstrictor agonists in both endothelium-denuded and endothelium-intact aortic strips (Zemel et al. 1990b & 1991b), while these effects were substantially lost in aortic strips from insulin resistant (Zucker obese) rats. Thus, these observations suggest that hyperinsulinemia *per se* does not induce hypertension, that insulin appears instead to exert a direct vasodilatory role.

Indeed, insulin has been shown to exert direct vasodilatory effects on vascular smooth muscle from several blood vessels, including rat aortic and human pulmonary artery (Kim and Zemel 1993, Zemel et al. 1990a, 1991a & 1993), canine femoral artery (Kahn et al. 1993), rabbit efferent renal arteriole (Juncos et al. 1992), and rat mesenteric resistance arteries (Touyz and Schiffrin 1994, Touyz et al. 1994). Further evidence demonstrated that

insulin increases forearm blood flow and decrease vascular resistance (Neahring et al. 1993), reduces the forearm vasoconstriction induced by reflex sympathetic activation (Lembo et al. 1992), and causes a dose-dependent increase in venous distensibility in phenylephrine pre-constricted vessels (Feldman and Bierbrier 1993). Treatment with pioglitazone (Dubey et al. 1993), ciglitazone (Pershadsingh and Kurtz 1992) or metformin (Morgan et al. 1992) which all amplify peripheral insulin sensitivity, results in attenuation of arterial pressure. Consequently, impaired VSMC response to insulin, rather than hyperinsulinemia, may be responsible for hypertension in insulin resistant states.

3. Insulin Attenuation of Peripheral Vascular Resistance : Role of $[Ca^{2+}]_i$ on Insulin Action

Enhanced peripheral vascular resistance and exaggerated vasoconstrictor response to various agonists have been implicated in the pathogenesis of hypertension in both IDDM and NIDDM (Sowers and Zemel 1990, Weidman et al. 1985). Chronic hyperinsulinemia has been shown to increase peripheral vascular tone and blood pressure, whereas acute or subacute insulin results in reduced blood pressure (Ferrari and Weidmann 1990, Baron et al. 1993, Ferrannini 1992, Mathias et al. 1987) as discussed previously. Insulin also attenuates vasoconstrictor responses to angiotensin II,

serotonin, norepinephrine, and KCl in rat mesenteric arteries and rabbit femoral vessels (Wambach and Liu 1992, Yagi et al. 1988). Exaggerated vasoconstrictor responses to vasoactive agonists have been reported in vessels from insulinopenic and insulin-resistant rats (Reddy et al. 1990, Shehin et al. 1989). Thus, insulin appears to have a "protective" effect on the vascular contractile responses to various vasoactive agonists and may thereby exert antihypertensive actions.

Although the underlying cellular mechanisms responsible for this phenomenon are unclear, altered VSM cation transport may play a key role in insulin regulation of agonist-induced responses. The acute insulin-induced rise in cytosolic Ca^{2+} is considered a possible mechanism for insulin stimulated glucose uptake (Pershadsingh et al. 1987, Draznin et al. 1987 & 1988). Maximal insulin sensitivity has been shown to be optimal within a certain range of intracellular Ca^{2+} . In cultured adipocytes, $[Ca^{2+}]_i$ either below or above an optimal range (140-370 nM) were found to blunt the transduction of the insulin signal (Draznin et al. 1987). Accordingly, alterations in cation transport could contribute to increases in VSM $[Ca^{2+}]_i$, which is a primary determinant of VSM contractility and tone.

Insulin modulates $[Ca^{2+}]_i$ by stimulating many membrane cation transport systems, including Na^+/K^+ -ATPase, Na^+/H^+ exchanger, Ca^{2+} -ATPase, and Na^+ -coupled amino acid transport

(Lytton et al. 1985, Moore 1981, Putnam 1985, Zemel et al. 1992a, Wong et al. 1988). The Na^+/H^+ antiporter is involved in regulation of intracellular pH, cell volume, cell proliferation, and Ca^{2+} exchange and is stimulated by physiological concentration of insulin (Aviv and Livne 1988, Pontrememali et al. 1991, Moore 1981). Increased Na^+/H^+ antiporter activity by insulin is associated with enhanced $[\text{Ca}^{2+}]_i$, characteristic of hypertension in insulin resistant states and promote intracellular alkalinization, which then stimulates protein synthesis and cell growth (Aviv and Livne 1988, Wray 1988). Further, the activity of Na^+/H^+ exchange was elevated in lymphocytes from spontaneously hypertensive rats (Feig et al. 1987) or from patients with essential hypertension and insulin resistance (Livne et al. 1987, Canessa et al. 1991). Insulin also regulates two important membrane pumps. the Na^+/K^+ -ATPase and the Ca^{2+} -ATPase. Decreased activity of these, resulting from insulin resistance or insulin deficiency, could lead to increases in VSM cytosolic Ca^{2+} and vascular resistance (Levy et al. 1989, Lytton et al. 1985).

Decreases in Na^+/K^+ -ATPase activity have been observed in diabetic rats (Levy et al. 1986), human obesity (DeLuise et al. 1980) and hypertensive patients (Cooper et al. 1987, Zemel et al. 1987). Kahn et al. (1992) reported that insulin attenuation of vasoconstriction in canine femoral artery VSMCs was inhibited by Na^+/K^+ -ATPase inhibition with

ouabain. Recently, Tirupattur et al. (1993) found insulin to increase both gene transcription and activity of Na^+/K^+ -ATPase in cultured VSMCs.

Alternatively, insulin may reduce vasoconstrictor responses via stimulating Ca^{2+} -ATPase-mediated Ca^{2+} efflux. Membrane Ca^{2+} -ATPase defects associated with increased $[\text{Ca}^{2+}]_i$ have been reported in skeletal muscle cells, bone cells and erythrocytes of diabetic patients (Zemel et al 1987, Levy et al. 1990, Shaefer et al. 1987). Moreover, Zemel et al. (1993) found insulin to stimulate Ca^{2+} -ATPase-mediated Ca^{2+} efflux in an insulin-dose-dependent manner. Touyz et al. (1994) recently reported that physiological concentrations of insulin attenuated $[\text{Ca}^{2+}]_i$ transient responses to arginine vasopressin, angiotensin II and norepinephrine in VSMCs.

Consequently, it has been proposed that this insulin effect may be mediated by increased Ca^{2+} -ATPase gene transcription. In support of this concept, insulin has recently been shown to stimulate both PM Ca^{2+} -ATPase and SR Ca^{2+} -ATPase gene expression in cultured rat VSMCs (Zemel et al. 1993), while expression of both pumps is blunted in VSMCs from insulin resistant (Zucker obese) rats (Zemel et al. 1991a). Further, we have also found insulin to markedly accelerate $[\text{Ca}^{2+}]_i$ recovery to baseline following stimulation of cultured rat aortic VSMCs and human pulmonary artery VSMCs (Kim and Zemel 1993) with either angiotensin II or

arginine vasopressin. In addition, the effect of insulin on this recovery rate after agonist stimulation was impaired in VSMCs freshly isolated from insulin resistant (Zucker obese) rats (Abel and Zemel 1993). Therefore, insulin-stimulated acceleration of $[Ca^{2+}]_i$ removal following agonist stimulation is likely to contribute to insulin attenuation of vasoconstrictor responses and acceleration of vascular relaxation. Figure 2.3 represents a hypothetical scheme for a chain of events leading to increased peripheral vascular resistance and essential hypertension.

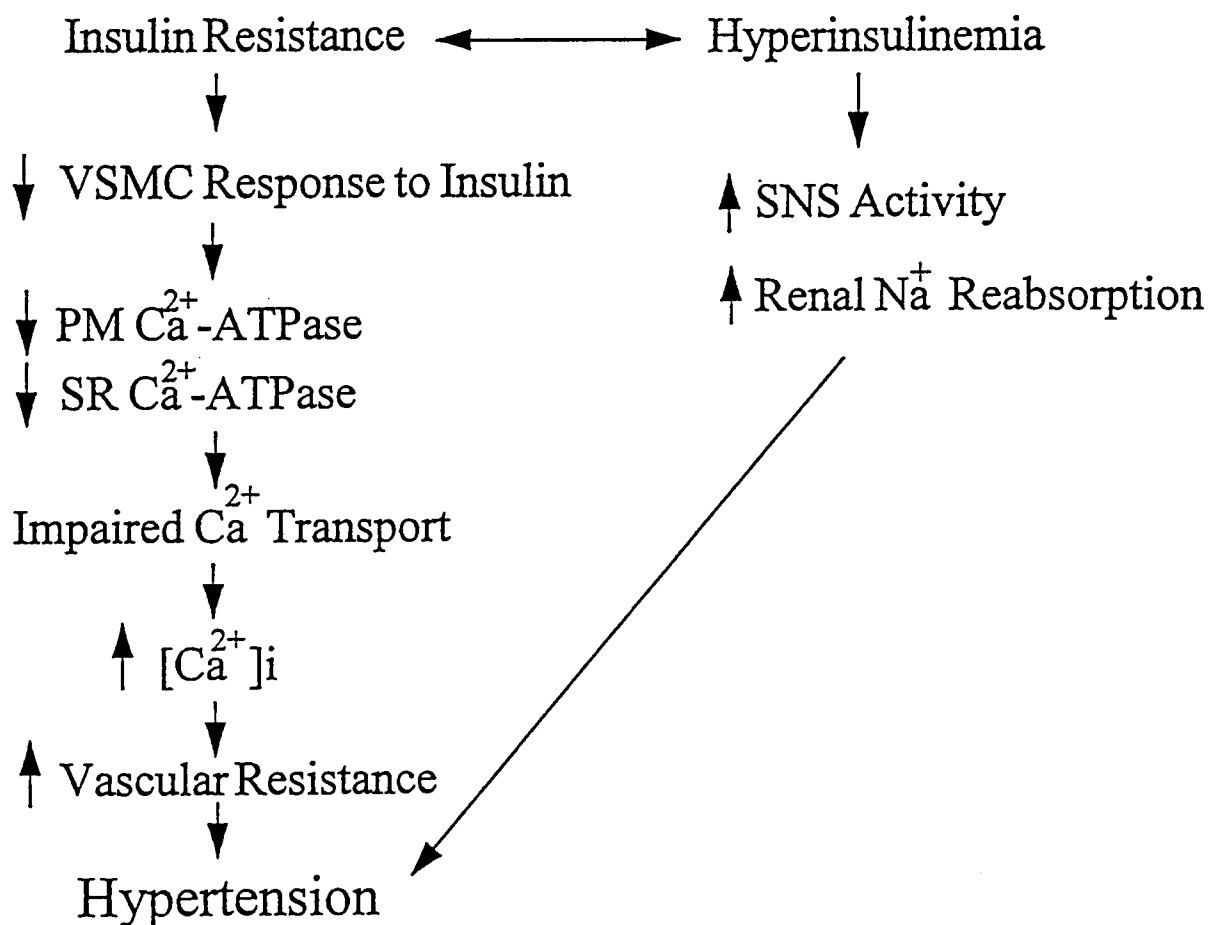


Figure 2.3 Proposed Mechanism of Associations Between Abnormal Cellular Ca²⁺ Homeostasis, Insulin Resistance, and Essential Hypertension.

This chart represents a hypothetical scheme for a chain of events leading to increased peripheral vascular resistance and hypertension.

VSMC, vascular smooth muscle cell; PM, plasma membrane; SR, sarcoplasmic reticulum; SNS, sympathetic nervous system; [Ca²⁺]_i, intracellular calcium.

Literature Cited

- Abel, M.A., Zemel, M.B. (1993) Impaired recovery of vascular smooth muscle intracellular calcium following agonist stimulation in insulin resistant (Zucker obese) rats. *Am J Hypertension*. 6:500-504.
- Adam, L.P., Hathaway, D.R. (1993) Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian *h-caldesmon*. *FEBS Lett*. 322, 56-60.
- Alkon, D.L., Rasmussen, H. (1988) A spatial-temporal model of cell activation. *Science* 239, 998-1005.
- Anderson, E.A., Hoffman, R.P., Balor, T.W., Sinkey, C.A., Mark, A.L. (1991) Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J. Clin. Invest*. 87, 2246-2252.
- Anderson, E.A., Balor, T.W., Hoffman, R.P., Sinkey, C.A., Mark, A.L. (1992) Insulin increases sympathetic activity but not blood pressure in borderline hypertensive humans. *J. Clin. Invest*. 19, 621-627.
- Andrea, J.E., Walsh, M.P. (1992) Protein kinase C of smooth muscle. *Hypertension Dallas* 20, 585-595.
- Andres, R., Crabbe, J. (1966) Stimulation by insulin of active sodium transport across toadskin: influence of aldosterone and vasopressin. *Arch. Int. Physiol. Biochim*. 74, 538-540.
- Ashley, C.C., Ridgway, E.B. (1970) Simultaneous recordings of membrane potential, calcium transient and tension in single muscle fibres. *Nature (London)* 219, 1168-1169.
- Aviv, A., Livne, A. (1988) The Na^+/H^+ antiport, cytosolic free Ca^{2+} and essential hypertension: A hypothesis. *Am. J. Hypertension*. 1, 410-413.
- Baron, A.D., Brechtel-Hook, G., Johnson, A., Hardin, D. (1993) Skeletal muscle blood flow: a possible link between insulin resistance and blood pressure. *Hypertension* 21, 129-135.
- Barzilai, A., Spanier, R., Rahaminoff, H. (1984) Isolation, purification and reconstitution of the Na^+ gradient-dependent Ca^{2+} transporter ($\text{Na}^+-\text{Ca}^{2+}$ exchanger) from brain synaptic plasma membranes. *Proc. Natl. Acad. Sci. USA*. 81, 6521-6525.

- Baum, M. (1987) Insulin stimulates volume absorption in the rabbit proximal convoluted tubule. *J. Clin. Invest.* 79, 1104-1109.
- Bennett, J., Weeds, A. (1986) Calcium and the cytoskeleton. *Br. Med. Bull.* 42, 385-390.
- Berridge, M.J. (1985) The molecular basis of communication within the cell. *Sci. Am.* 253, 124-134.
- Berridge, M.J. (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* 56, 159-193.
- Berridge, M.J., Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (London)* 312, 315-321.
- Best, P.M., Kwok, W.M., Xu, X. (1991) Calcium channels in excitation-contraction coupling. In: Hurwitz, L., Donald Patridge, L., Leach, J.K. eds. *Calcium channels: their properties, functions, regulation, and clinical relevance.* 6, 69-85.
- Bevan, J.A., Bevan, R.D., Hwa, J.J., Owen, M.P., Tayo, F.M., Winquist, R.J. (1982) Calcium, extrinsic and intrinsic (myogenic) vascular tone. In: Godfraind, T., Albertini, A., Paoletti, R. eds. *Calcium modulators.* Amsterdam: Elsevier, 125-132.
- Blaustein, M.P., Hamlyn, J.M. (1984) Sodium transport inhibition, cell calcium and hypertension. *Am. J. Med.* 77, 45-59.
- Blaustein, M.P. (1985) In "Calcium in biological systems" (Rubin, R.P., Weiss, G.B., Putney, J.W., Jr., eds.), Plenum, New York, pp23-33.
- Borle, A.B. (1981) Control, modulation and regulation of cell calcium. *Rev. Physiol. Biochem. Pharmacol.* 90, 14-153.
- Bookchin, R.M., Lew, V.L. (1980) Progressive inhibition of the Ca^{2+} pump and Ca^{2+} - Ca^{2+} exchange in sickle red cells. *Nature (London)* 284, 561-563.
- Brenner, B., Leepo, C.Y., Chalovich, J.M. (1991) Parallel inhibition of active force and relaxed fiber stiffness in skeletal muscle by caldesmon: implications for the pathway to force generation. *Proc. Natl. Acad. Sci. USA* 88, 5739-5743.

- Butler, T.M., Siegman, M.J., Mooers, S.U., Narayan, S.R. (1990) Myosin-product complex in the resting state and during relaxation of smooth muscle. *Am. J. Physiol.* 258, C1092-C1099.
- Bygrave, F.L. (1966) Studies on the interaction of metal ions with pyruvate kinase from Ehrlich ascites-tumour cells and from rabbit muscle. *Biochem. J.* 101(2), 488-494.
- Bygrave, F.L. (1967) The ionic movement and metabolic control. *Nature*. (London) 214(89), 667-671.
- Canessa, M., Falkner, B., Hulman, S. (1991) Red blood cell Na/H exchanger (EXC) activity is elevated in young hypertensive blacks. (abstract) *Hypertension* 18, 378.
- Carafoli, E. (1987) Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56, 395-433.
- Carafoli, E., Verma, A.K., James, P., Strehler, E., Penniston, J.T. (1989) The calcium pump of the plasma membrane: structure-function relationships. *Adv. Exp. Biol.* 255, 61-70.
- Carafoli, E., James, P. (1990) Structure-function relationships in the calcium pump of plasma membranes. *Prog. Clin. Biol. Res.* 332, 181-193.
- Carafoli, E. (1991) Calcium pump of the plasma membrane. *Physiol. Rev.* 71, 129-152.
- Cauvin, C., Loutzenhiser, R., van Breemen, C. (1983) Mechanisms of calcium antagonist-induced vasodilation. *Ann. Rev. Pharmacol. Toxicol.* 23, 373-396.
- Campbell, A.K. (1983) "Intracellular calcium: Its universal role as regulator." Wiley New York.
- Cobbold, P.H., Rink, T.J. (1987) Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem. J.* 248(2), 313-328.
- Cooper, R.S., Shamsi, N., Katz, S. (1987) Intracellular calcium and sodium in hypertensive patients. *Hypertension* 9, 224-229.
- Dean, W.L. (1989) Structure, function and subcellular localization of a human platelet Ca^{2+} -ATPase. *Cell Calcium* 10, 289-297.
- DeFronzo, R.A., Cooke, C., Andres, R., Faloona, G.R., Davis,

- P.J. (1975) The effect of insulin in renal handling of sodium, potassium, calcium, and phosphate in man. *J. Clin. Invest.* 55, 845-855.
- DeFronzo, R.A., Ferrannini, E. (1991) Insulin resistance: A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia and atherosclerotic cardiovascular disease. *Diabetes Care* 14, 173-194.
- DeLuise, M., Blackburn, G.L., Flier, J.S. (1980) Reduced activity of red-cell sodium-potassium pump in human obesity. *N. Engl. J. Med.* 303, 1017-1022.
- Dillon, P.F., Aksoy, M.O., Driska, S.P., Murphy, R.A. (1981) Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science* 211, 495-497.
- Dohm, G.L., Tapscott, E.B., Pories, W.J., Dabbs, D.J., Flickinger, E.G., Meelheim, D., Fushiki, T., Atkinson, S.M., Elton, C.W., Caro, J.F. (1988) An in vitro human muscle preparation suitable for metabolic studies: decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. *J. Clin. Invest.* 82, 486-494.
- Draznin, B., Sussman, K.E., Kao, M., Lewis, D., Shereman, N. (1987) The existence of an optimal range of cytosolic free calcium for insulin-stimulated glucose transport in rat adipocytes. *J. Biol. Chem.* 262, 14385-14388.
- Draznin, B., Sussman, K.E., Eckel, R.H., Kao, M., Yost, T., Sherman, N.A. (1988) Possible role of cytosolic free calcium concentrations in mediating insulin resistance of obesity and hyperinsulinemia. *J. Clin. Invest.* 82, 1848-1852.
- Driska, S.P., Aksoy, M.O., Murphy, R.A. (1981) Myosin light chain phosphorylation associated with contraction in arterial smooth muscle. *Am. J. Physiol.* 240, C222-C233.
- Droogmans, G., Raeymaekers, L., Casteels, R. (1977) Electro- and pharmacomechanical coupling in the smooth muscle cells of the rabbit ear artery. *J. Gen. Physiol.* 70, 129-148.
- Dubey, R.K., Zhang, H.Y., Reddy, S.R., Boegehold, M.A., Kotchen, T.A. (1993) Pioglitazone attenuates hypertension and inhibits growth of arteriolar smooth muscle in rats. *Am. J. Physiol.* 265, R726-732.
- Ebashi, S., Mikawa, T., Hirata, M., Noromura, Y. (1978) The regulatory role of calcium in muscle. *Ann. N.Y. Acad.*

Sci. 307, 451-461.

- Eisenbarth, G. (1986) Type I diabetes: a chronic autoimmune disease. *N. Engl. J. Med.* 314, 1360-1368.
- Feig, P.U., D'Occhio, M.A., Boylan, J.W. (1987) Lymphocyte membrane sodium-proton exchange in spontaneously hypertensive rats. *Hypertension* 9, 282-288.
- Feldman, R.D., Bierbrier, G.S. (1993) Insulin-mediated vasodilation: impairment with increased blood pressure and body mass. *Lancet* 342, 707-709.
- Ferrannini, E., Buzzigoli, G., Bonadonna, R., Giorico, M.A., Oleggini, M., Graziadei, L., Pedrinelli, R., Brandi, L., Bevilacqua, S. (1987) Insulin resistance in essential hypertension. *N. Engl. J. Med.* 317, 350-357.
- Ferrannini, E. (1992) Insulin and blood pressure : possible role of haemodynamics. *Clin. Exp. Hypertens. A.* 14, 271-285.
- Ferrari, P., Weidmann, P. (1990) Insulin, insulin sensitivity and hypertension. *J. Hypertension.* 8, 491-500.
- Fleckenstein, A. (1983) Calcium antagonism in heart and smooth muscle. Wiley, New York.
- Ford, L.E., Podolsky, R.J. (1972) Calcium uptake and force development by skinned muscle fibres in EGTA buffered solutions. *J. Physiol. (London)* 223, 1-19.
- Furukawa, K-I., Tawada, Y., Shigekawa, M. (1989) Protein kinase C activation stimulates plasma membrane Ca^{2+} pump in cultured vascular smooth muscle cells. *J. Biol. Chem.* 264, 4844-4849.
- Furchgott, R.F. (1983) Role of endothelium in responses of vascular smooth muscle. *Circ. Res.* 53, 557-573.
- Garrahan, P.J., Rega, A.F. (1978) Activation of partial reactions of the Ca^{2+} -ATPase from human red cells by Mg^{2+} and ATP. *Biochim. Biophys. Acta.* 513, 59-65.
- Garvey, W.T., Maianu, L., Huecksteadt, T.P., Birnbaum, M.J., Molina, J.M., Ciaraldi, T.P. (1991) Pretranslational suppression of a glucose transporter protein caused insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. *J. Clin. Invest.* 87, 1072-1081.

- Golay, A., Felber, J.P., Jequier, E., DeFronzo, R.A., Ferrannini, E. (1988) Metabolic basis of obesity and non-insulin dependent diabetes mellitus. *Diabetes Metab. Rev.* 4, 727-747.
- Goldfine, I.D. (1987) The insulin receptor. *Endo. Rev.* 8, 235-255.
- Gopinath, R.M., Vincenzi, F.F. (1981) ($\text{Ca}^{2+} + \text{Mg}^{2+}$)ATPase activity of sickle cell membranes decreased activation by red blood cell cytoplasmic activator. *Am. J. Hematol.* 7, 303-312.
- Green, N.M. (1989) ATP-driven cation pumps, alignment of sequences. *Trans. Biochem. Soc.* 17, 970-972.
- Gunter, T.E., Pfeiffer, D.R. (1990) Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* 258, C755-C786.
- Grynkiewicz, G, Poenie, M., Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J Biol Chem.* 260:3440-3450.
- Hai, C.M., Murphy, R.A. (1988) Cross-bridge phosphorylation and regulation of latch state in smooth muscle. *Am. J. Physiol.* 254, C99-C106.
- Hall, J.E., Coleman, T.G., Mizelle, H.L. (1989) Does chronic hyperinsulinemia cause hypertension ? *Am. J. Hypertension* 2, 171-173.
- Hall, J.E., Brands, M.W., Dixon, W.N., Mizelle, H.L., Hildebrandt, D.A. (1991) Hyperinsulinemia does not elevate blood pressure in obese hypertensive dogs. *FASEB* 5, A737.
- Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391, 85-100.
- Hartshorne, D.J., Gorecka, A. (1980) Biochemistry of the contractile proteins of smooth muscle. In: Bohr, D.F., Somlyo, A.P., Sparks, H.V. Jr., eds. *Handbook of physiology: the cardiovascular system*. Bethesda, MD: American Physiological Society. 4, 93-120.
- Hartshorne, D.J. (1987) Biochemistry of the contractile process in smooth muscle, in Johnson LR (ed): *Physiology of the Gastrointestinal Tract*. New York, Raven Press, Publishers, pp 423-482.

- Hasselbach, W., Makinose, M. (1961) Die calciumpumpe der "Erschlaffungsgrana" des muskels und ihre Abhangigkeit von der ATP-Spaltung. *Biochem Z.* 333, 518-528.
- Hasselbach, W. (1964) Relaxing factor and the relaxation of muscle. *Prog. Biophys. Mol. Biol.* 14, 167-222.
- Huxley, A.F., Niedergerke, R. (1954) Interference microscopy of living muscle fibres. *Nature (London)* 173, 971-973.
- Huxley, H.H. (1990) Sliding filaments and molecular motile systems. *J. Biol. Chem.* 256, 8347-8350.
- Inesi, G., Kirtley, M.E. (1990) Coupling of catalytic and channel function in the Ca^{2+} transport ATPase. *J. Membr. Biol.* 116, 1-8.
- Inesi, G. (1972) Active transport of calcium ion in sarcoplasmic membranes. *Annu. Rev. Biophys. Bioeng.* 1, 191-210.
- Ito, Y., Kitamura, K., Kuriyama, H. (1979) Effects of acetylcholine and catecholamines on the smooth muscle cell of the porcine coronary artery. *J. Physiol. (Lond)* 294, 595-611.
- James, P., Inui, M., Tada, M., Chiesi, M., Carafoli, E. (1989) Nature and site of phospholamban regulation of the Ca^{2+} pump of the sarcoplasmic reticulum. *Nature* 342, 90-92.
- Jeffery, D., Roufogalis, B.D., Katz, S. (1981) The effect of calmodulin on the phosphoprotein intermediate of Mg^{2+} -dependent, Ca^{2+} -stimulated adenosine triphosphatase in human erythrocyte membranes. *Biochem. J.* 194, 481-486.
- Johansson, B., Somlyo, A.P. (1980) Electrophysiology and excitation-contraction coupling. In: Bohr, D.F., Somlyo, A.P., Sparks, H.V. (eds) *The cardiovascular system. Am. Physiol. Soc., Bethesda, Maryland*, pp 301-321 (Handbook of physiology, sect. 2, vol. II).
- Juncos, L.A., Ito, S., Carretero, O.A. (1992) Disparate effects of insulin on isolated rabbit afferent and efferent arterioles. *Hypertension* 20, 403.
- Kahn, C.R. (1978) Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. *Metabolism* 27, (Suppl. 2): 1893-1902.
- Kahn, C.R., White, M.F. (1988) The insulin receptor and the molecular mechanism of insulin action. *J. Clin. Invest.*

82, 1151-1156.

- Kahn, A.M., Allen, J.C., Seidel, C.L., Shelat, H., Song, T. (1992) Physiological insulin concentrations cause ouabain- and verapamil-sensitive inhibition of vascular smooth muscle contraction. *Hypertension* 20, 409.
- Kahn, A.M., Seidel, C.L., Allen, J.C., O'Neil, R.G. (1993) Insulin reduces contraction and intracellular calcium concentration in vascular smooth muscle. *Hypertension* 22, 735-742.
- Katz, A.M., Blostein, R. (1975) Ca^{2+} -stimulated membrane phosphorylation and ATPase activity of the human erythrocyte. *Biochim. Biophys. Acta.* 389, 314-324.
- Katz, A.M. (1979) Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: a historical review. *Adv. Cyclic Nucleotide Res.* II, 303-343.
- Khalil, R.A., Lodge, N.J., Saida, K., Grlband, C.H., van Breemen, C. (1990) Calcium mobilization in vascular smooth muscle and its relevance to the etiology of hypertension. *Hypertension: Pathology, Diagnosis, and Management*, edited by Laragh, J.H. and Brenner, B.M., Raven Press, Ltd., New York. 36, 547-563.
- Kim, H.W., Steenaart, N.A.E., Ferguson, D.G., Kranias, E.G. (1990) Functional reconstitution of the cardiac sarcoplasmic reticulum Ca^{2+} -ATPase with phospholamban in phospholipid vesicles. *J. Biol. Chem.* 265, 1702-1709.
- Kim, Y-C., Zemel, M.B. (1993) Insulin increases vascular smooth muscle recovery from intracellular calcium loads. *Hypertension* 22, 74-77.
- Knauf, P.A., Proverbio, F., Hoffman, J.F. (1974) Electrophoretic separation of different phosphoproteins associated with Ca-ATPase and Na, K-ATPase in human red cell ghosts. *J. Gen. Physiol.* 63, 324-336.
- Kofuji, P., Lederer, W.J., Schulze, D.H. (1994) Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca Exchanger. *J. Biol. Chem.* 269, 5145-5149.
- Kojima, I., Kojima, K., Rasmussen, H. (1985) Role of calcium fluxes in the sustained phase of angiotensin II-induced aldosterone secretion from adrenal glomerulosa cells. *J. Biol. Chem.* 260(16), 9177-9184.

- Krisanda, J.M., Paul, R.J. (1983) Phosphagen and metabolite content during contraction in porcine carotid artery. *Am. J. Physiol.* 244, 385-390.
- Lagnado, L., McNaughton, P.A. (1990) Electrogenic properties of the Na : Ca exchange. *J. Membr. Biol.* 113, 177-191.
- Lehninger, A.L. (1950) *Physiol. Rev.* 30, 393-129.
- Lembo, G., Rendina, V., Lamenza, F., Iaccarino, G., Volpe, M., Trimarco, B. (1992) Insulin reduces forearm vasoconstriction evoked by reflex sympathetic activation in healthy humans. *Hypertension* 20, 409.
- Levy, J., Avioli, L.V., Roberts, M.L., Roberta, M.L., Gavin, J.R. (1986) (Na⁺/K⁺)-ATPase activity in kidney basolateral membranes of non-insulin-dependent diabetic rats. *Biochem. Biophys. Res. Commun.* 139, 1313-1319.
- Levy, J., Zemel, M.B., Sowers, J.R. (1989) Role of cellular calcium metabolism in abnormalities in glucose metabolism and diabetic hypertension. *Am. J. Med.* 87(suppl. 6A) 7-16.
- Levy, J., Sowers, J.R., Zemel, M.B. (1990) Abnormal Ca²⁺-ATPase activity in erythrocytes of non-insulin-dependent diabetic rats. *Horm. Metab. Res.* 22, 138-142.
- Livne, A., Veitchi, R., Grinstein, S., Balfe, J.W., Marquez-Julio, A., Rothstein, A. (1987) Increased platelet Na⁺-H⁺ exchange rates in essential hypertension: application of a novel test. *Lancet* 533-536.
- Lytton, J., Lin, J.C., Guidotti, G. (1985) Identification of two molecular forms of (Na⁺, K⁺)-ATPase in rat adipocytes: Relation to insulin stimulation of the enzyme. *J. Biol. Chem.* 260, 1177-1184.
- Marston, S.B., Lehman, W. (1985) Caldesmon is a Ca²⁺-regulatory component of native smooth-muscle thin filaments. *Biochem. J.* 231, 517-522.
- Marston, S.B. (1989) What is latch ? New ideas about tonic contraction in smooth muscle. *J. Muscle Res. Cell. Motil.* 10, 97-100.
- Mathias, C.J., daCosta, D.F., Fosbraey, P., Christesen, N.J., Bunmister, R. (1987). Hypotensive and sedative effects of insulin in autonomic failure. *Br. Med. J.* 295, 161-163.
- Mauger, J.P., Poggioli, J., Claret, M. (1985) Synergistic

- stimulation of the Ca^{2+} influx in rat hepatocytes by glucagon and the Ca^{2+} -linked hormones vasopressin and angiotensin II. *J. Biol. Chem.* 260(21), 11635-11642.
- McGarry, J.D. (1992) What if Minkowski had been ageusic ? An alternative angle on diabetes. *Science.* 258, 766-770.
- Meissner, G. (1975) Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta.* 389, 51-68.
- Mering, J.V., Minkowski, O. (1990) Diabetes mellitus nach pankreasextirpation. *Arch. Exp. Pathol. Pharmacol.* (Leipzig) 26, 371-387.
- Modan, M., Halkin, H., Almog, S., et al. (1985) Hyperinsulinemia: a link between hypertension, obesity and glucose intolerance. *J. Clin. Invest.* 75, 809-817.
- Moody, C., Lehman, W., Craig, R. (1990) Caldesmon and the structure of smooth muscle thin filaments: Electron microscopy of isolated thin filaments. *J. Muscle Res. Cell. Motil.* 11, 176-185.
- Moore, R.D. (1981) Stimulation of Na : H exchange by insulin. *Biophys. J.* 33, 203-210.
- Morgan, D.A., Ray, C.A., Balor, T.W., Mark, A.L. (1992) Metformin increases insulin sensitivity and lowers arterial pressure in spontaneously hypertensive rats. *Hypertension* 20, 421.
- Murphy, R.A. (1989) Specific topic: Contraction in smooth muscle cells. *Annu. Rev. Physiol.* 51, 275-283.
- Neahring, J.R., Stepniakowski, K., Greene, A.S., Egan, B.M. (1993) Insulin does not reduce forearm alpha-vasoreactivity in obese hypertensive or lean normotensive men. *Hypertension.* 22(4), 584-590.
- Niggli, V., Adunyah, E.S., Cameron, B.F., Bababunmi, E.A., Carafoli, E. (1982) The Ca^{2+} pump of sickle cell plasma membranes. Purification and reconstitution of the ATPase enzyme. *Cell Calcium* 3, 131-151.
- Niggli, V., Ronner, P., Carafoli, E., Penniston, J.T. (1979) Effect of calmodulin on the $(\text{Ca}^{2+} \text{ Mg}^{2+})$ -ATPase partially purified from erythrocyte membranes. *Arch. Biochem. Biophys.* 198, 124-130.
- Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* 233(4761), 305-312.

- Olefsky, J.M., Reaven, G.M., Farquhar, J.W. (1974) Effects of weight reduction on obesity: studies of carbohydrate and lipid metabolism. *J. Clin. Invest.* 53, 64-76.
- Olson, S., Wang, M.G., Carafoli, E., Strehler, E.E., McBride, O.W. (1991) Localization of two genes encoding plasma membrane Ca^{2+} -transporting ATPase to human chromosomes 1q25-32 and 12q21-23. *Genomics* 9(4), 629-641.
- Pedersen, P.L., Carafoli, E. (1987) Ion motive ATPases *Trends. Biochem. Sci.* 12, 186-189.
- Pershad Singh, H.A., Shade, D.L., Delfert, D.M., McDonald, J.M. (1987) Chelation of intracellular calcium blocks insulin action in the adipocyte. *Proc. Natl. Acad. Sci. USA.* 84, 1025-1029.
- Pershad Singh, H.A., Kurtz, T.W. (1992) Pharmacological reversal of hyperinsulinemia is associated with a reduction in blood pressure: A novel approach to treatment of hypertension. *Hypertension* 20, 410.
- Pontrememali, R., Rivera, A., Canessa, M. (1991) Insulin and cytosolic Ca^{++} (Ca_i) modulate the human red cell Na/H exchanger (EXC). (abstract) *Clin. Res.* 39, 192A.
- Popescu, L.M., Panoiu, C., Toescu, E.C., Nutu, O. (1984) Prostaglandins E_2 and $\text{F}_{2\alpha}$ inhibit the sarcolemmal Ca^{2+} extrusion pump of human myometrium at term. I: Braquet, P. et al., eds. *Prostaglandins and membrane ion transport*. New York: Raven Press, 217-222.
- Pritchard, K., Ashley, C.C. (1986) $\text{Na}^+/\text{Ca}^{2+}$ exchange in isolated smooth muscle cells demonstrated by the fluorescent calcium indicator fura-2. *FEBS Lett.* 195, 23-27.
- Putnam, R.W. (1985) Effects of insulin on intracellular pH in frog skeletal muscle fibers. *Am. J. Physiol.* 248, C330-C336.
- Rapp, J.P. (1981) Aortic response of vanadate: independence from (Na, K)-ATPase and comparison of Dahl salt-sensitive and salt-resistant rats. *J. Hypertension* 3(suppl I) 1168-1172.
- Rasmussen, H. (1970) Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* 170, 404-412.
- Rasmussen, H. (1986) The calcium messenger system. *N. Engl. J. Med.* 314, 1094-1101.

- Rasmussen, H., Takuwa, Y., Park, S. (1987) Protein kinase C in the regulation of smooth muscle contraction. *FASEB J.* 1, 177-185.
- Rasmussen, H., Barrett, P.Q. (1984) Calcium messenger system: an integrated view. *Physiol. Rev.* 64, 938-984.
- Reaven, G.M. (1988) Role of insulin resistance in human disease. *Banting Lecture Diabetes* 37, 1595-1607.
- Reaven, G.M., Chen, Y-D. (1988) Role of insulin in regulation of lipoprotein metabolism in diabetes. *Diabetes/Metab. Rev.* 4, 639-652.
- Reaven, G.M., Hoffman, B.B. (1987) A role for insulin in the aetiology and course of hypertension ? *Lancet* ii, 435-437.
- Reaven, G.M. (1991a) Insulin resistance, hyperinsulinemia and hypertriglyceridemia in the etiology and clinical course of hypertension. *Am. J. Med.* 8 (Suppl. 2A): 2A-7S-2A-12S.
- Reaven, G.M. (1991b) Insulin resistance and compensatory hyperinsulinemia: Role in hypertension, dyslipidemia, and coronary heart disease. *Am. Heart J.* 121, 1283-1288.
- Reddy, S., Shehin, S., Sowers, J.R., Dardas, G., Zemel, M.B. (1990) $^{45}\text{Ca}^{2+}$ flux and blood pressure regulation in streptozotocin-induced diabetic rats. *J. Vasc. Med. Biol.* 2, 47-51.
- Rega, A.F., Garrahan, P.J. (1975) Calcium ion dependent phosphorylation of human erythrocyte membranes. *J. Membr. Biol.* 22, 313-327.
- Rembold, C.M. (1992) Regulation of contraction and relaxation in arterial smooth muscle. *Hypertension* 20, 129-137.
- Rembold, C.M., Murphy, R.A. (1986) Myoplasmic calcium, myosin phosphorylation, and regulation of the crossbridge cycle in swine arterial smooth muscle. *Circ. Res.* 58, 803-815.
- Rembold, C.M., Murphy, R.A. (1988) Myoplasmic $[\text{Ca}^{2+}]$ determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. *Circ. Res.* 63, 593-603.
- Reuter, H., Blaustein, M.P., Hausler, G. (1973) Na-Ca exchange and tension development in arterial smooth muscle. *Philos. Trans. R. Soc. London [Biol]* 265, 87-94.

- Resink, T., Tkachuk, V.A., Erne, P., Buhler, F.R. (1986) Platelet membrane calmodulin-stimulated calcium-adenosine triphosphatase. Altered activity in essential hypertension. *Hypertension* 8, 159-166.
- Ringer, S. (1883) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J. Physiol. (London)* 4, 29-42.
- Rocchini, A.P. (1991) Insulin resistance and blood pressure regulation in obese and non-obese subjects. *Hypertension* 17, 837-842.
- Rosen, O.M. (1987) After insulin binds. *Science*. 237, 1452-1458.
- Rowe, J.W., Young, J.B. Minaker, K.L., Stevens, A.L., Pallotta, J., Landsberg, L. (1981) Effect of insulin and glucose infusion on sympathetic nervous system activity in normal man. *Diabetes* 30, 219-225.
- Ruegg, J.C. (1992) Calcium in muscle contraction : cellular and molecular physiology. Second edition, Springer-Verlag.
- Saida, K., van Breemen, C. (1987) GTP requirement for inositol 1,4,5-triphosphate-induced Ca^{2+} release from sarcoplasmic reticulum in smooth muscle. *Biochem. Biophys. Res. Commun.* 144, 1313-1316.
- Sawicki, P.T., Heinemann, L., Starke, A., Berger, M. (1992) Hyperinsulinaemia is not linked with blood pressure elevation in patients with insulinoma. *Diabetologia* 35, 649-652.
- Schatzmann, H.J. (1966) ATP-dependent Ca^{2+} extrusion from human red cells. *Experientia* 22, 364-368.
- Shaefer, W., Prieben, J., Mannhold, R., Gries, A.F. (1987) Ca^{2+} - Mg^{2+} -ATPase activity of human red blood cells in healthy and diabetic volunteers. *Klin. Wochenschr.* 65, 17-21.
- Shehin, S.E., Sowers, J.R., Zemel, M.B. (1989) Impaired vascular smooth muscle $^{45}\text{Ca}^{2+}$ efflux and hypertension in Zucker obese rats. *J. Vasc. Med. Biol.* 1, 278-282.
- Sheu, S., Blaustein, M.P. (1986) Sodium/calcium exchange and regulation of cell calcium and contractility in cardiac muscle, with a note about vascular smooth muscle. In: Fozzard, H.A., Haber, E., Jennings, R., Katz, A., Morgan,

- H. (eds) The heart and cardiovascular system. Raven, New York, pp 509-535.
- Siegmán, M.J., Butler, T.M., Mooers, S.U. (1985) Energetics and regulation of crossbridge states in mammalian smooth muscle. *Experientia* 41, 1020-1025.
- Smallwood, J.I., Gugi, B., Rasmussen, H. (1988) Modulation of erythrocyte Ca^{2+} pump activity by protein kinase C. *J. Biol. Chem.* 263, 2195-2202.
- Sobue, k., Kanda, K., Tanaka, T., Ueki, N. (1988) Caldesmon: A common actin-linked regulatory protein in smooth muscle and nonmuscle contractile system. *J. Cell. Biochem.* 37, 317-325.
- Sobue, K., Sellers, J.R. (1991) Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin system. *J. Biol. Chem.* 266, 12115-12118.
- Somlyo, A.P. (1985) Excitation-contraction coupling and the ultrastructure of smooth muscle. *Circ. Res.* 57, 497-507.
- Somlyo, A.P., Himpens, B. (1989) Cell calcium and its regulation in smooth muscle. *FASEB J.* 3, 2266-2276.
- Somlyo, A.P., Somlyo, A.V. (1968) Vascular smooth muscle. I. Normal structure, pathology, biochemistry, and biophysics. *Pharmacol. Rev.* 20, 197-272.
- Somlyo, A.V., Somlyo, A.P. (1968) Electromechanical and pharmacomechanical coupling in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* 159, 129-145.
- Somlyo, A.V., Bond, M., Somlyo, A.P., Scarpa, A. (1985) Inositol triphosphate-induced calcium release and contraction in vascular smooth muscle. *Proc. Natl. Acad. Sci. USA* 82, 5231-5235.
- Somlyo, A.P., Somlyo, A.V. (1986) Smooth muscle structure and function. New York : Raven Press. 845-864.
- Sowers, J.R., Zemel, M.B. (1990) Clinical implication of hypertension in the diabetic patient: A Review. *Am. J. Hypertension* 3, 415-424.
- Sowers, J.R., Standley, P.R., Ram, J.L., Zemel, M.B., Resnick, L.M. (1991) Insulin resistance, carbohydrate metabolism, and hypertension. *Am. J. Hypertension* 4, 466S-472S.
- Sperelakis, N., Caulfield (eds) (1984) Calcium antagonists.

- Mechanisms of action of cardiac muscle and vascular smooth muscle. Martinus Nijhoff, Boston.
- Stout, R.W. (1990) Insulin and atheroma: 20-year perspective. *Diabetes Care* 13, 631-654.
- Stull, J.T., Gallagher, P.J., Herring, B.P., Kamm, K.E. (1991) Vascular smooth muscle contractile elements: cellular regulation. *Hypertension* 17, 723-732.
- Sutherland, C., Walsh, M.P. (1989) Phosphorylation of caldesmon prevents its interaction with smooth muscle myosin. *J. Biol. Chem.* 264, 578-583.
- Szasz, I., Sarkadi, B., Schubert, A., Gardas, G. (1978) Effects of lanthanum on calcium-dependent phenomena in human red cells. *Biochim. Biophys. Acta.* 512, 331-340.
- Tirupattur, P.R., Ram, J.L., Standley, P.R., Sowers, J.R. (1993) Regulation of Na⁺, K⁺-ATPase gene expression by insulin in vascular smooth muscle cells. *Am. J. Hypertension* 6, 626-629.
- Tobey, T.A., Greenfield, M., Kraemer, F., Reaven, G.M. (1981) Relationship between insulin resistance, insulin secretion, very low density lipoprotein kinetics and plasma triglyceride levels in normotriglyceridemic man. *Metabolism* 30, 165-171.
- Touyz, R.M., Tolloczko, B., Schiffrin, E.L. (1994) Insulin attenuates agonist-evoked calcium transients in vascular smooth muscle cells. *Hypertension* 23(Suppl.I): I-25-I-28.
- Touyz, R.M., Schiffrin, E.L. (1994) Insulin-induced Ca²⁺ transport is altered in vascular smooth muscle cells of spontaneously hypertensive rats. *Hypertension* 23(part 2): 931-935.
- Tsien, R.Y., Rink, T.J., Peonie, M. (1985) Measurement of cytosolic free Ca²⁺ in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium* 6, 145-157.
- van Breemen, C., Aaronson, P., Loutzenhiser, R. (1979) Na-Ca interaction in mammalian smooth muscle. *Pharmacol. Rev.* 30, 606-718.
- van Breemen, C., Cauvin, C., Johns, A., Leijten, P., Yamamoto, H. (1986) Ca²⁺ regulation of vascular smooth muscle. *Fed. Proc.* 45, 2746-2751.
- Vezzoli, G., Elli, A.A., Tripodi, G., Bianchi, G., Carafoli,

- E. (1985) Calcium ATPase in erythrocytes of spontaneously hypertensive rats of the Milan strain. *J. Hypertension* 5, 645-648.
- Vionnet, N., Stoffel-M, Takeda-J, Yasuda-K et al. (1992) Nonsense mutation in the glucokinase gene causes early-onset non-insulin-dependent diabetes mellitus. *Nature* 356, 721.
- Wambach, G.K., Liu, D. (1992) Insulin attenuates vasoconstriction by noradrenaline, serotonin and potassium chloride in rat mesenteric arteries. *Clin. Exp. Hypertens. A.* 14, 733-740.
- Weidman, P., Beretta-Piccoli, C., Trost, B.N. (1985) Pressor factors and responsiveness in hypertension accompanying diabetes mellitus, *Hypertension* 79(suppl. II): II-33-II-42.
- Williams, D.A., Fogarty, K.E., Tsien, R.Y., Fay, F.S. (1985) Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. *Nature (London)* 318, 558-561.
- Winder, S.T., Walsh, M.P. (1993) Calponin: thin filament-linked regulation of smooth muscle contraction. *Cell Signalling* 5, 677-686.
- Wong, E.C., Sacks, D.B., Laurino, J.P., McDonald, J.M. (1988) Characteristics of calmodulin phosphorylation by the insulin receptor kinase. *Endocrinology* 123, 1830-1836.
- Wray, S. (1988) Smooth muscle intracellular pH: Measurement, regulation, and function. *Am. J. Physiol.* 254, C213-C225.
- Yagi, S., Takata, S., Kiyokawa, H., Yamamoto, M., Noto, Y., Ikeda, T., Hattori, N. (1988) Effects of insulin on vasoconstrictive responses to norepinephrine and angiotensin II in rabbit femoral artery and vein. *Diabetes* 37, 1064-1067.
- Yarden, Y., Ullrich, A. (1988) Growth factor receptor tyrosine kinases. *Ann. Rev. Biochem.* 57, 443-478.
- Zavaroni, I., Dall'Aglio, E., Alpi, O., et al. (1985) Evidence for an independent relationship between plasma insulin and concentration of high density lipoprotein cholesterol and triglyceride. *Atherosclerosis* 55, 259-266.
- Zavaroni, I., Bonora, E., Pagliara, M., et al. (1989) Risk

factors for coronary artery disease in healthy persons with hyperinsulinemia and normal glucose tolerance. N. Engl. J. Med. 320, 702-706.

Zeller, S., Bliss, M., Minkowski, O. (1990) Dictionary of Scientific Biography. Suppl. 2, New York, Scribner's, pp626-633.

Zemel, M.B., Kraniak, J., Standley, P., Gualdoni, S.M., Komanicky, P., Sowers, J.R. (1987a) Effects of dietary sodium and calcium on cellular calcium, magnesium, sodium and potassium metabolism in hypertensive blacks. Clin. Res. 35, 452A.

Zemel, M.B., Bedford, B.A., Zemel, P.C., Gualdoni, S.M., Felicetta, J.V., Suntheimer, C., Sowers, J.R. (1987b) Altered cation transport in diabetic hypertension. Clin. Res. 35, 853A.

Zemel, M.B., Bedford, B.A., Zemel, P.C., Marwah, O., Sowers, J.R. (1988) Altered cation transport in non-insulin-dependent diabetic hypertension: Effects of dietary calcium. J. Hypertension 6(suppl 4): S228-S230.

Zemel, M.B., Iannucci, A., Johnson, B.A.B., Sowers, J.R. (1991a) Reduced Ca^{2+} -ATPase expression in insulin resistant (Zucker obese) rats. Hypertension 18, 442.

Zemel, M.B., Johnson, B.A.B., Ambrozy, S.A. (1992a) Insulin-stimulated vascular relaxation: role of Ca^{2+} -ATPase. Am. J. Hypertension 5, 637-641.

Zemel, M.B., Zemel, P.C., Bijlani, S., Johnson, B.A.B., Sowers, J.R. (1990a) Relative effects of maxzide and hydrochlorothiazide on erythrocyte cations and forearm and vascular resistance. In: Diuretics III: Chemistry, Pharmacology and Clinical Applications. ed. J.B. Puschett and A. Greenberg. Elsevier, New York.

Zemel, M.B., Reddy, S., Shehin, S., Lockette, W., Sowers, J.R. (1990b) Vascular reactivity in Zucker obese rats. Role of insulin resistance. J. Vasc. Med. Biol. 2, 81-85.

Zemel, M.B., Reddy, S., Sowers, J.R. (1991b) Insulin attenuation of vasoconstrictor responses to phenylephrine in Zucker lean and obese rats. Am. J. Hypertension 4, 537-539.

Zemel, M.B., Peuler, J.D., Sowers, J.R., Simpson, L. (1992b) Hypertension in insulin-resistant Zucker obese rats is independent of sympathetic neural support. Am. J. Physiol. 262, E368-E371.

- Zemel, M.B., Iannucci, A., Moore, J.W. (1993) Role of insulin in regulating vascular smooth muscle Ca^{2+} -ATPase expression. J. Vasc. Med. Biol. 4, 79-84.
- Zvaritch, E., James, P., Vorherr, T., Falchetto, R., Modyanov, N., Carafoli, E. (1990) Mapping of functional domains in the plasma membrane Ca^{2+} pump using trypsin proteolysis. Biochemistry 29, 8070-8076.

PART III

INSULIN INCREASES VASCULAR SMOOTH MUSCLE RECOVERY FROM
INTRACELLULAR CALCIUM LOADS

Abstract

Insulin has previously been shown to attenuate vasoconstrictor responses to pressor agonists and accelerate vascular smooth muscle relaxation and vascular smooth muscle Ca^{2+} efflux. To further determine the role of insulin in regulating vascular smooth muscle Ca^{2+} , quiescent A7r5 cultured vascular smooth muscle cells and human vascular smooth muscle cells were incubated with or without 10^{-7} or 10^{-8} M insulin for 1 hour and then loaded with fura 2-AM; intracellular Ca^{2+} responses to and rates of recovery from angiotensin II (AII) (200 nM) and arginine vasopressin (AVP) were studied fluorometrically in stirred suspension. Insulin caused an increase in the peak intracellular Ca^{2+} response to angiotensin II (Peak/baseline $\times 100 = 469 \pm 96$ versus $288 \pm 74\%$, $p < 0.05$) and a decrease in the peak Ca^{2+} response to vasopressin (288 ± 50 versus $389 \pm 33\%$, $p < 0.025$). However, insulin also caused a marked increase in the rate of intracellular Ca^{2+} recovery to baseline after stimulation with both angiotensin II (77.3 ± 13.8 versus 30.6 ± 6 nM/min, $p < 0.03$) and vasopressin ($p < 0.05$), such that the cumulative exposure to elevated intracellular Ca^{2+} after stimulation with either agonist (i.e., area under the intracellular Ca^{2+} response curve) was reduced with insulin treatment. Insulin also caused comparable effects on Ca^{2+} recovery in the human cells but was without significant effect on peak Ca^{2+}

responses to AVP. It is concluded that accelerated removal of cytosolic Ca^{2+} after agonist stimulation is likely to contribute to insulin attenuation of vasoconstrictor responses and acceleration of vascular relaxation.

Introduction

Although hypertension in insulin-resistant states is most often attributed to increased sympathetic neural output and renal sodium retention secondary to hyperinsulinemia (Reaven 1988 and Rocchini 1991), several recent lines of evidence suggest otherwise. Insulin appears to exert direct vasodilator effects on vascular smooth muscle (Zemel et al. 1990, 1991a & 1993, Kahn et al. 1993). It has been suggested that vascular smooth muscle manifestations of insulin resistance, leading to loss of insulin-mediated vasodilation, may be responsible for the hypertension in insulin-resistant states (Zemel et al. 1990 & 1991a). Insulin was also found to attenuate in vitro vascular reactivity responses to vasoconstrictor agonists in endothelial-denuded aortic strips, and to accelerate endothelium-independent relaxation after vasoconstriction, whereas these effects are substantially lost in aortic strips from insulin-resistant (Zucker obese) rats (Zemel et al. 1990 & 1991a). Further, physiological concentrations of insulin were recently reported to inhibit angiotensin II (AII) and serotonin-induced contraction of canine femoral artery smooth muscle cells (Kahn et al. 1993).

Anderson et al. (1991 & 1992) found that insulin infusion with maintenance of euglycemia caused significant decreases in peripheral vascular resistance in both

normotensive and hypertensive individuals despite observed increases in muscle sympathetic nerve activity. Similarly, insulin infusion was recently reported to increase forearm blood flow during concomitant norepinephrine infusion in humans (Neahring 1993). Moreover, Dubey et al. (1993) reported that treatment with pioglitazone, a thiazolidinedione compound that amplifies peripheral cellular responses to insulin (Hofmann et al. 1991), resulted in attenuation of hypertension in both Dahl salt-sensitive and one-kidney, one clip hypertensive rats.

Insulin has been shown to decrease peak intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) responses to vasopressin in cultured rat aortic smooth muscle cells by reducing Ca^{2+} influx through receptor-operated channels and to decrease voltage-mediated Ca^{2+} influx (Standley et al. 1991). Moreover, insulin has also been reported to increase the expression of both sarcoplasmic reticulum and plasma membrane Ca^{2+} -ATPase (Zemel et al. 1993) and to cause a corresponding increase in Ca^{2+} efflux (Zemel et al. 1992). Accordingly, the present study was conducted to further define the role of insulin in regulating vascular smooth muscle cell (VSMC) intracellular calcium responses to vasoconstrictor agonists. We have studied the effects of insulin on AII and arginine vasopressin-induced $[\text{Ca}^{2+}]_i$ transients, since AII-induced Ca^{2+} transients are mediated by release from sarcoplasmic reticulum stores rather than transmembrane Ca^{2+} flux and may

therefore be regulated differently by insulin than is the vasopressin-induced $[Ca^{2+}]_i$ transient. This report demonstrates that although insulin exerts markedly different effects on peak Ca^{2+} responses to the two agonists, insulin accelerates the rate of recovery from both agonists and reduces the area under the $[Ca^{2+}]_i$ response curve after stimulation with either agonist.

Materials and Methods

Vascular Smooth Muscle Cell Preparation

A clonal VSMC line (A7r5) from thoracic aorta of BD1X rats was obtained from American Type Culture Collection, Rockville, Md. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10,000 U/mL penicillin, 10 mg/mL streptomycin, and 8 mg/mL tylosin tartrate and were maintained in a 95% O₂/5% CO₂, 100% humidity atmosphere at 37°C. In a separate series of studies, human pulmonary artery VSMCs were obtained (Clonetics, Inc., San Diego, Calif.) in passage 3 and grown as described above in a medium containing 5% fetal calf serum, 10 ng/mL epidermal growth factor, 1 mM dexamethasone, 2 ng/mL human fibroblast growth factor, gentamicin, and amphotericin-B (Hoshi et al. 1988). For sequential passage or study, confluent cells were rinsed with Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO.) and treated with 0.5 mg/mL trypsin for 5 minutes at 37°C. Released cells were centrifuged at 200 x g for 5 minutes, and the pellet was then resuspended in DMEM. The growth medium of confluent cells was replaced with serum-free DMEM and antibiotics (as above) for 24 hours before VSMC Ca²⁺ studies. A7r5 cells were studied in passages 4 to 22, and human cells were studied in passage 5 to 6.

Ca²⁺ Determination

The fluorescent dye fura-2/AM (acetylmethoxy ester, Calbiochem, San Diego, CA.) was dissolved in DMSO to yield a final fura-2 concentration of 10 μ M. AII, AVP were dissolved in serum-free HEPES-buffered salt solution (HBSS) containing (in mM): NaCl 138, CaCl₂ 1.8, MgSO₄ 0.8, NaH₂PO₄ 0.9, NaHCO₃ 4.0, D-glucose 25, glutamine 6.0, HEPES 20, and 5% bovine serum albumin, pH 7.4.

Confluent cell monolayers were maintained in serum-free DMEM for 24 hours, and quiescent cells were then rinsed with Hanks' solution, trypsinized, and centrifuged. The pellet was resuspended in HBSS, and cell suspensions were prechilled on ice for approximately 10 minutes before fura-2/AM loading. Fura-2-loaded cells were incubated in a shaking water bath in the dark for 20 minutes at 37°C and then sedimented by centrifugation and resuspended in HBSS. Cells were further incubated for 60 minutes in a 37°C shaking water bath in the absence of insulin or 10⁻⁷ or 10⁻⁸ M insulin. Cells were then washed, centrifuged, and resuspended at a concentration of approximately 10⁶ cells/mL. [Ca²⁺]_i levels were then determined fluorometrically in suspensions using dual excitation (340 and 380 nm) per single emission (510 nm) fluorometer (Hitachi F-2000, Naperville, Il.).

Maximal and minimal fluorescent signals were obtained

with 40 μM digitonin and pH 8.7 Tris (100 mM) containing 100 mM EGTA to calibrate the intracellular Ca^{2+} signal.

Intracellular Ca^{2+} was then calculated by the computer in the fluorometer using the equation of Grynkiewicz et al. (Grynkiewicz et al. 1985).

Peak Ca^{2+} response to AII (200 nM) and AVP (10 μM) were evaluated after establishing a stable baseline (within the first 2 minutes of study) for each cell suspension. The rate of $[\text{Ca}^{2+}]_i$ recovery (i.e., rate of return to baseline) after stimulation with each agonist was calculated from the slope of the $[\text{Ca}^{2+}]_i$ tracings in the recovery (i.e., post-peak) phase. Recovery rate was calculated by regression analysis. To determine the effects of insulin on releasable sarcoplasmic reticulum Ca^{2+} stores, 1 μM thapsigargin, a specific sarcoplasmic reticulum Ca^{2+} -ATPase inhibitor, was added to some control and insulin-treated preparations.

All data were captured in a microcomputer using a data acquisition program, and data points were converted to a spreadsheet format (QUATTRO PRO 4.0, Borland International, Inc., Scotts Valley, CA.). Regression analysis was performed on the first 30-second period after each peak response. The data were uniformly linear for at least 45 seconds in all cases. Attempts to fit the full recovery data set from each curve with an exponential regression resulted in qualitatively comparable data, but the exponential regressions resulted in a poorer fit (i.e., larger error

term).

All experiments were replicated eight times, and data were analyzed for differences between control and insulin-treated VSMCs using the unpaired t test.

Results

Baseline $[Ca^{2+}]_i$ levels were not significantly affected by insulin incubation. Figure 3.1 and 3.2 depict typical tracings of the effect of insulin on the A7r5 VSMC line $[Ca^{2+}]_i$ response to AII and AVP, respectively, in A7r5 cells, and the data are summarized in Table 3.1.

The 10^{-7} M insulin-treated preparations exhibited a significantly greater peak $[Ca^{2+}]_i$ response to AII but also markedly accelerated the rate of $[Ca^{2+}]_i$ recovery to baseline (77.3 ± 13.8 versus 30.6 ± 6 nM/min, $p < 0.03$). Consequently, although the insulin caused an increase in peak response to AII, the accelerated Ca^{2+} recovery resulted in a reduction in the area under the $[Ca^{2+}]_i$ response curve (Table 3.1). Treatment with 10^{-8} M insulin resulted in an intermediate recovery rate (56.9 ± 6 nM/min) and peak response ($p < 0.03$).

In contrast to the insulin-induced augmentation of the peak $[Ca^{2+}]_i$ response to AII, insulin treatment reduced the peak $[Ca^{2+}]_i$ response to AVP (Figure 3.2, Table 3.1). However, the rate of recovery to baseline was still significantly increased in the insulin-treated cells compared with control (Table 3.1), resulting in a decrease in the area under the AVP-induced $[Ca^{2+}]_i$ response curve (Table 3.1).

Recovery rates were slower in the human pulmonary

artery VSMCs than in the A7r5 cells, but insulin exerted a comparable effect on increasing recovery (AII recovery rate was 15.4 ± 1.6 versus 11.2 ± 1.2 nM/min in insulin-treated and control cells, respectively, $p < 0.025$; AVP recovery rate was 13.7 ± 2.5 versus 6.9 ± 0.8 nM/min, $p < 0.02$) (Table 3.2) and decreasing the area under the $[Ca^{2+}]_i$ response curve to both agonists (Table 3.2). However, insulin was without effect on the peak response to either agonist (Table 3.2).

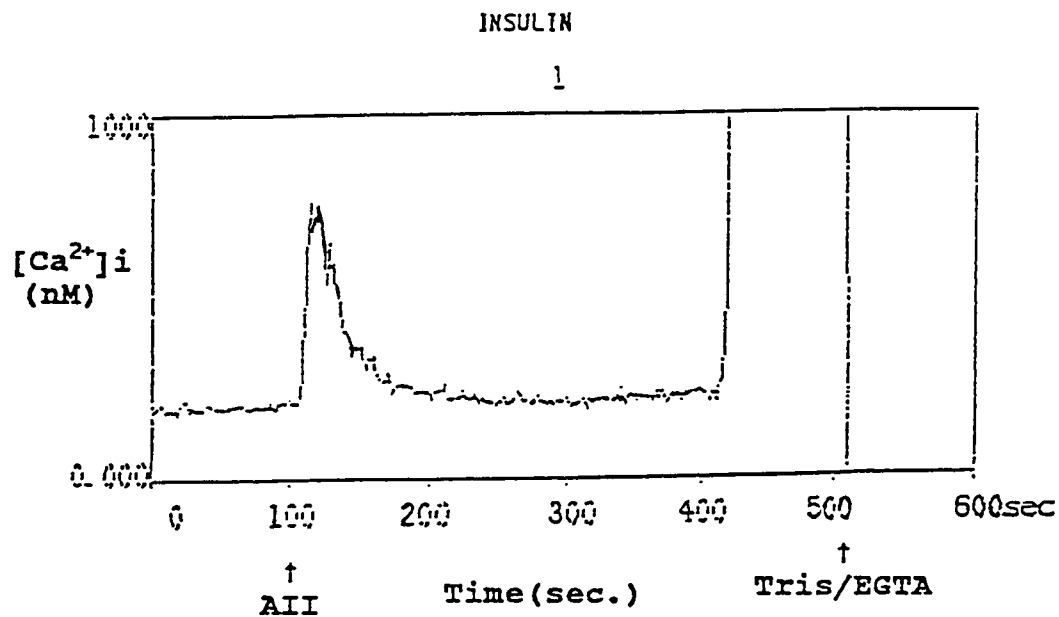
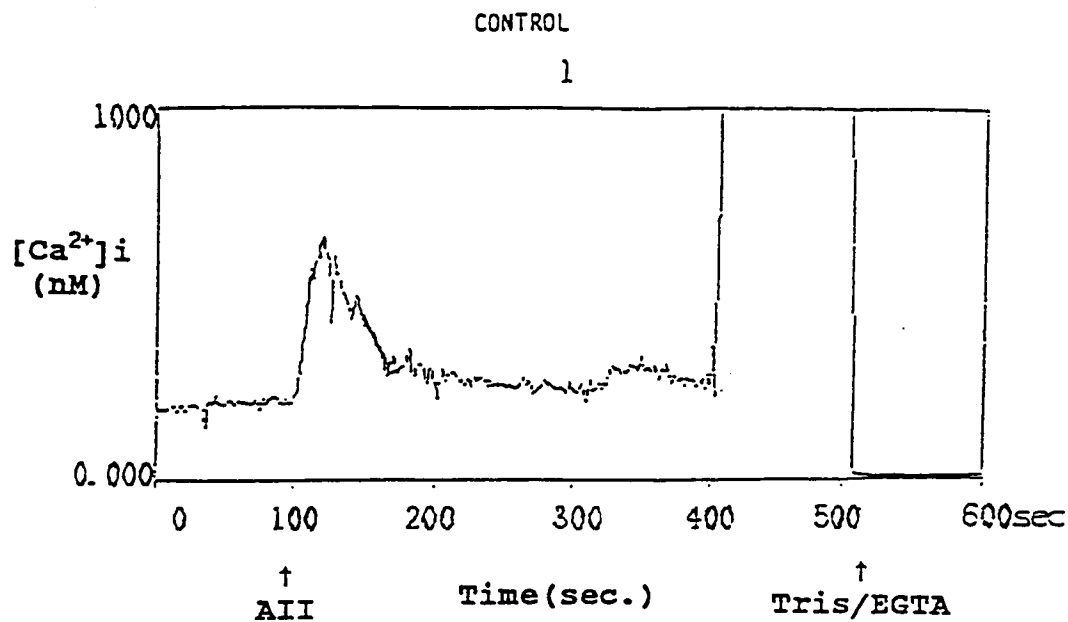


Figure 3.1 Characteristic fluorometric tracings of intracellular Ca²⁺ ([Ca²⁺]_i) responses to angiotensin II (AII) in control (top panel) and 10⁻⁷ M insulin-treated (bottom panel) A7r5 vascular smooth muscle cell suspensions.

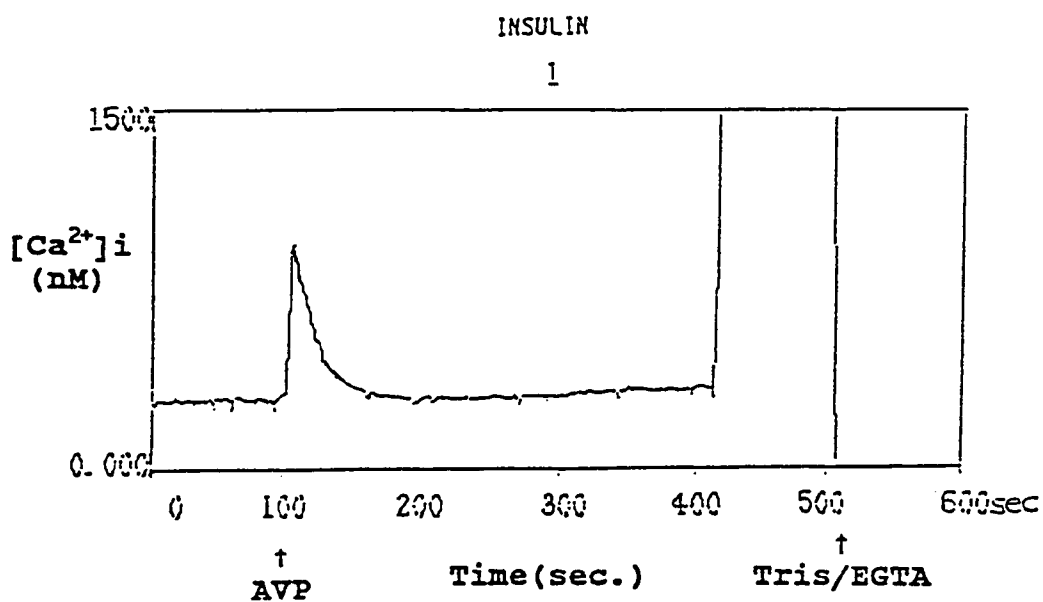
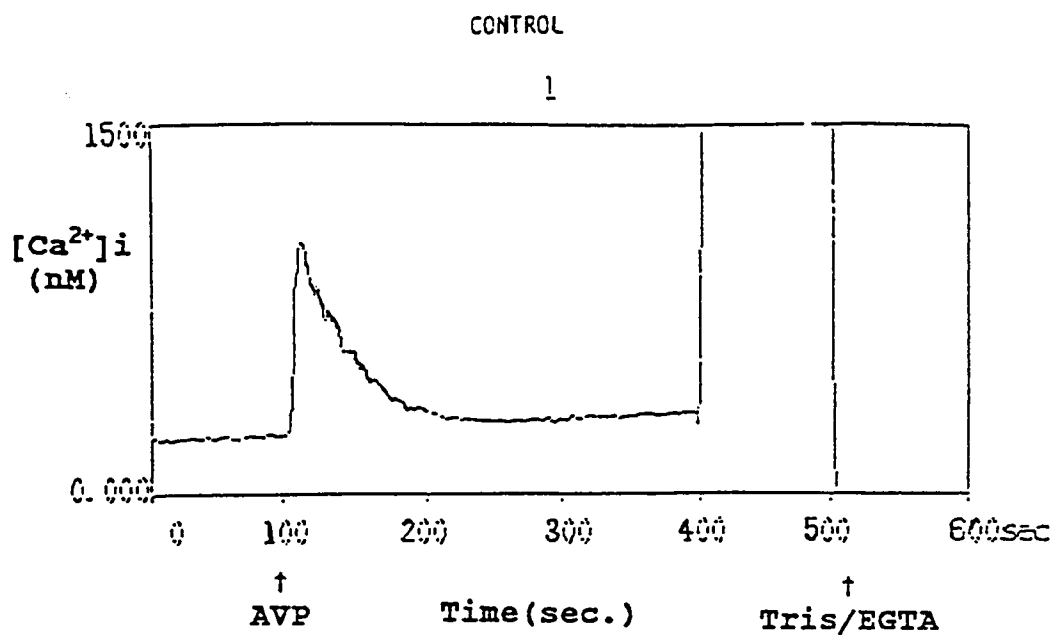


Figure 3.2 Characteristic fluorometric tracings of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) responses to arginine vasopressin (AVP) in control (top panel) and 10^{-7} M insulin-treated (bottom panel) A7r5 vascular smooth muscle cell suspensions.

Table 3.1 Effects of Insulin on A7r5 Vascular Smooth Muscle Intracellular Calcium Responses to Angiotensin II (AII) and Arginine Vasopressin (AVP).

Parameter	Control (n=8)	Insulin(10^{-7} M) (n=8)
Response to AII (%) ¹	288 ± 74	469 ± 96*
Response to AVP (%) ¹	386 ± 33	288 ± 50**
Recovery from AII (nM/min) ²	30.6 ± 6	77.3 ± 13.8**
Recovery from AVP (nM/min) ²	18.46 ± 4.7	23.44 ± 4.7*
Area under AII Response ³ curve (nM · sec)	19,865 ± 515	16,890 ± 258*
Area under AVP Response ³ curve (nM · sec)	37,556 ± 920	14,399 ± 689**

All data are expressed as mean ± SEM.

¹Response (%) = Peak/Baseline x 100.

²Recovery = rate of return to baseline $[Ca^{2+}]_i$ following AII and AVP stimulation; baseline $[Ca^{2+}]_i$, 141 ± 14.

³Area, the net area under the agonist-induced $[Ca^{2+}]_i$ response curve.

*P < 0.05

**P < 0.03

Table 3.2 Effects of Insulin on Human Pulmonary Artery Vascular Smooth Muscle Intracellular Calcium Responses to Angiotensin II (AII) and Arginine Vasopressin (AVP).

Parameter	Control (n=8)	Insulin(10^{-7} M) (n=8)
Response to AII (%) ¹	113 ± 5.87	112 ± 5.96
Response to AVP (%) ¹	119 ± 11.07	116 ± 4.7
Recovery from AII (nM/min) ²	11.2 ± 0.12	15.4 ± 0.16*
Recovery from AVP (nM/min) ²	0.69 ± 0.08	1.37 ± 0.25**
Area under AII Response ³ curve (nM · sec)	16,400 ± 310	12,125 ± 215*
Area under AVP Response ³ curve (nM · sec)	29,250 ± 250	14,100 ± 632**

All data are expressed as mean ± SEM.

¹Response (%) = Peak/Baseline x 100.

²Recovery = rate of return to baseline $[Ca^{2+}]_i$ following AII and AVP stimulation; baseline $[Ca^{2+}]_i$, 206.96 ± 6.9.

³Area, the net area under the agonist-induced $[Ca^{2+}]_i$ response curve.

*P < 0.025

**P < 0.013

Discussion

Data from the present study demonstrate that insulin accelerates the rate of $[Ca^{2+}]_i$ recovery to baseline in cultured VSMCs after stimulation with either AII or AVP. It has been recently reported that insulin incubation also stimulates the expression of plasmalemma Ca^{2+} -ATPase in A7r5 cultured VSMCs (Zemel et al. 1992) and causes a corresponding increase in Ca^{2+} -ATPase-mediated Ca^{2+} efflux. Insulin was also found to increase the expression of sarcoplasmic reticulum (SR) Ca^{2+} -ATPase in these cells (Zemel et al. 1993), although the time course of this stimulation was somewhat delayed in comparison with stimulation of the plasmalemma Ca^{2+} -ATPase. Accordingly, the increase in $[Ca^{2+}]_i$ recovery rate in the present study probably reflects insulin stimulation of plasmalemma Ca^{2+} -ATPase, SR Ca^{2+} -ATPase, or both.

In support of this concept, Zemel et al. (1992) previously reported that insulin stimulates rat aortic $^{45}Ca^{2+}$ efflux in a dose-dependent fashion. Moreover, it has also been recently reported decreases in both plasmalemma Ca^{2+} -ATPase expression (Zemel et al. 1991b) and the rate of $[Ca^{2+}]_i$ return to baseline in VSMCs obtained from Zucker obese rat aortas compared with Zucker lean rat aortas (Abel and Zemel 1993). Since the Zucker obese rat exhibits insulin resistance, it is concluded that these observations result

from a vascular smooth muscle manifestation of insulin resistance and a consequent failure of insulin to stimulate Ca^{2+} -ATPase expression and Ca^{2+} efflux.

Insulin attenuation of vasoconstrictor responses may also result, in part, from blunting of the peak $[\text{Ca}^{2+}]_i$ response to vasoconstrictor agonists. Insulin has been shown to decrease peak $[\text{Ca}^{2+}]_i$ responses to AVP in cultured rat aortic smooth muscle cells by reducing Ca^{2+} influx through receptor-operated channels and to decrease voltage-mediated Ca^{2+} influx (Standley et al. 1991). Results of the present study are consistent with these observations since insulin incubation resulted in a significant reduction in the peak $[\text{Ca}^{2+}]_i$ response to AVP in A7r5 VSMCs. However, the physiological significance of this observation is not clear because this laboratory found no difference in the peak $[\text{Ca}^{2+}]_i$ response to AVP in VSMCs isolated from Zucker lean and obese rats (Abel and Zemel 1993). Thus, despite other vascular smooth muscle manifestations of insulin resistance in Zucker obese rats, insulin regulation of AVP receptor-operated vascular smooth muscle Ca^{2+} channels appears to be preserved in this model of insulin resistance. Moreover, insulin attenuation of the peak $[\text{Ca}^{2+}]_i$ response was not evident in human VSMCs in the present study.

In contrast to the effects of insulin on peak $[\text{Ca}^{2+}]_i$ responses to AVP, insulin incubation caused a marked increase in the peak $[\text{Ca}^{2+}]_i$ response to AII in the A7r5

cells. However, insulin incubation also caused a twofold increase in the rate of recovery from the AII-induced $[Ca^{2+}]_i$ load and consequently caused a decrease in net cellular exposure to elevated $[Ca^{2+}]_i$ (i.e., area under the $[Ca^{2+}]_i$ curve). Since insulin attenuates the vasoconstrictor responses to AII (Kahn et al. 1993), it appears that the effects on recovery rate and area under the $[Ca^{2+}]_i$ response curve may be more important to the regulation of vasoconstrictor responses than are insulin effects on peak $[Ca^{2+}]_i$ responses to vasoconstrictor agonists. Moreover, since insulin increased the $[Ca^{2+}]_i$ recovery rate in the cultured human VSMCs without affecting the peak response to either agonist, the relevant effect of insulin on human VSMC $[Ca^{2+}]_i$ regulation is likely limited to stimulation of $[Ca^{2+}]_i$ recovery. However, whether these differences between rat and human cells will be sustained in human cells cultured from other vessels remains to be determined.

The mechanism of insulin augmentation of peak $[Ca^{2+}]_i$ responses to AII in A7r5 cells is not clear. However, insulin has been found to increase SR Ca^{2+} -ATPase expression; because AII-induced $[Ca^{2+}]_i$ transients are dependent on SR Ca^{2+} -ATPase release, we speculated that insulin stimulation of SR Ca^{2+} -ATPase may have resulted in increased AII-releasable stores. To explore this possibility, experiments were performed to evaluate the effects of inhibiting SR Ca^{2+} -ATPase on $[Ca^{2+}]_i$ in insulin-

treated and control cells. Treatment of cells with 1 μ M of the SR Ca^{2+} -ATPase inhibitor thapsigargin resulted in a substantially greater $[\text{Ca}^{2+}]_i$ response in insulin-treated cells than in controls as well as a faster recovery rate (Figure 3.3). This increased release of Ca^{2+} by thapsigargin supports the concept that insulin may increase peak $[\text{Ca}^{2+}]_i$ responses to AII by stimulating SR Ca^{2+} -ATPase and thereby increasing AII-releasable stores. In addition, the observed increase in $[\text{Ca}^{2+}]_i$ recovery rate in the presence of an SR Ca^{2+} -ATPase inhibitor is likely to reflect insulin stimulation of plasma membrane Ca^{2+} -ATPase.

Several lines of recent evidence suggest that insulin exerts vasodilator effects and protects against vasoconstriction and that loss of these normal vascular smooth muscle cellular responses to insulin in insulin resistance results in predisposition to hypertension (Zemel et al. 1991b, 1992 & 1993, Anderson et al. 1991 & 1992, Standley et al. 1991). Insulin attenuates *in vitro* vasoconstrictor responses to vasoconstrictor agonists (Zemel et al. 1990 & 1991a), and accelerates vascular relaxation (Zemel et al. 1992), whereas these effects are blunted in vascular smooth muscle from insulin-resistant animals (Zemel et al. 1991). Moreover, insulin infusion increases forearm blood flow (Neahring et al. 1993) and reduces peripheral vascular resistance and blood pressure in both normotensive and hypertensive individuals (Anderson et al. 1991 & 1992).

Several additional reports also support the concept that elevated circulating insulin per se does not result in blood pressure elevations and that VSMC resistance to the actions of insulin may instead be responsible for hypertension in insulin-resistant states (Gans et al. 1992, Joffe et al. 1992 and Sawicki et al. 1992). For example, Gans et al. (1992) recently reported that induction of acute hyperinsulinemia in a euglycemic clamp study of non-insulin-dependent diabetic patients resulted in blood pressure reductions despite observed reductions in the fractional excretion of sodium. Data from the present study, demonstrating an acceleration in $[Ca^{2+}]_i$ recovery rate after stimulation with vasoconstrictor agonists, appear to provide a physiological rationale for these observations.

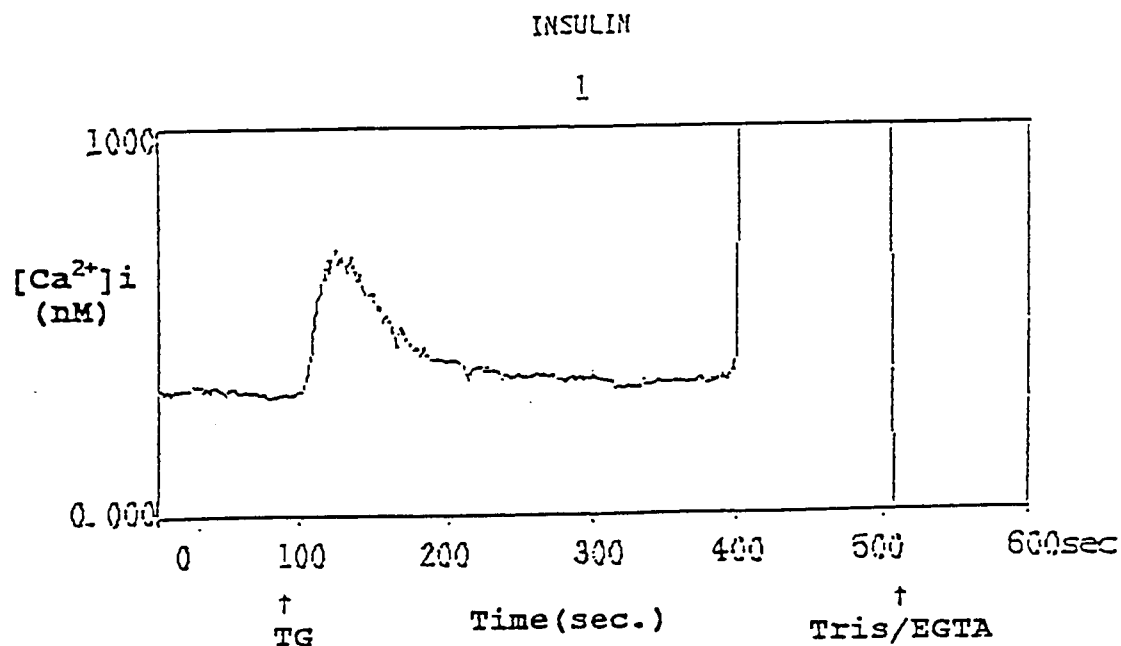
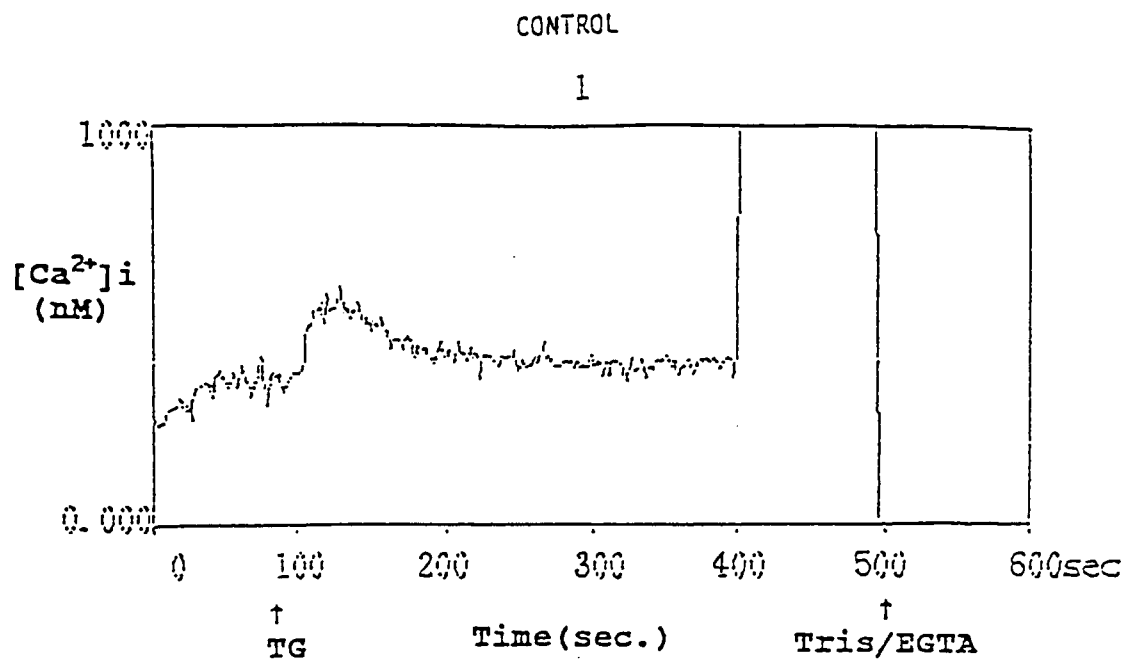


Figure 3.3 Characteristic fluorometric tracings of intracellular Ca²⁺ ([Ca²⁺]_i) responses to thapsigargin (TG) in control (top panel) and 10⁻⁷ M insulin-treated (bottom panel) A7r5 vascular smooth muscle cell suspensions.

Literature Cited

- Abel, M.A., Zemel, M.B. (1993) Impaired recovery of vascular smooth muscle intracellular calcium following agonist stimulation in insulin resistant (Zucker obese) rats. *Am. J. Hypertension* 6, 500-504.
- Anderson, E.A., Hoffman, R.P., Balor, T.W., Sinkey, C.A., Mark, A.L. (1991) Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J Clin Invest.* 87:2246-2252.
- Anderson, E.A., Balor, T.W., Hoffman, R.P., Sinkey, C.A., Mark, A.L. (1992) Insulin increases sympathetic activity but not blood pressure in borderline hypertensive humans. *Hypertension.* 19:621-627.
- Dubey, R.K., Zhang, H.Y., Reddy, S.R., Boegehold, M.A., Kotchen, T.A. (1993) Pioglitazone attenuate hypertension and inhibits growth of arteriolar smooth muscle in rats. *Am. J. Physiol.* 265, R726-732.
- Grynkiwicz, G., Poenie, M., Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J Biol Chem.* 260:3440-3450.
- Gans, R.O.B., Bilo, H.G.J., Nauta, J.J.P., Heine, R.J., Donker, A.J.M. (1992) Acute hyperinsulinemia induces sodium retention and a blood pressure decline in diabetes mellitus. *Hypertension.* 20:199-209.
- Hofmann, C., Lorenz, K., Colca, J.R. (1991) Glucose transport deficiency in diabetic animals is corrected by treatment with the oral antihyperglycemic agent pioglitazone. *Endocrinology.* 129:1915-1925.
- Hoshi, H., Kan, M., Chen, J-K., Mckeehan, W.L. (1988) Comparative endocrinology-autocrinology of human adult large vessel endothelial and smooth muscle cells. *In Vitro Cell Dev Biol.* 4:309-320.
- Joffe, B.I., Panz, V.R., Wing, J.R., Raal, F.C.P., Seftel, H.C. (1992) Relationship between plasma insulin and blood pressure in South African black women in Johannesburg. *Diabetes Care.* 4:556-558.
- Kahn, A.M., Seidel, C.L., Allen, J.C., O'Neil, R.G. (1993) Insulin reduces contraction and intracellular calcium concentration in vascular smooth muscle. *Hypertension.* 22:735-742.

- Neahring, J.R., Stepniakowski, K., Greene, A.S., Egan, B.M. (1993) Insulin does not reduce forearm alpha-vasoreactivity in obese hypertensive or lean normotensive men. *Hypertension*. 22(4), 584-590.
- Reaven, G.M. (1988) Role of insulin resistance in human disease. *Diabetes*. 37:1596-1607.
- Rocchini, A.P. (1991) Insulin resistance and blood pressure regulation in obese and non-obese subjects. *Hypertension*. 17:837-842.
- Sawicki, P.T., Heinemann, L., Berger, M. (1992) Hyperinsulinemia is not linked with blood pressure elevation in patients with insulinoma. *Diabetologia*. 35:649-652.
- Standley, P.R., Zhang, F., Ram, J.L., Zemel, M.B., Sowers, J.R. (1991) Insulin attenuate vasopressin-induced calcium transients and voltage-dependent calcium current in rat vascular smooth muscle cells. *J Clin Invest*. 88:1230-1236.
- Zemel, M.B., Johnson, B.A., Ambrozy, S.A. (1992) Insulin-stimulated vascular relaxation: role of Ca^{2+} -ATPase. *Am J Hypertens*. 5:637-641.
- Zemel, M.B., Iannucci, A., Moore, J.W. (1993) Role of insulin in regulating vascular smooth muscle Ca^{2+} -ATPase expression. *J. Vasc. Med. Biol*. 4:79-84.
- Zemel, M.B., Reddy, S., Shehin, S., Lockette, W., Sowers, J.R. (1990) Vascular reactivity in Zucker obese rats. Role of insulin resistance. *J Vasc Med Biol*. 2:81-85.
- Zemel, M.B., Reddy, S., Sowers, J.R. (1991a) Insulin attenuation of vasoconstrictor responses to phenylephrine in Zucker lean and obese rats. *Am J Hypertension*. 4:537-539.
- Zemel, M.B., Iannucci, A., Johnson, B.A.B., Sowers, J.R. (1991b) Reduced Ca^{2+} -ATPase expression in insulin resistant (Zucker obese) rats. *Hypertension*. 18:442. Abstract. rats. *Am J Hypertension*. 6:500-504.

PART IV

INSULIN STIMULATION OF INTRACELLULAR FREE CALCIUM
RECOVERY AND Ca^{2+} -ATPase GENE EXPRESSION IN
CULTURED VASCULAR SMOOTH MUSCLE CELLS:
ROLE OF GLUCOSE-6-PHOSPHATE.

Abstract

We have previously reported that insulin accelerates $[Ca^{2+}]_i$ recovery from pressor agonist-induced Ca^{2+} loads and stimulates both plasmalemma (PM) and sarcoplasmic reticulum (SR) Ca^{2+} -ATPases gene expression in cultured and freshly isolated VSMCs, suggesting that insulin attenuation of vascular tone may result from modulation of $[Ca^{2+}]_i$. Accordingly, we have now evaluated the linkage between this insulin-regulation of VSMC $[Ca^{2+}]_i$ to classical actions of insulin (ie. glucose transport and metabolism). Cultured VSMCs were incubated in the presence or absence of insulin in a medium containing either pyruvate, glucose, 3-O-methylglucose (3-O-MG) or 2-deoxyglucose (2-DG). Insulin caused a 87% increase in $[Ca^{2+}]_i$ recovery rate following stimulation with arginine vasopressin ($p < 0.01$) and caused a marked increase in both PM and SR Ca^{2+} -ATPases expression in the presence of glucose. Comparable increases in both $[Ca^{2+}]_i$ recovery and Ca^{2+} -ATPase expression were found when glucose was replaced by 2-deoxyglucose. In contrast, no stimulation was found in either the glucose-free or 3-O-methylglucose-containing medium. Since both glucose analogues are transported, but only 2-DG is phosphorylated, this indicates that glucose transport and metabolism to glucose-6-phosphate is essential for insulin regulation of VSMC $[Ca^{2+}]_i$, possibly via a glucose-6-phosphate-dependent

carbohydrate response element in the Ca^{2+} -ATPase gene.

Introduction

Insulin resistance and compensatory hyperinsulinemia have been implicated in the pathogenesis of hypertension, and there is a significant association between hypertension and insulin resistance in both obese and non-obese individuals (Sowers et al. 1991, Ferrannini et al. 1987). Further, both insulin resistance and hyperinsulinemia are associated with hypertension even in the absence of obesity or overt glucose intolerance (Ferrannini et al. 1987, Modan et al. 1985), indicating a direct relationship between insulin resistance and hypertension. However, the underlying cellular link to hypertension remains unclear.

Although hyperinsulinemia may contribute to hypertension via stimulation of sympathetic neural output (Reaven 1988) and renal sodium retention (Rocchini 1991), recent evidences from our laboratory and others strongly suggest that insulin exerts direct vasodilatory effects which may be impaired in insulin resistant states. We have shown that insulin attenuates *in vitro* vascular reactivity responses to pressor agonists (Zemel et al. 1990 & 1991a), accelerates vascular smooth muscle relaxation and Ca^{2+} efflux (Zemel et al. 1992), and inhibits vascular smooth muscle cell (VSMC) intracellular free calcium ($[\text{Ca}^{2+}]_i$) responses to arginine vasopressin (AVP) (Standley et al. 1991). Further, we have recently found insulin to accelerate

[Ca²⁺]_i recovery from agonist-induced Ca²⁺ loads in both cultured rat VSMC and human pulmonary artery VSMC (Kim and Zemel 1993) and to stimulate gene expression for both plasmalemma and sarcoplasmic reticulum Ca²⁺-ATPases (Zemel et al. 1993). Moreover, all these insulin effects were blunted in VSMCs freshly isolated from an animal model of insulin resistance and hypertension, the Zucker obese rat and from insulinopenic rats (Zemel et al. 1991a & 1991b, Abel and Zemel 1993). Insulin has also shown to exert vasodilatory effects on VSMC from canine femoral artery (Khan et al. 1993) and rabbit efferent arterioles (Juncos et al. 1992).

Accordingly, we have proposed that insulin-induced modulation of Ca²⁺ transport results in vascular relaxation and reduced vascular tone. Further, blunting of this modulation may be responsible for hypertension in insulin resistant states. However, it is unclear whether these effects of insulin are related to or dependent upon the classical actions of insulin, such as glucose transport and metabolism. Therefore, to further investigate whether insulin-regulation of vascular smooth muscle cell [Ca²⁺]_i and attenuation of vasoconstriction is linked to the insulin regulation of glucose metabolism, we studied the effects of glucose transport and metabolism on insulin regulation of VSMC [Ca²⁺]_i responses to a pressor agonist (AVP) and of Ca²⁺-ATPase gene expression. We report here that insulin

regulation of vascular smooth muscle cell $[Ca^{2+}]_i$ and attenuation of vasoconstriction appears to depend upon insulin-induced glucose transport and phosphorylation to glucose-6-phosphate.

Materials and Methods

Materials : Cells from the A7r5 vascular smooth muscle cell line were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's modified eagles medium (DMEM), fetal bovine serum (FBS) and fetal calf serum (FCS) were obtained from Gibco BRL (Gaithersburg, MD). Insulin, mannitol, pyruvate, 2-deoxy-D-glucose (2-DG), 3-O-methyl-D-glucose (3-O-MG), fructose, fluorescent dye Fura-2-acetylmethoxy ester (Fura-2-AM) and cytochalasin B were purchased from Sigma Chemicals Co. (St. Louis, MO.). Insulin and digitonin were prepared in 10 mM acetic acid and in distilled water, respectively. AVP was dissolved in serum-free HEPES-buffered salt solution (HBSS) containing 138 mM NaCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 0.9 mM NaH_2PO_4 , 4.0 mM NaHCO_3 , 25 mM D-glucose, 6.0 mM glutamine, 20 mM HERES and 5% bovine serum albumin, pH 7.4.

Glucose-depleted HBSS was prepared with either glucose or pyruvate as energy source and mannitol for osmotic adjustment with or without 3-O-MG or 2-DG, as detailed below. Guanidine isothiocyanate (GTC) and cesium chloride were purchased from Gibco BRL (Gaithersburg, MD).

Cell Culture : A7r5 VSMCs were maintained under 95% O_2 /5% CO_2 and 90-95% humidity at 37°C in growth medium (DMEM supplemented with 5% FBS, 5% FCS, 5,000 U/mL penicillin G

sodium and 5,000 $\mu\text{g/mL}$ streptomycin sulfate). Upon reaching confluence, cells were incubated for 24 hrs in a 0.2% FBS medium and antibiotics as above. Quiescent cell monolayers were then rinsed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' balanced salt solution (Sigma Chemicals) and were released by treatment with 0.05% trypsin, 0.53 mM EDTA/4Na. For fluorometric ionized Ca^{2+} measurement, the released cells were pelleted by centrifugation at 200 X g for 5 min and the pellet was resuspended in glucose-depleted HBSS with substitutions as follows; 1) 25 mM D-glucose; 2) 4 mM pyruvate, 16 mM mannitol and 1 mM 2-DG; 3) 4 mM pyruvate and 17 mM 2-DG; 4) 4 mM pyruvate and 17 mM 3-O-MG; 5) 4 mM pyruvate and 17 mM mannitol; 6) 25 mM fructose; 7) 25 mM D-glucose and 25 μM cytochalasin B.

[Ca^{2+}]_i Measurements : Intracellular calcium response to and rates of recovery from AVP were performed spectrofluorometrically as previously described (Standley et al. 1991, Kim and Zemel 1993, Abel and Zemel 1992). Cells resuspended in each glucose-depleted HBSS were prechilled at least ten minutes before Fura-2-AM loading. Fura-2 loaded cells were incubated in the dark for a further 60 minutes in the presence and absence of 10^{-7} M insulin in a 37°C shaking water bath. After 1 hr incubation, cells were centrifuged and resuspended in the indicated buffer to remove extracellular fura-2 dye prior to [Ca^{2+}]_i determination.

Intracellular calcium levels were then determined using a dual excitation (340 and 380 nm) and single emission (510 nm) spectrofluorometer (Hitachi F-2000, Naperville, IL). The intracellular calcium signal was calibrated using a maximum ($F_{\max}$) and minimum ($F_{\min}$) fluorescence ratio signal obtained by 40 μM digitonin and Tris/EGTA (100 mM/100 mM, pH 8.7), respectively. $[\text{Ca}^{2+}]_i$ was then calculated using the K_d of 224 nM (Grynkiecz et al. 1985). After establishing a stable baseline, $[\text{Ca}^{2+}]_i$ responses to and rates of recovery from AVP were evaluated. The rate of $[\text{Ca}^{2+}]_i$ recovery was determined for 10 seconds from the peak by AVP stimulation, as previously described (Kim and Zemel 1993).

Preparation of Total Cellular RNA : To evaluate the expression of Ca^{2+} -ATPases, quiescent VSMC monolayers (250 cm^2 flask / determination) were incubated in the buffer as indicated above in the presence and absence of insulin (10^{-7} M) for 4 hrs, and the cells were then immediately harvested by treating with guanidine thiocyanate. The released cells were shortly sonicated and centrifuged at 143,164 X g for 18 hrs (Sorvall Ultraspeed Centrifuge, DuPont). Total cellular RNA was isolated from cells (using CsCl_2 density gradient) using the guanidine thiocyanate/cesium chloride procedure (Sambrook et al. 1989). RNA concentration was measured spectrofluorometrically ($A_{260/\lambda 280}$).

Northern Blot Analysis : Total RNA (20 μ g) were denatured with 6.5% formaldehyde/0.05% formamide and its integrity was assessed by running on a 1% (w/v) agarose gel containing formaldehyde (0.66 M) in a 20 mM MOPS/5 mM sodium acetate/1 mM EDTA (pH 7.0) buffer at 75 volts. The gels were rinsed, visualized under an UV light and photographed. The separated RNA was then transferred to nylon filters (Genescreen Plus, DuPont) by Northern blot for approximately 16 hrs.

Hybridization and Analysis : The filters were prehybridized in a prehybridization solution (Gibco BRL) containing 6X SSC, 5X Denhardt's solution [0.1% (w/v) Ficoll Type 400, 0.1% (w/v) FBS], 0.5% (w/v) SDS and 100 μ g/ml sheared, denatured salmon sperm DNA (ssDNA) for 3 hrs at 42°C, followed by hybridization for 20 hrs in hybridization solution (Gibco BRL) containing 6X SSC, 0.01 M EDTA (pH 8.0), 5X Denhardt's solution, 0.5% (w/v) SDS and 100 μ g/ml ssDNA with ^{32} P-labeled oligonucleotide probes (described below), washed with 2X SSC twice, and then with 0.1X SSC, 0.1% SDS at 65 °C. Autoradiography was then performed with Kodak XAR-5 film and intensifying screen at -80°C. The resulting autoradiographs were quantitated densitometrically.

Labeling of cDNA probes of Ca²⁺-ATPases : The probes used were a 40-base, a 45-base and a 40-base oligonucleotide probes derived from human plasma membrane Ca²⁺-ATPase

(Oncogene Science, Inc), rat sarcoplasmic reticulum Ca^{2+} -ATPase (DuPont/NEN) and β -actin (DuPont/NEN), respectively. Hybridization probes were labeled with α - ^{32}P -dATP using a oligonucleotide 3'-end labeling kit (DuPont/NEN) and were used for hybridization as described above.

Data Interpretation and Analysis : All experiments were repeated at least eight times, and data were statistically analyzed via analysis of variance. For Ca^{2+} -ATPase expression, all data were normalized to β -actin mRNA.

Results

Role of Insulin on VSMC $[Ca^{2+}]_i$ Recovery and on Glucose

Transport: In the presence of glucose, insulin significantly accelerated the rate of $[Ca^{2+}]_i$ recovery following stimulation with AVP (Figure 4.1 and Figure 4.4), as we have previously reported (Kim and Zemel 1993, Abel and Zemel 1992). However, when A7r5 VSMC were incubated in the absence of glucose, but in the presence of 4 mM pyruvate and 17 mM mannitol as oxidative substrates and osmotic control, respectively, insulin did not significantly affect either peak $[Ca^{2+}]_i$, the rate of recovery to baseline or the area under the $[Ca^{2+}]_i$ response curve to AVP (Figure 4.3).

Similarly, insulin was without effect when glucose transport was inhibited with cytochalasin B or when the non-metabolizable glucose analogue 3-O-MG, which is transported into the cell but not phosphorylated, was substituted for glucose (Figure 4.2 and Figure 4.3). In contrast, insulin markedly accelerated the rate of $[Ca^{2+}]_i$ recovery to basal level following AVP stimulation in the presence of either 1 or 17 mM 2-DG (Figure 4.2 and 4.4, and Table A.1), which is phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate but not further metabolized.

Effects of Carbohydrates on Insulin Stimulation of Ca^{2+} -

ATPase Pumps : We have previously demonstrated that insulin

stimulates both plasmalemma and sarcoplasmic reticulum Ca^{2+} -ATPase mRNA levels in cultured VSMC (Zemel et al. 1993). Moreover, this increase in mRNA resulted in acceleration of cytosolic Ca^{2+} removal following VSMC calcium loads (Kim and Zemel 1993, Zemel et al. 1993). In the present study, the 4-hour insulin (10^{-7} M) incubation of cultured VSMC significantly ($p < 0.05$) increased both plasmalemma and sarcoplasmic reticulum Ca^{2+} -ATPase expression in the presence of either glucose or 2-DG (glucose; 243 and 244% stimulation of plasmalemma and sarcoplasmic reticulum Ca^{2+} -ATPase, respectively; 2-DG: 701 and 660% stimulation, respectively) (Table 4.1 and 4.2). However, when cells were incubated with 3-O-MG substituted for glucose or with 4 mM pyruvate and 17 mM mannitol, insulin was without effect on the expression of both calcium pumps (Table 4.1 and 4.2). Further, incubation with 25 mM fructose which is independent of insulin and inhibition of glucose transport with cytochalasin B resulted in both a marked diminution of Ca^{2+} -ATPase expression and a lack of insulin stimulation of expression. A representative autoradiograph of depicting these data is found in Figure 4.5. Insulin treatment was without effect on actin mRNA in all of the experimental treatments (Table 4.3).

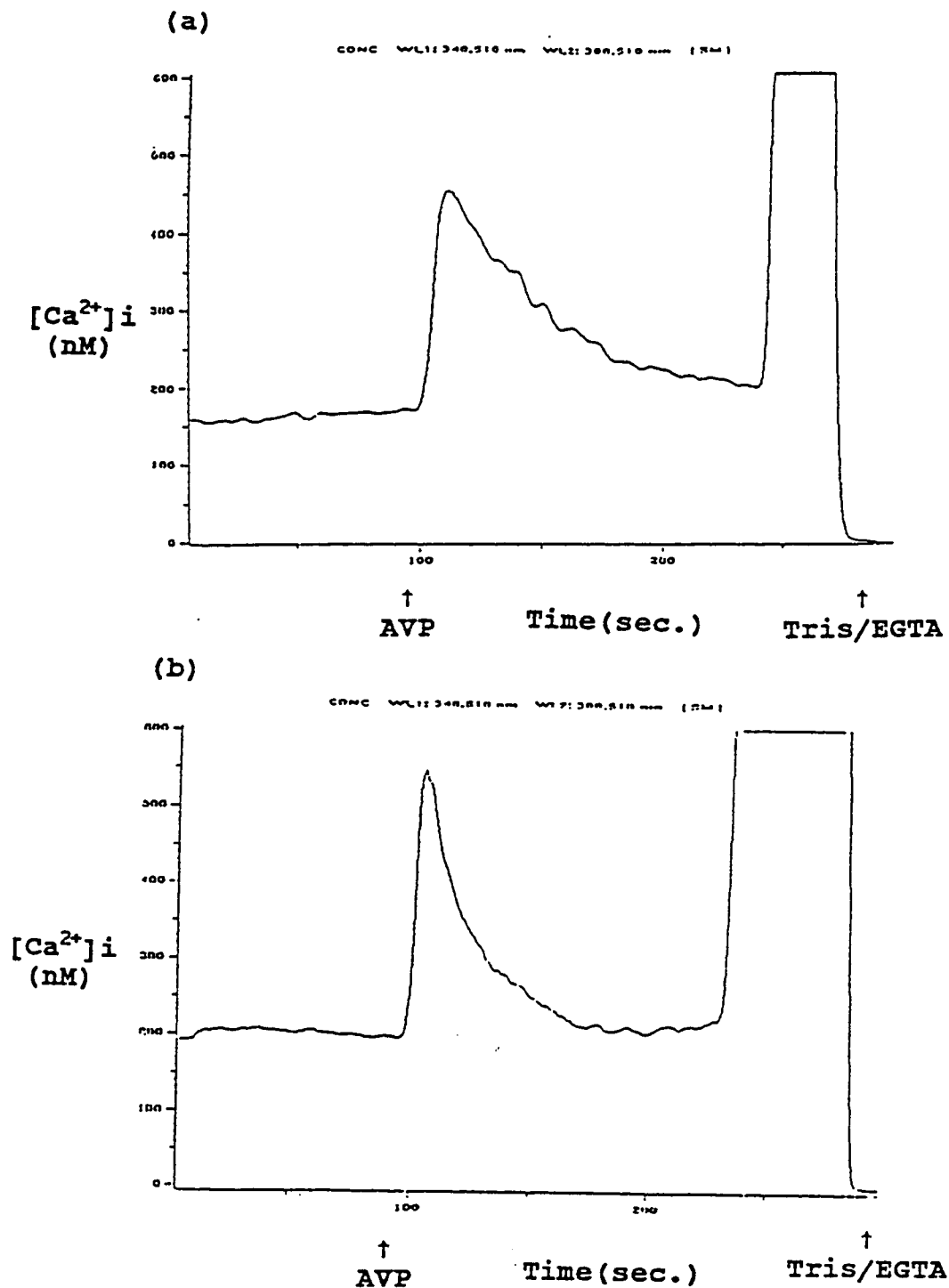


Figure 4.1 Representative Fluorometric [Ca²⁺]_i Responses to arginine vasopressin (AVP) in Cultured A7r5 Vascular Smooth Muscle Cells. (a) in the presence of glucose; (b) in the presence of glucose and 10⁻⁷ M insulin.

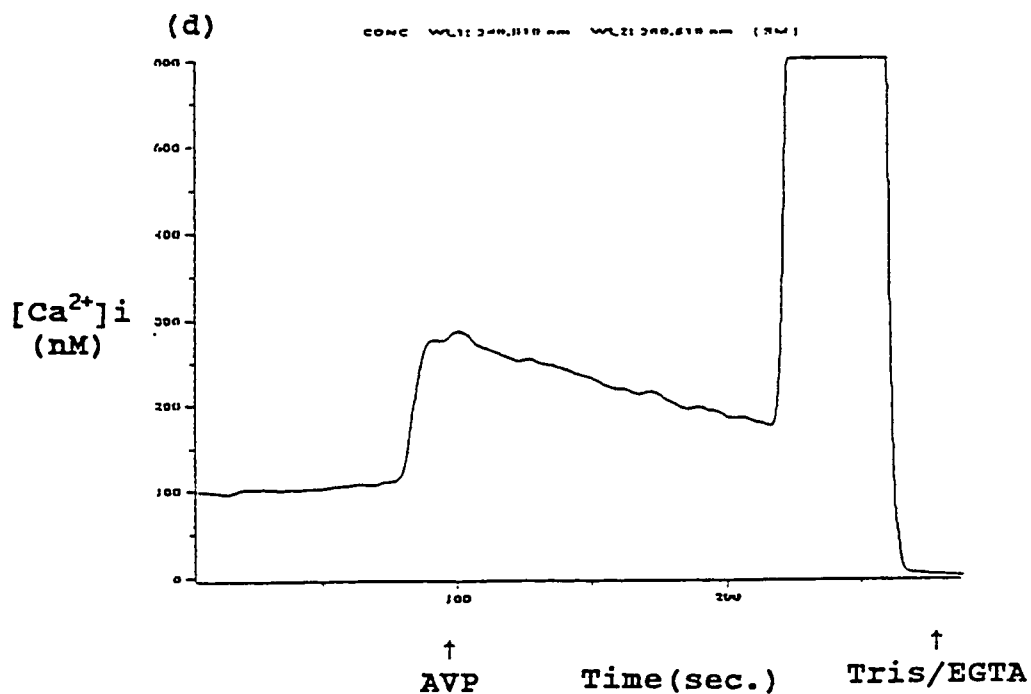
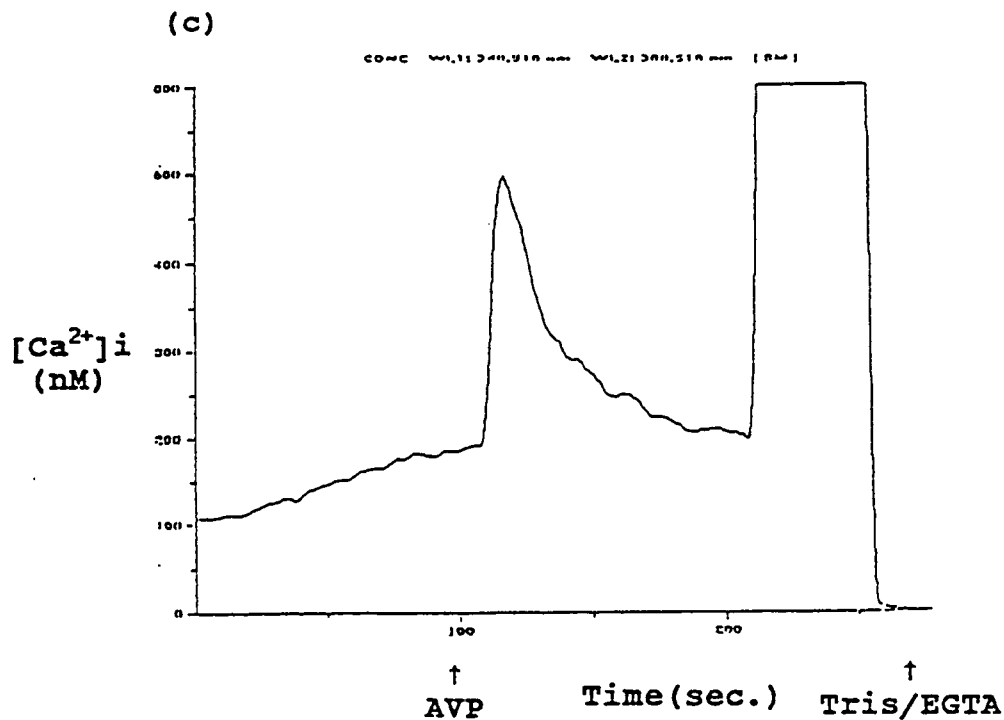


Figure 4.2 Representative Fluorometric [Ca²⁺]_i Responses to arginine vasopressin (AVP) in Cultured A7r5 Vascular Smooth Muscle Cells. (c) in the presence of 2-deoxyglucose and 10⁻⁷ M insulin; (d) in the presence of 3-O-methylglucose and 10⁻⁷ M insulin.

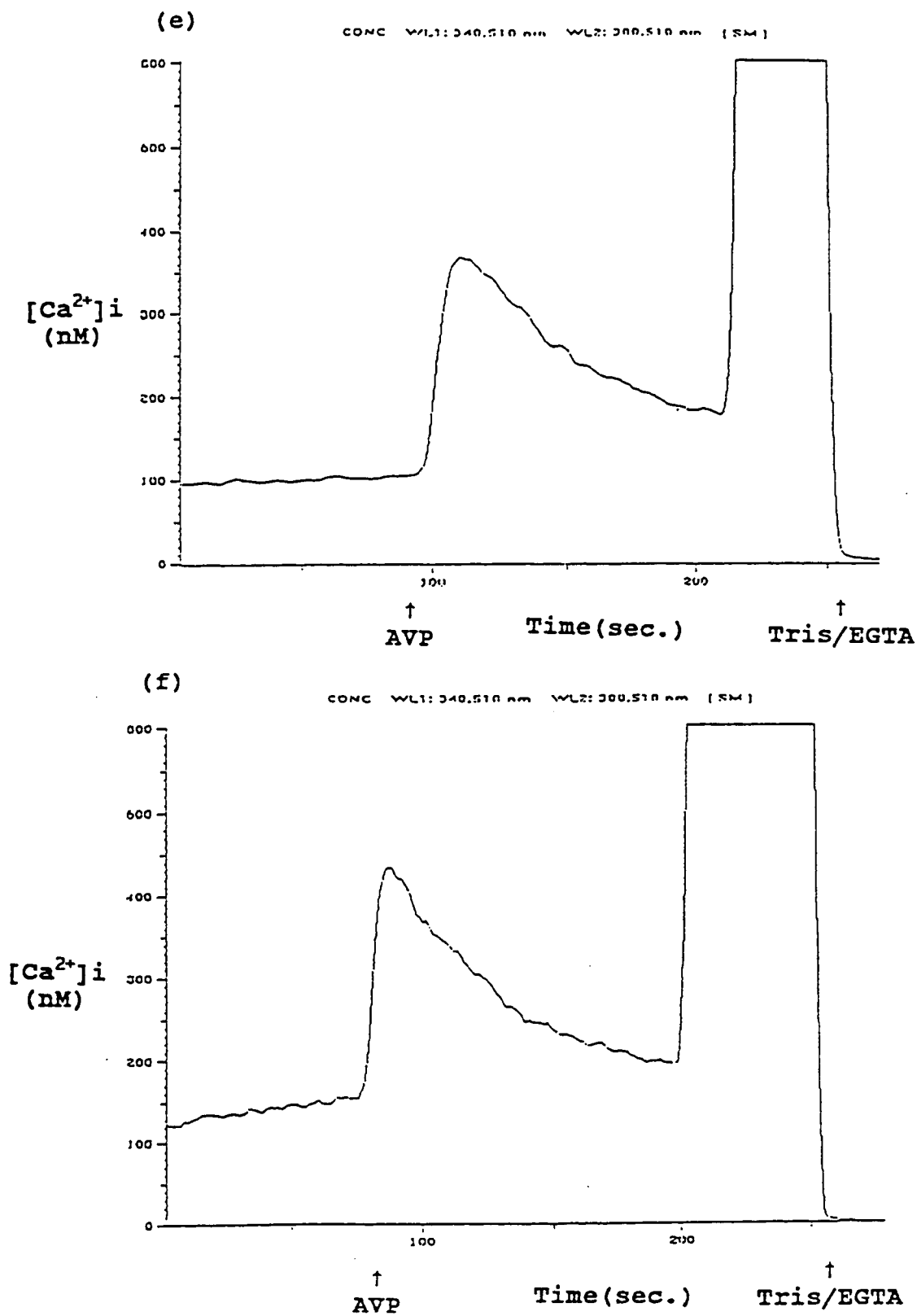


Figure 4.3 Representative Fluorometric [Ca²⁺]_i Responses to arginine vasopressin (AVP) in Cultured A7r5 Vascular Smooth Muscle Cells. (e) in the presence of mannitol, pyruvate and 10⁻⁷ M insulin; (f) in the presence of cytochalasin B and 10⁻⁷ M insulin.

Table 4.1 Effects of insulin (10^{-7} M) on plasmalemma Ca^{2+} -ATPase mRNA in the absence of glucose and in the presence of glucose and glucose analogues.

Treatment	Control	Insulin
25 mM Glucose	2.364 ± 0.29	$5.733 \pm 0.50^*$
17 mM 2-DG ¹	0.420 ± 0.20	$2.946 \pm 1.03^{**}$
17 mM 3-O-MG ²	2.293 ± 0.93	2.455 ± 1.14
Glucose Free ³	1.867 ± 0.71	1.878 ± 0.87
25 mM Fructose	4.296 ± 0.51	3.918 ± 0.58
Cytochalasin B ⁴	3.989 ± 0.71	3.140 ± 0.41

All data are expressed as mean $\pm$ SEM.

¹2-DG, 2-deoxyglucose.

²3-O-MG, 3-O-methylglucose.

³Mannitol and pyruvate were added to all glucose free preparations for osmotic control and energy, respectively.

⁴Cytochalasin B in the presence of 25 mM glucose.

* $p < 0.04$ and ** $p < 0.04$

Table 4.2 Effects of insulin (10^{-7} M) on sarcoplasmic reticulum Ca^{2+} -ATPase mRNA in the absence of glucose and in the presence of glucose and glucose analogues.

Treatment	Control	Insulin
25 mM Glucose	2.368 ± 0.26	$5.783 \pm 0.51^*$
17 mM 2-DG ¹	0.568 ± 0.27	$3.746 \pm 0.93^{**}$
17 mM 3-O-MG ²	2.545 ± 0.90	2.376 ± 0.86
Glucose Free ³	2.178 ± 0.83	1.732 ± 0.67
25 mM Fructose	4.680 ± 0.24	4.391 ± 0.26
Cytochalasin B ⁴	3.892 ± 0.50	3.319 ± 0.46

All data are expressed as mean $\pm$ SEM.

¹2-DG, 2-deoxyglucose.

²3-O-MG, 3-O-methylglucose.

³Mannitol and pyruvate were added to all glucose free preparations for osmotic control and energy, respectively.

⁴Cytochalasin B in the presence of 25 mM glucose.

* $p < 0.03$ and ** $p < 0.05$

Table 4.3 Effects of insulin (10^{-7} M) on β -Actin mRNA in the absence of glucose and in the presence of glucose and glucose analogues.

Treatment	Control Actin mRNA ⁵	Insulin Actin mRNA ⁵
25 mM Glucose	7.33 $\pm$ 1.77	7.69 $\pm$ 1.92
17 mM 2-DG ¹	7.72 $\pm$ 1.72	8.27 $\pm$ 1.78
17 mM 3-O-MG ²	7.21 $\pm$ 2.05	7.16 $\pm$ 2.04
Glucose Free ³	6.45 $\pm$ 1.13	6.60 $\pm$ 0.97
25 mM Fructose	8.05 $\pm$ 1.12	7.12 $\pm$ 1.23
Cytochalasin B ⁴	6.86 $\pm$ 0.88	6.27 $\pm$ 1.08

All data are expressed as mean $\pm$ SEM.

¹2-DG, 2-deoxyglucose.

²3-O-MG, 3-O-methylglucose.

³Mannitol and pyruvate were added to all glucose free preparations for osmotic control and energy, respectively.

⁴Cytochalasin B in the presence of 25 mM glucose.

⁵Arbitrary densitometric units.

In all groups, the effect of insulin on actin mRNA was not significantly different compared to control group.

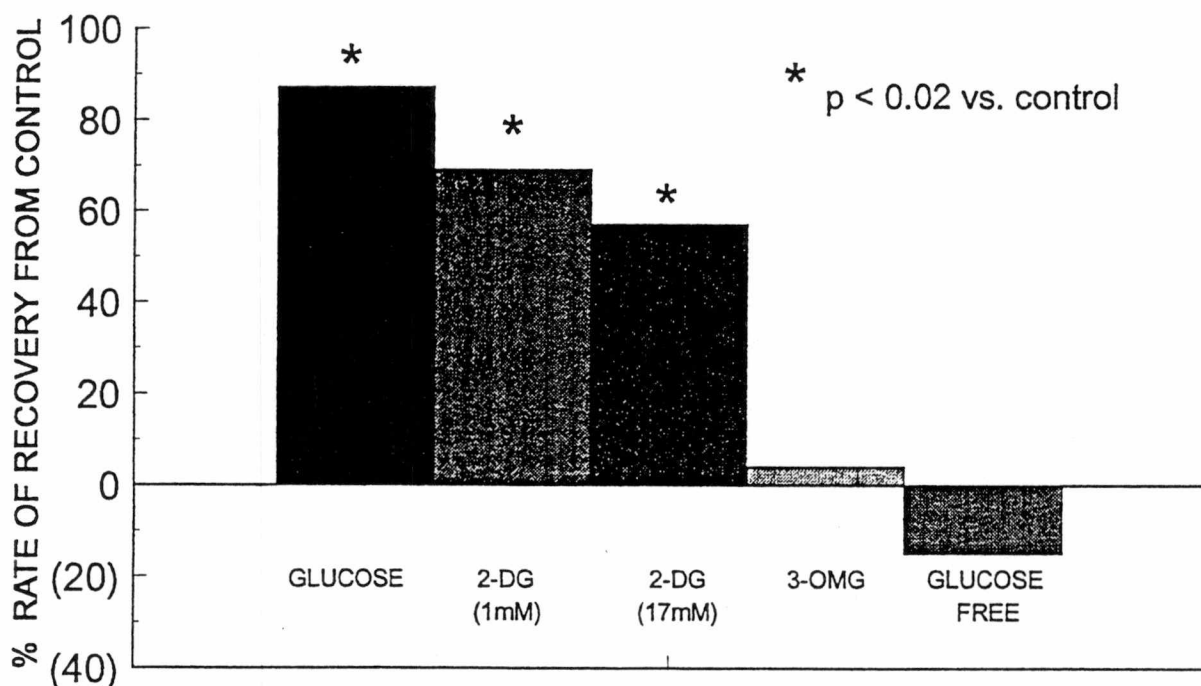


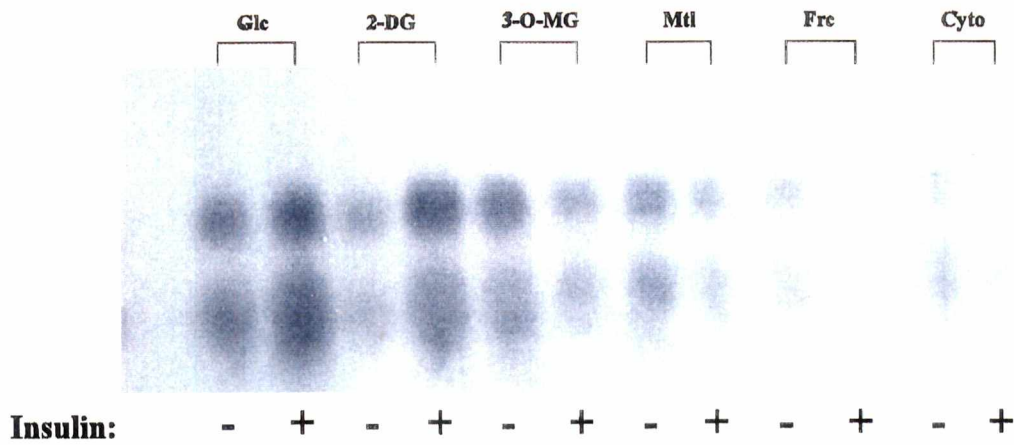
Figure 4.4 Effects of Insulin on the Rate of $[Ca^{2+}]_i$ Recovery to Following arginine vasopressin (AVP)-stimulation in the presence or absence of glucose or glucose analogues. Since each experiment contained it's own internal control, data are normalized to those controls and presented as % insulin stimulation.

* Denotes significant ($P < 0.02$) insulin (10^{-7} M) stimulation.

2-DG, 2-deoxyglucose; 3-O-MG, 3-O-methylglucose;

Glucose-free, pyruvate and mannitol were added as energy and osmotic control, respectively.

PM Ca-ATPase



SR Ca-ATPase

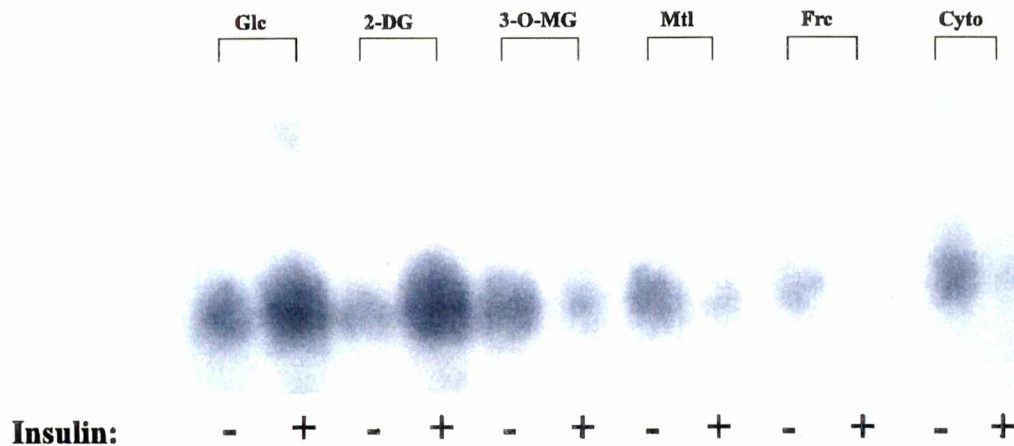


Figure 4.5 Representative autoradiograph of plasma membrane Ca^{2+} -ATPase mRNA (top panel) and sarcoplasmic reticulum Ca^{2+} -ATPase mRNA (bottom panel) in A7r5 vascular smooth muscle cells. Glc: glucose; 2-DG: 2-deoxyglucose; 3-O-MG: 3-O-methylglucose; Mtl: mannitol; Frc: fructose; Cyto: cytochalasin B.

Discussion

Data from the present study suggest that VSMC calcium transport is dependent upon glucose transport and metabolism. These data demonstrate that this regulation occurs in the presence of glucose and a glucose analogue (2-DG) which undergoes phosphorylation. Accordingly, it appears that insulin regulation of Ca^{2+} metabolism is linked to classical actions of insulin. Indeed, although VSMCs are not generally thought to be insulin responsive, it has been recently found insulin regulation of glucose transport in VSMCs and that this regulation is impaired in insulin resistant states (unpublished data).

Consequently, it appears likely that vascular smooth muscle cell manifestations of resistance to classical actions of insulin may be responsible for hypertension in insulin resistant states, as these actions are essential for insulin regulation of calcium transport. Insulin has been previously demonstrated that insulin attenuates *in vitro* vasoconstrictor responses to pressor agonists (Zemel et al. 1990 & 1991a), stimulates VSMC $^{45}\text{Ca}^{2+}$ efflux (Zemel et al. 1992), and accelerates vascular relaxation following contraction of endothelium-denuded aortic strips (Zemel et al. 1990 & 1992). We have also found that insulin accelerates the rate of $[\text{Ca}^{2+}]_i$ recovery to baseline after stimulation with either angiotensin II (AII) or arginine

vasopressin (AVP) (Kim and Zemel 1993) and increases the expression of both plasmalemma and sarcoplasmic reticulum Ca^{2+} -ATPase (Zemel et al. 1991b & 1993). Moreover, these effects are blunted in insulin resistant states (Zemel et al. 1990, 1991a & 1991b, Liu et al. 1993). Thus, it appears that insulin regulation of Ca^{2+} transport contributes to vascular relaxation following agonist stimulation and that impairment of this process may lead to hypertension.

Our present observations suggest that glucose-6-phosphate regulates Ca^{2+} -ATPase expression, possibly at the transcriptional level, via a carbohydrate response element (Vaulont and Kahn 1994). Several lines of evidence have shown that glucose increases the mRNA concentrations and the transcription of glycolytic or lipogenic enzymes such as aldolase B (Decaux et al. 1991), spot 14 (Mariash et al. 1986), and L-type pyruvate kinase (Decaux et al. 1989). Since glucose induces mRNAs of enzymes, catalyzing intermediate or distal reactions in the glycolytic or lipogenic pathway and the regulation of glucokinase transcription by insulin is independent of glucose (Matsuda et al. 1990, Nouspikel and Iynedjian 1992), it is speculated that insulin may have only a permissive role by allowing glucose phosphorylation to glucose-6-phosphate. Indeed, recent evidence (Foufelle et al. 1992) demonstrates that a low concentration of 2-DG (1 mM) very efficiently stimulated the expression of lipogenic enzymes (i.e., fatty acid

synthase and acetyl CoA carboxylase) in cultured adipocytes, and L-type pyruvate kinase in insulinoma INS-1 cells (Marie et al. 1993). Moreover, there was a clear correlation between intracellular glucose-6-phosphate concentration and mRNA expression of lipogenic enzyme.

In contrast, 3-O-MG was unable to induce transcription of genes for glycolytic or lipogenic enzymes. It seems, therefore, that activation of glycolytic and lipogenic genes by glucose requires accumulation of glucose-6-phosphate but not necessarily further entry into the glycolytic pathway. Similarly, the present study indicates that phosphorylation of glucose appears necessary for insulin-regulation of VSMC $[Ca^{2+}]_i$ and for specific activation of Ca^{2+} -ATPase gene transcription.

Although the mechanism by which carbohydrate induces gene transcription is not well known, it appears to involve an intracellular signal resulting from increased glucose metabolism that can affect nuclear events involving *cis*-acting elements and *trans*-acting factors (Vaulont and Kahn 1994). Several glucose response elements (GRE) responsible for the glucose-dependent regulation of glycolytic and lipogenic genes have been identified. The box L4 in a 183-bp minimal promoter fragment of L-pyruvate kinase (L-PK) gene has been shown to function as a glucose/insulin response element, *ex vivo*, by transient expression assays in hepatocytes in primary culture (Bergot et al. 1992, Diaz-

Guerra et al. 1993), and in vivo in transgenic mice (Cuif et al. 1993). This element was also necessary for the inhibitory action of glucagon and cyclic AMP (Bergot et al. 1992, Diaz-Guerra et al. 1993 and Cuif et al. 1993). The imperfect E boxes CACGGG of the L-PK GRE were also indispensable for the GRE activity.

Further, in addition to the GRE in the regulatory regions of the L-PK, a similar CACGTG core consensus element has been observed in the regulatory region of the spot 14 gene (Shih and Towle 1992) and in the first intron of the fatty acid synthase gene (Vaulont and Kahn 1994). Accordingly, data from the present study suggest that glucose-6-phosphate may similarly act either on a primary responsive gene, which mediates its effect on the Ca^{2+} -ATPase gene or directly activate Ca^{2+} -ATPase transcription. Such a carbohydrate response element has not yet been identified in the Ca^{2+} -ATPase gene. However, a GenBank search of the 5'-flanking region of both human (Latif et al. 1993) and rat (Shull and Greeb 1988) plasmalemma calcium pumps demonstrates similar imperfect E box motifs (e.g., CATGGG, CCCGGG, CAGGGG, CAAGTG, GACGTG, CGCGTG), suggesting that Ca^{2+} -ATPase may indeed contain such a glucose-6-phosphate response element.

Literature Cited

- Abel, M.A., Zemel, M.B. (1993) Impaired recovery of vascular smooth muscle intracellular calcium concentration following agonist stimulation in insulin resistant (Zucker obese) rats. *Am J Hypertension*. 6:500-504.
- Bergot, M.O., Diaz-Guerra, M.J.M., Puzenat, N., Raymondjean, M., and Kahn, A. (1992) *Cis*-regulation of the L-type pyruvate kinase gene promoter by glucose, insulin and cyclic AMP. *Nucleic Acid. Res.* 20:1871-1878.
- Cuif, M.H., Porteu, A., Kahn, A., and Vaulont, S. (1993) Exploration of a liver-specific glucose/insulin-responsive promoter in transgenic mice. *J. Biol Chem.* 268:13769-13772.
- Decaux, J.T., Marcillat, O., Pichard, A.L., Henry, J., and Kahn, A. (1991) Glucose-dependent and -independent effect of insulin on gene expression. *J. Biol. Chem.* 266:3432-3438.
- Diaz-Guerra, M.J.M., Bergot, M.O., Martinez, A., Cuif, M.H., Kahn, A. and Raymondjean, M. (1993) Functional characterization of the L-pyruvate kinase gene glucose response complex. *Mol. Cell. Biol.* In press
- Ferrannini, E., Buzzigoli, G., Bonadonna, R., Giorico, M.A., Oleggini, M., Graziadei, L., Pedrinelli, R., Brandi, L., Bevilacqua, S. (1987) Insulin resistance in essential hypertension. *N Engl J Med.* 317:350-357.
- Foufelle, F., Gouhot, B., Pegorier, J.P., Perdereau, D., Girard, J., and Ferre, P. (1992) Glucose stimulation of lipogenic enzyme gene expression in cultured white adipose tissue. *J. Biol. Chem.* 267:20543-20546.
- Grynkiewicz, G.M., Poenie, M., Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J Biol Chem.* 260:3440-3450.
- Juncos, L.A., Ito, S., Carretero, O.A. (1992) Disparate effects of insulin on isolated rabbit afferent and efferent arterioles. *Hypertension*. 20:403.
- Kahn, A.M., Seidel, C.L., Allen, J.C., O'Neil, R.G. (1993) Insulin reduces contraction and intracellular calcium concentration in vascular smooth muscle. *Hypertension*. 22: 735-742.

- Kim, Y-C., Zemel, M.B. (1993) Insulin increases vascular smooth muscle recovery from intracellular calcium loads. Hypertension. 22:74-77.
- Latif, F., Duh, F-M., Gnarr, J., Tory, K. Kuzmin, I., Yao, M., Stackhouse, T., Modi, W., Geil, L., Schmidt, L., Orcutt, M.L., Maher, E., Richards, F., Phipps, M., Ferguson-Smith, M., Le Paslier, D., Linehan, W.M., Zbar, B., Lerman, M.I. (1993) A 1Mb YAC and 400kb cosmid-phage contigs containing the von Hippel-Lindau tumor suppression and Ca^{++} transporting ATPase isoform 2 genes. Cancer Research. 53, 861-867
- Liu, Z., Thompson, K.S., and Towle, H.C. (1993) Carbohydrate regulation of the rat L-type pyruvate kinase gene requires two nuclear factors: LF-A1 and a member of the c-myc family. J. Biol. Chem. 268:12787-12795.
- Marie, S., Diaz-Guerra, M.J.M., Miquerol, L., Kahn, A., and Iynedjian, P.B. (1993) The pyruvate kinase gene as a model for studies of glucose-dependent regulation of gene expression in the endocrine pancreatic β -cell-type. J. Biol. Chem. In press.
- Mariash, C.N., Seelig, S., Schwartz, H.L., and Oppenheimer, J.H. (1986) Rapid synergistic interaction between thyroid hormone and carbohydrate on mRNA-S14 induction. J. Biol. Chem. 261:9583-9586.
- Matsuda, T., Noguchi, T., Yamada, K., Takenaka, M., and Tanaka, T. (1990) Regulation of the gene expression of glucokinase and L-type pyruvate kinase in primary culture of rat hepatocytes by hormones and carbohydrates. J. Biochem. 108:778-784.
- Modan, M., Halkin, H., Almay, S., Lusky, A., Eshkil, M., Shitrit, A., Fuchs, A. (1985) Hyperinsulinemia: a link between hypertension, obesity and glucose intolerance. J Clin Invest. 75:809-817.
- Nouspikel, T., and Iynedjian, P.B. (1992) Insulin signalling and regulation of glucokinase gene expression in cultured hepatocytes. Eur. J. Biochem. 210:365-379.
- Reaven, G.M. (1988) Role of insulin resistance in human disease. Diabetes. 37:1595-1607.
- Decaux, J.T., Antoine, B., and Kahn, A. (1989) Regulation of the expression of the L-type pyruvate kinase gene expression in adult rat hepatocytes in primary culture. J. Biol. Chem. 264:11584-11590.

- Rocchini, A.P. (1991) Insulin resistance and blood pressure regulation in obese and non-obese subjects. *Hypertension*. 17:837-842.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1989.
- Sower, J.R., Standley, P.R., Ram, J.L., Zemel, M.B., Resnick, L.M. (1991) Insulin resistance, carbohydrate metabolism, and hypertension. *Am J Hypertension*. 4:466S-472S.
- Shih, H.M., and Towle, H.C. (1992) Definition of the carbohydrate response element of the rat S14 gene. Evidence for a common factor required for carbohydrate regulation of hepatic genes. *J. Biol. Chem.* 267:13222-13228.
- Shull, G.E., Greeb, J. (1988) Molecular cloning of two isoforms of the plasma membrane Ca^{2+} -transporting ATPase from rat brain: Structural and functional domains exhibit similarity to Na^+ , K^+ - and other cation transporting ATPase. *J Biol. Chem.* 263, 8646-8657.
- Standley, P.R., Zhang, F., Ram, J.L., Zemel, M.B., Sowers, J.R. (1991) Insulin attenuates vasopressin-induced calcium transients and voltage-dependent calcium current in rat vascular smooth muscle cells. *J Clin Invest*. 88:1230-1236.
- Vaulont, S. and Kahn, A. (1994) Transcriptional control of metabolic regulation genes by carbohydrates. *FASEB J.* 8: 28-35.
- Zemel, M.B., Reddy, S., Shehin, S., Lockette, W., Sowers, J.R. (1990) Vascular reactivity in Zucker obese rats. Role of insulin resistance. *J Vasc Med Biol.* 2:81-85.
- Zemel, M.B., Reddy, S., Sowers, J.R. (1991a) Insulin attenuation of vasoconstrictor responses to phenylephrine in Zucker lean and obese rats. *Am J Hypertension*. 4:537-539.
- Zemel, M.B., Johnson, B.A., Ambrozy, S.A. (1992) Insulin-stimulated vascular relaxation: role of Ca^{2+} -ATPase. *Am J Hypertension*. 5:637-641.
- Zemel, M.B., Iannucci, A., Johnson, B.A.B., Sowers, J.R. (1991b) Reduced Ca^{2+} -ATPase expression in insulin resistant (Zucker obese) rats. *Hypertension*. 18:442.

Zemel, M.B., Iannucci, A., Moore, J.W. (1993) Role of insulin in regulating vascular smooth muscle Ca^{2+} -ATPase expression. J Vasc Med Biol. 4: 79-84.

PART V.

CONCLUSION

Part V. Conclusion

Recent observations have suggested that defects in insulin secretion or insulin action in target tissues are associated with the increased vascular smooth muscle tone which is characteristic of hypertension in both IDDM and NIDDM.

Hypertension in insulin resistant states has generally been ascribed to hyperinsulinemia, resulting in increased renal sodium retention and sympathetic nervous activity. However, impaired cellular responses to insulin, rather than hyperinsulinemia, have been suggested as an alternative mechanism responsible for increased peripheral vascular resistance, leading to hypertension. In support of this, observations of the current study show insulin attenuation of vascular smooth muscle cell Ca^{2+} , which is a primary determinant of contractility and vascular tone. Thus, insulin attenuation of vascular smooth muscle cell Ca^{2+} responses to vasoactive agonists may explain how insulin regulates blood pressure.

Data from the present studies also indicate that the vasodilatory effects of insulin are associated with stimulation of both calcium pumps, and that these effects are mediated by classical actions of insulin. Furthermore, the present data suggest that phosphorylation of glucose to glucose-6-phosphate may be indispensable for its transcriptional activation of calcium pumps (Figure 5.1),

similar to insulin stimulation of transcription of several glycogenic and lipogenic enzymes. Further investigation identifying one or more specific glucose-6-phosphate response elements in either Ca^{2+} -ATPase gene is necessary to confirm this as a mode of transcriptional regulation of Ca^{2+} -ATPases.

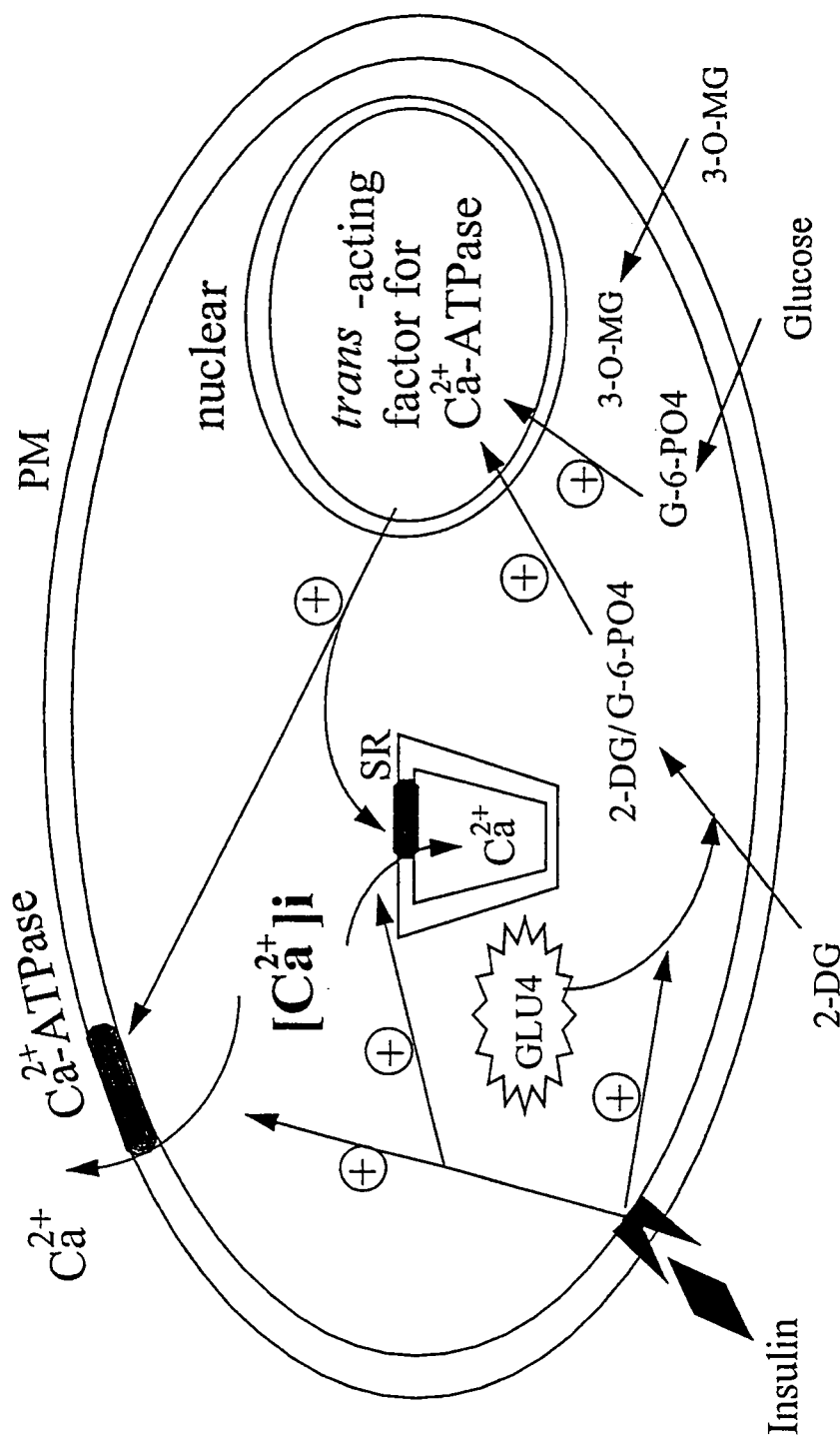


Figure 5.1 Schematic Representation for the Role of Glucose-6-phosphate in Regulating Intracellular Calcium. PM, plasma membrane; 2-DG, 2-deoxyglucose; 3-O-MG, 3-O-methylglucose; G-6-P, glucose-6-phosphate; GLUT4, glucose transporter 4; SR, sarcoplasmic reticulum Ca²⁺-ATPase.

APPENDIX

Table A.1 Effects of insulin on arginine vasopressin (AVP)-elicited $[Ca^{2+}]_i$ recovery rate¹ in fura-2-loaded A7r5 vascular smooth muscle cell.

	Control	Insulin (10^{-7} M)	%Insulin stimulation
Mannitol & Pyruvate	21.98 $\pm$ 5.79	18.62 $\pm$ 2.97	-15
17 mM 3-O-MG ²	10.94 $\pm$ 1.89	11.35 $\pm$ 1.50	4
1 mM 2-DG ³	5.67 $\pm$ 0.80	9.57 $\pm$ 1.45*	57*
17 mM 2-DG ³	5.67 $\pm$ 0.90	8.89 $\pm$ 1.47**	69**
25 mM Glucose	5.83 $\pm$ 0.90	10.91 $\pm$ 1.84***	87***

All data are expressed as mean $\pm$ SEM.

¹Recovery (nM/min), rate of return to baseline following AVP stimulation.

²3-O-MG, 3-O-methylglucose.

³2-DG, 2-deoxyglucose.

p value, % stimulation of insulin treatment versus control.

* $p < 0.005$, ** $p < 0.02$ and *** $p < 0.01$

VITA

Young-Cheul Kim was born in Kangwon-Do, South Korea on November 13, 1961. He attended elementary and middle school there and graduated from the High School affiliated to the College of Education, Kyungpook National University in Taegu, in 1979. He received a Bachelor of Science Degree in Biology from Keimyung University in Taegu, 1986 and during this time he also served in the Korean Army.

Upon graduation, he entered the graduate school of Korea University and received a Master of Science Degree in Food Technology and Science in August 1988. During his M.S. studies, he received a gold medal of honor for outstanding graduate work. From November 1988 to March 1989, he worked in the Planning and Research Division of Shany Co., LTD. In April 16, 1989, he married Chun-Hee Hyun and they now have a daughter, Mee-ji born on June 18, 1990 and a son, Sehyun who was just born on February 9, 1995.

In 1990, he came to the United States to pursue his Ph.D with a major in Nutritional Science at the University of Tennessee, Knoxville and earned the Doctor of Philosophy Degree in May of 1995. He was also given an award for outstanding research as a graduate student in Nutritional Science in April 1994.

He has recently received a post-doctoral appointment in the Medical School of the University of Wisconsin-

Madison. He has publications and abstracts oral-presented in several national meetings.

PUBLICATIONS

Kim Y-C, Zemel M.B. (1993) Insulin increases vascular smooth muscle recovery from intracellular calcium loads. Hypertension 22:74-77.

ABSTRACTS PRESENTED AND ORAL PRESENTATIONS

Kim Y-C, Zemel M.B: Insulin Potentiates vascular smooth muscle cell (VSMC) intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) responses to angiotensin II but accelerates recovery from $[\text{Ca}^{2+}]_i$ loads. FASEB, Anaheim, CA., April, 1992.

Kim Y-C, Zemel M.B: Insulin accelerates intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) recovery following agonist stimulation of human vascular smooth muscle cells (VSMCs). Experimental Biology 93, Anaheim, CA., March, 1993.

Kim Y-C, Zemel M.B: Insulin accelerates intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) recovery without affecting peak $[\text{Ca}^{2+}]_i$ responses following agonist stimulation of human vascular smooth muscle cells (VSMCs). The Inter-American Society of Hypertension, Lajolla, CA., April, 1993

Kim Y-C, Zemel M.B.: Insulin stimulation of intracellular free calcium ($[\text{Ca}^{2+}]_i$) recovery and Ca^{2+} -ATPase gene expression in cultured vascular smooth muscle cells (VSMCs) : Role of Glucose-6-Phosphate. Accepted for Experimental Biology 95, Atlanta, GA., March, 1995

MANUSCRIPTS IN PREPARATION

Kim, Y-C, Zemel M.B. (1995) Insulin stimulation of intracellular free calcium ($[\text{Ca}^{2+}]_i$) recovery and Ca^{2+} -ATPase gene expression in cultured vascular smooth muscle cells (VSMCs) : Role of Glucose-6-Phosphate.