THE REGULATION OF GLUCOSE TRANSPORT IN CULTURED VASCULAR SMOOTH MUSCLE CELLS BY ANGIOTENSIN II AND GLUCOSE

DISSERTATION

Submitted to the Graduate School

of

Marshall University

In Partial Fulfillment of the Requirements for

The Degree of Doctor of Philosophy

by

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1997

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ABSTRACT

Glucose transport was assessed in vascular smooth muscle (VSM) cells by measuring the uptake of a radiolabeled non-metabolizable glucose analog, [^{3}H]-2-deoxglucose. VSM cells, isolated from rat aortae by enzymatic digestion, were maintained in culture in Dulbecco's Modified Eagle Medium supplemented with 10% newborn calf serum at 37°C with 5% CO_2 and air. Angiotensin II (AII) increased glucose transport by 84%. Significant stimulation occurred by two hours of exposure with the maximum effect being observed between six and eight hours. AII effects were concentration dependent with a threshold response being detected at 0.1 nM. AII-stimulated transport was blocked by an AII receptor antagonist. AII stimulation was shown to require protein synthesis. A specific protein synthesized in response to AII stimulation was the GLUT 1 glucose transporter as assessed by western blot analysis, using an antibody generated against the carboxyl terminus of the GLUT 1 transporter. Protein kinase C (PKC) stimulation with phorbol esters significantly increased glucose uptake. The majority of evidence from PKC inhibition and downregulation studies however, suggest AII is capable of stimulating glucose transport through a PKC-independent mechanism. Extracellular calcium and calmodulin appear to be required for AII stimulation of glucose transport. Hyperglycemic conditions reduced basal glucose transport. The maximum rate of AII-stimulated transport was not appreciably altered by the glycemic state; however, the relative effectiveness of AII at different concentrations of glucose varied with media glucose concentration.

AKNOWLEDGEMENTS

I wish to express my deepest appreciation to the following people for the role that each played throughout all phases of this research:

My mentor, Dr. William McCumbee, for his thoughtful encouragement, insightful wisdom, and excellent advice.

Each of my committee members, for providing the advice and expertise necessary to make this project a reality.

My husband, Kevin, for his devotion, humor, and encouragement during the best and most difficult of times.

My parents, for providing the powerful roots necessary for emotional and intellectual growth and for providing the confidence of knowing that they will fully encourage and support my endeavors.

Finally, My Father in heaven, for the following promise, "I can do all things through Christ who gives me strength (Phillipians 4:13)."

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LITERATURE REVIEW

Essential hypertension is a condition of raised arterial pressure characterized by increased peripheral resistance and medial vascular hypertrophy. Hypertension is prevalent in the United States and other industrialized nations affecting 15 - 20 % of the adult population (James and Baker, 1995). Hypertensive individuals have a higher than average risk of developing coronary heart disease, heart failure, stroke, kidney disease, renal failure, atherosclerosis, and myocardial infarction (Wilson and Kannel, 1995; Davis et al., 1977). On examining the prevalence and consequences of essential hypertension, the importance of understanding the mechanisms involved in initiating and maintaining this condition and of studying potential treatment regimens that may be successful in its control becomes exceedingly clear.

Essential hypertension was described in 1836 by Richard Bright who noted left ventricular hypertrophy of the heart and aortic wall thickening (medial hypertrophy) associated with this condition (Folkow, 1982). Later, in 1868, George Johnson observed wall thickening of arterioles, a finding later confirmed by Ewald in 1877 (Folkow, 1982). While these initial descriptions were hallmarks in hypertension research, further advances in understanding the pathogenesis of hypertension were not possible without an appropriate animal model of essential hypertension in humans. Harry Goldblatt provided the first successful animal model that was reasonably similar to essential hypertension in man in the early 1930's. Dr. Goldblatt was able to induce hypertension in dogs by clamping the renal artery of one kidney and either removing or leaving intact the contralateral kidney (Goldblatt

et al., 1934). The former model was referred to as one-kidney, one-clip renovascular hypertension and the latter as two-kidney, one-clip renovascular hypertension. The Goldblatt model lead to the discovery of angiotensin by Braun-Menendez and colleagues in 1939 and Page and Helmer in 1940 (Fasciolo, 1990; Page and Helmer, 1940), as well as many other advances in hypertension research. Other models of experimentally induced hypertension now exist, including the DOC/salt model, the aortic coarctation model, and a genetic model known as the spontaneously hypertensive rat (SHR).

DOC-induced hypertension in animals was first described in 1939 by Loeb after discovering that treatment of patients having Addison's disease with synthetic deoxycorticosterone (DOC) or its acetate (DOCA) led to hypertension (Loeb et al., 1939; Ryan and McCullagh, 1940; Thorn et al., 1942). His results were later confirmed in dogs (Swingle et al., 1941) and rats (Grollman et al., 1940). Selye and colleagues (1943) described hypertension resulting from DOCA in conjunction with a high salt diet in rats, dogs, and monkeys. Further studies have demonstrated that DOCA-induced hypertension in rats requires a sodium component (Brownie, 1995). In the DOC/salt model, hypertension is induced by treating rats with deoxycorticosterone (DOC), a mineralocorticoid, and providing saline as the sole drinking fluid. Mineralocorticoids act at the level of the kidney to stimulate sodium and water reabsorption. In this model there is an initial rise in plasma volume and blood pressure. The increase in blood pressure is maintained with continued DOC/salt treatment. The precise mechanism leading to the development of hypertension in this model is unclear.

The aortic coarctation (also referred to as aortic stenosis or ligature) model of hypertension is unique in providing a means of studying the hypertensive process in vascular beds independent of high intravascular pressure. In this model, a small metal clip is placed around the aorta, above the renal arteries, to restrict blood flow. High arterial blood pressure occurs above the clip while normal blood pressure occurs below the point of coarctation. Using this model, several investigators have observed increased wall thickness of the normotensive abdominal aorta (Overbeck, 1979) and an increase in arterial resistance in the normotensive hindlimb vascular bed under resting conditions (Nolla-Panades, 1963) as well as conditions of maximal vasodilation (Bell and Overbeck, 1979). In addition, Plunkett and Overbeck (1985) noticed an increase in wall thickness and the wall-to-lumen ratio in arterioles of the normotensive cremaster vascular bed. These results provide indirect evidence that vascular structural changes have occurred independent of high intravascular pressure in coarctation-induced hypertension. Although interesting, these results have to be interpreted with caution because contradictory evidence has been obtained by other laboratories. Mangiarua and associates (1985) did not observe vascular hypertrophy in the normotensive abdominal aortae of rats with aortic coarctation. Moreover, Bevan and colleagues (1976) did not observe changes in wall thickness of normotensive saphenous arteries of coarctated rabbits.

The production of animals genetically susceptible to the occurrence of spontaneous hypertension has been achieved in rats (Smirk and Hall, 1958; Okamoto and Aoki, 1963 and rabbits (Alexander et al, 1954). The strain developed by Okamoto and Aoki is referred to as the "spontaneously hypertensive rat" (SHR). Okamoto and Aoki mated a hypertensive

male Wistar rat (150 - 175 mmHg) with a female Wistar rat having mildly elevated blood pressure (130 - 140 mmHg). Successive generations were achieved by mating siblings who had high blood pressure persisting for at least one month. With the spontaneously hypertensive rat strain, researchers have observed a rise in blood pressure with age (Okamoto and Aoki, 1963), cardiac hypertrophy (Yamabe and Lovenberg, 1974; Fischer, 1976), medial hypertrophy in conductance (Owens, 1985) and resistance vessels (Warshaw et al., 1979; Lee et al., 1983), and an increase in aortic connective tissue content (Yamabe and Lovenberg, 1974; Fischer, 1976). This animal model is possibly the closest experimental model to essential hypertension in man.

Since the initial observations of hypertension, it has become clear that an outstanding characteristic of essential hypertension is medial vascular hypertrophy. This term refers to an increase in the thickness of the medial layer of vessels of hypertensive subjects as compared with vessels of normotensive subjects. Medial vascular hypertrophy occurs in arteries of all sizes ranging from conductance to resistance vessels in subjects with essential and experimentally-induced hypertension. In conductance vessels the hypertrophy is predominately due to an increase in cell size, while in resistance vessels hypertrophy occurs primarily through an increase in cell number (Mulvany et al., 1985).

Medial hypertrophy was described by Johnson in 1868 and later confirmed by Ewald in 1877 (Folkow, 1982). Furuyama (1962) noted medial hypertrophy in the superior mesenteric artery and the renal artery from patients with primary hypertension. Medial hypertrophy has been observed in the caudal artery of rats with DOC/salt hypertension (Friedman et al., 1971) and in the thoracic aorta of renal hypertensive rats (Wolinsky, 1970;

Wiener et al. 1977) and rats with aortic coarctation-induced hypertension (Olivetti et al. 1980). Warshaw et al. (1979) and Lee et al. (1983) found an increase in medial thickness of large and small mesenteric resistance arteries from SHR.

In larger conductance vessels, medial vascular hypertrophy is characterized by cellular hypertrophy and an increase in the content of specific macromolecules. Direct chemical analysis of aortae of hypertensive animals as compared to normotensive animals has revealed an increase in the content of DNA, protein, collagen, and elastin (Wolinsky, 1972; Yamabe and Lovenberg, 1974; Fischer, 1976; Foidart et al., 1978; Mangiarua et al., 1981; Mangiarua et al., 1982). Smooth muscle cell hypertrophy occurs predominantly in conductance vessels like the aorta and is commonly associated with an increase in the incidence of polyploidy. Polyploid cells have a chromosome number that is a multiple of the normal complement of chromosomes. For instance, Olivetti et al. (1980) have demonstrated that aortic coarctation-induced hypertension in male Wistar rats is characterized by a 54% increase in vessel wall hypertrophy that can be accounted for by smooth muscle cell hypertrophy. In this model, the volume of the thoracic aorta occupied by smooth muscle cells increased by 57% and the mean smooth muscle cell volume increased 55%. There was no change in the number of smooth muscle cells and collagen content rose 136% (Olivetti et al., 1980). In a separate study, Owens et al. (1981) found an increased average smooth muscle cell size with increased frequency of polyploidy in SHR aortae compared to normotensive Wistar-Kyoto (WKY) rats. There was no change in smooth muscle cell number suggesting that the increase in smooth muscle cell mass could be accounted for by cellular hypertrophy rather than hyperplasia. These results were further

substantiated using a different model of experimentally-induced hypertension. The thoracic aortae of rats made hypertensive by the Goldblatt 2 kidney-1 clip method were found to have a 40% increase in smooth muscle mass with a greater frequency of polyploid cells as compared to sham-operated controls (Owens and Schwartz, 1983). In addition, the vascular hypertrophy in this animal model occurred in association with smooth muscle cell hypertrophy rather than hyperplasia as there was no change in smooth muscle cell number.

There is evidence that has been interpreted as suggesting that hyperplasia may also occur in conductance vessels. Bevan et al. (1976) found an increase in DNA content and ^{3}H-thymidine incorporation in the aortae of rabbits with hypertension induced by aortic coarctation. These researchers correlated the increased DNA content to an increase in cell number based on the assumption that smooth muscle cells remain diploid; however, cell number was not measured in this study. It is possible that Bevan's results could be explained by hypertrophy of polyploid cells rather than hyperplasia, if rabbits with aortic coarctation-induced hypertension respond similarly to rats made hypertensive by the experimental manipulations described above.

Although the precise mechanisms controlling vascular hypertrophy and the pathogenesis of hypertension have not been elucidated, at least two feasible hypotheses have been promulgated. Folkow's original hypothesis suggests that intermittent rises in blood pressure are met by structural adaptations of the vasculature, such as medial hypertrophy, in order to normalize the increase in vessel wall stress resulting from increases in intravascular pressure. Folkow hypothesized that as the vessels hypertrophy the increase in smooth muscle mass encroaches upon the lumen of the vessels thereby reducing the radius of the

vessel and further increasing intravascular pressure. A cycle would occur with increases in pressure leading to further hypertrophy. Once established, Folkow (1978) believed vascular hypertrophy could maintain a chronic elevation of blood pressure. An alternative hypothesis outlined by Lever provides for the role of trophic factors in initiating medial vascular hypertrophy which in turn would lead to an increase in blood pressure, further promoting medial hypertrophy and hence maintenance of hypertension (Lever et al., 1992). One such candidate trophic factor is angiotensin II (AII).

AII, an endogenous vasoconstrictor, is involved in normal blood pressure regulation (Sealey and Laragh, 1995). In response to a reduction in blood pressure which is translated into reduced renal arterial pressure, the kidneys secrete renin. Renin is an enzyme that cleaves its substrate, angiotensinogen, to release angiotensin I (AI). AI is then converted to AII by angiotensin converting enzyme, located predominately in the lung vasculature. AII raises blood pressure by both direct and indirect mechanisms. Directly, AII causes vasoconstriction of vascular smooth muscle cells. Indirectly, AII plays a role in sodium and water balance to expand plasma volume and subsequently raise blood pressure. AII stimulates the synthesis and secretion of aldosterone, a mineralocorticoid that stimulates sodium and water reabsorption by renal tubules. AII also exerts a negative feedback effect on renin synthesis and secretion by the kidneys. Under normal homeostatic conditions, AII potently regulates blood pressure.

AII binds to a specific plasma membrane receptor in order to elicit cellular responses. Based on pharmacological characteristics, three receptors have been identified for AII: AT_1, AT_2, and AT_3 (Harris and Inagami, 1995). While the signal transduction mechanisms for the

AT_2 and AT_3 receptors have not yet been determined, there is a wealth of information available for the secondary signaling events mediated by the AT_1 receptor. The AT_1 receptor is coupled to a G-protein. AII binding to this receptor activates a G-protein, which in turn activates phospholipase C (Duff et al., 1995). Phospholipase C hydrolyses phosphatidylinositol bisphosphate to yield diacylglycerol (Griendling et al., 1986) and inositol trisphosphate. Inositol triphosphate binds to ligand-operated calcium channels in the endoplasmic reticulum to promote an initial, rapid rise in cytosolic calcium (Alexander et al., 1985). This is followed by a plateau phase of elevated calcium levels which appears to be dependent upon the influx of extracellular calcium (Brock et al., 1985). Calcium can initiate cellular events by binding to calcium-binding proteins that function as regulators of cell function. The most ubiquitously distributed of these proteins is calmodulin. Calcium-calmodulin complexes modulate the activity of a variety of cellular proteins including enzymes and membrane transport proteins to promote specific cellular activities (Head, 1992; O'Neil and DeGrado, 1990). The second product of phosphatidylinositol diphosphate hydrolysis, diacylglycerol, remains associated with the plasma membrane to promote the association of protein kinase C (PKC) with the inner surface of the plasma membrane (Andrea and Walsh, 1992) where it is activated. Activated PKC elicits a cellular response by promoting the phosphorylation of target proteins that mediate specific cell functions. Multiple isoforms of PKC exist. The isoforms can be grouped into three broad categories based upon their substrate specificity, structure, and activation requirements (Williams, 1995; Newton, 1995). The conventional PKCs (α, β_I, β_{II}, and γ) are calcium-dependent and require DAG and membrane phospholipid for activation. The novel PKCs (δ, ϵ, θ, μ, and

η) are calcium-independent and require DAG and membrane phospholipid for activation. The atypical PKCs (ζ, λ, and ι) are calcium-independent and do not require DAG or membrane phospholipid for activation. Several isoforms of PKC have been identified in vascular smooth muscle including, α, β, ϵ, and ζ (Andrea and Walsh, 1992).

Pathologically, AII has been implicated in the development of vascular hypertrophy associated with hypertension. This evidence stems from animal studies using captopril, an angiotensin-converting enzyme inhibitor routinely used to reduce blood pressure in hypertensive patients and from studies involving the chronic infusion of subpressor doses of AII. In an experiment where antihypertensive drugs working through different mechanisms were evaluated according to their efficiency in reducing medial hypertrophy, only captopril was able to reduce the degree of vascular hypertrophy to an extent greater than that predicted by its blood pressure-lowering effect (Owens, 1987). Captopril reduced the tetraploidy, volume, and content of smooth muscle cells in the thoracic aortae of SHR. In another study, AII infusion was shown to promote vascular hypertrophy in normotensive Sprague Dawley rats even when hydralazine, a blood pressure lowering agent, was given to prevent the AII-induced rise in blood pressure (Griffin et al., 1991). These data suggest that AII is able to promote vascular hypertrophy by a mechanism that is unrelated to increased wall stress resulting from elevated blood pressure.

In vitro studies with cultured cells support the hypothesis that AII has a direct trophic effect on vascular smooth muscle. AII has been shown to cause an increase in cell volume (Geisterfer et al., 1988), protein synthesis (Berk et al., 1989), and DNA synthesis (Mangiarua and McCumbee, 1990) relative to unstimulated cells. Similarly, AII has been shown to

stimulate the synthesis of extracellular matrix proteins (Scott-Burden et al., 1990; Kato et al., 1991) and the transport of amino acids (McCumbee et al., 1996) by cultured vascular smooth muscle cells.

In that AII induces a number of growth promoting activities, it follows that AII stimulation of vascular smooth muscle cells will increase cellular demands for metabolic energy. Hypothetically, AII could help the cells in meeting this requirement by simultaneously stimulating glucose transport into those cells while promoting cellular hypertrophy.

Glucose is a major energy substrate utilized by mammalian cells. The cell membrane is impermeable to glucose. Glucose entry into the cell is made possible by a family of structurally related facilitative glucose transporter proteins that are expressed in a tissue-specific manner (reviewed by Mueckler, 1994; Bell et al, 1990; Baldwin, 1993). The GLUT 1 transporter protein and/or message has been found in cells of blood-tissue barriers (Kalari et al., 1988; Kasanicki et al., 1987; Pardridge et al., 1990; Tal et al., 1990) including vascular endothelial and smooth muscle cells (Kaiser et al, 1993) and in all cell lines (Bell et al, 1990; Mueckler, 1994). The GLUT 2 transporter protein is abundant in liver, pancreatic beta cells, kidney, and the small intestine (Hacker et al., 1991; Thorens et al., 1988; Thorens et al., 1990). The GLUT 3 transporter protein is abundant in the brain (Maher et al., 1992; Nagamatsu et al., 1992). The GLUT 4 transporter protein is found primarily in insulin-sensitive tissues, such as skeletal muscle, heart and adipose tissue (Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989; James et al., 1989; Kaestner et al., 1989). The GLUT 5 transporter protein is abundant in the small intestine and recent evidence suggests that this

transporter is primarily a fructose transporter (Burant et al., 1992; Davidson et al., 1992). GLUT 6 is a pseudogene that does not encode a functional protein (Kayano et al., 1990). The GLUT 7 transporter protein is located in the endoplasmic reticulum of hepatocytes and releases free glucose into the cytoplasm of these cells which, in turn, may exit the cells via a different glucose transporter (Waddell et al., 1991). Transporter proteins contain from 492 to 524 amino acids and there is 39-65% identity and 50-76% similarity among amino acid sequences of the transporter isoforms (Bell et al., 1990). The region of amino acids that is most different among the transporter isoforms is located in the carboxyl terminus (Bell et al., 1990). The carboxyl terminal region is routinely used to generate antibodies specific for the individual transporters.

GLUT 1 is the best characterized of the glucose transporters. It has been purified from human erythrocytes and cloned from human hepatoma cells and rat brain (Mueckler, 1994). This transporter is believed to be responsible for basal glucose transport (Mueckler, 1990). Stimulation of glucose transport activity in many types of cells has been attributed to regulation of GLUT 1 by changes in transcription, translation, degradation, translocation and intrinsic activity. GLUT 1 expression has been shown to be regulated by glycemia (Haspel et al., 1986; Kaiser et al., 1993), growth factors (Hiraki et al., 1988; Rollins et al., 1988), oncogene transformation (Flier et al., 1987), insulin (DeHerreros and Birnbaum, 1989), and angiotensin II (Low et al., 1992). A decrease in GLUT 1 degradation has been observed with glucose deprivation (Haspel et al., 1986; Ortiz et al., 1992). Translocation of GLUT 1 to the cell surface has been observed with glucose deprivation (Koska et al., 1991; Greco-Perotto et al., 1992), and insulin stimulation (Gould et al., 1989). Regulation of

GLUT 1 intrinsic activity has been implied in several studies where an increase in glucose transport occurs with a reduction in Km value (Clancy et al., 1991; Harrison et al., 1991) and where other regulatory mechanisms such as translocation or expression cannot fully account for the increase in glucose transport activity (Koska et al., 1991; and Kitzman et al., 1993). For instance, Clancy et al. (1991) observed a maximal 7-fold increase in glucose uptake by 3T3-L1 adipocytes using the protein synthesis inhibitors, anisomycin and cycloheximide. The anisomycin effect occurred with a reduction in Km. The effect of both protein synthesis inhibitors on glucose transport did not involve an increase in GLUT 1 or GLUT 4 proteins in the plasma membrane of the cells suggesting that the intrinsic activity of the glucose transporters is altered in response to this treatment. Further, Harrison et al. (1991) detected an increase in glucose transport in 3T3-L1 fibroblasts in response to cadmium through a mechanism involving a change in glucose transporter intrinsic activity. Cadmium treatment increased glucose transport into the cells 5- to 6-fold with a reduction in Km and no change in cell surface GLUT 1 protein. In summary, regulation of glucose transport activity through the GLUT 1 transporter appears to vary with the type of cell and the nature of the stimulus.

The role of glucose deprivation in the regulation of the GLUT 1 transporter has been extensively investigated. Glucose deprivation has been shown to stimulate glucose transport through several different mechanisms such as increased GLUT 1 expression (Kletzien and Perdue, 1975; Kumagai et al., 1995; Van Putten and Krans 1985; Yamada et al., 1983), decreased GLUT 1 degradation (Haspel et al., 1986; Ortiz et al., 1992; and Yamada et al., 1983), increased GLUT 1 mRNA (Koivisto et al., 1991; Kumagai et al., 1995; Walker et al., 1988; and Walker et al., 1989), and enrichment of GLUT 1 at the cell surface through

possible control of cellular trafficking (Koska et al., 1991; and Greco-Perotto et al., 1992). Intrinsic regulation of the transporter by glucose deprivation has also been implicated (Kitzman et al., 1993; Koska et al., 1991). Recently, Kaiser and associates (1993) demonstrated that low glucose stimulated transport in cultured vascular smooth muscle cells by an increase in GLUT 1 and a change in Vmax with no apparent changes in Km or GLUT 1 mRNA abundance.

Low et al. (1992) have demonstrated that AII stimulates glucose transport in cultured VSM cells 1) through a rapid response that is protein synthesis-independent and 2) through a more prolonged response that involves an increase in GLUT 1 mRNA synthesis without a change in stability of the message. The mechanism underlying the rapid response was not explored by these researchers and could be explained by an increase in intrinsic activity and/or translocation of GLUT 1. There has not been an attempt to explore the second messenger events associated with AII binding and the resultant increase in glucose transport nor has the effect of the glycemic state on AII-stimulated glucose uptake been investigated.

The purpose of this research was to further explore the role of AII in the stimulation of glucose transport in cultured vascular smooth muscle cells. Specific emphasis was placed on the role of PKC activation and extracellular calcium in mediating the effects of AII. In addition, the effects of AII on the expression of the GLUT 1 transporter were investigated as was the role of the cellular glycemic state on AII stimulation of glucose transport.

METHODS

CELL CULTURE

Vascular smooth muscle (VSM) cells were isolated from rat thoracic aortae via enzymatic digestion as described by Gunther et al. (1982). Briefly, thoracic aortae were excised from six anesthetized (ketamine HCl and xylazine, 45:5 mg/kg) 275-350 gram male Sprague-Dawley rats via midline incision under aseptic conditions. The aortae were rinsed in sterile Isolation Buffer [Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) containing 0.2 mM $CaCl_2$, 15 mM HEPES, and 14 mM glucose and adjusted to pH 7.4] and cut longitudinally. Endothelial cells were removed by gentle rubbing with the back of a curved pair of tweezers. The tunica media was separated from the tunica adventitia with the aid of a dissecting scope, cut into small pieces, and then subjected to enzymatic digestion for 30-40 minutes at 37°C using type II collagenase (700 U/ml), type II elastase (160 U/ml), type II DNase (300 U/ml), and a soybean trypsin inhibitor (1 mg/ml) suspended in 6 ml of Isolation Buffer in a siliconized flask. The resultant digest was then aspirated into and out of a siliconized, glass Pasteur pipet 20 times. The cell suspension was passed through a sterile nylon mesh filter and the remaining pieces were subjected to digestion for 10 minutes in 2 ml of fresh enzyme solution. The cells were recovered by centrifugation, rinsed once with DMEM, resuspended in 4 ml of serum-free DMEM and placed into two T-25 tissue culture flasks. Two hours later, 8 ml of DMEM containing 10% newborn calf serum were added to each flask. The medium was changed again on the following day and twice a week thereafter.

VSM cells were grown in Dulbecco's Modified Eagle Medium (DMEM) [with 25 mM HEPES and 25 mM glucose, pH 7.4] that was supplemented with 10% heat-inactivated newborn calf serum and penicillin (100 U/ml) / streptomycin (100 U/ml) at 37°C in a humidified atmosphere of 5% CO_2 and air. The medium was changed twice weekly. For passaging, cells were detached from the flasks with 0.05% trypsin/ 0.53 mM EDTA in HBSS. Cells from passages 3-10 were used for the experiments.

[^{3}H]-2-DEOXYGLUCOSE TRANSPORT MEASUREMENTS

Glucose transport was assayed by measuring the uptake of radiolabeled 2-deoxyglucose (2-DG). This assay is based on the principle that 2-DG is transported by the same carrier as D-glucose and then phosphorylated to 2-deoxyglucose-6-phosphate (2-DGP) by hexokinase (Wick et al., 1957). The 2-DGP does not exit the cell nor is it metabolized further.

Cultured VSM cells were seeded at a density of 4.5 - 5 x 10^5 cells/well in 6-well culture plates. The cells were grown in DMEM containing 10% heat-inactivated newborn calf serum for 7-9 days. Forty-eight hours prior to the start of an experiment, the medium was changed to DMEM containing 0.4% heat-inactivated newborn calf serum to ensure quiescence. At the beginning of a given experiment, the low serum medium was replaced with fresh medium containing specific agents. The cells were exposed to these agents for various time periods.

To assess glucose transport, the low serum medium was removed and the cells were rinsed twice with and then equilibrated for 30 minutes in a Kreb's Ringer Phosphate buffer

(136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM Na_2HPO_4, and 10 mM NaH_2PO_4, pH 7.4). The specific agents added at the start of the experiment or an equivalent volume of carrier were replenished at the beginning of the equilibration period. Then 50 μl of a solution containing unlabeled 2-deoxyglucose (20 μM) and [^{3}H]-2-deoxyglucose (0.5 μCi) were added to each well for 20 minutes. During this time glucose transport is linear (Appendix A). To terminate the experiment, the radioactive incubation buffer was rapidly aspirated from the wells and the cells were rinsed twice with ice-cold phosphate-buffered saline (20 mM Na_2HPO_4/ NaH_2PO_4 and 140 mM NaCl, pH 7.4). The cells from each well were harvested using trypsin/ EDTA in HBSS, dissolved in Universol ES (ICN Biochemicals, Irvine, CA), and radioactivity levels detected using a Beckman LS 5801 liquid scintillation counter. In several different experiments, nonspecific transport was assessed by adding 15 μM cytochalasin B to half of the wells in each treatment group 20 minutes into the incubation period. Cytochalasin B is a fungal metabolite used widely to inhibit glucose transporter activity (Czech et al., 1973; Kletzien and Perdue, 1973). Glucose uptake in the presence of cytochalasin B occurs through nonspecific means. Nonspecific transport accounted for less than 7% of total glucose transport per treatment group (Appendix B). In the studies to determine the level of nonspecific transport, each treatment group consisted of either three or six samples incubated in the presence of cytochalasin B and either three or six samples incubated in the absence of cytochalasin B. In the remainder of the experiments, each treatment group consisted of six samples. In each plate, there was a well representing each experimental treatment. The results were expressed as pmoles of glucose transported/ 10^6 cells. Cells were removed from the wells using trypsin/ EDTA and were

counted using a hemacytometer. Each experiment was repeated a minimum of three times using cells derived from different primary isolations. The experiments presented are representative experiments.

PEPTIDE SYNTHESIS AND ANTIBODY PRODUCTION

A peptide corresponding to the last 15 amino acids (TPEELFHPLGADSQV, see Appendix C for an abbreviation key) of the carboxyl-terminus of the GLUT 1 transporter was synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Fields and Noble, 1990) using the "standard scale" protocol of the Applied Biosystems (ABI) Model 431A Peptide Synthesizer. Specific Fmoc amino acids were sequentially added from cartridges, each containing 1 mmole of derivatized amino acid, to 0.1 mmole of ABI 8-branch Multiple Antigenic Peptide (MAP) resin. With this procedure, several identical peptides can be synthesized onto the branched lysine residues of the MAP resin. Once cleaved from the resin, the antigen used to immunize rabbits will be a complex of numerous peptides attached to lysine. This product is large enough to be completely immunogenic making a carrier protein unnecessary and eliminating antibodies generated against a carrier.

Following synthesis, the immunogenic product was cleaved from the resin and isolated as follows. The newly synthesized peptide-resin mixture was washed with dichloromethane and dried under high vacuum for 30 to 60 minutes prior to cleaving the synthesized peptides from the resin. The dried peptide-resin was placed into a flask and cooled in an ice bath. The cleavage solution [0.25 ml 1,2-ethanedithiol, 0.25 ml deionized and distilled water, plus 9.5 ml trifluoroacetic acid] was cooled using an ice bath and then

added to the cooled peptide-resin to give a final volume of 10 ml per 0.1-1.5 g of peptide-resin. The flask was removed from the ice bath and the contents were allowed to warm to room temperature. The flask was stoppered and the peptide-resin was exposed to the cleavage solution for 1.5 hours with stirring. At the end of the cleavage period, the peptide was isolated from the resin using vacuum filtration through a medium-porosity, fritted glass funnel. The peptide remained in the filtrate while the resin was too large to be filtered. In order to isolate the peptide from the filtrate, the filtrate was concentrated on a rotary evaporator to a volume of approximately 1 to 2 ml. Approximately 50 ml of cold ether was added to the concentrated filtrate to precipitate the peptide. The peptide was collected by filtering the mixture through a fine-porosity, fritted glass funnel. The precipitate was dried in a desiccator. After drying, the peptide was dissolved in a few ml of deionized/ distilled water and lyophilized. The yield of each of two cleaved and isolated batches of peptide was 25.8 mg and 51 mg of material. This represents approximately 50% of the theoretically possible yield.

The multiple peptide antigen complex was emulsified in 50% (v/v) Hunter's Titer Max™. Each rabbit was relaxed prior to injection using approximately 0.75 cc acepromazine administered intramuscularly in the hindlimb. The injection sites were shaved and cleaned with alcohol to reduce the presence of material that could cause infection. Two intradermal injections (0.05 cc/ site) of emulsified peptide were administered on the back of each rabbit. Each rabbit received approximately 1.5 to 2 mg of peptide. Blood samples were taken periodically to assess the level of serum antibodies. To obtain blood samples, the rabbit was relaxed using 0.75 cc acepromazine. An ear was shaved, swabbed with alcohol, and blood

withdrawn from the central ear artery. The sample size did not exceed one percent of the body weight of the animal. The animals received five booster doses of peptide in Hunter's Titer Max™. The serum antibody titer obtained using Hunter's Titer Max™ as adjuvant was never greater than 1:10. Therefore, the protocol was adjusted to employ Freund's complete adjuvant (FCA). FCA is the adjuvant widely used to generate glucose transporter antibodies. The Titer Max™ was used initially to approach the animals with a procedure offering fewer side effects than FCA. The peptide was emulsified in 50% (v/v) FCA and injected intradermally (0.05 cc/ site) into five shaved, alcohol swabbed sites on the back of each rabbit. Each animal received approximately 2 mg of peptide. Five booster doses of antigen, approximately 1 mg/ animal, in Freund's incomplete adjuvant were administered at 30 day intervals and two weeks prior to serum collection for antibody titer assessment. Prior to Freund's complete and incomplete adjuvant administration and blood collection the animals received 0.75 cc acepromazine. The health and comfort of the rabbits was attended to by a veterinarian.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to determine the titer of peptide-specific antibodies in immunized rabbit sera. The assay was based on a modification of the procedure described by Kennett (1980). The GLUT 1 peptide (antigen) was suspended in deionized/ distilled water at a final concentration of 10 μg/ ml. Wells of polystyrene micro titer plates were coated with 100 μl of peptide solution per well during an overnight incubation at 4°C. The remaining incubations were carried out at room temperature with

shaking using a rocking platform. The remaining protein-binding sites were blocked using 100 μl of 10 mM dibasic sodium phosphate with 0.9% NaCl and 1% bovine serum albumin (BSA) per well. The blocking solution was removed by inverting the plate and the wells were washed five times with approximately 200 μl per well of phosphate-buffered saline (PBS, 10 mM Na_2HPO_4 / NaH_2PO_4 and 140 mM NaCl, pH 7.2). Next, antiserum (100 μl, primary antibody), diluted 1:100 with PBS containing 1% BSA, was added to each peptide-coated well and allowed to bind for two hours at room temperature. Wells were washed five times with approximately 200 μl per well PBS to remove unbound primary antibody. One hundred μl of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (secondary antibody) diluted 1:1000 in PBS with 1% BSA were added to each well and incubation continued for two hours at room temperature. Wells were washed five times with approximately 200 μl per well PBS to remove unbound secondary antibody. The presence of the bound secondary antibody and therefore the interaction of primary antibody with antigen was assessed by measuring HRP activity of the conjugated secondary antibody. HRP activity was assessed by adding to each well 200 μl of a solution containing 1 mg/ml O-phenylenediamine, 1% hydrogen peroxide, and 0.1 M sodium citrate (pH 4.5). The presence of HRP is indicated by a change in the color of the assay solution from clear to yellow which takes approximately 10-15 minutes. The color intensity was then determined by measuring the absorbance of the solution at 450 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). The intensity values were recorded as optical density measurements. Nonspecific antibody-binding was determined using a 1:100 dilution of primary antibody in the absence of the peptide antigen. Optical density measurements

obtained for each experimental group were corrected for nonspecific antibody-binding.

MEMBRANE ISOLATION

Cells were seeded at a density of 10-12 x 10^6 cells/ flask in T-225 tissue culture flasks and grown in DMEM containing 10% heat-inactivated newborn calf serum for 7-9 days. Forty-eight hours prior to the start of the experiment, the medium was changed to DMEM containing 0.4% heat-inactivated newborn calf serum. At the start of the experiment, the low serum medium was removed and the cells were rinsed twice with 0.9% NaCl. The medium was replaced with medium containing either 2.5 mM glucose or 25 mM glucose and the incubation was allowed to proceed for an additional 24 hours. Six hours prior to the end of the incubation period, AII (final concentration 100 nM) or an equivalent volume of vehicle (0.9% NaCl) was added to the culture medium. At the end of the experiment, the medium was removed and the cells were rinsed twice with ice-cold 0.9% NaCl. Medium glucose concentration was measured at the beginning and at the end of the 24 hour incubation period using an enzymatic-colorimetric glucose assay (Stanbio Laboratory, Inc., San Antonio, TX). During the course of this assay, the glucose present in the medium is oxidized by glucose oxidase to form gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminophenazone in the presence of peroxidase to yield a red-violet quinone complex. The absorbance of the resulting colored product was measured at 500 nm within 60 minutes using a Beckman DU-8 spectrophotometer. The concentration of glucose in the sample tested was calculated using the following formula:

mg/ dl glucose = [absorbance of unknown ÷ absorbance of standard] × the concentration of the standard (100 mg/ dl).

Crude membranes were isolated by the method of Klip et al. (1987) with modifications. The cell monolayer was removed by scraping, centrifuged at 3,000 rpm for 10 minutes at 4°C in a Beckman GS-6R refrigerated centrifuge, resuspended in approximately 1 ml of Buffer A [10 mM $NaHCO_3$, 250 mM sucrose, 5 mM NaN_3, pH 7.0] plus 5 mM phenylmethylsulfonylfluoride (PMSF) and 10 μg/ ml pepstatin A, and disrupted with several brief bursts of an Ultrasonic Processor (Cole-Parmer Instrument Co.). The disrupted cells were centrifuged at 1,200 *x*g in a Sorvall RC-5B refrigerated centrifuge for 10 minutes at 4°C. The supernatant fraction was saved and the pellet resuspended in Buffer A, resonicated as above, and centrifuged at 1,200 *x*g for 10 minutes at 4°C. Both 1,200 *x*g supernatant fractions were pooled and centrifuged at 9,000 *x*g for 10 minutes at 4°C. The pellet was discarded and the supernatant was collected and centrifuged at 190,000 *x*g for 60 minutes at 4°C using a SW41 rotor in a Beckman Model L5-65 Ultracentrifuge. The resulting pellet contained the crude membranes which were resuspended in Buffer B [10 mM $NaHCO_3$, 5 mM NaN_3, pH 7.0] plus 5 mM PMSF and 10 μg/ ml pepstatin A. Protein determinations were made using the bicinchoninic acid assay (Pierce, Rockford, IL).

WESTERN BLOT ANALYSIS

Fifty microgram quantities of crude membrane proteins from each experimental group were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) according to the method of Laemmli (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes. Briefly, the proteins were separated through 15% polyacrylamide separating gels at 60 volts applied overnight using a PROTEAN® II xi Cell

Apparatus (BIO-RAD, Richmond, CA). The proteins were then transferred to nitrocellulose membranes using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). Each gel was removed from the gel apparatus and allowed to equilibrate in transfer buffer (48 mM Trizma base, 39 mM glycine, 20 % methanol, 0.00375% SDS, pH 9.2; Bjerrum and Schafer-Nielsen, 1986) for approximately 30 minutes with shaking using a rocking platform. Enough transfer buffer was added to a glass dish containing the gel to completely submerge the gel. After 10 minutes, the transfer buffer was replaced with fresh buffer and the incubation continued. The nitrocellulose membrane was cut to the dimensions of the gel and soaked in transfer buffer for at least 30 minutes with shaking. Four pieces of thick filter paper were cut to the dimensions of the gel and soaked in transfer buffer for approximately 15 minutes with shaking. Following soaking in transfer buffer, two pieces of filter paper were placed upon the platinum anode plate followed sequentially by the nitrocellulose membrane, the equilibrated gel, two final pieces of filter paper, and the platinum cathode plate. Care was taken to remove all air bubbles between the layers of filter paper, gel, and membrane to ensure an uninterrupted transfer. The transfer was accomplished using a current of 23 V applied for 30 minutes.

Specific proteins were detected on the nitrocellulose membranes using the procedure described by Green et al. (1992). The nitrocellulose membranes were blocked for one hour with shaking using enough Tris-saline [10 mM Tris, 150 mM NaCl, 0.2 mM EDTA, pH 8] plus 0.05% Tween 20 and 5% (w/v) nonfat dried milk to cover the membrane. At the end of the one hour incubation period, the blocking buffer was replaced with fresh buffer. Polyclonal antibodies (primary antibodies) against synthetically prepared GLUT 1 carboxyl-

terminus were added to this buffer to give a dilution of 1:1,000, unless otherwise noted, and incubation proceeded for 2.5 hours with shaking. After thorough washing in enough Tris-saline with 0.05 % Tween 20 (Tween-Tris-saline) to sufficiently cover the membrane, the nitrocellulose membranes were exposed to alkaline phosphatase-conjugated goat anti-rabbit lgG (secondary antibody) diluted 1:1000 with 5% (w/v) nonfat dried milk in Tween-Tris-saline for 1.5 hours, and washed twice in Tween-Tris-saline followed by a final wash in Tris-saline. The presence of alkaline phosphatase was assessed by exposing the membranes to a color developing solution (10 ml of 1 M Tris, pH 9.5; 5 ml of 2 M NaCl; 0.5 ml of 1 M $MgCl_2$; 84.5 ml deionized and distilled water; 0.4 ml of 75 mg/ ml nitroblue tetrazolium dissolved in 70% N,N-dimethylformamide; and 0.3 ml of 50 mg/ ml 5-bromo-4-chloro-3-indolyl phosphate dissolved in 100% N,N-dimethylformamide) for approximately 30 minutes with shaking. The 5-bromo-4-chloro-3-indolyl phosphate served as substrate. The inclusion of nitroblue tetrazolium in the reaction mixture allowed for visualization of the presence of alkaline phosphatase. The result of the alkaline phosphatase reaction was a purple precipitate (McGadey, 1970). The color development reaction was terminated by removing the color developing solution and rinsing the membranes with deionized and distilled water. The membranes were placed between two pieces of filter paper to dry. The membranes were analyzed using a scanning densitometer (Molecular Dynamics) to determine relative amounts of the GLUT 1 protein. The level of the GLUT 1 protein was expressed as arbitrary units.

MATERIALS

[^{3}H]-2-deoxyglucose (26.2 Ci/mmol) was purchased from NEN/ DuPont (Boston, MA). DMEM, newborn calf serum, trypsin/ EDTA, penicillin/streptomycin, amino acids, and vitamins were purchased from Gibco BRL (Grand Island, NY). Other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Calcium-free and glucose-free media were prepared based on the formulation of DMEM #12430 (Appendix D) supplied by Gibco BRL (Grand Island, NY). The osmolality of these solutions was adjusted to that of the standard DMEM formulation by the addition of an appropriate quantity of NaCl. Gibco BRL-formulated solutions of amino acids and vitamins were used to provide these components for the prepared media.

ANIMALS.

Male Sprague-Dawley rats were obtained from Hilltop Lab Animals, Scottdale, PA and were housed in plastic cages with wood-chip bedding. Male New Zealand white rabbits were obtained from Robinson Services, Inc., Winston-Salem, NC and were housed in stainless steel rabbit cages. Both animal species were maintained in rooms having an ambient temperature of 23 $\pm$ 2 °C and a 12 hour light/ dark cycle for 5-7 days prior to use. All animals had free access to food and tap water. Protocols involving animal use were approved by the Institutional Animal Care and Use Committee.

STATISTICAL ANALYSIS

Each type of experiment was repeated a minimum of three times using cells from different cell lines. The individual experiments presented in the paper are representative experiments. Statistical analyses were made using SuperANOVA™ software (Abacus Concepts, Berkeley, CA). Significant differences among the means in an experiment were determined by one-way analysis of variance followed by Newman-Keuls post hoc test or by two-way analysis of variance where appropriate. Following two-way analysis of variance, "contrasts" (Abacus Concepts, Berkeley, CA) was used to make specific, preplanned comparisons.

RESULTS

To determine if AII stimulated glucose transport in a time-dependent manner, cultured VSM cells were exposed to 100 nM AII for various time intervals. After one hour of incubation, the rate of glucose transport in cells exposed to AII was significantly greater than the rate of transport in the corresponding control (Figure 1). The rate of transport continued to rise with increasing time of exposure with the maximum AII response being observed between six and eight hours. The maximum rate of transport was maintained through 20 hours, the longest time period for which a measurement was made.

To determine if the AII effect on glucose transport was concentration-dependent, VSM cells were exposed to various concentrations of AII for six hours. VSM cells responded to AII at concentrations as low as 0.1 nM (Figure 2). The maximum response was observed at 100 nM AII.

To determine whether the AII effect on glucose transport was mediated by the AII receptor, VSM cells were incubated with a 100 molar excess of saralasin, a competitive inhibitor of AII receptor binding. As shown in Figure 3, saralasin reduced AII-stimulated transport to a level not significantly different from that induced by saralasin alone. This data suggests that AII is binding to its receptor to stimulate glucose transport.

The long period of time required to elicit a maximal AII effect on glucose transport suggests that this response may require protein synthesis. To test this hypothesis, VSM cells were incubated for six hours with and without AII in the presence or absence of 10 μM cycloheximide, an inhibitor of protein synthesis. Under these conditions, cycloheximide

Figure 1 Time course for AII-stimulated glucose transport. Cultured vascular smooth muscle cells were exposed to 100 nM AII for 1, 2, 4, 6, 8, 16, and 20 hours. The AII was dissolved in deionized/ distilled water to make a stock solution of 1 mM (1 mg/ ml). The AII stock solution was diluted 1:100 with deionized/ distilled water and added as 25 μl aliquots to the incubation medium to give a final concentration of 100 nM. Cells incubated with an equivalent volume of diluent alone served as controls. Each point is the mean $\pm$ SEM of six replicates. Data are expressed as percent stimulation above control. At each time point, AII-stimulated glucose transport was significantly ($p < 0.05$) greater than the corresponding control.

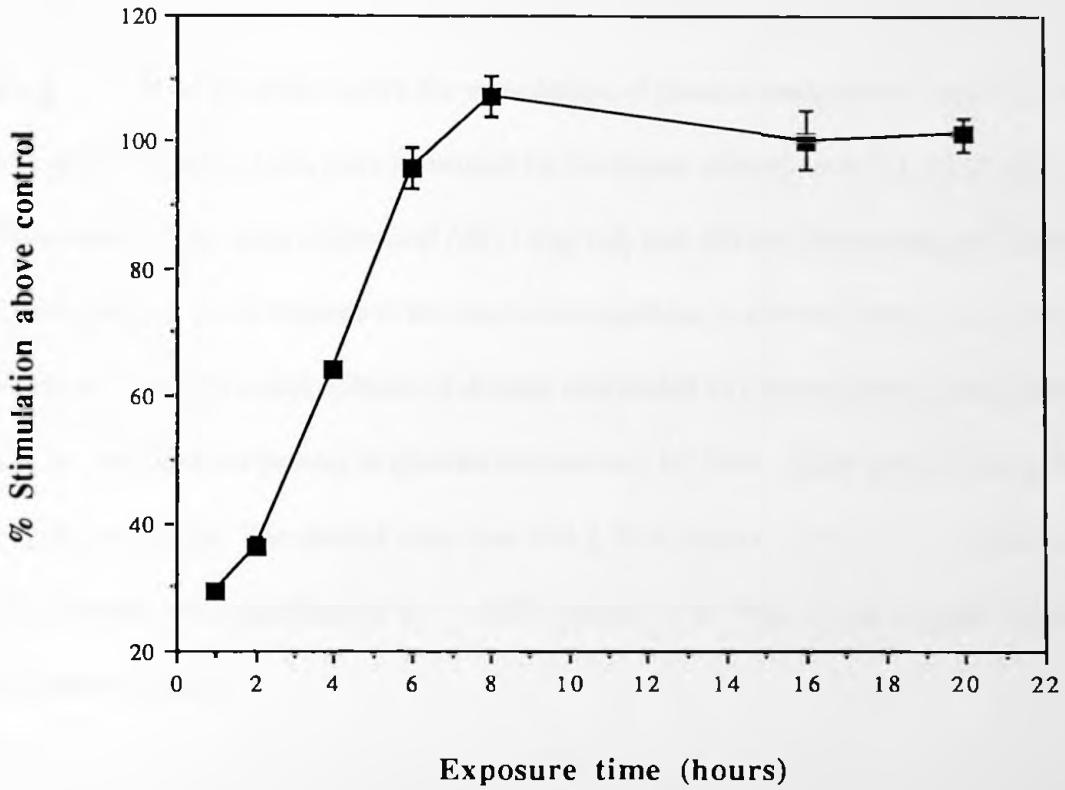

% Stimulation above control
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
Exposure time (hours)

Figure 2 Dose response curve for stimulation of glucose transport by AII. Cultured vascular smooth muscle cells were incubated for six hours with diluent, 0.1, 1, 10, 50, 100, or 1000 nM AII. The stock solution of AII (1 mg/ ml) was diluted with deionized/ distilled water and added as 25 μl aliquots to the incubation medium to give the final concentrations listed above. An equivalent volume of diluent was added to cells to serve as the control. Results are expressed as pmoles of glucose transported/ 10^6 cells. Each point is the mean $\pm$ SEM of six replicates. The control value was 616 $\pm$ 22.9 pmoles / 10^6 cells. AII-stimulated glucose uptake was significantly ($p < 0.05$) greater than that of the control for each concentration of AII.

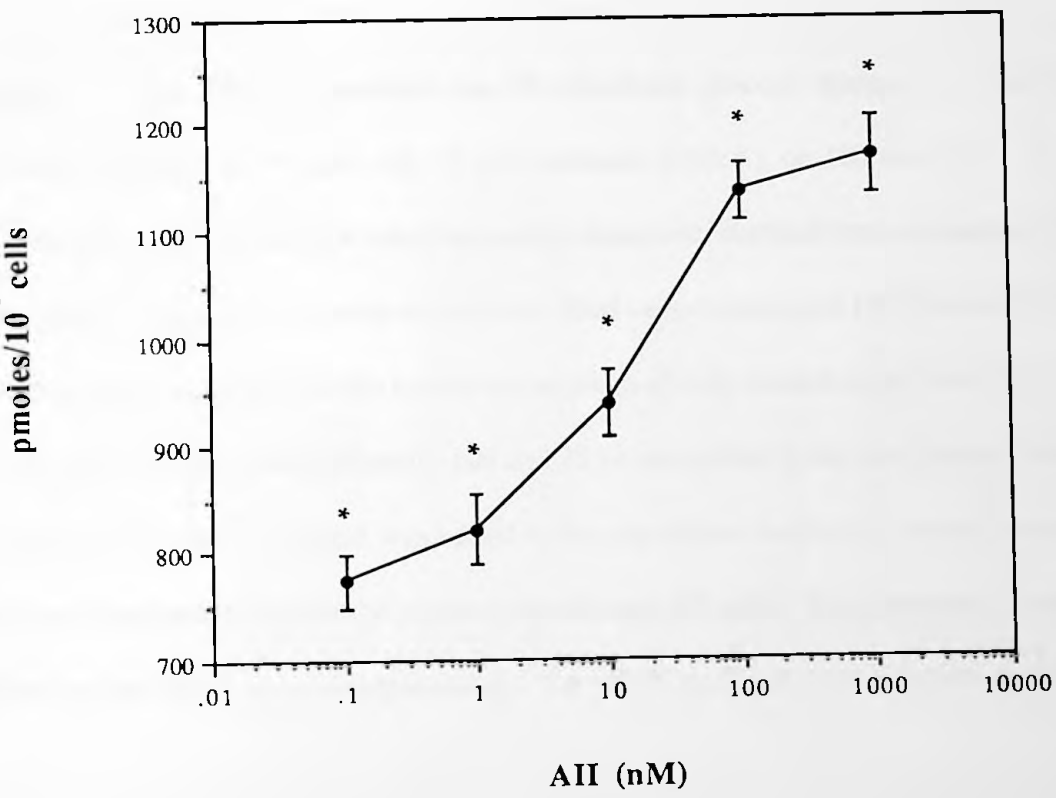
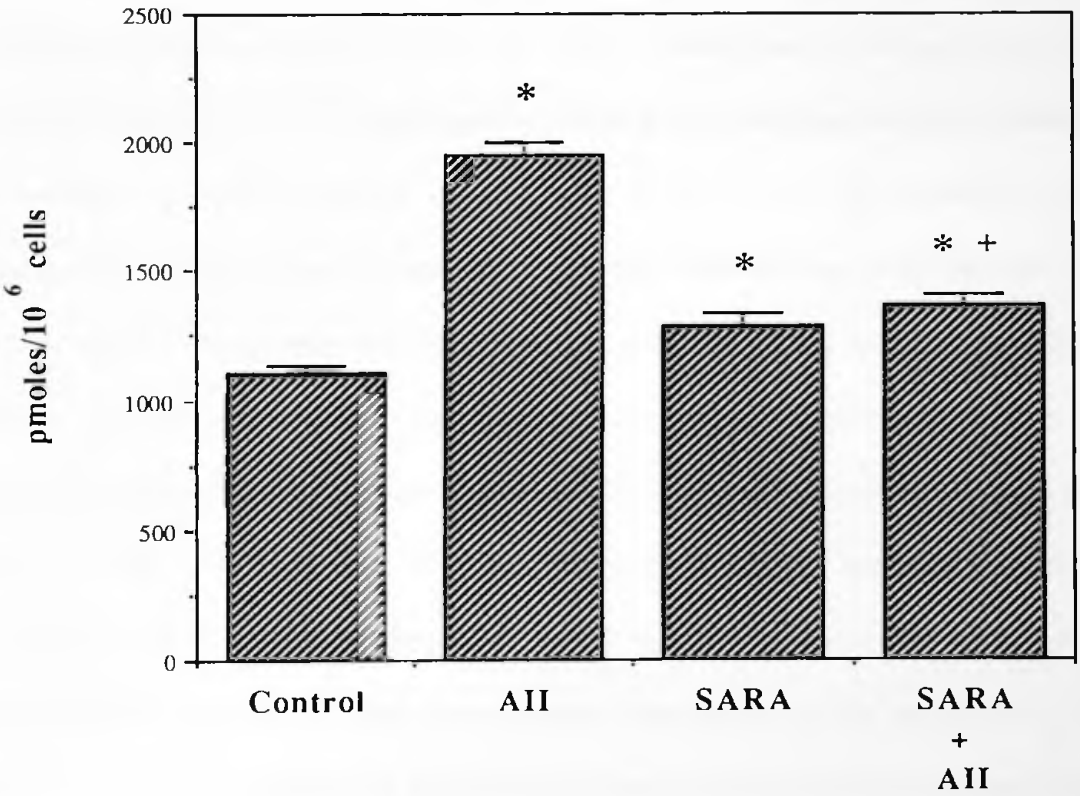

1300
1200
1100
1000
900
800
700
pmoles/10 6 cells
.01
.1
1
10
100
1000
10000
AII (nM)
*
*
*
*
*

Figure 3 The effect of saralasin on AII-stimulated glucose transport. Cells were incubated with diluent, 100 nM AII, 10 μM saralasin (SARA), or 100 nM AII + 10 μM SARA for six hours. The SARA was dissolved in deionized/ distilled water to make a 1 mM stock solution (1 mg/ ml). In order to achieve a final concentration of 10,000 nM, 25 μl of the stock solution was added to the incubation medium of cells treated with SARA. The AII stock solution (1 mg/ ml) was diluted 1:100 and 25 μl was added to the incubation medium. An equivalent volume of diluent was added to the incubation medium to serve as control. Results are expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six determinations. * $p < 0.05$ vs. Control; + $p < 0.05$ vs. AII.

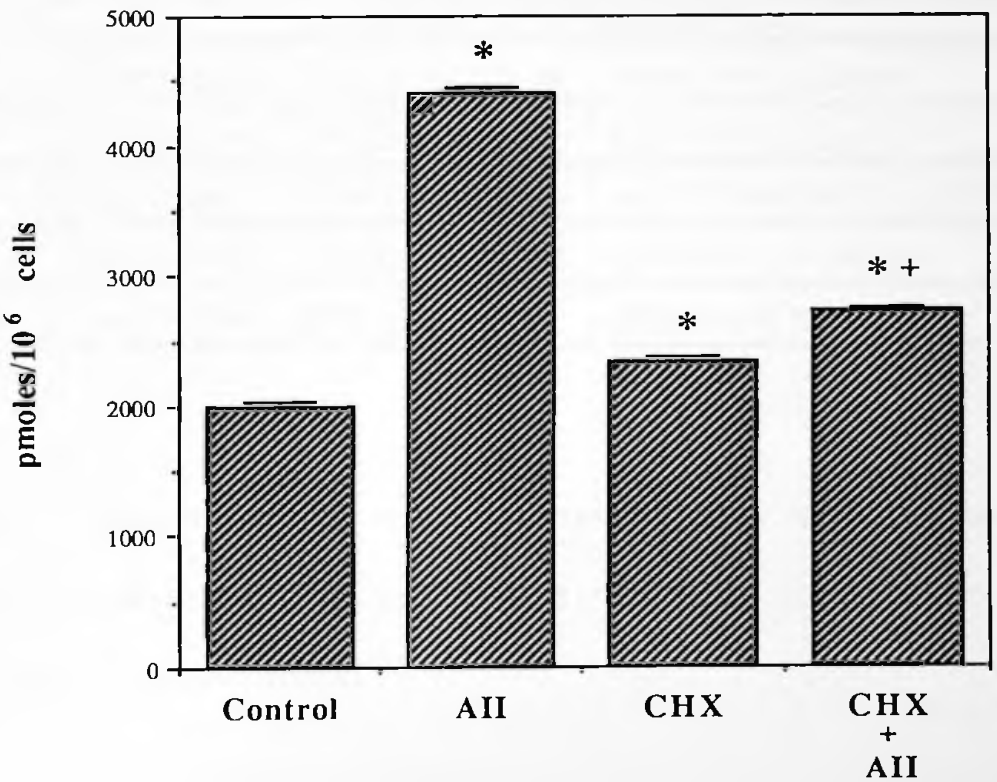

2500
2000
1500
1000
500
0
pmoles/10 6 cells
*
*
* +
Control
AII
SARA
SARA
+
AII

reduced AII-stimulated transport by 70% (Figure 4) suggesting that protein synthesis is involved in AII stimulation of glucose transport.

A specific protein that could be affected by AII stimulation is the GLUT 1 transporter. Western blot analysis was utilized to assess this possibility. For these studies, a polyclonal antibody was generated against the carboxyl terminus of the GLUT 1 transporter: this region exhibits the lowest degree of homology among the glucose transporter isoforms (Table 1; Bell et al., 1990). Subsequently, the specificity of this antibody (3406 GLUT 1 Ab) was assessed by using an enzyme-linked immunosorbent assay to compare the cross-reactivity of the 3406 GLUT 1 Ab with synthetic peptides corresponding to the carboxyl termini of four different glucose transporter isoforms (GLUT 1, 2, 4, and 7). In addition, the degree of cross-reactivity of the synthetic peptides to a second, commercially available, anti-GLUT 1 antibody was determined. With a 1:100 dilution, the 3406 GLUT 1 Ab reacted strongly with the GLUT 1 peptide and much less so with the other GLUT peptides (Table 2). When the dilution of antibody was raised to 1:1,000, the optical density recordings for GLUT 2, 4, and 7 were less than the background measurement (Appendix E). The data support the hypothesis that the 3406 GLUT 1 Ab is specific for GLUT 1. Moreover, this study provided additional evidence that the GLUT 1 peptide synthesized in our hands was an appropriate antigen due to the intense reaction observed with the commercially available anti-GLUT 1 antibody (East Acres Biologicals, Southbridge, MA).

To further ensure that the 3406 GLUT 1 Ab recognized the GLUT 1 transporter, red blood cell membranes were used as positive controls when VSM cell membranes were

Figure 4 The effect of cycloheximide on AII-stimulated glucose transport. Cultured VSM cells were incubated with 10 μM cycloheximide (CHX) or vehicle in the presence of 100 nM AII or vehicle for six hours. The cycloheximide was dissolved in deionized/ distilled water to prepare a stock solution with a concentration of 10 mM (2.8 mg/ ml). The stock CHX solution was diluted 1:100 and 50 μl aliquots were added to the incubation medium of cells receiving CHX to give a final concentration of 10 μM. The stock AII solution (1 mg/ ml) was diluted 1:100 and 25 μl aliquots were added to the incubation medium of cells receiving AII at a final concentration of 100 nM. Control cells received an equivalent volume of diluent. Results are expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six determinations. * $p < 0.05$ vs. Control. + $p < 0.05$ vs. AII.


5000
4000
3000
2000
1000
0
pmoles/10 6 cells
*
*
* +
Control
AII
CHX
CHX
+
AII

	Intracellular COOH-Terminal Domain		
GLUT 1	KGRTFDEIAS	GFRQGGASQSDK	TPEELFHPLGADSQV
GLUT 2	KGKSFEEIAA	EFQKKSGSAHRP	KAAVEMKFLGATETV
GLUT 3	RGRTFEDITR	AFEGQAHGADRS	GKDGVMEMNSIEPAKETTTNV
GLUT 4	RGRTFDQISA	AFHRTPSLLEQE	VKPSTELEYLGPDEND
GLUT 5	KAKTFIEINQ	IFTKMNKVSEVY	PEKEELKELPPVTSEQ

Table 1 Comparison of the amino acid sequences of the intracellular carboxyl termini of human glucose transporters (Bell, et al., 1990; see Appendix C for a key to the amino acid abbreviations).

	Optical density measurements	
Synthetic peptide	3406 GLUT 1 Ab	Commercial GLUT 1 Ab
GLUT 1	1.632	>3
GLUT 2	0.155	0.127
GLUT 4	0.027	0.021
GLUT 7	0.196	0.332

Table 2 Detection of cross-reactivity of 3406 GLUT 1 Ab with GLUT 1, 2, 4, and 7 synthetic peptides. The antibody dilution was 1:100 and the optical density measurements were corrected for background. The optical density measurements obtained for nonspecific binding of the antibody in the absence of antigen served as the background measurement. This measurement was subtracted from those measurements obtained for each of the experimental groups. The corrected values are presented.

subjected to western blot analysis (Figure 5). Red blood cell membranes are enriched in GLUT 1 transporters (Carruthers, 1990). The 3406 GLUT 1 Ab recognized protein bands from red blood cell membranes and smooth muscle cell membranes that correspond to published molecular weights for the GLUT 1 transporter (Gorga et al., 1979; Kumagai et al., 1994).

To assess the effects of AII on the GLUT 1 transporter, membranes prepared from VSM cells incubated with and without AII were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and then probed with the 3406 GLUT 1 Ab. There was a 5-fold increase in the level of immunoreactive GLUT 1 following a six hour exposure to AII (Figure 6). These results support the conclusion that AII is capable of altering the level of the GLUT 1 glucose transporter.

AII elicits a response in target cells by increasing the concentration of free cytosolic calcium and by activating protein kinase C (PKC) (Griendling et al., 1989). The role of PKC activation in mediating AII-stimulated glucose transport was assessed using phorbol esters. In one experiment PKC was activated by a short-term (six hour) exposure of VSM cells to 50 nM phorbol-myristate-acetate (PMA) or 100 nM phorbol-dibutyrate (PDB) prior to measuring glucose uptake. As shown in Figure 7, both PMA and PDB significantly stimulated glucose transport under these conditions suggesting that PKC activation can promote glucose transport. In another experiment, VSM cells were exposed to 1 μM PDB for 48 hours in order to promote PKC downregulation. Subsequently, the cells were stimulated with AII for six hours and glucose transport was measured. Under these conditions, AII was capable of fully stimulating glucose transport in the presence of PDB

Figure 5 Detection of the GLUT 1 transporter in smooth muscle and red blood cell membranes using the prepared 3406 GLUT 1 Ab. Dilutions of 1:2,500 and 1:1,000 of the antibody were used for analysis.

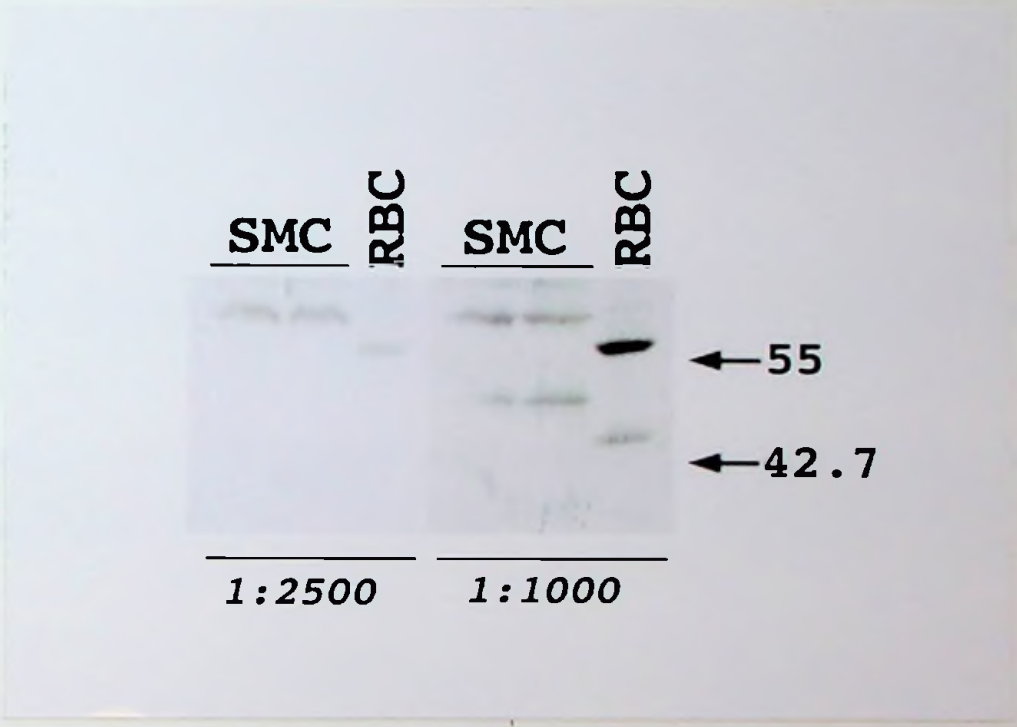

SMC
RBC
SMC
RBC
55
42.7
1:2500
1:1000

Figure 6 The effect of AII on GLUT 1 protein levels. VSM cells were incubated with 100 nM AII or diluent for six hours. Crude membranes were isolated and subjected to western blot analysis using a 1:1,000 dilution of the 3406 GLUT 1 antibody. The level of immunoreactive GLUT 1 was assessed using densitometric analysis. The results are expressed as arbitrary units.

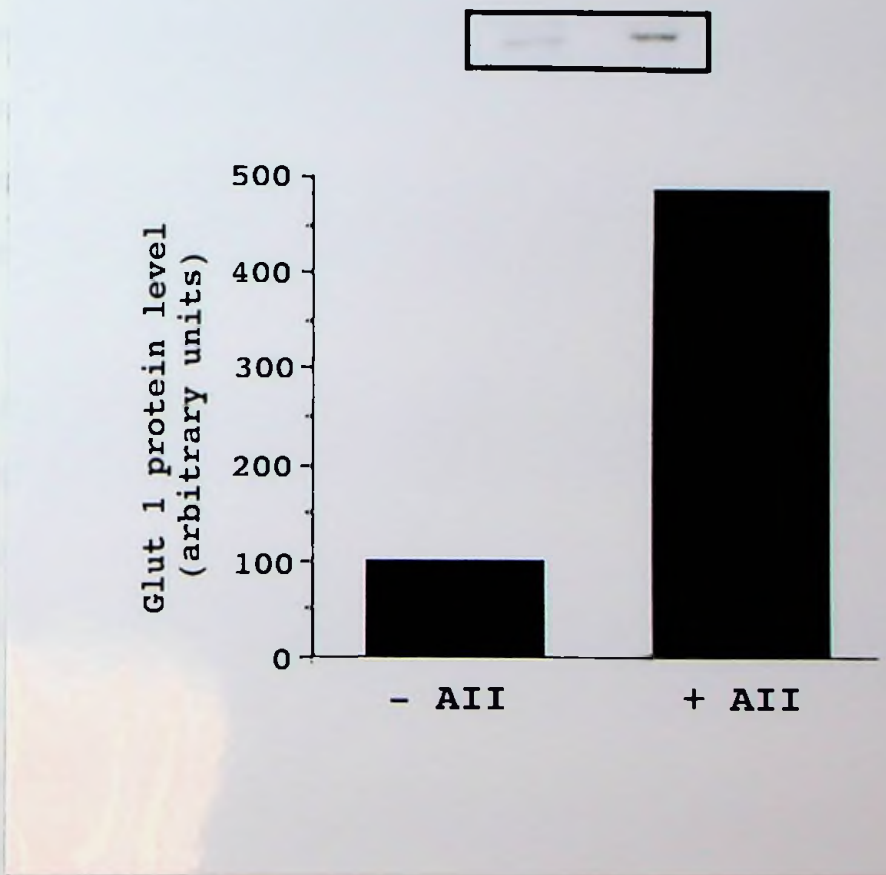
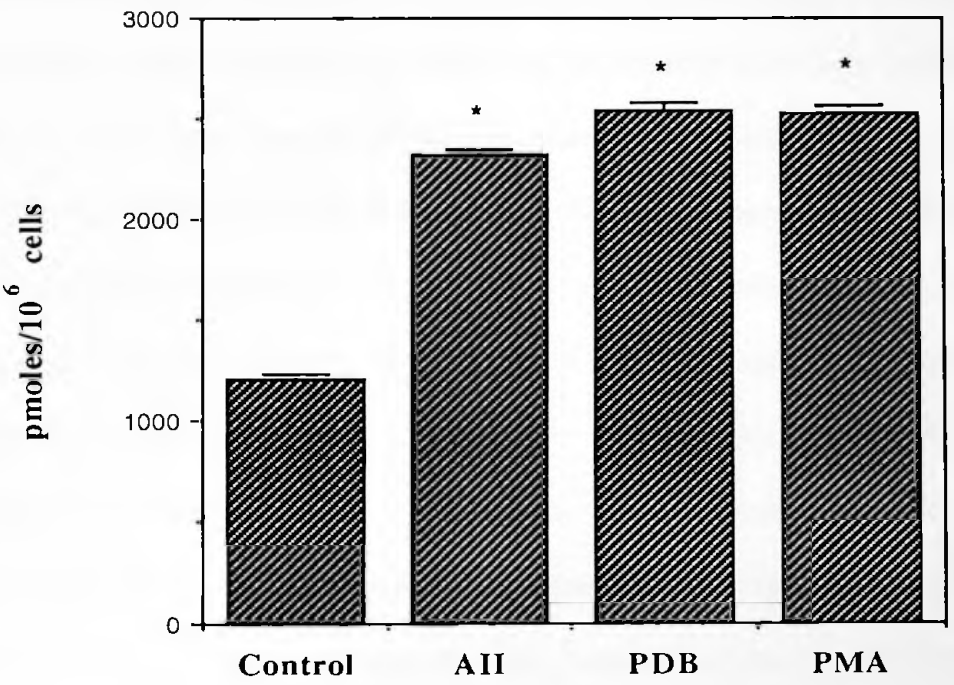

Glut 1 protein level
(arbitrary units)
500
400
300
200
100
0
- AII
+ AII

Figure 7 The effect of PKC stimulation with phorbol-12,13-dibutyrate (PDB) or phorbol-12,14- myristate acetate (PMA) on glucose transport. Cells were incubated with 100 nM AII, 100 nM PDB, 50 nM PMA, or diluent for six hours. The PDB was dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 10 mM (5mg/ 1 ml). The stock solution was diluted 1:200 and then 1:9 with 0.9% NaCl and 50 μl aliquots were added to the incubation medium of cells treated with PDB to give a final concentration of 100 nM. The cells treated with PDB alone also received 25 μl of 0.9% NaCl. The PMA was dissolved in DMSO at a concentration of 10 mM (5mg/ 0.81 ml). This stock solution was diluted 1:200 and then 1:19 with 0.9% NaCl and 50 μl aliquots were added to the incubation medium of cells treated with PMA to give a final concentration of 50 nM. The cells treated with PMA alone also received 25 μl of 0.9% NaCl. The stock AII solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl was added to the incubation medium of cells receiving AII at a final concentration of 100 nM. The cells treated with AII alone also received 50 μl of DMSO diluted 1:200 and then 1:19 with 0.9% NaCl. Control cells received 25 μl 0.9% NaCl plus 50 μl of DMSO diluted 1:19 with 0.9% NaCl. The final concentration of DMSO in the incubation buffer was less than 0.05%. Glucose transport is expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six observations.* $p < 0.05$ vs. control.

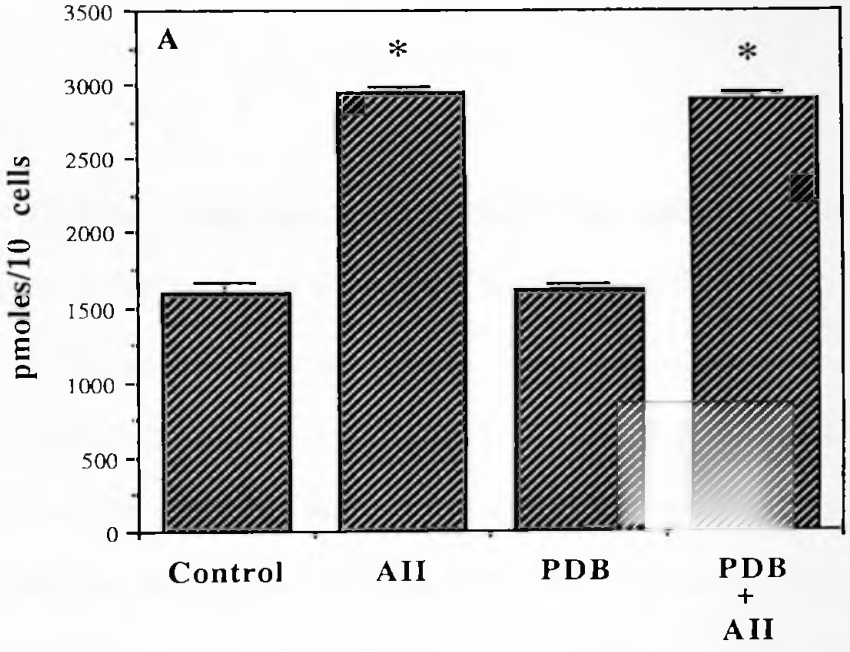

3000
2000
1000
0
pmoles/10^6 cells
*
*
*
Control
AII
PDB
PMA

(Figure 8A) in half of the experiments; while in the other half, AII-stimulated glucose transport was significantly reduced by 31.9% in the presence of PDB (Figure 8B).

To further examine the role of PKC in AII-stimulated glucose transport, three inhibitors of PKC were employed: staurosporine, calphostin C, and bisindoylmaleimide. VSM cells were exposed to 50 nM staurosporine for one hour prior to the addition of AII. AII was capable of fully stimulating glucose transport in the presence of this nonspecific PKC inhibitor (Figure 9). Calphostin C is considered to be a more specific inhibitor of PKC. VSM cells were exposed to 200 nM calphostin C for one hour prior to the addition of AII. In one experiment AII was able to fully stimulate glucose transport in the presence of calphostin C, while in another experiment AII-stimulated glucose transport was reduced (Table 3). Bisindoylmaleimide is also considered a specific inhibitor of PKC. VSM cells were exposed to 100 nM BIM for 30 minutes prior to the addition of AII (Figure 10). AII was capable of fully stimulating glucose transport in the presence of BIM. In summary, attempts to downregulate or inhibit PKC activity produced results that were somewhat inconsistent; however, the preponderance of evidence supports the conclusion that AII is capable of stimulating glucose transport through a mechanism that is independent of PKC.

To determine whether extracellular calcium plays a role in mediating the effect of AII on glucose transport, VSM cells were incubated in a calcium-free medium or in a medium containing 1.8 mM $CaCl_2$. Under conditions where basal transport was unaffected by the medium calcium content, the rate of AII-stimulated glucose transport was 2.5 times greater in the medium containing 1.8 mM $CaCl_2$ than in a medium containing no calcium (Figure 11). To determine if membrane integrity was compromised in the cells exposed to zero

Figure 8 The effect of downregulating PKC with phorbol dibutyrate (PDB) on AII-stimulated glucose transport. VSM cells were exposed to 1 μM PDB or diluent for 48 hours prior to the addition of AII or diluent for an additional six hours. Prior to adding AII or diluent, the medium was removed and replaced with fresh medium containing PDB or diluent. The PDB was dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 10 mM (5 mg/ 1 ml). The PDB stock solution was diluted 1:200 with 0.9% NaCl and 50 μl aliquots were added to the incubation medium of cells to give a final concentration of 1 μM. The cells treated with PDB alone also received 25 μl of 0.9% NaCl. The AII stock solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl was added to the incubation medium to give a final concentration of 100 nM. The cells treated with AII alone also received 50 μl of DMSO diluted 1:200 in 0.9% NaCl. Control cells received 25 μl of 0.9% NaCl plus 50 μl of DMSO. The final concentration of DMSO in the incubation buffer was less than 0.5%. Data are expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six observations. Panel A shows no effect on AII-stimulated glucose transport with PDB. Panel B shows that PDB reduced AII stimulation of glucose transport. * $p<0.05$ vs. control; + $p< 0.05$ vs. AII.


A
3500
3000
2500
2000
1500
1000
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0
pmoles/10 6 cells
*
*
Control
AII
PDB
PDB
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AII

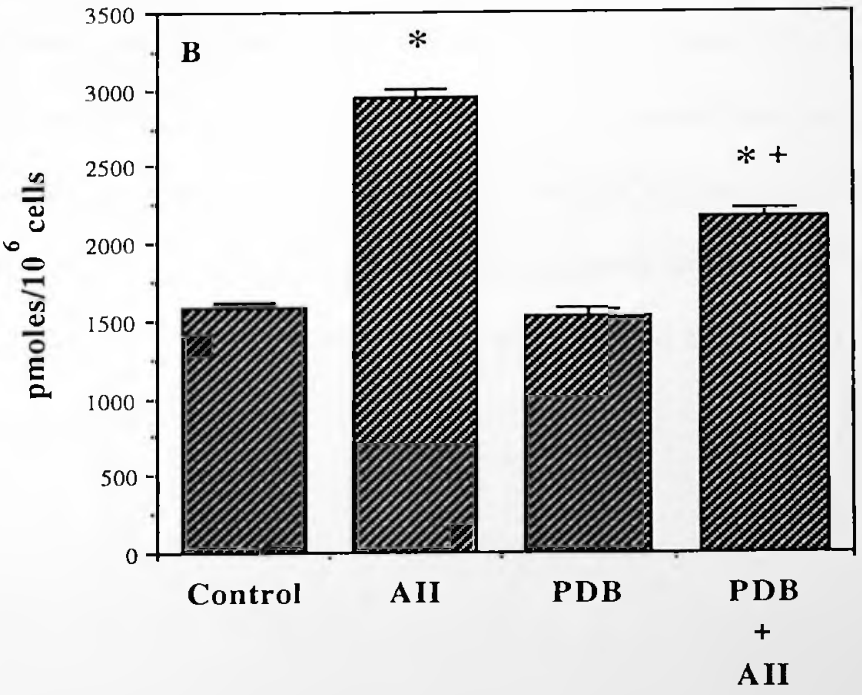
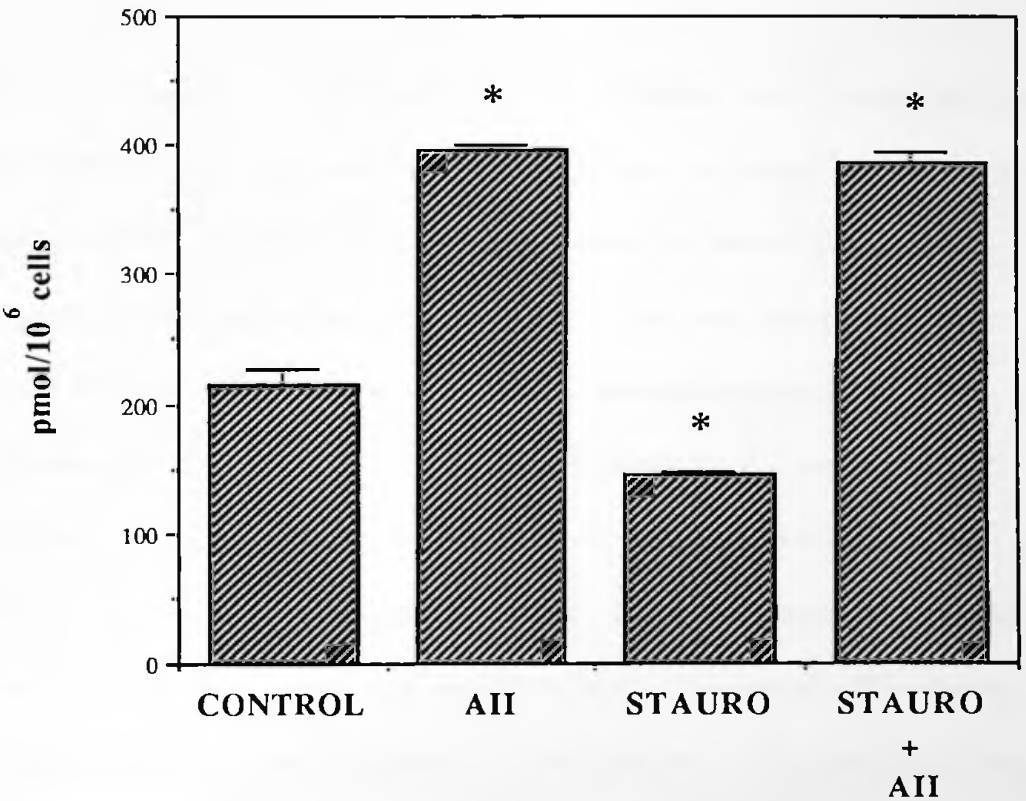

B
3500
3000
2500
2000
1500
1000
500
0
pmoles/10 6 cells
*
* +
Control
AII
PDB
PDB
+
AII

Figure 9 The effect of staurosporine (STAURO), a PKC inhibitor, on AII-stimulated glucose transport. VSM cells were exposed to 50 nM STAURO, 100 nM AII, 50 nM STAURO + 100 nM AII, or diluent for six hours. STAURO was dissolved in DMSO to prepare a stock solution of 1 mM (0.1 mg/ 0.214 ml). The stock solution was diluted 1:400 with 0.9% NaCl and 50 μl aliquots were added to the incubation medium to give a final concentration of 50 nM one hour prior to the addition of AII or vehicle. The cells treated with STAURO alone also received 25 μl of 0.9% NaCl. The stock solution of AII (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 100 nM. The cells treated with AII alone also received 50 μl of DMSO diluted 1:400 with 0.9% NaCl. The control cells received 25 μl of 0.9% NaCl and 50 μl of DMSO diluted 1:400 with 0.9% NaCl. The final concentration of DMSO in the incubation buffer was less than 0.05%. Results are expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six observations. * $p < 0.05$ vs. control


500
400
300
200
100
0
pmol/10 cells
6
*
*
*
CONTROL
AII
STAURO
STAURO
+
AII

	AII stimulation	
	Experiment 1	Experiment 2
- Calphostin C	78%	122%
+ Calphostin C	62%	22%*

Table 3 The effect of calphostin C, a PKC inhibitor, on AII-stimulated glucose transport. VSM cells were exposed to 200 nM calphostin C or diluent for one hour prior to the addition of 100 nM AII or diluent for an additional six hours. The calphostin C was dissolved in DMSO to make a 1 mM stock solution. The stock solution was diluted 1:50 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 200 nM. The cells incubated with calphostin C alone also received 25 μl of 0.9% NaCl. The AII stock solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium. The cells treated with AII alone also received 25 μl of DMSO diluted 1:50 with 0.9% NaCl. The control cells received 25 μl 0.9% NaCl and 25 μl of DMSO diluted 1:50 with 0.9% NaCl. In experiment 2, the cells were exposed to direct fluorescent lighting during the one hour preincubation with calphostin C or diluent; while in experiment 1, the cells were not. Results are expressed as percent stimulation above control. * $p < 0.05$ vs. percent stimulation of AII in the absence of calphostin C.

Figure 10 The effect of bisindoylmaleimide (BIM), a PKC inhibitor on AII stimulated glucose transport. Cells were exposed to 100 nM BIM or vehicle for 30 minutes prior to the addition of 100 nM AII or vehicle for an additional six hours. The BIM was dissolved in DMSO to prepare a stock solution of 5 mM (2 mg/ ml). The stock solution of BIM was diluted 1:49 with 0.9% NaCl, then further diluted 1:9 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 100 nM. The cells treated with BIM alone also received 25 μl of 0.9% NaCl. The AII stock solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 100 nM. The cells treated with AII alone also received 25 μl of DMSO diluted 1:49 then 1:9 with 0.9% NaCl. The control cells received 25 μl 0.9% NaCl and 25 μl of DMSO diluted 1:49 then 1:9 with 0.9% NaCl. The final concentration of DMSO in the incubation buffer was less than 0.05%. Results are expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six observations. * $p < 0.05$ vs. control.

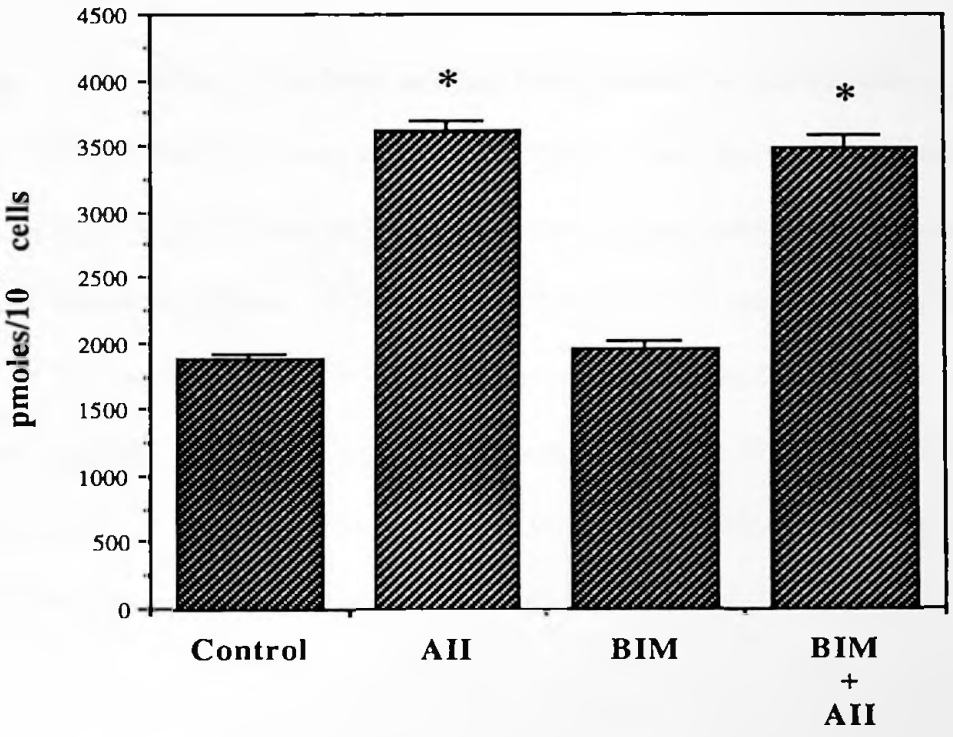
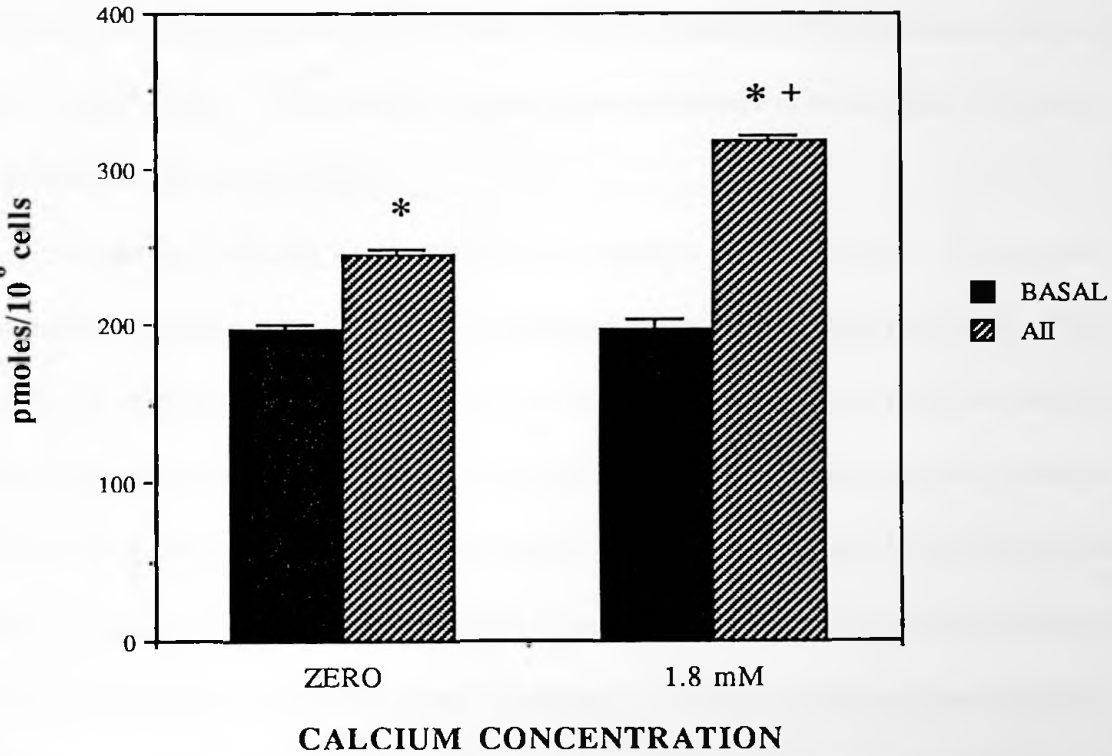

4500
4000
3500
3000
2500
2000
1500
1000
500
0
pmoles/10 6 cells
*
*
Control
AII
BIM
BIM
+
AII

Figure 11 The effect of medium calcium concentration on AII-stimulated glucose transport. Cultured VSM cells were incubated in DMEM with either zero or 1.8 mM $CaCl_2$ for eight hours. Six hours prior to the end of the incubation period, AII or an equivalent volume of diluent were added to the incubation medium. The AII stock solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium. Results are expressed as pmoles of glucose transported/ 10^6 cells. Bars represent the mean and brackets the SEM of six observations. * $p < 0.05$ compared with corresponding control; + $p < 0.05$ compared with AII-stimulation in a Ca^{2+}-free medium.


400
300
200
100
0
pmoles/10^6 cells
* +
*
BASAL
AII
ZERO
1.8 mM
CALCIUM CONCENTRATION

calcium, cell viability and nonspecific glucose transport were assessed. Cell viability was determined using trypan blue exclusion. Zero calcium did not alter percent viability of the cells (Table 4). Nonspecific transport was examined by incubating cells in zero or 1.8 mM $CaCl_2$-containing medium with an excess of cytochalasin B to block specific glucose transport. In studies where cytochalasin B was present in the incubation medium, there was no change in the percent of glucose transport due to nonspecific uptake in cells incubated in a medium containing zero or 1.8 mM $CaCl_2$ (Table 4) suggesting that membrane integrity was not compromised. These results emphasize the importance of extracellular calcium in AII-stimulated glucose transport.

Intracellular calcium binds calmodulin, a calcium binding protein. The calcium-calmodulin complex, in turn, is capable of mediating many cellular processes (Head, 1992; O'Neil and DeGrado, 1990). To determine whether or not calmodulin is involved in AII-stimulated glucose transport, VSM cells were incubated with AII in the presence or absence of 5 μM calmidazolium, a calmodulin inhibitor. Glucose transport in response to AII was reduced 57% in the presence of calmidazolium (Figure 12) suggesting that calmodulin plays a role in mediating AII-stimulated glucose transport. This data is further supported by the results of an experiment using a different calmodulin inhibitor, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, 50 μM). W-7 reduced AII-stimulated transport by 46% (Figure 13).

Since medium glucose concentration has been reported to affect hormone-induced increases in glucose transport (Cooper et al., 1993), the effect of medium glucose concentration on AII-stimulated glucose transport was assessed (Figure 14). Clearly, basal

Medium calcium concentration (mM)	Cell viability	Nonspecific transport
0	53.5 ± 7.1 %	4.6 ± 0.96 %
1.8	47.7 ± 5.1 %	4.2 ± 0.21 %

Table 4 The effect of medium calcium concentration on cell membrane integrity. Cultured VSM cells were incubated in DMEM with either zero $CaCl_2$ or 1.8 mM $CaCl_2$ for eight hours. Cell viability was assessed using trypan blue exclusion and is expressed as the percent of cells that are viable and was calculated as follows:

(number of cells excluding trypan blue ÷ total number of cells) × 100.

Nonspecific glucose transport was measured using cytochalasin B to inhibit the glucose transporter (refer to Methods: [^{3}H]-2-deoxyglucose transport assay). The glucose transport data is expressed as the percent of glucose uptake due to nonspecific transport and was calculated as follows:

[(pmoles of glucose transported in the presence of cytochalasin B / 10^6 cells) ÷

(pmoles of glucose transported in the absence of cytochalasin B / 10^6 cells)] × 100.

Figure 12 The effect of calmidazolium, a calmodulin inhibitor, on AII-stimulated glucose transport. VSM cells were exposed to 5 μM calmidazolium (CDZ) or an equivalent volume of vehicle for one hour prior to the addition of 100 nM AII or vehicle for an additional six hours. The CDZ was diluted with DMSO to prepare a stock solution of 1 mM (5 mg/ 7.25 ml). The CDZ stock solution was diluted 1:1 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 5 μM. The cells treated with CDZ alone also received 25 μl 0.9% NaCl. The AII stock solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 100 nM. The cells treated with AII alone also received 25 μl of 50% (v/v) DMSO in 0.9% NaCl. The control cells received 25 μl 0.9% NaCl and 25 μl of DMSO diluted 1:1 with 0.9% NaCl. The results are expressed as pmoles of glucose transported /10^6 cells. Bars represent the mean and brackets the SEM of six observations. * $p < 0.05$ vs control; + $p < 0.05$ vs AII.

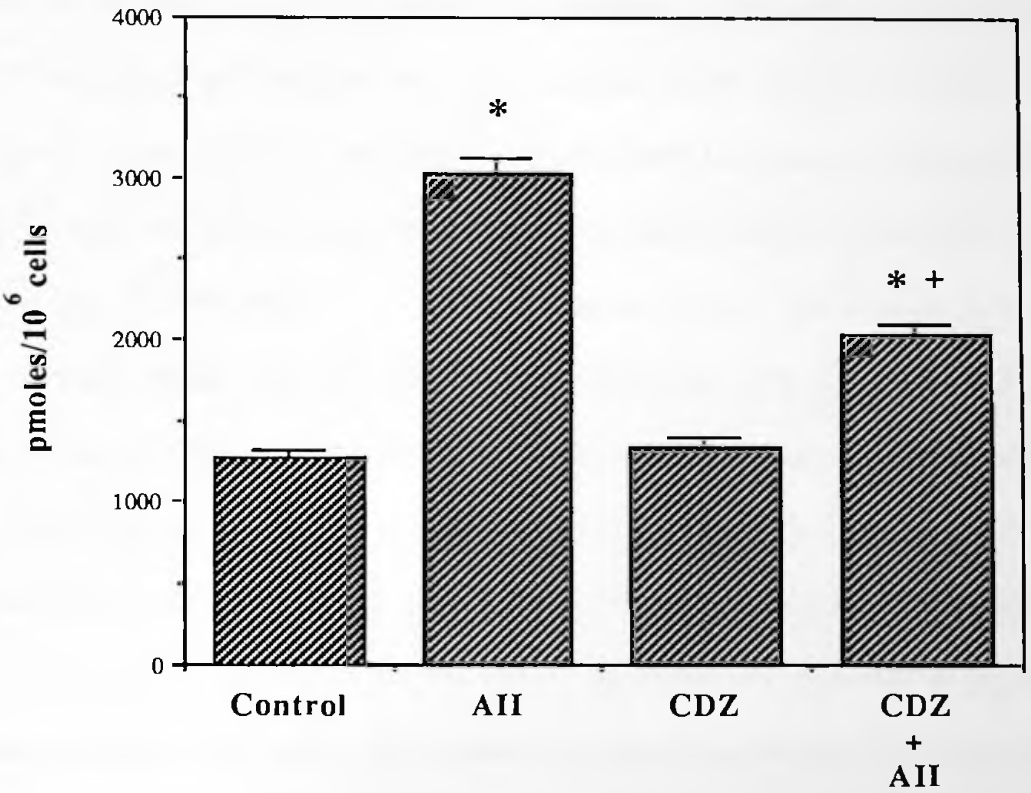

4000
3000
2000
1000
0
pmoles/10 6 cells
*
* +
Control
AII
CDZ
CDZ
+
AII

Figure 13 The effect of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin inhibitor, on AII-stimulated glucose transport. VSM cells were exposed to 50 μM W-7 or vehicle for one hour prior to the addition of 100 nM AII or vehicle for an additional six hours. The W-7 was diluted with 10% DMSO to prepare a stock solution of 5 mM (3.1 mg/ 1.63 ml). The stock solution was then further diluted 1:1 with 11% DMSO and 50 μl aliquots were added to the incubation medium to give a final concentration of 50 μM. The cells treated with W-7 alone also received 25 μl 0.9% NaCl. The AII stock solution (1 mg/ ml) was diluted 1:100 with 0.9% NaCl and 25 μl aliquots were added to the incubation medium to give a final concentration of 100 nM. The cells treated with AII alone also received 50 μl of 10% DMSO in 0.9% NaCl. The control cells received 25 μl 0.9% NaCl and 50 μl of 10% DMSO in 0.9% NaCl. The results are expressed as pmoles of glucose transported /10^6 cells. Bars represent the mean and brackets the SEM of six observations. * $p< 0.05$ vs control; + $p< 0.05$ vs AII.

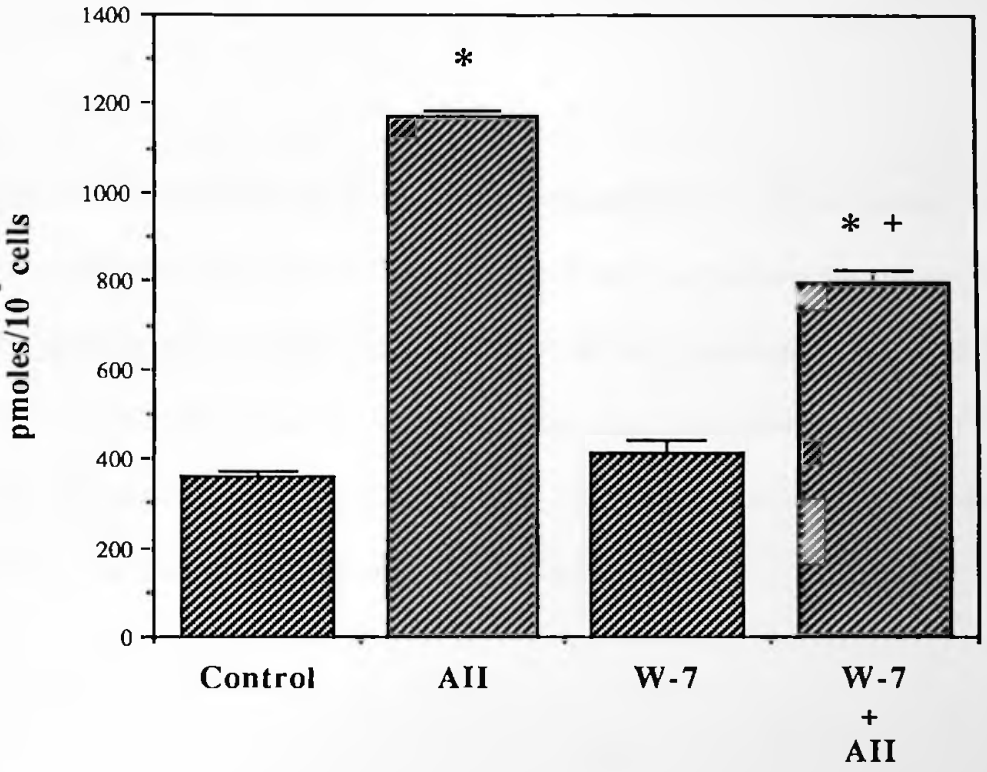
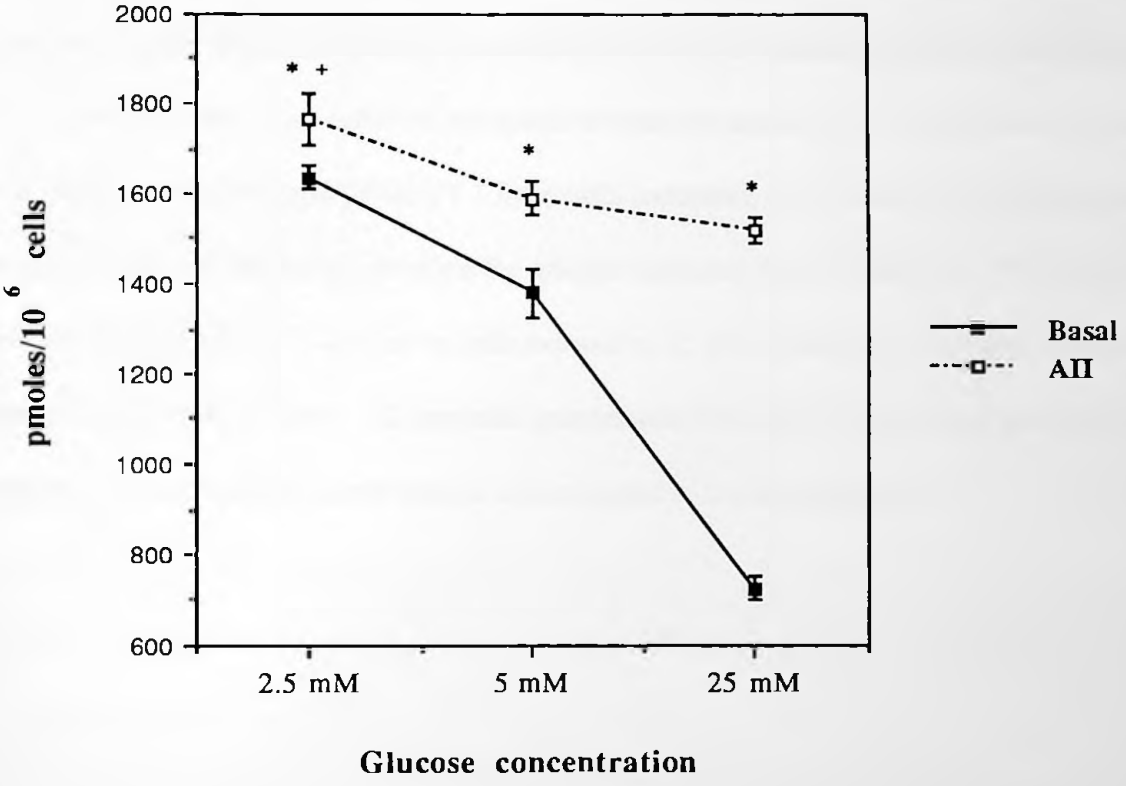

1400
1200
1000
800
600
400
200
0
pmoles/10 6 cells
*
* +
Control
AII
W-7
W-7
+
AII

Figure 14 The effect of medium glucose concentration on AII-stimulated glucose transport. Cultured VSM cells were incubated for 18 hours in medium containing either 2.5 or 25 mM glucose prior to the addition of 100 nM AII or an equivalent volume of diluent (Basal) for an additional six hours. Transport is expressed as pmoles of glucose/ 10^6 cells. Each point is the mean $\pm$ the SEM of six replicates. *$p < 0.05$ vs. corresponding basal values; + $p < 0.05$ vs. AII stimulation in 25 mM glucose medium.

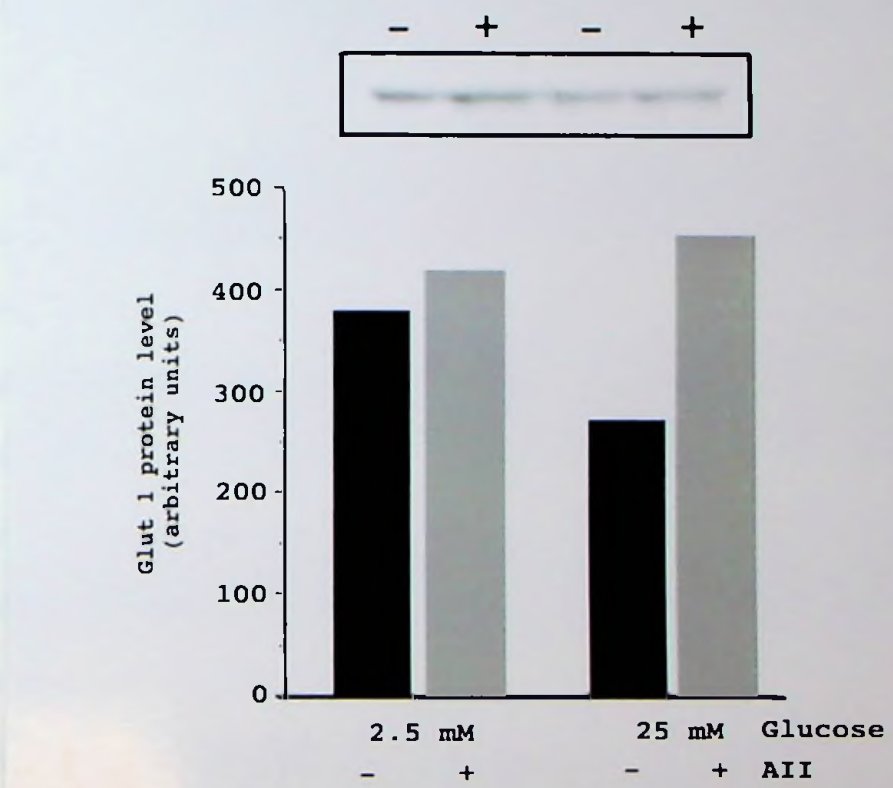

2000
1800
1600
1400
1200
1000
800
600
pmoles/10 6 cells
2.5 mM
5 mM
25 mM
Glucose concentration
Basal
AII

glucose transport was increased in cells exposed to a medium containing 2.5 mM glucose for 24 hours compared to cells incubated in a medium containing 25 mM glucose for the same period of time. During the incubation period, the concentration of glucose in the 2.5 mM and the 25 mM glucose media decreased by 92% and 20%, respectively (Table 5). Cell number was not affected by medium glucose concentration (Table 5). While there was not an appreciable change in the maximum level of glucose uptake achieved with AII at different glucose concentrations, the percent stimulation above basal transport with AII was greater in cells incubated in 25 mM glucose compared to those incubated in 2.5 mM glucose (Figure 14). Western blot analysis of GLUT 1 from cells incubated in 2.5 mM or 25 mM glucose medium with or without AII parallels the glucose transport data (Figure 15). The level of immunoreactive GLUT 1 was less in cells exposed to 25 mM glucose as compared to those exposed to 2.5 mM glucose. AII increased immunoreactive GLUT 1 in cells exposed to 25 mM glucose to a greater extent than in cells exposed to 2.5 mM glucose.

Predicted glucose concentration (mM)	Incubation time (hours)	Actual glucose concentration (mM)	Cell number
2.5 mM	0	2.6 ± 0.1	-
	24	0.2 ± 0.2	$1.41 \times 10^6 \pm 0.09$
25 mM	0	22.2 ± 1.7	-
	24	17.8 ± 1.7	$1.23 \times 10^6 \pm 0.18$

Table 5 The effect of a 24 hour exposure to 2.5 mM and 25 mM glucose medium on medium glucose concentration and cell number. VSM cells were incubated in medium containing 2.5 mM or 25 mM glucose. The concentration of glucose in the stock medium was measured for a time zero value. The concentration of glucose in the medium at the end of the 24 hour incubation period was also measured. In addition, cell number was also measured after a 24 hour exposure to 2.5 mM and 25 mM medium glucose.

Figure 15 The effect of AII on GLUT 1 protein levels from cells incubated in 2.5 mM or 25 mM glucose medium. The cells were treated as in Figure 14 and crude membranes were isolated and subjected to western blot analysis. Relative levels of GLUT 1 in the experimental groups were assessed by densitometric analysis and expressed as arbitrary units.


- + - +
500
400
300
200
100
0
Glut 1 protein level
(arbitrary units)
2.5 mM
25 mM
Glucose
- + - +
AII

DISCUSSION

The results of the present investigation support the hypothesis that AII can help VSM cells meet the increased metabolic demands of the growth processes that it initiates by concurrently stimulating glucose transport. It is clear that AII stimulates glucose transport in these cultured cells in both a time- and dose-dependent manner (Figures 1 and 2).

It can also be concluded that AII-stimulated glucose transport is initiated by the binding of AII to specific AII receptors because AII-stimulated glucose transport was reduced by 90% in the presence of saralasin (Figure 3), a competitive inhibitor of AII binding. In addition to being a competitive inhibitor of AII binding, saralasin, [(1-sarcosine, 8-threonine) AII], also exhibits partial agonist activity. It was selected for this study primarily because its agonist activity is low relative to other available receptor antagonists. This was demonstrated by Munoz-Ramirez (1976) and colleagues who examined a variety of one- and eight- position amino acid substituted analogs that are routinely used as AII receptor antagonists. They showed that saralasin was a potent antagonist for the AII receptor in the presence of AII even though it exhibited partial agonist activity when used by itself. Of the receptor antagonists that they examined, saralasin had the lowest level of agonist activity. The very modest stimulation of glucose uptake by saralasin alone in the present study (Figure 3) is consistent with this observation.

The involvement of protein synthesis in AII stimulation of glucose transport was also examined. The concentration of cycloheximide, a potent inhibitor of protein synthesis, used to affect AII-stimulated glucose transport was in the concentration range (0.5 μg/ ml) used

by others to inhibit glucose transport in different tissues as opposed to larger concentrations (50 μg/ ml) which affect transporter synthesis as well as transporter degradation (Yamada et al., 1983). Cycloheximide reduced AII-stimulated glucose transport by 70% (Figure 4), suggesting that glucose transport in response to a six hour exposure to AII involves new protein synthesis. In some, but not all experiments with cycloheximide, a small, yet significant increase in basal transport was also observed. This is consistent with the observations of others. Significant stimulation of glucose uptake has been observed in several cell lines in response to large concentrations of cycloheximide (Clancy et al., 1991; Jones and Cushman, 1989). Because cycloheximide-induced cell death could explain the reduction in AII-stimulated glucose uptake in response to cycloheximide, cell viability was assessed under the experimental conditions used to examine the effect of cycloheximide on glucose transport. Cycloheximide treatment did not reduce cell viability as assessed by trypan blue exclusion (84.7% viable for control and 92.3% viable for cycloheximide). In summary, the results of these studies suggest that AII increases glucose transport at least in part through a mechanism that requires protein synthesis.

Further support for the role of protein synthesis in AII-stimulated glucose uptake is provided by the observation that AII increased the level of a specific protein, the GLUT 1 transporter, in VSM cells (Figure 6). Many investigators have reported an increase in glucose transport that can be attributed to mechanisms involving an increase in GLUT 1 protein levels (Haspel et al., 1986; Kaiser et al., 1993). One must also consider the possibility that an increase in immunoreactive GLUT 1 transporter could be due to a reduction in GLUT 1 degradation as opposed to synthesis. The western blot analysis study

did not delineate the precise mechanism responsible for the increase in GLUT 1 protein levels.

AII elicits a cellular response by binding to specific plasma membrane receptors. The binding of AII to the AT_1 receptor in vascular smooth muscle activates phospholipase C via a G-protein mediated mechanism (Duff et al., 1995). Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate and diacylglycerol (Alexander et a ., 1985; Griendling et al., 1986). This, in turn, leads to a rise in intracellular Ca^{2+} (Brock et al., 1985) and the activation of PKC (Griendling et al., 1989; Williams and Schrier, 1992). The present investigation examined the role of PKC, extracellular calcium, and calmodulin in mediating AII-stimulated glucose transport in cultured VSM cells.

Three separate experimental designs were used to assess the role of PKC in AII-stimulated glucose transport. In one experiment PKC was activated by the addition of phorbol esters to the incubation medium. In a second, PKC was downregulated by prolonged exposure to phorbol esters prior to AII stimulation. In a third, PKC activity was inhibited using specific inhibitors.

Since phorbol esters are known to promote PKC activity by binding to the diacylglycerol (DAG) binding site of the PKC molecule (Newton, 1995), these agents were used to stimulate PKC activity in VSM cells in the present study. A six hour exposure of VSM cells to the phorbol esters PMA and PDB resulted in an increase in glucose uptake, suggesting that glucose transport can be stimulated by the activation of PKC (Figure 7). Using these same experimental conditions, other researchers have directly shown that PKC activity is increased. Williams and Schrier (1992) exposed cultured VSM cells to 1 μM and

100 μM PMA for six hours and observed a significant increase in PKC activity. Additionally, Longo (1992) and colleagues demonstrated that 100 nM PDB increased glucose transport in human cultured skin fibroblasts after a six hour exposure. Even in light of these observations, however, a conclusion from the present study that PKC is involved in mediating AII-stimulated glucose transport must be regarded with considerable caution for several reasons. First, even though the phorbol esters stimulated glucose uptake, there was no simultaneous measurement of phorbol ester stimulation of PKC activity. Moreover, the use of phorbol esters to activate PKC does not reliably indicate PKC involvement in an AII-mediated response in that it has been observed that phorbol esters are more potent activators of PKC than the physiological activator, diacylglycerol (Newton, 1995). Further, phorbol esters can activate PKC independently of calcium (Rasmussen et al., 1995). The conventional PKC isoforms (α, β_I, β_{II}, γ) require both DAG and calcium for activation whereas the novel (δ, ϵ, θ, μ, η) PKC isoforms require DAG but not calcium for activation. A third category of PKC, the atypical isoforms (ζ, λ, ι), require neither DAG nor calcium for activation. Therefore, treatment with phorbol esters (a pharmacological activator of PKC) may demonstrate a positive cellular response via activation of PKC isoforms that are not normally activated by the agonist in question.

To further examine the potential role of PKC in mediating glucose uptake in response to AII, VSM cells were treated chronically with PDB to reduce cellular PKC levels. This treatment is thought to prevent PKC activation by an agonist due to the relative lack of PKC. The involvement of PKC in the AII response was not clearly delineated in this study, in part, because the numerous PKC downregulation experiments produced variable results. In some

experiments, a 48 hour exposure of VSM cells to PDB partially suppressed AII-stimulated glucose uptake, whereas in other experiments PDB did not have an effect on this parameter (Figure 8). The dichotomy of results can be interpreted in light of two hypotheses: 1) the treatment employed in this study may not fully downregulate all PKC isoforms and 2) there may be differences in the responsiveness of cell populations to the treatment.

Addressing the first hypothesis, McCumbee and colleagues (1996) demonstrated that a 48 hour exposure to 50 nM PDB significantly reduced, but did not abolish, the level of immunoreactive PKC α in cultured VSM cells. It is possible that the PKC α remaining after the treatment was sufficient to fully stimulate glucose transport in response to AII. Also, VSM cells express several PKC isoforms including α, β, ϵ, and ζ (Williams, 1995). The susceptibility to downregulation is different for some of the individual isoforms. For instance, PKC ζ is not downregulated by chronic phorbol ester exposure (Huwiler et al., 1992). It is possible that when an isoform that is normally activated by AII is not present in a quantity sufficient to accomplish the effect or is inhibited, another PKC isoform might be activated to accomplish the task.

The above results are also consistent with the hypothesis that cultures derived from vascular smooth muscle yield subpopulations of cells that vary in their responses to a specific experimental manipulation (Kimes and Brandt, 1976; Absher et al., 1989). The experiments presented throughout this project were performed a number of times using cells derived from different populations. In three separate cell populations, exposure of cells to PDB to downregulate PKC did not affect AII-stimulated glucose transport. In three additional and separate cell populations, PDB downregulation did reduce glucose uptake in response to AII.

These results may reflect the resistance of some populations of VSM cells to PKC downregulation with phorbol esters , as well as reflecting the possibility that in some cell populations AII-stimulated glucose transport can be achieved via a PKC-independent pathway. The data also suggest, at least in some VSM cell populations, that full expression of AII-stimulated transport requires PKC activation. While PKC downregulation appears to be a nice approach to assessing the involvement of PKC in intracellular events, the data must be interpreted with caution. As Rasmussen and colleagues (1995) have suggested, chronic phorbol ester treatment or downregulation of PKC likely results in pharmacologically altered cells that may respond nonphysiologically to specific stimuli. Given the nature of the treatment and the dichotomy of results obtained from the experiments, downregulation of PKC by PDB does not appear to be a very reliable technique in the context of this study.

PKC inhibitors were used in an attempt to provide a better assessment of the involvement of PKC activation in AII stimulation of glucose transport. Using the nonspecific PKC inhibitor, staurosporine, AII-stimulated glucose transport was not affected (Figure 9). It is possible that the 50 nM concentration used in the studies was not enough to fully inhibit PKC. However, Baldo (1995) and colleagues were able to significantly inhibit PKC-mediated triglyceride synthesis in human skin fibroblasts in response to PMA using a six hour exposure to 50 nM staurosporine. While higher concentrations of staurosporine might have achieved a complete reduction in AII stimulation of glucose transport in the present experiment (Figure 9), basal transport would also be reduced since the concentration of staurosporine used in this study already reduced basal transport by approximately 30%.

Such an alteration in baseline transport would further compromise a meaningful interpretation of the data.

Calphostin C was used as an additional PKC inhibitor to complement the preceding study. The results, however, were not conclusive (Table 3). In one experiment, calphostin C did not have an effect on AII-stimulated glucose uptake when measures were taken to prevent its exposure to light. In contrast, calphostin C significantly reduced AII-stimulated transport when the inhibitor was light-activated. VSM cells were exposed to calphostin C for one hour in direct fluorescent lighting prior to the addition of AII to induce light-activation. In that PKC activity was not directly measured in these experiments, it is not clear whether calphostin C requires light-activation in order to be effective. Some studies indicate that calphostin C needs to be light-activated before it can inhibit PKC (Ali et al., 1994). On the other hand, many studies, including the original paper describing the specificity of calphostin C for PKC (Kobayashi et al., 1989), do not indicate that light-activation is necessary for calphostin C to function effectively as a PKC inhibitor. In addition, studies have successfully used calphostin C to inhibit PKC-mediated responses in whole arteries (Henrion et al., 1992; Shimamoto et al., 1993) and cultured vascular smooth muscle cells (Rao et al., 1994) without specifying light-activation in the protocol.

Bisindoylmaleimide (BIM), another specific PKC inhibitor, was also employed in this investigation. As shown in Figure 10, 100 nM BIM did not alter AII-stimulated glucose transport. To summarize the PKC inhibitor studies, staurosporine and BIM did not significantly alter AII-stimulated transport. The evidence derived from the calphostin C studies was not conclusive. Even so, the results gained using two inhibitors, staurosporine

and BIM, support the conclusion that AII can stimulate glucose transport independently of PKC.

Taken together, the majority of the evidence from the PKC downregulation and the PKC inhibitor studies suggests that AII is capable of stimulating glucose transport through a mechanism independent of PKC. Of the two mechanisms employed, PKC inhibition is probably the most reliable indicator of PKC involvement in a given response. PKC downregulation requires a prolonged exposure (forty-eight hours) to PDB and likely results in a pharmacologically and physiologically altered cell. PKC inhibitor studies require a shorter exposure (six hours) of the cells to the inhibitor. The staurosporine data demonstrated that AII-stimulated glucose transport was not altered. However, staurosporine does not discriminate among protein kinases and binds the catalytic domain of PKC which is common to protein kinases (Tamaoki et al., 1986). Calphostin C is a more specific inhibitor of PKC, binding to the regulatory domain of the enzyme (Kobayashi et al., 1989). Bisindolylmaleimide is a PKC inhibitor that is structurally related to staurosporine but is very selective for PKC (Toullec et al., 1991). The use of calphostin C did not appear to be productive as it gave conflicting results. The use of BIM as a more specific inhibitor of PKC supported the data obtained using staurosporine.

To further investigate the signaling mechanisms involved in AII-stimulated glucose transport, the role of extracellular calcium was assessed by incubating VSM cells in medium with zero $CaCl_2$ or 1.8 mM $CaCl_2$. While there is evidence suggesting that AII causes a rapid rise in intracellular calcium through the release of stored intracellular calcium (Alexander et al., 1985), evidence also supports the role of extracellular calcium in maintaining a plateau

phase of elevated intracellular calcium in response to AII (Brock et al., 1985). The results presented support the hypothesis that extracellular calcium is important for AII-stimulated glucose transport (Figure 11) in that there was a significant attenuation of glucose uptake in response to AII in the absence of extracellular calcium. Occasionally, basal transport was significantly increased in cells incubated in medium with zero calcium. AII did not further stimulate glucose transport in these cells. It is possible that cell permeability was compromised, due to the relative lack of calcium, leading to a leaky cell membrane. In such a case, the increase in basal transport could be due to glucose entering the cells through nonspecific transport; however, this did not appear to be the case. There was no difference in the percent of glucose transport due to nonspecific uptake in cells incubated in medium containing zero $CaCl_2$ or 1.8 mM $CaCl_2$ (Table 4). These results suggest that the occasional increases in basal transport seen in the absence of extracellular calcium were not simply due to a leakiness of the membrane. Further, zero calcium did not alter percent viability of the cells as evidenced by the capacity of cells in zero $CaCl_2$ to exclude trypan blue as effectively as cells in 1.8 mM $CaCl_2$ (Table 4).

One mechanisms through which intracellular calcium can alter cellular function is via the activation of calmodulin (Head, 1992; O'Neil and DeGrado, 1990). Evidence supports a role for Ca^{2+}/calmodulin in AII-stimulated processes in VSM cells (Kubalak and Webb, 1993; Nantel et. al., 1991). Calmodulin-dependent processes can be blocked using calmodulin inhibitors, such as W-7 (Hidaka et al., 1981) and calmidazolium (Gietzen et al., 1981). The concentrations of inhibitors used in this study were consistent with concentrations used by other investigators (Gietzen et. al., 1981; Kindmark et al., 1995;

Hidaka et al., 1981). Both inhibitors (Figures 12 and 13) significantly reduced AII-stimulated glucose uptake in the present study, suggesting that calmodulin is involved in AII stimulation of glucose transport.

Medium glucose has been shown to affect basal glucose transport in many cell lines, including cultured chicken embryo fibroblasts (Kletzien and Perdue, 1975), cultured diploid human skin fibroblasts (Germinario et al., 1982), and cultured L6 muscle cells (Koivisto et al., 1991). In addition, Cooper and associates (1993) have observed changes in hormone-induced glucose transport with changes in medium glucose concentration using A-10 vascular smooth muscle cells. The current investigation demonstrates that basal glucose transport increases when medium glucose concentrations decrease (Figure 14). Greco-Perotto et al. (1992) and Yamada et al. (1983) have reported similar observations using L_8 myocytes and chicken embryo fibroblasts, respectively. In addition, the percent stimulation of glucose transport by AII is considerably less in a low glucose medium than in a high glucose medium. The data suggest that the maximum rate of glucose transport in response to AII does not change appreciably with various glucose concentrations and that the relative effectiveness of AII at different concentrations of glucose is a function of glucose concentration on the transport process. This conclusion is supported by the observation that the GLUT 1 transporter level mimics that of the glucose transport activity in response to AII and glucose levels (Figure 15).

The western blot experiments presented within this study used the anti-GLUT 1 antibody (3406 GLUT 1 Ab) to detect the GLUT 1 transporter. This antibody was prepared in the laboratory and appears to be specific for the GLUT 1 transporter. Enzyme-linked

immunosorbent analysis demonstrated that the 3406 GLUT 1 Ab reacted strongly with the GLUT 1 peptide and not the GLUT 2, GLUT 4, and GLUT 7 peptides (Table 2). Additional support for the recognition of GLUT 1 by the 3406 GLUT 1 Ab stems from studies using red blood cell membranes as positive controls during western blot analysis of VSM cell membranes (Figure 5). Red blood cell membranes are enriched in GLUT 1 transporters (Carruthers, 1990). The 3406 GLUT 1 Ab recognized protein bands from both sources of membranes that correspond with published molecular weights for GLUT 1 (Gorga, et al., 1979; Kumagai, et al., 1994). The recognition of more than one protein band by GLUT 1 Ab can be explained. The GLUT 1 transporter has a sugar moiety bound to it which accounts for almost one-fourth of the molecular mass of GLUT 1 (Gorga, et al., 1979). Glycosylated GLUT 1 has been reported to run as a broad band of 45- to 60-kDa (Asano, et al., 1991). Other investigators have reported apparent molecular weights of 52-kDa and 55-kDa for glycosylated GLUT 1 and 38-kDa (Asano, et al., 1991), 42-kDa, and 46-kDa (Kumagai, et al., 1994; Gorga, et al., 1979) for deglycosylated GLUT 1. It is possible that the two protein bands detected by 3406 GLUT 1 Ab represent glycosylated and deglycosylated GLUT 1. Occasionally, 1:1,000 dilutions of 3406 GLUT 1 Ab detected a protein band with a molecular weight of approximately 30-kDa which could possibly represent a partially degraded GLUT 1.

In summary, this research project has provided evidence that AII stimulates glucose transport in cultured VSM cells in a time and concentration-dependent manner. AII stimulation of glucose transport was protein synthesis dependent and resulted in an increase in the membrane GLUT 1 transporter content. The evidence presented supports a role of

extracellular calcium and calmodulin in mediating intracellular events leading to increased glucose transport in response to AII. While the role of PKC is less clear, the evidence suggests that AII is capable of stimulating glucose transport through a mechanism independent of PKC. The medium glucose concentration affected both basal glucose transport and the magnitude of the response to AII. The rate of basal transport decreases with increasing media glucose content. The magnitude of the response to AII appeared to increase with increasing glucose content of the incubation medium, whereas the maximum level of glucose transport did not change appreciably. This can probably be explained in light of the observation that media glucose concentration affects the basal rate of transport. With increasing medium glucose concentration, the rate of basal transport decreases while the maximum level of glucose transported in response to AII remains unchanged. Therefore, the actual percent stimulation in response to AII increases with increasing medium glucose content. The decrease in glucose transport into the cell with increasing extracellular glucose content may be a protective mechanism to prevent the excessive glycosylation of cellular proteins in vascular smooth muscle during hyperglycemic states like diabetes mellitus. If this is indeed the case, then the response of vascular smooth muscle cells to AII in a hyperglycemic environment could lead to some potentially serious deleterious effects within the cell and contribute even further to the pathogenesis of hypertension. It is interesting to note that diabetic patients are more commonly hypertensive than nondiabetic patients. This relationship may reflect the interaction of hyperglycemia and the factors that lead to hypertension in the absence of hyperglycemia.

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APPENDIX A

TIME COURSE OF [^{3}H]-2-DEOXYGLUCOSE UPTAKE

TIME (minutes)	pmoles glucose transported/ 10^6 cells
10	231.12 $\pm$ 8.52
20	387.99 $\pm$ 15.56
30	499.81 $\pm$ 32.99
60	664.51 $\pm$ 42.20

APPENDIX B

NONSPECIFIC GLUCOSE TRANSPORT MEASUREMENTS

TREATMENT	% OF TOTAL*
EXPERIMENT 1	
0 nM AII	3.51 ± 0.10
0.10 nM AII	7.10 ± 0.45
1.0 nM AII	2.73 ± 0.12
10 nM AII	3.04 ± 0.29
100 nM AII	2.13 ± 1.07
1000 nM AII	3.84 ± 0.26
EXPERIMENT 2	
Control	3.01 ± 0.53
100 nM Saralasin	3.36 ± 0.42
100 nM AII	2.63 ± 0.30
100 nM Saralasin + 100 nM AII	3.63 ± 0.67
EXPERIMENT 3	
Control	4.15 ± 0.04
1 μM CHX	2.88 ± 0.05
100 nM AII	2.35 ± 0.16
1 μM CHX + 100 nM AII	2.94 ± 0.13

Continued....

APPENDIX B, continued

TREATMENT	% OF TOTAL*
EXPERIMENT 4	
Control	2.98 ± 0.50
100 nM PDB	2.60 ± 0.09
100 nM AII	2.26 ± 0.31
100 nM PDB + 100 nM AII	2.78 ± 0.45
EXPERIMENT 5	
Control	2.56 ± 0.09
50 nM Staurosporine	3.61 ± 0.26
100 nM AII	3.30 ± 0.08
50 nM Staurosporine + 100 nM AII	2.24 ± 0.12
EXPERIMENT 6	
2.5 mM glucose	3.56 ± 0.52
2.5 mM glucose + 100 nM AII	3.78 ± 0.84
25 mM glucose	3.10 ± 0.39
25 mM glucose + 100 nM AII	3.56 ± 0.15

* [nonspecific transport ÷ total transport] × 100

APPENDIX C

SINGLE LETTER ABBREVIATIONS OF THE AMINO ACIDS

AMINO ACID	SINGLE LETTER ABBREVIATION	AMINO ACID	SINGLE LETTER ABBREVIATION
alanine	A	Leucine	L
Arginine	R	Lysine	K
Asparagine	N	Methionine	M
Aspartate	D	Phenylalanine	F
Cysteine	C	Proline	P
Glutamate	E	Serine	S
Glutamine	Q	Threonine	T
Glycine	G	Tryptophan	W
Histidine	H	Tyrosine	Y
Isoleucine	I	Valine	V

From: Halkerston, 1988.

APPENDIX D

DMEM # 12430 FORMULATION

COMPONENT	mg/ L
$CaCl_2$ (anhydr.)	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.10
KCl	400.00
$MgSO_4$ (anhydr..)	97.67
NaCl	4750.00
$NaHCO_3$	3700.00
$Na_2HPO_4 \cdot H_2O$	125.00
D-Glucose	4500.00
HEPES	5958.00

APPENDIX E

SYNTHETIC PEPTIDE CROSS-REACTIVITY WITH A 1:1,000 DILUTION OF THE 3406 GLUT 1 Ab

	Optical density measurements*	
Synthetic peptide	**3406 GLUT 1 Ab**	**Commercial GLUT 1 Ab**
GLUT 1	+0.396	+1.057
GLUT 2	-0.234	-0.561
GLUT 4	-0.290	-0.268
GLUT 7	-0.445	-0.281

*corrected for nonspecific antibody binding