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RAPID AND REVERSIBLE VASCULAR EFFECTS OF PROGESTERONE AND OTHER STEROID HORMONES

BY

Meili Zhang

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfilment of the requirements for the degree of

Master of Science

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING, 1996

ISBN 0-612-10773-6

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Degree:	**Master of Science**
Year This Degree Granted:	**1996**

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Date: April 4, 1996

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Rapid and Reversible Vascular Effects of Progesterone and Other Steroid Hormones" submitted by Meili Zhang in partial fulfilment of the requirements for the degree of **Master of Science**.

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ABSTRACT

Progesterone induced rapid relaxation in KCl-contracted rat aortic rings and tail artery helical strips. The effects of progesterone on aortic rings are partially endothelium-dependent. Fura-2 was used to measure intracellular calcium ($[Ca^{2+}]_i$) of vascular smooth muscle cells (VSMC). Incubation of cultured VSMC for 15 min with progesterone resulted in an inhibition of $[Ca^{2+}]_i$ increase in the cells stimulated with KCl. The whole cell patch clamp technique was used to examine Ca^{2+} channel activities in the membrane of VSMC. Progesterone suppressed L-type Ca^{2+} channel currents at a holding potential of -40 mV. The progesterone effects were quickly reversible by washout in all three preparations suggesting that these effects on vascular tissues are non-genomic. The correlation of the effects on all these preparations, their time course, and reversibility suggested that the actions of progesterone are physiological. Effects of other steroids on vascular tissues were studied and compared to the progesterone effects.

ACKNOWLEDGMENTS

I would like first to thank my supervisor, Dr. Peter K.T. Pang for his excellent guidance, cordial encouragement and consistent understanding. His enlightened comments and creative advice made it a valuable experience for me to pursue my graduate studies and improve my confidence as well. This work could not have been done without his great enthusiasm and generous help.

Many thanks to Dr. Edward Karpinski for his instruction in patch clamp techniques, his constructive suggestions, and concern for my study. His friendly and supportive attitude made hard work enjoyable.

Special thanks to Dr. Christina Benishin for her advice and concern for my work.

I wish to express my great appreciation to Dr. Rosemary Pang for her excellent editing of my thesis and her patience in improving my English. I learned from her not only the language but also conscientiousness and earnestness towards work.

I am grateful to Dr. Richard Lewanczuk and Dr. Susan Donn for their constructive criticisms in the writing and completion of my thesis.

I am also indebted to Dr. Loren Kline and Professor Jingming Gang who helped me in experimental techniques and English. My sincere thanks to Bing Li and Lei Zhang for the valuable discussion and sharing of their knowledge with me. My thanks to everyone in Dr. Pang, Dr. Karpinski and Dr. Benishin's labs for their help and the enjoyable warm atmosphere they created for the last few years, especially to Kou-Jong Lin for his help.

Thanks to Annette Aarbo and Michelle Evans for their administrative assistance.

My deep gratitude goes to my father, bother, sister and her family for their persistent support, understanding and belief in me. My sincere appreciation is expressed to Julius Zou for his help and support. His encouragement and care gave me great strength to finish my work. Finally, I dedicate this work to the memory of my mother, Tinglian Li.

TABLE OF CONTENTS

CHAPTER 1 INTRODUCTION

CHAPTER II MATERIALS AND METHODS

CHAPTER III THE EFFECTS OF PROGESTERONE ON TENSION IN RAT AORTA AND TAIL ARTERY

CHAPTER IV THE EFFECTS OF PROGESTERONE ON $[Ca^{2+}]_i$ AND Ca^{2+} CHANNELS IN VSMC AND ON $[Ca^{2+}]_i$ IN ENDOTHELIAL CELLS

CHAPTER V EFFECTS OF OTHER STEROID HORMONES ON VASCULAR TISSUE

CHAPTER VI GENERAL DISCUSSION AND SUMMARY

LIST OF FIGURES

LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
ACh	acetylcholine
ADP	adenosine diphosphate
AR	acrosome reaction
ATP	adenosine triphosphate
bPTH	bovine parathyroid hormone
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclicmonophosphate
CGB	corticosteroid-binding globulin
cGMP	guanosine 3',5'-cyclicmonophosphate
cNOS	constitutive NO synthase
CNS	central nervous system
DHP	dihydropyridine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
E_2	estradiol
EDRF	endotheliun-derived relaxing factor
EGTA	ethyle. glycol-bis-(-aminoethylether)
ER	endoplasm reticulum
FBS	fetal bovine serum

FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GC	glucocorticoids
Hb	hemoglobin
HBSS	Hank's Balanced Salt Solution
HBSS-CMF	Ca^{2+}- and Mg^{2+}-free HBSS
HDL	high density lipoprotein
HEPES	N,N,N',N',-tetraacetic acid
I-V relationship	current-voltage relationship
iNOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-triphosphate
L-NMMA	N^G-monomethyl-L-arginine
LDL	low density lipoprotein
MC	mineralocorticoids
NGS	normal goat serum
NO	nitric oxide
NOase	nitric oxide synthase
NRS	normal rabbit serum
O_2Hb	oxyhemoglobin
PBS	phosphate-buffered saline
PKA	cAMP-dependent protein kinase
PKC	protein kinase C

ROC	receptor operated Ca^{2+} channel
SD	Sprague-Dawley
SHBG	sex hormone-binding globulin
SMOC	second message-operated Ca^{2+} channel
SR	sarcoplasmic reticulum
T	testosterone
TTX	tetrodotoxin
VDCC	voltage dependent Ca^{2+} channel
VSMC	vascular smooth muscle cells
vWF	von Willebrand Factor

Chapter I

INTRODUCTION

1.1. SYNTHESIS AND ACTION OF STEROID HORMONES

Steroid hormones are commonly classified into five groups according to the biological processes they regulate: (1) glucocorticoids, (2) mineralocorticoids, (3) androgens, (4) estrogens and (5) progestins. All steroid molecules have three six-carbon rings attached to one five-carbon ring.

1.1.1. SYNTHESIS OF STEROID HORMONES

In many species, steroid hormones are derived from cholesterol in steroidogenic tissues, such as adrenal glands, ovaries, placenta and testes. The tissues take up cholesterol from plasma lipoproteins through a receptor-mediated low density lipoprotein (LDL) pathway or a high density lipoprotein (HDL) pathway. Inside the steroidogenic cells cholesterol is transported into mitochondria and converted into pregnenolone by the cholesterol side-chain cleavage enzyme system (cytochrome P_{450}). After the synthesis, pregnenolone passes from the mitochondria to the endoplasmic reticulum (ER) where it is further converted into progesterone or 7-hydroxyprogesterone. Pregnenolone and progesterone are the common precursors in the formation of all other steroid hormones. Steroidogenic tissue for specific steroids contains the necessary enzymes to convert pregnenolone or progesterone into specific

steroid molecules. The production of each steroid is controlled by those special enzymes in the steroidogenic tissue. Steroids formed inside cells can be released into the peripheral circulation by passive diffusion, activation of Na^+/K^+-ATPase or other transport vehicles (Gower 1988)

1.1.2 TRANSPORT OF STEROID HORMONES

Steroids are small, nonpolar and water insoluble molecules. In order to circulate in the blood and not be excreted by the kidneys into the urine, steroids are bound to large "carrier" proteins . In human plasma there are two types of highly specific carrier proteins that bind steroids with high affinity. Corticosteroid-binding globulin (CBG) binds cortisol and progesterone. Sex hormone-binding globulin (SHBG) binds testosterone and estradiol (Rosner 1991) . There are also albumin and prealbumin bound steroids in the blood stream. This binding has less specificity and less affinity for steroids.

After the discovery of CBG and SHBG in human plasma (Rosenbaum *et al.* 1966) the concepts of "free" and reversibly bound steroids were formed. It is believed that free steroids diffuse passively out of the plasma compartment where they were synthesised and across the cell membranes to initiate hormone action. Bound steroids are not available for transport to the target cell. They can, however, be quickly transferred from the bound to the free state and thus be available for hormone action (Mendel 1989). According to this free hormone concept, the function of the high affinity binding proteins, CBG and SHBG, in plasma is to provide a reservoir of the hormones and to regulate the magnitude of the free fraction.

Studies measuring bioavailable testosterone (T) and estradiol (E_2) in human serum that was taken up by rat brain showed that the biologically active T or E_2 is nearly equal to the free plus albumin-bound fraction as measured *in vitro* (Pardridge *et al.* 1980). This investigation suggested that while SHGB-bound T or E_2 is not available for transport, albumin-bound T and E_2 are nearly freely available for transport into the brain. Considering the large amounts of steroid hormones bound to the albumin fraction, free hormone measurements might underestimate the exchangeable hormones in the circulation.

CBG is synthesised and secreted by hepatocytes. CBG consists of a single polypeptide chain and five carbohydrate side chains. The steroid-binding site is within the peptide chain and the carbohydrate side chains are important in the interaction of CBG and membrane receptors (Akhrem *et al.* 1982; Hammond *et al.* 1987). Estrogen is the most important substance which can increase the concentration of CBG in plasma. In late pregnancy, when plasma estrogen secreted by the placenta is several hundred times higher than the highest concentration found during the ovarian cycle, CBG concentration will increase to 2 to 2.5 times that of nonpregnant levels (Doe *et al.* 1964). Glucocorticoid decreases the CBG concentration in plasma (Frairia *et al.* 1988). The half-life of CBG in plasma is 5 days. CBG itself can bind to a membrane receptor and cause the rapid activation of adenylate cyclase and production of cAMP formation (Nakhla *et al.* 1988; Siger *et al.* 1988). These actions depend on the presence of cortisol or progesterone at the CBG binding site. CBG not bound to the steroids does not have this effect. The purpose of these actions is unknown.

Like CBG, SHBG is a plasma glycoprotein synthesised in, and secreted by, the liver . It has one steroid-binding site per molecule and its half-life in plasma is 6 days. Studies showed

that thyroxin and estrogen lead to an increase in SHBG secretion by a hepatocyte cell line (Hep G2) (Rosner *et al.* 1984; Mercier-Bodard *et al.* 1989). SHBG also interacts with a cell membrane receptor. The unbounded form of SHBG can bind to its membrane receptor (Strel'chyonok *et al.* 1984). The binding of SHBG and its receptor will be prevented if the steroid binds SHBG before SHBG binds the cell membrane receptor. On the other hand, after the unbounded SHBG binds the membrane receptor, it maintains the ability to bind steroids and cause intracellular cAMP accumulation (Hryb *et al.* 1990).

1.1.3. INTRACELLULAR RECEPTORS AND ACTIONS OF STEROID HORMONES

A recent review by Brasch and Ochs (1995) discussed intracellular receptors for steroid hormones. Steroid hormone receptors are proteins. Each receptor has a central DNA-binding domain that is well conserved, a ligand-binding C-terminal domain that is moderately sequence conserved, and an extremely variable N-terminal domain. The receptors become functionally active after binding with the ligand and will then influence gene transcription. Although mineralocorticoid and glucocorticoid receptors may reside in the cytoplasm when they are not bound to the ligands, estrogen receptors and progesterone receptors are located inside the nucleus even when they are not bound to the steroids.

The cDNAs for each of the steroid receptors have been cloned and sequenced revealing that there are several highly conserved regions in the receptors. It has been confirmed that one of the conserved regions is, in fact, the DNA binding domain of the nuclear

receptors (McDonnell *et al.* 1993). All steroid intracellular receptor proteins are both steroid- and tissue-specific. The binding of the receptors and steroid is reversible but with high affinity. The equilibrium dissociation constants (K_d) are between 10^{-10} to 10^{-8} M (Katzenellenbogen 1980).

There is an original model describing the intracellular events after the steroids diffuse into the cells. The model shows a two-step process in which the receptors for the steroids are located in the cytoplasm and transfered to the nucleus after binding with the steroid. A conformational change occurs after binding with the steroids resulting in increased affinity for specific DNA sequences which will ultimately lead to alteration of specific gene expression.

A current model of the general mechanism of steroid action on gene expression (Landers and Spelsberg 1992) is not greatly different from the mechanisms mentioned above. Steroid molecules passively diffuse into the cells and bind to their receptors. The binding activates the receptors and enables the receptors to bind to the specific sites on chromatin. The activated steroid-receptor complex will ultimately modulate the transcription of steroid-responsive genes resulting in changes in the production of mRNAs and proteins. Since there are receptors located in the nucleus even when they are not bound to the steroids, the new model suggests that translocation of the receptor from the cytosol to the nucleus does not always happen. The time course of the process is as follows: the steroid molecule binds to its receptor 4 min after entering the cell, and the steroid-receptor complex binds to the DNA binding site in 5 min. After this binding, 1-4 hours are needed for pre-mRNA synthesis and another 1-4 hours for the pre-mRNAs to be modified into mRNA. Four-8 hours after mRNA

formation, the protein molecules and modified protein molecules are produced. Typically the whole process of the steroid hormone action will take 12 to 24 hours.

1.2. PHYSIOLOGICAL EFFECTS OF STEROID HORMONES

1.2.1. EFFECTS OF PROGESTERONE

Progesterone is synthesised by the corpus luteum during the second half of the menstrual cycle and by the placenta during pregnancy. Progesterone mediates many aspects of reproduction in female mammals, egg white protein synthesis in the oviduct of oviparous animals and oocyte maturation in Amphibia. The most prominent function of progesterone in mammals is the establishment and maintenance of pregnancy. For the maintenance of pregnancy, progesterone inhibits myometrial contraction and the synthesis of prostaglandin. Progesterone also has roles in immunological protection of the embryo and maintenance of uterine growth and plasticity (Rothchild 1983). Inhibition of myometrial contraction by progesterone is believed to be related to prostaglandin inhibition of calcium metabolism in the sarcolemma (Currie and Jeremy 1979). Mammary glands are target tissue for progesterone which acts as an anti-prolactin. Progesterone levels will drop at the end of pregnancy to facilitate prolactin-induced lactogenesis (Ganguly *et al.* 1982).

It was recognised over half a century ago that progesterone was required for full expression of female sexual behaviour in female rats. Treatment of ovariectomized female rats with progesterone and estradiol resulted in the full expression of sexual behaviours while

neither of the hormones alone was adequate (Fadem *et al.* 1979). Evidence has suggested that the hypothalamus, specifically the ventromedial nucleus of the hypothalamus (VMH) is the region necessary for the receptor mediated genomic facilitation of sexual behaviour by progesterone (Bayliss *et al.* 1991).

Combined with estrogen, progesterone has been used for hormonal therapy in postmonopausal women to reduce the risk of endometrial cancer.

1.2.2. EFFECTS OF ESTROGEN

Estrogens are produced by the ovary, the placenta during pregnancy, the adrenal cortex in both males and females and the male gonads. Some tissues can produce estrogen from its precursor. The aromatase present in the brain can convert androgens into estrogens.

Estrogen stimulates the development of the sex organs, influences breast development, the "female" pattern of fat distribution and plays an important role in the hypothalamic-hypophyseal-gonadal regulatory circuit. In the human body there are three most important estrogens: estrogen, 17β-estradiol (E_2) and estriol. E_2 is the main physiological estrogen in the menstrual cycle.

Menopause occurs in women who reach a certain age (average 50 years of age) when menstruation ceases. Along with the decline of follicles in the ovary with age, estrogen secretion will decrease and finally stop. Estrogen deficiency at the menopause can cause type I (postmenopausal) osteoporosis, reduction of total bone mass or cone density due to the increase in bone turnover and resorption (for reviews see Melton 1994; Breslau 1994).

Another incidence in postmenopausal women is the increase of cardiovascular disease (Kannel 1976). Numerous studies have been conducted on the efficacy of hormone replacement therapy for preventation of menopausal osteoporosis and cardiovascular disease (Breslau 1994; Kafonek 1994). Estrogen has been used as the main hormone in this therapy. Progestin was added to the treatment to eliminate the risk of endometrial cancer which is increased by estrogen administration. Hormone replacement therapy can reduce the risk of heart ischemia and potentiate endothelium-dependent vasodilation (Gilligan *et al.* 1994) while also reducing bone remodelling to premenopausal levels and, thus, reducing the rate of loss of skeletal tissue (Lindsay 1991, 1993). In studies conducted on female monkeys, replacement of E_2 or E_2 plus progesterone reduced the extent of coronary artery atherosclerosis (Adams *et al.* 1990), relaxed coronary artery vasomotion (Williams 1992) and prevented postmenopausal bone loss (Breslau 1994). Clarkson (1994) suggested that about 20%-25% of the cardiovascular benefits of estrogen replacement are mediated by the favourable changes in the plasma lipoprotein profiles.

1.2.3. EFFECTS OF ANDROGEN

Testosterone is the major androgen produced by the Leydig cells in the testis and the zona reticularis in the adrenal cortex. Testosterone stimulates the development of male sexual organs and male secondary sex characteristics. Unlike estrogen, testosterone has far broader biologic effects on almost every tissue. It is responsible for erythropoiesis (red blood cell formation) and the growth of other organs. In addition to playing an important role in

spermatogenesis, testosterone has strong anabolic effects such as stimulating protein synthesis and bone growth. Androgen is thus also referred to as an anabolic steroid. Synthetic anabolic steroids have been used to stimulate the increase of muscle strength or muscle mass and physical performances of athletes (for review see Celotti and Negri 1992). Researchers have attempted to separate the anabolic from the androgenic effect of testosterone since the abuse of this hormone in the sports community has caused concern. Anabolic steroid abuse-related cardiac problems and death have been reported (Kennedy and Lawrence 1993).

1.2.4. EFFECTS OF ADRENAL CORTICAL STEROIDS

Mineralocorticoids are produced in the zona glomerulosa and glucocorticoids are produced in the zona fasciculata in the adrenal cortex. Effects of mineralocorticoid include regulation of blood pressure, electrolytes and water balance.

Aldosterone is the most important mineralocorticoid in the human body and the major mineralocorticoid in rats. Administration of aldosterone enhances sodium reabsorption into the circulation and potassium excretion by the kidney in mammalian species (for review see Ross 1975). The net effect of aldosterone is the increase of extracellular fluid volume which can lead to increased blood pressure and blood flow. The secretion of aldosterone is controlled by the renin-angiotension system.

Mineralocorticoid excess can cause hypertension. One of the factors for mineralocorticoid-salt hypertension is changes in the sensitivity of vascular smooth muscle to vasoactive stimuli.

Glucocorticoid is an essential hormone for human beings in response to the environmental changes in their daily life. Cortisol is a typical glucocorticoid. It is a catabolic hormone which stimulates protein degradation in muscle and increases glycogen synthesis in the liver. Cortisol is also anti-inflammatory and immunosuppressive. Like cortisol, dexamethasone is a glucocorticoid and has anti-inflammatory effects.

1.3. RAPID NON-GENOMIC EFFECTS OF STEROID HORMONES

The conventional genomic action of steroid hormones starts inside the cell. The steroid hormones enter the target cells by simple diffusion and bind to the specific intracellular receptors . The steroid-receptor complex then undergoes activation and transfer to the nucleus where it binds to DNA. Cellular actions after the formation of the hormone-receptor complex include gene transcription, translation and protein synthesis. The actions take place inside the cell and the effects usually appear with a latency of a few hours. The induced macromolecular synthesis may continue for several hours or days after steroid removal (Russo-Marie and Duval 1982). A block at any of the steps, such as RNA synthesis or protein synthesis, will inhibit steroid-induced responses.

During the last two decades, numerous studies on the rapid, non-genomic effects of steroids have been published for almost every group of steroid hormones. These effects are presumably non-genomic because they are rapid in onset and not modified by inhibitors of protein synthesis. The earliest finding of steroids affecting the cell membrane instead of binding to intracellular receptors was that of the anaesthetic steroids reported over 50 years ago (for

review, see Duval *et al.* 1983). Anaesthetic steroids from a few groups showed a rapid action (less than 3 min). The concentrations necessary for the expression of the anaesthetic effects are relatively high and the effects were considered to act through a membrane process (for review see Duval *et al.* 1983).

After the above observation, numerous studies were conducted on the rapid, non-genomic effects of steroid hormones. In this chapter the focus will be on studies of the rapid effects of steroid hormones on the nervous system, the maturation of human sperm, and the effects of estrogen and aldosterone.

1.3.1. RAPID NON-GENOMIC EFFECTS OF HORMONES ON THE NERVOUS SYSTEM

Investigations in this area include the responses of preparations (1) lacking intracellular receptors, (2) in the presence of RNA and/or protein synthesis inhibitors, and (3) having steroids coupled to large molecules which block access to intracellular receptors (for review see McEwen 1991). These rapid responses are believed to be mediated by membrane bound receptors.

Studies on hypothalamic neurones revealed that changes in electrical activity of the neurones appeared almost instantaneously after intravenous injection of estradiol benzoate (Dufy *et al.* 1976, 1979). A recent study demonstrated that corticosterone stimulated $^{45}Ca^{2+}$ uptake by brain synaptosomes within 60 seconds (Sze and Iqbal 1994).

GABA is a major inhibitory neurotransmitter in the brain. GABA mediated rapid synaptic inhibition by activating receptor-gated Cl^- channels. There is evidence showing some neuroactive steroids modulated the inhibitory events mediated by GABA (Paul and Purdy 1992).

1.3.2. RAPID NON-GENOMIC EFFECTS OF PROGESTERONE ON THE MATURATION OF HUMAN SPERM.

It has long been known that sperm must undergo the acrosome reaction (AR) before they can penetrate the zona pellucida and fertilize the egg. It is also believed that Ca^{2+} influx is necessary for the occurrence of the AR. Progesterone and 17α-hydroxyprogesterone were shown to specifically stimulate a rapid and dose-dependent elevation of $[Ca^{2+}]_i$ in human sperm (Blackmore and Lattanzio 1991; Blackmore 1993; Mendoza *et al.* 1995). The increase in $[Ca^{2+}]_i$ was due to the Ca^{2+} influx from the extracellular solution through a plasma membrane channel and was blocked by the Ca^{2+} chelator EGTA and the Ca^{2+} channel antagonist La^{3+}. The effect of progesterone was specific. Corticosterone and pregnenolone produced minimal effects. Further studies showed that the Ca^{2+} influx stimulated by progesterone was not sensitive to verapamil and pertusis toxin. The authors believed that progesterone activated a membrane ion channel that is permeable to monovalent cations as well as to Ca^{2+} (Föresta *et al.* 1993). The concentrations of progesterone used were 1-10 μM. Cell-surface binding sites for progesterone have been found and are believed to be responsible for mediating the calcium uptake (Blackmore and Lattanzio 1991). Recent studies demonstrated that the progesterone

effect could be inhibited by suppression of protein-kinase C (Föresta *et al.* 1995) and that progesterone activated a protein tyrosine kinase independently of the Ca^{2+} influx process (Mendoza *et al.* 1995).

1.3.3 RAPID NON-GENOMIC EFFECTS OF ESTROGEN.

Change in Ca^{2+} flux was observed 3 min after estrogen application in rat uterine cells (Pietras and Szego 1975). In cultured breast cancer and uterine cells and intact uterus of rats, estrogen increased the concentration of cAMP without the formation of new RNA or protein synthesis (Aronica *et al.* 1994). Estrogen was found to specifically stimulate the activity of adenylate cyclase in human endometrium (Bergamini *et al.* 1985). Estrogen also affected $[Ca^{2+}]_i$ levels in rat hepatocytes (Sanchez-Bueno *et al.* 1991), human oocyte maturation (Tesarik and Mendoza 1995), and gonadotropin-releasing hormone activities (Ravindra and Aronstam 1992). Estrogen superfusion caused hyperpolarization due to increased K^+ conductance in the presence of cycloheximide in brain neurones (Nabekura *et al.* 1986) and other types of neurons (Zyzek *et al.*1981). 17β-estradiol induced an increase in IP_3 and DA formation in rat osteoblasts and the effect could not be blocked by tamoxifen, a inhibitor of genomic steroid responses.

The first evidence for estrogen receptors on the surface of cells was discovered in chicken and pig ovarian granulosa cells. A recent study applied microscopy using confocal laser scanning and a specific antibody to the intracellular estrogen receptor to recognise a membrane

estrogen receptor. The author suggested that the rapid or non-genomic steroid actions are mediated by a modified form of the intracellular receptor (Pappas *et al.* 1995).

1.3.4. RAPID NON-GENOMIC EFFECTS OF ADRENAL CORTICAL STEROIDS, ANDROGEN AND VITAMIN D

Aldosterone rapidly influences the transport of Na^+ and K^+ in classic genomic target tissues. It has rapid effects on the Na^+/H^+ exchanger in renal tubule cells, hippocampus, smooth muscle cells and mammary glands (Christ *et al.*1995a). Aldosterone also affected Na^+, K^+ and Ca^{2+} concentration and cell volume of human mononuclear leukocytes (Wehling *et al.* 1987, 1989) and the activity of the sodium-proton exchanger in human lymphocytes (Wehling *et al.* 1989). Aldosterone specific membrane receptors have been demonstrated (Wehling *et al.* 1993).The effects of aldosterone on the cardiovascular system will be discussed in the next section.

Androgens have been reported to cause a rapid increase in $[Ca^{2+}]_i$ in Sertoli cells (Gorczynska and Handelsman 1995), an increase in $[Ca^{2+}]_i$ and IP_3 in rat osteoblasts (Lieberherr and Grosse 1994) and $[Ca^{2+}]_i$ increase in human prostate cancer cells (LNCaP) (Steinsapir *et al.* 1991). Testosterone reduced the contractility of isolated rat myometrium (Perusqúa *et al.*1990).

Vitamin D is a seco-steroid hormone and is believed to be the precursor of a steroid hormone (Norman *et al.* 1982). Vitamin D and its active metabolite 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2\text{-}D_3$) play an important role in the regulation of mineral metabolism and calcium

homeostasis. It has been demonstrated that $1\alpha,$-25-$(OH)_2D_3$ exerts non-genomic actions on osteoblasts and osteoblast-like cells by increasing $[Ca^{2+}]_i$ and nuclear calcium, cellular Ca^{2+} uptake, and phospholipase C activity (for review see Baran 1994). 1,25 D_3 increased $[Ca^{2+}]_i$ by increasing Ca^{2+} entry via voltage-dependent Ca^{2+} channels and Ca^{2+} mobilization from intracellular stores in rat islet cells which led to insulin secretion (Billardel *et al.* 1993). It also has been shown that 1,25-D_3 stimulates Ca^{2+} uptake in vascular smooth muscle cells (VSMC) (Shan *et al.* 1993).

1.4. EFFECT OF STEROID HORMONES ON THE CARDIOVASCULAR SYSTEM

1.4.1. GENOMIC EFFECTS

In the heart, direct genomic effects occur for estradiol, testosterone, adrenal cortical steroids and vitamin D. In general, estrogen has a vasodilating and anti-atherogenic action; progesterone is a smooth muscle relaxant; androgen promotes atherogenesis and vitamin D lowers the blood pressure.

1.4.1.1. *Estrogen*

Estrogen has direct and indirect effects on the cardiovascular system. It has long been known that premenopausal women have a lower risk for cardiovascular disease (CVD) than do men. But this advantage exists only before the menopause. The risk increases progressively in postmenopausal women. Hormone replacement therapy with estrogen reduced the

cardiovascular mortality and morbidity in postmonopausal women indicating that estrogen has a protective effect on the cardiovascular system (Stevenson *et al.* 1994).

Estrogen at high doses caused beneficial changes in plasma lipoprotein levels, i.e. lowering LDL and raising HDL (Walsh *et al.*,1991). These changes reduced the incidence of arteriosclerosis. Experiments using autoradiography showed nuclear labelling of estrogen is conspicuous in the atrium and in muscle and connective tissue cells in the walls of large, intermediate and small arteries and in capillary pericytes (for review see Stumpf 1990). The location of the binding sites to estrogen in the central nervous system (CNS) is close to the regulatory sites of cardiovascular function (Henry and Calaresu 1972). These data suggested that the cardiovascular system is a direct target of estrogen. Experiments on VSMC derived from rat aorta found that there were estrogen receptors in the cells. In response to 17β-estradiol, ER and mRNA increases in these cells (Orimo *et al.* 1993).

1.4.1.2. *Progesterone*

Progestin nuclear binding has been demonstrated by autoradiography in the central nervous system and heart of rats and guinea pigs (Stumpf 1990). Compared to estrogen, little is known about progesterone which usually modulate estrogen effects.

Progesterone has been used combined with estrogen in hormone replacement therapy to reduce the incidence of adenocarcinoma of the endometrium which is increased in women using estrogen alone (Gambrell 1981; Samsioe 1994).

1.4.1.3. *Testosterone*

There is a correlation between testosterone and plasma lipoprotein. Low plasma testosterone levels are always associated with low levels of high density lipoprotein cholesterol (HDL-C) (Hämäläinen *et al.* 1987). In terms of the effects on the plasma lipoproteins, the function of testosterone is opposite to that of estrogen. Studies in adult men suggested an inverse relationship between testosterone and blood pressure (Khaw and Barrett-Connor 1988).

Abuse of anabolic steroids is believed to cause death by the possible increase in heart size and responsiveness to catecholamines (Kennedy and Lawrence 1993).

1.4.1.4. *Adrenal cortical steroids*

Mineralocorticoids and glucocorticoids have effects on the cardiovascular system by regulating blood electrolytes and the renin-angiotensin system.

Aldosterone and dexamethasone binding sites in CNS and heart muscle and in walls of blood vessels have been reported (Stumpf 1990). It has been recognised for many years that mineralocorticoids can cause hypertension. Studies indicated that mineralocorticoid excess is associated with changes in vascular smooth muscle membrane permeability to ions and increased responsiveness to vasoactive agents (Kornel *et al.* 1987; Angeli *et al.* 1988). Aldosterone levels have a corelation with cardiac mass in patients with essential hypertension and animals with experimentally induced mineralocorticoid hypertension. With long term treatment both adrenal cortical steroids can increase contractility of vascular smooth muscle. Studies in rabbit arterial smooth muscle cells showed that these effects may be due to

increased Na^+ and Ca^{2+} influx and may be mediated by the intracellular receptors for the two adrenal cortical hormones (for review see Gómez Sánchez 1995).

1.4.1.5. *Vitamin D*

Studies indicate the presence of vitamin D receptors in cardiomyocytes and endothelial cells of certain blood vessels and in CNS regions associated with cardiovascular regulation(Stumpf 1990). Dietary studies have shown that a vitamin D-deficient diet caused an increase in systolic blood pressure (Weishaar Simpson 1987).

1.4.2. NON-GENOMIC EFFECTS

1.4.2.1. *Effects of steroid hormones on the heart*

17β-E_2 caused a dose-dependent decrease in the sinoatrial rate in rat right atria. 17β-E_2 at a concentration of $5x10^{-6}$ M caused a significant decrease in heart rate in the isolated beating rat heart. The same concentration of 17β-E_2 produced a significant increase in coronary flow (Eckstein *et al.* 1994). An inhibitory effect of 17β-E_2 perfusion on heart rate was also observed in rabbit (Raddino *et al.* 1986). It remains uncertain, however, whether these results are due to an effect on the myocardium or on coronary circulation. Studies on isolated guinea-pig heart demonstrated that 17β-E_2 ($3x10^{-6}$M) caused a decrease in cell shortening and decreased the peak inward Ca^{2+} current (Jiang *et al.* 1992a). This result indicates that 17β-E_2 has a negative inotropic effect on the heart.

1.4.2.2. *Effects of steroid hormones on blood vessels*

Acute administration of physiological concentrations of 17β-E_2 selectively potentiated endothelium-dependent vasodilation in healthy postmenopausal women and potentiated both endothelium-dependent and endothelium-independent vasodilation in postmenopausal women with risk factors for atherosclerosis and evidence of impaired vascular function (Gilligan *et al.* 1994). These effects were suggested to be partly responsible for the long-term benefit of estrogen therapy on cardiovascular health in postmenopausal women. 17β-E_2 was suggested to be a vasodilator.

Several investigations have demonstrated that 17β-E_2 has acute relaxant effects on blood vessels in the rabbit, rat, dog and human (Gisclard *et al.* 1988 ; Jiang *et al.* 1991; Ravi *et al.* 1994; Shan *et al.* 1994; Sudhir *et al.* 1995). The results of these experiments are not consistent with the endothelium-dependence of the relaxant effects. Some of the studies indicated that the effect of 17β-E_2 was regulated by calcium influx through voltage-dependent calcium channels (Shan *et al.* 1994; Zhang *et al.* 1994). Experiments on isolated cardiac myocytes and VSMC proved that 17β-E_2 decreased the voltage-dependent calcium currents across the cell membrane (Sheldon and Argentieri 1995).

Steroid hormones from other groups also have effects on blood vessels. Progesterone induced an endothelium-independent relaxation in rat coronary artery (Jiang *et al.* 1992b). 1,25-vitamin D was found to increase cytosolic free calcium and calcium channel currents in

VSMC (Shan *et al.* 1993). Dexamethasone inhibited vascular contraction in isolated rabbit ear artery (Miyahara *et al.* 1993).

There are many investigations concerning the effect of aldosterone on the cardiovascular system. Aldosterone increased Na^+ influx in the VSMC (Christ *et al.* 1995a), and increased intracellular free calcium in VSMC and endothelial cells (Wehling *et al.* 1994, 1995). Recent evidence suggests that the rapid effects of aldosterone are mediated through specific membrane receptors and the intracellular signalling pathway phospholipase C and protein kinase C (Christ *et al.* 1995b; Wehling 1994).

1.5. BLOOD VESSELS AND BLOOD PRESSURE

1.5.1 BLOOD VESSELS AND BLOOD PRESSURE CONTROL

The cause of hypertension in most clinical forms and experimental models is an increase in peripheral resistance. Blood pressure is dependent on cardiac output and the resistance in the circulation system

$$MAP = CO \times TPR$$

where MAP is mean arterial pressure; CO is cardiac output and TPR is total peripheral resistance. The resistance in the blood vessel is determined by Poiseulle's law:

$$R = P_1\text{-}P_0 / Q = 8hl / pr^4$$

Where R is resistance; P_1-P_0 is pressure difference; Q is blood flow, r is the radius of the blood vessel; h is viscosity and l is the length of the blood vessel. Poiseulle's law points out that vascular resistance depends on the change of arteriolar diameter.

There are two processes which could contribute to the change of the diameter of the blood vessel: (1) changes in the contractility and the response to vasoactive agents which regulate vascular tone and (2) structural changes which lead to an increase in wall to lumen ratio in blood vessels. The control of vascular function is a central issue in hypertension. There are two mechanisms to control arterial tone: intrinsic control and extrinsic control.

Intrinsic factors include local chemical dilators such as the pressure of oxygen or carbon dioxide (PO_2 , PCO_2) , PH, and endothelium-derived relaxing factor(EDRF) which regulate vascular smooth muscle contraction leading to changes in blood flow in response to metabolic demand. Extrinsic factors include nervous control (sympathetic), adrenaline and noradrenaline secreted from the adrenal glands, the angiotensin-renin system, and vasopressin. They are also important for the regulation of vascular smooth muscle contraction.

1.5.2. CALCIUM AND VASCULAR SMOOTH MUSCLE CONTRACTION

VSMC are spindle-shaped and contain more actin and less myosin than do skeletal muscle cells (Murphy *et al.* 1974). The mechanism of smooth muscle contraction by phosphorylation of regulatory myosin light-chains has been reviewed by Hartshorne and Mrwa (1982). When the $[Ca^{2+}]_i$ increases to 10^{-5} M, calmodulin which is capable of sensing free Ca^{2+} concentrations in the range of 10^{-7} to 10^{-5} M will bind with the ion and increase the affinity for myosin light-chain kinase. Activated by calmodulin, myosin light-chain kinase in turn catalyzes the phosphorylation of myosin. Actin then interacts with phosphorylated myosin and thus increases its ATPase activity to generate force as the cross bridges undergo a cyclic process.

Purified myosin light chain kinase from smooth muscle can be phosphorylated by protein kinase A and protein kinase C. The phosphorylation can desensitize myosin light-chain kinase to respond to stimulation by Ca^{2+}/calmodulin. Thus, the effect of PKA, PKC phosphorylation could play an important role in the regulation of smooth muscle contractility (Jiang and Stephens 1994).

Calcium plays an important role in the regulation of vascular smooth muscle contraction. Simultaneous measurement of Ca^{2+} levels and tension revealed that the peak of the Ca^{2+} increase occurred during the period of the force development in ferret portal vein strips(Morgan and Morgan 1982, 1984). The fluorescent calcium indicator, Quin-2 , was used in animal studies to show that the concentration of cytosolic free Ca^{2+} ranged from 120 - 270 nM in smooth muscle cells at rest and 500 -700 nM in activated cells

(Williams and Fay 1986). The evidence also showed that the calcium increase is transient even in the continued presence of the contractile agents. $[Ca^{2+}]_i$ increased to 3-5 μM after the addition of a stimulus and decreased back to the resting level in 120-150 seconds. Relaxation agents caused a transient decrease in resting $[Ca^{2+}]_i$ levels from 135 to 94 nM (Williams and Fay 1986). Intracellular Ca^{2+} is maintained at a low concentration compared to the concentration of extracellular Ca^{2+} which is 1-2 mM for a typical mammalian cell. When the Ca^{2+} channels open either in the plasma membrane or intracellular Ca^{2+} stores, calcium ions will enter the cytoplasm causing a transient increase in $[Ca^{2+}]_i$. Cells extrude excess Ca^{2+} by an ATP-dependent Ca-pump and a Na^{+}-Ca^{2+} exchanger system on the surface membrane and ATP- dependent pumps in the membrane of intracellular Ca^{2+} stores (Williams and Fay 1986).

1.5.3. CALCIUM AND CALCIUM CHANNELS IN SMOOTH MUSCLE CELLS

Elevation of $[Ca^{2+}]_i$ is due to entry of extracellular Ca^{2+} (Ca^{2+} influx) or Ca^{2+} released from intracellular Ca^{2+} stores, in each of which the concentration of Ca^{2+} is 10,000 fold that of $[Ca^{2+}]_i$.

There are several cellular pathways for Ca^{2+} influx (for review see Tisen and Tisen 1990): (1) Voltage dependent Ca channels (VDCC). These channels will be discussed later. (2) Receptor operated Ca channels (ROC). Ca^{2+} channels in this group will open in direct response to binding of the appropriate ligand. The response is rapid and without diffusible cytosolic messenger involvement. These channels are less specific for Ca^{2+} in

preference to Na^+ than are voltage-dependent Ca^{2+} channels. There are ROC found in arterial smooth muscle and activated by ATP (Benham and Tsien 1987). (3) Second messenger-operated Ca^{2+} channels (SMDC). These channels are opened by cytosolic messengers. (4) Other channels such as mechanically operated channels and gap junction channels.

VDCC are more selective for Ca^{2+} than for Na^+ or K^+ (1000-fold). There are four major types of VDCC: L, T, N and P.

L-type Ca^{2+} channels are activated by high voltage (HVA) and are 1,4-dihydropyridine (DHP)-sensitive. L-channels are insensitive to ω-conotoxin GVIA(ω-CgTx). L stands for large and long lasting (little inactivation). The L-channel has a large channel conductance of 25 pS. L-type channels are ubiquitous in excitable tissues and exist in many non-excitable cells. They are the major pathway for voltage-gated Ca^{2+} entry into the cell. L-channels play a key role in excitation-contraction coupling in smooth and cardiac muscle (Lubic *et al.* 1995), modulating membrane excitability and the release of some hormones and neurotransmitters.

T-type channels are opened by relatively low voltage (LVA). T refers to tiny and transient. The T-channel has a single-channel conductance of 8 to 10 pS. T channels inactivate rapidly and require a very negative voltage to de-inactivate. There are no specific blockers of T-channels. The most obvious functions of T-type channels are to support pacemaker activity in the heart (Bean 1989).

N-channels are high voltage activated. They are insensitive to DHPs and largely blocked by ω-conotoxin. N-channels occur in neurons (Tsien *et al.* 1988). They are responsible for Ca^{2+} entry into sensory, sympathetic neurons.

The physiology of P-channels is between that of T- and N- type channels. P-channels are not sensitive to either DHP or ω-CgTx. These channels are found in cerebellar Purkinje cells.

All excitable and some sensory cells exhibit VDCC. Due to the great electrochemical gradient across the cell membrane, a net inward current will occur when the channels are activated to open. Ca^{2+} ions will rapidly enter the cell through the channels.

VDCC have been found and characterized in vascular smooth muscle. There are two types of Ca^{2+} channels found in primary cultured single VSMC from rat tail artery: T-channels and L-channels (Wang *et al.* 1989; Loirand *et al.* 1986). These two components could be separated by the application of a specific L-channel blocker such as nifedipine and by using different holding potentials. Results showed that a parathyroid hormone fragment [bPTH-(1-34), the active fraction of bovine parathyroid hormone] reduced the magnitude of L-channel current but not T-channel current. This inhibitory effect could be reversed by removal of the drug. Further investigation indicated that the inhibitory effect of bPTH was partly dependent on the cAMP-PKA signal transduction pathway (Wang *et al.* 1991a, b).

The sarcoplasmic reticulum (SR) in muscle cells is a highly specia. zed smooth endoplasmic reticulum. The principal function of SR is sequestering Ca^{2+} from the cytosol. It is the major intracellular Ca^{2+} storage site. Ca^{2+}-pump ATPase is the major protein in the SR membrane to move Ca^{2+} back to the SR from the cytosol.

There are two kinds of Ca^{2+} release channels in the SR in smooth muscle: the Ca^{2+}-induced Ca^{2+} release channel and the inositol 1,4,5-triphosphate (IP_3)-induced Ca^{2+} release channel (Chadwick *et al.* 1990). These channels mediate the Ca^{2+} release from the SR to the cytosol in response to stimulation.

1.6. ENDOTHELIUM AND VASODILATION

1.6.1. ENDOTHELIUM AND NITRIC OXIDE

The endothelium is a monolayer of squamous cells lining the inner surface of the entire cardiovascular system. In major vessels, the endothelium occurs in the lumen and is continuous with the cardiac endothelium. Capillaries consist solely of endothelium and basement membranes. Endothelial cells retain the capacity to divide. This characteristic facilitates maintenance of the lining layer of the circulatory system and enables new capillaries to sprout whenever it is necessary. The mesoderm-derived cells that line the blood vessels, lymphatic system and heart are, by convention, called endothelium although they are true epithelium.

Endothelium has long been known as a preventive barrier to the formation of thrombi because of its location and physical characteristics (smooth surface, dense cell arrangement).

However, this traditional view has changed in the past two decades. It is now well established that the endothelium plays an active role in a variety of physiological functions in the circulation and cardiovascular system such as maintenance of the fluidity of the blood, participation in inflammatory and immunological processes, and modulation of underlying vascular smooth muscle tone.

Endothelium was first considered as a modulator of vascular tone by a "cornerstone observation" reported by Furchgott and Zawadzki (1980). They demonstrated that vascular rings of rabbit aorta, pre-constricted with norepinephrine, relaxed in response to the muscarinic agent acetylcholine (ACh) and that the relaxation was dependent on the presence of a functionally intact endothelium. Therefore, the authors proposed that endothelial cells released a diffusible, labile factor which was later termed endothelium-derived relaxing factor (EDRF). They also reported that when the aortic preparation was hypoxic, ACh produced little or no relaxation. That is, hypoxia would cause an effect similar to that of endothelial denudation.

Biochemical assay studies have suggested that EDRF may be the inorganic free-radical gas, nitric oxide (NO) or a closely related species (Ignarro *et al.* 1987a,b).There are great similarities in their pharmacological properties: the interactions with oxyhemoglobin and the inactivation by superoxide (Palmer and Moncada 1989). Studies with cultured endothelial cells provided more direct evidence. Now this hypothesis is widely accepted although there are contradictory results showing that EDRF and NO have different stabilities during chromatographic analysis and are differently retained by anionic exchange columns (Augus *et al.* 1987).

Studies have indicated that NO is synthesised from L-arginine by oxidation of the guanidine-nitrogen terminal of the amino acid. The enzyme responsible for this reaction in the cell is NO synthase. NO synthase is a soluble, Ca^{2+}-, NADPH- and calmodulin-dependent enzyme (Bredt and Snyder 1990). The subsequent discovery that L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) can inhibit the effect of EDRF in a dose-dependent manner facilitated investigations on the physiological, pharmacological and biochemical properties of EDRF. Experiments showed that L-NMMA alone caused vasoconstriction in rat carotid, mesenteric and renal arteries (Archer *et al.* 1989). Application of L-NMMA into the brachial artery in humans caused both direct vasoconstriction and inhibition of the vaso-dilatory responses to ACh or bradykinin (Vallance *et al.* 1989). These results suggested that there is basal NO production in the artery, increased NO production in response to certain stimuli, and modulation by EDRF/NO of both basal vascular tone and endothelium-dependent dilation. Recent studies have demonstrated that there are two levels of NO activities in endothelial cells: continuous synthesis of NO in unstimulated endothelial cells and the synthesis of NO stimulated by receptor-dependent stimuli such as ATP, bradykinin and ACh and receptor-independent stimuli such as Ca^{2+} ionophores or by physical stimuli such as shear stress (Rubanyi *et al.* 1986; Newby and Henderson 1990; Korenaga *et al.* 1993).

Increases in intracellular Ca^{2+} in endothelial cells appear to play a key role in the production and release of NO. In rabbit and rat aorta and human coronary artery, depletion of intracellular Ca^{2+} inhibited endothelium-dependent relaxation in response to ACh and the Ca^{2+} ionophore A23187, but did not affect endothelium-independent vasodilatation (Winquist *et al.*

1985). The rise of $[Ca^{2+}]_i$ from 100 to 500 nM increases NO synthesis from <5% to >95% of maximum (Pollock *et al.* 1991).

NO synthesised in endothelial cells will rapidly diffuse both within the endothelium and across the membrane to nearby cells (e.g. SMC) due to its gaseous nature. One limiting factor of NO action is its extremely high affinity to oxyhemoglobin : $NO + O_2Hb \Rightarrow NO^{3\cdot} + Hb^{\cdot}$. Thus, NO diffused into the lumen of blood vessels will be rapidly inactivated and NO/EDRF acts only as a local mediator.

VSMC represent the most widely known and studied target for the physiological actions of NO. In VSMC, NO regulates the soluble guanylate cyclase in the cytosol (Moncada *et al.* 1991). Activated guanylate cyclase will produce the second messenger cGMP in a time and concentration-dependent manner. An increase in cGMP levels in VSMC will inhibit Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx through receptor-operated channels in VSMC (Collins *et al.* 1986). It will also inhibit phosphatidylinositol metabolism and cGMP-dependent protein kinase activities which control the phosphorylation and dephosphorylation of myosin-light chains (Rapoport and Murad 1983). This results in the relaxation of VSMC.

1.6.2 RAT ENDOTHELIAL CELL CULTURE

Endothelial cells have long been cultured for research purposes (Gimbrone 1976). Early attempts involved culturing endothelial cells from large animals and humans (Pomerat and Slick 1963; Fryer *et al.* 1966; Jaffe *et al.* 1973; Lewis *et al.* 1973). Due to the small size

and the special arrangement of the aorta wall, there were fewer reports on the culture of rat endothelial cells.

Two kinds of methods were routinely used to culture rat endothelial cells: enzymatic dissociation and explantation. Collagenase was commonly used to dissociate the aortic tissue and collect the endothelial cells (Booyse *et al.* 1975; Gordon *et al.* 1991). Other enzymes including elastase, dispase and hyaluronidase were also employed (Cole *et al.* 1986). The most difficult part of the enzyme dissociation method is to control the enzyme incubation time to avoid the inclusion of other types of cells. After enzyme dissociation in some experiments the cells underwent gradient centrifugation to separate the endothelial cells from non-endothelial cells (Gordon *et al.* 1991). Rat aorta lacks the subendothelial layer found in the human and other large animals. This layer contains a network of collagen and elastic fibres arranged loosely adjacent to the endothelial surface. Rat endothelium is attached directly to the irregular inner surface of the internal elastic lamina (Pease and Puale 1960; French *et al.* 1965). Enzymes usually employed to isolate endothelial cells digest the subendothelial layer. This is partly why rat aorta was found resistant to enzymatic dispersal (Merrilees and Scott 1981). In explant techniques, endothelial cells migrate from the explant to form colonies. The explants are then removed to reduce contamination by smooth muscle cells and fibroblast outgrowths. For this method, the key was the time duration in which endothelial cells were allowed to migrate before the explants were removed from the culture. For both methods, application of certain basement membranes to the culture dishes was quite helpful in supporting the growth of the endothelial cells. Commonly used materials are fibronectin, type I collagen or Matrigel (McGuire and Orkin 1987; Hashimoto *et al.* 1991; Gordon *et al.* 1991). Growth factors such

as endothelial cell growth supplement, endothelial mitogen or endothelial growth factor were used in some of the culture processes to stimulate the growth of endothelial cells (McGuire and Orkin 1987; Gordon *et al.* 1991; Hashimoto *et al.* 1991).

Endothelial cells in culture were previously reported to show a "cobblestone monolayer" The morphology of a cell type may, however, be influenced to some extent by the culture conditions. Therefore, the appearance of cells under light microscopy can not be used to clearly identify their origin. The Von Willebrand Factor (vWF, or Factor VIII) is one of the most widely accepted criteria for the positive identification of endothelial cells (Jaffe 1977). Immunofluorescence staining is commonly used to demonstrate the presence of the vWF in cultured endothelial cells (Cole *et al.* 1986; McGuire and Orkin 1987). vWF is also produced by megakaryocytes and platelets, but these cells are not normal vascular wall constituents and, thus, should not be present in the cultures.

1.7. OVERALL HYPOTHESIS

The two hypotheses of this investigation are: 1. The non-genomic effects of steroid hormones are rapid in onset and reversible quickly by removing the hormones. 2. The vascular system is the direct target of some steroid hormones.

Based on these hypotheses, the present studies were conducted to examine whether the vascular effects of the steroid hormones are rapid and non-genomic ones.

Experiments were carried out to monitor the effects of the hormones on three physiological vascular preparations: tissue tension generation, intracellular calcium change, and calcium channel activities in the cell membrane.

The time course and reversibility of these effects were considered as the criteria to recognise the existence of non-genomic effects.

1.8. SPECIFIC AIMS

1. To study the effects of steroid hormones on tension generation in vascular tissue preparations of rat aorta and tail artery.

2. To investigate intracellular calcium change in VSMC and endothelial cells in response to progestereone, one of the steroid hormones.

3. To examine the effects of steroid hormones on voltage-dependent calcium channel activities in the membrane of VSMC.

4. To examine closely the time course of these steroid effects, as well as their reversal by washout, in the three above-mentioned tissue preparations.

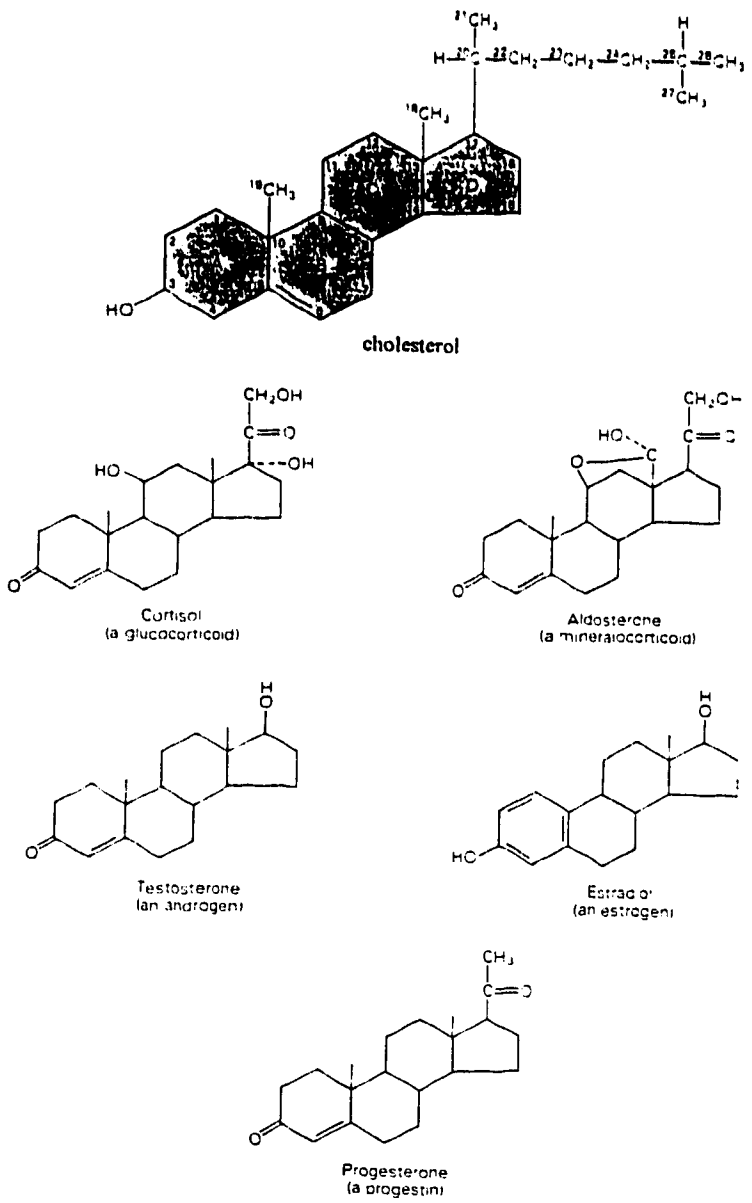

Fig. I-1 Structure of cholesterol and steroids from the five groups of steroid hormones (adapted from Rhoades and Pflanzer 1989).

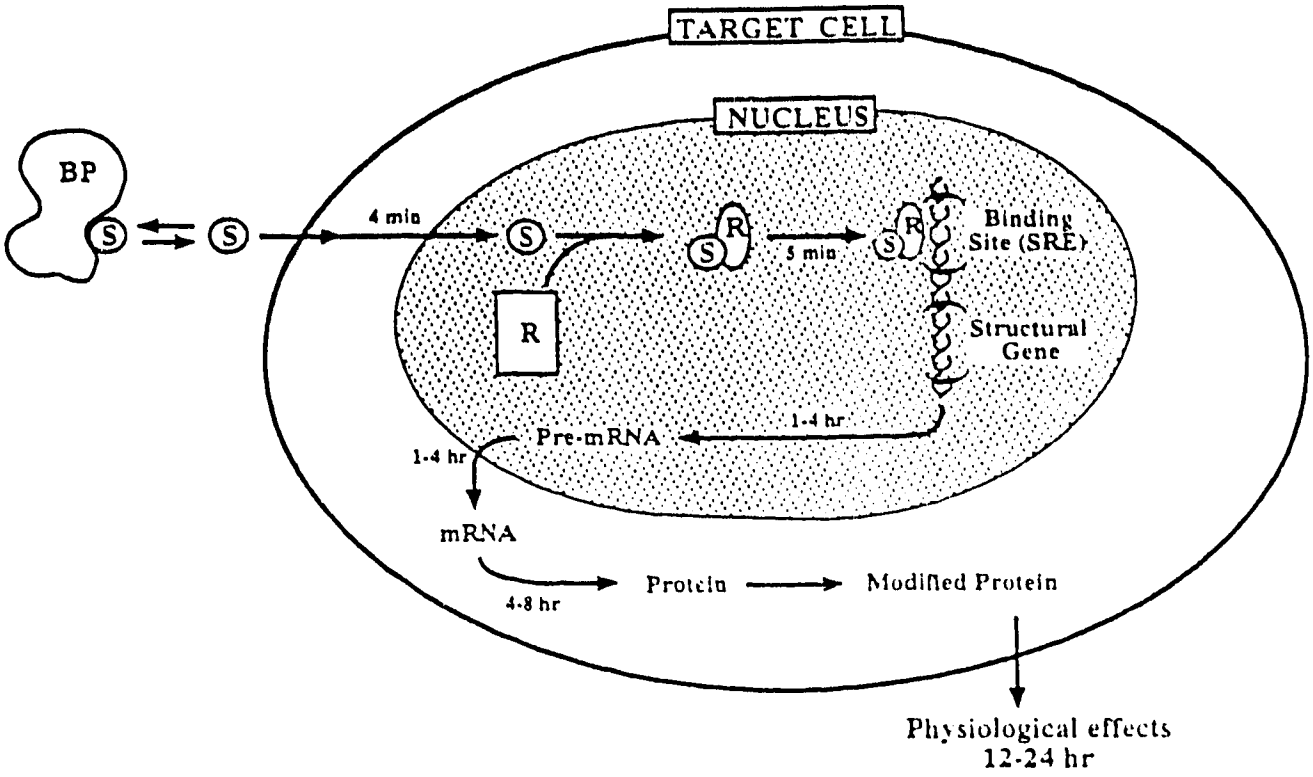

Fig. I-2 Mechanism of action for steroid hormones. The times represent the interval after exposure of the cell to the steroid. BP = steroid-binding protein; R = receptor; S = steroid; RS = receptor-steroid complex; SRE = steroid regulatory element (adapted from Landers and Spelsberg 1992).

Chapter II

MATERIALS AND METHODS

2.1. TENSION STUDY

Isometric contraction was measured in rat aortic rings and tail artery helical strips according to the method of Pang *et al.* (1985). Male Sprague-Dawley (SD) rats weighing 250-350 g were anaesthetized with pentobarbital (65mg/kg, i.p.) and the thoracic aortas and/or tail arteries were removed. Under a dissecting microscope the tissues were cleaned of adherent connective tissue. The tail arteries were cut into helical strips approximately 1.5 cm in length before being mounted in a 10-ml Sawyer-Bartlestone tissue bath chamber. The aortas were cut into rings 2-4 mm in length. Some of the rings were denuded of endothelium. A long strand of cotton was rubbed into approximately the same diameter as the aortic ring and inserted into the lumen of the rings. By carefully sliding the aortic rings back and forward along the cotton strand, the aortic ring endothelium was removed. Particular care was taken during this procedure to prevent any damage to the tissue. The aortic rings were then hooked in the Sawyer-Bartleston tissue bath chamber. The bath solution used to suspend the tissues was Krebs-Henseleit solution (KHS) with the following composition: 115 mM NaCl, 5 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose. The tissues inside the bath chamber were attached to a FT.03 Grass force displacement transducer and the tension of the tissues was recorded on a Grass 79 D polygraph (Grass Instrument Co., Mass, USA) or a

Gould polygraph (Gould Inc., Cleveland, Ohi, USA). The tissues were continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Before experimentation, the tissues were allowed to equilibrate for 60-120 min under a resting tension of 0.7 g for tail artery and 1 g for aorta. During the equilibration period, the tissues in the bath chamber were washed every 15 min. After equilibration, the tissues were tested for responsiveness by the application of KCl, 60 mM for tail artery helical strips and 30 mM for aortic rings. Only those tissue preparations showing repeatable contractions were used for the studies. An additional experiment was conducted with the aortic rings to determine whether the endothelium had been completely removed. After force generation following stimulation by KCl reached a stable stage, aortic rings were given ACh (10^{-5} M). Aortic rings with intact endothelium exhibited a 40-50% decline in tension while aortic rings completely denuded of endothelium showed no change.

2.2. PATCH CLAMP STUDY

Primary cultured VSMC (for culture procedure see "cell culture" in this chapter) were used and voltage-dependent inward calcium channel currents of the cell membrane were measured using the whole cell version of the patch clamp technique (Hamill *et al.*, 1981).

Primary cultured VSMC were allowed to grow in a CO_2 incubator over night prior to experimentation. The cells placeded in a 3 cm Petri dish were washed 2-3 times with the extracellular bath solution and only those cells with the following characteristics were

chosen for the electrophysiological experiments: spherical cells with a clear border and firm attachment to the bottom of the dishes. The Petri dishes with primary cultured VSMC were then placed on the stage of an inverted phase contrast microscope (Diaphot-TMD, Nikon, Tokyo, Japan).

The patch microelectrode pipettes were pulled from borosiliate thin wall glass capillary tubes (OD 1.2mm, ID 0.9mm, FHS, Brunswick, Maine, USA) with a two stage microelectrode puller (Narishige pp-83, Tokyo, Japan). The tips of the pipettes were fire polished with a microforge (Narishige MF-83, Tokyo, Japan). The tip diameter was approximately 1 μm with a resistance of 2-8 mΩ.

The intracellular solution used to fill up the microelectrode pipettes was composed of (in mM): Cs_2-aspartate 70, EGTA 10, ATP-Na_2 2, $MgCl_2$ 5, K-pyruvate 5, K-succinate 5, Phosphocreatine-Na_2 5, Creatine kinase 15 units/ml, HEPES 15 and Glucose 5. Among the components, Cs^+ was used to block the potassium channels; EGTA and zero Ca^{2+} minimized intracellular Ca^{2+} concentration so that the calcium-induced inactivation of calcium currents could be eliminated.

The extracellular solution buffering the cells contained (in mM): $BaCl_2$ 20, Tris 110, CsCl 5, KCl 5, Glucose 20, HEPES 20 and Tetrodotoxin (TTX) 0.5 mM. Ba^{2+} was the charge carrier moving through the Ca^{2+} channels to measure the currents since it is believed to be more permeable than is Ca^{2+} through L-type calcium channels (Fox *et al.*, 1987; Tsien and Tsien 1990). It has been also established that Ba^{2+} inhibits potassium currents and prevents calcium induced calcium channel inactivation (Brown *et al.*1982, Akbarali and Giles 1993). TTX and zero Na^+ concentration diminished sodium currents.

Above the stage of the microscope a micropipette filled with intracellular solution was connected to a head stage via an Ag/AgCl electrode. The patch pipette, controlled by a micromanipulator (Narishige Co., LTD, Tokyo, Japan), was pressed onto the chosen cell membrane. Once the "gigaseal" was established at the contact area by suction from the pipette, further suction was applied to rupture the patch.

The holding potential was set at -40 mV to measure the L-type inward calcium channel currents. A voltage commend of +10 mV was applied for 250 milliseconds at intervals of 5 seconds to monitor the current change. Test pulses from -30 to +80 mV (at 10 mV intervals) were used to depolarize the membrane and the data were recorded in order to plot the current-voltage relationship (I-V relationship). The membrane currents were monitored using a List EPC-7 patch clamp amplifier (Lis-Medical-Electronic, Darmstadt, Germany) and a digital oscilloscope (Nicolet Instrument Co., Madison, WI, USA). The data were sampled using pClamp software (version 5.5) and an Axolab 1100 (Axon Instruments, Inc., Burlingame, CA, USA) analog-to-digital converter and stored on the floppy disk of a personal computer (Zenith data system). Data were analyzed and the I-V relationship curves were plotted using the pClamp and the PSI software. Peak currents were always used when analyzing the data.

2.3. INTRACELLULAR CALCIUM MEASUREMENT

Fura-2 is a commonly accepted fluorescent Ca^{2+} indicator. The acetoxymethyl ester of the fluorescent tetracarboxylated chelator was used to measure the cytosolic free

calcium ($[Ca^{2+}]_i$) in cultured VSMC and endothelial cells. Primary cultured rat VSMC or subcultured endothelial cells (passage 3-7) were planted on circular glass coverslips in Petri dishes (4-6 days for VSMC and 2-3 days for endothelial cells). When the cells were confluent on the cover slips, fresh culture medium was used to replace the old one and Fura-2/AM dissolved in dimethyl sulfoxide (DMSO) was added to a final concentration of 3 μM. This loading process was carried out in the dark at room temperature for 50-60 min for VSMC and 30-45 min for endothelial cells. The cells were then washed and kept in recording buffer solution containing (in mM): NaCl 145, KCl 5, $MgCl_2$ 2, D-Glucose 10, NaH_2PO_4 0.5, HEPES 10, and $CaCl_2$ 2. The coverslips with Fura-2 loaded cells were mounted in a 1 ml Sykes-Moore chamber on the stage of an inverted phase contrast microscope (Diaphot-TMD, Nikon, Tokyo, Japan).

A Spex excitation fluorescence spectrophotometer and an IBM computer using CM3000DM program were used to measure the intensity of fluorescence of Fura-2 and to collect data during the experiments. The cells were irradiated alternately by excitation lights at 340 nM and 380 nM changing every second. The intensity of fluorescence emitted from the Fura-2 loaded cells was recorded at 510 nM. With an increase of $[Ca^{2+}]_i$ the intensity of fluorescence would increase at an excitation wavelength of 340 nM and decrease at 380 nM. $[Ca^{2+}]_i$ could be determined by using the following formula (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}]_i = K_d\,(R-R_{min})/(R_{max}-R) \times b$$

where K_d is the dissociation constant of Fura-2 for Ca^{2+} and is assumed to be 224 nM; R is the ratio of the intensity of fluorescence measured at 340 nM and 380 nM. R_{max} is the

value of ratio R when the indicator is saturated with Ca^{2+} and R_{min} is the value of ratio R when the indicator is in Ca^{2+} free form; b is the ratio of fluorescence intensities measured at 380 nM under conditions of very low and saturating concentrations of Ca^{2+} . R_{max} and R_{min} were obtained by applying 2 μM ionomycin or 10 mM EGTA to the bath solution, respectively.

2.4. CELL CULTURE

2.4.1 CULTURE OF VASCULAR SMOOTH MUSCLE CELLS

VSMC were cultured following the method of Wang *et al.* (1989). Male SD rats weighing 150-350 g were anaesthetized and the tail arteries were removed. Under the dissecting microscope, the arteries were cleaned of the surrounding connective tissues. The clean arteries were cut open longitudinally into 2-4 mm strips. The tissue was immersed in chilled Hank's Balanced Salt Solution (HBSS) and washed occasionally during the cleaning. After a 30 min rest at 4°C, the tissue was transferred to the first enzyme solution (EI) composed of: collagenase/dispase (1.5mg/ml), elastase (0.5mg/ml), trypsin inhibitor (1mg/ml) and bovine serum albumin (BSA, 2mg/ml) in HBSS with a low calcium concentration (0.2mM). The tissue was incubated in EI solution for 60 min in a CO_2 incubator (5% CO_2 and 95% air) at 37°C before being washed with Ca^{2+}-and Mg^{2+}-free HBSS (HBSS-CMF) 3 times. The second enzyme solution (EII) contained

collagenase (1mg/ml), trypsin inhibitor (0.3mg/ml) and BSA (2mg/ml) in HBSS-CMF. The tissue was incubated in the EII solution for 50-60 min. After the enzyme incubation, the tissue was washed with HBSS-CMF and triturated for 3 min using a fire polished Pasteur pipette. This was done to dissociate the smooth muscle cells from the tissue. The cells were placed at rest for 30 min at 4°C. Ca^{2+} was then gradually added to the cells to a final concentration of 2 mM.

The cells were seeded in 3cm Petri dishes in the Dulbecco's modified Eagle medium (DMEM) solution with insulin (31μg/ml). Four-6 hours later, the DMEM solution was replaced by the full culture medium: 10% fetal bovine serum (FBS) in DMEM. The cells were placed in the CO_2 incubator overnight. For patch clamp studies, the cells were used within 48 hours. For intracellular calcium measurement, the cells were seeded on circular glass coverslips and cultured for 4-6 days before use.

2.4.2. CULTURE AND CHARACTERIZATION OF RAT ENDOTHELIAL CELLS

2.4.2.1. Culture of rat endothelial cells

2.4.2.1.1. Explantation method

Rat endothelial cells were cultured following Hashimoto *et al.* (1991) with modification. The thoracic aorta were removed from male rats (SD 150-350 g) anaesthetized by pentobarbital (65mg/kg, i.p.).The procedure was conducted with extreme care and immediately following removal, the aortas were immersed in chilled Hank's

solution. Inside the tissue culture hood, the tissue was rinsed three times with HBSS + 3x antibiotic mixture solution. The periadventitial fat and connective tissue were carefully separated from the aorta and the aorta was cut open longitudinally into flat segments 3-4 mm in length. After being washed several times with the same HBSS solution, the aortic segments were planted endothelial-side down in Petri dishes moistened with culture medium (10% FBS in DMEM). The dishes were incubated in a CO_2 incubator with 5% CO_2 and 95% room air at 37°C. Culture medium was added to the dishes 24 hours later. Forty eight-72 hours after the beginning of the culture, the explants (aortic segments) were removed from the culture dishes to avoid the growth of smooth muscle cells. Upon confluence, the cells were trypsinized with 0.02% trypsin and 0.05% EDTA. Harvested cells were seeded into Petri dishes. At intervals, the cells were seeded on glass coverslips for endothelial cell identification with the immunofluorescence method.

2.4.2.1.2. Endothelial cell culture by enzyme dissociation

Rat aorta was cleaned with the procedure described in the explantation studies. The culture method was modified from Gordon *et al.* (1991). The cleaned aortas were then cut into rings 2-4 mm in length. The aortic rings were rinsed several times with dissecting medium (DM) containing 5% horse serum (EPDS). Collagenase/dispase (3.5mg/10ml) in DM was used to dissociate the aortic rings in a 50 ml, screw-topped Erlenmeyer flask in a shaking water bath for 3-4 hours at 37°C. After incubation, the tissue was transferred to a 15 ml tube and vortexed at high speed for 2-3 minutes to separate the endothelial cells from the rest of the vessel wall. The cells were washed and

suspended in 2 ml of DM before being layered onto the top of the Percoll gradient. A wide mouth pipette was used. The Percoll gradient containing the cells was centrifuged at 1650 xg for 10 min at 4°C. The middle part (about one third of the total amount) of the Percoll gradient, assumed to contain endothelial cells and some of the aortic rings, was collected and washed before being plated into Petri dishes. The aortic rings were removed and the culture medium was changed the next day. The culture dishes were coated with fibronectin ($5mg/cm^2$) for 30 min at 37°C and the fibronectin solution was aspirated in the tissue culture hood just before the cells were seeded. The culture medium used contained EPDS 15%, FBS 4%, sodium pyruvate 1 mM, L-glutamine 2 mM, and 1x antibiotic mixture solution in DMEM. The Percoll gradient was formed by centrifuging a mixture of: Percoll 43ml, 10x M119 5ml, 1M HEPES 1.5ml, antibiotic mixture solution 0.5ml, DM 50ml with 10% EPDS at 25,100 xg in a fixed angle rotor (Beckman JA-17, Fullerton, California, USA) for 70 min.

2.4.2.1.3. Explantation method with application of the Matrigel

Rat thoracic aorta was removed and cleaned as described above. The culture method was modified from McGuire and Orikin (1987). The flat aortic segments were placed endothelial-side down on top of the Petri dishes coated with Matrigel. Just enough medium was used to keep the preparation moist. This initial incubation was carried out in a humidified CO_2 incubator at 37°C in 5% CO_2 and 95% air. When the explants contacted the substratum (in approximately 24 hours) more complete medium was added to the dishes. Four days after beginning the culture, the explants were removed. Confluent cells

were passed with 2% dispase in HBSS-CMF. The subcultured cells were routinely passed with 0.05% trypsin and 0.02% EDTA and seeded in Petri dishes without coating. Cells from various passages were plated on circular glass coverslips for the immunofluorescence study. Using an inverted phase contrast microscope with camera attached, photographs were taken at different stages of cell outgrowth. Complete culture medium contained 20% FBS in RPMI 1640 plus endothelial cell growth supplement (100mg/ml).

Matrigel is a solubilized basement membrane to support the outgrowth of the endothelial cells. It rapidly and irreversibly gels at 22-35°C. The dish-coating procedure was carried out on ice. Matrigel was thawed and kept overnight at 4°C. All pipettes, plates, dishes and tubes were prechilled before use. After being coated with the Matrigel, the dishes were incubated for 30-60 min at 37°C. Complete medium was added into the dishes to equilibrate prior to the seeding of the cell.

The results from the three culture methods are as follows: (i) Endothelial cells cultured by the simple explantation method were always mixed with other types of cells. After selecting and culturing the cell clusters separately, the results were still not satisfactory. (ii) There were only a few cells produced by culture with the enzymatic method. Collagenase/dispase digestion did not separate enough endothelial cells from the aortic tissue. After centrifugation on the Percoll gradient, there were not many endothelial cells separated from other types of cells. In the bottom 1/3 of the centrifuge tube were red blood cells and small particles. The middle 1/3 of the centrifuge tube contained endothelial cells and aortic segments. From culture of this portion, endothelial cells grew out slowly. Aortic segments were removed 24 hours after the culture. (iii) Endothelial cells grew out well when he

explantation method was used along with Matrigel and endothelial cell growth supplement. Cells started to grow out from the aortic segments 48 hours after the culture began. The aortic segments were removed from the dishes before 4 days of culture. Endothelial cells were harvested upon confluence and then passed to subculture.

2.4.2.2 Identification and characterization of rat endothelial cells

Endothelial cells were identified by an immunofluorescence method (Booye *et al.* 1975) with antibodies specific for the von Willebrand Factor (vWF). Cells were allowed to grow to confluence on the circular glass coverslips. Culture medium was removed and the cells were rinsed three times with phosphate-buffered saline (PBS) to remove residual serum. The cells were then fixed with 100% acetone for 5 min and washed with PBS. To block any nonspecific binding, the cells were incubated with normal goat serum (1:20) in PBS for 20 min at room temperature. The first antibody, anti-human vWF developed in rabbit (at 1:50 in PBS) was applied on the top of the coverslips for 60 min at room temperature. Some coverslips were incubated with normal rabbit serum and used as a control group. After washing with PBS, the second antibody, anti-rabbit IgG FITC conjugate was added to the cells at 1:10 in PBS for 30 min at room temperature. After being washed three times, the coverslips were dried and mounted with buffered glycerin. The cells were examined by fluorescence microscopy using a blue excitation filter and a green barrier filter. Photographs were taken.

Endothelial cell identification with the antibody against vWF suggested that the culture using the method of explantation with Matrigel and endothelial growth supplement resulted in most of the cells being positive to the antibody. Fig.II-1 shows the result of the immunofluorescence study. A strong specific fluorescence is shown in a. The fluorescence is localised in the cytoplasm with little or no specific staining in the nucleus.

Effects of ATP, ACh, thrombin, washout (sheer stress) and KCl on $[Ca^{2+}]_i$ in the cultured rat endothelial cells were tested using Fura-2 as the Ca^{2+} indicator. Figs.II-2 and 3 represent the original recordings and the results. ATP (10^{-5} M), ACh (10^{-5} M), thrombin (3 unit/ml) and washout increased $[Ca^{2+}]_i$ in the cells while KCl (30 mM) did not change $[Ca^{2+}]_i$.

Rat endothelium has a very particular structural arrangement. In rat blood vessels there is no subendothelial layer which would serve as a target for enzymes to digest (Merrilees and Scott 1981). Although there are reports where enzymatic methods were used to culture rat endothelial cells (Gordon *et al.*1991), in more successful methods explantation was applied. One of the problems in the enzymatic methods is the risk that other types of cells may be digested out of the tissue as well. In the present study, the enzyme (collagenase/dispase) did not digest many endothelial cells from the aortic tissue segments. Centrifugation with Percoll gradient did not separate endothelial cells from other types of cells. Of the three methods used, the method of explantation with application of Matrigel and endothelial cell growth factor was the most successful and convenient method for culture of rat endothelial cells. The key points in this method are: (i) tissue segments should be removed from the culture within 4 days to avoid outgrowth of VSMC, (ii) Matrigel should be applied to selectively support the

outgrowth of endothelial cells, (iii) endothelial cell growth factors should be supplemented in the culture medium to promote endothelial cell growth.

The presence of the von Willebrand factor in most of the cells indicated that the cells cultured under the present condition were, indeed, endothelial cells. The results from $[Ca^{2+}]_i$ studies further suggested that like other typical endothelial cells these endothelial cells would exhibit an increase in $[Ca^{2+}]_i$ in response to stimulation by ATP, ACh, thrombin and sheer stress. KCl did not increase $[Ca^{2+}]_i$ in these cultured endothelial cells indicating that, again like other endothelial cells, no voltage-dependent Ca^{2+} channel existed in the membrane of these cells.

2.5. LIST OF CHEMICALS AND THEIR SOURCES

17β-: Sigma Chemical Co., Louis, MO, USA

ACH: Sigma Chemical Co., St. Louis, MO, USA

Aldosterone: Sigma Chemical Co., St. Louis, MO, USA

ATP: Sigma Chemical Co., St. Louis, MO, USA

ATP-Na_2: Sigma Chemical co., St. Louis, MO, USA

BSA: Sigma Chemical Co., St. Louis, MO, USA

Collagenase (Type II): Sigma Chemical Co., St. Louis, MO, USA

Creatine kinase: Sigma Chemical Co., St. Louis, MO, USA

Creatine phosphate-Na_2: BDH Inc., Toronto, Ontario, Canada

Cs-aspartate: Department of Chemistry, University of Alberta, Edmonton, AB, Canada

Dexamethasone: Sigma Chemical Co., St. Louis, MO, USA

DM : Gibco, Grand Island, NY, USA

DMEM: Gibco, Grand Island, NY, USA

DMSO: Fisher Scientific, Fair Lawn, NJ, USA

EGTA: Sigma Chemical Co., Louis, MO, USA

Elastase (Type I): Sigma Chemical Co., Louis, MO, USA

Endothelial cell growth supplement: Collaborative Biomedical Products, Two Oaks Park, MA, USA

EPDS (horse serum): Sigma Chemical Co., St. Louis, MO, USA

FBS : Sigma Chemical Co., St. Louis, MO, USA

Fibronectin: Sigma Chemical Co., St. Louis, MO, USA

Fura-2/AM: Molecular Probes Inc., Eugene, OR, USA

HBSS: Gibco, Grand Island, NY, USA

Insulin : Sigma Chemical Co., St. Louis, MO, USA

Ionomycin : Sigma Chemical Co., St. Louis, MO, USA

K-pyruvate: Pfalts & Bauer Inc., Waterbury, CT, USA

K-succinate: Pfalts & Bauer Inc., Waterbury, CT, USA

L-glutamine: Sigma Chemical Co., St. Louis, MO, USA

L-NMMA: Research Biochemicals International, Natick, MA, USA

Matrigel: Collaborative Biomedical Products, Two Oaks Park, MA, USA

NGS: Sigma Chemical Co., St. Louis, MO, USA

NRS: Sigma Chemical Co., St. Louis, MO, USA

Pentobarbital sodium: M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada.

Progesterone: Sigma Chemical Co., St. Louis, MO, USA

PSN antibiotic mixture: Gibco, Grand Island, NY, USA

RPMI 1640: Gibco, Grand Island, NY, USA

Sodium pyruvate: Sigma Chemical Co., St. Louis, MO, USA

Testosterone: Sigma Chemical Co., St. Louis, MO, USA

Thrombin: Sigma Chemical Co., St. Louis, MO, USA

Trypsin EDTA: Gibco, Grand Island, NY, USA

Trypsin inhibitor (Type I): Sigma chemical Co., St. Louis, MO, USA

Trypsin (Type III): Sigma Chemical Co., St. Louis, MO, USA

TTX: Sigma Chemical Co., St. Louis, MO, USA

2.6. STATISTICS

The data were presented as mean ± S.E.. The paired or non-paired Student's t test was used for comparisons between two groups. The Newman Keuls test was applied when the comparison was among multiple groups. P values less than 0.05 were considered statistically significant and shown as "*". P values less than 0.01 were showed as "**".

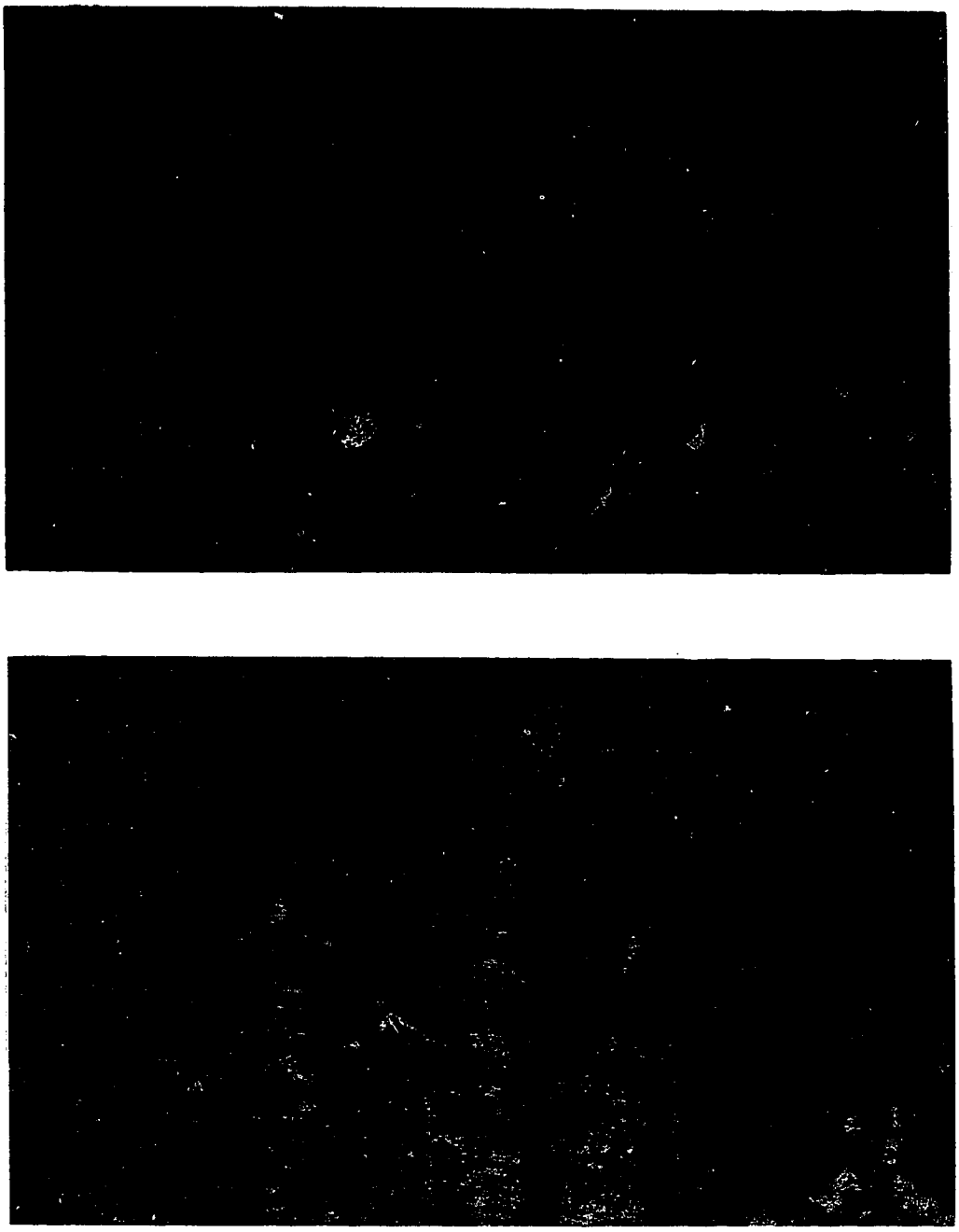

Fig. II-1 Immunofluorescence staining of rat endothelial cells. Upper: demonstrating the presence of vWF in endothelial cells (passage 4). Lower: control staining (anti-vWF was not applied) for endothelial cells.

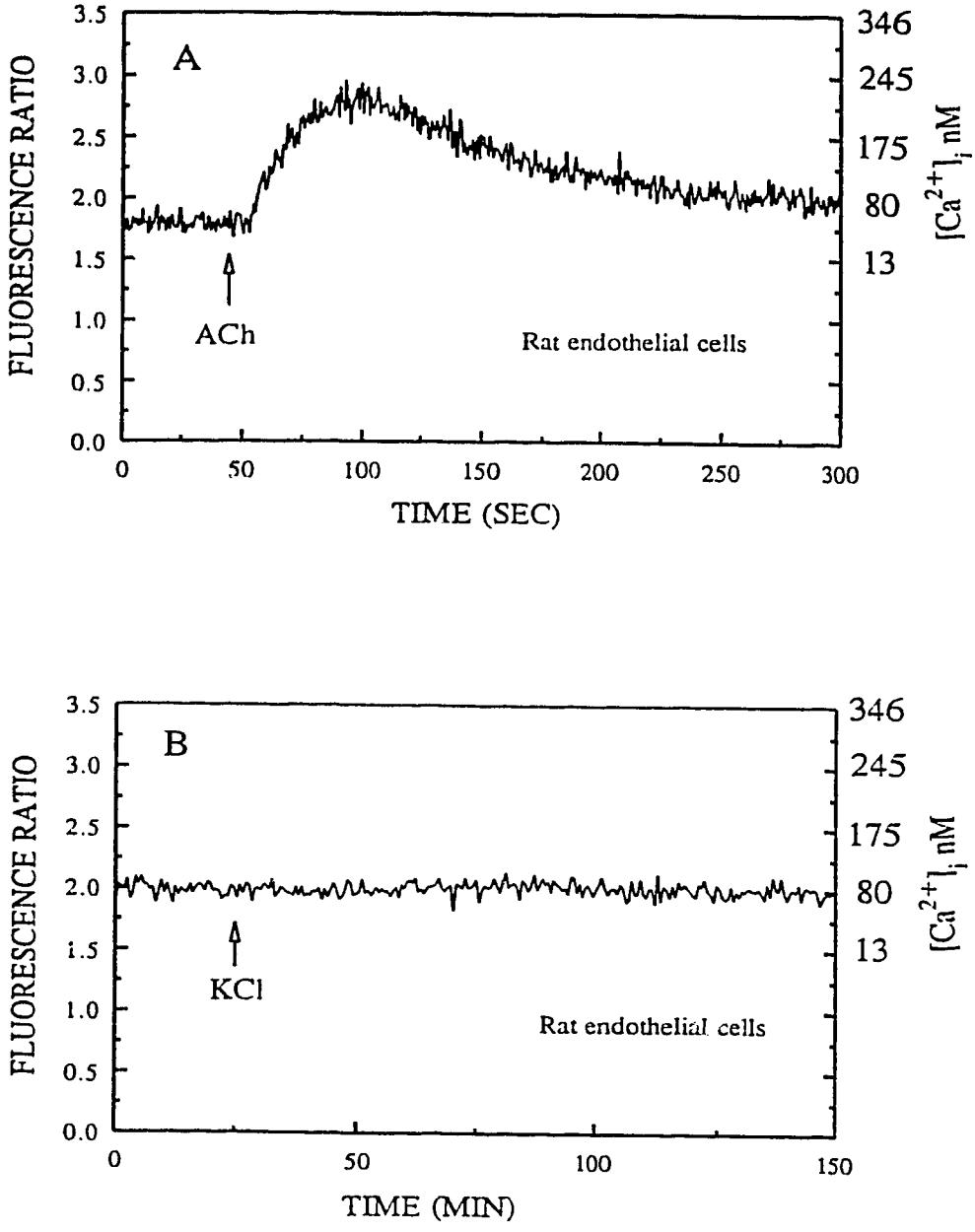

Fig.II-2 Original records of $[Ca^{2+}]_i$ in cultured rat aortic endothelial cells. A, effect of ACh (10^{-5} M). B, effect of KCl (30 mM).

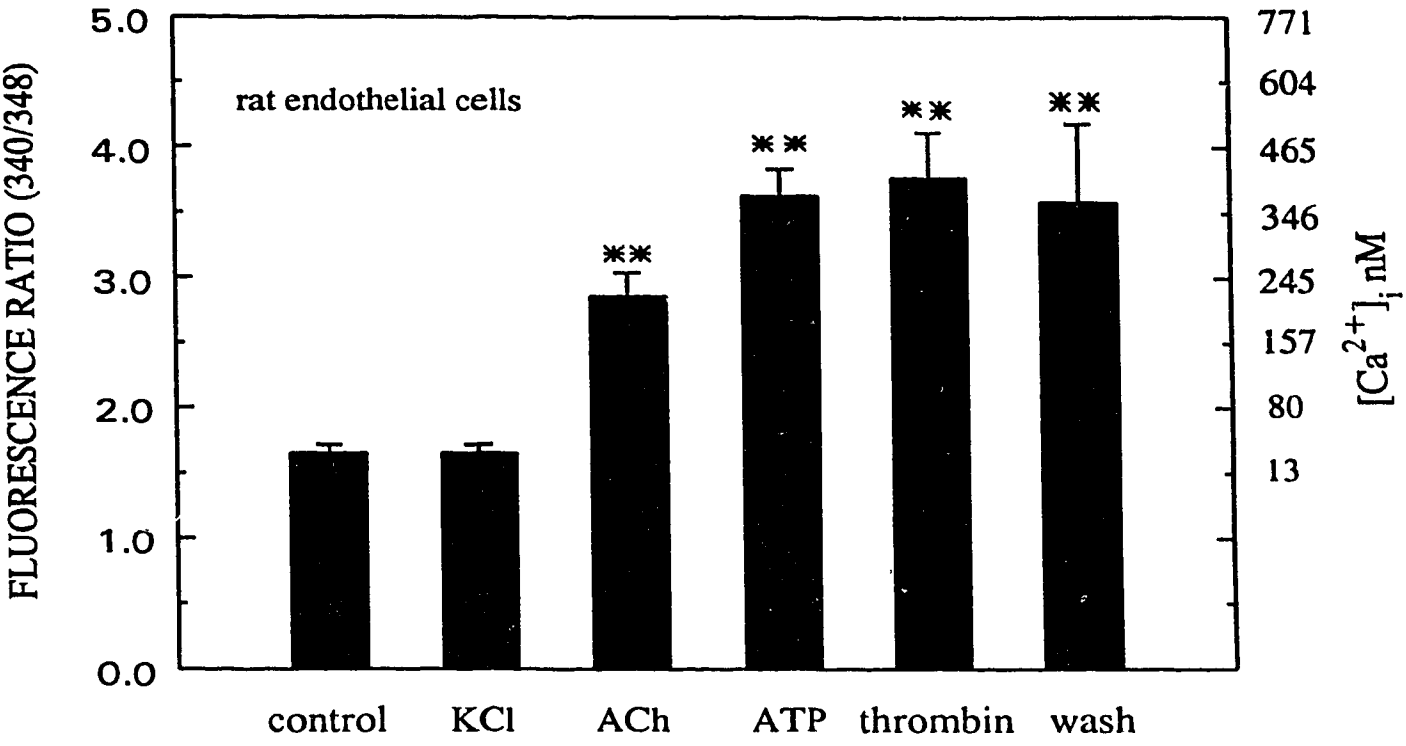

Fig. II-3 Effects of KCl (30 mM), ACh (10^{-5} M), ATP (10^{-5} M), thrombin (3 units/ml) and wash (sheer-stress) on $[Ca^{2+}]_i$. in rat endothelial cells.

Chapter III

THE EFFECTS OF PROGESTERONE ON TENSION GENERATION IN RAT AORTA AND TAIL ARTERY

3.1 INTRODUCTION

Progesterone is an important reproductive hormone in the female and is secreted regularly by the corpus luteum. Apart from its function in the full expression of female sexual behaviour, progesterone is essential to the maintenance of pregnancy by inhibiting myometrial contraction. In the late stages of pregnancy, the placenta secretes a large amount of progesterone. Prior to delivery, the circulating level of progesterone will decline in some women while a progesterone binding protein will be secreted by the placenta to reduce the available free progesterone in the circulation in preparation for labor.

Intensive studies on the effect of progesterone on myometrial contractility demonstrated that progesterone inhibits uterine contraction (Perusqúa *et al.* 1990; Putnam *et al.* 1991; Sanchez Aparicio *et al.* 1992; Cabral *et al.* 1994; Gutierrez *et al.* 1994). Some studies have also indicated that progesterone had effects on vasculature. Progesterone caused relaxation in canine and rabbit coronary arteries (Miller and Vanhoutte 1991; Jiang *et al.* 1992b). Experiments on rabbit basilar arteries mimicking the progesterone surge during the luteal phase showed that progesterone withdrawal increased contractility of the tissue to serotonin (Futo *et al.* 1992).

More studies have been carried out on the effects of estrogen on the vasculature. Estrogen was reported to have relaxant effects on arteries (Jiang *et al.* 1991; Futo *et al.* 1992; Gilligan *et al.* 1994; Shan *et al.* 1994) and to augment vasoconstriction (Miller and Vanhoutte 1990).

The effects of progesterone and estrogen on vascular smooth muscle and uterine smooth muscle were suggested to occur through the production of NO and the cGMP system. They were believed to be most likely a non-genomic process (Goetz *et al.* 1994; Weiner *et al.* 1994; Yallampalli *et al.* 1994a).

The aims of the present study were (1) to examine the effects of progesterone on the contractility of rat aortic ring and tail artery, (2) to investigate the time course of progesterone effects and the reversibility of the effects by washout to determine whether the effects are mediated by a conventional genomic mechanism or by a rapid non-genomic mechanism, and (3) to examine if the progesterone effects are endothelium-dependent.

3.2 EXPERIMENTAL DESIGN

3.2.1. *Effects of progesterone on tension generation in isolated rat aortic ring and tail artery helical strips.*

3.2.1.1. The relaxant effects of progesterone on tonic tension

KCl (30 mM) was used to generate tonic tension in aortic rings with the endothelium intact or denuded. After the tension become stable, cumulative doses (10^{-10}-10^{-5} M) were applied to the bath solution to obtain a dose response curve.

KCl at a concentration of 60 mM was used to challenge the tonic tension in tail artery helical strips. A single dose of progesterone (10^{-5} M) was applied during the contraction.

3.2.1.2. The inhibitory effects of progesterone on phasic tension

Phasic tension was generated in aortic rings by cumulative doses of KCl (30 mM - 40mM). This tension was taken as the control value. After washout of KCl containing bath solution and a recovery period, the tissue was challenged with the same cumulative doses of KCl.

3.2.2. *Function of endothelium in the effects of progesterone on tension in aorta*

3.2.2.1. Comparison of progesterone relaxant effects on endothelium-denuded and endothelium-intact aortic rings

The endothelium layer was gently removed from the aortic rings and ACh was used to test if the removal was complete. Tonic tension was produced by 30 mM KCl and progesterone was added during the contraction.

Each pair of endothelium-denuded and -intact aortic rings are from the same rat.

3.2.2.2. The reversal effects of L-NMMA on progesterone pre-treated aortic rings

To study the mechanism of the endothelium-dependent relaxation effect of progesterone, L-NMMA, a NO synthase antagonist, was used. Both endothelium-denuded and -intact aortic rings were challenged by KCl followed by progesterone. After the relaxation

effect of progesterone reached its maximum point, L-NMMA (10^{-5} M) was added to reverse the proposed effect of NO which is produced by endothelium cells in response to vasodilators.

The results from endothelium-denuded and -intact tissues were compared to the control values.

3.2.3. *Reversal of the inhibitory effects of progesterone by washout*

These experiments were designed to investigate whether the inhibitory effect of progesterone is a rapid, non-genomic action or a conventional genomic one.

Rat aortic rings with endothelium-intact or -denuded were stimulated by KCl, the maximum point of contraction was taken as the control value. After a rest period (20-30 min), progesterone (10^{-5}M) was given. The tissue was incubated for 15 min which is, as shown by previous experiments, sufficient time for progesterone to act. Progesterone then was removed by changing the progesterone-containing bath solution. The tissue in the normal bath solution was washed several times during the time period of 5, 10, 15, or 30 min. When the washing within the various time periods was complete, KCl was given again to test the degree to which the contractility of the tissue was restored after inhibition by progesterone.

Repeated KCl stimulation and solvent application were carried on as for the control values.

3.3. RESULTS

3.3.1. *The relaxant effects of progesterone on tonic tension in aortic rings and tail artery helical strips.*

In fig.III-1 shows the relaxation effect of progesterone on tail artery helical strips. The tension was decreased by 55.6 ± 4% at a progesterone concentration of 10^{-5} M.

Fig. III-2 presents the relaxation effect of progesterone on aortic rings with and without endothelium. The tissue was first stimulated by 30 mM KCl to generate a tonic contraction. Progesterone was then administered. In aortic rings which had an intact endothelium layer, the relaxation effects began when progesterone concentration increased to 10^{-7} M (decrease in tension by 11 ± 2%). In the aortic rings without endothelium, the effective concentration of progesterone was 10^{-5} M (decrease in tension by 27 ± 2%). In this study, the highest concentration used was 10^{-5} M. The difference in the extent of the relaxation effect between endothelium-intact and -denuded tissues should be noted.

Fig.III-3, typical original recording traces of A, aortic ring and B, tail artery are shown. In B, only one dose of progesterone (10^{-5} M) was applied. From Fig.III-3 two significant points should be noticed. First, the effects of progesterone on aortic rings were concentration -dependent. Second, the effects of progesterone on aortic rings and tail arteries were fast in onset. They occurred immediately after drug application.

3.3.2. *The inhibitory effects of progesterone on phasic tension development in aortic rings*

Progesterone also inhibited phasic tension development in aortic rings. The tissue was first challenged by cumulative concentrations of KCl (30-40 mM) to cause a phasic contraction. This was recorded as the control value. After KCl washout and tissue recovery, one dose of progesterone (10^{-5} M) was applied to the chamber and incubated with the tissue for 15 min. The KCl challenge procedures were then repeated for the control group. Fig.III-5 shows the results of phasic tension change after progesterone incubation in endothelium-intact and -denuded aortic rings. The tension was expressed as grams of force.

3.3.3. *Rapid in onset of the relaxant and inhibitory effects of progesterone on tension generation in aortic rings and tail artery helical strips*

It is obvious from Fig.III-3 that the effects of progesterone on aortic rings and tail artery occurred immediately. Fig.III-6 presents original traces of phasic tension generation in aortic rings with and without endothelium. The time duration for the incubation of progesterone with the tissue was 15 min. The inhibitory effects of progesterone occurred immediately after incubation. Fig.III-4 shows the different extent of the effects of progesterone on endothelium-intact and -denuded aortic rings. It also indicates the rapid onset of these effects.

3.3.4. *The endothelium-dependent effects of progesterone on tension generation in aortic rings.*

3.3.4.1. The relaxant and inhibitory effects of progesterone on aortic rings with intact or denuded endothelium

It is clear from Fig.III-2 that progesterone had a greater relaxant effect in endothelium-intact tissue than in endothelium-denuded tissue stimulated by KCl. Fig.III-4 presents the original recording traces showing the relaxant effects of progesterone in endothelium-intact and -denuded aortic tissue, respectively. The relaxation effect of progesterone in this experiment was about 30% greater in endothelium-intact tissue than in endothelium-denuded tissue. The tissues were prepared from the same rat and the completeness of removal of the endothelial layer was tested by applying ACh (10^{-5} M) before the experiments started. Fig.III-6 shows the difference in the effect of progesterone on phasic tension change in aortic rings with and without endothelium.

3.3.4.2. The involvement of NO in the effects of progesterone on endothelium-intact aorta.

L-NMMA, a NO synthase antagonist, was applied once the relaxant action of progesterone had been established. The lower panel in Fig.III-7 is an actual experimental recording showing the partial reversal by L-NMMA of the relaxant effect of progesterone in endothelium-intact tissue. However, the upper panel in Fig.III-7 shows that endothelium-denuded tissue did not respond to L-NMMA.

Fig.III-8 presents the results of a group of experiments which indicate that L-NMMA could partially reverse the relaxant effects of progesterone in endothelium-intact tissue but not in the endothelium-denuded one.

3.3.5. *Rapid reversal of the effects of progesterone on tension generation in aortic rings*

In both endothelium-intact and -denuded tissue pre-treated with progesterone, phasic contraction development could be restored. As soon as 5 min after washout it returned to control levels.

Fig.III-9 displays the results of a set of experiments with different time periods for washout. Fig.III-10 shows the recording traces of the reverse of the inhibitory effect after a shorter washout of less that 10 min

3.3.6. *Effects of repeated KCl stimulation and solvent for steroids on the contractility of aortic rings*

Fig.III 11 shows repeated KCl stimulation within 60 min to endothelium-intact and -denuded aortic tissue. There was no significant difference detected.

Fig.III-12 presents the solvent effect on both endothelium-intact and -denuded aortic rings. No significant difference is indicated between either the experimental groups and the control groups or between the two experimental groups.

3.4. DISCUSSION

3.4.1. *The relaxant and inhibitory effects of progesterone on vasoconstriction*

It has been established that progesterone maintains pregnancy in mammals by inhibiting the contraction of the myometrium. During pregnancy circulating levels of progesterone increase to 10 times the levels found during the ovarian cycle. Studies on rat uterine contraction showed clearly that progesterone, not estradiol, was responsible for the inhibition of uterine smooth muscle contraction during pregnancy to maintain uterine quiescence. These effects were mediated by the NO-cGMP producing system and accompanied by inhibition of Ca^{2+} influx (Cabral *et al.* 1994; Gutierrez *et al.* 1994; Yallampalli *et al.* 1994). The mechanism of these effects was suggested to be a non-genomic membrane-related process. There has also been a report that progesterone had a relaxing effect on human myometrium contraction (Kostrzewska *et al.* 1993).

However, contrary results were obtained in studies conducted on the vascular system. Investigations with human uterine arteries showed that both estrogen and progesterone had inhibitory effects on contractions induced by K^+ depolarisation, but at a low dose (0.2 μM) the arterial response to vasopressin was enhanced (Kostrzewska *et al.* 1993). In studies on canine coronary arteries, results indicated that progesterone generated greater relaxation of smooth muscle in response to ADP (Miller and Vanhoutte 1991). Progesterone also inhibited the contraction of rabbit common iliac arterial rings (Hidaka *et al.* 1991). However, experiments with rat aorta showed that estrogen, but not progesterone, induced an increase in nitric oxide

synthase (NOs-III), which is believed to be responsible for the production of the endothelium-derived vasodilator, NO (Goetz *et al.* 1994).

In regard to the endothelium-dependency of the relaxation effect of progesterone, the reports are inconsistent. In investigations using human arteries, some results showed that the effect was endothelium-dependent (Woolfson *et al.* 1992) while others showed that it was endothelium-independent (Omar *et al.* 1995). Studies on animals also gave different results. Among the experiments, those which showed an endothelium-dependent effects were conducted with rabbit common iliac arteries (Hidaka *et al.* 1991) and canine coronary arteries (Miller and Vanhoutte 1991) while those showing endothelium-independent effects were conducted with rabbit coronary artery (Jiang *et al.* 1992). Therefore, the endothelium may have different functions depending on the species and the vascular bed investigated.

In the present study, progesterone showed obvious relaxant and inhibitory effects on vasoconstriction stimulated by K^+ depolarisation. These effects were concentration dependent. Differences in the extent of the effects between endothelium-intact and -denuded tissue indicated that the effects were endothelium-dependent. By application of the NO synthase antagonist L-NMMA after progesterone treatment, the contraction was partially reversed. This suggests that the relaxation effect of progesterone was partially endothelium-dependent and may be mediated through NO synthesis.

3.4.2. *Rapid onset and reversal of the effects of progesterone*

Previous reports have shown that inhibitory effects of progesterone on myometrium contraction are fast in onset (approximately 10 min) and related to the inhibition of Ca^{2+} influx

(Perusquia *et al.* 1990; Cabral *et al.* 1994; Gutierrez *et al.* 1994). These effects were not modified by inhibitors of protein synthesis (such as cycloheximide) or inhibitors of transcription (such as actinomycin).

Rapid effects of estrogen on the vasculature have been investigated in rat, dog and rabbit (Jiang *et al.* 1991; Ravi *et al.* 1994; Shan *et al.* 1994; Sudhir *et al.* 1995). In the studies on canine coronary arteries, the vasodilatory effect was not inhibited by classic intracellular estrogen-receptor antagonists.

However, there are fewer reports of the rapid effects of progesterone on the vasculature. Studies on rabbit coronary artery indicated that progesterone at micromolar or higher concentrations induced relaxation in K^+, prostaglandin or Bay K 8644 precontracted arteries (Jiang *et al.* 1992b).

Steroid hormones diffuse across the cell membrane into the cytosol. Hormone binding to intracellular receptors leads to gene transcription, translation, and protein synthesis. These genomic effects occur within hours or days. Once the reaction starts, it will continue even in the absence of the hormones. These reactions can be inhibited by inhibitors of protein synthesis. In contrast, non-genomic effects of steroid hormones are defined as rapid in onset and not modified by inhibitors of protein synthesis (Schumacher 1990; Cabral *et al.* 1994).

The fast effects of progesterone on myometrial contraction were suggested to be non-genomic because they occurred rapidly, could not be modified by inhibitors of protein synthesis and could be reversed quickly by $CaCl_2$ (Cabral *et al.* 1994). The effect of estrogen on canine arteries was also believed to be a non-genomic process because it is rapid in onset and unmodified by classic intracellular estrogen-receptor antagonists. In both examples of the non-

genomic effects, the investigators suggested that these effects were possibly mediated through membrane receptors different from the classic intracellular receptors.

In the present study, the effects of progesterone occurred rapidly in both endothelium-intact and endothelium-denuded aortic rings and in rat tail artery. In the original trace recordings presented in Fig.III-3 and -4, it was obvious that the effect of progesterone took place with no latency. After the establishment of the effect of progesterone, application of L-NMMA could partially restore the contraction. This reaction also happened immediately. This result suggests that progesterone exerts its effect partially through the endothelium.

A more significant finding of the present studies was that the effect of progesterone could be reversed by washout of the steroid hormones from bath solutions. Fig.III-9 indicates these results. After washout and recovery in various periods of time from 5-30 min, the contractility of the aortic tissue was fully restored. This was true of both endothelium-intact and -denuded tissue.

In summary, progesterone had relaxant and inhibitory effects on tonic and phasic tension generation in rat aortic ring and tail artery helical strips. These effects were concentration-dependent. The effects of progesterone on rat aortic tissue were partially endothelium-dependent and could be reversed by the NO synthase antagonist L-NMMA.

The effects of progesterone on aortic ring and tail artery were fast in onset. The effect of progesterone on aortic rings could be quickly reversed by washout. Therefore, the effects were likely mediated by a non-genomic mechanism and may occur through a membrane process. Progesterone acted on both vascular smooth muscle and endothelium.

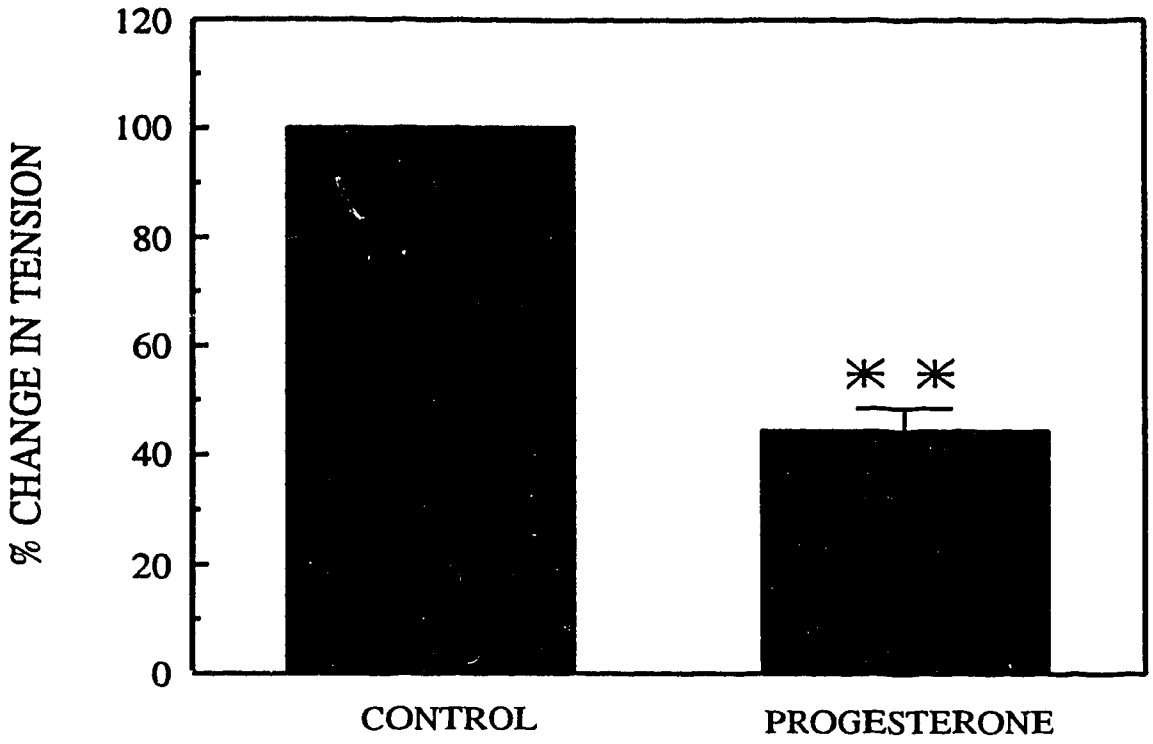

Fig.III-1 Effect of progesterone (10^{-5} M) on tail artery helical strips. The tissue was pre-contracted by 60 mM KCl before the addition of progesterone. ** significantly different from control values.

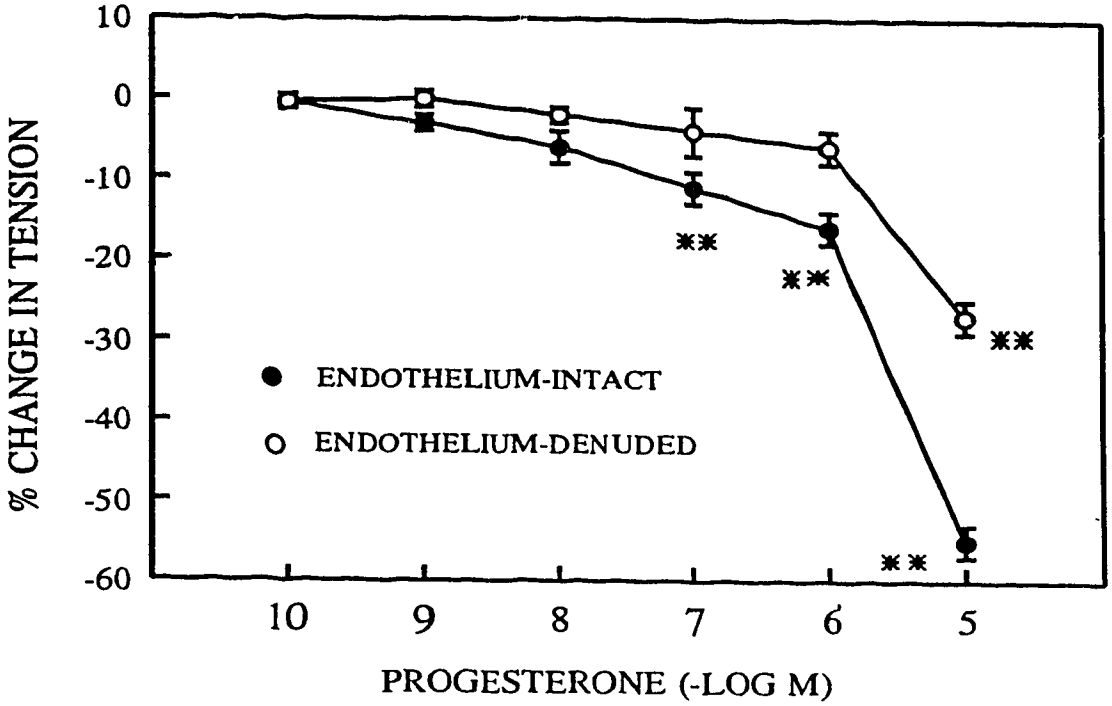

Fig. III-2. The effect of progesterone on tension in aortic rings. Cumulative concentrations(10^{-10} - 10^{-5} M) were applied after the tissue was stimulated with KCl (30 mM).** significantly different from control values.

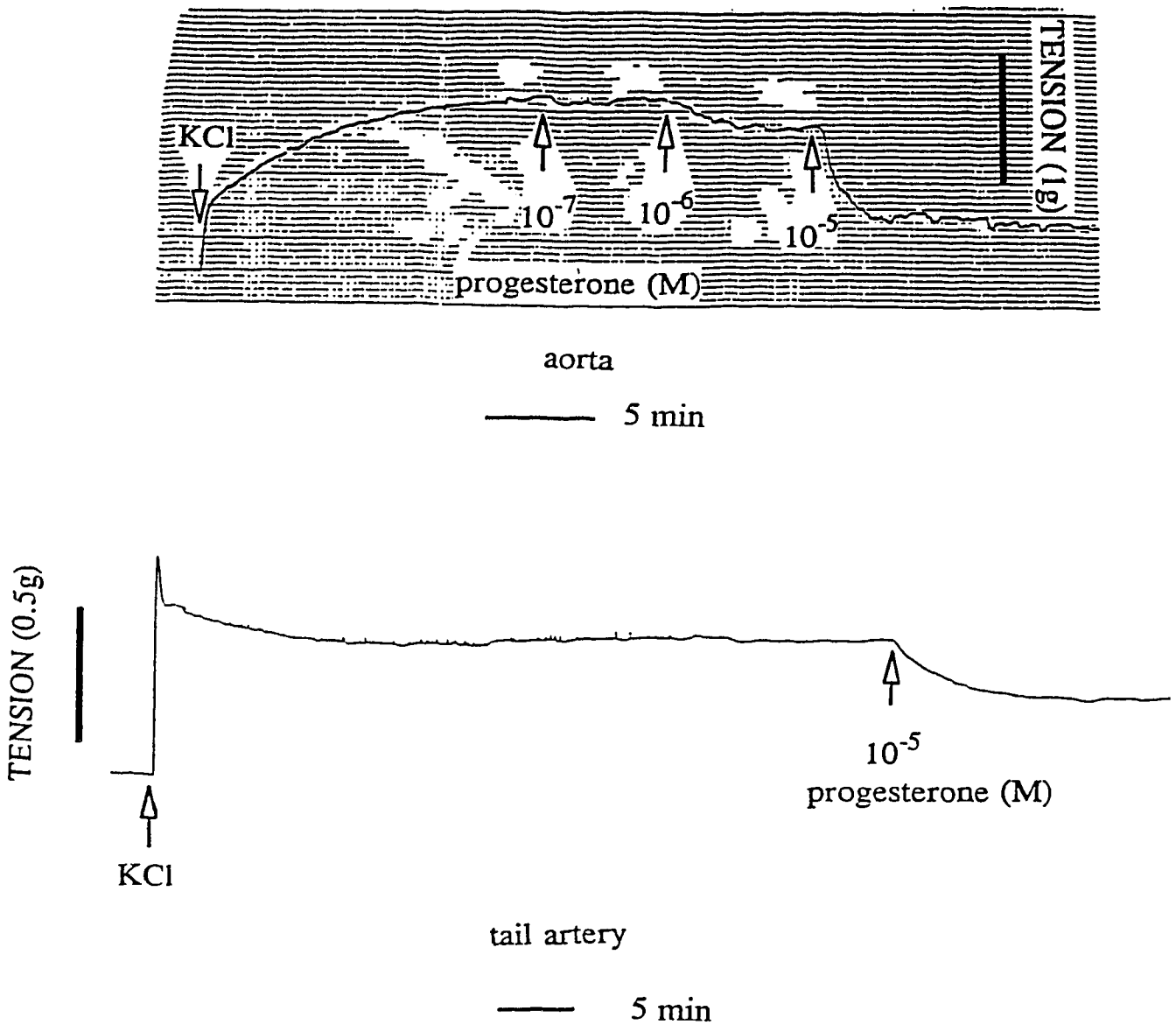

Fig.III 3. The effect of progesterone on tension in aortic rings and tail artery helical strips. Upper panel: original tracing of one typical experiment showing the relaxation effect of progesterone on an aortic ring. Lower panel: original tracing of one typical experiment showing the relaxation effect of progesterone on tail artery helical strip.

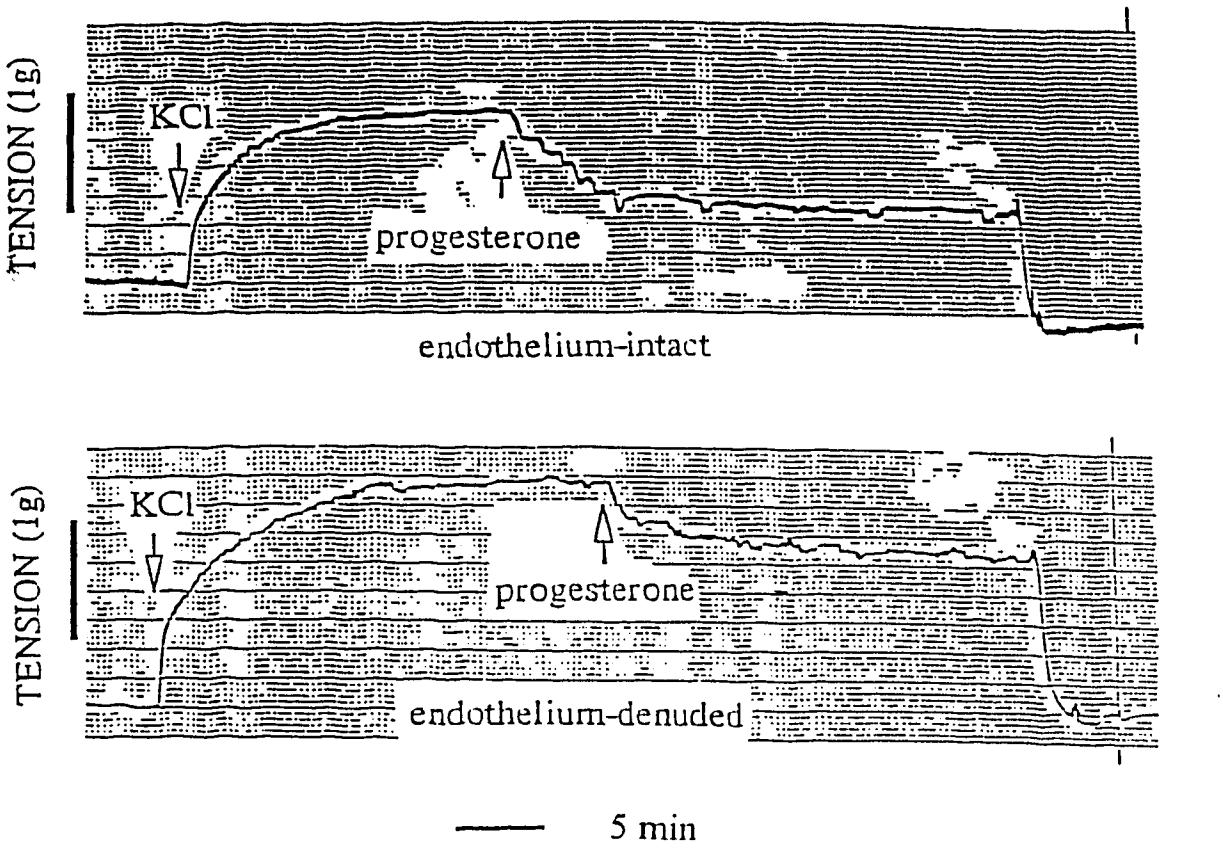

Fig. III-4. Relaxation effect of progesterone (10^{-5} M) on tonic tension in aortic rings. Upper panel: original tracing of a typical recording from an endothelium-intact aortic ring. Lower panel: original tracing of a typical recording from an endothelium-denuded aortic ring.

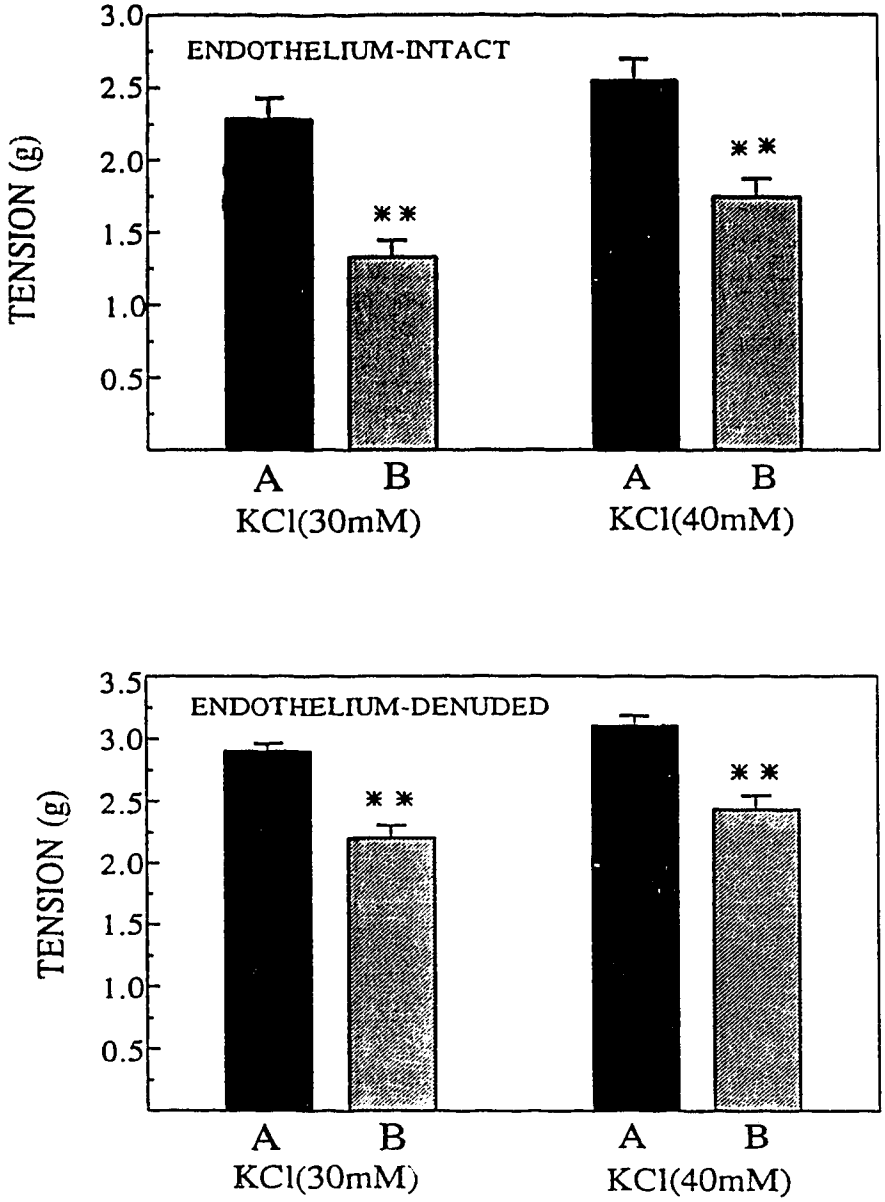

Fig.III-5. Effects of progesterone (10^{-5} M) on phasic tension generation in aortic rings. The tissue was challenged by KCl before and after incubation with progesterone for 15 min. A, KCl. B, progesterone + KCl. Upper pannel: endothelium-intact tissue. Lower pannel: endothelium-denuded tissue. ** significantly different from A.

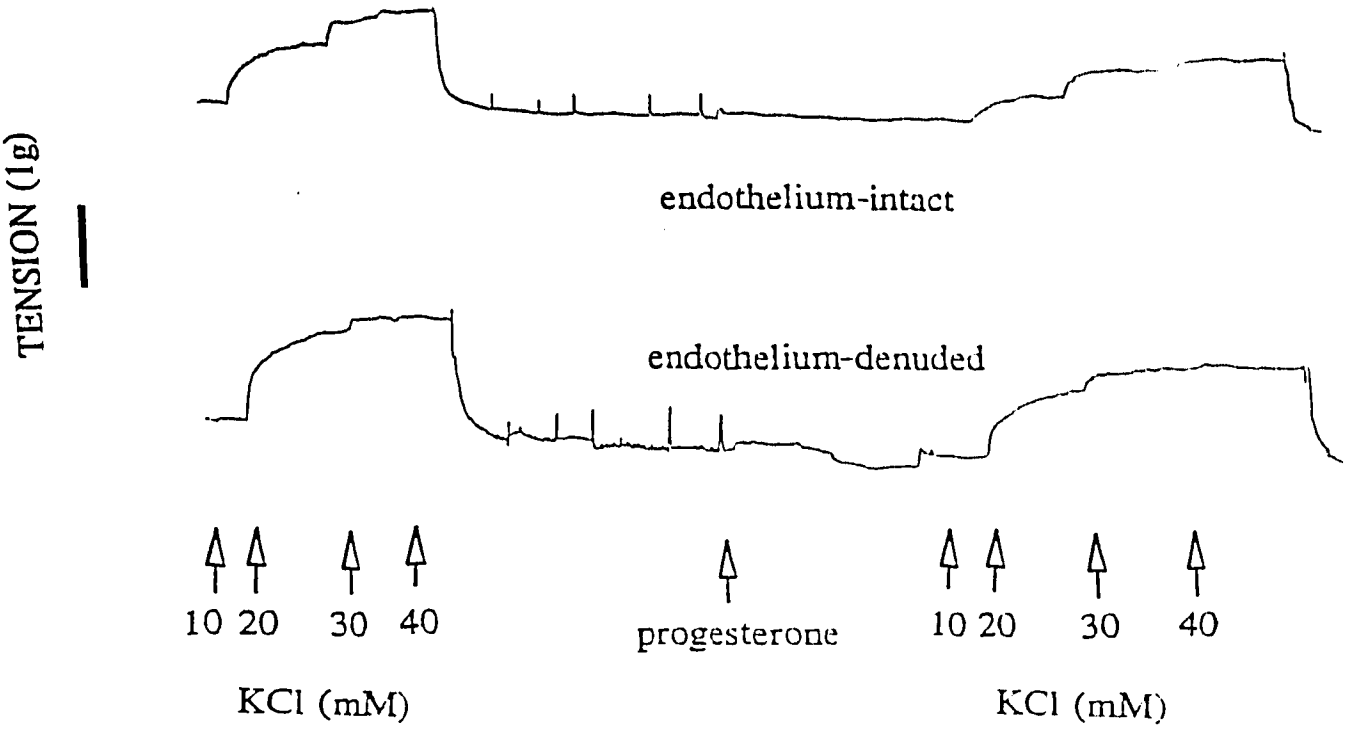

Fig.III-6. Original traces showing the effect of progesterone (10^{-5} M) on phasic tension generation in aortic rings.

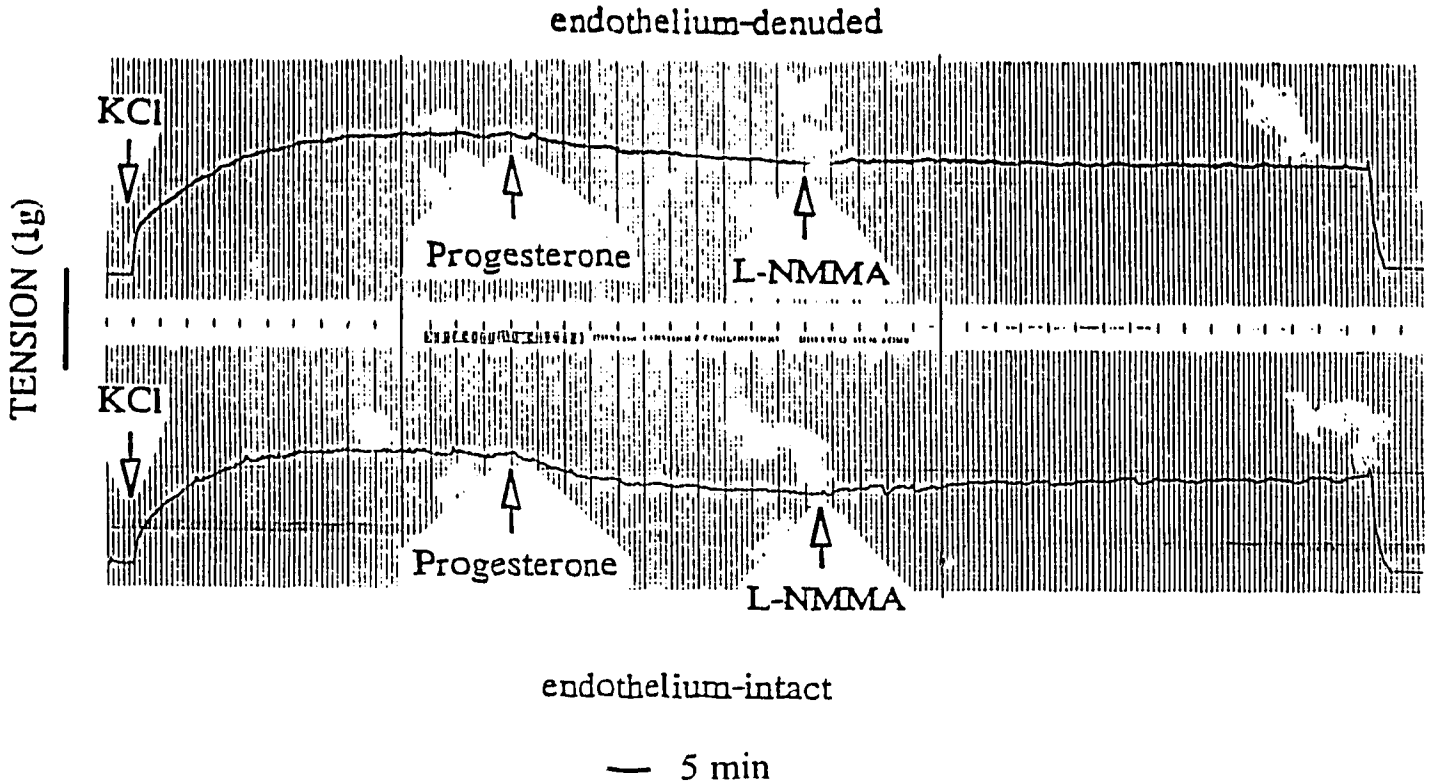

Fig.III-7 Original traces showing the reversal of the effect of progesterone by L-NMMA in aortic rings. Upper panel: endothelium-denuded aortic ring. Lower panel: endothelium-intact aortic ring. The tissue was stimulated by KCl (30mM) before the application of progesterone (10^{-5} M). L-NMMA was added when the effect of progesterone reached its maximum point.

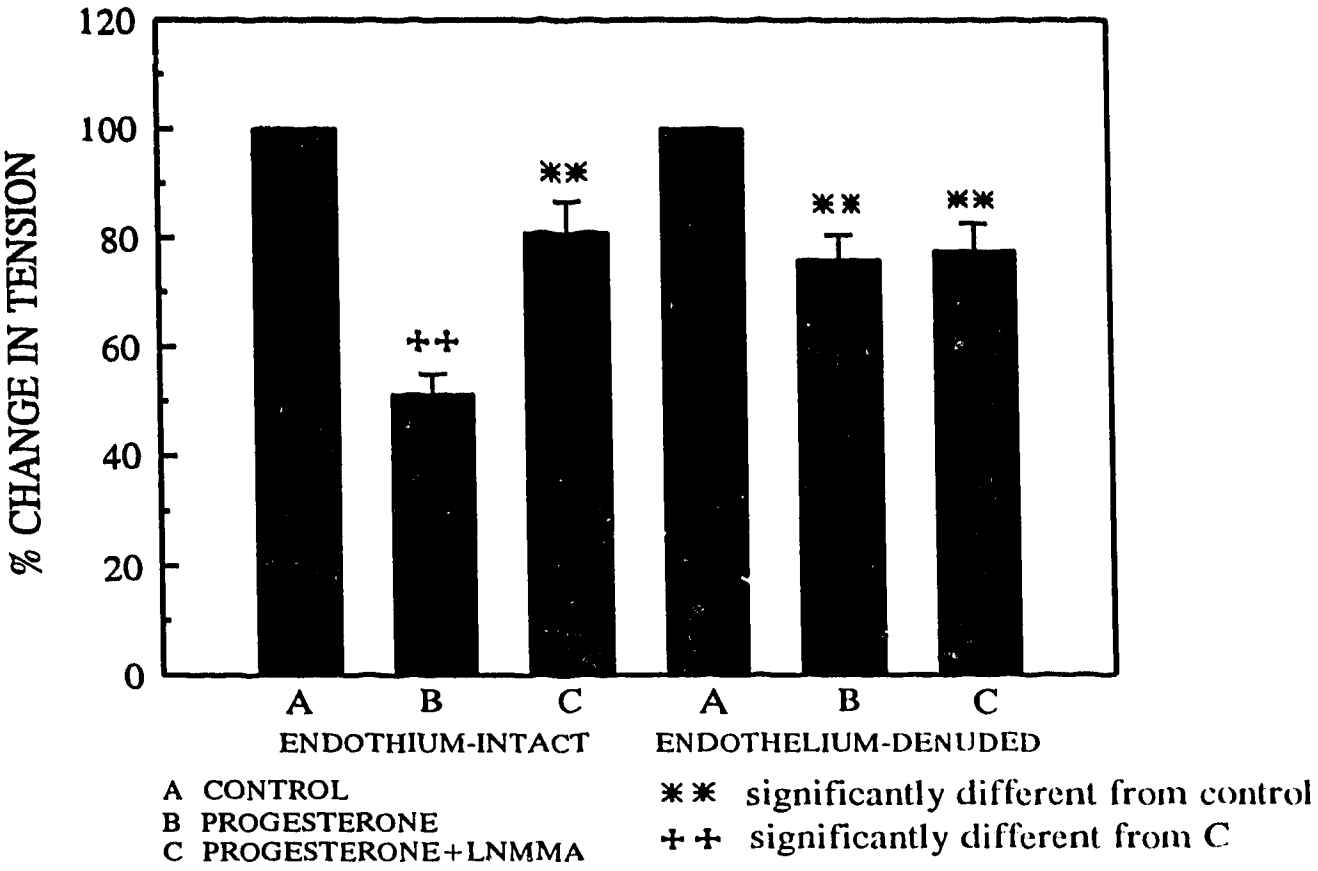

Fig.III-8 Reversal of the effect of progesterone by L-NMMA on tonic tension in aortic rings, showing a partially endothelium-dependent effect. L-NMMA (10^{-5} M) was applied after the effect of progesterone occurred .

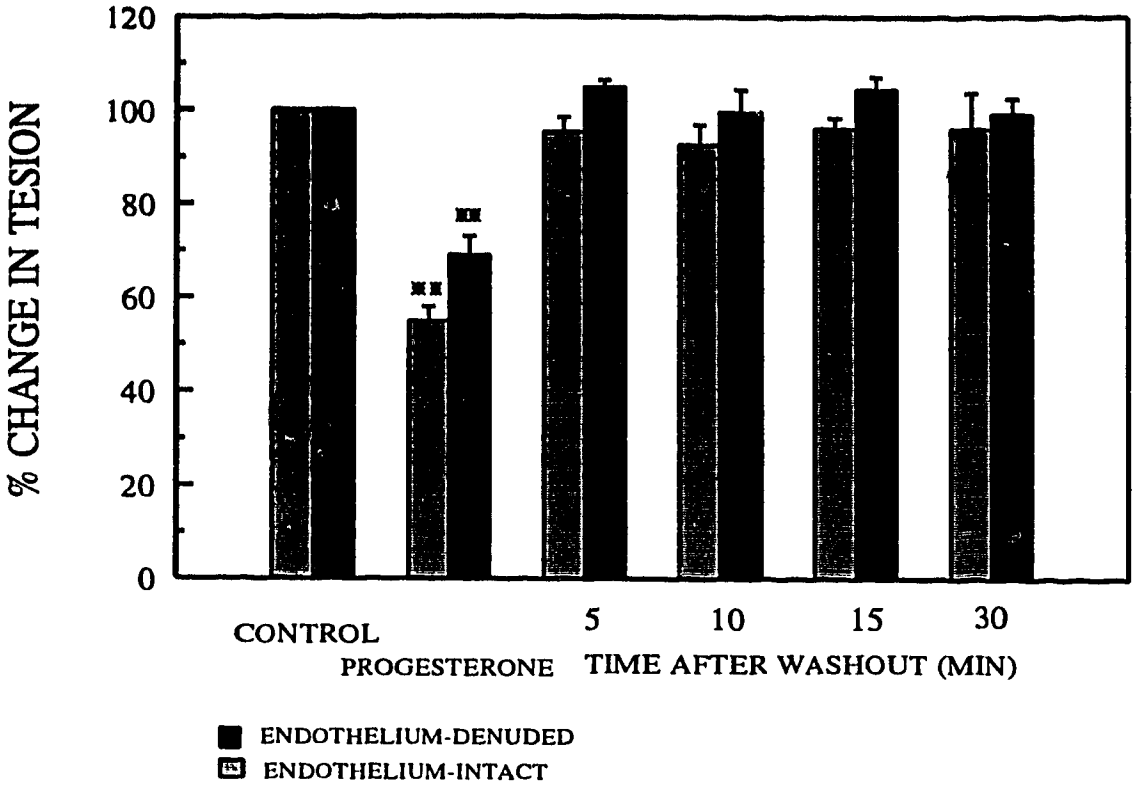

Fig.III-9 Reversal by washout of the effect of progesterone on tension generation in aortic rings. Different periods of time are shown. ** significantly different from the control values.

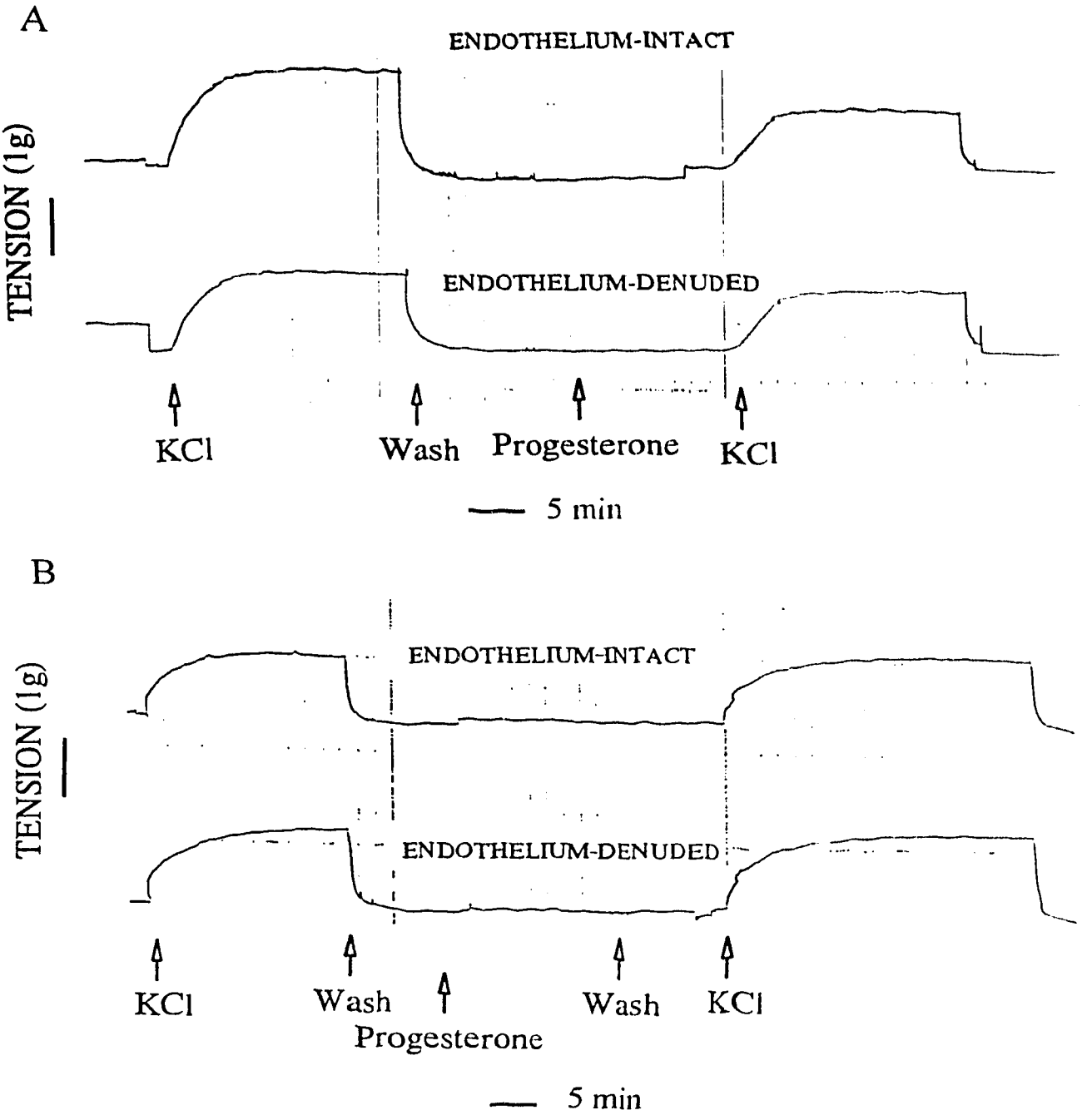

Fig.III-10 Original traces showing the reversal by washout of progesterone effects on tension generation in aortic rings. A, progesterone effect. B, reversal of progesterone effect by washout.

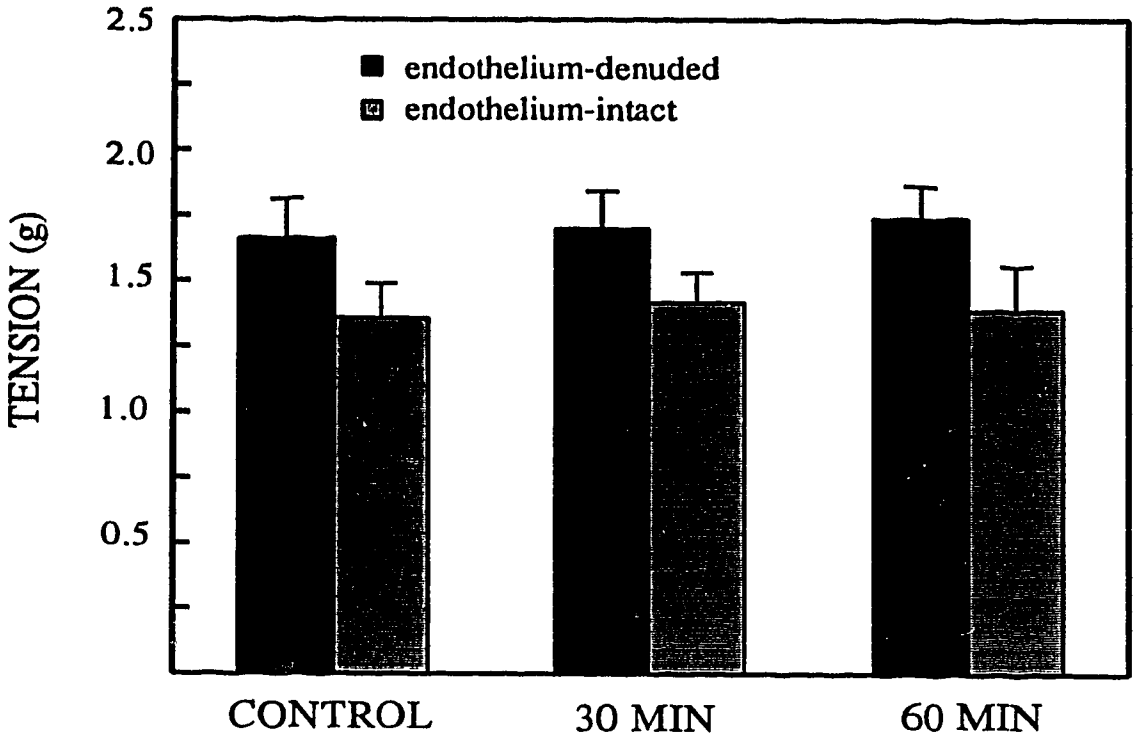

Fig.III-11 Effect of repeated stimulation by KCl (30 mM) on tension generation in aortic rings.

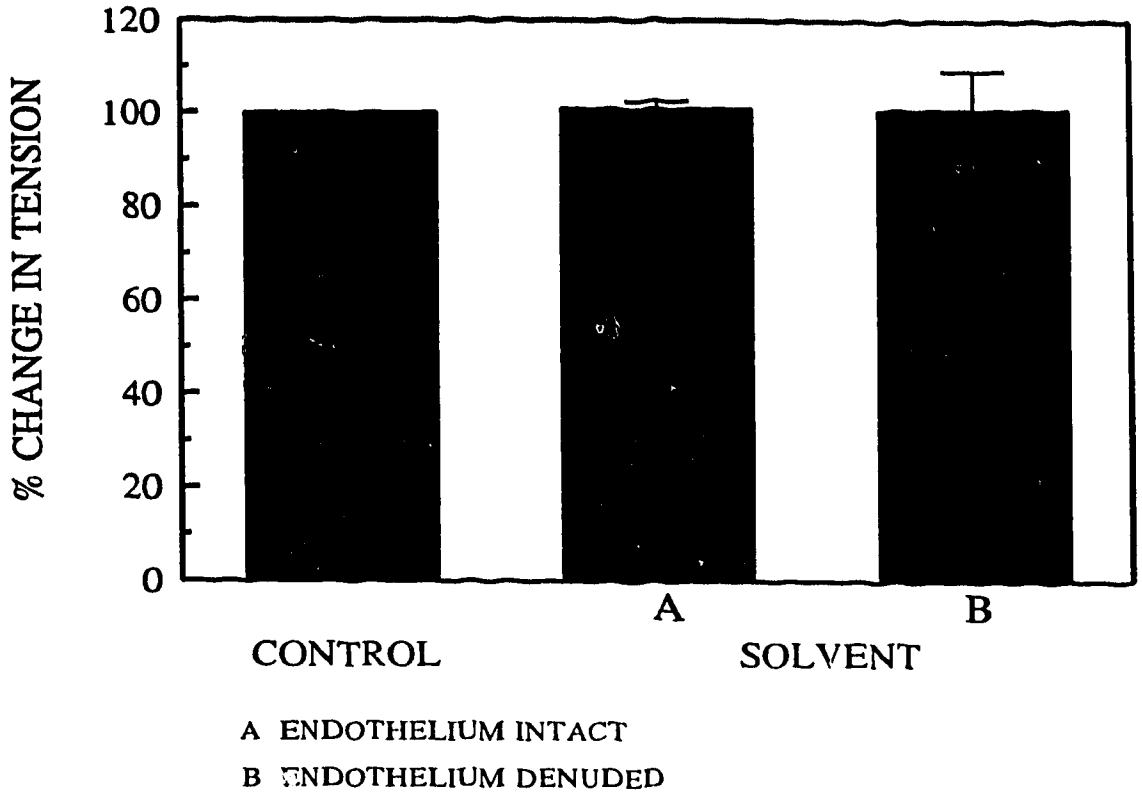

Fig.III-12 Effect of solvent on tension generation in aortic rings. The tissue was incubated with the solvent for 30-40 min.

Chapter IV

THE EFFECT OF PROGESTERONE ON $[Ca^{2+}]_i$ AND Ca^{2+} CHANNELS IN VSMC AND ON $[Ca^{2+}]_i$ IN ENDOTHELIAL CELLS

4.1 INTRODUCTION

The involvement of calcium in the generation of vascular smooth muscle contraction is well recognised. In general, an increase in intracellular Ca^{2+} causes the binding of four Ca^{2+} ions to one calmodulin molecule to form a Ca^{2+}/calmodulin complex. The Ca^{2+}/calmodulin complex will activate the myosin light chain kinase which phosphorylates the 20-kDa regulatory light chain of myosin and, with the presence of actin, an increase in myosin-ATPase activity will follow. The consequence of this series of events is cross-bridge cycling and force development (Lucchesi 1989; Stull *et al.* 1991). The Ca^{2+} appearing in the cytoplasm has two origins: an intracellular origin through Ca^{2+} stores (SR) and an extracellular origin through the opening of membrane Ca^{2+} channels. Evidence has revealed that vasoconstrictors such as phenylephrine and serotonin evoke a transient increase in $[Ca^{2+}]_i$ followed by a sustained plateau phase in VSMC. When phenylephrine is administered, 70-80% of the increase is due to the influx of extracellular Ca^{2+} and the plateau phase is only due to Ca^{2+} entry through membrane Ca^{2+} channels (Semenchuk and Di Salvo 1995).

Two types of Ca^{2+} channel currents in VSMC in short term culture have been identified (Loirand *et al.* 1986; Wang *et al.* 1989). These are the rapidly inactivating current (T-type Ca^{2+}

channel current) and the slowly inactivating current (L-type Ca^{2+} channel current). T-type channels are very sparse in adult VSMC and cardiac muscle cells. Therefore, the major inward Ca^{2+} channel current is through the L-type slow Ca^{2+} channels by which Ca^{2+} enters the cell during the regulation of muscle contraction (Xiong and Sperelakis 1995). There is growing evidence showing that the activities of L-type Ca^{2+} channels can be regulated by various vasoactive substances such as neurotransmitters and hormones. This regulation may be mediated by the interaction of protein kinases (such as PKA, PKC or PKG) and the channel proteins either directly or indirectly (Spedding 1987; Mironneau 1991; Clapp and Gurney 1991; Wang *et al.*. 1991a, b; Ishikawa *et al.* 1993). Both cAMP-PKA and cGMP-PKG systems were found to have predominately inhibitory effects on inward Ca^{2+} channel currents and thus lead to vasodilation. PKC exhibited mainly stimulatory effects on cardiac muscle cells (Dosemeci *et al.* 1988; Liu *et al.* 1993;) but it had different or no effects on the Ca^{2+} channels in VSMC (Campbell *et al.* 1985; Spedding 1987; Walsh *et al.* 1994).

Ovarian steroid hormones were found to be active in regulating the contraction of smooth muscle in the cardiovascular system and uterus. One possible mechanism of these effects is believed to be the modulation of Ca^{2+} channel activity in the cell membrane. It has been reported that 17β-estradiol decreased $[Ca^{2+}]_i$ in porcine coronary artery (Han *et al.* 1995) and inhibited the activity of Ca^{2+} channels in VSMC (Shan *et al.* 1994; Zhang *et al.* 1994), cardiac myocytes (Jiang *et al.* 1992a; Sheldon and Argentieri 1995) and neurones (Joëls and Karst 1995). Progesterone increased $[Ca^{2+}]_i$ by opening Ca^{2+} channels in human sperm (Mendoza *et al.* 1995) and rat myometrium (Rendt *et al.* 1992).

Endothelium plays an important role in the control of vascular tone. One of the most important endothelium-derived relaxing effects is duo to nitric oxide. NO is produced by endothelial cells in response to various stimuli such as ATP, ACh, bradykinin (BKN), thrombin and Ca^{2+} ionophore (A23187) (Schmidt *et al.* 1989; Korenaga *et al.* 1993). An isoform of the NO synthesis enzyme, constitutive NO synthase (cNOS), was found to be $[Ca^{2+}]_i$-dependent and constitutively present in endothelial cells (Davies and Hagen 1993; Zhang *et al.* 1994). Another type of NO synthase, inducible NO synthase (iNOS), is Ca^{2+}-independent and is induced by immunological stimuli (Stuehr *et al.* 1991). cNOS plays a primary role in the regulation of blood pressure (Rees *et al.* 1989). In cardiac muscle and vascular muscle NO may stimulate the accumulation of cGMP in the cytosol of the muscle cells. This would inhibit L-type Ca^{2+} channel currents (Méry *et al.* 1991; Ishikawa *et al.* 1993). The result of the action of NO is vasodilation.

In the present study the effects of progesterone on intracellular Ca^{2+} levels in VSMC and endothelial cells were examined. The reversal of the effects of progesterone on $[Ca^{2+}]_i$ was also tested in order to determine whether the effects were rapid non-genomic or genomic ones. In addition, the effects of progesterone on voltage dependent Ca^{2+} channels in VSMC were investigated. The onset and reversal by washout of the effects were closely observed.

4.2. EXPERIMENTAL DESIGN

4.2.1. *Effects of progesterone on $[Ca^{2+}]_i$ in VSMC*

Fura-2, a commonly used intracellular calcium indicator, was chosen for intracellular calcium increment measurements. Primary cultured VSMC from rat tail artery were used.

Confluent smooth muscle cells were first challenged by 30mM KCl several times to obtain a stable, repeatable calcium increase curve to serve as the control values. After washout with normal bath solution, the cells were incubated with progesterone (10^{-5} M) for 15 min. Without additional washing, the cells were stimulated again by KCl in the presence of progesterone. The results were recorded. After the cells were washed with normal bath solution, calcium increment was measured again for comparison with the two previous results.

The data were calculated and presented as the ratio of fluorescent intensity and concentration of intracellular calcium.

4.2.2. *Effect of progesterone on $[Ca^{2+}]_i$ in aortic endothelial cells*

Subcultured aortic endothelial cells (passage 3 to 7) were examined with the same methods used for VSMC. Fura-2 was used as the Ca^{2+} indicator and was loaded into the cells. Progesterone was applied to the confluent cells alone or after the addition of KCl (30 mM). Control values were obtained before the experiments were initiated.

4.2.3. *Effect of progesterone on voltage-dependent Ca^{2+} channels in VSMC*

The whole cell version of the patch clamp technique was employed to examined the change in inward Ca^{2+} channel currents through voltage-dependent Ca^{2+} channels in the cell membrane.

Primary cultured VSMC were held at -40mV to monitor the L-type Ca^{2+} channel currents. Progesterone was applied to the external solution after the current became stable and the control I-V record was obtained. Various doses of progesterone (10^{-10} - 10^{-5} M) were added to examine the dose dependence of the agent. The inward currents were recorded every 5 min after the addition of progesterone.

To test the reversibility of the inhibitory effect of progesterone on Ca^{2+} channels, the cells were washed several times after the progesterone action occurred and the currents were continuously recorded. The effects of the solvent were then tested.

4.3. RESULTS

4.3.1. *Progesterone inhibition of the KCl-stimulated $[Ca^{2+}]_i$ increase in VSMC*

Fig.IV-1 presents the experimental recording traces showing the inhibitory effects of progesterone on $[Ca^{2+}]_i$ in primary cultured VSMC. The cells were incubated with progesterone for 15 min in between the stimulation by KCl. After progesterone incubation, 15-min washout was applied to the cells. The measurement of the first KCl stimulation was taken as the control value. Data are represented as increase in ratio of fluorescence at 340 and 380 nm and $[Ca^{2+}]_i$ at a nanomolar concentration. Fig IV-1 indicates that progesterone has a rapid

inhibitory effect on $[Ca^{2+}]_i$ increase and this inhibitory effect can be rapidly reversed by washout.

Fig.IV-2 gives a summary of a group of experiments indicating that progesterone incubation caused a smaller $[Ca^{2+}]_i$ increase in response to the KCl challenge compared to the control values. This inhibition effect could be reversed by washout of the progesterone containing bath solution.

4.3.2. *Lack of effect of progesterone on $[Ca^{2+}]_i$ in endothelial cells*

There was no significant effect by progesterone detected on $[Ca^{2+}]_i$ change in cultured aortic endothelial cells. Progesterone was applied to the cells both at rest and after the addition of KCl (30 mM). Fig.IV-9 presents the results of progesterone administration to rat endothelial cells.

4.3.3. *Effect of progesterone on L-type Ca^{2+} channel currents in VSMC*

The L-type Ca^{2+} channel currents started to decrease 5 min after the application of progesterone. The inhibitory effect reached a maximum point in an average time of 15 min. In Fig.IV-3, at the left there are two records from the same VSMC of the L-type inward Ca^{2+} channel currents before and after the application of progesterone (10^{-5} M) . The currents appeared after the membrane was depolarized to +10 mV from the holding potential of -40 mV. At the right are the current-voltage (I-V) relationship curves plotted from the same cell as

at the left. These curves show the inhibitory effect of progesterone on the Ca^{2+} channel currents at different membrane potentials. Fig.IV-4 indicates the concentration-dependent effects of progesterone on the Ca^{2+} channel currents. The effect of progesterone started to show significantly at a concentration of 10^{-6} M. At this concentration, currents were decreased by about 16±5% from the control values and by 37±2.5% at a concentration of 10^{-5} M.

To investigate if the inhibitory effect of progesterone on the Ca^{2+} channels occurred via the traditional nuclear receptors and DNA transcription pathway, the washout procedure was employed. In Fig.IV-5, the dots represent peak current records from a representative cell during the washout procedure. In this experiment, the cell was treated with progesterone (10^{-5} M) after the currents were initially recorded to obtain control values. When the inhibitory effects of progesterone reached a maximum (approximately 15 min after progesterone application) the bath solution was washed out and replaced by normal extracellular solution. As the washout procedure continued, the inward Ca^{2+} current started to increase until it reached control levels. This recovery process took about 20 min. When the current remained stable, progesterone (10^{-5} M) was applied once again. Over a time course of approximately 25 min, the current was suppressed to about the same level observed during initial progesterone treatment. At the top of the figure are current records at the time of A, control; B, after the first progesterone treatment; C, after washout and D, after the second progesterone incubation.

Fig.IV-6 shows current records and I-V relationship curves from one cell under control conditions, after progesterone treatment and after washout. Fig.IV-7 presents the results of a group of experiments indicating the reversal of the progesterone inhibitory effect by washout.

The solvent for steroids used in these studies was a mixture of ethanol and DMSO (2:1). This solvent was tested in tension measurement and Ca^{2+} channel current measurement. The solvent alone had no significant effect on either of these measurements. Fig.IV-8 shows the effects of the solvent on Ca^{2+} channel current measurement.

4.4. DISCUSSION

That $[Ca^{2+}]_i$ increase is related to smooth muscle contraction has long been widely accepted. Abnormal elevation of $[Ca^{2+}]_i$ was found in VSMC from hypertensive rats (Losse *et al.* 1984; Papageorgiou and Morgan 1991). This could be one of the logical explanations of the altered contractility of blood vessels in hypertension. On the other hand, decrease in $[Ca^{2+}]_i$ caused vasodilation in, for example, cyclic nucleotide dependent-relaxation in vascular smooth muscle (Abe and Karaki 1989; Chen *et al.* 1992).

In the present study, it was shown that progesterone at a concentration of 10^{-5} M inhibited $[Ca^{2+}]_i$ increase stimulated by high K^+. The incubation time of VSMC with progesterone was 15 min. After washout the progesterone containing bath solution within 15 min, $[Ca^{2+}]_i$ increased to the control levels when the cells were again stimulated with K^+.

These results were similar to those obtained with 17β-E_2 and progesterone on VSMC and hepatocytes (Sanchez-Bueno *et al.* 1991; Jiang *et al.* 1992b; Shan *et al.* 1994). In the above studies 17β-E_2 showed a rapid inhibitory effect on $[Ca^{2+}]_i$ in VSMC stimulated by KCl and in cardiac myocytes activated by changing the membrane holding potential. Progesterone increased $[Ca^{2+}]_i$ in rat hepatocytes within a few minutes.

Our data showed rapid onset of the progesterone effect. In addition to the rapid onset, our data also showed that the effect of progesterone was reversed by washout. Fig.IV-2 shows the results of a group of experiments. KCl was given to stimulate VSMC and the fluorescence ratio was taken as a control value. After washout, progesterone was added and incubated with the cells for 15 min. KCl was then added again. It is clear from this figure that after incubation with progesterone, $[Ca^{2+}]_i$ did not increase as much as in the control group. This result suggested that progesterone inhibited the increase in $[Ca^{2+}]_i$. With the same set of cells washout was applied before KCl was once more added. The $[Ca^{2+}]_i$ increase produced by KCl administration was significantly greater than that in the presence of progesterone.

Voltage-dependent Ca^{2+} channels are the principal entry pathways for Ca^{2+} ions into smooth muscle cells. An increase in $[Ca^{2+}]_i$ in turn initiates contraction development. In this study, progesterone exhibited a concentration-dependent inhibitory effect on the L-type Ca^{2+} channel currents activated by depolarisation of the cell membrane. At a concentration of 10^{-5} M, progesterone significantly suppressed inward currents by 37±2.5%. There have been reports indicating that Ca^{2+} channel currents in smooth muscle cells could be regulated by different agents. Among these reports noradrenaline showed stimulating effect (Loirand *et al.* 1990) while 17β-E_2 and parathyroid hormones showed inhibitory effects on Ca^{2+} currents (Wang *et al.* 1991a; Jiang *et al.* 1992a; Shan *et al.* 1994; Sheldon and Argentieri 1995). Other steroids were also found to exert their effects by regulating $[Ca^{2+}]_i$ and Ca^{2+} channels. Progesterone was reported to affect human sperm maturation via the increase of $[Ca^{2+}]_i$ (Blackmore *et al.* 1990). The increase in $[Ca^{2+}]_i$ was entirely due to the influx of Ca^{2+} from the extracellular compartment. This effect was very rapid (occurring within several seconds). The

mechanism of progesterone action was proposed to be through the mediation of a progesterone receptor resident in the plasma membrane. Binding of progesterone to this receptor activated the Ca^{2+} channel or inhibited the Ca^{2+}-ATPase pump (Blackmore *et al.* 1990). Since voltage-dependent Ca^{2+} channel blockers such as verapamil and diltiazem did not block the Ca^{2+} influx efficiently, it was suggested that the Ca^{2+} channel was not one of the voltage-dependent types but was more likely to be similar to the receptor operated Ca^{2+} channels (Blackmore 1993). Certain neurosteroids also showed depression of voltage-gated Ca^{2+} channels in adult mammalian hippocampal neurones (ffrench-Mullen and Spence 1991). By tail current analysis, the steroid and the typical Ca^{2+} channel blocker CgTX appeared to block the same fraction of Ca^{2+} current.

In this study, the Ca^{2+} channel recording procedure followed that previously reported by Wang and co-workers (1989). The slow-inactivation segments of inward current appearing at a holding potential of -40 mV was the L-type Ca^{2+} channel current. It was activated at a test pulse of -20 mV and saturated at +20 mV. The inhibitory effect of progesterone was on the L-type Ca^{2+} channel current, although there is no data available from the present study to show whether progesterone acts directly on the channel protein or through other intracellular messengers.

Human sperm cell has been widely used as a model to study the mechanism of non-genomic effects of steroids (Blackmore and Lattanzio 1991; Mendoza *et al.* 1995). The effects of progesterone on $[Ca^{2+}]_i$ and Ca^{2+} channels in human sperm were suggested to be non-genomic. In rat hepatocytes, progesterone and estradiol increased cytosolic Ca^{2+} in minutes or seconds. It is believed that the steroid effects on the nucleus or on protein synthesis were not

involved in this mechanism (Sanchez-Bueno *et al.* 1991). The inhibitory effects of certain neurosteroids on voltage-gated Ca^{2+} channels in hippocampal neurones were also suggested to involve an interaction with the cell membrane or the channel protein because the effects were rapid and reversible (ffrench-Mullen and Spence 1991).

In the present study, the inhibitory effect of progesterone started to show 5 min after the addition of the agent and reached its maximum point at 15 min. Fig.IV-6 shows that after washout Ca^{2+} channel currents increased. If the effect was really membrane-associated it should be eliminated by washout. To determine if this is true, an experiment was conducted to apply and washout progesterone repeatedly. Fig.IV-5 shows records of the time course of this effect. The suppression effect of the progesterone effect began at about 5 min. Fifteen min after the addition of progesterone, washout was employed. After washout the inhibited current returned to control values. When the current remained stable, progesterone was added again. Not surprisingly, the current once again was inhibited. These results suggested that progesterone exerted its inhibitory effect on L-type Ca^{2+} channel current rapidly and that the effect lasted only in the presence of progesterone. In other words, the inhibitory effect of progesterone was both rapid in onset and reversible. Progesterone thus acted on VSMC possibly by a rapid membrane-associated mechanism other than the traditional gene expression and protein synthesis pathway of steroid hormones.

In the present study, our data did not directly show whether the inhibition of $[Ca^{2+}]_i$ by progesterone was due to the inhibition of Ca^{2+} influx from the extracellular compartment or due to the inhibition of Ca^{2+} release from intracellular Ca^{2+} stores. Considering the results, however, from Ca^{2+} channel studies, progesterone did rapidly inhibit the Ca^{2+} channel currents.

Therefore, it is not illogical to presume that the inhibitory effect of progesterone on $[Ca^{2+}]_i$ increase was, at least in part, due to the inhibition of Ca^{2+} influx from Ca^{2+} channels in the plasma membrane.

In the previous chapter, we reported the inhibitory effect of progesterone on blood vessel contraction. That effect occurred immediately after the addition of progesterone and reached its maximum point within approximately 15 min. The time course for the effects of progesterone in tissue tension studies, cellular Ca^{2+} studies and Ca^{2+} channel studies were very comparable and on all three levels the progesterone effect could be reversed by washout of the bath solution. This makes the inference reasonable that the inhibitory effects of progesterone on tension generation may be mediated by an $[Ca^{2+}]_i$ decrease resulting from the decrease of Ca^{2+} influx from Ca^{2+} channels in the cell membrane.

Under the experimental condition of the present study, no significant effect of progesterone on $[Ca^{2+}]_i$ in aortic endothelial cells was detected. When the endothelial cells were cultured and characterised they reacted to various stimulators (ACh, ATP, thrombin) by rapidly increasing $[Ca^{2+}]_i$ (results presented in chapter II).

From the literature, at least two isoforms of NOS appear to exist. cNOS (or CeNOS, constitutive nitric oxide synthase) and iNOS (inducible nitric oxide synthase). cNOS is expressed in endothelial cells. NO synthesised by cNOS in vascular endothelial cells plays a vital role in the control of vascular tone. NO dilates blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells (Rapoport and Murad 1983; Förstermann *et al.* 1986). NO is produced by this enzyme under basal conditions and can be stimulated by various agonists as well as by shear stress from the blood flow. cNOS is

dependent on the increase of $[Ca^{2+}]_i$ (Pollock *et al.* 1991). Agonists elicited a rapid increase of $[Ca^{2+}]_i$ due to release of Ca^{2+} from intracellular stores followed by a lower sustained increase due to entry of external Ca^{2+} through the cell membrane (Weintraub *et al.* 1992; Korenaga *et al.* 1993). In contrast, activation of iNOS is not regulated by Ca^{2+} and requires a much longer time to show its activity (several hours) after exposure to cytokines or bacterial endotoxins (Stuehr *et al.* 1991; Xie *et al.* 1992).

In tension experiments in the present study, an endothelium-dependence of the progesterone effect was found. This effect was reversed by the NOS blocker, L-NMMA. This implied the existence of NOS activation. In our studies, only the rapid effects of progesterone were monitored. It is, therefore, unlikely that iNOS activity occurred. However, an increase in $[Ca^{2+}]_i$ was not detected to prove the existence of cNOS in endothelial cells. In studies with human placenta, Ca^{2+}-independent NOS activity was detected. It was further demonstrated that the enzyme was not an inducible isoform of NOS (Kukor and Toth 1994). Lacking further evidence, it can not be determined by our study whether the NOS was a $[Ca^{2+}]_i$-independent isoform of NOS or, alternatively, under these experimental condition a $[Ca^{2+}]_i$ increase was not recorded.

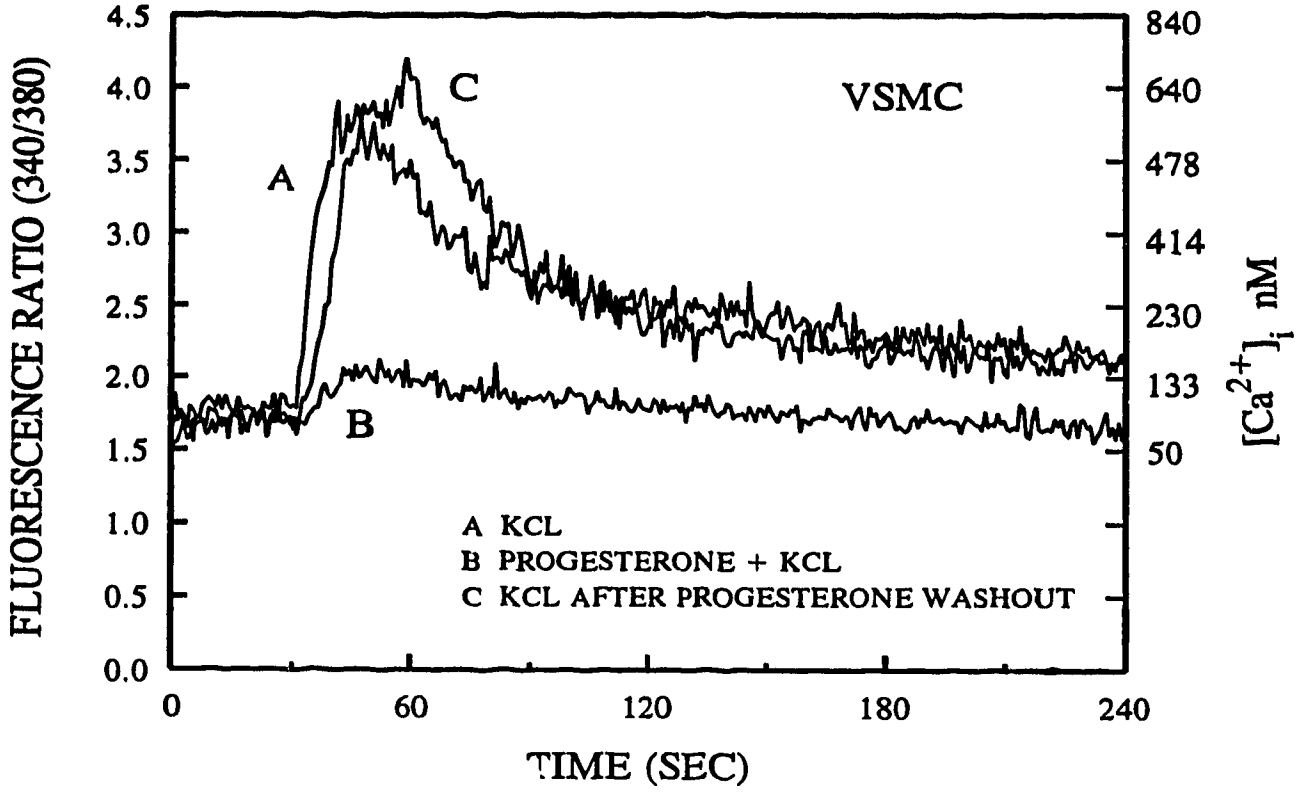

Fig.IV-1 Original recordings showing the effect of progesterone on $[Ca^{2+}]_i$ in VSMC. The smooth muscle cells were challenged with 30 mM KCl (A) , washed and incubated with progesterone (10^{-5} M) for 15 min before KCl was applied again (B). KCl then was finally applied after washout and recovery (C).

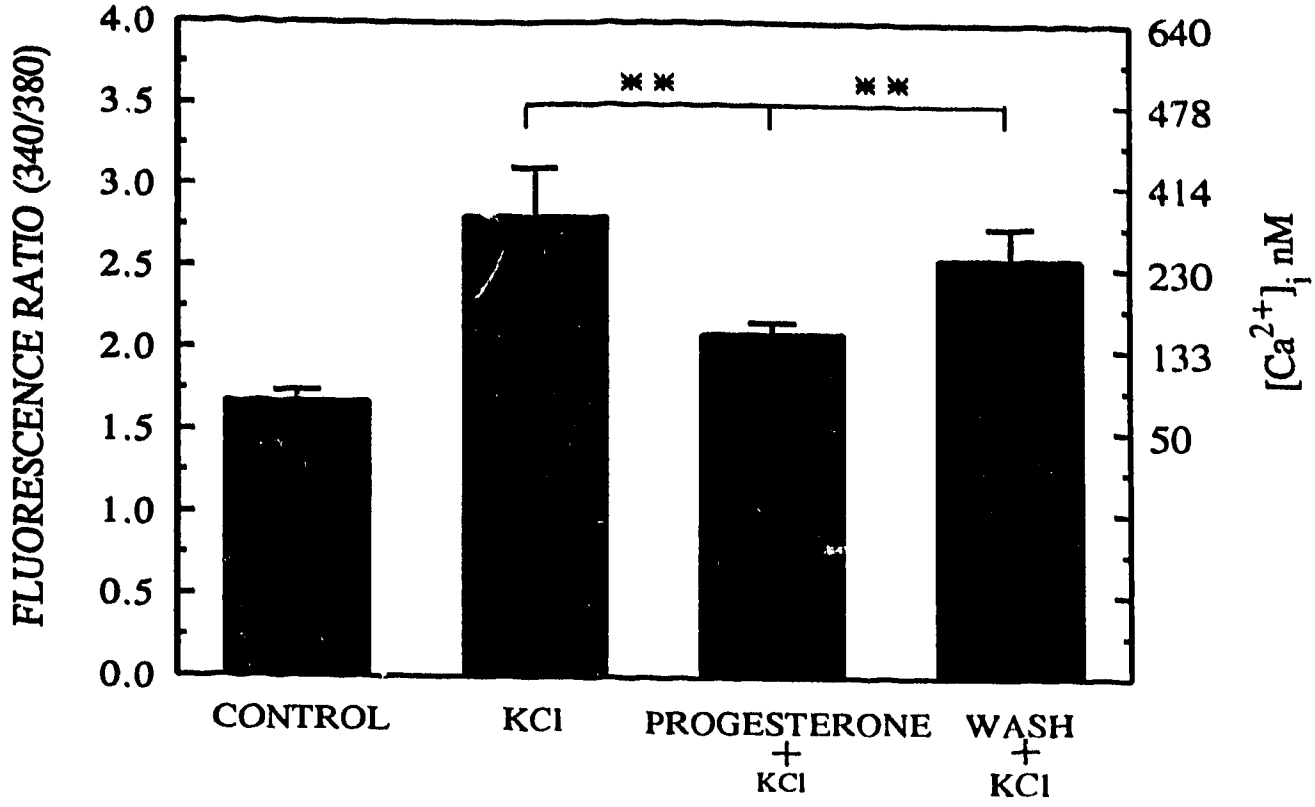

Fig.IV-2 Effect of progesterone on $[Ca^{2+}]_i$ increase in VSMC. ** significantly different

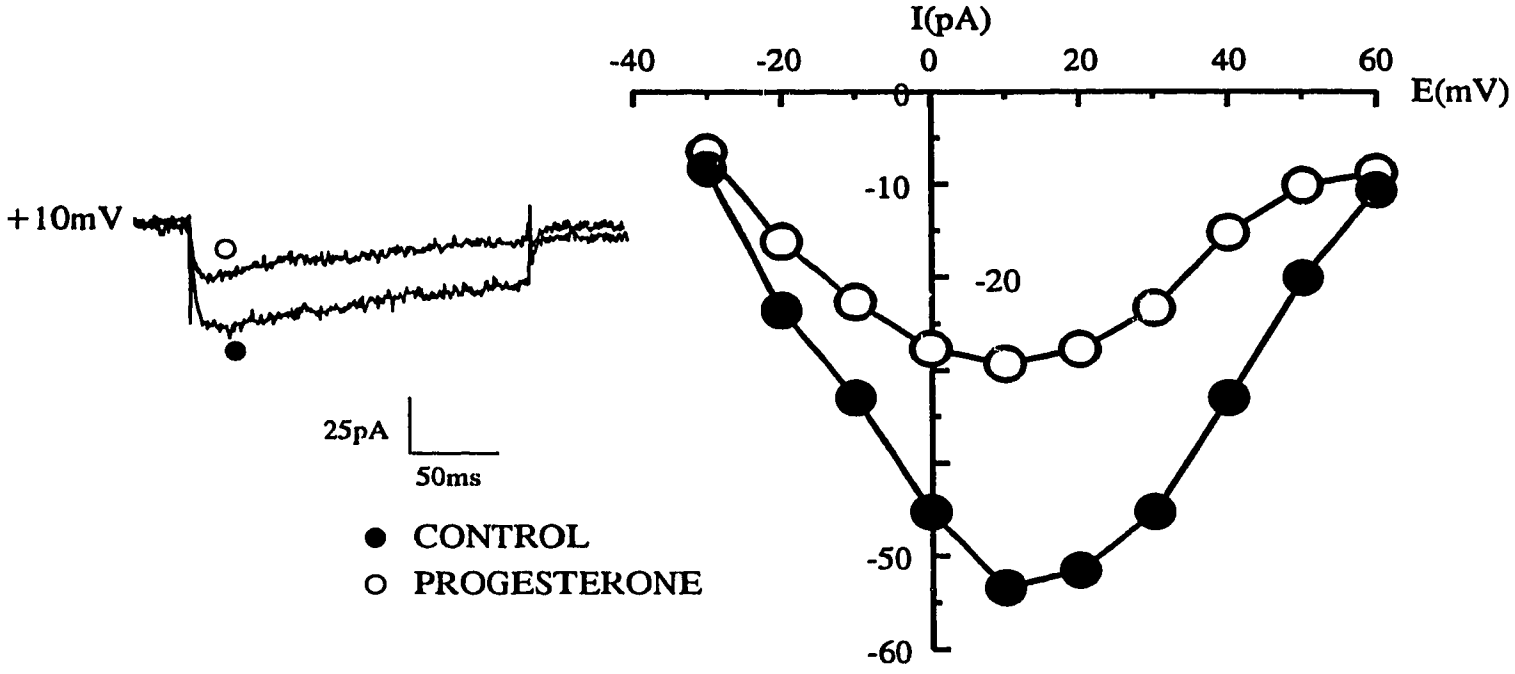

Fig.IV-3 Effect of progesterone on L-type Ca^{2+} current in VSMC. Left: original current records before and after addition of progesterone (10^{-5} M). The inhibitory effect may be observed. The cell was activated by depolarizing the cell membrane to 10 mV from a holding potential of -40 mV. Right: Current (I) and voltage (V) relationships plotted from the same cell before and after progesterone incubation.

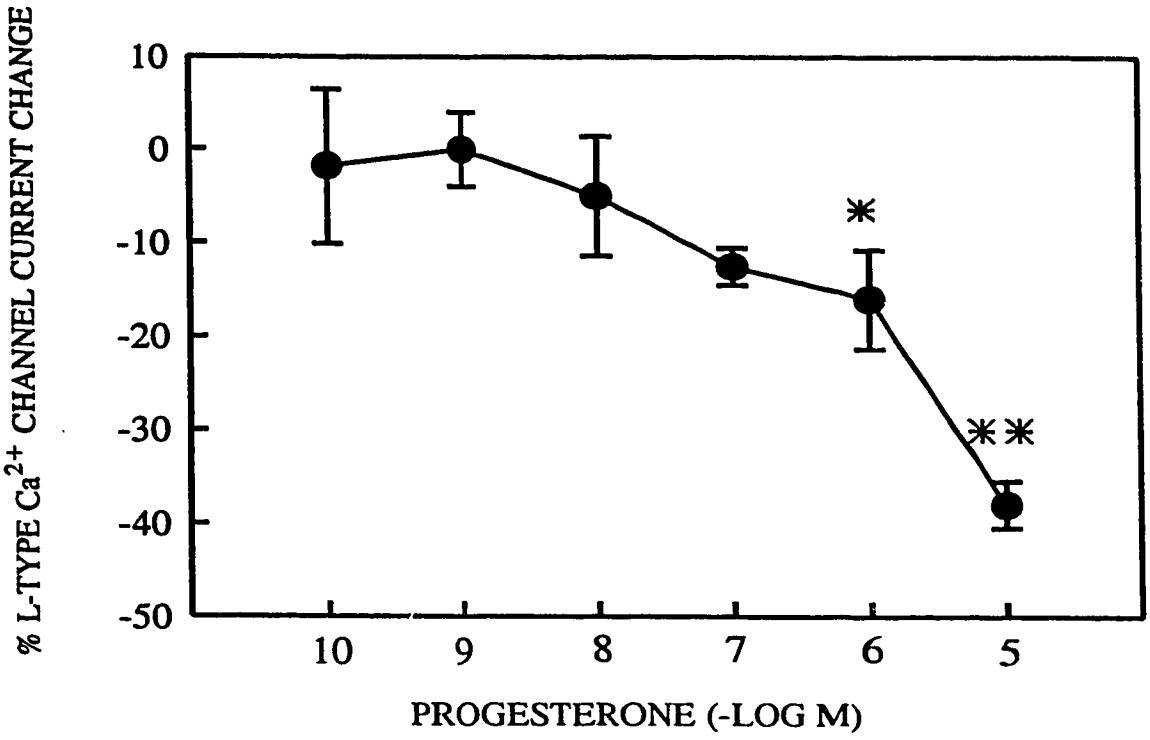

Fig.IV-4 Effect of progesterone at different concentrations on L-type Ca^{2+} current in VSMC. The effect is concentration-dependent. * significantly different from the control values. * at $p<0.05$, ** at $p<0.01$

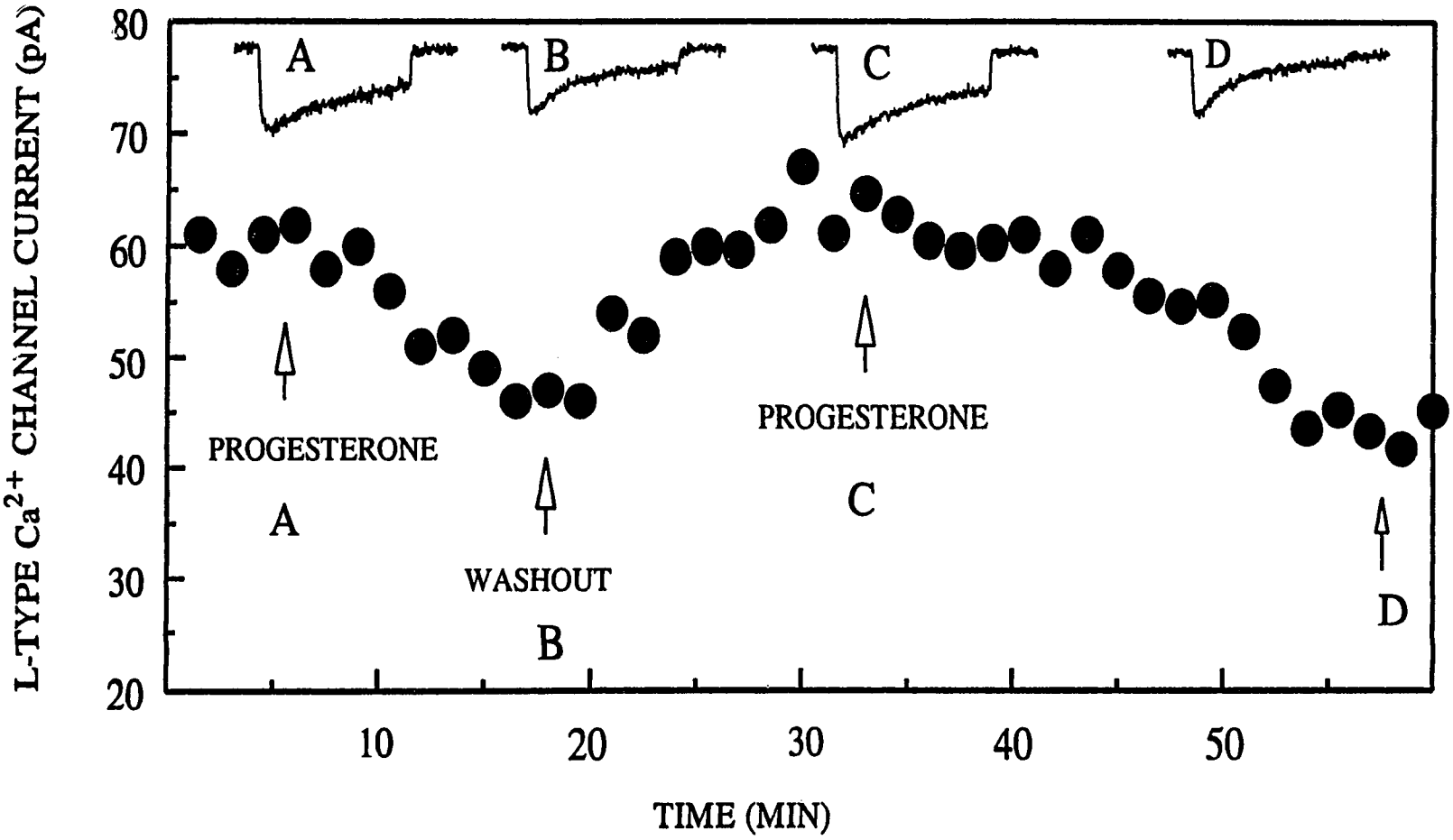

Fig.IV-5 The effect of progesterone and the reversal of this effect by washout on the L-type Ca^{2+} current in VSMC. solid circles: continued recording of the inward current. Original traces of the current recorded at +20 mV at the times indicated below are given at the top of the figure.

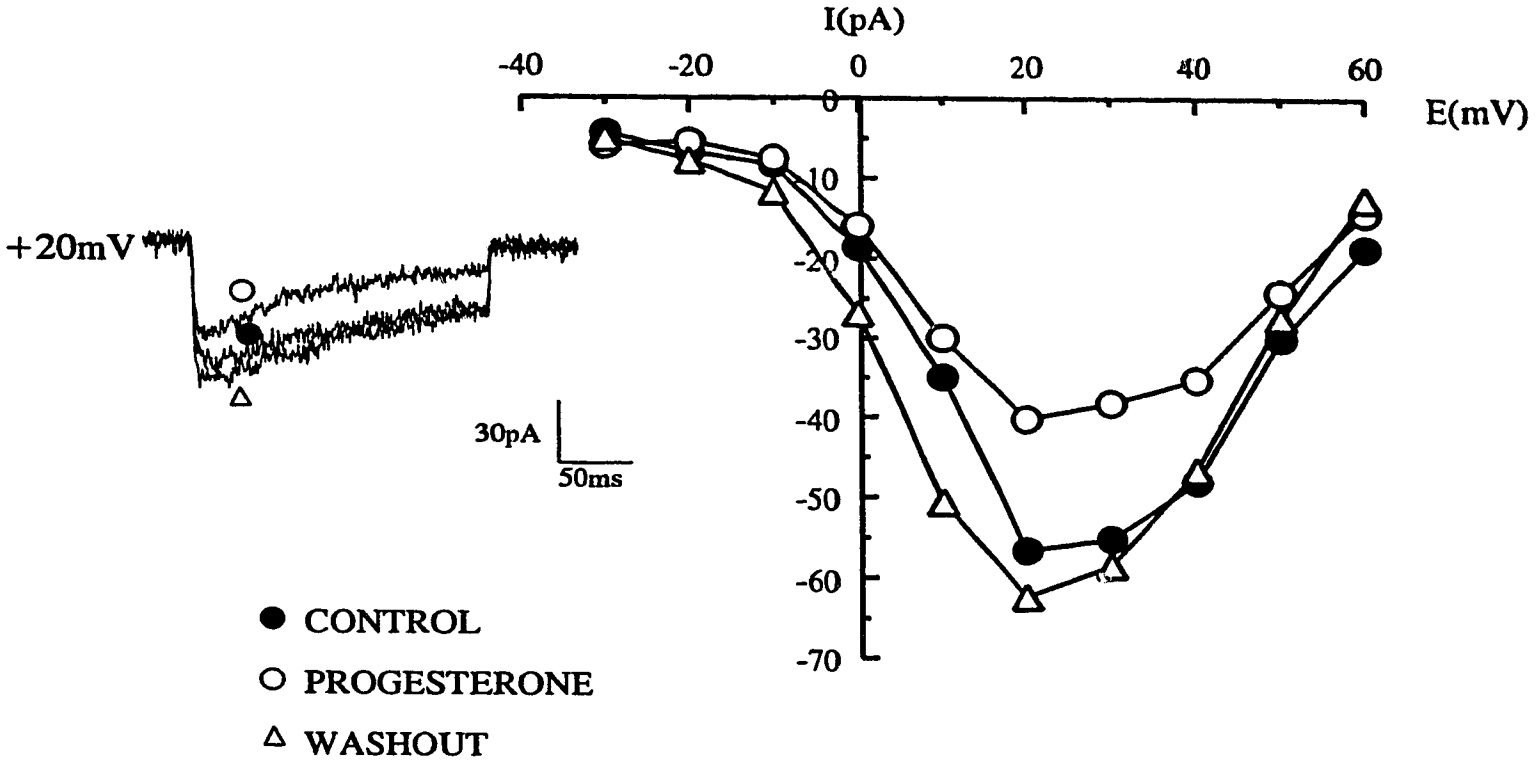

Fig.IV-6 Reversal by washout of the effect of progesterone on the L-type Ca^{2+} current. Left: current records at a holding potential of -40 mV and depolarized to +20 mV showing the recovery of the current from the inhibitory effect of progesterone. Right: the current voltage relationship was plotted from the same cell shown on the left.

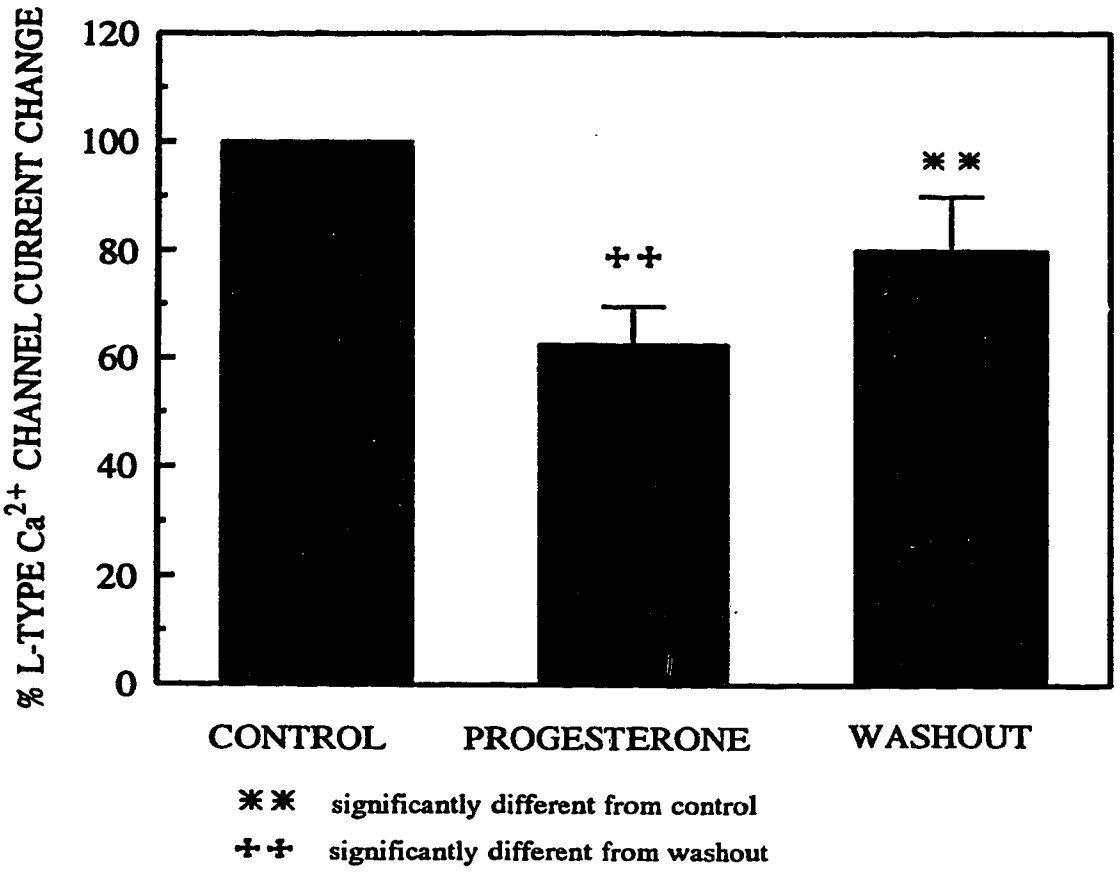

Fig.IV-7 The effect of wash-out on the L-type Ca^{2+} current in a group of cells incubated with progesterone (10^{-5} M).. ** significantly different from the control values.

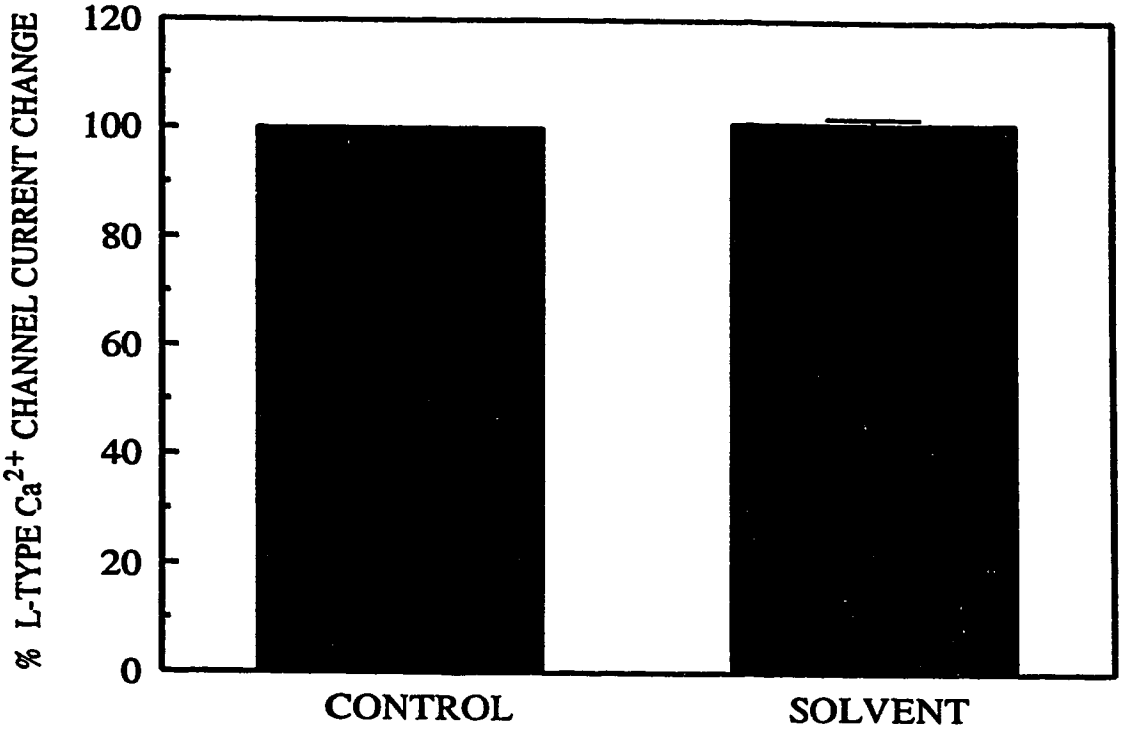

Fig.IV-8 Effect of solvent on the L-type Ca^{2+} current in the VSMC

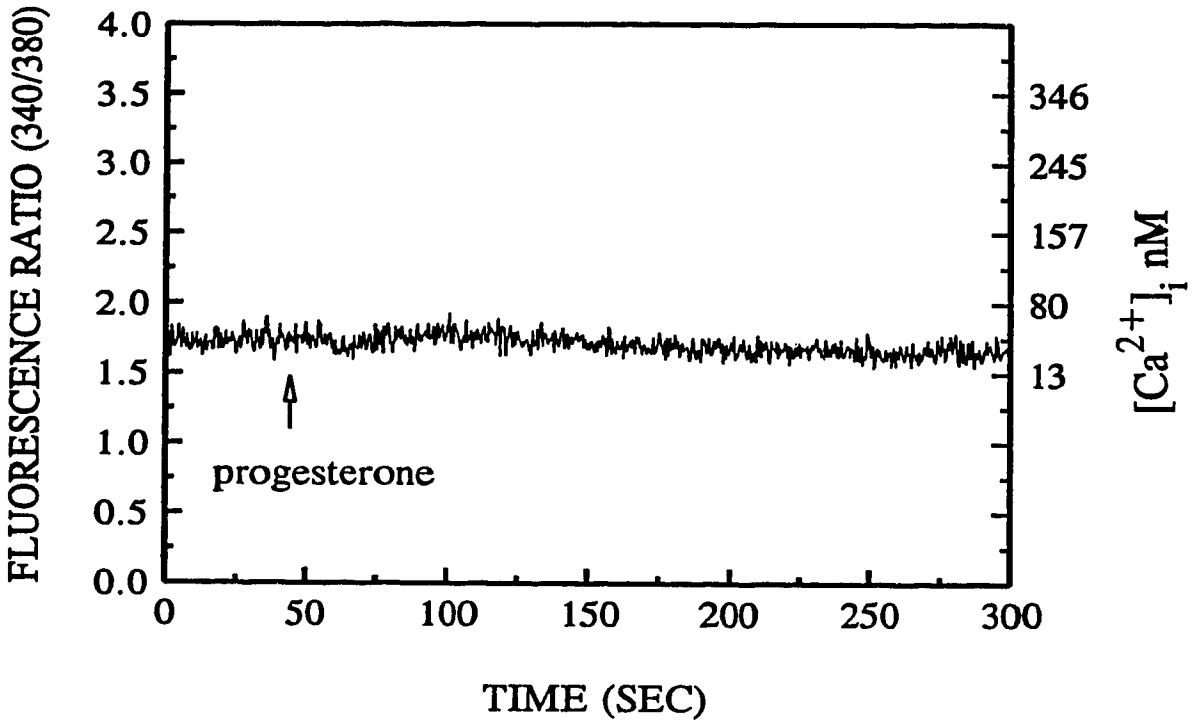

Fig.IV-9 Effect of progesterone on $[Ca^{2+}]_i$ in rat endothelial cells.

Chapter V

EFFECTS OF OTHER STEROID HORMONES ON VASCULAR TISSUE

5.1 INTRODUCTION

Epidemiological studies have shown that estrogen replacement therapy reduced the incidence of coronary heart disease in postmenopausal women (Kafonek 1994). Further *in vivo* studies demonstrated that acute (20 min) infusion of 17β-E_2 selectively potentiated endothelium-dependent vasodilation in healthy postmenopausal women and potentiated both endothelium-dependent and endothelium-independent vasodilation in response to acetylcholine chloride or sodium nitroprusside in postmenopausal women with risk factors for cardiovascular disease (Gilligan *et al.* 1994). Animal studies indicated that 17β-E_2 decreased the pressor response to norepinephrine in rats (Shan *et al.* 1994), inhibited the contraction of cardiac and vascular smooth muscle cells (Jiang *et al.* 1992a; Shan *et al.* 1994), and attenuated the voltage-dependent Ca^{2+} currents in cardiovascular smooth muscle cells (Jiang *et al.* 1992a; Shan *et al.* 1994; Zhang *et al.* 1994).

There are few reports on the effects of androgen on the cardiovascular system. Studies on monkeys demonstrated that atherosclerosis was more extensive in the testosterone-treated monkeys than in the controls group (Adams *et al.* 1995). Chronic treatment with anabolic steroid (4-12 weeks) inhibited both vasodilator responses and vasoconstrictor responses in rabbit arteries (Ferrer *et al.* 1994a,b). Testosterone was found to increase $[Ca^{2+}]_i$ in rat Sertoli

cells (Gorczynska *et al.* 1995) and to increase $[Ca^{2+}]_i$, IP_3 and DAG formation in rat osteoblasts (Lieberherr and Grosse 1994).

The effects of adrenal cortical steroids on blood pressure regulation has been known for many years. These effects include both mineralocorticoids (GC) and glucocorticoids (MC). Studies indicated that adrenal steroids play an important role in the development and maintenance of arterial hypertension (Mendlowitz *et al.* 1979; Garwitz *et al.* 1982; Grunfeld *et al.* 1987). The administration of MC or GC has been shown to increase the rate of Na^+ influx into cultured VSMC (Kornel *et al.* 1993; Christ *et al.* 1995a). Treatment of cultured VSMC with MC or GC was also reported to increase Ca^{2+} influx (Wehling *et al.* 1994; Christ *et al.* 1995a). Application of dexamethasone to rabbit ear produced an initial vasodilation followed by vasoconstriction with a long delay (120 min). Dexamethasone also reduced contraction of isolated rabbit ear arteries (Miyahara *et al.* 1993).

In the present studies, in order to understand the profile of the effects of various steroid hormones on the vascular system, the effects of estrogen (17β-E_2), androgen (testosterone), mineralocorticoid (aldosterone) and glucocorticoid (dexamethasone) on vascular tissue and /or VSMC were examined. The results of these experiments were compared to those from the studies on progesterone reported in the previous chapters.

5.2. EXPERIMENTAL DESIGN

5.2.1. *17β-Estradiol*

Ca^{2+} channel activities of primary cultured VSMC were examined using the whole cell version of the patch clamp technique. The holding potential was set at -40 mM to monitor the L-type Ca^{2+} channel currents.

Different concentrations of 17β-estradiol (10^{-10}, 10^{-6}, 10^{-5} M) were applied to the external bath solution to investigate the inhibitory effect of this hormone on the Ca^{2+} channel activities.

5.2.2. *Testosterone*

Effects of testosterone on tension generation were studied. Testosterone at a concentration of 10^{-5} M was applied to precontracted aortic rings stimulated by KCl. In some experiments, L-NMMA was used after the effect of testosterone took place to determine the endothelium-dependence of the effect.

5.2.3. *Aldosterone*

The effects of aldosterone on tonic tension generation in aorta were tested. Aldosterone (10^{-5} M) was added to the aorta rings stimulated by KCl. The effects of aldosterone on voltage-dependent Ca^{2+} channel currents were then examined. Two doses (10^{-5}, 10^{-6} M) of aldosterone were tested on VSMC.

5.2.4. *Dexamethasone*

The effects of dexamethasone (10^{-5}M) were tested in tonic tension generation in aortic rings.

5.3. RESULTS

5.3.1. *Effect of 17β-E_2 on L-type Ca^{2+} channel currents in VSMC*

17β-E_2 at a concentration of 10^{-5} M decreased L-type Ca^{2+} channel currents. As in the experiment with progesterone, the inhibitory effects became evident 5 min after the addition of 17β-E_2 and reached a maximum approximately 15 min later. Fig.V-1 shows the results from a group of patch clamp experiments. At the concentrations of 10^{-10} M and 10^{-6} M, there were no significant changes detected.

5.3.2. *Effects of testosterone on tension generation in aortic rings*

Fig.V-2 indicates that testosterone relaxed KCl-stimulated aortic tension. The relaxation effect occurred immediately after the addition of testosterone. From Fig.V-2 it is also apparent that there is a difference between the responses to testosterone of endothelium-intact and -denuded tissue. After the application of L-NMMA, a NOS blocker, the relaxation effect was partially reversed in endothelium-intact tissue. There was no response to L-NMMA in endothelium-denuded tissue.

5.3.3. *Effects of aldosterone on tension generation in aortic rings and Ca^{2+} channel currents in VSMC*

There was no significant change in tension generation detected after application of aldosterone at a concentration of 10^{-5} M. Fig.V-3 shows the results from a group of experiments.

There was no significant change in L-type Ca^{2+} channel currents recorded after application of aldosterone at a concentration of 10^{-5} M. FigV-4 shows results from a group of experiments.

5.3.4. *Effect of dexamethasone on tension generation in aortic rings*

There was no significant change in tension generation detected after the application of dexamethasone at a concentration of 10^{-5} M. Fig.V-5 shows results from a group of experiments.

5.4. DISCUSSION

Estrogen is known to have acute effects on the cardiovascular system. 17β-E_2 has been reported to inhibit Ca^{2+} channel currents in cardiac myocytes (Jiang *et al.* 1992a), Primary cultured VSMC (Shan *et al.* 1994), and VSMC lines (Zhang *et al.* 1994). 17β-E_2 also inhibited Ca^{2+} current in guinea pig detrusor myocytes (Sheldon *et al.* 1995). In the present study, different concentrations of 17β-E_2 (10^{-10}, 10^{-6} and 10^{-5} M) were tested on Ca^{2+} channel currents

in VSMC. Only at a concentration of 10^{-5} M did significant inhibitory effects appear. In the previous chapter of this study, the inhibitory effects of progesterone on Ca^{2+} channel currents were investigated. The effects of progesterone appeared at a concentration of 10^{-6} M. Under the present experimental conditions, progesterone showed a stronger inhibitory effect than did 17β-E_2 on Ca^{2+} channel currents in VSMC.

While estrogen treatment reduced atherosclerosis and cardiovascular risk, testosterone was thought to have adverse effects on atherosclerosis. Direct evidence to support this hypothesis is, however, lacking. Testosterone-treated ovariectomized female monkeys developed atherosclerosis (Adams *et al.* 1995). However, in the same study testosterone treatment also improved the relaxation response of the coronary artery to acetylcholine. This response was endothelium-dependent. Our results showed that testosterone relaxed the contraction of aorta stimulated by high K^+ concentrations. The relaxation effect was partially endothelium-dependent. The NOS blocker, L-NMMA, partially reversed the relaxation effect of endothelium-intact tissue. This result is similar to those obtained in our studies on progesterone. It indicates that part of the testosterone effect may be mediated through the endothelium and probably involves NOS.

It has long been acknowledged that an excess of mineralocorticoids or glucocorticoids causes hypertension. Aldosterone was found to rapidly increase Na^+ transport (Christ *et al.* 1995a; Komel *et al.* 1995) and $[Ca^{2+}]_i$ (Wehling *et al.* 1995). In the present studies, aldosterone application did not have a rapid and direct effect on tension generation in aorta. Results from Ca^{2+} channel studies showed that aldosterone did not affect Ca^{2+} channel currents. In addition, dexamethasone showed no effect on tension generation in the aorta.

The results from these investigations with aldosterone and dexamethasone supported the hypothesis that different steroid molecules have different effects on the same tissue i.e. the vascular tissue in the present study. They further supported the hypothesis that the effects of progesterone on tension generation, $[Ca^{2+}]_i$ levels and Ca^{2+} channel activities of vascular tissues were specific. They were, in other words, not non-specific effects of steroids.

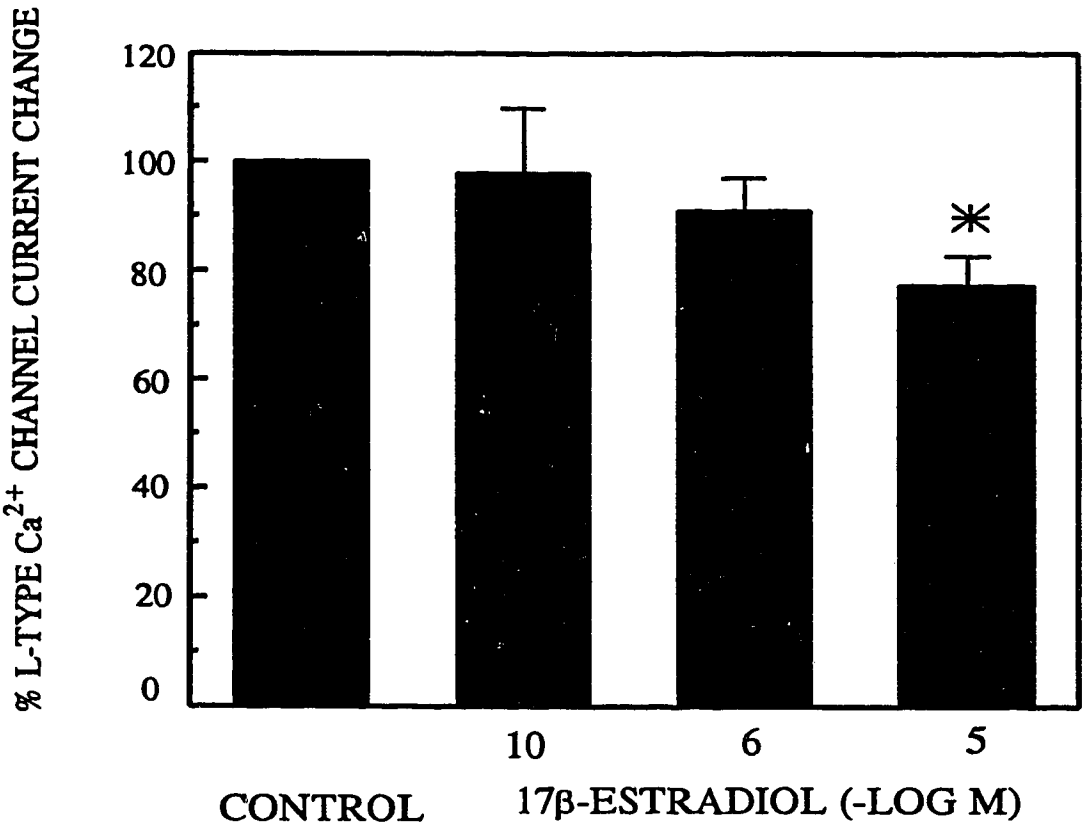

Fig. V-1 Effects of different concentrations of 17β-estradiol on L-type Ca^{2+} currents in VSMC. * significantly different from the control group ($p<0.05$).

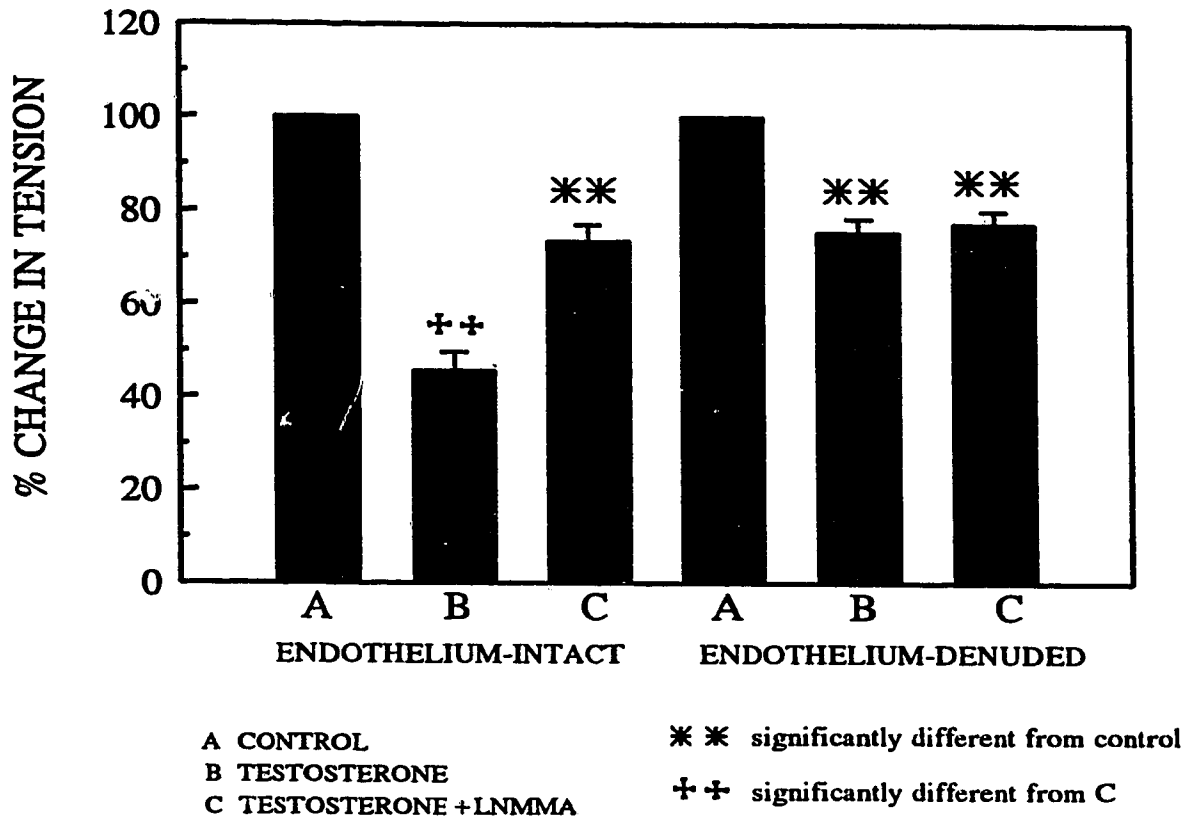

Fig.V-2 Effect of testosterone (10^{-5} M) and L-NMMA(10^{-5} M) on tension generation in endothelium-denuded and -intact aortic rings. The results indicate that the effect of testosterone was endothelium-dependent.

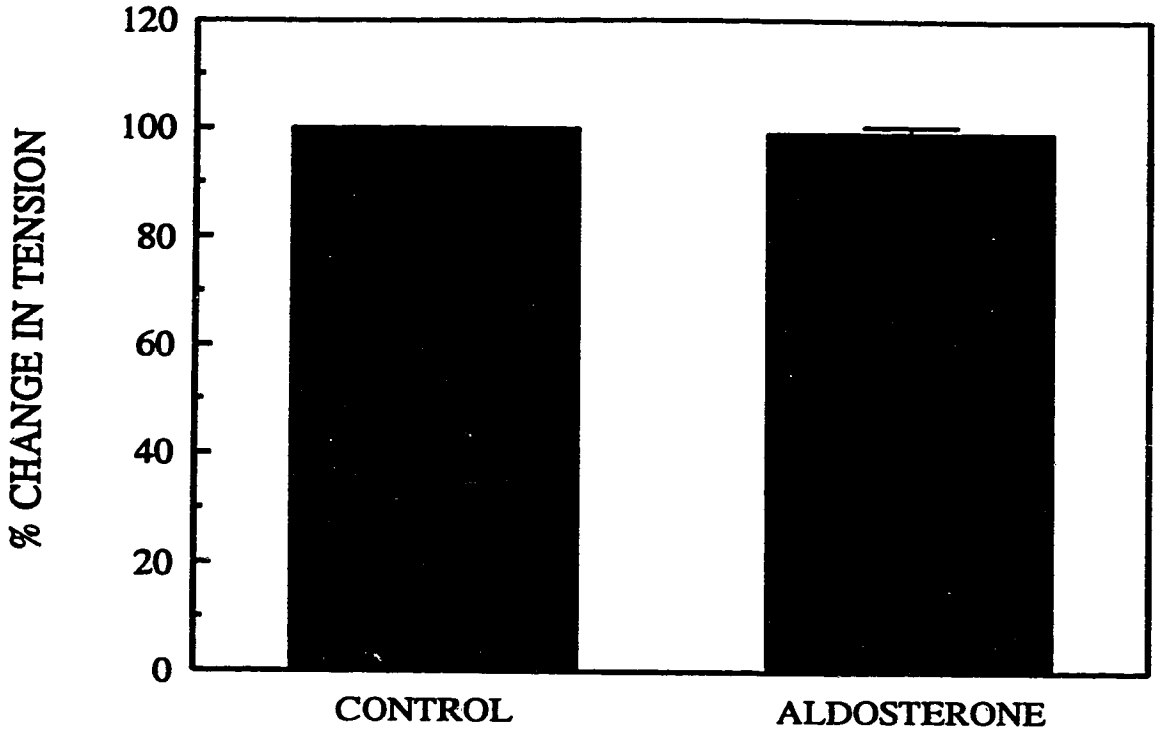

Fig.V-3 Effect of aldosterone (10^{-5} M) on tension generation in aortic rings

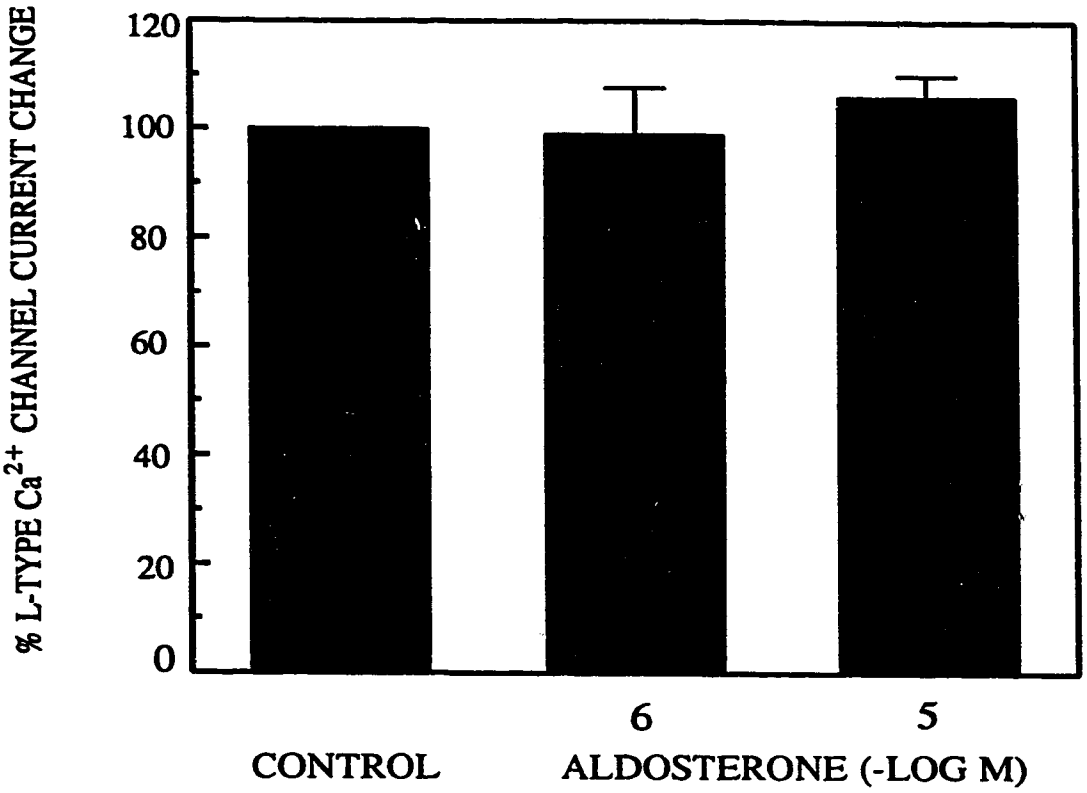

Fig. V-4 Effect of aldosterone (10^{-6}, 10^{-5} M) on inward L-type Ca^{2+} current in VSMC

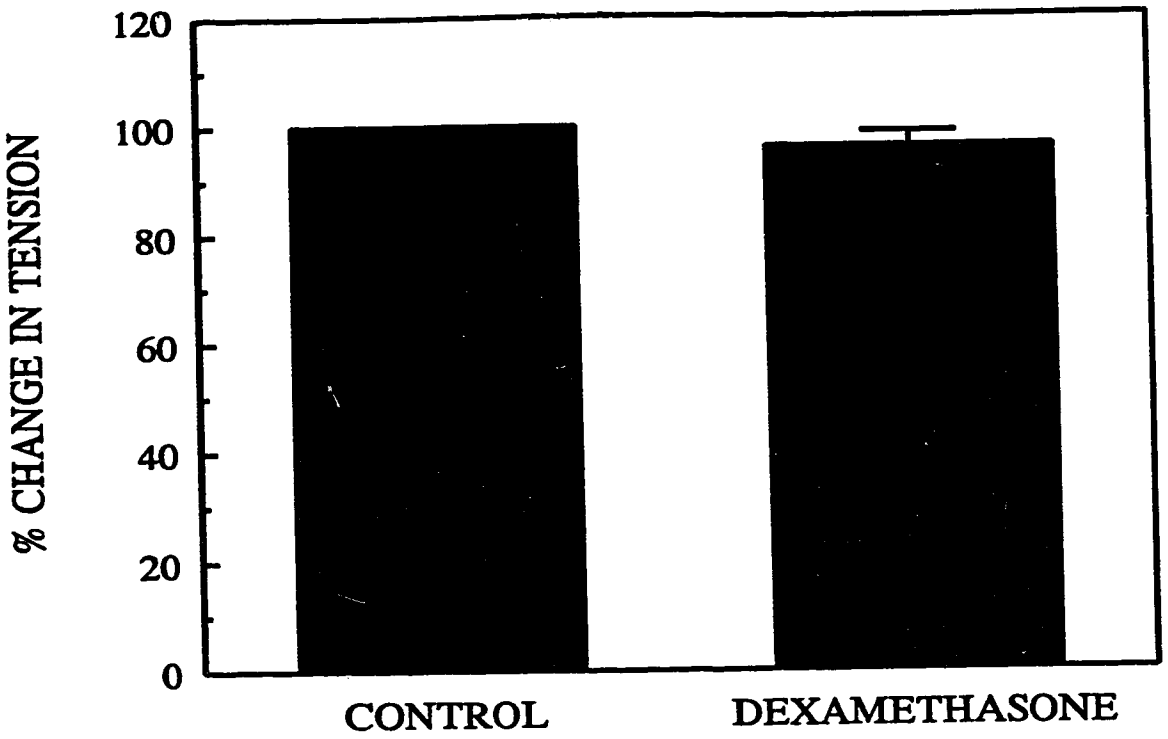

Fig. V-5 Effect of dexamethasone (10^{-5} M) on tension generation in aortic rings

Chapter VI

GENERAL DISCUSSION AND SUMMARY

6.1 PROGESTERONE AS A VASODILATOR

The difference between atherosclerosis in men and premenopausal women indicates that steroid hormones influence the vascular system. Cytoplasmic receptors for estradiol and progesterone have been detected in dog vascular tissues and human endometrial blood vessels (Horwitz *et al.* 1982; Perrot-Applanat *et al.* 1994). Steroid hormones are believed to have a direct effect on vascular tissue which is mediated by specific receptors present in arterial blood vessel walls (Perrot-Applanat *et al.* 1994). Long-term administration of progesterone was found to reduce the response to angiotensin in rats and it was suggested that progesterone rather than estrogen is responsible for the decrease in pressor response in pregnancy when both hormones are increased in the circulation (Hettiaratchi and Pickford 1967; Nakamura *et al.* 1988). In studies of the short-term effects of progesterone, it was also demonstrated that progesterone produced a negative inotropic effect in rabbit heart and that this effect resembled Ca^{2+} entry blocker activity (Raddino *et al.* 1989). Application of combined estrogen and progesterone resulted in lower blood pressure in postmenopausal women (Regensteiner 1991). In isolated tissue studies, progesterone produced relaxation in precontracted coronary arteries (Jiang *et al.* 1992b). In the present study, progesterone showed a concentration-dependent (10^{-10}-10^{-5} M) relaxing effect on precontracted rat aortic rings and a relaxing effect (at a concentration of 10^{-5} M) on tail artery helical strips. Progesterone showed relaxing effects on

tonic tension generation and inhibitory effects on phasic tension generation in aortic rings. These results indicate that the relaxing effect of progesterone acted directly on the blood vessel wall. Considering the results from the literature mentioned above, progesterone appears to lower blood pressure, decrease presser response to angiotensin and relax precontracted arteries from different vascular beds. Although the relaxing effects of progesterone was apparent only when it was combined with other agents, e.g. when the blood vessels were precontracted with KCl or angiotensin, progesterone itself in fact is a vasodilator.

6.2. ENDOTHELIUM-DEPENDENT EFFECTS OF PROGESTERONE ON BLOOD VESSEL CONTRACTION

Our results show that the effect of progesterone on tension generation in rat aortic tissue was partially endothelium-dependent. Both endothelium-intact and endothelium-denuded tissue responded to progesterone. When endothelium-intact and -denuded aortic rings are compared, the relaxing of inhibitory effects of progesterone were always greater in the former. L-NMMA, a NOS blocker, reversed the relaxing effect of progesterone in endothelium-intact tissue while no response was observed in endothelium-denuded tissue. This result suggested that endothelial NO was involved in the mediation of the vasodilation effect of progesterone. The reversal of the progesterone effect by L-NMMA was partial. Fig.III-7 shows that after L-NMMA action, the relaxing effect of progesterone was partially diminished. Tension then was increased to approximately the same level as in endothelium-denuded tissue. In other words, the effect of L-NMMA in this case was relevant to denudation of the endothelium. This result

indicates that the effect of progesterone was mediated both directly through smooth muscle and indirectly through endothelium. In the studies of the vascular effects of female sex hormones, progesterone and estrogen were found to cause endothelium-independent relaxation in rabbit coronary artery (Jiang *et al.*1991;1992b), rat aorta (Thomas *et al.*1995), and canine coronary artery (Sudhir *et al.*1995). Estrogen was also found to cause endothelium-dependent relaxation in rabbit coronary artery (Collins *et al.*1994) and femoral arteries (Gisclard *et al.*1988;). Studies examining the effect of estrogen on the expression of NOS in endothelial cells showed that NOS was either increased (Schray-Utz *et al.*1993) or unchanged (Sayegh *et al.*1993) by the application of estrogen. Thus, involvement of endothelium or NOS in relaxation of vascular tissues by female hormones is not clear. This may be due to the differences between species, vascular beds or experimental conditions. Our data showed that under the present experimental conditions, the relaxation effect of progesterone on rat aorta precontracted by KCl was partially endothelium-dependent.

6.3. Ca^{2+} ANTAGONISTIC EFFECT OF PROGESTERONE

There are reports that estrogen has Ca^{2+} antagonist properties in uterine arterial smooth muscle (Stice *et al.*1987), rabbit coronary artery (Jiang *et al.*1991) and canine coronary artery (Sudhir *et al.*1995). In the present study, progesterone showed an inhibitory effect on Ca^{2+} increase in VSMC stimulated by KCl. Patch clamp studies further indicated that the Ca^{2+} antagonistic effect of progesterone was mediated, at least in part, through inhibition of voltage-dependent Ca^{2+} channel activity in the cell membrane.

It has been demonstrated that estrogen and progesterone exert their effects on $[Ca^{2+}]_i$ and Ca^{2+} channels in various types of cells. In different cell types the hormones alter $[Ca^{2+}]_i$ in a different manner. In human sperm, progesterone increased Ca^{2+} influx through membrane Ca^{2+} channels which were not sensitive to dihydropyridine and did not involve a pertussis toxin-sensitive G protein, i.e. they were not voltage-dependent Ca^{2+} channels (Blackmore *et al.* 1991; Foresta *et al.* 1993; Mendoza *et al.* 1995). Progesterone also increased cytosolic Ca^{2+} in rat hepatocytes (Sanchez-Bueno 1991). Long-term treatment by progesterone increased voltage-dependent Ca^{2+} channel currents in rat myometrial cells (Rendt *et al.* 1992) and rat CA1 hippocapal neurones (Joëls and Karst 1995). 17β-E_2 rapidly decreased Ca^{2+} channel currents in rat VSMC (Shan *et al.* 1994). Our results show that progesterone inhibited $[Ca^{2+}]_i$ increase at least partially through inhibition of the voltage-dependent Ca^{2+} channel in cell membrane.

6.4. RAPID AND REVERSIBLE EFFECTS OF PROGESTERONE ON TISSUE TENSION, $[Ca^{2+}]_i$, AND Ca^{2+} CHANNEL ACTIVITIES

The genomic pathway of steroid action involves profound effects on protein synthesis. The processes include receptor activation, translocation, binding to DNA and subsequent regulation of mRNA production. These genomic effects are considered to have a latency of 2-8 hours and are sensitive to inhibitors of transcription or translation (Wehling 1994). Some investigators believe that the latency from the time of steroid application to the appearance of physiological effects should be 12-24 hours (Landers *et al.* 1992). Therefore an immediate

steroid effect that appears within a few seconds is considered to almost certainly be a non-genomic rather than genomic effect (Wehling 1994).

Non-genomic effects were studied extensively in human sperm. Progesterone was first found to increase $[Ca^{2+}]_i$ immediately through Ca^{2+} influx from the extracelluar medium. The effect was postulated to be mediated by a receptor resident in the plasma membrane of sperm since the Ca^{2+} influx effect was rapid in onset (Blackmore *et al.* 1990). Cell surface-binding sites for progesterone to mediate Ca^{2+} influx were subsequently detected and found to be distinct from the cytosolic nuclear receptor in their steroid specificity. Progesterone linked to BSA (which is too large to diffuse through the cell membrane) was still found effective in eliciting Ca^{2+} influx (Blackmore *et al.* 1991). This provided additional proof for the existence of a membrane progesterone receptor. The rapid and membrane-associated effect of progesterone was considered to be a non-genomic effect.

Non-genomic effects were also demonstrated in studies of vitamin D on Ca^{2+} uptake in skeletal and cardiac muscles and in osteoblasts (for reviews, see Baran 1994; DeBoland and Boland 1994). Vitamin D and its metabolites increased $[Ca^{2+}]_i$ and Ca^{2+} channel currents in osteoblasts and VSMC (Khoury *et al.* 1995; Shan *et al.* 1993). Aldosterone showed rapid stimulatory effects on sodium transport and $[Ca^{2+}]_i$ in VSMC (Christ *et al.* 1995a; Wehling *et al.* 1995). Aldosterone-specific membrane receptors were detected in human mononuclear leukocytes (Wehling *et al.* 1991).

In the present investigation, progesterone produced rapid effects on all three levels studied: tissue tension generation; intracellular Ca^{2+} concentration and Ca^{2+} channel in the cell membrane. In tension studies, progesterone exerted a rapid relaxing effect on KCl-stimulated

aorta. It is clear from Fig.III-4 that the effect of progesterone occurred almost immediately after the application of progesterone and reached its maximum in a short period of time (approximately 15 min average time). In studies on $[Ca^{2+}]_i$, the incubation time of progesterone with VSMC was 15 min. Inhibition of KCl-stimulated $[Ca^{2+}]_i$ increase occurred immediately after incubation. As for the Ca^{2+} channel current, the inhibitory effect of progesterone could be detected 5 min after addition of the hormone and reached a maximum at approximately 15 min. These results demonstrated that progesterone has inhibitory effects on tissue tension generation, $[Ca^{2+}]_i$ increase and Ca^{2+} channel activities. The time course of progesterone action at the three levels is quite compatible. Another significant finding from the present studies was that the rapid effects of progesterone on the three levels could be reversed by washout of the bath solutions. Fig. III-10 shows the reversal of the inhibitory effect of progesterone on tension by washout. As rapidly as 5 min after washout, aortic tension generation was restored. Fig. IV-1 represents the results of $[Ca^{2+}]_i$ measurement experiments. After progesterone incubation, the VSMC were washed for 15 min and stimulated again by KCl. $[Ca^{2+}]_i$ then increased to the level of the control value. Fig. IV-5 shows continuous recording of Ca^{2+} channel currents in VSMC. Progesterone was given followed by washout and subsequent administration of progesterone. It is obvious that the inhibitory effect produced by progesterone on Ca^{2+} channel currents only occurred in the presence of that hormone and that the effect was rapid and reversible by washout. Collectively, the effects of progesterone on tissue tension generation, $[Ca^{2+}]_i$ and Ca^{2+} channel currents were fast in onset and reversible by washout. Therefore, the mechanism of progesterone action is not likely to be mediated by genomic pathways. The onset of the effects was too rapid to involve interactions between hormones and intracellular receptors, gene

transcription and protein synthesis. Fast reversal of the effects by washout suggested that the effects might be mediated by a membrane process rather than an intracellular steroid receptor.

Therefore, we suggest that the effects of progesterone on aortic tissue contractility are most likely non-genomic effects and are probably mediated through a membrane process.

The data presented in these studies also demonstrated that progesterone and testosterone at a concentration of 10^{-5} M caused significant relaxation of KCl-stimulated aortic tension generation while aldosterone and dexamethasone at the same concentration did not affect tension generation. These results suggested that different steroid hormones have different effects on the same tissue. They thus indicated that the effects of steroid hormones on vascular tissue were not merely non-specific steroid effects.

In the bloodstream, steroid hormones are usually found in very low concentrations (i.e. 10^{-10} - 10^{-7} M). We noted that in the present study the effective concentrations of the steroids used were relatively high (10^{-7} -10^{-5} M). In investigations of the non-genomic effects of steroids, vitamin D has been shown to affect $[Ca^{2+}]_i$ and Ca^{2+} channels in VSMC and osteoblasts (Shan *et al.* 1993; Khoury *et al.* 1995) at concentrations of 10^{-10}-10^{-7} M; aldosterone altered the sodium-proton exchanger and $[Ca^{2+}]_i$ at concentrations of 10^{-10}-10^{-8} M, and estrogen increased $[Ca^{2+}]_i$ in osteoblasts (Lieberherr 1993) and potentiated vasodilation in the human at concentrations of 10^{-12}-10^{-9} M (Gilligan *et al.* 1994). Many other reports have shown the effective concentrations of steroids to be much higher. Concentrations of progesterone were 10^{-6}-10^{-3} M for studies of smooth muscle contractility (Jiang *et al.* 1992b; Cutiérriz *et*

al. 1994; Thomas *et al.* 1995) and $[Ca^{2+}]_i$ mobilization (Blackmore *et al.* 1990; Sanchez-Bueno *et al.* 1991; Foresta *et al.* 1993,1995). Estrogen concentrations of 10^{-7}- $5x10^{-5}$ M were used in studies of cardiovascular contraction and $[Ca^{2+}]_i$ (Jiang *et al.* 1992a; Ecksterin *et al.* 1994; Ravi *et al.* 1994; Shan *et al.* 1994; Zhang *et al.* 1994). In their comprehensive review, Duval and colleagues (1983) discussed the reasons for the requirement of supraphysiological doses in investigations of the non-genomic effects of steroids: circulating hormone concentrations do not accurately reflect those in target tissues. Tissue concentrations may well be higher than plasma concentrations. High levels of steroids could occur under either pathological or physiological conditions. The concentration of free steroids in the blood may also vary as a function of plasma steroid-binding proteins. In these preliminary studies, we were seeking substantial changes in tissues and cells *in vitro*. The concentrations needed to produce these effects may be higher than those required for physiological actions *in vivo*.

6.5. SUMMARY

6.5.1. Progesterone has rapid relaxant and inhibitory effects on KCl-contracted rat vascular tissue preparations. These effects can be quickly reversed by washout of the hormone.

6.5.2. The effect of progesterone on endothelium-intact aortic tissue may partially be mediated by stimulating the production of NO in the endothelium.

6.5.3. Progesterone inhibits KCl-stimulated $[Ca^{2+}]_i$ increase in VSMC. This effect is rapid in onset and reversible by washout.

6.5.4. Progesterone rapidly decreases L-type Ca^{2+} channel currents in VSMC. The effects were concentration-dependent and can be quickly reversed by washout.

6.5.5. 17β-E_2 decreases L-type Ca^{2+} channel currents in VSMC.

6.5.6. Testosterone has a relaxant effect on aortic tissue contraction.

6.5.7. Aldosterone does not have effect on either aortic tissue tension generation or L-type Ca^{2+} channel currents.

6.5.8. Dexamethasone dose not have effect on aortic tissue contraction.

6.5.9. The effects of progesterone on vascular tissue preparations are most likely specific and non-genomic.

6.6. SIGNIFICANCE

This study demonstrates for the first time that the effects of progesterone on tension generation of vascular tissue, $[Ca^{2+}]_i$ and Ca^{2+} channel activity in VSMC are fast in onset and rapidly reversible by washout. The effects are most likely non-genomic ones.

It is also the first time to show that the effect of progesterone on aortic tension is partially endothelium-dependent.

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