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Postsynaptic Cholinergic Control Of Cardiac Function In Aging

Na Su

POSTSYNAPTIC CHOLINERGIC CONTROL OF CARDIAC FUNCTION IN AGING

by

Na Su

Department of Physiology
Faculty of Medicine

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ABSTRACT

Cardiac performance is determined by the integrated function of multiple interdependent variables: heart rate, contractility, coronary flow, preload and afterload. Each of them is subject to autonomic modulation. There exists an impressive body of evidence showing that adrenergic control of heart function declines with aging in humans and animals due, in part, to impaired β-adrenergic signal transduction at the postsynaptic level. In contrast, information about age-related changes in the cholinergic control of heart function is scanty and limited *in vivo* studies have produced conflicting results. The present study using isolated constant flow-perfused heart from adult (8 month-old) and aged (26 month-old) Fischer 344 rats first time demonstrated a striking enhancement of the negative chronotropic, inotropic and coronary vasoconstriction responses of the aging heart to postsynaptic cholinergic stimulation.

To study the underlying mechanisms of these age-related changes in the heart, the approaches used and the results demonstrated were the following. (i) Acetylcholinesterase activity measured by the method of Ellman et al (1961) declined significantly in the atria and ventricles of the aged compared to adult rats. (ii) No age-related difference was seen in muscarinic receptor number in the atria and ventricles as assessed by [^{3}H]QNB binding, but the muscarinic receptor binding affinity for carbachol, a cholinergic agonist, was increased in the atria but not ventricles of aged compared to adult rats. (iii) The relative amount of Giα protein measured by Western immunoblotting and ADP-ribosylation techniques was significantly greater in the atria and ventricles of the aged compared to adult rats. The fidelity of the signal transduction through muscarinic receptor-linked Gi protein, as judged from the guanine nucleotide-induced decrease in receptor affinity for carbachol, was unaltered with aging in atria and ventricles. (iv) Using standard microelectrode techniques, it was found that aging was accompanied by (a) enhancement in carbachol-induced hyperpolarization of the resting membrane potential in atria but not ventricles and (b) more pronounced carbachol-induced shortening of the action potential duration measured at 50% of repolarization in the ventricles but not in

the atria. (v) Aging did not alter the carbachol-induced prolongation of atrioventricular conduction time measured from electrocardiograms. All of the age-related differences mentioned above may contribute to the increased negative chronotropic and inotropic responses of the aged heart to cholinergic stimuli. The age-associated increase in the coronary vascular response to a cholinergic stimulus may be caused by a relatively greater influx of extracellular Ca^{2+}, since the age-related difference in the coronary vascular response was attenuated in the presence of verapamil, a calcium channel blocker. Neither impaired synthesis of endothelium-derived relaxing factor (EDRF) nor reduced sensitivity of coronary vascular smooth muscle to EDRF contributes to the enhanced coronary vasoconstrictive effect to cholinergic stimulation since there was no age-related difference in the L-NMMA (an inhibitor of EDRF synthesis)-induced coronary vasoconstriction in the absence and presence of carbachol. The age-related increase in the coronary response was specific for the cholinergic receptor as the α-adrenergic response of the coronary vasculature was not altered with aging.

In summary, in contrast to the age-related decrease in the β-adrenergic response, the cholinergic response of the heart is increased in the aged compared to the adult rats. The mechanisms for the enhanced postsynaptic response is multi-factorial, and may be attributable to the age-related changes in acetylcholinesterase activity, characteristics of the muscarinic receptor, content of the Gi protein, and electrophysiological properties of the atria and ventricles.

DEDICATED TO:

My husband GuangHue Li, M.D. and my son James Li.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 GENERAL REVIEW

Our understanding of the neural pathways, receptor properties, the guanine nucleotide regulatory protein (G protein), and ionic currents responsible for vagally induced inhibition of cardiac function has been significantly advanced in the past 10 years. Improvements in electrophysiological techniques, cell isolation methods, protein chemistry, and molecular biology have played a very important role in allowing us to examine molecular interactions in great detail so as to better reconstruct the reaction steps between release of acetylcholine (ACh), the vagal transmitter, and the eventual electrophysiological and contractile changes in various cardiac cells. The purpose of this chapter is to review the important advances in uncovering the mechanisms for the postsynaptic cholinergic control of cardiac function in the normal and aging heart. This chapter will start with the general overview of autonomic regulation of cardiac function and end with the specific objectives of this Ph.D. thesis.

1.1 Autonomic nervous control of cardiac function

Cardiac performance is determined by the integrated function of multiple interdependent variables: heart rate, contractility, coronary blood flow, preload, and afterload. Each of these variables is subjected to autonomic regulation. In the resting heart, the major influence is parasympathetic, whereas during exercise the situation is reversed and sympathetic stimulation becomes predominant (Katz, 1992). Inherent in this autonomic control system is the ability of the myocardium to alter its activity rapidly and reversibly in accordance with variations in physiological demands and thus maintain circulatory homeostasis.

1.1.1 Sympathetic and parasympathetic innervations of the heart

The heart is innervated by both sympathetic and parasympathetic subdivisions of the autonomic nervous system (Norris and Randall, 1977). The former arise mainly from the fourth and fifth segments of the thoracic spinal cord and from synaptic connections

sympathetic fibers are distributed to all regions of the heart. Parasympathetic innervation of the heart originates in the dorsal efferent nuclei of the medulla oblongata. Parasympathetic fibers arising in these brainstem nuclei reach the heart via the cardiac branches of the vagus nerve, where they impinge on ganglion cells generally located within the heart. Until recently, it was generally thought that the parasympathetic fibers only innervated the SA node, the AV node, the atria and the ventricular blood vessels, but not the ventricular myocardium. However, it is now apparent that the cholinergic nerves supply the ventricles and conducting tissues of the His-Purkinje system (Loffenholz and Pappano, 1985; Randall and Ardell, 1985; Katz, 1992).

Sympathetic fibers appear to traverse the ventricles within the epicardium before penetrating to the endocardium, whereas the parasympathetic innervation of the ventricles is distributed from their endocardial surface (Barber et al., 1984).

1.1.2 Sympathetic control of cardiac function

Norepinephrine released from postganglionic sympathetic nerve endings of the heart enhances heart rate (chronotropy), contractility (inotropy), and coronary vasodilation through interaction with β-adrenergic receptors on the sarcolemma (Tsien, 1977). In addition to activation of β-adrenergic receptors, norepinephrine potentiates cardiac contractility, heart rate and coronary vasoconstriction via α-adrenoceptors (Flavahan and McGrath, 1981; Tung et al, 1982). Although both β- and α-adrenergic stimulation produce positive inotropic effects, the underlying mechanisms differ (Benfey, 1982; Scholz, 1980). The positive inotropic effects of β-adrenergic stimulation are associated with a shortening of the time to peak tension and potentiated rate of myocardial relaxation (positive lusitropy), whereas α-adrenergic stimulation prolongs the time to peak tension and the duration of contraction (negative lusitropy) (Toda and Miyazaki, 1987; Ledda et al., 1975). The α-adrenergic stimulated prolongation in the time to peak force is accompanied by an increase in action potential duration (Vogel and Terzic, 1989) and an inhibition of transient outward current (I_{to}) and therefore slow repolarization (Hartzell, 1988). Different ionic and biochemical mechanisms are involved in mediating these α- and β-adrenergic responses as well (Lindemann and Watanabe,

1990), as will be discussed in sections 1.1.3 and 1.1.4.

Sympathetic control of cardiac function depends on various types of receptors in the cell membrane to translate signals to particular cellular responses (Watanabe and Lindemann, 1984). The heart contains mainly β-adrenergic receptors although α-adrenergic receptors are also present; coronary vessels, on the other hand, contain more vasoconstrictor α- than vasodilator β-adrenergic receptors (Katz, 1992). In the heart, in most circumstances, the responses are predominantly of the β-adrenergic agonist type. Under conditions in which the β-adrenergic response is compromised, or in response to myocardial ischemia, α-adrenergic responses may predominate (Lindemann and Watanabe 1990), as a backup inotropic system. For the subtypes of the adrenergic receptors, generally speaking, norepinephrine mainly stimulates α- and β_1- adrenergic receptors in the heart, whereas epinephrine released from the adrenal medulla, mainly stimulates α- and both classes of β-receptors (β_1- and β_2-subtypes), in the heart (Lindemann and Watanabe 1990). Both β_1- and β_2-adrenergic receptors in cardiac tissue are distributed throughout the mammalian heart. In general, in the atrial and ventricular myocardium of normal heart, β_1-receptors account for 60-80% of the total β-receptor population; β_1 and β_2 receptors have similar function in the heart, mediating positive inotropic and chronotropic effects through cyclic AMP pathway (Brodde, 1988). Several studies reveal that the α-receptors are predominantly of the α_1-subtype in mammalian myocardium (Bode and Brunton, 1989; Colucci et al., 1984; Karliner et al., 1979).

1.1.3 Mechanisms of β-adrenergic signal transduction in the heart

It is generally accepted that the predominant effects of β-adrenergic stimulation of a variety of cell types are mediated by cyclic AMP (cAMP) (Drummond and Severson, 1979; Tsien, 1977), through stimulatory G protein (G_s) (Gilman, 1987; Levitzki, 1988). There are, however, additional mechanisms by which activation of G_s by β-adrenergic stimulation can modify cardiac function: these involve (1) direct interactions between the activated G_s and specific effector systems (Brown and Birnbaumer, 1988; Yatani and Brown, 1989; Brown 1990; Katz, 1992), and (2) phosphorylation of specific membrane proteins by Ca^{2+}/calmodulin-dependent protein

kinase (CaM kinase) (LePeuch et al, 1979; Takasago et al, 1991; Witcher et al, 1991; Xu et al, 1993).

1.1.3.1 Cyclic AMP-dependent mechanisms

Increases in cAMP levels by the β-adrenergic system may augment protein phosphorylation by activation of the cAMP-dependent protein kinase (PKA) and inhibition of type I phosphatase activity (Ahmad et al., 1989). The specificity of the cellular responses depends upon the protein substrates phosphorylated. In myocardium, a number of cytosolic and integral membrane protein substrates of PKA have been identified (Jones et al., 1986; Kameyama et al., 1986). Phosphorylation of these proteins may change the contraction of cardiac muscle cells by promoting Ca^{2+} entry from the extracellular space and Ca^{2+} release and uptake by the sarcoplasmic reticulum, and by decreasing Ca^{2+} affinity of troponin I (a contractile protein).

1.1.3.1.1 Regulation of sarcolemma ion channels

The major electrical effects of β-adrenergic stimulation in ventricular myocardium include an increase in the amplitude of the plateau and an increase in the rate of phase 3 repolarization, with an overall shortening of action potential duration (Lindemann and Watanabe, 1990). The increase in the plateau of the action potential is generally attributed to an augmentation of L-type Ca^{2+} channel activity (I_{Ca}), and the shortening of the action potential duration is attributed to increasing the delayed outward K-current (I_K), through PKA (Walsh and Kass, 1988; Trautwein and Hescheler, 1990; Schult et al., 1990). Decreasing the rate of dephosphorylation by inhibition of phosphatase has also been demonstrated in the augmentation of I_{Ca} in guinea pig ventricular cells and this result support the participation of phosphatase in regulation of the I_{Ca} (Hescheler et al, 1987; Sperelakis et al, 1992).

In addition, β-adrenergic stimulation has been reported to increase pacemaker-current (I_f) (Hagiwara and Irisawa, 1989), transient outward current (I_{to}) (Nakayama and Fozzard, 1988), and activate a Cl^- current (Bahinski and Nairn, 1989; Harvey and Hume, 1989), all through the cAMP-dependent pathway. Thus, cAMP-dependent protein kinase

phosphorylates several membrane proteins, each associated with an ion channel, to exert the positive chronotropic and inotropic effects (Hartzell, 1988). β-adrenergic stimulation also increase sodium current in rabbit ventricular myocytes through both direct and cAMP-dependent pathways (Matsuda et al, 1993). In the heart the importance of ion channel modulation is quite obvious: regulation of frequency and force of the heart beat by neurotransmitters depends on gating ion channels (Reuter, 1979; Reuter, 1987).

1.1.3.1.2 Regulation of sarcoplasmic reticulum (SR)

The Ca^{2+} uptake and release functions of SR are well known to be subject to modulation by cAMP-mediated protein phosphorylation.

β-adrenergic receptor-stimulated cAMP-dependent phosphorylation of phospholamban (PL) has been well documented (Hicks 1979; Tada et al., 1983; James et al 1990). PL is a pentamer made up of five identical subunits having molecular weights of 6,000 (Fujii et al., 1987). The C-terminal domain of PL is hydrophobic and lies within the sarcoplasmic reticulum membrane bilayer, whereas the N-terminal region contains two sites that, when phosphorylated, stimulate calcium transport. The substrate for cAMP-dependent phosphorylation is serine at position 16. Phosphorylation of PL by cAMP-dependent protein kinase stimulates calcium transport into the cardiac sarcoplasmic reticulum. This effect is due both to an increase in calcium sensitivity of the cardiac calcium pump, which accelerates calcium removal at low [Ca^{2+}], and more rapid pump turnover (Hicks et al, 1979; Sasaki et al, 1992; Tada et al, 1974; 1982; 1983). Both effects accelerate relaxation; the increased calcium sensitivity of the calcium pump may also increase the extent of relaxation by reducing cytosolic [Ca^{2+}] in the fully relaxed heart.

It is also postulated that increased Ca^{2+} uptake by calcium pumps, as described in previous paragraph, would increase the sarcoplasmic reticulum Ca^{2+} loading; more Ca^{2+} will be available for subsequent Ca^{2+} release from sarcoplasmic reticulum, thus contributing to the positive inotropy of β-adrenergic stimulation (Tada and Katz, 1982).

Sarcoplasmic reticulum Ca^{2+} release channel proteins have recently been isolated from cardiac muscle as ryanodine receptor (for a review see Fleisher and Inui, 1989).

PKA phosphorylates the ryanodine receptor (Takasago et al., 1991), which may promote Ca^{2+} release from sarcoplasmic reticulum and contribute to the positive inotropic effect of β-adrenergic stimulation.

1.1.3.1.3. Regulation of myofibrillar proteins and modulation of contractility

PKA also phosphorylates the troponin I of the thin filaments and C protein of the thick filaments in intact heart (Solaro, 1980; Garvey et al., 1988). Phosphorylation of troponin I decreases the calcium affinity of the contractile proteins, and so facilitates the ability of the calcium pump to relax the heart by removing calcium from troponin C. However, the physiological significance of the phosphorylation of C protein of the thick filaments is not known (Garvey et al., 1988). β-Adrenergic stimulation also increases the rate of cross-bridge cycling; the mechanism responsible for this effect, which appears to be mediated by cAMP, is not yet clear (Hoh et al., 1988).

1.1.3.2. Cyclic AMP-independent mechanisms

Direct activation of cardiac L-type Ca^{2+}-channels by $Gs\alpha$ has been suggested recently (Brown, 1990; Brown, 1991). This direct G-protein activated calcium current is proposed to be important in the ability of cardiac sympathetic nerves to change heart rate within a single beat (Yatani and Brown, 1989). Nevertheless, several lines of evidence suggest a minor role for this direct mechanism (Kameyama et al., 1985). Hartzell and Fischmeister (1992) have evaluated evidence for this direct pathway and conclude that although G-proteins affect cardiac Ca^{2+} channels in bilayer and excised patches, there is little evidence that this pathway is physiologically significant. Additional studies will be needed to establish the physiological relevance of direct G-protein regulation of Ca^{2+}-channels in myocardium.

It is also suggested that there are direct G_s -linked and /or phosphorylation of sarcolemmal Na^+ channels by PKA to effectively close them when membranes are depolarized (Schubert et al., 1989). However, the effects were inhibitory and may only be important in pathophysiological states (Schubert et al., 1989; Schubert et al., 1990).

CaM kinase-dependent phosphorylation of SR proteins such as PL (LePeuch et

al, 1979; Tada and Katz, 1982), Ca^{2+} pumping ATPase (Xu et al, 1993), and Ca^{2+} release channel (Witcher et al, 1991; Takasago et al, 1991), has been demonstrated in the heart. The phosphorylation of PL by CaM kinase relieves the inhibition of pump function. The phosphorylation of the cardiac pumping ATPase by CaM kinase can stimulate its enzymatic activity and therefore Ca^{2+} transport function. The phosphorylation of the SR release channel by CaM kinase may increase in the open time of the single channels.

1.1.4 Mechanisms of α-adrenergic effect on the heart

In the heart, activation of α_1-adrenergic receptors potentiates the force of contraction, while in vascular smooth muscle, stimulation of these receptors initiates contraction. Both of these effects are thought to be mediated by an increase in the free cytosolic Ca^{2+} concentration through its action on a number of Ca^{2+}- and Ca^{2+}/calmodulin-dependent proteins (Brown and Birnbaumer 1990). The exact mechanism leading to the increase in the cytosolic Ca^{2+} concentration is still unclear. Current evidence implicates the following pathways. Agonist-receptor binding stimulates the phospholipase C hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) through pertussis toxin-insensitive G protein, resulting in the formation of 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) (Berridge 1988; Minneman, 1988). An increase in IP_3 results in the mobilization of intracellular Ca^{2+} (Berridge, 1988), while the increase in DAG in combination with an elevated Ca^{2+} concentration may stimulate protein kinase C. This will phosphorylate a number of cellular proteins (Nishizuka, 1984), and therefore increase cellular calcium levels by promoting the opening of calcium channels in the sarcolemma (Döşemeci et al, 1988), and increase calcium affinity of the contractile apparatus (Gwathmey and Hajjar, 1990). A recent study (Otani and Das, 1988) has shown that the rapid formation of the 1,4,5-isomer of IP_3 was associated with the rapid, transient, positive inotropic effect of α-adrenergic stimulation in papillary muscle; while the sustained phase of the positive inotropic effect is related to and involves voltage gated Ca^{2+} channels. These transient effects were not affected by nifedipine, suggesting that IP_3 may have resulted in the mobilization of Ca^{2+} from

intracellular stores (Otani and Das, 1988). Although studies in skinned (Fabiato, 1986) or permeabilized (Nosek et al, 1986), cardiac cells have shown that IP_3 can release Ca^{2+} from sarcoplasmic reticulum, how these small IP_3-induced increases in $[Ca^{2+}]_i$ mediate the functional effects of α-adrenergic stimulation is not known (Lindemann and Watanabe, 1990).

Myocyte α_1-adrenergic receptors are also coupled to other effector proteins through a pertussis toxin-sensitive G protein (G_i). These include inhibition of an outward K^+ current, either I_K or I_{to} (Apkon and Nerbonne, 1988; Szabo and Otero, 1990) and inhibition of β-adrenergic-stimulated adenylate cyclase by activation of a cAMP phosphodiesterase (Buxton and Brunton, 1985; Steinberg et al, 1989). However, how these effector pathways link to the α-adrenergic effects on the heart is not clear yet. The occurrence of both pertussis toxin-sensitive and pertussis toxin-insensitive effects suggests that multiple G-proteins may be involved in mediating their effects and that different G-proteins may be linked to specific α_1-adrenergic receptor subtypes (Minneman, 1988).

1.2 Parasympathetic control of cardiac function

Parasympathetic nerves innervating the heart release acetylcholine (ACh), which acting *through* the cardiac muscarinic receptors (MAChR), exerts powerful negative chronotropic (heart rate), inotropic (force of contraction), and dromotropic (atrioventricular conduction) effects on the heart, and coronary vasoconstriction (Doods et al, 1989).

1.2.1 Muscarinic cholinergic receptors in the heart

1.2.1.1 Receptor subtypes

Cholinergic effects on the heart are mediated by muscarinic cholinergic receptors. At the present time the amino acid sequences of five muscarinic receptor species (m1-m5) are known, each subtype being encoded by different cellular genes (Hammer, 1989; Bonner, 1989). These include : (i) M_1-subtype with high affinity for pirenzepine, found mainly in autonomic ganglia and the central nervous system; (ii) M_2-subtype with a high

affinity for AFDX-116 (11-[{2[(diethylamino)methyl]-1-piperidinyl}acetyl]-5,11-dihydro 6H-pyrido[2,3-b][1,4]benzodiazepine-6-one) found in the heart; and (iii) M_3-subtype with high affinity for 4-DAMP (4-diphenylacetoxy-1-methylpiperidine methiodide) in smooth muscle and secretory cells. There is a candidate M_4 receptor, found in the striatum and some cell lines (e.g. NG108-15), which inhibits adenylate cyclase. The pharmacology of this receptor, as well as that of the expressed m4 and m5 species, whilst being different from M_1, M_2, and M_3 receptors is not sufficiently distinctive or well characterized to allow an unambiguous assignment of a pharmacological M_4 (or M_5) receptor subtype (Bonner, 1989).

In the heart, ACh released from postganglionic neurons of the vagus nerves activates M_2 muscarinic receptors on the myocardial cells to reduce the force of contraction, beating frequency, and slow the atrioventricular conductance. Furthermore, ACh may stimulate presynaptic muscarinic receptors on cholinergic and noradrenergic nerve fibers (Starke et al., 1989. Muscholl, 1980; Bogar et al., 1990). Activation of these presynaptic receptors in the heart inhibits the release of ACh and norepinephrine, respectively (Dammann et al 1989; Bognar et al., 1990). There is evidence for a vagal innervation of large and small coronary arteries in several species (Feigl, 1983; Kalsner 1989). Isolated coronary arteries from most species, including human, sheep, pig and rat, contract in response to ACh and other cholinergic agonists (Doods et al., 1989; Kalsner, 1989). The contraction of coronary vasculature leads to an atropine-sensitive decrease in coronary flow (Van Charldorp et al., 1987). The coronary vasoconstriction is mediated by M_3 muscarinic receptors (Eglen and Whiting, 1990).

1.2.1.2 Receptor structure

Rapid advances in molecular biology have led to the recognition that most cardiac receptors are members of an extensive family of membrane proteins containing seven hydrophobic membrane-spanning regions (reviewed by Katz, 1992). This family includes all known subtypes of the α- and β-adrenergic receptors, the muscarinic cholinergic receptors, receptors for a number of peptide hormones and rhodopsin, which responds not to chemical transmitters, but to photons. Homologies among the different members

of the family of receptor proteins provide clues regarding structure-function relationships in their amino acid sequences (Katz, 1992). Although many details of these structure-function relationships are still unclear, it appears that binding of extracellular messengers occurs within a "pocket" formed by the receptors within the membrane bilayer. This leads to a conformational change of the receptors that affects the next step in signal transduction, interaction of ligand-receptor complex with G proteins.

1.2.2 Muscarinic receptor-linked signal transduction

ACh is recognized by muscarinic receptors and forms a ligand-receptor complex. The extent to which ACh modifies cardiac function depends on its concentration, increasing when more ACh is present (Katz, 1992).

The signal transduction occurs when the ligand-receptor complex interacts with another class of membrane proteins called G proteins. The G proteins should be viewed as providing a vital step in a series of "translations" that begins when a ligand arrives at the cell surface and binds to its specific receptor. It is the coupling proteins, rather than the receptor, that interact with various intracellular effector systems to produce the cellular physiological responses (Fleming et al., 1992).

1.2.2.1 G protein subtypes

At least three types of G protein have been identified in cardiovascular tissue (Robishaw and Foster, 1989). G_s protein mediates the β-adrenergic stimulated cAMP production and modulates the activity of voltage-dependent ion channels as described in section 1.1.3. G_i protein mediates inhibition of β-adrenergic-stimulated adenylate cyclase activity by muscarinic cholinergic, α-adrenergic, and adenosine A_1-receptor agonists (Neumann et al., 1989). G_i also directly couples muscarinic receptors to various effectors which will be discussed in section 1.2.3. G_o is present in heart and blood vessels, but its role is unclear. There is ample evidence that G_o regulates Ca^{2+} channels in brain where G_o is very abundant. G_q is a recently identified 42-kDa protein involved in muscarinic and α-adrenergic receptor stimulation of phospholipase C (Pappano et al, 1988), and

phospholipase A_2 (Kim et al., 1989).

For the parasympathetic control of cardiac functions, the major three systems involved in the transmembrane signaling pathway are the muscarinic cholinergic receptor (MAChR), pertussis toxin sensitive G_i-G_o proteins and pertussis toxin insensitive G_q proteins, and various effectors which will be discussed later.

1.2.2.2 Subunits of the G proteins

Each of these G proteins is a heterotrimer consisting of α-, β-, and γ-subunits (G_α, $G\beta$ and $G\gamma$) (Gilman, 1987). $G\alpha$-subunits contain the binding site for guanine nucleotides and a specific GTP hydrolytic enzyme (GTPase). $G\alpha$ interacts reversibly with its respective receptor and effector molecules, and confers specificity to the holoprotein by virtue of α-subunit structural heterogeneity relative to the more homogeneous structures of β- and γ-subunits. Another characteristic of $G\alpha$-subunits is that most of them possess one or two sites for nicotinamide adenine dinucleotide (NAD)-dependent ADP-ribosylation. This covalent modification of α-subunits is catalyzed by bacterial toxin-cholera toxin in the case of $G_{s\alpha}$ and pertussis toxin in $G_{i\alpha}$, $G_{o\alpha}$. $G_{q\alpha}$ appears not to be ADP-ribosylated by either toxin. ADP-ribosylation of $G_{s\alpha}$ inhibits its GTPase activity, thus irreversibly activating the subunit to stimulate adenylate cyclase and ion channels (Milligan, 1988). ADP-ribosylation of $G_{i\alpha}$ and $G_{o\alpha}$ inhibits the interaction between the subunits and receptors (Milligan 1988). These functional modifications make ADP-ribosylation a means for detecting, quantifying, and localizing G proteins.

1.2.2.3 The G proteins undergo a regulatory cycle

The G proteins undergo a regulatory cycle consisting of binding of GTP to the $G_{s\alpha}$-subunit of the $G_{\alpha\beta\gamma}$ heterotrimeric complex, dissociation of the inhibitory $G_{\beta\gamma}$ complex, and interaction of $G_{s\alpha}$·GTP with the effectors (Gilman, 1987). The binding of $G_{s\alpha}$ to GTP is vital to the ability of the G proteins to activate the effectors (reviewed by Lindemann and Watanabe, 1990). The signal transduction occurs when activated receptor-agonist complex interacts with $G_{\alpha\beta\gamma}$·GDP in the presence of GTP. Termination of the cycle occurs with the hydrolysis of GTP to GDP (by GTPase in G_α) and

reassociation of $G_{s\alpha}$·GDP with $G_{\beta\gamma}$ to form the inactive complex. G_i protein inhibits the effect of $G_{s\alpha}$ (Hartzell, 1988). Receptor affinity for agonists is relatively high when the agonist-receptor complex is associated with a G protein complex with GDP (Caron and Lefkowitz, 1993). Upon binding of the agonist-receptor complex to the G protein, GDP is released, freeing the nucleotide site on the α-subunit for interaction with GTP. Binding of GTP to the G protein reduces the ligand-binding affinity of the receptor. Because the rate of dissociation of the agonist-receptor complex from the G protein is faster than the rate of G protein inactivation (GTPase activity), each agonist-receptor complex catalytically activates multiple G proteins (signal amplification) (Fleming et al 1992). When not complexed to a G protein, receptors have low affinity for agonists; thus, observation of a GTP-mediated decrease in agonist affinity for receptor is a common characteristic of receptors coupled to G proteins (Flemming et al, 1992).

1.2.2.4 Muscarinic receptor-linked G proteins and their functions in the heart

The heterogeneity of the α subunit(s) of G_i has recently been documented (See Gilman, 1987 for a review). Three distinct α subunits of G_i have been designated α_{41} of G_{i1}, α_{40} of G_{i2}, and α_{41} of G_{i3}. $G_{i\alpha}$ has been shown to inhibit adenylate cyclase (Fleming et al., 1992) and directly couple cell membrane receptors to ion channels (Christie and North, 1988). $G_{i\alpha3}$ has been called G_K, when it is preactivated with GTPγS (a nonhydrolyzable GTP analog), mimicked the MAChR effect on I_{KACh} channel (Brown and Birnbaumer, 1990). Although three distinct α-subunits ($G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$) were demonstrated to open single I_{KACh} (Yatani et al., 1988), the G_K was effective at pM concentrations even in the absence of Mg^{2+} (Kurachi et al., 1986). However, the linkage between the other $G_{i\alpha}$ subunits ($G_{i\alpha1}$ and $G_{i\alpha2}$) and their effector(s) is not yet clear. It is reported that $G_{i\alpha1}$ is absent in the heart (Jones and Reed, 1987).

G_o (G "other") was first observed as a 39-KDa pertussis toxin substrate in addition to G_i in brain, and G_o has now been cloned (Itoh et al, 1986). G_o is very similar to G_i, binds GTP, and has been shown to regulate muscarinic receptor affinity for agonists in brain (Florio and Sternweis, 1985). Although functions have not been well defined for G_o, many possibilities exist (Fleming et al, 1992).

$G_{q\alpha}$ of the G_q is a newly purified G protein subunit that is refractory to ADP-ribosylation by cholera toxin or pertussis toxin (Pang and Sternweis, 1990). It migrates on polyacrylamide gel electrophoresis as a 42-KDa protein and has been identified in a number of mammalian tissues, including brain, lung (greatest concentrations), and heart (Fleming et al 1992). Isolated $G_{q\alpha}$ exhibits slow rates of GDP-GTP exchange and GTP hydrolysis in comparison to other G_α-subunits. The $G_{q\alpha}$ protein may be considered a candidate for the pertussis toxin-insensitive protein that couples muscarinic and α_1-adrenergic receptors to phosphoinositide hydrolysis (Fleming et al., 1992).

1.2.3 Functional effects of muscarinic receptor in the heart

ACh initiates inhibitory signals in the heart by activating MAChR of the M_2 type as described in chapter 1.2.1.1. Since the inhibitory action of carbachol in the absence of isoproterenol, and its attenuation by pertussis toxin were not associated with any change in tissue cAMP level, the inhibitory action of carbachol elicited via MAChR is generally accepted as a cAMP-independent subcellular process (direct action) (Endoh, 1987). Carbachol decreased the isoproterenol-induced positive chronotropic and inotropic responses by preventing rises in cAMP level. The inhibitory action of carbachol on the isoproterenol-induced functional and cAMP responses was also reduced by pertussis toxin treatment (Endoh, et al, 1985). This inhibitory action of carbachol via MAChR is regarded as cAMP-dependent subcellular process (indirect action) (Endoh, 1987).

In the heart, the MAChR is coupled directly through Gi-Go proteins to the opening of ligand-regulated K^+ channels (I_{KACh}), and mainly indirectly (i.e. through the regulation of a second messenger, cAMP), to the closing of L-type Ca^{2+} channels (I_{Ca}). There also appears to be a dual (direct and/or indirect) coupling of MAChR to pacemaker channels (I_f). By virtue of the signal transduction function of G_i and/or G_o, ACh exerts negative chronotropic, inotropic and dromotropic actions on the heart (reviewed in Hartzell, 1988; Katz, 1992).

1.2.3.1 Direct stimulation of an inward rectifying outward K^+ channel by Gi protein

One of the major effects of muscarinic agonists in the heart is to decrease the

heart rate set by specialized pacemaker cells in the right atrium. Muscarinic agonists appea to decrease the heart rate by activating K^+ channels (I_{KACh}) in pacemaker cells, thereby stimulating K^+ efflux and hyperpolarizing these cells (Sakmann et al., 1983). This viewpoint has been confirmed and extended to atrial cells (Soejima and Noma, 1984), and Purkinje fibers (Carmeliet and Mubagwa, 1986). Ventricular tissue generally has a lower concentration of I_{KACh} channels than does atrium or pacemaker tissue. In frog ventricle, I_{KACh} is about 25% as large as it is in atrium (Hartzell and Simmons, 1987). I_{KACh} has also been reported in ferret ventricular muscle (Boyett et al., 1988), but I_{KACh} may be present only at very low levels in ventricle of other species (Hartzell, 1988). Since the potassium currents carried by these channels promote repolarization and shorten the plateau of action potential, the opening of I_{KACh} also reduces contractility by limiting the Ca^{2+} entry during the action potential (Katz, 1992).

It was puzzling to learn that the α-subunit of G_K was proposed as the transducing element by one group of investigators (Yatani et al., 1987; Codina et al., 1987), whereas the $\beta\gamma$-subunit was thought to serve this function by another group (Logothetis et al., 1987). In the several subsequent reports that addressed the apparent discrepancy (Kirsch et al., 1988; Clapham and Neer, 1988), both groups of investigators agree that either α- or $\beta\gamma$-subunits can activate the I_{KACh} and that there are physiological roles for both the α- and the $\beta\gamma$-subunits in regulating the I_{KACh} function. The G-protein-K^+ channel interaction is obviously more complex than originally envisioned (Neer and Clapham, 1988; Logothetis et al., 1988; Logothetis, et al., 1988; Kim et al., 1989). It is thought that although the α-subunit may be the principal transducer for muscarinic agonist activation of I_{KACh}, stimulation of phospholipase A_2 activity by $\beta\gamma$-subunits may represent another important, but as yet poorly undefined, pathway (Kim et al., 1989).

Recently, it has been reported that adenosine triphosphate-Mg^{2+} (ATP-Mg^{2+}) can activate Muscarinic K^+ channels in the absence of ACh (Heidbuchel et al, 1992). This activation is the result of the association of a membrane-bound nucleoside diphosphate kinase (NDPK) to the muscarinic signaling transduction triad "receptor-G protein-K^+ channel". Direct transformation of G_K-bound GDP into GTP by the enzyme-complex G_K-NDPK without passing by a phase of free cytosolic GDP or GTP can act as an alternative

for exchange of one GDP molecule for a second GTP molecule to activate the G protein and the channels. Hence, it is postulated that the main functional role of NDPK under physiological conditions is to provide a local supply of GTP (using GDP and ATP), in the immediate vicinity of the G protein, thereby maintaining a high local GTP/GDP ratio and ensuring adequate receptor-mediated regulation of muscarinic K^+ channel activity.

1.2.3.2 Inhibition of L-type Ca^{2+} current (I_{Ca}) by MAChR

Another important effect of muscarinic stimulation is to close (L-type), I_{Ca} through G_i protein, especially when it is under the stimulatory influence of β-adrenergic agonists (Robishaw and Foster, 1989; Pappano et al., 1988). Inhibition of calcium channel opening will shorten the plateau of an action potential, and this has been held responsible for the negative inotropic effect of ACh (Ten Eick et al, 1976). The inhibition of the I_{Ca} may also produce negative chronotropic and dromotropic effects in the sinoatrial (SA), and atrioventricular (AV), nodes respectively (Katz, 1992; Hartzell, 1988).

It is important to know whether ACh has effects on I_{Ca} in the absence of catecholamines, because in most studies on isolated atrial and ventricular cells from guinea pig, frog or ferret, ACh alone has no effect on basal I_{Ca} even though it has very potent inhibitory effects on I_{Ca} in the presence of adrenergic stimulation (Fischmeister and Hartzell, 1986; Hescheler et al, 1986; Boyett et al., 1988). In contrast, most studies in multicellular tissue have shown that ACh does inhibit basal I_{Ca} and inhibit the contractility in mammalian atrial and ventricular muscles (Ten Eick et al, 1976; Giles and Noble, 1976; Hino and Ochi, 1980; Biegon and Pppano, 1980; Nargeot et al, 1981). Interestingly, in Biegon and Pappano's experiments (1980), ACh reduced Ca^{2+} - dependent action potential durations of chick ventricle markedly (without altering membrane potential) even after treatment with propranolol and reserpine or 6-hydroxydopamine. Thus, they suggested that ACh might have direct G protein effects on an ion channel that produces the inhibition of the Ca^{2+} dependent action potential under these conditions. Also, it has been reported recently that ACh depressed basal I_{Ca} in rabbit sino-atrial myocytes without previous β-adrenergic stimulation (Petit-Jacques et al, 1993).

1.2.3.3 Inhibition of hyperpolarization activated I_f by MAChR

The hallmark of a pacemaker cell is its ability to depolarize spontaneously during diastole (DiFrancesco et al, 1989; Katz, 1992). The pacemaker current I_f was first described as an outward K^+ current (I_{K2}) (Noble and Tsien, 1968). Subsequently, it has been recognized that this current is not a slowly decaying outward K^+ current, but rather is a slowly activating inward current carried by both Na^+ and K^+ ions that is activated by hyperpolarization (Noble, 1984; DiFrancesco, 1985). ACh shifts the activation curve of I_f to negative potentials with no change in maximum activated current (DiFrancesco and Tromba, 1987, 1988a) through $G_{o\alpha}$ and $G_{i\alpha}$, with $G_{i\alpha}$ being more potent (DiFrancesco and Tromba, 1988b; Brown, 1990). The effect of ACh is blocked by muscarinic antagonists and pertussis toxin, suggesting that the effect of ACh is mediated by a muscarinic-receptor-coupled G_i protein.

1.2.3.4 Muscarinic receptor induced other effects in the heart

Muscarinic receptors are also coupled to phosphoinositide turnover through a pertussis toxin-insensitive G_q protein, like α-adrenergic receptors in the heart (Fleming et al, 1992). Muscarinic effects directed through the phosphoinositide pathway appear to be positive inotropic; thus, these effects are opposite to the predominant negative inotropic effects that usually result from stimulation of muscarinic receptors. Muscarinic stimulation via this pathway leads to generation of IP_3 and DAG. The physiological significance of the muscarinic-phosphoinositide connection is not yet clear (Robishaw and Foster, 1989).

In addition, muscarinic receptor stimulation increases production of cyclic GMP (Brown, 1989). Of the two forms of guanylate cyclase that exist, soluble and particulate, the MAChR is thought to activate the soluble form (Levy et al, 1994). The effects of cyclic GMP have been suggested to account for the negative inotropic response to cholinergic stimulation (Nawrath 1976), and are considered to be due to down modulation of calcium channels (Sperelakis, 1985), by phosphorylating an inhibitory subunit of the channel complex (Sperelakis and Wahler, 1988).

Very recently, it has been reported that M_2 receptor occupancy by carbachol induces a tetrodotoxin- and pertussis toxin-resistant Na^+ current which underlies positive inotropic effect (through Na-Ca exchanger), in guinea pig ventricular cells (Shirayama et al, 1993). The inability of guanine nucleotides to modulate the carbachol-induced Na^+ current indicates that the transmembrane signaling system of this current does not involve guanine nucleotides in the manner usually described for their regulation of agonist action (Shirayama et al, 1993).

1.2.4 Summary of muscarinic cholinergic control of myocardial function

Rapid improvements in electrophysiological techniques, cell isolation methods, protein chemistry, and molecular biology have played a very important role in the significant advance of our understanding of the cholinergic control of myocardial functions in the past decade. It is now clear that mammalian cardiac muscarinic receptors (M_2) serve as inhibitory physiological modulators of myocardial functions: negative chronotropic, inotropic, and dromotropic effects. Dual inhibitory regulation of myocardial function via stimulation of these receptors is established through cAMP-dependent and cAMP-independent subcellular processes. The inhibitory signals triggered by agonist binding to the respective receptors are transmitted to the subsequent biochemical, electrophysiological and functional changes through activation of the G_i and/or G_o. The G proteins then couple either directly to I_{KACh} or indirectly to the catalytic subunit of adenylate cyclase in the actions mediated by cAMP-dependent processes or to I_f and I_{Ca} through both indirect and direct pathways. Inhibition of I_{Ca} and activation of I_{KACh} will reduce the amplitude and duration of the action potential and accelerate repolarization leading to the negative inotropic effect in atria and ventricles (Giles and Taien, 1975; Goto et al, 1979; Pott, 1979; Katz, 1992). In addition, activation of I_{KACh} also hyperpolarizes the resting membrane potential, whereas inhibition of I_f decreases the slope of diastolic depolarization. Inhibition of I_f and I_{Ca} and activation of I_{KACh} will lead to the negative chronotropic effect in the sinoatrial node. Inhibition of I_{Ca} can result in negative dromotropic effect in the atrioventricular node (Katz, 1992).

1.2.5 Mechanisms of muscarinic cholinergic control of coronary vasculature

It is well established that α-adrenergic receptor stimulation induces coronary vasoconstriction (Edwards et al., 1989) and β-adrenergic receptor stimulation induces coronary vasodilation (Toda and Miyazaki, 1987). Based mainly on evidence from experiments performed on dogs (Hashimoto et al, 1960; Glaviano et al, 1977, Gross et al, 1981; Cox et al 1983), it has long been assumed that cholinergic agonists are vasodilators of coronary arteries and that parasympathetic innervation of coronary vessels mediates vasodilation (reviewed at Feigl, 1983). However, in recent years it has become clear that the dog is an exception, and in most species, cholinergic stimulation in fact produces coronary vasoconstriction. Thus, isolated coronary arteries from most species including human, sheep, pig, rabbit and rat contract in response to ACh and other cholinergic agonists (Kalsner, 1985; Doods et al, 1989; Van Winkle and Feigl, 1989). Furthermore, in isolated rat heart, perfused at constant pressure, electrical stimulation of the vagus nerve leads to a decrease in coronary flow (due to coronary vasoconstriction), suggesting a functional parasympathetic innervation of the coronary arteries in this species (Van Charldorp et al, 1987). The muscarinic stimulated coronary vasoconstriction in isolated human coronary arteries is comparable to histamine and serotonin and stronger than that of noradrenaline (Doods et al., 1989; Kalsner, 1985), and mediated by M_3 receptors (Bognar et al. 1990; Van Charldorp and Van Zwieten, 1989).

The postreceptor mechanisms governing cholinergic coronary vasoconstriction are not yet clearly understood. It has been suggested that ACh augments Ca^{2+} release from sarcoplasmic reticulum (Ganitkevich and Isenberg, 1990), through activation of muscarinic receptor-G_q-linked phospholipase C and production of IP_3 (Berridge and Irvine, 1989). A partial cellular depolarization by the increase in intracellular Ca^{2+} (Suyama and Kuriyama, 1984), may automatically trigger the opening of dihydropyridine-sensitive Ca^{2+} channels (Cavero and Spedding, 1983; Meldolesi et al, 1991). Hence receptor-mediated Ca^{2+} entry can be partially blocked by dihydropyridine sensitive Ca^{2+} blocker such as verapamil (Cavero and Spedding, 1983; Rink, 1990). However, muscarinic receptor mediated increase in intracellular Ca^{2+} is considered

essential for the vasoconstrictor action of cholinergic agonists (Nuutinen et al., 1985).

1.3. Sympathetic and parasympathetic interactions in the heart

Pronounced interactions among the adrenergic and cholinergic limbs of the autonomic nervous system, occurring both peripherally and centrally, play an important role in the integration of the autonomic control of heart function, and therefore, in the maintenance of circulatory homeostasis (Levy, 1984; Muscholl, 1980; Wurster, 1984). The antiadrenergic action of ACh involves both presynaptic and postsynaptic mechanisms (Levy, 1984; Mace and Levy 1983; Watanabe et al, 1984). Presynaptically, interaction of ACh with muscarinic receptors on postganglionic sympathetic nerve endings causes inhibition of the norepinephrine (NE) release; postsynaptically, interaction of ACh with cardiac muscarinic receptors causes inhibition of β-adrenergic activation of heart function through inhibition of adenylate cyclase (Narayanan and Tucker, 1986; Watanabe et al, 1984).

On the other hand, NE has anticholinergic action both presynaptically and postsynaptically. Presynaptically, interaction of NE with α_1-adrenoceptor receptors on postganglionic parasympathetic nerve endings causes inhibition of ACh release (Wetzel and Brown, 1985), and bradycardia induced by vagal stimulation (Starke, 1972; McGrattan et al, 1987). Postsynaptically, the β-adrenergic agonists enhance the muscarinic-activated K^+ channel activity in rat atrial cells at the single-channel level (Kim, 1990). It has been suggested that β-adrenergic agonists may modulate their positive chronotropic action on atrial cells by cAMP-dependent phosphorylation of the muscarinic K^+ channel to increase potassium conductance (Kim, 1990). In the absence of such a negative feedback mechanism to increase the muscarinic K^+ channel activity, strong sympathetic stimulation of the heart may result in arrhythmias. Thus, the integrated and coordinated control of cardiac function by both adrenergic and cholinergic systems are central to the maintenance of circulatory homeostasis.

1.4. Regulation of cardiac function by autonomic nervous system in aging

Aging is defined as the sum of all the changes that occur continuously with the

passage of time and lead to functional impairment and death sometime after maturation (Kenney, 1982; Geokas et al, 1990). Aging is a normal process occuring in all members of a population, while disease is a pathological process, occuring in only a fraction of a population. In the present study, aging refers to postmaturational aging, from adulthood to senescence.

Age-associated diminution in the ability to respond to various forms of stress is one of the well recognized functional changes in the heart in aging humans and animals (Lakatta, 1980; Xiao and Lakatta, 1992 and Lakatta, 1993). Age-related changes at the level of cardiac autonomic receptors and their effector systems may contribute to impaired stress-response of the aging heart. The mechanism for the age effects appears to be attributable to multiple changes in molecular and biochemical receptor-coupling and postreceptor events, and not to a major modification of a single rate-limiting step, as might occur, for example, in a genetic defect. Evidence supporting this possibility has been provided by previous studies as reviewed below.

1.4.1 Adrenergic control of the heart in aging

Increments in heart rate, contractile force and cardiac output in response to various forms of stress, eg. physical (exercise), emotional (fear, anxiety), environmental (hypoxia, hypercapnia), normally depend upon adrenergic stimulation of the heart. With increasing age, electrically induced β-adrenergic-mediated release of NE is markedly diminished at the cardiac neuroeffector junction (Mortimer et al, 1991). Several studies in intact animals and in cardiovascular tissues, cells and subcellular organelles isolated from animal hearts, confirm the hypothesis that responsiveness of the heart to adrenergic mediated stress declines in aging humans and animals (Guarnieri et al, 1980; Lakatta, 1980; Xiao and Lakatta, 1992; Lakatta, 1993). This observation strongly suggests that postsynaptic changes most likely occur in aging myocardium, and such changes can contribute, at least in part, to the decline in adrenergic control, and therefore stress-responsiveness, of the aging heart (Scarpace et al, 1991). The most remarkable change within the β-adrenergic receptors is the decrease in the receptor affinity for agonists and guanine nucleotide regulation of receptor affinity for agonist (Narayanan and Derby,

1982), in the absence of substantial changes in β-adrenergic receptor density (Guarnieri et al, 1980, Xiao and Lakatta, 1992). However, there is very little information available regarding age-associated changes on β-adrenergic receptor subtype density, affinity, or functional regulation. The postreceptor changes include a decrease in the activity of G_s-protein, decreased activities of catalytic unit, and cAMP-dependent protein kinase A (PKA)-induced protein phosphorylation (Jiang et al, 1993, Narayanan and Derby, 1982; Scarpace, 1986; Shu and Scarpace, 1994; Xiao and Lakatta, 1992).

Besides the β-adrenergic pathway, the positive inotropic response of the heart to adrenergic stimulation is mediated, in part, through activation of cardiac α-adrenoceptor. One study has indicated an age-related decrease in α receptor density in rat ventricular myocardium (Partilla et al, 1982; Gascon et al, 1993). However, no information is available concerning the effects of aging on the contractile response of the heart to α adrenergic stimulation or α-receptor functions in the myocardium.

1.4.2 Cholinergic control of the heart in aging

Very little is known about the impact of aging on the responses of the heart to cholinergic stimulation or MAChR mediated cellular processes in the myocardium. Limited *in vivo* studies in this regard have produced conflicting results. One study reported a decreased negative chronotropic response of the heart to vagal stimulation and methacholine (a MAChR agonist), administration in aged compared to adult rats (Kelliher and Conahan, 1980). Another study reported increased negative chronotropic response of the heart to acetylcholine given intravenously (Ferrari et al, 1989). A greater hypotensive response to acetylcholine in aged compared to adult rat was reported as well (Frolkis et al, 1973). It is not clear whether cardiac and vascular responses to cholinergic stimulation are altered differentially with aging. In any case, the interplay of multiple factors controlling cardiovascular functions in the intact organism makes it difficult (if not impossible) to identify age-related alterations intrinsic to the myocardium.

Studies reported to date utilizing cardiac muscle from rats of various ages have suggested the following. (i) One study reported that the density of MAChR is increased significantly (24%) in aged rat atria while no age-related change is seen in receptor

density in the ventricles or in agonist and antagonist binding affinities of atrial and ventricular receptors (Narayanan and Derby 1983). (ii) Another study, while confirming the lack of age-related changes in ventricular receptor density and antagonist affinity of ventricular receptors, has reported an age-associated decrease in agonist binding affinity of ventricular receptors (Baker et al, 1985). This study did not examine the effects of aging on atrial receptors. Clearly, more detailed and systematic studies on the effects of age on cholinergic responses of the heart, as well as on various parameters of muscarinic receptor functions, are required to assess the impact of aging on postsynaptic cholinergic control mechanisms in the heart.

1.4.3 Autonomic control of the coronary arteries in aging

Age-associated alterations in vasoregulatory responses of the coronary vasculature to autonomic stimuli may also influence autonomic modulation of cardiac rhythm and contractile performance. While *in vitro* measurements did not show age-related changes in coronary blood flow (Friberg et al, 1985; Weisfeldt et al, 1971), recent evidence from *in vivo* measurements has revealed decrements in coronary blood flow as well as coronary vascular reserve and increments in coronary vascular resistance in aging rats (Hachamovitch et al, 1989). Whether or not age-related changes in autonomic tone in the coronary vasculature contribute to the alterations in coronary vascular function parameters measured in vivo remains unclear.

Very little is known about the impact of aging on the responses of the coronary arteries to autonomic stimulation. A recent study reported a diminished coronary relaxant responses to beta-adrenergic stimulation and an enhanced coronary vasoconstriction to alpha adrenergic stimulation in aged beagles (Toda and Miyazaki, 1987). The effect of aging on the coronary vascular response to cholinergic stimulation is not known. To fully understand age-related alteration in the cholinergic control of heart function, it is necessary to study the age-associated changes in the responsiveness of coronary arteries to cholinergic stimulation.

1.4.4 Adrenergic-cholinergic interactions in the aging heart

Attenuation of the adrenergic activation of the heart by the cholinergic neurotransmitter, acetylcholine, is a well known example of autonomic interactions governing the regulation of heart function (Levy, 1984; Muscholl, 1980; Wurster, 1984). Age-related changes in autonomic interactions in the heart, such as exaggeration of anti-adrenergic action of cholinergic stimulus, can also contribute to impairments in adrenergic control and stress-responsiveness of the aging heart. To my knowledge, the only published work describing the influence of aging on autonomic interactions in the heart, is that reported from our laboratory. This study (Narayanan and Tucker, 1986), has shown that the ability of the cholinergic agonist, carbachol, to inhibit β-adrenergic activation of adenylate cyclase is significantly reduced in aged heart, suggesting an age-associated diminution in adrenergic-cholinergic interactions in the heart. Thus, postsynaptically, exaggerated cholinergic antagonism of adrenergic stimulation does not appear to contribute to the decline in adrenergic control of the heart in aging. On the other hand, the age-dependent decline in the anti-adrenergic action of cholinergic stimulation may be construed as a protective adaptation occurring in the face of diminished adrenergic control of the aging heart. Extensive studies are required to understand the cellular basis and pathophysiological implications of the apparent age-related changes in autonomic interactions in the heart.

1.4.5 Summary of current knowledge and future perspectives

From the forgoing review, the current knowledge regarding the impact of aging on autonomic control of the heart can be summarized as follows. (1) The contractile response of the heart to β adrenergic stimulation, as well as β adrenergic stimulation of adenylate cyclase declines with aging. The density of β adrenergic receptors in the myocardium does not appear to be altered with aging. The postreceptor changes include a decrease in the activity of G_s protein, catalytic unit, and cAMP-dependent PKA induced protein phosphorylation. (2) The density of α adrenoceptor in the myocardium reportedly diminishes with aging but the impact of aging on α adrenoceptor modulation of heart function remains obscure. (3) The density of muscarinic cholinergic receptors in the

myocardium is not diminished with aging. There is little information available with regard to the effect of aging on the responses of the heart to cholinergic stimulation or muscarinic receptor functions in the myocardium. (4) There appears to be a decrease in β adrenergic mediated coronary vasodilation and an increase in α adrenergic mediated coronary vasoconstriction in aging. The impact of aging on coronary vasculature responses to cholinergic stimulation is not known. (5) There is an apparent decline in adrenergic-cholinergic interactions in the heart as evidenced by a diminished ability of cholinergic agonists to attenuate β adrenergic activation of adenylate cyclase. Clearly, extensive future studies on the effects of age on (1) muscarinic receptor functions in the myocardium, (2) muscarinic receptor-linked coronary vascular responses and (3) alpha receptor-mediated cellular processes are required to identify the postsynaptic components of the autonomic control systems affected by age, the molecular nature of age-associated changes, and the relationship of such changes to impairment in cardiac function.

1.5 Scope and specific objectives of the present studies

The research work presented in this thesis focuses on the impact of aging on cholinergic control of the heart at the postsynaptic level. The present studies undertaken were designed to achieve the following specific objectives. (1). To determine the influence of postmaturational aging on the chronotropic and inotropic responses of the heart to cholinergic stimulation. (2). To identify the mechanisms underlying the age-associated changes in cardiac cholinergic responses. (3). To assess the impact of aging on coronary vascular responses to cholinergic stimulation.

CHAPTER 2

CHRONOTROPIC AND INOTROPIC RESPONSES OF THE RAT MYOCARDIUM TO CHOLINERGIC STIMULUS WITH AGING

2.1 Introduction

Evidence from several studies indicates that the responsiveness of the heart to adrenergic stimulation declines with aging in humans and animals and this is due, in part, to impaired β-adrenoceptor function (Lakatta 1980, 1993; Xiao and LaKatta, 1992; Narayanan and Derby 1982; Scarpace 1986). On the other hand, relatively little is known about the responses of the heart to cholinergic stimulation. *In vivo* studies in this regard have produced conflicting results. Thus, Kelliher and Conahan (1980) reported that the negative chronotropic response of the heart to vagal stimulation and administration of cholinergic agonists is reduced in aged compared with adult rats. In contrast, Ferrari et al (1991), reported markedly larger bradycardia in the aged compared with young rats in response to vagal stimulation and intravenous injections of acetylcholine. Frolkis and co-workers (1973), observed a greater decline in cardiac output and blood pressure in the aged compared with adult animals (rats and rabbits), following a low dose (0.05 μg/kg), of acetylcholine, but at a higher dose (2 μg/kg), the magnitude of the hemodynamic responses was lower in aged compared with adult animals. Data obtained in human studies have shown that aging is accompanied by an attenuation of the tachycardia induced by atropinization (Nafelski and Brown 1950), of the bradycardia induced by baroreceptor stimulation (Gribben et al. 1971), and of the spontaneous heart rate variability (Mancia et al. 1983). The interplay of multiple factors controlling cardiovascular functions in the intact organism makes it difficult (if not impossible), to identify age-related alterations intrinsic to the myocardium. In view of this, the present study was undertaken using isolated perfused intact hearts from adult and aged Fischer 344 rats to determine whether aging alters the chronotropic and inotropic responses of the heart to muscarinic receptor stimulation by cholinergic agonists.

2.2 Materials and methods

2.2.1 Animals

Male virgin Fischer 344 rats, the most commonly used animal model for the study of myocardial function in aging, were used in all the studies for this Ph.D. thesis. The Fischer 344 rats reach adulthood by 6 months and become senescent by 24 months when the mortality of the colony is 50%. It is recognized that all aged rats do not die of old age and the aged rats are more susceptible to disease. Upper respiratory tract infections are among the most common diseases in rats. They are also sensitive to mold and bacterial infections. Pathology of the myocardium is rare (Coleman et al, 1977). A previous study (Coleman et al, 1977), showed that only a few coronary artery lesions in aged male Fischer 344 rats were found, which seemed to have no correlation to age. None of the lesions noted were considered of sufficient severity to impair cardiac function.

Fischer 344 rats of 6-8 month-old, 12, 20 month-old, and 26-30 month-old were obtained from the National Institute of Aging (NIA), rat colony maintained at Harlan Industries, Indianapolis, U.S.A. The 12 month-old and 20 month-old rats were only used in the studies of negative chronotropic and inotropic responses of the heart to cholinergic stimulation. All the other experiements were done only with the 6-8 month-old (adult) and 26-30 month-old (aged), rats. Upon arrival, the rats were housed individually in the Health Sciences Animal Care Facility at University of Western Ontario with free access to food (Purina Chow containing 20% protein), and water, and were used for experiments within two weeks. The body weight of all these rats ranged from 343 to 380 g by the time they were used. All animals used in the present studies were certified free of disease (including coronary artery disease), by NIA, and only hearts free of obvious pathology were used in the experiments. All the experiment approaches were approved by the Health Sciences Animal Care Committee of the University of Western Ontario.

2.2.2 Chemicals

Acetylcholine chloride was purchased from BDH Chemicals, Toronto. Carbachol chloride, atropine sulphate, acetyl-(β-methyl)thiocholine iodide, 5, 5'-dithio-bis(2-nitrobenzoic acid), and eserine were obtained from Sigma Chemical Co., St. Louis, MO. AFDX-116(11-[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6Hpyrido-[2,3-b][1, 4]benzodiazepine-6-one) was a generous gift from Boehringer Ingelheim Ltd., Burlington, Ontario. All the other chemicals were from BDH chemicals, Toronto.

2.2.3 Heart perfusion

The rat was decapitated and hearts was rapidly excised and immediately cannulated for retrograde aortic perfusion of coronary arteries with modified freshly made Krebs-Henseleit buffer consisting of (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 10 and sodium succinate 5. The buffer solution was saturated with 95% O_2-5% CO_2, which maintained a pH of 7.4; perfusion temperature was set at 37°C $\pm$ 0.2°C. The hearts were perfused at a constant flow rate of 9 ml/g ventricular weight/min in a nonrecirculating manner using a peristaltic pump (Gilson Minipulse 2, Mandel Scientific Co.). As the ventricular mass is about 23% higher in the aged heart compared with that of adult (mean $\pm$ SEM: 0.78 $\pm$ 0.05 g for adult, n=13; and 0.96 $\pm$ 0.06 g for aged, n = 13, unpublished observation), the flow rate was adjusted accordingly. After cannulation the hearts were allowed to stabilize for 20 min before the experiments were begun. All pharmacological agents were dissolved in Krebs-Henseleit buffer on the day of the experiment. Each concentration of pharmacological agent was used to perfuse the heart for 3 min (at the constant flow rate indicated above), to reach its maximal effect. Perfusion with varying concentrations of pharmacological agents was carried out in ascending order with a washing out period of 3-6 min between each concentration. Heart rate and contractile force were recorded throughout the perfusion of the heart as described below.

2.2.4 Measurement of heart rate and contractility

To determine heart rate and contractile force, contractions were recorded on a Grass 79D polygraph using a Grass FT.03C force displacement transducer connected to

a stainless steel hook and attached to the apex of the heart and adjusted to provide a diastolic tension of 2 g. Chronotropic responses were studied using spontaneously beating heart preparations and inotropic responses were assessed using electrically paced hearts. Prior to electrical pacing, both atria were removed and the ventricular myocardium was paced at 240 beats per min with a Grass SD9 stimulator via platinum wire electrodes inserted into the epicardium, at 2X threshold voltage and a pulse duration of 5 ms.

2.2.5 Assay of Cholinesterase Activity

Acetylcholinesterase is an enzyme which hydrolyzes the ACh in the heart. Change in the acetylcholinesterase activity in the aged heart may alter the concentration of ACh that reaches the heart. The activities of acetylcholinesterase and non-specific cholinesterase (pseudocholinesterase), in atrial and ventricular homogenates were determined by the method of Ellman et al. (1961). The atria and ventricles were freed of blood by retrograde aortic perfusion of coronary arteries with 40 ml ice-cold 0.1 M sodium phosphate buffer (pH 8.0). Tissue homogenates were prepared in 20 volumes (based on tissue weight) of 0.1 M sodium phosphate buffer (pH 8.0) using a Polytron PT-10 homogenizer (15 sec x 3 with 30 sec interval between bursts, setting 9) and aliquots were used for enzyme assay. The assay mixture (total volume 3.22 ml), in a cuvette, contained 0.1 M sodium phosphate buffer (pH 8.0), 30 μM dithiobisnitrobenzoate (DTNB), tissue homogenate (250-350 μg protein), and one of the substrates viz acetylthiocholine (1 mM) or acetyl-(β-methylthio)-choline (1 mM). The reaction was initiated by the addition of substrate and absorbance changes were monitored at 412 nm in a Beckman model 35 spectrophotometer. The enzyme activity was calculated from the linear portion of the hydrolysis curve obtained during the first 5 min of reaction. Appropriate assay blanks containing the cholinesterase inhibitor, eserine (5 μM, maximum effective concentration), were included to correct for any non-enzymatic substrate hydrolysis. The enzyme activity determined with acetylthiocholine was taken to represent total cholinesterase activity. Since acetyl-(β-methyl)-choline is regarded as virtually specific for acetylcholinesterase in mammals (Silver 1974), enzyme activity determined with this substrate was taken to represent acetylcholinesterase activity. The

non-specific cholinesterase (pseudocholinesterase) activity was defined as the difference between total cholinesterase activity and acetylcholinesterase activity.

2.2.6 Statistical analysis of the data

The data are presented as mean ± S.E.M. of the number (n) of observations. The age-related differences in cholinesterase activities were tested using unpaired two tailed Student's *t* test. The basal function parameters (heart rate and contractile force) were analyzed by one way analysis of variance (ANOVA).

The data on chronotropic and inotropic responses have been standardized to depict percentage changes from predrug (base-line) values. Dose response curves were analyzed using nonlinear curve fitting technique as described by DeLean et al. (1978) and, where appropriate, EC_{50} values were derived using Graph PAD program (Graph PAD Software Version 2.0, Serial #10916, available from Graph PAD, San Diego, CA). The EC_{50} data from individual rats were averaged separately for the adult and aged groups and expressed as mean ± SE of mean. Statistical comparisons of the EC_{50} values between age groups were performed by one way ANOVA followed by Tukey-Kramer test for multiple comparisons. The level of significance was set at $P<0.05$.

2.3 Results

2.3.1 Basal Heart Rate and Contractile Force

In spontaneously beating preparations, the basal heart rate did not differ significantly with age [230 ± 8 beats/min for adult (n=24); 229 ± 8 beats/min for 12 month-old rats (n=6); 222 ± 8 beats/min for 20 month-old rats (n=6); 210 ± 7 beats/min for aged (n=24)]. When the hearts were paced at 240 beats/min, the contractile force, expressed per gram wet weight of ventricle, was not statistically different with age (adult: 12.9 ± 2.0 g/g, n=9; 12 month-old rats: 9.3 ± 1.6 g/g, n=5; 20 month-old rats: 9.2 ± 1.3 g/g, n=5; Aged: 9.8 ± 2.4 g/g, n=9).

2.3.2 Comparison of chronotropic responses to acetylcholine and carbachol

The results shown in Fig. 2-1 compare the chronotropic response elicited by acetylcholine and carbachol in spontaneously beating isolated perfused hearts from adult and aged rats. At all concentrations tested, acetylcholine produced much larger (up to 4-fold higher), bradycardia in hearts from aged compared to adult rats (Fig. 2-1, panel A). Cessation of heart beat occurred with 10 $\pm$ 0.5 μM acetylcholine in the aged as opposed to 100 $\pm$ 10 μM in the adult (estimated EC_{50} values for acetylcholine were: adult, 5.4 $\pm$ 0.03 μM; aged, 0.32 $\pm$ 0.02 μM; $P<0.001$). Since an analogous observation (Kennedy and Seifen, 1990) has attributed the age-related difference in their preparation to age-related decline in acetylcholinesterase activity, although no direct measurement of the enzyme activity was performed, We did the experiment in the presence of a cholinesterase inhibitor, eserine (5μM which inhibits the enzyme activity completely). The age-related difference in EC_{50} of the acetylcholine-induced bradycardia was smaller (Fig. 2-1, panel B), compared to that in the absence of eserine (Fig. 2-1, panel A), but still significant ($P<0.02$). In other experiments, it was found that inclusion of higher concentrations ($>5\mu$M) of eserine in the perfusate did not further reduce the age-related difference (results not shown). The cholinesterase-resistant cholinergic agonist, carbachol, also produced significantly greater bradycardia (up to 3-fold higher, $P<0.01$), in hearts from the aged compared to adult rats (Fig. 2-1, panel C). Complete dose-response curves for carbachol-induced bradycardia in adult and aged hearts are presented in Fig. 2-2. The aged heart was clearly more sensitive to the negative chronotropic effect of carbachol (cessation of heart beat occurred with 0.86 $\pm$ 0.03 μM carbachol in the aged as opposed to 5.7 $\pm$ 0.3 μM in the adult; estimated EC_{50} values for carbachol were: adult, 622 $\pm$ 33 nM; aged, 139 $\pm$ 35 nM; $P<0.001$).

To assess the time of onset and progression of the age-related difference, the chronotropic response of the heart to varying concentrations (1 nM - 10 μM) of carbachol was also determined in two other age groups *viz* 12 month-old and 20 month-old rats. The EC_{50} values for the negative chronotropic response to carbachol derived for all four age groups studied are summarized in Tables 2-1 and 2-2. A statistically significant age-related increase in carbachol sensitivity was evident at 20 months of age and this age-related difference became more pronounced with further progression of age.

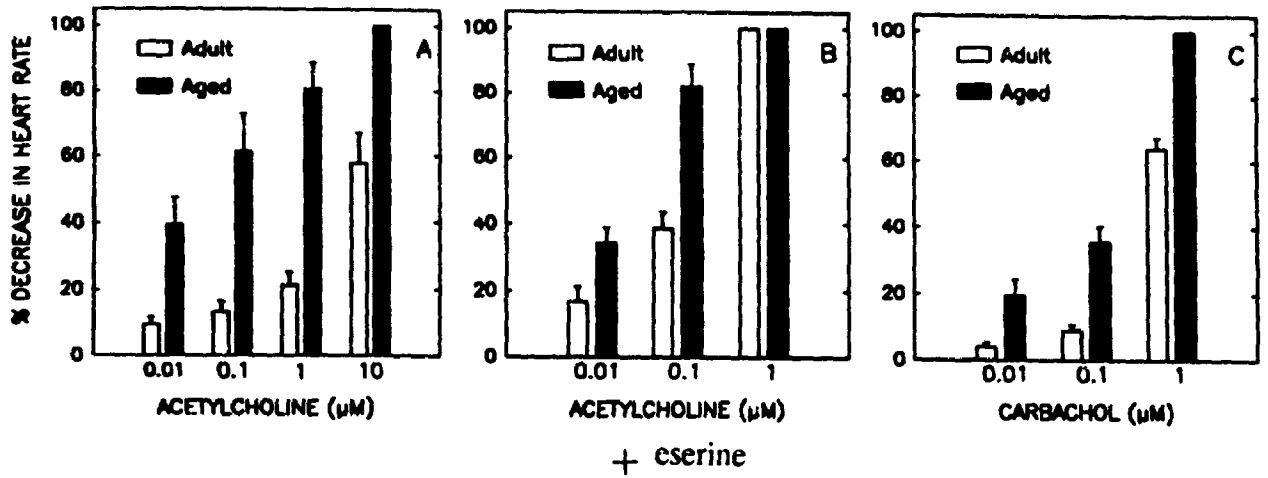

Fig. 2-1 Comparison of the negative chronotropic response of isolated perfused spontaneously beating adult and aged rat hearts to acetylcholine (panels A and B) and carbachol (panel C). The results shown in panel B were obtained in the presence of eserine (5 μM), a cholinesterase inhibitor. The data represent mean $\pm$ SE of experiments using 10 adult and 10 aged rats for each panel. The age-related differences in the estimated EC_{50} values are statistically significant in panel A, panel B, and panel C (see text for the EC_{50} values). The EC_{50} values for the ACh-produced bradycardia in the presence of eserine was similar to that for the carbachol-induced bradycardia.

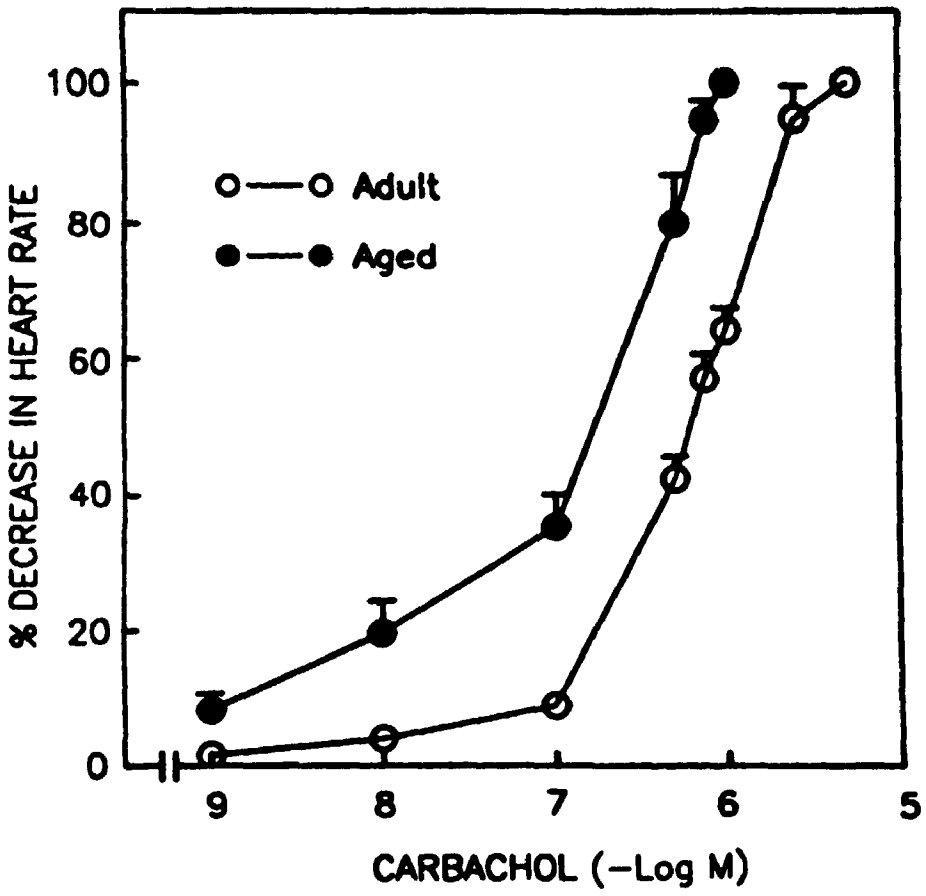

Fig. 2-2 Concentration-dependence of the negative chronotropic response of isolated perfused spontaneously beating adult and aged rat hearts to carbachol. Results represent mean ± SE of experiments using 10 adult and 10 aged rats. The age-related difference in the sensitivity of the heart to carbachol is statistically significant ($P < 0.001$; see Table 2-1 and 2-2 for EC_{50} values).

Table 2-1 EC_{50} values for the negative chronotropic response to carbachol

Age: months	6-8	12	20	26-30
EC_{50}: nM	622±33	595±45	353±93	139±35
N	10	6	6	10

Comparison of EC_{50} values for the negative chronotropic response to carbachol in the 6-8 month-old, 12 month-old, and 26-30 month-old rats. The results from the one way analysis of variance showed significant age-related difference among these groups ($p<0.0001$).

Table 2-2 Tukey-Kramer Multiple Comparisons

6-8 vs 12 month-old	NS (not significant)
6-8 vs 20 month-old	$p<0.05$
6-8 vs 26-30 month-old	$p<0.001$
12 vs 20 month-old	$P< 0.05$
12 vs 26-30 month-old	$p<0.001$
20 vs 26-30 month-old	$P<0.05$

Tukey-Kramer multiple comparisons of the data obtained from Table 2-1. The results showed that the onset of the age-related increase in carbachol sensitivity became evident at 20 months of age, and this age-related difference became more pronounced with further progression of age.

2.3.3 Comparison of inotropic response to carbachol in adult and aged hearts

Fig. 2-3 shows the results of a typical experiment demonstrating the effects of selected concentrations of carbachol on myocardial contractility in constant flow-perfused

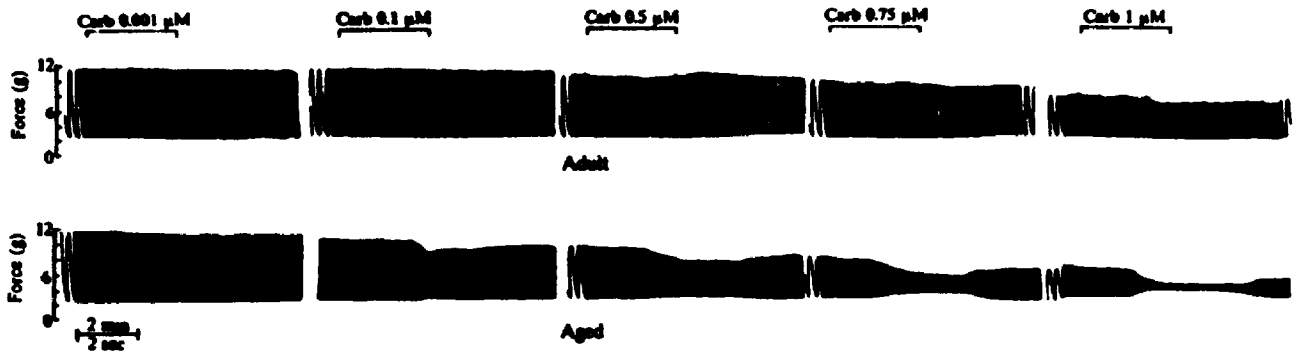

Fig. 2-3 Original recordings showing negative inotropic response of isolated perfused, electrically paced (240 beats/min), an adult and aged rat hearts to varying concentrations of carbachol. The period of carbachol infusion is shown on top and the horizontal time bar indicates interval at either fast or slow recorder speed.

hearts from adult and aged rats paced at 240 beats/min. In both adult and aged hearts, carbachol caused a concentration-dependent negative inotropic effect which was strikingly more pronounced in the aged. At higher concentrations of carbachol (>0.5 μM), recovery of contractile force during washing out was incomplete, and there was an increase in resting tension. In these experiments, the latency for the onset of inotropic response (i.e. time from the entry of drug into coronary circulation to the beginning of inotropic response) was less than 30 sec in both adult and aged hearts at the various concentrations of carbachol used. No significant age-related difference was evident in the time required to reach maximum inotropic response (Fig. 2-4). Composite data from several experiments depicting changes in contractile force as a function of carbachol concentration are presented in Fig. 2-5. At all concentrations tested, the carbachol-induced decrease in contractile force was significantly more pronounced ($\sim$2-fold) in the aged compared to adult heart. Assuming that the maximum negative inotropic response occurred at the highest concentration (10 μM) of carbachol used (saturation was evident in the aged but not adult heart), the EC_{50} values were estimated to be 408 $\pm$ 95 nM and 71 $\pm$ 28 nM, respectively, for adult and aged heart ($P<0.005$).

The inotropic response of the heart to varying concentrations (1 nM - 10 μM) of carbachol was also determined in two other age groups *viz* 12 month-old and 20 month-old rats, so as to assess the time of onset and progression of the age-related difference. The EC_{50} values for the negative inotropic response to carbachol derived for all four age groups studied are summarized in Table 2-3 and 2-4. Significant age-related increase in carbachol sensitivity was evident at 20 months of age and this age-related difference became more pronounced with further progression of age.

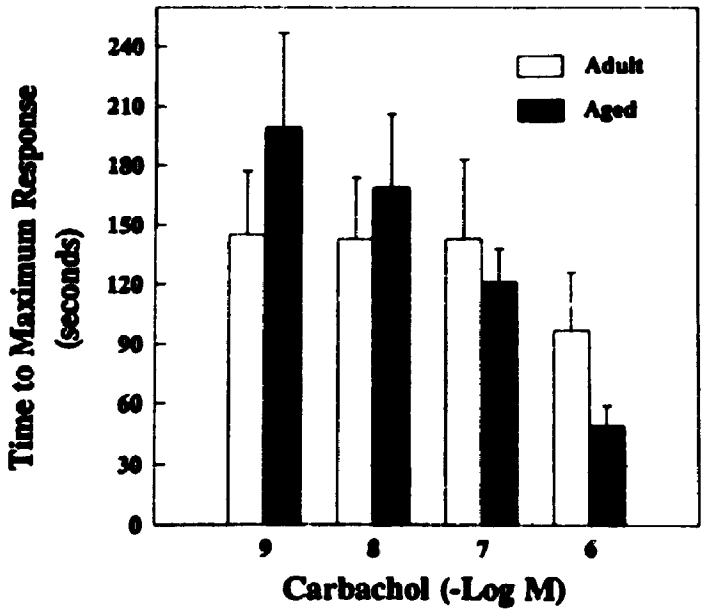

Fig. 2-4 Comparison of the time required to elicit maximal negative inotropic response to varying concentrations of carbachol in isolated perfused, electrically paced (240 beats/min), adult and aged rat hearts. "Time to maximal response" was defined as the time required to reach maximal negative inotropic response following the onset of the negative inotropic response. The data represent mean $\pm$ SE of experiments using 9 adult and 9 aged hearts. The age-related differences in time to maximal response are not statistically significant.

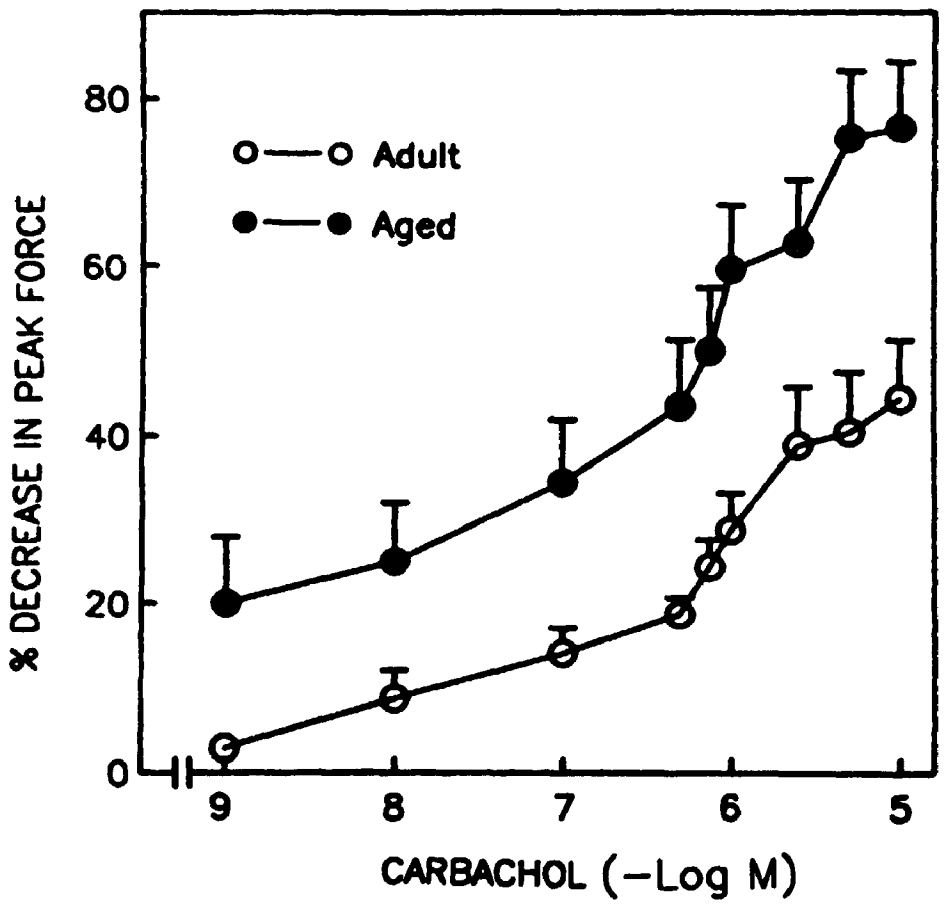

Fig. 2-5 Concentration-dependence of the negative inotropic response of isolated perfused, electrically paced (240 beats/min), adult and aged rat hearts to carbachol. Results represent mean ± SE of experiments using 9 adult and 9 aged rats. The age-related differences in carbachol sensitivity (and maximum response) is statistically significant ($P < 0.005$; see Table 2-3 and 2-4 for EC_{50} values).

Table 2-3 **EC_{50} values for the negative inotropic response to carbachol**

Age: months	6-8	12	20	26-30
EC_{50}: nM	408±95	275±33	109±25	71±28
N	9	5	5	9

Comparison of the EC_{50} values for the negative inotropic response to carbachol in 6-8 month-old, 12 month-old, 20 month-old, and 26-30 month-old rats. The result from one way analysis of variance showed significant age-related difference among these groups ($p < 0.0024$).

Table 2-4 Tukey-Kramer Multiple Comparisons

6 vs 12 month-old	NS
6 vs 20 month-old	$P < 0.05$
6 vs 26 month-old	$P < 0.01$
12 vs 20 month-old	NS
12 vs 26 month-old	NS
20 vs 26 month-old	NS

Tukey-Kramer multiple comparisons of the data obtained in Table 2-3. The results showed that the age-related increase in the carbachol sensitivity was evident at 20 months of age and progressed with further progression of aging.

2.3.4 Inhibition of cholinergic responses by muscarinic receptor antagonists

As shown in Fig. 2-6, in both adult and aged hearts, the negative chronotropic response to carbachol (0.5 μM) was antagonized by the cardioselective (M_2) muscarinic receptor antagonist, AFDX-116 (Giachetti et al. 1986) in a concentration-dependent

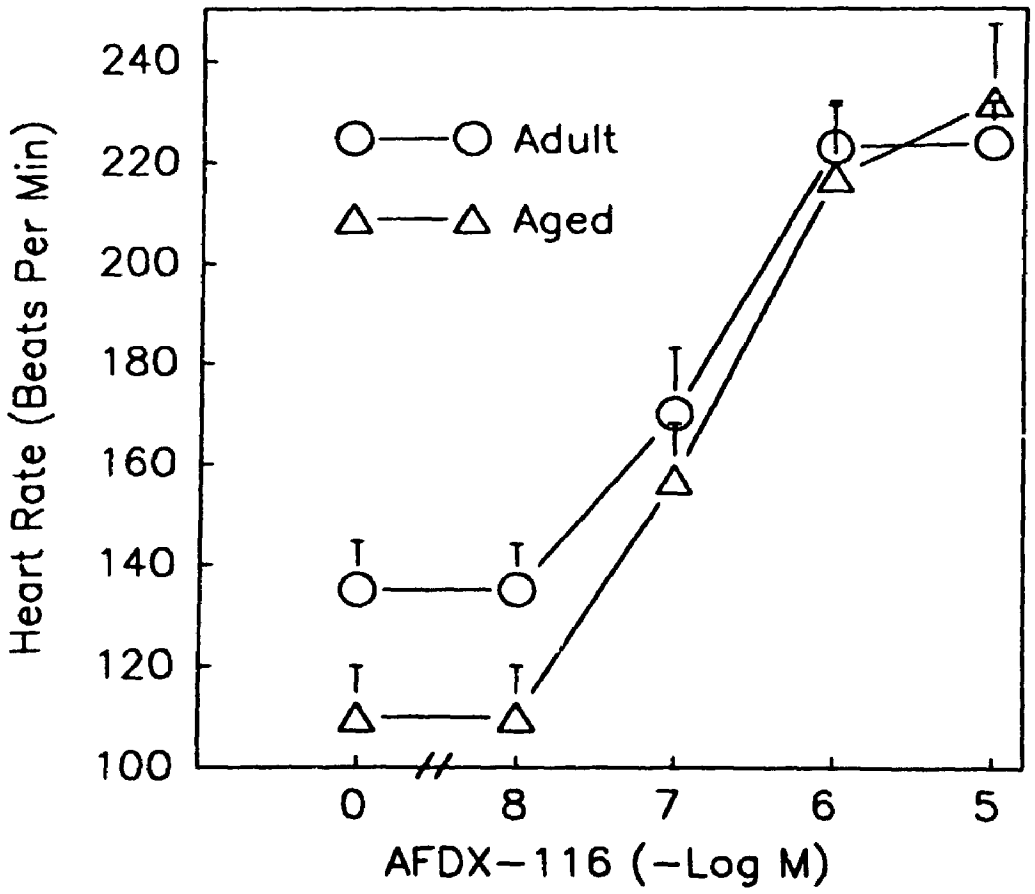

Fig. 2-6 Effect of varying concentrations of AFDX-116 on carbachol (0.5 μM)-induced decrease in heart rate in isolated perfused spontaneously beating adult and aged rat hearts. Results represent mean $\pm$ SE of experiments using 6 adult and 6 aged rats. The heart rate measured in the absence of carbachol was 220 $\pm$ 7 beats/min for adult and 230 $\pm$ 12 beats/min for aged rats.

manner which confirms that the carbachol-induced cardiac responses are mediated by muscarinic receptors. No age-related difference was evident in the effectiveness of AFDX-116. Similar findings were obtained using the non-subtype selective muscarinic receptor antagonist, atropine. Atropine also antagonized the negative inotropic effect of the carbachol.

2.3.5 **Cholinesterase Activities in Atria and Ventricles**

Acetylcholinesterase is an enzyme which hydrolyzes the ACh in the synaptic clef between the postsynaptic nerve ending of the vagus and cardiac tissue. To further determine if there is an age-related difference in the enzyme activity in our preparations, the acetylcholinesterase and psedocholinesterase activity were studied. The results showed that acetylcholinesterase activity was significantly lower (50-60%), in atria ($P<0.001$) and ventricles ($P<0.05$) of the aged compared to adult rats (Fig. 2-7, panel A). No significant age-related difference was seen in non-specific cholinesterase (pseudocholinesterase) activities in atria or ventricles (Fig. 2-7, panel B).

2.4 Discussion

The results of the present study demonstrate that in isolated, constant flow-perfused, beating rat hearts, the negative chronotropic and inotropic responses to cholinergic agonists are strikingly enhanced with aging. To our knowledge, this study provides the first documentation of age-associated alterations in cholinergic responses of isolated intact functioning myocardium. The age-associated enhancement in chronotropic response of the isolated heart to cholinergic agonists is consistent with a recent *in vivo* study showing markedly larger bradycardia in aged compared to adult rats in response to vagal stimulation and intravenous injection of acetylcholine (Ferrari et al. 1991). Our findings provide direct evidence of age-associated alteration in the cholinergic control of heart function at the postsynaptic level.

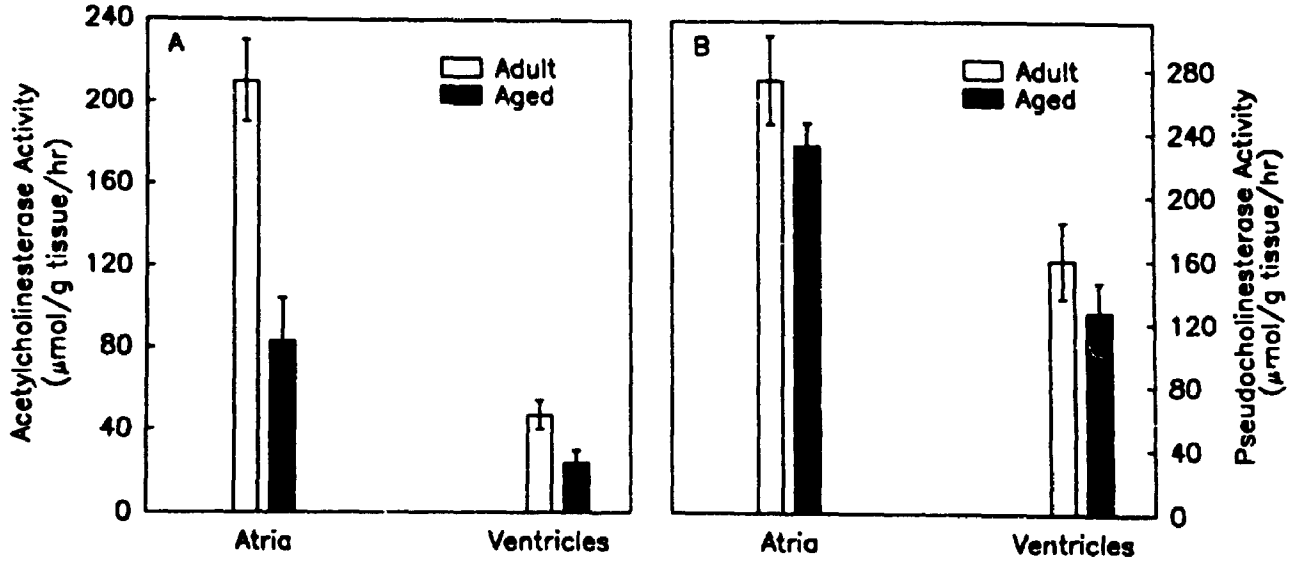

Fig. 2-7 Comparison of acetylcholinesterase (panel A) and pseudocholinesterase (panel B) activities in atria and ventricles from adult and aged rats. Results represent mean $\pm$ SE of experiments using 7 adult and 7 aged rats. The aged-related difference in acetylcholinesterase (but not pseudocholinesterase) activity is significant in the case of atria ($P<0.001$) and ventricles ($P<0.05$).

It is clear from our findings that aging is not associated with a generalized depression of the cardiovascular system but that cardiovascular functions may even be enhanced during this physiological process. It is also clear that the effects of aging on the autonomic system are characterized by a marked nonuniformity. Possibly, the age-related muscarinic receptor hyper-responsiveness is due to a reduction in tonic vagal outflow to the aged compared to the adult hearts (Alicandric et al, 1987; Lakatta 1993). This pattern opposes that in the sympathetic nerve system, where tonic outflow is increased by aging (Wallin and Sundlof, 1979; Fleg et al, 1985), with consequent down regulation of β-adrenergic signal transduction (Lakatta, 1993; Shu and Scarpace, 1994).

The cholinergic hypersensitivity of the heart in aging may have physiological, pathophysiological and clinical implications. In the pathophysiological setting, this phenomenon implies that an age-related decline in baroreceptor control of heart rate (Gribben et al. 1971; Lowenthal et al. 1983; Pfeifer et al. 1983), a function largely mediated by the vagus (Mancia and Mark 1983), is not due to effector organ hyporesponsiveness. Consequently, the defect is likely located elsewhere in the reflex arc. In the clinical setting, cholinergic hypersensitivity of the heart may contribute to inordinate sinus node depression and carotid sinus syndrome prevalent in the elderly (Thomas 1969). Whether observations made in the aging rat model is applicable to humans is uncertain. However, these findings suggest need for caution in the therapeutic use of cholinomimetics in the elderly so as to guard against the occurrence of exaggerated bradycardia and heart failure.

An age-related change in acetylcholinesterase activity appears to be one of the factors contributing to the cholinergic hypersensitivity of the heart in aging. Thus, in agreement with early reports (Frolkis et al. 1973; Mayer et al. 1985; Sket and Brzin 1985), our results showed a substantial decline in acetylcholinesterase activity in the atria and ventricles of aged compared to adult rats. Decreased activity of this enzyme would increase the effective synaptic (junctional), concentration of acetylcholine at any given dose of the neurotransmitter, and this may explain in part, the enhanced bradycardic response to acetylcholine in the aged heart. Such a possibility is supported by our observation that the magnitude of the age-related difference in the bradycardic response

to acetylcholine is smaller in the presence of the cholinesterase inhibitor eserine. An analogous observation has been reported recently by Kennedy and Seifen (1990). These investigators reported that right atria isolated from aged rats exhibited a greater negative chronotropic response to acetylcholine compared to similar preparations from adult rats, and pretreatment with an irreversible inhibitor of acetylcholinesterase abolished the age-related difference. They attributed the age-related difference in their preparations to an age-related decline in acetylcholinesterase activity although no direct measurement of enzyme activity was performed. The results of our study showed that the enhanced negative chronotropic and inotropic responses of the aged heart to cholinergic agonists can be attributed in part, but not exclusively, to an age-associated decline in acetylcholinesterase activity. Such a view is supported by the findings that: (a) at a maximally effective concentration of the cholinesterase inhibitor, eserine (5 μM), a significant age-related difference in the chronotropic response to acetylcholine persisted, and (b) marked age-related differences could be observed in the chronotropic and inotropic responses to carbachol, a cholinesterase-resistant cholinergic agonist. Thus, it is likely that an enhanced sensitivity of the postsynaptic effectors for cholinergic agonists also contributes to the exaggeration in cholinergic responses of the heart. Chapter 3 will present further studies focused on the postsynaptic mechanisms underlying the age-related increase in cholinergic responses of the heart.

SUMMARY

The findings presented in this chapter have shown the following: In isolated perfused spontaneously beating rat hearts, the negative chronotropic response to acetylcholine (10^{-9}-10^{-5}M) was up to 4-fold greater in the aged compared to adult rat; this age-related difference was less marked (2-fold), but not abolished in the presence of a maximally effective concentration of the cholinesterase inhibitor (5 μM), eserine. The cholinesterase-resistant agonist, carbachol (10^{-9}-2.5x10^{-6}M), elicited 2 to 3-fold greater negative chronotropic response in the aged compared to adult hearts. In isolated perfused, electrically paced (4Hz), rat hearts, carbachol (10^{-9}-10^{-5}M), elicited concentration-dependent negative inotropic response which was 2-fold greater in the aged compared to

adult heart at all carbachol concentrations. Acetylcholinesterase activities (μmol/g/hr) were 50 to 60% lower in the aged atria (83 ± 21), and ventricles (24 ± 6), than in adult atria (210 ± 20), and ventricles (47 ± 7). These findings demonstrate a striking enhancement in the responses of the heart to cholinergic stimulus with aging which can be attributed in part, but not solely, to an age-associated decline in cholinesterase activity. Age-associated alterations in muscarinic receptor linked events may also underlie the cholinergic hypersensitivity of the aging heart.

CHAPTER 3

MECHANISMS UNDERLYING THE ENHANCED CHOLINERGIC RESPONSES OF RAT MYOCARDIUM WITH AGING

3.1 Introduction

The findings described in the preceding chapter (Chapter 2) have shown that, in contrast to the age-related decline in β-adrenergic responses of the heart, cardiac muscarinic cholinergic responses at posynaptic level are strikingly enhanced with aging. Mechanisms underlying the enhanced cholinergic responses of rat myocardium with aging are not fully known. Since the muscarinic cholinergic signal transduction system has three components (receptors, G proteins, and effectors), at postsynaptic level, the age-related alterations in cholinergic responses can result from changes in the number and /or properties of muscarinic receptors (MAChR), G-proteins or their effector components. In attempting to further define the mechanisms underlying the age-related exaggeration of myocardial cholinergic responses, the present study investigated the muscarinic receptor linked biochemical events and electrophysiological responses to muscarinic receptor stimulation by carbachol in atria and ventricles of adult and aged Fischer 344 rats.

In examining the biochemical mechanisms underlying the age-related increase in cholinergic responses of the heart, this study focused on the effects of aging on characteristics of muscarinic receptors, on the expression of the Gi-Go proteins and the efficacy of the G protein-mediated signal transduction. To characterize the muscarinic receptors in the hearts, the muscarinic receptor number and its binding affinity to its agonist were studied. The expression of Gi-Go proteins were studied using both Western blotting and ADP-ribosylation techniques. Binding of GTP to carbachol-MAChR-Gi protein complex results in dissociation of the complex and liberation of the $Gi\alpha GTP$ subunit. The dissociation of the complex is due to a GTP-induced decrease in the affinity of the MAChR for the carbachol (Baker et al, 1985; Fleming et al, 1992).

Experimentally, the GTP or Gpp(NH)p induced decrease in the receptor binding affinity for its agonist can be used as a parameter to determine the efficacy of the signal transduction (Baker et al, 1985; Fleming et al 1992). Therefore, the efficacy of the signal transduction through the MAChR linked Gi protein was assessed by determining the influence of Gpp(NH)p on carbachol competition for MAChR binding sites labelled with [^{3}H]QNB.

Electrophysiological approaches were used to examine the possible age-related changes in the MAChR-G protein-linked effector system. In the heart, the well recognized electrophysiological effects of muscarinic receptor stimulation include (a) activation of a potassium current (I_{KACh}) (Giles and Noble, 1976; Noma and Trautweun, 1978; Pfaffinger et al, 1985; Loffelholz and Pappano, 1985; Noma, 1987; DiFrancesco et al, 1989), and (b) decrease of the L-type calcium current (Giles and Noble, 1976; Ten Eick et al, 1976; Hino and Ochi, 1980; Ochi, 1981; Noma, 1987; DiFrancesco et al, 1989). Activation of I_{KACh} results in marked hyperpolarization of the resting membrane potential and shortening of action potential duration leading to a negative chronotropic effect in sinoatrial node, and a negative inotropic effect in atria and ventricles. Decrease of the L-type calcium current will result in marked shortening of action potential duration leading to a negative dromotropic effect in the atrioventricular node and a negative inotropic effect in atria and ventricles. Using standard microelectrode techniques, we determined the effect of aging on carbachol-induced alterations in transmembrane action potential parameters *viz* (a) hyperpolarization of maximum diastolic potential (MDP), and (b) shortening of action potential duration (APD), in electrically paced, normally polarized, atrial and ventricular cells. Since it has been suggested that the magnitude of muscarinic effects may differ in ventricular epicardium and endocardium (Litovsky and Antzelevitch, 1990), the ventricular electrophysiological responses to carbachol were assessed in both epicardium and endocardium. Additionally, carbachol-induced changes in atrioventricular node conduction time (AVT) were monitored, in order to differentiate if the age-related difference in the negative chronotropic effect observed in Chapter 2 is caused by blockage of atrioventricular conduction or by inhibition of sinoatrial nodal function.

3.2 Materials and methods

3.2.1 Materials

Pertussis toxin was obtained from Sigma Chemical Co., St. Louis, MO. (-)-[^{3}H]Quinuclidinyl benzilate ([^{3}H] QNB), [^{32}P] NAD^+ , and antibodies of Giα (AS/7 anti Gi1α and anti Gi2α) and Goα were purchased from New England Nuclear, Montreal, Canada. Electrophoresis and autoradiography reagents were from Bio-Rad Laboratories, Mississauga, Ontario. Supplies for Western blotting were from Amersham Canada, Oakville. All other chemicals were available from Sigma, St. Louis, MO, or BDH Chemicals, Toronto.

3.2.2 Preparation of Homogenates and Particulate Fractions and [^{3}H] QNB Binding Assay

Atria and ventricles were freed of blood by retrograde aortic perfusion of coronary arteries with 40 ml of ice cold 10 mM sodium-potassium-phosphate buffer (mixture of Na_2HPO_4 8.1 mM and KH_2PO_4 1.9 mM in distilled water), pH 7.4. The tissues were minced, and then homogenized in 10 (ventricle) or 20 (atria) volumes (based on tissue weight) of the phosphate buffer using a Polytron PT-10 homogenizer (10 sec x 3 with 30 sec interval between bursts; setting 8). The homogenates were filtered through a double layer of cheese cloth and centrifuged at 40,000 x g for 60 min. The supernatants were discarded and the pellets were washed by resuspension in the phosphate buffer followed by centrifugation at 40,000 x g for 30 min. The final pellets were resuspended in the phosphate buffer to yield a protein concentration of 2 to 3 mg/ml (protein concentration was determined as described in section 3.2.4).

[^{3}H] QNB binding to muscarinic receptor sites in homogenates and total particulate fractions of atria and ventricles was determined as described previously (Narayanan and Derby 1983). The reaction mixture (final volume 0.5 ml) contained 10 mM sodium-potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, tissue fraction (100-150 μg protein) and varying concentrations (0.01-1 nM) of [^{3}H] QNB. All assays were

performed in the absence and presence of atropine (1 μM). The binding reaction was carried out for 60 min at 37°C. At the end of incubation, the reaction mixture was diluted with 4.5 ml of the ice cold phosphate buffer containing 10 mM $MgCl_2$ (stop solution), vortexed and 4 ml of the suspension was filtered through Whatman GF/C glass fiber filters (1.2 μm), under mild suction. The filters were washed with 20 ml (5 ml x 4), of stop solution, placed in scintillation vials, dried at 60°C for 3 h, and ^{3}H-radioactivity was determined by liquid scintillation spectrometry. The specific binding of [^{3}H]QNB was calculated from the difference in counts obtained in the absence and presence of atropine (1 μM). The specific binding was 90-95% of the total binding. All results reported here represent specific binding. The concentration of muscarinic receptors (Bmax) and the dissociation constant (Kd), for [^{3}H]QNB binding were determined by hyperbolic plot using Graph PAD, Inplot, vertion 4, San Diego, CA (Bylund, 1980).

The ability of the muscarinic agonist carbachol to compete for the [^{3}H]QNB binding sites was determined to assess the agonist binding affinity of the muscarinic recepotors. Agonist competition assays were performed in the same way except the concentration of [^{3}H]QNB used was 1 nM and the concentration of carbachol varied from 1 nM - 1 mM. The competition assays were also performed in the presence of 10 μM Gpp(NH)p to study the efficacy of the G protein-mediated signal transduction (Baker et al, 1985). All assays were performed in triplicate and the variability was less than 4%.

3.2.3 Preparation of atrial and ventricular membranes, ADP-ribosylation, and Western blotting for detecting Giα proteins

Atria and ventricles were washed free of blood, minced and then homogenized in 20 volumes of buffer (10 mM Tris-HCl/ 2 mM DTT, pH 7.5), using a polytron PT-10 homogenizer (10 s X 2 with a 30-s interval between burst; setting 10). KCl was added to the homogenates to give a final concentration of 0.6 M. Following incubation at 4°C for 20 min the homogenates were centrifuged at 40,000 X g for 30 min. The pellets were washed and centrifuged the same way as above in the homogenizing buffer. The final pellets were resuspended in the homogenizing buffer, and filtered through a nylon mesh to remove any large particles. The protein concentration of the sample was determined.

They were then diluted to yield final protein concentration of 3 mg/ml for ventricular membranes and 2 mg/ml for atrial membranes. The aliquots were quick-frozen in liquid nitrogen and stored at -70^{0}C.

Analysis of Gi protein by ADP-ribosylation. The α-subunits of Gi protein were identified by the ability of pertussis toxin to transfer an ADP-ribose moiety from [^{32}P]NAD to the α-subunit of the Gi protein. The toxins were activated by incubation with 50 mM dithiothreitol for 20 min at 32^{0}C. The ADP-ribosylation medium (total volume 150 μl) contained 16.7 mM Tris-HCl/0.67 mM EDTA, pH 7.8, 6.7 mM thymidine, 0.67 mM ATP, 0.67 mM GTP, 1.3 mM DTT, and 2.5 μg activated pertussis toxin; 6.3 μM [^{32}P]NAD and atrial (60 μg protein) or ventricular (90 μg protein) membranes. The mixture was incubated at 37^{0}C for 1 h and the reaction was stopped by adding 150 μl of ice-cold 20% trichloroacetic acid. The samples were centrifuged for 5 min in a microfuge. The pellets were washed with ether and dissolved in 40 μl of SDS-sample buffer containing 0.125M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.01% bromophenol blue. Following the ADP-ribosylation reaction, an equal amounts of membrane protein (60 μg atrial protein and 90 μg ventricular protein) from adult and aged groups was fractionated on a 7-15% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The ribosylated α-subunit of Gi protein which has an apparent molecular weight of 41 Kd was identified following autoradiography of the gel. The relative amounts of Giα subunits in the cardiac membranes from adult and aged rats were determined from two dimensional scanning densitometry of the autoradiograms using an LKB ultroscan XL laser densitometer.

Western blotting. The advantage of "Western" blotting is that it can quantify all the cardiac Gi protein even if the Gi protein is not accessible for ADP-ribosylation. Equal amounts of atrial (34 μg), and ventricular (51 μg), membranes from adult and aged hearts were solubilized in SDS-sample buffer, resolved on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose paper (Hybond-ECL), at 250 mA for 100 min. After transfer, the membranes were incubated in the washing buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl and 0.025% Tween-20), containing 5% casein for 4 hr. The blots were washed in the washing buffer 3 times for 1 hr and then incubated with antisera

against Giα in the washing buffer (1:1000 dilution), at room temperature for 1 hr. The antigen-antibody complexes were detected by incubating the blots with goat anti-rabbit IgG (1:1500 dilution), conjugated with horseradish peroxidase for 70 min at room temperature. The blots were washed 4 times with washing buffer before reacting with enhanced-chemiluminescence (ECL), Western-blotting detection reagents. The immunoreactive protein band representing Gα subunit of the G proteins was quantified by two dimensional scanning densitometry using an LKB ultroscan XL laser densitometer.

3.2.4 Determination of Protein

The amount of protein in tissue fractions was determined by the method of Lowry et al (1951), with a spectronic 21 Spectrophotometer at the wavelength of 660 nm, using bovine serum albumin as a standard.

3.2.5 Recording of action potentials from atria and ventricles

The right atria and right ventricular free wall preparations were obtained and mounted as described previously (Duan and Moffat, 1991). Briefly, rats were sacrificed by cervical dislocation and their hearts were quickly excised and rinsed in prewarmed (37°C) Krebs-Henseleit buffer containing (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-Glucose 10, and Na Succinate 5, pH 7.4, bubbled with a 95% O_2, 5% CO_2 gas mixture. The right atrium, 0.3 to 0.5 cm^2, or the right ventricular free wall, approximately 0.7 cm^2 in area, was dissected in a bath containing the same solution and maintained at 37°C. The preparation was transferred to a 5 ml tissue bath and superfused with Krebs-Henseleit solution at a rate of 15 $\pm$ 1 ml/min using a Watson-Marlow peristaltic pump. The atrial preparation was held approximately 1 mm above the bottom of the bath, while the ventricular preparation was held in position with the natural curve retained and the endocardium facing upward (Duan and Moffat, 1991). The preparations were stimulated through bipolar silver electrodes that were applied to the surface of the atria or to the endocardial surface of the ventricle. Rectangular pulses, 3 msec in duration at 1.5 times threshold, were

delivered using an optically isolated digital stimulator (Pulsar 4I and 6I, Frederick Haer and Co., Brunswick, MN, USA). Stimuli were delivered at a basic cycle length (BCL) of 500 msec.

The preparations were equilibrated for 30 min prior to impalement. Following equilibration, transmembrane action potentials were recorded from the atrial surface, or from both endocardial and epicardial surfaces of the ventricular preparations using standard microelectrode techniques. The glass microelectrodes (tip diameter $< 1\ \mu m$), were filled with 2.7 M KCl (resistance 10-30 MΩ). Transmembrane electrical activity was displayed on a Tektronix 5110 oscilloscope and recorded on a Gould 2200S pen chart recorder through a WPI amplifier. Concentration-dependent effects of carbachol (10^{-10} - 10^{-5} M) were examined after 30 min of equilibration following impalement.

3.2.6 Measurement of atrioventricular conduction time (AVT)

In experiments designed to study the effect of carbachol on AVT in spontaneously beating heart preparations using the Langendorff preparation as described in chapter 2, the entire atria and ventricles were left intact, and extraneous tissue was trimmed away. A bipolar extracellular electrode was positioned at the right atria and another bipolar extracellular electrode with a ground electrode was positioned on the ventricles. Atrial and ventricular extracellular cardiac electrocardiograms were recorded on separate channels of a Grass 79 polygraph.

In hearts in which AVT was measured at a constant rate of atrial pacing, the sinoatrial nodal region (including vena cava), and part of right atrium were excised to facilitate electrical stimulation. Besides the electrodes indicated above for spontaneously beating hearts, one more bipolar extracellular electrode was placed on the remaining part of right atrium for delivery of electrical stimulation. The right atrium was paced at 240 beats/min using a Grass SD5 stimulator at a duration of 3 msec at 2X the threshold intensity. The atrial and ventricular ECGs were recorded in the same way as for the spontaneously beating heart. The atrioventricular interval, ie, AVT was defined as the time difference between the beginning of the atrial wave and the beginning of ventricular wave in both spontaneously beating heart and electrically paced hearts.

After completion of dissection and instrumentation, all hearts were allowed to stabilize for 20 min before the experiments were begun. Perfusion with varying concentrations of carbachol (10^{-10} - 10^{-5} M), was carried out in ascending order for 3 min (at constant flow rate of 9 ml/g ventricular weight/min in nonrecirculating manner), with a washing-out period of 3-6 min between each concentration.

3.2.7 Data analysis

The data are presented as the mean ± S.E.M. The age-related differences of B_{max}, K_D and IC_{50} values, quantity of the Giα protein, and the control MDP, APD, and AVT values were tested using unpaired two tailed Student's *t* test. Statistical significance for the differences in carbachol responses between adult and aged rats was tested using two way analysis of variance with repeated measures (SPSS/PC+$_{TM}$, version 3.0, SPSS Inc; logarithmic transformation was used to make the variance homogeneous). A P value of < 0.05 was regarded as significant.

3.3 Results

3.3.1 Characteristics of muscarinic receptors in atria and ventricles

Fig. 3-1 shows the equilibrium binding data for [^{3}H]QNB in atrial and ventricular tissues of adult and aged rats. [^{3}H]QNB is a MAChR antagonist and its binding at equilibrium provides an estimate of the density of MAChR. Specific [^{3}H] QNB binding in total particulate fractions of atria and ventricles indicated no significant age-related differences in the number of muscarinic receptor sites or their antagonist binding affinities (Fig. 3-1). At a saturating concentration (1 nM), specific [^{3}H] QNB binding in atrial and ventricular homogenates also did not differ significantly with age [specific ^{3}H-QNB binding (fmol/mg protein): adult atria 360 ± 38; aged atria 334 ± 35; adult ventricle 214 ± 8; aged ventricle 193 ± 22; n = 5 in each case).

The ability of the muscarinic agonist carbachol to compete for the [^{3}H]QNB binding sites was determined to assess the agonist binding affinity of muscarinic receptors. As shown in Fig. 3-2, carbachol caused concentration-dependent inhibition of

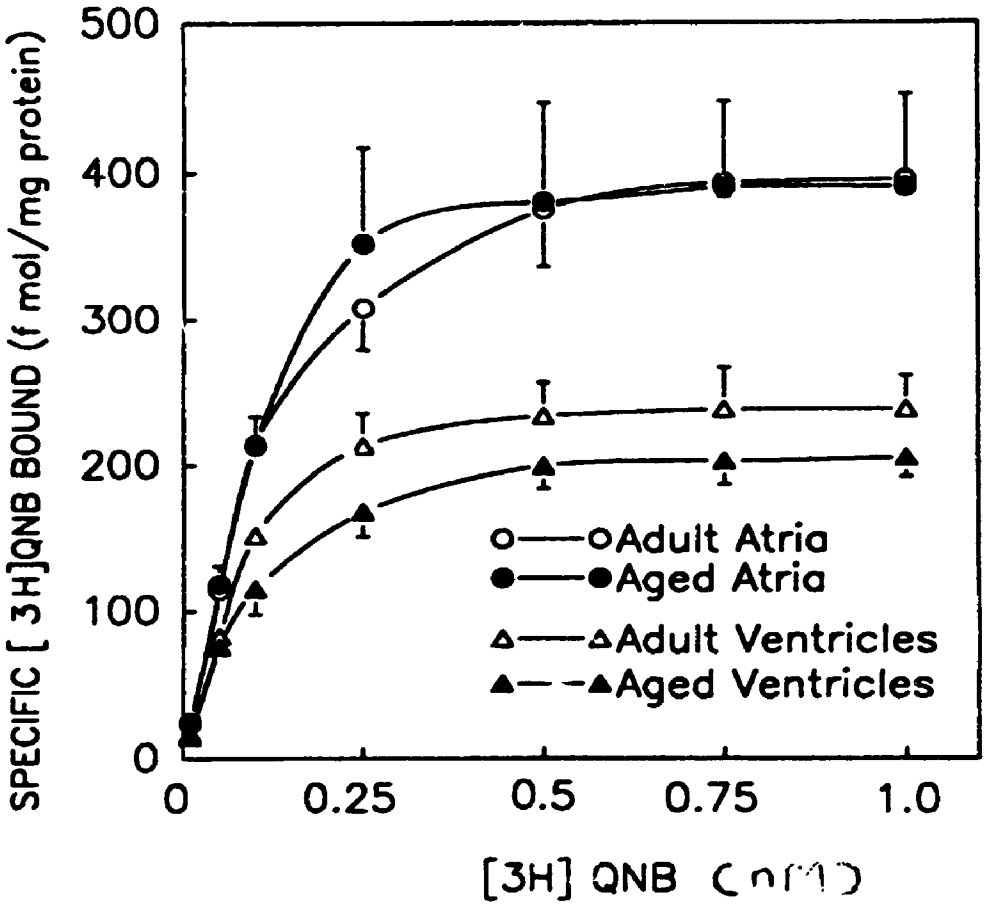

Fig. 3-1 Specific [^{3}H]QNB binding to total particulate fraction of atria and ventricles from adult and aged hearts as a function of varying concentrations of [^{3}H]QNB. Results represent mean $\pm$ SE of 4 separate experiments. No significant age-related difference was evident in B_{max} [B_{max}, (fmol/mg protein): adult atria 463 $\pm$ 15; aged atria 437 $\pm$ 81; adult ventricle 268 $\pm$ 34; aged ventricle 240 $\pm$ 12] or K_D values [K_D (pM): adult atria 135 $\pm$ 13; aged atria 112 $\pm$ 11; adult ventricle 88 $\pm$ 10; aged ventricle 128 $\pm$ 32] determined by hyperbolic plot using Graphpad. Inplot, version 4, SanDiego, CA (Bylund, 1980).

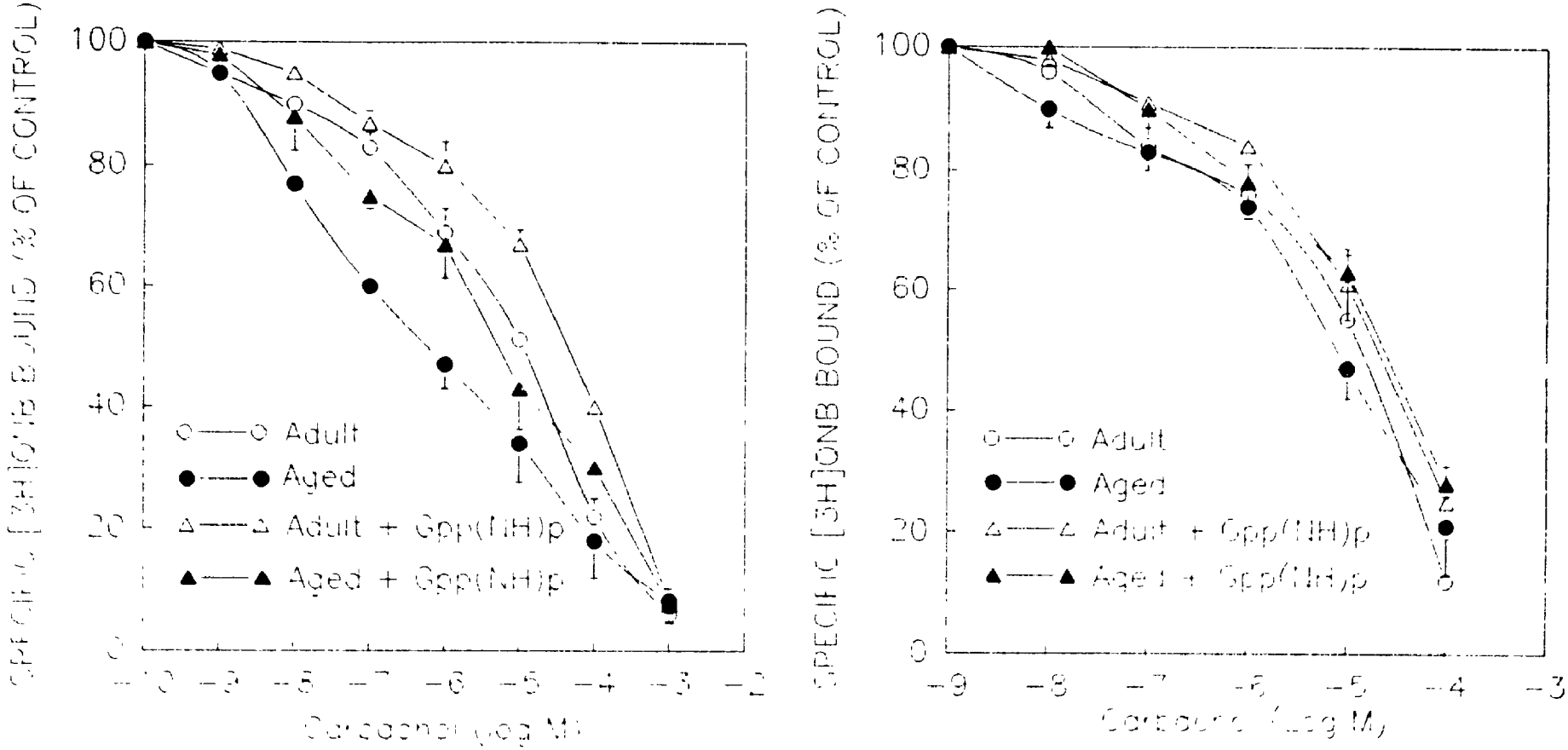

Fig. 3-2 Inhibition of specific [^{3}H]QNB binding by carbachol in atria (left panel) and ventricles (right panel) in the absence and presence of 10 μM Gpp(NH)p. Results represent mean $\pm$ SE of 4 separate experiments in the absence of Gpp(NH)p and 3 separate experiments in the presence of Gpp(NH)p. See Table 3-1 for the IC_{50} values.

the [^{3}H]QNB binding to receptor sites in atrial and ventricular fractions. In the case of the atria, the dose response curve was shifted to the left in the aged compared with the adult (Fig. 3-2, left panel). The IC_{50} for aged atria was 7 fold lower compared with adult ($P<0.05$; Table 3-1). In the ventricle, no age-related difference was evident in IC_{50} values (Fig. 3-2, right panel).

Table 3-1 The IC_{50} values for carbachol in the absence and presence of Gpp(NH)p

	IC_{50} for Carbachol (μM)	
	- Gpp(NH)p	+ Gpp(NH)p (10 μM)
Adult Atria	6.5 $\pm$ 1	30 $\pm$ 13
Aged Atria	0.87 $\pm$ 0.1	5 $\pm$ 0.8
Adult Ventricles	8.2 $\pm$ 1.1	18 $\pm$ 5
Aged Ventricles	7 $\pm$ 1	19 $\pm$ 8

Comparison of IC_{50} values for carbachol in the absence and presence of 10 μM Gpp(NH)p. The data from Fig. 3-2 were analyzed using a nonlinear curve fitting technique as described by DeLean et al (1978). The IC_{50} values were derived using Graph PAD program (Graph PAD Software Version 2.0, Serial #10916, available from Graph PAD, San Diego, CA). The IC_{50} data from individual rats were averaged separately for the adult and aged groups and expressed as mean $\pm$ SE of mean. Statistical comparisons of the IC_{50} values between adult and aged rats were tested using unpaired two tailed Student's *t* test. The folds of Gpp(NH)p-induced shift in receptor affinity for agonist (compared to the IC_{50} values in the absence of Gpp(NH)p) is similar in adult and aged atria and ventricles.

3.3.2 The efficacy of signal transduction through MAChR-linked Gi protein

The data presented in Fig. 3-2 compare the ability of carbachol to compete for muscarinic receptor sites in the absence and presence of Gpp(NH)p. Gpp(NH)p induced a 5- to 6-fold increase in the carbachol IC_{50} values in the adult and aged atrial membranes whereas Gpp(NH)p only induced a 2- to 3-fold shift compared to the carbachol IC_{50} values in the absence of Gpp(NH)p in adult and aged ventricular membranes (Table 3-1). However, no significant age-related difference was seen in the magnitude of the guanine nucleotide-induced shift in IC_{50} for carbachol (Table 3-1) in the atrial or in the ventricular membranes.

3.3.3 Effects of aging on expression of the Giα-Goα protein levels

The biochemical mechanisms studied so far indicated that there was no age-related difference in the density or the antagonist binding affinities of muscarinic receptors, or in the efficacy of signal transduction through MAChR linked Gi proteins of the atrial and ventricular membranes. However, a selective enhancement in the affinity of MAChR for its agonist carbachol in aged atria but not ventricles was observed, which may be of relevance to the enhanced negative chronotropic response in the aged heart.

The muscarinic effects on the heart are thought to be mediated largely by the signal transducing G proteins, Gi and Go (Katz, 1992). The magnitude of muscarinic effects may be influenced by the amount of these G proteins. Therefore, the effect of aging on the expression of Gi and Go in atrial and ventricular tissues was assessed by Western immunoblot analysis using α subunit specific antibodies (Shu and Scarpace, 1994) of these G proteins. The results showed that the steady state levels of Giα were significantly greater (2- to 3-fold), in the atria and ventricles from the aged compared to adult rats (Fig. 3-3 and 3-4). An age-related increase in Giα levels in atria and ventricles could also be observed in experiments where ^{32}P-ADP ribosylation was used to quantify this G protein (Fig. 3-5). Levels of Goα as evidenced by immunoblot analysis, were very low and sufficiently close to background levels to make quantitative analysis difficult (data not shown). However, no substantial difference in the signal was apparent between adult and aged groups in both atrial and ventricular membranes.

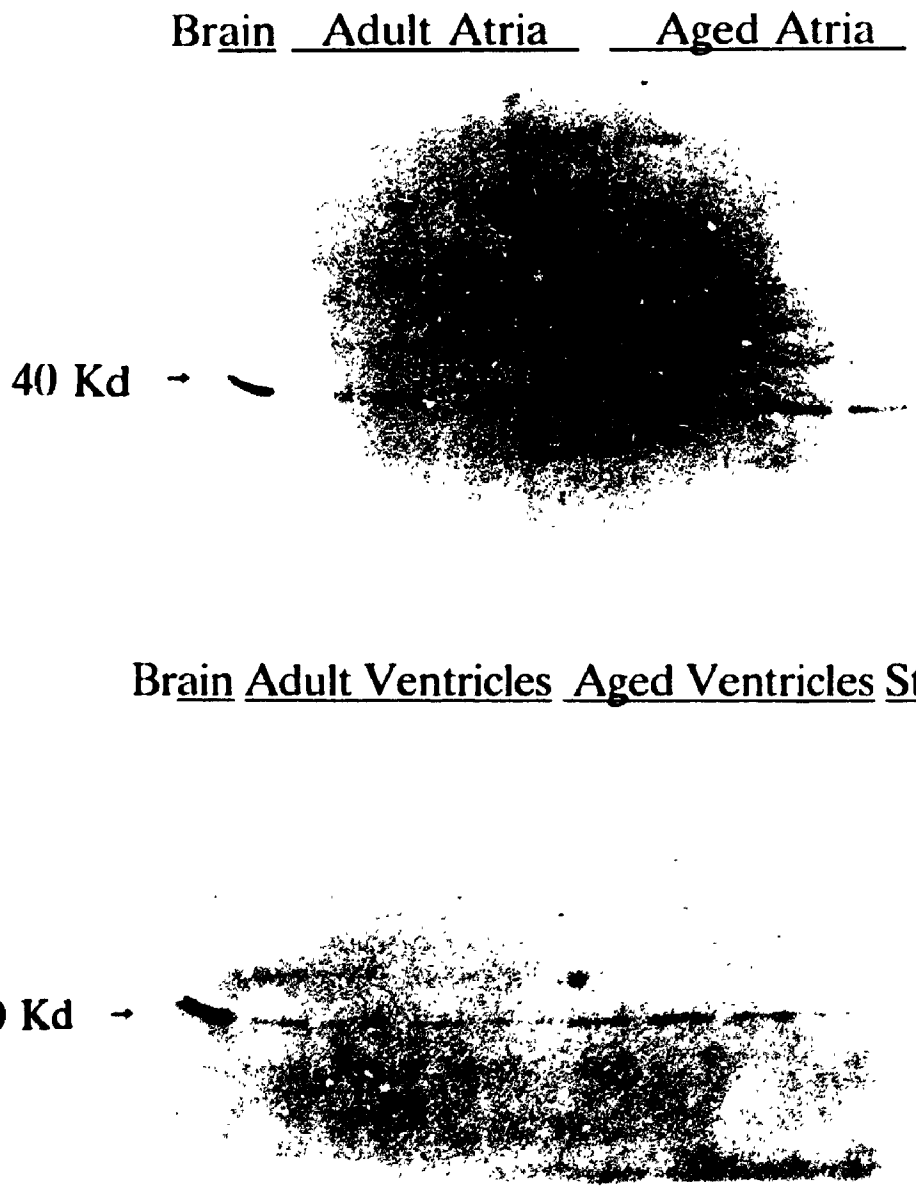

Fig. 3-3 Immunoblots of Giα in atrial (upper panel) and ventricular (lower panel) membranes of adult and aged rats. 34 μg atrial and 51 μg ventricular protein were loaded onto each well of the gels. Note that the positive control sample obtained from brain indicated 40 Kd Giα subunit. The reaction product for the positive control was denser than those obtained with the samples, indicating that the concentrations used were below saturation. The concentration of protein loaded onto each gel was equal between the adult and aged samples, well below saturating concentrations in order to permit accurate quantitative comparison by densitometry. This experiment was duplicated with similar results.

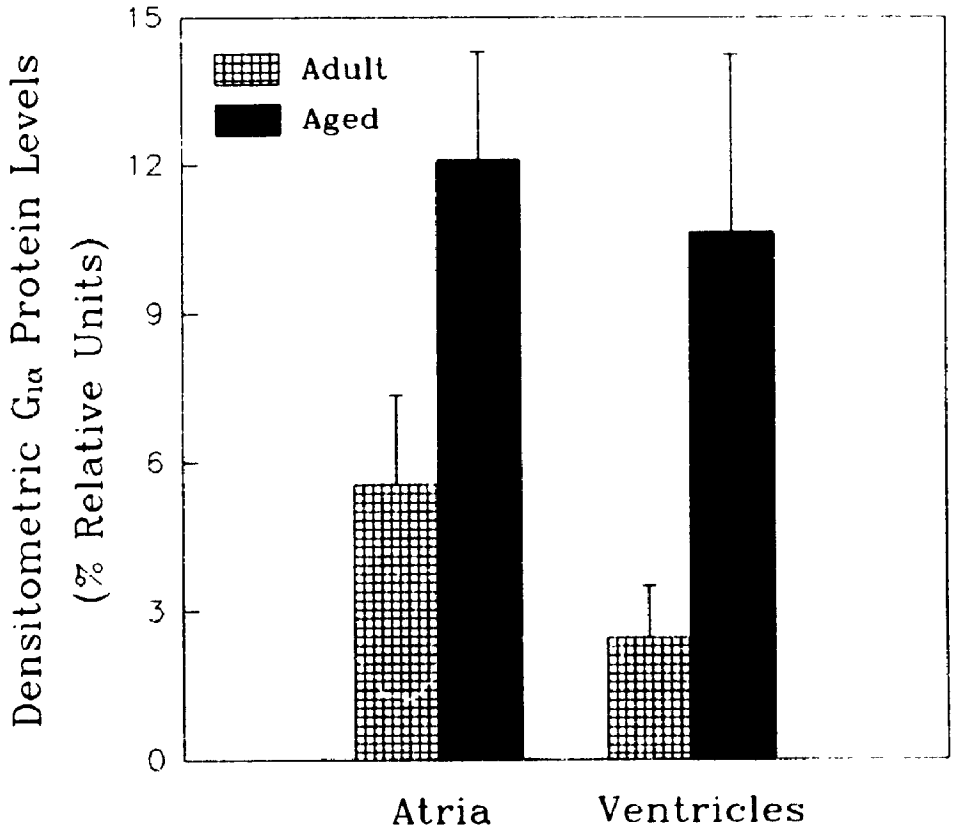

Fig. 3-4 Comparison of Giα protein levels of atrial and ventricular membranes from adult and aged rats. The Western immunoblots shown in Fig. 3-3 were subjected to laser densitometric scanning to quantify the relative amount of Giα protein ($n=4$). The age-related difference is significant in both atria and ventricles ($P<0.01$).

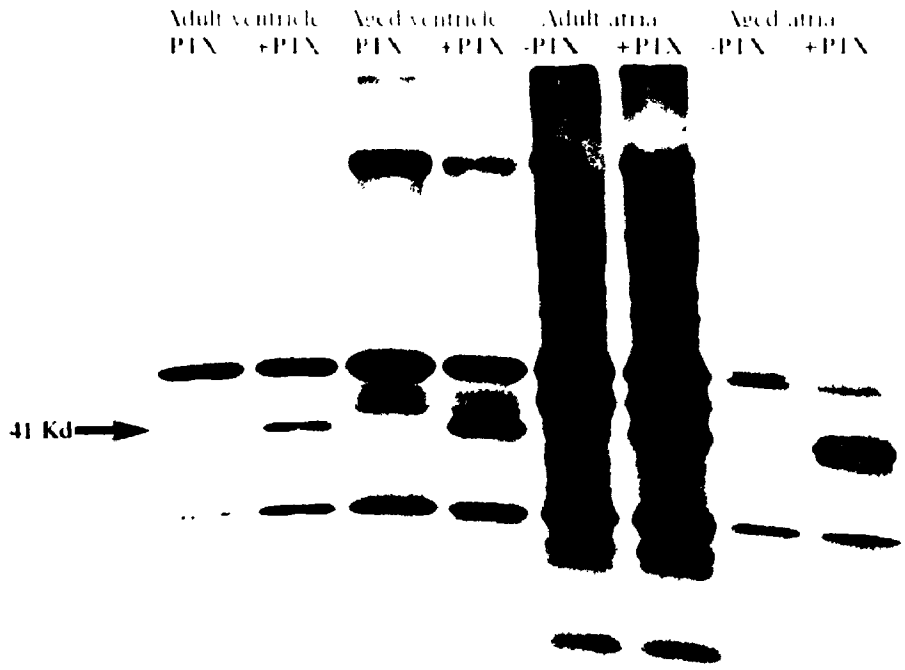

Fig. 3-5 Quantification of pertussis toxin sensitive G protein by ADP-ribosylation in ventricular and atrial membranes of adult and aged rats. The concentration of protein loaded onto each well was 90 μg for adult and aged ventricular samples and 60 μg for adult and aged atrial samples. Densitometric scan showed that the relative units of this peptide is 5.28 for adult ventricles, 32.5 for aged ventricles, 22.1 for adult atria, and 40.1 for aged atria.

3.3.4 Effect of carbachol on atrial action potentials of adult and aged rats

Having examined the mechanisms underlying the enhanced cholinergic responses of rat myocardium with aging at the receptor level and MAChR-G prtein mediated siginal transduction pathway, we also studied the MAChR-G protein linked effector systems using electrophysiological approaches. Five adult and five aged rats were used in these experiments. The atrial action potential parameters measured prior to superfusion with carbachol are shown in Table 3-2. The maximum diastolic potential (MDP) was significantly smaller in amplitude (~ 13%, $P < 0.05$), in atria of aged compared to adult rats. The atrial action potential duration measured at 50% (APD_{50}) or 95% (APD_{95}), repolarization did not differ significantly with age. Carbachol (10^{-10} - 10^{-5} M) caused concentration-dependent hyperpolarization of the MDP and shortening of the APD_{95} in atria from adult and aged rats (Fig. 3-6; Fig. 3-7). The carbachol-induced hyperpolarization was significantly greater (2 to 4-fold, $P < 0.01$), in atria from aged compared to adult rat at the varying carbachol concentrations used (Fig. 3-7, left panel). Although, the carbachol-induced shortening of APD_{95} tended to be greater in the aged compared to adult rat atria (especially at carbachol concentrations below 10^{-6} M), this age-related difference was not statistically significant (Fig. 3-7, right panel).

Table 3-2 **Comparison of action potential parameters in atria and ventricles of adult and aged rats**

		MDP (mV)		APD_{50} (msec)		APD_{95} (msec)	
Tissue	N	Adult	Aged	Adult	Aged	Adult	Aged
Atria	5	-76.4±1.8	-66.8±1.5*	11.3±1.6	12.4±2.7	40.0±5.0	47.4±6.7
Ventricle (Epicardium)	6	-69.8±0.5	-69.0±1.1	8.12±0.4	13.0±2.3	32.3±1.6	45.0±6.5
Ventricle (Endocardium)	6	-72.5±1.4	-73.0±1.2	11.6±2.2	23.0±4.6*	31.5±4.8	49.0±7.4

Values are mean ± SE; N, number of animals; MDP, maximum diastolic potential; APD_{50}, action potential duration measured at 50% of repolarization; APD_{95}, action potential duration measured at 95% of repolarization.

* $P < 0.05$ vs. corresponding value for the adult.

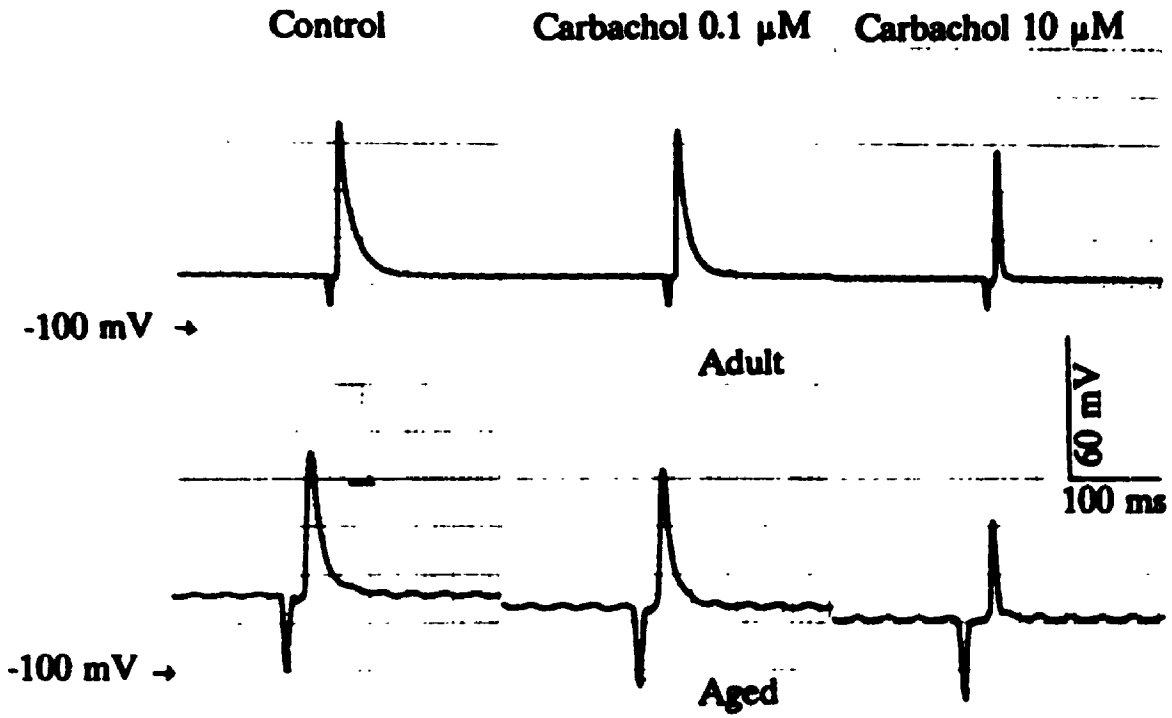

Fig. 3-6 Original recordings of action potentials showing effects of carbachol (10^{-8}M and 10^{-5}M) on atrial membrane potentials of an adult (upper tracing) and an aged rat (lower tracing).

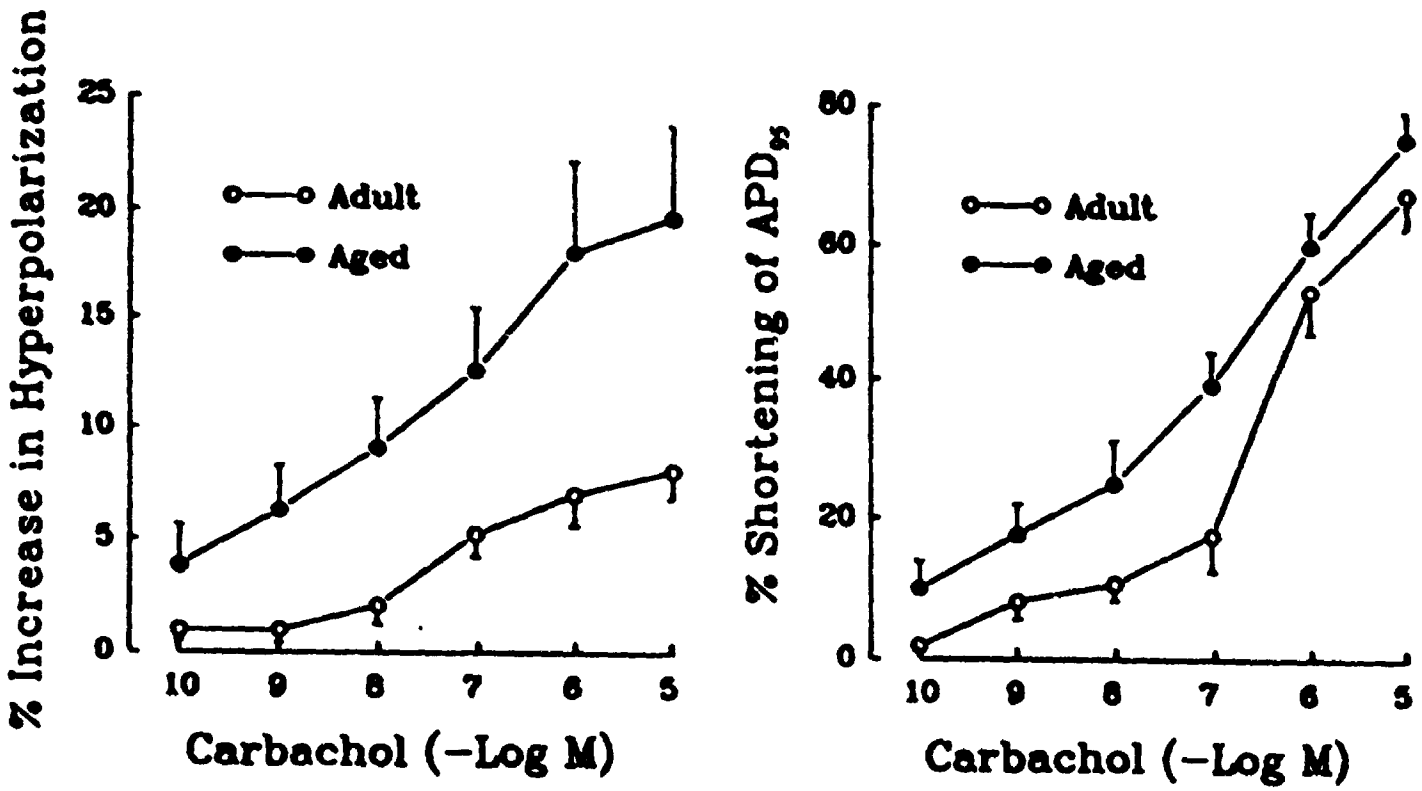

Fig. 3-7 Comparison of the effects of carbachol on resting membrane potential (left panel) and action potential duration (right panel) in atria from adult and aged rats. Results represent mean ± SEM of experiments using 5 adult and 5 aged rats. The age-related difference in membrane hyperpolarization is statistically significant ($P < 0.01$) at all carbachol concentrations tested. The age-related difference in APD_{95} is not statistically significant. The baseline (control) values for maximum diastolic potential (MDP) and action potential duration (APD_{95}) prior to carbachol superfusion are given in Table 3-2.

3.3.5 Effects of carbachol on ventricular action potentials in adult and aged rats

Six adult and six aged rats were used in these experiments. The action potential parameters measured prior to superfusion with carbachol are shown in Table 3-2. The MDP of ventricular epicardium and endocardium were essentially similar in adult and aged rats. The ventricular action potential duration (APD_{50} and APD_{95}), tended to be prolonged in both epicardium and endocardium of the aged compared to adult rat; this age-related difference was most pronounced and statistically significant with respect to APD_{50} measured in endocardium (2-fold increase in the aged; $P < 0.05$).

When the ventricular epicardium and endocardium were superfused with carbachol, the drug shortened the APD_{50} in a concentration-dependent fashion without any significant effect on the MDP (Fig. 3-8; Fig. 3-9; Fig. 3-10). The carbachol-induced shortening of APD_{50} was strikingly more pronounced ($>$ 4-fold, $P < 0.001$), in the aged in comparison with the adult ventricular epicardium and endocardium across the concentrations of carbachol used.

3.3.6 Comparison of dromotropic response to carbachol

In spontaneously beating rat hearts (basal heart rate varied between 205-220 beats per min in the adult, and between 200-210 beats per min in the aged), the basal AVT was significantly greater in the aged compared to the adult (adult 32.5 $\pm$ 1.6 msec; n=3; aged 44.3 $\pm$ 1.4 msec, n=3; $p < 0.05$). In other experiments where the atria were paced at 240 beats per min, the age-related difference in the basal AVT persisted (adult 31.7 $\pm$ 1.4 msec, n=3; aged 43.3 $\pm$ 1.4 msec, n=3; $p < 0.01$).

In the spontaneously beating hearts, carbachol caused concentration-dependent bradycardia in the absence of any noticeable increase in AVT (Fig. 3-11). When the atria were paced at 240 beats per min, carbachol caused a concentration-dependent increase in AVT (Fig. 3-12, left panel); the magnitude of carbachol-induced increase in AVT did not differ significantly with age (Fig. 3-12, right panel). There was no degree II or III AV block when the adult and aged hearts were perfused with 0.1 nM-0.1 μM carbachol.

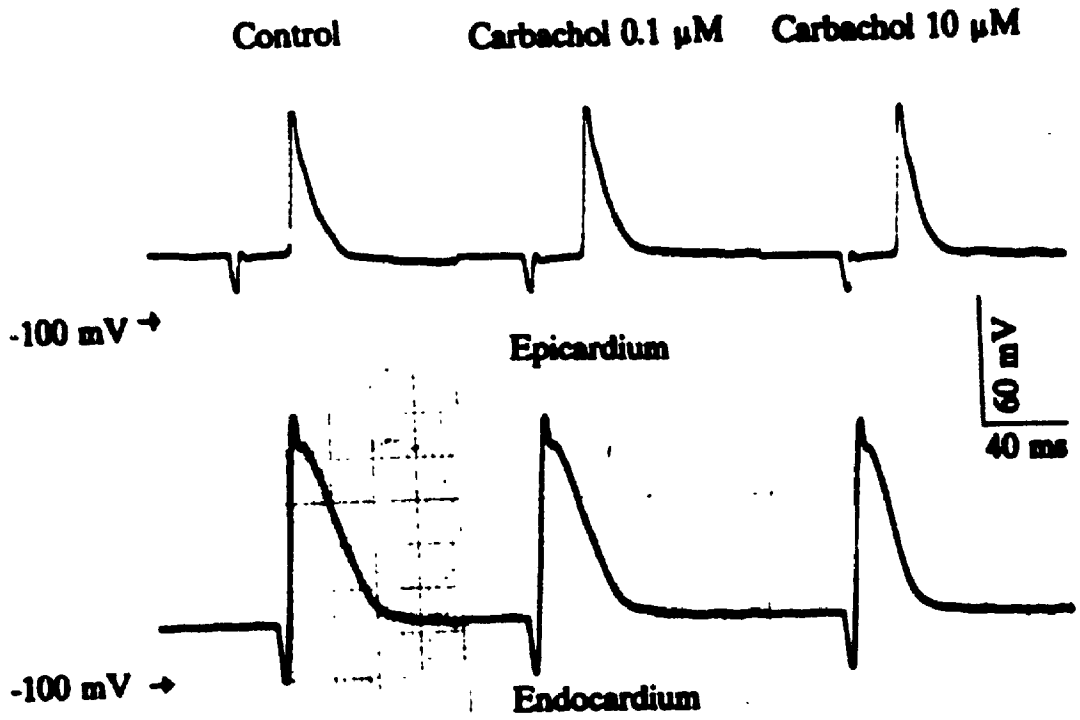

Fig. 3-8 Experimental recordings of action potentials showing the effect of carbachol on the epicardial (Upper panel) and endocardial (Lower panel) APD_{50} in an adult rat.

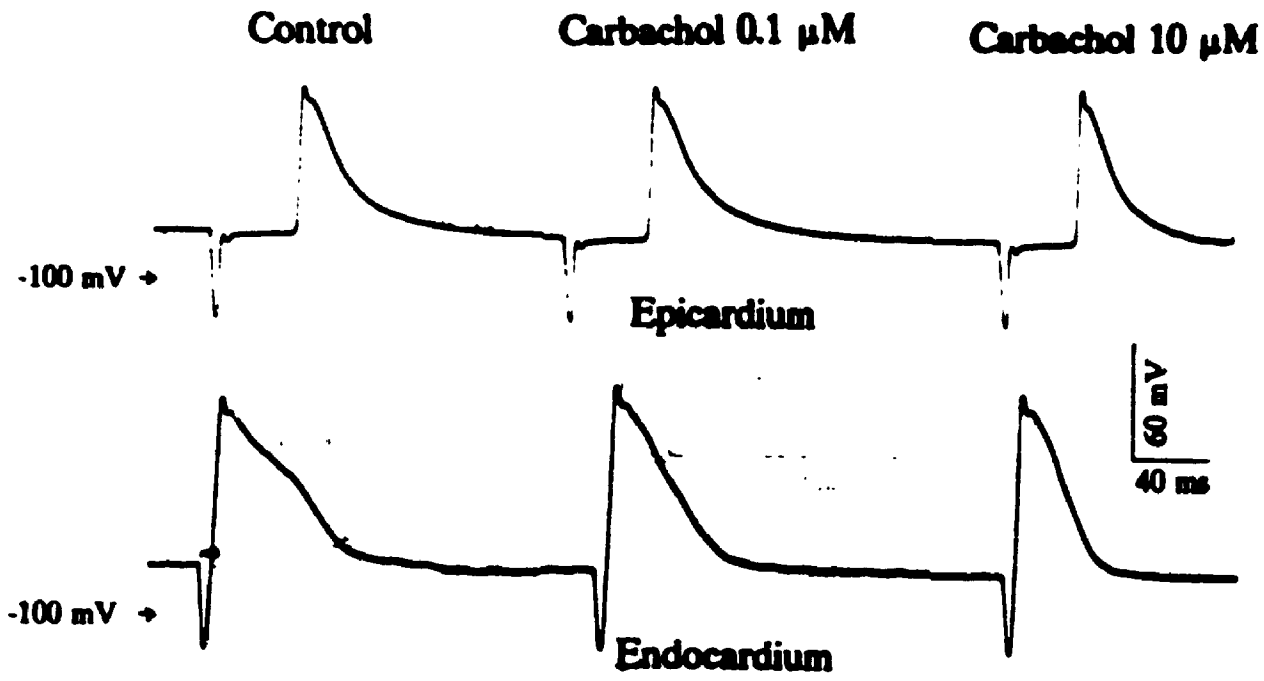

Fig. 3-9 Experimental recordings of action potentials showing the effect of carbachol on the epicardial (Upper panel) and endocardial (Lower panel) APD_{50} in an aged rat.

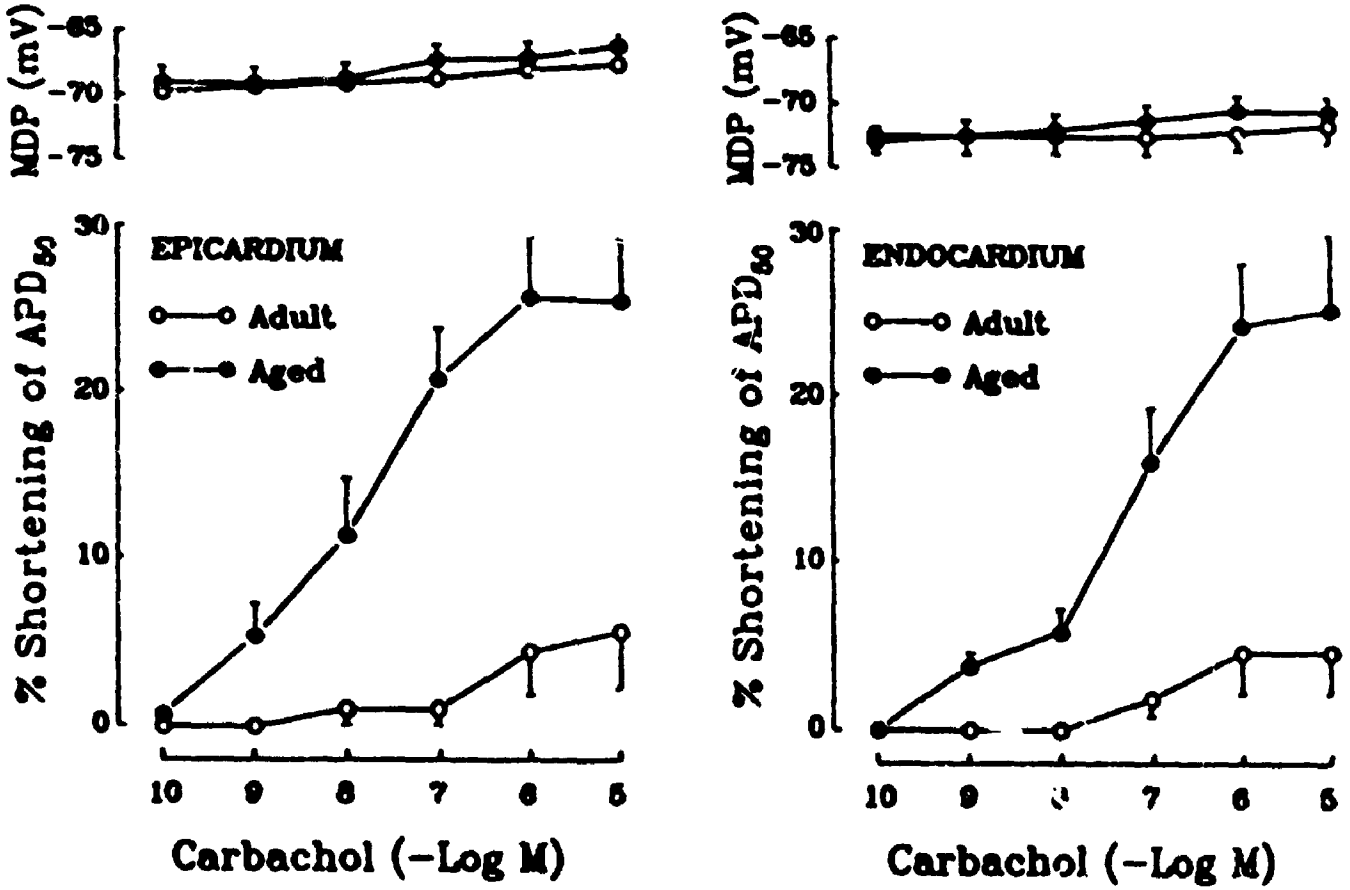

Fig. 3-10 Comparison of the effects of carbachol on MDP and action potential duration measured at 50% of repolarization (APD_{50}) in ventricular epicardium (left panel) and endocardium (right panel) from adult and aged rats. Results represent mean ± SEM of experiments using 6 adult and 6 aged rats. Carbachol had no significant effect on MDP in epicardium or endocardium. The age-related difference in carbachol-induced shortening of APD_{50} is statistically significant ($P < 0.05$) in epicardium and endocardium. The baseline (control) values for MDP and APD_{50} prior to carbachol superfusion are given in Table 3-2.

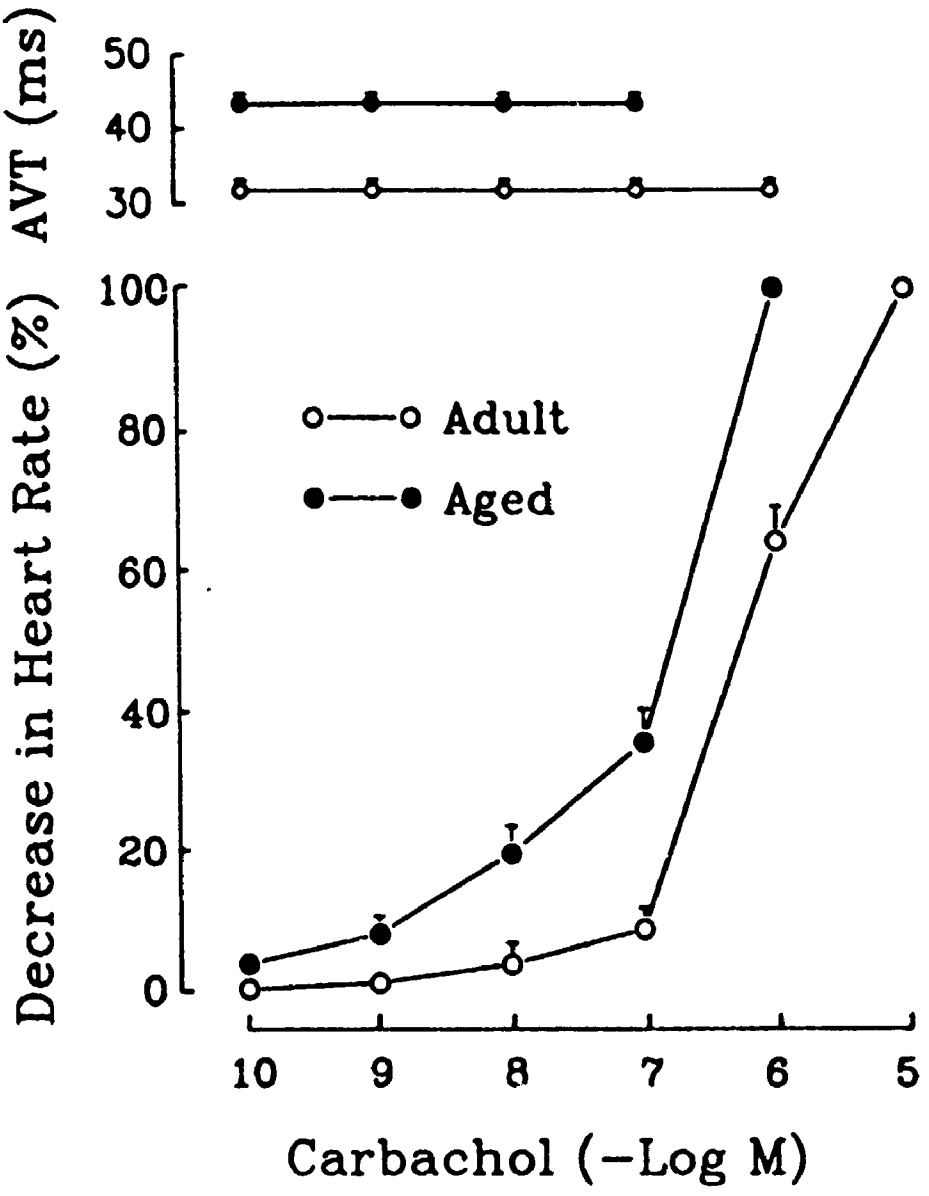

Fig. 3-11 Comparison of the effects of carbachol on heart rate (upper panel) and atrioventricular conduction time (AVT, lower panel) in isolated perfused spontaneously beating hearts from adult and aged rats. Results represent mean ± SEM of experiments using 3 adult and 3 aged heart preparations. Note that carbachol had no significant effect on AVT in these preparations. The age-related differences in heart rate response to carbachol are statistically significant ($P < 0.05$). The baseline (control) values for AVT and heart rate are given in the text.

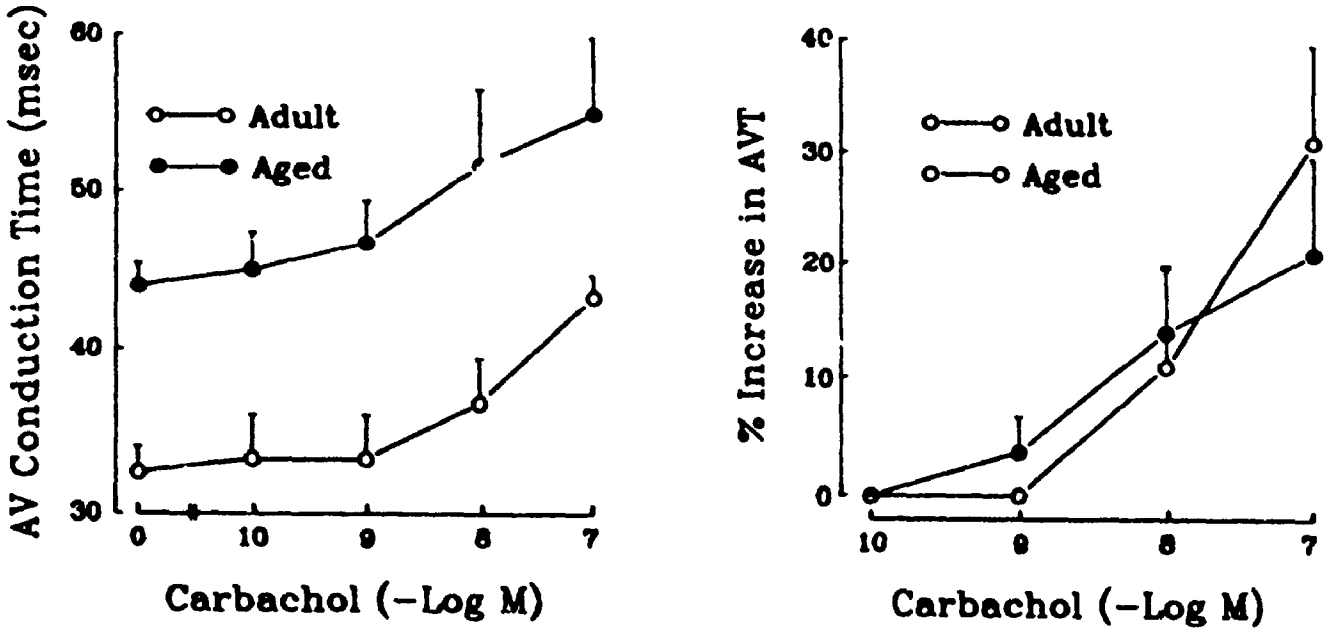

Fig. 3-12 Comparison of the effect of carbachol on atrioventricular conduction time (AVT) in isolated perfused atrial-paced (240 beats per min) hearts from adult and aged rats. Results represent mean ± SEM of experiments using 3 adult and 3 aged heart preparations. No statistically significant age-related difference was evident in the carbachol-induced increase in AVT. When hearts were perfused with 1 μM carbachol, second-degree AV block occurred in two of three adult hearts, and third-degree AV block occurred in two of three aged hearts.

3.4 Discussion

The muscarinic cholinergic signal transduction system has three basic components: the receptors, G proteins, and the effectors. In this chapter, age-related changes in these three components were examined. It is possible that enhanced cholinergic responses of the heart in aging may be associated with age-related alterations in the number of muscarinic receptors and/or their functional properties. The present study showed that no age-related difference was seen in receptor number and antagonist binding affinity in atria or ventricles (Fig. 3-1). However, a selective enhancement in the affinity of MAChR for its agonist carbachol was observed in aged atria but not ventricles (Fig. 3-2). A previous study using Sprague Dawley rats did not indicate age-related differences in agonist or antagonist binding affinity of muscarinic receptors in atria or ventricles although a modest increase ($<30\%$), in atrial but not ventricular receptor density was observed (Narayanan and Derby 1983). Since they used Sprague Dawley rats and we used Fischer 344 rats in the present study, different strains of rats used may explain the slightly different data. However, it appears that age-related alterations in muscarinic receptor linked cellular processes (e.g. Gi-Go protein, activation of potassium channels, inhibition of Ca^{2+} influx), rather than receptor density may underlie the exaggerated cholinergic responses of the aging heart. It is worth noting here that previous studies have also documented lack of age-related changes in β-adrenoceptor density in rat myocardium. Age-related alterations in functional properties of the receptors and/or post receptor mechanisms are thought to underlie the diminished β-adrenergic responses of the heart in aging (Lakatta 1985; Narayanan and Derby 1982; Scarpace 1986).

Binding of GTP to carbachol-MAChR-Gi protein complex decreases the affinity of the MAChR for carbachol (Baker et al, 1985). The GTP or Gpp(NH)p induced decrease in the receptor binding affinity for its agonist can be used as a parameter to determine the efficacy of the signal transduction (Baker et al, 1985). Current evidence suggests that there is no age-related difference in the ability of Gpp(NH)p to influence carbachol competition for MAChR binding sites. Thus, it appears that the efficacy of the signal transduction through the MAChR linked Gi protein is not changed with aging.

Another experimental approach used to study signal transduction involved determination of the expression of Giα subunits in atrial and ventricular membranes of adult and aged rats. The results demonstrated a significant increase in the content of Giα of aged atrial and ventricular membranes using both immunoblot and ADP-ribosylation techniques. This change may be partially responsible for the age-related enhancement in the negative chronotropic and inotropic responses of the heart to cholinergic stimulation. A recent study in Fischer 344 rats aged 6 and 24 months showed by Western blotting that the amount of ventricular Gsα and Giα was unchanged with age (Shu and Scarpace, 1994). However, their study did not examine the effect of aging on atrial Giα protein. Since they used crude membrane preparation (27,000 g pellets) and partially purified membranes, while we used total particulate fractions of the atria and ventricles, different ways of purifying the membrane may explain the different results. Since it has been suggested that the Fischer 344 rats has a mortality rate 50% by 24 months, 95% by 30 months (Masoro 1993), the different population of the aged rats used in experiments may explain the different results as well. It is worth noting that previous studies have documented increased amount of Giα protein in hypertensive rats (Anand-Srivastava, 1992), human and hamster failure heart which, like the aging heart, are hypertrophic heart model. The increased amount of Giα protein was accompanied by increased potency for the negative inotropic effect of carbachol (Bristow and Feldman, 1992; Urasawa et al, 1992; Eschenhagen et al, 1992). In our study, the increased amount of Giα proteins in the the aged atria and ventricles may contribute to the age-related enhancement in the negative chronotropic and inotropic responses to carbachol.

The results from the electrophysiological studies seem to provide insights into the potential mechanisms underlying the cholinergic hypersensitivity of the aging heart at the postreceptor-G protein level. Our results show that, in the absence of extrinsic stimulus (i.e. in the absence of carbachol), myocardial aging is associated with (a) partial depolarization of maximum diastolic potential (MDP) in the atrium but not the ventricle, (b) increase in AVT and (c) prolongation of APD_{50}. The age-associated prolongation of ventricular APD in the absence of age-related change in MDP, confirms to previous findingf using rat cadiac muscle preparations inclusing right ventricle (Wey et al, 1984),

left ventricle (Capasso et al, 1983), papillary muscles and left ventricular myocytes (Walker and Houser, 1990). We are not aware of any report on the effect of aging on the MDP in atria, but an age-associated increase in AVT has been observed in humans as well as in animals (Bachman et al, 1981; Dubell et al, 1991).

The mechanisms underlying these age-related electrophysiological alterations are not well understood at present. Presumably, significant age-related alterations occur in ionic homeostasis of the myocardial fibers with aging contributing to the changes in electrophysiological properties. Recent studies suggested that decrement in the rate of inactivation of the Ca^{2+} current (Walker and Houser, 1990) and the magnitude of outward directed K^+ current (I_{to}) (Walker et al, 1991) contribute to the prolonged APD in the ventricles. Cytosolic Ca^{2+} transient has been shown to modulate action potential (Dubell et al, 1991) and it is prolonged with aging in the rat heart (Orchard and Lakatta, 1985). Therefore, the observed prolongation of APD with aging may also related to prolongated Ca^{2+} transient. The partial depolarization of atrial resting membrane potential may be associated with the modified ionic homeostasis of the myocardial fibers. For example, the activity of the Na^+-K^+-ATPase has been reported to decrease with aging (Frolkis et al, 1984; Ruth et al, 1991). Reduction in the activity of Na^+-K^+-ATPase may result in elevation of intracellular Na^+ contributing to the age-associated depolarization of MDP in atrium. The underlying mechanisms for the prolonged AVT are not well understood at present. Since AV node depolarization is due primarily to a slow inward calcium current (Katz, 1992), prolongation of AVT may be caused by depressed conduction of the calcium-dependent impulses generated in the AV node.

The major electrophysiological responses to muscarinic receptor stimulation by carbachol observed in the present study included: (i) hyperpolarization of MDP (in atria); (ii) shortening of APD (in atria and ventricles); and (iii) increase in AVT. Such muscarinic receptor-dependent effects of acetylcholine and carbachol have been reported previously (Endoh et al, 1985; Loffelholz and Pappano, 1985; Difrancesco et al, 1989; Nonia and Trautwein, 1978; Ten Eick et al, 1976; Ochi, 1981; Hino and Ochi, 1980; Litovsky and Antzelevitch, 1990). The most striking novel observation made in the present study is that certain of these electrophysiological responses to muscarinic receptor

stimulation are selectively and significantly modified with aging. Thus, our results demonstrate that aging is accompanied by (i) enhancement in carbachol-induced hyperpolarization of the MDP in atria, (ii) more pronounced carbachol-induced shortening of APD (particularly APD_{50}) in the ventricle, and (iii) unaltered negative dromotropic response to carbachol (as shown by essentially similar magnitude of carbachol-induced increase in AVT of adult and aged heart). The age-associated enhancement in carbachol-induced hyperpolarization in atria may be due to a greater increase in I_{KACh} in the aged atria since the key mechanism for hyperpolarization of MDP is the activation of I_{KACh} through stimulation of muscarinic cholinergic receptors in the atria (Giles and Noble, 1976; Noma and Trautwein, 1978; Pfaffinger et al, 1985; Loffelholz and Pappano, 1985; Noma, 1987; DiFrancesco et al, 1989). The above action of muscarinic agonists results in depression of excitability and pace maker activity at the sinoatrial node. Therefore, the greater bradycardic response of the aged compared to adult heart to carbachol appears to be linked to an age-associated difference in muscarinic modulation of I_{KACh}.

The present observation that aging is associated with a more pronounced effect of carbachol on the shortening of APD_{50} in the ventricle (epicardium and endocardium), has mechanistic relevance to the age-associated enhancement in the negative inotropic response of the heart to carbachol reported in chapter 2. The ionic basis of the shortening of APD_{50} by muscarinic agonists likely involves a reduction in L-type calcium currents ($I_{Ca(L)}$) (Giles and Noble, 1976; Ten Eick et al, 1976; Hino and Ochi, 1980; Noma, 1987), and perhaps, activation of I_{KACh}, as well (Ochi, 1981). Reduction of $I_{Ca(L)}$ and /or activation of I_{KACh} will shorten APD_{50} thus shortening the plateau phase and diminishing Ca^{2+} influx during the action potential. This effect has been suggested to be responsible for the negative inotropic response of the heart to muscarinic cholinergic stimulation (Giles and Noble, 1976; Ten Eick et al, 1976; Hino and Ochi, 1980; Ochi, 1981, Noma, 1987). Thus, the age-related enhancement in carbachol-induced shortening of APD_{50} may have resulted from a more potent inhibition of $I_{Ca(L)}$ in the aged compared to the adult ventricle, and this would contribute to the enhanced negative inotropic response of the aged heart to carbachol. Since carbachol did not appreciably alter the MDP in ventricular

tissues (epicardium and endocardium), from adult and aged rats, it is unlikely that carbachol caused significant activation of I_{KACh} in these tissues. However, the precise ionic mechanisms for the age-related alteration in carbachol-induced hyperpolarization and shortening of APD_{50} need to be further investigated using voltage or patch clamp techniques.

It is noteworthy that in the present study, the effect of carbachol in shortening of APD_{50} did not differ appreciably between epicardium and endocardium in adult and aged rats. However, it has been reported recently that acetylcholine caused significant shortening of APD_{50} in canine ventricular epicardium but had little or no effect on APD_{50} in canine ventricular endocardium (Litovsky and Antzelevitch, 1990). It has been suggested that the difference in electrophysiological characteristics of epicardium and endocardium is due to the presence of a prominent transient outward current (I_{to}), in the epicardium but relatively weak in the endocardium (Litovsky and Antzelevitch, 1990; Bohm et al, 1981). I_{to} is believed to be carried predominantly by K^+ ions and shows voltage-dependent activation, inactivation and reactivation. By exerting an effect on $I_{Ca(L)}$ and I_{to} in epicardium, and $I_{Ca(L)}$ alone in the endocardium, muscarinic agonists may produce differential electrophysiological changes in these two areas of the ventricle (Litovsky and Antzelevitch, 1990; Bohm et al, 1989). Species-related differences may account for the differential effect of carbachol on ventricular epicardium and endocardium in rat and canine.

In agreement with our finding described in chapter 2, carbachol elicited concentration-dependent bradycardia in spontaneously beating rat heart, and the aged heart was strikingly more sensitive to this action of carbachol. Interestingly, the carbachol-induced bradycardia in the spontaneously beating adult or aged hearts was not accompanied by any noticeable change in AVT. In contrast, in atrial paced hearts beating at a rate (240 beats per min), carbachol caused pronounced concentration-dependent increase in AVT. The increased AVT due to carbachol (0.1 nM-0.1 μM) was not accompanied by either II or III degree AV block in both adult and aged hearts. Since the AVT measured in the atria-paced heart possibly prologed the time spent in the atrial cell to cell conduction as well, the age-related difference in the atrial cell to cell conduction

can not excluded from this study. However, the above findings show that in rats, the muscarinic inhibition of AV nodal conduction is heart rate-dependent, and is manifested only when muscarinic inhibition of the intrinsic pace maker activity is overridden by electrical pacing. It is noteworthy that in another recent study, adenosine-induced bradycardia in rats was also found to occur in the absence of significant prolongation of AVT in the spontaneously beating heart (Froldi and Belaedinelli, 1990). They suggested that slowing in AVT may have a higher threshold than slowing atrial rate in rats, which may well explain our results. Since (a) II or III degree AV block did not occur in the carbachol-induced increase in AVT in atrial paced hearts, and (b) the bradycardic response to carbachol in spontaneously beating hearts occurred in the absence of a significant change in AVT, it can be concluded that the exaggerated cholinergic-triggered bradycardia in the aged heart stems largely, if not exclusively, from greater muscarinic inhibition of the pacemaker activity at the sinoatrial node rather than from greater slowing of AV nodal conduction.

Whether or not observations made in the aged rat model is applicable to humans is uncertain, these findings may have potential significance. The age-related prolongation of AVT itself has little effect on the pumping action of the heart (Katz, 1992), but it may contribute to the development of the heart block (Frolkis et al, 1984). It will be extremely dangerous in the patients with SA nodal dysfunction. Beside, the impairment in AV conduction in old age can promote the development of spontaneous activity in the underlying areas (ventricles) of automatism and initiation of arrhythmias (Frolkis et al, 1984).

SUMMARY

Studies exploring the biochemical and electrophysiological mechanistic basis of the age-related enhancement of MAChR-mediated negative chronotropic and inotropic responses of the heart suggested the following: (1) Specific [^{3}H]QNB binding in total particulate fractions of atria and ventricles indicated no significant age-related differences in the number of muscarinic receptor sites or their antagonist binding affinity. (2) Carbachol caused concentration-dependent inhibition of the [^{3}H]QNB binding to receptor

sites; the receptor binding affinity to its agonist is greater in the atria but not ventricles of aged rat compared to adult. (3) The magnitude of Gpp(NH)p-induced shift in receptor affinity for agonist is similar in adult and aged atria or ventricle. (4) The amount of Giα protein measured by Western blotting and ^{32}P-ADP-ribosylation was significant greater in the atria and ventricles of aged rat compared to adult. (5) Carbachol (10^{-10} - 10^{-5}M) caused greater hyperpolarization of the MDP in the atria but not ventricles of aged rat compared to adult. (6) Carbachol (10^{-10} - 10^{-5}M) induced shortening of APD_{50} was strikingly more pronounced in the aged in comparison with adult ventricular epicardium and endocardium. (7) There is no age-related difference in carbachol-induced prolongation of AVT. The carbachol-induced increase in AVT did not accompany any II or III degree AV block in both adult and aged hearts.

Conclusions: (a) The increased negative chronotropic response of the aged heart to cholinergic stimulus is likely associated with age-related decrease in acetylcholinesterase activity in the atria, age-related increase in the affinity of MAChR for its agonist and in the Giα content of the atria, and enhancement in the magnitude of MAChR-mediated maximal diastolic hyperpolarization likely in the sinoatrial node. (b) The increased negative inotropic response of the aging heart to cholinergic stimulus may be due to an age-related decrease in acetylcholinesterase activity in the ventricles, increase in the Giα content and enhancement in the magnitude of MAChR-mediated abbreviation of action potential duration in the ventricular myocytes which limits transsarcolemmal Ca^{2+} influx.

CHAPTER 4

ENHANCED CHOLINERGIC RESPONSE OF THE CORONARY VASCULATURE IN AGING RAT

4.1 Introduction

The studies described in preceding chapters 2 and 3, have demonstrated greatly enhanced negative chronotropic and inotropic responses of rat myocardium to cholinergic agonists with aging. Furthermore, these studies have provided evidence suggesting that certain age-associated alterations occurring at the level of cardiocytes [such as age-related decreased acetylcholinesterase activity in the atria and ventricles, increased binding affinity of muscarinic receptor to its agonist in the atria, greater amount of Gi protein in the atria and ventricles, enhanced MAChR-mediated maximal diastolic hyperpolarization in the sinoatrial node, and exaggerated MAChR-mediated abbreviation of action potential duration in the ventricular myocytes (which limits transsarcolemmal calcium influx)], can contribute to the enhanced cholinergic responses of the aging heart. In addition to age-induced changes at the level of cardiomyocytes, age-associated alterations in coronary blood flow and vasoregulatory responses of the coronary vasculature to autonomic stimuli may also influence autonomic modulation of cardiac rhythm and contractile performance. In the rat heart, an age-related change in coronary vascular function has been suggested by structural studies showing decreased capillary density due to inadequate capillary growth in the face of excessive hypertrophic enlargement of the myocyte compartment of the ventricle, and subendocardial ischemia under stressful conditions (Anserva et al, 1986; Guideri et al, 1987; Tomanek, 1970; Weisfeldt et al, 1971). While *in vitro* measurements did not show age-related changes in resting coronary blood flow (Weisfeilt et al, 1971; Friberg et al, 1985), recent evidence from *in vivo* measurements has revealed decrements in coronary blood flow as well as coronary vascular reserve and increments in coronary vascular resistance in aging rats (Hachamovitch et al, 1989). Whether or not age-related changes in autonomic tone in the coronary vasculature contribute to

alterations in coronary vascular function parameters measured *in vivo* remains unclear.

In view of this, the present study was undertaken to assess potential age-related alterations in coronary vascular response to cholinergic stimulation and the underlying mechanisms. The impact of aging on the responsiveness of the coronary vasculature to α adrenergic stimulation was also assessed in parallel studies.

4.2 Materials and methods

4.2.1 Animals

Strain and source of adult (6-8 month-old), and aged (26-30 month-old), rats and conditions of maintenance were the same as described in chapter 2.

4.2.2 Chemicals

Phenylephrine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO. Phentolamine mesylate was from CIBA Pharmaceuticals, Mississauga, Ontario and N^G-monomethyl-L-arginine (L-NMMA), was from Calbiochem, San Diego, CA. All other chamicals were obtained from the same sources as listed in chapter 2. All other chemicals were from Sigma or BDH Chemicals, Toronto. All pharmacological agents were dissolved in Krebs-Henseleit buffer (see below), on the day of the experiment.

4.2.3 Measurement of coronary perfusion pressure, heart rate and contractility

Isometric contractions were recorded, heart rate and contractile force were measured using the Langendorff preparation as described in chapter 2. Coronary perfusion pressure was monitored by means of a pressure transducer (COBE CDX3) attached to the side arm of the aortic cannula. Since the hearts were perfused at a constant flow rate (9 ml/g ventricular weight), changes in perfusion pressure were regarded as reflecting alterations in coronary vascular resistance. As the ventricular mass was about 23% higher in the aged heart compared with that of the adult heart (0.78 $\pm$ 0.05 g for adult; n=13 and 0.96 $\pm$ 0.06 g for aged, n=13), the actual flow rate is 7.0

± 0.5 ml/min/g for adult and 8.6 ± 0.5 ml/min/g for aged heart. Experiments were performed using spontaneously beating heart preparations as well as electrically paced hearts. Prior to electrical pacing, both atria were removed and interatrial septa crushed. The ventricular myocardium was then paced at 250 beats per min with a Grass SD9 stimulator via a platinum wire electrode inserted into the epicardium, at double threshold voltage and a duration of 5 ms. The pacing rate used was slightly higher than the spontnaeous heart rate *in vitro* for both adult and aged rats. In some experiments coronary perfusion pressure was also monitored in potassium-arrested hearts. For this, the concentration of KCl in the perfusion medium was increased to 18 mM while proportionately decreasing the concentration of NaCl.

4.2.4 Presentation of results and statistical analysis

All data have been standardized to depict percentage changes from predrug (base-line) values. The results are expressed as mean ± S.E. of mean. Dose response curves were analyzed using a nonlinear curve fitting technique as described by DeLean et al (1978), and where appropriate, EC_{50} values were derived using the Graph PAD program (Graph PAD Software Version 2.0, available from Graph PAD, San Diego, CA). The EC_{50} data from individual rats were averaged separately for the adult and aged groups and expressed as mean ± SE of mean. Statistical comparisons between adult and aged rats were performed by two tailed Student's *t* test for unpaired values with the level of significance set at $P<0.05$.

4.3 Results

4.3.1 Baseline coronary perfusion pressure

Usually, both adult and aged hearts stabilized about 15 min after the start of perfusion. Baseline heart rate and contractile force were similar as described in chapter 2. No age-related difference was observed in the base-line coronary perfusion pressure in these constant flow-perfused heart preparations (adult 51 ± 3 mmHg, n=7; aged 49 ± 3 mmHg, n = 7).

4.3.2 Effects of carbachol on coronary perfusion pressure and contractile force

Fig. 4-1 shows the results of a typical experiment demonstrating the effects of selected concentrations of carbachol, a cholinesterase-resistant cholinergic agonist, on coronary perfusion pressure and contractility in constant flow-perfused hearts from adult and aged rats paced at 250 beats/min. In both adult and aged hearts, carbachol caused a concentration-dependent increase in coronary perfusion pressure; this effect of carbachol was strikingly more pronounced in aged hearts. Consistent with our previous findings (chapter 2), under these experimental conditions, carbachol also induced a relatively greater negative inotropic effect on the aged compared to adult heart. Composite data from several experiments depicting changes in coronary perfusion pressure as a function of carbachol concentration are presented in Fig. 4-2, left panel. At all concentrations tested, the carbachol-induced increase in coronary perfusion pressure was over two-fold greater in the aged heart compared to adult heart (carbachol concentration required for 50% increase in coronary perfusion pressure: adult 916 $\pm$ 210 nM; aged 21 $\pm$ 7 nM; $P<0.01$). Since the hearts were perfused in the constant flow mode, changes in coronary perfusion pressure reflect changes in coronary vascular resistance. Thus, these results demonstrate a striking enhancement in carbachol-induced coronary vasoconstriction in the aged compared to adult heart. While the data presented in Fig. 4-2, left panel were obtained with hearts paced electrically at 250 beats/min, essentially similar findings were also obtained in experiments with spontaneously beating hearts as well (Fig. 4-2, right panel).

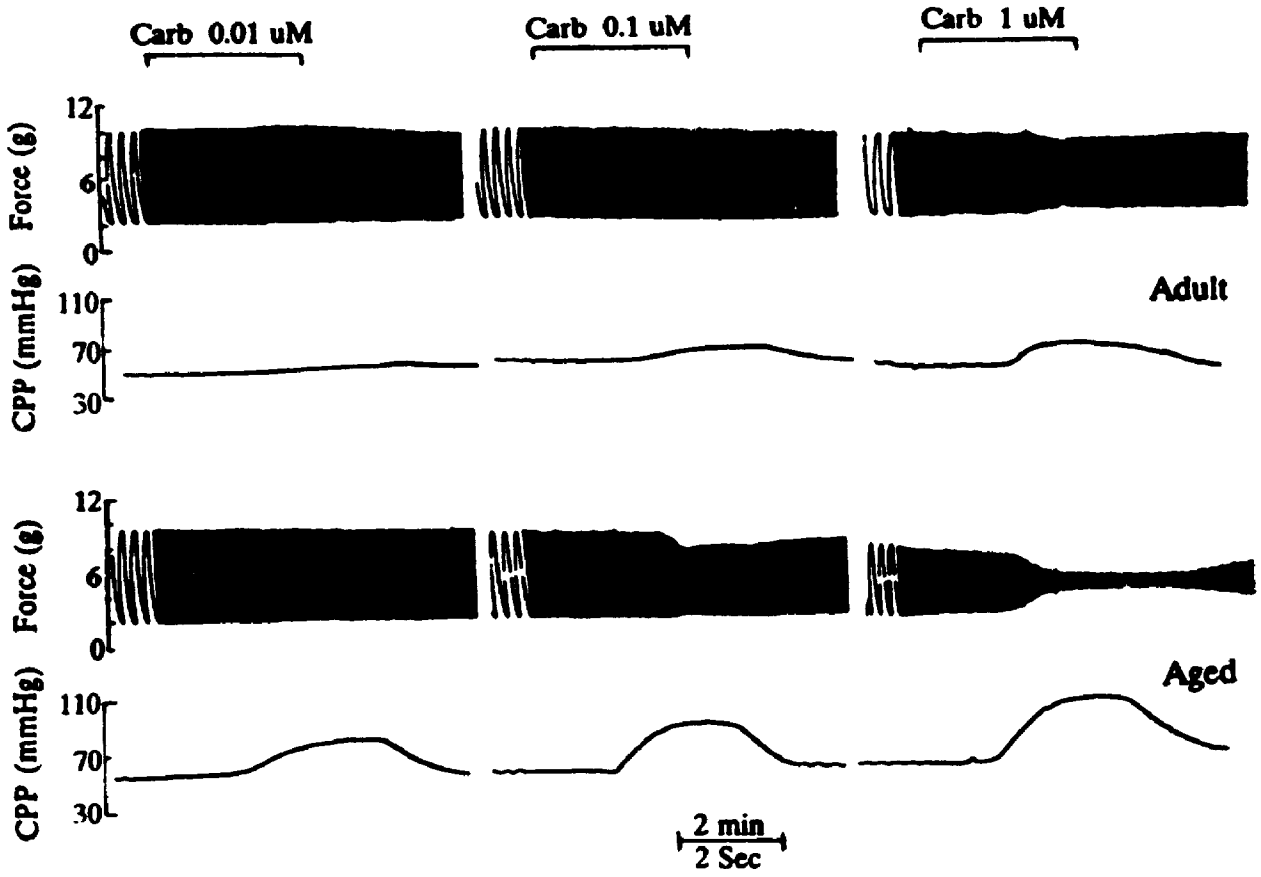

Fig. 4-1 Experimental records showing changes in coronary perfusion pressure (CPP) and contractile force in isolated, constant flow-perfused, electrically paced (250 beats/min) hearts from adult and aged rats following infusion of different concentrations of carbachol. The period of carbachol (Carb) infusion is shown on top and the horizontal time bar indicates interval at either fast or slow recorder speed. See "Methods" for experimental details.

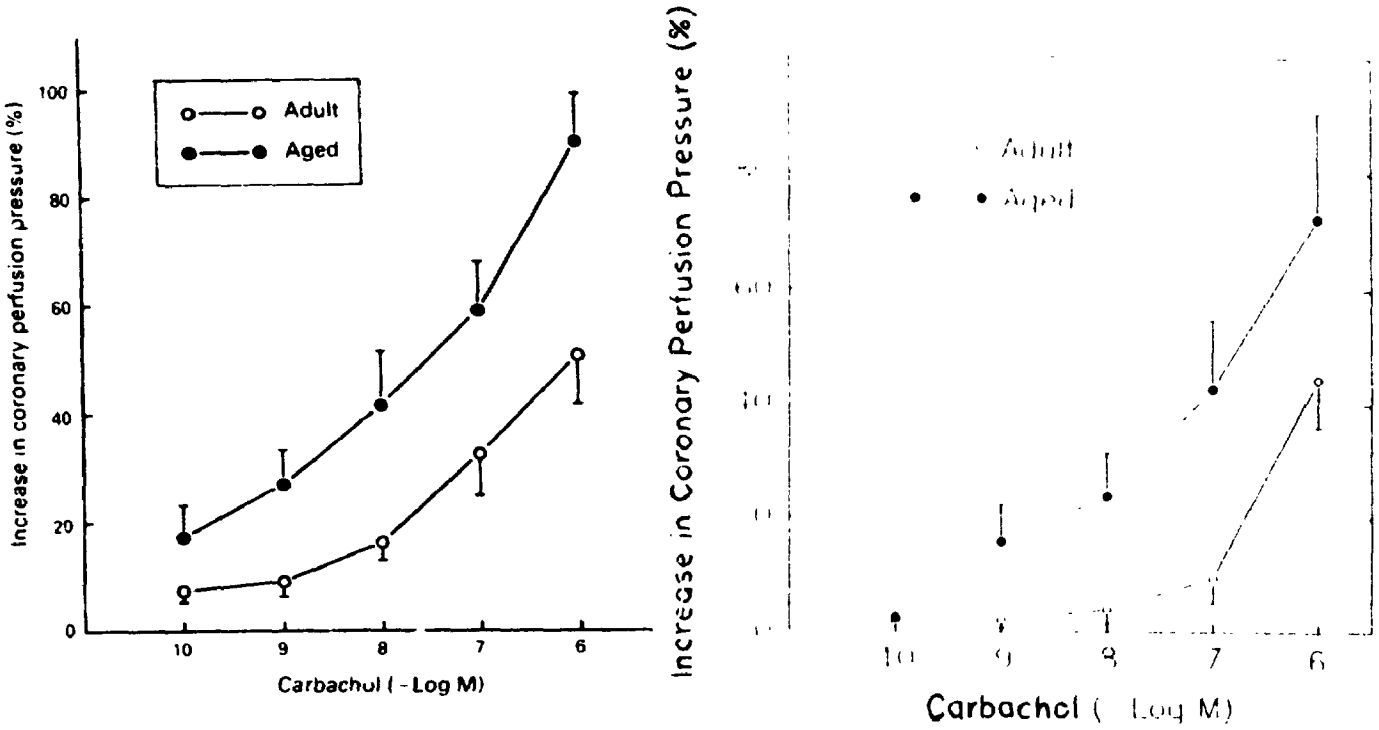

Fig. 4-2 Comparison of the effects of varying concentrations of carbachol on coronary perfusion pressure in isolated, constant flow-perfused, electrically paced (250 beats/min) (left panel) and spontaneously beating hearts (right panel) from adult and aged rats. In left panel, each point represents mean $\pm$ SE (n = 7 for adult and aged). In the right panel, the number for adult heart is 5 and that for aged heart is 4. See "Methods" for experimental details.

4.3.3 Effect of carbachol on coronary perfusion pressure in KCl-arrested hearts

When isolated hearts were perfused with buffer containing 18 mM KCl, instead of 4.7 mM KCl, they ceased to beat in 1 min and there was a moderate (20-30% above base-line) increase in coronary perfusion pressure for 2-3 min; the pressure then fell back to the base-line value where it remained stable. No age-related difference was seen in this transient increase in coronary perfusion pressure induced by 18 mM KCl. Fig. 4-3 compares the effects of varying concentrations (10^{-10} - 10^{-4}M) of carbachol on coronary perfusion pressure in KCl-arrested, constant flow-perfused hearts from adult and aged rats. Carbachol caused concentration-dependent increases in coronary perfusion pressure in these preparations; at each concentration of carbachol, the pressor response was nearly 2-fold greater in the aged compared to adult heart (the carbachol concentration required for 50% increase in coronary perfusion pressure was adult 224 $\pm$ 54 nM; aged 8 $\pm$ 0.7 nM; $P < 0.01$; Fig. 4-3, left panel). However, after transfering the data to percentage of maximum, the EC_{50} values were 217 $\pm$ 52 nM for adult and 144 $\pm$ 65 nM for aged hearts. There was no age-related difference in the EC_{50} values ($p > 0.05$ between adult and aged hearts; Fig. 4-3, right panel). The results from KCl-arrested hearts confirm the observations made using beating heart preparations (Fig. 4-1 and 4-2), and further show that the direct vasoconstrictor action of carbachol on the coronary vasculature increases in the aged compared to adult hearts.

4.3.4 Inhibition of carbachol-induced coronary vasoconstriction by atropine but not AFDX-116

The effects of muscarinic cholinergic receptor antagonists on carbachol-induced changes in coronary perfusion pressure were examined in spontaneously beating, constant flow-perfused hearts from adult and aged rats. As shown in the left panel of Fig. 4-4, the non-subtype selective muscarinic receptor antagonist, atropine, inhibited the carbachol (0.1 μM)-induced increase in coronary perfusion pressure in a concentration-dependent

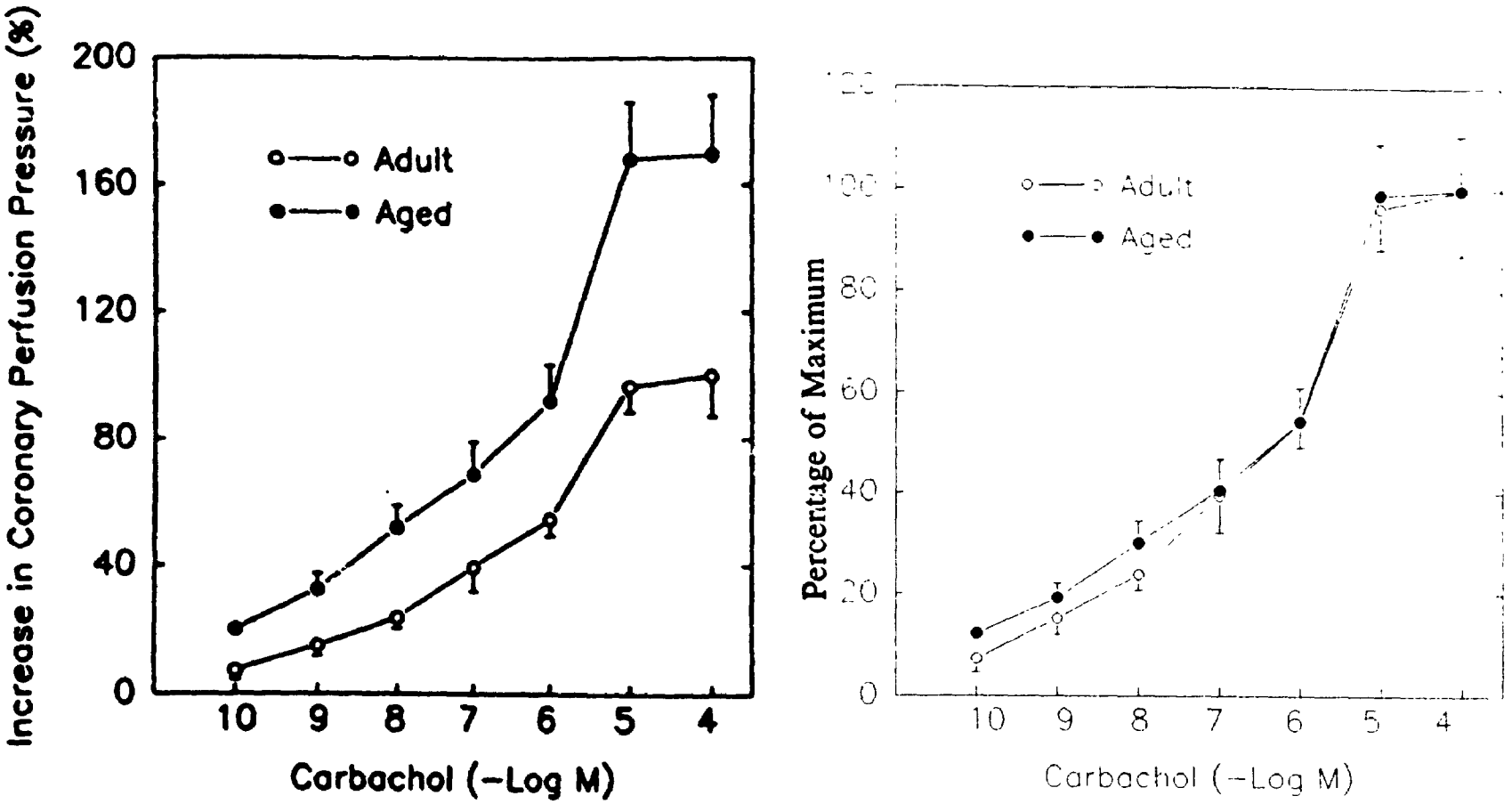

Fig. 4-3 Comparison of the effects of varying concentrations of carbachol on coronary perfusion pressure in isolated, KCl-arrested, constant flow-perfused hearts from adult and aged rats (Left panel). Each point represents mean ± SE (n = 5 for adult; n = 4 for aged). Right panel is showing the same data after transfered to percentage of maximum. See "Methods" for experimental details.

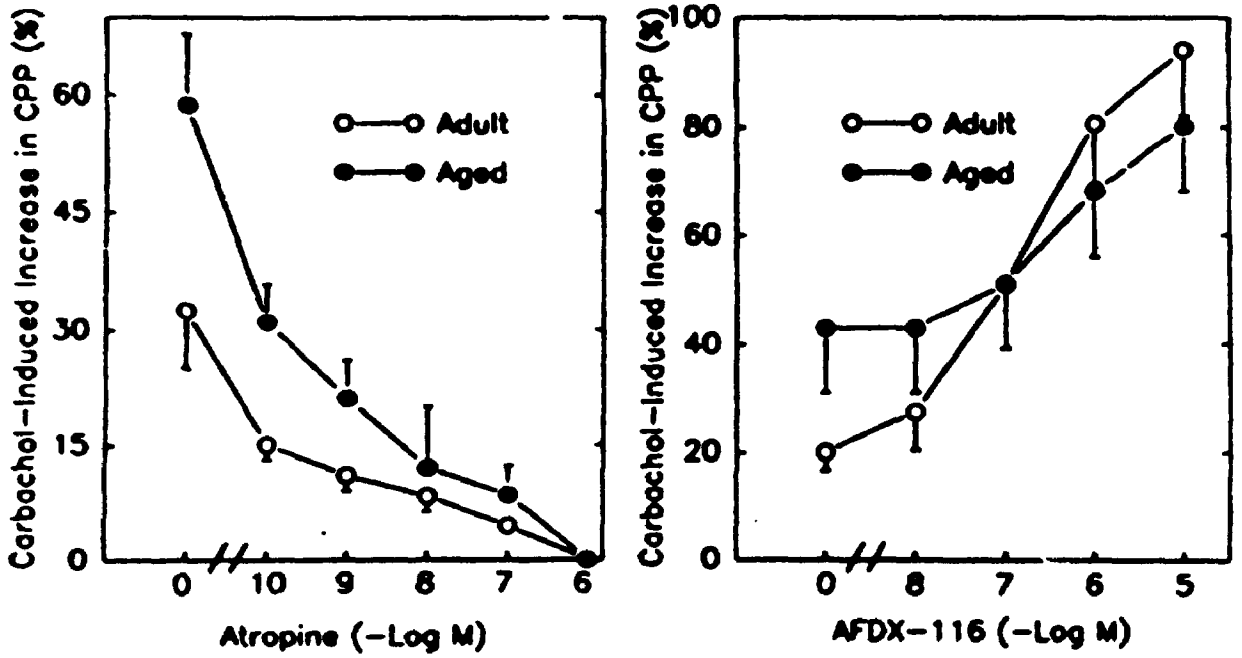

Fig. 4-4 Effects of varying concentrations of atropine (left panel) or AFDX-116 (right panel) on carbachol (0.1 μM)-induced increase in coronary perfusion pressure (CPP) in isolated, constant flow-perfused, spontaneously beating hearts from adult and aged rats. Each point represents mean $\pm$ SE (left panel: n = 6 for adult and aged; right panel : n = 4 for adult and aged). See "Methods" for experimental details.

manner. No statistically significant age-related difference was evident in the ability of atropine to antagonize the coronary vascular pressor response to carbachol (EC_{50} values for atropine: adult 0.27 $\pm$ 0.03 nM; aged 0.34 $\pm$ 0.03 nM). In contrast to the effect of atropine, the cardioselective muscarinic receptor antagonist AFDX-116 failed to inhibit carbachol (0.1 μM)-induced increase in coronary perfusion pressure; in fact, AFDX-116 potentiated the coronary vascular pressor response to carbachol in adult and aged rats (Fig. 4-4, right panel). As shown previously, the negative chronotropic and inotropic responses elicited by carbachol were antagonized by both atropine and AFDX-116 (chapter 2). These findings demonstrate that the pressor response to carbachol is mediated by vascular muscarinic receptors (M_3 subtype), distinct from muscarinic receptors of cardiomyocytes (M_2 subtype), mediating the chronotropic and inotropic response to carbachol.

4.3.5 Effect of verapamil on carbachol-induced coronary vasoconstriction in KCl-arrested hearts

The extracellular Ca^{2+}-dependence of the coronary vascular response to carbachol was investigated by determining the effect of verapamil (a Ca^{2+} channel antagonist), on carbachol-induced changes in coronary perfusion pressure in constant flow-perfused, KCl-arrested hearts. The results are summarized in Fig. 4-5. Administration of 2.5 μM verapamil (Wolfe et al, 1991) to hearts arrested with KCl caused only a slight, transient decrease in coronary perfusion pressure. However, infusion of verapamil at this maximal effective concentration, markedly attenuated the pressor response induced by 1 μM carbachol in both adult and aged hearts (60 and 75% inhibition of pressor response, respectively, in adult and aged hearts).

4.3.6 Effect of L-NMMA on the coronary vascular response to carbachol

The present study using rats, and several recent studies using rats and other species, have shown coronary vasoconstriction in response to cholinergic stimulation (Kalsner, 1989; Woods et al, 1989; Van Charldorp et al, 1987). On the other hand,

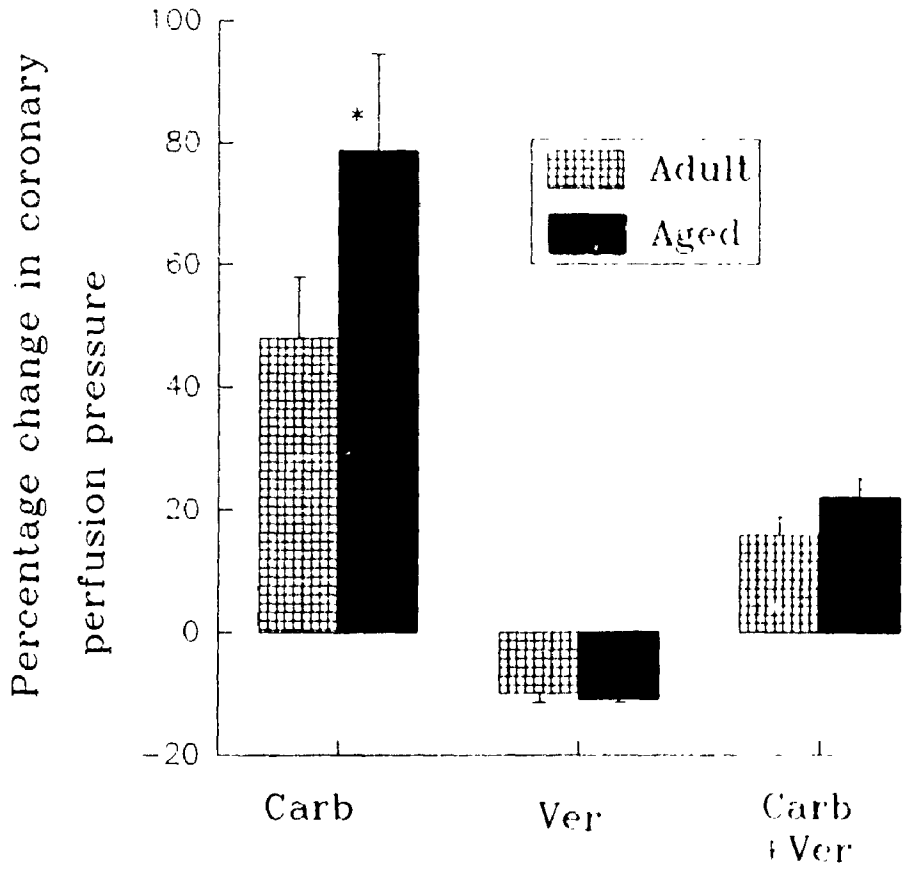

Fig. 4-5 Effect of verapamil on the carbachol-induced increase in coronary perfusion pressure in isolated, constant flow-perfused, KCl-arrested hearts from adult and aged rats. The isolated hearts were perfused with high KCl (18 mM) buffer throughout the experiments. Infusion of carbachol (Carb, 1 μM) or verapamil (Ver, 2.5 μM), separately or in combination was continued for 3 min. Values are mean $\pm$ SEM (n = 6 for adult and aged hearts).

coronary vasodilation due to endothelium-derived relaxing factor [EDRF, which has been identified as nitric oxide (Palmer et al, 1987)], has been reported in other studies (Stewart et al, 1987; Amezcua et al, 1989; Klem and Schrader, 1990). Therefore, it was deemed important to determine whether an age-associated dysfunction of endothelial cells (e.g. decreased rate of synthesis of EDRF), and/or a diminished responsiveness of vascular smooth muscle to EDRF contributed to the enhanced vasoconstrictive action of carbachol on the coronary vasculature in the aged rat heart. Thus, we examined the effects of L-NMMA, an inhibitor of EDRF (nitric oxide) synthesis (Palmer et al, 1988), on the coronary vasculature in adult and aged rat hearts. Further, the effects of carbachol were assessed in the presence of L-NMMA. The results are presented in Fig. 4-6. In the absence of carbachol, L-NMMA (30 and 100 μM; Tschudi et al, 1991), caused concentration-dependent increase in coronary perfusion pressure in constant flow-perfused hearts; no significant age-related difference was evident in this vasoconstrictive action of L-NMMA. On the other hand, in the presences of L-NMMA (30 or 100 μM), carbachol (1 μM) caused a significantly greater increase in coronary perfusion pressure in the aged compared to adult heart; the pressor response to L-NMMA and carbachol was nearly additive in both adult and aged hearts. Taken together, these findings suggest that the enhanced vasoconstrictive response of the coronary vasculature to carbachol cannot be attributed to age-associated decrements in the rate of synthesis of EDRF or diminished responsiveness of the vascular smooth muscle to EDRF.

4.3.7 Alpha adrenergic response of coronary vasculature

The coronary vascular response to the α_1 adrenergic receptor agonist phenylephrine was determined in spontaneously beating, constant flow perfused hearts from adult and aged rats to see if the enhanced coronary vasoconstrictor response in aged rats was specific to cholinergic agonists. As shown in the left panel of Fig. 4-7, phenylephrine caused a concentration dependent increase in coronary perfusion pressure; no age related difference was evident in the pressor response to phenylephrine [estimated

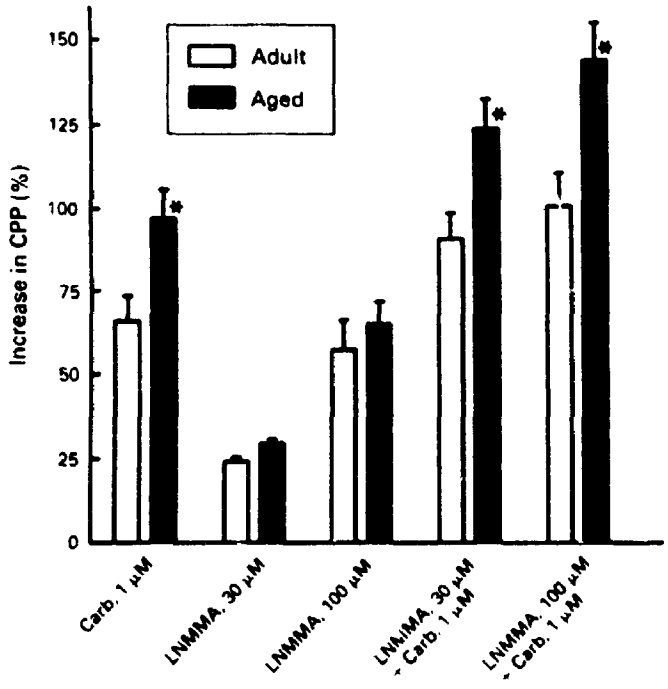

Fig. 4-6 Comparison of the effects of N^G-monomethyl-L-arginine (LNMMA) on coronary perfusion pressure (CPP) in isolated, constant flow-perfused, electrically paced (250 beats/min) hearts from adult and aged rats in the absence and presence of carbachol (Carb). Data represent mean $\pm$ SE; asterisk denotes significant difference between adult and aged ($P<0.05$; $n=4$ for adult and aged). See "Methods" for experimental details.

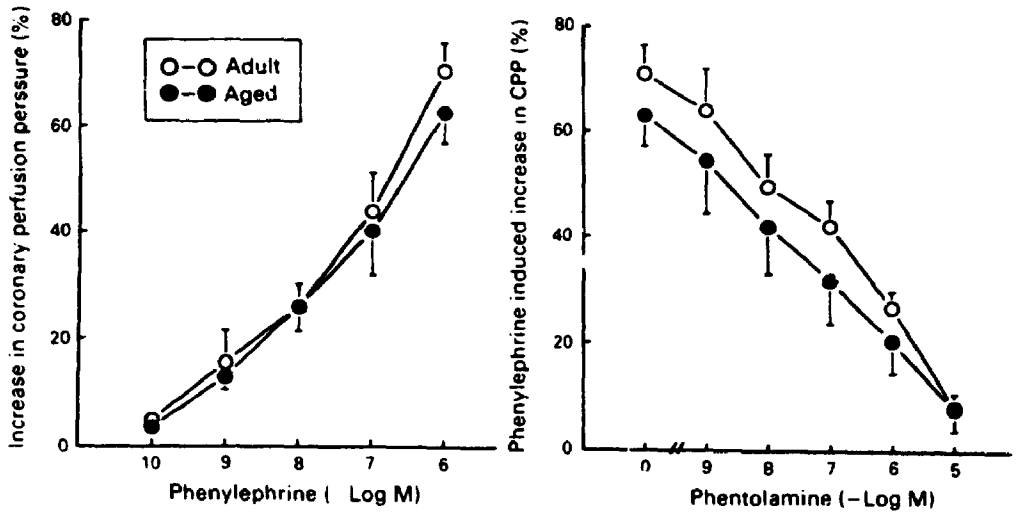

Fig. 4-7 Comparison of phenylephrine induced increase in coronary perfusion pressure and antagonism by phentolamine in isolated, constant flow perfused, spontaneously beating hearts from adult and aged rats. Left panel (n=8 for adult and aged) shows pressor response to varying concentrations of phenylephrine; right panel (n=6 for adult and aged) shows inhibition of the pressor response induced by 1 μM phenylephrine by varying concentrations of phentolamine. Values are means, Bar= SEM.

EC_{50} values were 371 ± 17 nM (n=8) and 405 ± 20 nM (n=8), respectively for adult and aged hearts]. In both adult and aged hearts, the pressor response to phenylephrine (1μM), was blocked by the α-adrenoreceptor antagonist phentolamine [Fig. 4-7, right panel; estimated EC_{50} values for phentolamine were 96 ± 30 nM (n=6) and 54 ± 20 nM (n=6), respectively, for adult and aged hearts].

4.4 Discussion

The results of the present study demonstrate that (a) carbachol, a cholinergic agonist, causes coronary vasoconstriction in the isolated constant flow-perfused rat heart and (b) at each concentration of carbachol, the pressor response was nearly two fold greater in the aged compared to adult hearts. Based mainly on evidence from experiments performed on dogs (Hashimoto et al, 1960; Blesav and Ross, 1970; Glaviano et al, 1977; Gross and Buck, 1981; Cox et al, 1983), it has long been assumed that cholinergic agonists are vasodilators of coronary arteries and that parasympathetic innervation of coronary vessels mediates vasodilation (Feigl, 1983). However, in recent years it has become clear that the dog is an exception and in most species, cholinergic stimulation, in fact, produces coronary vasoconstriction. Thus, isolated coronary arteries from most species including human, sheep, pig, rabbit and rat, contract in response to acetylcholine and other cholinergic agonists (Kalsner, 1989; Doods et al, 1989). Furthermore, in isolated rat heart preparations perfused at constant pressure, electrical stimulation of the vagus nerve leads to a decrease in coronary flow (due to coronary vasoconstriction) suggesting a functional parasympathetic innervation of the coronary arteries in this species (Van Charldorp et al, 1987). Our results showing a pressor response to carbachol in isolated, constant flow-perfused heart provide further confirmation of the vasoconstrictor action of cholinergic agonists in the coronary vasculature. More importantly, to our knowledge, this study provides the first documentation of alteration in vasoregulatory response of the coronary vasculature to cholinergic stimulus in aging. Further, our findings show that perfusion with L-NMMA, an inhibitor of EDRF (nitric oxide) synthesis, results in similar degree of vasoconstriction in adult and aged hearts

while the age-related difference in carbachol-induced vasoconstriction prevails in the presence of L-NMMA. Therefore, it is unlikely that impaired generation of EDRF by coronary endothelial cells or reduced sensitivity of vascular smooth muscle to EDRF contributes to the enhanced coronary vasoconstrictive effect of cholinergic stimulus in the aged rat heart.

In isolated coronary arteries (Kalsner, 1989; Doods et al, 1989; Nuutinen et al, 1985; Van Charldorp and Van Zwieten, 1989; Entzeroth et al, 1990; Duckles and Garcia-Villalon, 1990) and other blood vessels (Kalsner, 1989; Doods et al, 1989; Duckles and Garcia-Villalon, 1990; Kuriyama and Suzuki, 1978; Vanhoutte, 1974), cholinergic agonists induce vasoconstriction by direct activation of vascular muscarinic receptors (M_3 subtype) which are distinct from muscarinic receptors in cardiomyocytes (M_2 subtype). However, it has been suggested that in the intact beating heart, cholinergic coronary vasoconstriction may also result indirectly from decrease in cardiac work owing to activation of the M_2 muscarinic receptors in cardiomyocytes (Nuutinen et al, 1985). The carbachol-induced coronary vasoconstriction in adult and aged hearts observed in this study appears to result mainly, if not exclusively, from the activation of vascular muscarinic receptors as the non-subtype selective muscarinic receptor antagonist atropine, but not the cardioselective muscarinic receptor antagonist AFDX-116, blocked this response. Minimal involvement of carbachol-induced decrease in cardiac metabolism in the coronary vascular response to carbachol is also suggested by the observations that: (a) carbachol-induced coronary vasoconstriction in spontaneously beating and electrically paced hearts was similar to that in potassium-arrested hearts, and (b) despite the greater negative chronotropic and inotropic effects of carbachol on aged compared to adult heart, the age-related difference in coronary vascular response to carbachol could be observed in beating and non-beating heart preparations.

The post receptor mechanisms governing cholinergic coronary vasoconstriction are not yet clearly understood. In earlier studies, vasoconstriction of coronary arteries by acetylcholine was reported to occur with membrane depolarization (Suyama and Kuriyama, 1984), hyperpolarization (Kitamura and Kuriyama, 1979), or constant membrane potential (Ito et al, 1982). This inconsistency of results has been attributed,

in part, to the presence or absence of endothelium in the multicellular tissue and it was shown recently that in isolated coronary smooth muscle cells, acetylcholine induces hyperpolarization through stimulation of Ca^{2+}-activated potassium channels (Ganitkevich and Isenberg, 1990). In any case, muscarinic receptor-mediated increase in intracellular Ca^{2+} is considered essential for the vasoconstrictor action and the hyperpolarizing effect of cholinergic agonists (Nuutinen et al, 1985; Ganitkevich and Isenberg, 1990). Our finding that the maximal dose of Ca^{2+} channel antagonist verapamil markedly attenuates (but does not abolish), the coronary vasoconstriction induced by carbachol (Fig. 4-5) suggests that a large component of the contractile Ca^{2+} mobilized by muscarinic receptor activation is derived via influx of extracellular Ca^{2+}. However, since verapamil (at 2.5 μM), did not abolish the vasoconstrictor response to carbachol (1 μM), it is also likely that muscarinic receptor activation also leads to Ca^{2+} mobilization from intracellular sources. This possibility is supported by the recent observation that in isolated coronary smooth muscle cells, acetylcholine augments Ca^{2+} release from sarcoplasmic reticulum (Ganitkevich and Isenberg, 1990), apparently through activation of muscarinic receptor-linked phospholipase C and production of inositol triphosphate (Berridge and Irvine, 1989). The mechanisms underlying the age-associated enhancement in coronary vasoconstriction in response to muscarinic receptor activation remains to be determined. Nevertheless, it is noteworthy that attenuation of carbachol-induced coronary vasoconstriction by verapamil resulted in abolition of the age-related difference (Fig. 4-5). This observation suggests that relatively greater influx of extracellular Ca^{2+} upon muscarinic receptor activation in the aged compared to adult heart may be a factor contributing to the age-related enhancement in coronary vascular response to cholinergic stimulus.

In the present study no age-related difference was observed in alpha receptor mediated vasoconstriction in coronary vasculature of the rat. Another study has reported enhanced contractile response to alpha receptor stimulation in isolated coronary arteries from aged compared to young beagles (Toda and Miyazaki, 1987). We are not aware of other studies on coronary vascular response to alpha adrenergic stimulation in aging. Studies on other vascular beds have shown no age-related alteration in alpha adrenoceptor

response (Pan et al, 1986; Duckles et al, 1985). On the other hand, diminished vascular smooth muscle relaxation in response to beta adrenergic stimulation has been observed in several vascular beds including the coronary vasculature (Toda and Miyazaki, 1987; Pan et al, 1986; O'Donnel and Wanstall, 1986; Tsujimoto et al, 1986; Fleish and Hooker, 1976; Deisher et al, 1989).

The pathophysiological and clinical implications of the age-associated increase in coronary vascular response to cholinergic stimulus can only be speculated at this time. In the pathophysiological setting, cholinergic hypersensitivity may result in greater coronary vasoconstriction at any level of cholinergic tone, thus compromising coronary blood flow, energy metabolism and contractility in the aging heart. Thus, age-related decrease in coronary blood flow and increase in coronary vascular resistance encountered *in vivo* (Hachamovitch et al, 1989), but not *in vitro* (Weisfeldt et al, 1971; Friberg et al, 1985), can be explained, in part, on the basis of age-associated change in vasoregulatory response to endogenously released acetylcholine. In the clinical setting, cholinergic constriction is recognized to be an underlying factor in the pathogenesis of coronary spasm (Kalsner, 1989; Yasue et al, 1986). Hence, cholinergic hypersensitivity of coronary vasculature in aging may contribute to higher incidence of coronary spasm in the elderly. Although whether the observations made in the aging rat model is applicable to humans is uncertain, these findings suggest need for caution in the therapeutic use of cholinomimetic agents in the elderly. The clinical implication of our finding that AFDX-116 can potentiate cholinergic vasoconstriction is also worth noting. This phenomenon, which has also been observed in another recent study (Bognar et al, 1990), is apparently related to the ability of AFDX-116 to block prejunctional M_2 muscarinic receptors (autoreceptors), in cholinergic nerve endings. Interaction of acetylcholine with the autoreceptors serves to inhibit excessive acetylcholine release and thus helps to moderate postsynaptic M_3 muscarinic receptor mediated vasoconstriction. Blockade of the autoreceptors by AFDX-116 and consequent increase in endogenous acetylcholine release may serve to enhance coronary tone and perhaps even elicit anginal attacks in predisposed individuals.

SUMMARY

In electrically paced hearts, perfused at constant perfusate flow rate, the cholinergic agonist carbachol (10^{-10} - 10^{-6} M) elicited concentration-dependent coronary vasoconstriction. The maximum response was greater in the aged than in the adult heart. A similar age-related difference in coronary vascular response to carbachol was also observed in potassium (18 mM KCl) arrested, constant flow perfused hearts. The carbachol-induced vasoconstriction was mediated by vascular M_3 muscarinic receptors. There was no age-related difference in the coronary vasoconstriction produced by α-adrenergic agonist phenylephrine (10^{-10} - 10^{-6} M). Thus we concluded that there is striking enhancement of coronary vascular response to cholinergic but not α-adrenergic stimuli with aging. A relatively greater influx of extracellular calcium upon muscarinic receptor activation in the aged compared to adult heart may be a factor contributing to the age-related enhancement in coronary vascular response to cholinergic stimulation. Further, it is unlikely that impaired generation of EDRF by coronary endothelial cells or reduced sensitivity of vascular smooth muscle to EDRF contributes to the enhanced coronary vasoconstrictor effect of cholinergic stimulus in the aged rat heart. Such age-related cholinergic hypersensitivity may contribute to the high incidence of coronary artery spasm and impairment of coronary blood flow, cardiac energy metabolism, and contractile function that occurs with aging.

CHAPTER 5 SUMMARY AND CONCLUSION

5.1 Introduction

A striking diminution in the capacity to respond to various forms of stress (physical, emotional and environmental), is one of the well recognized defects of the heart in aging humans and animals. Age-related changes at the level of cardiac autonomic receptors and their effector systems may contribute to impaired stress-response of the aging heart. As summarized below, evidence supporting this possibility has emerged from previous studies, centered largely on β-adrenergic control of the heart. (1) The contractile response of the heart to β-adrenergic stimulus as well as β-adrenergic stimulation of adenylate cyclase, declines with aging. The density of β-adrenergic receptors in myocardium does not appear to be altered with aging and the precise mechanisms underlying the age-associated functional impairment in β-receptor-adenylate cyclase system remain to be established. (2) The density of α-adrenoceptor in the myocardium reportedly diminishes with aging but the impact of aging on α-adrenoceptor modulation of cardiac function remains obscure. (3) The density of muscarinic cholinergic receptors in myocardium is not diminished with aging. The very few studies on the impact of aging on cardiac cholinergic responses, performed *in vivo*, have produced conflicting results. (4) There appears to be a decrease in β-adrenergic mediated coronary vasodilation and an increase in α-adrenergic mediated coronary vasoconstriction in aged beagles. The effect of aging on coronary vascular responses to cholinergic stimulus is not known. (5) There is an apparent decline in adrenergic-cholinergic interactions in the aged heart as evidenced by a diminished ability of cholinergic agonist to attenuate β adrenergic activation of adenylate cyclase. Clearly, extensive future studies on effects of aging on: (1) β-adrenergic receptor linked signal transduction pathways; (2) α-adrenoceptor-mediated cellular processes; and (3) muscarinic receptor functions in myocardium and coronary vasculature are required to identify the postsynaptic components of the autonomic control systems affected by age, the molecular mature of age-associated changes, and the relationship of such changes to impairment in cardiac

function.

5.2 The aim of this thesis

A maior limitation of previous *in vivo* studies is that the interplay of multiple factors controlling cardiovascular functions in the intact organism make it difficult (if not impossible) to identify age-related alterations intrinsic to the myocardium. The studies described in this thesis utilized isolated, constant flow-perfused, beating heart preparations to investigate the impact of aging on cardiac cholinergic responses at the postsynaptic level. These studies were aimed to determine whether aging alters the responsiveness of the heart and coronary vasculature to muscarinic cholinergic stimulation and if so, to characterize the underlying mechanisms by examining potential age-associated alterations in acetylcholinesterase activity, and muscarinic receptor linked events in the signal transduction pathway which culminate in physiological responses.

5.3 Findings and conclusions from the present study

In the present study, isolated, Langendorff-perfused hearts from 6-8 month-old (adult), 12 month-old, 20 month-old and 26-30 month-old (aged) Fischer 344 rats were used to determine the negative chronotropic and inotropic responses to cholinergic agonists. In contrast to the age-related diminution in myocardial responses to β-adrenergic stimulation, the negative chronotropic and inotropic responses to cholinergic muscarinic receptor stimulation are strikingly enhanced with aging which is evident in the 20 month-old rat.

Studies investigating the mechanisms underlying the age-related increase in cholinergic responses of the heart suggested the following.

(1) Acetylcholinesterase (but not pseudocholinesterase), activity declined substantially in the atria and ventricles of aged compared to adult rats. Decreased activity of this enzyme would increase the effective synaptic concentration of acetylcholine at any given dose of the neurotransmitter, and this may explain, in part, the enhanced negative chronotropic and inotropic response to acetylcholine in the aged heart. Interestingly, (a) at a maximally effective concentration of the cholinesterase inhibitor eserine (5μM),

significant age-related difference in the chronotropic response to acetylcholine persisted and (b) marked age-related difference could be observed in the chronotropic and inotropic responses to carbachol, a cholinesterase-resistant cholinergic agonist. These observations suggest that age-associated alterations in postsynaptic muscarinic receptor linked events also underlie the cholinergic hypersensitivity of the aging heart.

(2) It is possible that enhanced cholinergic responses of the heart in aging may be associated with age-related alterations in the number of the muscarinic receptors and/or their functional properties. In the present study, no age-related difference was seen in receptor number and antagonist binding affinity in atria or ventricles. However, the muscarinic receptor binding affinity for its agonist was increased in the atria but not ventricles of the aged compared to adult rats. This may be a factor contributing to the enhanced bradycardic response in the aged heart.

(3) Western immunoblotting and ADP-ribosylation techniques were used to study the effect of aging on Gi protein levels in the hearts. Both studies demonstrated that the relative amount of Giα was significantly greater in the atria and ventricles of the aged compared to adult rats, and this may serve to intensify the muscarinic effects on the heart. The fidelity of the signal transduction through muscarinic receptor-linked Gi protein, as judged from guanine nucleotide-induced decrease in muscarinic receptor affinity for carbachol, was unaltered with aging in atria and ventricles.

(4) The electrophysiological studies demonstrated that aging was accompanied by: (a) enhancement in carbachol-induced hyperpolarization of the maximal diastolic potential in atria, and (b) more pronounced carbachol-induced shortening of action potential duration measured at 50% of repolarization (APD_{50}) in the ventricles. Aging did not alter the carbachol-induced prolongation of atrioventricular conduction time during sinus rhythm. The age-associated enhancement in carbachol-induced hyperpolarization in atria may be due to a greater increase in I_{KACh} in the aged atria since the key mechanism for membrane hyperpolarization is the activation of I_{KACh} through stimulation of muscarinic cholinergic receptors in the atria. This action of muscarinic agonist may result in depression of excitability and pace-maker activity at the sinoatrial node. Therefore, the greater bradycardic response of the aged compared to adult heart to carbachol appears

to be linked to an age-associated difference in muscarinic modulation of I_{KACh}. The present observation that aging is associated with a more pronounced effect of carbachol on shortening of APD_{50} in the ventricle (epicardium and endocardium), has mechanistic relevance to the age-associated enhancement in the negative inotropic response of the heart to carbachol. The ionic basis of the shortening of APD_{50} by muscarinic agonists likely involves a reduction in $I_{Ca(L)}$. Reduction of $I_{Ca(L)}$ will shorten APD_{50} thus shortening the plateau phase and diminishing Ca^{2+} influx during the action potential. This effect has been suggested to be responsible for the negative inotropic response of the heart to muscarinic cholinergic stimulation. However, the precise ionic mechanisms for the age-related alterations in carbachol-induced membrane hyperpolarization and shortening of APD_{50} need to be further investigated using voltage or patch clamp techniques.

Since (a) II or III degree AV block did not occur in the carbachol-induced increase in atrioventricular conduction time in atrial paced hearts, and (b) the bradycardic response to carbachol in spontaneously beating hearts occurred in the absence of a significant change in atrioventricular conduction time, it can be concluded that the exaggerated cholinergic-triggered bradycardia in the aged heart stems largely, if not exclusively, from greater muscarinic inhibition of the pacemaker activity at the sinoatrial node rather than from greater slowing of atrioventricular nodal conduction.

(5) Age-induced changes in vasoregulatory responses of the coronary vasculature to autonomic stimuli may also influence autonomic modulation of cardiac rhythm and contractile performance. The present study evaluated the impact of aging on coronary vascular response to cholinergic stimulation. The results showed that cholinergic stimulation evokes vasoconstriction in the rat coronary vasculature and this response is strikingly enhanced with aging. This age-related difference in coronary vascular response was attenuated in the presence of verapamil, a Ca^{2+} channel blocker. This implies that relatively greater influx of extracellular Ca^{2+} upon muscarinic receptor activation in the aged compared to adult heart may be a factor contributing to the age-related enhancement in coronary vascular response to cholinergic stimulus. Since L-NMMA (an inhibitor of EDRF synthesis) -induced vasoconstriction did not show age-related difference, the generation of the EDRF by coronary endothelial cells seems not compromised in the aged

heart. In the presence of L-NMMA (30 or 100 μM), carbachol (1 μM), caused significantly greater increase in coronary perfusion pressure in the aged compared to adult heart. The pressor response to L-NMMA and carbachol was nearly additive in both adult and aged hearts. Thus, neither impaired synthesis of EDRF nor reduced sensitivity of coronary vascular smooth muscle to EDRF contributes to the enhanced coronary vasoconstrictive effect of cholinergic stimulus in the aged rat heart. It seems that the age-related difference in response to carbachol is selective because we also demonstrated that α-adrenergic response, another vasoconstriction action, is not altered with aging.

In conclusion, in the rat model, aging is accompanied by greatly enhanced negative chronotropic and inotropic responses of the heart to cholinergic stimulation. The vasoconstriction response of the coronary vasculature to cholinergic stimulation is also enhanced with aging. (1) The increased negative chronotropic response of the aging heart to cholinergic stimulation is likely associated with an age-related decrease in acetylcholinesterase activity, increase in the affinity of muscarinic receptor for its agonist, increase in the Giα protein content of in atria, and enhancement in the muscarinic receptor-mediated hyperpolarization in the atria. (2) The increased negative inotropic response may be due to an age-related decrease in acetylcholinesterase activity, increase in Giα content of ventricles and an enhancement in the muscarinic receptor-mediated abbreviation of ventricular action potential duration which limits transsarcolemmal Ca^{2+} influx. (3) The increased coronary vasoconstriction response to cholinergic stimulation may be caused by relatively greater influx of extracellular Ca_{2+} upon muscarinic activation.

5.4 Physiological, pathophysiological, and clinical implications

The cholinergic hypersensitivity of the heart in aging may have physiological, pathophysiological, and clinical implications. In the physiological setting, the enhanced negative chronotropic and inotropic responses of the heart to cholinergic stimulation may contribute to the depression in heart rate and contractility, thus, compromising cardiac output under condition of stress. In pathophysiological setting, this phenomenon implies that age-related decline in baroreceptor control of heart rate, a function largely mediated

by the vagus nerve, is not due to effector organ hyporesponsiveness. Consequently, the defect is likely located elsewhere in the reflex arc. Cholinergic hypersensitivity may result in greater coronary vasoconstriction at any level of cholinergic tone, thus compromising coronary blood flow, energy metabolism, and cor ac ility in the aging heart. In the clinical setting, cholinergic hypersensitivity of the heart may contribute to the higher incidence of coronary spasm, inordinate sinus node depression and carotid sinus syndrome in the elderly. Whether the observations made in the aging rat model are applicable to humans is uncertain; these findings suggest need for caution in the therapeutic use of cholinomimetic agents in the elderly so as to guard against the occurrence of exaggerated bradycardia, heart failure, and cardiac ischemia.

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