CELLULAR MECHANISMS OF ATRIAL MECHANOTRANSDUCTION

Interacting mechanisms in stretch-induced changes of rat atrial function and their modulation by intracellular acidosis

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Department of Physiology

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To Marjo and Perttu

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Abstract

Stretch of the cardiac muscle activates several physiological processes leading to changes in the function of the muscle. These changes include increase of the contraction force accompanied by changes in the intracellular calcium concentration. This phenomenon is known as Frank-Starling relation of the heart. In addition to this, stretch also influences the membrane voltage of individual myocytes predisposing the cardiac muscle to arrhythmias. In atrial muscle stretch augments the secretion of the atrial natriuretic peptide (ANP). Although several cellular components are known to be sensitive to mechanical stimulus the precise mechanisms participating to these stretch-induced changes are not known in detail. Further it is not known if these changes are causally related or if they share a common causal factor. This research was aimed to study the stretch-induced changes in the rat atrium. The possible interactive mechanisms were studied by recording intracellular action potentials, changes in the intracellular calcium concentration, contraction force and ANP secretion during stretch. The plausible mechanosensitive cellular components were incorporated into a mathematical model that was used to further study the mechanisms. The role of intracellular acidosis as a possible modulator of the mechanotransduction was of special interest.

In isolated rat left atrium moderate stretch produced by increasing the intra-atrial pressure increased the contraction force in a biphasic manner. The immediate increase of the force was caused by altered properties of the contractile element, but the following slow increase was accompanied by an increase of the Ca^{2+} transient. These changes were followed by lengthening of the late phase of action potentials and augmented secretion of the ANP. Intensive sustained stretch was also found to induce delayed afterdepolarizations (DADs). Gadolinium (Gd^{3+}), blocker of stretch-activated ion channels reduced the stretch-dependent activation of the contraction and inhibited the stretch-induced DADs. The mathematical model simulated the experimental findings at best when stretch-activated channel (SA-channel) activation and increased troponin-C affinity were used to mimic the stretch. The modelling data suggested that the SA-channel current increases the sarcoplasmic reticulum calcium content in a time dependent manner leading to Ca^{2+} transient augmentation during systole. Bigger Ca^{2+} transients induce a depolarizing current during the late phase of the action potential (AP) repolarization via the Na^{+}/Ca^{2+} exchanger causing the lengthening of the action potentials. A small reduction of the intracellular pH (0.18 units) with 20 mM propionate was found to modulate the stretch-induced changes in the rat atrium. Acidosis leads to an increase in the diastolic $[Ca^{2+}]_i$ during stretch, inhibits the stretch-induced changes in action potentials and slows down the contraction development during stretch by inhibiting the fast component of the force increase. These changes in E-C-coupling (excitation-contraction-coupling) were accompanied by a simultaneous augmentation of the ANP secretion. Furthermore, it was shown that contraction force and diastolic $[Ca^{2+}]_i$ of the stretched tissue are more sensitive to acidosis than in non-stretched tissue.

In conclusion, the stretch-induced changes in rat atrial myocytes are mediated by at least two mechanisms; stretch-activated Ca^{2+} influx and change in the properties of the contractile element. The action potential changes can be largely explained by modulation of the membrane voltage by intracellular calcium via Na^{+}/Ca^{2+}-exchanger. The co-occurrence of the changes in the $[Ca^{2+}]_i$ and ANP secretion suggests that the stretch-induced ANP secretion can be mediated by $[Ca^{2+}]_i$.

Keywords: intracellular calcium, action potential, contraction, ANP secretion

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Abbreviations

A^-	anion
ADP	adenosine diphosphate
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AP	action potential
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CICR	calcium-induced calcium release
DAD	delayed afterdepolarization
DAG	1,2-diacylglyserol
E-C-coupling	excitation-contraction coupling
EAD	early afterdepolarization
E_m	equilibrium potential (membrane)
F-S-relation	Frank-Starling relation
HPLC	high performance liquid cromatography
$I_{Ca,B}$	background calcium current
$I_{Ca,L}$	L-type calcium current
$I_{Ca,T}$	T-type calcium current
I_K	delayed rectifier potassium current
I_{K1}	inward rectifier potassium current
I_{Na}	voltage-activated sodium current
$I_{Na,B}$	background sodium current
IP_3	inositol (1, 4, 5) triphosphate
I_{to}	transient outward current
JSR	junctional sarcoplasmic reticulum
M-ATP	myosin-adenosine triphosphate-complex
MAP	monophasic action potential
NSR	non-junctional sarcoplasmic reticulum
pH_i	intracellular pH
PKC	protein kinase C
PLC	phospholipase C
PVE	premature ventricular excitation
r.p.	resting potential
RyR	ryanodine receptor

SA	stretch-activated
SAPK	stress-activated protein kinase
SR	sarcoplasmic reticulum
T-tubule	transverse tubule
TnC	troponin C
TnI	troponin I
TnT	troponin T
VACR	voltage-activated calcium release
V_m	membrane potential
$[Ca^{2+}]_i$	intracellular calcium concentration

List of original papers

This thesis is based on the following articles, which are referred to in the text by their Roman numerals I-IV:

I Tavi P, Laine M & Weckström M (1996) Effect of gadolinium on stretch-induced changes in contraction and intracellularly recorded action- and afterpotentials of isolated rat atrium. Br J Pharmacol 118: 407- 413.

II Tavi P, Han C & Weckström M (1998) Mechanisms of stretch-induced changes in $[Ca^{2+}]_i$ in rat atrial myocytes: Role of increased TnC affinity and stretch-activated ion channels. Circ Res 83: 1165-1177.

III Tavi P, Han C & Weckström M (1999) Intracellular acidosis modulates the stretch-induced changes in E-C coupling of the rat atrium. (submitted for publication).

IV Tavi P, Laine M, Voutilainen S, Lehenkari P, Vuolteenaho O, Ruskoaho H & Weckström M (1999) Potentiation of the stretch-induced atrial natriuretic peptide secretion by intracellular acidosis. Am J Physiol (in press).

Contents

1. Introduction

The ability of cardiac muscle contraction to respond to increase in the mechanical load is the well known Frank-Starling law, which states that contraction force is increased upon an increase of the ventricular volume (see, e.g. Lakatta, 1986). Among other effects of stretch on the cardiac muscle function are changes in the electrical behaviour of the myocytes (Lab, 1978), changes in the intracellular calcium concentration (Allen & Kurihara, 1982) augmented secretion of atrial natriuretic peptide (ANP)(Lang *et al.* 1985) and onsets of the expression of genes (Sadoshima *et al.* 1992). Thus, the mechanotransduction of the cardiac muscle is a process transforming a mechanical stimulus into a form of definite changes in the cell membrane voltage, contraction force, ion balance, exocytosis and gene expression.

The concept of mechanosensitivity requires that the cells posses a component sensitive to the input of mechanical energy. For the role of mechanosensitive component in the heart cells several molecules have been proposed. The increase of contraction force by stretch has been explained by stretch sensitivity of the contractile element (Babu *et al.* 1988). The changes in the ion balance and electrical behaviour of the myocytes can be explained by activation of stretch-activated ion channels (Kim, 1993). The stretch-induced exocytosis (Ruskoaho, 1992) and gene expression seem to require increases in the enzymatic activity of regulatory enzymes (Komuro *et al.* 1991). Although mechanosensitive molecules have been found from the cardiac muscle, the role of each of these molecules in the process of mechanotransduction is not known. Also, the interactions of the cellular mechanosensitive elements during stretch-induced modulation of the myocardial function are largely unknown.

The present study was aimed to examine the role of the potential mechanosensitive elements in the rat atrium leading to changes in the contraction force, electrical properties and calcium balance of the myocytes, and also to activation of the exocytosis of the ANP. Further, the interactions of these effects were also studied to see whether they share a common causal factor.

2. Review of the literature

2.1. Electrical properties of the cardiac myocytes

2.1.1. Origin of the resting potential

All living cells are separated from their surroundings by a lipid bilayer, plasmalemma. Semipermeable nature of this membrane creates the physical foundation for the existence of an electrical potential difference (voltage) across plasma membrane. Cytoplasm contains negatively charged proteins, organic polyphosphates and other ionized substances (A^-) that cannot permeate through the plasma membrane, but also water, K^+, Cl^-, and other ions, to which the plasma membrane is more or less permeable. The principle of electroneutrality states that any macroscopic region of a solution must have an equal number of positive and negative charges. In the case of living cells the permeant ions compensate the unbalance generated by the non-permeant ions. The steady-state of this kind of mixture of permeant and non-permeant ions is given by the Gibbs-Donnan equilibrium. For two permeant ions (K^+, Cl^-) the Gibbs-Donnan equilibrium is:

$$[K^+]_{in}[Cl^-]_{in}=[K^+]_{out}[Cl^-]_{out} \qquad (1)$$

The ion movements across the membrane would create an osmotic gradient. In the situation described by equation (1) water movement into the cell due the osmotic gradient would destroy the cell, and so an equilibrium cannot be achieved (see, e.g. Baumgarten & Feher, 1995). More reasonable equilibrium can be obtained if one ionic species is restricted to the extracellular compartment to compensate the effects of the ions restricted to the intracellular compartment. This situation is referred to as a double-Donnan (Leaf, 1959) or pump-leak system (Tosteson & Hoffman, 1960). Assuming that the cell interior contains ions (A^-) with a restricted permeability but also that the extracellular space contains ions that cannot permeate the cell membrane, a steady-state equilibrium can be achieved. Since animal cell membranes are relatively impermeable to Na^+, extracellular [Na^+] could readily compensate the osmotic gradient caused by $[A^-]_{in}$. Because only the impermeant ions contribute to the osmotic pressure, in osmotic equilibrium $[Na^+]_{out}=[A^-]_{in}$. This holds true only in the situation where the cell membrane is totally impermeable to Na^+. Although the plasma membrane of

living cells is relatively impermeable to Na^+, still a small amount of Na^+ ions leaks into the cell, even at rest. This leak is compensated by the Na^+/K^+-ATPase, which extrudes Na^+ from cells and transports K^+ ions into the cells while hydrolysing ATP. The stoichiometry of this transporter is $3Na^+:1K^+$. Thus, this transporter participates to the maintenance of both the electrical gradient and osmotic balance across the membrane. As a consequence, the electrical potential difference across the plasma membrane (E_m) is ideally the same as the equilibrium potentials of the permeant ions, K^+ and Cl^-, i. e. $E_m=E_k=E_{Cl}$, there is no net osmotic pressure across the membrane, and the system is stable. Because the electrical potential difference (E_m) is also present in the resting cells, it is usually called the resting potential (r.p.).

The relationship between membrane voltage and concentration of any ion at both sides of the membrane at equilibrium is given by Nernst equation (e.g. Hille, 1992):

$$E_{ion} = \frac{RT}{zF} \ln \frac{[Ion]_o}{[Ion]_i} \qquad (2)$$

where E_{Ion} is the equilibrium potential, z is the charge of the ion, R is the gas constant, T is the absolute temperature, F is the Faraday's constant and $[Ion]_o$, $[Ion]_i$ are the concentrations of the ion outside and inside of the cell, respectively. Usually many ions contribute to the formation of the membrane potential. Each one of these ions drifts the membrane potential towards their equilibrium potential. Assuming that ions do not interact with each other, that the potential drops linearly across the membrane, and that the total current through the membrane is zero, the membrane potential can be calculated to univalent ions by using the Goldman-Hodgkin-Katz voltage equation (Goldman 1943, Hodgkin & Katz 1949):

$$E_m = \frac{RT}{zF} \ln \frac{P_{Ion}[Ion]_o^z}{P_{Ion}[Ion]_i^z} \qquad (3)$$

where E_m is the membrane potential, z is the valence of the ion and P_{Ion} is the permeability of the membrane to the particular ion. In cardiac myocytes E_m at rest (resting potential) is mostly due to the permeability and concentrations of K^+, but the r.p. is little higher than the equilibrium potential of potassium. This is mainly caused by the leak of Na^+. Thus the equation (3) can be simplified and given in a different form for cardiac cells (e.g. Berne & Levy, 1993):

$$E_m = -\frac{RT}{F} \ln \frac{[K]_i + \frac{P_{Na}}{P_K}[Na]_i}{[K]_o + \frac{P_{Na}}{P_K}[Na]_o} \qquad (4)$$

This equation shows that at constant concentrations of K^+ and Na^+ the r.p. is determined by the P_{Na}/P_K ratio, the relative permeability of the membrane to Na^+ and K^+.

2.1.2. Membrane excitability

Excitability is an intrinsic membrane property that allows a cell to generate an electrical signal or action potential (AP) in response to stimuli of sufficient magnitude. This feature of excitable cells is intrinsic in a sense that the energy source for the generation of action potentials is stored in the excitable cell itself. The energy for the action potentials comes from the transmembrane ionic gradients created by the existence of the resting potential and the uneven distribution of ions across the membrane (e.g. Wahler, 1995). The electrochemical driving force for each species of an ion is the difference between its equilibrium potential and the membrane potential (E_m-E_{Ion}). This driving force is manifested in a current (I) that obeys Ohm's law;

$$I = \frac{V}{R} = g \cdot V \qquad (5)$$

where V is the voltage, R is the resistance and g is the conductance (1/R). For any individual ion current,

$$I_{Ion} = g_{Ion}(E_m - E_{Ion}) \qquad (6)$$

where I_{Ion} is the current generated by the movement of an ionic species with a given conductance (g_{Ion}) and driving force (E_m-E_{Ion}). Equations 5 and 6 demonstrate that a change in the membrane voltage can be induced by increasing or decreasing the conductance of ions. In all excitable cells the conductance of each ionic species is regulated by transmembrane proteins with selective permeabitity to certain ion, called ion channels (Hille, 1992)

2.1.3. Ion channels regulating the action potential waveform in rat atrial myocytes

Cardiac cells have certain properties in common with other excitable cells, like nerve and skeletal muscle cells, but significant differences also exist. In nerve and skeletal muscle cells the AP is short (usually< 10 ms), and the waveform of AP is due to activation of only few ion channel types. In contrast, during the cardiac AP more than ten different ion channel types may contribute to the final shape of the AP (Carmeliet, 1993) and the duration of the AP can vary from tens to several hundreds of milliseconds (Varro *et al.* 1993b). Furthermore, AP shape has species specific differences, but it also varies among the cell types in the cardiac muscle of the same species (Bers, 1993). For example, the rat atrial AP differs from the ventricular AP and even more from the AP of the nodal cells (Wahler, 1995). A common feature of all cardiac action potentials is that they are all due to concerted activation and inactivation of wide variety of ion channels (Boyett *et al.* 1996).

In cardiac myocytes, the upstroke phase (depolarization) of APs is caused by an increased conductance of Na^+ through voltage-activated Na^+ channels. The depolarization caused by the Na^+ current opens many types of potassium channels, all contributing to the

repolarization of the membrane potential (e.g. Standen, 1993). The first phase of repolarization is largely due to a transient outward current (I_{to}). The I_{to} current seems to have two distinct components, I_{to1} and I_{to2} (e.g. see Carmeliet, 1993). The I_{to1} is purely a voltage activated K^+ current whereas I_{to2} is a Ca^{2+}-activated Cl^- current (Duan *et al.* 1992, Zygmunt 1994). It has also been shown that intracellular Ca^{2+}-transients modulate the I_{to2} (Sipido *et al.* 1993, Kawano *et al.* 1995), linking the Ca^{2+}-release and membrane potential together. Depolarization of the E_m during the initial phase of the AP activates also the voltage-dependent L-type Ca^{2+} current and, at more negative voltages, the T-type Ca^{2+} channels (eg. see Katz 1997). However, the T-type Ca^{2+} channel current is not prominently present in rat cardiac myocytes (Bogdanov *et al.* 1995), so the calcium influx relies on L-type Ca^{2+} current. Although the L-type current is activated during the upstroke of the AP, it contributes most prominently to the shape of the AP at the repolarization phase (Mitchell *et al.*1984, Schouten & TerKeurs, 1985, 1991). In the species with a long AP the L-type current affects the duration of the AP by regulating the so called "plateau" phase of AP. In the rat cardiac myocytes the plateau is almost nonexistent, so the duration of AP is mainly regulated by K^+ conductances. Together with the I_{to} current another K^+ conductance is first activated by the depolarization of the V_m, but due to the delayed activation of this channel type the current is substantially activated only during the repolarization of the AP. This current, called delayed rectifier (I_K), generates the final repolarization together with the inward rectifier K^+ current (I_{K1}), which also contributes to the r.p. of the myocytes. A schematic graph showing the typical rat atrial AP and the timing of the main contributions of different currents to the shape of the AP is presented as figure 1.

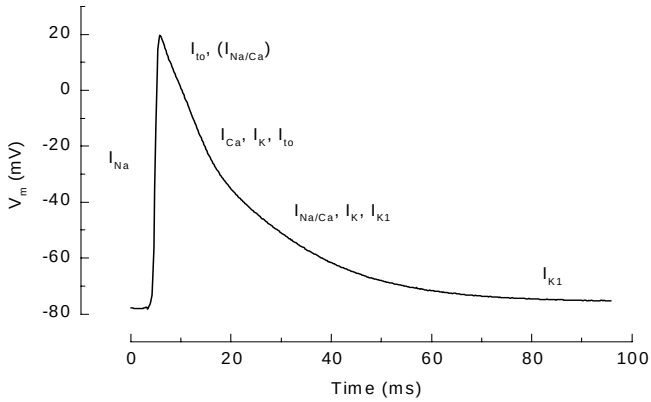


Fig. 1. Contribution of different ion channels and exchangers to the action potential waveform of rat atrial myocytes. Abbreviations: I_{Na}; Sodium current, I_{to}; transient outward current, $I_{Na/Ca}$; sodium-calcium exchanger current, I_{Ca}; voltage activated L-type Ca^{2+}-current, I_K; delayed rectifier potassium current, I_{K1}; Inward rectifier potassium current.

The primary function of Na^+/Ca^{2+}-exchanger in the cardiac myocytes is Ca^{2+} handling. It moves three Na^+ ions for one Ca^{2+} ion, thereby generating a current. Depending on whether Na^+ ions are moved in or out, the current is inward or outward. The polarity of the

current depends on the chemical gradient and on the membrane potential (e.g. see Janvier &Boyett 1996). During the upstroke of the AP the V_m moves to be positive in reference to the equilibrium potential of the exchanger, and the current becomes transiently positive with Ca^{2+} ions moving into the cell. The prominent rise of $[Ca^{2+}]_i$ during systole caused by calcium-induced calcium release forces the exchanger current to carry Ca^{2+} out from the cell and thus, to be an inward current. Because of the timing of these events, Na^+/Ca^{2+}-exchanger generates inward current at the late phase of repolarization causing lengthening of the action potential (Schouten & TerKeurs 1991, DuBell *et al.* 1991). However, the role of the Na^+/Ca^{2+} exchanger current varies between species, depending on the AP duration and sodium concentration inside the cell (Bers, 1991, Sham *et al.* 1995b).

2.2. Regulation of the intracellular calcium balance

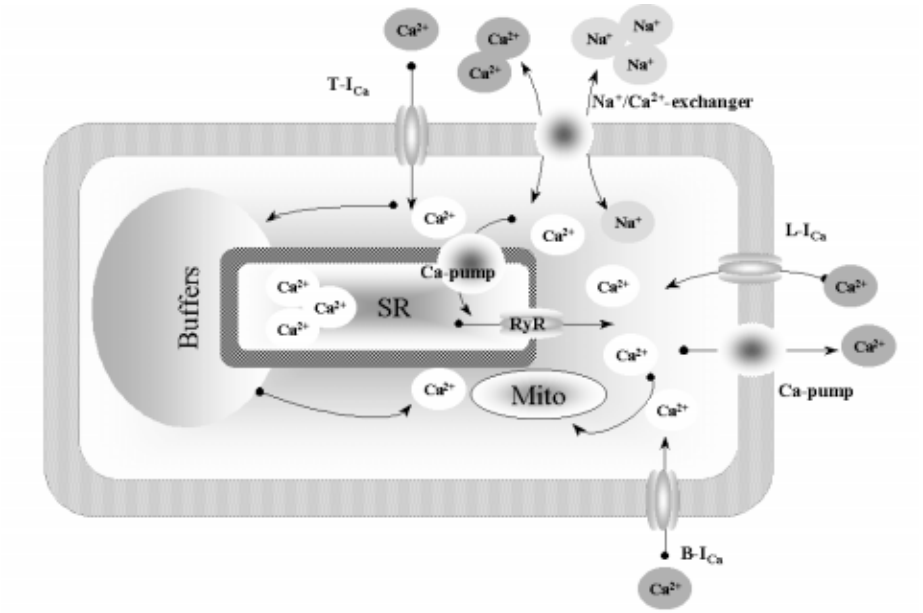
2.2.1. Sources of calcium

The free intracellular calcium concentration in the resting cardiac myocyte is very low, usually between 75-200 nM (e.g. see Bers, 1993). Low $[Ca^{2+}]_i$ with the membrane potential gives an electrochemical gradient favouring Ca^{2+} entry at rest (ca. 200 mV). Thus, any leak of Ca^{2+} ions trough the cell membrane affects the $[Ca^{2+}]_i$. Two main routes by which Ca^{2+} is known to enter the cell are by voltage-dependent Ca^{2+} channels (L-type and T-type) and the Na^+/Ca^{2+}-exchanger. In addition to this, rat ventricular myocytes posses also a B-type (Background) Ca^{2+}-permeable channels (Coulombe *et al.* 1989). These channels are open at negative membrane potentials, providing a route for calcium to enter the cell even during diastole (Coulombe *et al.* 1989, Lefevre *et al.* 1995). Besides extracellular sources also the intracellular compartments contribute to $[Ca^{2+}]_i$. At diastole the calcium leaks from the intracellular stores, (although e.g. the leak from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR) is modest), and this may contribute to the loss of Ca^{2+} from the SR during long periods of rest. This may cause a phenomenon known as rest decay, in which some mammalian cardiac muscle preparations exhibit smaller contractions after longer periods of rest (Bers, 1985).

2.2.2. Calcium buffers

Calcium ions are moved from the cytosol by several transporters and exchangers. Efflux of Ca^{2+} ions from the cell is controlled by the sarcolemmal Ca^{2+}-ATPase-pump and the Na^+/Ca^{2+} -exchanger. It has been approximated that Na^+/Ca^{2+} -exchanger contributes ca. 77% of the calcium extrusion at the resting levels of $[Ca^{2+}]_i$ in rat trabeculae (Lamont & Eisner, 1996). The major intracellular Ca^{2+} store is the smooth sarcoplasmic reticulum, which is an entirely intracellular, membrane bound compartment that is not continuous with the sarcolemma. The main function of this organelle is sequestration and release of calcium to the myoplasm. Ca^{2+} ions are moved from the cytosol to the SR by high affinity Ca-

ATPase distinct from the sarcolemmal Ca^{2+}-pump. The interior of the SR contains a low affinity, high capacity calcium binding protein called calsequestrin (Ostwald & MacLennan, 1974). The amount of Ca^{2+} in the SR is of course variable. It has been estimated that the $[Ca^{2+}]$ inside the rat ventricular SR is ca. 120 µM, when the SR is maximally loaded (Varro *et al.* 1993a). The other major store of Ca^{2+} ions is the mitochondria. It has been shown that isolated mitochondria can accumulate large amounts of Ca^{2+} (Lehninger et al 1967, Carafoli & Lehninger, 1971). However, the mitochondria are about 50-fold slower than the Na^+/Ca^{2+} -exchanger at removing the Ca^{2+} from the cytosol (Bassani et al, 1992). It has also been shown that the mitochondria do not take up detectable amounts of Ca^{2+}, unless the $[Ca^{2+}]_i$ exceeds 300-500 nM (Zhuan *et al.* 1998). So, in theory, mitochondria can contribute to the resting $[Ca^{2+}]_i$ at least when resting $[Ca^{2+}]_i$ is high. Cardiac myocytes also contain several other Ca^{2+} binding molecules, which can be considered as buffers, including Troponin C, calmodulin, phosphocreatine, ATP, outer SR surface and inner sarcolemmal surface. These buffers maintain low resting $[Ca^{2+}]_i$ and induce fast removal of free Ca^{2+} ions from the cytosol during transient rise in $[Ca^{2+}]_i$. Taken together, the resting $[Ca^{2+}]_i$ is formed by a complex sum of leaks of calcium from extracellular space and intracellular compartments (SR, mitochondria), the action of transporters and pumps and binding by the Ca^{2+} buffers with varying affinity and capacity. An overview of the Ca^{2+} balance in the cardiac myocyte



is presented in figure 2.

Fig. 2. Schematic view of the sources and buffers of Ca^{2+} ions in the cardiac myocytes. Abbreviations: SR; sarcoplasmic reticulum, B-I_{Ca}; background calcium current, RyR; ryanodine receptor, Mito; mitochondria, T-I_{Ca}, L-I_{Ca}; T-and L-type calcium currents, respectively. "Buffers" indicates the buffering of Ca^{2+} by macromolecules, membranes,

contractile elements and other Ca^{2+} binding sites not specified in the figure. Direction of arrow indicates the direction of the calcium flux in a resting myocyte.

2.3. E-C-coupling in cardiac myocytes

2.3.1. Calcium-induced calcium release (CICR)

The muscle cell membrane is folded to form specific T-tubular (Transverse tubules) system. In cardiac muscle cells the T-tubular system allows close connection between plasmalemmal ion channels and channels in the sub-cellular components, namely the ryanodine sensitive calcium channels (RyR) in the SR (e.g. Sommer & Jennings, 1986). Electrical excitation of the surface membrane leads to an action potential which propagates as a wave of depolarization along the surface and along the T-tubules. Depolarization of the T-tubule overlying the terminal cisternae opens L-type Ca^{2+} channels in the plasmalemma. Since the L-type channels and RyR channels are closely connected (Sham *et al.* 1995a, Sham, 1997), the Ca^{2+} flux through L-type channels induces the release of Ca^{2+} ions from the SR. This process is known as calcium-induced calcium release (CICR) (Fabiato & Fabiato, 1972,1975, Fabiato, 1983). CICR leads to a transient rise in the $[Ca^{2+}]_i$. During this so-called calcium transient $[Ca^{2+}]_i$ rises from the diastolic level (75-200 nM) up to levels that can activate the contraction (0.5-3 µM, see, e.g. Blinks, 1986) of the muscle.

2.3.2. Subcellular features of calcium-induced calcium release

For many years the theory of CICR in cardiac muscle cells has been the so called common pool model. The basic idea of this theory is that there is one cytosolic Ca^{2+} "pool" to which calcium enters both through L-type Ca^{2+} channels and ryanodine receptors (e.g. Stern 1992). This theory can explain the macroscopic events seen in experiments, e.g. that membrane depolarization precedes the increase in cytosolic $[Ca^{2+}]$ causing contraction. In this scheme the Ca^{2+} release from SR would be a positive feedback mechanism, where the Ca^{2+} release would, once triggered, evolve autonomously. However, there is no experimental evidence that Ca^{2+} release becomes autonomous, but it is smoothly graded as a function of the calcium trigger, i.e. the Ca^{2+} current through plasmalemma (Stern, 1992). On the light of recent experimental data another theory of calcium release has been developed. This so-called local control theory suggests that Ca^{2+} entering through a single L-type Ca^{2+} channel induces a high local $[Ca^{2+}]_i$ which activates closely associated Ca^{2+} release channels in the SR (Niggli & Lipp, 1995). The localization of RyR into "clusters" provides independently functioning units controlling the SR Ca^{2+} release. According to this theory, Ca^{2+} transients underlying the normal E-C- coupling are caused by spatial and temporal summation of these independent and local Ca^{2+} transients (sparks) triggered by L-type Ca^{2+} channel currents (Cannell *et al.* 1994, López-López *et al.* 1995, Shacklock *et al.* 1995). The local Ca^{2+} transients may also occur spontaneously resulting from opening of multiple Ca^{2+} release channels clustered within discrete SR junctional regions (Blatter *et al.* 1997).

The local control theory of the Ca^{2+} release has been developed on the basis of

experiments done with the ventricular myocytes (e.g. Stern & Lakatta, 1992). However, atrial myocytes have several anatomical and functional differences compared to the ventricular myocytes. Atrial myocytes lack the T-tubular system (Sommer & Jennings, 1986) which, in ventricular myocytes, enables the functional coupling between L-type Ca^{2+} channels and Ry-receptors (Fenoglio *et al.* 1979). Therefore, in atrial myocytes the subcellular events leading to CICR are at least somewhat different from the local control theory developed for ventricular myocytes. In myocardial cells which are lacking a T-tubular system, like in atrial myocytes, the close associations of SR compartments with the surface membrane, the so-called peripheral couplings, are considered to be functionally homologous to diads and triads in T-tubular system containing cells (McNutt & Fawcett, 1969). It has been shown that, in atrial myocytes, the voltage-dependent Ca^{2+} entry triggers Ca^{2+} release from these peripheral couplings of SR, subsequently inducing further Ca^{2+} release from stores in more central regions of the myocyte (Lipp *et al.* 1990, Hüser *et al.* 1996). Based on these findings a two-compartment model for atrial Ca^{2+} release was developed (Hatem *et al.* 1997). The basic idea of this two-step Ca-release model is that the calcium entering through L-type channels triggers calcium release from the first release compartment (peripheral SR). Thereafter the Ca^{2+} released from the first compartment triggers release from the second compartment (corbular SR) in an all-or-none manner (Hatem *et al.* 1997). So, according to this model, the I_{Ca} only partially controls the activation of RyRs. The prolonged Ca^{2+} transients in the atrial cells (compared to ventricular cells) reflect the activation of RyRs not coupled to L-type Ca^{2+} channels (Hatem *et al.* 1997). These fundamental differences between atrial and ventricular myocytes may explain the fact that in atrial cells the SR Ca^{2+} uptake is faster and the amount of Ca^{2+} released during excitation is smaller (Minajeva *et al.* 1997) although the releasable pool of Ca^{2+} has a similar capacity in both ventricular and atrial myocytes (Minajeva *et al.* 1997).

2.3.3. Voltage activated calcium release (VACR)

Although CICR seems to be the main mechanism of E-C-coupling in cardiac myocytes, other alternative or perhaps complementary hypothesis for calcium release has also been presented. The activation of skeletal muscle is strongly voltage dependent as related to intramembrane charge movement, the so-called charge movement coupling (Schneider & Chandler, 1973). According to this hypothesis the Ca^{2+} channel in the surface membrane acts as a voltage sensor, communicating directly with the Ca^{2+} release channel of the SR to initiate Ca^{2+} release (e.g. Callewaert, 1992). It has been proposed that calcium release in the cardiac cells is also sensitive to voltage, since repolarization can switch off calcium release from SR (Cannel *et al.* 1987). In guinea-pig ventricular myocytes this voltage-activated calcium release (VACR) was found to be present only when cells were dialyzed with cAMP (Hobai *et al.* 1997). Quite recently, it was showed that under certain conditions voltage can induce a Ca^{2+} influx through fast Na^{+}-channels. Interestingly, this promiscuous permeability, called slip-mode conductance, can be activated by protein kinase A (PKA) which is known to be activated by cAMP (Santana *et al.* 1998). If this is true, and VACR is indeed modulated by intracellular cyclic nucleotides, it is not surprising that other studies have failed to see the VACR (Näbauer *et al.* 1989). This finding clearly challenges the idea that Ca^{2+} entry through L-type Ca^{2+} channels is an absolute requirement for Ca^{2+} release in

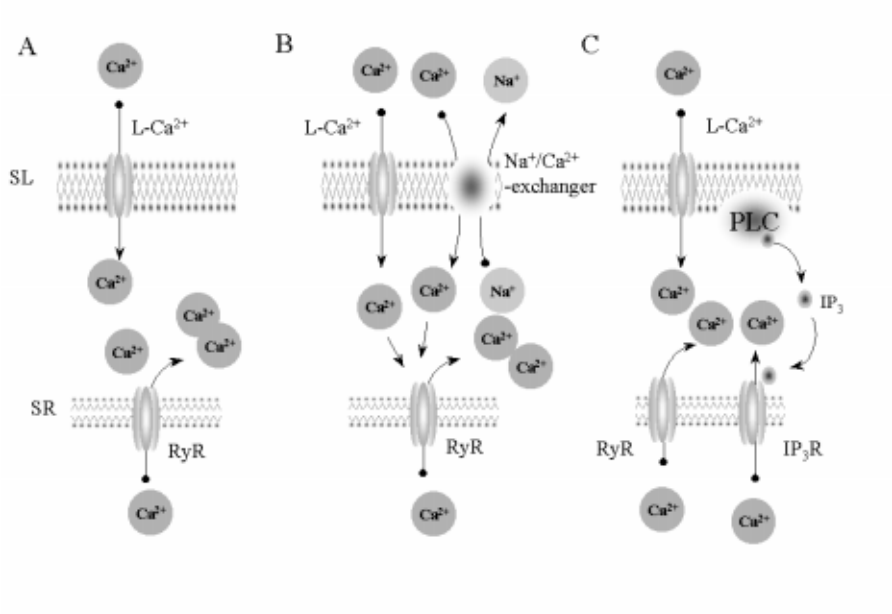
cardiac muscle (e. g. see Bers, 1993), but it is still unlikely that VACR is a major mechanism.

2.3.4. Modulators of the CICR

The role of the Na^+/Ca^{2+}-exchanger in modulating the E-C-coupling has been under intensive study for many years (e.g. Schulze *et al.* 1993) but it is still controversial. In theory, the Na^+/Ca^{2+}-exchanger can contribute to the Ca^{2+} release by inducing a Ca^{2+} influx when activated in the reverse mode, carrying Ca^{2+} ions in and Na^+ ions out (e.g. see Callawaert, 1992). Because the turnover rate of the Na^+/Ca^{2+}-exchanger is clearly voltage dependent, during the upstroke of the AP the Na^+/Ca^{2+}-exchanger might cause a Ca^{2+} influx triggering the Ca^{2+} release through RyR (Levi *et al.* 1993, Levi *et al.* 1994, Wasserstrom & Vites, 1996). In some studies the role of Na^+/ Ca^{2+}-exchanger as a trigger of Ca^{2+} release was found to be negligible (Sipido *et al.* 1997). The fact that calcium flux through Na^+/Ca^{2+}-exchanger alone is able to trigger Ca^{2+} transients in cardiac myocytes (Hancox & Levi, 1995), suggests that the Na^+/Ca^{2+}-exchanger is located nearly opposite the RyRs in the plasma membrane and both could be controlled by the same subcellular ion gradients, as suggested (Janiak *et al.* 1996). This is in line with the results from immunofluorescence labelling of the Na^+/Ca^{2+}-exchanger, showing that the exchanger protein is present in the T-tubular system and the intercalated discs (e.g. see Schulze *et al.* 1993). It is also possible that the reverse mode of the Na^+/Ca^{2+}-exchanger is directly activated by sodium current during the upstroke of the action potential. This would cause I_{Na} -induced calcium signals whenever sodium channels are activated, as demonstrated previously (Leblanc & Hume, 1990, Lipp &Niggli, 1994, Levesque *et al.* 1994). In some studies Ca^{2+} release is neither initiated by sodium current nor by sodium accumulation (Sham *et al.* 1992). However, if Na^+/Ca^{2+}-exchanger is part of the CICR, it makes the control of the E-C coupling more complex, since the amount Na^+/Ca^{2+}-exchanger induced Ca^{2+} release would depend on several subcellular ion gradients (Na^+, Ca^{2+}) and on the membrane voltage. For example, if the $[Ca^{2+}]_o$ or $[Na^+]_i$ is increased, the contribution of the Na^+/Ca^{2+}-exchanger to the calcium transient is greater, and vice versa (Evans & Cannell, 1997, Bers *et al.* 1988). Recent results suggest that the activities of the Na^+/K^+ pump and the Na^+/ Ca^{2+}-exchanger are tightly correlated via changes in $[Na^+]_i$ in restricted space near the plasmalemma (Fujioka *et al.* 1998). In this space $[Na^+]$ can be seven times higher than the mean $[Na^+]_i$ (Fujioka *et al.* 1998). This would naturally have an effect on the Na^+/Ca^{2+}-exchanger induced changes in the E-C-coupling.

Inositol (1,4,5)-trisphosphate (IP_3) can induce Ca^{2+} release from the endoplasmic reticulum of many cell types (e.g. see Berridge, 1987). The IP_3 formation is initiated by an activation of the phospholipase C (PLC) resulting in the generation of 1,2-diacylglyserol (DAG) and IP_3 (e.g. see Woodcock, 1995). The G-protein linked PLC was shown to be Ca^{2+} dependent in isolated cardiac membrane preparations (Renard & Poggioli, 1990, DeJonge *et al.* 1995). Hirata *et al.* (1984) first showed that IP_3 can induce a slow release of Ca^{2+} from cardiac SR vesicles, but opposite results also exist (Movesian *et al.* 1985). More recently, it has been shown quite convincingly that IP_3 can induce Ca^{2+} release in skinned cardiac preparations (Vites & Pappano, 1990, 1995). This is not surprising, because IP_3 receptors are present in both ventricular and atrial myocytes (Kijima *et al.* 1993) and also in cultured neonatal cardiac cells (Fitzgerald *et al.* 1994). Kentish *et al.* (1990) used flash photolysis of "gaged" IP_3 to rapidly release biologically active IP_3, and showed that IP_3 can activate contraction. They also found that high concentrations of IP_3 could induce SR Ca^{2+} release but that the rate and extent was much lower than for CICR. These studies led to the conclusion that IP_3 is not the primary mechanism releasing Ca^{2+} from the SR, but may be

physiologically important in the modulation of the $[Ca^{2+}]_i$ by increasing the Ca^{2+} sensitivity of the CICR (Nosek *et al.* 1986) or modulating the Ca^{2+} oscillations (Zhu & Nosek, 1991). The PLC activity is under hormonal control in the heart. For example, activation of cardiac α_1-adrenergic receptors has been reported to increase the IP_3 production (Schmitz *et al.* 1987, Poggioli *et al.* 1986), preceding the increase in the contraction force (Scholz *et al.* 1992). Thus, IP_3 may be an important physiological mechanism modulating the cardiac $[Ca^{2+}]_i$ and contractile force in response to hormones and pharmacological agents. A



simplified overview of the CICR modulation is presented in figure 3.

Fig. 3. Modulation of the calcium-induced calcium release in the cardiac myocytes. (A) A prototypical calcium-induced calcium release, where incoming calcium from L-type Ca^{2+} channels triggers further release from SR by activating ryanodine receptors (RyRs). (B) A scheme where Na^+/Ca^{2+} exchanger participates to CICR by providing additional Ca^{2+} influx during the action potential, able to augment the SR Ca^{2+} release. (C) Activation of phospholipase C (PLC) in the cell membrane leading to formation of IP_3, which releases calcium from SR by activating IP_3 receptors (IP_3R) in the SR.

2.3.5. Function and regulation of the myofilaments

The E-C coupling in cardiac myocytes involves a transient rise in the $[Ca^{2+}]_i$ by CICR followed by the Ca^{2+} binding of the contractile element and subsequent contraction of the muscle. The effector in this scheme, the contractile element, consists of several proteins which interact with each other (e.g. Warber & Potter, 1986). The functional unit in the muscle cell, called the sarcomere, consists basically of two types of myofilaments (Fig. 4.).

The thin filament contains two major proteins. A globular protein called actin is polymerized to form twisted, two stranded filaments (Fig.4.). Tropomyosin molecules are located along each strand of the thin filament, which is composed of two separate polypeptide chains. The regulatory unit of the thin filament is troponin complex containing three regulatory subunits, TnT (Tropomyosin binding), TnI (Inhibitory) and TnC (Calcium binding) (e.g. Farah & Reinach, 1995).

The thick filament contains merely myosin, having heavy and light chains. The tails of myosin heavy chains form the main axis of the thick filament. Most of the heavy chain has an α-helical structure, and two strands are twisted around each other in a supercoil that forms a long, rigid " tail". The heads form the crossbridges, which interact with the thin filaments, contain the site of ATP hydrolysis and have two light chains associated with each head (Figure. 4. C.). In the presence of sufficient $[Ca^{2+}]_i$, myosin can interact with actin, which greatly increases the ability of myosin ATPase to hydrolyze ATP and also allows transformation of chemical energy stored in ATP to mechanical energy and work. At rest the myosin heads (or crossbridges) extend from the thick filament perpendicular to the filament axis. Upon activation the crossbridges can interact with the thin filament. Force generation, or relative filament movement, is produced by rotation of the myosin head. The chemical steps involved in the crossbridge cycle are illustrated in the Figure. 4. C. At rest myosin (M) is mostly complexed with ATP (M-ATP) or in the rapidly equilibrated M-ADP-Pi, where ATP is hydrolysed, but energy has not been used. When $[Ca^{2+}]_i$ rises, the M-ADP-Pi interacts with actin (A) and phosphate is released. The actin-myosin passes through at least two energetic states where ADP remains bound. During these transitions the so-called "power-stroke" i.e. myosin head rotation takes place. The affinity of myosin for actin increases along this series of steps and is strongest after the ADP dissociates from the A-M complex. At normal $[ATP]_i$ this complex rapidly binds ATP and dissociation of actin and the M-ATP ensues. The cycle can then continue until the $[Ca^{2+}]_i$ declines, thereby stopping the myofilament interaction (in the M-ADP-Pi state) or until the ATP is depleted (rigor).

The part creating the link between $[Ca^{2+}]_i$ and the myofilament movement is the troponin, a complex consisting of three regulatory subunits (TnC, TnI and TnT), interacting during the step where Ca^{2+} ions activate the contraction. At rest when $[Ca^{2+}]_i$ is low, the Ca^{2+} binding sites of TnC are unoccupied. In this condition the TnI interacts strongly with actin thereby preventing the actin-myosin interaction. When $[Ca^{2+}]_i$ rises, Ca^{2+} binds to the Ca^{2+} specific sites in the TnC (Pan & Solaro, 1987). This may then strengthen the interactions between TnC and TnI and destabilize the interaction of TnI with actin (e.g. Parmacek & Leiden, 1991). This induces a shift of the troponin-tropomyosin to a more axial position, allowing the interaction between the myosin and actin, and contraction ensues. The tight interaction between the TnT and the tropomyosin is probably important in the transmission of these conformational changes along the thin filament (Fig. 4.D.) (e.g. Bers, 1993).

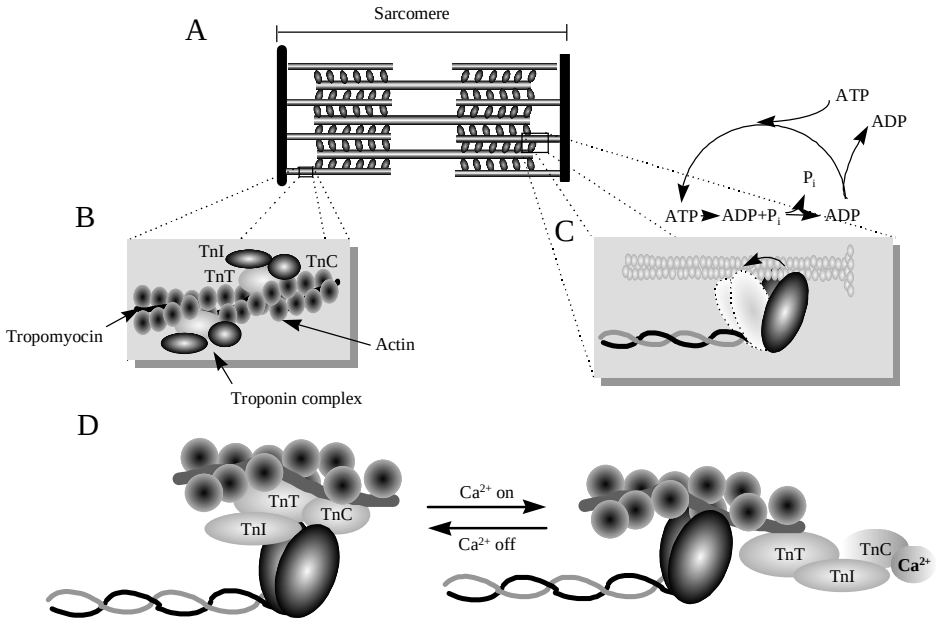


Fig. 4. Ultrastructure of the sarcomere and the molecular interactions during contraction. (A) Sarcomere ultrasructure, including thin and thick filaments. (B) Detailed structure of the thin filament consisting of tropomyosin, troponin complex and actin. (C) Chemical steps involved in the "power stroke" where myosin head rotates due to the hydration of the ATP molecules. (D) Calcium regulation of the actin-myosin interaction. For details and abbreviations see text.

The systolic $[Ca^{2+}]_i$ is the main determinant of the contraction force in the heart muscle. All modulation of the contraction force, however, is not due to $[Ca^{2+}]_i$. It is evident that contractile proteins themselves are physiologically regulated. For example, the inhibitory subunit of the troponin complex (TnI) is regulated by cAMP-dependent protein kinase (see e.g. Winegrad, 1984), affecting the Ca^{2+} sensitivity of the contractile element and thus the calcium-dependent force production.

2.4. Calcium-dependence of the ANP exocytosis

2.4.1. Calcium dependent exocytosis

The secretory process called the exocytosis includes transport and fusion of secretory vesicles into the plasmamembrane and the subsequent release of the vesicular content to the extracellular space. There appears to be two distinct types of exocytosis; constitutive and triggered exocytosis. The former seems to lack of specific control but it may well be due to

low threshold of the triggering machinery. In contrast, triggered exocytosis occurs in response to the generation of second messengers (e.g. Knight *et al.* 1989, Almers 1990). The dominant role of Ca^{2+} as a second messenger in the exocytosis of many excitable cells lead to so-called calcium hypothesis as early as 1968 (Douglas, 1968). This hypothesis was based on the following observations 1) Extracellular calcium is required to evoke transmitter release, 2) Ca^{2+} channels allow influx of Ca^{2+}, the magnitude of which determines the amount of transmitter release, and 3) procedures that elevate intracellular calcium concentration induce exocytosis and blocking the Ca^{2+} influx abolishes secretion. In addition to the extracellular sources of calcium needed for exocytosis also intracellular stores may elevate $[Ca^{2+}]_i$ during exocytosis (Penner & Neher, 1988b). The concept of the Ca^{2+} control of exocytosis also appears to apply to non-excitable cells which are lacking voltage-gated Ca^{2+} channels, including mast cells (Foreman *et al.* 1977, Kanno *et al.* 1973, Penner & Neher, 1988a), neutrophils (Rubin *et al.* 1981) and platelets (Feinman & Detwiler, 1974). In addition to this, when stimulated by secretagogues, several types of non-excitable cells show increase in $[Ca^{2+}]_i$ (Tsien *et al.* 1984). The Ca^{2+} sensitivity of the exocytotic process can also be regulated. The production of DAG increases the Ca^{2+} sensitivity of the process, when excocytosis can be triggered at very low $[Ca^{2+}]_i$ (Knight *et al.* 1989). In rat pituitary gonadotropes the luteinizing hormone can even be released by either calcium or PKC activity increase (Billiard *et al.* 1997). This would allow secretion from a single population of vesicles to be initiated by alternative stimuli, allowing secretion from different pools of vesicles to be controlled differentially by different stimuli (Billiard *et al.* 1997).

2.4.2.Calcium regulation of the ANP secretion

ANP is a cardiac hormone that is secreted primarily by atrial myocytes in response to a variety of stimuli including stretch, high external $[Ca^{2+}]$ and several hormones (see e.g. DeBold *et al.* 1996). In atrial myocytes ANP is stored and transported in specific secretory vesicles (e.g. Ruskoaho, 1992). In this respect the ANP release is a typical exocytotic process. Parallel to other secretory systems it has been suggested that calcium is also the second messenger in the ANP secretion. However, ANP secretion from the cardiac myocytes differs from the prototypical Ca^{2+} regulated exocytosis. In cardiac myocytes, $[Ca^{2+}]_i$ fluctuates following the heart rhythm, triggering contraction during each systolic calcium peak (e.g. Reiter, 1988). Thus sustained Ca^{2+} signals for exocytosis as they would normally be interpreted, are not present. The role of Ca^{2+} ions in the ANP secretion has been studied in different animal models by measuring ANP secretion and simultaneously manipulating $[Ca^{2+}]_i$. In isolated spontaneously beating rat heart the calcium ionophore A23187 induces ANP secretion (Ruskoaho *et al.* 1985). ANP secretion can also be induced by Bay K8644, a substance which can directly activate L-type Ca^{2+} channels in isolated beating heart (Ruskoaho *et al.* 1986), paced atria (Schiebinger, 1989) and in isolated myocytes (Matsubara *et al.* 1988). Supporting this, acute elevation of the extracellular calcium concentration alone is able to induce ANP secretion (Wong *et al.* 1991). On the other hand, inhibition of the voltage-activated calcium channels with nifedipine or verapamil inhibits the ANP secretion (Iida & Page, 1989). The basal ANP secretion can also be reduced by ryanodine (Katoh *et al.* 1990), indicating that SR may have a role in the secretion process.

On the basis of experiments where extracellular Ca^{2+} has been reduced by Ca^{2+} chelators it has been suggested that Ca^{2+} acts as a negative modulator in the ANP secretion (DeBold &DeBold, 1989, Iida & Page, 1989). In these experiments the rat atrium or cultured rat myocytes are subjected to Ca^{2+} -free medium or Ca^{2+} ions are rapidly removed from extracellular space by Ca^{2+} chelators, and rise in the ANP secretion has been observed (DeBold &DeBold, 1989, Deng & Lang, 1992). The experimental design in these experiments is, however, rather obscure if the aim is to reduce the $[Ca^{2+}]_i$, because it is known that removing the Ca^{2+} ions from the extracellular space causes liberation of Ca^{2+} ions from intracellular stores (Penner &Neher, 1988a). This will lead to an increase in $[Ca^{2+}]_i$ and naturally increase the ANP secretion. Despite the growing amount of evidence supporting the idea that ANP secretion is a Ca^{2+} dependent process, direct evidence linking ANP secretion and calcium together is still lacking.

2.5. Regulation and actions of intracellular pH in cardiac myocytes

2.5.1. Control of pH_i in cardiac myocytes

Protons tend to bind to macromolecules and thus are usually present at very low concentrations in biological solutions. This property is the basis for buffering power of the cell interior (e.g. Putnam, 1995). A variety of weak acids and bases can bind H^+ ions through reversible equilibrium binding reactions. Thus, a weak acid in solution obeys the equilibrium reaction,

$$HA \rightleftarrows H^+ + A^- \qquad (7)$$

where HA is the weak acid and A^- is the conjugate weak base. This equilibrium is described by an apparent equilibrium constant, K'_a, as

$$K'_a = aH \cdot \frac{[A-]}{[HA]} \qquad (8)$$

in its logarithmic form this equation is called Henderson-Hasselbach equation,

$$pH = pK'_a + \log\frac{[A-]}{[HA]} \qquad (9)$$

where pK'_a is $-\log K'_a$. In the living cells the main acid-base pair involves hydration of CO_2 and the dissociation of the resulting carbonic acid into H^+ and bicarbonate as

$$pH = pK'_a + \log\frac{[HCO_3^-]}{\alpha \cdot P_{co_2}} \qquad (10)$$

where α is the solubility coefficient of CO_2 in a given solution and partial pressure of CO_2 (PCO_2). The intracellular pH is not, however, solely determined by the equation (10). Several processes can contribute to the acid loading of the cell, including metabolic production of the acid, passive flux of H^+ trough plasmamembrane and to and from intracellular organelles. So, in order to maintain the steady-state pH_i, the rate of acid loading and extrusion have to be equal. On the other hand, pH_i is not simply due to the passive distribution of protons across the membrane, but in many cell types even the steady state pH_i is regulated. Assuming that the extracellular pH is 7.4 and V_m is -60mV, passive distribution of protons would cause the pH_i to be ca. 6.4 according the Nernst equation. It is evident that the pH_i of the cells is more alkaline than 6.4, usually between 6.8-7.2, indicating active regulation of the pH_i of the cells (Putnam, 1995). This active regulation is due to the activity of several integral proteins in the surface membrane of the cells, specialised for the active transport of acids and bases across the membrane. Several mechanisms are reported for cellular regulation of pH_i in cardiac myocytes. These include Na^+/H^+ antiport and the Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchangers (Putnam, 1995). The Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger are activated by intracellular acidosis (Ellis & MacLeod, 1985, Lagadic-Gossmann *et al.* 1992, Grace *et al.* 1993) and Na^+-independent Cl^-/HCO_3^- exchanger by intracellular alkalinization (Kusuoka *et al.* 1994).

2.5.2. pH as a modulator of the function of the cardiac myocytes

Intracellular pH is an important aspect of the intracellular environment. Virtually all cellular processes can be affected by changes in the intracellular pH, including metabolism, membrane potential, cell growth, calcium balance and contraction. Changes of intracellular pH are also often one of the responses to several agents like hormones, transmitters and pharmacological agents. Acidosis causes a significant decrease of the contraction force in isolated cardiac muscle (Cingolani *et al.* 1970, Ricciardi *et al.* 1986, Vaughan-Jones *et al.* 1987, Bountra & Vaughan-Jones, 1989), in isolated whole heart (Eisner *et al.* 1987) and even in neonatal cultured myocytes (Kohmoto *et al.* 1990). The main mechanism of the acidosis-induced contraction decline seems to be the reduction of the Ca^{2+} sensitivity of the contractile element by protons. It has been shown that much of the shift of the myofilament sensitivity could be attributed to a decrease in the affinity of Ca^{2+} binding to cardiac troponin C (Blanchard & Solaro, 1984). This pH effect is also amplified by a pH-sensitive change in the affinity of troponin I for troponin C (El-Saleh & Solaro, 1988, Solaro *et al.*1989). On the basis of the reduction of the contraction force by acidosis one would expect that the systolic $[Ca^{2+}]_i$ would also be diminished during acidosis. It is therefore quite surprising that acidosis causes an increase of the systolic and diastolic $[Ca^{2+}]_i$ (Allen & Orchard, 1983). Both simulated metabolic acidosis produced by application of lactate (Cairns *et al.* 1993, Terracciano & MacLeod, 1997), and respiratory acidosis produced by manipulating CO_2 (Allen & Orchard, 1983), produce similar changes in the $[Ca^{2+}]_i$ (Orchard & Kentish, 1990).

Intracellular protons interact with the calcium binding molecules by means of

competitive binding to the same binding sites. Therefore it is natural that protons inhibit calcium handling molecules in the cardiac myocytes. Protons inhibit calcium influx by blocking L-type Ca^{2+}-channels in a dose-dependent manner (Kaibara &Kameyama, 1988, Irisawa & Sato, 1986, Chen *et al.* 1996). The reduced influx of calcium is at least partly compensated by the simultaneous block of Na^+/Ca^{2+}-exchanger (Doering & Lederer, 1993, 1994), inhibiting the Ca^{2+} efflux during diastole. The block of Na^+/Ca^{2+}-exchanger would also cause Na^+ loading of the cells, amplified by activation of the Na^+/H^+ antiport during acidosis. Increased $[Na^+]_i$ would move the reversal potential of the Na^+/Ca^{2+}-exchanger towards more positive potential. This compensates the reduced calcium translocation capacity of Na^+/Ca^{2+}-exchanger leading to increased $[Ca^{2+}]_i$. These interacting mechanisms would explain the transient nature of the acidosis-induced changes in the contraction force and $[Ca^{2+}]_i$(Bers, 1993). Opposing these changes that act to rise the $[Ca^{2+}]_i$, acidosis inhibits the Ca^{2+} release from SR (Orchard, 1987) by inhibiting the calcium release channels in the SR (Xu *et al.* 1996, Kentish & Xiang, 1997) via modulation of the conducting and gating behaviour of the single RyR channels (Rousseau & Pinkos, 1990). The wide variety of other effects of acidosis include modulation of the cell-to-cell coupling (Reber & Weingard, 1982) and inhibition of the transient outward potassium current (I_{to}) (Xu & Rozanski, 1997), making the evaluation of the effects of acidosis even more complicated.

As a result, acidosis causes reduction of the contraction force together with increased $[Ca^{2+}]_i$. The most prominent change in the electrical behaviour of the cardiac myocytes caused by acidosis is the shortening of the action potential (Gasser & Vaughan-Jones, 1990) accompanied by oscillatory afterpotentials (Kurachi, 1982). The afterpotentials are probably caused by the calcium overload during acidosis, predisposing the cardiac muscle to arrhythmias (e.g. see Orchard & Cingolani, 1994).

2.6. Mechanosensors in the cardiac myocytes

2.6.1. General features of mechanotransduction in biological systems

Mechanotransduction is a process where mechanical energy is transformed into electrical signals or enzymatic activity enabling the cell to respond to the stimulus. Many senses rely on the mechanotransduction like senses of touch, hearing and balance, but mechanotransduction is also involved in the control of muscle contraction, joint rotation, cardiovascular function and many other physiological mechanisms. Mechanotransduction is conventionally viewed as a three-stage process; 1) the stimulus is mechanically coupled to the receptor cell, 2) the deformation is transduced into an electrical signal, and 3) electrical signal is encoded into action potentials for transmission to the nervous system (e.g. see French, 1992). Cardiac myocytes cannot respond to the mechanical stimulation in a conventional way, but rather the mechanotransduction involves modulation of several physiological processes like contraction force, action potential shape, calcium balance, exocytosis and perhaps enzymatic activity.

2.6.2. Stretch-activated ion channels

The mechanosensitivity could be explained by the activation of specific ion channels gated by mechanical stimuli. After the initial discovery of these channels in skeletal muscle cells (Guharay & Sachs, 1984), they have been found from wide variety of species and cell types (e.g. see Morris, 1990, Sackin, 1995), also from the cells not specialised to mechanotransduction, like cardiac myocytes. The stretch-activated channels (SA-channels) present in cardiac myocytes can be divided into three distinct types on the basis of their ion selectivity. Cation selective SA-channels are relatively non-selective over cations, including Na^+, K^+ and Ca^{2+}, and non-permeant to anions (Kim, 1993, Ruknudin *et al.* 1993, Bustamante *et al.* 1991). The second group of channels is more selective, passing through mainly potassium ions (Kim, 1992, VanWagoner, 1993). Third channel type is selective to anions over cations, and in physiological situations permeable mainly to chloride ions (Hagiwara *et al.* 1992).

Although SA-channels are present in the cardiac myocytes, the physiological role of these channels is not known. In theory, stretch of the cell membrane produces a stretch-dependent current if the SA-channels operate at the physiological range of stretch. This kind of stretch-activated cation current has been documented in rat atrial myocytes (Kim, 1993). Current showed clear stretch-dependence in the whole-cell patch clamp configuration with the reversal potential at -3.2 mV (Kim, 1993). Activation of this current would cause a sodium and calcium influx during stretch. Simultaneous activation of the potassium selective SA-channels would stabilize the membrane potential (Kim, 1992).

One of the problems in the SA-channel studies at the tissue level is the lack of specific blockers of these channels. Several pharmacological agents like aminoglycoside antibiotics (Winegar *et al.* 1996), Gd^{3+} (Yang & Sachs, 1989) and amiloride (Lane *et al.* 1991) block SA-channels. However, all of these are rather non-specific in their actions. Aminoglycoside antibiotics block also voltage activated Ca^{2+} channels (Haws *et al.* 1996), Gd^{3+} the L-type Ca^{2+} channels (Lansman, 1990) and delayed rectifier potassium channels (Hongo *et al.* 1997). Amiloride inhibits the Na^+/H^+ exchanger (Simchourtz & Gragoe, 1986). Despite the non-specificity of these agents, they have been used in studying the role of the SA-channels in the stretch-induced changes in different cardiac preparations. It has been shown that SA-channel blockers, like Gd^{3+}, inhibit the stretch-induced changes in the function of the heart muscle including arrhytmias (Hansen *et al.* 1991), changes in contraction force (Lab *et al.* 1994) and rise in the $[Ca^{2+}]_i$ (Sigurdson *et al.* 1992).

2.6.3. Myofilaments

On the premise that mechanical load results in an increase in the contraction force in the cardiac myocytes, stretch sensitivity of the contractile element could serve as an additional mechanosensitive mechanism in the cardiac muscle. Tension developed by isolated cardiac muscle increases with increasing the sarcomere length from ca. 1.4 to 2.5 µm (Fabiato & Fabiato, 1975). The molecular basis of this so called Frank-Starling behaviour of cardiac muscle is not known in detail. The role of the calcium binding part of the contractile element, troponin C (TnC) (Pan & Solaro, 1987), has been studied as a plausible candidate of the stretch sensitive component in the cardiac muscle (TerKeurs *et al.* 1980, Hibberd &

Jewell, 1982). The data obtained from the skinned cardiac preparations indicates that the Ca^{2+} affinity of the TnC is sensitive to sarcomere length (Kentish *et al.* 1986). Supporting this, it has been found that by exchanging cardiac TnC for skeletal muscle TnC the stretch-sensitivity of skinned cardiac preparation could be abolished (Babu *et al.* 1988, Gulati *et al.* 1990). The precise mechanism of the stretch-induced affinity change of the cardiac TnC is still not resolved, but if true, it explains a part of the F-S-behaviour of the cardiac muscle (for review see, e.g. Parmacek & Leiden, 1991). It has also been suggested that variations in the number of interacting cross-bridges may be the critical factor (Hofmann & Fuchs, 1988, Allen & Kentish, 1988), because actin-myosin interactions can modulate the troponin C affinity for Ca^{2+} (Bremel & Weber, 1972). Supporting this, when actin-myosin interactions were inhibited, Ca^{2+} binding to TnC diminished and length-dependence of Ca^{2+} binding disappeared (Hofmann & Fuchs, 1987). Thus, based on the actin-myosin interaction studies, it was suggested that the true length-sensing structure in the cardiac contractile element is the cross-bridge attachment, rather than the TnC (Fuchs & Wang, 1997). They concluded that the cross-bridges communicate information about muscle length to the regulatory proteins in the thin filament. This is a justified hypothesis, because it seems that sarcomere length also increases the actomyosin ATPase Ca^{2+} sensitivity (Kuhn *et al.* 1990) and ATP consumption (Wandenburg *et al.* 1997). However, the question how information about the muscle length is conveyed to the TnC remains to be answered.

2.6.4. Stretch-sensitive enzymatic cascades

In addition to the immediate or fast cardiac responses to mechanical load, long-lasting stretch seems to trigger several sustained cellular signals, leading to e.g. cardiac hypertrophy (e.g. Sadoshima & Izumo, 1997). The immediate stretch-induced changes could be explained by changes in the calcium balance and/or function of the contractile element, but the sustained long-term responses require usually changes in the enzymatic activity of the cells. In cultured neonatal cardiac myocytes stretch activates several enzymes, like phospholipases C, D and A_2, tyrosine kinase, mitogen-activated protein kinases (MAP), protein kinase C (Sadoshima & Izumo, 1997) and probably many others (Komuro *et al.* 1991, Sadoshima & Izumo, 1993). A group of stretch-activated kinases are even named after their ability to respond to different forms of stress, among these mechanical stretch, the stress-activated protein kinases (SAPK, Komuro *et al.* 1996). Despite their seeming stretch-sensitivity the actual mechanotransduction process probably involves more complex mechanisms than direct activation of the SAPKs by mechanical load.

In adult frog ventricle stretch stimulates the production of both cGMP and cAMP but, more importantly, the ratio of the amount of cAMP/cGMP decreases upon stretch (Singh, 1982). Recently Todaka *et al.* (1998) reported that $[cAMP]_i$ is increased when canine heart is stretched. The mechanism of this cyclic nucleotide regulation by stretch is not known. It was proposed that this might contribute to the calcium sensitivity change of the contractile element during stretch, but both cGMP and cAMP regulate also Ca^{2+}-channels thus participating to the $[Ca^{2+}]_i$ regulation of the cells (Hartzell & Fischmeister, 1986, Hove-Madsen *et al.* 1996).

In smooth muscle cells stretch increases the phospholipase C (PLC) activity, evidently via the influx of Ca^{2+} ions through a gadolinium-sensitive pathway (Matsumoto *et al.* 1995).

The PLC activity regulates the IP_3 and DAG concentrations in the cells (e.g. Woodcock, 1995). In cardiac myocytes this kind of ion channel-PLC coupling has not been reported. If stretch increases the PLC activity in the cardiac myocytes, it might be manifested as an increase of $[IP_3]_i$. It is known that, at least in cultured cardiac myocytes, stretch does increase the IP_3 formation (Dassouli *et al.* 1993). If the $[IP_3]_i$ of the cardiac myocytes is increased by stretch this would have a great impact on the calcium balance of the cells, since IP_3 is able to release Ca^{2+} from the SR in cardiac myocytes (Vites & Pappano, 1990, 1995).

2.7. Stretch-induced changes in the function of the cardiac myocytes

2.7.1. Changes in the calcium balance of the myocytes by stretch

Allen & Kurihara (1982) first showed that increase of the ventricular muscle length augments the Ca^{2+}-transients during systole, measured in preparations injected with aequorin. The change in the Ca^{2+}- transients showed similar slow time course over a period of minutes as the development of the contraction force after a step-like increase of the muscle length. Later this finding has been verified by using ratiometric Ca^{2+} indicators like Fura-2 (Hongo *et al.* 1996, Kentish & Wrzosek, 1998). The slow augmentation of the systolic $[Ca^{2+}]_i$ is a property of individual myocytes, since it is present also in isolated cardiac myocytes (Hongo *et al.* 1996). The effects of stretch on the diastolic $[Ca^{2+}]_i$ leads to more variable results. It was originally suggested that an increase of the diastolic $[Ca^{2+}]_i$ during stretch might augment the Ca^{2+} transients during systole (Allen *et al.* 1988). Some of the recent studies have, however, somewhat compromised this idea, showing no change in the diastolic $[Ca^{2+}]_i$ during augmentation of the systolic $[Ca^{2+}]_i$ by stretch (Hongo *et al.* 1996, Kentish & Wrzosek, 1998). With or without change in the diastolic $[Ca^{2+}]_i$, augmentation of the calcium transients requires increased calcium influx from extracellular space and/or increased calcium release from the SR. Increase of the systolic $[Ca^{2+}]_i$ might be due to 1) augmentation of the triggering Ca^{2+} through L-type channels, 2) increase of the stored Ca^{2+} in the SR or 3) increased amount of open Ry-receptors during excitation. The stretch-sensitivity of the L-type current has been described (Langton, 1993) but this feature is not present in the rat ventricular myocytes (Hongo *et al.* 1996) or in guinea-pig ventricular myocytes (White *et al.* 1995). Using so called rapid cooling method Bluhm & Lew (1995) showed that the amount of stored calcium is increased during stretch, explaining at least partly the augmentation of the calcium transients.

Accepting that stretch augments the systolic $[Ca^{2+}]_i$ the underlying mechanism has to be considered. The simplest mechanism would be the activation of the Ca^{2+} permeable SA-channels like the cation selective SA-channels (Kim, 1993). Allen *et al.* (1988) suggested that activation of the SA-channels leads to increase in the diastolic $[Ca^{2+}]_i$ which is known to augment the Ca^{2+} transients (Frampton *et al.* 1991) by increasing the SR calcium content in a time dependent manner (Orchard *et al.* 1998). Whether calcium influx via SA-channels is large enough to rise the diastolic $[Ca^{2+}]_i$ would naturally dependent on the amount of stretch applied. Stretch increases $[Ca^{2+}]_i$ in cultured chick cardiac myocytes and this increase

can be blocked by 20 µM gadolinium (Sigurdson *et al.* 1992), a trivalent lanthanide known to block SA-channels (Yang & Sachs, 1989). In isolated guinea pig ventricular myocytes stretch causes an increase in the resting $[Ca^{2+}]_i$ (LeGuennec *et al.* 1991, White *et al.* 1993). This increase in the $[Ca^{2+}]_i$ can be inhibited by streptomycin (Gannier *et al.* 1994), blocker of SA-channels (Winegar *et al.* 1996). It has also been proposed that this calcium flux during stretch depolarizes the membrane potential promoting more calcium influx with subsequent depolarization and so creating a positive feed-back loop (Gannier *et al.* 1996). However, this would lead to immediate calcium overload of the cell and malfunction of the Ca^{2+} dependent processes, which cannot be the case, since cardiac muscle responds to stretch by an increase of the contraction force. If present, this kind of calcium overload is not part of the normal physiology, but rather a pathological phenomenon. Although SA-channels may have a role in the stretch-induced changes in the calcium balance of cardiac myocytes, this question clearly warrants more investigation. On the premise that no change in the diastolic $[Ca^{2+}]_i$ was observed during stretch, Kentish & Wrzosek (1998) suggested that the Ca^{2+} influx needed for Ca^{2+} transient augmentation occurs during systole. This could involve cAMP or IP_3 dependent mechanism (Kentish & Wrzosek, 1998). A cAMP-dependent mechanism is one of the plausible candidates mediating the stretch -induced changes in contraction and Ca^{2+} transient, because the $[cAMP]_i$ is increased upon stretch in the canine ventricle (Todaka *et al.* 1998). However, the role of these different mechanisms is still controversial.

2.7.2. Frank-Starling relation in the heart

The contraction force of cardiac muscle depends, among other things, on the mechanical load to which the muscle is subjected (Frank-Starling relation). Although this intrinsic property of cardiac muscle has been known over a century (see, e.g. Levick, 1995), still the mechanisms are mostly unknown. The response of whole heart (Lew, 1988), isolated cardiac preparations (Kentish & Wrzosek, 1998), and isolated cardiac myocytes (White *et al.*1995) to the mechanical load are very much alike, indicating that the F-S-relation arises from the function of individual myocytes. A step-like increase of the cardiac muscle length results in a two-phasic increase in the contraction force (Parmley & Chuck, 1973). Immediately after the length change the contraction force is increased, followed by a secondary, slow increase of force, with a time course of several minutes (Parmley & Chuck, 1973, Chuck & Parmley, 1980). Originally this two-step process was considered to be due to the increased affinity of the TnC to calcium when the muscle was stretched (Allen & Kurihara, 1982, Allen & Kentish, 1988). Later the role of the changes in the $[Ca^{2+}]_i$ were recognized (Allen *et al.* 1988). Quite recently it was shown that, during the slow phase of the contraction development, the affinity of the TnC is unaltered, although it is changed by the initial length change (Kentish & Wrzosek, 1998). The result shows that the affinity change of TnC can explain the immediate response of the cardiac muscle to stretch, but not the slow part of the contraction development, as previously suggested (Hongo *et al.* 1996). The slow part of the contraction development is accompanied by an increase of the systolic $[Ca^{2+}]_i$ (e.g. Allen & Kurihara, 1982, Hongo *et al.* 1996), solely causing the slow contraction development (Kentish &Wrzosek, 1998). This is supported by the notion that the stretch-induced increase in contraction force is augmented with higher external $[Ca^{2+}]$ in isovolumic rat heart

(Stefanon *et al*. 1990). It can be concluded that the F-S-relation consist at least two different mechanisms, change in the myofilament function and augmentation of the systolic $[Ca^{2+}]_i$.

2.7.3. Mechanoelectrical feedback in the cardiac myocytes

Mechanoelectric feedback, where electrophysiological changes follow mechanical stimulation of the cardiac muscle, has been widely studied (for reviews see, Lab, 1996, Crozatier, 1996, Lab & Dean, 1991, Taggart, 1996, Dean & Lab, 1989, Nazir & Lab, 1996, Franz, 1996). Stretch has a number of effects on the electrical parameters in the myocardium, including changes in the action potentials (Lab, 1978, 1980, Boland & Troquet, 1980, Nakagawa *et al.* 1988), changes in the excitability (Dean & Lab, 1990, Tung & Zou, 1995) and changes in the conduction properties of the muscle (Penefsky & Hoffman, 1963, Spear & Moore, 1972, Dominguez & Fozzard, 1979). In intracellular voltage recordings it has been considered difficult to separate genuine stretch-induced voltage changes from the artifactual ones (Lab, 1978). Mechanical stimulus during intracellular recording may cause artifacts by damaging the cell membrane and/or by unstabilizing the cell-electrode impalement. Considering the technical difficulties, it is not surprising that the results from stretch-induced electrical changes have lead to variable results (see, Crozatier, 1996). For example, the results of stretch-induced changes in resting potential (r.p.) of the cardiac myocytes are controversial. It has been shown that stretch causes depolarization of the r.p. up to -30 mV (White *et al.* 1993). Opposing this, some studies have shown that even the direction of the change varies depending on the amount of stretch applied. Moderate stretch may depolarize the r.p., but severe stretch could cause an opposite change, a hyperpolarization of the r.p. (Nakagawa *et al.* 1988).

In very few studies the stretch-induced changes have been linked to the normal physiological consequences of stretch, like the phasic increase of the contraction force (F-S-relation). From the physiological point of view, the electrical changes in the myocyte function are not independent phenomena, but rather linked to the complex modulation of the contraction force, calcium balance and other effects of stretch. As an indication of this, it has been shown that action potential shape modulates the Ca^{2+} transient (Bouchard *et al.* 1995) and vice versa (DuBell *et al.* 1991). On the relationship between the slow development of contraction force and action potential shape, Allen (1977) showed that the contractile changes are accompanied by the lengthening of the late duration of the AP in cat papillary muscle. The slow time course and co-occurrence of these changes suggest that the physiological modulation of action potentials by stretch may include also other mechanisms than direct stretch-activated conductances of the cell membrane.

The stretch-induced changes in the myocardial electrical function have also been of some clinical interest, because stretch can induce arrhythmias directly or indirectly (for review see, Reiter, 1996). Extracellular, so called MAP-recordings (Monophasic Action Potentials) have been used to study the acute stretch-induced electrical changes in the heart (see e.g. Franz, 1996). Increase in ventricular volume and/or pressure in canine and rabbit heart results in a decrease of the amplitude and plateau of the action potential (Franz *et al.* 1989, 1992) when measured by MAP electrodes from the surface of the ventricle. These changes are parallelled by a decrease in the refractory periods (Calkins *et al.* 1989). Furthermore, stretch seems to trigger afterdepolarizations i.e depolarizations occurring before complete repolarization of the membrane after the AP (Franz *et al.* 1989). Several studies have demonstrated that rapid stretch of the whole ventricle can elicit premature ventricular excitations (PVEs) (Hansen *et al.* 1990, Stacy *et al.* 1992). The timing of the stretch pulse applied seems to be an important factor ruling the direction of the voltage change (Zabel *et al.* 1996). If the pulsatile stretch is applied during the MAP, the voltage response is

repolarizing, and if the stretch pulse is applied during the diastolic period, a depolarization ensues (Zapel *et al.* 1996). The fact that the sign of the stretch-induced voltage change could be reversed by voltage implies that the current generating the voltage change has a reversal potential between the tip of the AP (ca. +20-30 mV) and r.p. (-80–90 mV). Given that the reversal potential of the SA-channel current is between those voltages, it was considered that these voltage changes were caused by the direct activation of SA-channels (Zapel *et al.* 1996, for review see, Franz 1996). An additional evidence supporting this idea is given by the fact that the stretch-induced arrhythmias can be inhibited by gadolinium (Hansen *et al.* 1991, Stacy *et al.* 1992), a blocker of SA-channels (Yang & Sachs, 1989).

2.7.4. Stretch-induced ANP secretion

Although it is evident that mechanical load (or stretch) is one of the main triggers of the ANP secretion, less evident are the underlying mechanisms (see e.g. DeBold *et al.* 1996). When stretch is applied to the heart atrium, an increase of the ANP secretion occurs. However, when the stretch is sustained, the ANP secretion shows a decline within minutes (Schiebinger & Linden 1986a). This transient nature of the ANP secretion suggests that the ANP stores contain a readily releasable pool of ANP, released immediately as response to adequate stimuli (Dowsley *et al.* 1995). This also shows that although stretch is a trigger for ANP secretion, other mechanisms are likely to be involved in the sustained ANP responses. These may include adaptational mechanisms, or mechanisms triggered by stretch like activation of enzymatic cascades leading to enhanced synthesis and release of ANP (see e.g. Ruskoaho 1992).

Several mechanisms have been proposed for the transient stretch-induced ANP secretion. The stretch-induced changes in $[Ca^{2+}]_i$ may trigger ANP secretion, but the source of this "extra" calcium influx during stretch is not known (for review see, DeBold *et al.* 1996). The augmentation of stretch-induced ANP secretion by an increase of the extracellular $[Ca^{2+}]$ (Laine *et al.* 1996) suggests that the calcium needed for exocytosis comes through the plasmalemma. It has been suggested that SA-channel activation during stretch is able to induce ANP secretion, supported by the fact that Gd^{3+}, a blocker of SA-channels, inhibits the stretch-induced ANP secretion dose-dependently (Laine *et al.* 1994b). Since neither diltiazem (L-type Ca^{2+} channel blocker) nor $NiCl_2$ (T-type Ca^{2+} channel blocker) inhibit the stretch-induced ANP secretion, it can be concluded that the Gd^{3+} effects are not mediated by the block of voltage-activated Ca^{2+} channels. Furthermore, this also indicates that the ANP secretion is not mediated by modulation of L-or T-type channels during stretch. Calcium release from the SR seems to also have a prominent role since ryanodine (blocker of CICR channels in the SR) can inhibit the stretch-induced ANP secretion in the paced and non-paced rat atrium (Laine *et al.* 1994a).

Many of the studies concerning the stretch-induced ANP secretion are based on the hypothesis that stretch is sensed by the myocytes themselves, but cardiac muscle contains also many other cell types like endothelial cells. Stretch of the endothelial cells causes a production of endothelin (Sumpio & Widman, 1990), which is a potent stimulator of ANP secretion (Mäntymaa *et al.* 1990). Interestingly, endothelial cells contain SA-channels sensitive to Gd^{3+} (Naruse & Sokabe 1993), indicating that the stretch-induced ANP secretion block by Gd^{3+} does not rule out the possibility that the stretch-induced ANP secretion is

mediated by an interaction between myocytes and e.g. endothelial cells. However, this question clearly warrants more investigation.

3. Aims of the research

The effects of mechanical stimulation on the function of the heart muscle have been studied previously on the premise that stretch (pressure, length) changes the electrical behaviour of the myocytes leading to e.g. arrhythmias. In other studies the contractile function of the heart during stretch has been the main interest. The stretch-induced ANP secretion has been studied largely independently of the growing amount of data concerning other effects of stretch. This is partly due to the methodological difficulties faced in the heart mechanotransduction studies. As a result, there is only few studies where mechano-transduction is studied in its widest sense, including changes in ionic balance, contraction, ANP secretion and electrical behaviour of the myocytes. This study was designed to obtain a more holistic point of view on the mechanotransduction. Specifically, factors linking the different effects of stretch together were studied, including possible modulation of the mechanosensitivity by acidosis on the premise that it could reveal crucial information about the underlying mechanisms. Following specific questions were addressed:

1. What is the role of the SA-channels in the stretch-dependent activation of the rat atrium? This was studied by using Gd^{3+} to block SA-channels and by utilizing a mathematical model into which a SA-channel conductance could be incorporated.

2. Are the stretch-induced changes in AP, Ca^{2+}-balance and contraction causally linked to each other and what are the mechanisms activated upon stretch? Is stretch activated Ca^{2+} influx needed and what is the role of contractile element stretch sensitivity? These questions were studied by a mathematical model, and by experiments where simultaneous measurement of intracellular $[Ca^{2+}]_i$, action potentials and contraction force were performed from the rat atrium following stretch.

3. Is the stretch-induced ANP secretion accompanied by changes in the $[Ca^{2+}]_i$? This question was studied by measuring the calcium changes in the same time when the ANP secretion is augmented by stretch.

4. Can interactions of protons and Ca^{2+} in the cytosol modulate the stretch-induced changes in the rat atrium? By producing intracellular acidification with propionate the interactions of stretch and acidosis were studied with reference to $[Ca^{2+}]_i$, action potentials, contraction and ANP secretion.

4. Materials and methods

4.1. Chemicals

HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethansulfonic acid) and propionate (propionic acid) were obtained from Sigma Chemical Co, St.Louis, MO, USA; KCl, glucose, $CaCl_2$, $MgCl_2$ from E. Merck, Darmstadt, Germany; NaCl from FF-Chemicals AB, Sweden; BCECF (2´,7´-bis-(2-carboxyethyl loading)-5-carboxyfluorescein, tetraacetoxymethyl ester), Nigericin, Indo-1 AM, Pluronic, Probenecid (p-[Dipropylsulfamoyl]-benzoic acid) from Molecular Probes Europe BV, Netherlands. $GdCl_3$ from Aldrich Chemical Company, Milwaukee, USA and diltiazem from Orion-Farmos Pharmacological Company, Turku, Finland.

4.2. Animals, in vitro atrial preparation, and measurement of contraction force

Male Sprague-Dawley rats weighing 290-400 g were used. The rats were decapitated, and the hearts were rapidly removed and placed in oxygenated (ca. 10 ^{o}C) buffer solution (137 mM NaCl; 5.6 mM KCl; 2.2 mM $CaCl_2$; 5.0 mM HEPES; 1.2 mM $MgCl_2$; 2.5 mM glucose: pH 7.4), which was also used at 37 ^{o}C for superfusion of the atrium. After this, an X-branch polyethylene adapter was inserted into the lumen of the left atrium appendix, and the tissue was placed in a constant temperature (37 ^{o}C) organ bath. Another tube with smaller diameter was inserted inside the adapter in order to carry perfusate inflow into the lumen of the atrium. The outflow from the lumen came from one cross branch of the X-cannula. The stretch of the tissue was produced by increasing the intra-atrial pressure simply by rising the height of the tip of the outflow tube. The other cross branch of the X-cannula was connected to a pressure transducer (TCB 100, Millar instruments, Inc.,USA), so that the pressure in the lumen of the atrium could be recorded. Inflow and outflow (3 ml/min) both to auricle lumen and to organ bath with constant temperature were controlled by a peristaltic pump (7553-85, Cole-Parmer Instrument Co., USA). The contraction force (pressure generated by the contraction) was recorded concomitantly with the sampling of perfusate (in ANP secretion experiments) or with the measurement of intracellular Ca^{2+}.

4.3. Measurement of the ANP secretion

The rat left atria were pre-incubated in normal HEPES buffer for 30 minutes before starting any interventions. When propionate was used in ANP experiments, it was applied 16 minutes before stretching the muscle, which was a sufficient time to achieve a steady-state pH_i. In all experiments the perfusate samples were collected with 4 minutes interval. The immunoreactivity of ANP from perfusate samples was determined by radioimmunoassay (RIA). Briefly, duplicate samples (100µl) of each perfusate fraction were incubated with the specific rat ANP antiserum (100µl) at the final dilution of 1/200000. Synthetic rat $ANP_{99\text{-}126}$ in the range 0-500pg/tube was used as a standard. $ANP_{99\text{-}126}$ was radioiodinated by chloramine-T technique followed by desalting on a sephadex G25 column (Pharmacia) and final purification by reverse phase HPLC using a Vydak C18 column (Separations Group, Hesperia, CA, USA). The tracer was added after 18-24 h incubation. After further incubation at +4 °C overnight, immunocomplexes were precipitated by double antibodies in the presence of polyethyleneglycol, followed by centrifugation (4000 g, 20 min). The radioactivity in the precipitates was counted with 12-channel Gamma counter (LKB-W, Turku, Finland). The sensitivity of the assay was 2 pg/tube and the intra-and interassay coefficients of variation were <10 % and <15 % respectively. The ANP antiserum cross-reacts 100 % with rat proANP but it does not recognize the N-terminal fragment of proANP, Brain Natriuretic peptide, C-type natriuretic peptide, vasopressin, angiotensin of endothelins (cross-reactivity <0.01%).

4.4. Molecular form of secreted ANP

The molecular form of secreted ANP was determined with HPLC. Lyophilized samples of the perfusates were dissolved in 0.4 ml 40 % acetonitrile-0.1 % trifluoroacetic acid in water. The samples were applied to a 7.8 x 300-mm ProteinPak-125 gel permeation HPLC column (Waters, Milford, MA) and eluted with the same solvent. The flow rate was 1 ml/min and fractions of 0.5 ml were collected. The fractions were dried in a Speed-Vac concentrator (Savant, Hicksville, NY), dissolved in buffer, and subjected to ANP radioimmunoassay. The column was calibrated with bovine serum albumin, purified rat proANP 1-126, synthetic ANP 99-126 and radioiodine. The recovery of immunoreactivity from the column was 55-80 %.

4.5. Electrophysiological recordings and data analysis

Membrane potentials were recorded using glass microelectrodes filled with a solution with 2 M K-acetate and 5 mM KCl, pH 7.0. The resistance of the electrodes in tissue was ca.100 MΩ. The electrode holder connected to a micromanipulator was a spring made of chlorided silver wire. Electrical stimulation (steps of 1 ms duration, 50 % over threshold voltage) was provided by a voltage stimulator (S44, Grass Instruments Co., USA) through bipolar platinum electrodes. Recorded membrane potentials (with 3 kHz sampling frequency) were amplified with an intracellular amplifier (Dagan 8100-1, Dagan Co., USA). Data was

analyzed with DT VEE (Data Translation Inc., USA) and MATLAB (The Math Inc. Natick, MA. USA) programs.

4.6. Calcium measurements

To record intracellular Ca^{2+} transients from the Indo-1 loaded rat atria, a fiber optic silica cable was lead through the bottom of the perfusion chamber to contact with the tissue. In the detection end, the fiber cable consists of ca. 100 unorganized fibers (diameter 100 µm), which are further divided into three individual fiber bunches consisting ca. 30 individual fibers each. One branch guided the 355 nm filtered excitation light to the tissue provided by 75 W xenon light source (Hamamatsu), and two remaining branches conducted the emitted light which was filtered (405±5nm and 495±5nm) and detected with photomultiplier tubes (Hamamatsu). The emission signal was further amplified (8x) and filtered with an adjustable filter Kemo (Kemo Copr., USA) at 50 Hz. The Indo-1 emission ratio (405/495) was calculated online from A/D converted (Data Translation) signal by Testpoint (Testpoint, Capital Equipment Corp., Mass., USA) custom created software. The atrium was paced with two platinum electrodes at 1 Hz.

For Ca^{2+} measurements the left atrial preparation was attached, prior to loading, to the perfusion system in order to measure the autofluorescence from each atria. The autofluorescence at both emission wavelengths was determined and was then subtracted from the signals after loading. Together with the autofluorescence, the contraction force (developed pressure) was measured in the low pressure (1 mmHg) which was compared to the contraction force after loading. For Indo-1AM loading the preparation connected to the plastic tube was attached to a separate loading chamber. In this chamber the atria were superfused for 25-40 min (flow 7 ml/min) with HEPES buffer (4 ml) containing; 10µM Indo-1 AM dissolved to 100 µl DMSO with 20 % Pluronic, 0.5 mM Probenecid and 1.5 % BSA. To avoid loading of the intracellular organelles such as SR and mitochondria the temperature in the chamber was kept between 25 -30 ºC during the entire loading period. After loading the fluorescence was ca. 20 times greater than the autofluorescence prior loading. The contraction force (developed pressure) in 1 mmHg before loading was 3.1±0.4 mmHg and 3.17±0.4 mmHg after the loading (ns. n=15). This indicates that Indo-1, as used here, does not itself buffer calcium ions enough to influence the contraction. It has been shown previously that when epifluorescence of the heart muscle is used in estimation of the intracellular calcium of the myocytes, part of the fluorescence signal may originate from cells other than myocytes (Shinozaki *et al.* 1993), forming a possible source of error. When cardiac tissue is loaded with fluorescence indicators like Fura-2 and Indo-1 through coronary arteries, a great portion of the epifluorescence comes from the endothelial cells (Shinozaki *et al.* 1993). Here this was tried to avoid by not utilizing the coronary circulation in loading, but instead used direct perfusion of the tissue. The source of fluorescence was also under visual control, i.e. when the atria were attached to the perfusion system the areas of clearly greater fluorescence intensity were avoided and the atria were measured from the areas with low total fluorescence intensity and no visually detectable “hot spots”. In some preparations the contraction and stretch of the tissue caused a prominent movement artifact, and these atria were not used.

When Indo-1 ratio is used to estimate the free $[Ca^{2+}]_i$, like in this study, possible sources of errors have to be specified. First, the relationship between Indo-1 ratio and $[Ca^{2+}]_i$ is not linear (Grynkiewicz *et al.* 1985). This non-linearity causes an underestimation of the true $[Ca^{2+}]_i$ at high Ca^{2+} levels. The difference between Indo-1 ratio and true $[Ca^{2+}]_i$ is about 9% at the peak value of Ca^{2+} during normal systolic Ca^{2+} transient in rat heart (Brandes *et al.* 1993). The nonlinearity results in larger underestimation when the Ca^{2+} transients are bigger. Thus, when the Ca^{2+} transients are observed to get bigger by some intervention, the true effect is always larger. Secondly, the intracellular pH has reportedly an effect on the Ca^{2+} binding by the fluorescent indicators, among them the indo-1 (Lattanzio, 1990). This was evaluated in the present study by determining the Indo-1 K_d at two different pH (7.0, 7.2) with and without propionic acid (8 mM) (see, III, IV). Figure 5. shows a schematic drawing from the setup used in the present study to measure action potential, contraction force, Indo-1 fluorescence and ANP secretion.

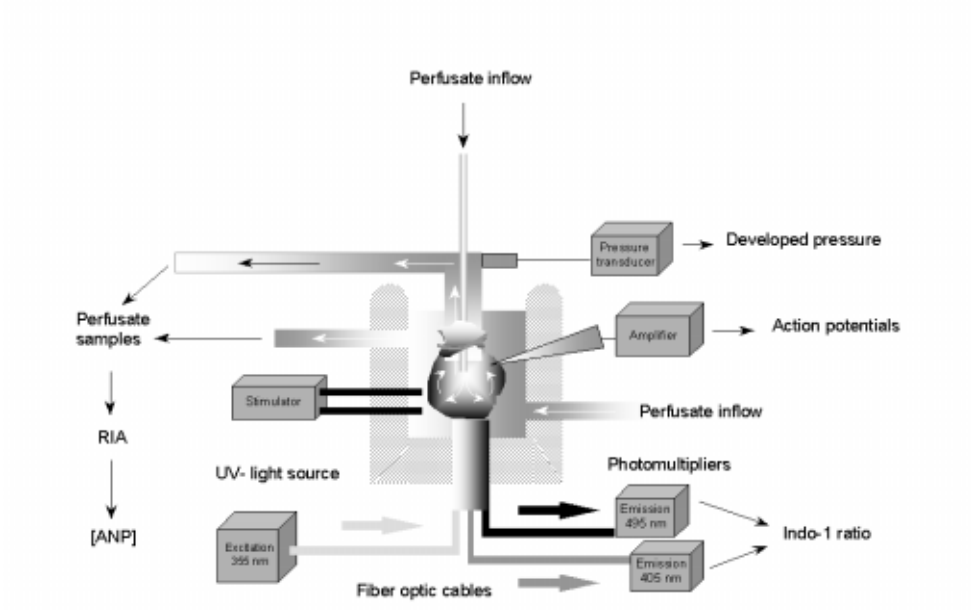


Fig. 5. Experimental setup used for measuring action potentials, contraction force, Indo-1 fluorescence and ANP secretion from isolated rat left atria.

4.7. pH measurements

For pH_i measurements, isolated atria were loaded with cell permeable AM-ester of BCECF (2´,7´-bis-(2-carboxyethyl loading)-5-carboxyfluorescein, tetraacetoxymethyl ester) at a concentration of 5 µM for 30 minutes. After 20 minutes washout the intracellular pH measurements were performed with an image analysis system (MCID/M2, Imaging

Research Inc., Brock University, Ontario, Canada) consisting of an Intel 403 E microcomputer linked to an Image 1280 image processor (Matrox Dorral, Quebec, Canada). The buffer exchange was performed with perfusion equipment providing gradual addition of solutions. Atria and solutions were kept at 37 °C in a thermostat incubation hood (Nikon, Tokyo, Japan). Sony CCD 72E camera (Dage-MTI Inc. Michigan City, USA) and Videoscope KS-1381 signal amplifier (Washington, USA) were used to collect the data. Atrial strips were excited at 495 and 440 using a computer-driven filter wheel (MAC 2000, Ludl Electronic Products Ltd, NY, USA), and emitted light was collected through a dichroid mirror and interference filter at 510 nm. The 495/440 ratio were obtained on a pixel-by-pixel basis, spatially averaged over a large (multicellular) area, and converted to pH values. Calibration of the fluorescence ratio versus pH was performed using the K^+/H^+ ionophore nigericin. The atria were equilibrated in high K^+ solution (140 mM) of varying pH (6.8, 7.0 and 7.2) in the presence of 5 µM nigericin and the calibration curves were constructed by plotting the extracellular pH against the corresponding fluorescence ratio. The resulting curve was sigmoidal with the inflection point at ca. 7.0 as expected from the reported pKa of BCECF.

4.8. Mathematical model

4.8.1 Properties of the model

The model used in this study is based on the model developed by Luo and Rudy (Luo &Rudy, 1991, 1994 a,b). Since the original model was designed to model the guinea-pig ventricular cell, extensive modification had to be made in order to fit the model to present results and previously published experimental results from rat atrial myocytes. Together with the species specific modifications, a more complex Ca^{2+} handling was introduced into the model on the basis of recent reports. The calcium release in atrial myocytes is a combination of the release from two different compartments of sarcoplasmic reticulum (Hüser *et al.* 1996, Hatem *et al.* 1997). The basic idea of this two-step Ca-release model is that the calcium entering through L-type channels triggers calcium release from the first release compartment (peripheral SR) and the Ca^{2+} released from the first compartment triggers release from the second compartment (corbular SR) in all-or-none manner. The trigger threshold of the second compartment was set at the value equal to the half of the calcium peak of normal release from the first compartment, with the time constants of activation and inactivation 40 ms and 10 ms respectively. Time delay between the release from the first and second compartments was 5 ms based on the calcium wave propagation velocity and calcium gradients in atrial myocytes (Hüser *et al.* 1996, Takamatsu & Wier, 1990, Cheng *et al.* 1996). These adjustments made the calcium release from the second compartment slower than from the first compartment, consistent with experimental data (Hatem *et al.* 1997). The components of the model cell are presented in Fig.6.

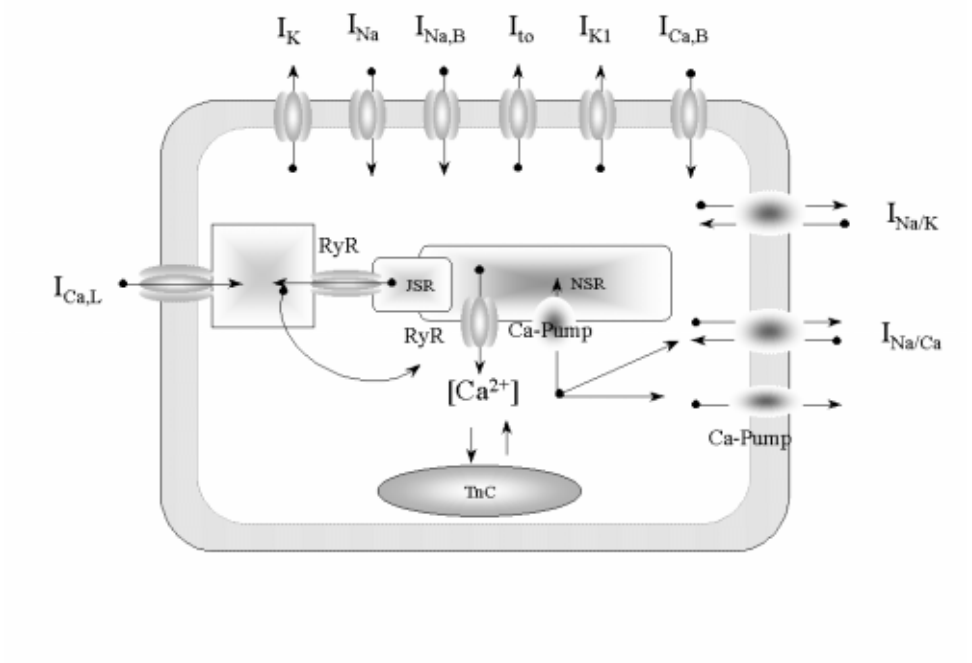


Fig. 6. Outline of the components in the mathematical model used in the study. Abbreviations: I_{Na}; voltage-activated sodium current, $I_{Na,\ B}$; background Na-current (leak), I_{to}; transient outward current, $I_{Na/Ca}$; sodium-calcium exchanger current, $I_{Na/K}$; sodium -potassium pump current, $I_{Ca,L}$; voltage activated L-type Ca^{2+}-current, I_K; delayed rectifier potassium current, I_{K1}; inward rectifier potassium current, $I_{Ca,B}$; background calcium current, RyR; ryanodine receptor, TnC ;troponin-C, JSR; junctional sarcoplasmic reticulum, NSR; non-junctional sarcoplasmic reticulum. For details see text.

4.8.2. Simulation of stretch by the model

The SA-channels in the cardiac myocytes have reportedly a near linear voltage dependence (Ruknudin *et al.* 1993), no adaptation (Sasaki *et al.* 1992) and non-selectivitity over cations (Kim, 1993). The reversal potential of the SA-channel current was -3.2 mV in rat atrial myocytes (Kim, 1993). The SA-channel formalism was adapted from previous models (Sachs, 1994), with small modifications:

$$I_{sac} = \frac{(V - V_{rev})\gamma\rho}{1 + K\exp\left[-\alpha(L - L_0)\right]} \qquad (11)$$

where I_{SAC} is the current density (μA/μF), V= membrane potential, V_{rev}= reversal potential (-3.2 mV), K= equilibrium constant (100), L= sarcomere length, L_0 =minimum sarcomere

length (1 µm), α = parameter defining the stretch sensitivity (3), ρ=channel density ($0.015/\mu m^2$), γ=single channel conductance (25 pS). The iteration of the parameters for the SA-current was based on the experimental data. To simulate stretch of the rat atrium caused by the increase of the intra-atrial pressure from 1 to 3 mmHg, increase of the current density during diastole from -0.03 to -0.32 µA/µF was used. In the model this SA-channel current increase corresponds to an increase of sarcomere length from 1.2 to 1.9 µm, increasing the open probability of the SA-channels from 0.03 to 0.16.

Gulati *et al.* (1990) showed that the calcium affinity of the contractile element increased after a length change, whereby K_d (for Ca^{2+}) of the troponin C decreased by 42% when the sarcomere length of skinned cardiac muscle was increased from 1.7 to 2.2 µm, which almost corresponds to the overall sarcomere length change during the rising phase of the F-S-relation in rat trabeculae (TerKeurs et al, 1980). According to these observations it was approximated that the overall increase of the TnC affinity during rising phase of the F-S-relation is ca. 50%. Knowing that the rising phase of the F-S relation in rat left atrium is within intra-atrial pressure between 0 and ca. 10 mmHg (see, I) it was approximated that the increase of the intra-atrial pressure in rat atrium from 1 to 3 mmHg produces a 25 % decrease of the K_d in the TnC. This value was used in modeling the stretch caused by increase of the intra-atrial pressure from 1 to 3 mmHg. The simulation of the stretch in the model was based on the following assumptions. First, the relation of the TnC affinity change between 0 and 50% increase and sarcomere length is linear. Secondly, the SA-channel current density (open probability of the channel) increases with increased sarcomere length giving the maximum open probability of the channel at a sarcomere length which produces maximal developed tension. Thus, the current density increase from 0 to 100 % is achieved during the rising phase of the F-S-relation in the model. In extrapolating to pathological stretch levels, SA-channel current density from 0.2 to 1.54 µA/µFwas used and at the same time the TnC affinity was increased from 20 to 50 %. The assumptions are based on the scarce published data on the mechanisms, and, if the relationships between the modeled components of stretch sensitivity are more complex, it probably introduces more complex behavior into the model, but does not change the basic results.

4.9. Statistical testing

Statistical testing was done by the SPSS program (SPSS Inc. USA) and by SigmaStat program (Jandel Scientific, USA). The action potential data were tested with one-way ANOVA. The data from contraction, ANP secretion and Indo-fluorescence were tested with the paired t-test. The time dependent changes in Indo-1 transients were tested by one-way ANOVA followed by the Student-Newman-Keuls test. In all cases p-values less than 0.05 were considered statistically significant. Variances are expressed by±SEM.

5. Results of original papers (I-IV)

5.1. Stretch-induced changes in the function of rat atria (I-IV)

In isolated rat atrium moderate (+2 mmHg) stretch (increase of intra-atrial pressure) causes increase in the Ca^{2+}-transients (II-IV) and decrease of the time constant of the transients' decay (II) without a significant change in the diastolic $[Ca^{2+}]_i$ (II-IV). These changes are accompanied by lengthening of the action potentials of the myocytes (II, III) and a biphasic increase of the contraction force of the atrium consisting fast and slow part (II, III, IV). For mechanisms the present study suggested that the slow increase of the contraction force is caused by augmentation of the Ca^{2+} transients. Bigger transients during systole lead to increased Na^+/Ca^{2+}-exchanger current during the repolarization phase of the action potential, solely causing the AP lengthening (II). Parallel with the changes in the E-C-coupling induced by stretch the ANP secretion is also increased (IV). However, the ANP secretion is clearly adapting. After reaching a peak value (ca. 8 min after onset of the stretch) the ANP secretion shows a time-dependent reduction despite the sustained stretching stimulus. In addition to this, when intensive stretch (+5 mmHg) is applied to atrium, delayed afterpolarizations ensues (I). The main effects of stretch on the function of rat atrium are summarized in figure 7.

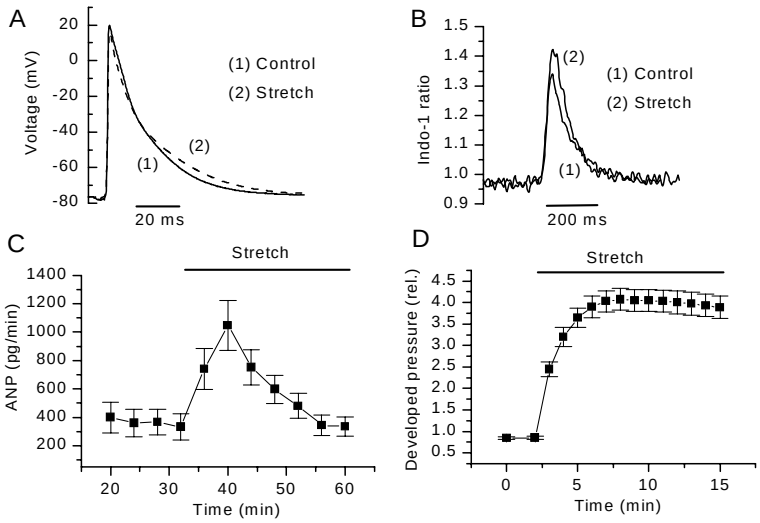


Fig. 7. Examples of the stretch-induced changes in the function of rat left atrium. (A) Action potential change induced by rising the intra-atrial pressure from 1 (control) to 3 mmHg (stretch). (B) Intracellular Ca^{2+} transients (Indo-1 fluorescence) in 1 mmHg (control) and 4 min in the 4 mmHg intra-atrial pressure (stretch). (C) Stretch-induced ANP secretion after changing the intra-atrial pressure from 1 mmHg to 4 mmHg (stretch). (D) developed pressure (contraction force) after changing the diastolic intra-atrial pressure from 1 to 4 mmHg (stretch). For statistics, see the text in results section.

5.2. Effect of Gd^{3+} on the stretch-induced changes in the contraction force and afterpotentials (I)

To study the effect of intensive stretch on the electrical properties of atrial myocytes, stimulated action potentials were recorded under low (1 mm Hg, n = 14) and high pressure (7 mm Hg, n = 10). Rise in the intra-atrial pressure significantly increased the action potential amplitude ($p < 0.001$), and relative conduction speed ($p < 0.001$). Gd^{3+} inhibited (at 80 µM) the stretch-induced changes in action potential parameters ($p < 0.05$). During 7 mmHg pressure stimulation delayed afterdepolarizations were observed following each action potential. Occasionally afterdepolarizations resulted in extra action potentials and contractions. Application of 80 µM Gd^{3+} blocked the stretch-induced afterdepolarisations entirely in all experiments (n = 12). No extra action potentials nor extra contractions were observed when gadolinium was used.

To study the effects of Gd^{3+} and diltiazem on the stretch-dependent increase in the contraction force, pressure pulses were recorded at five different pressure levels (between 1 and 8 mmHg). Diltiazem at 5 µM blocked almost 50 % of the contraction compared to

control, at every basal pressures inside the atria ($n = 4$, $p < 0.05$). The block of contraction by 80 µM Gd^{3+} was highly pressure-dependent: at 2 mmHg basal pressure blocked only 17 ± 12 % of the contraction ($n = 4$) while at 7 mmHg pressure Gd^{3+} blocked 64 ± 14 % compared to control ($p < 0.05$, $n = 8$). The effect of Gd^{3+} was thus significantly different at low pressure from that with higher pressures and from the effects of diltiazem at any pressure.

5.3. Stretch induced changes in the rat atrium; modelling the mechanotransduction (II)

Knowing that sustained physiological stretch changes the shape of action potentials (Fig. 7, for statistics see II and III), the time course of the AP changes was compared to the time course of the contraction development after onset of the stretch. It was seen that AP lengthening (increase in $APD_{90\%}$) corresponded to the development of the contraction force after stretch with a similar time course. Furthermore, the increase of contraction force was accompanied by an increase of calcium transients, without any change in the diastolic Indo-1 fluorescence ratio (ns, $n=4$). Although increase of the contraction force is fast, i.e. 10 seconds of stretch more than doubles the force, there is still a significant increase between 10 s and 2 min after stretch ($p<0.01$, $n=4$). This slower increase of force is likely to be due to the increase of Ca^{2+}-transients that were also augmented during the same time scale ($p<0.01$, $n=4$).

Because it is evident that most of the effects of stretch are actually manifested during 10 s after the onset of stretch, the first ten Ca^{2+}-transients and contraction pulses from each recording ($n=4$) were analysed. The development of contraction force is faster than the increase of the calcium transient amplitude. This indicates that the fast component of the contraction force development is not due to the increase of the calcium transients, but is probably caused by the increased sensitivity of the contractile element to Ca^{2+}. If this were true, increased buffering of the Ca^{2+} by the contractile element should alter the shape of the Ca^{2+} transients. The one-exponential fits to the decay of Ca^{2+} transients revealed that in fact the decay becomes faster after stretch. However, it has been shown that the decay of the Ca^{2+} transient is accelerated by increased Ca^{2+} transients (Bers & Berlin, 1995). Because of this, the effect of the size of the Ca^{2+} transients had to be estimated in order to use the decay as an indicator of the Ca^{2+} buffering of the cells. As the rate of free Ca^{2+} decline increases in a parabolic manner (Bers & Berlin, 1995), proportional to $[Ca^{2+}]^2$, the ratio between the decay and the square of the Ca^{2+} transients' amplitude was calculated. In theory, any intervention that increases buffering of the Ca^{2+} should reduce the ratio, but if the changes in the decay are caused by bigger Ca^{2+} transients this ratio is not changed. The decay of the Ca^{2+} transient was accelerated during the first 10 s after the onset of the stretch with no significant change thereafter (ns., $n=4$), independently of the Ca^{2+} transients' amplitude. This is likely to be caused by the stretch sensitivity of the contractile element.

The second part of this study was aimed to reproduce similar changes in the Ca^{2+}-balance and action potentials in the model as seen in experiments by modulating the TnC affinity and/or calcium influx. Increase of the TnC affinity to Ca^{2+} by decreasing the dissociation constant (K_d) of TnC for 25% in the model (see Methods) caused a biphasic behavior of the

Ca^{2+} transients. First transients were smaller, but amplitude stabilized near to the control value within 1 minute. Even when the systolic $[Ca^{2+}]_i$ had recovered back to the control value, the decay of the Ca^{2+} transients was faster (time constant decreased from 118 ms to 105 ms). When control action potentials were compared to steady-state action potentials after simulated stretch, only modest changes were observed, the most prominent change being the small increase of the $APD_{90\%}$ (+2.4 ms). The increased Ca^{2+} affinity of the TnC modulated slightly the Ca^{2+} dependent currents, the peak value of the L-type Ca^{2+} current was increased from -10.3 to -10.5 µA/µF and the inward current of the Na^{+}/Ca^{2+}-exchanger was decreased from -1.09 to -1.05 µA/µF. This result indicates that the TnC affinity change alone can not produce similar changes as observed in experiments, since the TnC affinity change could not augment the Ca^{2+} transients or lengthen the action potentials.

When stretch of the myocyte was simulated by applying the SA-channel conductance into the cell membrane of the model, the amplitude of the calcium transients were gradually increased. The activation of a cation selective SA-channel increased the systolic $[Ca^{2+}]$ gradually from 0.88 to 1.04 µM within 1 minute (+15.4%). The decay of the calcium transients decreased (as measured by the time constant of the one exponential fits) from 115 ms to 114 ms. Greater Ca^{2+}-transients increased the late duration of the APs', the time course of the AP lengthening being related to the increase of the calcium transients' amplitude. The $APD_{90\%}$ increased from 52.2 ms to 61.4 ms within 1 min after the increased SA-channel current. This was probably due to the increase of the inward current carried by the Na^{+}/Ca^{2+} exchanger which was increased from -1.09 to -1.16 µA/µF. Bigger Ca^{2+} transients decreased the peak value of the L-type Ca^{2+}current (by increasing inactivation) from -10.3 to -10.1 µA/µF and made the decay of the current a little faster.

Neither of the two candidates of the mechanosensation (SA-channels and TnC) could faithfully produce similar changes in the model cell as seen in experiments. In order to better fit the model predictions to experimental findings, these two mechanisms were combined in the next round of modelling. When stretch was simulated by increasing the TnC affinity together with the activation of the SA-current, the Ca^{2+} transient amplitude gradually increased by 20.2 % from 0.88 to 1.09 µM. The increase of the Ca^{2+}-transients was accompanied by a decrease of the time constant of the decay of the transients from 119 to 96 ms. Increased calcium mobilization during the action potential caused a simultaneous lengthening of the AP. The $APD_{90\%}$ increased from 52.2 ms to 63.8 ms within a minute and time course of the AP lengthening was related to the increase of the calcium transients. Since the peak value and the decay of the L-type Ca^{2+}current was unaffected, it was concluded that the increase of the inward current carried by Na^{+}/Ca^{2+} exchanger from -1.09 to -1.19 µA/µF increased the late duration of the action potentials. Since the combination of the SA-channel activation and the increased affinity of TnC simulated fairly well the effects of stretch observed in the experiments, it was studied if this approach causes simultaneous changes in Ca^{2+} transients and action potentials, and if the time course of this change is similar to the experiments. The augmentation of the Ca^{2+} transients lengthened the action potentials by 10 ms within 1 minute, as indicated by the $APD_{90\%}$, in line with the experimental findings.

When stretch was simulated solely by increasing the TnC affinity by 25% the SR Ca^{2+} content was affected only slightly. The diastolic Ca^{2+} in the SR increased from 1.65 mM to 1.69 mM within 1 minute. Activation of SA-channels without any change in TnC affinity increased the diastolic SR Ca^{2+} content from 1.65 to 1.79 mM. The effects of combined TnC

affinity change and activation of SA-channels were additive, and SR calcium content then increased from 1.65 to 1.88 mM. This modeling result indicates that augmentation of the Ca^{2+} transients in the model is due to the increased amount of Ca^{2+} in the SR. Furthermore, the SA- channel activation does not cause directly the increase of the Ca^{2+} transients, but the effect is mediated by storage and release of the Ca^{2+} into and from the SR.

Next it was studied, if the same mechanisms that cause the physiological effects of stretch could be responsible for the pathological stretch-induced changes in the function of the heart. In order to do this the effects of incremental stretch on the function of the model cell were simulated. It is natural to assume that if a cationic current through SA-channels with reversal potential more positive than the r.p. is activated, it should cause a depolarization of the membrane potential. Because SA-channels pass also Ca^{2+} ions, the depolarization would be followed by an increase of the $[Ca^{2+}]_i$. Both of these effects were seen when SA-current density was increased gradually from 0.1 to 1.6 µA/µF (see also the Methods for details), at the same time when the Tnc affinity was increased from 20 to 50 %, respectively. The r.p. depolarized up to -68 mV (from -80.5 mV) at the same time when diastolic $[Ca^{2+}]_i$ was increased from 0.1 µM to 0.16 µM. Similarly as in other simulations, augmentation of the Ca^{2+} transients modulated the action potentials through increased Na^+/Ca^{2+} current. When 80% of the channels present in the model were open, the augmented Ca^{2+} transient reduced the L-type Ca^{2+} current from -10.4 to -8.2 µA/µF. Because the Na^+/ Ca^{2+} exchanger inward current was increased from -1.2 to -2.6 µA/µF, it generated an afterpotential that resembles the early afterdepolarizations of the atrial myocytes. These results show that the same mechanisms that in the model mimic the physiological effects of stretch can also produce the arrhythmogenic changes in myocytes' function.

5.4. Effect of intracellular acidosis on the stretch-induced changes in the rat atrium (III)

To study how and by which mechanisms intracellular acidosis changes the atrial responses to stretch, intracellular acidification (analogously with lactate) with organic acid propionate was produced. Propionate is readily transported to the cytosol in a protonated form. Previously ischemia-induced pH changes have been simulated by lactate (Allen *et al.*, 1989) but the use of propionate was preferred here to avoid the possible lactate-specific effects on the metabolism of the cardiac muscle. Propionate (20mM) in HEPES-buffer (pH 7.4) caused a 0.18 ± 0.02 (n=3) drop in pH_i, as determined by a BCECF based imaging system. This method was used to study the interactions between stretch and intracellular acidification on basic functional parameters of the rat atria, contractile force, intracellular action potentials, and intracellular Ca^{2+} balance.

When the intra-atrial pressure was increased from 1 to 4 mmHg, the contraction force was increased biphasically. The immediate increase was followed by a secondary, slower increase in force. In control experiment the half time of this contraction development was 27.0 ± 6.1s but when tissue was pre-exposed to acidosis the development of the contraction force was significantly slower with half time of 56.9 ± 8.1 s ($p<0.01$, n=6). The slower development of the contraction during acidosis resulted in smaller contraction force after 4 min of continuous stretch. When acidotic tissue was stretched, the contraction force

increased from 1 in non-acidotic non-stretched control to 3.4 ± 0.2 whereas in control situation the overall increase of the contraction force was significantly larger (4.7±0.4, n=6, $p<0.05$).The averaged Ca^{2+}-transients show that the increase of the contraction force was accompanied by an increase of the calcium transients. In 1 mmHg the amplitude was 0.26±0.09 (n=6). After 1.5 min of continuous stretch (4 mmHg) the amplitude was increased to 0.36±0.13 ($p< 0.05$, n=6), and after 4 min of continuous stretch 0.37±0.15 ($p< 0.05$, n=6). The diastolic Indo-fluorescence ratio was not significantly altered by stretch (ns, n=6). When the same atria were pre-exposed to 20 mM of propionate for 15 minutes in the low pressure, the amplitude of the calcium transients did not change significantly (0.3±0.08, ns, n=6), but stretch increased the transients amplitude similarly as in control. One and a half minutes after onset of stretch the amplitude of the Ca^{2+} transient was 0.38±0.1 ($p<0.05$, n=6) and after 4 min it was 0.4±0.13 ($p<0.05$, n=6). However, application of the stretch during acidosis increased the diastolic Ca^{2+} levels compared to non-acidotic control ($p<0.05$, n=6). The diastolic Ca^{2+} level after 4 min of stretch was 1.06±0.06 (n=6) in control and 1.16±0.07 (n=6) during propionate load. In addition to changes in the contraction and $[Ca^{2+}]_i$, propionate induced acidosis shortened the action potentials also in low pressure ($APD_{60\%}$, $p<0.05$, n=10), and hyperpolarized the r.p. ($p<0.05$, n=10). The surprising effect of acidosis was the inhibition of the effects of stretch on the action potentials, including inhibition of both, the increase of the $APD_{90\%}$ ($p<0.05$, n=10) and the decrease of the $APD_{15\%}$ ($p<0.05$, n=10).

To study the development of the acidosis in stretched tissue Indo-1 loaded pre-stretched (intra-atrial pressure 5 mmHg, 10 minutes) left atrium were exposed to 20 mM propionate. The development of intracellular acidosis after application of 20 mM propionate was accompanied by changes in systolic and diastolic $[Ca^{2+}]_i$ as indicated by Indo-1 fluorescence ratio (405/495). Application of propionate gradually increased the calcium transients. Up to 7.5 minutes the amplitude increased from 0.40±0.08 to 0.55±0.12 ($p<0.05$, n=5). Between 7.5 minutes and 15 minutes the transients became smaller, but remained bigger than in control (10 min, 0.50±0.10, $p<0.05$ and 15 min, 0.47±0.08, $p<0.05$, n=5). The propionate application slowed the decay of the Ca^{2+}-transients from 46.8±2.3 ms to 56.2±3.4 ms within 5 min ($p<0.05$, n=5), to 55.2±5.0 ms after 7.5 min ($p<0.05$, n=5) and to 54.2±5.1 ms after 10 minutes ($p<0.05$, n=5). However, between 10 and 15 minutes the decay was speeded up to the same level as in control (47.2±2.6 ms, p=ns., n=5). When the measured diastolic $[Ca^{2+}]_i$ were normalized to 1 in control experiments, the diastolic $[Ca^{2+}]_i$ was 1.28±0.02 after 15 minutes ($p<0.05$, n=5) when pH_i reached the steady state value (ca. 7.0). The diastolic $[Ca^{2+}]_i$ increase was gradual since the change of diastolic $[Ca^{2+}]_i$ was statistically significant between each time step ($p<0.05$).The change in contraction force during the development of the acidosis also seems to include time-dependent and transient changes. The initial decrease of the contraction force is followed by a transient augmentation of the force after the resulting decline of the force. The initial decrease is probably caused by the inhibition of the contractile element by acidosis whereas the following transient augmentation is due to the simultaneous augmentation of the Ca^{2+} transients. This suggests that the effects of acidosis are mediated by at least two different mechanisms. One of these is the inhibition of the contractile element by acidosis and the other is related to the Ca^{2+} handling of the myocytes.

Finally, the possibility that stretched atrium is more sensitive to acidosis that the non-stretched atria was evaluated. By further analysing the data it was observed that the

contraction force is indeed inhibited more in stretched tissue (5 mmHg, n=5) than in non-stretched tissue (1 mmHg, n=6, $p<0.01$). In 1 mmHg 15 minutes exposure caused reduction of the relative (to contraction before application of propionic acid) contraction force from 1 to 0.77±0.04 (n=6). When similar experiments were done in pre-stretched (5 mmHg) tissue the reduction of the contraction was significantly bigger, from 1 to 0.46±0.06 (n=5, $p<0.01$). In 1 mmHg diastolic indo-1 ratio changed from 1 to 1.05±0.02 (n=6), but in 5 mmHg the increase of the diastolic indo-1 ratio was significantly bigger, from 1 to 1.27±0.02 (n=5, $p<0.001$)

5.5. Modulation of stretch-induced ANP secretion by intracellular acidification (IV)

Application of 20 mM propionate 16 minutes before the onset of stretch potentiated the stretch-induced ANP secretion into the perfusate when the intra-atrial pressure was raised from 1 to 4 mmHg. Propionate-induced acid load caused a two-fold increase in cumulative ANP secretion (during 32 min) from 8.0 ± 1.4 to 16.8 ± 3.0 ng ($p<0.05$, n=7), without any significant effect on the basal level of ANP secretion. The molecular form of the secreted ANP was the processed form of ANP, the ANP 99-126, and virtually no pro-ANP 1-126 (the storage form) was found, as determined by HPLC. This indicates that the ANP was secreted via the normal physiological exocytotic cascade (Vuolteenaho *et al.* 1985). The changes in the ANP secretion were accompanied by changes in the $[Ca^{2+}]_i$ in the same time scale similarly as in (IV).

On the premise that acidosis has a prominent effect on the contraction force of the heart muscle it should also modulate the stretch-induced changes in the contraction force. To study this, the contraction force throughout the ANP experiments was recorded. The speed of the time-dependent development of the contraction force was analysed by measuring the half times of the contraction curves. In control measurements the half times were 1.17±0.14 min and in propionate group 2.25±0.73 ($p<0.01$, n=7), without a change in the contraction at low pressure (ns., n=7). Acidosis suppressed the stretch-induced increase in contraction force by inhibiting the fast component of the force development (1 min, n=7, $p<0.05$).

6. Summary of the results

1.Moderate stretch has a number of effects in the isolated rat atrium. It produces: a) gradual augmentation of Ca^{2+} -transients, with faster decay without a change in diastolic $[Ca^{2+}]_i$, b) two-phasic increase in the contraction force, c) lengthening of late phase of action potentials of myocytes, and d) augmentation of the ANP secretion.

2. Gd^{3+} inhibits the stretch-induced changes in contraction force and stretch-induced delayed afterdepolarizations, with a mechanism different from L-type Ca^{2+} channel block.

3. In a mathematical model stretch-induced changes in $[Ca^{2+}]_i$ and AP shape can be reproduced by increasing the TnC Ca^{2+} affinity with a simultaneous activation of a cationic current mimicking SA-channel activation. This causes augmentation of Ca^{2+} transients which modulates the shape of APs through an increase in the Na^+/Ca^{2+} inward current. The SA-channel activation alone or in combination with the TnC affinity change leads to a Ca^{2+} accumulation into the SR and a subsequent augmentation of Ca^{2+} transients during systole. When the SA-channel activation is increased, depolarization of the r.p. and an increase in the diastolic $[Ca^{2+}]_i$ ensues leading to arrhythmogenic afterpotentials via the Na^+/Ca^{2+} exchanger current.

4. A small reduction of the intracellular pH (0.18 units) with 20 mM propionate modulates the stretch-induced changes in the rat atrium. Acidosis leads to an increase in the diastolic $[Ca^{2+}]_i$ during stretch, inhibits the stretch-induced changes in action potentials and slows down the contraction development during stretch by inhibiting the fast component of the force development. These changes in E-C-coupling were accompanied by a simultaneous augmentation of the ANP secretion. Furthermore, it was shown that contraction force and diastolic $[Ca^{2+}]_i$ of the stretched tissue are more sensitive to acidosis-induced changes than in non-stretched tissue.

7. Discussion

7.1. Mechanosensitive components in rat atrium (I-IV)

7.1.1. The role of contractile element in stretch-induced changes

The stretch-induced contractile changes in heart muscle are manifested as an increase in the contraction force, the well known Frank-Starling mechanism. The calcium binding part of the contractile machinery, the troponin C (TnC) (Pan & Solaro, 1987), is known to be sensitive to muscle length (Babu *et al.* 1988; Gulati *et al.* 1990). The stretch sensitivity of TnC can explain the fast increase in contraction force, causing additional buffering of Ca^{2+} by the contractile element (Saeki *et al.* 1993, Kurihara & Komukai, 1995) and the subsequent, rapid increase in contraction force. Previously it has been shown that myofilament sensitivity does not change during the slow increase of the contraction force after stretch (Hongo *et al.* 1996, Kentish & Wrzosek, 1998), further supporting the idea that TnC affinity change upon stretch is very fast. If Ca^{2+} sensitivity of TnC increases with stretch, this might influence the function of the myocytes several ways. First, fast increase of the affinity would increase the contraction force and decrease the systolic $[Ca^{2+}]_i$. Secondly, the decay of the Ca^{2+} transients during stretch would be faster than what would be expected on the basis of enzyme kinetics alone, as previously demonstrated (Komukai & Kurihara, 1996), because the calcium binding "eats into" the calcium transient. Thirdly, the affinity change would favour the formation of the TnC-Ca^{2+} complex leading to a slower off-rate of the complex, and so the time course of the contraction would be prolonged. The experimental data demonstrates that stretch decreases the decay of the calcium transients significantly and the model simulation shows that TnC affinity increase leads to similar changes (II). The decline of the Ca^{2+} transients immediately after the onset of stretch was not seen in the experiments, suggesting that rise in systolic $[Ca^{2+}]_i$ and increased TnC affinity are smoothly graded. However, in the model a transient decrease of the Ca^{2+} transients amplitude was produced when the TnC affinity was increased. According to the results of the present study the TnC sensitivity increase contributes to the stretch-induced changes by providing an additional buffering of the Ca^{2+} ions during stretch. Increased buffering by TnC leads to an increase of the contraction force during systole, most

prominently manifested during the fast increase in contraction force following stretch. During diastole this mechanism would balance the $[Ca^{2+}]_i$ or even slightly lower the diastolic$[Ca^{2+}]_i$.

7.1.2 The role of SA-channels

The TnC affinity change can explain the fast part of contraction development during stretch, but the mechanism of the slow part is different. It is accompanied by the augmentation of the Ca^{2+} transients, suggesting that an additional Ca^{2+} influx may be activated upon stretch (II, III, IV). Opening of cation selective SA-channels during stretch would cause this kind of Ca^{2+} influx. In order to study the role of SA-channels in the stretch-induced changes in rat atrium Gd^{3+} was used to block these channels (I). Gadolinium seems to be very effective blocker of the stretch-induced changes in rat atrial function, including a block of delayed afterdepolarizations and increase in contraction force (I). However, gadolinium has a number of effects in the cardiac myocytes, which are not necessarily related to the stretch-sensing machinery of the cells. As an example, a high dose of gadolinium blocks the delayed rectifier (I_K) potassium channels (Hongo *et al.* 1997) and causes a slower repolarization of the AP. It is known that Gd^{3+} also blocks the L-type Ca^{2+}-channels in isolated heart cells (Lacampagne *et al.* 1994).

It is clear that the above mentioned unspecific actions contribute to these gadolinium-induced changes as suggested previously (Ward & White, 1994). The prominent reduction of contraction force by Gd^{3+} (I) suggests that the actions of gadolinium may be mediated by the changes in the Ca^{2+} balance of the myocytes. This might be caused by the block of SA-channels contributing to the SR Ca^{2+} loading during stretch. The time-dependent reduction of the Ca^{2+}-transients by Gd^{3+} would then be due to the depletion of the SR calcium (Ward & White, 1994). When considered that stretch increases the amount of calcium in the SR (Bluhm & Lew, 1995), this mechanism would explain the apparent stretch-sensitivity of the gadolinium block. In addition to this, the modelling part of the present study reproduced a gradual increase of the calcium transients only when an additional calcium influx through plasma membrane was activated (II). The "SA-channel" used in the model has a permeability ratio of 1:1:1 to Na^+:Ca^{2+}:K^+, similar to what has been reported earlier in rat atrial myocytes (Kim,1993). Activation of the SA-channels leads to an increase in $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ is pumped to the SR by the Ca^{2+}-pump, causing a greater release of Ca^{2+} during systole. However, no change in the diastolic $[Ca^{2+}]_i$ in the experiments (II-IV) was observed during stretch which would occur if SA-channels permeable to Ca^{2+} would have been activated. This may be due to relative insensitivity of the fluorescent dye to such low levels of Ca^{2+} as prevail during diastole, but other possibilities also exists. As it seems that Ca^{2+} signals in the heart muscle are locally controlled, it would be a fascinating idea that the Ca^{2+} flux induced by SA-channels would also be restricted to a certain local pool inside the cells. This idea has been presented earlier (Sigurdson *et al.* 1992), but the data supporting this is still lacking. If, for example, SA-channels pass current to a subcellular space near the SR Ca^{2+}-pump, SA-channel activation would not increase the mean diastolic $[Ca^{2+}]_i$, but more or less directly increase the amount of Ca^{2+} pumped into the SR. This would, of course, cause a Ca^{2+} transient augmentation without any change in the diastolic

$[Ca^{2+}]_i$.

Although SA-channels are one of the most plausible candidates for generating a stretch-induced Ca^{2+} influx during stretch, the data supporting this idea clearly warrants a critical examination, and several points compromising this idea can be put forth. First, SA-channels can be activated in the patch-clamp configuration by applying a positive or negative pressure to the membrane (e.g. Sigurdson *et al.* 1992), but also by osmotic manipulation of the cell volume (Kim, 1993). Very little is known whether either one of these stimuli is actually adequate for these channels. Since the structure of the channels is not known, the primary transduction prosess may well include a link between SA-channels and the cytoskeleton, or even between the neighbouring cells (Ingber, 1997). If the transduction process includes a more complex regulation of the channel open probability than the direct effect of membrane stretch, all the above mentioned stimuli are inadequate, because of the use of isolated cells in all patch-clamp studies. Secondly, all the electrophysiological methods, including patch-clamp, intracellular voltage recordings and MAP-recordings, are sensitive to mechanical artifacts. In patch-clamp measurements a small membrane rupture may readily cause a leak of cations into the cell which would resemble a stretch-activated cationic conductance. During the present work I observed that stretch of rat atrium often causes a "depolarization" of the membrane potential in intracellular voltage recordings. This depolarization was not of biological origin but in every case caused by unstability of the cell-electrode connection (impalement). It is, however, possible to distinct these artifacts from genuine currents by monitoring the resistance seen by the measuring electrode, because these changes were always associated with drastic changes in the resistance. When omitting those artefactual responses, I never observed stretch-induced sustained depolarizations similar to what has been reported earlier (White *et al.* 1993). The MAP-recording is a sum of potentials of number of cells in the surface of the heart muscle. It is a result of current flowing extracellularly in an area of a large number of cells. Stretch might cause an artefact in MAP-recordings simply by reducing the amount of cells in the measuring area due to the distension of the muscle or by changing the properties of the extracellular space. Thirdly, all the pharmacological agents used to block SA-channels are relatively non-specific. Aminoglycoside antibiotics, like streptomycin, block voltage activated Ca^{2+} channels (Haws *et al.* 1996), amiloride blocks Na^+/H-transporter (Simchourtz & Gragoe, 1986) and Gd^{3+}, as other lanthanides, block potassium channels and Ca^{2+} channels (Hongo *et al.* 1997, Lansman, 1990).

Despite the criticism opposing the idea about SA-channels participating to the stretch-induced changes in heart muscle, these channels would serve a route for calcium influx needed for Ca^{2+} transient augmentation during stretch. Whether the Ca^{2+} influx during stretch comes through some of the SA-channels characterized from heart myocytes or not, remains to be clarified. On the basis of the present study, SA-channels can be at least considered as a concept, where Ca^{2+} influx to the myocyte is increased upon stretch. Bearing in mind the complexity of the mechanotransduction of the cardiac muscle, explaining the stretch-induced changes in cardiac E-C-coupling solely by means of the identified SA-channels would be an oversimplification.

7.1.3. Interactions of stretch sensitive elements in rat atrium

In a prototypical physiological mechanism a stimulus changes the function of a system in a way that would suppress the initial stimuli. This so-called negative feedback is a fundamental property of physiological regulation. What then would serve as such a mechanism in the mechanotransduction of heart muscle? Previously is has been proposed that the SA-channel current activation would lead to Ca^{2+} influx causing release of Ca^{2+} from the SR, amplifying the Ca^{2+} loading during stretch (Gannier *et al.* 1996). This mechanism is a typical positive feedback mechanism. According to the present study the stretch introduces a Ca^{2+} flux into the cell, but this is preceded by an increased Ca^{2+} affinity of the contractile element (II). This affinity change would make the cardiac muscle more rigid (less compliant) at any given $[Ca^{2+}]_i$. If the stretch-induced Ca^{2+} influx is proportional to membrane stretch, the rigidity of the muscle would reduce the stretch (stimulus) by opposing the distension of the tissue (i.e. stretch), which would thus constitute a negative feedback. This scheme suggests that the TnC affinity change would serve as a way to rapidly adjust the contraction force to increased mechanical load, but also as a mechanism protecting the tissue against pathological distension.

7.2. Consequences of the mechanosensitivity in rat atrium

7.2.1. Action potential changes induced by stretch

The effect of stretch on the shape of action potentials (AP) leads to variable results. In some animal models stretch causes a shortening of AP (Lab, 1978; Lab, 1980). Usually these cells have long APs with a prominent plateau. It has also been generally considered to be difficult to separate genuine currents (or changes in voltage) from artifactual ones which might be due to damage of the cell membrane by mechanical factors (Crozatier, 1996). Since calcium transient modulates the action potential (DuBell *et al.* 1991), the changes in $[Ca^{2+}]_i$ during stretch should change the shape of the action potentials of the myocytes. Inotropic interventions, like stretch, increase the late duration of the APs' in cardiac myocytes with short plateau (Schouten, TerKeurs, 1985), via the increased Na^+/Ca^{2+}-exchanger inward current that is boosted by augmentation of the calcium transients (DuBell *et al.* 1991). In rat myocytes the Na^+/Ca^{2+}-exchanger current is proportional to $[Ca^{2+}]_i$ (Janvier &Boyett, 1996). The increase of the contraction force after stretch is also accompanied by an increase of the late duration of the AP (Allen, 1977). The present study demonstrates that stretch influences the action potentials of the rat atrial myocytes. The action potential duration ($APD_{90\%}$) was lengthened during the slow phase of the contraction development (II, III). The mathematical model was able to reproduce similar changes in APs when the simulation included SA-channel activation (II). The mechanism suggested by the modelling was the increased Na^+/Ca^{2+}-exchanger inward current generated by bigger Ca^{2+} transients (II). Calcium-induced calcium release (CICR) in cardiac myocytes can be modulated by the trigger, the L-type current through membrane, or by the release of the Ca^{2+} from SR. On the

basis of previous results, the amount of the trigger current is not significantly changed by stretch (Hongo *et al.* 1996). In the present study stretch did not induce such changes in action potentials that would be expected if L-type Ca^{2+} current is significantly augmented (II, III). This leads to a conclusion that the Ca^{2+} transient augmentation is mediated by the release of the Ca^{2+} from the SR. Any modulation of the RyRs is able to increase the amount of Ca^{2+} released, but due to the compensatory changes in SR Ca^{2+} content, only transiently (Eisner *et al.* 1998). More sustained augmentation of the SR Ca^{2+} release is mediated by changes in the SR Ca^{2+} content (Eisner *et al.* 1998). It has been reported that SR calcium content is increased slowly after a step increase of cardiac muscle length (Bluhm & Lew, 1995). Increased amount of Ca^{2+} in the SR will directly increase the amount of Ca^{2+} released during systole (Janczewski *et al.* 1995). This is not surprising because the SR release channels (RyRs) are modulated by intraluminal Ca^{2+} (Lukyanenko *et al.* 1996). The model produced an increase in SR content concomitantly with the augmentation of the Ca^{2+} transient when SA-channels were introduced to the model cell (II). Based on experimental and modelling data (II) it can be concluded that during the normal stretch-dependent activation in rat atrium the SR has a crucial role in augmentation of the Ca^{2+}transients and thus, modulation of the action potentials.

7.2.2. Pathological effects of stretch in the rat atrium

The effects of stretch on the function of the heart muscle have previously been studied on the premise that stretch can cause many pathological phenomena. These effects include changes in conduction (Dominguez & Fozzard, 1979), excitability (Dean & Lab, 1990, Lerman *et al.* 1985), and generation of afterpotentials (e. g. Nazir & Lab, 1996). Present study shows that the same mechanism which produces typical Frank-Starling responses during moderate stretch in the rat atrium can also induce pathological changes in cardiac myocytes when subjected to more intense stretch (I, II). These effects were manifested in the model as a depolarization of the r.p. and an increase of the diastolic $[Ca^{2+}]_i$. In the model the stretch-induced Ca^{2+} overload causes a Ca^{2+} induced inactivation of the L-type Ca^{2+} current which opens a time window where the increased Na^+/Ca^{2+}-exchanger current can trigger early afterpotentials (II). In the experiments (I) sustained stretch was able to induce delayed afterdepolarizations. The stretch-induced delayed afterdepolarizations (DADs) resemble the ones seen during digitalis poisoning, caused by inhibition of the Na^+/K^+-pump, leading to an increase in the $[Na^+]_i$ (Ebner *et al.* 1986). The increased $[Na^+]_i$ is compensated by the Na^+/Ca^{2+} exchanger causing a Ca^{2+} overload via compensation of the Na^+ load by Na^+/Ca^{2+} exchanger (Sheu & Fozzard, 1982). In these Ca^{2+} overload situations afterpotentials are frequently seen, caused by a current called transient inward current (I_{Ti}) that is triggered by intracellular Ca^{2+} oscillations (e.g. January & Fozzard, 1988). It is natural that intracellular Ca^{2+} oscillation induces a membrane current, since Ca^{2+} is able to activate several ion channels in the membrane, like Ca^{2+} -activated cation channels (Ehara *et al.* 1988) and Ca^{2+} activated Cl^- channels (Kawano *et al.* 1995,) and, naturally, Na^+/Ca^{2+}-exchangers. Several mechanisms have been proposed for the role of the mediator of the I_{Ti}, including activation of Ca^{2+} -activated cation channels (Ehara *et al.* 1988) and activation of Na^+/Ca^{2+} exchangers (Kass *et al.* 1978). More recently it was shown that the I_{Ti} in rabbit purkinje cells

consists two channel components, cationic and anionic, wherein the latter was the Ca^{2+}-activated Cl^- channel (Han & Ferrier, 1996). The specific mechanism of I_{Ti} will, of course, depend on the ion channels present in the species used in the study. Thus, it is probable that in some species the I_{Ti} will be carried mostly by Ca^{2+} activated channels whereas in others e.g. by Na^+/Ca^{2+}-exchanger. The modelling part of the present study shows that in rat atrial myocytes, augmentation of Ca^{2+} transients may cause early afterdepolarizations (EAD) without any oscillations of the $[Ca^{2+}]_i$. According to this finding it is possible that the underlying mechanism of the delayed and early afterdepolarizations is not the same. Nazir and Lab (1996) suggested that the AP length could define whether the afterdepolarization is early or delayed. In APs with longer duration, an afterdepolarization would occur as an EAD, and in APs with shorter duration as a DAD. This would lead to a conclusion that in species with short AP duration (like in rat atrium) EADs would not be seen since afterdepolarizations would always be delayed. On the basis of the results of the present study (I, II) it is, however, possible that both EADs and DADs are present in rat atrial myocytes. The trigger of these is events is probably different, the EADs being caused by the interaction of the timing of the L-type current inactivation on the one hand and by the activation of Na^+/Ca^{2+}-exchanger current on the other, in the situation where Ca^{2+} transients are greatly augmented. DADs are probably due to Ca^{2+} oscillations reflected back to membrane voltage by a Ca^{2+} -activated current.

7.2.3. Stretch-induced ANP secretion

One of the consequences of myocardial stretch is the increase of the ANP secretion rate. The question if this exocytosis is somehow linked to the stretch-induced changes in the E-C-coupling remains to be answered. On the basis of the present study the evident link would be the increase of the systolic $[Ca^{2+}]_i$ during stretch (IV). The Ca^{2+} sensitivity of the ANP exocytosis is not known in quantitative terms i.e. in terms of K_d of the exocytotic cascade to Ca^{2+}. However, the co-occurrence of Ca^{2+} transient augmentation and increase of the ANP secretion during stretch suggests that the hypothesis about Ca^{2+} regulation of the ANP secretion is not totally unwarranted (IV). If calcium indeed does regulate the ANP secretion, several features of the exocytotic cascade can be addressed. First, the ANP exocytosis is sensitive to relatively small augmentation of the Ca^{2+} transients during stretch (IV). Secondly, the process shows clear adaptation, indicated by the fact that the peak value of ANP secretion is achieved after 8 min of continuous stretch, with a decline of the secretion thereafter (IV, Fig. 1. A.). This seems to correspond to the contraction development after stretch (peak value ca. 6 min, IV, Fig. 3.A.), so the decline of the ANP secretion occurs at the same time when a steady-state contraction force after onset of the stretch is achieved. Thirdly, the adaptation seems to affect only the stretch-induced ANP secretion, but not the basal secretion, since there is a continuous ANP secretion also in the unstretched tissue (IV, Fig. 1.). Some exocytotic processes are controlled by low affinity Ca^{2+} binding sites sensitive to the time integral of $[Ca^{2+}]_i$ (Thomas *et al.* 1993). This kind of mechanism would explain not only the stretch-induced ANP secretion but also the frequency-stimulated ANP secretion (Schiebinger & Linden, 1986b, Schiebinger *et al.* 1994). In both cases the time integral of the $[Ca^{2+}]_i$ is increased. Stretch increases it by augmenting the Ca^{2+} transients and increased

pacing naturally by increasing the number of transients per time. This mechanism does not explain the adapting behaviour of the ANP secretion, indicating that some other factors may regulate the Ca^{2+} affinity of this cascade. The seeming adaptation of the ANP secretion during sustained stretch can be due to readily releasable pool of ANP vesicles which are released by the stimulus (Dowsley *et al.* 1995), but long-lasting augmentation of the secretion may require additional mechanisms to be activated. These may well be some of the suggested enzymatic cascades involved in ANP secretion like the PKC activity (see, e.g. Ruskoaho, 1992).

7.3. Modulation of the stretch-sensitivity in rat atrial myocytes by acidosis (III-IV)

Intracellular acidification stimulates proton extrusion via the Na^+/H^+ exchange when pH_o is relatively normal (Ellis & MacLeod, 1985). Thus, extrusion of protons by Na^+/H^+ exchanger increases the intracellular sodium concentration ($[Na^+]_i$) (Bountra &Vaughan-Jones, 1989). Knowing that increased $[Na^+]_i$ causes Ca^{2+} accumulation to the cytosol via Na^+/Ca^{2+} exchanger, it might be possible that this mechanism contributes to the increased diastolic $[Ca^{2+}]_i$ during acidosis. On the other hand, the inhibition of the Na^+/Ca^{2+} exchanger by protons (Doering & Lederer, 1993) would reduce this Ca^{2+} loading. Supporting these considerations, no change in the systolic or diastolic $[Ca^{2+}]_i$ were observed in the present study when the rat atrium was exposed to 20 mM propionate (III, IV), indicating that the calcium extrusion systems can cope with small acidosis when the diastolic $[Ca^{2+}]_i$ is near normal, as in unstretched atrium. Acidosis decreases cardiac SR Ca^{2+} uptake (Kentish & Xiang, 1997), leading to a reduction of SR Ca^{2+} content during acidosis (Terracciano & MacLeod, 1997). The release of calcium from the SR is also decreased during acidosis since isolated SR Ca^{2+} release channels are inhibited by low pH_i (Rousseau & Pinkos, 1990). Together with the inhibition of the L-type Ca^{2+} current (Irisava & Sato, 1986; Kaibara & Kameyama, 1988; Chen *et al.* 1996), these inhibitory effects of acidosis should reduce the size of the Ca^{2+} transients. However , these effects are compensated by a rise in cytoplasmic $[Ca^{2+}]_i$ (Hulme & Orchard, 1998). According to the results of this study protons are likely to interact with several Ca^{2+} binding sites in the cytosol. First, modulation of the development of the stretch-induced increase in contraction force (III, IV) indicates the binding of H^+ into Ca^{2+} sites in the contractile element, as previously suggested (Blanchard & Solaro, 1984), resulting in a reduction of the TnC affinity to Ca^{2+} in acidosis (Palmer & Kentish, 1994). It is known that H^+ ions cause a shift of the force-pCa^{2+} curve to the right by reduction of the Ca^{2+} sensitivity of the contractile element at all sarcomere lengths (Ricciardi *et al.*, 1986). In the present study the contraction force of the pre-stretched atria was more sensitive to acidosis than the contraction of the non-stretched atria (III). This suggests that acidosis has a more profound effect on the contractile element when Ca^{2+} affinity is high, like during stretch. It is also possible that protons interact with the mechanism causing the stretch-induced TnC affinity change. This is supported by the fact that acidosis inhibits the stretch-induced development of the contraction merely by inhibiting the immediate (fast) phase of the development (IV). Secondly, slowing the decay of the calcium transients during development of the acidosis (IV) can be interpreted to be

partly due to the inhibition of the Ca^{2+} extrusion mechanisms by elevated $[H^+]_i$, including the inhibition of Na^+/Ca^{2+}-exchanger (Doering & Lederer, 1993). When the Ca^{2+} transients are augmented during stretch, acidotic cells are unable to efficiently remove the additional Ca^{2+} from the cytosol. This leads to the accumulation of Ca^{2+}-ions as manifested as an increase of the diastolic $[Ca^{2+}]_i$ (III).

Propionate application shortens the action potentials probably via the activation of the pH sensitive K_{ATP}-channels (Koyano *et al.* 1993), which also causes hyperpolarization (III) of the diastolic r.p., similarly as during ischemia (Gasser & Vaughan-Jones, 1990). The fact that acidosis inhibits the stretch-induced lengthening of the action potentials (III) suggests that other mechanisms are also involved. If the lengthening of the late phase of the APs during stretch is caused by the increased Na^+/Ca^{2+}- exchanger current due to bigger Ca^{2+} transients (DuBell *et al.* 1991), the inhibition of the Na^+/Ca^{2+} -exchanger by protons would make APs more insensitive to stretch. Shorter action potentials by themselves should make the Na^+/Ca^{2+}-exchanger inward current cause a more prominent depolarization, because the membrane voltage would now be more negative during the peak of the Ca^{2+} transient (Janvier & Boyett, 1996). This was not seen in the present study (III), which gives further support to the hypothesis of significant inhibition of the Na^+/Ca^{2+}-exchanger in acidosis. On the basis of the present work, modulation of the action potentials by pH_i can largely be explained by the concerted effects of intracellular protons on changes in systolic and diastolic $[Ca^{2+}]_i$, Na^+/Ca^{2+}-exchanger activity and potassium conductance.

Stretch of the cardiac muscle as such increases the amplitude of the Ca^{2+} transients (II, III, IV). Inhibition of the Ca^{2+} binding sites by H^+ potentiates this increase apparently by decreasing the Ca^{2+} buffering power, leading to elevated systolic and diastolic $[Ca^{2+}]_i$ (III, IV) and increased secretion of ANP (IV). Based on the present results the stretch-induced ANP secretion seems to be sensitive to the increase of the systolic $[Ca^{2+}]_i$ since stretch as such increases the Ca^{2+} transients (IV). Potentiation of the stretch-induced ANP by acidosis is likely to be caused by the increase of diastolic $[Ca^{2+}]_i$, indicating that the effects of diastolic and systolic $[Ca^{2+}]_i$ on the ANP secretion are additive (IV). This kind of modulation of the exocytotic processes is typical to the described low-affinity Ca^{2+} dependent mechanisms, where exocytosis is controlled by the integral $[Ca^{2+}]_i$ (Thomas *et al.* 1993). It is, however, interesting to note that among the Ca^{2+} dependent processes of the cardiac myocytes, the ANP secretion is far more tolerable to acidic shifts of pH_i than e.g. the Ca^{2+} extrusion mechanisms or the contractile element, indicating a different pH optimum of the exocytotic process. Intracellular acidosis, in addition to, or in combination with stretch, seems to be one of the main physiological stimuli of the secretion.

8. Conclusions

1. The stretch-induced changes in rat atrium are mediated via the activation of two separate mechanisms, the calcium affinity change of the contractile element and changes in the intracellular calcium concentration. An additional stretch-activated Ca^{2+} influx is required for the $[Ca^{2+}]i$ changes, whereas the contractile element's response to stretch seems to be an insintric property of the troponin C.

2. Rat atrium responses to stretch by two-phasic augmentation of the contraction force. Immediately after the onset of stretch the TnC Ca^{2+} affinity increases, leading to fast increase of the contraction force. This is followed by a secondary increase in the force, which is caused by augmentation of the systolic Ca^{2+} transients.

3. Action potential changes caused by stretch depend on the amount of stretch applied. Moderate stretch causes changes in the AP duration, but when severe stretch is applied, delayed afterpolarizations ensue. The AP changes caused by moderate stretch are due to an increased Na^{+}/Ca^{2+}-exchanger current during the repolarization phase of action potential. Exchanger current is activated by the increase of the Ca^{2+} transients during systole.

4. The stretch-induced ANP secretion can be due to the augmentation of the Ca^{2+} transients during stretch, without a change in the diastolic calcium concentration. The normal stretch-induced ANP secretion follows the slow part of the contraction development.

5. Intracellular acidosis modulates the stretch-sensitivity of the rat atrium by several ways. During co-incidence of stretch and acidosis the diastolic $[Ca^{2+}]_i$ is increased, due to the inhibition of calcium extrusion mechanisms and inhibition of the calcium binding by the contractile element. As a result, acidosis inhibits the fast development of the contraction force during stretch and the stretch-induced changes in the action potentials. In the same time-scale the stretch-induced ANP secretion is significantly augmented.

Conclusions are summarised in figure 8.

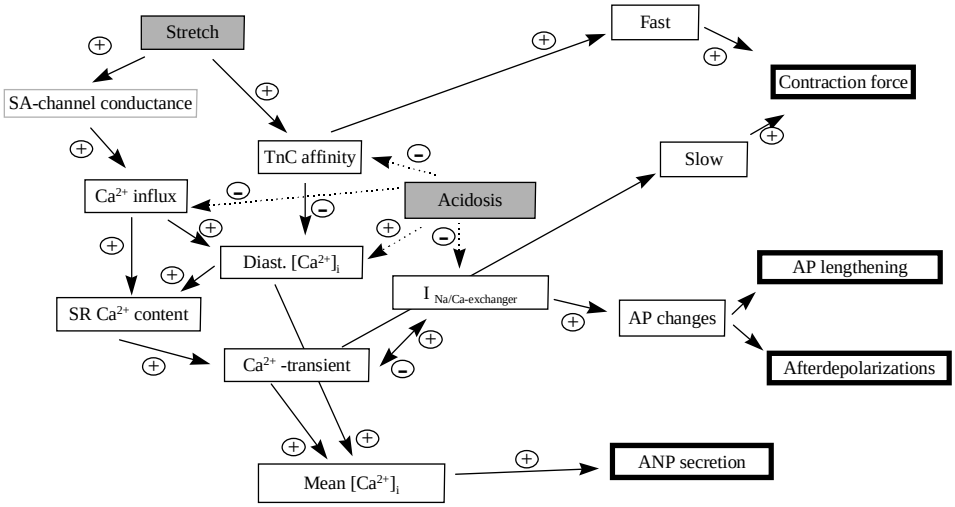


Fig. 8. Suggested mechanisms for stretch-induced changes in the function of the rat atrium. + and - signs indicate activation or inhibition, respectively.

9. References

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