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SARCOPLASMIC RETICULUM FUNCTION: COMPARISON OF ATRIAL AND VENTRICULAR MYOCARDIUM

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Minajeva, A., Kaasik, A., Paju, K., Seppet, E. K., Lompré, A.-M., Veksler, V., Ventura-Clapier, R. Sarcoplasmic reticulum function in determining atrio-ventricular contractile differences in rat heart. Am. J. Physiol. 273: H2489–H2507, 1997.

II Kaasik, A., Minajeva, A., Paju, K., Eimre, M., Seppet, E. K. Thyroid hormones differentially affect sarcoplasmic reticulum function in rat atria and ventricles. Mol. Cell. Biochem. 176: 119–126, 1997.

III Minajeva, A., Ventura-Clapier, R., Veksler, V. Ca^{2+} uptake by cardiac sarcoplasmic reticulum ATPase in situ strongly depends on bound creatine kinase. Pflügers Arch. 432: 904–912, 1996.

ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ANT	adenine nucleotide translocase
ATP	adenosine 5'-triphosphate
B.W.	body weight
$[Ca^{2+}]_o$	extracellular Ca^{2+} concentration
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
Cals	calsequestrin
CK	creatine kinase
Cr	creatine
DT	developed tension
+dT/dt	maximal rate of tension development (contraction)
-dT/dt	maximal rate of tension fall (relaxation)
FFR	force-frequency relationship
MHC	myosin heavy chain
Mi-CK	mitochondrial isoform of creatine kinase
P_i	inorganic phosphate
PCr	phosphocreatine
PEP	phosphoenolpyruvate
PK	pyruvate kinase
PLB	phospholamban
RFA	recirculation fraction of activator Ca^{2+}
RT	relaxation time
RT50	half-relaxation time (from peak tension)
RyR	ryanodine receptor/Ca^{2+} release channel
SERCA	sarco(endo)plasmic reticulum calcium pump
SL	sarcolemma
SR	sarcoplasmic reticulum
$S[Ca^{2+}]_f$	Ca^{2+}-time integral of caffeine-induced tension transient
S(T)	area under the tension transient of caffeine-induced contracture
T-tubules	transverse tubules
T_{max}	maximal Ca^{2+}-activated tension
TPT	time to peak tension
T_3	L-triiodothyronine
T_4	L-thyroxine

INTRODUCTION

Sarco(endo)plasmic reticulum (SR) is an intracellular membraneous organelle, which main function in muscle cells is the sequestration and release of Ca^{2+} to the myoplasm. By releasing Ca^{2+}, SR controls the activation of contraction in adult mammalian myocardium. On the other hand, diastolic relaxation largely depends on SR Ca^{2+} sequestration (Fabiato and Fabiato, 1979).

Differences in SR structure and function appear to underlie numerous differences in cardiac function between animal species, cardiac compartments or stages of development (Lompré *et al.*, 1991; Koss *et al.*, 1995; Moorman *et al.*, 1995; Harrer *et al.*, 1997). For example, atria contract and relax faster than ventricular chambers (Korecky and Michael, 1974; Urthaler *et al.*, 1975; Agata *et al.*, 1994; Asgrimsson *et al.*, 1995), and have a higher amount of SR in their myocytes (Bossen *et al.*, 1981; McNutt and Fawcett, 1969). In this work, SR function and protein expression in rat cardiac compartments will be addressed, since in atria they have not been sufficiently studied. The rat heart represents a valuable tool for studying the SR function in different cardiac compartments without interference of myofibrillar properties, since there is predominantly the same MHC isoform expressed in both atria and ventricles (Samuel *et al.*, 1986).

SR function is also altered under pathological conditions. For example, changes in thyroid state are known to impair the cardiac contractility (Buccino *et al.*, 1967, Taylor, 1970; Smithermann *et al.*, 1979; Wei *et al.*, 1982; Morkin *et al.*, 1983; Holubarsch, 1985; Seppet *et al.*, 1990; 1991). This is in great part determined by alterations in the SR function (Rohrer and Dillman, 1988; Beekman *et al.*, 1989; Nagai *et al.*, 1989; Arai *et al.*, 1991; Kimura *et al.*, 1994). In contrast to ventricles, the change in SR function under altered thyroid state is much less studied in atria. Therefore, in this work, the SR function in ventricles and atria in control and under altered thyroid state will be compared.

Adequate energy supply for Ca^{2+}-ATPase in the SR is a prerequisite for effective Ca^{2+} pumping and heart relaxation. In cardiomyocytes, the creatine kinase (CK) isoenzymes are bound specifically at the sites of energy production and utilisation and have an important role in the intracellular energy transfer (Wyss, *et al.*, 1992; Wallimann *et al.*, 1992, Saks *et al.*, 1994). In SR, there is evidence for the functional interaction between the cytosolic form of CK and Ca^{2+}-ATPase. In isolated vesicles of SR, bound CK could support ATP-driven Ca^{2+} uptake at the expense of PCr and ADP (Baskin and Deamer, 1970; Levitsky *et al.*, 1978; Rossi *et al.*, 1990). ATP, regenerated by CK is not in free equilibrium with ATP in the surrounding medium, but is used preferentially by the Ca^{2+}-ATPase for Ca^{2+} uptake (Korge *et al.*, 1993). However, in isolated vesicles, CK-driven Ca^{2+} uptake was always less than that driven by exogenous ATP (Levitsky *et al.*, 1978; Rossi *et al.*, 1990). In this work, the functional role of SR-bound CK *in situ* is studied by using the skinned-fibre technique.

REVIEW OF LITERATURE

1. Role of SR and myofilaments in excitation-contraction coupling in cardiomyocytes

Cell differentiation and maturation lead to a highly complex specialization and organization of cellular functions within structural and functional compartments. In adult mammalian cardiomyocytes, the depolarisation and subsequent Ca^{2+} entry via SL are the first events in the chain called excitation-contraction coupling, i.e. the process transforming the electrical excitation of the surface membrane into mechanical contraction. Ca^{2+} entering the cell releases an additional Ca^{2+} from the SR through the Ca^{2+} release channels. Contraction is ensured in the myofibrillar compartment (which occupies ~50–60% of muscle cell volume) by Ca^{2+}, leading to the sliding of the filaments. The ATPase of the SR is pumping up Ca^{2+} from myoplasm, resulting in muscle relaxation.

1.1. Ultrastructure and function of SR

SR is an intracellular membrane system, which in mammalian cardiomyocytes is composed of at least three structurally distinct regions (Sommer and Jennings, 1986; Forbes and Sperelakis, 1983; Segretain *et al.*, 1981; Lompré *et al.*, 1994): the network (longitudinal or free) SR, the junctional SR, and the specialized non-junctional SR (extended or corbular SR) (Figure 1). The network SR is composed of an anastomosing network of sarcotubules, surrounding

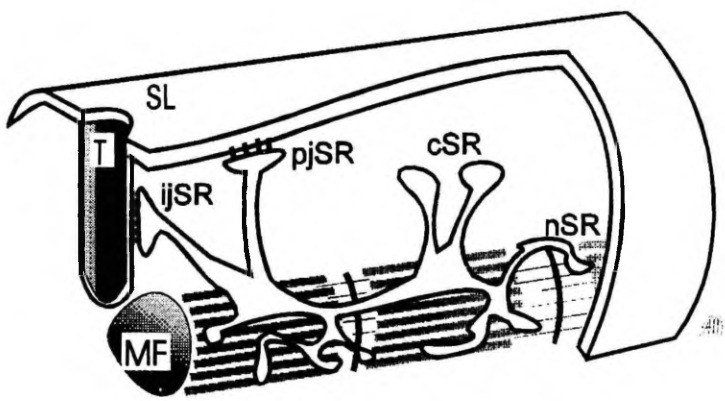

Figure 1. The diagram of heart sarcoplasmic reticulum illustrating the internal junctional SR (ijSR), peripheral junctional SR (pjSR), corbular SR (cSR) and network SR (nSR). SL, sarcolemma; T, transverse tubule; MF, myofilaments.

the myofibrils (Sommer and Jennings, 1986; Forbes and Sperelakis, 1983). The junctional and corbular SR are structurally specialized domains extending from the network SR and containing electron-dense material in their lumen (Sommer and Jennings, 1986). The structural difference between these two SR regions is that the junctional SR is connected to either the transverse (T) tubules (interior junctional SR) or the SL (peripheral junctional SR) via "feet" structures (Somlyo, 1979; Franzini-Armstrong, 1980) whereas this is not the case for corbular SR (Jorgensen *et al.*, 1988). These types of the SR have different proteins on their surface and in the lumen, summarized in Table 1. It is believed that distinct regions of SR perform different functions in cardiac cells.

1.1.1. Proteins involved in SR Ca^{2+} uptake

Sarco(endo)plasmic reticulum calcium pump (SR Ca^{2} -ATPase, SERCA) was first described as a Mg-activated ATPase in microsomal fraction from muscle by Kielley and Meyerhof (1948). Later, it was identified as the membrane associated Ca^{2+}-ATPase or "relaxing factor," responsible for lowering the cytosolic [Ca^{2+}] (Ebashi, 1961; Hasselbach and Makinose, 1961). SERCA is composed of a single large polypeptide of 100 kDa, that represents up to 40% of the total protein in the cardiac SR (Tada *et al.*, 1978). It catalyses Ca^{2+} transport to the lumen of the SR with stoichiometry of two Ca^{2+} ions for each ATP molecule hydrolyzed (Tada *et al.*, 1978; Tada and Katz, 1982; Inesi and Kirtley, 1990). In the presence of Ca^{2+}, the γ phosphate of ATP is covalently transferred to the aspartate 351 residue of the SR Ca^{2+} pump. Phosphorylation of the enzyme and ATP hydrolysis result in the translocation of the two Ca^{2+} ions bound to the enzyme from the high-affinity sites (E1-P state) to the low-affinity sites (E2-P). The two Ca^{2+} ions are then released into the lumen of SR.

Five Ca^{2+}-ATPase isoforms, encoded by three different genes have been identified. SERCA1 gene encodes two alternatively spliced transcripts: SERCA1a, present in adult fast skeletal muscle, and SERCA1b, transiently detected in neonatal fast skeletal muscle (Brandl *et al.*, 1987). The SERCA2 gene also encodes two alternatively spliced Ca^{2+}-ATPase mRNA. In ventricular myocardium of mammals (rat, rabbit, pig and human), the SERCA2a is the predominant isoform (Brandl *et al.*, 1987; MacLennan, 1990; Lompré *et al.*, 1989; Zarain-Herzberg *et al.*, 1990; Lytton *et al.*, 1992). SERCA2b has been observed at low levels in all cell types but mainly in smooth muscle (De la Bastie *et al.*, 1988; Eggermont *et al.*, 1989) and non-muscle tissues (Gunteski-Hamblin *et al.*, 1988; Lytton and MacLennan, 1988). SERCA3, encoded by an independent gene, is found in particular cell types, such as endothelial cells, some epithelial cells (Anger *et al.*, 1993), platelets (Bobe *et al.*, 1994) and in heart tube at early stages of development (Anger *et al.*, 1994).

Table 1. Ultrastructural localization of SR proteins in cardiomyocyte.

	Ca^{2+} uptake		Ca^{2+} release and storage	
	SERCA	PLB	RyR	Cals
network SR	+ (Jorgensen *et al.*, 1982; Jorgensen and McGufee, 1987)	+ (Jorgensen and Jones, 1987)	– (Jorgensen *et al.*, 1993)	– (Jorgensen and McGufee, 1987)
junctional SR	– (Jorgensen *et al.*, 1982)	– (Jorgensen and Jones, 1987)	+ (Jorgensen *et al.*, 1993)	+ (Jorgensen and Campbell, 1984; Jorgensen *et al.*, 1985; Jorgensen and McGufee, 1987)
corbular SR	– (Jorgensen *et al.*, 1982)	+ (Jorgensen and Jones, 1987)	+ (Jorgensen *et al.*, 1993)	+ (Jorgensen *et al.*, 1985; Jorgensen and McGufee, 1987)

SERCA, sarco(endo)plasmic reticulum calcium pump; PLB, phospholamban; RyR, Ryanodine receptor/Ca^{2+} release channel; Cals, calsequestrin; +, presence; – absence.

Phospholamban (PLB). In the short-term, the activity of SR is regulated by activation of the Ca^{2+}-pumps, but in the long-term by modulation of gene expression. PLB is present in tissues that express SERCA2 isoform and in physiological conditions, only SERCA2 isoform of SR Ca^{2+}-pump is regulated by PLB (Tada *et al.,* 1982; Tada and Katz, 1982; Colyer, 1993; James et al., 1989). PLB is a pentameric transmembrane protein complex of 51 amino acids, which has only one isoform (Wegener and Jones, 1984; Fujii *et al.,* 1987; 1991). In its nonphosphorylated form, PLB binds to SERCA and thereby inhibits its activity by decreasing the affinity of the pump to Ca^{2+}. Phosphorylation of PLB prevents the binding and allows the pump to exhibit its full activity (Tada *et al.,* 1975; Kranias *et al.,* 1980; 1985; Movsesian *et al.,* 1984; Luo *et al.,* 1994).

Ca^{2+}-ATPase and PLB are colocalized uniformly in the network SR, but are absent from the junctional membrane (Jorgensen *et al.,* 1982; Jorgensen and Jones, 1987). Thus, the function of the vast majority of the surface of the SR is likely to remove Ca^{2+} from the cytoplasm. In the corbular SR, however, there is PLB present, but not Ca^{2+}-ATPase (Jorgensen and McGufee, 1987).

1.1.2. Proteins involved in SR Ca^{2+} release and storage

The terminal cisternae of junctional SR in skeletal muscle were demonstrated to be the sites of Ca^{2+} release from the SR (Winegrad, 1965). The structural basis for this are the "feet" structures (Franzini-Armstrong, 1975), the complex of the highly specialized spanning proteins on the junctions of SR with SL. Similar structures are found in cardiac muscle at the junction of SR with SL (surface or T-tubules) and have been identified as the sites of Ca^{2+} release relevant to the excitation-contraction coupling (Fleischer and Inui, 1989; Wier, 1991). The biochemical evidence for a high molecular weight protein, which builds up the junctional "feet", was first provided by Caldwell and Caswell (1982) and identified later as a ryanodine receptor.

Ryanodine receptor/Ca^{2+} release channel (RyR) is a tetrameric protein of ~565 kDa, to which ryanodine is a specific ligand. Upon incorporation into planar lipid bilayers, the purified RyR forms a high conductance cation channel, which retains the characteristics of the channel from the native SR (Imagawa *et al.,* 1987). There are 3 RyR isoforms, encoded by 3 separate genes. RyR1 appears to be expressed predominantly in skeletal muscle (Takeshima *et al.,* 1989; Imagawa *et al.,* 1987), whereas RyR2 is predominantly expressed in heart (Inui *et al.,* 1987; Otsu *et al.,* 1990; Nakai *et al.,* 1990), but is detectable also in brain (Otsu *et al.,* 1990), stomach (Nakai *et al.,* 1990), and endothelial cells (Lesh *et al.,* 1993). RyR3 is expressed in brain, epithelial and smooth muscle cells (Giannini *et al.,* 1992; Hakamata *et al.,* 1992). The cardiac and skeletal RyR have similar conductance properties and pharmacological regulation (Inui *et al.,*

1987; Anderson *et al.,* 1989). An important functional difference between them is that in skeletal muscle, SR Ca^{2+} release is voltage-dependent, whereas in cardiomyocytes the SR Ca^{2+} release is dependent on trans-sarcolemmal Ca^{2+} influx (Näbauer and Morad, 1990; Niggli and Lederer, 1990). The cardiac RyR therefore is thought to be a Ca^{2+}-induced Ca^{2+}-release channel (Fabiato, 1983).

In addition to the junctional SR, corbular SR in cardiomyocytes has also been shown to contain RyR (Jorgensen *et al.,* 1993), which means that in cardiomyocytes, corbular SR also represents the site of SR Ca^{2+} release.

Calsequestrin (Cals) was first described in the skeletal muscle SR by MacLennan and Wong (1971). Cals in cardiac muscle was first identified and purified by Campbell *et al.* (1983). Two isoforms of this protein have been deduced by cDNA cloning: a "cardiac" Cals, expressed also in slow-twitch skeletal muscle (Scott *et al.,* 1988), and a fast-twitch skeletal muscle Cals (Fliegel *et al.,* 1987). Cals is a highly acidic glycoprotein which binds Ca^{2+} with high capacity (each molecule binds ~35–40 Ca^{2+} ions or ~900 nmol Ca^{2+}/mg protein, Mitchell *et al.,* 1988) and moderate affinity (an apparent Km (Ca) ~500 μM). Cals has been proposed to be the major protein to sequester and concentrate Ca^{2+} in the SR (Yano and Zarain-Herzberg, 1994). Comparison of biochemical and immunocytochemical studies with electron probe analysis of the subcellular distribution of Ca^{2+} in rabbit (Wheeler-Clark and Tormey, 1987) and rat (Jorgensen *et al.,* 1988) cardiac muscle suggested that both corbular (Jorgensen *et al.,* 1988) and junctional SR (Wheeler-Clark and Tormey, 1987; Jorgensen *et al.,* 1988) contain relatively high [Ca^{2+}], as well as Cals (Jorgensen and Campbell, 1984; Jorgensen *et al.,* 1985; Jorgensen *et al.,* 1988), whereas the network SR has a relatively low [Ca^{2+}] and lacks Cals (Jorgensen and Campbell, 1984; Jorgensen *et al.,* 1985; Jorgensen *et al.,* 1988). These observations support the idea that both the junctional and corbular SR containing Cals, are able to sequester Ca^{2+}, accumulated during relaxation, and represent the potential sources of Ca^{2+} release into the cytosol during contraction in mammalian myocardium.

Cals is bound to the junctional face of SR at least by two proteins, triadin (Guo and Campbell, 1995) and Cals binding protein (junctin, Jones *et al.,* 1995). Cals and RyR are functionally coupled: binding of ryanodine to RyR induces a rapid conformational change of Cals, which leads to the dissociation of Ca^{2+} from Cals, allowing the free Ca^{2+} to be released (Ikemoto *et al.,* 1991; Ohkusa *et al.,* 1991). On the other hand, conformational transitions of Cals have also been suggested to reciprocally regulate the transitions between open and closed states of RyR (Ikemoto *et al.,* 1989; Gilchrist *et al.,* 1992; Kawasaki and Kasai, 1994). Triadin has been proposed to be involved in the functional coupling between RyR and Cals, since monoclonal antibodies against it inhibit depolarization-induced Ca^{2+} release in skeletal muscle (Brandt *et al.,* 1992; Guo and Campbell, 1995).

1.2. Myofilament proteins in determining contractile function

1.2.1.Contractile proteins

Myofilaments are the end-effectors responsible for transducing chemical energy into mechanical work. The contractile proteins of myofilaments are thick (myosin) and thin (actin) filaments.

Myosin is composed of two heavy chains (MHC) and four light chains (MLC). Different MHC isoforms of mammalian sarcomeres are encoded by a multigene family consisting of at least seven different genes (Mahdavi *et al.*, 1987). Two classes of MHC have been found in cardiac tissue: α and β-MHC, which associate to form three isomyosins that exhibit different Ca^{2+}- and actin-stimulated ATPase activities and can be separated on polyacrylamide gel electrophoresis (Hoh *et al.*, 1978; Lompré *et al.*, 1984). The high ATPase myosin V_1 contains two α-MHCs; the intermediate form V_2 is an αβ heterodimer, whereas myosin V_3 contains two β-MHCs and has a lower activity (Hoh *et al.*, 1978; Litten *et al.*, 1982; Lompré *et al.*, 1981; 1984). Tension development is a result of a complex process (the "cross-bridge cycle") in which ATP is split by myosin ATPase. There is a correlation among myosin isoenzyme composition, myosin ATPase activity, shortening velocity and heart rate (Bárány, 1967; Delcayre and Swynghedauw, 1975; Schwartz *et al.* 1981; Cappelli *et al.*, 1989). Accordingly, V_3 is the principal isoform in the slowly beating hearts of larger mammals (e.g., rabbit), whereas V_1 is the prevailing isoform in the faster beating hearts of small laboratory mammals (mice, rat) (Swynghedauw, 1986; Syrovy *et al.*, 1979).

The thin filament is composed of two chains of the globular G-actin proteins, which form a helical double-stranded F-actin polymer. Muscle contraction occurs when the myosin and actin filaments slide past each other. According to the widely accepted theory proposed by Huxley (1957), muscle contraction or shortening occurs as a result of cyclic interactions between thick and thin filaments and cross-bridge extensions from myosin heads to actin molecules with the consumption of ATP energy in the presence of Ca^{2+}.

1.2.2. Regulatory proteins

Each myosin head contains a regulatory light chain (LC2) and an alkali light chain (LC1). Phosphorylation of LC2 by myosin light chain kinase increases the myofilaments Ca^{2+} sensitivity in pig skinned ventricular muscle (Morano *et al.*, 1985), but decreases the rate of myosin cross-bridge cycling (Franks *et al.*, 1984). Thus, myosin light chains have been suggested to modify the contractile velocity (Greaser *et al.*, 1988; Bottinelli *et al.*, 1995).

In the groove between the two actin helices, there is tropomyosin attached to the thin filament. At every seventh actin there is a troponin complex attached to

tropomyosin. The troponin complex is made up of three subunits: troponin T (TnT, or the tropomyosin binding subunit), troponin C (TnC, or the Ca^{2+} binding subunit), and troponin I (TnI, or the inhibitory subunit which can also bind actin). TnI interacts specifically with TnT and also binds specifically to actin. By binding to actin, TnI prevents myosin from interacting with actin. TnC is the site to which Ca^{2+} is bound to activate contraction (Zot and Potter, 1987). In heart, TnC has only one Ca^{2+}-specific binding site with Kd ~500 nM, which is appropriate to the activation of contraction of isolated myofibrils or the native actomyosin (Holroyde *et al.*, 1980; Johnson *et al.*, 1980). At myocardial relaxation when $[Ca^{2+}]_i$ is low, the Ca^{2+}-specific sites of TnC are unoccupied. In this condition the interaction between TnI and TnC at the critical region of TnI is weak and this region of TnI appears to interact more strongly with actin. When $[Ca^{2+}]_i$ rises, Ca^{2+} binds to the Ca^{2+}-specific site of TnC. This may then strengthen the specific interaction of TnC with TnI and destabilize the interaction of TnI with actin. The resulting conformational change of troponin-tropomyosin complex relieves the sites on the backbone of the actin filament to which the myosin heads of the thick filaments can bind enabling, thus, force production and shortening.

1.3. Mechanism of excitation-contraction coupling in cardiomyocyte

Since the classic experiments of Ringer (1883) it has become clear that cardiac contraction depends on the extracellular $[Ca^{2+}]$. By different estimations, the resting free $[Ca^{2+}]$ in cardiomyocytes is in the range of ~75–200 nM. The Ca^{2+} requirements for activation of contraction have been estimated to be ~30–50 μmol/kg wet wt (Fabiato, 1983; Blinks, 1986; Alpert *et al.*, 1989). In adult mammalian cardiomyocytes, electrical excitation at the surface membrane leads to an action potential which propagates as a wave of depolarization along the surface and along the T-tubules leading to the transsarcolemmal Ca^{2+} entry (Figure 2). The vast majority of the Ca^{2+} entry (10 μmol/kg wet wt, Bers, 1983) in ventricular muscle is through the L-type Ca^{2+} channel (DHPR, dihydropyridine receptor) during the action potential plateau (Morad and Goldman, 1973; London and Krueger, 1986; Näbauer *et al.*, 1989; Cannell *et al.*, 1994). Ca^{2+} can enter the cell also due to the reversal of the Na^+/Ca^{2+} exchanger during the upstroke of the action potential (Eisner *et al.*, 1984; Kimura *et al.*, 1987; Bers, 1987; Blaustein, 1988). The Ca^{2+} influx via Na^+/Ca^{2+} exchange is, however only less than 30% of the Ca^{2+} entry by the L-type Ca^{2+} channel (Grantham and Cannell, 1996). To which extent Ca^{2+} influx via SL participates in direct activation of the myofilaments remains under debate. By estimates, the Ca^{2+} entering the cardiac cell via SL Ca^{2+} channels would by itself be sufficient to activate only about 4–5% of the maximal force (Bers, 1991). However, the transsarcolemmal Ca^{2+} influx both by the L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchange have two important effects: the Ca^{2+} entering the cell induces Ca^{2+}

release from the SR and contributes to the replenishment of the SR Ca^{2+} stores (Fabiato and Fabiato, 1975; 1978a,b; 1979; Fabiato, 1983; 1985; Berlin *et al.*, 1987; Banijamali *et al.*, 1991; Lipp and Niggli, 1994; Kohmoto *et al.*, 1994).

SR represents a huge intracellular Ca^{2+} store, sufficient to fully activate all the myofilaments. The maximal Ca^{2+} content of the SR has been estimated to be from 100 to 300 μmol Ca^{2+} /kg wet wt. (Solaro and Briggs, 1974; Levitsky *et al.*, 1981, Bers *et al.*, 1989). Apparently, the intra-SR $[Ca^{2+}]$ is buffered to a significant extent by Cals. The mechanism of Ca^{2+}-induced Ca^{2+}-release from the SR was first demonstrated in cardiac cells with mechanically skinned sarcolemma by simulating the transsarcolemmal Ca^{2+} entry by the rapid Ca^{2+} application (Endo *et al.*, 1970; Fabiato and Fabiato, 1975; 1978a;b; 1979; Fabiato, 1983; 1985). It was revealed, that the amount of Ca^{2+} released was graded with the amount of the "trigger" Ca^{2+} (Fabiato, 1983; 1985). In skeletal muscle, the SL L-type Ca^{2+} channel and the RyR are colocalized (Block *et al.*, 1988) and the SL Ca^{2+} channel itself may transmit the signal for Ca^{2+} release to the SR. In cardiac muscle, direct physical connection between the L-type Ca^{2+} channels and the RyR have not been demonstrated. Instead, there is an evidence from intact cardiac myocytes about cross-signalling between L-type Ca^{2+} channels and RyR (Sham *et al.*, 1995; Adachi-Akahane, 1996): the Ca^{2+} transport through the L-type Ca^{2+} channels was much more effective to trigger the Ca^{2+}-induced Ca^{2+} release from the SR than the Ca^{2+} entry via Na^{+}/Ca^{2+} exchange (Sham *et al.*, 1995).

When $[Ca^{2+}]_i$ rises, it binds to TnC and relieves the sites on the backbone of the actin filament to which the myosin heads of the thick filaments can bind and perform the work (Zot & Potter, 1987). Relaxation occurs when Ca^{2+} is removed from the cytoplasm such that it will dissociate from TnC. Ca^{2+} is removed from the cytoplasm into the SR by the SR Ca^{2+}-pump and by the extrusion out of the cell. Two most important mechanisms responsible for the extrusion of Ca^{2+} from cardiac myocytes are the SL Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger. The SL Ca^{2+}-pump, first described in myocardium by Caroni and Carafoli (1980) can have high affinity for $[Ca^{2+}]_i$ but the Ca^{2+} transport rate is too slow (Dixon and Haynes, 1989) for it to be important to the Ca^{2+} fluxes during the cardiac cycle. It might, however be more important in long-term extrusion of Ca^{2+} by the cell (Bers, 1991). Already in 1958, Lüttgau and Niedergerke suggested that Na^{+} and Ca^{2+} compete for a site at the SL responsible for bringing Ca^{2+} into the cell. The Na^{+}/Ca^{2+} exchange system was first described in heart SL vesicles by Reeves and Sutko (1979). By now it is clear that the Na^{+}/Ca^{2+} exchanger is the main route for Ca^{2+} extrusion of the myocardial cell. 20–30% of the Ca^{2+} (at a rate of 150 μmol/kg wet wt/sec) is removed from the cytoplasm by this mechanism (Reeves and Philipson, 1989).

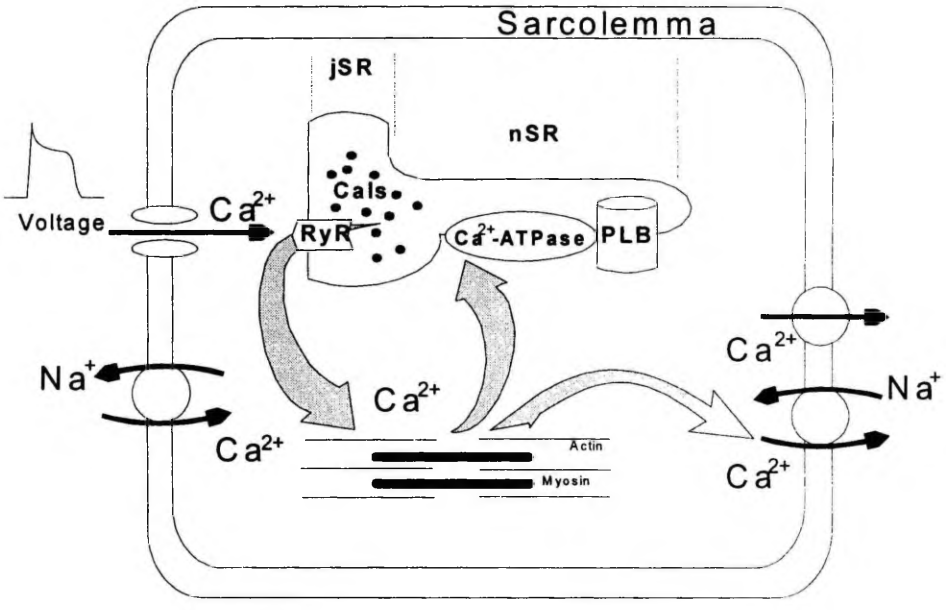

Figure 2. The scheme illustrating Ca^{2+} movements during excitation-contraction coupling in mammalian cardiomyocyte. jSR, junctional SR; nSR, network SR; RyR, ryanodine receptor-Ca^{2+} release channel; Cals, calsequestrin; PLB, phospholamban.

2. Differences between atria and ventricles

Atrial myocytes differ morphologically from ventricular ones: they are smaller, branch less, have fewer if any T-tubules, and contain specific granules that are not found in ventricular myocytes (Palade, 1961; McNutt and Fawcett, 1969; Hibbs and Ferrans, 1969; Bossen *et al.,* 1981). The major difference in atrial and ventricular contractility is that atria contract and relax faster than ventricles (Korecky and Michael, 1974; Urthaler *et al.,* 1975; Agata *et al.,* 1994; Asgrimsson *et al.,* 1995). Also, there is a shorter action potential in atria than in ventricles (Korecky and Michael, 1974; Asgrimsson *et al.,* 1995).

In some animals, the faster kinetics of atrial contraction can be attributed to a higher proportion of the fast α-MHC isoform in atria compared with ventricles (Cappelli *et al.,* 1989). However, it cannot be an explanation in small mammals such as rat, in which fast α-MHC is the predominant isoform in both atria (>90–95% ; Samuel *et al.,* 1986) and ventricles (~70–80% α-MHC, Hoh *et al.,* 1978; Cappelli *et al.,* 1988; 1989). Myosin molecules present in atrial and ventricular myocytes differ in their two associated light chains, which are tissue specific (LC1a and LC2a in atrium and LC1v and LC2v in ventricle) (Dalla-Libera, 1983; Cummins and Russel 1986; Swynghedauw 1986). Despite the differences

in LC expression, the muscle stiffness and maximal Ca^{2+}-activated tension of atrial and ventricular tissues are similar (Vannier *et al.* 1996). Also, the Ca^{2+} sensitivity of tension development is not different between these two types of myocardium (Vannier et al 1996; Palmer and Kentish, 1996).

In atrial myocytes, there is a more developed SR network than in ventricular ones. The amount of the interior junctional SR is significantly lower in atria, but the volume fraction and the surface area of network, peripheral junctional and corbular SR, as well as of total SR is higher in atria than in ventricles (Bossen *et al.*, 1981; McNutt and Fawcett, 1969). In atrial SR, there is higher SERCA2 expression but less PLB than in ventricles, the relative ratio of PLB to SERCA2 mRNA being 4.2-fold lower in the atrium than in the ventricle (Koss *et al.*, 1995). There is no study comparing RyR or Cals expression in both tissues.

In myocardium from several animal species, atrial contraction is always more dependent on SR Ca^{2+} release than on SL Ca^{2+} influx and has more active Ca^{2+}-induced Ca^{2+}-release than ventricular one (Bers *et al.*, 1989, Fabiato 1982, Agata *et al.*, 1994; Asgrimsson *et al.*, 1995). The complex study relating the SR function to the protein expression in atria compared to ventricles is to be carried out.

3. Thyroid hormones in regulation of cardiac contractile function

Thyroid status has profound effects on the cardiovascular system with major actions on the myocardium (Morkin *et al.*, 1983; Forfar and Caldwell, 1985; Klein, 1990). Long term excess of thyroid hormones is associated with an abbreviated duration and increased velocity of contraction in ventricular myocardium (Buccino *et al.*, 1967, Taylor, 1970; Smithermann *et al.*, 1979; Wei *et al.*, 1982; Morkin *et al.*, 1983; Cappelli *et al.*, 1988; Kolár *et al.*, 1992). Hypothyroidism has the opposite effects (Buccino *et al.*, 1967; Morkin *et al.*, 1983; Holubarsch, 1985; Taylor, 1970; Seppet *et al.*, 1990; 1991). Recently, higher rates of contraction and relaxation under hyperthyroidism and lower ones under hypothyroidism compared to euthyroidism were also demonstrated in rat atria (Kaasik *et al.*, 1997).

Many of the known cellular effects of thyroid hormones are mediated by the specific high-affmity nuclear receptors (Evans, 1988; Oppenheimer, 1985; Sap *et al.*, 1986; Samuels *et al.*, 1988; Weinberger *et al.*, 1986), that interact with the cis-acting DNA elements of the specific genes to either promote (Markham *et al.*, 1987; Ojamaa and Klein, 1991; Samuels *et al.*, 1988) or inhibit (Chin *et al.*, 1985) the DNA transcription. In ventricular myocardium of several animal species, the hypothyroid state favours the expression of β-MHC at the expense of α-MHC, while the opposite is true for hyperthyroidism (Litten *et al.*, 1981;

1982; Lompré *et al.,* 1984; Everett *et al.,* 1983; 1984; Gustafson *et al.,* 1986; Cappelli *et al.,* 1988; 1989), resulting in a shift towards higher ATPase activity (Buccino *et al.,* 1967; Suko, 1973; Goodkin, *et al.,* 1974; Skelton *et al.,* 1976; Flink *et al.,* 1979; Hoh *et al.,* 1978; Morkin *et al.,* 1983; Holubarsch *et al.,* 1985). In the mature rat heart, where myosin ATPase activity is already high in euthyroid state (Morkin *et al.,* 1983), the myosin ATPase activity and myofilaments Ca^{2+} sensitivity remain relatively unchanged under hyperthyroidism in both atria and ventricles (Rovetto *et al.,* 1972; Yazaki and Raben, 1975). However, under hypothyroidism, the isomyosin pattern is shifted towards β-MHC and the normal isomyosin pattern can be reproduced only with daily administration of thyroid hormone (Hoh *et al.,* 1978; Chizzonite and Zak, 1984; Samuel *et al.,* 1986). The important difference between atria and ventricles is that in atria, there is only a slight accumulation of β-MHC (<5% of total myosin) under hypothyroidism (Samuel *et al.,* 1986) whereas in ventricles α-MHC can be almost entirely replaced by β-MHC (Chizzonite and Zak, 1984; Chizzonite *et al.,* 1984; Gibson *et al.,* 1992). Thus, atrial MHC expression is almost insensitive to thyroid hormones.

The effect of thyroid hormone on the speed of diastolic relaxation is thought to result from the changes in the velocity of cytoplasmic Ca^{2+} sequestration by the SR (Alpert *et al.,* 1987). SR isolated from the ventricular muscle of hyperthyroid animals exhibits increased rates of Ca^{2+} sequestration and Ca^{2+}-ATPase activity (Limas, 1978; Suko, 1973; Kiss *et al.,* 1994; Kimura *et al.,* 1994); in contrast, these rates are decreased in SR from hypothyroid animals (Suko, 1973; Kiss *et al.,* 1994). This is associated with an increase in the SR Ca^{2+}-ATPase gene expression as evidenced by a higher level of Ca^{2+}-ATPase under hyperthyroidism and decreased level in thyroidectomized rats (Arai *et al.,* 1991; Kimura *et al.,* 1994; Kiss *et al.,* 1994) (Table 2). Similarly, the SR Ca^{2+}-ATPase mRNA is low in hypothyroid animals whereas injection of thyroid hormone to hypothyroid (Rohrer and Dillman, 1988) or euthyroid rabbits (Nagai *et al.,* 1989; Arai *et al.,* 1991) up-regulates the SERCA2 gene to euthyroid or hyperthyroid levels.

PLB gene and Ca^{2+}-ATPase genes are inversely regulated by thyroid hormone. The level of PLB is increased in hypothyroidism and decreased in hyperthyroidism compared with euthyroidism (Kiss, 1994). However, alterations in the level of PLB did not correspond to the changes in the PLB mRNA level, which was lower than in controls in both hypothyroid and hyperthyroid animals (Arai *et al.,* 1991; Kimura *et al.,* 1994; Nagai *et al.,* 1989). These discrepancies between PLB mRNA and protein level have been explained by longer half-life of the mRNA and/or increased stability of the protein in hypothyroidism (Kiss, 1994). Recently, it was shown that the decreased amount of PLB in hypothyroidism and increase under hyperthyroidism were much more pronounced in atria (Kaasik *et al.,* 1997) than it has been reported in ventricles (Kiss *et al.,* 1994).

Table 2. Thyroid state dependent shifts in different SR proteins and the corresponding mRNA levels.

	SERCA2		PLB		RyR	Cals
shift relative to euthyroidism	mRNA	protein	mRNA	protein	mRNA	mRNA
hyperthyroidism	+67% (Nagai *et al.*, 1989) +86% (Arai *et al.*, 1991) + 80% (Kimura *et al.*, 1994)	+99% (Arai *et al.*, 1991) +34% (Kiss *et al.*, 1994)	–39% (Nagai *et al.*, 1989) –28% (Arai *et al.*, 1991) – 50% (Kimura *et al.*, 1994)	–25% (Kiss *et al.*, 1994)	+47% (Arai *et al.*, 1991)	= (Arai *et al.*, 1991)
hypothyroidism	–49% (Nagai *et al.*, 1989) –25% (Arai *et al.*, 1991)	–14% (Arai *et al.*, 1991) –26% (Kiss *et al.*, 1994)	= (Nagai *et al.*, 1989) –23% (Arai *et al.*, 1991)	+35% (Kiss *et al.*, 1994)	–29% (Arai *et al.*, 1991)	= (Arai *et al.*, 1991)

SERCA2, sarco(endo)plasmic reticulum calcium pump type2; PLB, phospholamban; RyR, Ryanodine receptor/Ca^{2+} release channel; Cals, calsequestrin.

The expression of RyR is co-ordinately regulated with SERCA2 by thyroid hormones (Arai *et al.*, 1991). The Cals gene is not influenced by thyroid hormone (Arai *et al.*, 1991).

At the level of SL, thyroid hormones restrict the expression of Na^+/Ca^{2+} exchanger (Vetter and Kott, 1995) and of the slow Ca^{2+} channels (Hawthorn *et al.*, 1988). Also, thyroid hormones activate SL ATP-dependent Ca^{2+}-pump (Rudinger *et al.*, 1984; Seppet *et al.*, 1990; 1991). Therefore, under hyperthyroidism, the role of the SR Ca^{2+} source for activating contraction is increased as compared to ventricular myocardium (Seppet *et al.*, 1990; 1991). Also, thyroid hormones accelerate the developmental shift from predominantly SL to predominantly SR mechanism of providing activator Ca^{2+} (Kolár, 1992). However, the study comparing the contribution of the SR and SL sources of activator Ca^{2+} under altered thyroid state in atrial and ventricular myocardium of the same animal species is still lacking.

4. Role of coupled creatine kinases in metabolic compartmentation and the concept of PCr energy shuttle

Adult mammalian cells, exhibiting highly differentiated and organized structure, are characterized by compartmentation of enzymes implicated in cellular energy production, utilization and transfer. ATP may be derived from two major synthetic pathways, that is, from fatty acid oxidation in mitochondria and from glycogenolytic or glycolytic reactions. The relative importance of the two metabolic pathways of energy production (mitochondria and glycolysis) varies between muscle types depending on the contractile pattern. The family of creatine kinase (CK) isoenzymes is functioning as a pathway to optimize the production, transfer and utilization of energy in the muscle cells.

4.1. CK isoenzymes

The CK reaction was discovered by Karl Lohman (1934). This reaction:

$$MgADP^- + PCr^{2-} + H^+ \Leftrightarrow MgATP^{2-} + Cr$$

is catalysed by different isoenzymes of CK, which have the same conserved structure of the active centre (Mühlebach *et al.*, 1994). A major part of muscle CK exists as dimers composed of two subunits, M and B, giving three isoenzymes, MM, BB and MB. In addition, there is a fourth isoenzyme in the mitochondria (mi-CK), which differs biochemically and immunochemically from the cytosolic forms and can form both octameric and dimeric structures

(Wallimann *et al.,* 1992; Wyss *et al.,* 1992). Mi-CK is coded by two different genes in a tissue specific manner. One form (mi-CK_s or mi_b-CK) is present in tissue presenting sarcomeric structures (striated muscles) and is co-expressed with M-CK, while the other (mi-CK_u or mi_a-CK) is ubiquitous (in non-muscle cells) and co-expressed with B-CK (Payne *et al.,* 1991; Wyss *et al.,* 1992). Four CK isoenzymes described do not differ in their kinetic parameters. (Wallimann *et al.,* 1992; Wyss *et al.,* 1992). However, they mainly differ in their subcellular distribution: they are present in the cytosol, or bound to the intracellular compartments in a tissue-specific manner. In fast twitch skeletal muscles which exhibit a high and fluctuating ATP consumption, the PCr/CK system functions as a reserve of energy rich phosphates, but also transfers energy from glycolytic complexes to ATPases (Kupriyanov *et al.,* 1980). Accordingly, most of the CK activity in these muscles is cytosolic with only 5–10% being associated with the SR and myofibrils and the amount of mi-CK being negligible (<5%). These muscles develop a burst of intensive activity at the expenses of the energy reserves and are highly fatiguable. In contrast, myocardium represents an oxidative muscle with cyclic activity. Oxidative muscles rely more on the simultaneous energy production and energy transfer from mitochondria than on energy reserves. Accordingly, they possess a high amount of mitochondria (up to 40% of the cell volume), a high specific activity of mi-CK and a high relative proportion of bound CK, but with lower total CK and creatine contents compared with glycolytic muscles. In ventricular muscle, at least 20% of total CK is associated with myofibrils (Ventura-Clapier *et al.,* 1987) whereas mi-CK represents 20–30%. Additional binding sites in the SR, SL and nucleus results in soluble CK being at most 50% of total activity.

4.2. Subcellular and functional compartmentation of CK isoenzymes in cardiomyocytes

In mitochondria, positively charged mi-CK are fixed to the negatively charged cardiolipin molecules at the surface of the inner membrane, which surround adenine nucleotide translocase (ANT), therefore connecting these proteins into one complex (Müller *et al.,* 1985; Stachowiak, *et al.,* 1996). In addition, mi-CK is present in dimeric or octameric forms and, due to their identical top and bottom faces, octamers can simultaneously interact with two opposing membranes (Rojo and Wallimann, 1994), forming contact sites between the inner and outer mitochondrial membranes. Mi-CK is functionally coupled to the ANT so that ATP generated by oxidative phosphorylation, after transport through the inner mitochondrial membrane, is transphosphorylated to PCr with the ADP production. This ADP is trasported back to the mitochondrial matrix (Bessman and Carpentier, 1985; Wallimann *et al.,* 1992; Saks *et al.,* 1994). In this reaction, by increasing the local ATP concentration and removing the product ADP, ANT drives the CK reaction towards PCr production.

Suprisingly, rat atrial tissue does not exhibit mi-CK coupling to oxidative phosphorylation (Anflous *et al.*, 1997). Mi-CK_s is present in rat atria, (Anfous *et al.*, 1997), although its activity is about 7 times lower than in ventricles (Vannier *et al.*, 1996; Savabi and Kirsch, 1991). Also, in atrial myocytes significantly lower mitochondrial fraction and oxidative capacity has been reported (Bossen *et al.*, 1981; Sylven *et al.*, 1991; Vannier *et al.*, 1996).

In extramitochondrial compartment of heart and skeletal muscles, MM-CK is the predominant isoform.

In myofibrils, the MM-CK has been described as a structural protein of the M-band participating in the connections between myosin filaments inside the muscle fibres (Wallimann, *et al.*, 1983; 1985; Wallimann and Eppenberger, 1985). This bound M-CK is found near the location of ATPase activity (Saks *et al.*, 1976) and has been shown to be biochemically and functionally coupled to myosin ATPase (Bessman *et al.*, 1980; Ventura-Clapier *et al.*, 1994). Myofibrillar CK can use PCr to rephosphorylate all of the ADP produced by myosin ATPase and can produce enough energy for maximal force and normal contractile kinetics even in the absence of MgATP (Wallimann *et al.*, 1985; Saks *et al.*, 1984; Ventura-Clapier, 1987; Ventura-Clapier *et al.*, 1994). This functional interaction of CK with myosin ATPase provides a high local ATP/ADP ratio and low proton concentration favourable to the myofibrillar function (Wallimann *et al.*, 1992; Ventura-Clapier *et al.*, 1994). Furthermore, it allows the myosin ATPase reaction to use preferentially ATP supplied by the CK reaction rather than cytosolic ATP (Bessman *et al.*, 1985; Saks *et al.*, 1984). In atria, the M-CK bound to myofibrils rephosphorylates ADP as efficiently as in ventricle (Vannier et al 1996).

Sarcoplasmic reticulum. The presence of CK activity in SR was first described in fragmented SR of rabbit skeletal muscle (Baskin and Deamer 1970) and cardiac muscle (Levitski, 1978). In chicken breast skeletal muscle, Rossi *et al.* (1990) showed that SR vesicles contain about 1 IU of CK/mg SR protein. Highly purified fraction of pigeon heart SR vesicles contains 0.6 IU/mg SR protein CK activity with a SR-ATPase/CK ratio of 4.5 (Levitsky et al 1978). In 1990 it was confirmed by Rossi *et al.* that MM-CK is indeed strongly anchored to highly purified SR vesicles of skeletal muscle. In isolated SR vesicles, bound CK could produce sufficient energy to support a significant portion of the maximal Ca^{2+} uptake rate (Baskin, 1970; Levitski 1978; Rossi *et al.*, 1990).

Later it has been shown that the ATP regenerated by CK is not in free equilibrium with the ATP in the surrounding medium, but is used preferentially by the Ca^{2+} ATPase for Ca^{2+} uptake (Korge *et al.*, 1993). In rat ventricles, it was shown that PCr could efficiently abolish the inhibitory effect P_i exerts on the Ca^{2+} accumulation into the SR (Steele *et al.*, 1995). However, few data exist concerning the functional role of the SR-bound CK *in situ*. Moreover, in atria,

nothing is known about functioning of the CK system at the sites of SR Ca^{2+} uptake.

MM-CK has also been found at the *sarcolemma* (Sharov, 1977). The membrane-bound CK ATP-regenerating system was found to be the most effective energy source for active Ca^{2+} transport, indicating thus the functional coupling of bound CK to the Na^{+}/K^{+}-pump (Saks *et al.*, 1977; Grosse *et al.*, 1980).

4.3. Concept of PCr energy shuttle

Characterisation of the roles of different CK isoenzymes has led to development of a concept of PCr energy shuttle. This concept assumes that the diffusion of ATP and ADP in the cells is restricted (Nagle, 1970a;b; Saks *et al.*, 1994). Therefore, energy is transferred from the sites of its production to the ATPases via enzymatic equilibrium CK reactions (PCr energy shuttle) in the cytosol (Figure 3) (Bessman and Geiger, 1981; Bessman and Carpenter, 1985; Mitchell, 1991; Wallimann *et al.*, 1992; Saks *et al.*, 1994). One role of the PCr shuttle therefore is to maintain sufficient level of [ATP] at the sites of energy consumption (ATPases). This is achieved via functional coupling between MM-CK and ATPases that ensures continuous rephosphorylation of ADP at the expense of PCr (Bessman and Geiger, 1981; Bessman and Carpenter, 1985; Saks *et al.*, 1994; Wallimann *et al.*, 1992). On the other hand, PCr energy shuttle provides a feedback signal in the form of [Cr] to mitochondria. Reaching the mitochondria, the local change in [Cr] stimulates coupled reactions of its rephosphorylation in the intermembrane space of mitochondria. If mitochondria receive a signal of a local change in [ADP], this signal is manifold amplified by the coupled mitochondrial systems that again results in a rapid PCr production (Saks *et al.*, 1994; Gellerich, 1994; Brdiczka and Wallimann, 1994). As a result, the metabolic rates and ATP production in mitochondria would be precisely matched to the myocardial workload and the ATP consumption.

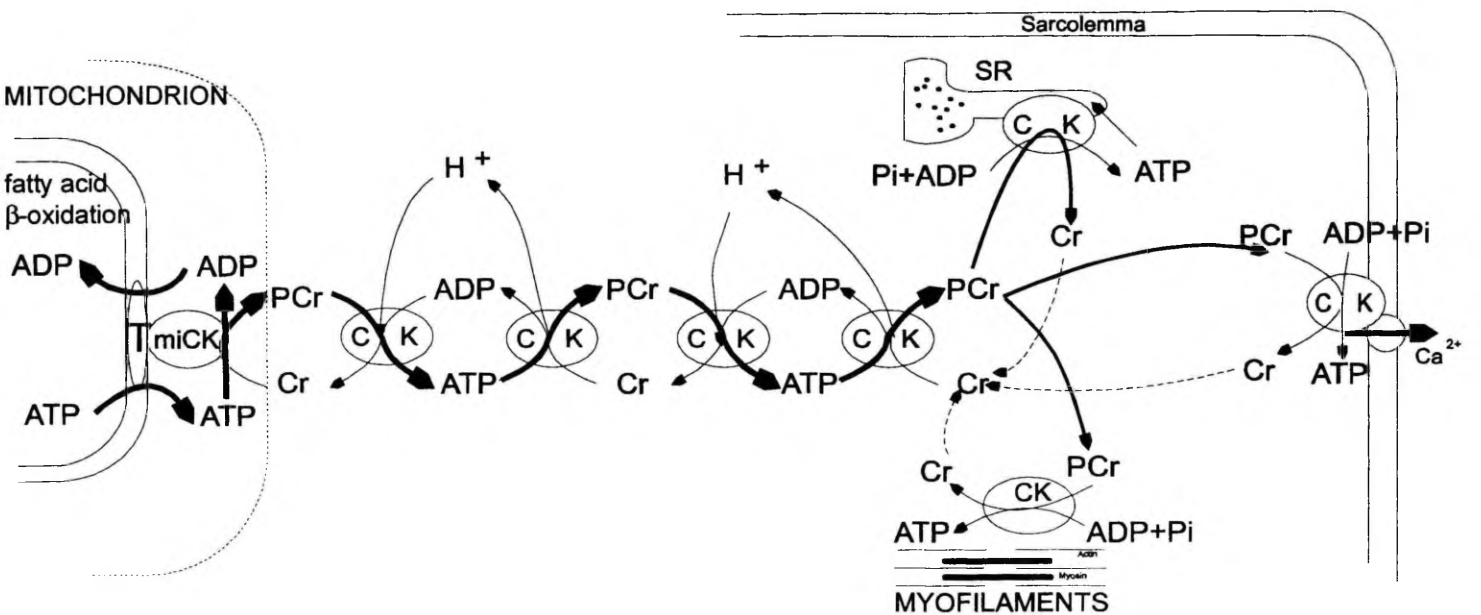

Figure 3. Phosphocreatine pathway for intracellular energy transport in muscle cells.

PURPOSES OF THE STUDY

The present study was performed to elucidate the functional and metabolic properties of SR in atrial and ventricular myocardium and their relation to the contractile function of both tissues under control and altered thyroid state.

In detail, the aims of the study were:

1. To relate differences in contractile parameters between atria and ventricles to the SR function and expression of the SR proteins in both tissues. For this purpose, the following characteristics of atria and ventricles were compared: (1) the twitch tension parameters of isolated trabeculae; (2) the SR function *in vivo*; (3) the SR Ca^{2+} uptake in saponin-skinned fibres; (4) the SR Ca^{2+} uptake rate in tissue homogenates and (5) the expression of mRNA of the SR proteins (SERCA, RyR, Cals) relative to MHC mRNA (I).
2. To study the effects of hypothyroidism and hyperthyroidism on SR Ca^{2+} -pump activity, and the functional role of SR as a source of activator Ca^{2+} under these altered thyroid states in atria and ventricles. For this purpose, the following characteristics of atria and ventricles from rats of different thyroid status were compared: (1) the twitch tension parameters; (2) the role of SR as a source of Ca^{2+} for twitch contraction *in vivo* and (3) the Ca^{2+} uptake rate in tissue homogenates (II).
3. To study the energy dependence of SR Ca^{2+} uptake and the role of bound CK *in situ* in atrial and ventricular myocardium by using saponin-skinned atrial and ventricular fibres (I, III).

MATERIALS AND METHODS

1. Animals, modification of their thyroid state

For all experiments, adult Wistar strain rats of either sex (B. W. 200–400 g) were used. Animals were treated and sacrificed accordingly to the European Convention for Protection of Vertebrate Animals. Hypothyroidism of animals was induced by addition of 6-n-propyl-2-thiouracil to then drinking water in a concentration of 0.05% during 6 wk. Hyperthyroidism was caused by the subcutaneous administration either of L-thyroxine (T_4) to euthyroid animals at daily doses of 1 μg/g B. W. for 1 week or L-triiodothyronine (T_3) to hypothyroid animals at daily doses of 50 ng/g B. W. for 5 days.

2. Chemicals

Caffeine was purchased from Merck-Clevenot. PCr (Neoton, Schiapparelli Searle, Turin, Italy) was a kind gift of Prof. E. Strumia. Other chemicals were obtained from Sigma Chemical Co.

3. Registration of twitch tension parameters

The steady-state isometric twitch tension parameters (DT, TPT, +dT/dt, -dT/dt, CT, RT50), normalized to average cross section area, were registered in electrically stimulated fibres (300–600 μm wide), dissected from papillary muscles and left atria as detailed in I. The average cross-section area were calculated by dividing the weight of trabeculae by their length.

4. Assays of the function of SR *in vivo*

To reveal the contribution of SR in providing activator Ca^{2+} *in vivo*, several approaches were used. One possibility for this is to inhibit the SR Ca^{2+} release by ryanodine.

Effect of ryanodine (II) on twitch tension was studied by applying 30 μM of ryanodine to the perfusion medium. Ryanodine is a neutral plant alkaloid which

in submicromolar concentrations activates the cardiac SR RyR (Rousseau *et al.*, 1986; Smith *et al.*, 1986), at concentrations ≤ 30μM it opens the channel to a stable subconducting state (Rousseau *et al.*, 1987; Meissner, 1986). At very high ryanodine concentrations (> 100 μM) the Ca^{2+} release channel appears to be locked in a closed state (Meissner, 1986; Lai *et al.*, 1989). Under inhibition by ryanodine, twitch tension will depend on transsarcolemmal Ca^{2+} influx. The extent of inhibition of twitch tension by ryanodine correlates with the abundance of SR and the activity of the Ca^{2+}-induced Ca^{2+} release of the tissue (Fabiato, 1978a; Fabiato, 1982).

Twitch tension-interval relationship. It has been known for more than a century that changes in the frequency of contraction affect the tension generated by the heart (Bowditch, 1871; Woodworth, 1902). In rat cardiac trabeculae, the twitch tension-interval relation is characterized by three phases: (1) an increase of the twitch tension to the steady state (early recovery), which occurs in response to the diminishing of the interbeat interval shorter than the refractory period (<1s), followed by (2) a secondary slow increase of the twitch tension above the steady state up to an interval of ~100 s (rest potentiation), and (3) subsequent decrease in twitch tension at intervals >100 s (rest depression) (Schouten et al, 1987). The basis of these contractile phenomena lies in the dynamic changes of SR Ca^{2+} content and release and SL Ca^{2+} fluxes and have frequently been used for estimating the contribution of SR and SL Ca^{2+} sources for contraction *in vivo.*

Early contractile recovery (I, II), named also mechanical restitution (Kruta and Braveny, 1961) lies on the observation that immediately after a contraction has been activated, a time period is required before another contraction of the same amplitude can be activated (Gibbons and Fozzard, 1975; Wolfhart, 1979; Lipsius *et al.*, 1982; Yue *et al.*, 1985; Wier and Yue, 1986). It is analogous to a relative refractory period. The recovery of the SR Ca^{2+} release channel has been shown to limit the restitution of twitch tension (Fabiato, 1985; Banijamali *et al.*, 1991).

Post-extrasystolic potentiation (II) is a functional response in cardiac muscle to the activation prior the mechanical restitution (refractory period) such that only a weak contraction occurs (extrasystole), but the subsequent contraction is potentiated. If SL Ca^{2+} channel has recovered (even partially) before the extrasystole, some Ca^{2+} will enter the cell at the extrasystole. If the SR Ca^{2+} release channel is refractory, there will not occur normal Ca^{2+} release, which results in a weak contraction. The Ca^{2+} which entered during the extrasystole will however, contribute to a larger Ca^{2+} accumulation by the SR. Now when the RyR has recovered by the next contraction, a greater SR Ca^{2+} release and contraction occurs. This phenomenon has been used for estimation of the recirculation fraction of activator Ca^{2+}.

Recirculation fraction of activator Ca^{2+} (RFA, II) is an index of the fraction of the internally released Ca^{2+} sequestered by the SR during each contraction-relaxation cycle (Wohlfart, 1979; Schouten *et al.*, 1987; Urthaler, 1975, 1990; Banijamali, 1991). It was estimated in atrial and ventricular myocardium to compare the thyroid state dependent changes in the contribution of the SR in providing the activator Ca^{2+}. This was done by inserting 10 stimuli at the frequency of 10 Hz rather than one regular stimulus into the basic (0.2 Hz) stimulation protocol. This led to a marked potentiation of the twitch due to the liberation of a high amount of Ca^{2+} from the SR, which was accumulated during the extrasystoles. Part of this Ca^{2+} is extruded via SL during relaxation, and part will be resequestered by the SR. The amount of Ca^{2+} liberated by each subsequent twitch will therefore decline gradually, leading to the decay of each following beat. The decay of the tension can be described by a single exponential function, and serves as the basis for calculation of the RFA (Urthaler, 1975, 1990; Banijamali, 1991).

Rest potentiation (I). The dependence of post-rest potentiation on rest interval was compared in trabeculae dissected from left atria and papillary muscles at 1 and 2.5 mM $[Ca^{2+}]_o$ by interrupting the basic stimulation at 1 Hz for 3 to 600 s. In each preparation, the values of the DT of the first post-rest twitches were normalized to the maximal value of potentiation, in order to compare the two tissues. In rat heart, the increase of the rest interval up to ~100 s results in the potentiation of the twitch tension above steady state. Rest potentiation in rat heart has been attributed to the gain of Ca^{2+} of the SR during the rest (Bers, 1989; Lewartowski and Zdanowski, 1990; Banijamali *et al.*, 1990). One reason for this is that in rat, there is higher resting intracellular Na^+ activity compared to other animal species (Wang *et al.*, 1993). The low transsarcolemmal $[Na^+]$ gradient is limiting Ca^{2+} extrusion via Na^+/Ca^{2+} exchange. However, in most mammalian species, the twitch tension declines when the period of rest is increased (Allen *et al.*, 1976. Bers, 1989). This has been attributed to a gradual decline in the SR Ca^{2+} content. The Ca^{2+} which leaks out of the SR is extruded from the cell mainly via Na^+/Ca^{2+} exchange (Allen *et al.*, 1976. Bers, 1989)

Force-frequency relationship (II) was estimated in atrial and papillary muscles from animals of altered thyroid state in order to evaluate the thyroid state dependent changes in the SR function *in vivo*. The stimulation frequency was increased from the basal value of 0.2 Hz to the test frequencies 0.5, 1.0 or 2.0 Hz for 1 minute. The contractile parameters were registered at the basal pacing frequency and at the end of the test periods.

5. Evaluation of SR Ca^{2+} uptake in skinned fibres (I, III)

The fibres (100–250 μm diameter) were dissected from left ventricular papillary muscles and atria in Ca^{2+}-free Krebs solution and the SL made permeable (skinned) by incubation with a detergent saponin (50 μg/ml) in strongly Ca^{2+}-buffered solution (Endo and Iino, 1980). In skinned preparations, the SR and myofilaments retain their architecture and natural interactions between cellular compartments (Endo, 1975; Endo and Kitazawa, 1977; Endo and Iino, 1980), enabling, thus, to study their function *in situ*, separately from sarcolemmal Ca^{2+} fluxes.

5.1. Using caffeine to evaluate SR function *in situ*

In order to study the SR Ca^{2+} uptake in situ, the SR was loaded with Ca^{2+} for various periods of time or/and under different test conditions. To evaluate the SR Ca^{2+} uptake, the Ca^{2+} release was induced by 5 mM caffeine. Caffeine can produce a transient contracture in cardiac and skeletal muscle (Chapman and Miller, 1974; Endo, 1975; Chapman and Leoty, 1976). In preparations with permeabilized sarcolemma, this results from a combination of the two main actions of caffeine: (i) it releases Ca^{2+} from the SR (Weber and Herz, 1968; Endo, 1875; Endo and Kitazawa, 1977; Endo and Iino, 1981; Fabiato, 1982; Fabiato and Fabiato, 1975; 1976; 1978a; b; Su, 1992) and (ii) directly sensitizes the myofilaments to Ca^{2+} in a concentration-dependent manner (Fabiato and Fabiato, 1975; 1976; Wendt and Stephenson, 1983, Eisner and Valdeolmillos, 1985, De Beer *et al.*, 1988). The model of saponin-skinned fibres enabled us to evaluate both principal actions of caffeine and by taking into account the Ca^{2+}-sensitizing effect on myofilaments, to recalculate the $[Ca^{2+}]$ released by the SR.

Analysis of the caffeine-induced contracture. The time from the onset of contracture to half-relaxation, T_{r50}, was taken to describe the time course of caffeine-induced contracture. The peak, normalized to T_{max}, and area under the tension transient of caffeine-elicited contracture, S(T) were used to evaluate the amount of Ca^{2+} taken up by the SR.

The Ca^{2+}-sensitising effect of caffeine on the myofilaments was estimated by the pCa-tension relationship in the presence of 5 mM caffeine in conditions identical to those of the release, by sequentially exposing the fibres to a set of solutions with stepwise decreasing pCa until T_{max} was reached (at pCa 4.5). The developed tension at each pCa was normalized with respect to T_{max}. The data from each fibre were fitted with Hill equation using linear regression analysis, and pCa_{50} and Hill coefficient (nH) were determined. The pCa-tension relationship characterizes the graded manner by which cardiac myofilaments are

activated by Ca^{2+} (Fabiato, 1982; Hibberd and Jewell, 1982). Caffeine, by sensitizing the myofilaments to Ca^{2+}, shifts this curve to the left.

Recalculation of free [Ca^{2+}] close to myofibrils during caffeine-induced contracture was carried out by using the pCa/tension dependence of each fibre as an internal calibration. The [Ca^{2+}] at each step of the tension-time integral was recalculated from the Hill equation to obtain the [Ca^{2+}]-time integrals, $S[Ca^{2+}]_f$, which were taken to evaluate the SR Ca^{2+} loading capacity.

5.2. Energy dependence of SR Ca^{2+} uptake *in situ* (I, II)

The skinned fibre technique described above was used to evaluate the efficiency of MM-CK bound to the SR in providing the SR Ca^{2+} pump with ATP *in situ.* For this purpose, we compared the SR Ca^{2+} loading in the presence of MgADP and PCr (when ATP generated by the bound CK was the only source of energy for the Ca^{2+} pump) with that supported by MgATP alone. The level of SR Ca^{2+} loading in the presence of 3.16 mM MgATP and 12 mM PCr served as a control. To permit comparison between different loading conditions, the Ca^{2+} release by 5 mM caffeine was induced always at constant conditions of substrates and ions. Evaluation of the Ca^{2+} uptake under different conditions was done by an analysis of tension transients due to caffeine-induced Ca^{2+} release as described above.

6. Determination of the oxalate-supported SR Ca^{2+} uptake in tissue homogenates (I, II)

To compare the SR Ca^{2+} pump function in atria and ventricles, the Ca^{2+} accumulation in the homogenates (Pagani and Solaro, 1984) of both tissues were determined at 30°C, in the stirred medium containing in mM: ATP 6; $MgCl_2$ 6; KCl 120; NaN_3 3; imidazole 60 (pH 7.0), K-oxalate 6 and a ^{45}Ca-labeled $CaCl_2$-EGTA buffer containing 0.58 mM EGTA. Appropriate concentrations of $CaCl_2$ were added to the medium to obtain different free [Ca^{2+}], corresponding to the range of cytosolic free [Ca^{2+}] during contraction-relaxation cycle. The reaction was started by an addition of 50 µl homogenate per 0.5 ml medium. After 2 min. incubation, the samples were filtered. Radioactivity associated with the membranes was counted.

To estimate thyroid state dependent alterations in the SR function, atrial and ventricular homogenates (30 µl) from different thyroid states were incubated for 5 min at 30°C in the stirred medium, to which 100 µM H-89 was added, in order to block the endogenous PKA (Chijiwa *et al.,* 1990). After incubation

period, Ca^{2+} uptake was started by addition of ^{45}Ca-labelled $CaCl_2$-EGTA buffer to obtain 0.1 μM free [Ca^{2+}]. The Ca^{2+} uptake was stopped after 3 min by filtering the samples and radioactivity associated with the membranes counted.

7. Isolation of total RNA and mRNA slot-blot analysis (I)

For total RNA isolation, both auricles and a piece of ventricular tissue from eight rat hearts were collected. Total RNA was extracted by the guanidinium isothiocyanate procedure (Chomzinsky and Sacci, 1987) using RNA quick™ kit (Bioprobe). Specific mRNA species were quantified by slot-blot hybridization. One, 2 and 4 μg of total RNA from atria and ventricles as well as from liver, fast skeletal muscle (*extensor digitorum longus*) and yeast tRNA were denatured in 15× standard saline citrate (SSC) (1×SSC contained 0.15 M sodium chloride and 0.015 M sodium citrate) and 3% formaldehyde at 65°C for 15 min and rapidly cooled on ice. Liver, skeletal muscle RNA and tRNA were used as negative controls to check for specificity of the various probes. The samples were directly spotted onto the nylon membrane with use of a Minifold apparatus (Schleicher & Schuell, Keene, NH). The RNA was cross-linked to the membrane by ultraviolet irradiation, and the membranes were prehybridized at 42°C for > 4 hours in the presence of 50% formamide, 0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.05 M sodium phosphate (pH 6.5), 5×SSC, 0.1% sodium dodecyl sulphate (SDS) and 250 μg/ml salmon sperm DNA.

The specific SERCA 2 mRNA was detected using a probe (nt 2616 to 3120) of the rat heart Ca^{2+}-ATPase mRNA (Lompré *et al.*, 1989). The rat ryanodine receptor probe (RyR 2) was obtained by reverse transcription of rat cardiac total RNA and subsequent amplification of the RyR 2 mRNA using primers derived from the sequence of the rabbit cardiac RyR 2 (Otsu *et al.*, 1990). The probe extents from nt 8604 to 9144 of the rabbit sequence (Otsu *et al.*, 1990) with few silent mutations. The Cals probe was isolated from a canine cDNA library (Scott *et al.*, 1988) and used as described by Lompré *et al.* (1991). The α-MHC probe has been previously described by Schiaffino *et al.* (1989). The cDNA probes were labelled by use of random primers, DNA polymerase I (klenow fragment) and [α-^{32}P]dATP (3000 Ci/mmol) and the specific activity was 1–3×10^9 dpm/μg. Hybridization was done in the same conditions than prehybridization. Excess of probe was eliminated by washing in 0.5×SSC at 55°C for SERCA 2, 60°C for RyR 2, 42°C for Cals and in 1×SSC at 45°C for MHC.

After each hybridization, the blots were dehybridised by boiling in 0.1% SDS and then rehybridized as described above. To normalize to the amount of total RNA present on the membrane, the blots were rehybridized with a 24-mer oligonucleotide complementary to the rat 18S ribosomal RNA. The oligo-

nucleotide was labelled at its 5' end by use of T4 polynucleotide kinase and [γ-^{32}P] ATP and diluted with cold oligonucleotide to a specific activity of 105 dpm/μg. It was hybridised in the medium described above but in the absence of formamide. The washing conditions were 2×SSC at room temperature. After washing, the membranes were exposed to X-ray film for 1 day to 1 week. Unsaturated autoradiograms were analyzed by densitometry (Molecular Dynamics). The specific mRNA level was corrected for the total RNA present on the membrane by calculating the ratio of the signals obtained with the specific probe and the 18S probe for the three dilution of each sample. The relative proportion of RERCA 2 and SERCA 2b mRNA was determined by RNase protection assay using SERCA 2 probe described above and the Ambion kit protocol (Clinisciences, France).

8. Statistical analysis

The data are expressed as means ±SEM or as representative tracings in a single experiment. The values were analysed by Students t test or using ANOVA followed by Dunnett test. Differences at $P<0.05$ were considered significant.

RESULTS

1. Contractile function

1.1. Comparison of atrial and ventricular myocardium (I)

Table 3 shows that atrial contractions were characterized by weaker twitch DT but significantly shorter TPT and RT50 as compared with ventricles. Also, maximal +dT/dt and -dT/dt values if normalized to DT (+dT/dt/DT and -dT/dt/DT) were higher in atria than in ventricles. These data indicate that peak isometric tension in atria reached its maximum and relaxed faster than in ventricles.

Table 3. Comparison of the mean twitch tension parameters of atrial and ventricular trabeculae.

Parameter	atria	ventricles
DT, mN/mm^2	7.4±0.8	26.1±1.6 ***
TPT, ms	71±1	145±3 ***
RT50, ms	32±2	104±5 ***
(+dT/dt)/DT, s^{-1}	26.2±4.6	11.4±0.3 **
(+dT/dt)/DT, s^{-1}	18.7±2.9	6.7±0.5 ***

**, ***, $P < 0.001$, < 0.0001 respectively for comparison with atria.

Post-rest potentiation of twitch tension was compared in atria and papillary muscles at two $[Ca^{2+}]_o$ to evaluate the function of SR in providing activator Ca^{2+} *in vivo*. At 1 mM $[Ca^{2+}]_o$, inserting the rest periods into the basic stimulation protocol of 1 Hz was accompanied by potentiation of the first post-rest twitch in both atrial and ventricular preparations. However, in atrial fibres the maximal level of potentiation was achieved after significantly shorter rest intervals (15 s) than in ventricular ones (60 s). Further increase in rest duration led to a significant reversal of potentiation in ventricular fibres, but not in atria. These differences between atria and ventricles were abolished if the $[Ca^{2+}]_o$ was increased from 1 mM to 2.5 mM. Thus, SR filling in atrial cardiomyocytes is saturated faster than in ventricular ones and the loss of Ca^{2+} from the SR during the rest is less pronounced than in ventricles.

1.2. Effect of altered thyroid state on myocardial function (II)

Contractile parameters. In comparison with euthyroid state, hypothyroidism was associated with decreases in DT, +dT/dt and -dT/dt, but with increased TPT and RT in both atria and papillary muscles. In contrast, hyperthyroidism led to increases in DT, +dT/dt and -dT/dt together with shortened TPT and RT (Table 4).

Table 4. Isometric contraction parameters under different thyroid states.

	Atria			Papillary muscles		
	Hypothyroid	Euthyroid	Hyperthyroid	Hypothyroid	Euthyroid	Hyperthyroid
n	7	7	8	11	7	8
DT, mN	0.98±0.10***	3.92±0.59	4.21±0.28+++	3.34±0.59	5.49±0.88	7.65±0.795 +++
+dT/dt, mN/s	14.7±2.9***	94.2±12.7	133.4±9.8* +++	35.3±5.9 **	72.6±11.8	122.6±13.7 * +++
-dT/dt, mN/s	11.8±2.0***	68.7±7.8	101.0±10.8* +++	21.6±3.9 *	39.2±4.9	82.4±29.4 +
TPT, ms	101±3***	75±3	57±1*** +++	157±3 ***	129±4	98±3*** +++
RT, ms	132±6***	87±8	68±3* +++	217±4	202±9	141±8 *** +++

*, **, ***, P<0.01, <0.001, <0.0001 respectively for comparison with euthyroid state; +, ++, +++, P<0.01, <0.001, <0.0001 respectively for comparison with hypothyroid state.

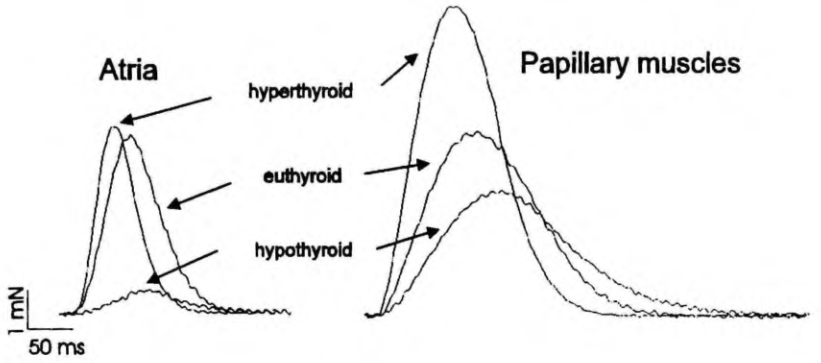

Figure 4. Effect of thyroid state on isometric twitches of left atria and papillary muscles.

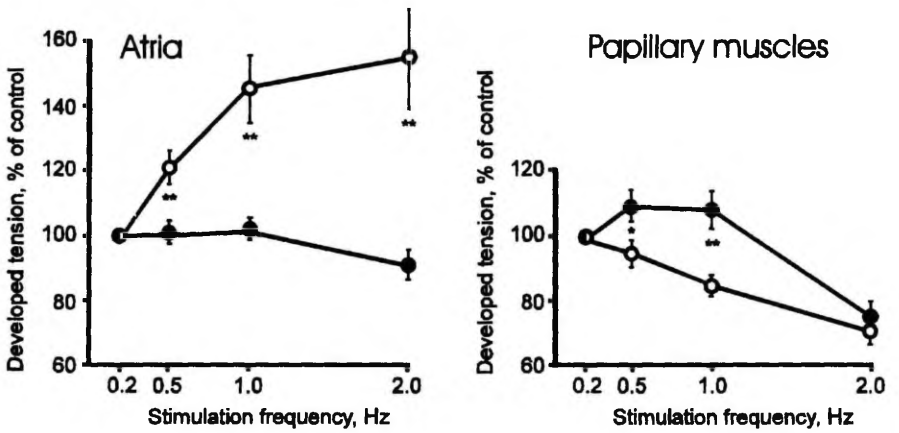

Figure 5. Effect of thyroid state on force-frequency relationships in atria and papillary muscles at 2.5 mM $[Ca^{2+}]_o$. *,** Significant difference at $P<0.05$, <0.01 between 7 hypothyroid (O) and 8 hyperthyroid (●) preparations.

A remarkable difference between atria and ventricles was that in atria, a shift from hypothyroid to hyperthyroid state was associated with the 8–9-fold increases in +dT/dt and -dT/dt, whereas in ventricles it lead only to 3–4-fold increase in these parameters (Figure 4). Also, the increase in DT, corresponding to the shift from hypothyroidism to hyperthyroidism was more pronounced in atria than in ventricles.

Recirculation fraction of activator Ca^{2+}. The RFA was estimated as an index of the fractional amount of Ca^{2+}, which is sequestered during the diastole and released for the subsequent systole by the SR (Morad and Goldman, 1973; Urthaler *et al.*, 1988; 1990; Wohlfart, 1979). The RFA was significantly lower in atria than in papillary muscles in hypothyroid conditions, whereas hyperthyroidism increased this parameter up to the equal values in both muscle types. Thus, in response to a shift from hypothyroid to hyperthyroid state, the RFA increased to a far greater extent in atria (4-fold) than in papillary muscles.

Effect of ryanodine. Under hypothyroidism, the percentage of inhibition of DT by ryanodine was 70±7 in atria and 86±2 in papillary muscles ($p<0.05$). Under hyperthyroidism, ryanodine almost entirely inhibited the DT in both atria (97±2%) and papillary muscles (95±2%). Thus, thyroid hormones increased the inhibition of DT by ryanodine more in atria than in papillary muscles. This points that the control of thyroid state over the role of SR as a Ca^{2+} source is much more pronounced in atria than in ventricles.

Force-frequency relationship. The effects of stimulation frequency on the DT of atria and papillary muscles of hypo- and hyperthyroid rats is presented in Figure 5. In atria of hypothyroid animals, increasing the stimulation frequency stepwise from 0.2 Hz (control level) to 2.0 Hz, led to a rise of the DT more than 50% above the control level, showing a positive FFR (Figure 5, left panel). Because at higher stimulation frequencies more Ca^{2+} is transported into the cell via SL, this result suggests that the contraction development in hypothyroid atria is critically dependent on the SL Ca^{2+} source and less on the SR Ca^{2+} pool. Thyroid hormones converted the FFR from positive into negative, suggesting that the predominant role of the SR as a pool of Ca^{2+} for contractile activation was restored under the influence of thyroid hormones. In contrast to hypothyroid atria, hypothyroid papillary muscles showed a negative FFR (Figure 5, right panel). In the hyperthyroid papillary muscles, after enhancing the stimulation frequency from 0.2 Hz to 0.5 Hz, the DT tended to increase, but maintained its level at 1.0 Hz so that the DT exceeded the value of that parameter in the hypothyroid muscles. Further increase in stimulation frequency, however, led to a negative inotropic response which was equivalent to that in hypothyroid papillary muscles. As it could be seen by comparing ventricular FFR with atrial one, the role of SR as a source of activator Ca^{2+} was less dependent on thyroid state than in atria.

2. SR Ca^{2+} uptake in skinned cardiac fibres

2.1. Comparison of atrial and ventricular myocardium (I)

The SR Ca^{2+} handling in atrial and ventricular myocardium was compared *in situ* by using saponin-permeabilized fibres with similar mean diameters. Atrial and ventricular fibres exhibited similar values of maximal Ca^{2+}-activated tension (T_{max}), normalized per cross-section area: 21.9±5.1 mV/mm^2 and 19.1± 1.4 mV/mm^2, respectively. Also, the myofilaments sensitivity to Ca^{2+} in the presence of 5 mM caffeine was similar in both tissues, pCa_{50} being 5.85±0.03 in atria and 5.82±0.01 in ventricles.

The similar responsiveness of myofibrils from atrial and ventricular preparations to Ca^{2+} in the presence of 5 mM caffeine allows us to compare the time courses of caffeine-induced tension transients in the tissues. The SR Ca^{2+} uptake was estimated by loading it with Ca^{2+} for different time periods and by subsequent liberation of Ca^{2+} by caffeine. The caffeine-induced contractures, taken to evaluate the SR Ca^{2+} uptake, appeared to have lower peak tension and a substantial tonic component in atrial fibres while ventricular muscle promptly returned to the baseline tension. This was evidenced by an analysis of the parameters of caffeine induced tension transient as a function of the loading time. The maximal peak tension values, normalized to T_{max}, appeared to be 50% in atrial and 80% in ventricular fibres. The time to half-relaxation (from onset of tension transient) of caffeine induced contracture reached significantly higher levels in atrial than in ventricular preparations at times of loading 10 min and more. Further analysis of tension-time integrals revealed that area under caffeine-induced tension transient was the same in atrial and ventricular fibres. The total amount of free [Ca^{2+}], liberated to myofilaments under caffeine, could be compared in atrial and ventricular fibres by calculating the free [Ca^{2+}]-time integrals ($S[Ca^{2+}]_f$) on the basis of the tension-time integrals and Ca-sensitivity of each fibre. The $S[Ca^{2+}]_f$ appeared to be similar in both atrial and ventricular fibres at any time of loading. Thus, in spite of the different shape of the caffeine-elicited tension transients, both tissues exhibited equivalent SR Ca^{2+} capacity *in situ*.

2.2. Energy dependence of SR Ca^{2+} uptake (I; III)

For evaluation of the efficiency of the MM-CK bound to the SR in providing SR Ca^{2+} pump with ATP *in situ*, we compared the SR Ca^{2+} loading in the presence of MgADP and PCr (when ATP generated by the bound CK was the only energy source for the Ca^{2+} pump) with that supported by MgATP alone. The level of SR Ca^{2+} loading in the presence of 3.16 mM MgATP and 12 mM PCr served as a control. The Ca^{2+} -releasing conditions were kept constant.

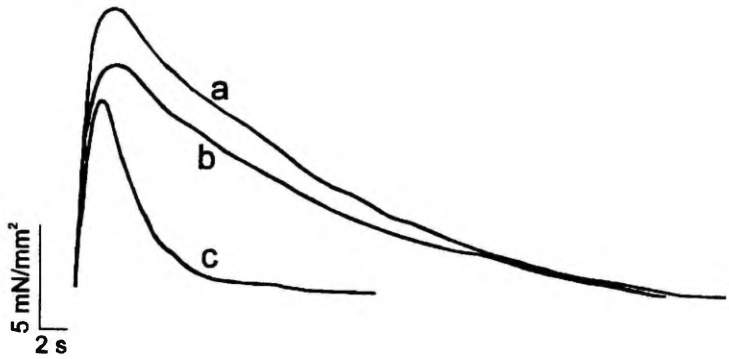

Figure 6. Superimposed tension transients obtained after 5 min SR loading in different conditions in atrial skinned fibre. (a) in the presence of MgATP and PCr, (b) in the presence of MgADP and PCr, (c) in the presence of MgATP alone.

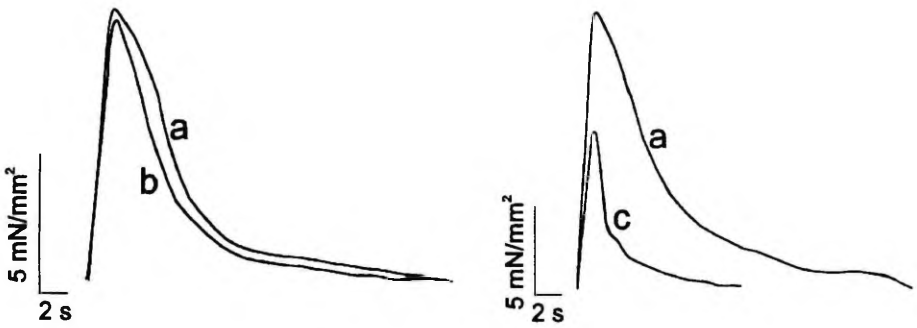

Figure 7. Superimposed tension transients obtained after 5 min SR loading in different conditions in two ventricular skinned fibres: (a) in the presence of MgATP+PCr, (b) in the presence of MgADP and PCr, (c) in the presence of MgATP alone.

Figure 6 shows superimposed tracings of caffeine-elicited tension transients obtained after 5 min period of SR loading at three test conditions in an atrial skinned fibre. The peak and area under tension transient, obtained following ADP and PCr-supported loading were slightly less than in control-loading (ATP+PCr), but the difference was not significant. After loading in the presence of exogenous MgATP alone, the peak of the subsequent caffeine-induced tension transient was remarkably less than in control. Also, the time to half-relaxation of the tension transient was notably shorter than in control, leading, thus, to a significant differences in area under the tension transient. The mean data of caffeine-induced contraction, obtained for 5 min of SR Ca^{2+} loading in control and in the two test conditions in atrial skinned fibres are compared in Table 5.

Figure 7, left panel shows superimposed tracings of caffeine-elicited tension transient obtained after 5 min period of SR control-loading (ATP+PCr) and 5 min CK-supported loading (ADP+PCr) in a ventricular skinned fibre. In right panel, the tracings of caffeine-elicited tension transient following 5 min control-loading and 5 min ATP-loading are superimposed. Like in atria, the SR load in the presence of ADP and PCr was nearly as efficient, but in the presence of ATP alone significantly less than in control. The mean parameters of caffeine-induced contraction, obtained for 5 min of SR Ca^{2+} loading in control and in the two test conditions are presented in Table 6. From tables 5–6 it can be seen, that in both atria and ventricles, the parameters of caffeine-elicited contractures (T/T_{max}, S(T) and $S[Ca]_f$), obtained after SR loading in the presence of MgADP and PCr were similar to those obtained following the load at the control conditions (in the presence of MgATP 12 mM PCr). On the other hand, when the Ca^{2+} uptake was supported by the external ATP alone, all measured parameters were significantly lower than in the control-loading or in the ADP+PCr-supported loading.

We also studied the dependency of the SR Ca^{2+} load on the time of loading in atrial and ventricular skinned fibres at different conditions. In both atria and ventricles, the time-dependent curves of T/T_{max} in control- and CK-loading had very similar shape and stable maximal values. Instead, while SR was loaded in the presence of externally added ATP alone, the maximal values of T/T_{max} appeared to be less than in the two abovementioned conditions. Moreover, loading in the presence of ATP for more than 3 min led to the decrease of T/T_{max}. The inability of externally added ATP to support maximal SR Ca^{2+} load became even more evident when time-dependent curves of S(T) and $S[Ca^{2+}]_f$ were analysed.

To test the hypothesis that the failure of ATP-loading to support effective Ca^{2+} uptake into the SR might result from a diffusion gradient of ATP and ADP inside the fibres, we added 50 or 100 IU/ml of pyruvate kinase (PK) and 1 mM of phosphoenolpyruvate (PEP) to the conditions of loading, supported by MgATP alone in some ventricular fibres. The loading in the presence of 3.16 mM MgATP and 12 mM PCr served as a control. The peak of caffeine-elicited contracture in ATP-loading was 72% of the control value and 79% of it if 50 IU/ml PK and PEP were added. Addition of 100 IU/ml of PK instead of 50 did not significantly increase the SR loading, T/T_{max} being 81% of control. At the same time, the loading of SR in the presence of ADP and PCr, was much more efficient (the peak value being 96% of control) than any condition where ATP was provided exogenously. The same type of relation between the groups became even more relevant if the S(T) or $S[Ca^{2+}]_f$ were compared to evaluate the SR Ca^{2+} load. The S(T) of caffeine-elicited contracture obtained in ATP-loading condition was 25% of the control value, 31% and 34% respectively, if obtained in the presence of 50 or 100 IU/ml PK, but 82% of control if obtained in the ADP+PCr-supported loading conditions. Thus, the addition of PK and PEP to the loading protocol in the presence of ATP did not substantially increase the Ca^{2+} uptake, indicating thus, that the diffusion gradient for ATP inside the fibre was not likely.

Table 5. The parameters of caffeine-induced Ca^{2+} release, obtained from experiments on atrial skinned fibres after 5 min SR loading under different conditions. Data are means±SEM for tension, normalized to maximal Ca^{2+}-activated tension, T/T_{max}; the area under the tension transient, S(T); and the integral of the free $[Ca^{2+}]$, calculated from tension transient according to myofilaments Ca^{2+} sensitivity, $S[Ca^{2+}]_f$. The significance of difference from control, i.e. ATP+PCr (asterisks) and from loading in the presence of ADP and PCr (crosses) are indicated, *,+, $p<0.05$; **,++, $p<0.01$; ***,+++, $p<0.001$.

Condition of SR loading	n	T/Tmax	S(T), arbitrary units	$S[Ca]_f$, μM×s
ATP+PCr	6	0.477±0.037	7.5±2.1	31.5±6.6
ADP+PCr	4	0.449±0.043	5.3±0.7	23.5±3.1
ATP	5	0.304±0.013**,+	1.5±0.1*,++	8.4±0.8**,++

Table 6. The parameters of caffeine-induced Ca^{2+} release, obtained from experiments on ventricular skinned fibres after 5 min SR loading under different conditions. Data are means±SEM for tension, normalized to maximal Ca^{2+}-activated tension, T/T_{max}; the area under the tension transient, S(T); and the integral of the free $[Ca^{2+}]$, calculated from tension transient according to myofilaments Ca^{2+} sensitivity, $S[Ca^{2+}]_f$. The significance of difference from control, i.e. ATP+PCr (asterisks) and from loading in the presence of ADP and PCr (crosses) are indicated, *,+, $p<0.05$; **,++, $p<0.01$; ***,+++, $p<0.001$.

Condition of SR loading	n	T/Tmax	S(T), arbitrary units	$S[Ca]_f$, μM×s
ATP+PCr	14	0.716±0.033	3.8±0.3	18.4±1.9
ADP+PCr	6	0.674±0.031	3.0±0.3	15.7±2.1
ATP	6	0.385±0.071***,++	0.9±0.2***,+++	5.8±0.8***,+++

induced tension was remarkably less than in control. Also, the time to half-relaxation of the tension transient was notably shorter than

3. Oxalate-supported $^{45}Ca^{2+}$ uptake in tissue homogenates (I; II)

The net SR Ca^{2+} uptake rate in tissue homogenates of both atria and ventricles were assayed over a wide range of free [Ca^{2+}], corresponding to the range of cytosolic free [Ca^{2+}], during contraction-relaxation cycle. The rate of maximal net Ca^{2+} uptake was more than 2-fold higher in atria than in ventricles (4.17±0.72 and 2.03±0.42 nmoles Ca/mg tissue protein/min, respectively; $p<0.05$). However, the K_{50} value of the SR Ca^{2+} pump for free Ca^{2+} and the Hill coefficient were similar in atria and in ventricles.

To estimate the thyroid state dependent alterations in SR function, we analysed the oxalate-supported Ca^{2+} uptake rate by the SR in cardiac homogenates in different thyroid state in the presence of 100 μM H-89, in order to block the endogenous protein kinase A (Chijiwa, 1990). Both in atrial and in ventricular homogenates, the Ca^{2+} uptake rates were higher in hyperthyroidism and lower in hypothyroidism compared to euthyroid state. The shift from hypothyroid to hyperthyroid state resulted in nearly 10-fold increase in the net SR Ca^{2+} uptake rate in atria (from 0.004±0.001 to 0.039±0.004 nmol/Ca^{2+}/mg tissue/min), whereas in ventricles the shift was only 1.7-fold (from 0.020±0.003 to 0.034±0.005 nmol/Ca^{2+}/mg wet tissue/min). Thus, the SR Ca^{2+}-pump in atrial tissue was activated under the influence of thyroid hormones in greater extent than in ventricles.

4. Expression of mRNA (I)

In order to relate the differences in SR function between atria and ventricles to the level of expression of the principal SR proteins (SERCA, RyR, Cals) and the fast myosin heavy chain α-MHC, the tissue amounts of the corresponding mRNA relative to 18S were estimated by slot-blot. The results show that the expression of the α-MHC relative to 18S was not significantly different between atria and ventricles (1000±90 and 974±81 a.u. respectively). Relative to 18S RNA, the content of RyR and Cals mRNAs in the two tissues was not different whereas the amount of SERCA 2 mRNA was 38% higher in atria than in ventricles (220±21 and 160±18 a.u. respectively, $p<0.05$). No variation in SERCA 2a/SERCA 2b ratio was detected by RNase protection analysis between the two tissues. As already shown in ventricle, the majority of the SERCA 2 mRNA was of the 2a type (Lompré *et al.*, 1991). An identical pattern was observed in atria. Relative to α-MHC mRNA, the amount of SERCA 2 mRNA was lower in ventricles than in atria (0.165±0.010 and 0.234±0.049 a.u. respectively) but the difference did not reach significance. This suggests that higher SR Ca^{2+}-pump activity was due to a higher expression of pump protein in atria than in ventricles.

DISCUSSION

1. Relationships between contractile parameters and SR function in atria and ventricles (I)

In comparison with papillary muscles, atrial preparations exhibited smaller absolute values of DT, normalized to muscle cross-section area. At the same time, the kinetic parameters such as TPT, RT50, and the first derivative of tension development and tension fall normalized to maximal tension showed that the peak isometric tension in atria reached its maximum and relaxed faster than in ventricles. This is in accordance with earlier studies (Agata and Tanaka, 1994; Asgrimsson *et al.*, 1995; Koss *et al.*, 1995). At the same time, both the maximum Ca^{2+} activated tension and the Ca^{2+}-sensitivity of myofibrils were similar in atrial and ventricular saponin-treated preparations, which is in agreement with others (Vannier *et al.*, 1996; Palmer and Kentish, 1996). To study the role of the SR in determining contractile differences between atrial and ventricular myocardium, the SR function, Ca^{2+} handling and protein expression were compared in both tissues.

1.1. Differences in SR Ca^{2+} release

Experiments on saponin-permeabilized fibres enabled us to study the SR function *in situ*. In both atria and ventricles, caffeine-induced tension transient rapidly gained the peak tension, indicating that the initial phase of SR Ca^{2+} release was fast in both tissues. However, the peak of caffeine-induced tension transient, normalized to T_{max} was lower in atrial than in ventricular fibres. This could be associated with the lower DT observed in intact atrial preparations compared to papillary muscles.

The tension development upon caffeine application was followed by the relaxation. The increase in the loading time progressively induced the prolongation of this phase, as evidenced by the increased time to half-relaxation and area under caffeine-induced tension transient. As a result, the tension transient prolonged, being more pronounced in atria than in ventricles. The time course of caffeine-induced tension transient will depend on 1) the amount of released Ca^{2+}, 2) the rate of SR Ca^{2+} release, 3) the diffusion of Ca^{2+} away from the myofibrils, 4) the Ca^{2+} buffering by EGTA and proteins, 5) the possible reuptake and release of Ca^{2+} and, 6) the Ca^{2+}-sensitising effect of caffeine. Since atrial and ventricular fibres had similar diameter and composition, it is unlikely that the differences will arise from different Ca^{2+} buffering. Also, atrial and ventricular fibres exhibited the similar myofilaments Ca^{2+} sensitivity. Thus, the pro-

longation of the tension transient in atrial fibres could be considered as a result of a prolonged Ca^{2+} release, suggesting different mechanisms of Ca^{2+} release in atria and ventricles.

The total Ca^{2+}-time integral, calculated from caffeine-elicited tension transient was applied to estimate the total amount of Ca^{2+}, released to the myofilaments. The results indicate that the total amount of the sequestrable Ca^{2+} appeared to be the same in the two tissues irrespectively of the speed of Ca^{2+} release or the time of SR loading. These results, together with the similar amount of mRNA for Cals, suggest that the capacity of SR Ca^{2+} pool was similar in atrial and ventricular myocardium, and, hence the prolonged Ca^{2+} release in atria could not be related to different Ca^{2+} capacity of the SR. The same expression of RyR mRNA in both atria and ventricles suggests a close amount of Ca^{2+} release channels in both tissues. Thus, prolonged SR Ca^{2+} release in atria compared to ventricles could not be attributed to a smaller amount of RyR either.

The prolonged SR Ca^{2+} release in atria compared to ventricles may be attributed to morphological differences between these two tissues. In human atrial cells, it was shown that the transsarcolemmal inward Ca^{2+} current activated two components of intracellular [Ca^{2+}] signals instead of one signal in ventricular cells (Hatem *et al.,* 1996). Inhomogenity of intracellular [Ca^{2+}] transient has also been demonstrated by Berlin (1995), using confocal microscopy. In intact rabbit ventricular cardiomyocytes, Bassani *et al.* (1995) have shown that one-half of the Ca^{2+} in the SR is released during a twitch but the beat-dependent depletion of SR Ca^{2+} was biexponential. They suggest that the slower phase might represent a caffeine-sensitive pool of Ca^{2+} not normally released during a twitch, and speculated that the twitch Ca^{2+} in the corbular SR could represent such a pool. Atrial myocytes, devoid of T-tubular system (McNutt and Fawcett, 1969) contain only peripheral junctional SR connected to SL, and a higher proportion of corbular SR within the cytoplasm (Bossen *et al.,* 1981). Because corbular SR is not connected to the SL membrane, Ca^{2+} release from these stores can be triggered by a diffusible agent (Jorgensen *et al.,* 1985). The delayed phase of Ca^{2+} release in atrial skinned fibres could thus represent Ca^{2+} released from these internal cisternae by a slower Ca^{2+} induced Ca^{2+} release mechanism (Figure 8).

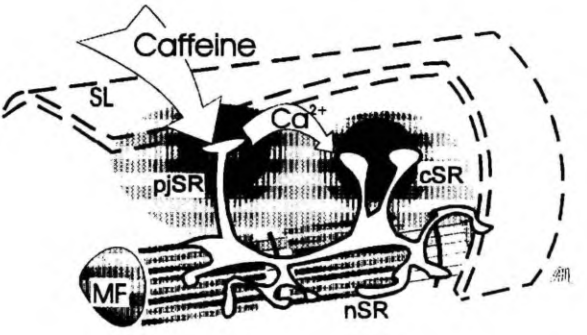

Figure 8. A diagram illustrating hypothetical two pools of caffeine-elicited Ca^{2+} release in atrial skinned fibres. Caffeine induces Ca^{2+} release from peripheral junctional SR (pjSR), which, diffusing in the intracellular environment, triggers Ca^{2+}-induced Ca^{2+} release from corbular SR (cSR). SL, sarcolemma; MF, myofilaments; nSR, network SR.

1.2. Differences in SR Ca^{2+} resequestration

Differences in contractile function between atria and ventricles could also be associated with the limited Ca^{2+} release for contraction in atria due to higher rate of SR Ca^{2+} re-sequestration in atria compared to ventricles. The SR function *in vitro* was compared in atria and ventricles by determining the oxalate-supported Ca^{2+} uptake rate in tissue homogenates, that reflects the intrinsic Ca^{2+} transport by the SR Ca^{2+} -ATPase (Pagani and Solaro, 1984). The results indicate that the oxalate supported Ca^{2+} uptake rate, expressed per mg protein, is two times higher in atria than in ventricles. This could be partly explained by 30% higher level of the SERCA 2 mRNA/18S RNA observed in atria. This, together with the lower expression of PLB in atria as compared to ventricles (Koss *et al.,* 1995) is in favour of a higher SR Ca^{2+} uptake rate in atria than in ventricles.

The SR function in intact atrial and ventricular preparations was also compared by studying the rest potentiation of both tissues. In rat cardiomyocytes, rest potentiation has been suggested to result from the increase in Ca^{2+} release from the SR due to either the increase in SR Ca^{2+} content (Banijamali *et al.,* 1991; Bassani *et al.,* 1993; Bers, 1989; Lewartowski and Zdanowski, 1990), or the increase in fractional SR Ca^{2+} release (Bassani and Bers, 1994; Bouchard and Bose, 1989). The results of our experiments indicated that, at low $[Ca^{2+}]_o$, the mechanisms responsible for maximal rest potentiation needed about 4 times less time to become saturated in atrial than in ventricular myocardium. This suggests that the SR Ca^{2+} filling saturates earlier and faster in atria. These features of atrial myocardium could be associated with a faster SR Ca^{2+} uptake. An increase in rest duration for 10 min led to a significant reversal of potentiation

in papillary muscles, but not in atria. Since the decline in rest potentiation and the rest decay have been attributed to the SR Ca^{2+} loss due to its extrusion through the sarcolemmal Na^+/Ca^{2+} exchanger (Bassani and Bers, 1994; Bers, 1991), it indicates that the SR in rat ventricular myocardium looses more Ca^{2+} during pauses than in atrial one. Faster saturation of the rest potentiation in atria could be attributed to faster SR Ca^{2+} uptake rate in atria. On the other hand, atrial myocytes are characterized by higher intracellular $[Na^+]$, due to a lower Na^+/K^+ ATPase content compared to ventricular cells (Wang *et al.,* 1993). Thus, the Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger could be less pronounced in atria, resulting in a smaller rest decay. This explanation is supported by the finding that under high $[Ca^{2+}]_o$, which minimizes the Ca^{2+} extrusion via Na^+/Ca^{2+} exchange, there were no differences in rest potentiation between the atrial and ventricular myocardium.

Taken together, the results indicate that atrial myocardium is characterized by a faster SR Ca^{2+} uptake rate, but limited or slow Ca^{2+} release and diffusion for contraction compared to ventricular one. These differences in SR Ca^{2+} handling could be proposed as the main modulators of the shorter time course and lower tension development in rat intact atrial myocardium compared with that in ventricular myocardium, despite the similar total SR capacity of releasable Ca^{2+} in both tissues.

2. Effect of thyroid state on SR Ca^{2+} pump activity and cardiac muscle contractile function (II)

In accordance with previous studies (Morkin *et al.,* 1983; Seppet *et al.,* 1990; 1991), a shift from euthyroid state to hypothyroidism was associated with decreases in +dT/dt and -dT/dt and prolonged TPT and RT, whereas hyperthyroidism led to increases in +dT/dt and -dT/dt together with shortened TPT and RT both in atria and in ventricles (Figure 4, Table 4.). In atria, however, a shift from hypothyroid to hyperthyroid state was associated with 8–9-fold increases in +dT/dt and -dT/dt, whereas in ventricles it led only to 3–4-fold increase in these parameters. Thus, the thyroid state dependent changes in contraction and relaxation rate are more pronounced in atria than in ventricles.

Thyroid hormones are known to abbreviate the intracellular Ca^{2+} transient (MacKinnon and Morgan, 1986; Beekman *et al.,* 1990; Gwathmey and Hajjar, 1990), that has been attributed to a faster removal of cytoplasmic Ca^{2+} by the SR Ca^{2+}-pump. Our estimation of the oxalate-supported Ca^{2+} uptake rate in tissue homogenates showed that thyroid hormones increased the activity of the SR Ca^{2+}-pump in atria to a much greater extent than in ventricles. Earlier, in ventricular myocardium, increased SR Ca^{2+}-pump activity has been considered to be a reason for enhanced rate of relaxation under hyperthyroidism (Suko,

1971; 1973; Limas, 1978; Nagai *et al.,* 1989; Beekman *et al.,* 1990; Gwathmey and Hajjar, 1990; Kiss *et al.,* 1994). Kiss (1994) have shown that a shift from hypothyroid to hyperthyroid state was associated with a 2.4-fold increase in SR Ca^{2+}-pump amount, and with a 1.8-fold decrease in PLB amount in ventricular tissue. Recently, we have found a 2.4-fold increase in expression of SR Ca^{2+}-pump in atria. However, the PLB content decreased much more (6.7-fold) in atria than it was reported for ventricles by Kiss et al. (1994). Since unphosphorylated PLB acts as an inhibitor of SR Ca^{2+}-pump (Colyer, 1993; Luo *et al.,* 1994), the larger suppression of PLB expression should result in a bigger increase in SR Ca^{2+}-ATPase activity in atria. Thus, it is likely that the observed differences between the SR Ca^{2+} -pump activity are due to different regulation of PLB in atria and ventricles, i.e. the expression of PLB is downregulated to a higher extent in atria than in ventricles by thyroid hormones.

Considering these differences in SR Ca^{2+} pump activities, it was of interest to compare the physiological role of SR in providing the activator Ca^{2+} in atria and ventricles. For this purpose the contractile sensitivity to ryanodine was assessed, and the registrations of RFA and FFR were carried out. The extent of suppression of DT by ryanodine is proportional to the cellular content of SR membranes and to the importance of the Ca^{2+}-induced Ca^{2+} release for activation of myofilaments (Bers, 1991). The RFA is an index of the fractional amount of Ca^{2+}, which is sequestered during the diastole and released for the subsequent systole by the SR (Morad and Goldman, 1973; Urthaler *et al.,* 1988; 1990; Wohlfart, 1979). Compared to ventricles, atria showed a bigger increase in RFA and greater sensitivity to ryanodine, in response to thyroid hormone. This could be attributed to increased susceptibility of atrial SR Ca^{2+} -pump to thyroid hormones. Another mechanism may localize at the level of SL, for thyroid hormones stimulate the in ward Ca^{2+} transient, I_t that shortens the duration of action potential in ventricles, but not in atria (Shimoni *et al.,* 1992; Shimoni and Banno, 1993). Therefore, under hyperthyroidism, Ca^{2+} influx through the slow Ca^{2+} channels may decrease due to a shortened action potential duration in ventricles. Hence, the atrial SR could be replenished at the expense of the transsarcolemmal Ca^{2+} influx more effectively than in ventricles. As a result, the atrial SR would exert larger control over activator Ca^{2+} that could explain the higher RFA and ryanodine effects compared to ventricles. However, considering the drastic augmentation of the SR Ca^{2+}-pump activity *in vitro* where the SL Ca^{2+} fluxes are unable to control the SR Ca^{2+} uptake, the increased SR Ca^{2+}-pump activity appears to be the main reason for augmented contribution of SR Ca^{2+} for activating the myofilaments.

Normally, increasing stimulation frequency causes negative inotropy in rat myocardium (Koch-Weser and Blinks, 1963; Benforado, 1958; Stemmer and Akera, 1986) due to increased refractory state of the SR Ca^{2+}-release channels under higher stimulation rates (Bers, 1991). Hypothyroid atria showed a positive FFR (Figure 5), that is characteristic of a normal myocardium in species like guinea-pig, rabbit and human, where a significant amount of activator Ca^{2+}

enters the myocytes not from the SR, but by the transsarcolemmal Ca^{2+} fluxes (Koch-Weser and Blinks, 1963; Handberg *et al.*, 1984; Bers, 1991; Stemmer and Akera, 1986). Thyroid hormone treatment, known to stimulate the development of SR, converted the positive FFR into negative one in the atria. In contrast to hypothyroid atria, hypothyroid papillary muscles showed a negative FFR (Figure 5, right panel), indicating that in these muscles SR still supplied significant amount of Ca^{2+} to contraction. Thyroid hormone treatment had notably less effect on FFR in papillary muscles as compared with atria. These results suggest that the role of SR was lower, and of SL higher in providing activator Ca^{2+} in hypothyroid atria than in other muscle preparations. It could also be concluded that thyroid hormones increase the relative importance of SR in providing activator Ca^{2+} to a greater extent in atria than in ventricles. This effect of thyroid hormones is based on the larger stimulation of SR Ca^{2+}-pump in atria compared to ventricles, due to differential regulation of the expression of SR Ca^{2+}-pump and PLB proteins in these tissues.

Recently, it was proposed that the SR Ca^{2+} uptake rate could determine not only -dT/dt, but +dT/dt as well, since the increased Ca^{2+} uptake, due to the ablation of PLB gene, was associated with increased contractility (Luo *et al.* 1994). These authors suggested that the increased Ca^{2+} uptake rate may lead to a higher amounts of Ca^{2+} sequestered by the SR, which would be available for release, resulting in the higher rate of contraction. Our results support this hypothesis, because in both atria and ventricles there were strongly positive correlations between the changes in +dT/dt and -dT/dt. Considering that the expression of α-MHC gene is insensitive to thyroid hormones in atria (Everett *et al.*, 1983; Banerjee, 1983; Chizzonite *et al.*, 1984; Samuel *et al.*, 1986) and thus could not interfere in our studies, the enhanced SR Ca^{2+}-pump activity due to the down-regulation of PLB expression appears to be the most likely determinant for changes in both +dT/dt and -dT/dt in atria.

3. Energy dependence of SR Ca^{2+} uptake *in situ* (I, III)

In this work, the role of bound CK in providing SR Ca^{2+} uptake with energy was studied by using fibres with saponin-permeabilized SL. This enabled us to study the SR uptake *in situ*, in close colocalization with myofibrils by applying modified conditions of substrates and ions. In both atrial and ventricular skinned fibres, it was shown that when Ca^{2+} uptake was supported by ADP+PCr it was as efficient as when supported by ATP+PCr. In contrast, external ATP alone was not able to sustain the loading of the SR to the same extent as localized regeneration of ATP in the presence of ADP and PCr or ATP and PCr. These results indicate that sufficient amount of CK was still bound after the fibres were skinned in saponin and that local ATP generated by the CK reaction

was sufficient to completely meet the ATP requirements of the Ca-ATPase. In ventricular trabeculae from rat heart, Steele et al. (1995) also reported that PCr+ADP could sustain efficient Ca^{2+} uptake and that PCr can prevent the inhibition of Ca^{2+} uptake by P_i. This points to another function of bound CK which is to prevent a rise in intracellular [ADP] by its rephosphorylation of ADP.

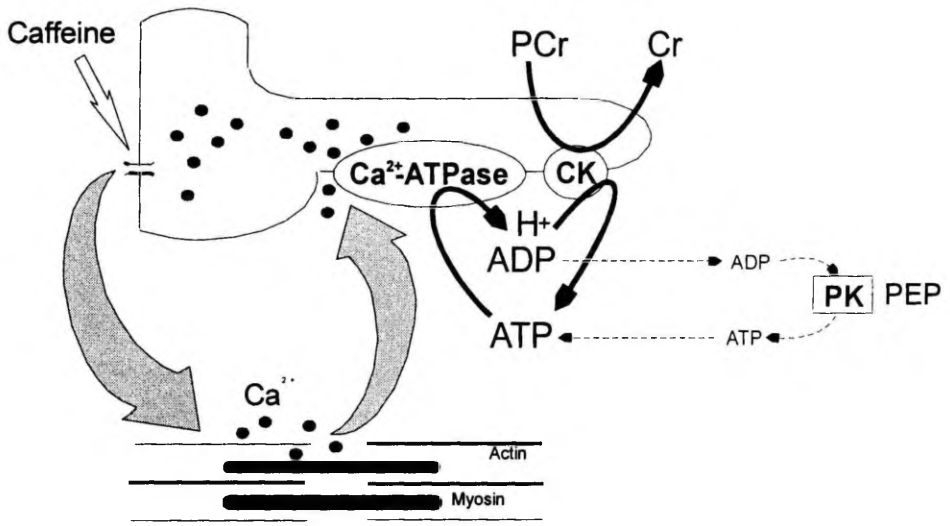

Figure 9. A diagram illustrating functional interaction of Ca^{2+}-ATPase and CK on SR membrane, leading to compartmentation of ADP and ATP in vicinity of Ca^{2+}-ATPase, not readily accessible for pyruvate kinase (PK). PEP, phosphoenolpyruvate.

As reported in isolated SR vesicles, ADP has a depressive effect on Ca^{2+} uptake with a Ki of 180 μM (Jacobus and Lehninger, 1973; Saks *et al.*, 1976; Iyengar *et al.*, 1982; Korge *et al.*, 1993). Due to the high reverse gradient between inside and outside of SR, ADP accumulation would favour Ca^{2+} leakage outside the SR (Korge and Campbell, 1994). The depressive effect of ADP on Ca^{2+} loading has also been confirmed in saponin-skinned fibres (Xiang and Kentish, 1995).

That the ADP+PCr, but not ATP alone could sustain nearly maximal SR Ca^{2+} loading in skinned fibres seems to be in contradiction with earlier experiments on SR vesicles, isolated from chicken breast skeletal muscle (Rossi *et al.*, 1990) and pigeon heart (Levitsky *et al.*, 1978), where the SR-bound CK was not able to support the maximal velocity of Ca^{2+} uptake. Several explanations can be put forward like tissue and species differences to explain the contradiction.

Also, it is highly possible that the procedure of preparation of SR vesicles induces detatchment of CK from SR. Furthermore, the intact intracellular architecture may preserve a spatial arrangement of SR Ca^{2+}-ATPase and CK more favourable for their interaction, i.e. for CK to rephosphorylate nearly all ADP released in the ATPase reaction to ATP.

In ventricular fibres, our study demonstrated, that externally added ATP-regenerating system of PK and PEP was not as effective as CK in supporting SR Ca^{2+} uptake. This confirms that the low SR Ca^{2+} load in the presence of ATP alone could not result from diffusion gradients of ATP and ADP inside the fibres. Instead, the results confirm that the increase in [ADP] takes place close to the active site of ATPase in a microenvironment not readily accessible to PK (Figure 9). The limited access of PK itself to the intracellular space is not likely, as it has been demonstrated by Kraft *et al.* (1995) that PK can equilibrate in skinned skeletal fibres within minutes. Also, it has been reported previously in isolated SR vesicles, that PK is not as effective as CK in supporting SR Ca^{2+} uptake (Levitsky *et al.,* 1978; Saks *et al.,* 1994). As a conclusion, the greater efficiency of CK to provide the SR Ca^{2+}-ATPase with ATP and to maintain the local [ADP] near the ATPase may be due to the close localization of CK to Ca^{2+}-ATPase and functional coupling of the two enzymes.

Taken together, the results show that localized regeneration of ATP is more efficient than external ATP to meet the requirement of Ca^{2+}-ATPase for efficient Ca^{2+} uptake. These results suggest that ATP and ADP are locally constrained within an effective micro-environment defined by the spatial proximity of the two enzymes, thus creating a compartmentalized high ATP/ADP ratio favourable to the Ca^{2+}-ATPase activity (Korge *et al.,* 1993; Korge and Campbell, 1994; Rossi *et al.,* 1990).

CONCLUSIONS

1. Atrial myocardium is characterized by a faster SR Ca^{2+} uptake rate, but limited or slow Ca^{2+} release and diffusion for contraction compared to ventricular one. This difference in SR Ca^{2+} handling could be proposed as the main modulator of the shorter time course and lower tension development in rat intact atrial myocardium compared with that in ventricular myocardium, despite the similar total SR capacity of releasable Ca^{2+} in both tissues.

2. Thyroid hormones increase the relative importance of SR in providing activator Ca^{2+} to the greater extent in atria than in ventricles. This effect of thyroid hormones is based on larger stimulation of SR Ca^{2+}-pump in atria compared to ventricles, due to differential regulation of expression of SR Ca^{2+}-pump and PLB proteins in these tissues and may underlie the observation that the thyroid-dependent changes in contraction and relaxation rate are more pronounced in atria than in ventricles.

3. In both atria and ventricles, the SR Ca^{2+} uptake is fueled by ATP produced in the endogenous bound-CK reaction in preference to exogenous ATP or to ATP generated by a soluble ATP-regenerating system. This suggests that CK is functionally coupled to Ca^{2+}-ATPase of SR and that bound CK is necessary for optimal functioning of the SR Ca^{2+}-ATPase *in situ*.

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SARKOPLASMAATILSE RETIIKULUMI FUNKTSIOON: Kodade ja vatsakeste müokardi võrdlev uuring

Kokkuvõte

Sarkoplasmaatiline retiikulum (SR) on membranoosne rakusisene struktuur, mis imetajate müokardirakkudes reguleerib rakusisest kaltsiumioonide kontsentratsiooni: Ca^{2+}-ioonide vabastamine SR-st müofilamentidele loob aluse lihase kontraktsiooniks ning Ca^{2+}-ioonide haare SR poolt tekitab lihase lõõgastumise. On alust arvata, et kodade müokardis on SR roll kontraktsiooni aktivaatorkaltsiumi allikana veelgi suurem kui vatsakestes, sest kodade müotsüütides on SR üldhulk suurem kui vatsakestes. Türeoidse staatuse nihked mõjutavad oluliselt müokardi kontraktiilseid omadusi ning suur osa nende muutuste tekkel on SR funktsioonihäiretel, mistõttu SR roll aktivaatorkaltsiumi allikana rakus muutub. Täiskasvanud roti süda, kus nii kodades kui ka vatsakestes domineerib müosiini raske ahela kiire isovorm, mille ekspressiooni türeoidhormoonide liig ei mõjuta, annab suurepärase võimaluse uurida SR funktsiooni erinevuste mõju kodade ja vatsakeste kontraktiilsetele omadustele.

Efektiivne SR kaltsiumihaare ja seega ka müokardi lõõgastumine sõltuvad SR Ca^{2+}pumba adekvaatsest varustamisest ATP energiaga. Südamelihasrakkude mitokondrites oksüdatiivsel fosforüülimisel vabaneva energia transpordis ATPaasideni osalevad fosfokreatiin, kreatiin ja spetsiifiliselt rakustruktuuridega seostunud kreatiinkinaasi (CK) erinevad isovormid. SR membraanidel on seostunud MM-CK, kus ta on funktsionaalses seoses Ca^{2+}-ATPaasiga, tootes fosfokreatiini ja ADP juuresolekul ATP-d. See ATP ei ole ümbritseva keskkonnaga vabas tasakaalus, vaid kasutatakse eelisjärjekorras SR Ca^{2+}-ATPaasi poolt. Ometi on isoleeritud SR vesiikulites kaltsiumihaare, mis toimub CK reaktsioonis toodetud ATP toel, olnud alati väiksem kui eksogeenselt lisatud ATP toimel. Seetõttu uurisime oma töös SR kaltsiumihaaret erinevate energeetiliste substraatide juuresolekul *in situ*, kasutades lihaskiude, kus kardiomüotsüütide rakumembraan on saponiiniga permeabiliseeritud.

Käesoleva töö eesmärgiks oli uurida SR funktsiooni, selle sõltuvust energeetilistest substraatidest ja peamiste proteiinide ekspressiooni võrdlevalt kodade ja vatsakeste müokardis, et lahti mõtestada nende parameetrite seoseid kummagi müokarditüübi kontraktiilsete omadustega normi tingimustes ja türeoidse staatuse häirete korral. Töö konkreetsed ülesanded olid:

1. Leida seoseid kodade ja vatsakeste kontraktiilsete parameetrite erinevuste ning SR funktsiooni ja proteiinide ekspressiooni vahel mõlemas koes. Selleks kõrvutati järgnevaid kodade ja vatsakeste omadusi: (1) isoleeritud trabeekulite kontraktsiooniparameetreid; (2) SR funktsiooni *in vivo*; (3) SR

kaltsiumi haaret saponiinis permeabiliseeritud lihaskiududes; (4) SR kaltsiumi haarde kiirust koehomogenaadis ja (5) SR proteiinide (SERCA, RyR, Cals) ekpressiooni, normaliseerituna müosiini raske ahela (MHC) mRNA suhtes .

2. Uurida hüper- ja hüpotüreoidismi mõju SR Ca^{2+}pumba aktiivsusele ning SR osatähtsust rakusisese aktivaator-kaltsiumi allikana nende türeoidse staatuse häirete korral kodades ja vatsakestes. Selleks võrreldi erineva türeoidse staatuse tingimustes alljärgnevaid kodade ja vatsakeste omadusi: (1) kontraktsiooniparameetreid; (2) SR rolli kontraktsiooni aktivaatorkaltsiumi allikana *in vivo* ning (3) SR kaltsiumi haarde kiirust koehomogenaadis.

3. Uurida SR Ca^{2+} haarde energeetilist sõltuvust ning SR-ga seotud CK osalust Ca^{2+}-ATPaasi varustamisel energiaga *in situ*, kasutades saponiinis permeabiliseeritud sarkolemmiga kodade ja vatsakeste lihaskiude.

Töö tulemused võimaldavad teha järgmisi järeldusi:

1. Kodade müokardi iseloomustab suurem SR kaltsiumi haarde kiirus, kuid limiteeritud või aeglane kaltsiumi vabanemine ning difusioon kontraktsiooniks, võrreldes vatsakestega. Seda erinevust kaltsiumi metabolismis mõlemas koes võib pidada peamiseks modulaatoriks, mis tingib intaktsete kodade lühema kontraktsiooniaja ja madalama kontraktsioonijõu võrreldes vatsakestega, ning seda vaatamata mõlema koe lähedasele SR kaltsiumi reservile.

2. Türeoidhormoonid tõstavad SR osatähtsust aktivaatorkaltsiumi allikana kodade müokardirakus tunduvalt suuremal määral kui vatsakestes. See türeoidhormoonide toime põhineb SR Ca^{2+}pumba võimsamal stimulatsioonil kodades võrreldes vatsakestega, sest SR Ca^{2+}pumba ja fosfolambaani (PLB) proteiinide ekpressioon neis kudedes on erinevalt reguleeritud, ning võib olla põhjuseks, miks türeoidsest staatusest sõltuvad kontraktsiooni- ja lõõgastumiskiiruse muutused on kodades väljendunud enam kui vatsakestes.

3. Nii kodades kui ka vatsakestes kasutab SR Ca^{2+}pump kaltsiumihaardeks eelistatult endogeenset, s.o. CK reaktsioonis produtseeritud ATP-d, mitte nii suurel määral eksogeenset ATP-d või lahustuva ensüümisüsteemi produtseeritud ATP-d. See lubab järeldada, et CK ja SR Ca^{2+}pump on funktsionaalses seoses ning et SR-ga seotud CK on vajalik SR Ca^{2+}pumba optimaalseks funktsioneerimiseks.

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PUBLICATION

I

Minajeva, A., Kaasik, A., Paju, K., Seppet, E. K., Lompré, A.-M., Veksler, V., Ventura-Clapier, R. Sarcoplasmic reticulum function in determining atrio-ventricular contractile differences in rat heart. Am. J. Physiol. 273: H2489–H2507, 1997.

Sarcoplasmic reticulum function in determining atrioventricular contractile differences in rat heart

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Minajeva, Ave, Allen Kaasik, Kalju Paju, Enn Seppet, Anne-Marie Lompré, Vladimir Veksler, and Renée Ventura-Clapier. Sarcoplasmic reticulum function in determining atrioventricular contractile differences in rat heart. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H2498–H2507, 1997.—The relationships between the contractile characteristics and the sarcoplasmic reticulum (SR) function of rat atrial and ventricular trabeculae were compared. The isometric developed tension (DT) and the rates of contraction ($+dT/dt$) and relaxation ($-dT/dt$) normalized to cross-sectional area were 3.7, 2.2, and 1.8 times lower, respectively, in intact atrial strips compared with ventricular strips, whereas $+dT/dt$ and $-dT/dt$ (normalized to DT) were 2.3 and 2.8 times higher, respectively, in atria. Atria exhibited a maximal potentiation of DT after shorter rest periods than ventricles and a lower reversal for prolonged rest periods. Caffeine-induced tension transients in saponin-permeabilized fibers suggested that the Ca^{2+} concentration released in atrial myofibrils reached a lower maximum and decayed more slowly than in ventricular preparations. However, the tension-time integrals indicated an equivalent capacity of sequestrable Ca^{2+} in SR from both tissues. In atrial, as in ventricular myocardium, the SR Ca^{2+} uptake was more efficiently supported by ATP produced by the SR-bound MM form of creatine kinase (CK; MM-CK) than by externally added ATP, suggesting a tight functional coupling between the SR Ca^{2+} adenosinetriphosphatase (ATPase) and MM-CK. The maximal rate of oxalate-supported Ca^{2+} uptake was two times higher in atrial than in ventricular tissue homogenates. The SR Ca^{2+}-ATPase 2a mRNA content normalized to 18S RNA was 38% higher in atria than in ventricles, whereas the amount of mRNA encoding the α-myosin heavy chain, calsequestrin, and the ryanodine receptor was similar in both tissues. Thus a lower amount of readily releasable Ca^{2+} together with a faster uptake rate may partly account for the shorter time course and lower tension development in intact atrial myocardium compared with ventricular myocardium.

sarcoplasmic reticulum calcium uptake; skinned fibers; calsequestrin; ryanodine receptor; calcium pump

IT IS KNOWN THAT atria contract and relax faster than ventricles, both isotonically and isometrically (1, 2, 16), but the underlying mechanisms for these differences are not yet completely understood. Besides a shorter action potential (2, 16), the faster kinetics of atrial contraction have been attributed to a higher proportion of the fast α-myosin heavy chain (MHC) isoform compared with ventricles. However, this seems not to be an explanation in small mammals such as rats, in which both atria and ventricles express α-MHC in a similarly high proportion (28), but atria still contract faster than ventricles (16). While addressing this issue, we recently observed that skinned left atrial and ventricular preparations from adult rat heart developed similar levels of maximal force and stiffness, displaying also similar Ca^{2+} sensitivity and tension kinetics (31). Conversely, faster cross-bridge kinetics have been observed in right atria of hyperthyroid rats compared with ventricles and have been attributed to the differences in myosin light chain content between the two tissues (11). Several observations, however, suggest that differences in Ca^{2+} handling at the level of the sarcoplasmic reticulum (SR) may contribute to the atrioventricular differences in contractile function. In mouse heart, both volume fraction and surface area of total SR per cell volume are higher in atria, mainly due to a higher longitudinal SR content (10). Therefore, atrial contraction has been proposed to be more dependent on Ca^{2+} release by the SR than ventricular contraction (1, 9, 22). Atrial SR has been shown to exhibit a 4.2-fold lower ratio of phospholamban to Ca^{2+}-adenosinetriphosphatase (ATPase) than ventricular SR, and the lower phospholamban-to-Ca^{2+}-ATPase ratio has been suggested to increase SR Ca^{2+} uptake and, consequently, lead to faster relaxation in rat atria (17). Unlike in ventricular cardiomyocytes, a nonsynchronous and biphasic Ca^{2+}-induced Ca^{2+} release has been observed in atrial cardiomyocytes (8, 14).

Atrioventricular differences in contractile function may also reflect the different metabolic profiles of these tissues. In comparison with ventricles, atria are characterized by lower activities of glycolytic and citric acid cycle enzymes (4). Lower oxidative capacities in atria are associated with decreased activities of both total creatine kinase (CK) and mitochondrial CK (mito-CK) compared with ventricles (31). It has also been shown in atria that mito-CK is not coupled to the adenine nucleotide translocase (31). These findings suggest fundamentally different mechanisms of energy transport in atrial compared with ventricular cells. In this respect, a question arises concerning the interactions between CK and the SR Ca^{2+}-ATPase in atria. The MM form of CK (MM-CK) bound to the myofilaments exhibited the same efficacy in controlling the ATP-to-ADP ratio in atria and ventricles (31). Characterization of SR function in isolated membrane preparations and in

skinned fibers from ventricular myocardium has revealed a functional coupling between SR Ca^{2+} uptake and MM-CK bound to the SR membranes (18, 24). However, nothing is known about the role of CK in SR Ca^{2+} uptake in atria.

This work was undertaken to study and compare the relationships between the contractile parameters, the expression of SR proteins, the SR function, and the functional characteristics of MM-CK bound to SR in atria and ventricles from rats. It was found that, compared with ventricles, atria exhibit a higher expression of SR Ca^{2+}-ATPase (SERCA) 2a associated with faster SR Ca^{2+} uptake but prolonged caffeine-induced Ca^{2+} release. These properties of SR are accompanied by a faster rate of filling during interbeat pauses as well as a reduced loss of SR Ca^{2+} during pause decay in intact atria. At the same time, the SR calsequestrin (Cals) and ryanodine receptor (RyR) expression as well as the SR capacity of releasable Ca^{2+} appear similar in atria and ventricles. Like in ventricles, the SR Ca^{2+} uptake is highly dependent on ATP generated locally in the CK reaction, thus indicating a coupling of MM-CK to the SR Ca^{2+} pump in atria.

MATERIALS AND METHODS

Muscle preparations. Wistar rats of both sexes (average body wt 260 g) were treated according to the recommendations of the institutional animal care committee (INSERM, Paris, France). They were anesthetized by intraperitoneal injection of urethan (0.2 g/100 g body wt). For contractility measurements, papillary muscles from right ventricles or fibers from papillary muscles of left ventricles and atria with similar cross-sectional areas were dissected in a solution containing (in mM) 120 NaCl, 5.4 KCl, 0.6 $CaCl_2$, 0.42 NaH_2PO_4, 1.05 $MgCl_2$, 5 glucose, 30 2,3-butanedione monoxime, 0.05 Na_2 EDTA, and 20 tris(hydroxymethyl)aminomethane (Tris)·HCl, gassed with 100% oxygen, pH 7.4.

Fibers to be used for permeabilization of the sarcolemma (diameter 150–240 μm) were dissected from left ventricular papillary muscle or from left atria in ice-cold zero-Ca^{2+} Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 1.2 $MgSO_4$ equilibrated with 95% O_2-5% CO_2. Specific permeabilization of the sarcolemma was obtained by incubating the fibers in relaxing *solution A* (see Table 1) containing 50 μg/ml saponin in the presence of 20 μM leupeptin at +4°C for 30 min (13).

Contractile function measurements. Muscle preparations with silk thread tied to one end were placed horizontally in a 0.2-ml perfusion chamber between a force transducer (6MX1C) and a needle that was attached to a length adjustment device. Preparations were field stimulated by a pair of platinum-plate electrodes using rectangular current pulses (1 Hz, 5 ms, 1.5-threshold voltage) generated by an electronic stimulator (SC-50–1). During an equilibration period of 90 min, the muscles were superfused at the rate of 5 ml/min with a solution containing (in mM) 145 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose, 1.1 mannitol, and 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4 at 30°C, aerated with 100% O_2. The isometric contractile parameters of muscle preparation were registered at the peak of the length-tension curve using on-line PC-AT 486 with Atrium software designed by Dr. U. Braun. The length of each fiber was measured by means of a micrometer in a dissecting microscope. At the end of the experiment, the fiber was cut off between the silk thread and the needle and weighed. Mean cross-sectional area for each preparation was calculated from the weight and length of the trabeculae, assuming a muscle density of one. Tension values were expressed in millinewtons per square millimeter.

The dependence of postrest potentiation on the rest interval at extracellular Ca^{2+} concentrations ($[Ca^{2-}]_o$) of 1 and 2.5 mM was used to compare the SR function in intact myocardium. In these experiments, the basic stimulation at 1 Hz was interrupted for 3–600 s. In each preparation, the values of the developed tension (DT) of the first postrest twitches were normalized to the maximal value of potentiation, usually gained after a 60- to 120-s pause.

Preparation of tissue homogenates and estimation of SR ^{45}Ca uptake. Rats were anesthetized with thiopental sodium (50 mg/kg), and hearts were excised and rinsed rapidly in ice-cold isotonic saline solution. The whole atrial auricles and ventricular apex region were isolated and weighed. The homogenization was carried out with an Ultra-Turrax homogenizer (3 × 20 s, 24,000 revolutions/min) in 50 vol of ice-cold homogenization buffer containing (in mM) 250 sucrose, 20 Tris (pH 6.8), 2 $MgCl_2$, 0.01 leupeptin, 0.01 phenylmethylsulfonyl fluoride, 1 dithiothreitol, and 2 benzamidine. The final homogenate was further treated with a glass-glass homogenizer (10 strokes).

The Ca^{2-} accumulation in the tissue homogenate was determined at 30°C in stirred medium containing (in mM) 6 ATP, 6 $MgCl_2$, 120 KCl, 3 sodium azide (NaN_3), 60 imidazole (pH 7.0), 6 potassium oxalate, and a ^{45}Ca-labeled $CaCl_2$-ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) buffer containing 0.58 mM EGTA. Appropriate concentrations of $CaCl_2$ were added to the medium to obtain different free Ca^{2+} concentrations ($[Ca^{2+}]$). The reaction was started by addition of 50 μl of homogenate per 0.5 ml of medium. After a 2-min incubation, the samples were filtered through 0.45-μm Schleicher & Schuell (Keene, NH) glass microfiber filters using a vacuum pump. Radioactivity associated with the membranes was counted in Optiphase "HiSafe" 3 (Wallac, Turku, Finland). After subtraction of unspecific binding, the Ca^{2+} uptake by the homogenate was entirely blocked by 30 μM cyclopiazonic acid, a specific inhibitor of SR Ca^{2+}-ATPase that shows that Ca^{2+} uptake was restricted to the SR Ca^{2+} pump. Protein concentration in the homogenate was determined by the biuret method and was between 104 and 166 μg per uptake assay.

Estimation of SR Ca^{2+} uptake in saponin-permeabilized fibers. SR Ca^{2+} uptake in permeabilized fibers was estimated by analyzing the tension transients due to caffeine-induced Ca^{2-} release after various periods of SR loading (13, 30), as described recently (24). The fibers were mounted between a length-adjustment device and a force transducer (AE 801; Aker's Microelectronics, Horton, Norway). Fibers were immersed in 2.5-ml chambers arranged around a disk. The chambers were placed in a temperature-controlled bath positioned on a magnetic stirrer. All experiments were performed at 22°C. The immersion solutions were calculated as described previously and are listed in Table 1. All solutions contained (in mM) 0.8 free Mg^{2+}, 30.6 Na^+, 60 *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 7.1), 3.16 MgATP, 12 phosphocreatine (PCr), and 0.3 dithiothreitol. The ionic strength was adjusted to 160 mM with potassium methanesulfonate. All solutions contained 2 mM NaN_3 to inhibit possible Ca^{2+} uptake by mitochondria and 20 μM leupeptin to inhibit the proteases. To study the energy requirement for SR Ca^{2+} loading, solutions with modified adenine nucleotides and PCr content were designed.

Table 1. *Composition of solutions for estimating Ca^{2+} uptake in skinned cardiac fibers*

Solutions	pCa	EGTA, mM	Caffeine, mM
Relaxing			
A	9.0	10	
A_1	9.0	10	5
Activating			
B	4.5	10	
Loading			
C	6.5	10	
Prerelease			
D	9.0	0.25	
R	9.0	0.2	
Release			
R_1	9.0	0.2	5

All solutions contained (in mM) 0.8 free Mg^{2+}, 30.6 Na^+, 60 *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 7.1), 3.16 MgATP, 12 phosphocreatine, 0.3 dithiothreitol, 2 sodium azide, and 20 μM leupeptin. The ionic strength was adjusted to 160 mM with potassium methanesulfonate.

At the beginning of each experiment, the fiber was stretched to 120% of the slack length in the relaxing *solution A*. The maximal Ca^{2+}-activated tension (T_{max}) was estimated in the activating *solution B*, pCa 4.5. The fiber was then relaxed in *solution A*. To empty the SR of Ca^{2+}, 5 mM caffeine was applied for 2 min in the relaxing solution (A_1) followed by the washout of caffeine for 2 min in the same solution (*A*). SR loading was carried out at pCa 6.5 and 10 mM [EGTA] (*solution C*) for different time periods, so that under control conditions the peak force never reached the upper saturating part of the pCa-tension relationship and the Ca^{2+} release was in the quasi-linear part of the pCa-tension relationship. To wash out Ca^{2+} and EGTA after loading, the prerelease solution (*D*) containing 0.25 mM [EGTA] was applied for 1.5 min. The fiber was then passed into another prerelease solution (*E*) with 0.2 mM [EGTA] for 30 s. Finally, Ca^{2+} was released from the SR by applying 5 mM caffeine in a solution of the same composition (R_1), which resulted in a tension transient. The peak of relative tension (T/T_{max}) and the area under isometric tension (S_T) were measured. The pCa-tension relationship in the presence of 5 mM caffeine in conditions identical to those of the release (except that 10 mM EGTA was present to adequately buffer free Ca^{2+}) was obtained at the end of each experiment by sequentially exposing the fibers to a set of solutions with decreasing pCa until T_{max} was reached (at pCa 4.5). DT at each pCa was normalized with respect to T_{max}. The data from each fiber were fitted by the Hill equation using linear regression analysis, and the pCa required to produce 50% of maximal activation (pCa_{50}) and the Hill coefficient (n_H) were determined. The same Ca^{2+}-release protocol was always applied to enable comparison of SR Ca^{2+} uptake in different fibers or loading conditions.

Evaluation of the role of bound CK in SR Ca^{2+} uptake by saponin-permeabilized atrial fibers. To evaluate the role of bound CK in providing the energy for SR Ca^{2+} uptake in situ, SR loading was carried out at pCa 6.5 in the presence of 0.25 mM MgADP and 12 mM PCr, so that ATP generated in the CK reaction was the only source of energy for the Ca^{2+} pump. Alternatively, the load in the presence of 3.16 mM MgATP alone was carried out to compare the efficacy of externally added ATP with the internally produced ATP. Loading in the presence of 3.16 mM MgATP and 12 mM PCr served as a control. [EGTA] in all test solutions was 10 mM. Before loading, a 2-min preequilibration period was used at pCa 9, with the same conditions of substrates as those of loading, to equilibrate the concentration of compounds inside the fiber. To permit comparison between different loading conditions, Ca^{2+} release by 5 mM caffeine was always induced at constant conditions of substrates and ions. Evaluation of the Ca^{2+} uptake under different conditions was done by analyzing tension transients due to caffeine-induced Ca^{2+} release, as described above.

Isolation of total RNA and mRNA dot-blot analysis. Eight hearts were collected from adult Wistar rats. The atria were isolated, and the ventricles were cut into pieces. The tissues were blotted dry, frozen in liquid nitrogen, and kept at −80°C until RNA preparation. Both atria from each rat and a piece of ventricle with approximately the same weight were used for RNA preparation. Total RNA was extracted by the guanidinium isothiocyanate procedure using RNA quick (Bioprobe) and kept at −20°C in 70% ethanol, 0.3% sodium acetate, pH 5.2. Specific mRNA species were quantified by slot-blot hybridization. One, two, and four micrograms of total RNA from atria and ventricles as well as from liver and fast skeletal muscle (extensor digitorum longus) and yeast tRNA were denatured in 15× standard saline citrate (SSC; 1× SSC contained 0.15 M sodium chloride and 0.015 M sodium citrate) and 3% formaldehyde at 65°C for 15 min and rapidly cooled on ice. Liver, skeletal muscle RNA, and tRNA were used as negative controls to check for specificity of the various probes. The samples were directly spotted onto the nylon membrane using a minifold apparatus (Schleicher & Schuell). The RNA was cross-linked to the membrane by ultraviolet irradiation, and the membranes were prehybridized at 42°C for >4 h in the presence of 50% formamide, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.05 M sodium phosphate (pH 6.5), 5× SSC, 0.1% sodium dodecyl sulfate (SDS), and 250 μg/ml salmon sperm DNA.

The specific SERCA2 mRNA was detected using a probe [from nucleotide (nt) 2616 to 3120] of the rat heart Ca^{2+}-ATPase mRNA (20). The rat RyR probe (RyR-2) was obtained by reverse transcription of rat cardiac total RNA and subsequent amplification of the RyR-2 mRNA using primers derived from the sequence of the rabbit cardiac RyR-2 (25). The probe extends from nt 8604 to 9144 of the rabbit sequence (25) with few silent mutations. The dog Cals probe was a gift from Dr. B. Nadal-Ginard and was used as described by Lompré et al. (21). The α-MHC probe has been previously described by Schiaffino et al. (29). The cDNA probes were labeled by use of random primers, DNA polymerase I (Klenow fragment) and [α-^{32}P]dATP (3,000 Ci/mmol), and the specific activity was 1–3 × 10^9 disintegrations/min (dpm)/μg. Hybridization was done in the same conditions as prehybridization. Excess of probe was eliminated by washing in 0.5× SSC at 55°C for SERCA2, at 60°C for RyR-2, and at 42°C for Cals and in 1× SSC at 45°C for MHC.

After each hybridization, the blots were dehybridized by boiling in 0.1% SDS and then rehybridized as described above. To normalize to the amount of total RNA present on the membrane, the blots were rehybridized with a 24-mer oligonucleotide complementary to the rat 18S ribosomal RNA. The oligonucleotide was labeled at its 5′ end by use of T4 polynucleotide kinase and [γ-^{32}P]ATP and diluted with cold oligonucleotide to a specific activity of 10^5 dpm/μg. It was hybridized in the medium described above but in the absence of formamide. The washing conditions were 2× SSC at room temperature. After washing, the membranes were exposed to X-ray film for 1 day to 1 wk. Unsaturated autoradiograms were analyzed by densitometry (Molecular Dynamics). The specific mRNA level was corrected for the total RNA present on the membrane by calculating the ratio of the signals obtained with the specific probe and the 18S probe for the

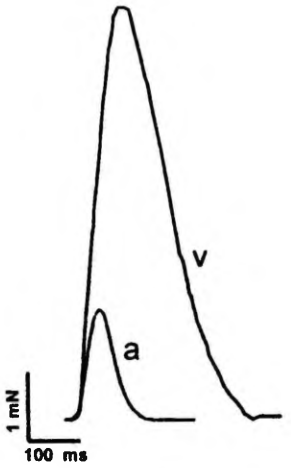

Fig. 1. Superimposed twitch tension recordings from atrial (a) and ventricular (v) trabeculae at a steady-state stimulation rate of 1 Hz with 2.5 mM extracellular Ca^{2+} concentration. Diameter of atrial trabecula, 325 μm; diameter of ventricular trabecula, 350 μm.

three dilutions of each sample. Specific mRNA levels were expressed in arbitrary units (AU) as means ± SE. The relative proportion of SERCA2 and SERCA2b mRNA was determined by ribonuclease (RNase) protection assay using the SERCA2 probe described above and the Ambion kit protocol (Clinisciences, Montrouge, France).

Chemicals. Caffeine was purchased from Merck-Clevenot. PCr (Neoton, Schiapparelli Searle, Turin, Italy) was a kind gift from Prof. E. Strumia. Other chemicals were obtained from Sigma Chemical.

Statistical analysis. The data are expressed as means ± SE or as representative tracings in a single experiment. Statistical analysis is presented in RESULTS. Differences at $P < 0.05$ were considered significant.

RESULTS

Contractile parameters. Figure 1 shows that atrial contractions are characterized by a weaker twitch DT but a faster time course than ventricular contractions. The mean values of contractile characteristics are presented in Table 2 and compared using unpaired Student's *t*-test. DT in atrial preparations was 3.7 times less than that in ventricular preparations. The time to peak tension (TPT) and the half-relaxation time (RT_{50}) were significantly less in atria. The maximal rates of contraction ($+dT/dt$) and relaxation ($-dT/dt$) also appeared to be lower in atria than in ventricles. However, if normalized to DT, these parameters [$(+dT/dt)/DT$ and $(-dT/dt)/DT$] became higher in atria, evidencing faster contractile kinetics.

Postrest potentiation. Postrest potentiation of twitch tension was compared in atria and papillary muscles at two external $[Ca^{2+}]$ (1 and 2.5 mM) to assess the function of SR in providing activator Ca^{2+} in vivo (3, 19; for review see Ref. 9). The absolute mean values of pretest steady-state and maximal potentiation in both tissues are presented in Table 3. In Fig. 2, values were normalized to maximal potentiation to compare the two tissues. The values were analyzed using analysis of variance (ANOVA) followed by Dunnett's test. Figure 2*A* shows that, at 1 mM $[Ca^{2+}]_o$, increasing the rest duration was accompanied by potentiation of the first postrest twitch in both atrial and papillary muscle preparations. However, the maximal level of potentiation was achieved after significantly shorter rest intervals (15 s) in atrial than in papillary muscles (60 s). In addition, the magnitude of potentiation was less in atria than in ventricles. Further increase in rest duration led to significant reversal of potentiation in papillary but not in atrial muscles. Figure 2*B* demonstrates that these differences between atrial and papillary muscles were abolished when $[Ca^{2+}]_o$ was increased from 1 to 2.5 mM.

Oxalate-supported $^{45}Ca^{2+}$ uptake in tissue homogenates. The oxalate-supported $^{45}Ca^{2+}$ uptake rates were estimated in tissue homogenates in conditions (see MATERIALS AND METHODS) under which the Ca^{2+} uptake has been previously defined to be restricted to SR vesicles (26). In our experiments, the specific SR Ca^{2+}-ATPase inhibitor cyclopiazonic acid (30 μM) inhibited ~98% of Ca^{2+} uptake. This also confirms that the intrinsic SR Ca^{2+} uptake was assayed.

The net SR Ca^{2+}-uptake rates in tissue homogenates of both atria and ventricles were assayed in the presence of oxalate over a wide range of free $[Ca^{2+}]$, corresponding to the range of cytosolic free $[Ca^{2+}]$ during the contraction-relaxation cycle (Fig. 3). Data were fitted using the Hill equation, and the results were compared using Student's *t*-test. The net maximal Ca^{2+}-uptake rate was more than twofold higher in atria than in ventricles (4.17 ± 0.72 and 2.03 ± 0.42 nmol Ca^{2+}·mg tissue protein^{-1}·min^{-1}, respectively; $P < 0.05$). However, the free Ca^{2+} concentration that produces

Table 2. *Mean characteristics and contractile parameters obtained at 1-Hz stimulation, $[Ca^{2+}]_o$ 2.5 mM, of fibers dissected from rat atria and ventricles*

	n	*S*, mm^2	DT, mN/mm^2	Resting Tension, mN/mm^2	TPT, ms	RT_{50}, ms	$+dT/dt$, (mN/s)/mm^2	$-dT/dt$, (mN/s)/mm^2	$(+dT/dt)/DT$, s^{-1}	$(-dT/dt)/DT$, s^{-1}
Atria	13	0.19 ± 0.02	7.4 ± 0.8	2.1 ± 0.3	71 ± 1	32 ± 2	173 ± 24	120 ± 13	26.2 ± 4.6	18.7 ± 2.9
Ventricles	11	0.19 ± 0.02	26.1 ± 1.6	5.7 ± 0.8	145 ± 3	104 ± 5	295 ± 16	173 ± 13	11.4 ± 0.3	6.7 ± 0.5
P		NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.001	<0.01	<0.01	<0.001

Values are means ± SE; *n*, no. of fibers. $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; *S*, cross-sectional area; DT, developed tension; TPT, time to peak tension (from the moment of stimulation to peak twitch tension development); RT_{50}, half-relaxation time; $+dT/dt$, maximal rate of tension development; $-dT/dt$, maximal rate of relaxation; NS, not significant.

Table 3. *Absolute mean values of maximally potentiated and steady-state twitch amplitudes in atria and ventricles*

		Atria			Ventricle	
$[Ca^{2+}]_o$	n	Steady state	Potentiated	n	Steady state	Potentiated
1 mM	8	3.6 ± 0.6	8.27 ± 2.0	10	12.6 ± 2.9*	48 ± 17*
2.5 mM	7	8.4 ± 0.9	12.7 ± 2.1	9	31.0 ± 4.8‡	64 ± 12†

Values are means ± SE expressed in mN/mm²; n, no. of fibers. *P < 0.05; †P < 0.01; ‡P < 0.001, ventricles vs. atria.

half-maximal activation of the SR Ca^{2+} pump (0.22 ± 0.03 and 0.22 ± 0.04 μM in atria and ventricles, respectively) as well as the n_H (1.55 ± 0.13 and 1.68 ± 0.34 in atria and ventricles, respectively) were similar in atria and ventricles.

Ca^{2+} uptake in skinned cardiac fibers. To compare the SR Ca^{2+} handling in atrial and ventricular myocardium in situ, saponin-permeabilized fibers with similar mean diameters were used. Table 4 shows that atrial and ventricular preparations exhibited similar values of T_{max} normalized per cross-sectional area and of myofilament Ca^{2+} sensitivity in the presence of 5 mM caffeine. In addition, no effect of caffeine on T_{max} was observed (results not shown).

The similar responsiveness of myofibrils from atrial and ventricular preparations to Ca^{2+} in the presence of 5 mM caffeine allows us to compare the time courses of caffeine-induced tension transients in the two tissues. The SR Ca^{2+} uptake was estimated by loading tissues with Ca^{2+} for different time periods and subsequent liberation by caffeine. Figure 4 shows that, after a 10-min load at pCa 6.5, a typical caffeine-induced contracture in atrial fibers was characterized by lower peak tension and a substantial tonic component of the contracture, whereas ventricular muscle promptly returned to baseline tension. In Fig. 5, mean data as a function of loading time are presented and compared

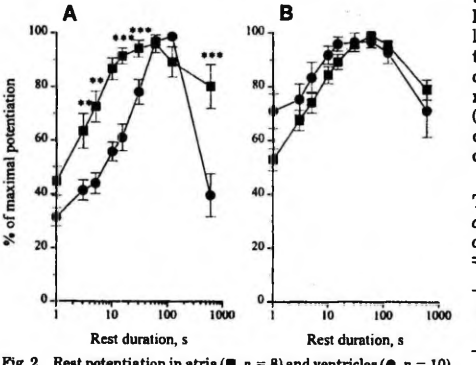

Fig. 2. Rest potentiation in atria (■, n = 8) and ventricles (●, n = 10) at 1 (*A*) and 2.5 mM (*B*) external Ca^{2+} concentrations. Values have been normalized to maximal potentiation. **P < 0.01; ***P < 0.001, ventricles vs. atria.

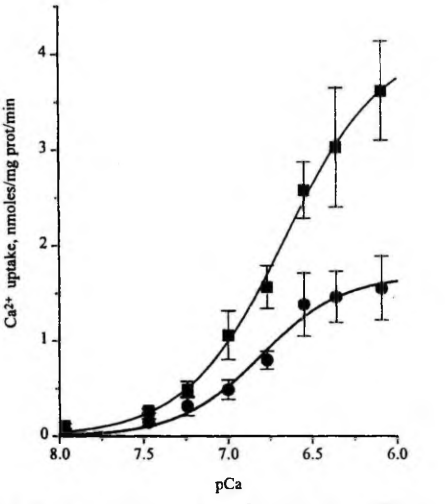

Fig. 3. Sarcoplasmic reticulum (SR) Ca^{2+} uptake rate in 4 different preparations of atrial (■) and ventricular (●) homogenates at different pCa (expressed in nmol $Ca^{2+} \cdot min^{-1} \cdot mg\ protein^{-1}$). Each curve was drawn using mean values of 4 preparations. Continuous curves obtained from Hill equation were fitted by least-square fitting procedure.

using ANOVA followed by Dunnett's test. Figure 5*A* shows that, in response to increased periods of loading, the peak tension of caffeine-induced contractures increased in both atrial and ventricular fibers. However, the peak tension values normalized to T_{max} reached a lower level (50%) in atrial than in ventricular fibers (80%). The RT_{50} of caffeine-induced contracture increased progressively with the loading time in both tissues (Fig. 5*B*), reaching, however, significantly higher levels in atrial than in ventricular preparations at loading times ≥10 min. To eliminate the possibility that these differences were due to limited diffusion of caffeine at a concentration of 5 mM, application of 25 mM caffeine was used in some experiments. The results (not shown) indicated that the higher caffeine did not diminish the atrioventricular differences in the time course of tension transient. In addition, when a second

Table 4. *Characteristics of saponin-permeabilized atrial and ventricular fibers in presence of 5 mM caffeine*

	n	Diameter, μm	T_{max}, mN/mm²	pCa_{50}	n_H
Atria	15	210 ± 14	21.9 ± 5.1	5.85 ± 0.03	2.95 ± 0.41
Ventricles	20	207 ± 7	19.1 ± 1.4	5.82 ± 0.01	3.26 ± 0.24
P		NS	NS	NS	NS

Values are means ± SE; n, no. of fibers. T_{max}, maximal Ca^{2+}-activated tension; pCa_{50}, pCa for half-maximal activation; n_H, Hill coefficient. pCa_{50} and n_H have been calculated using nonlinear fit of the Hill equation for each fiber. NS, difference not significant.

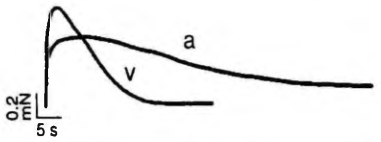

Fig. 4. Superimposed recordings of atrial (a) and ventricular (v) tension transients elicited by 5 mM caffeine in saponin-permeabilized fibers after loading at pCa 6.5 for 10 min. Diameter of atrial fiber, 240 μm; diameter of ventricular fiber, 160 μm.

application of 5 mM caffeine was made immediately after the first, no increase in tension was observed in either tissue, suggesting that the slow phase of tension transient in atria was due to a slow phase of Ca^{2+} release. Further analysis of tension-time integrals (S_T) calculated over the whole tension transient revealed that the area under the caffeine-induced tension transient was the same in atrial and ventricular fibers (Fig. 5*C*). Thus, despite the different shape of the caffeine-elicited tension transients and a faster SR Ca^{2+}-uptake rate in atria (Fig. 3), atria seemed to exhibit at least equal SR Ca^{2+} capacity in situ as ventricles.

Energy dependence of SR Ca^{2+} uptake in situ in atria. To evaluate the efficiency of the MM-CK bound to SR in providing the SR Ca^{2+} pump with ATP in situ, we compared the SR Ca^{2+} loading in the presence of MgADP and PCr (when ATP generated by the bound CK was the only source of energy for the Ca^{2+} pump) with that supported by MgATP alone. The level of SR Ca^{2+} loading in the presence of 3.16 mM MgATP and 12 mM PCr served as control. Data were compared using ANOVA followed by Dunnett's test. Figure 6*A* shows that when SR was loaded either in the presence of ADP and PCr or ATP and PCr the time dependence of relative peak tension for these two conditions had similar shape, both reaching their equivalent maximal values (51 and 47% of T_{max}, respectively) within 1 min. Further increase in loading time did not alter the values of T/T_{max}. In contrast, the curve obtained in the presence of ATP alone tended to decrease if the time of loading was prolonged for >1 min. As a result, the values of T/T_{max} became significantly less than those in control conditions.

Figure 6*B* shows the dependency of the tension-time integral on the time of loading for different conditions of energy supply. It can be seen that S_T increased progressively with the time of loading, being 11.2 ± 3.2 $mN \cdot s/mm^2$ in the presence of ATP and PCr and not significantly different when ATP was replaced by ADP for a 7-min load. In contrast, when SR was loaded in the presence of ATP alone, the value of S_T dramatically decreased (1.31 ± 0.16 $mN \cdot s/mm^2$, $P < 0.05$) for the same loading time. These results show that the amount of Ca^{2+} released from the SR by caffeine was much higher in conditions when SR Ca^{2+} uptake was supported by ATP produced by the SR-bound CK than when externally added ATP was used as the only source of energy for the SR Ca^{2+} pump.

Expression of mRNA. To relate the observed differences in SR function between the two cardiac tissues to the level of expression of the principal SR proteins (SERCA, RyR, and Cals) and the α-MHC, the tissue amounts of corresponding mRNA relative to 18S were estimated by slot blot (Fig. 7, *A* and *B*). Figure 7*A* shows that no signal was observed with any of our probes with liver RNA, whereas 18S RNA was present, indicating no nonspecific binding of the probes. This is also attested by the negative signal in tRNA samples. As expected, the α-MHC and the RyR-2 probes were specific for cardiac RNA and were not detected in skeletal muscle, whereas the SERCA2 and Cals mRNA were present in both muscle types. Quantification of slot blot (Fig. 7*B*) indicates that the expression of the α-MHC relative to 18S was not significantly different between atria and ventricles [1,000 ± 90 and 974 ± 81 AU, respectively]. Relative to 18S RNA, the content of RyR and Cals mRNAs in the two tissues was not different, whereas the amount of SERCA2 mRNA was

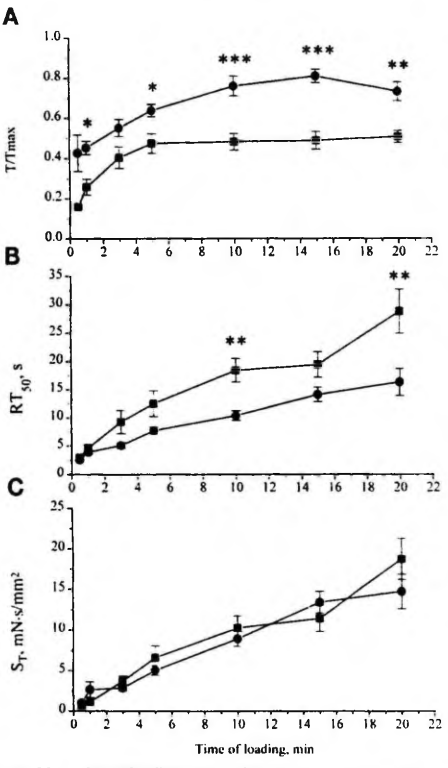

Fig. 5. Mean data of caffeine-elicited tension transients from atrial (■) and ventricular (●) saponin-permeabilized fibers, as a function of loading time at pCa 6.5. *A*: peak tension of caffeine-induced contracture normalized to maximal Ca^{2+}-activated tension (T/T_{max}) at pCa 4.5 for each fiber. *B*: time to half-relaxation of tension transient (RT_{50}). *C*: tension-time integral (S_T). Each value is mean of 3–7 determinations in different fibers. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ventricles vs. atria.

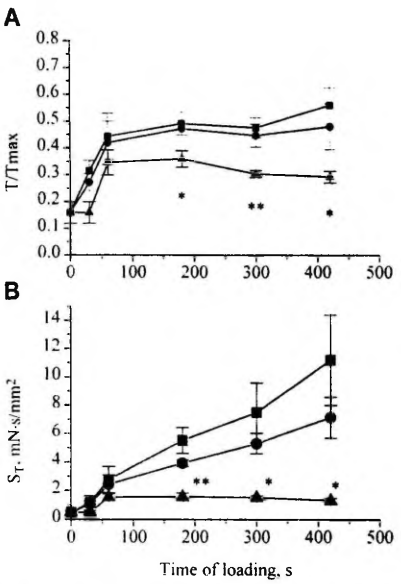

Fig. 6. Parameters of caffeine-elicited tension transient in saponin-permeabilized atrial fibers as a function of loading time. Loading was performed in presence of 12 mM phosphocreatine (PCr) and 3.16 mM MgATP (control, ■), 12 mM PCr and 0.25 mM MgADP (●) and 3.16 mM MgATP (▲) in atrial skinned fibers. *A*: T/T_{max} of each fiber. *B*: S_T. Each value is mean of 3–7 determinations in different fibers. * and **, $P < 0.05$ and $P < 0.01$, respectively, vs. control (ATP-PCr).

38% higher in atria than in ventricles (220 ± 21 and 160 ± 18 AU, respectively, $P < 0.05$). The relative proportion of SERCA2a and SERCA2b mRNA was determined by RNase protection analysis. As already shown in the ventricle, the majority of the SERCA2 mRNA was of the 2a type (21). An identical pattern was observed in the atria (data not shown). Relative to α-MHC mRNA, the amount of SERCA2 mRNA was lower in ventricles than in atria (0.165 ± 0.010 and 0.234 ± 0.049 AU, respectively), but the difference did not reach significance. This suggests that higher SR Ca^{2+}-pump activity was due to a higher expression of pump protein in atria than in ventricles.

DISCUSSION

Contractile parameters in atria and ventricles. In comparison with papillary muscles, atrial preparations exhibited smaller absolute values of DT. However, the kinetic parameters (TPT and RT_{50}) and $+dT/dt$ and $-dT/dt$ normalized to DT showed that peak isometric tension in atria reached its maximum and relaxed faster than in ventricles, in accordance with earlier studies (1, 2, 17). A low DT in left atria is strikingly in contrast to the observation that both maximum Ca^{2+}-activated tension and Ca^{2+} sensitivity of myofibrils were similar in atrial and ventricular saponin-treated preparations (Table 3) (31). This could be the result of a smaller amount of activator Ca^{2+} in intact atria compared with ventricles. On the other hand, the process could be limited also by a higher rate of Ca^{2+} resequestration by SR.

Rest potentiation. Rest potentiation has been suggested to result from the increase in Ca^{2+} release from the SR as a result of either the increase in SR Ca^{2+} content in rat cardiomyocytes (3, 7, 10, 19) or the increase in fractional SR Ca^{2+} release (6, 12). Our data indicate that, at low $[Ca^{2+}]_o$, although relative potentiation is lower in atria, the mechanisms responsible for maximal postrest potentiation need about four times less time to become saturated in atrial (15 s) than in ventricular (60 s) myocardium. This suggests that SR Ca^{2+} filling saturates earlier and faster in atria. These features of atrial myocardium could be associated with a faster SR Ca^{2-}-uptake rate, although it is not clear whether the amount of SR-releasable Ca^{2+} is indeed increased. An increase in rest duration for 10 min led to significant reversal of potentiation in papillary muscles but not in atria. The decline in rest potentiation and rest decay has been attributed to SR Ca^{2+} loss due to its extrusion through the sarcolemmal Na/Ca exchanger (6, 9). This may indicate that the SR in rat ventricular myocardium loses more Ca^{2+} during pauses. As for rest potentiation, this could be attributed to a faster SR Ca^{2+} uptake rate in atria. On the other hand, atrial myocytes are characterized by higher intracellular sodium concentration due to a lower Na^+-K^+ ATPase content (33). This may lead to less pronounced Ca^{2+} extrusion via the Na/Ca exchanger, resulting in the less pronounced rest decay. Indeed, under high $[Ca^{2+}]_o$, which minimizes the Ca^{2+} extrusion via Na/Ca exchange, there were no differences in pause-dependent potentiation between the atrial and ventricular myocardium. This may suggest that Na/Ca exchange plays an important role in the reversal of rest potentiation.

Differences in SR Ca^{2+} handling in atrial and ventricular myocardium. The oxalate-supported Ca^{2+} uptake rate in tissue homogenate, which reflects the intrinsic Ca^{2+} transport by SR Ca^{2+}-ATPase (26), was taken to estimate the SR function in vitro. Our data indicate that the oxalate-supported Ca^{2+} uptake rate (expressed per milligram of protein) is two times higher in atria than in ventricles. This may be partly explained by the 30% higher level of the SERCA2 mRNA relative to 18S RNA observed in atria (Fig. 7) and by the lower expression of phospholamban (17), favoring a more efficient Ca^{2+} uptake rate in atria than in ventricles. The similar free Ca^{2+} concentration that produces half-maximal activation of the SR Ca^{2+} pump in atria and ventricles is in agreement with the observation of an unchanged SERCA2a-to-SERCA2b ratio.

To evaluate the interaction of SR with the contractile apparatus, the SR function was analyzed in situ, in saponin-permeabilized fibers. In both atria and ventricles, caffeine-induced Ca^{2+} release was observed to occur in two phases. The fast phase of Ca^{2+} release was evidenced by T/T_{max}, which saturated faster and appeared lower in atria than in ventricles. This smaller

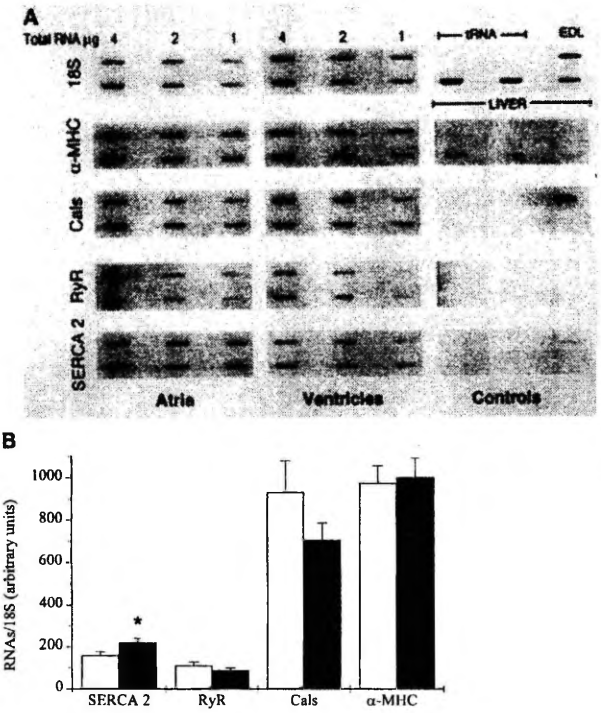

Fig. 7. Quantification of 18S RNA, SR Ca^{2+}-ATPase 2 (SERCA2) mRNA, ryanodine receptor (RyR) mRNA, calsequestrin (Cals) mRNA, and α-myosin heavy chain (α-MHC) mRNA levels in atria and ventricles by slot-blot analysis. *A*: representative results of slot blot with 2 samples from atria and 2 samples from ventricles. Liver RNA, tRNA, and RNA from extensor digitorum longus (EDL) muscle were used as negative controls. *B*: level of each mRNA species relative to 18S RNA. Specific mRNA levels were expressed in arbitrary units as means ± SE. Differences between independent samples were tested for significance by a nonparametric transformation of unpaired *t*-test: Wilcoxon-Mann-Whitney *U*-test. $*P < 0.05$, atria vs. ventricles.

"ast phase of Ca^{2+} release might be the basis for the lower DT observed in atria. However, a further increase in the loading time induced a prolongation of the tension transient as evidenced by the progressive increase in the S_T. This prolongation of the tension transient was more marked in atria than in ventricles. The time course of the caffeine-induced tension transient will depend on *1*) the amount of released Ca^{2+}, *2*) the rate of SR Ca^{2+} release, *3*) the diffusion of Ca^{2+} away from the myofibrils, *4*) the Ca^{2+} buffering by EGTA and proteins, *5*) the possible reuptake and release of Ca^{2+} and, *6*) the Ca^{2}-sensitizing effect of caffeine. Because atrial and ventricular fibers have similar diameter and composition, it is unlikely that differences will arise from different Ca^{2+} buffering. Similarly, atrial and ventricular fibers exhibited the same sensitivity to Ca^{2+} (31) and similar sensitivity to Ca^{2+} in the presence of caffeine (this study), allowing the comparison of force transients. Moreover, increasing caffeine concentration or reapplying caffeine quickly after the first application did not modify atrioventricular differences, suggesting that these differences did not arise from incomplete Ca^{2+} release or differences in caffeine sensitivity. The appearance of a second slow phase of tension could be considered as a result of prolonged Ca^{2+} release, suggesting different mechanisms of Ca^{2+} release in atria and ventricles. On the other hand, the similar tension-time integrals suggested that the amount of releasable Ca^{2+} appeared to be the same in the two tissues, whatever the speed of Ca^{2+} release or the duration of SR loading. These results, together with the similar amount of mRNA for Cals, the main Ca^{2+}-buffering protein in SR, suggest that the capacity of the SR Ca^{2+} pool is similar in atrial and ventricular myocardium.

Slow SR Ca^{2+} release in atria appeared not to be due to a lower amount of SR Ca^{2+} channel, because the same degree of expression of RyR mRNA suggested a similar amount of Ca^{2+} release channels in both tissues. An alternative explanation may be morphological differences between atria and ventricles. In human atrial cells, it was shown that the intracellular Ca^{2+} transient triggered by membrane depolarization is not entirely controlled by the Ca^{2+} current and results from the activation of two components of Ca^{2+} signals (14). Berlin (8), using confocal microscopy, showed that, in guinea pig atrial myocytes devoid of t tubules, stimulated increases in internal Ca^{2+} can be observed to arise

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in focal regions of the cell before spreading to the cell interior. In intact rabbit ventricular cardiomyocytes, Bassani et al. (7) showed that one-half of the Ca^{2+} in the SR is released during a twitch, whereas the beat-dependent depletion of SR Ca^{2+} is biexponential. They suggest that the slower phase might represent a caffeine-sensitive pool of Ca^{2+} not normally released during a twitch and speculated that the Ca^{2+} in the corbular SR could represent such a pool. In comparison with ventricular cardiomyocytes, atrial cells are characterized by the absence of a t tubular system (23). They contain only peripheral junctional SR connected to sarcolemma and a higher proportion of corbular SR within the cytoplasm. Because corbular SR is not connected to sarcolemmal membrane, Ca^{2+} release from these stores must be triggered by a diffusible agent (15). The delayed phase of Ca^{2+} release in atria could thus represent Ca^{2-} released from these internal cisternae by a slower Ca^{2+}-induced Ca^{2+}-release mechanism.

CK and SR Ca^{2+} uptake in atria. The rapid work of the SR Ca^{2+} pump in atria critically depends on adequate energy supply and effective withdrawal of ATPase reaction products. The isolated SR membranes of several muscle types have been shown to contain strongly anchored MM-CK that is functionally coupled to the Ca^{2+} pump (32). Recently, we have confirmed the coupling between bound CK and SR Ca^{2+}-ATPase in situ in saponin-skinned ventricular fibers with preserved local architecture by showing that localized regeneration of ATP at the expense of PCr and ADP is more efficient to meet the requirement of Ca^{2+}-ATPase than external ATP (24). The role of CK in providing energy for the SR Ca^{2-} pump in situ could be demonstrated by efficient loading of the SR in the presence of PCr and ADP. These results showed that local ATP generated by the CK reaction was sufficient to completely meet the ATP requirements of the SR Ca^{2+}-ATPase in atria. In contrast, ATP alone was not able to sustain control loading of the SR. Our study clearly demonstrates that, in atria, the CK system bound to structures at sites of energy utilization is at least as efficient as in ventricles to provide energy for SR Ca^{2+} uptake and to maintain a favorable local ATP-to-ADP ratio in the vicinity of the ATPase.

In conclusion, fast and efficient CK-supported SR Ca^{2+} uptake together with limited or slow Ca^{2+} release and diffusion for contraction could be proposed as the main modulator of the shorter time course and lower tension development in rat intact atrial myocardium compared with that in ventricular myocardium, despite a similar total capacity of releasable Ca^{2+} in both tissues.

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Thyroid hormones differentially affect sarcoplasmic reticulum function in rat atria and ventricles

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Abstract

The present study was undertaken to compare the effects of hypothyroidism and hyperthyroidism on sarcoplasmic reticulum (SR) Ca^{2+}-pump activity, together with assessment of the functional role of SR in providing activator Ca^{2+} under these altered thyroid states. In response to a shift from hypothyroid to hyperthyroid state, a 10 fold and 2 fold increase in SR Ca^{2+}-pump activity in atria and ventricles, respectively, were observed. This was associated with the 8–9 fold increases in atrial contractility (+dT/dt) and relaxation (–dT/dt), but only with a 3–4 fold increase in their ventricular counterparts. Also, the recirculation fraction of activator Ca^{2+} (RFA) increased to a far greater extent in atria (4 fold) than in papillary muscles, and the relative increment in inhibition of developed tension by ryanodine became 3 times larger in atria than in papillary muscles. A positive force-frequency relationship (FFR) was observed in hypothyroid atria, whereas the hyperthyroid atria, hypothyroid and hyperthyroid papillary muscles showed a negative FFR. These results suggest the greater role of transsarcolemmal (SL) Ca^{2+} and smaller role of SR Ca^{2+} in activating contraction in hypothyroid atria compared to other preparations. Thyroid hormones decrease the contribution of SL and increase that of SR in providing activator Ca^{2+} to the greater extent in atria than in ventricles. This effect of thyroid hormones is based on larger stimulation of SR Ca^{2+}-pump in atria compared to ventricles. (Mol Cell Biochem **176**: 119–126, 1997)

Key words: atria, ventricles, thyroid hormones, sarcoplasmic reticulum, phospholamban, Ca^{2+}-pump, force-frequency relationships, ryanodine effects, recirculation fraction of activator Ca^{2+}

Introduction

The twitch force of cardiac muscle is approximately proportional to the amount of Ca^{2+} released into sarcoplasm to activate the myofilaments [1]. In most mammalian species, Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) is a predominant mechanism for providing the activator Ca^{2+}, whereas only a small part of Ca^{2+} enters the cell via transsarcolemmal (SL) routes, through voltage dependent Ca^{2+} channels and Na^{+}-Ca^{2+} exchange [2].

Contribution of SR and SL sources of activator Ca^{2+} is known to change under several physiological (age) and pathological (pressure overload hypertrophy) factors [3–5]. With this respect, the thyroid hormones that markedly stimulate the expression of SR Ca^{2+}-pump protein and decrease the expression of its inhibitor, phospholamban (PLB) [6–8], appear to be of particular importance. In fact, a direct relationship between thyroid hormone-induced changes in expression of SR proteins and contractile parameters have been recently shown in ventricular myocardium of neonatal rats [9]. Increased expression of SR Ca^{2+}-ATPase and decreased expression of Na^{+}-Ca^{2+} exchanger [10] under thyroid hormones were combined with stronger inhibitory effects of ryanodine, and weaker effects of verapamil both on contractility and relaxation [9]. It was asserted that thyroid hormones accelerate the developmental shift from predominantly SL to predominantly SR mechanism of providing activator Ca^{2+} [9].

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Unlike ventricular myocardium, the effects of thyroid hormones on SR function have not been studied in atria yet. However, both in guinea-pig and mouse heart, the SR is more proliferated in atria than in ventricles [11, 12]. Therefore, SR is considered to play a greater role in atria than in ventricles in providing activator Ca^{2+} [2, 11, 12]. Moreover, the murine atria are characterized by higher levels of SR Ca^{2+}-ATPase, but lower levels of PLB than ventricles [13]. These data suggest that thyroid hormones may differentially regulate the expression of SR proteins in atria and ventricles. In contrast to ventricular myocardium, thyroid hormones do not affect myosin heavy chain expression in atria [14–17]. Therefore, atria appear to be the favourable objects for studying the relationships between the thyroid hormone-dependent changes in SR Ca^{2+}-pump and contractile function, without involvement of alterations in myosin ATPase activity. The present study was undertaken to compare the effects of hypothyroidism and hyperthyroidism on SR Ca^{2+}-pump activity, together with an assessment of the functional role of SR in providing activator Ca^{2+} under these altered thyroid states. The results show that thyroid hormones increase the contribution of SR Ca^{2+} in activation of myofilaments by increasing the SR Ca^{2+}-pump activity to a much larger extent in atria than in ventricles.

Materials and methods

Animals

White adult Wistar rats of either sex, weighing 200–400 g, were made hypothyroid by adding 0.05% 6-n-propyl-2-thiouracil in their drinking water for 6 weeks. Hyperthyroidism was induced by daily subcutaneous injections of L-thyroxine to euthyroid animals at a dose of 1 μg/g body weight for 1 week. In other experiments, L-triiodothyronine was injected to hypothyroid animals at a dose of 50 ng/g body weight for 5 days. Both of these thyroid hormone treatment schedules showed similar effects on parameters such as increase in heart weight and contractile function, demonstrating the development of hyperthyroidism [18]. Therefore, the results obtained with these two thyroid hormone treatment schedules were combined and presented as a hyperthyroid group.

Preparation of homogenates and determination of SR Ca^{2+} uptake

The rats were anaesthetized with sodium thiopental, and the hearts were excised and rinsed rapidly in ice-cold isotonic saline solution. The atria and apex of ventricles were isolated and weighed. All subsequent procedures were carried out at 4°C. Preparations were homogenized with Ultra-Thurrax (Janke and Kunkel, Germany) (3 × 30 sec, 24,000 rpm) in 19 vol ice-cold buffer containing (in mM): sucrose 250, Tris 20 (pH 6.8), leupeptin 0.01, PMSF 0.1, DTT 1. The homogenates were further treated with glass-glass homogenizer (10 strokes). The samples of atrial homogenate (30 μl) containing SR vesicles were incubated for 5 min at 30°C with constant shaking in the medium (in mM): ATP 2, $MgCl_2$ 2, KCl 100, NaN_3 2, imidazole 30 (pH 7.0), K_2-oxalate 2, and protein kinase A (PKA) inhibitor H-89 [19] (100 μM). After incubation period, the Ca^{2+} uptake was started by addition of ^{45}Ca-labeled $CaCl_2$-EGTA buffer into the media containing 0.58 mM EGTA and 117 μM of $CaCl_2$ to give the free Ca^{2+} concentrations 0.1 μM. The Ca^{2+} uptake was stopped after 3 min by filtering the samples through Whatman glass microfibre filters GF/A using a vacuum pump. Radioactivity associated with the membranes was counted in Optiphase 'HiSafe' 3 (Wallac, Finland).

Muscle preparation and contractile studies

The rats were anaesthetized with sodium thiopental (50 μg/kg body weight), the hearts were removed, rinsed and the left atria or papillary muscles were dissected in the medium that consisted of (in mM): NaCl 120, KCl 5.4, $CaCl_2$ 0.6, NaH_2PO_4 0.42, $MgCl_2$ 1.05, D-glucose 11, Na_2EDTA 0.05, 2,3-butanedione-monoxime 30, HEPES 5.0 (pH 7.4, 22°C) gassed with 100% O_2. The muscle preparations were inserted into a 0.2 ml perifusion chamber between a force transducer and a length adjustment device. The perifusion medium that contained (in mM) NaCl 120, KCl 5.4, $CaCl_2$ 1.0, NaH_2PO_4 0.42, $MgCl_2$ 1.05, D-glucose 11, Na_2EDTA 0.05, HEPES 5.0 (pH 7.4, at 30°C) was gassed with 100% O_2, and continuously recirculated at a rate of 3–5 ml/min. The muscle preparations were electrically field-stimulated by platinum electrodes (rectangular pulses of 5 msec duration at a voltage of 1.5 times of threshold). The output signals were amplified, recorded and analysed with an on-line computer using software ATRIUM designed in our laboratory to obtain the parameters of isometric contraction. During the adaptation period, the muscle preparations, stimulated at the rate of 1.0 Hz, were perifused without recirculation and stretched stepwise to the length at which the maximal isometric tension was achieved. After 45 min, the perifusion was switched to a recirculating system and the muscles were allowed to adapt for additional 15 min before the control values of the contractile parameters were registered.

Force-frequency relationships (FFR)

To estimate the FFR, the muscles were allowed to contract at 0.2 Hz during the last 15 min of the adaptation. Thereafter, the stimulation frequency was increased from the frequency

0.2 Hz to the test frequencies 0.5, 1.0 or 2.0 Hz for 1 min. At the end of the period, the contractile parameters were recorded. Testing at each of the given frequencies was followed by a 2 min period stimulation at 0.2 Hz. In these experiments, the $[Ca^{2+}]_o$ was 2.5 mM.

Recirculation fraction of activator Ca^{2+}

Recirculation fraction (x) of activator Ca^{2+} (RFA) was calculated on the basis of potentiation of the first postextrasystolic contraction and its decay as described earlier [20–23]. In our experiments, 10 stimuli at the frequency of 10 Hz rather than one regular stimulus were inserted into the basic (0.2 Hz) stimulation protocol. For each muscle, several cycles of potentiation were implemented at 2.5 mM $[Ca^{2+}]_o$, and a reliable value of x was obtained by averaging the data.

Statistics

Results are indicated as mean ± SEM. Statistical significance of differences between the averages was estimated by a two-tailed unpaired Student's *t*-test.

Reagents

6-n-propyl-2-thiouracil and L-thyroxine was purchased from Sigma Chemical Co (St. Louis, MO, USA), L-triiodothyronine from Fluka AS (Germany), ryanodine from Calbiochem (La Jolla, CA), ^{45}Ca from Amersham, and H-89 from BIOMOL (PA, USA).

Results

Effect of thyroid state on SR Ca^{2+}-pump activity

The effect of thyroid state on SR Ca^{2+}-pump function was assessed by registering the oxalate-supported Ca^{2+}-uptake *in vitro* in homogenates with different thyroid states, in the presence of the inhibitor of PKA (H-89, 100 μM) that blocks the endogenous PKA [19]. In our experimental conditions (see Materials and methods), the Ca^{2+}-uptake is restricted to SR, and therefore represents the intrinsic Ca^{2+}-uptake by SR vesicles in the homogenate [24]. Figure 1 shows that the Ca^{2+}-uptake rates were higher in hyperthyroid and lower in hypothyroid preparations compared to euthyroid ones. It is evident that in comparison with hypothyroid state, hyperthyroidism caused a 10 fold increase in SR Ca^{2+}-pump activity in atria, but only a 2 fold increase in ventricles. Further we attempted to determine if the differences between the atrial and ventricular SR

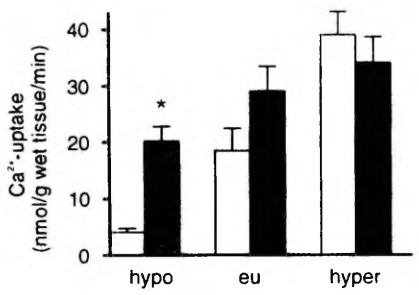

Fig. 1. Effect of thyroid state on oxalate supported Ca^{2+} uptake rates in atrial (open columns) and ventricular (dashed columns) homogenates estimated in the presence of H-89 at pCa 7.0. Hypo – hypothyroid; eu – euthyroid; hyper – hyperthyroid. The means ± SEM are given for 5 experiments. *Difference between atria and ventricles at $p < 0.01$.

Ca^{2+}-pump function observed *in vitro* could also be reflected in contractile function *in vivo*, at the intact muscle level. For this purpose, the effects of thyroid state on contractile parameters, FFR, RFA, and myocardial sensitivity to ryanodine were compared in atria and papillary muscles.

Effect of thyroid state on contractile parameters

Original mechanograms (Fig. 2) show that in comparison with euthyroid state, hypothyroidism was associated with decreases in developed tension (DT), contractility (+dT/dt) and relaxation (–dT/dt), but with increased time to peak tension (TPT) and relaxation time (RT) both in atria and papillary muscles. In contrast, hyperthyroidism led to increases in DT, +dT/dt and –dT/dt together with shortened TPT and RT. This agrees well with previous reports on thyroid hormone effects [25–29]. A novel finding, however, is that a shift from hypothyroid to hyperthyroid state was associated with the 8–9 fold increases in atrial +dT/dt and –dT/dt, together with a 3–4 fold increase in their ventricular counterparts (Fig. 3). Thus, the effects of thyroid hormones on the rates of contraction and relaxation, were about 2–3 fold higher in atria than in papillary muscles.

Effect of thyroid state on force-frequency relationship (FFR)

Normally, increasing stimulation frequency causes negative inotropy in rat myocardium [30–32] due to increased refractory state of SR Ca^{2+}-release channels under higher stimulation rates [2]. However, Fig. 4 shows that FFR in hypothyroid atria entirely differs from that in normal rat myocardium. In response to increasing the stimulation frequency stepwise from 0.2 Hz (control level) to 2.0 Hz, the DT of hypothyroid atria rose more

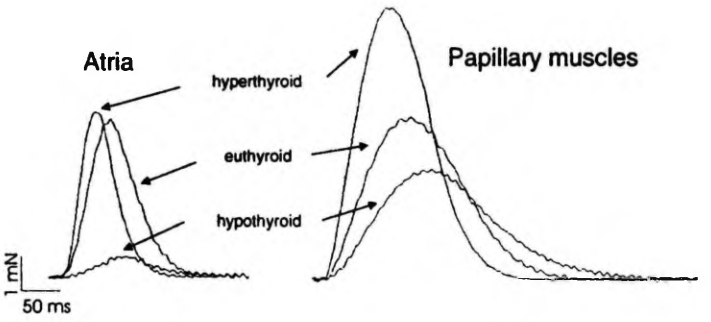

Fig. 2. Effect of thyroid state on isometric twitches of left atria and papillary muscles.

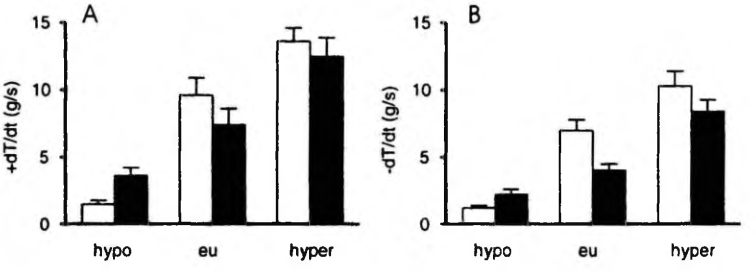

Fig. 3. Effect of thyroid state on contractility (A) and relaxation (B) in atria (open columns) and papillary muscles (dashed columns). Hypo – hypothyroid; eu – euthyroid; hyper – hyperthyroid. The means ± SEM for 5–9 experiments are given.

than 50% above the control level. Thus, hypothyroid atria showed a positive FFR, that is characteristic to species like guinea-pig and rabbit, where a significant amount of activator Ca^{2+} enters the myocytes via transsarcolemmal routes [2, 30, 32]. Thyroid hormones converted the FFR from positive into negative. In contrast to hypothyroid atria, hypothyroid papillary muscles showed a negative FFR (Fig. 4, right panel). In the hyperthyroid papillary muscles, after enhancing the stimulation frequency from 0.2 Hz to 0.5 Hz, the DT tended to increase, but maintained its level at 1.0 Hz so that the DT exceeded the value of that parameter in the hypothyroid muscles. Further increases in stimulation frequency, however, led to a negative inotropic response which was equivalent to that in hypothyroid papillary muscles. These results suggest that the role of SR was less important, and the sarcolemma more important in providing activator Ca^{2+} in hypothyroid atria than in other muscle preparations.

Effect of thyroid state on RFA and myocardial sensitivity to ryanodine

The RFA is an index of the fractional amount of Ca^{2+} which is sequestered during diastole and released for the subsequent systole by SR [20–23]. Figure 5 shows that the RFA was significantly lower in atria than in papillary muscles in hypothyroid conditions, whereas hyperthyroidism increased this parameter up to the equal values in these muscle types. In response to a shift from hypothyroid to hyperthyroid state, the RFA increased to a far greater extent in atria (4 fold) than in papillary muscles. This could be interpreted [20–23] to suggest that the role of SR in providing activator Ca^{2+} rose more in atria than in papillary muscles under thyroid hormones.

The degree of cardiac contractility inhibition by ryanodine, a SR Ca^{2+} release channel inhibitor, positively correlates with the relative contribution of SR Ca^{2+} for activating the myofilaments [2]. We found that, under hypothyroidism, the atria were less sensitive than papillary muscles to ryanodine, as the percentage of inhibition of DT by ryanodine was 70 ± 7 and 86 ± 2 ($p < 0.05$), respectively. Under hyperthyroidism, ryanodine almost entirely inhibited the DT both in atria (97 ± 2%) and papillary muscles (95 ± 2%). Thus, the relative increment in inhibition of DT by ryanodine became 3 times larger in atria than in papillary muscles. The results are compatible with the data on RFA, both indicating that thyroid hormones increased the contribution of SR in supplying activator Ca^{2+} more in atria than in papillary muscles.

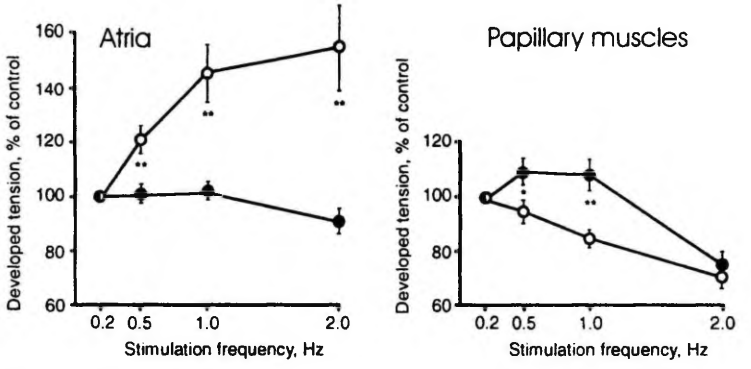

Fig. 4. Effect of thyroid state on force-frequency relationships in atria and papillary muscles, at 2.5 mM $[Ca^{2+}]_o$. The means ± SEM are given for n = 7 and n = 8 in hypothyroid (○) and hyperthyroid (●) groups, respectively. *Difference between hypothyroid and hyperthyroid groups at $p < 0.05$. **Difference between hypothyroid and hyperthyroid groups at $p < 0.01$.

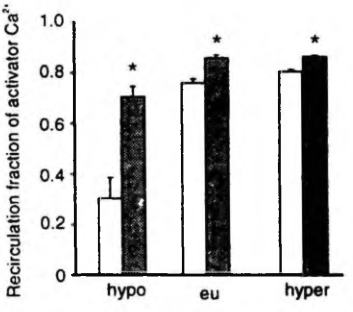

Fig. 5. Effect of thyroid state on recirculation fraction of activator Ca^{2+} in atria (open columns) and papillary muscles (dashed columns) at 2.5 mM $[Ca^{2+}]_o$. The means ± SEM, are given for n = 4–5 in each group. *Difference from hypothyroid group at $p < 0.01$.

Discussion

This study is first to demonstrate that thyroid hormones increased the activity of SR Ca^{2+}-pump in atria, and that such a stimulation was higher in atrial than in ventricular homogenates (Fig. 1). The thyroid hormone receptor levels in atria are known to be half the amount of that in ventricular myocardium [33]. Therefore, the increased effect of thyroid hormone on SR Ca^{2+}-pump in atria cannot be related to changes in hormone receptor parameters. It has been shown that thyroid hormones increase the tissue levels of SR Ca^{2+}-pump mRNA both in atria and ventricles [34]. Enhanced SR Ca^{2+}-pump mRNA is associated with increases in SR Ca^{2+}-pump protein and SR Ca^{2+}-pump activity [8, 34]. Kiss *et al.* 1994 [8] have shown that the shift from hypothyroid to hyperthyroid state was associated with a 2.4 fold increase in SR Ca^{2+}-pump amount, and with a 1.8 fold decrease in PLB amount in ventricular tissue. Recently, we also have found a 2.4 fold increase in expression of SR Ca^{2+}-pump in atria (unpublished results). However, the PLB content decreased much more (6.7 fold) in atria than for ventricles [8]. Since unphosphorylated PLB acts as an inhibitor of SR Ca^{2+}-pump [35, 36], the larger suppression of PLB expression should result in a bigger increase in SR Ca^{2+}-ATPase activity in atria. Indeed, this is supported by the present data (Fig. 1). Thus, it is likely that observed differences between SR Ca^{2+}-pump activity are due to different regulation of PLB in atria and ventricles, i.e. the expression of PLB is downregulated to the higher extent in atria than in ventricles by thyroid hormones.

Although thyroid hormones cause a number of biochemical changes in Ca^{2+}-handling mechanisms, their relationship with contractile function has remained rather unclear. Thyroid hormones are known to abbreviate the intracellular Ca^{2+} transient [37–39], that has been attributed to faster removal of cytoplasmic Ca^{2+} by SR Ca^{2+}-pump. Therefore, increased SR Ca^{2+}-pump activity has frequently been considered to be a reason for enhanced rate of relaxation in ventricular myocardium under thyroid hormones [6, 8, 37–40]. This opinion is confirmed by the present results, since greater changes in the SR Ca^{2+}-pump rates were associated with larger alterations in relaxation rates of atria than in papillary muscles, in response to shift from hypothyroidism to hyperthyroidism (Fig. 3). The interesting finding of our study, however, was that in both atria and ventricles, strongly positive correlations were observed between the changes in +dT/dt and –dT/dt (Fig. 6). This suggests that the relaxation and contractility are equally affected by changes in SR function under thyroid hormones. That SR exerts control not only over the

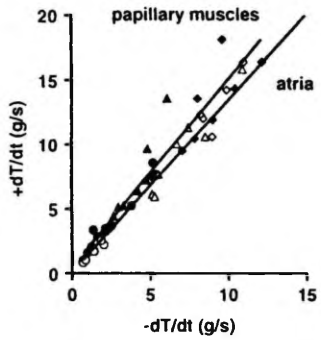

Fig. 6. Linear regressions between the relaxation (–dT/dt) and contractility (+dT/dt) in atria (open symbols) and papillary muscles (solid symbols) from hypothyroid (○), euthyroid (△) and hyperthyroid (✧)rats. Linear regression analysis revealed the values of $r \geq 0.95$ for both dependencies.

relaxation, but also the contractility, is shown in the experiments using transgenic mice by Luo *et al.* [36]. Increased Ca^{2+} uptake due to the ablation of SR Ca^{2+}-ATPase inhibitor phospholamban gene, was associated with increased contractility. They suggested that increased Ca^{2+} uptake rate may lead to higher amounts of Ca^{2+} sequestered by the SR, which would be available for release, resulting in a higher rate of contraction. For two reasons, the similar mechanism seems to be the only explanation for increased +dT/dt under thyroid hormone in atria (Fig. 6). (i) Like in PLB-deficient mice [36], the expression of PLB is downregulated by thyroid hormones (as discussed above). (ii) Since the expression of α-myosin gene is insensitive to thyroid hormones [14–17], the thyroid hormone-dependent changes in +dT/dt cannot be attributed to changes in myosin isoenzyme profile in atrial myocardium. However, thyroid hormones cause a significantly augmented SR Ca^{2+}-pump also in ventricles [6, 7, 9, 28, 40]. Thus, our results suggest the intriguing possibility that in both atria and ventricles, the thyroid hormone-induced increases in contractility are mediated by enhanced SR Ca^{2+} uptake.

Two indices, the contractile sensitivity to ryanodine and RFA were assessed to compare the thyroid hormone-dependent changes in contribution of SR in providing the activator Ca^{2+} in atria and ventricular myocardium. The extent of suppression of DT by ryanodine is proportional to the cellular content of SR membranes and to the importance of Ca^{2+}-induced Ca^{2+} release for activation of myofilaments [2]. The assumption that RFA also serves as a physiological index for SR function, is confirmed by numerous facts. Developmental proliferation of SR membranes is associated with increased RFA in rat ventricles [5], In guinea-pigs the atria, containing more SR than ventricles do, also exhibit higher RFA values [41]. Impairment of SR function by ryanodine is accompanied by decreased RFA [41]. Increase in $[Ca^{2+}]_i$ that accelerates the replenishment of SR Ca^{2+} stores is reflected by enhanced RFA [21]. Compared to ventricles, the atria showed a bigger increase in RFA (Fig. 5) and greater sensitivity to ryanodine, in response to thyroid hormone. Principally, two mechanisms may be responsible for such a difference. First, as discussed above, this could be attributed to increased susceptibility of atrial SR Ca^{2+}-pump to thyroid hormones (Fig. 1). Another mechanism may localize at the level of sarcolemma for thyroid hormones stimulate I_t, that shortens the duration of action potential in ventricles, but not in atria [42, 43]. Therefore, under thyroid hormones, Ca^{2+} influx through slow Ca^{2+} channels may decrease due to a shortened action potential duration in ventricles. Hence, the atrial SR could be replenished at the expense of the transsarcolemmal Ca^{2+} influx more effectively than its ventricular counterpart. As a result, the atrial SR would exert larger control over activator Ca^{2+} that could explain the increased RFA and ryanodine effects compared to ventricles. However, considering the drastic augmentation of SR Ca^{2+}-pump activity *in vitro* where the SL Ca^{2+} fluxes are unable to control the SR Ca^{2+} uptake (Fig. 1), the increased SR Ca^{2+}-pump activity appears to be the main reason for augmented contribution of SR Ca^{2+} for activating the myofilaments.

Also a new finding in this study is that hypothyroid rat atria, showing decreased SR Ca^{2+}-pump activity, exhibited a positive FFR (Fig. 4). Thus, hypothyroid atria behaved in a similar manner to normal myocardium of rabbit, guinea-pig, and human that are characterized by a less important role for the SR and a more important role for the transsarcolemmal Ca^{2+} fluxes for activation of the contractile elements than is observed in rats. In contrast, thyroid hormone treatment which stimulated the development of SR, also converted the positive FFR into negative one in the atria (Fig. 4). Thus, our results support the view that the activity of SR is an important factor for determining the character of FFR in the myocardium: low activity of SR is associated with a positive FFR, but high activity with a negative FFR [2, 32]. The mechanism by which the SR controls FFR may be based on time-dependent inactivation of the SR Ca^{2+} release mechanism [1, 2] that is more expressed in myocardium with the high SR activity, and less in the myocardium with a low activity.

In conclusion, the major implications of this study are that thyroid hormones increase the relative importance of SR in providing activator Ca^{2+} to a larger extent in atria than in ventricles. This effect of thyroid hormones is based on larger stimulation of SR Ca^{2+}-pump in atria compared to ventricles.

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III

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Ca^{2+} uptake by cardiac sarcoplasmic reticulum ATPase in situ strongly depends on bound creatine kinase

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Abstract The role of creatine kinase (CK) bound to sarcoplasmic reticulum (SR), in the energy supply of SR ATPase in situ, was studied in saponin-permeabilised rat ventricular fibres by loading SR at pCa 6.5 for different times and under different energy supply conditions. Release of Ca^{2+} was induced by 5 mM caffeine and the peak of relative tension (T/T_{max}) and the area under isometric tension curves, S_T, were measured. Taking advantage of close localisation of myofibrils and SR, free $[Ca^{2+}]$ in the fibres during the release was estimated using steady state $[Ca^{2+}]$/tension relationship. Peak $[Ca^{2+}]$ and integral of free Ca^{2+} transients ($S[Ca^{2+}]_i$) were then calculated. At all times, loading with 0.25 mM adenosine diphosphate, Mg^{2+} salt (MgADP) and 12 mM phosphocreatine (PCr) [when adenosine triphosphate (ATP) was generated via bound CK] was as efficient as loading with both 3.16 mM MgATP and 12 mM PCr (control conditions). However, when loading was supported by MgATP alone (3.16 mM), T/T_{max} was only 40% and $S[Ca^{2+}]_i$ 31% of control ($P < 0.001$). Under these conditions, addition of a soluble ATP-regenerating system (pyruvate kinase and phosphoenolpyruvate), did not increase loading substantially. Both S_T and $S[Ca^{2+}]_i$ were more sensitive to the loading conditions than T/T_{max} and peak $[Ca^{2+}]$. The data suggest that Ca^{2+} uptake by the SR in situ depends on local ATP/ADP ratio which is effectively controlled by bound CK.

Key words Skinned myocardial fibres · Calcium uptake · Compartmentation · Heart · Sarcoplasmic reticulum · Ca^{2+}-transporting ATPase · Creatine kinase · Caffeine · pCa/tension relationship

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Introduction

Muscle cells of adult mammals exhibit a highly organised architecture. Such an organisation has been shown to increase the potential for fine tuning and the efficiency of cell work [8]. In heart muscle, contractile activity is graded by the amount of Ca^{2+} liberated by the sarcoplasmic reticulum (SR), which is located around the myofibrils, being in close contact with the T-tubular system and controlling the local $[Ca^{2+}]$. The activation of myofibrils is achieved by the Ca^{2+}-induced opening of the Ca^{2+} channels of the surrounding SR [6,7]. In turn, relaxation occurs mainly due to the uptake of Ca^{2+} by the Ca^{2+}-stimulated adenosine triphosphatase (Ca-ATPase)-driven pump of the SR. Ca^{2+} uptake by the SR is regulated by phospholamban; calmodulin- or protein kinase A-dependent phosphorylation of phospholamban has been shown to relieve the inhibitory effect of phospholamban on the Ca^{2+}-ATPase activity.

The Ca pump efficiency strongly depends on an adequate energy supply and the effective withdrawal of ATPase reaction products. Numerous studies have shown the important role of creatine kinase (CK) in regulation of the intracellular concentrations of adenosine triphosphate (ATP) and diphosphate (ADP) in cardiomyocytes (for review see [21]). Isoenzymes of CK are bound to the sites of energy production and energy utilisation. CK catalyses the reversible transfer of a phosphate moiety between phosphocreatine (PCr) and MgADP according to the reaction:

$$MgADP + PCr + H^+ \Leftrightarrow MgATP + Cr$$

In muscle cells, a fraction of cytosolic MM-CK (a dimer of the muscle-specific CK subunit) is specifically bound

to the intracellular structures at sites of energy consumption: myofilaments (for review see [20]), sarcolemma ([15], for review see [21]), as well as SR [1, 13, 14]. The mitochondrial isoenzyme of CK is located at the external face of the inner mitochondrial membrane and is functionally coupled to the ATP/ADP translocator (for review see [16, 21]). In the myofibrillar compartment, the binding of MM-CK and its functional interaction with myosin ATPase provides a high local ATP/ADP ratio and low proton concentration favourable for myofibrillar function (for review see [19, 20]).

The presence of CK in membrane vesicles of SR isolated from skeletal muscle [1] and heart muscle has been well documented [13]. Rossi et al. [14] have shown that MM-CK is indeed strongly bound to highly purified SR vesicles of skeletal muscle. Although the exact location of CK is not known, there is evidence for the functional interaction between CK and ATPase. In isolated SR vesicles, PCr could support ATP-driven Ca^{2+} uptake [1, 13]. In vitro, SR-bound CK activity was sufficient to support a significant portion of a maximal Ca^{2+} uptake rate in the presence of PCr and ADP [14]. Recently, it has been shown that PCr can abolish the inhibitory effect that inorganic phosphate exerts on Ca^{2+} accumulation within the SR [17]. In experiments on SR vesicles isolated from skeletal muscle it was demonstrated that ATP regenerated by CK is not in free equilibrium with the ATP in the surrounding medium, but is used preferentially by Ca^{2+}-ATPase for Ca^{2+} uptake [11]. However, in isolated vesicles, CK-driven Ca^{2+} uptake was always less than that driven by ATP. Few data are available concerning the functional role of SR-bound CK in situ. Yet, cellular functioning is highly dependent on the interaction between intracellular compartments. The skinned-fibre technique preserves local architecture and natural interactions between such compartments and thus allows the study of SR function in situ [4].

The aim of this work was to study the energy dependence and role of bound CK in calcium loading in situ. In this study we compare, under constant Ca^{2+}-releasing conditions, the amount of Ca^{2+} liberated in the myofibrillar space, after loading the SR in the presence of different concentrations of adenine nucleotides and PCr. We show that the ATP produced in the CK reaction is used for Ca^{2+} uptake in preference to exogenous ATP or to ATP generated by a soluble ATP-regenerating system. This suggests a functional interaction between the Ca^{2+} uptake by SR and bound CK.

Materials and methods

Isolation and preparation of skinned muscle fibres

Adult male Wistar rats weighing 250–400 g were anaesthetised by injection of urethane (2 g/kg). The hearts were removed immediately and rinsed in ice-cold free Ca^{2+} Krebs solution containing (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, equilibrated with 95% O_2–5% CO_2. Cardiac fibres (diameter 140–300 µm) were dissected from the left ventricular papillary muscles in the same solution. Specific permeabilisation of the sarcolemma was obtained by incubating the fibres for 30 min in solution A (see Table 1) containing 50 µg/ml saponin in the presence of 20 µM leupeptin at temperature +4 °C.

Table 1 Composition of solutions (mM except where stated). In addition, all solutions contained (in mM): free Mg^{2+} 0.8; Na^+ 30.6; *N,N*-bis(2-Hydroxyethyl)-2-aminoethanesulphonic acid 60 (pH 7.1); dithiothreitol 0.3; sodium azide 2 and leupeptin 0.02. The ionic strength was adjusted to 160 mM with potassium methanesulphonate (EGTA ethyleneglycolbis(β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid, MgATP adenosine triphosphate, Mg salt, PCr phosphocreatine, MgADP adenosine diphosphate, Mg salt)

Type of solution		Constituents					
		pCa	EGTA	MgATP	PCr	MgADP	Caffeine
relaxing	A	9.0	10	3.16	12		
	A_1	9.0	10	3.16	12		5
activating	B	4.5	10	3.16	12		
loading	C	6.5	10	3.16	12		
	D	6.5	10		12	0.25	
	E	6.5	10	3.16			
washing	G	9.0	10				
	H	9.0	0.05				
	R	9.0	0.05	3.16	12		
release	R_1	9.0	0.05	3.16	12		5

Experimental apparatus

The fibre was mounted on stainless-steel hooks between a length adjustment device and a force transducer (AE 801, Aker's Microelectronics, Horton, Norway). The transducer elements formed the two arms of a Wheatstone bridge and the output signal for recording was amplified. Fibres were immersed in 2.5-ml chambers arranged around a disc. The chambers were placed into a temperature-controlled bath, positioned on a magnetic stirrer. All experiments were performed at 22 °C.

Solutions

Solutions were calculated by use of the computer program of Fabiato [5] and are listed in Table 1. All solutions contained (in mM): free Mg^{2+} 0.8, Na^+ 30.6, *N,N*-bis(2-Hydroxyethyl)-2-aminoethanesulphonic acid 60 (pH 7.1), dithiothreitol 0.3. The ionic strength was adjusted to 160 mM with potassium methanesulphonate. All solutions contained 2 mM sodium azide to inhibit possible Ca^{2+} uptake by mitochondria and 20 µM leupeptin to inhibit proteases. To study the energy requirement for SR Ca^{2+} loading, solutions with modified concentrations of adenine nucleotides and PCr were made.

Chemicals

Caffeine was purchased from Merck-Clevenot. PCr (Neoton, Schiapparelli Searle, Turin, Italy) was a kind of gift of Prof. E. Strumia. Other chemicals were obtained from Sigma.

Estimation of Ca^{2+} loading by SR

The technique for assessing SR function using saponin-skinned fibres has been used widely for cardiac, smooth and skeletal muscle [4, 9, 18]. The modified protocol, designed to compare the Ca^{2+} load under different test conditions, is presented in the Results. At the beginning of each experiment, the fibre was stretched to 20% of the slack length in solution A, mean resting tension was 3.0 ± 0.3 mN/mm^2 ($n = 47$). After this, the fibre was exposed to activating solution (B) with pCa 4.5, to obtain the maximal tension (T_{max}), the mean value of which was 17.9 ± 1.1 mN/mm^2 ($n = 47$).

SR was loaded with Ca^{2+} for various periods of time and under different test conditions. To allow comparison between different loading conditions, Ca^{2+} release was induced by 5 mM caffeine in the presence of fixed concentrations of substrates and ions. The tension transient was used to assess the amount of Ca^{2+} taken up by the SR. Several parameters were used to estimate the SR Ca^{2+} capacity. First of all, the traditional parameters peak of tension and tension-time integral of the caffeine-induced contracture curves were used. The time course of free $[Ca^{2+}]$ close to myofibrils during the release was then calculated from the pCa/tension dependence. For this purpose the pCa/tension relationship in the presence of 5 mM caffeine under conditions identical to those of the release [except that 10 mM ethyleneglycolbis (β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) was present instead of 0.05 to buffer free Ca^{2+} adequately] was obtained at the end of each experiment. The fibres were exposed sequentially to a set of solutions in which the pCa decreased stepwise until maximal tension was reached (at pCa 4.5; Fig. 1). The tension developed at each pCa was normalised with respect to T_{max}. The data from each fibre were fitted to the Hill equation using linear regression analysis, and the pCa for half-maximal tension (pCa_{50}) and the Hill coefficient (n_H) determined. This served as internal calibration for $[Ca^{2+}]$ in the extrareticular space during Ca^{2+} release in the presence of caffeine. The $[Ca^{2+}]$ at each step of the tension-time integral was recalculated from the Hill equation to obtain $[Ca^{2+}]$-time integrals, which were taken to evaluate the SR Ca^{2+} loading capacity.

Statistical analysis

The data are expressed as means ± SEM or as representative tracings in a single experiment. Student's *t*-test for paired or unpaired data was used when appropriate. Differences at $P < 0.05$ were considered significant.

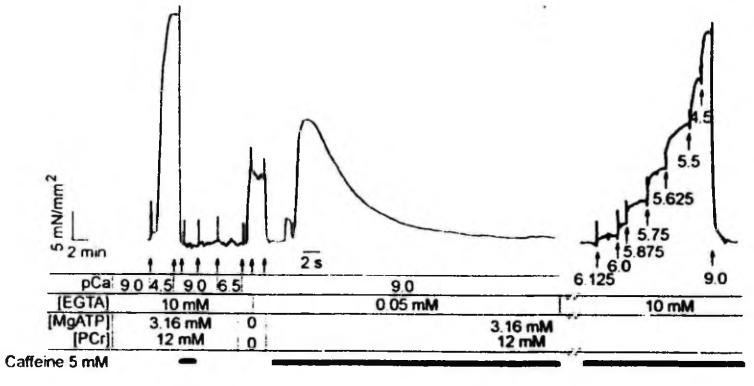
Fig. 1 Original tension recording of force developed by rat ventricular saponin-skinned fibre. The changes in pCa, total concentrations of ethyleneglycolbis(β-aminoethylether) *N,N,N',N'*-tetraacetic acid ([EGTA]), phosphocreatine ([PCr]) and adenosine triphosphate, Mg salt ([MgATP]) are indicated. *Artefacts* on the tracing (*upper panel*) and *arrows* in the *lower panel* indicate the change of experimental solution. First, maximal Ca-activated tension was obtained at pCa 4.5. After relaxing the fibre, SR was emptied of Ca^{2+} by application of 5 mM caffeine for 2 min. Next, caffeine was washed out and the SR was loaded at pCa 6.5. After loading, Ca^{2+} was washed out in solution without ATP and PCr. This was followed by the washout of excess EGTA. The withdrawal of ATP and PCr caused the rigor tension, which was relaxed by readmitting of the substrates. Ca^{2+} was released from SR by application of 5 mM caffeine. At the end of each experiment, the fibre was sequentially exposed in the presence of caffeine to a set of solutions with stepwise decreasing pCa to obtain pCa/tension dependence in order to estimate the amount of Ca^{2+} released to myofibrils by caffeine after loading of SR.

Results

Experimental protocol

The protocol used in this study was a heavily modified version of that described by Su [18]. To compare the release of Ca^{2+} from SR in situ under different loading conditions it was necessary: (1) to have a high [EGTA] solution for precise control of the pCa during loading, (2) to achieve efficient control loading conditions, but avoid saturation of tension at release, (3) to obtain a real zero load of the SR at zero loading time, (4) to use the lowest caffeine concentration in order to minimise the sensitising effect of caffeine on myofilaments and (5) to keep the concentrations of substrates and ions constant during release.

Figure 1 shows the experimental protocol. After determination of T_{max}, the fibre was relaxed in solution A (see Table 1). The SR was then emptied of Ca^{2+} by application of 5 mM caffeine (solution A_1) for 2 min. Next, caffeine was washed out in solution A for 2 min (pCa 9), under the same conditions of substrates as the loading, to equilibrate the concentrations of compounds inside the fibre before starting the load (not



shown). Loading of SR was carried out in strongly buffered (10 mM EGTA) test solutions at pCa 6.5 to maintain a constant [Ca^{2+}] near the Ca^{2+} pump (solutions C, D or E). This resulted in a peak of caffeine-induced contracture close to 70% of T_{max} under optimal energetic conditions. The tension transient due to the release of Ca^{2+} from SR into the myofibrillar space by the application of caffeine was elicited at a low [EGTA] (0.05 mM, solution R_1). After the loading was completed, therefore, excess EGTA was washed out before application of caffeine.

To avoid Ca^{2+} uptake by the SR after the loading period, the washout period was divided into three steps: (1) washout of Ca^{2+} in a high EGTA solution G (2 min), followed by (2) washout of EGTA in solution H (0.05 mM EGTA) for 1.5 min. To prevent SR loading, both solutions contained neither ATP nor PCr. Elimination of high energy phosphates was followed by rigor tension development. Finally, (3) substrates were readmitted for 30 s in solution R (there was no marked load during this short period) before inducing Ca^{2+} release by the application of 5 mM caffeine in the same solution (solution R_1). There was no Ca^{2+} added to any of these solutions. Indeed, with this complicated pre-release protocol, application of caffeine when the test solution contained no energetic compounds did not elicit any tension ("zero load", results not shown).

Evaluation of Ca^{2+} uptake by SR

The accumulation of Ca^{2+} by the SR during the loading period was estimated by calculating free [Ca^{2+}], seen by the myofilaments during caffeine application, by using the pCa/tension relationship in the presence of 5 mM caffeine as an internal calibration (see Materials and methods). The validation of this approach is shown in Fig. 2, in which the amounts of calcium released by the application of 5 or 15 mM caffeine following 3 min of loading at pCa 6.5 are compared. The maximal tension of the caffeine-induced contractures, normalised to T_{max} (pCa 4.5) was 0.724 and 0.776 respectively (panel A). Panel B shows the pCa/tension dependence for both caffeine concentrations. Each curve was drawn using the pCa/tension relationship obtained in the presence of 5 or 15 mM caffeine. The pCa_{50} for 5 and 15 mM caffeine was 5.75 and 5.91 respectively, indicating the leftwards shift of the curve. Caffeine thus dose-dependently increased the sensitivity of the myofilaments to Ca^{2+}. Taking into consideration the different Ca^{2+} sensitivity of the myofilaments, the free [Ca^{2+}]-time transients for either contracture were calculated (panel C). The [Ca^{2+}]-time integrals, obtained from these transients were 8.93 and 8.98 $\mu M \times s$ for 5 and 15 mM caffeine respectively. Thus, the amount of Ca^{2+} released from the SR into the myofibrillar space by application of 5 or 15 mM caffeine was the same and depended only upon the conditions of loading. It could be concluded that the difference in the tension transients was due to the sensitising effect of caffeine on myofilaments, but not to additional Ca^{2+} release by 15 mM caffeine. The mean values for pCa_{50} and n_H in the presence of 5 mM caffeine were 5.74 ± 0.02 and 2.68 ± 0.10 respectively (n = 35). That 5 mM caffeine was sufficient to release all Ca^{2+} from SR was further confirmed by application of 25 mM caffeine subsequent to the contracture induced by 5 mM caffeine. This additional application of 25 mM caffeine failed to induce any tension transient (results not shown).

Energy dependence of SR loading

To study the energy requirement of Ca^{2+} uptake by SR and the role of bound CK, we compared the load at pCa 6.5 under three different conditions (1) in the presence of 3.16 mM MgATP and 12 mM PCr for control loading (solution C in Table 1), (2) in the presence of 3.16 mM MgATP alone (solution E, ATP loading) and (3) in the presence of 0.25 mM MgADP and 12 mM PCr, when ATP generated in the CK reaction was the only source of energy for the Ca^{2+} pump (solution D, CK loading). The release conditions in all cases were as described above.

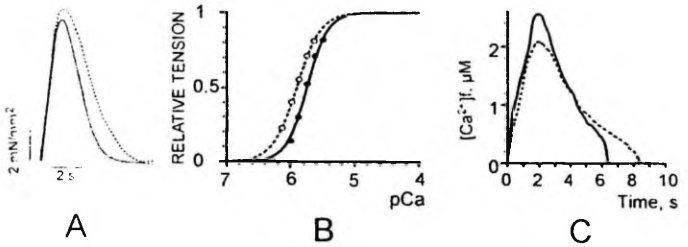


Fig. 2A–C Comparison of caffeine-induced calcium release by the application of 5 mM (*solid lines*) and 15 mM (*dotted lines*) caffeine in one saponin-skinned fibre. **A** Original superimposed tension recordings. **B** pCa/tension dependence for both concentrations of caffeine. Each *curve* was drawn using the pCa-tension relationship obtained in the presence of 5 or 15 mM caffeine. From the calcium sensitivity of the myofilaments at each caffeine concentration the free [Ca^{2+}] transients for both contractures are calculated **C**)

Table 2 The parameters of caffeine-induced Ca release, obtained after 5 min sarcoplasmic reticulum (SR) loading under different conditions. Data are means ± SE. T/T_{max} tension, normalised to maximal Ca-activated tension, S_f the area under the tension transient, $S[Ca^{2+}]_f$ the integral of the free $[Ca^{2+}]_f$ calculated from tension transient using myofilament Ca sensitivity, $[Ca^{2+}]_{f,peak}$ the peak free $[Ca^{2+}]_f$, RT_{50} half-relaxation time of tension transient

Condition of SR loading	n	T/T_{max}	S_f, arbitrary units	$S[Ca^{2+}]_f$, μM × s	$[Ca^{2+}]_{f,peak}$, μM	RT_{50}, s
ATP + PCr	14	0.716 ± 0.033	3.78 ± 0.33	18.44 ± 1.85	2.51 ± 0.23	2.81 ± 0.13
ADP + PCr	6	0.674 ± 0.031	3.01 ± 0.28	15.70 ± 2.06	2.71 ± 0.23	2.22 ± 0.15*
ATP	6	0.385 ± 0.071***, ++	0.88 ± 0.21***, +++	5.75 ± 0.82***, +++	1.40 ± 0.24***, ++	1.08 ± 0.13***, +++

*, *** $P < 0.05$, < 0.001 respectively for comparison with control (ATP + PCr)
++, +++ $P < 0.01$, < 0.001 respectively for comparison with ADP + PCr

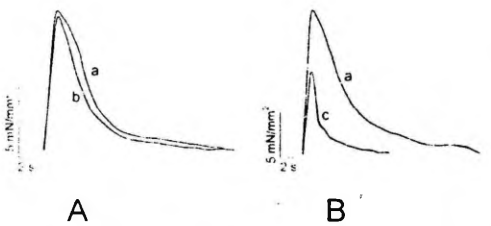


Fig. 3A,B Superimposed tension transients obtained after 5 min SR loading in different conditions. **A** *a* control (MgATP+PCr), *b* in the presence of MgADP and PCr. **B** *a* control (MgATP+PCr), *c* in the presence of MgATP alone

Figure 3A shows superimposed tracings of caffeine-elicited tension transients obtained after a 5-min period of control loading or 5 min CK loading. There were no significant differences in peak and area of tension transients between the two. In Panel B, the tracings of the caffeine-elicited tension transient following 5 min control loading and 5 min ATP loading are superimposed. After loading in the presence of exogenous MgATP alone, the peak of the subsequent caffeine-induced tension was markedly smaller than in control. As the relaxation of the tension transient was notably more rapid than in control, there was a significant difference in the area under the tension transient.

The parameters of caffeine-induced contraction, obtained for 5 min of SR Ca^{2+} loading in control and in the two test conditions are compared in Table 2. While Ca uptake by SR was supported by the CK-loading solution, most of the parameters describing the subsequent caffeine-induced Ca release did not differ significantly from the control, except half-relaxation time, which was decreased by 0.6 s ($P < 0.05$). On the other hand, when the Ca^{2+} uptake was supported by external ATP alone, all measured parameters appeared significantly lower than with control or CK loading. The difference in the tension-time integral was the most striking, being more than 4 times lower than in the control, and 3.4 times lower than with CK loading. The $[Ca^{2+}]$-time integrals obtained for evaluation of the amount of Ca^{2+} released confirmed this difference between conditions: the integral was highest in control, somewhat less with CK loading, but significantly (3.2 times) less with ATP loading. With ATP loading, the peak of caffeine-elicited contracture, normalised to maximal Ca-activated tension (T/T_{max}), was nearly 2 times less than in the control. The free $[Ca^{2+}]$ at the peak confirmed the same difference between the groups. From these data it is concluded that SR Ca^{2+} load is more readily supported by internal ATP generated in the CK reaction than by externally added ATP.

The peak and area of the caffeine-elicited contracture, as well as the amount of free Ca^{2+} calculated from myofilament Ca^{2+} sensitivity, were dependent on the time of loading. The mean values expressed as a function of loading time are plotted in Fig. 4. Time-dependent curves of relative peak tension for control and CK loading had very similar shapes and could be fitted to a first-order rate equation. They rose within 1 min to their maximal values, 68% and 65% of T_{max} respectively. The curve of ATP loading exhibited a different behaviour. After reaching its maximal value (39% of T_{max}) after 2 min loading, tension tended to decrease if the loading time was prolonged further.

The free $[Ca^{2+}]$ values at the peak of caffeine-elicited Ca^{2+} release in control and with CK loading showed the same time dependency as the tension from which they were calculated. The maximal free $[Ca^{2+}]$ values were 2.48 ± 0.56 and 2.53 ± 0.12 μM respectively. However, when SR was loaded in the presence of MgATP alone, the maximal free $[Ca^{2+}]$ value at peak (1.88 ± 0.23 μM) was obtained in 3 min loading, even decreasing to 1.26 ± 0.38 μM after longer periods of loading. The shape of the time-dependent curves for area under caffeine-elicited contracture were different from the curves for peak tension. It took at least 5 min to reach the maximal value in control and with CK loading. The maximal values, calculated from the first order rate equation were 3.86 ± 0.13 and 3.01 ± 0.11 arbitrary units for these two conditions. If SR was loaded in the presence of externally added ATP alone the area under the caffeine-induced tension transient was as low as 0.90 ± 0.26 units (the mean value for a 3-min load). The $[Ca^{2+}]$-time integral of caffeine-induced Ca^{2+} release exhibited the same dependency upon time for control and CK loading. The maximal

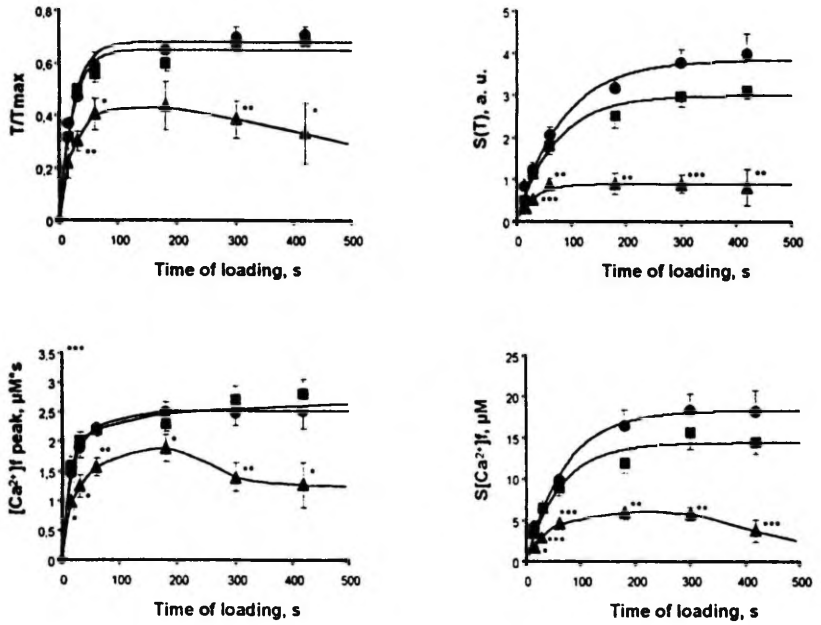


Fig. 4 Parameters of caffeine-elicited Ca^{2+} release as a function of loading time in the presence of 12 mM PCr and 3.16 mM MgATP (●), 12 mM PCr and 0.25 mM ADP (■) and 3.16 mM MgATP (▲). *Upper left* peak tension of caffeine induced contracture, normalised to maximal calcium activated tension (T/T_{max}). *lower left* free $[Ca^{2+}]$ at the peak of the caffeine-elicited Ca^{2+} release ($[Ca^{2+}]_{f,peak}$). *upper right* tension-time integral (S_T). *lower right* free $[Ca^{2+}]$-time integral of the caffeine-elicited Ca^{2+} release ($S[Ca^{2+}]_f$). Each *curve* was drawn using the mean values of 10, 14, 4, 6 and 5, 9 fibres respectively.
*, **, *** $P < 0.05$, < 0.01, < 0.001 respectively vs control (ATP + PCr)

values for both were 18.4 ± 0.4 and 14.4 ± 0.7 μM $Ca^{2+} \times$ s respectively. The maximal value of the $[Ca^{2+}]$-time integral for loading in the presence of MgATP alone was reached in 3 min and was as low as 5.87 ± 0.73 μM × s. When loaded longer than 5 min, the value tended to decrease.

Theoretically, the failure of ATP-loading to support effective Ca^{2+} uptake into the SR might result from diffusion gradients for ATP and ADP inside the fibres. To test this hypothesis, we added an additional ATP-regenerating and ADP-consuming system consisting of pyruvate kinase (PK) and phosphoenolpyruvate (PEP) to the conditions of loading, supported by MgATP alone. The preincubation at pCa 9 was prolonged in all test conditions to 5 min to equilibrate the concentration of the enzyme in the fibre before the load was started. A 3-min period of SR loading was chosen as it had been shown that this was sufficient for almost maximal Ca^{2+} uptake. Data were normalised to control conditions (3.16 mM MgATP and 12 mM PCr). The addition of 50 IU/ml PK and 1 mM PEP increased calcium uptake with ATP loading only slightly (Fig. 5). The peak of the caffeine-elicited contracture with ATP loading was 72% of control and 79% in the presence of 50 IU/ml PK and PEP. To test if 50 IU/ml PK was sufficient activity, the enzyme concentration in some experiments was increased twofold. The calcium uptake in the presence of 100 IU/ml PK was not significantly different from that with 50 IU/ml, the peak of the caffeine-elicited contracture being 81% of control. Additionally, we compared the ATP- and PK (100 IU/ml)-supported loading in the presence of 1 and 10 mM PEP (Fig. 6). Increasing PEP concentration 10 times did not increase the Ca^{2+} load significantly as could be expected, since the Michaelis-Menten constant (K_m) of PK for PEP is 8.6×10^{-5} M [3]. This indicates that the activity of the ATP-regenerating system in the fibres was not the limiting factor for SR ATPase energy supply. Additionally, the loading of SR in the presence of ADP and PCr, while normalised to control, was much more efficient (the peak value being 96% of control) than any condition where ATP was provided exogenously. Similar relations between the groups were observed when the free $[Ca^{2+}]$ at the peak was compared. However, the difference between the groups became even more striking if the area or $S[Ca^{2+}]_f$ were compared to evaluate the SR Ca^{2+} load. The area of the caffeine-elicited contracture obtained under

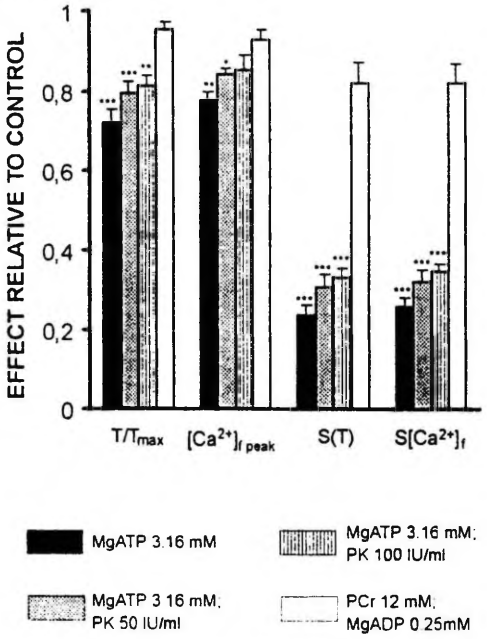


Fig. 5 The parameters of caffeine-elicited Ca^{2+} release, obtained after loading of SR for 3 min in following conditions: (1) MgATP 3.16 mM; (2) MgATP 3.16 mM + pyruvate kinase (*PK*) 50 IU/ml + phosphoenolpyruvate (*PEP*) 1 mM; (3) MgATP 3.16 mM + PK 100 IU/ml + PEP 1 mM and (4) MgADP 0.25 mM + PCr 12 mM, expressed relative to control (MgATP 3.16 mM, PCr 12 mM).
*, **, *** $P < 0.05$, < 0.01, < 0.001 respectively vs. MgADP and PCr

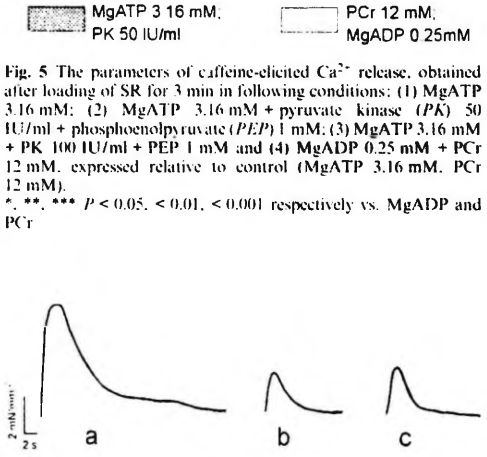


Fig. 6a–c Traces of caffeine-elicited tension recordings obtained after loading SR for 3 min in the following conditions: **a** MgATP 3.16 mM, PCr 12 mM; **b** MgATP 3.16 mM + PK 100 IU/ml + PEP 1 mM; **c** MgATP 3.16 mM + PK 100 IU/ml + PEP 10 mM

ATP-loading conditions was 25% of the control value, and 31% and 34% respectively, when obtained in the presence of 50 or 100 IU/ml PK, but 82% of control if obtained under the CK-loading conditions. With respect to $S[Ca^{2+}]_f$, CK-supported loading was more than twice as efficient as that with exogenous ATP.

Discussion

We used saponin-treated ventricular fibres to assess the functional importance of bound creatine kinase for calcium uptake by the SR in situ. We compared three loading protocols: loading in the presence of ATP and PCr (control loading), loading in the presence of ADP and PCr (CK loading) and loading in the presence of ATP alone (ATP loading). The main results of this study are: (1) $[Ca^{2+}]$ during caffeine-induced calcium release can be estimated by using myofibrils as internal sensor, (2) there is a PCr and ADP supported SR calcium uptake mediated by bound creatine kinase, (3) this CK-mediated Ca^{2+} uptake is close to the control (ATP and PCr supported) calcium uptake and much higher than the ATP-supported one and (4) addition of an ATP regenerating system (PK and PEP) during ATP-supported calcium uptake is not able to increase calcium uptake to control level.

Experimental protocol and analysis of the data

The skinned fibre technique is largely used to assess the properties of the sarcoplasmic reticulum of muscle. In these preparations, the amplitude of the caffeine-induced tension transient can be used to assess the amount of calcium loaded into the SR under different loading conditions. However, this method has several drawbacks. Among them, is the fact that tension development is not a linear function of the free $[Ca^{2+}]$ and that caffeine or any substance tested for its effects on calcium-induced calcium release may have direct effects on myofibrillar calcium sensitivity, thus biasing the estimation of calcium uptake from the tension transient. For these reasons we attempted to use the pCa/tension relationship, established in the presence of caffeine, as an internal calibration for the calcium released by the SR. This approach is justified by the fact that myofibrils are the final recipient of calcium released by the SR. The calibration was obtained by using the steady state calcium/tension relationship obtained for each fibre and used to recalculate $[Ca^{2+}]$ seen by the fibre during the transient release. This approach is valid if caffeine diffusion in the fibre is not rate limiting and if caffeine concentration is sufficient to produce complete Ca release. This was confirmed by the fact that there is no difference in the ascending phase and in the area under the transient when 5 or 15 mM caffeine was used to induce the release, and when the caffeine effect on myofibrils is taken into account. That 5 mM caffeine is sufficient to induce complete release of calcium from the SR was further confirmed by the fact that a second application of 25 mM caffeine failed to induce any additional tension. Since caffeine was present throughout the release, it prevented the reaccumulation of calcium into the SR, and it could be concluded that the descending phase of the transient was mainly

determined by binding of calcium to EGTA and by diffusion out of fibre. This allowed us to compare, within each fibre, the area of tension transients obtained in different loading conditions as an index of the relative amount of calcium released.

Two distinct parameters, the peak and the area of the tension transient, have been used to estimate calcium content in the SR. However, the loading-time dependence of both parameters is different, the peak tension being achieved for shorter loading times than the area of the tension curve. The same is true when comparing recalculated peak calcium and the area of calcium transient. This could be the result of the existence of two calcium pools within the SR, one pool readily releasable and saturable, while the second pool would be more slowly releasable and would take part in the prolongation of the force or calcium transient. Different compartments in the SR can be represented by junctional or corbular SR [2]. Alternatively, the existence in the SR of calcium binding proteins such as calsequestrins which bind calcium with high capacity and low affinity, may also represent a distinct pool of calcium [23]. More work is needed to identify the reasons for such behaviour. The existence of a more slowly releasable Ca^{2+} pool emphasises the importance of analysis of the whole caffeine-induced tension and Ca^{2+} transients, rather than peak values.

Creatine kinase and SR

In skinned fibres, we showed that calcium uptake supported by ADP and PCr was as efficient as Ca uptake supported by ATP and PCr and greater than by ATP alone. This was demonstrated either by studying the peak of force or calcium, as well as the area of both force and calcium transients. These results showed that sufficient creatine kinase was still bound after the fibres were permeabilised in saponin and that local ATP generated by the CK reaction was sufficient to meet completely the ATP requirements of the Ca-ATPase. In contrast, ATP alone was not able to sustain control loading of the SR. Steele et al. have also reported that PCr and ADP sustains efficient calcium uptake and that PCr prevents the inhibition of calcium uptake by Pi [17]. They propose that this inhibition is mediated by the capacity of CK and PCr to rephosphorylate ADP.

Addition of pyruvate kinase and phosphoenolpyruvate has often been used as an external ATP-regenerating system to assess the efficiency of SR-bound creatine kinase. The use of pyruvate kinase is validated by the fact that the K_m of creatine kinase and pyruvate kinase for ADP are in the same range [15]. Levitsky et al. have found that PK is not as effective as CK in supporting Ca^{2+} uptake [13]. This suggests that the increase in ADP takes place close to the active site of the ATPase in a microenvironment not readily accessible to pyruvate kinase. Steele et al. have attributed the same observation made in skinned fibres to the limited access of pyruvate kinase to the intracellular space [17]. However, this explanation is not supported by the fact that the same observation has been reported in cardiac [13] and skeletal muscle [16] isolated vesicles where the accessibility of pyruvate kinase to the SR is not restricted. In addition, Krafi et al. has reported recently that pyruvate kinase can equilibrate in skinned skeletal fibres within minutes [12]. The seemingly greater efficiency of CK in decreasing local ADP concentration near the ATPase and inhibiting reversal of the ATPase or calcium leakage may be due to the close localisation of CK to calcium ATPase and functional coupling of the two enzymes.

The presence of CK activity in SR was first described in fragmented sarcoplasmic reticulum of rabbit skeletal muscle and cardiac muscle [1]. In chicken breast skeletal muscle, Rossi et al. has shown that SR vesicles contain about 1 IU CK/mg SR protein and that SR-bound CK is able to sustain a SR Ca loading 25%–40% of that measured in the presence of ATP alone [14]. Highly purified fractions of pigeon heart SR vesicles contain 0.6 IU/mg SR protein CK activity with a SR-ATPase /CK ratio of 4.5 [13]. In these conditions, bound CK is not able to support the maximal velocity of calcium uptake. Despite the fact that the experiments by Rossi et al. suggest a strong association between this enzyme and SR membranes [14], it is highly possible that the thorough procedure of preparation of SR vesicles induces detachment of creatine kinase from SR. This could lead to underestimation of the importance of this coupling in the intact cells [11]. It is also possible that, in skinned fibers, the intactness of intracellular architecture, by preserving the spatial arrangement of SR ATPase and CK, favours their interaction. It should also be taken into consideration that, in saponin-skinned fibres, myofibrillar-bound CK is still present and due to the close location of longitudinal SR wrapped around myofibrils, myofibrillar CK can participate in the regulation of the local ATP/ADP ratio near the Ca-ATPase.

The results show that localised regeneration of ATP is more efficient than external ATP in fueling the Ca-ATPase. One role of the CK reaction is to maintain a low ADP concentration close to the ATPase. ADP depresses Ca-uptake by SR vesicles with an inhibitory constant (K_i) of 180 μM. This effect is reversed by PCr [11]. ADP has been shown also to depress calcium loading and this was attributed to a fall in the free energy of ATP hydrolysis available for the calcium pump [22]. Additionally, due to the high reverse gradient between inside and outside of SR, ADP accumulation would favour calcium leakage outside the SR [10]. These results suggest that ATP and ADP are locally constrained within an effective microenvironment defined by the spatial proximity of the two enzymes thus creating a compartmentalised high ATP/ADP ratio favourable to the Ca-ATPase activity [10, 11, 14].

Finally, the prevention of protons accumulation near the Ca-ATPase may also be one of the role of SR-bound creatine kinase.

Our results confirm the presence and efficiency of SR-associated CK as demonstrated in isolated vesicles. In addition, in situ experiments using skinned fibres, by preserving the local architecture, allowed us to show that ATP regenerated in the vicinity of the SR Ca-ATPase by bound CK is indeed more efficient than externally added ATP in sustaining SR calcium uptake. These results show that CK is functionally coupled to Ca-ATPase of the SR and that bound CK is necessary for optimal functioning of the Ca-ATPase. The SR of the adult mammalian heart is a complex unit comprising structural, functional and regulatory aspects and should be considered as a complex multienzyme system. All these aspects contribute to the efficiency of calcium pumping and release and to its fine regulation in living cells. Local control of ATPase substrate and products by the CK reaction has an important part in this function. Any defect in this system could impair the efficiency of calcium homeostasis, and be deleterious for regulation of cardiac contraction.

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