Levosimendan: Studies on its mechanisms of action and beyond

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to the text by the Roman numerals I-V, and some unpublished data:


ABBREVIATIONS

4-AP 4-aminopyridine
α₁-AR adrenergic alpha-1 receptor
AMI acute myocardial infarction
ANCOVA analysis of covariance
ANOVA analysis of variance
AC adenylate cyclase
ACE angiotensin converting enzyme
ACEI angiotensin converting enzyme inhibitors
ADHF acutely decompensated heart failure
ADP adenosine diphosphate
AE adverse effect
AF atrial fibrillation
AHF acute heart failure
ARB angiotensin receptor antagonist
ATP adenosine triphosphate
β₁-AR adrenergic beta-1 receptor
β₂-AR adrenergic beta-2 receptor
BKCa calcium-activated potassium channel
BIM bisindolylmaleimide
BP blood pressure
CABG coronary artery bypass graft
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
[Ca²⁺],i intracellular calcium concentration
CAD coronary artery disease
CBP cardiopulmonary bypass
CCB calcium channel blockers
CF coronary flow
CHD coronary heart disease
CHF congestive heart failure
CI cardiac index
CIP Cahn Ingold Prelog priority rules
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CMK</td>
<td>calmodulin-kinase</td>
</tr>
<tr>
<td>CN</td>
<td>calcineurin</td>
</tr>
<tr>
<td>CP</td>
<td>cardiac power</td>
</tr>
<tr>
<td>CRT</td>
<td>cardiac resynchronization therapy</td>
</tr>
<tr>
<td>CS</td>
<td>cardiogenic shock</td>
</tr>
<tr>
<td>cTn</td>
<td>cardiac troponin complex</td>
</tr>
<tr>
<td>DCFV</td>
<td>diastolic coronary flow velocity</td>
</tr>
<tr>
<td>DCM</td>
<td>dilated cardiomyopathy</td>
</tr>
<tr>
<td>DHF</td>
<td>diastolic heart failure</td>
</tr>
<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>EF</td>
<td>ejection fraction</td>
</tr>
<tr>
<td>ESC</td>
<td>European Society of Cardiology</td>
</tr>
<tr>
<td>ETB</td>
<td>endothelin-receptor type-B</td>
</tr>
<tr>
<td>GC</td>
<td>guanylate cyclase</td>
</tr>
<tr>
<td>hBNP</td>
<td>human B-type natriuretic peptide</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>IBTX</td>
<td>iberiotoxin</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IRAG</td>
<td>cGMP kinase substrate</td>
</tr>
<tr>
<td>IRK</td>
<td>inwardly rectifying potassium channels</td>
</tr>
<tr>
<td>Kₐ₅TP</td>
<td>ATP-sensitive potassium channels</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>KV</td>
<td>voltage-gated potassium channels</td>
</tr>
<tr>
<td>LV +dP/dtₘₐₓ</td>
<td>left ventricular maximal positive pressure derivative</td>
</tr>
<tr>
<td>LV -dP/dtₘₐₓ</td>
<td>left ventricular maximal negative pressure derivative</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>M₁</td>
<td>muscarinic type-1 receptor</td>
</tr>
<tr>
<td>M₃</td>
<td>muscarinic type-3 receptor</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>mitoKₐ₅TP</td>
<td>mitochondrial Kₐ₅TP channels</td>
</tr>
<tr>
<td>MOF</td>
<td>multiorgan organ failure</td>
</tr>
</tbody>
</table>
MVO2  oxygen consumption
Na\textsuperscript{+}-K\textsuperscript{+} pump  sodium–potassium ATPase
NCX  sodium-calcium exchanger
NO  nitric oxide
NOS  nitric oxide synthase
NRP  natriuretic peptide receptor
PCWP  pulmonary capillary wedge pressure
PDE  phosphodiesterase
PDEI  phosphodiesterase inhibitor
PCI  percutaneous coronary intervention
PKA  cAMP-dependent protein kinase
PKG  cGMP-dependent protein kinase
PKC  protein kinase C
RAAS  renin angiotensin aldosterone system
S1  subfragment 1
sarcK\textsubscript{ATP}  sarcolemmal K\textsubscript{ATP} channels
SBP  systolic blood pressure
SERCA-2  sarcoplasmic reticulum calcium ATPase isoform 2
SNS  sympathetic nervous system
SR  sarcoplasmic reticulum
SVT  supraventricular tachycardia
SUR  sulfonylurea receptor
SVR  systemic vascular resistance
Tm  tropomyosin
Tn  troponin complex
TnC  troponin C
TNF- \alpha  tumor necrosis factor-\alpha
TnI  troponin I
TnT  troponin T
VT  ventricular tachycardia
ABSTRACT

Acute heart failure syndrome represents a prominent and growing health problem all around the world. Ideally, medical treatment for patients admitted to hospital because of this syndrome, in addition to alleviating the acute symptoms, should also prevent myocardial damage, modulate neurohumoral and inflammatory activation, and preserve or even improve renal function. Levosimendan is a cardiac enhancer having both inotropic and vasodilatory effects. It is approved for the short-term treatment of acutely decompensated chronic heart failure, but it has been shown to have beneficial clinical effects also in ischemic heart disease and septic shock as well as in perioperative cardiac support.

In the present study, the mechanisms of action of levosimendan were studied in isolated guinea-pig heart preparations: Langendorff-perfused heart, papillary muscle and permeabilized cardiomyocytes as well as in purified phosphodiesterase isoenzyme preparations. Levosimendan was shown to be a potent inotropic agent in isolated Langendorff-perfused heart and right ventricle papillary muscle. In permeabilized cardiomyocytes, it was demonstrated to be a potent calcium sensitizer in contrast to its enantiomer, dextrosimendan. It was additionally shown to be a very selective phosphodiesterase (PDE) type-3 inhibitor, the selectivity factor for PDE3 over PDE4 being 10000 for levosimendan. Irrespective of this very selective PDE3 inhibitory property in purified enzyme preparations, the inotropic effect of levosimendan was demonstrated to be mediated mainly through calcium sensitization in the isolated heart as well as the papillary muscle preparations at clinically relevant concentrations. In the isolated Langendorff-perfused heart, glibenclamide antagonized the levosimendan-induced increase in coronary flow (CF). Therefore, the main vasodilatory mechanism in coronary veins is believed to be the opening of the ATP-sensitive potassium (K_{ATP}) channels. In the paced hearts, CF did not increase in parallel with oxygen consumption (MVO_{2}), thus indicating that levosimendan had a direct vasodilatory effect on coronary veins. The pharmacology of levosimendan was clearly different from that of milrinone, which induced an increase in CF in parallel with MVO_{2}.

In conclusion, levosimendan was demonstrated to increase cardiac contractility by binding to cardiac troponin C and sensitizing the myofilament contractile proteins to calcium, and further to induce coronary vasodilation by opening K_{ATP} channels in vascular smooth muscle. In addition, the efficiency of the cardiac contraction was shown to be more advantageous when the heart was perfused with levosimendan in comparison to milrinone perfusion.
Acute heart failure syndromes are a most common cause of hospitalization all over the world with a dismal long-term prognosis (Goldberg et al. 2007). According to the European Society of Cardiology (ESC) guidelines for the diagnosis and treatment of acute and chronic heart failure (HF) issued in 2008, acute heart failure is defined as ‘rapid onset or change in the signs and symptoms of HF, resulting in the need for urgent therapy’ (Dickstein et al. 2008). HF is defined as a syndrome in which patients should have the following features: Typical symptoms like breathlessness at rest or on exercise, fatigue, tiredness and ankle swelling; typical signs like tachycardia, tachypnoea, pulmonary rales, pleural effusion, raised jugular venous pressure, peripheral oedema and hepatomegaly and; objective evidence of a structural or functional abnormality in the heart at rest like cardiomegaly, third heart sound, cardiac murmurs, abnormality on the echocardiogram and raised natriuretic peptide concentration (Dickstein et al. 2008). Acute heart failure (AHF) may be either new HF or worsening of pre-existing chronic HF. Patients differ in terms of their clinical presentation, pathophysiology, prognosis, and therapeutic options (Flaherty et al. 2009). Primarily the symptoms of AHF are the result of severe pulmonary congestion due to elevated left ventricular filling pressure and/or diastolic dysfunction, which can be related to low cardiac output. AHF patients can have both preserved and reduced ejection fraction (EF) and be suffering from a variety of cardiovascular conditions such as coronary heart disease (CHD), hypertension, valvular heart disease and atrial arrhythmias (Gheorghiade et al. 2005c). In addition, noncardiac conditions like renal dysfunction, diabetes and anaemia are often present (Gheorghiade et al. 2005c).

AHF can be categorized into 6 clinical distinct entities, which can overlap with each other: worsening or decompensated chronic HF, pulmonary oedema, hypertensive HF, cardiogenic shock, isolated right HF and, acute coronary syndrome (ACS) and HF (Dickstein et al. 2008) (Fig. 1.). The majority of patients presenting with AHF have coronary artery disease (CAD) (Flaherty et al. 2009). These patients can be divided into two groups, those with acute CAD and those with underlying or chronic CAD. Thus, therapeutic strategies can be designated according to appearance of CAD.

The most common entities of symptoms of AHF patients admitted to the intensive care unit are acutely decompensated chronic heart failure and pulmonary edema with elevated systemic blood pressure (Gheorghiade et al. 2005c). Conventionally, the primary therapeutic management for acute HF deterioration are reduction of PCWP and/or increase of cardiac output, but blood pressure control, myocardial protection, neurohormonal modulation, and preservation of renal function may also be needed. According to Finnish Acute Heart Failure Study (FINN-AKVA), acute congestion
(63.5%) was the most common manifestation of AHF and over one quarter of the patients (26.3%) displayed pulmonary oedema. The rest had either cardiogenic shock (2.3%) or hypertensive crisis (3.1%) or right ventricular failure (4.8%). Furthermore, half of the patients had a history of HF (Siirila-Waris et al. 2006).

According to the ESC Guidelines (Dickstein et al. 2008), inotropic agents should be considered in patients with low output state, in the presence of signs of hypoperfusion or congestion despite the use of vasodilators and/or diuretics to alleviate the symptoms. Traditionally inotropes are categorised into three classes: cardiac glycosides (e.g. digitalis); sympathomimetic amines having β-adrenergic activity (e.g. dopamine, dobutamine, norepinephrine and epinephrine) and; phosphodiesterase inhibitors (PDEIs) (e.g. milrinone and enoximone) (Braunwald 1992). These inotropic agents generally improve the hemodynamic status of the AHF patients, but on the other hand, have not been shown to improve survival, in fact may even worsen survival (Amidon and Parmley 1994; Thackray et al. 2002; Felker et al. 2003) probably due to elevating intracellular calcium levels, an action which is crucial to their mechanism of inotropic action (Papp et al. 2005). Therefore, these drugs should be withdrawn as soon as the desired hemodynamic effects are reached (e.g. adequate organ perfusion or reduced congestion) (Dickstein et al. 2008).

One of the latest entries in the family of cardiac enhancers is levosimendan, a novel compound having both inotropic and vasodilatory effects (Parissis et al. 2008). The present studies were designed to characterize the mechanisms of action of levosimendan and to compare its inodilatory effects to those of phosphodiesterase inhibitors, acting via a different mechanism of action. The ex-vivo experiments were done in isolated heart preparations, a technique which allows untangling the inotropic and vasodilatory effects of the drugs studied. Levosimendan increases cardiac contractility by enhancing the sensitivity of the contractile proteins to calcium ions (Pollesello et al. 1994), and exerts a vasodilatory effect by opening adenosine triphosphate ATP-sensitive potassium (K_{ATP}) channels in vascular smooth muscle (Yokoshiki et al. 1997). Due to its novel mechanism of action, which is not dependent upon an increase in the intracellular level of calcium, it is believed to be more beneficial than the other inodilators in being able to reduce both morbidity and mortality.
Figure 1. Clinical classification of AHF. Acute heart failure (AHF); Acute coronary syndrome (ACS). Modified from (Dickstein et al. 2008) Eur Heart J 29(19): 2388-442.
2 REVIEW OF THE LITERATURE

2.1 Regulation of the cardiac contraction

Two characteristic intrinsic mechanisms, independent of neural and humoral influences, determine the contractile regulation of cardiac myocytes; the Frank-Starling mechanism and the positive force-frequency relationship. The Frank-Starling mechanism is defined as the ability of the heart to change its force of contraction and therefore the stroke volume in response to changes in venous return, i.e. an increase in ventricular wall stretch, leading to an increase in contractile force. The positive force-frequency relationship, i.e. increased heart rate which induces an increase in contractile force, also leads to an increase in stroke volume. The immediate response to the stretch (Frank-Starling mechanism) does not increase the $\text{Ca}^{2+}$ transient, but is followed by slowly developing increase in intracellular calcium concentration $[\text{Ca}^{2+}]_i$, probably due to the increase in $\text{Ca}^{2+}$ binding affinity to troponin C induced by the force generation (Endoh 2008). The force-frequency relationship is, in turn, associated with the mobilization of $[\text{Ca}^{2+}]_i$. Furthermore, cardiac muscle contracts phasically, so that the difference between systolic and diastolic $[\text{Ca}^{2+}]_i$, determine the effect on the left ventricular function. Thus, heart muscle contraction can be enhanced by increasing the $[\text{Ca}^{2+}]_i$ in the cardiomyocytes. Relaxation, in turn, depends on how fast $\text{Ca}^{2+}$ is extruded or taken back into the sarcoplasmic reticulum (SR). Myocyte $[\text{Ca}^{2+}]_i$, can be increased by increasing intracellular levels of cyclic adenylate monophosphate (cAMP) or by affecting the sodium-calcium exchanger (NCX). The increased amount of $\text{Ca}^{2+}$ is accumulated into the cytosol and stored into the SR. This $\text{Ca}^{2+}$ is then released from SR by each heart-beat-cycle, which leads to increased contractility of the heart (Braunwald 1992).

Contraction of the cardiac cell

Skeletal and cardiac muscles are both composed of striated muscle cells and the sarcomere structure and the general mechanism involved in the muscle contraction is the same. The striated muscle cell contains myofibrils formed by repeating units of sarcomeres. They are arranged in series, which are composed of two type parallel filaments, a thin filament and a thick filament. The thick filament is a polymer, composed of myosin molecules which in turn consists of two heavy and four light chains. A bundle of myosin heavy chain coiled tails forms the backbone of the thick filament. The globular heads of the heavy chain N-terminus named as subfragment 1 (S1) are located in the thick filament at regular intervals and interact with the thin filaments to form strong crossbridges. The thin filament, in turn, has a two-stranded helical structure. The backbone of the thin filament is
composed of polymerized globular actin monomers (G-actin). Actin monomers consist of two equal-sized domains which are available for myosin interaction or interact with the corresponding sub-domains of the adjacent strand (Holmes et al. 1990).

A series of regulatory proteins modulate the interaction between the thick and thin filaments. The most elongated of them, tropomyosin (Tm) overlaps with the neighboring tropomyosins in a head-to-tail configuration. The overlapping regions of adjacent tropomyosins are mainly responsible for the affinity of Tm for actin. It binds to the actin filament by electrostatic interaction (Lorenz et al. 1995). Instead of being fixed in one position, Tm rolls over the surface of the thin filament depending on the phase of the contraction cycle. This movement is influenced by Ca\(^{2+}\) and it affects myosin S1 binding to actin (Gordon et al. 2000; Gordon et al. 2001; Sorsa et al. 2004). Every tropomyosin is spatially coupled to a troponin complex (Tn), and together they form the calcium dependent trigger of the contractile apparatus. Tn consists of a Tm binding unit troponin T (TnT), an actomyosin ATPase inhibitory unit troponin I (TnI), and a calcium-binding unit troponin C (TnC). Striated-muscle troponin C is expressed in two isoforms in vertebrates, in fast skeletal muscle there is skeletal troponin C (sTnC) and in slow skeletal and cardiac muscles it is cardiac troponin C (cTnC). TnT is an asymmetric protein that attaches the Tn complex to a defined position on the thin filament. It is needed for full, Ca\(^{2+}\)-dependent activity of the thin filament. The interaction of TnT with Tm is calcium sensitive. Calcium-binding to TnC initiates the cascade of events leading to muscle contraction. The interaction between TnC and TnI is essential for further transmission of the contraction signal to the other components of the thin filament (Sorsa et al. 2004).

**Mechanism of action of cardiotonic agents**

The adrenergic beta1-receptor (β\(_{1}\)-AR) is the main adrenergic receptor type in heart cells. It is stimulated by the endogenous norepinephrine (NE), released from the sympathetic nerve-endings. Drugs that enhance NE release can be inotropic, but mainly the receptor is stimulated by the beta-adrenergic agonists. Stimulation of β\(_{1}\)-AR activates adenyl cyclase (AC) due to the G-stimulatory protein (G\(_{s}\)). The activation of AC increases the intracellular levels of cAMP, which activates cAMP dependent protein kinase (PKA). PKA, in turn, phosphorylates L-type calcium channels leading to their opening and increase in \([\text{Ca}^{2+}]\). Increased \([\text{Ca}^{2+}]\) triggers the release of Ca\(^{2+}\) from SR via ryanodine receptors and furthermore, there is increased force generation by the actin-myosin apparatus when Ca\(^{2+}\) binds to the cTnC (Sorsa et al. 2004).

PDE inhibitors increase the amount of intracellular cAMP by inhibiting its breakdown. Thus, this mechanism is downstream to β\(_{1}\)-AR stimulation and acts in a parallel manner with it. In all, 11
families of PDE enzymes have been identified, 5 of them in heart tissue. They differ in terms of their affinity for cAMP and cGMP, cellular expression, intracellular localization, and mechanisms of regulation. Their function is dependent on their compartmentation with protein kinases and other proteins related to signal transduction cascades (Fischmeister et al. 2006; Vandecasteele et al. 2006). Two families, PDE3 and PDE4, are the most important in the regulation of the cardiac contractility. PDE4 is cAMP specific, but PDE3 has a very high affinity for both cAMP and cGMP. However, its turnover rate for cGMP is much lower than its turnover rate for cAMP (Shimizu et al. 2002). Thereby, PDE3 may function primarily as a cGMP-inhibited cAMP phosphodiesterase. Inhibition of the cAMP-hydrolytic activity of PDE3 by cGMP contributes to the potentiation of delayed rectifier K⁺ currents and L-type Ca²⁺ currents in cardiac myocytes (Shimizu et al. 2002; Movsesian et al. 2008).

Cardiac glycosides, like digoxin, bind to the α-subunit of the sodium/potassium ATPase (Na⁺/K⁺ pump) in the extracellular membranes of the myocytes and inhibit its function. The inhibition of the Na⁺/K⁺ pump causes an increase in the intracellular sodium concentration [Na⁺]i in the myocytes, which leads further to an elevation in [Ca²⁺]i by the NCX. Basically, NCX that normally extrudes Ca²⁺ from the cell (forward mode), also brings Ca²⁺ into the cell (reverse mode) during membrane depolarization in the resting phase of the cardiac action potential (Iwamoto et al. 2007). NCX can run in the reverse mode also when Na⁺ accumulates into the cytosol. As a result of the inhibition of the Na⁺/K⁺ pump, Ca²⁺ accumulates in the cytosol and is then stored in the SR. In principle, the inhibition of the forward mode of NCX can also cause an inotropic effect.

The above-mentioned mechanisms increase the cytosolic calcium concentration [Ca²⁺]i and thus, result in higher demands for oxygen and elevated risk of arrhythmias, cell injury, apoptosis or necrosis due to Ca²⁺ overload. An alternative idea is to enhance the response of myofilaments to calcium without an increase in the [Ca²⁺]i, and thus, have a positive inotropic effect on cardiac contractility. Calcium sensitizers can increase the Ca²⁺ affinity to troponin, like pimopendan. They can stabilize the Ca²⁺ bound conformation of troponin C, like levosimendan or furthermore, they can have a direct effect on the cycling rate of actinmyosin cross-bridges, lowering the threshold of [Ca²⁺]i needed for actin sliding, like MCI-154 (Kass and Solaro 2006).
Role of stereoisomerism on the pharmacodynamic action

The symmetry of a molecule determines its chirality. A molecule is chiral when it is non-superposable on its mirror image. Enantiomers are two stereoisomers that are mirror images of each other. However, a chiral molecule is not necessarily asymmetric. Different enantiomers of a compound have the same physical properties, but they rotate polarized light in different directions and interact differently with optical isomers of other compounds. Therefore, the enantiomers of a compound may have substantially different biological effects. A mixture of equal parts of the enantiomers is called racemic.

Many drugs are chiral and only one of the enantiomers are biologically active or more effective than the other. A good example is adrenaline of which the natural form is the (R)-(−)-L-adrenaline. An enantiomer can be named by the direction in which it rotates the plane of polarized light. If it rotates the light clockwise the enantiomer is labeled (+), if counter-clockwise it is labeled (−). An optical isomer can be named by the D/L system, a spatial configuration of its atoms relating the molecule to glyceraldehyde. The R/S system is the most important nomenclature system for symbolizing enantiomers, which does not involve a reference molecule such as glyceraldehyde. It labels each chiral center R or S according to the CIP system (Cahn Ingold Prelog priority rules) (Cahn et al. 1966). The R/S system has no fixed relation to the (+)/(−) or the D/L systems.

Classical examples of cardiotonic drugs, which have two enantiomers are propranolol and carvedilol. Only L-propranolol is a β₁-AR antagonist, whereas D-propranolol has little binding affinity. However, both isomers possess a local anesthetic effect. Furthermore, S(-) isomer of carvedilol is 100 times more potent as β₁-AR antagonist than R(+) isomer. However, both of the isomers are approximately equipotent as adrenergic α₁-receptor (α₁-AR) antagonists.

2.2 Regulation of the vascular resistance

Vascular resistance is dependent on contraction and relaxation of smooth muscle myocytes. The molecular mechanisms involved in the contraction-relaxation process in the smooth muscle cells and its modulation can be manipulated pharmacologically in many ways. Smooth muscle contracts tonically, thereby the vascular tone is in direct proportion to a function of $[Ca^{2+}]_i$ itself, not to the difference between systolic and diastolic $[Ca^{2+}]_i$. Agents that increase $[Ca^{2+}]_i$ in vascular smooth muscle cells are vasoconstrictors, while agents that decrease $[Ca^{2+}]_i$ in vascular smooth muscle cells are vasodilators.
Mechanism of action of vasodilatory agents

The inhibition of L-type calcium channels leads to a decrease in vascular myocyte $[Ca^{2+}]_i$. Less $Ca^{2+}$ is bound to calmodulin, which decreases the activation of myosin kinase and further reduces the phosphorylation of myosin thick filament. L-type calcium channels are voltage-dependent and are inhibited by three types of chemical substances, the phenylalkylamines (like verapamil), the dihydropyridines (like nifedipide) and by benzothiazepines (like diltiazem). They are vasodilators, but verapamil is used also as an antiarrhythmic agent.

Calcium release via inositol trisphosphate (IP3)-sensitive $Ca^{2+}$ channels from SR is another source of $[Ca^{2+}]_i$ in vascular myocytes. IP3 is generated from phosphatidylinositol when phospholipase C is activated by $G_{aq}$-coupled receptors i.e. $\alpha_1$-adrenergic, angiotensin, and endothelin receptors. M1/M3 muscarinic receptors and ET$_B$ endothelin-receptor are also coupled to inositol trisphosphate (IP3) generation, but in endothelial cells. Increased endothelial $[Ca^{2+}]_i$ leads to vasodilatation contrary to the situation in vascular smooth muscle cells (see below, cGMP-dependent vasodilatation). The main drug categories acting ultimately through IP$_3$ generation are angiotensin converting enzyme inhibitors (ACEIs), angiotensin II receptor antagonists (ARBs), endothelin receptor antagonists and $\alpha_1$-AR antagonists.

The formation of cGMP from GTP by guanylate cyclase (GC) is analogous to the formation of cAMP from ATP by AC. Two forms of GC exist, a soluble enzyme and a membrane-bound enzyme. GC activates cGMP-dependent protein kinase (PKG), which phosphorylates the IP$_3$ receptor-associated cGMP kinase substrate (IRAG). Phosphorylation of IRAG further reduces IP$_3$-induced $Ca^{2+}$ release, leading to relaxation of vascular myocytes. PKG also phosphorylates $K^+$ channels and thereby, hyperpolarizes the cell membrane and decreases $Ca^{2+}$ influx through L-type channels. In addition, PKC phosphorylates myosin light chain phosphatase and so, dephosphorylates the myosin ATPase and reduces the contraction of vascular myocytes. Furthermore, it phosphorylates $G_{aq}$-protein and thus, inhibits IP$_3$ formation. Soluble GC is a target for nitric oxide (NO), synthesised endogenously from arginine and oxygen by nitric oxide synthase (NOS) enzymes or formed by reduction from organic nitrates and then released from endothelial cells. Nitroglycerin (or glyceryl trinitrate) and sodium nitroprusside, are both sources of NO and thus, common vasodilatory agents. The natriuretic peptides (ANP, BNP and CNP), instead, bind to the specific NP receptors (NPRs). Three subtypes of NPRs have been characterized, namely NPR1, NPR2 and NPR3. Two of them, NPR1 and NPR2, contain the GC catalytic domain (membrane bound GC). ANP and BNP selectively stimulate NPR1, whereas CNP activates primarily NPR2. All three NPs
bind to NPR3, which clears them from the circulation through receptor-mediated internalization and degradation (Potter et al. 2006; Potter et al. 2009). Moreover, PDE5 is essentially the most cGMP specific of the cardiac phosphodiesterases. The benefits of PDE5 inhibition in heart failure originate from a decrease of the right ventricle afterload resulting from pulmonary vasodilatation (Movsesian et al. 2008).

The increase in the cAMP content in smooth muscle cells evokes vasodilatation. The cAMP level increases when vascular β2-adrenergic receptors are stimulated. Instead of PKA, cAMP activates PKG in vascular myocytes. PKG has a much lower affinity for cAMP than for cGMP, but cAMP levels can rise high enough to activate PKG in smooth muscle cells, despite its relatively low affinity for cAMP. In this manner, vasodilatation occurs when PDE3 is inhibited and cAMP hydrolysis is restricted.

Activation of potassium channels in vascular smooth muscle cells results in membrane hyperpolarization and thus, a decrease Ca2+ influx through L-type channels and further vasodilatation (Brayden 2002). Four major classes of potassium channels have been classified: calcium-activated potassium channel (BKCa), inwardly rectifying potassium channels (IRK), two-pore-domain potassium channels and voltage-gated potassium channels (KV). KATP channels belong to the IRK channels, containing four pore-forming Kir6.x subunits and a large regulatory sulfonylurea receptor (SUR) (Aguilar-Bryan et al. 1998; Brayden 2002). They appear to be tonically active in some vascular beds, e.g. in the coronary veins, and contribute to the physiological regulation of vascular tone and blood flow. KATP channels are expressed also in the vascular endothelium (Yoshida et al. 2004) and may modulate vasodilatation in response to shear stress and pathophysiological conditions, such as hypoxia, ischemia, acidosis and septic shock as well as some vasodilators, e.g. adenosine (Yoshida et al. 2004; Adebiyi et al. 2008).

2.3 Acute Heart Failure Syndromes

2.3.1 Pathophysiology and risk markers of AHF

Multiple reasons are involved in the pathogenesis of AHF, starting from nonadherence to diet, pharmacologic therapy or fluid restriction and ending in cardiogenic shock and multiorgan failure. However, fluid accumulation is the most important factor causing hospitalization of patients with AHF and traditionally it has been considered simply as the major cause of the syndromes (Metra et al. 2008a). This opinion has now been challenged. Most patients with AHF can be divided into two main types according to their symptoms, especially according to their blood pressure (Gheorghiade
et al. 2005a). Furthermore, Cotter et al (Cotter et al. 2008) proposed that the mechanisms behind AHF are more complex than simply fluid accumulation. They distinguished two categories of symptoms of ADHF. Usually ADHF is the end result of a relatively slow (days to weeks) deterioration of severe chronic HF. However, it can be a rapidly progressive disorder of high blood pressure (BP) seen in the emergency rooms, accompanied by severe acute dyspnoea. Moreover, ADHF may be the result of the decrease in myocardial contractility due to ischemia. On the other hand, it can be caused by a combination of increased vascular resistance with decreased cardiac contractility, even though EF is relatively well preserved. This leads to severe hypertension with increased diastolic left ventricular failure (Cotter et al. 2008).

Volume overload is typical for AHF and is traditionally believed to be the main cause of congestion. Thus, treatment with diuretics results in recovery of the fluid homeostasis. However, recent studies do not necessarily support this concept. The symptoms of HF e.g. fatigue and increased shortness of breath as well as an increase in pulmonary pressure have been shown to occur days to weeks before any weight gain was first observed (Cotter et al. 2008). Furthermore, there is evidence that even a dramatic rise in cardiac filling pressure (preceding an AHF hospitalization) often occurs without any significant change in weight gain in patients with chronic hemodynamic monitoring devices (Cotter et al. 2008). In addition, even treatment with a high dose diuretic has not been associated with improved outcome (Cotter et al. 1997). In fact, a high dose of loop diuretics has been associated with a higher risk of renal failure (Butler et al. 2004).

Myocardial ischaemia is considered as an important trigger of AHF and more than a half of the patients hospitalized with AHF have a history of chronic ischemic heart disease in the USA, in Europe as well as in Finland (Siirila-Waris et al. 2006; Alla et al. 2007). Furthermore, Gheorghiade et al (2005) in PRESERVD-HF study (Gheorghiade et al. 2005b) studied 51 patients with AHF who were admitted with worsening HF and a history of CAD but not with an acute coronary event. Most of the patients had detectable levels of cardiac troponin (cTn) already at the initial measurement, and some patients displayed cTn release during hospitalization. The release of cTn is thought to be a marker for myocardial injury, and therefore the study raised the possibility that injury occurred in most patients admitted with AHF.

According to FINN-AKVA, elevated cTn levels are common in AHF patients without ACS, cTnI being more often elevated than cTnT. Increase in both cTnI and cTnT correlated with increased mortality. However, they did not act as independent risk markers (Ilva et al. 2008). Cystatin C is a novel marker of renal function, which has been discovered as a prognostic marker in AHF.
According to FINN-AKVA, cystatin C was a strong and independent predictor of outcome at 12 months in AHF. In spite of normal plasma creatinine, cystatin C identifies patients with poor prognosis (Lassus et al. 2007).

Arrhythmias are common in chronic heart failure. In congestive heart failure (CHF), atrial fibrillation (AF) is accompanied by a marked attenuation of the cardiac sympathetic response to acute hemodynamic stress in isometric exercise (Gould et al. 2008). Previously, impairment of the baroreceptor responses has been associated with abnormal hemodynamic responses to isometric exercise (Fukuma et al. 2004). This indicates that AF could be associated with the impairment of the baroreceptor response in CHF. According to the EuroHeart Failure Survey II (EHFS II) (Nieminen et al. 2006), 38.7% of the patients had AF. However, the role of new arrhythmias in acute heart failure is largely unknown. Nevertheless, AF has been shown to be a strong predictor of the reappearance of symptoms and death in patients admitted for AHF (Benza et al. 2004).

Rupture of chordae tendineae or a papillary muscle can lead to acute mitral regurgitation, which in turn, may be a cause of acute pulmonary edema and a rapid decrease in myocardial contractility, leading further to AHF. Instead, the role of chronic ischemic mitral regurgitation as a cause for AHF is less clear. However, major increases in mitral regurgitation and systolic pulmonary-artery pressure during exercise have been observed a few days after acute pulmonary edema (Pierard and Lancellotti 2004). This suggests that acute pulmonary edema is associated with the dynamic changes in ischemic mitral regurgitation and the further development of AHF.

Recent studies have revealed that elevated BP plays a central role in the pathogenesis of AHF in most patients. Initial BP measurements in the emergency room before any treatment has shown that BP is elevated prominently at the onset of AHF (Milo-Cotter et al. 2007). A marked increase in systemic vascular resistance (SVR) may also be an important trigger for AHF. The extreme vasoconstriction combined with impaired cardiac power (CP), defined as the product of CO and systemic arterial pressure, induces a vicious cycle of afterload mismatch leading to a reduction of CO and elevated left ventricular end-diastolic pressure. This affects the pulmonary capillaries resulting in pulmonary edema. However, the basic reason for the increase in SVR is still unknown. Increased arterial stiffness could be one reason for the increased SVR in AHF, since the incidence of both AHF and arterial stiffness increases with age (Redfield et al. 2005).

Diastolic heart failure (DHF) is defined as heart failure with preserved systolic function accompanied with other signs of heart failure. DHF is diagnosed by echocardiography as the normal ejection fraction (EF >40-45%) combined with diastolic dysfunction. Preserved EF is detected in
24% to 55% of HF patients depending on the study population (Gorelik et al. 2009). DHF patients are usually older females. They are more often hypertensive, obese, and suffering from chronic obstructive pulmonary disease or atrial fibrillation, but not from coronary artery disease. They may well receive calcium antagonists, but are less likely to be treated with ACEIs or digoxin (Gorelik et al. 2009). A study on patients with hypertensive pulmonary edema has shown that EF during an episode of acute hypertensive pulmonary edema is similar to that measured after the treatment of the hypertension. (Gandhi et al. 2001). Thus, a normal EF after the BP has been controlled, indicates with high probability that the pulmonary congestion was due diastolic dysfunction. However, in patients with AHF, echocardiographic EF may correlate poorly with hemodynamic measures of the left ventricular contractility and outcome (Uriel et al. 2005). One possibility is that heart failure in patients with diastolic dysfunction might be paroxysmal rather than chronic (Banerjee et al. 2004). Thus, an acute worsening of diastolic function may be one of the main echocardiographic findings in patients with AHF, present predominantly in emergency units.

Chronic renal dysfunction is one of the strongest risk factors for mortality in patients with chronic HF. In this respect, renal function is considered as useful indicator for heart failure prognosis. Worsening of renal function has been diagnosed also in patients hospitalised for AHF (Metra et al. 2008b) and it may be a marker of the disease severity. The best single predictor for mortality in AHF patients admitted to hospital according to ADHERE registry in the United States was the presence of concomitant high admission levels of blood urea nitrogen followed by low admission systolic blood pressure and then by high levels of serum creatinine (Fonarow et al. 2005).

Activation of neurohormonal pathways and inflammatory cascades has been known to contribute to the chronic HF for a long time. The participation of neurohormones and inflammation in AHF is more complex and has been less extensively studied. However, it has been shown that the levels of both neurohormones and inflammatory markers increase during the acute phase of AHF (Milo et al. 2003). In addition, a high lymphocyte ratio has been observed to be related to high BP (Cotter et al. 2008). A low lymphocyte ratio, on the other hand, was related to higher troponin and more severe recurrent HF and death (Cotter et al. 2008). The parallel increase in systolic BP and lymphocytes supports the idea that there is an interaction between inflammatory activation and increased arterial stiffness, further evidence for a link between inflammatory and hemodynamic events in AHF (Vlachopoulos et al. 2005).

The Cardiac Resynchronization-Heart Failure (CARE-HF) trial (Cleland et al. 2001) showed that patients with chronic heart failure and cardiac dyssynchrony benefitted from cardiac resynchronization therapy (CRT). Not only were the cardiac function and symptoms improved and
the hospitalization and complications reduced, but also the mortality was reduced (Cleland et al. 2005). In addition, a recent study utilizing exercise Doppler echocardiography, showed that the response to CRT largely depended on the presence of contractile reserve collaterally with the extent of LV dyssynchrony and the severity of mitral regurgitation (Moonen et al. 2008). However, the importance of dyssynchrony in AHF is obscure. The only study on dyssynchrony in AHF, showed that the intraventricular conduction delay was associated with more adverse outcome in patients admitted with AHF (Brophy et al. 1994). Subsequently, a multicenter, international, observational study, Karolinska-Rennes (KaRen), has been designed to characterize electrical and mechanical dyssynchrony and to assess its prognostic impact in patients presenting acute heart failure with preserved ejection fraction (Donal et al. 2009).

2.3.2 Current pharmacological management of AHF

Diverse pharmacological agents are used in the treatment of AHF. Nevertheless, there is sparse data from clinical trials, mostly done with heterogenous patient populations without appropriate randomization. These trials have shown improvements in hemodynamics, but none of the agents used have reduced mortality. The multiple mechanism of action of the drugs in use are listed in table 1.

**Opiates**

Traditionally morphine has been used in the treatment of acute pulmonary oedema and it is still in practise for relieving the symptoms of AHF. ESC guidelines states that morphine should be considered in the early stage of the treatment of patients with severe AHF, particularly in patients with restlessness, anxiety, or chest pain (Dickstein et al. 2008). However, morphine can cause many adverse side-effects, including depressed respiration, which need to be monitored.

**Diuretics**

Administration of diuretics is an effective treatment for symptoms secondary to congestion and volume overload and is recommended for the sodium and water retention in AHF. The loop diuretics are the most effective and most frequently used diuretics in treating of AHF (Somberg and Molnar 2009) and are therefore recommended either alone or in combination with a vasodilator as initial therapy in patients with volume overload (Amin 2008). *Furosemide*, in turn, is the most often used loop diuretic. Before the increase in urinary sodium and water output, *i.v.* furosemide provides rapid symptomatic relief. This beneficial effect is due to the dilatation of peripheral capacitance vessels, consequently there is a decrease in left ventricular filling pressure and immediate relief of
the symptoms of pulmonary congestion (Jhund et al. 2000; Somberg and Molnar 2009). Plasma vasopressin concentrations are elevated in patients with worsening heart failure due to fluid overload and LV systolic dysfunction. This may lead to fluid retention and hemodynamic abnormalities. Vasopressin antagonists, which are able to treat congestion without causing electrolyte abnormalities or worsening renal function, can relieve the symptoms in AHF. Tolvaptan is one of the most extensively investigated oral vasopressin V2-receptor antagonist that decreases body weight and increases urine volume without inducing renal dysfunction or hypokalemia (Gheorghiade et al. 2005c; Udelson et al. 2008).

Vasodilators

Intravenous vasodilators (nitrates, sodium nitroprusside and nesiritide) are used as a continuous infusion in the treatment of hospitalized patients during the early stages of AHF and ESC guidelines recommend their use for patients without symptomatic hypotension (SBP <90mmHg) or serious obstructive valvular disease (Dickstein et al. 2008). Nitroglycerin is most commonly used intravenous vasodilator in ADHF. It is predominantly a venous vasodilator compared to nesiritine, which dilates both venous and arterial blood vessels and has a combined diuretic and natriuretic effect. The most common adverse effect of nitroglycerin treatment is headache, which occurred in 20% of the patients with ADHF in the VMAC study (VMAC 2002). This was followed by asymptomatic hypotension (8%) and nausea (6%) and there were also some cases of symptomatic hypotension (Elkayam et al. 2008). Nesiritide is a recombinant 32 amino acid human B-type natriuretic peptide (hBNP) and therefore chemically and structurally identical to the endogenous hormone. It was introduced into clinical practice in the USA in 2001. Nesiritide increases cytosolic cGMP levels by binding to NP receptors. It has venous and arterial vasodilatory effects that reduce preload and afterload, and induces coronary vasodilatation. In addition, nesiritide increases cardiac output without having any direct inotropic effects. The use of nesiritide is normally restricted to patients hospitalized with acutely decompensated heart failure and who have dyspnea at rest (Colucci 2001; Maisel et al. 2008).

Inotropic agents

The infusion rate of the inotropic drugs needs to be modified according to symptoms. Also blood pressure should be continuously monitored. Furthermore, weaning patients from the drug infusion needs to be done carefully. Dobutamine is a positive inotropic agent, acting via stimulation of the β1-ARs. It causes dose-dependent positive inotropic and chronotropic effects, starting rapidly after the beginning of the i.v. infusion. Dopamine also stimulates β1-ARs, both directly and indirectly and
is used as an additional inotropic agent. PDEIs inhibit the breakdown of cyclic AMP causing inotropic and peripheral vasodilating effects. Milrinone and enoximone are two PDEIs widely used in clinical practice. They are administered by continuous infusion, evoking an increase in cardiac output and stroke volume, and a simultaneous decrease in pulmonary artery and wedge pressures as well as pulmonary vascular resistance (Dickstein et al. 2008). Compared to beta-agonists, PDEIs preserve hemodynamic effects fully during complete beta-blockade, since their site of action is beyond the beta-adrenergic receptor. Thus, PDEIs have been recommended for inotrope-requiring patients with decompensated heart failure who are undergoing long-term beta-blocking therapy, where a beta-agonist such as dobutamine would be less effective (Bristow et al. 2001). Cardiac glycosides produce a small increase in CO and a reduction of filling pressure. According to ESC guidelines, in AHF, cardiac glycosides like digitalis, may be useful in slowing the vetricular rate in rapid AF (Dickstein et al. 2008). However, a meta-analysis of digitalis studies has revealed that there is no evidence for any benefits in terms of mortality between treatment and control groups. Nonetheless, digitalis therapy lowered the rate of both hospitalization and clinical decline (Hood et al. 2004). Levosimendan is an inodilator compound, having both inotropic and vasodilatory effects. It belongs to the group of calcium sensitizers and its clinical use is discussed in detail in chapter 2.4.

**Vasopressors**

Norepinephrine, the recommended vasopressor in cardiogenic shock, is not a first-line agent and it should be used only if other inotropic agents and fluids do not restore the systolic blood pressure. In addition, sepsis patients with AHF may need vasopressor therapy. Norepinephrine can be used with other inotropic agents, but must be withdrawn as soon as possible. Epinephrine is not recommended as vasopressor or inotropic agent in cardiogenic shock. Its use should be limited to rescue therapy in cardiac arrest (Dickstein et al. 2008).

**Renin angiotensin aldosterone system (RAAS) inhibitors and \( \beta \)-blockers**

Beta-blockers, ACEIs, ARBs as well as aldosterone antagonists have been success stories in drug development for chronic HF. However, there is no consensus about the advantages of ACEI/ARB therapy in AHF. In general, the ESC guideline recommends that treatment with these agents should be initiated before discharge from hospital (Dickstein et al. 2008). In particular, in patients at high risk for development chronic HF, ACEIs and ARBs have an important role in the early management of AHF and acute MI (Dickstein et al. 2008). These agents attenuate remodelling and reduce morbidity and mortality. Patients on ACEI/ARB therapy admitted with worsening HF should have treatment continued with the highest tolerated dose whenever possible (Dickstein et al. 2008).
In patients admitted with acutely decompensated HF, the dose of β-blockers may need to be reduced temporarily. In general, treatment should not be stopped, unless the patient is clinically unstable with signs of low output. In patients admitted with de novo AHF, β-blockers should be considered when the patient has become stabilized on an ACEI or ARB and treatment with the β-blocker preferably initiated before hospital discharge (Dickstein et al. 2008). Data from the Italian survey on acute heart failure, established that in patients hospitalized for worsening HF, non-use or discontinuation of β-blockers was associated with a significant higher mortality (Orso et al. 2009). Furthermore, intravenous β-blockers are not contraindicated in patients with AHF who present with hypertension and/or atrial fibrillation with a rapid ventricular response (Khan et al. 2008).

Cardioselective (β₁-selective) β-blockers are usually used in HF patients, because many of the side-effects of the non-selective blockers i.e. bronchospasm, peripheral vasoconstriction, alteration of glucose and lipid metabolism of these drugs are attributable to their blockade of β₂-receptors. Therefore, β-blockers that selectively block β₁-receptors are thought to produce fewer adverse side-effects than those drugs that non-selectively block both β₁- and β₂-receptors.
<table>
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<tr>
<td>Loop diuretics</td>
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<td>Vasodilators</td>
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<td>Beta-agonists</td>
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<td>Need of inotropic support, low CO, hypotension</td>
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<td></td>
<td>Dopamine</td>
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<td>Tachycardia, arrhythmias, vasoconstriction</td>
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<td>Phosphodiesterase inhibitors</td>
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<td>Cardiac glycosides</td>
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<td>Carvedilol</td>
<td>Adrenergic $\beta_1$- and $\alpha_1$-receptor antagonism</td>
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<td>Cough, hyperkalemia, hypotension, dizziness</td>
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<tr>
<td>Angiotensin converting enzyme</td>
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<td>Inhibition of the conversion of Ang I to Ang II</td>
<td>Attenuation of remodelling and reduction of morbidity and mortality</td>
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<td>Aldosterone antagonists</td>
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<td>Mineralocorticoid receptor antagonism</td>
<td>Attenuation of remodelling and reduction of morbidity and mortality</td>
<td>Hyperkalemia, hypotension, dizziness</td>
</tr>
</tbody>
</table>
2.3.3 Unmet needs for the treatment of AHF

Undoubtedly, therapy for AHF should improve symptoms and hemodynamics of acutely hospitalized patients without adverse effects and the risk of mortality. However, patients with AHF have usually been diagnosed with other cardiac and also noncardiac contributing disorders, such as coronary artery disease, chronic ischemic heart disease, hypertension, atrial fibrillation, type 2 diabetes mellitus. In severe types of the syndrome, there may be cardiorenal or multiorgan failure which needs to be taken into account in the choice of appropriate medication. Ideal medical treatment should prevent myocardial damage, modulate neurohumoral and inflammatory activation, and preserve or even improve renal function (Figure 2.). Moreover, costs of index hospitalization increase depending on the severity of the different classes AHF (Harjola et al. 2009). This has created a growing need for effective treatment for the most severe classes of the syndrome.

There is a vigorous debate of the role of inotropic therapy in the management of AHF and counterarguments have been postulated (Petersen and Felker 2008). Nevertheless, the use of conventional inotropic agents has been shown to have favourable haemodynamic effects in patients with decreased cardiac contractility. According to Acute Decompensated Heart Failure National (ADHERE) Registry, 9.6% of patients hospitalized for AHF in the United States receive intravenous inotrope therapy, either PDEIs or beta1-adrenergic agonists (β1-AR agonists) (Abraham et al. 2005). These patients tended to have a clinical profile associated with more severe disease, including lower blood pressure, lower ejection fraction, and higher blood urea nitrogen. The use of PDEIs and β1-AR agonists in heart failure has consistently been associated with increased myocardial oxygen demand, more cardiac arrhythmias, and worsened mortality in many clinical trials. This unsatisfactory outcome is believed to be related to the fact that these agents increase myocardial concentrations of cAMP, leading to an increase in the level of intracellular calcium which in turn can trigger myocardial cell death and provoke lethal arrhythmias. Furthermore, according to FINN-AKVA, the mortality increased in proportion to the number of inotropes used (Rossinen et al. 2008). The administration of inotropes was related to low BP, low ejection fraction, elevated C-reactive protein and cardiac markers and was a marker of increased mortality in patients with AHF. However, the use of a single inotrope during hospitalization appeared to be rather safe (Rossinen et al. 2008).

For these reasons, there is a clear need for improvements in therapy. Some new drugs have been recently introduced for the treatment of AHF. One of them is istaroxime, a new agent with inotropic and especially powerful lusitropic properties, that is improvement in relaxation. It inhibits the sodium–potassium ATPase (Na⁺-K⁺ pump) and stimulates the sarcoplasmic reticulum calcium
ATPase isoform 2 (SERCA-2) and is consequently believed to improve contractility as well as causing diastolic relaxation (Khan et al. 2009). Data from human phase II study (HORIZON-HF) indicated that istaroxime decreases PCWP and possibly improves diastolic function without evoking any significant change in heart rate (HR), blood pressure, ischemic or arrhythmic events (Blair et al. 2008; Gheorghiade et al. 2008). However, the mechanism of action of istaroxime means that it can contribute to an increase in cytosolic calcium [Ca\textsuperscript{2+}], in a manner similar to the cardiac glycosides, and to this extent, harmful effects attributable to increased [Ca\textsuperscript{2+}], cannot be excluded. In addition, the enhanced calcium uptake in the SR causes a greater amount of calcium release in every diastole-systole cycle. Cardiac-specific myosin ATPase activators are another novel class of agents designed to improve myocardial contractility. They accelerate the productive phosphate-release step of the crossbridge cycle (Bragadeesh et al. 2007). CK-1827452 is a new agent that directly activates myosin, prolonging the duration of time that myosin remains in a force-generating reaction with actin and enhancing the extent of myocyte shortening, with no effect on the Ca\textsuperscript{2+} transient (Solaro 2009).

Calcium sensitization is a mechanism that is thought to resolve many of the disadvantages of the therapies that increase the cytosolic calcium concentration [Ca\textsuperscript{2+}]. Calcium sensitizers do not increase the energy required for Ca\textsuperscript{2+} handling and are able to reverse contractile dysfunction under pathophysiological conditions. However, calcium sensitizers carry a potential risk of the diastolic dysfunction due to the impaired relaxation, which will reduce the rate of ventricular filling (Endoh 2008). A set of compounds has been under development as calcium sensitizers during the past two decades. Most of them have a combination of mechanism of action of calcium sensitization and PDE3 inhibition. Some of them have only weak PDE3 inhibitory effect like EMD 57033, MCI-154, Org 30029 and SCH00013, but also pure calcium sensitizers, like CGP 48506, have been developed (Endoh 2002; Kass and Solaro 2006). However, none of them have been a success story in the clinic. Pimobendan is a calcium sensitizer with potent PDE3 inhibitory properties in the same concentration range (Endoh 2002). It is approved in HF patients in Japan only (Kass and Solaro 2006). However, pimobendan has been used in the clinical management of CHF secondary to both dilated cardiomyopathy (DCM) and chronic degenerative valvular disease in dogs (Gordon et al. 2006).
2.4 Levosimendan

2.4.1 Physicochemical properties

Levosimendan is commercially available as a powder, to be dissolved in a solution of glucose 5% in water and administered as an intravenous infusion. The chemical name of levosimendan is (-) (R)-[[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl) phenyl] hydrazono] propanedinitrile (WHO Drug Information Vol. 7, No. 3, 1993). Its molecular weight is 280.291 and the molecular formula C_{14}H_{12}N_{6}O (Figure 4). Levosimendan, is a weak acid and a moderately lipophilic compound with a pKa value of 6.2.
2.4.2 Pharmacokinetics

The molecule has a short elimination half-life of approximately 1 h, and is 95–98% bound to plasma proteins. The drug is mostly metabolized through glutathione conjugation at one of its nitrile groups followed by amino acid cleavage and cyclization or acetylation (Lehtonen et al. 2004; Koskinen et al. 2008). Even though it is administered intravenously, levosimendan is excreted into the small intestine and reduced by intestinal bacteria to an amino phenolpyridazinone metabolite (OR-1855), which is further metabolised by acetylation to N-acetylated conjugate (OR-1896). The circulating metabolites OR-1855 and OR-1896 are formed slowly, and their maximum concentrations are seen on average 2 days after cessation of a 24 h infusion. The haemodynamic effects after levosimendan infusion appear to be similar between fast and slow acetylators. OR-1896, an active metabolite, is approximately 40% bound to plasma proteins. It has a longer half life (75 to 80 h) than levosimendan and it is responsible for the prolonged hemodynamic effects, which can be detected for 7–9 days after a 24 h continuous infusion of levosimendan (Antila et al. 2007; Puttonen et al. 2007). The hemodynamic effects after levosimendan infusion are thus, combination of the acute effects by levosimendan itself and the long lasting effects by OR-1896.

2.4.3 Mechanism of action

2.4.3.1 Positive inotropic effect

Since it is a calcium sensitizer, levosimendan does not increase $[\text{Ca}^{2+}]_i$. It rather increases myofilament calcium sensitivity by binding to cTnC in a calcium-dependent manner. Levosimendan binds to the calcium-saturated N-terminal domain of troponin C and stabilizes the troponin molecule with subsequent prolongation of its effect on the contractile proteins (Pollesello et al. 1994). The heart contracts when calcium binds to cTnC and it relaxes when calcium dissociates from cTnC. Therefore, the calcium saturated form of cTnC is an ideal target for calcium sensitisers (Haikala and Linden 1995). Furthermore, a desirable feature of a calcium sensitizer is that it should not impair the relaxation of cardiac muscle, i.e. it must detach from cTnC when $\text{Ca}^{2+}$ dissociates. In fact,
Levosimendan behaves in this way (Figure 5.) (Pollesello et al. 1994; Levijoki et al. 2000; Sorsa et al. 2004; Antoniades et al. 2007). In addition, levosimendan has been shown to actually enhance diastolic function in pacing induced heart failure dogs during exercise (Tachibana et al. 2005). It is also essential that an efficacious calcium sensitizer must not prevent or inhibit any of the protein–protein interactions required for muscle contraction and relaxation, and furthermore, it should not exert any detrimental actions on troponin’s functions, levosimendan does not (Sorsa et al. 2004).

2.4.3.2 Stereoselective interaction with cardiac troponin C

Lefvosimendan has a chiral carbon atom in its pyridazine ring (Figure 4.). Therefore, it has an optical stereoisomer, dextrosimendan. Levosimendan and dextrosimendan are the (R)-(−) and (S)+(+) enantiomers of simendan. The interaction of simendan stereoisomers on cTnC has been shown to be different in the absence of cardiac troponin I (cTnI) (Sorsa et al. 2004). Both stereoisomers interacted with both, C- and N-domains of the isolated cTnC. However, levosimendan has been shown to have an order of magnitude higher affinity for the N-domain than dextrosimendan (Sorsa et al. 2004). The binding of calcium ion to the N-domain of cTnC appropriately initiates the contraction of the cardiac muscle, and thus, explains the stereospecific mode of action of the simendans in cardiac preparations.

The positive inotropic effect of levosimendan has been demonstrated in many in vitro experiments. Levosimendan has been shown to increase cardiac contractility in isolated cardiomyocytes, papillary muscles, atrial muscle strips, ventricular muscle strips, and in isolated hearts (Haikala et al. 1995; Lancaster and Cook 1997; Hasenfuss et al. 1998; Janssen et al. 2000). In in-vivo experimental models, levosimendan has shown positive inotropic effects in normal rats, dogs, cats and rabbits. In addition, levosimendan has been proven to be even more potent in animal model of heart failure (Levijoki et al. 2001; Louhelainen et al. 2007; Masutani et al. 2008).

One plasma metabolite of levosimendan which possesses biological activity, OR-1896, is also a potent positive inotropic compound. In anaesthetised dogs, the overall cardiovascular pharmacodynamic profile of OR-1896 was shown to be rather similar to that of levosimendan (Banfor et al. 2008). Levosimendan and OR-1896 were intravenously infused at 30 min intervals in cumulatively increasing doses. They were hemodynamically active in anaesthetised dogs and elicited equivalent cardiovascular, whereas another metabolite, OR-1855 was inactive (Banfor et al. 2008). In anesthetized rats, levosimendan and OR-1896 have also been shown to have similar hemodynamic effects (Banfor et al. 2008; Segreti et al. 2008). The relatively long-lasting duration
of levosimendan’s positive inotropic effect and the possible accumulation of OR-1896 after prolonged administration of levosimendan may modify the effects of levosimendan.

Figure 5. Mechanism of levosimendan as an inotropic agent. (A) Levosimendan binds to troponin C during systole, increasing the sensitivity of myofilaments to Ca$^{2+}$ levels. This phenomenon increases the contractility of myocardium during systole, but it does not affect diastolic function. (B) The presence of levosimendan leads to an opening of the active sites of troponin C, increasing in this way its sensitivity to Ca$^{2+}$. Reprinted from Pharmacol Ther, 114 (2), Antoniades, C., D. Tousoulis, N. Koumallos, K. Marinou and C. Stefanadis, “Levosimendan: beyond its simple inotropic effect in heart failure”, Pages 184-97, Copyright (2007), with permission from Elsevier.

2.4.3.3 Vasodilatory effect

Levosimendan has a pronounced vasodilatory effect. The opening of the K$_{ATP}$ channels is believed to be the main mechanism of the vasodilatory effect of levosimendan. This has been shown in arterial and venous preparations as well as in coronary arteries. In rat mesenteric arterial myocytes, levosimendan hyperpolarized the cells, probably through the activation of K$_{ATP}$ channels, i.e. an effect which is known to have a vasodilating action (Yokoshiki et al. 1997). Furthermore, in the isolated rat small mesenteric artery preparation, levosimendan had a relaxant response in KCl precontracted arteries. Glibenclamide, a K$_{ATP}$ channel blocker, inhibited the response, supporting
the theory that the opening of $K_{ATP}$ channels was the important vasodilatory mechanism. The response did not differ significantly between endothelium-intact and endothelium-denuded preparations (Ozdem et al. 2006). In isolated human portal vein, levosimendan was found to be about 16-fold more potent as a relaxing agent than cromakalim in noradrenaline-precontracted portal venous preparations. Glibenclamide totally inhibited the cromakalim-induced relaxation of the portal vein. However, the effect of levosimendan was only partially prevented (Pataricza et al. 2000), indicating that levosimendan might also have some other vasodilatory effects in addition to opening of the $K_{ATP}$ channels.

Subsequently, in isolated porcine epicardial coronary arteries, it has been shown that the specific $BK_{Ca}$ channel blocker, iberiotoxin (IBTX), could suppress the maximum effect of levosimendan, and the KV channel blocker, 4-aminopyridine (4-AP) significantly shifted the concentration-response curve to the right. This indicated that the vasorelaxing mechanism of levosimendan involves the activation of voltage-sensitive and, at large concentrations, calcium-activated potassium channels (Pataricza et al. 2003). However, IBTX and 4-AP were not able to suppress the vasodilatory effect of levosimendan in isolated rat small mesenteric arteries (Ozdem et al. 2006). In addition, studies with porcine coronary arteries suggest that levosimendan may also interact with smooth muscle EF-hand proteins, such as, calmodulin, the regulatory myosin light chains, or S100 proteins and thus, relax coronary smooth muscle through calcium desensitization (Bowman et al. 1999). Furthermore, the role of PDE3 inhibition cannot be excluded, since levosimendan has been shown to potentiate the relaxant effect of a cAMP-stimulating drug, isoprenaline in isolated porcine arteries (Gruhn et al. 1998).

The vasodilatory effect of OR-1896 has been shown to be comparable to that of levosimendan in isolated, pressurized rat coronary and skeletal muscle arterioles. The vasodilatation was proposed to be mediated primarily by activation of $BK_{Ca}$ and $K_{ATP}$ channels, respectively (Erdei et al. 2006). In anaesthetised rats and dogs, the vasodilatory effect of OR-1896 is rather similar to that of levosimendan (Banfor et al. 2008; Segreti et al. 2008).

Thus, it can be concluded that levosimendan predominantly stimulates $K_{ATP}$ channels in small resistance vessels. In large conductance vessels, the vasodilatation may be mediated mainly through opening of KV as well as $BK_{Ca}$ channels. Moreover, the duration of the vasodilatory effect is proportionately long-lasting, because of the formation of an active metabolite, OR-1896.
2.4.3.4 Anti-ischemic effect

Endogenous protective mechanisms in heart cells, so called preconditioning, have been previously claimed to be mediated via $K_{ATP}$ channels. Preconditioning is known to exist in many mammalian species and tissue types. It is described as a cardiac phenomenon in which a short period of ischemia will protect the heart from a subsequent, much longer, ischemic episode (Grover and Garlid 2000).

The opening of the sarcolemmal $K_{ATP}$ (sarc$K_{ATP}$) channels in vascular smooth muscle cell, resulting in membrane hyperpolarization and vasodilatation, was previously believed to be behind this protective mechanism. Subsequently, mitochondria $K_{ATP}$ (mito$K_{ATP}$) channels have been described (Liu et al. 1999; O'Rourke 2004) and the mitochondrial hypothesis has become more generally accepted. Furthermore, it has been shown that $K_{ATP}$ channel openers can induce vasodilatation by activating two different signaling mechanisms, one pathway that is mitochondrial and another pathway that involves sarc$K_{ATP}$ channel activation (Adebiyi et al. 2008). That study indicated that the $K_{ATP}$ channel openers, pinacidil and diazoxide, could induce vasodilatation by two distinct mechanisms. The action of pinacidil required the sulfonylurea receptor (SUR 2B) whereas diazoxide targeted the mitochondrial electron transport chain (ETC).

$K_{ATP}$ openers have been shown to mimic preconditioning and the results from several studies with a variety of $K^+$ channel openers have demonstrated their effects on mitochondrial function (O'Rourke 2004). This is evidence for a link between the mito$K_{ATP}$ channels and protection against ischemic injury in intact hearts (Garlid et al. 1997) and isolated myocytes (Liu et al. 1998). The mechanisms believed to be behind this protective effect are prevention of mitochondrial calcium overload, preservation of high energy phosphates and regulation of mitochondrial volume (O'Rourke 2004). Mitochondrial Ca$^{2+}$-activated $K^+$ (mito$K_{Ca}$) channels also exist in cardiac myocytes, and are known to play a key role in cardioprotection. Potassium influx through mito$K_{ATP}$ or mito$K_{Ca}$ channels occurs independently of each other and induces cardioprotection in a similar manner (Nishida et al. 2009). Activation of mito$K_{ATP}$ channel is enhanced by protein kinase C (PKC), whereas mito$K_{Ca}$ channel is activated by protein kinase A (PKA) (Nishida et al. 2009).

Levosimendan has been demonstrated to open mito$K_{ATP}$ channels in liver, but also in cardiac preparations, indicating that it may have a cardioprotective effect. In the rat liver mitochondria, levosimendan at a concentration of 0.7-2.6 μM could open the mito$K_{ATP}$ channels (Kopustinskiene et al. 2001). The effect was abolished by the selective mitochondrial $K_{ATP}$ channel blocker 5-hydroxydecanoate. In addition, levosimendan up to 2.2 μM had no effect on the respiration rate of rat liver mitochondria. In the rat cardiac cells, levosimendan activated potassium flux to the mitochondrial matrix (median effective concentration, EC$_{50}$, 0.83 +/- 0.24 μM) (Kopustinskiene et
al. 2004). The anti-ischemic effect has been seen also in a dog-infarction model, where levosimendan (24 μg/kg bolus followed by an infusion of 0.4 μg/kg/min), when given before ischemia, reduced the infarct size by 50%. This reduction of infarct size was abolished by treatment with the K<sub>ATP</sub> channel blocker glibenclamide, indicating that levosimendan had an anti-ischemic effect, which was mediated via opening of the K<sub>ATP</sub> channels (Kersten et al. 2000).

In addition, oral simendan (the racemate of levosimendan) has been shown to improve survival in rats with healed myocardial infarction during the follow-up period of 312 days. The incidences of mortality in control, simendan, enalapril and milrinone groups were 81%, 53%, 56% and 63%, respectively. The calculated mean daily dose of simendan was 2-2.5 μg/kg from the 10th week onwards. The mean plasma concentration of simendan was 97 ng/ml at the end of the follow-up period. The hemodynamic responses to simendan, measured prior to sacrifice, had been well preserved i.e. no tolerance developed to the hemodynamic effects of simendan (Levijoki et al. 2001).

2.4.3.5 **Antiaggregatory, anti-inflammatory and antiapoptotic effects**

Levosimendan has been shown to inhibit concentration-dependently the platelet aggregation induced by ADP (5 and 10 μM), and collagen (2 and 5 μg/mL) in human platelet-rich plasma. Levosimendan inhibited also the secondary wave of platelet aggregation induced by ADP, indicating that it may inhibit platelet signal transduction, e.g. thromboxane A2 formation (Kaptan et al. 2008). In vitro levosimendan was able to reverse interleukin (IL)-5-related survival of human eosinophils i.e. it increased the number of apoptotic eosinophils in the presence of 10 pM IL-5 with an EC50 value of 6.5 ± 0.7 μM. The increase in the number of apoptotic cells was confirmed by the percentage of annexin V-positive cells, which were in the absence or presence of levosimendan (10 μM) 7 ± 1 and 86 ± 4%, respectively (Kankaanranta et al. 2007). Moreover, levosimendan and dextrosimendan decreased NO production in a dose-dependent manner in macrophages and in fibroblasts exposed to inflammatory stimuli. They reduced IL-6 production slightly but they had no effect on TNF-α synthesis (Sareila et al. 2008).

2.4.4 **Effects in combined heart and renal failure model**

When administered via the drinking water, levosimendan dose-dependently prevented cardiomyocyte apoptosis and normalized salt-induced increased expression of natriuretic peptide, and decreased urinary noradrenaline excretion in salt-sensitive hypertensive Dahl/Rapp rats (Louhelainen et al. 2007). Preventative treatment with levosimendan for seven weeks in rats fed a high-salt diet, improved also survival, increased cardiac function, and ameliorated cardiac
hypertrophy. In addition, levosimendan also corrected salt-induced declines in myocardial SERCA2a protein expression and myocardial SERCA2a/NCX-ratio. In conclusion, the modulation of pro-inflammatory and pro-apoptotic pathways by levosimendan may be part of its beneficial effects in the progression of decompensated heart failure.

2.4.5 Effects in stroke models

In addition to the above mentioned studies, there is strong experimental evidence that levosimendan may have preventive effects on stroke. Recent unpublished studies in experimental models of primary and secondary prevention of stroke, confirmed by using MRI and/or histology, have demonstrated this effect. Oral treatment with levosimendan prevented strokes and mortality in Dahl salt-sensitive and stroke-prone spontaneously hypertensive rats. The effects were seen at low daily doses of levosimendan alone and in combination with the angiotensin II receptor blocker, valsartan. An international patent for a combination treatment with levosimendan and ACE inhibitor or AngII receptor blocker has been accepted and published (Sallinen et al. 2009).

2.5 Clinical use of levosimendan

Levosimendan is the only calcium sensitizer, which is widely used in the clinical management in humans. Today it has an established place in the treatment of AHF. Levosimendan increases myofilament calcium sensitivity and has a highly selective PDE3 inhibitory action. It has been shown to enhance cardiac contraction without increasing oxygen consumption, and thus, having an energetically advantageous effect on the contraction (Ukkonen et al. 1997; Ukkonen et al. 2000). Levosimendan induces vasodilatation by opening the sarcolemmal K_{ATP} channels in the resistance arteries. There is some evidence that it may be also a venodilator and therefore, reduce the central venous pressure and improve the pulmonary congestion, and this may be the mechanism behind the observed improvement in renal function (Yilmaz et al. 2007). Moreover, levosimendan-evoked mitochondrial K_{ATP} channel opening may have cardioprotective effects due to the ATP synthesis during ischemia. In summary, the positive effects on the energy balance could be of major significance in the use of levosimendan in the treatment of AHF (Nieminen et al. 2009).

2.5.1 Use in acute heart failure

To date, levosimendan has been approved in 47 countries worldwide for short-term treatment of AHF. According to ESC guidelines, levosimendan infusion is recommended in acutely decompensated heart failure and may be effective in patients with decompensated chronic HF, too (Dickstein et al. 2008). The effects of levosimendan on the symptoms of AHF have been evaluated
in several clinical trials. The effects on overall hemodynamics in different trials are presented in table 3. Some of these trials have suggested that levosimendan might improve prognosis and mortality in patients with decompensated heart failure. There are three trials, LIDO, RUSSLAN and CASINO, which were carried out in patients with high filling pressures, where intravenous levosimendan was compared with placebo or dobutamine. The mortality rate of levosimendan was statistically significantly lower in the levosimendan group than in the dobutamine group (LIDO) or placebo group (RUSSLAN) or in both (CASINO) (Follath et al. 2002; 2002; Cleland et al. 2004; Lehtonen 2004; Lehtonen and Poder 2007). However, two larger trials (SURVIVE and REVIVE II) in patients who were hospitalized for worsening of heart failure, but in whom it was not essential to measure the filling pressure, indicated that though levosimendan did improve the symptoms of HF, it did not improve survival (Cleland et al. 2006; Lehtonen and Poder 2007; Mebazaa et al. 2007) (Table 2.). Nevertheless, levosimendan proved beneficial compared to dobutamine in specific subgroups of patients in the SURVIVE trial. Levosimendan treatment lowered all-cause mortality during the first weeks following treatment, in patients admitted with acute decompensated heart failure and with a known history of CHF and/or currently treated with oral β-blockers (Mebazaa et al. 2009).
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Number of patients (total / LS)</th>
<th>Type of patients</th>
<th>Levosimendan dose</th>
<th>Primary end point</th>
<th>Secondary end points</th>
<th>Prespecified follow-up time</th>
<th>Effects on primary end point</th>
<th>Main outcome on mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>European dose finding study</td>
<td>Randomized, placebo controlled, parallel group (fixed-dose)</td>
<td>151/95</td>
<td>NYHA III–IV CHF* with ischemic etiology (EF &lt;40%)</td>
<td>Bolus 3–36 μg/kg + infusion of 0.05, 0.1, 0.2, 0.4 or 0.6 μg/kg/min for 24 hrs</td>
<td>Hemodynamic improvement (response criteria based on SV and PCWP)</td>
<td>Individual hemodynamic parameters</td>
<td>7 days</td>
<td>Levosimendan was well tolerated and leads to favorable hemodynamic effects</td>
<td>No difference compared to placebo and dobutamine</td>
</tr>
<tr>
<td>US dose duration study</td>
<td>Randomized, placebo controlled, parallel group (forced uptitration + withdrawal)</td>
<td>146/98</td>
<td>Decompensated CHF (PCWP ≥15 mmHg, CI ≤2.5 L/min/kg)</td>
<td>Repeated bolus of 6 μg/kg and infusion of 0.1–0.4 μg/kg/min for 24 or 48 hrs</td>
<td>Hemodynamic improvement (response criteria based on SV and PCWP)</td>
<td>Individual hemodynamic parameters, symptoms of heart failure, 14-day outcome</td>
<td>14 days</td>
<td>Levosimendan was well tolerated and leads to favorable hemodynamic effects</td>
<td>No difference compared to placebo</td>
</tr>
<tr>
<td>LIDO</td>
<td>Randomized, double blind (double dummy) comparison between levosimendan and dobutamine</td>
<td>203/103</td>
<td>Severe low-output heart failure requiring inotropic support</td>
<td>Levosimendan bolus of 24 μg/kg and infusion of 0.1–0.2 μg/kg/min versus dobutamine 6-12 μg/kg/min for 24 hrs</td>
<td>Hemodynamic improvement (response criteria based on CO and PCWP)</td>
<td>Symptoms of heart failure, 31-day outcome</td>
<td>31 days*</td>
<td>Levosimendan improved haemodynamic performance more effectively than dobutamine</td>
<td>Levosimendan was superior compared to dobutamine</td>
</tr>
<tr>
<td>RUSSLAN</td>
<td>Randomized, placebo controlled, parallel group</td>
<td>504/402</td>
<td>Heart failure after acute myocardial infarction and clinical need for inotropic support</td>
<td>Levosimendan bolus of 6, 12 or 24 μg/kg and infusion of 0.1, 0.2 or 0.4 μg/kg/min for 6 hrs</td>
<td>Safety (clinically significant ischemia or hypotension)</td>
<td>Symptoms of heart failure, 14-day mortality</td>
<td>14 days*</td>
<td>Levosimendan did not induce hypotension or ischaemia.</td>
<td>Levosimendan was superior compared to placebo</td>
</tr>
<tr>
<td>CASINO</td>
<td>Randomized, comparison between levosimendan, dobutamine and placebo</td>
<td>299/100</td>
<td>Patients hospitalized with NYHA class IV HF, LVEF ≤35%</td>
<td>Levosimendan bolus of 16 μg/kg and infusion of 0.2 μg/kg/min versus placebo / dobutamine 10 μg/kg/min for 24 hrs</td>
<td>Mortality at 1 month, 6 months and 1 year</td>
<td>Death or rehospitalization due to worsening heart failure</td>
<td>1 year (the study was prematurely halted by the data safety monitoring board, citing the superior results of levosimendan)</td>
<td>Levosimendan was superior compared to placebo and dobutamine</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Number of patients (total / LS)</td>
<td>Type of patients</td>
<td>Levosimendan dose</td>
<td>Primary end point</td>
<td>Secondary end points</td>
<td>Prespecified follow-up time</td>
<td>Effects on primary end point</td>
<td>Main outcome on mortality</td>
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<tr>
<td>REVIVE-2</td>
<td>Randomized, placebo controlled, parallel group (fixed dose)</td>
<td>600/299</td>
<td>Primary or secondary heart failure, LVEF &lt;35%, breathlessness at rest despite diuretic and vasodilator therapy</td>
<td>Levosimendan bolus of 6–12 μg/kg infusion of 0.1–0.2 μg/kg/min versus placebo for 24 hrs</td>
<td>Death or worsening heart failure over 5 days with a composite end point consisting of the patient’s self-assessment of symptoms together with a physician’s assessment of the occurrence of clinical deterioration</td>
<td>Mortality at 90 days, duration of hospital stay, change in BNP at 24 hours, patient global assessment and dyspnea at 6 hours</td>
<td>90 days</td>
<td>Levosimendan was superior on the composite primary outcome compared to placebo</td>
<td>No difference compared to placebo</td>
</tr>
<tr>
<td>SURVIVE</td>
<td>Randomized, double blind (double dummy) comparison between levosimendan and dobutamine</td>
<td>1327/664</td>
<td>Heart failure with features of low cardiac output (oliguria), LVEF ≤30%, breathlessness at rest despite diuretic and vasodilator therapy</td>
<td>Levosimendan bolus of 12 μg/kg and infusion of 0.1–0.2 μg/kg/min versus dobutamine 5 μg/kg/min for 24 hrs</td>
<td>180-day all-cause mortality, 31-day all-cause mortality, 180-day cardiovascular mortality, days alive and out of hospital for 180 days, change in BNP at 24 hours, symptoms of heart failure</td>
<td>180 days</td>
<td>No overall difference compared to dobutamine, however, levosimendan was superior in patients on β-blockers compared to dobutamine</td>
<td>No difference compared to placebo</td>
<td></td>
</tr>
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</table>

1 (Nieminen et al. 2000), 2 (Slawsky et al. 2000; Kivistikko et al. 2003), 3 (Follath et al. 2002), 4 (Moiseev et al. 2002), 5 (Cleland et al. 2004; Lehtonen 2004), 6 (Cleland et al. 2006), 7 (Mebazaa et al. 2007); a Six-month follow-up data has been collected for both LIDO and RUSSLAN as a part of the drug approval process.

BNP = b-type natriuretic peptide; CHF = congestive heart failure; CI = cardiac index; CO = cardiac output; EF = ejection fraction; HF = heart failure; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; PCWP = pulmonary capillary wedge pressure; SV = stroke volume.

2.5.2 Additional clinical use

In addition to its use in ADHF, as recommended according to ESC guidelines, levosimendan has found increasing off-label use also for other cardiovascular symptoms in acute cardiovascular events and in intensive care units. Ischemic heart disease and septic shock as well as perioperative cardiac support are conditions where the use of levosimendan has been shown to have beneficial clinical effects.

2.5.2.1 Ischemic heart disease and cardiogenic shock

Cardiogenic shock (CS) is the leading cause of death in patients hospitalized for acute myocardial infarction (AMI) (Hochman et al. 2000). The syndrome has been defined as the inability of the heart, with its impairment of pumping function, to deliver sufficient blood flow to the tissues to meet resting metabolic demands (Califf and Bengtson 1994). The symptoms of CS are diagnosed as the combination of low mean arterial blood pressure, low cardiac index (CI), elevated PCWP, and an increase in systemic vascular resistance index (Hochman et al. 2000). Moreover, there is believed to be a systemic inflammatory response owing to the release of inflammatory cytokines and the expression of inducible nitric oxide synthase and extraneous vasodilatation may also play an important role (Kohsaka et al. 2005). Furthermore, patients with large MIs often have signs of inflammation like elevation of body temperature, white blood cell count, complement, interleukins, C-reactive protein, and other inflammatory markers (Hochman 2003) (Figure 3.). Early revascularization, either by percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG) surgery, has been shown to increase the long-term survival (Hochman et al. 2006).

Levosimendan infusion was shown to improve hemodynamics in critically ill patients with CS requiring catecholamine therapy (Delle Karth et al. 2003). In patients with AMI complicated by severe and refractory CS, therapy with levosimendan added to current therapy resulted in a better outcome and may have contributed to improved survival compared with phosphodiesterase inhibitor, enoximone (Fuhrmann et al. 2008). Particular, in the levosimendan-treated patients, no deaths because of multiorgan failure (MOF) were observed compared to mortality of 25% in the enoximone group. It is worth noting that in the multicenter trial SURVIVE, where levosimendan failed to improve mortality in acute heart failure compared with dobutamine, all patients with CS were excluded (Mebazaa et al. 2007). In addition, clinical data demonstrate that levosimendan does not increase markers of oxidative and nitrosative stress, in contrast to placebo treatment, in patients with advanced chronic heart failure (Parissis et al. 2008). Thus, the severity of heart failure may be
quite different in different patient populations and the occurrence of MOF and the systemic inflammatory response can be variable. Furthermore, in patients having ADHF with ischemic cardiomyopathy and LV ejection fraction (LVEF) <40%, left atrial functions, calculated from left atrial volume i.e. the active emptying fraction, the passive emptying fraction, and the reservoir fraction were respond better to levosimendan than to dobutamine (Duygu et al. 2008). In conclusion, pharmacological modulation of mitochondrial $K_{ATP}$ channels may be beneficial in patients at risk of myocardial ischemia, particularly those requiring inotropic support.

Figure 3. Shock paradigms. Classic shock paradigm is shown in black. The influence of the inflammatory response syndrome initiated by a large MI is illustrated in gray. Left ventricular end diastolic pressure (LVEDP); Cardiac output (CO); Stroke volume (SV); Systemic vascular resistance (SVR); Inducible nitric oxide synthase (iNOS); Nitric oxide (NO). Modified from (Hochman 2003) Circulation 107(24): 2998-3002.
2.5.2.2 Sepsis and septic shock

Sepsis is referred to as a systemic disease caused by microbial invasion of normally sterile parts of the body. More accurately defined, sepsis is a set of non-specific inflammatory responses with evidence, or suspicion, of a microbial origin (Lever and Mackenzie 2007). Severe sepsis is accompanied by evidence of hypoperfusion or dysfunction of at least one organ system. Septic shock, in turn, is sepsis accompanied by hypotension or the need for vasopressors, despite adequate fluid resuscitation. Severe sepsis and septic shock together are some of the leading causes of death in noncoronary intensive care units. Increasing severity of sepsis correlates with increasing mortality, i.e. 25-30% for severe sepsis up to 40-70% for septic shock (Lever and Mackenzie 2007).

Myocardial depression is related to symptoms of sepsis, although cardiac output (CO) may be preserved or even increased. This is explained by a maintained stroke volume due to a dilated ventricle and an increased heart rate (Rabuel and Mebazaa 2006). Sepsis can induce myocardial dysfunction which is seen in both ventricles. Echocardiography studies have noted that 40% to 50% of patients with prolonged septic shock have reduced ejection fraction as a sign of develop myocardial depression (Rudiger and Singer 2007). Alterations in sympathetic beta-adrenoceptor signalling are considered to impair the myocardial response to endogenous and exogenous catecholamines. Moreover, sepsis-induced cardiomyopathy can affect calcium homeostasis in the cardiomyocytes. The calcium current can be disturbed and myofilament calcium sensitivity be decreased (Rudiger and Singer 2007). Calcium is also related to mitochondrial dysfunction, which is believed to be important in the pathophysiology of sepsis, and seems to correlate to the severity of the disease. In brief, calcineurin (CN), activated by calmodulin-kinase (CMK), interacts with the mitochondrial permeability transition pore, triggering mitochondria-related death pathways (Rudiger and Singer 2007). Also circulatory abnormalities related to vasodilatation and vascular leakage are often encountered in sepsis.

The use of levosimendan in patients with septic shock has been described in a few case reports. The drug was reported to improve hemodynamics, global oxygen transport, pulmonary circulation, metabolism and vasopressor requirements, mostly in patients with long-lasting refractory septic shock (Pinto et al. 2008; Salmenpera and Eriksson 2009). Levosimendan can exert beneficial effects in both the left and right ventricles. As a calcium sensitizer, it does not influence the beta-adrenoceptor signalling or evoke changes in the intracellular Ca\(^{2+}\) concentration. Therefore, it was postulated that levosimendan could theoretically be an ideal therapy for sepsis-induced myocardial dysfunction (Pinto et al. 2008). In addition, levosimendan enhances pulmonary and intestinal microcirculation a priori due to its vasodilatory actions and therefore, prevents multi-organ failure.
Moreover, some recent experimental studies have shown that levosimendan has a positive effects in the rat sepsis model of cecal ligation and puncture. Treatment with levosimendan had a marked effect on attenuating or decreasing apoptosis and inflammation in the lung (Erbuyun et al. 2009). In other recent study, levosimendan equally attenuated arterial hypotension, metabolic acidosis and prolonged survival on mortality (Scheiermann et al. 2009).

2.5.2.3 Perioperative cardiac support

Inotropic support is frequently required to wean patients from cardiopulmonary bypass (CBP). In particular, in patients with preoperative impaired ventricular function, weaning failure without medical or mechanical support is a common occurrence occurring in as many as 70% to 80% of cases (Eriksson et al. 2009). Moreover, postoperative renal dysfunction and acute renal failure are frequent and serious complications of cardiac surgery and these are associated with prolonged intensive care unit stay and hospitalization, as well as significant increases in mortality (Mangano et al. 1998).

Levosimendan has been demonstrated to have beneficial effects on postoperative conditions and it has been postulated as a bridge therapy for the perioperative phase of cardiac surgery (Nieminen et al. 2009). Levosimendan has been shown to increase cardiac output without increasing myocardial

Figure 4. Sepsis-induced organ dysfunction. Acute respiratory distress syndrome (ARDS); Acute renal failure (ARF); Disseminated intravascular coagulation (DIC). Modified from (Pinto et al. 2008) Curr Opin Anaesthesiol 21(2): 168-77.
oxygen consumption in patients early after weaning from CPB following coronary artery bypass grafting (CABG) (Lilleberg et al. 1998; Nijhawan et al. 1999). Moreover, the effect of prophylactic levosimendan administration on weaning from and early recovery after CPB in patients with preoperatively impaired left ventricular function undergoing CABG was studied recently. Levosimendan showed a positive outcome and significantly facilitated the primary weaning from CPB compared with placebo (Eriksson et al. 2009). Furthermore, a comprehensive meta-analysis of existing trials to determine the impact of levosimendan on cardiac troponin release in patients undergoing cardiac surgery was done by the senior training project in Center for Overview, Meta-analysis, and Evidence-based Medicine Training (COMET) in Milano (Zangrillo et al. 2009). The results suggested that the use of levosimendan was associated with a significant reduction in cardiac troponin I (cTnI) release in patients undergoing cardiac surgery. The authors hypothesized that the better preservation of early cardiac function with levosimendan may have been the result of the improved global tissue perfusion leading to better recovery from surgery (Zangrillo et al. 2009).

2.5.2.4 Effects in calcium channel poisoning

Poisoning with calcium channel blockers (CCB), accidental or intentional, has become an increasing caseload in the poisoning centers in recent years. This is associated with the more frequent use of these drugs. CCB and beta-blockers account for approximately 40% of the cardiovascular drug exposures reported to the American Association of Poison Centers (DeWitt and Waksman 2004). Verapamil causes the most severe cases and tends to produce the most hypotension, bradycardia, and conduction disturbances and deaths from the calcium channel blockers seen in emergency rooms. The adverse effects of verapamil consist of its negative effects on the sino-atrial and atrio-ventricular nodes reducing heart rate and atroventricular conduction, reduction of myocardial contractility by antagonism of myocardial L-type calcium channels, and antagonism of L-type calcium channels in vascular smooth muscle, resulting in peripheral vasodilatation. The toxicity of calcium channel blockers can lead also to a wide variety of manifestations in the central nervous system, gastrointestinal system, endocrine-metabolic, hematologic and respiratory systems.

Levosimendan is believed to improve the cardiac depression and prognosis of the patients. There is some experimental evidence which supports these claims. In anaesthetized guinea-pigs, levosimendan markedly decreased mortality in the diltiazem intoxicated group (12% versus 100%) and in the verapamil-intoxicated group (0% versus 89%) ((Levijoki, unpublished data). However, in a rat model, levosimendan failed to cause any improvement in the survival time (Abraham et al. 2009). In one other rat study, levosimendan increased CO to a similar degree as CaCl₂ alone, but it
did not improve BP from the time of maximal toxicity. The addition of CaCl₂ to levosimendan did not confer any further improvement in CO and BP compared to CaCl₂ alone. There was either no mortality benefit observed with levosimendan compared to CaCl₂ treated animals. However, a recent case report did reveal a beneficial hemodynamic effect of levosimendan in CCB overdose patients (Varpula et al. 2009). Clearly, more studies are needed to evaluate the place of levosimendan in the treatment of verapamil poisoning in humans.

2.5.3 Other clinical effects

Short-term levosimendan therapy of heart failure has been shown to have no tendency to increase the incidence of cardiac arrhythmias. A meta-analysis of cardiac arrhythmias by analysing ECG recordings from clinical studies on intravenously administered levosimendan in heart failure patients showed no difference between levosimendan and control groups in the occurrence of atrial fibrillation (AF), supraventricular tachycardia (SVT), or ventricular tachycardia (VT). In addition, the frequency of VT was similar and no torsade de pointes or sustained VT occurred (Lilleberg et al. 2004). In a recent open-label study in 50 patients with heart failure, given a 24-hour intravenous protocol once monthly for 6 months, no increase was seen in the incidence of supraventricular or ventricular beats or SVT and VT episodes in levosimendan group, compared with controls (Mavrogeni et al. 2007). However, in REVIVE II and SURVIVE trials, ventricular tachycardia as an adverse effect (AE) was statistically significantly more frequent with levosimendan than with placebo (Cleland et al. 2006), or occurred at a similar frequency with both levosimendan and dobutamine (Mebazaa et al. 2007). To sum up all of the i.v. studies, levosimendan seems to increase the occurrence of atrial fibrillation according to AEs (Levijoki, unpublished data). However, with respect to ventricular tachycardia, contradictory results have been reported in clinical trials.

Levosimendan (0.5 mg four times daily) orally for 9 days in healthy subjects had no effects on blood coagulation (Antila et al. 2000). In addition, levosimendan has been shown to possess some anti-inflammatory and antiapoptotic properties. In patients with decompensated advanced heart failure after 48 hours compared with placebo, levosimendan induced a significant reduction in the concentration of interleukin-6 and a slight decrease in the level of tumor necrosis factor-α (TNF-α). It also reduced soluble apoptosis mediators, such as soluble Fas and Fas ligand (Parissis et al. 2004; Paraskevaidis et al. 2005).

2.5.4 Drug interactions

Levosimendan seems to have no serious interactions with most of the drugs used in the treatment of CHF. The combination of levosimendan and dobutamine has been shown to be relatively safe and
Levosimendan is effective in patients with severe heart failure (Nanas et al. 2004; Nanas et al. 2005). In cardiogenic shock patients requiring catecholamines, additive levosimendan infusion was shown to improve hemodynamics (Delle Karth et al. 2003). No pharmacodynamic interactions between levosimendan and warfarin, were seen in healthy volunteers (Antila et al. 2000). Levosimendan in combination with isosorbide-5 mononitrate was shown to have no major additive haemodynamic effects compared with each drug alone at rest. However, the response to an orthostatic heart rate test was potentiated with the combination of levosimendan and isosorbide-5-mononitrate in healthy subjects (Sundberg and Lehtonen 2000). When levosimendan was given in addition to carvedilol in healthy subjects, no additive positive inotropic, positive chronotropic, or effects on diastolic blood pressure were seen though the effect on systolic blood pressure was attenuated (Lehtonen and Sundberg 2002). No major haemodynamic interactions between levosimendan and captopril, an ACE inhibitor, (Antila et al. 1996) or between levosimendan and felodipine, a calcium channel blocker, (Poder et al. 2003) have been observed in humans. Moreover, no pharmacodynamic interactions between levosimendan and ethanol, used as a diluent in the intravenous formulation, were found (Antila et al. 1997). Furthermore, levosimendan did not demonstrate any interaction with itraconazole (Antila et al. 1998), a potent inhibitor of CYP3A4 isoenzyme and an antifungal agent.

**2.6 Summary of the effects of levosimendan on the cardiovascular system**

Levosimendan is defined as a inodilator agent based on its combined positive inotropic and vasodilator effects related to calcium sensitization and potassium channel opening. In addition to these properties it can evoke mitochondrial $K_{ATP}$ channel opening, a unique assortment of mechanisms, which may be responsible for a positive synergistic effects on the whole cardiovascular system (Figure 6.). The abilities of levosimendan to improve myocardial function without increasing oxygen consumption practically at all and its property to enhance the perfusion of peripheral organs generates energetically favourable conditions for the restoration of impaired cardiac performance in addition to AHF also in ischemic heart disease, sepsis and perioperative cardiac support.
Figure 6. Summary of the effects of levosimendan on cardiovascular functions. ATP-dependent potassium channels ($\text{K}_{\text{ATP}}$); Large conductance calcium-activated potassium channels ($\text{BK}_{\text{Ca}}$); Voltage gated potassium channels ($\text{KV}$); Phosphodiesterase type-3 (PDE3); Troponin C (TnC); Ischemia-reperfusion (I-R); Left ventricle (LV); Right ventricle (RV). Modified from (Pinto et al. 2008) Curr Opin Anaesthesiol 21(2): 168-77.
3 AIMS OF THE STUDY

The inodilatory effects of levosimendan have been described in many of the above-mentioned *in vitro* and *in vivo* studies. It is a calcium sensitizer and an opener of ATP-dependent potassium channels and exerts its primary pharmacological effects via this unique double mechanism of action. There is another family of drugs with an inodilatory effect, the PDEIs, of which milrinone is a classical example. However, these drugs exert their inotropic and vasodilatory effects by a mechanism of action based on the increase of intracellular calcium, which has been associated with undesirable effects such as increased myocardial oxygen demand, cardiac arrhythmias, and mortality. However, it has been shown that levosimendan is an effective and potent PDE3 inhibitor, *in vitro*. The consequence of this PDE3 inhibition, *in vivo* is obscure, since levosimendan enhances cardiac contraction without increasing oxygen consumption, in contrast to the PDEIs.

The present *ex-vivo* experiments were designed to further characterize the mechanisms of action of levosimendan and to differentiate it from the inodilatory effects of the PDEIs. The model of isolated heart preparation was selected in order to study the dual effects of levosimendan in the target organ, possibly untangling the inotropic and vasodilatory effects. The experiments were conducted in guinea-pig isolated hearts because the cardiomyocytes of this species have a calcium handling similar to human myocytes.

Five different study settings were designed in order to answer five main questions about the mechanisms of action of levosimendan:

1. What is the role of cAMP- and cGMP-dependent protein kinases in the effects of levosimendan on cardiac contraction and coronary vasodilatation compared to the effects of the PDE inhibitor milrinone?

2. How potent a inotropic agent and Ca$^{2+}$-sensitizer is the active plasma metabolite of levosimendan, OR-1896, compared to levosimendan, and how selective inhibitors are these two agents on phosphodiesterase isoforms (PDE3 and PDE4)?

3. Is the difference between the positive inotropic effects of levosimendan and its stereoisomer, dextrosimendan, correlated primarily to their stereoselective Ca$^{2+}$-sensitizing effect or to their stereoselective phosphodiesterase inhibitory functions?

4. What is the main vasodilatory mechanism of levosimendan in the coronary arteries?
5. Is the inotropic mechanism of levosimendan energetically more advantageous than the mechanism of the PDE inhibitor, milrinone?
4 MATERIALS AND METHODS

4.1 Experimental animals

Adult guinea pigs of either sex (MOL-DUHA in studies I and III-V and Duntley Hartley in study II, purchased from Mollegaard Breeding Center, Denmark), weighing 300–400 g were used. The guinea-pigs were housed in polycarbonate cages (Macrolone IV, Scanbur A/S, Denmark), in a thermostatically controlled room at 20 ± 1°C at a relative humidity of 50 ± 10%. The room was artificially illuminated from 06.00 to 20.00 hours. The guinea pigs received commercial, pelleted, guinea-pig feed (Altromin 3120, Chr.Petersen A/S, Denmark) autoclaved at 120°C and sterile, filtered tap water ad libitum. The study was conducted with the permission of the Animal Ethic Committee of Orion Pharma, in accordance with Finnish law and government regulations complying with the European Community guidelines for the use of experimental animals.

4.2 Preparations

4.2.1 Isolated heart

The guinea-pig isolated heart was the most common preparation used in this study. Langendorff-perfused heart is described in four studies, I, II, IV and V. Briefly, the guinea-pig was sacrificed and the heart was rapidly excised and rinsed in ice-cold oxygenated perfusion buffer. A cannula was inserted into the aorta and retrograde perfusion of the heart began as soon as the heart was placed in a thermostatically controlled moist chamber of the Langendorff apparatus. Modified Tyrode solution was used as a perfusion buffer. Most of the experiments were carried out under constant pressure conditions (50 mmHg). A latex balloon, attached to a stainless-steel cannula coupled to a pressure transducer, was placed in the left ventricle. The isovolumetric left ventricular pressure was recorded with a pressure transducer. Coronary flow was continuously recorded by an electromagnetic flow meter with a flow probe inserted above the aortic cannula. The HR and the left ventricular systolic and end-diastolic pressure of the heart were obtained from the digitised pressure signals. The left ventricular pressure signals were used to calculate the maximal positive and negative pressure derivatives (LV + dP/dt_{max} and LV − dP/dt_{max}, respectively). In studies II and V, the hearts were overpaced at 5 Hz. Furthermore, in study II, the experiment was carried out in the presence of constant coronary flow. Under these conditions, myocardial contractility could be increased either by an increase in the Ca^{2+}-sensitivity of the contractile system and/or by an increase in the amplitude of the Ca^{2+} transient due to phosphodiesterase inhibition.
4.2.2 Papillary muscle

The papillary muscle preparation is described in studies I and III. Briefly, the right ventricular papillary muscle of the guinea-pig heart was mounted in an organ bath containing modified Tyrode solution for measurement of isometric tension. For pacing of the papillary muscle, the electrical pulses were conducted through platinum wire electrodes inserted on both sides of the muscle. The field stimulation occurred at 20% above threshold or at double the threshold voltage. A force-displacement transducer was connected to a driver amplifier and a programmable scanner. The amplified signal was digitised at 1 kHz frequency by a programmable digitizer and the twitch tension was measured.

4.2.3 Permeabilized cardiomyocytes

Force measurements in permeabilized myocyte-sized preparations (skinned fibres) are described in studies I, II and III. Briefly, the calcium-sensitizing effect of levosimendan was investigated in two types of skinned fibers obtained either from unpretreated or isoprenaline-pretreated isolated, spontaneously beating guinea-pig hearts. Fast and complete perforation of the cell membranes was achieved by retrogradely conducting saponin solution through the aorta into the coronary arteries of the heart. The fibers dissected from the papillary muscles were further mildly sonicated and treated with saponin. A slightly acidic pH of 6.7 was chosen in order to mimic the pH in the ischemic myocardium in which the calcium sensitivity is decreased and in which the benefits of levosimendan should be most prominent. The fibers were induced to contract in the calculated desired free pCa (Fabiato and Fabiato 1979). The absolute stability constants used were as reported by Fabiato (Fabiato 1981). At the beginning of the experiment, the fiber was stretched in ‘relaxing’ calcium free solution until the resting tension amounted to approximately 2% of the maximum force able to produce by the fiber. Then, the ‘relaxing’ solution was replaced with the ‘activating’ pCa 5.8. solution which roughly corresponds to the intracellular cytoplasmic calcium during muscle contraction. When the tension produced by the fiber had reached a steady state, various concentrations of levosimendan were successively added to the solution. The maximum tension which was produced by the fiber at pCa 4.8 was determined after washing out the test compound.

4.2.4 Inhibition of phosphodiesterase isoenzymes

Inhibition of PDE is described in studies II and III. PDE III and IV were isolated from guinea pig hearts and from a human myeloid leukemia promonocytic cell line (U-937), respectively, to achieve high level PDE purity. The isolation procedure of PDE and the determination of their activities have been described earlier (Alajoutsijarvi and Nissinen 1987; Frodsham and Jones 1992; Torphy et al.
1992). Briefly, tissues were homogenized and the homogenate was centrifuged and further applied to DEAE cellulose columns and subsequently eluted with a linear gradient of sodium buffers. The collected fractions were subsequently assayed for cAMP and cGMP phosphodiesterase activity. The fractions containing phosphodiesterase activity were pooled to form isoenzyme specific pools. The effects of levosimendan, dextrosimendan, milrinone and OR-1896 on PDE activity were measured by using a liquid chromatograph system for on-line radiochemical detection of the formation of [\(^{3}\)H]cAMP. PDE inhibitory potential determinations were performed in duplicate and the results are given as the means of the data from the individual test runs. The IC\(_{50}\)-values for PDE inhibition were calculated.

### 4.3 Statistical analysis

In study I, ANCOVA (repeated measures) was used in order to verify that the selected concentration range for levosimendan and milrinone produced positive inotropy to the same extent in the isolated heart. The same statistical test was used when the hearts treated with the protein kinase inhibitors were compared with those exposed to levosimendan or milrinone alone. In all studies ANOVA (repeated measures), followed by Dunnett’s \(t\)-test was used, when the measured parameters were compared to the initial values. Statistical significance was accepted at \(P<0.05\). The EC\(_{50}\) values from the sigmoidal dose-response data were calculated by using the curve-fitting algorithm of Microsoft® Office Excel program.

### 4.4 Ethical statement

The study was conducted with the permission of the Animal Ethic Committee of Orion Pharma, in accordance with Finnish law and government regulations complying with the European Community guidelines for the use of experimental animals.
5 RESULTS

The main results are summarized in table 3.

5.1 Brief summary of the main effects in the separate studies

Study I

In the isolated Langendorff-perfused spontaneously beating guinea-pig heart, levosimendan and milrinone increased the left ventricular systolic peak pressure to almost the same extent. The inotropic and lusitropic effects of milrinone were mediated via PKA mediated phosphorylation, while the effects of levosimendan were not mediated via PKA up to its EC\textsubscript{50}. Furthermore, the stimulation of AC potentiated the positive inotropic effect of milrinone but not that of levosimendan in the papillary muscle.

Study II

In the isolated Langendorff-perfused paced guinea-pig heart, in the presence of constant coronary flow, levosimendan and OR-1896 were equally potent as positive inotropes. In the permeabilized cardiomyocytes, levosimendan and OR-1896 both increased isometric force production similarly. Furthermore, levosimendan was 40-fold more potent and a 3-fold more selective PDE3 inhibitor than OR-1896.

Study III

In the isolated guinea-pig papillary muscle preparation, levosimendan increased the twitch tension at 47 times lower EC\textsubscript{50} value than dextrosimendan and in the permeabilized cardiomyocytes, levosimendan and dextrosimendan increased isometric force production due to Ca\textsuperscript{2+}-sensitization with a similar relative potency difference of 76. However, levosimendan was 427 times more potent PDE3 inhibitor than dextrosimendan.

Study IV

In the isolated Langendorff-perfused spontaneously beating guinea-pig heart, levosimendan increased diastolic coronary flow velocity (DCFV) concentration-dependently, and this effect was noncompetitively antagonized by glibenclamide, whereas the effects of pinacidil were antagonized competitively by glibenclamide. In the presence of glibenclamide, the positive inotropic and
chronotropic effects of levosimendan were unaltered. The effect of a PKC inhibitor, BIM and levosimendan on DCFV was additive.

Study V

In the isolated Langendorff-perfused paced guinea-pig heart, perfusion with levosimendan consumed proportionally less oxygen than perfusion with milrinone, while the compounds increased the mechanical performance of the heart equally.

5.2 Isolated Langendorff-perfused heart

The inotropic and vasodilatory effects of levosimendan and its active metabolite, OR-1896, were studied in retrogradely perfused isolated guinea-pig hearts and compared to the effects of milrinone and pinacidil. In the paced hearts, levosimendan and OR-1896 were equally potent positive inotropes with very similar concentration dependences with a slightly lower EC_{50} value for levosimendan than that for OR-1896 (15 ± 2 nM vs 25 ± 1 nM, P < 0.05). They were also equal efficacious, increasing the left ventricular + dP/dt_{max} by 26 ± 4% and 25 ± 3% (mean ± S.E.M.), respectively (study II). Compared to milrinone, levosimendan was 10 to 30 times more potent than milrinone as a positive inotropic and lusitropic agent. The maximum increase in left ventricular systolic pressure (LVSP) was 24 ± 10% in the levosimendan-treated group and 20 ± 10% in the milrinone-treated group. The maximum increase in contractility (+dP/dt max) was 30 ± 10% and 28 ± 11% and the maximum increase in relaxation (−dP/dt max) was 36 ± 17% and 37 ± 14% in the levosimendan and milrinone groups, respectively (study V). In the spontaneously beating hearts, levosimendan was 10 to 20 times more potent as an inotropic agent than milrinone. The EC_{50} concentration for the elevation in LVSP by levosimendan was about 0.03 μM and that for milrinone was about 0.6 μM. However, levosimendan and milrinone increased the left LVSP almost equally, levosimendan maximally by 17 mmHg (+19%) at 0.3 mM and milrinone maximally by 14 mmHg (+15%) at 3 mM (study I). The PKA inhibitor, KT5720 (1 μM) inhibited the contraction (LVSP and +dP/dt_{max}) of levosimendan at the highest studied concentrations (≥ 0.1 μM). Instead the inotropic effects of milrinone, were inhibited totally by KT5720. The effects of KT5720 on relaxation (−dP/dt_{max}) of levosimendan and milrinone were similar to those on contraction. The PKG inhibitor, KT5823 (1 μM), did not affect statistically significantly the increasing effect of levosimendan or milrinone on the contraction or relaxation (study I). The PKC inhibitor, BIM, did not have any effects either on the contraction or relaxation in the levosimendan group (study IV).
In spontaneously beating guinea-pig hearts, levosimendan concentration-dependently increased the heart rate (HR) to the same extent in the absence and presence of KT5720 or KT5823. In all groups, a maximum increase of 27–30% was seen at 1 μM levosimendan. The magnitude of the positive chronotropic effect of milrinone (0.1–10 mM) was comparable to that of levosimendan (0.01–1 mM), but unlike the situation with levosimendan, the maximum effect was not reached at the concentrations used (study I). Moreover, the PKC inhibitor, BIM, did not have any effects on the HR in the levosimendan group (study IV).

Levosimendan alone increased the coronary flow concentration-dependently and maximally by 64% at 1 μM. The increase in coronary flow by milrinone alone did not differ from that induced by levosimendan except that the maximum effect was not reached at the milrinone concentrations used (study I). Also the increase in the DCFV by levosimendan reached a maximum effect at 1 μM. The effect of pinasidil on DCFV was equal with levosimendan (study IV). The total change in the oxygen consumption was significantly lower during levosimendan than the corresponding value during milrinone perfusion. Furthermore, the maximum increase in oxygen consumption was 10 ± 4% in the levosimendan group compared with 38 ± 15% in the milrinone group ($P = 0.031$ between the concentration-dependent effects of the two drugs on VO$_2$) (study V). The response to levosimendan or milrinone was slightly and to the same extent decreased in the presence of KT5720 (1 μM). In contrast, the levosimendan-induced increase in coronary flow was almost doubled in the presence of KT5823 (1 μM), whereas KT5823 did not alter the milrinone-induced increase in coronary flow (study I). The effect of levosimendan on DCFV was noncompetitively (nonparallel shift of the concentration-response curve to the right) antagonized by glibenclamide on the contrary to pinacidil, which was antagonized competitively (parallel shift of the concentration-response curve to the right) by glibenclamide. Furthermore, the concentration-response curve of levosimendan in the presence of the BIM, was markedly potentiated (study IV). In addition, it should be mentioned that glibenclamide alone decreased the DCFV and BIM increased it, while KT5720 and KT5823 had no effects on the baseline DCFV.

5.3 Papillary muscle

The concentration–response curve of levosimendan on twitch tension displayed a bell-shaped form and was markedly dependent on the stimulus strength used in the pacing of the papillary muscle. No such similar phenomenon was seen with milrinone (study I). The EC$_{50}$ concentrations for levosimendan were 0.1 and 0.3 μM when the papillary muscles were paced at 100% or at 20% above threshold voltage, respectively. The maximum increases of 330 mg (+127%) and 90 mg
(+45%) in twitch tension were reached at 1 and 3 μM, respectively. The EC50 value for milrinone was about 2 μM independently of the stimulus strength used. The maximum increase of 240 mg in twitch tension induced by milrinone was also not affected by the stimulus strength (study I). Moreover, twitch tension increased with EC50 values of 60 nM for levosimendan compared to 2.8 μM for dextrosimendan. Hence, the two enantiomers exhibited a 47 times potency difference in their positive inotropic effects (study III).

In guinea-pig papillary muscles paced at twofold threshold voltage, forskolin (0.1 μM) increased twitch tension due to stimulation of the cAMP synthesis. The levosimendan-induced increase in twitch tension was not markedly changed in the presence of forskolin, while the positive inotropic effect of milrinone was almost doubled when it was combined with forskolin (study I). The initial twitch tension changed less than 10% by treatment with the β1-AR antagonist, atenolol (1 μM). However, atenolol abolished the stimulus sensitivity of the twitch tension and thereby eliminated the stimulus strength dependency of the inotropic response to levosimendan. Furthermore, levosimendan at 1 μM and milrinone at 10 μM increased twitch to the same extent (+180% to +190%) when these concentrations were tested before isoprenaline. After wash-out of isoprenaline, in percentage terms, milrinone was as efficacious as before isoprenaline, but the levosimendan-induced increase in twitch tension was reduced to 78% of pre-isoprenaline values (study I).

5.4 Permeabilized cardiomyocytes

In permeabilized guinea-pig cardiomyocytes, levosimendan (0.3–10 μM) concentration-dependently increased the calcium-induced tension at pCa 5.8 from 7% to 26% of the maximum tension produced by the skinned fibers at pCa 4.8 (study I). In the comparison of levosimendan with OR-1896, both compounds increased isometric force production at pCa 6.2 by 51% ± 7% and 52% ± 6%, with EC50 values of 8 ± 1 nM and 36 ±7 nM, respectively (study II). In addition, levosimendan and dextrosimendan increased isometric force production (at pCa 6.2) with EC50 values of 8.4 nM and 0.64 μM, respectively, i.e. a relative potency difference of 76 (study III). However, when the permeabilized cardiomyocytes were obtained from isoprenaline-pretreated (0.1 μM, for 10 min) hearts, the levosimendan-induced increase in tension of the skinned fibres was only from 6% to 16% of the maximum tension (study I).

5.5 Purified PDE enzymes

Increasing concentrations of levosimendan or OR-1896 decreased the PDE3 activity in a dose-dependent manner. The half-maximal inhibition of PDE3 was achieved at a concentration (IC50) of
2.5 nM by levosimendan, while in the case of OR-1896, the IC₅₀ was 94 nM. Similarly to the results with PDE3, increasing concentrations of levosimendan or OR-1896 also progressively decreased the PDE4 activity. However, to achieve half maximal inhibition of PDE4, significantly higher drug concentrations were required. The IC₅₀ value for levosimendan was 25 μM, comparison to 286 μM for OR-1896 (study II). Furthermore, levosimendan was a 427 times more potent PDE3 inhibitor than dextrosimendan, with IC₅₀ values of 7.5 nM, and 3.2 μM, respectively (study III).
<table>
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<th>Modulatory agent</th>
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<td>isolated Langendorff-perfused guinea-pig heart</td>
<td>constant pressure (50mmHg), spontaneously beating heart</td>
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<td>levosimendan &gt; milrinone</td>
<td>levosimendan &gt; milrinone</td>
<td>BIM + levosimendan ↑</td>
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<td>constant pressure (50mmHg), paced heart at 5 Hz</td>
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<td>KT5720 + milrinone</td>
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<td>levosimendan &gt; milrinone</td>
<td>BIM + levosimendan ↑</td>
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<td>PDE3 enzyme from guinea pig hearts</td>
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<td>levosimendan &gt; OR-1896</td>
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<td>PDE4 enzyme from a human myeloid leukemia promonocytic cell line (U-937)</td>
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<td>levosimendan OR-1896</td>
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<td>levosimendan &gt; OR-1896</td>
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1 study I, 2 study II, 3 study III, 4 study IV, 5 study V

pinacidil = K_{ATP} channel opener, KT5720 = PKA inhibitor, KT5823 = PKG inhibitor, BIM (bisindolylmaleimide) = PKC inhibitor, glibenclamide = K_{ATP} channel antagonist, atenolol = β1-AR antagonist, forskolin = adenylate cyclase activator, isoprenaline = β1-AR agonist

↑ = additive effect, ↓ = antagonistic effect
6 DISCUSSION

The main conclusion emerging from the present study is the concept that levosimendan possesses of two main mechanisms of action, an inotropic effect via calcium sensitization and a vasodilatory effect mediated through the opening of the K<sub>ATP</sub> channels. However, levosimendan was found to be a potent PDE3 inhibitor, a finding which is at odds with the fact that levosimendan does not increase intracellular calcium concentration [Ca<sup>2+</sup>]<sub>i</sub> and oxygen consumption in proportion to its inotropic effect. This dilemma is discussed in the following chapters.

6.1 Inotropic effects

In the present study, the economy of the contraction was shown to be more advantageous in levosimendan-perfused hearts than in milrinone-perfused hearts (study V). Previously, Lancaster and Cook have shown that levosimendan produced an increase in cell shortening in guinea-pig cardiomyocytes without affecting the [Ca<sup>2+</sup>]<sub>i</sub> transient or the Ca<sup>2+</sup> content of the sarcoplasmic reticulum at clinically relevant concentrations ≤ 0.1 μM (Lancaster and Cook 1997). However, other studies have revealed that levosimendan can increase the calcium transient already at low inotropic concentrations and emphasized its dual mechanism of action in its positive inotropic effects (Sato et al. 1998; Takahashi and Endoh 2005). In canine ventricular trabeculae loaded with aequorin, the positive inotropic effect of levosimendan up to 10 μM was associated with an increase in Ca<sup>2+</sup> transients (Takahashi and Endoh 2005). On the other hand, in isolated failing human myocardium, levosimendan induced moderate inotropic effects without any increased intracellular calcium transients (Hasenfuss et al. 1998). Levosimendan increased twitch tension statistically significantly and aequorin light emission statistically nonsignificantly at a concentrations of 0.3 and 1μM. In comparison, at concentrations of 10 μM and 100 μM, milrinone increased both twitch tension and aequorin light emission statistically significantly. Therefore, these findings support the concept that the positive inotropic effect originates predominantly from myofilament calcium sensitization.

In the present study, levosimendan was found to be 10–20 times more potent as a positive inotropic compound than the PDE inhibitor, milrinone, in isolated guinea-pig heart. Previously, the positive inotropic effect of levosimendan has been shown to be sensitive to carbachol (Sato et al. 1998). The inhibitory action of carbachol may be exerted selectively on the cAMP mediated positive inotropic effect, because it is virtually unaffected by the inotropic effect of other drugs e.g. α-adrenoceptor agonists and cardiac glycosides, that do not involve the generation of cAMP (Endoh 1995).
However, the PKA inhibitor, KT5720, did not antagonise the increase in LVSP produced by levosimendan at concentrations up to its EC$_{50}$. This effect of levosimendan contrasts with that of milrinone, which was devoid of the positive inotropic effect in the presence of KT5720, i.e. evidence that PDE inhibition is the major mechanism of action of milrinone. In heart failure patients, 95–98% of levosimendan is bound to plasma proteins and therefore its therapeutically active free plasma concentration is less than 0.02 μM (Sandell et al. 1995). Additionally, during the treatment of acutely decompensated heart failure, a maximal free concentration of 3–6 nM of levosimendan has been measured (Kivikko et al. 2002). Therefore, the concentration range used in the present study was at the clinically relevant level. In guinea-pig papillary muscle, the EC$_{50}$ value of levosimendan for the inotropic effect was about 3 times higher than in isolated heart, being comparable to the effects in isolated human cardiac muscle strips. In canine ventricular trabeculae, the EC$_{50}$ value of levosimendan to promote contraction was even higher.

Compartmentation of cAMP is thought to generate the specificity of Gs-coupled receptor activation in cardiac myocytes. The PDEs have a major role in this signal cascade by preventing cAMP diffusion. For example, selective inhibition of PDE3 by cilostamide and of PDE4 by Ro 20-1724 have both been shown to potentiate β$_1$-AR cAMP signals, whereas the Glu-R activated cAMP level was increased only by PDE4 inhibition. Furthermore, cAMP levels increased by PGE1-R and β$_2$-AR stimulation only when PDE3 and PDE4 were blocked (Rochais et al. 2006). In the recent study with cilostamide and Ro 20-1724 in adult rat ventricular myocytes, Leroy et al (Leroy et al. 2008) revealed that PDE3 inhibition has a direct inotropic effect on basal contractility and PDE4 inhibition causes inotropic responses in the presence of β-AR stimulation. Thus, they hypothesized that PDE3 can regulate a “constitutive” cAMP pool linked to contractility, whereas PDE4 regulates the cAMP microdomains mobilized by β-AR stimulation.

In study II, the selectivity factors for levosimendan and OR-1896 to inhibit PDE3 in preference to PDE4 were 10000 and 3000, respectively. The corresponding value for milrinone has been found to be only 14 (de Cheffy de Courcelles et al. 1992). Leroy et al observed that when PDE4 was inhibited, PDE3 became the predominant enzyme to hydrolyze membrane and cytosolic cAMP, as well as regulating $I_{Ca,L}$ recovery (Leroy et al. 2008). Therefore, PDE4 seems to be able to compensate for PDE3 inhibition, at least in the cytosol and in sites near the L-type Ca$^{2+}$ channels. Moreover, PDE4D subtype is related to the A kinase anchoring protein-organized signaling complexes (Dodge-Kafka et al. 2006), thus activation of PDE4 can be regulated by PKA. This could explain why KT5720 did not antagonize the effects of levosimendan, a very selective PDE3
inhibitor, and therefore, the role of PDE3 inhibition in levosimendan induced inotropy seems to be modest.

Thus, one could speculate that when only one phosphodiesterase isoenzyme is inhibited and one signal cascade is blunted, cAMP is presumably metabolized by the other phosphodiesterase isoenzymes and another inotropic signal cascade is active. In addition, PDE inhibitors with poor PDE selectivity have been shown to be more potent elevators of intracellular cAMP in cardiomyocytes, supporting the concept of additive effect on simultaneous activation of parallel inotropic signal cascades (Cone et al. 1999; Shakur et al. 2002). It is notable that in rapid pacing-induced heart failure in dog, PDE3 mRNA and activity had decreased, whereas PDE4D was unchanged, another indication that the role of PDE3 inhibition is not very significant in the effects of levosimendan (Smith et al. 1997). Moreover, dextrosimendan, the stereoisomer of levosimendan, had 47 times less positive inotropic effect in papillary muscle preparations and 76 times less effect on the contractile apparatus, the EC50-values being comparable in permeabilized cardiomyocytes. However, the PDE3 inhibitory activities of the two stereoisomers differed by 427 fold (study III).

Furthermore, the positive inotropy by levosimendan was found to depend on the strength of electrical stimuli. This phenomenon was not seen with milrinone, indicating that it was not due to PDE inhibition and cAMP. However, the β1-AR antagonist, atenolol, decreased the sensitivity of twitch tension to the stimulus strength. Thus, the high stimulus strength may have enhanced the release of noradrenaline from adrenergic nerve terminals, leading to activation of β1-ARs and to the direct Gs-protein-mediated opening of calcium channels (Rochais et al. 2006). In this respect, a contributory role for PDE3 inhibition cannot be excluded. Otherwise, the high stimulus strength finally increases intracellular calcium transient, which in turn increases the number of activated cTnC molecules. Since the binding of levosimendan to cTnC is calcium-dependent, there will be an increased number of binding sites when more cTnC molecules become activated. Therefore, the calcium sensitization and subsequently the positive inotropy caused by levosimendan can be potentiated when a high stimulus strength is used. Moreover, forskolin activates AC and further increases cAMP and the intracellular calcium transient. Theoretically, selective inhibition of PDE3 should potentiate the stimulatory effect of forskolin on β1-AR associated AC. However, milrinone, but not levosimendan, was able to potentiate the effect of forskolin. Alternatively, the increased intracellular calcium content may activate more cTnC molecules and this could then potentiate calcium sensitization evoked by levosimendan.
Isoprenaline, in turn, induces PKA-mediated phosphorylation of cTnI which leads to an alteration in the conformation of cTnC (Rapundalo et al. 1989; Wattanapermpool et al. 1995). This may inhibit the binding of levosimendan to cTnC and thereby antagonize its positive inotropy. The present study with isoprenaline showed that the positive inotropy induced by levosimendan at the concentration giving a maximum effect was decreased by 60–70% in isoprenaline-pretreated muscles. In contrast, the inotropic response to milrinone was not altered by similar isoprenaline pretreatment. Thus, cTnC seems to be the target protein for levosimendan also in the intact muscle and most of its positive inotropic properties may then be due to calcium sensitization. Furthermore, when levosimendan was added to the buffer, the positive inotropy evoked by isoprenaline had completely expired due to its wash-out. Therefore, the effect of levosimendan was affected only by the altered phosphorylation of contractile proteins and no longer by the increased intracellular calcium transient. When forskolin and levosimendan were investigated simultaneously, also the increased calcium transient induced by forskolin influenced the response to levosimendan. These differential experimental results explain why the action of levosimendan was altered in different ways in the forskolin and isoprenaline studies.

Acceleration of the relaxation induced by levosimendan could be due to cAMP accumulation induced by PDE3 inhibition, because like the contraction, it is inhibited by carbachol (Boknik et al. 1997; Sato et al. 1998). However, the present study revealed that in the presence of the PKA inhibitor, KT5720, levosimendan did not impair relaxation but increased the negative dP/dt\text{max}. The milrinone-induced increase in the negative dP/dt\text{max} was instead markedly antagonised by KT5720. However, the effect was not completely abolished probably because of the increase in HR, which was not abolished by KT5720 (study I). Moreover, levosimendan does not impair relaxation either under normal conditions or during acidosis (Takahashi and Endoh 2005). In the failing heart, cAMP-mediated signaling is weakened (Feldman et al. 1987), and therefore, it is probable that the increase in Ca\textsuperscript{2+}-sensitivity could be more pronounced than any PDE3 inhibitory action in failing ventricular myocardium (Hasenfuss et al. 1998). Furthermore, during acidosis, where cAMP loses its effectiveness, lack of impairment of relaxation due to the Ca\textsuperscript{2+}-dependent nature of the binding of levosimendan to troponin C is even more pronounced than in normal conditions (Haikala et al. 1995).

### 6.2 Vasodilatory effects

In the present study, levosimendan increased coronary flow both in the spontaneously beating heart (study I) and in the overpaced heart (study V). In their classical work, Braunwald et al. (Braunwald...
et al. 1958) demonstrated that oxygen consumption (MVO$_2$) is the major determinant of coronary blood flow. Heart rate (HR), in turn, is one of the main determinants of MVO$_2$. Therefore, any increase in HR is in proportion to the increase in CF and MVO$_2$. Even though levosimendan increased HR and CF in a parallel manner in the spontaneous beating hearts, MVO$_2$ was not increased in parallel with CF after levosimendan administration in the paced hearts. Thus, levosimendan had a direct vasodilatory effect on coronary veins. Moreover, the PKG inhibitor, KT5823 potentiated the levosimendan- but not the milrinone-induced increase in CF i.e. the coronary dilatory mechanism of action of levosimendan differs from that of milrinone and it does not seem to be mediated through the activation of PKG. The K$^+$ current through the delayed rectifier K$^+$ channels should be limited during PKG inhibition since these channels are normally activated by PKG (Sperelakis et al. 1994; Ko et al. 2008). In this situation, the efflux of K$^+$ ions may occur more through the K$_{ATP}$ channels, which may potentiate any vasodilatory effect mediated through these channels.

Patch clamp studies in rat mesenteric arterial cells have revealed that levosimendan can open K$_{ATP}$ channels (Yokoshiki et al. 1997). In study IV, it was shown that glibenclamide antagonized the increase in DCFV induced by levosimendan, demonstrating that the opening of the K$_{ATP}$ channels was the mechanism of action of the observed vasodilatory effect. Pinacidil was three times more efficacious and ten times less potent than levosimendan at increasing DCFV. Moreover, glibenclamide antagonized the coronary dilation induced by levosimendan and pinacidil in two different ways, suggesting that these two drugs may have different binding sites on the K$_{ATP}$ channels. The effect of pinacidil was antagonized competitively by glibenclamide, as expected (Nielsen-Kudsk et al. 1996), whereas levosimendan was inhibited in a non-competitive manner. One possible explanation for this result is that levosimendan opens the channel indirectly or, alternatively, interacts with different subtypes of glibenclamide-sensitive channels (Quast and Cook 1989; Quast et al. 1994; Adebiyi et al. 2008). However, the effect of levosimendan on K$_{ATP}$ channel was also revealed previously in single-channel recordings (Yokoshiki et al. 1997), which suggests that it does have a direct effect. An alternative explanation is that levosimendan has another vasodilatory mechanism.

As previously mentioned, the specific BK$_{Ca}$ channel blocker, IBTX suppressed the maximum effect of levosimendan, and the KV channel blocker, 4-AP significantly shifted the concentration-response curve to the right in isolated porcine epicardial coronary arteries, indicating that the vasorelaxing mechanism of levosimendan involves the activation of voltage-sensitive and, at large concentrations, of calcium-activated potassium channels (Pataricza et al. 2003). In addition,
levosimendan is believed to be able to relax porcine coronary arteries through calcium desensitization, interacting with smooth muscle EF-hand proteins, such as, calmodulin, the regulatory myosin light chains, or S100 proteins (Bowman et al. 1999). This effect was seen at ten times higher concentrations (0.1–10 μM) than those used in the present study (0.01–1 μM). However, this could explain the non-competitive inhibitory mode of action of glibenclamide on the increase in DCFV by levosimendan.

The role of PDE3 inhibition cannot be totally excluded, since levosimendan has been shown to potentiate the relaxant effect of cAMP-stimulation evoked by isoprenaline (Gruhn et al. 1998). On the other hand, the PKA inhibitor, KT5720 did not antagonize the effect of levosimendan on CF (study I). Nevertheless, the effect of milrinone on CF was also not antagonized by KT5720. This can be explained by the differences in the MVO₂ of the contraction-relaxation cycle between levosimendan and milrinone. In isolated heart, milrinone increased CF in parallel with MVO₂, in contrast to levosimendan, which increased CF independently of MVO₂ (study V). In addition, in patch clamp studies, a non-specific protein kinase inhibitor, H-7 did not prevent the hyperpolarisation and stimulation of K⁺ current by levosimendan in mesenteric arterial myocytes (Yokoshiki et al. 1997). Thus, it seems unlikely that PDE3 inhibition plays any major role in the vasodilatory effects of levosimendan.

The concentration response of levosimendan was markedly potentiated in the presence of the PKC inhibitor, bisindolylmaleimide (BIM). PKC is a mediator of the effects of several vasoconstrictors on K⁺ channels (Ko et al. 2008) and it is known that inhibition of the PKC-mediated phosphorylation of the K_ATP channels in the isolated rabbit heart can evoke a vasodilatory effect (Pongo et al. 2001). Therefore, it could be hypothesized that the potentiation of the increase in DCFV was due to a specific binding and action of levosimendan on the non-phosphorylated form of the K_ATP channels. In the present study, glibenclamide did not antagonize the effect of levosimendan on the contractile parameters of the heart, indicating that its action on the K_ATP channels was not indirectly mediated through the changes in the contraction parameters. Rather it is directly mediated via the coronary vasculature smooth muscle relaxation, since glibenclamide markedly antagonized the levosimendan-induced increase in DCFV. On the contrary, the negative inotropic responses of pinacidil were shown to competitively antagonized by glibenclamide (Lau 1992).

In addition, K_ATP channel activation is an important mediator of ischemic preconditioning (Gross and Auchampach 1992; O'Rourke 2004). The stimulation of K_ATP channels has been shown to
decrease myocardial infarct size (Gross and Auchampach 1992) and it can also enhance recovery of the stunned myocardium (Yao and Gross 1994). Moreover, the studies of Kersten et al. (Kersten et al. 2000) showed that levosimendan decreased the myocardial infarct size in coronary-ligated dogs and that the effect was mediated through $K_{ATP}$ channels. Although, vasodilatation may be beneficial in ischemia, it seems that mito$K_{ATP}$ channels are especially linked to the cardioprotective effects (Nishida et al. 2009). The effects of levosimendan on ischemia-reperfusion were not studied in the present experiments. However, we have noticed in guinea-pigs, that levosimendan could prevent the myocardial stunning in the isolated, Langendorff-perfused heart after global ischemia (Kaheinen, unpublished data).

6.3 Limitations of the methods and proposal for future studies

Isolated Langendorff-perfused heart was the most widely used preparation used in this study. It was also the most complex method and has a number of limitations, which have been previously reviewed extensively (Sutherland and Hearse 2000). Briefly, the lack of any oxygen carrier in the salt solution requires the use of high oxygen partial pressure to saturate the perfusion solvent and this causes considerably higher basal coronary flow than that normally present in vivo. A consequence of this unnatural coronary perfusion is a deterioration of the contractile function during the experiment. In study IV, the incomplete oxygen supply was seen as a decrease in basal CF in the presence of glibenclamide, indicating at least partially open $K_{ATP}$ channels. Moreover, use of salt-base buffer lacking plasma proteins can cause edema, which could induce a decrease in coronary flow. It took at least 1-2 h to reach a stable function of the heart and achieve a balance between oxygen supply and need (seen as stable coronary flow, oxygen consumption, and contractility parameters) in the present study. Subsequently, it was found that addition of pyruvate as well as glucose in the perfusate could shorten the stabilization period, but pyruvate was not used in the present study. Furthermore, in constant flow mode, unlike constant pressure perfusion, autoregulatory mechanisms are bypassed, which does not automatically ensure that the perfusate would be delivered uniformly to the whole heart as it responds to changes in heart rate or work. In addition, in the intact heart, the atria and sino-atrial node are not perfused by coronary vessels but by extracardiac vessels, which are damaged when the heart is excised for perfusion. This makes it difficult to maintain essential oxygen supply for the sino-atrial node and a stable heart rate. However, this problem can be avoided by pacing the heart, which was done in the part of the study (studies II and V).
Although, the stature of PDE3 inhibition on the inotropic and vasodilatory effects of levosimendan was shown to be slight in the experimental settings used in the present study, its final status still remains unclear. In addition, the intracellular calcium content was not measured. Simultaneous measurement of Ca\(^{2+}\) transient with alteration of contractile function in the isolated heart perhaps would have made it possible to elucidate the mode of action of levosimendan, especially the role of PDE inhibition. Interestingly, Sun et al (Sun et al. 2007) have recently published a study using PDE3A and PDE3B gene knockout (KO) mice. They proposed that PDE3A is the main subtype of PDE3 expressed in cardiac ventricular myocytes, and that it is responsible for the functional changes caused by PDE3 inhibition. Thus, further studies are warranted to examine the effects of levosimendan in these mice, though the species differences may complicate comparisons with the present studies. In addition, studies examining the mechanism of action behind the stroke-preventive effects of levosimendan would be scientifically interesting, in addition to being beneficial for future drug design.

6.4 Overall clinical aspects

In drug development, two characteristics come before all others, efficacy and safety in patients. In clinical use, the originally designed indication may be changed and/or new off-label indications stand out. That is what has happened to levosimendan. As mentioned above, it is approved for short-term treatment of acutely decompensated chronic heart failure. However, in the clinic, levosimendan has been demonstrated to have beneficial effects in ischemic heart disease and also against septic shock. It has been also demonstrated that levosimendan can be used as a bridge therapy for the perioperative cardiac support e.g. it facilitates weaning of patients from cardiopulmonary bypass (Eriksson et al. 2009). The last-mentioned indication is possible because of the ability of levosimendan to improve myocardial function without substantially increasing oxygen consumption.
7 SUMMARY AND CONCLUSIONS

The mechanisms of action of an inodilator, levosimendan, were studied in isolated guinea-pig heart preparations. Levosimendan was shown to be a potent inotropic agent in isolated Langendorff-perfused heart and right ventricle papillary muscle. In permeabilized cardiomyocytes, it was proved to be a potent calcium sensitizer in contrast to its positive enantiomer, dextrosimendan. Despite its very selective PDE3 inhibitory properties in purified enzyme preparations, the inotropic effect of levosimendan was observed to be mainly mediated through calcium sensitization also in the isolated heart as well as the papillary muscle preparations at clinically relevant concentrations. The effects of levosimendan differed considerably from the effects of milrinone. In particular, the energy efficiency of the contraction was shown to be advantageous in levosimendan-perfused hearts compared to milrinone-perfused hearts. Levosimendan binds to cardiac troponin C in a calcium-dependent manner and thus, does not impair the relaxation, as confirmed in the present study. Furthermore, the vasodilatory effect of levosimendan was shown to be mediated mostly through the opening of the K\textsubscript{ATP} channels in the isolated heart.

The main findings and conclusions of the present study are as follows:

1. The main inotropic and vasodilatory mechanisms of action of levosimendan are not related to the PKA mediated phosphorylation.

2. Levosimendan and OR-1896 are equally potent as positive inotropes and Ca\textsuperscript{2+}-sensitizers and reveal highly selective PDE3 inhibitory properties.

3. Levosimendan and its positive stereoisomer, dextrosimendan seem to exert their positive inotropic effects via a stereoselective Ca\textsuperscript{2+}-sensitizing mechanism and not via stereoselective inhibition of PDE3.

4. The main vasodilatory mechanism in coronary veins is the opening of the K\textsubscript{ATP} channels in the vascular smooth muscle.

5. The inotropic mechanism of levosimendan is energetically more advantageous than the mechanism of the PDE inhibitor milrinone, this being seen as low oxygen consumption in relation to the produced force of contraction.
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