PEPTIDERGIC REGULATION OF CARDIAC CONTRACTILITY: ROLE OF PROLACTIN-RELEASING PEPTIDE AND ENDOTHELIN-1

Ph. D. Thesis

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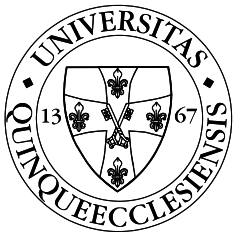

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1. LIST OF ABBREVIATIONS

AC.....	Adenylyl cyclase
APJ.....	Apelin receptor-angiotension receptor-like 1
BMK.....	Big mitogen-activated protein kinase
cAMP.....	cyclic adenosine monophosphate
GFP.....	Green fluorescent protein
DAG.....	Diacylglycerol
DT.....	Developed tension
EGFR.....	Epidermal growth factor receptor
ERK1/2.....	Extracellular signal-regulated protein kinase 1/2
ET-1.....	Endothelin-1
GPCR.....	G protein–coupled receptor
IBMX.....	3-Isobutyl-1- methylxanthine
IP3.....	Inositol trisphosphate
JNK.....	c-Jun N-terminal kinase
KACh.....	Acetylcholine sensitive potassium channel
LTD4.....	Leukotriene D4
MAPK.....	Mitogen-activated protein kinase
MEK.....	MAPK kinase 1
MKK3.....	MAPK kinase 3
MKK6.....	MAPK kinase 6
MLP.....	Muscle lim protein
NHE1.....	Na^+-H^+ exchanger-1
NPAF.....	Neuropeptide AF

5-oxo-ETE.....	5-oxo-6,8,11,14-eicosatetraenoic acid
PDE.....	Phosphodiesterase
PLN.....	Phospholamban
PLC.....	Phospholipase C
PKA.....	Protein kinase A
PKC.....	Protein kinase C
nPKC.....	Novel protein kinase C
PP.....	Protein phosphatase
PrRP.....	Prolactin-releasing peptide
p90RSK.....	p90 ribosomal S6 kinase
QRFP.....	Pyrogultamylated arginine-phenylalanineamide peptide
RyR.....	Ryanodine receptor
SERCA.....	Sarcoplasmic reticulum Ca^{2+} -ATPase-2
SR.....	Sarcoplasmic reticulum
7TMP.....	Seven transmembrane protein

2. INTRODUCTION

The major function of cardiomyocytes is to maintain the cardiac contraction-relaxation cycle. The contractile function of the heart is regulated by a number of acute and long-term mechanisms. The sympathetic-adrenergic activity, the Frank-Starling mechanism and the force-frequency relationship are the known possibilities of the acute regulation of cardiac contractility. Recently novel peptidergic mechanisms have been implicated in the acute and long-term regulation of cardiac contractility. These peptides (e.g. adrenomedullin, apelin), act via guanine nucleotide-binding regulatory (G) protein-coupled receptors (GPCRs), which represent the largest family of cell surface receptors and of all protein families.

2.1 G protein-coupled receptors

GPCRs are one of the most important modulators of organism function. They play a key role in cellular signaling pathways that regulate many basic physiological processes, such as neurotransmission, secretion, growth, cellular differentiation, inflammatory and immune responses. GPCRs are responsible for recognition and transduction of messages from the external environment of organisms or of cells within the body. GPCRs are the targets of a wide variety of ligands and can be divided into the chemosensory receptors (csGPCRs- are receptors for sensory signals of external origin that are sensed as odors, pheromones, or tastes) (*Buck L, Axel R, 1991*) and the “transmitter” receptors

which bind neuropeptides, opsins, biogenic amines, glycoprotein hormones, purine ligands, lipid mediators and a few other small molecules such as amino acids and their derivates (*Civelli O,et al., 2001*; *Hermans E*, 2003; *Baldwin J., 1994*).

GPCRs consist of a single protein chain that crosses the membrane seven times (seven transmembrane proteins-7 TM), they share a conserved core structure with extracellular amino termini (N-terminal tail), intracellular carboxyl termini (C-terminal tail), and seven transmembrane helices, which are connected by three extracellular and three intracellular loops (*Figure 1.*) (*Salazar NC, et al., 2007*). GPCR signaling is activated by ligand binding to an extracellular active site of the receptor. Depending on the ligand and type of GPCR, the active site is located on either the N-terminal tail, extracellular loops, or exofacial transmembrane helices. Ligand binding induces a conformational change in the GPCR, which disrupts the ionic interactions between the third cytoplasmic loop and the sixth transmembrane segment and allows for coupling with G-proteins. Trimeric G-protein links the GPCR to the intracellular signaling pathways, consists of three parts: Gα (the largest, GTP-binding subunit), Gβ and Gγ. Upon interaction with an agonist-activated receptor, Gα exchanges GTP for bound guanosine diphosphate (GDP), (*Salazar NC, et al., 2007*) thereby generating active GTP-bound G_{α} from the inactive GDP-bound state, followed by dissociation from the receptor and $G_{\beta\gamma}$. Thus, agonist-occupied receptors function as guanine nucleotide exchange factors (GEFs). Both the G_{α} subunit and $G_{\beta\gamma}$ dimer can modulate the activity of downstream effectors, including adenylyl cyclases (AC), phospholipases, phosphodiesterases, and ion channels. The rate and extent of signaling is determined by the lifetime of the active GTP-bound G_{α} subunit; G_{α} inactivates when the GTP is hydrolyzed to GDP. Currently known GPCRs include rhodopsin-

like family, secretin-like receptor family, glutamate-like receptor, pheromone-like receptors, cAMP-like receptors and frizzled family of receptors (*Sadowski M.I. and Parish J.H., 2003*). The rhodopsin-like receptors, which form a major superfamily of GPCRs, consist of 60 families and subfamilies each one of which has many types of receptors (*Horn F. et al., 2001*). The GPCRs are activated by a plethora of ''transmitters'', the first messengers that are either present in the environment or released from a cell to carry a message to a second one. These act in an endocrine, paracrine, or exocrine fashion to allow the organism to react to particular physiological challenges. A GPCR may bind more than one transmitter, but then these transmitters share structural similarities and are often part of the same synthesis pathway, as in the case of the neuropeptides synthesized from the same precursor (*Douglass J., Civelli O. et al., 1984*).

Yet, one tenet of the basic concept has remained constant over the last 20 years: all the small molecules that have evolved to direct intercellular interactions interact mainly with GPCRs. This positions the GPCRs at the center of signal transmission and endows them with an extraordinary importance in the organism's life and survival.

Because of their wide distribution, and the ability of GPCR subtypes to mediate particular responses, GPCRs have been, and are predicted to be in the future, important drug targets. Actually, more than 30% of currently available pharmaceutical agents are directed at GPCRs as agonists or antagonists (*Hopkins AL, Groom CR, 2002*).

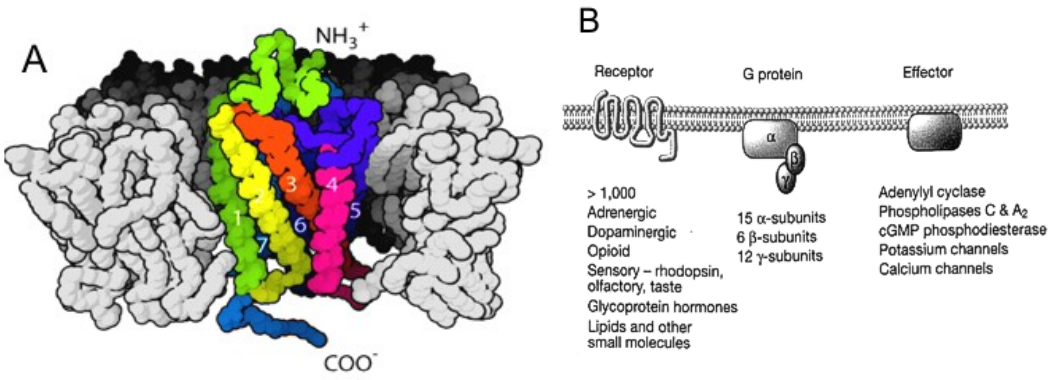

Figure 1: *Structure of the G-protein coupled receptors-* ***A****. GPCR signalling mechanism-* ***B.*** *(adopted from Salazar NC. et al., 2007)*

2.2. Orphan receptors, deorphanisation

The human genome encompasses more than 800 GPCRs, which probably represents the largest of all the gene families (*Levoye et al. 2008)*. Most of the GPCRs originally started as "orphan" because their transmitters were unknown. The discovery of their specific transmitters (deorphanizing these receptors) was prime importance to understand their function. During the last 20 years most of them were matched with their ligands using numerous different strategies. The first deorphanisation of orphan GPCRs (5- HT_{1A} receptors) were reported in 1988 (*Fargin A. et al. 1988*) and dopamine D2 (*Bunzow J.R. et al. 1988*) receptors and were followed during the next seven years by many others.

Homology screening techniques, low stringency hybridization (*Libert F. et al. 1989*) were soon followed by PCR-derived approaches. From the early days of GPCR cDNA cloning a strategy termed „reverse pharmacology" was used for the

deorphanisation of GPCRs (*Civelli O., 2005*). This approach is based on the exogenous expression of the orphan GPCR in a suitable cell system. Activation of the receptor by exposure to a variety of potential ligands is monitored by changes in intracellular second messenger levels involving heterotrimeric G proteins. A variation of the reverse pharmacology approach was developed in 1995 (*Figure 2.)* (*Civelli O. et al. 2001*). This strategy is also referred to as the 'tissue-extract based approach' where orphan GPCRs are exposed to tissue extracts instead of purified ligands. This approach was proven successful with the discovery of a new neuropeptide, nociceptin/orphanin, as the transmitter of the orphan GPCR ORL-1 (*Reinscheid R. et al. 1998*). During the next years, numerous new bioactive peptides were identified from tissue extracts (*Table 1.*). The other successful attempt at discovering novel transmitter through orphan GPCRs screened over 50 different orphan GPCRs by measuring their abilities to induce intracellular calcium release when subjected to peptidic extracts. One receptor did respond and led to the characterization of two peptides, the orexins (Oxs) (*Sakurai T. et al. 1998*), also identified through an RNA subtraction approach as hypocretins (Hcrts) (*de Lece et al.1998*). The discovery of orphanin demonstrated that orphan GPCRs could be used to find novel ransmitters and a rush for novel transmitters was initiated.

Table 1. *Identification of transmitters as ligands of orphan GPCRs*

Date	*Reverse farmacology approach*	*In tissue extracts*
1995	–	Nociceptin/ orpharin
1996	C3a	–
1997	Leukotriene B4	–
	Latrotoxin	–
1998	S1P (sphingosine-1-phosphate)	Hypocretins and orexins
	LPA (lysophosphatidic acid)	PrRP (prolactin-releasing peptide)
	–	Apelin
1999	Leukotriene D4	Ghrelin
	Melanin-concentrating hormone	Melanin-concentrating hormone
	Urotensin II	Urotensin II
	Motilin	–
2000	Neuromedin	Neuromedin
	Histamine	–
	Prostaglandin D2	–
	NPFF (neuropeptide FF)	–
	LPC (lysophosphatidylcholine)	Metastin
	ADP	–
2001	Psychosine	–
	Trace amines	–
	5-Oxo-ETE	neuropeptide B and W
	PK1 and PK2 (prokineticin)	PK1 and PK2 (prokineticin)
2002	BAM22	–
	Relaxin	–
	Bradykinin	Relaxin 3
	QRFP	–
	Cortistatin	–
2003	Nicotinic acid	–
2004	α-Ketoglutarate	neuropeptide S
	β-Alanine	Succinate

More recently, approaches based on the cellular translocation of proteins that participate in the GPCR desensitisation/internalisation cycle, that is b-arrestin, have been added to the toolbox. This system commercialised as 'Transfluor technology' monitors the change in distribution of a b-arrestin-GFP construct from homogenous and diffuse intracellular localisation to aggregated distribution as a consequence of ligand-dependent internalisation (*Mashukova A. et al. 2006*).

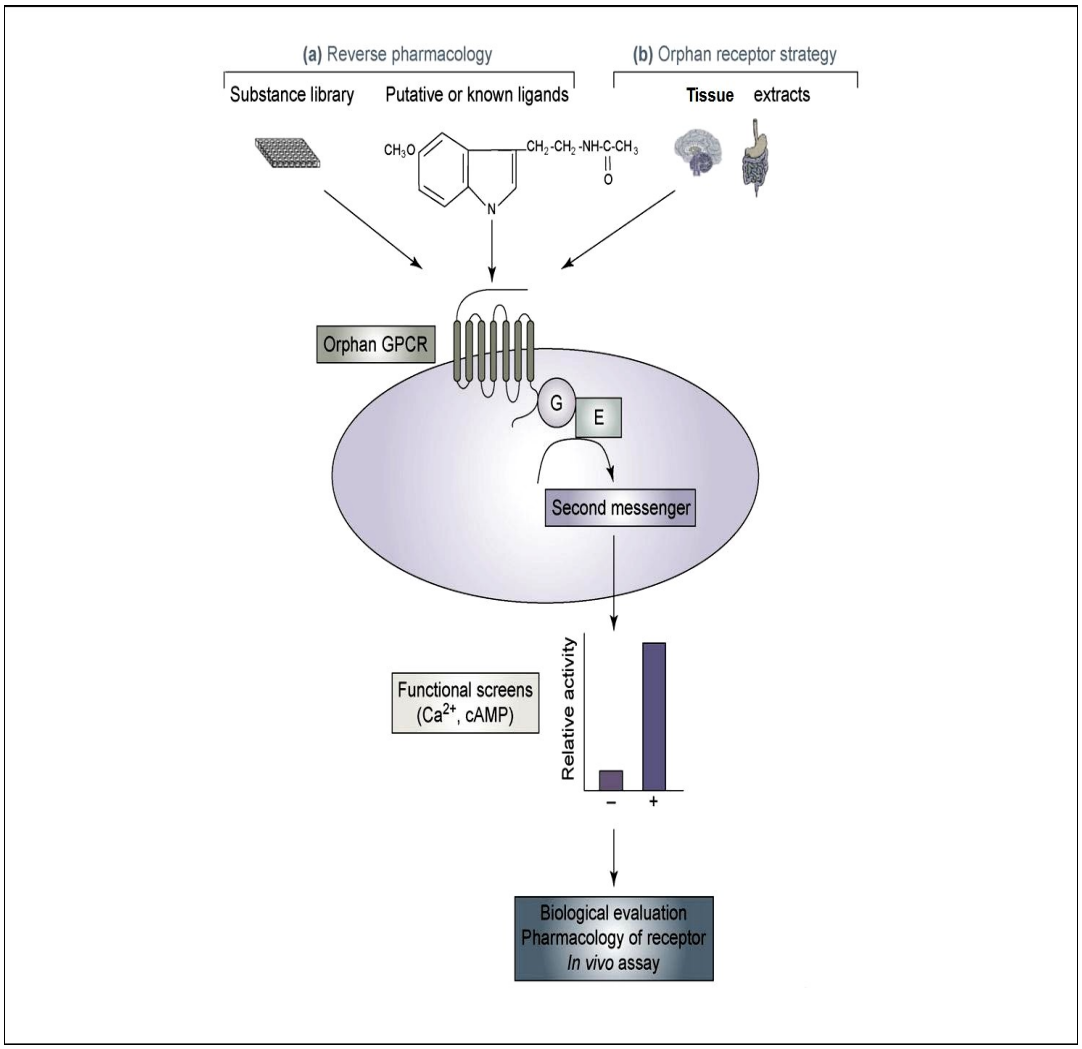

***Figure 2**. Schematic representation of current deorphanisation approaches. (Adapted from. Levoye et al. 2008)*

Today, one estimates that the number of GPCRs is over 800, of which more than half are olfactory GPCRs, and approximately 367 ”non-odorant” receptors exist in humans. Of these, over 100 GPCRs are still „orphan” (*Vassilatis D. et al 2003; Levoye A., Jockers R., 2008*), whose cognate ligands and their physiological roles have not yet been identified. Functional studies have implicated several recently paired peptide ligands in the regulation of cardiovascular homeostasis, including apelin (*Szokodi I. et al. 2002; Ashley EA. et al. 2005; Farkasfalvi K. et al. 2007*), ghrelin (*Nagaya N. et al. 2001*), urotensin II (*Cheriyan J. et al. 2009*), urocortins (*Wright SP., 2009*), motilin , neuromedin U, sphingosine-1-phosphate (*Kitazawa T. et al. 2009*) and nociceptin (*Giuliani S. et. al.1997*).

A recent update of the number of deorphanised GPCRs clearly shows that the deorphanisation rate slowed down since 2004, with 4–6 deorphanisations per year (*Figure 3.*) (*Table 2.*). Functional screening assays for orphan GPCRs traditionally focus on the monitoring of intracellular second messengers of G protein dependent pathways. However, some receptors may not rely on the activation of heterotrimeric G proteins but rather on the activation of G protein-independent pathways (*Lefkowitz RJ., Shenoy SK., 2005*). This could be the case for GPCR, which binds to C5a anaphylotoxin in binding assays, but which is completely inactive in functional G protein-dependent assays. Similarly, some chemokine receptors such as D6 receptors are known to bind to chemokines without inducing cell migration or G protein-dependent signaling (*Mantovani A. et al. 2001*).

The absence of specific GPCR-associated proteins in a given cell system may also represent a major obstacle for successful expression of functional receptors. It is clear that new assays have to be designed to deorphanise the remaining orphan receptors. This includes the enlargement of functional readouts

towards G protein-independent (i.e. b-arrestin-dependent) signalling pathways and the coexpression of appropriate interacting partners, which promote either cell surface expression or ligand binding.

Table 2. *The rate of deorphanisation after 1995*

	Ligand	**Amino acid**	**Receptor**
1995	Nociceptin	17	NOP
1998	Apelin	36	APJ
	PrRP-31	31	GPR10
	PrRP-20	20	GPR10
	Orexin A	33	OXR1
	Orexin B	28	OXR2
1999	Ghrelin	28	GHS-R
	MCH	19	MCHR1
2000	Neuromedin	25	FM-3 FM-4
	Neuropeptid FF	11	NPFF-R1
2001	Metastatin	54	CRF2
	Urocortin	38	CRF2
2002	BAM 22	22	SNSR3
	Relaxin	22	LGR7

In addition, recent evidence suggests that orphan GPCRs could also have ligand-independent functions. Such a concept would offer additional options for future drug screening strategies (*Levoye A. et al. 2006).* To overcome the increasing resistance of more than 100 remaining orphan 7TM proteins to current deorphanisation strategies, alternative approaches and concepts are needed.

Whereas some of these orphans must be matched with their endogenous ligands, others may be true orphans for which no natural ligand does exist. Elucidating the function of these orphans continues to be a priority for the understanding of their biological role and for drug discovery (*Table 3*.). Whereas the complete sequence of the human genome provided us with a fairly precise idea about the total number of orphan 7TM proteins, current progress in systems biology and proteomics will provide us with valuable insights in the orphan 7TMprotein-containing complexes, to ultimately elucidate the in vivo function of these proteins.

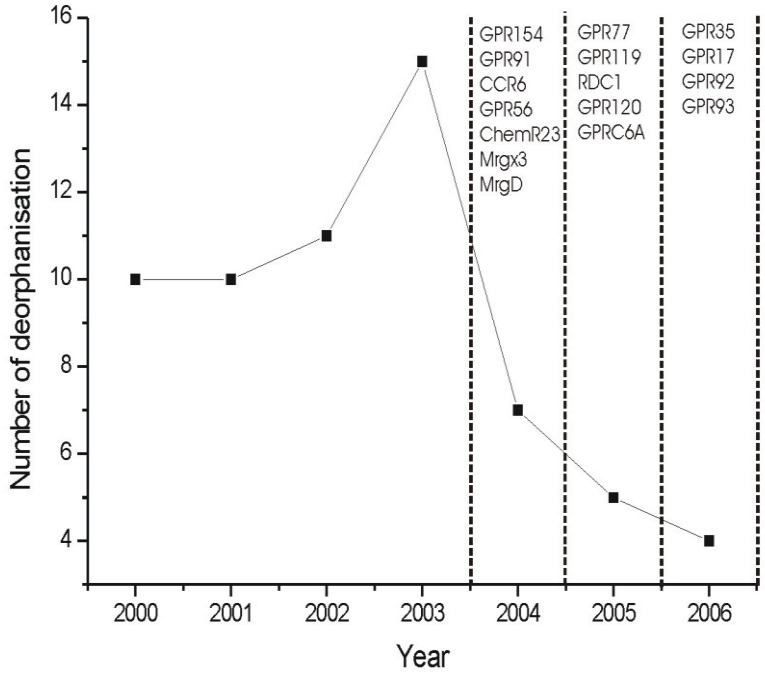

Figure 3. *Success rate of deorphanisation between 2002 and 2006. (Adapted from Oh da Y., 2006)*

Table 3. GPCRs with therapeuthic implications

Receptor	Ligand	Indication
GPR14	Urotensin II	Hypertension
P2Y12	ADP	Thrombosis
CysLT1	LTD4	Asthma bronchiale
NPGPR	NPAF	Pain
TA1	Trace amines	Depression
GPR40	M and L chain fatty acids	Diabetes
HM74	Nicotinic acid	Dyslipidaemia

2.3. Myocardial contractility

The major function of myocardial muscle cells is to accomplish the cardiac contraction and relaxation. The contractile function of the heart is regulated by a number of intrinsic and extrinsic mechanisms. Intrinsic mechanisms include the Frank-Starling mechanism and the force-frequency relation. Extrinsic mechanisms affecting cardiac function are the autonomic nervous system, hormones and peptides acting in autocrine/paracrine manner.

Contractile proteins

The contractile proteins of the heart lie within cardiomyocytes. The major molecules involved include the two chief contractile proteins, the thin actin filament and the thick myosin filament. During contraction, the filaments slide over each other without the individual molecules of actin or myosin actually shortening. As they slide, they pull together the two ends of the fundamental contractile unit called the sarcomere. On electron microscopy, the sarcomere is limited on either side by the Z line, to which the actin filaments are attached (*Figure 4*). The myosin heads interact with actin filaments when sufficient calcium is present. This process is the crossbridge cycling. As the actin filaments move toward the center of the sarcomere, drawing the Z lines closer together, the sarcomere shortens. Titin (also called connectin) acts as a third filament and provides elasticity. Between 0.6 and 1.2 mm in length, the titin molecule extends from the Z line, stopping just short of the M line. Titin has two functions: it tethers the myosin molecule to the Z line, and as it stretches its explains the stress-strain elastic relation of striated muscle elasticity (*Linke WA., et al.1994*).

Actin filaments are composed of two actin units, which intertwine in a helical pattern, both being carried on a heavier tropomyosin molecule. At regular intervals of 38.5 nm along this twisting structure is a closely bound group of three regulatory proteins called the troponin complex. Of these three, it is troponin C that responds to the calcium ions that are released in large amounts from the SR to start the crossbridge cycle. When the cytosolic calcium level is low, the tropomyosin molecule is twisted in such a way that the myosin heads cannot interact with actin. When more calcium ions present at the start of the contractile

cycle, interact with troponin-C, then the activated troponin C binds tightly to the inhibitory molecule, troponin I. This process repositions troponin M (tropomyosin) on the thin filament, which removes the inhibition exerted by tropomyosin on the actin-myosin interaction. Thus, the crossbridge cycle is initiated (*Opie LH., 1995*).

Structure of cardiomyocytes

Each cardiomyocyte is bounded by a complex cell membrane, the sarcolemma, and is filled with rodlike bundles of myofibrils. The sarcolemma invaginates to form an extensive tubular network (T tubules) that extends the extracellular space into the interior of the cell. Between the myofibrils and immediately beneath the sarcolemma are many mitochondria. The function of mitochondria is to generate the energy in the form of adenosine triphosphate (ATP) needed to maintain the heart's contractile function. Of the other organelles, the sarcoplasmic reticulum (SR) is most important (*Opie LH., 1995*).

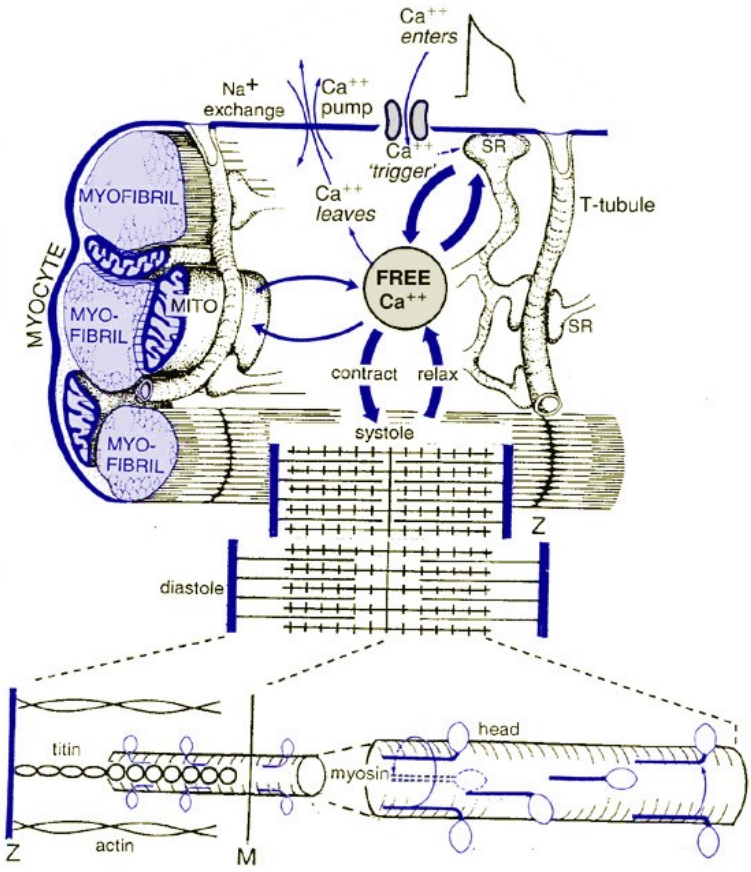

Figure 4. *Mechanisms of contraction of the heart. (Adapted from L.H.Opie.)*

Anatomically, the SR is a fine network spreading throughout the myocytes, demarcated by its lipid bilayer which is rather similar to that of the sarcolemma. Parts of the SR lie in very close apposition to the T tubules. Here the tubules of the SR expand into bulbous swellings. The name of these expanded areas is subsarcolemmal cisternae. Their function is to release calcium from the calcium release channel (also called the ryanodine receptor) to initiate the contractile cycle. The second part of the SR, the network SR, consists of ramifying and is concerned with the uptake of calcium that initiates relaxation. This uptake is achieved by the ATP-requiring calcium pump, (SERCA -sarcoendoplasmic

reticulum Ca^{2+}-ATPase). In the cytosol the concentrations of calcium ions rise and fall to cause cardiac contraction and relaxation.

Ion Channels

All current models of excitation-contraction coupling ascribe a crucial role to the voltage-induced opening of the sarcolemmal L-type calcium channels in the initiation of the contractile process. Ion channels have two major properties: gating and permeation. Ions can permeate through the channel only when both gates are open. In the case of the sodium and calcium channels the activation gate is shut at the normal resting membrane potential and the inactivation gate is open, so that the channels are voltage-gated. Depolarization opens the activation gate.

The alpha1-subunit (the organ-specific subunit) of the sarcolemmal calcium channel can be phosphorylated at several sites, especially in the C-terminal tail. Thereby, the electrical charges near the inner mouth of the nearby pores are altered to induce changes in the molecular conformation of the pores, so that there is an increased probability of opening of the calcium channel. Either the time that the channel remains in the open state is increased so that more calcium ions flow with the same degree of voltage activation, or phosphorylation activates calcium channels that were otherwise inactive(*Opie LH., 1995*).

There are two major subpopulations of sarcolemmal calcium channels relevant to the cardiovascular system, namely the T channels and the L channels. The T (transient) channels open at a more negative voltage, have short bursts of opening and do not interact with conventional calcium antagonist drugs. The T channels presumably account for the earlier phase of the opening of the calcium channel, which may also give them a special role in the early electrical depolarization of the

sinoatrial node, and hence of initiation of the heart beat. During relaxation, the sarcoplasmic calcium uptake pump and the Na^{+}/Ca^{++} exchanger compete for the removal of cytosolic calcium, with the SR pump normally being dominant.

The direction of ion exchange is responsive to the membrane potential and to the concentrations of sodium and calcium ions on either side of the sarcolemma. Because sodium and calcium ions can exchange either inward or outward in response to the membrane potential, there must be a specific membrane potential, called the reversal or equilibrium potential, at which the ions are so distributed that they can move as easily one way as the other. The reversal potential may lie about halfway between the resting membrane potential and the potential of the fully depolarized state. Changing the membrane potential from the resting value of, say, –85 mV to +20 mV in the phase of rapid depolarization of the action potential may therefore briefly affect the direction of Na^{+}/Ca^{2+} exchange.

2.4. Classical GPCR signaling in the heart

Cardiovascular homeostasis is regulated by a diverse type of hormones and neurotransmitters, many of which exert their physiological effects through activation of GPCRs. It is now clear that messenger RNAs for a broad spectrum of GPCRs are expressed in cardiac cells. It has been estimated that ~200 different GPCRs are expressed in various cardiac cell types including endothelial cells, fibroblasts, and myocytes of the atria, ventricles, and coronary vasculature. The G-protein coupled receptors are a large superfamily, among others the β-adrenergic, α-adrenergic, muscarinic, angiotensin, adenosine, cardiac opioid receptors belong to GPCRs. It is quite possible that many (if not all) of these neurohumoral signaling

systems play crucial roles, not only in regulating cardiac performance, but also in orchestrating cardiac growth (*Table 4*).

For example, the nine different adrenergic receptors ($\alpha_{1A, 1B, 1D}$; $\alpha_{2A, 2B, 2C}$; and $\beta_{1, 2, 3}$) play a key role in the control of cardiovascular function (*Table 5*) by mediating actions of sympathetic nervous system activation and circulating catecholamines. The mostly examined cardiac adrenerg receptor is the β receptor, which preferentially couples to the Gs-AC-cAMP signaling cascade. Activation of this cascade enhances the activity of protein kinase A (and probably other pathways (*Kopperud R. et al. 2003*); and, through phosphorylation, regulates activity of ‘‘downstream’’ enzymes and channels, ultimately impacting on metabolic pathways, contractile regulatory proteins, and L-type calcium channels (*Wallukat G., 2002; Xiang Y., 2003*).

Table 4. *G protein-coupled receptors for classical and nonclassical agonists expressed in cardiac myocytes*

Classical	
β adrenergic (β1, β2, β3)	Xiang and Kobilka 2003
Adenosine	Dougherty et al. 1998
Angiotensin II (AT1 & 2)	Regitz-Zagrosek et al. 1998
Glucagon	Mery et al. 1990
Prostanoid	Mendez and LaPointe 2002
Endothelin (ET-A, ET-B)	Asano et al. 2002
Muscarinic (M2, M3)	Wang et al. 2001
Bradykinin	Nakamura et al. 1996
α adrenergic (α1A, α1B, α1D)	Xiang and Kobilka 2003
Histamine (H1, H2, H3)	Genovese and Spadaro 1997
Opioid (A, n, y)	Barron 2000
Serotonin	Nebigil and Maroteaux 2001
Nonclassical	
Nucleotide (P2Y1, 2, 4, 6, 11)	Vassort 2001
Adrenomedullin/CGRP	Szokodi et al. 1998
"Protease-activated"	Sabri et al. 2002
Urotensin-II (GPR14)	Ames et al. 1999
CRF/urocortin (CRFR2h)	Kimura et al. 2002
PACAP/VIP (PAC1)	Henning and Sawmiller 2001
Sphingosine-1-phosphate/LPA (Edg1, 3, 5)	Karliner 2002
Glucagon-like peptide-1	Vila Petroff et al. 2001
Vasopressin (V1)	Xu et al. 1999
Cysteinyl leukotriene (CysLT2)	Hui and Funk 2002
Calcium sensing (Ca-SR)	Wang et al. 2003
Cannabinoid (CB1)	Bonz et al. 2003
Growth hormone secretagogue-receptor (GHS-R1a)	Gnanapavan et al. 2002
Melatonin receptor (MT-1, MT-2)	Sewerynek 2002
Oxytocin PTH-PTHrp (PTH-1)	Shojo and Kaneko 2000
Relaxin-H2 (LGR7)	Samuel et al. 2003
Melanin-concentrating hormone (SLC-1)	Kolakowski et al. 1996
Prolactin releasing peptide (rUHR-1, hGR3*)	Satoh et al. 2000
Neuromedin U (NMU1, NMU2)	Raddatz et al. 2000
Apelin (APJ)	Szokodi et al. 2002
Melanocortin (MC3R)	Schioth 2001

Adrenoceptors and adrenergic signaling

Heart cells express the β_1 and β_2 subtype of adrenoreceptors (AR), but the relative density of β_1-AR is much higher than that of the β_2-AR subtype. Both subtypes stimulate adenyl cyclase. The principal function of β-AR is to mediate positive inotropy and chronotropy. The occurrence of other β-AR subtype, β_3-AR and its mRNA has been described in heart ventricles and atria, with cardioinhibitive function mediated via Gi proteins (*Emorine L. et al. 1994*). Moreover, β_3-AR have shown substantial variable expression across species. The last subtype of β-AR, which has been discovered in the heart, is the fourth subtype, also called atypical cardiostimulatory β-AR, assumed as putative β4-AR (*Molenaar P., 1997)*. Heart cells also express α1-AR (*Brodde OE., Michel MC., 1999*). Functionally, α1-AR are able to increase cardiac contractility and excitability, and they also alter heart metabolism and they can (similarly to β-AR) induce cell growth (*Zimmer HG., 1997*).

Occupancy of the beta-adrenergic receptor is coupled by a G-protein complex to activation of a sarcolemmal enzyme, adenylate cyclase. The G-protein complex involved, being stimulatory, is called Gs. Situated in the sarcolemma, Gs passes on the signal from the beta-receptor to adenylate cyclase. The G protein composed of Gα, Gβ, and Gγ, which upon receptor stimulation splits into the alpha-subunit that is bound to GTP, and the beta-gamma-subunit. Either of these subunits may regulate differing effectors such as adenylate cyclase, phospholipase C, and ion channels. The activity of adenylate cyclase is controlled by two different G-protein complexes, namely G_S which stimulates and G_i which inhibits. The alpha-subunit of GS (α_S) combines with GTP and then separates from the other two subunits to enhance activity of adenylate cyclase.

***Table 5**. Important GCPRs in the heart*

Receptor	**β_1**	**β_2**	**β_3**	**α_{1ABD}/AT_1/ET**	**α_2**	**M_2**
Primer G protein	Gs	Gs/Gi	Gs/Gi	Gq/G11	Gi	Gi
Primary effector in heart tissue	AC	AC	AC	PLC-β	AC	AC
Signals	↑cAMP / PKA	↑cAMP/ PKA MAPK	↑cAMP/ PKA	↑PKC/MAPK	↓cAMP/ PKA	↓cAMP/ PKA
Endogenous agonist	NA/A	NA/A	NA/A	NA/A /AT-II/ET	NA/A	ACh

The β- and γ-subunits appear to be linked structurally and in function. Adenylate cyclase, stimulated by Gs, produces the second messenger, cyclic AMP, which then acts through a further series of intracellular signals and specifically the third messenger protein kinase A (PKA), to increase cytosolic calcium transients. When activated, PKA directly phosphorylates nodal Ca^{2+} regulatory proteins such as L-type Ca^{2+} channel, RyR and PLN. PLN function as a negative regulator of SERCA-2 activity in the SR by direct association. PKA-mediated phosphorylation of PLN at Serine16 causes its dissotiation from SERCA-2, permitting near-maximal Ca^{2+} ATP-ase activity and an increase in SR Ca^{2+} loading, which in turn generates larger action potentials during systole (*Xiao RP, et al., 2006*).

Other cardiac receptors, such as the alpha-adrenergic receptor, have an alternate dual messenger system involving inositol trisphosphate (IP3) and diacylglycerol, with the latter activating protein kinase C. Such signals are of established importance in controlling calcium flux in vascular smooth muscle, thereby regulating vascular tone and indirectly the blood pressure. In the case of cardiac myocytes, it is now appreciated that receptors coupled to protein kinase C, such as angiotensin II, may play a major role in the regulation of cardiac myocyte growth and sometimes have inotropic effects. Cardiac β-adrenergic receptors are chiefly the beta1-subtype, whereas most noncardiac receptors are β_2. There are also β_2-receptors in the human heart, about 20 per cent of the total β-receptor population in the left ventricle and about twice as high a percentage in the atria. These β_2-receptors appear to have greater efficacy in their capacity to activate the G-protein–adenylate cyclase system than do the β_1-receptors (*Xiao RP, et al., 2006*).

Muscarinic receptors

In the case of the parasympathetic system, signaling is again an extracellular first messenger (acetylcholine), a receptor system (the muscarinic receptor), and a sarcolemmal signaling system (the G-protein system) (*Fleisch JH., 1969*; *Hartzell HC., 1988; Cohen-Armon M., 1986*). The M2 subtype mostly represents MR in the mammalian myocardium. The function of MR is opposite to those of β-AR (i.e. negative inotropy and chronotropy). M2 receptors are coupled to the Gi protein (they inhibit AC), but a potent activation of the receptor permits interaction of the receptor with Gq and activation of phospholipase C (PLC). A further consequence of MR activation is its influence on ionic flow through the membrane via activation or inhibition of appropriate ion channels. M2-MR inhibit Ca^{2+} channels indirectly

via decreased production of cAMP and lower PKA activity; the main result of MR activation mediated via ion channels is the activation of inwardly rectifying acetylcholine sensitive potassium channels (KACh) (*Giraldo E. et al., 1988*) Activation of KACh leads to hyperpolarization and negative inotropy in the atria (*Baskin SI, Thomsen RH. et al., 1991*). In addition, these channels also participate in negative chronotropy (*Taniguchi T. et al., 1979*). Another situation occurs in a pacemaker (sinoatrial node), where the negative chronotropy is considerably affected by muscarinic inhibition of adenylylcyclase. The evidence that M2 receptors are not the only subtype of MR in the heart is gaining support, but the problem of identification and quantification has not been satisfactorily solved. Among all non-M2 receptors identified pharmacologically and/or electrophysiologically in the heart up to now (M1, M3, M4), the M1 subtype (cardiostimulating, coupled to the Gq/PLC) is the best documented (M1mRNA detection by single-cell reverse transcriptase-polymerase chain reaction (rT-PCR), (*Colecraft et al. 1998*). These „receptors"- better Ca^{2+}-sensitive channels - are the subjects of phosphoralytion by PKA as a result of β-AR stimulation. Moreover, they can also be activated via enhanced level of intracellular Ca^{2+}. Therefore they can be considered as one of the „targets of opposite action" of adrenoceptors vs. muscarinic receptors. Explanation of cardiostimulative effect of β1-AR and M1MR thus gains new importance.

Endothelin signaling

Endothelin is a potent peptide vasoconstrictor released by endothelial cells throughout the circulation. Firstly isolated by Yanagisawa et al. in 1988, and it has been found to be the most potent and long lasting endogenous vasoconstrictor known so far. (*Yanagisawa M, et al., 1988*). Three endothelin peptides (endothelin-1, endothelin-2, and endothelin-3) have been identified, all of which are potent constrictors. In humans ET-1, the predominant and biologically most relevant isoform is produced by the vascular endothelium and smooth muscle cells, cardiac myocytes, fibroblasts, macrophages, airway epithelial cells, macrophages, pancreatic islets and brain neurons among others. At least two subtypes of endothelin receptors (types A and B) have been recognized (*Arai H. et al.,* 1993).

Both receptors are G-protein-coupled heptahelical transmembrane proteins. In mammalian tissues, the 2 receptors, ET_A and ET_B, are responsible for the diverse actions of the three ET isoforms. The affinity of ET_A receptors for ET-1 and ET-2 is >100-fold higher than for ET-3, whereas ET_B receptors bind ET isopeptides with a similar affinity (*Masaki T,* 1995). The binding of ET-1 to ET_A receptors activates phospholipase C, which leads to an accumulation of inositol triphosphate, diacyl-glycerol and intracellular calcium (*Goto K. et al., 1989; Krämer BK., et al, 1991*). In contrast, the activation of endothelial ETB receptors stimulates the release of NO and prostacyclin (*Warner TD. et al., 1989; Piuhola J. et al., 2003*).

The production of ET is regulated at the gene level. ET-1 expression is stimulated by hypoxia, various growth factors, cytokines and vasoactive substances such as thrombin, transforming growth factor, angiotensin II, vasopressin. The expression of ET mRNA is inhibited by nitric oxide (NO), natriuretic peptides, prostacyclin, and heparin. Endothelin-1 linked positive inotropic effect in cardiac muscle has

been described in several mammalian species, including rat (*Krämer BK. et al., 1991*), guinea-pig (*Ishikawa T. et al., 1988*), rabbit (*Endoh M. et al., 1991*) and human (*Moravec CS* et al., 1989). ET-1 is one of the most potent positive inotropic agent (*Concas V. et al., 1989; Leppäluoto J. et al., 1992*) yet described in mammalian myocardium. In the spontaneously active right atria of the rat, endothelin induces a positive inotropic effect with no chronotropic effect. Endothelin does not modify intracellular levels of cAMP under basal conditions or after stimulation with isoproterenol. The positive inotropic effect of ET-1 is shown to be associated with stimulation of phospholipase C (PLC) and the resultant acceleration of the hydrolysis of phosphoinositide, leading to production of putative second messengers, 1,4,5-inositol trisphosphate and 1,2-diacylglycerol. ET-1 exerts its positive inotropic effect by increasing the sensitivity of the myofilament to Ca^{2+} ions, increasing intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$), by mobilization of intracellular Ca^{2+} ions and transmembrane Ca^{2+} currents, alkalinization of the intracellular milieu by stimulation of protein kinase C (PKC)-dependent Na^{+}/H^{+} exchanger. It has been shown that ET-1 just like phenylephrine and angiotensin II increases the Na^{+}/Ca^{2+} exchange activity in enriched sarcolemmal vesicles from rat heart via a PLC-PKC pathway, while isoprenaline does not have an effect on the Na^{+}/Ca^{2+} exchanger function *(Brunner F, et al., 2006*).

Adenosine signaling

Adenosine is a physiological vasodilator. It is formed from the breakdown of ATP both physiologically and pathologically (*Böhm M. et al., 1988*). Adenosine can diffuse from myocardial cells to act on coronary arterial smooth muscle to cause vasodilation. The mechanism of the latter effect is reasonably well understood and

involves the stimulation of vascular adenylate cyclase and cyclic AMP formation. A_2-Receptors mediate such vasodilation (*Romano FD. et al., 1989*). The A_1-receptors coupled to adenylate cyclase by the inhibitory G protein (alpha-subunit) are functional in the myocardium. A1-receptors couple to the acetycholine-sensitive potassium channel to stimulate channel opening and thereby to exert inhibitory effects on the sinus and AV nodes, the latter inhibition being the basis for the use of adenosine in the treatment of supraventricular nodal reentry arrhythmias. Second, A1-receptors may in some circumstances couple to phospholipase C, which hypothetically explains their role in preconditioning.

2.5. Novel GPCR candidates

The last twenty years it was demonstrated that numerous peptides had role in the regulation of cardiac contractility. For example in 1998 it was described that *adrenomedullin* at lower doses may enhance contractility via cAMP-independent mechanisms, and at higher doses, stimulate cAMP formation and induce the positive inotropic effect (*Szokodi I. et al., 1996; 1998; Sato A. et al, 1997*).

Some in vivo studies have demonstrated that *ghrelin* exerts cardiac actions in humans (*Enomoto M., 2003, Nagaya N.,2003*), and ghrelin administration induces hemodynamic effects in man either in normal subjects or in patients with dilated cardiomyopathy, reducing cardiac afterload and increasing cardiac output, without an increase in heart rate (*Nagaya N., 2003*). Vasopressin, urotensin II (*Cheriyan J. et al., 2009*), urocortins (*Wright SP. et al., 2009*) and motilin also have effect on cardiac functions in different degree.

In 1998 Tatemoto et al. discovered *apelin* as an endogenous peptide ligand for the apelin-angiotension receptor-like 1 (APJ), which receptor originally identified by *O'Dowd et al. in 1993*. It shares the closest identity to the angiotensin II type 1 (AT1) receptor ranging from 40% to 50% in the hydrophobic transmembrane regions, but does not bind angiotensin II. Intraperitoneal administration of apelin resulted in short-term increases in drinking behavior in rats (*Tatemoto K. et al., 1998*), in parallel with the thirst-promoting effect of angiotensin II. In contrast to the well-established vasopressor effect of angiotensin II, intravenous injection of apelin lowered blood pressure in anesthetized rats (*Tatemoto K. et al., 1998*).

The apelin-APJ system has been postulated to play an important role in cardiovascular homeostasis. Plasma apelin levels were found to be increased in patients with early stages of heart failure and decreased in late stages of heart failure (*Chen MM. et al., 2003*). The fact that apelin exerts the most potent positive inotropic action in normal hearts (*Szokodi I, et al., 2002*) suggests a role for reduced apelin levels in the pathogenesis of heart failure. Indeed, in rat failing hearts, administration of apelin augmented pressure development and cardiac output (*Berry MF. et al., 2004*). In normal hearts, the apelin-induced inotropic effect was attenuated by inhibition of PLC, PKC, Na^+-H^+ exchanger, and Na^+-Ca^{2+} exchanger (*Szokodi I. et al., 2002*). These data suggest that apelin causes activation of PLC-dependent signal transduction pathway, which ultimately affects Ca^{2+} availability and/or Ca^{2+} responsiveness of the myofilaments.

Prolactin- releasing-peptide

In 1998 a new bioactive peptide was identified from bovine hypothalamic tissue (*Hinuma S. et al., 1998*) as a potential ligand of G-protein-coupled receptor 10 (GPR 10, this receptor has also been designated as GR3, and the rat counterpart of hGR3/GPR10 is UHR-1). As this peptide can stimulate the release of prolactin from anterior pituitary cells, it was named prolactin releasing peptide (PrRP). It has been shown to stimulate the secretion of other hypothalamic-pituitary homones e.g. oxytocin, adrenocorticotropin both in-vivo and in-vitro. PrRP consists of 31 amino acids and it has a variant form (PrRP-20), which is posttranslational modification of the same gene product (*Figure 5*).

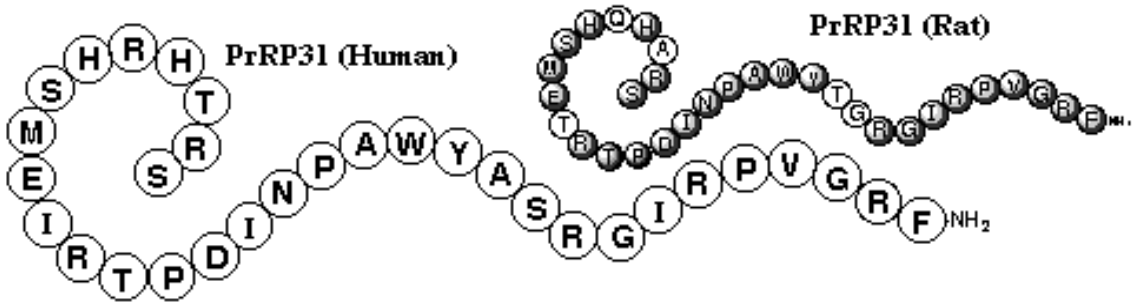

***Figure 5**. The structure of PrRP-31 in human and rat (Chang RC. et al., 1980).*

It is well established that PrRP plays role in the regulation of the central stress response (*Maruyama M. et al., 1998*), feeding behaviour (*Sun B. et al., 2005*) and sleep regulation (*Zhang SQ. et al., 2000*). Specific ^{125}I-PrRP binding sites were described in the rat. In addition to the expected binding sites in the brain there was also high affinity binding in the heart and the mainly red fibre soleus muscle (*Satoh*

F. et al., 2000). Intracerebro-ventricular administration of both PrRP-20 and PrRP-31 resulted in significantly increased mean arterial blood pressure in conscious, unrestrained rats (*Samson WK. et al., 2000*). It was also demonstrated that Ir-PrRP could be found in the plasma, but the source of circulating peptide remains unknown (*Matsumoto H. et al., 1999*). The plasma levels of PrRP in rats were not significantly different between male and female. The effect of PrRP was examined (*Nanmoku T. et al., 2003*) on catecholamine secretion and DNA synthesis in rat pheochromocytoma and the results had shown that PrRP directly stimulated both catecholamine synthesis and secretion in the adrenal medulla, and they suggested that the cAMP-PKA system may be involved in PrRP-induced dopamine release. On the other hand they have also demonstrated that PrRP31 significantly increase PKC activity in pheochromocytoma cells (*Nanmoku T. et al., 2005*).

Based on these results, one can anticipate that PrRP may have an important role in the regulation of cardiovascular homeostasis, however, it has not been addressed so far.

2.6. Novel signaling mechanisms in the heart

PKCα as a novel negative regulator of contractility

PKC comprises a multigene family of related serine/threonine kinases that sit at the crossroads of many signal transduction pathways and are implicated in a wide range of GPCR and other growth factor-dependent cellular responses including cell proliferation, differentiation and apoptosis (*Steinberg SF., 2008*). Approximately 10 different isozymes make up the PKC family, and they are broadly

classified by their activation and conformation characteristics. The conventional PKC isozymes (α, βI, βII, and γ) are Ca^{2+}- and DAG-activated, the novel PKC isozymes (δ, ε, θ, and η) are DAG-sensitive but Ca^{2+}-insensitive. The atypical forms (ζ, and λ) are Ca^{2+}- and DAG-independent (*Steinberg SF., 2008*). The activity of any given PKC isoform is dependent upon its expression level, its localization within the cell, and its phosphorylation state (*Dorn GW II, Force T., 2005; Steinberg SF., 2008*).

PKC isoforms in the myocardium are involved in numerous cellular processes including regulation of cytosolic [Ca^{2+}], myofilament Ca^{2+} sensitivity, and contractility of cardiac muscle cells (*Solaro RJ. , 2008*). The loss of contractility that accompanies heart failure is also associated with an increase in PKCα protein content and activity (*Bowling . et al., 1999; Braz JC. et al., 2004; Johnsen DD. et al., 2005*). Moreover, growing body of evidence suggest that PKCα acts as a critical regulator of myocardial contractility (*Braz JC. et al., 200; Hahn HS. et al., 2003*; *Hambleton M. et al., 2006*). Braz et al. have reported that cardiac-restricted deletion of *PKCα* gene results in increased cardiac contractility, whereas transgenic overexpression of the molecule leads to ventricular dysfunction (*Braz JC. et al., 2004*). Hypercontractility induced by *PKCα* gene deletion protected against pressure overload-induced heart failure and dilated cardiomyopathy associated with deletion of the *muscle lim protein (MLP)* gene in the mouse (*Braz JC. et al., 2004*). In agreement, long-term pharmacological inhibition of PKCα with Ro-31-8220 in $MLP^{-/-}$ mice dramatically improved left ventricular function (*Hambleton M. et al., 2006*). Furthermore, transgenic mice with cardiac-restricted inducible

expression of a dominant negative PKCα mutant also showed reduced failure progression after myocardial infarction (*Hambleton M. et al., 2007*).

Mechanistically, PKCα directly phosphorylates inhibitor-1, resulting in greater protein phosphatase type 1 (PP1) activity, which in turn leads to greater phospholamban dephosphorylation and less activity of the SERCA2 pump (*Braz JC. et al., 2004*). Less SERCA2 activity reduces SR Ca^{2+} load, leading to reduced Ca^{2+} release during systole, hence reduced contractility (*MacLennan DH. et al., 2003*). Alternatively, PKCα appears to directly phosphorylate key contractile proteins, leading to reduced force production in isolated, skinned myocytes (*Belin RJ . et al., 2007*). Although PKCα is emerging as a crucial negative modulator of cardiac function, its role in the acute regulation of contractility in a physiological milieu is unclear.

Mitogen-activated protein kinases as novel modulators of cardiac contractility

The evolutionarily conserved mitogen-activated protein kinase (MAPK) superfamily occupies a central position in intracellular signal transduction in all eukaryotic cells (*Widmann C. et al., 1999)*. The members of the classic MAPK family, including extracellular signal-regulated kinases (ERK1 and 2), p38-MAPK (α, β, γ, δ), c-Jun N-terminal kinases (JNK1, 2 and 3) and big MAPK (BMK or ERK5), have been implicated in the regulation of diverse cellular processes from cell growth and proliferation to apoptosis (*Johnson GL. et al., 2002; Qi M, Elion EA*., 2005; *Nishimoto S., Nishida E*., 2006; *Gerits N. et al., 2007; Wang Y., 2007*). The ERK1/2 pathway is activated mainly by growth stimuli (GPCR agonists, receptor tyrosine

kinases) (*Bueno OF, Molkentin JD., 2002; Rubinfeld H. et al., 2005*), JNK and p38-MAPK are called stress-activated kinases because of their selective responses to physical, chemical and physiological stressors (*Kyriakis JM, Avruch J.*, 2001), and the BMK is implicated in both growth and stress signaling (*Hayashi M, Lee JD., 2004*).

p38-MAPK signaling has been shown to be associated with various pathological conditions such as cardiac hypertrophy and heart failure in humans (*Cook SA, Sugden PH, Clerk A.*, 1999) and animal models (*Izumi Y. et al., 1998*). Initially, p38-MAPK signaling was considered to promote cardiomyocyte hypertrophy in response to GPCR agonists (e.g. angiotensin II and ET-1) in cultured neonatal cardiomyocytes (*Clerk A. et al., 1998)*. In line, specific activation of the p38-MAPK pathway using constitutively activated mutants of upstream kinases, MAPK kinase 3 (MKK3) and MKK6, was sufficient to provoke hypertrophic phenotype in cultered myocardial cells (*Zechner D. et al., 1997)*. However, subsequent studies using transgenic models revealed very different effects in vivo. Prolonged inhibition of p38-MAPK activity in mice expressing dominant negative mutants of MKK3 or MKK6 enhanced further pressure overload-induced cardiac hypertrophy, suggesting an inhibitory function of p38-MAPK on cardiac growth response (*Braz JC. et al., 2003).* Moreover, cardiac-specific conditional inactivation of the *p38α* gene indicated that p38α-MAPK is not an indispensable regulator for cardiac hypertrophy in response to pressure overload but exerts a protective effect against cardiomyocyte apoptosis and ventricular remodeling (*Nishida K. et al., 2004*).

Accumulating data suggest that p38-MAPK is directly involved in the regulation of cardiac contractility. Persistent activation of p38-MAPK signaling in mice with cardiac-specific expression of MKK3bE or MKK6bE was associated with compromised left ventricular systolic and diastolic function (*Liao P. et al., 2001).* Moreover, sarcomeric function was depressed in conjunction with decreased phosphorylation of α-tropomyosin and troponin I in response to chronic activation of p38α-MAPK in transgenic MKK6bE hearts (*Vahebi S. et al., 2007).* In adult rat cardiomyocytes, activation of p38-MAPK by adenoviral gene transfer of MKK3bE resulted in a significant reduction in contractility. Conversely, pharmacological inhibition of p38-MAPK activity increased contractile force in a dose-dependent manner, without altering L-type Ca^{2+} currents or intracellular Ca^{2+} transients (*Liao P. et al., 2002)*. Though chronic modulation of p38-MAPK activity clearly affects cardiac performance, the involvement of p38-MAPK signaling in the physiological control of cardiac contractility is less clear.

In contrast to the MEK3/6–p38-MAPK pathway, ample evidence indicate that activation of the MEK1/2–ERK1/2 pathway represents a pivotal protective mechanism in the heart (*Bueno OF, Molkentin JD., 2002; Wang Y., 2007*). ERK1/2 signaling has been reported to confer cardioprotection in vivo against ischemia-reperfusion injury. Genetic alterations in ERK activity correlated well with susceptibility to ischemic damage. *Erk2*$^{+/-}$ gene-targeted mice showed an increase in infarct area, enhanced apoptotic cell death and reduced left ventricular performance. On the contrary, mice with cardiac-specific overexpression of a constitutively active MAPK kinase 1 (MEK1), the upstream regulator of ERK1/2, were (*Lips DJ. et al., 2004)* significantly protected from ischemia-reperfusion

imjuryeover, MEK1 transgenic mice were reported to develop mild concentric left ventricular hypertrophy associated with enhanced cardiac function without signs of decompensation over time (*Bueno OF. et al., 2000)*. Consistent with these observations, inhibition of ERK1/2 activation by cardiac-specific expression of a dominant negative form of Raf-1, the upstream regulator of MEK1/2, resulted in blunted cardiac hypertrophy, increased apoptosis, and systolic dysfunction with concomitant increase in mortality in response to pressure overload (*Harris IS. et al., 2004)*. Though systolic function is clearly affected in these transgenic models, it has not yet been established if ERK1/2 can directly modulate cardiac contractility.

3. AIMS OF STUDY

1. To study the functional significance of PrRP in the myocardium by characterizing its direct effects on cardiac contractility.

2. To characterize the underlying signaling mechanisms of the cardiac effects of PrRP in the intact rat heart.

3. To characterize the role of ERK1/2 and the potential upstream regulators and downstream effectors in the regulation of cardiac contractility stimulated by ET-1 in the intact rat heart.

4. To characterize the role of p38-MAPK and the potential upstream regulators and downstream effectors in the regulation of cardiac contractility stimulated by ET-1 in the intact rat heart.

4. ROLE OF PROLACTIN RELEASING PEPTIDE IN THE REGULATION OF CARDIAC CONTRACTILITY

4.1. Introduction

PrRP, the endogenous ligand for the orphan GPCR, hGR3/GPR10 (*Hinuma S. et al., 1998*), has been recently implicated in the central control of cardiovascular function. Microinjection of PrRP into the most caudal ventrolateral medulla oblongata, recognized as the caudal pressor area, elicited dose dependent increases in mean arterial pressure, heart rate, and renal sympathetic nerve activity (*Horiuchi J. et al., 2002)*. Specific PrRP binding sites have been found in a number of rat peripheral tissues, with the highest level of binding present in the heart (*Satoh F. et al., 2000*), suggesting that the peptide may play a role in the regulation of cardiac function, however, to date there is no information available regarding the functional significance of PrRP in the myocardium. Therefore, the objective of our study was to characterize the direct cardiac effects of PrRP, as well as the underlying signaling mechanisms.

4.2 Materials and methods

4.2.1. Drugs

Drugs used were PrRP-31 (Phoenix Europe GmbH, Karlsruhe, Germany); dobutamine (Sigma-Aldrich Co, Saint Louis, Mo); calyculin A, 3-isobutyl-1-methylxanthine (IBMX), okadaic acid, and Ro32-0432 (Merck Chemicals Ltd., Nottingham, UK).

4.2.2. Isolated perfused rat heart preparation

All protocols were reviewed and approved by the Animal Use and Care Committees of University of Oulu and University of Pécs. Cardiac function was assessed ex vivo in an established isolated rat heart preparation (*Szokodi I. et al., 1998; 2002*) Hearts of male 7-week-old Sprague-Dawley rats (body weight: 260±31 g, n=138) were excised rapidly (wet left ventricular weight: 0.71± 0.13 g), and mounted on a Langendorff perfusion system (LF-01, Experimetria Ltd., Budapest, Hungary). The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer, pH 7.40, equilibrated with 95% O_2-5% CO_2 at 37°C. The composition of the buffer was (in mM) NaCl 113.8, $NaHCO_3$ 22.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.1, $CaCl_2$ 2.5, and glucose 11.0. Hearts were perfused at a constant flow rate of 5.5 mL/min with a peristaltic pump (Minipuls 3, model 312, Gilson, Villiers, France). Heart rate was maintained constant (305±1 beats per minute) by atrial pacing using a Grass stimulator (model S88, Grass Instruments, West Warwick, RI) (11 V, 0.5 ms). Contractile force (apicobasal displacement) was obtained by connecting a force displacement transducer (FT03, Grass Instruments, West Warwick, RI) to the apex of the heart at an initial preload stretch of 2 g. Perfusion pressure reflecting coronary vascular resistance was measured by a pressure transducer (model MP-15, Micron Instruments, Los Angeles, Calif) situated on a side arm of the aortic cannula.

4.2.3. Experimental design

A 40-minute equilibration period and a 5-minute control period were followed by addition of various drugs to the perfusate for 15 minutes. Initially, we determined the concentration-dependent effect of PrRP-31 (1 to 100 nM) on cardiac contractility. Next, we compared the effect of PrRP to the maximal response to the β-adrenergic agonist dobutamine (10 μM). For signal transduction studies, the concentrations of IBMX (10 μM) and Ro32-0432 (100 nM) were selected because these concentrations have been demonstrated to suppress the activity of phosphodiesterases (*Bian JS. et al., 2000*) and PKCα (*Hambleton M. et al., 2006*), respectively. Calyculin A blocks both protein phosphatase type 1 (PP1) and type 2A (PP2A) at the dose of 1 nM, whereas 10 nM okadaic acid inhibits only PP2A activity (*Herzig S. et al., 2000*). At the end of the experiments, the left ventricles were frozen in liquid nitrogen and stored at –80 C° until assayed.

4.2.4. Immunoblot analysis

Left ventricular tissue was homogenized in lysis buffer containing of 20 mM Tris, (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, supplemented with 1 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 μg/mL pepstatin, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 20 mM NaF. Samples (30 μg) were loaded onto SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk and incubated with indicated primary antibody overnight. Protein levels were detected using enhanced chemiluminescence. For a second staining, the membranes were stripped for 30

minutes at 60 °C in stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol). Western blotting was performed with antibodies specific for phospho(Ser16)-phospholamban (Santa Cruz Biotechnology, Santa Cruz, Calif) and p38 (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK).

4.2.5. Statistical analysis

Results are presented as mean±SEM. Two-way repeated-measures ANOVA was used to evaluate the statistical significance of differences among groups for cardiac contractility. When significant differences were detected in 2-way repeated measures ANOVA for the treatment-by-time interactions, a Bonferroni post hoc test was used for specific comparisons. All other parameters were analyzed with 1-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at the level of $P < 0.05$.

4.3. Results

4.3.1. Effect of PrRP on cardiac contractility

The intracoronary administration of PrRP (1 to 100 nM) for 15 minutes induced a dose-dependent slowly developing positive inotropic effect in the isolated rat heart preparation (*Figure 6*). Maximal response to PrRP was observed at the concentration of 10 nM. As shown in Figure 6B, the elevation of developed tension in response to PrRP was gradual and the maximal response occurred approximately at 15 minutes. The time course of the effect of PrRP was markedly

different from the rapidly developing, but short-lived effect of the β-adrenergic receptor agonist dobutamine (250 nM) (*Figure 7*).

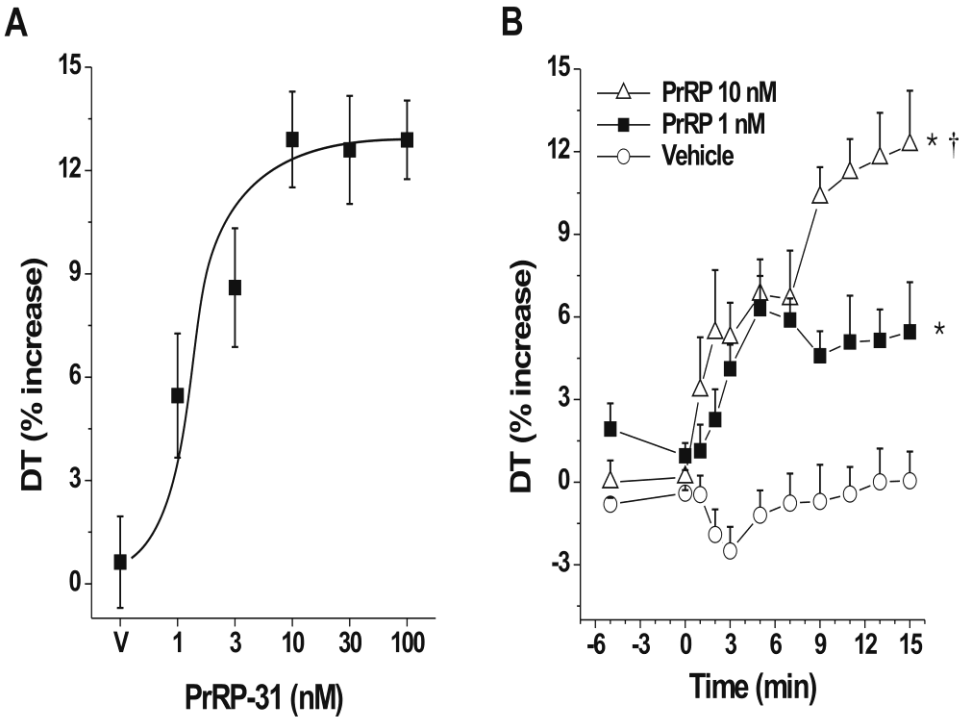

Figure 6. A: *Effect of PrRP-31 (1 to 100 nM) on developed tension (DT) in isolated perfused rat hearts.* ***B:*** *Time course of the inotropic effect of PrRP-31. Results are expressed as a percent change versus baseline values. Data were analyzed by 2-way repeated-measures ANOVA followed by multiple comparisons with the Bonferroni post hoc test and are reported as mean±SEM (n=4-6 for each group). *P<0.05, †P<0.001 vs. control and PrRP (1 nM).*

The resting tension (2.0±0.01 g) of the perfused hearts was not significantly affected by PrRP (1 to 30 nM), except that it induced a slight increase (2.2±0.03 g; $P<0.05$) at the highest concentration (100 nM). Overall, changes in perfusion pressure induced by PrRP were small (data not shown).

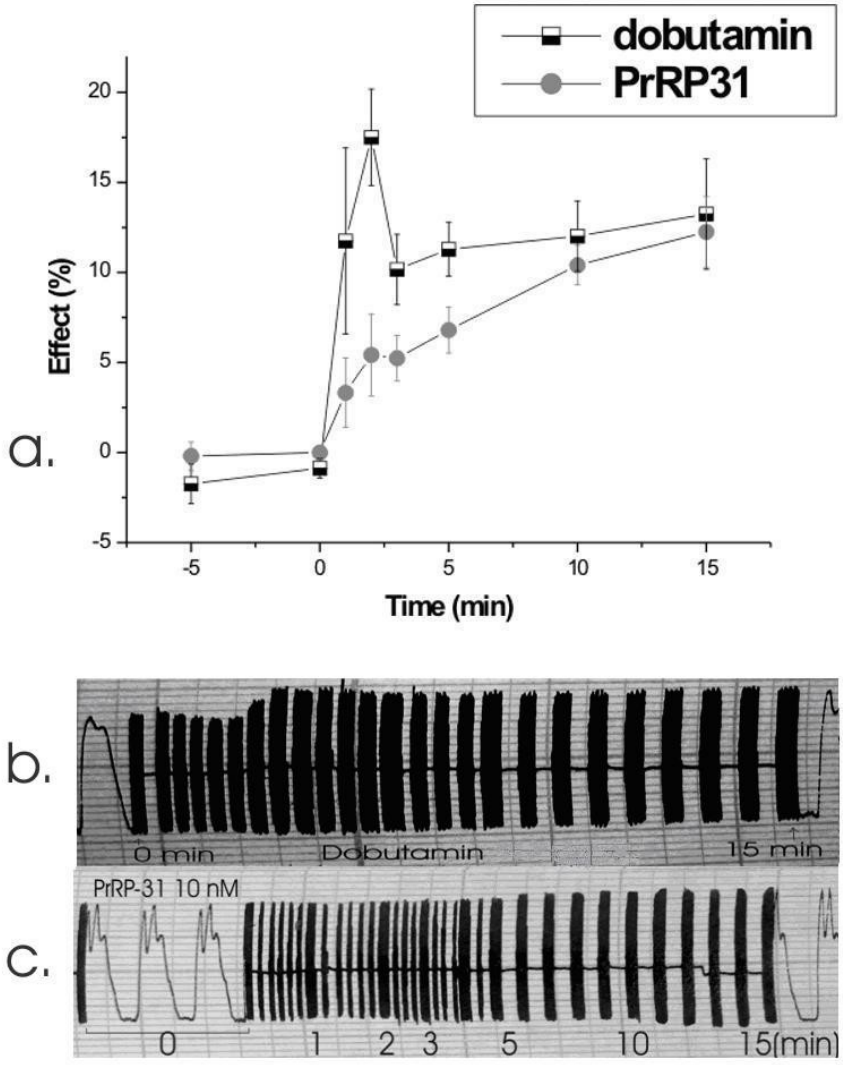

Figure 7. ***A:*** *Dobutamine (250 nM) has short-lived, rapidly developing inotropic effect in contrast to the slowly developing effect of PrRP (10 nM). Data are reported as mean±SEM (n=4-6 for each group).* ***B and C:*** *Original recordings of changes in developed tension (DT) in response to dobutamine and PrRP-31.*

4.3.2. cAMP and PrRP signaling

To assess the involvement of the cAMP-PKA pathway in PrRP signaling, we tested whether inhibition of cAMP catabolism by a non-selective phosphodiesterase inhibitor, IBMX, can augment the inotropic action of PrRP. As shown in *Figure 8A*, the effect of PrRP (10 nM) on contractility was not affected by IBMX (10 μM) (*P*=NS), whereas phosphodiesterase inhibition markedly enhanced the inotropic response to dobutamine (250 nM) (*P*<0.001) (*Figure 8B*). These data suggest that PrRP increases contractile force via cAMP-independent mechanisms.

Reversible phosphorylation of key elements in the contractile machinery plays an important role in regulation of cardiac contractility. Protein kinase-dependent phosphorylation processes are counterbalanced by dephosphorylating protein phosphatases. To study the contribution of protein phosphatases to PrRP signaling, we used calyculin A and okadaic acid as specific inhibitors.

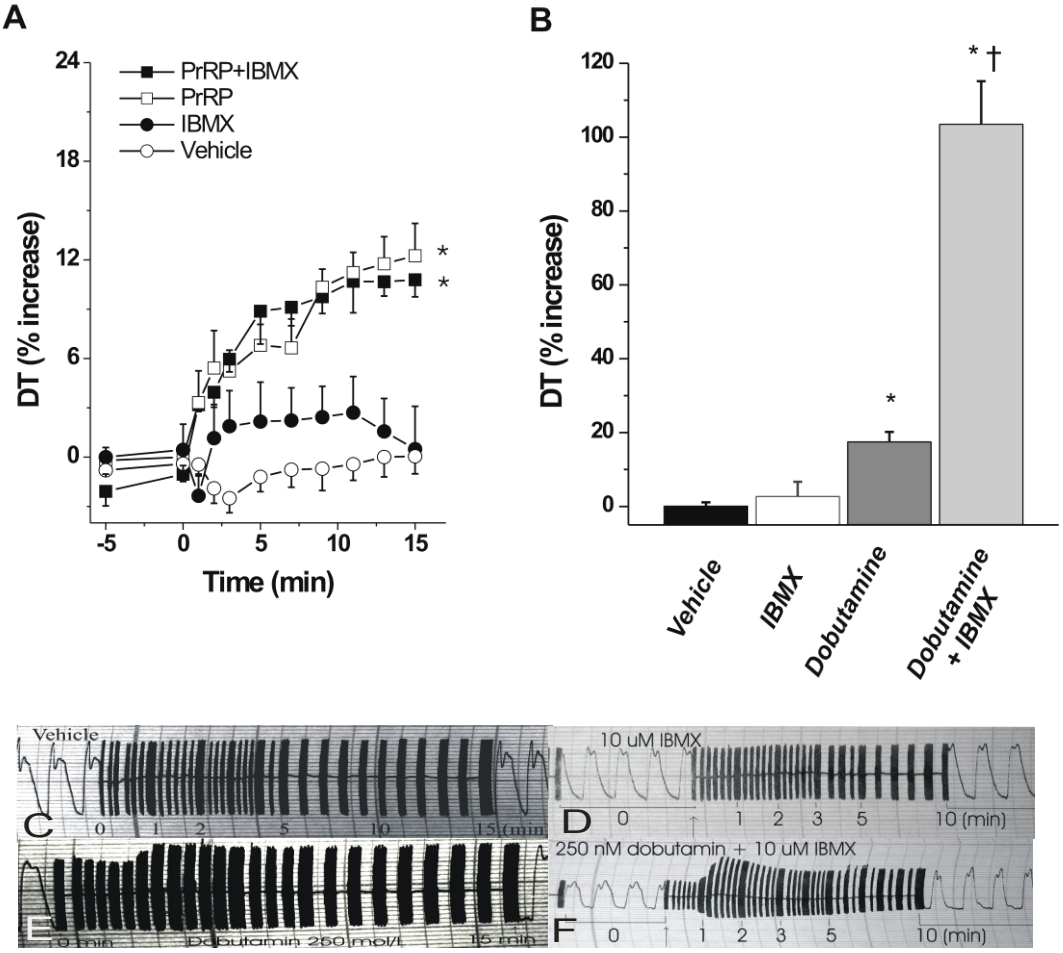

Figure 8. *The inotropic effect of PrRP is not augmented by phosphodiesterase inhibition.* ***A****: Effect of IBMX (10 µM), a non-selective phosphodiesterase inhibitor, on PrRP-induced (10 nM) developed tension (DT) elevation. Data are mean±SEM (n=4-8 for each group). *P<0.001 vs control; †P<0.001 vs PrRP (10 nM) (2-way ANOVA with Bonferroni post hoc analysis).* ***B****: Effect of IBMX on dobutamine–induced (250 nM) inotropic response. Results in bar graph are mean± SEM (n=4-8 for each group). *P<0.001 vs control; †P<0.001 vs dobutamine (1-way ANOVA with Bonferroni post hoc analysis).* ***C-F:*** *Original recordings of changes in developed tension in response to vehicle (C), IBMX (D), dobutamine (E) and dobutamine with IBMX.*

4.3.3. Protein phosphatases and PrRP signaling

Reversible phosphorylation of key elements in the contractile machinery plays an important role in regulation of cardiac contractility. Protein kinase–dependent phosphorylation processes are counterbalanced by dephosphorylating protein phosphatases. To study the contribution of protein phosphatases to PrRP signaling, we used calyculin A and okadaic acid as specific inhibitors. Calyculin A blocks both PP1 and PP2A at the dose of 1 nM, whereas 10 nM okadaic acid inhibits only PP2A activity (*Herzig S. et al., 2000*). When PrRP (10 nM) was infused in the presence of calyculin A, the inotropic effect of the peptide was markedly enhanced (P<0.001) (*Figure 9A*). In contrast, okadaic acid failed to alter the response to PrRP (P=NS) (*Figure 9A*). Notably, the inotropic actions of PrRP at various other doses (1 nM, 3 nM, and 30 nM) were influenced in a similar manner by the PP inhibitors (*Figure 9C*). These data suggest that the inotropic effect of PrRP is suppressed by PP1 but not PP2A.

4.3.4 Protein kinase Cα and PrRP signaling

Overexpression of PKCα in the heart depresses cardiac contractility by increasing PP1 activity (*Braz JC. et al., 2004*), therefore we examined whether PKCα modulates the PrRP-mediated increase in contractile function. As shown in *Figure 9B*, Ro32-0432 (100 nM), a PKCα inhibitor (*Hambleton M. et al., 2006*), significantly increased the inotropic response to PrRP (P<0.001). PKCα overexpression has been shown to decrease the phosphorylation of phospholamban, the inhibitory protein for SERCA2a (*Braz JC. et al., 2004*). In agreement, PrRP significantly

increased phospholamban phosphorylation at Ser-16 in the presence of Ro32-0432 (P<0.01), although the peptide had no effect on its own (*Figure 10*). Our observations indicate that PKCα inhibition may augment the inotropic response to PrRP by enhancing phospholamban phosphorylation in the myocardium.

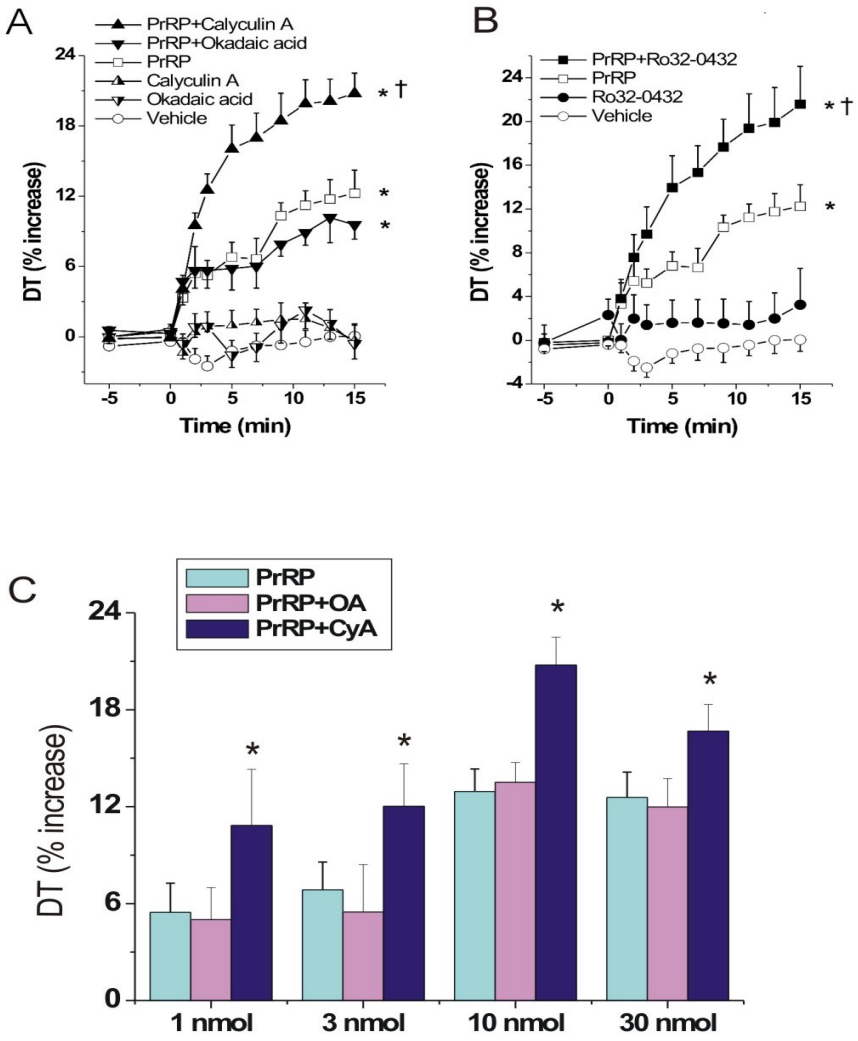

Figure 9. *The inotropic effect of PrRP is suppressed by PP1 and PKCα.* ***A and B:*** *Effects of calyculin A (1 nM), a PP1/PP2A inhibitor, okadaic acid (10 nM), a PP2A inhibitor (A), and Ro32-0432 (100 nM), a PKCα inhibitor (B) on PrRP-induced (10 nM) inotropic response. Data are mean±SEM (n=4-8 for each group). *P<0.001 vs control; †P<0.001 vs PrRP (10 nM) (2-way repeated-measures ANOVA with Bonferroni post hoc test).* ***C:*** *The responses of different doses of PrRP with (and without) calyculin A (1 nM) and ocadaic acid (10 nM).*

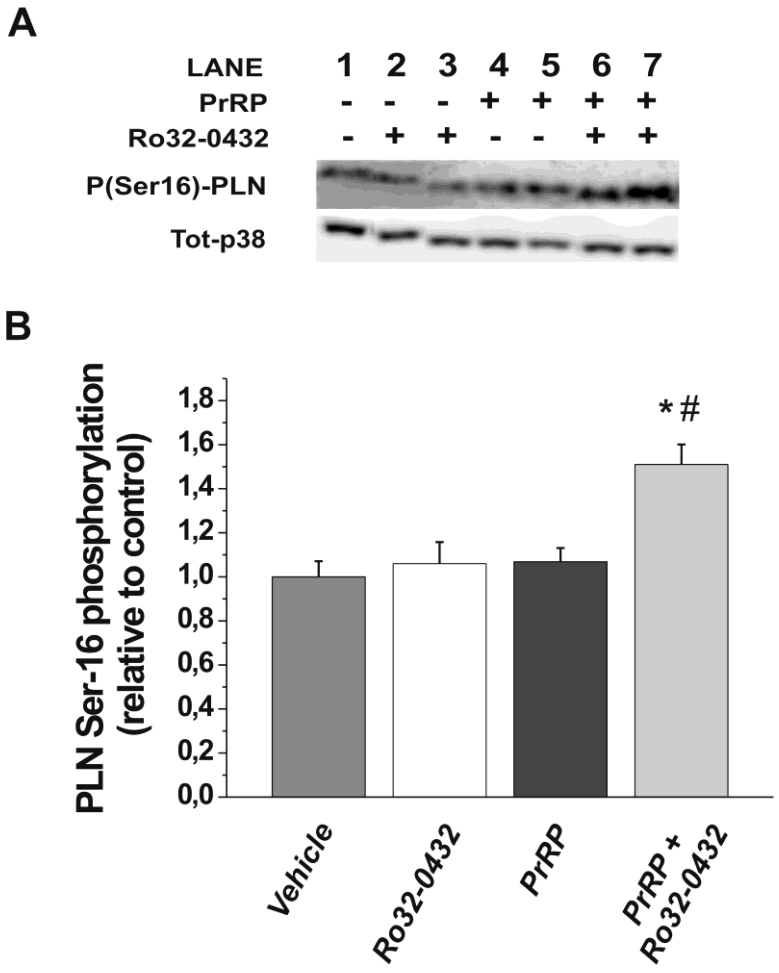

Figure 10. *PrRP increases phospholamban (PLN) phosphorylation at Ser-16 in the presence of Ro32-0432.* ***A:*** *Representative Western blot analysis. p38 was used as the loading control.* ***B:*** *Results in bar graph are mean±SEM (n=3-4 for each group). *P<0.05 vs control; #P<0.01 vs PrRP (1-way ANOVA with Bonferroni post hoc analysis).*

4.4. Conclusions

To our knowledge the present results provide the first evidence for the functional relevance of PrRP in the myocardium. Our data show that PrRP has a direct, dose-dependent, slowly developing, positive inotropic effect in the intact rat heart. Since the inotropic effect of PrRP was modest, we have hypothesized that it may be suppressed by simultaneous activation of certain counter-regulatory mechanisms. Indeed, the cAMP-independent positive inotropic effect of PrRPs is suppressed by enhanced activity of PKC-α and PP1. Based on these findings, one can anticipate that PrRP may have a hitherto unrecognized role in the regulation of cardiovascular homeostasis.

5. ROLE OF ENDOTHELIN-1 IN THE REGULATION OF CARDIAC CONTRACTILITY

5.1. Introduction

The members of the MAPK family, ERK1/2 and p38-MAPK, have been implicated in the development of various pathological states such as cardiac hypertrophy and heart failure by controlling cell growth and proliferation (*Bueno OF, Molkentin JD., 2002; Wang Y., 2007*). Initially, ERK1/2 and p38-MAPK signaling were both considered to promote cardiomyocyte hypertrophy in response to GPCR agonists such as angiotensin II and ET-1 in cultured cells (*Clerk A. et al., 1998*). However, recent studies using transgenic models have defined distinct roles for ERK1/2 and p38-MAPK in the hypertrophic response. Cardiac-specific overexpression of a constitutively active MEK1, the upstream regulator of ERK1/2, stimulates concentric left ventricular hypertrophy without signs of progression toward heart failure (*Bueno OF. et al., 2000*). In contrast, prolonged inhibition of p38-MAPK activity in mice expressing dominant-negative mutants of MKK3 or MKK6, the proximal regulatory kinases of p38-MAPK, facilitates progressive left ventricular hypertrophy, leading to dilation and functional decompensation and suggesting an inhibitory function of p38-MAPK on cardiac growth response (*Braz JC. et al., 2003*).

Transactivation of epidermal growth factor receptor has been established as a major mechanism for GPCR agonists to activate MAPKs (*Wetzker R., Böhmer FD.,2003)*. Stimulation of GPCRs induces metalloproteinase-mediated ectodomain

shedding of membrane-anchored proheparin-binding EGF-like growth factor (pro-HB-EGF). Soluble HB-EGF then binds to and activates EGFR, triggering MAPK phosphorylation (*Wetzker R., Böhmer FD.,2003*). Recent studies advocate that EGFR transactivation via HB-EGF shedding represents a vital step for GPCR agonist–induced cardiac hypertrophy in vitro and in vivo (*Asakura M. et al., 2002*).

In contrast to pathological conditions, the role of MAPKs and EGFR in the regulation of physiological cellular processes in the intact myocardium is not yet well understood. ET-1 is a potent stimulator of cardiac contractility (*Krämer BK.* et al., 1991; *Wang H.* et al., 2000; *Chu L. et al., 2003;2004; Kinnunen P. et al., 2000*) acting mainly via the ET_A GPCR subtype (*Takeuchi Y .* et al., 2001; *Piuhola J.*et al., 2003; *Zolk O. et al., 2004*). Previous studies demonstrated that stimulation of cardiomyocytes with ET-1 produces a robust increase in ERK1/2, p38-MAPK, and EGFR phosphorylation (*Bogoyevitch MA. et al., 1993; Sugden PH. et al., 2003; Asakura M. et al., 2002*) to date, however, no information is available on whether these signaling pathways are involved in the inotropic response to ET-1. Therefore our objective was to characterize the role of ERK1/2 and p38-MAPK and the potential upstream regulators (eg, EGFR) and downstream effectors of MAPK signaling (eg, Na^+-H^+ exchange, phospholamban) in the regulation of cardiac contractility stimulated by ET-1 in the intact rat heart.

5. 2. Materials and methods

5.2.1. Drugs

Drugs used were ET-1 (Phoenix Europe GmbH, Karlsruhe, Germany); AG1478, GF-109203X (bisindolylmaleimide I), LY 294002, ML-7, phorbol 12-myristate 13-acetate, SB239063, U0126 and U-73122 (Merck Chemicals Ltd., Nottingham, UK); zoniporide (generously supplied by Dr Ross Tracey, Pfizer Global Research and Development, Groton, Conn).

5.2.2. Isolated Perfused Rat Heart Preparation

Male 7-week-old Sprague-Dawley rats (n=112) were used. Hearts were excised rapidly, mounted on a Langendorff perfusion system (LF-01, Experimetria Ltd, Budapest, Hungary), and perfused under constant-flow conditions as described earlier (chapter 4.2.2.).

5.2.3. Experimental design

A 40-minute equilibration period and a 5-minute control period were followed by the addition of various drugs to the perfusate for 10 minutes. The concentrations of U0126 (1.5 μmol/L), SB239063 (3 μmol/L), U-73122 (100 nmol/L), GF-109203X (90 nmol/L), AG1478 (1 μmol/L), and zoniporide (1 μmol/L) were selected because they have been demonstrated to suppress ERK1/2 (*Tenhunen O. et al., 2004*), p38-MAPK (*Tenhunen O. et al., 2004*), phospholipase C

(PLC), (*Szokodi I. et al., 2002*; *Fulton D. et al., 1996*) PKC, (*Szokodi I. et al., 2002*) and EGFR tyrosine kinase activity (*Thomas WG. et al., 2002*) and to inhibit Na^+-H^+ exchanger-1 (NHE1), respectively (*Szokodi I. et al., 2002; Knight DR. et al., 2001*). At the end of the experiments, the LVs were frozen in liquid nitrogen and stored at -80°C until assayed.

5.2.4. Immunoblot analysis

Left ventricular tissue was homogenized, cytosolic and non-soluble fractions were separated by centrifugation and samples were subjected to immunoblot analysis as described earlier (chapter 4.2.4.). The antibodies used were anti-phospho-ERK1/2, anti-ERK1/2, anti-p38, and anti-phospho-p90 ribosomal S6 kinase (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK), anti-phospho-p38 (Chemicon International Inc., Temecula, Calif), anti-EGFR, anti-phospho(Ser16)-phospholamban (Santa Cruz Biotechnology, Santa Cruz, Calif), and anti-phospho-tyrosine (Upstate Biotechnology, Lake Placid, NY). Isoform-specific PKC antibodies (anti-PKCα, δ and ε) were from Sigma (Saint Louis, Mo).

5.2.5. p38-MAPK assay

Left ventricular tissue was homogenized in lysis buffer containing of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM benzamidine, 1 mM PMSF, 50 mM NaF, 1 mM DTT, and 10 μg/mL each of leupeptin, pepstatin, and aprotinin. Tissue homogenates were clarified at 14,000 x

g for 10 minutes and subjected to p38-MAPK activity assay by using ATF-2 as substrate. Briefly, 250 µg of protein extract was incubated for 4 hours with immobilized phospho-p38 antibody. The pellets were washed twice with lysis buffer and once with kinase buffer consisting of 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. Finally, the pellets were suspended in 40 µL of kinase buffer, including 200 µM ATP and 2 µg of ATF-2 fusion protein (Cell Signaling Technology Inc., Hitchin, Hertfordshire, UK). The kinase reaction was conducted at +30 °C for 30 minutes and stopped by placing the samples on ice and adding 30 µL of 2 x SDS. Next, the samples were boiled, microcentrifuged, and levels of phospho-ATF-2 were determined with immunoblot analysis as previously described (*Tenhunen O. et al., 2004*).

5.2.6. Statistical analysis

Results are presented as mean±SEM. Two-way repeated-measures ANOVA was used to evaluate the statistical significance of differences among groups for cardiac contractility. When significant differences were detected for the treatment-by-time interactions, a Bonferroni post hoc test was used for specific comparisons. All other parameters were analyzed with 1-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at the level of P=0.05.

5.3. Results

5.3.1. Role of ERK1/2 in the Regulation of Cardiac Contractility

In the isolated perfused rat heart preparation, intracoronary infusion of ET-1 (1 nmol/L) for 10 minutes increased developed tension by 40±2% (*Figure 11A*), which corresponds to the maximal response based on our previous results (*Szokodi I. et al., 2002; Kinnunen P. et al., 2000*). To determine ERK1/2 activation under our experimental conditions, Western blotting was performed. In agreement with earlier data obtained in cultured neonatal cardiac myocytes (*Bogoyevitch MA. et al., 1993; 1994*) administration of ET-1 (1 nmol/L) for 10 minutes increased phospho-ERK1/2 levels in isolated perfused adult rat hearts (*Figure 11B*). To examine whether activation of ERK1/2 contributes to the positive inotropic action of ET-1, we assessed the effect of U0126, which is a potent specific inhibitor of MEK1/2, the upstream regulator of ERK1/2 (91). Administration of U0126 (1.5 μmol/L) significantly reduced the levels of phospho-ERK1/2 in the ET-1–stimulated LVs (*Figure 11C and 11D*). Two-way repeated-measures ANOVA showed significant treatment-bytime interaction ($P<0.001$) in contractility among the 4 groups. Post hoc analysis revealed that U0126 significantly attenuated the inotropic response to ET-1, the maximal reduction being 57% at the end of 10 minutes' infusion time ($P<0.001$; *Figure 11A*). Infusion of U0126 alone had no effect on contractile force ($P=1.0$; *Figure 11A*).

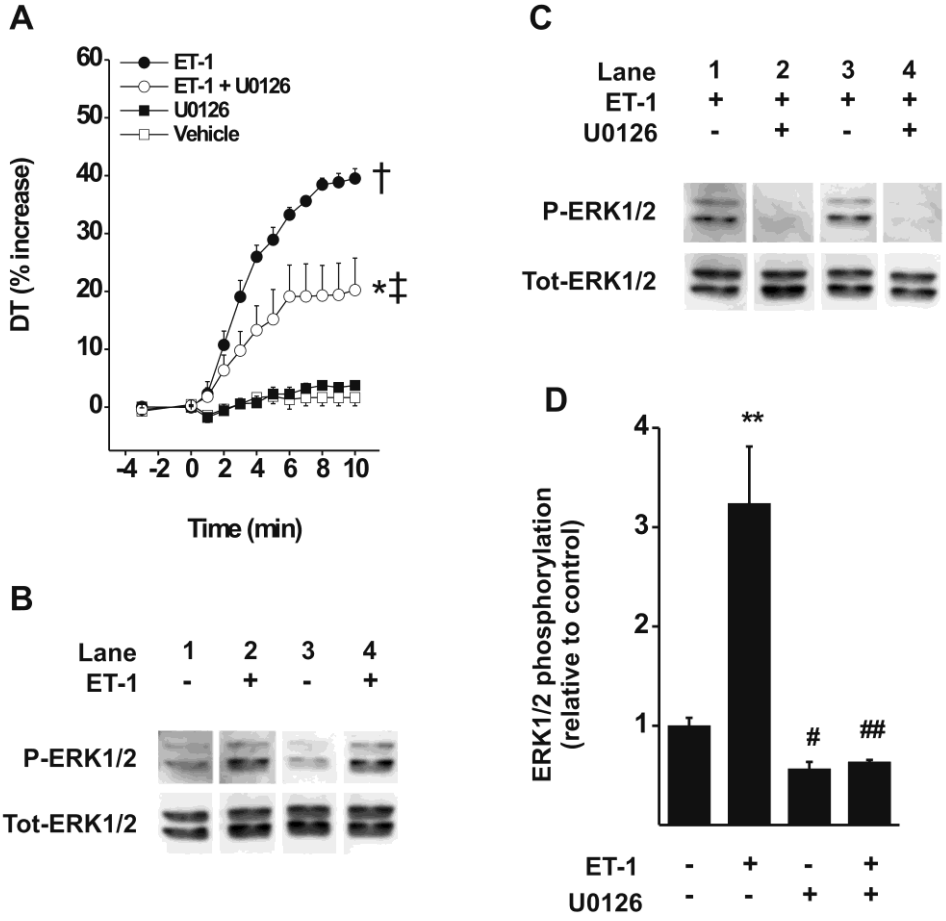

Figure 11. *ERK1/2 signaling is required for the ET-1–mediated increase in contractility.* ***A****: In isolated rat hearts, infusion of ET-1 (1 nM) for 10 minutes increased developed tension (DT), and U0126 (1.5 μM), an MEK1/2 inhibitor, attenuated ET-1–enhanced contractility. Results are expressed as a percentage change versus baseline values. Data were analyzed by 2-way repeated-measures ANOVA followed by multiple comparisons with the Bonferroni post hoc test and are reported as mean±SEM (n=4 to 6 for each group). *P<0.05, †P<0.001 vs. control and U0126; ‡P<0.001 vs ET-1.* ***B through D****: Western blot analysis of ERK1/2 phosphorylation in left ventricles. ET-1 increased phospho-ERK1/2 (P-ERK1/2) levels (B and D), and U0126 abolished ET-1–induced ERK1/2 phosphorylation (C and D). Results in the bar graph are expressed as the ratio of phospho-ERK1/2 and total (Tot) ERK1/2. Data were analyzed by 1-way ANOVA followed by Bonferroni posthoc test and are reported as mean±SEM (n=4 for each group). **P<0.01 vs control; #P<0.05, ##P<0.01 vs ET-1.*

5.3.2. Upstream Activators of ERK1/2 Regulating Cardiac Contractility

Previously, it has been suggested that ET-1 increases cardiac contractility (*Krämer BK. et al., 1991; Knight DR. et al., 2001*) and stimulates ERK 1/2 phosphorylation via a PKC-dependent pathway in cardiomyocytes (*Bogoyevitch MA. et al., 1994*). To study the activation of PKC in the adult rat heart, the translocation of PKC isoforms between cytosolic and particulate fraction was determined. In response to ET-1 infusion, no consistent increases in the translocation of PKCα, PKCδ, or PKCε into the particulate fraction were seen in Western analysis (*Figure 12A*). To assess the contribution of PKC to the effects of ET-1, we used the specific PKC inhibitor GF-109203X (*Szokodi I. et al., 2002)*. Infusion of GF-109203X (90 nMol/L) had no effect on the ET-1–stimulated increase in phospho-ERK1/2 levels (*Figure12B*). Moreover, GF-109203X did not alter ET-1–enhanced contractility (P=1.0; *Figure 12C*). In contrast, the inotropic response to phorbol 12-myristate 13-acetate (PMA; 40 nMol/L), a direct activator of PKC, was markedly reduced by GF-109203X (6.4±1.6% versus 29.2±4.9%, PMA with and without GF-109203X; n=4; P=0.01). In addition, we studied the role of PLC, the upstream activator of PKC. In agreement with the observation obtained using the PKC inhibitor, the potent PLC inhibitor U-7312213 (100 nmol/L) (*Fulton D. et al., 1996; Szokodi I. et al., 2002*) failed to alter the positive inotropic effect of ET-1 (P=1.0; *Figure 12D*). These data suggest that ET-1 increases contractile force through a PLC-PKC–independent pathway. Recent studies suggest that GPCR agonists can promote the growth of cardiomyocytes via transactivation of EGFR with subsequent activation of MAPKs (*Thomas WG. et al., 2002*). To examine whether ET-1 transactivates the EGFR, EGFR phosphorylation was measured by

immunoprecipitation with anti-EGFR antibody followed by immunoblotting of immunoprecipitates with antiphosphotyrosine antibody. ET-1 increased total tyrosine phosphorylation of EGFR in the left ventricles (*Figure 13A*), indicating EGFR transactivation. As shown in *Figure 13B*, ET-1–induced EGFR tyrosine phosphorylation was abolished by AG1478 (1 μmol/L), a specific EGFR tyrosine kinase inhibitor. (*Bogoyevitch MA. et al., 1994*) Moreover, AG1478 significantly reduced ET-1–induced ERK1/2 phosphorylation (*Figure 13C and 13D*), suggesting that EGFR transactivation is required for the activation of the ERK1/2 cascade. Furthermore, in the presence of AG1478, the inotropic response to ET-1 was significantly suppressed, the maximal reduction being 46% ($P<0.001$; *Figure 13E*). Infusion of AG1478 alone had no effect on developed tension ($P=1.0$; *Figure 13E*).

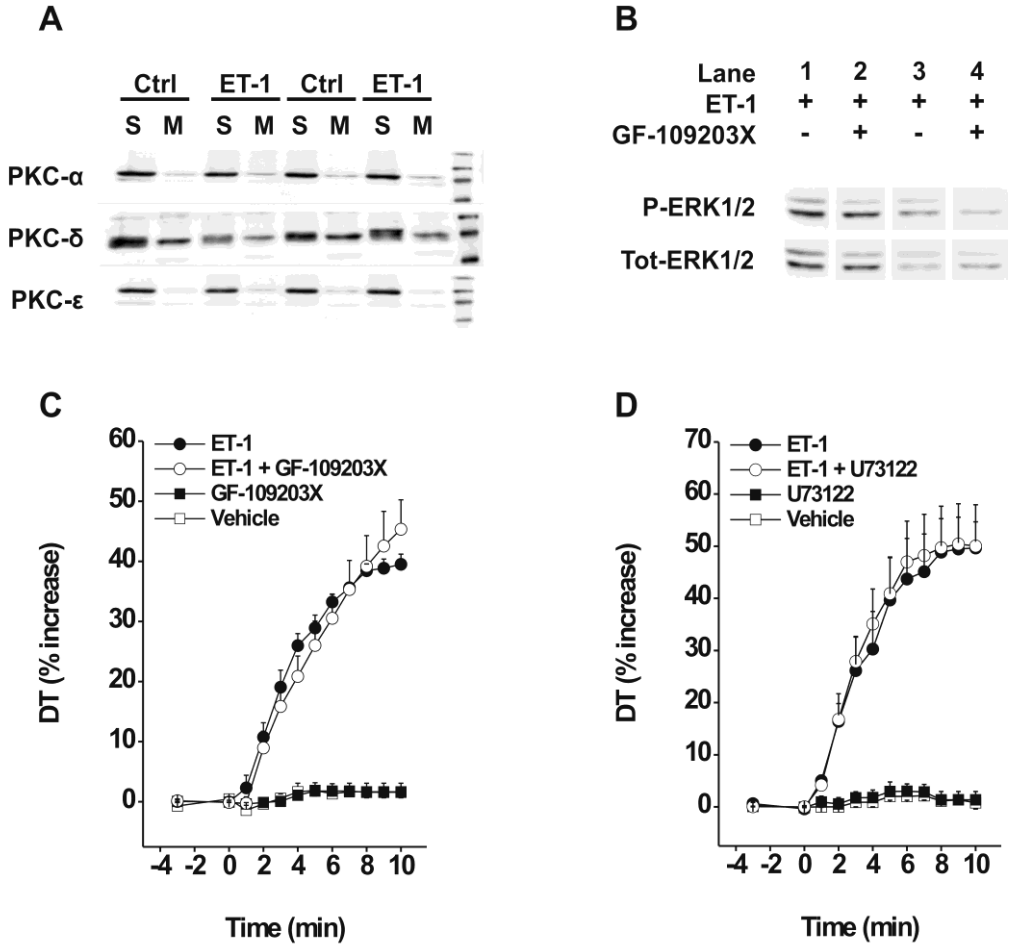

Figure 12. *PKC signaling is not involved in the inotropic response to ET-1.* ***A:*** *Immunoblot analysis shows that ET-1 had no consistent effects on translocation of PKCα, PKCδ, or PKCε into the particulate fraction.* ***B:*** *Western analysis shows that GF-109203X (90 nM), a PKC inhibitor, failed to suppress ET-1–induced ERK1/2 phosphorylation.* ***C and D:*** *GF-109203X (C) and the PLC inhibitor U-73122 (100 nM) (D) had no effect on the ET-1–mediated increase in contractility. Data are mean±SEM (n=4-6 for each group). S indicates soluble fraction; M, membrane fraction; DT, developed tension; Ctrl, control; P, phospho-; and Tot, total.*

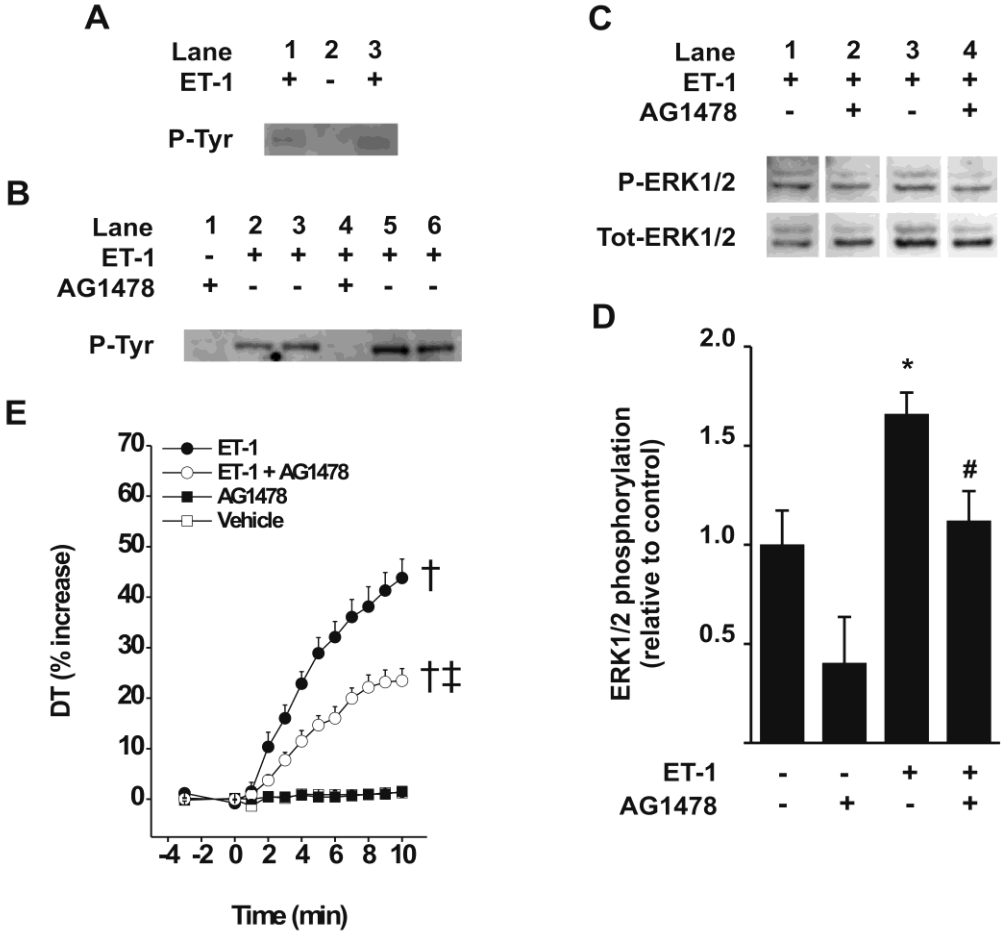

Figure 13. *EGFR transactivation contributes to the ET-1–mediated increase in contractility.* ***A and B****, EGFR phosphorylation was measured by immunoprecipitation with anti-EGFR antibody followed by immunoblotting of immunoprecipitates with antiphosphotyrosine (P-Tyr) antibody. ET-1 stimulation increased EGFR tyrosine phosphorylation (A), and AG1478 (1 μM), an EGFR tyrosine kinase inhibitor, abolished it (B).* ***C and D****: Western blot analysis shows that AG1478 reduced the ET-1–induced increases in ERK 1/2 phosphorylation. Results in the bar graph are expressed as the ratio of phospho-ERK1/2 and total ERK1/2. Data are mean±SEM (n=3-6 for each group). *P<0.05 vs control; #P<0.05 vs ET-1 (1-way ANOVA with Bonferroni posthoc analysis). P indicates phospho-; Tot, total.* ***E:*** *AG1478 reduced the ET-1–induced increase in contractility. Data are mean±SEM (n=4-6 for each group). A significant treatment-by-time interaction (P<0.001) was found among the groups (2-way repeated-measures ANOVA). †P<0.001 vs control and U0126; ‡P<0.001 vs ET-1 (Bonferroni post hoc analysis).*

5.3.3. Downstream Targets of ERK1/2 Regulating Cardiac Contractility

Several lines of evidence suggest that the sarcolemmal NHE1 is a pivotal mediator of the positive inotropic effect of ET-1 (*Krämer BK. et al., 1991*). Accordingly, zoniporide (*Szokodi I. et al., 2002; Knight DR. et al., 2001*) (1 μM), a potent and selective inhibitor of NHE1, attenuated the ET-1–induced inotropic response by 57% under our experimental conditions (P<0.001), whereas zoniporide alone had no effect on cardiac contractility (P=1.0; *Figure 14A*). Previously, it has been shown that ERK1/2 and one of its downstream effectors, p90 ribosomal S6 kinase (p90RSK), can phosphorylate and activate NHE1 in response to ET-1 in cardiac myocytes. (*Moor AN. et al., 1999*) As shown in *Figure 14B*, ET-1 increased the phospho-p90RSK levels in the membrane fraction. Importantly, both U0126 and AG1478 significantly attenuated ET-1–induced phosphorylation of p90RSK (*Figure 14C* and *14D*), suggesting that p90RSK-mediated activation of NHE1 may be the downstream target of the EGFR–ERK1/2 pathway.

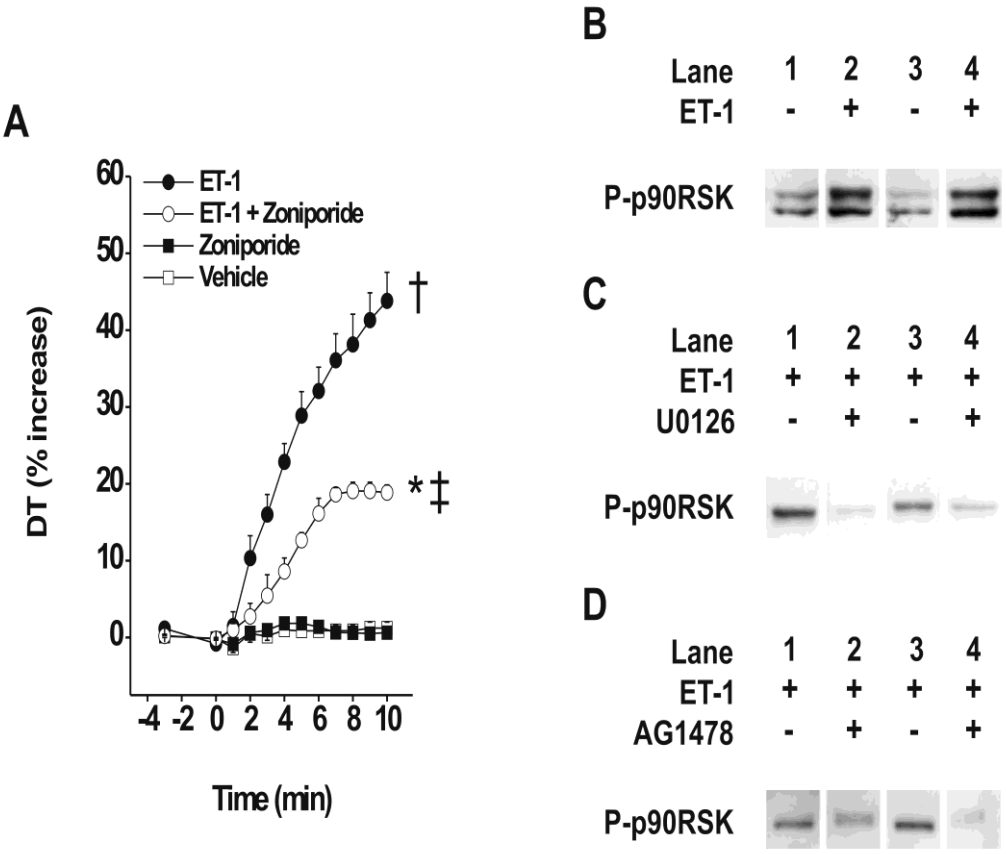

Figure 14. *NHE1 is involved in the inotropic response to ET-1.* ***A:*** *Zoniporide (1 μM), an NHE1 inhibitor, attenuated the ET-1– induced increase in contractility. Data are mean±SEM (n=4-6 for each group). A significant treatment-by-time interaction (P<0.001) was found among the groups (2-way repeatedmeasures ANOVA). *P<0.01, †P<0.001 vs control and U0126; ‡P<0.001 vs ET-1 (Bonferroni posthoc analysis).* ***B through D:*** *Western blot analysis for p90RSK phosphorylation (P-p90RSK) in left ventricles. ET-1 increased phospho-p90RSK levels in the membrane fraction (B), which was prevented by U0126 (C) and AG1478 (D). DT indicates developed tension; P, phospho-.*

5.3.4. Role of p38-MAPK in the Regulation of Cardiac Contractility

Previously, ET-1 has been shown to produce a robust increase in p38-MAPK phosphorylation in primary cultures of cardiac myocytes. (*Clerk A. et al., 1998*) In agreement, ET-1 infusion for 10 minutes increased phospho–p38-MAPK levels in isolated perfused adult rat hearts (*Figure 15A*). To assess the involvement of p38-MAPK in the positive inotropic effect of ET-1, we used a novel potent inhibitor, SB239063 (*Tenhunen O. et al., 2004*). Because p38-MAPK inhibitors, including SB239063, are known to affect the catalytic activity of p38-MAPK rather than the levels of phosphorylated p38-MAPK, a kinase assay that uses ATF-2 as a substrate was performed to confirm the inhibition of p38-MAPK. As shown in *Figure 15B* and *15C*, the ET-1–induced increase in p38-MAPK activity in the LV was abolished by SB239063 (3μM). Interestingly, administration of SB239063 augmented the ET-1–induced inotropic response maximally by 42% ($P<0.05$; *Figure 15D*). Infusion of SB239063 alone had no effect on developed tension ($P=1.0$; *Figure 15D*).

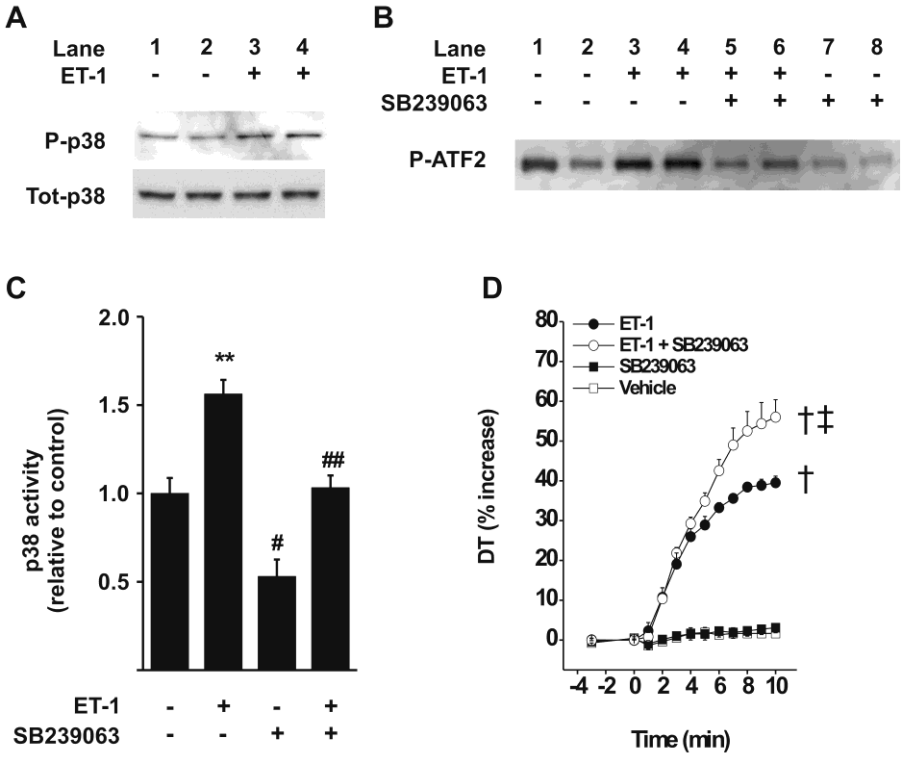

Figure 15. *p38-MAPK signaling counteracts the positive inotropic effect of ET-1.* ***A****: Western blot analysis shows increased phosphorylation of p38-MAPK in response to ET-1.* ***B and C****: SB239063 (3 μM), a p38-MAPK inhibitor, decreased ET-1–induced p38-MAPK activity measured by immunocomplex kinase assay with ATF-2 as a substrate. Results in the bar graph are mean±SEM (n=4 for each group). **P<0.01 vs control; #P<0.05, ##P<0.01 vs ET-1 (1-way ANOVA with Bonferroni post hoc analysis).* ***D****: ET-1–enhanced contractility was augmented by SB239063. Data are mean±SEM (n=4-6 for each group). A significant treatment-by-time interaction (P<0.001) was found among the groups (2-way repeated-measures ANOVA). †P<0.001 vs control and U0126; ‡P<0.05 vs ET-1 (Bonferroni post hoc analysis). Tot indicates total; P, phospho-.*

5.3.5. Cross-Talk Between p38-MAPK and ERK1/2 Signaling

Inhibition of p38-MAPK by SB239063 had no effect on ET-1–induced ERK1/2 phosphorylation (*Figure 16A*). Similarly, administration of U0126 did not affect phospho–p38-MAPK levels (*Figure 16B*). Interestingly, AG1478 significantly

reduced ET-1–induced increases in left ventricular levels of phospho–p38-MAPK (*Figure 16C*) in addition to its effect on ERK1/2 phosphorylation (*Figure 13C*). These data suggest that no direct cross-talk exists between ERK1/2 and p38-MAPK signaling. Moreover, EGFR transactivation appears to be involved in the activation of both pathways.

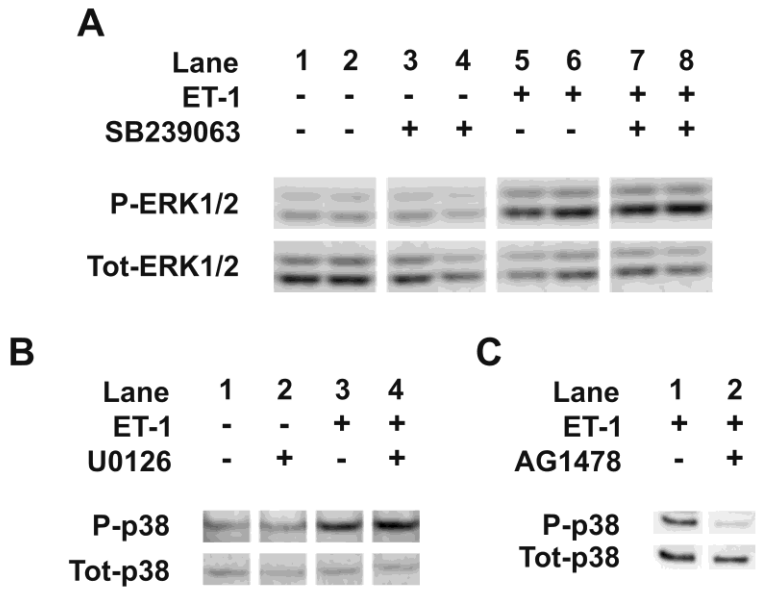

Figure 16. *Putative cross-talk between p38-MAPK and ERK1/2 signaling.* ***A****: Western blot analysis shows that inhibition of p38-MAPK by SB239063 (3 μM) had no effect on ET-1–induced ERK1/2 phosphorylation (P-ERK1/2).* ***B****: Inhibition of ERK1/2 by U0126 (1.5 μM) did not affect phospho–p38-MAPK levels.* ***C****: AG1478 (1 μM) significantly reduced ET-1–induced increases in left ventricular levels of phospho–p38-MAPK. Tot indicates total; P, phospho-.*

5.3.6. Downstream targets of p38-MAPK regulating cardiac contractility

Phospholamban is a crucial regulator of cardiac contractility. In its dephosphorylated state, phospholamban binds to and inhibits the activity of

sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA-2a). Phosphorylation of phospholamban on Ser-16 relieves SERCA-2a inhibition and enhances Ca^{2+} reuptake into the sarcoplasmic reticulum, leading to increased contractility (*MacLennan DH. et al., 2003*). ET-1 significantly increased phospholamban phosphorylation at Ser-16 in the presence of SB239063, although the peptide had no effect on its own (*Figure 17A and 17B*). Infusion of SB239063 alone did not increase phospholamban phosphorylation significantly (*P*=0.115; *Figure 17B*). In addition, U0126 failed to alter the phosphorylation of phospholamban (*Figure 17C*). These results suggest that p38-MAPK inhibition may enhance the inotropic response to ET-1 by augmenting phospholamban phosphorylation in the myocardium.

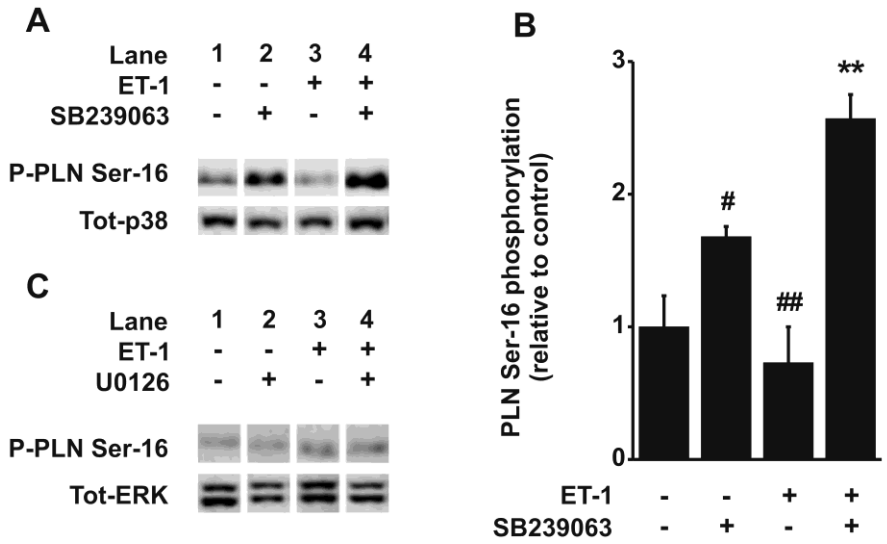

Figure 17. *p38-MAPK signaling regulates phospholamban phosphorylation (PLN).* ***A:*** *Western blot analysis shows that ET-1 increased phospholamban phosphorylation at Ser-16 in the presence of the p38-MAPK inhibitor SB239063 (3 μM).* ***B:*** *Results in the bar graph are mean±SEM (n=3-5 for each group). **P<0.01 vs control; #P<0.05, and ##P<0.01 vs ET-1 plus SB239063 (1-way ANOVA with Bonferroni post hoc analysis).* ***C:*** *U0126 (1.5 μM) failed to alter phosphorylation of phospholamban. Tot indicates total; P, phospho-.*

5.3.7. Effect of ET-1 on vascular tone

Overall, the changes in vascular tone were small. Importantly, inhibition of ERK1/2 or p38-MAPK significantly modified the inotropic response to ET-1 (*Figures 11A and 15D*) without altering the effect of the peptide on vascular tone (ET-1: from 32.0±1.8 to 38.8±3.3 mmHg, a 20.6±3.4% increase in perfusion pressure; ET-1 plus U0126: 16.8±2.7%; ET-1 plus SB239063: 18.73.8%; *P*=1.0 versus ET-1 alone). In line with previous observations (*McNair LL. et al., 2004*), the PKC inhibitor GF-109203X and PLC inhibitor U-73122 significantly attenuated the vasoconstrictor effect of ET-1 (ET-1: from 32.4± 1.0 to 40.3±2.0 mm Hg, a 23.9±2.8% increase; ET-1 plus GF- 109203X: 6.0±1.4%; ET-1 plus U-73122: 13.8±1.1%; *P*=0.001 and *P*=0.01 versus ET-1 alone, respectively). The other inhibitors had no significant effect on the ET-1–induced increase in perfusion pressure.

5.4. Conclusions

We present evidence for the functional importance of ERK1/2 and p38-MAPK in the acute regulation of cardiac contractility in the intact adult rat heart. Our results reveal that MAPKs play opposing roles in that the ERK1/2-mediated positive inotropic response to ET-1 is counterbalanced by simultaneous activation of p38-MAPK. EGFR may act as the upstream regulator and the p90RSK–NHE1 pathway as the downstream effector of ERK signaling. Moreover, p38-MAPK activation may suppress contractility by dephosphorylating phospholamban.

Identification of novel signaling pathways that promote cardiomyocyte survival while improving contractile function may offer an attractive approach for treating patients with heart failure. Therefore, further studies are required to test the hypothesis that activation of MEK1/2–ERK1/2 signaling, possessing such beneficial effects, can eventually rescue the failing heart.

6. DISCUSSION

6.1. Functional importance of PrRP in the myocardium

Recent studies suggest that PrRP, a putative ligand for the GPR10 orphan receptor, may act as an important neurotransmitter/neuromodulator in the central nervous system. Although PrRP is not a classic hypothalamic releasing factor (*Samson WK. et al., 2000; Sun B. et al., 2005*), intracerebroventricular administration of PrRP stimulates the secretion of several pituitary hormones such as adrenocorticotropic hormone (*Matsumoto H. et al., 2000*), oxytocin (*Marauyuma m. et al., 1999*), vasopressin (*Marauyama m. et al., 1999*), lutenizing hormone and follicle stimulating hormone (*Seal LJ. et al., 2000*). PrRP has also been implicated in the regulation of stress responses (*Maruyama M. et al., 2001*), feeding behavior (*Takayanagi Y, et al., 2008*), waking/sleeping states (*Lin SH. et al., 2002*), and nociception (*Laurent P. et al., 2005*). Recent studies propose that PrRP is involved in the central control of blood pressure (*Samson WK. et al., 2000; Horiuchi J. et al., 2002*). To our knowledge the present results provide the first evidence for the functional significance of PrRP in the myocardium. Our data show that PrRP has a direct, dose-dependent, slowly developing, positive inotropic effect in the intact rat

heart. Based on these findings, one can foresee that PrRP may have a thus far unrecognized role in the regulation of cardiovascular homeostasis.

PrRP has been shown to stimulate catecholamine biosynthesis in rat pheochromocytoma cells partly via the cAMP-PKA pathway *(Nanmoku T. et al., 2005)*. Therefore, activation of the adenylate cyclase–cAMP–PKA system, which is one of the major pathways for the regulation of cardiac contractility in the mammalian heart, may also mediate the cardiac effects of PrRP. Since the inotropic effect of PrRP was modest, we have hypothesized that it may be suppressed by simultaneous activation of certain counter-regulatory mechanisms. The inotropic response to cAMP-elevating agents can be limited by activation of phosphodiesterases hydrolyzing cAMP (*Fischmeister R. et al., 2006*). Indeed, the positive inotropic effect of the β_1-adrenergic receptor agonist dobutamine was strikingly enhanced by the non-selective phosphodiesterase inhibitor IBMX. In contrast, IBMX failed to augment the contractile effect of PrRP, ruling out the involvement of phosphodiesterases. Moreover, these data argue against a role for the adenylyl cyclase–cAMP–PKA pathway in mediating the inotropic effect of the peptide.

In the heart, Ca^{2+} cycling and contractility are controlled by a fine equilibrium of protein kinase and phosphatase activities in response to various GPCR agonists. PP1, PP2A and PP2B (calcineurin) are the major serine/threonine phosphatases in the myocardium (*Herzig S., Neumann J., 2000*). High doses of PP inhibitors such as okadaic acid, calyculin A, sodium fluoride, cantharidin, and flosequinoxan can increase the positive inotropic state by promoting the phosphorylation of phospholamban, troponin I, and myosin light chain without any change in cAMP

levels (*Gupta RC. et al., 2002)*. In the present study, low doses of calyculin A and okadaic acid had no effect on baseline contractility. Inhibiting PP1 and PP2A activity by calyculin A significantly augmented the inotropic response to PrRP, whereas PP2A inhibition by okadaic acid had no effect. Thus, our results indicate that activation of PP1 but not PP2A can counterbalance the inotropic effect of PrRP.

Serine/threonine kinases of the PKC family function downstream of many membrane-associated signal transduction pathways and are involved in numerous cellular processes including regulation of cytosolic [Ca^{2+}], myofilament Ca^{2+} sensitivity, and contractility of cardiac muscle cells (*Solaro RJ., 2008*). Approximately 10 different isozymes compose the PKC family, and they are generally classified by their activation characteristics. The conventional PKC isozymes (α, βI, βII, and γ) are Ca^{2+}- and lipid-activated, the novel isozymes (δ, ε, θ, and η) and atypical forms (ζ, and λ) are Ca^{2+} independent but activated by distinct lipids (*Steinberg SF. et al., 2008)*. Recent studies have established that PKCα plays a pivotal role in the regulation of myocardial contractility *(Braz JC. et al., 2004; Hahn HS. et al., 2003; Hambleton M. et al., 2006, Belin RJ. et al., 2007; El-Armouche A. et al., 2007; Molnár A. et al., 2008*) Braz et al. have reported that PKCα gene-deleted mice are hypercontractile, whereas transgenic mice overexpressing PKCα are hypocontractile (*Braz JC. et al., 2004*). Furthermore, enhancement in cardiac contractility associated with PKCα knockout or with pharmacological inhibition of PKCα protect against heart failure in several experimental models (*Braz JC. et al., 2004, Hambleton M. et al., 2006*). Although PKCα is emerging as a crucial negative modulator of cardiac function, its role in the acute regulation of contractility in a

physiological milieu is unclear. Of particular importance was our finding that Ro32-0432, a PKCα inhibitor (*Hambleton M. et al., 2006*), significantly augmented the inotropic effect of PrRP, implying that activation of PKCα provides a negative feedback to the PrRP-mediated inotropic response. Moreover, our results showed that PrRP promoted the phosphorylation of phospholamban at Ser-16 in the presence of PKCα inhibition. Our data are in agreement with previous observations that PKCα overexpression decreases the phosphorylation of phospholamban by increasing PP1 activity (*Braz JC. et al., 2004*). Phosphorylation of phospholamban relieves SERCA2a inhibition and enhances Ca^{2+} reuptake into the sarcoplasmic reticulum thereby increasing contractility (*MacLennan D., Kranias E., 2003)*. In addition to Ca^{2+} handling, PKCα has been shown to regulate myofilament function. Troponin I and troponin T are feasible substrates for PKCα-dependent phosphorylation. However, the functional consequences are contradictory, with findings that suggest that PKCα can either reduce (*Sumandea MP. et al., 2003;* Belin RJ. et al., 2007*)* or increase myofilament contractility (*Molnár A. et al., 2008*). Further experiments are needed to clarify whether PrRP modulates myofilament function. Taken together, our results suggest that the cAMP-independent inotropic response to PrRP is suppressed by concurrent activation of PKCα and PP1 leading to dephosphorylation of phospholamban (*Figure 18*). Moreover, these findings raise the possibility that activation of PKCα may have a universal homeostatic function by counterbalancing excess inotropic stimulation in the heart.

Our results revealed that PrRP was active in the low nanomolar range (1-10 nM), which is in agreement with the reported affinity (IC_{50} of 6.6±0.7 nM) of the specific binding sites in the myocardium (*Satoh F. et al., 2000*). Taking into account

that PrRP plasma levels are very low (0.13 pM) (*Matsumoto H. et al., 1999*), it is unlikely that the peptide can act as a circulating hormone. To date there is no information available whether PrRP is synthesized and/or released in functionally relevant concentrations in the myocardium. Notably, PrRP is coexpressed with tyrosine hydroxylase, the rate-limiting enzyme in noradrenalin synthesis, in A1 and A2 noradrenergic neurons of the rat medulla oblongata (*Maruyama M. et al., 2001; Horiuchi J. et al., 2002*), and PrRP acts synergistically with noradrenaline to stimulate the hypothalamo-pituitary-adrenal axis (*Maruyama M. et al., 2001*). In cultured rat adrenal medullary cells, PrRP is colocalized with tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase, which converts noradrenaline to adrenaline (*Fujiwara K. et al., 2005*). Moreover, PrRP has been reported to increase catecholamine secretion from rat pheochromocytoma PC12 cells (*Nanmoku T. et al., 2003*). Further experiments are warranted to elucidate whether nerve fibers innervating the heart may contain PrRP and whether the peptide may modulate noradrenaline release from sympathetic nerve terminals in the myocardium in a similar fashion to other neuropeptides including neurotensin, substance P and calcitonin gene-related peptide (*Osadchii O. et al., 2005; Seyedi N. et al., 1999)*

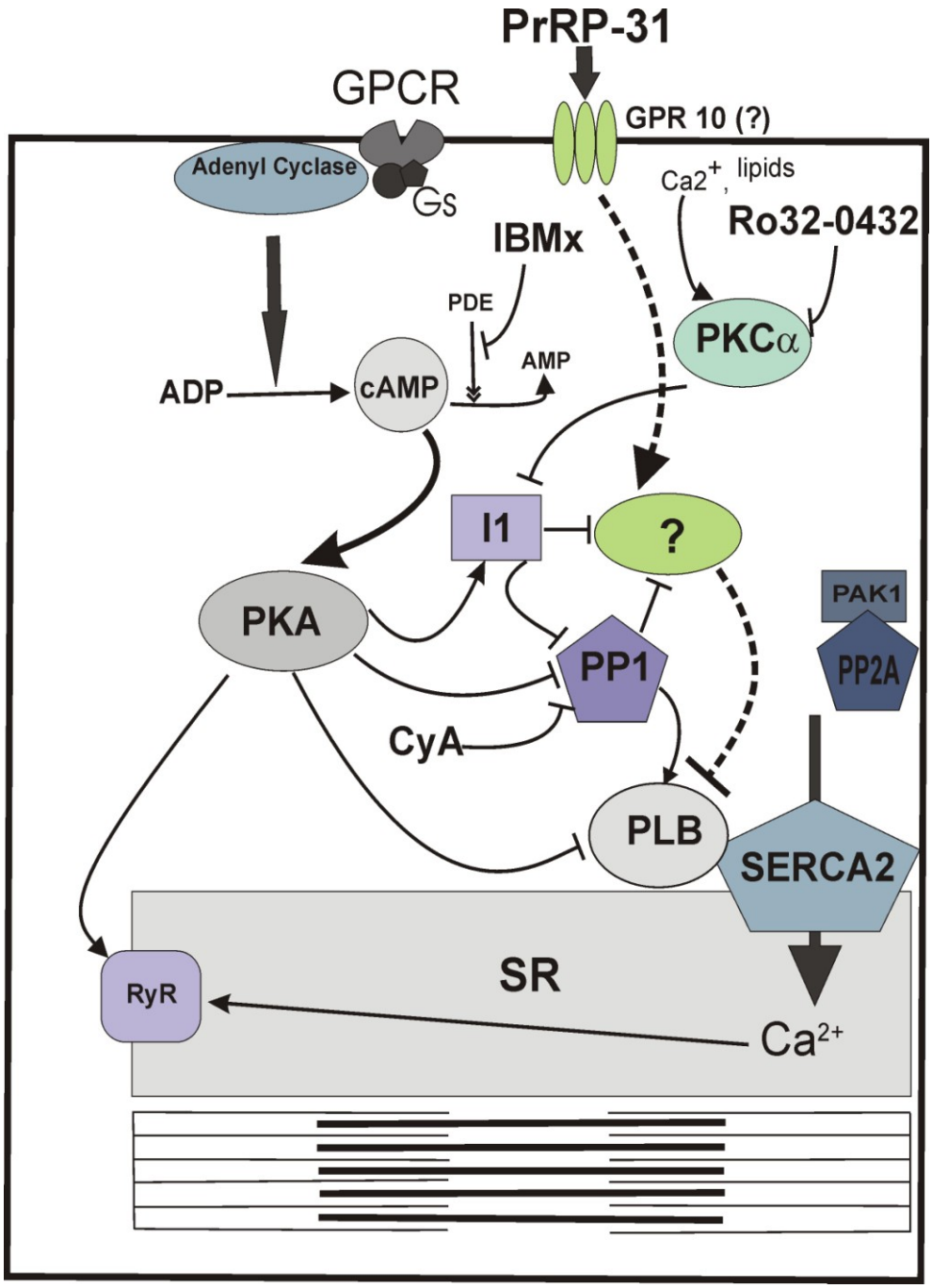

Figure 18. *Putative signaling mechanisms of PrRP in the heart. Stimulation of GPR10 by PrRP evokes a cAMP-independent inotropic response, which is suppressed by concurrent activation of PKCα and PP1 leading to dephosphorylation of phospholamban.*

6.2. Functional importance of ERK1/2 and p38-MAPK signaling in the acute regulation of cardiac contractility

As summarized in *Figure 19*, previous studies have suggested that ET-1 increases cardiac contractility via a PKC-dependent pathway (*Krämer BK. et al., 1991; Chu L. et al., 2003; Zolk O. et al., 2004*). However, our data indicate that PKC is unlikely to mediate the inotropic effect of ET-1 in the intact adult rat heart. We did not detect translocation of PKCα, PKCδ, or PKCε into the particulate fraction after a 10-minute infusion of ET-1, although we cannot exclude the possibility that translocation occurred earlier and the PKC isoforms already returned to the soluble fraction by that time. Moreover, GF-109203X, a specific PKC inhibitor, did not attenuate the inotropic response to ET-1, although it markedly reduced the inotropic effect of PMA. Furthermore, pharmacological inhibition of PLC, the upstream regulator of PKC, also failed to alter the inotropic action of ET-1. Of note, GF-109203X and U-73122 significantly attenuated the modest vasoconstrictor effect of ET-1, in line with previous observations (*McNair LL. et al., 2004*), indicating that the inhibitors were effective in our experimental system.

A growing body of evidence suggests that activation of the MEK1/2–ERK1/2 pathway constitutes an important adaptive mechanism in the myocardium (*Bueno OF, Molkentin JD., 2002; Wang Y., 2007*). ERK1/2 signaling has been reported to confer cardioprotection in vivo against ischemia-reperfusion injury by directly antagonizing myocyte apoptosis (*Lips DJ. et al., 2004*). Moreover, mice with cardiac-specific overexpression of a constitutively active MEK1 were characterized by long-standing moderate concentric left ventricular hypertrophy with enhanced

pump function (*Bueno OF. et al., 2000*). Consistent with these observations, inhibition of ERK1/2 activation by cardiac-specific expression of a dominant-negative form of Raf-1, the upstream regulator of MEK1/2, resulted in blunted cardiac hypertrophy, increased apoptosis, and left ventricular dysfunction with a concomitant increase in mortality in response to pressure overload (*Harris IS. et al., 2004*). More recently, the requirement of ERK1/2 signaling in stress adaptation has been addressed directly with *Erk1*$^{-/-}$ and *Erk2*$^{+/-}$ mice, as well as transgenic mice with inducible expression of an ERK1/2-inactivating phosphatase (dual-specificity phosphatase 6) in the heart. Although the hypertrophic growth is not attenuated in these models after long-term pressure overload, selective ablation of cardiac ERK1/2 signaling predisposes the heart to decompensation and failure in conjunction with an increase in myocyte apoptosis (*Purcell NH. et al., 2007*). Although systolic function is clearly affected in these transgenic models, it has not yet been established whether ERK1/2 can directly modulate cardiac contractility. In the present study, the GPCR agonist ET-1 produced a rapid increase in left ventricular phospho-ERK1/2 levels, and inhibition of ERK1/2 activation by U0126, a potent MEK1/2 inhibitor, markedly attenuated the ET-1–induced increase in contractile force in the intact rat heart. Hence, our results demonstrate a novel function for MEK1–ERK1/2 signaling whereby it regulates myocardial contractility in addition to influencing cell growth and survival (*Figure 20)*.

Recent advances indicate that EGFR transactivation is an important pathway that links GPCRs and ERK1/2 activation (*Thomas WG. et al., 2002*). Stimulation of GPCRs induces metalloproteinase-mediated ectodomain shedding of HB-EGF, which can activate EGFR, leading to ERK1/2 phosphorylation via recruitment of

the Ras–Raf1–MEK1/2 cascade (*Wetzker R, Böhmer FD., 200*3). Inhibition of HB-EGF shedding and EGFR transactivation can prevent GPCR agonist–induced left ventricular hypertrophy and cardiac dysfunction in vivo (*Asakura M. et al., 2002*). On the other hand, targeted deletion of HB-EGF results in a severe cardiac phenotype associated with dilated ventricular chambers and diminished cardiac function (*Iwamoto R. et al., 2003*). Although these studies have demonstrated the functional importance of the HB-EGF–EGFR pathway in hypertrophic and developmental growth processes, the relationship between EGFR signaling and cardiac contractility remains unknown. In our experiments, ET-1 increased total tyrosine phosphorylation of EGFR. Because considerable specificity is found between the sites in terms of recruitment of signaling molecules (*Jones RB. et al., 2006*), further experiments are warranted to identify the specific sites that have been tyrosine phosphorylated on the EGFR. However, inhibition of EGFR transactivation by the specific EGFR tyrosine kinase inhibitor AG1478 was accompanied by significant attenuation of the ET-1–induced increase in phospho-ERK1/2 levels and the inotropic response to ET-1. Thus, the present data define a previously unrecognized role for EGFR in the regulation of myocardial contractility, acting as a proximal component of MEK1/2–ERK1/2 signaling (*Figure 20)*.

Previous studies suggest that activation of sarcolemmal NHE1 contributes, at least in part, to the positive inotropic effect of ET-1 (*Krämer BK. et al., 1991; Wang H. et al., 2000; Takeuchi Y. et al., 2001; Chu L. et al., 2003; Zolk O. et al., 2004*). Stimulation of NHE1 can lead to intracellular alkalinization and sensitization of cardiac myofilaments to intracellular Ca^{2+}. On the other hand, NHE1-mediated

accumulation of intracellular Na^+ can indirectly promote a rise in intracellular levels of Ca^{2+} via a reverse-mode Na^+-Ca^{2+} exchanger (*Kentish JC. 1999; Aiello EA. et al., 2005*). In agreement with previous findings, our data showed that zoniporide, a highly selective inhibitor of NHE1, attenuated the inotropic response to ET-1. Activation of ERK1/2 can result in phosphorylation of the C-terminal regulatory domain of the NHE1, either directly by ERK1/2 itself (*Moor AN., Fliegel L., 1999*) or indirectly through p90RSK (*Takahashi E. et al., 1999*). p90RSK is located in the cytosol under basal conditions and translocates to the plasma membrane after stimulation where it becomes fully activated (*Richards SA. et al., 2001*). Maekawa et al have reported recently that expression of a dominant-negative p90RSK abolished oxidative stress–induced activation of NHE1 in cardiomyocytes, providing evidence for the essential role of p90RSK in the regulation of NHE1 activity (*Maekawa N. et al., 2006*). In the present study, ET-1 increased phospho-p90RSK levels in the membrane fraction, and both AG1478 and U0126 attenuated it. Therefore, it is conceivable that membrane-associated p90RSK can mediate the effect of ET-1 on NHE1 activity (*Figure 20)*.

The finding that ~40% of the ET-1–induced positive inotropic effect remained unaffected after inhibition of the EGFR–ERK1/2–NHE1 pathway indicates the existence of additional signaling mechanisms. Phosphoinositide 3-kinase plays an important role in the positive inotropic effect of insulin (*von Lewinski D. et al., 2005*). However, our preliminary results showed that inhibition of phosphoinositide 3-kinase by LY 294002 (3 μM) had no effect on ET-1–enhanced contractility (45.2±5.4% versus 49.7±5%, ET-1 with and without LY 294002; n=5; *P*=1.0). In contrast, ML-7 (1 μM), an inhibitor of myosin light-chain

kinase, significantly reduced the inotropic effect of ET-1 (20.7±1.7% versus 43.8±3.7%, ET-1 with and without ML-7; n=5; $P<0.001$). These results are in line with previous findings (*Andersen GO. et al., 2002; Chu L. Endoh M., 2004*) and suggest that myosin light-chain kinase is an important mediator of the inotropic effect of ET-1.

p38-MAPK signaling has been supposed long to be associated with various pathological conditions in the heart such as hypertrophy, extracellular matrix remodeling and cardiac decompensation [Wang Y, Circulation. 2007;116:1413-1423]. However, less information is available regarding the role of p38-MAPK in the regulation of physiological cellular processes in the myocardium. In adult rat cardiomyocytes, prolonged activation of p38-MAPK by adenoviral gene transfer of its upstream kinase, MKK3bE, resulted in a significant reduction in contractility. On the contrary, pharmacological inhibition of p38-MAPK activity augmented cardiac contractility (*Liao P. et al., 2002*). In the present study, ET-1 rapidly increased the activity of p38-MAPK in the intact adult rat heart, and inhibition of the enzyme by its potent inhibitor SB239063 markedly augmented the inotropic response to ET-1, whereas the inhibitor alone failed to alter baseline contractility. These data imply that p38-MAPK has no direct negative effect on contractility in unstressed adult ventricular myocardium, whereas physiological activation of p38-MAPK provides a negative feedback to the ET-1–mediated inotropic response (*Figure 20)*. This agrees with the report that the positive inotropic effect of β_2-adrenergic stimulation is considerably enhanced by pharmacological inhibition of p38-MAPK in isolated mouse cardiomyocytes (*Zheng M. et al., 2000)*.

Although the concept that p38-MAPK may act as an important regulator of cardiac function is well accepted (*Zheng M. et al., 2000; Liao P. et al., 2002; Bellahcene M. et al., 2006*), the molecular mechanism by which p38-MAPK affects cardiac contractility is still elusive. Previously, it has been suggested that p38-MAPK suppresses ERK1/2 signaling by a mechanism involving protein phosphatases in cardiomyocytes (*Liu Q., Hofmann PA. 2004*). However, our results showed that inhibition of p38-MAPK could not further augment ET-1–induced ERK1/2 phosphorylation, ruling out the existence of direct cross-talk between the ERK1/2 and p38-MAPK pathways in the intact heart. Previous observations suggest that p38-MAPK activation may lead to dephosphorylation of phospholamban, a crucial regulator of cardiac contractility. Liu and Hofmann have shown that adenosine A1 receptor activation can blunt β-adrenergic–stimulated phospholamban phosphorylation in a p38-MAPK–sensitive manner (*Liu Q., Hofmann PA. 2004*). Of particular importance was our finding that ET-1 promoted the phosphorylation of phospholamban at Ser-16 in the presence of p38-MAPK inhibition. Phosphorylation of phospholamban relieves SERCA2a inhibition and enhances Ca^{2+} reuptake into the sarcoplasmic reticulum. On subsequent beats, contractility is increased in proportion to the elevation in the size of sarcoplasmic reticulum Ca^{2+} store and the resulting increase in Ca^{2+} release from the sarcoplasmic reticulum (*MacLennan DH., Kranias EG., 2003*). Therefore, it is tempting to speculate that p38-MAPK may limit increases in contractility via dephosphorylation of phospholamban in the myocardium. In addition to Ca^{2+} handling, p38-MAPK may alter myofilament function. Chronic activation of p38α-MAPK in transgenic MKK6bE hearts has been reported to depress sarcomeric function in association with decreased phosphorylation of α-tropomyosin and

troponin I (*Vahebi S. et al., 2007*). Further experiments are required to elucidate whether p38-MAPK can modulate myofilament function in the acute setting. Taken together, in the normal heart, the dynamic transient activation of p38-MAPK may have an important homeostatic function by counterbalancing excess inotropic stimulation (*Figure 20*). Loss of this cardioprotective mechanism during chronic suppression of p38-MAPK signaling may be a pathogenic factor in the progression of heart failure in mice expressing dominant negative mutants of MKK3 or MKK6 (*Braz JC. et al., 2003*). On the contrary, substantial persistent activation of p38-MAPK signaling also may contribute to the evolution of heart failure by impairing myocardial contractility such as in mice with cardiac-specific expression of MKK3bE or MKK6bE (*Liao P. et al., 2001*). Therefore, cardiac function may be improved by normalization of upregulated p38-MAPK activity in the diseased heart.

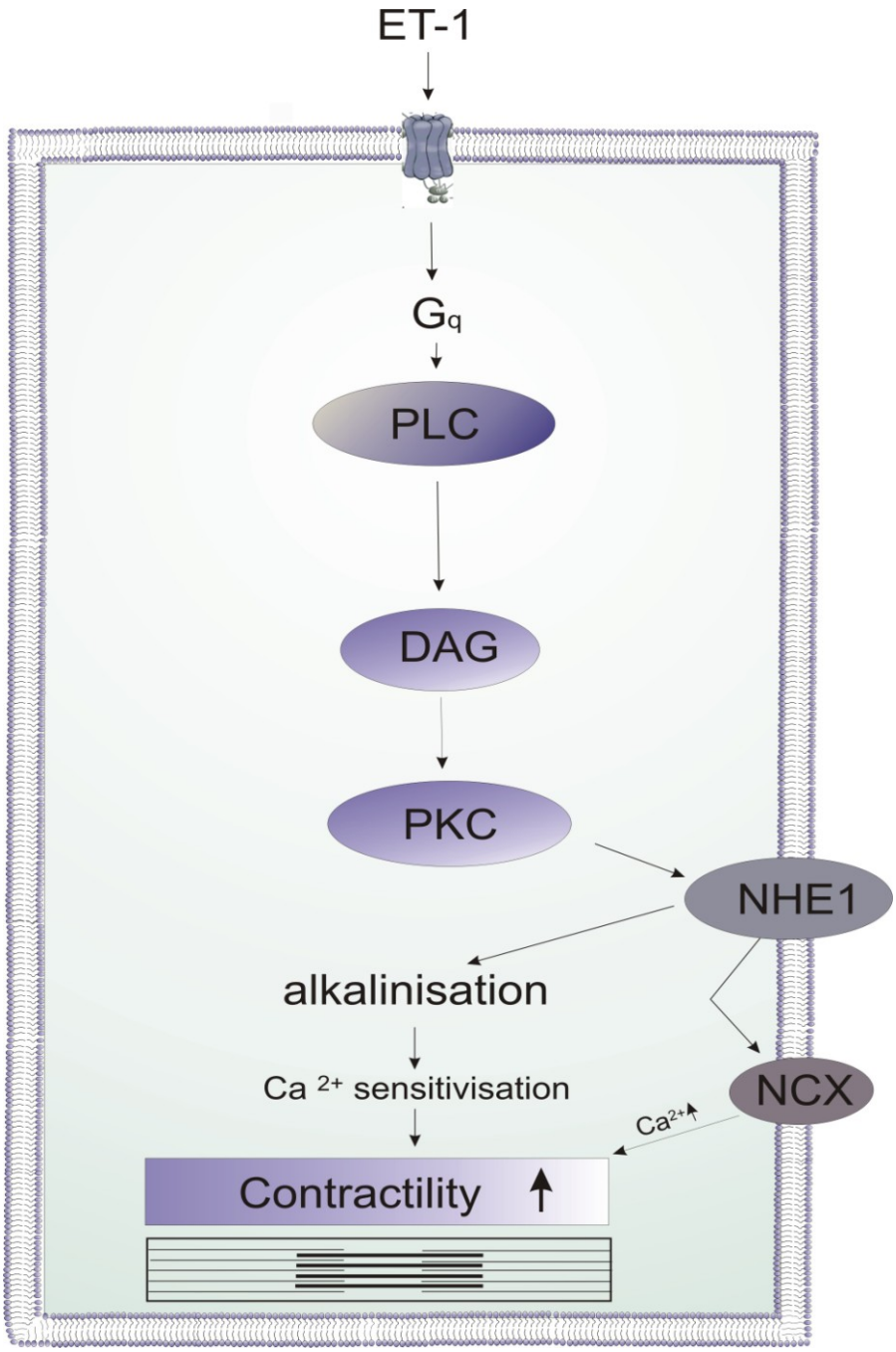

Figure 19. *The traditional view on the signaling mechanisms involved in ET-1–mediated positive inotropic effect. Stimulation of ET_A receptors causes Gq protein-directed activation of the PLC-PKC-NHE-NCX pathway. Increased contractile force is the result of (i) sensitization of cardiac myofilaments to Ca^{2+} due to intracellular alkalosis, and (ii) increased Ca^{2+} influx through the NCX operating in reverse mode (Brunner F, et al., 2006).*

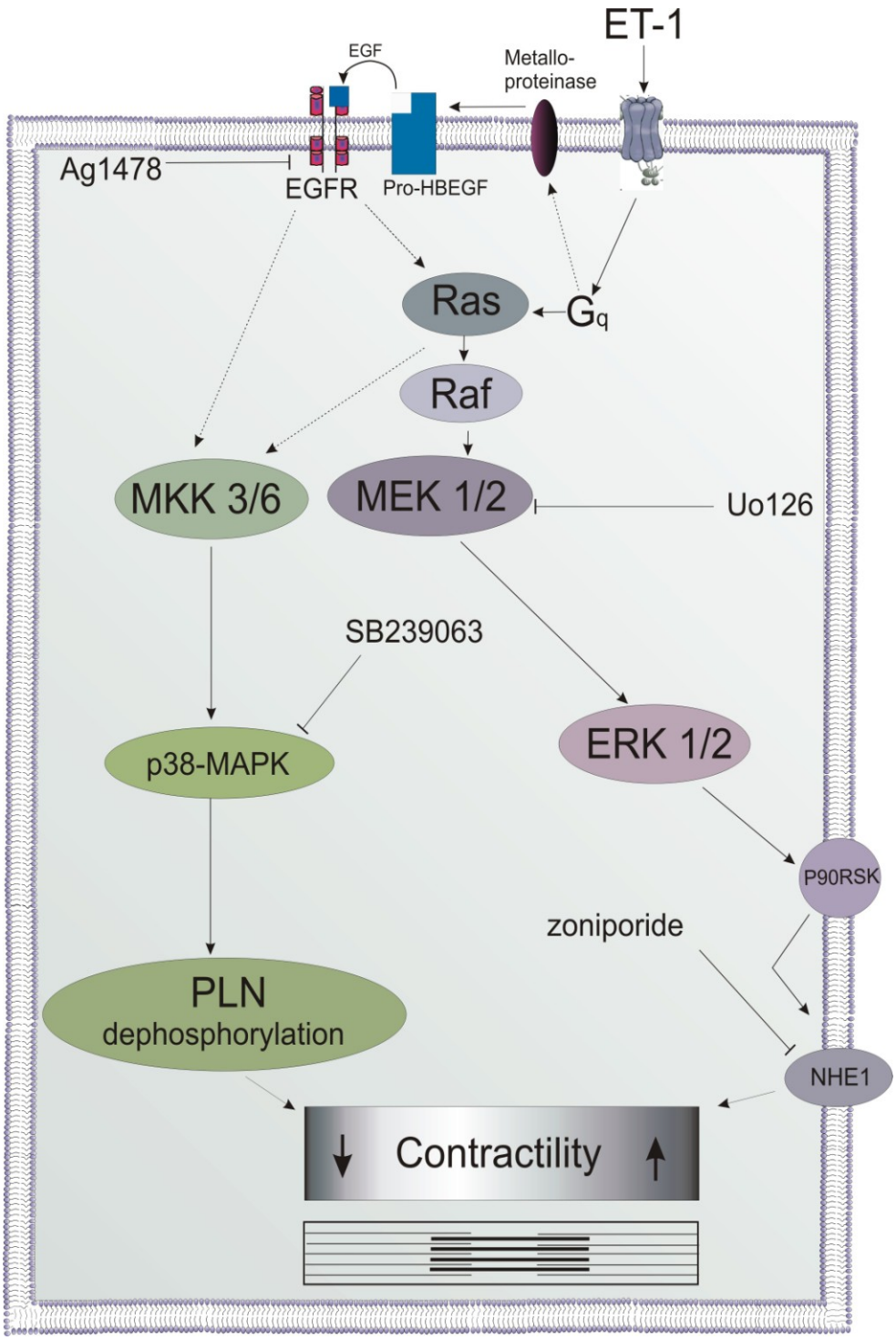

Figure 20. *The novel theory of signaling mechanisms activated by ET-1. MAPKs play opposing roles in that the ERK1/2–mediated positive inotropic response to ET-1 is counterbalanced by simultaneous activation of p38-MAPK. EGFR may act as the upstream regulator and the p90RSK–NHE1 pathway as the downstream effector of ERK signaling. Moreover, p38-MAPK activation may suppress contractility by dephosphorylating phospholamban (PLN), the inhibitory protein for the sarcoplasmic reticulum Ca^{2+} pump (SERCA).*

7. NOVEL FINDINGS

1. We present the first evidence for the functional significance of PrRP, a putative ligand for the GPR10 orphan receptor in the heart.

2. We have shown that PrRP has a direct, dose-dependent, slowly developing, positive inotropic effect.

3. Our results suggest that the cAMP-independent inotropic response to PrRP is suppressed by concurrent activation of PKCα and PP1 leading to dephosphorylation of phospholamban.

4. We present evidence for the functional importance of ERK1/2 and p38-MAPK in the acute regulation of cardiac contractility in the intact adult rat heart.

5. Our results demonstrate that MAPKs play opposing roles in that the ERK1/2-mediated positive inotropic response to ET-1 is counterbalanced by simultaneous activation of p38-MAPK.

6. We have shown that EGFR may act as the upstream regulator and the p90RSK–NHE1 pathway as the downstream effector of ERK signaling.

7. Our data indicate that p38-MAPK activation may suppress contractility by dephosphorylating phospholamban, the inhibitory protein for the sarcoplasmic reticulum Ca^{2+} pump int he intact heart.

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9. PUBLICATIONS

(**Cumulative IF: publications: 26.759; with abstracts: 61.006**)

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9.1.1 Full papers

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9.2.3. Book

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