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DISSERTATION

Direct functional effects of opioid agonists on the isolated perfused rat heart

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Table of Contents

1. INTRODUCTION

Opioids are widely used in clinical practice to relieve pain. Enhanced understanding of the potential mechanisms of opiate peptides, and their respective receptors has resulted in therapeutic advances. Furthermore, a growing body of evidence suggests that in addition to their anaesthetic and analgesic actions, opioids also play an important role as endogenous neurotransmitters by modulating organ function. Despite these findings, the direct local effects of opioids on the cardiovascular system have not been fully understood yet. It has been described that opioid agonists can have complex pharmacological actions on the cardiovascular system. A number of in vitro and in vivo studies have demonstrated that opioids can indirectly alter cardiac function via inhibitory effects at nerve endings of the first autonomic neuron,[1;2] or directly affect cardiac function via myocardial membrane opioid receptors without link to the automatic system.[3;4] However, other studies showed that non-opioid effects, e.g., opioid receptor-independent mechanisms, can also exert direct cardiac depressive actions.[5] For instance, this effect predominates in the model of arrhythmias induced by coronary arterial occlusion.[6] Therefore, it is important to clarify the detailed effects of each opioid receptor subtype on cardiac function. Moreover, given the close relationship between nicotinic acetylcholine receptors and the sympathetic system, the potential modulating effects of opioids on nicotine-mediated sympathetic neurotransmission also need more attention.

Based on above considerations, the present study was designed to explore the potential activities of three opioid agonists: [D-Ala^2, N-Me-Phe^4, Gly-ol^5] - enkephalin (DAMGO, preferentially μ-agonist), (trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U50488H, preferentially κ-agonist), or [D-Pen^{2,5}] enkephalin (DPDPE, preferentially δ-agonist) on the isolated perfused rat heart. In addition, the interaction between these opioid agonists and the transmitters of the autonomous nervous system, norepinephrine and acetylcholine, was investigated. Furthermore, to differentiate between effects on the parasympathetic ganglion and direct postganglionic effect on cardiac myocytes, opioid agonists were combined with nicotine.

1.1 Endogenous opioid systems and classification of opioid

1.1.1 Endogenous opioid system

Opioids such as morphine and heroin have been used for a long time to relieve pain and produce euphoria. Pharmacological investigations suggested an interaction of administered opiate alkaloids with a highly specific receptor. The earliest findings on an opioid receptor and endogenous opioid peptides were reported in 1973[7] and in 1975,[8] respectively. Since then the concept of an endogenous opioid system was gradually established. The complex effects, both beneficial and adverse, of opioid analgesics can now be linked with the interaction between these agents and opioid receptors.

1.1.2 Classification of opioid peptides and opioid receptors

Briefly, the endogenous opioid system includes three parts: Opioid receptors (ORs); endogenous opioid peptides that are ligands or agonists for ORs; and enzymes involved in the synthesis and decomposition of opioid peptides. Endogenous opiate peptides and their receptors exist throughout the central and peripheral nervous systems and in other tissues. The endogenous opioid system has multisystem functions essential for the maintenance of homeostasis and for the survival of the organism. Three distinct families of endogenous opioid peptides have been well characterized:[9] the endorphins, the enkephalins, and the dynorphins, which derive from the precursor polypeptides pro-opiomelanocortin (POMC, also known as beta-endorphin/ACTH precursor), preproenkephalin (ppEnk), and prodynorphin, respectively. The endogenous opioid peptides act via opiate receptors. Thus, three classic genetically and pharmacologically distinct primary opioid receptor types were also identified; they were named μ-, κ-, and δ-receptors and each receptor can also be divided into different subtypes (e.g. δ_1 and δ_2, κ_1 and κ_2). Recently, the fourth member of the endogenous opioid receptor family, which was named ORL_1 (opioid receptor-like), has been shown to be widely distributed in the brain and periphery, and the endogenous ligand was also identified, e.g. nociceptin/orphanin FQ peptide (N/OFQ).[10] Moreover, each type of opioid receptor possesses different yet overlapping ligand-binding properties and functional characteristics: the μ- and δ- receptors bind endorphins and enkephalins, κ-receptors bind dynorphin and ORL_1 binds nociceptin. Table 1 shows the proposed classification of opioid receptors with corresponding agonists and antagonists.

1.1.3 Signaling pathway of endogenous opioid receptor

Genes encoding for ORs have recently been cloned and experimental results indicated that the classical opioid receptors and ORL_1 are all members of the highly homologous superfamily of seven transmembrane domain-spanning G-protein–coupled receptors (GPCRs).[11] Both classical and non-classical opioid receptors bind to inhibitory, pertussis toxin-sensitive G-proteins and initiate a cascade of complex intracellular processes, which include: closing of voltage-sensitive calcium channels; opening of potassium channels with subsequent cellular hyperpolarization, and inhibition of cyclic adenosine monophosphate (cAMP) production through inhibition of adenylate cyclase. Besides, stimulation of ORs also increases the synthesis of NO, cyclic guanosine monophosphate (cGMP), inositol trisphosphate (IP_3), and diacylglycerol DAG), which in turn activates the phospholipase C / protein kinase C (PKC) pathway.[12-14] All these processes alter the cellular response characteristics and organ function.

1.2 Endogenous opioid system of the heart

In the past decade, biosynthesis, posttranslational processing, and anatomical distribution of endogenous opioids within the central and peripheral nervous systems as well as their powerful roles in the regulation of the cardiovascular response have been intensively investigated by means of intraventricular or systemic injection.[9;15] However, the peripheral mechanisms of the endogenous opioid system in modulating cardiovascular function have not been understood. Given the involvement of heterogeneous endogenous opioids in these actions,[16] a systemic investigation with the aim to clarify the different effects of selective opioid peptides acting on the different opioid receptor subtypes on cardiac function is needed.

1.2.1 Cardiac opioid peptides

The occurrence and distribution of an enkephalin-like immunoreactivity in the neuronal system including the brain, the spinal cord, sympathetic fibers and ganglia, parasympathetic preganglionic neurons, and splanchnic nerves have been described.[17-19] The heart is supplied by a dense network of sympathetic and parasympathetic nerve fibers and therefore may also be a site where opioid peptides interact with neurotransmission. Lange and colleagues[20] demonstrated that leucine^5-enkephalin

(Leu-ENK) and methionine[5]-enkephalin (Met-ENK) were present in isolated guinea pig hearts and that the majority of these peptides were of sympathetic origin, indicating an enkephalin-mediated innervation of the heart. Also, a discrepancy in the noradrenaline decrease and the relatively smaller reduction in enkephalins after chemical sympathectomy indicated that nerve cells and the terminals of the cardiac parasympathetic nervous system could be a potential site of enkephalin storage and synthesis, which was also consistent with previous observations.[18] However, Caffrey et al. recently reported that most myocardial enkephalins are non-adrenergic in origin.[21] Meanwhile, using immunohistochemistry or radioimmunoassay on tissue extracts of mammalian hearts demonstrated a significant amount of enkephalins.[22] Thus, local synthesis, storage and release of opioid peptides in non-neuronal tissues, such as cardiac myocytes, could be demonstrated. By demonstrating that the precursors POMC, ppEnk and prodynorphin, and the respective genes are expressed in cardiac tissue[23] and in isolated cardiac myocytes,[24;25] recent studies have revealed that the heart is able to synthesize all three types of opioid peptides, i.e. enkephalins, endorphins, and dynorphins. These studies support the notion that the heart is also a complex endocrine organ exerting complex myocardial action via autocrine or paracrine mechanisms.

Table 1. Classification of opioid peptides and opioid antagonists (Adapted from *van den Brink*[14]*, Waldhoer* [26])

Opioid receptor	Subtypes	Endogenous peptides	Synthetic agonists	Antagonists
μ	μ_1, μ_2, μ_3	Endomorphin-1 Endomorphin-2 β-endorphin β-neoendorphin Dermorphin	DAMGO, PL017, Fentanyl, Morphine, Sufentanil, Buprenorphin	Naloxone, β-FNA, Naloxazone, Naloxonazine ,CTOP, CTAP, SMS201995, TCTOP
δ	δ_1, δ_2	Leu^5- Enkephalin Met^5- Enkephalin Met^5- Enkephalin-Arg^6-Phe^7 Met^5- Enkephalin-Arg^{6}Gly^{7}Leu^8 Deltorphin Deltorphin-I Deltorphin-II	DADLE, DPDPE, DSLET [D-Ala^2]-deltorphin, DTLET, BW373U86, SIOM, SNC 80, TAN-67,	Naloxone, Nalotrindole, BNTX, NTB, NTI, NTII, ICI174864, TIPP, TIPP (ψ), ICI-154129, M154
κ	κ_1, κ_2, κ_3	Dynorphin A Dynorphin B	U-50488, Dynorphin 1a , Bremazocine, EKC, Ketocyclazocine, Spiradoline, U-62066, U-69593, ICI-199441, 6'-GNTI	nor BNI, DIPPA, WIN44441-3, Mr1452, Mr 2266, 5'-GNTI
ORL$_1$		Nociceptin/orphanin FQ	[Arg14, Lys15]nociceptin, [(pX)Phe^4]nociceptin (1-13) amide analogues, NC(1-13)NH2, Cyclo[Cys10, Cys14]NC(1-14)NH2, Ro 64-6198,UFP-102	[N-Phe^1]NC(1-13)NH2, UFP-101, Benzimidazolinone, J-113397, JTC-801, TRK-820, Trap 101

β-CNA: β-chlornaltrexamine , β-FNA: β-Funaltrexamine BNTX: 7-Benzylidenenaltrexone
BW373U86: 4-[(R)-[(2S,5R)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-hydroxyphenyl)methyl]-N,N-diethylbenzamide
CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 DADLE: [D-Ala2, D-Leu5]-enkephalin
DALCE: [D-Ala2, Leu5, Cys6]-enkephalin DAMGO: [D-Ala2, N-Me-Phe4, Gly-ol5]-enkephalin
DIPPA:2-(3,4-Dichlorophenyl)-N-methyl-N-[(1S)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl] acetamide hydrochloride
DPDPE: [D-Pen2,5]-enkephalin DSLET: [D-Ser2, Leu5, Thr6]-enkephalin DTLET: [D-Thr2]-Leu- Enkephalin-Thr
EKC: Ethylketocyclazocine
[FG]OFQ/N(1-13)-NH2 : [Phe1ψ(CH2-NH)Gly2]Nociceptin(1-13)NH2
GNTI: 5'-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5a-epoxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan dihydrochloride
J 113397: 1-[(3R*,4R*)-1-(Cyclooctylmethyl)-3-(hydroxymethyl) -4-piperidinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one
ICI-154129: N,N-bisallyl-Tyr-Gly-Gly-ψ-(CH2S)-Phe-Leu-OH. ICI-174864: N,N-diallyl-Tyr-Aib-Aib-Phe-Leu
ICI-199441:2-(3,4-Dichlorophenyl)-N-methyl-N-[(1S)-1-phenyl-2-(1-pyrrolidinyl)ethyl] acetaide hydrochloride
JTC-801: N-(4-Amino-2-methyl-6-quinolinyl)-2-[(4-ethylphenoxy)me thyl]benzamide
MEAP: Met5- Enkephalin –Arg6-Phe7 NIB: Natriben
Mr1452 : (-)-5,9-dimethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan methane sulphonate
Mr2266 : (-)-5,9α-diethyl -2'-(3-furylmethyl)-2'-hydroxy 6,7 benzomorphan
NNC 63-0532: 8-(1-Naphthalenylmethyl)-4-oxo-1-phenyl-1,3,8-triazaspi ro[4.5]decane-3-acetic acid
N-NTD: N-Benzylnaltrindole Nor-BNI: Nor-binaltorphimine NTB : Naltriben
NTI: Natrindole NTII: NTI 5’- isothiocyanate PLOI7 : [N-MePhe3,D-Pro4]-morphiceptin
Ro 64-6198 : [(1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one]
SIOM: spiroindanyloxymorphone SMS201995: Octreotide
SNC80: [(+)-4-[alpha(R)-alpha-[(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-(3-methoxybenzyl)-N,N-diethylbenzamide]
TAN-67:2-methyl-4aa-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12a_-octahydroquinolino[2,3,3-g]isoquinoline
TCTOP: D-Tic-CTOP TIPP: H-Tyr-Tic-Phe-Phe-OH
TRAP101: 1-[1-(Cyclooctylmethyl)-1,2,3,6-tetrahydro-5-(hydroxyme thyl)-4-pyridinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol- 2-one hydrochloride
TRK-820: (−)-17-cyclopropylmethyl-3,14b-dihydroxy-4,5a-epoxy-6b-[N-methyl-trans-3-(3-furyl)acrylamide]morphinan hydrochloride
UFP 101: [Nphe1,Arg14,Lys15]Nociceptin-NH2 UFP 102: [Arg14,Lys15]Nociceptin-NH2 WIN44441-3: guadazocine

1.2.2 Cardiac opioid receptors

Cardiac opioid receptors (ORs) are widely distributed between atria and ventricles, and most studies have demonstrated the activity of endogenous opioids at cardiac opiate receptors by means of opiate receptor antagonists. However, studies are controversial regarding receptor localization, and only few studies address the diversity in different species. In the human heart the δ-receptor is the most prevalent opioid receptor in the ventricle and the atrium, followed by the μ-receptor.[27] In the rat heart, however, only κ- and δ-ORs are present on the adult ventricular myocardium, with δ-ORs being the abundant receptor and μ-ORs being obviously absent.[4;28;29] Moreover, Zimlichman et al. reported the presence of κ- and δ-ORs in adult rat hearts, whereas in neonatal hearts only μ- and κ-ORs were observed.[30] Although it was suggested that μ-opioid agonists exert cardiac action via δ- or κ-Ors,[31] the heterogenous distribution of different opioid receptors within the heart remains unclear. It is still not known whether or how other heart tissues, such as His-Purkinje and nodal tissue, are involved in the distribution of opioid receptors.[32]

1.3 The cardiovascular function of the endogenous opioid system

1.3.1 Direct action of opioids

Many observations indicate that local administration of opiate receptor agonists can dose-dependently cause functional changes in the heart and cardiomyocytes. Stimulation of all the subtype opiate receptors will affect systemic vascular resistance, excitation-contraction coupling, ventricular contractility and heart rate. For instance, activation of κ- and δ-opioid receptors results in a reduction of sarcoplasmatic reticulum Ca^{2+} depletion, of Ca^{2+} transients, and of contractility.[3] These effects are reversed by the opioid antagonist, naloxone, indicating a receptor-mediated mechanism. In addition, ORs are able to directly regulate voltage-gated K^+ channel opening, and Ca^{2+} channel closing without involvement of a second messenger. Hence, besides central effects on cardiovascular centers of the brain, peripheral effects on the heart significantly contribute to the opioid-mediated effects on the cardiovascular system.

1.3.2 Interaction between endogenous opioid system and autonomic regulation

Besides the direct action of opioids on the heart, the close relationship between the cardiac endogenous opioid system and the autonomic nervous system has to be considered. It has been suggested that enkephalin is presynaptically co-released with the β-adrenergic receptor (β-AR) agonist norepinephrine from nerve terminals.[33] Moreover, it has been demonstrated that δ- and κ-opioid peptides coexist with catecholamines in the sympathetic nerve terminal.[34] In vivo and in vitro studies further demonstrated that activation of three subtypes of opioid receptors, especially κ- and δ-receptor, leads to inhibitory effects on the β_1-AR mediated positive inotropic responses. Eiden et al.[2] reported that the δ-opioid receptor agonist met-ENK (10^{-7}M), but not the μ-receptor agonist β-endorphin, markedly decreased the maximal chronotropic effect of 10^{-5}M norepinephrine in isolated rat atria, while 10^{-7}M naloxone completely blocked the action of met-ENK.[2] Parra and colleagues[35] reported that low concentrations of agonists of μ and δ-opioid receptors (10^{-7}-10^{-6}M morphine and DAMGO) may act as modulators of noradrenaline-induced responses. Vagish and colleagues[36] showed that morphine (10^{-4}M) decreased cardiac output in the presence of norepinephrine (10^{-9}M). This effect was attenuated by naloxone (10^{-5}M), whereas naloxone or norepinephrine alone had no effect on isolated heart at all. Recently, Pepe and colleagues[37] also reported that the specific δ-opioid receptor agonist Leu-ENK (10^{-8}M) significantly inhibited the β-AR-induced positive inotropic effect and increase of cAMP, which was reversed by naloxone (10^{-8}M). Similar results were also obtained by using the κ-agonist U50488H in rat ventricular myocytes.[1] It was demonstrated that the effects of norepinephrine (10^{-8}-10^{-5}M) on both contraction and electrically induced intracellular calcium ($[Ca^{2+}]_i$) transient current were attenuated in a dose-dependent manner by U50488H at 10^{-8}-10^{-6}M, which itself had no effect. Moreover, the inhibitory effects of U50488H on β-AR stimulation were completely blocked by pretreatment with 10^{-6}M norbinaltorphimine (nor-BNI), a specific κ-opioid receptor antagonist, or preincubation with pertussis toxin (PTX), an inhibitor of the opioid receptor signaling pathway (G-protein). On the other hand, adrenergic second messenger stimulation also increased enkephalin production in isolated cardiomyocytes.[38]

The interaction between the endogenous opioid system and the parasympathetic system has also been investigated in a number of studies. The administration of the opioid agonists morphine and met-enk-arg-phe (met-enkephaline) inhibited the release of

acetylcholine and reversed bradycardia induced by vagal stimulation.[39-41] As met-enk-arg-phe inhibited vagal bradycardia without affecting the response to the non-selective muscarinic receptor agonist methacholine,[41] the authors suggested that it may act via nicotinic acetylcholine receptors at the parasympathetic ganglion or at the sinoatrial node.

Taken together, these data suggest that the “cross-talk” between opioid receptors and the autonomous nervous system represents an important mechanism to maintain homeostasis and decrease harmful cardiovascular responses in normal or pathological conditions.

1.4 Relationship between the endogenous opioid system and nicotine

Nicotinic acetylcholine receptors play a vital role in ganglionic transmission within the cardiac autonomic system.[42] It has been demonstrated that the positive cardiovascular response to nicotine is transmitted by the multi-type nicotinic acetylcholine receptor (nAChR) resulting in sympathetic activation and catecholamine release at postganglionic sympathetic nerve endings.[43] Becker et al.[44] reported a biphasic haemodynamic response in isolated guinea pig hearts after adding nicotine in the perfusate: a 3-5 min positive response followed by a negative response. Fuder and colleagues[45] reported a weak release of [^{3}H]norepinephrine from Wistar rat hearts in response to nicotine administration, while Westfall and Saunders failed to observe an increase in the [^{3}H]noradrenaline overflow from Sprague-Dawley rat hearts following infusion of nicotine.[46] Theoretically, based on the aforementioned observations on the relationship between opioids and the β-adrenergic system, it could be postulated that administration of opioids attenuates the sympathomimetic effects of nicotine. Chittal et al[47] demonstrated that morphine blocked the stimulatory and inhibitory responses to nicotine in atria of rabbit. This response was associated with decreased release of catecholamines due to a presynaptic inhibition induced by acetylcholine. The small number of studies and the lack of evidence about the interaction between the endogenous opioid system and nicotinic receptors in the cardiovascular system show the need for further investigation on this topic.

1.5 Receptor-independent action of opioid peptides

Besides the well-described actions of opioid peptides on μ-, κ- and δ-opioid receptors,

there is increasing evidence on receptor-independent action of various opioids on cardiac tissues and the peripheral vascular system, which cannot be reversed be naloxone. Micol et al.[5] demonstrated that the μ-, κ- and δ- opioid agonists, DAMGO, U50488H, and DPDPE induced negative chronotropic effects on isolated rat atria strips. Neither naloxone, MR-2266 (selective κ-antagonist), nor atropine modified the effect of U50488H, while propranolol increased the inhibitory effect of U50488H. These results indicate, that a catecholaminergic mechanism, rather than an opioid receptor mediated or a cholinergic mechanism is involved in the cardiac inhibitory effect of opioids.[5] Similar results in human atria strips were described by Llobell et al.[48] Eimerl and colleagues[49] also found an opioid-receptor-independent mechanism in the haemodynamic modulation before and after electric stimulation of the spinal cord.

Consistent with the above findings, Utz et al.[50] and Pugsley et al.[51] described the interaction of opioids with cardiac ion channels and subsequent electrophysiological effects on the cardiac muscle action potential.[52] This may significantly contribute to the receptor-independent action of opioids on the heart. Furthermore, there is evidence that blockade of ion channels, including Na^+-, K^+- and L-type Ca^{2+}-channels, is involved in the naloxone-insensitive inhibitory effects of opiate peptides.[6;32] In addition to opioid agonists, also opioid antagonists, such as naloxone and MR-1452 (κ-antagonist) have similar effects on the cardiac muscle.[6;53] Thus, a large group of chemicals exist that are able to activate or block opioid receptors with relative specificity at low concentrations, yet, at high concentration, they mediate opioid receptor-independent actions on cardiac tissue.[32]

1.6 Aim of the study

Based on the results of the studies summarized above, we tested the hypothesis that the endogenous opioid system participates in the regulation of sympathetically- and parasympathetically-mediated cardiovascular responses, especially the nicotine-mediated sympathetic neurotransmission. To test these hypotheses, the following protocols were carried out in a model of Langendorff isolated rat heart perfusion system:

1. Investigate the basic cardiovascular responses of three opioid-agonist subtypes: [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO, preferentially μ-agonist), (trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U50488H, preferentially κ-agonist), and [D-Pen^{2,5}] enkephalin (DPDPE, preferentially δ-agonist).
2. Investigate cardiac responses of the three opioid agonists in the presence of norepinephrine, with or without an antagonist (naloxone).
3. Investigate cardiac responses of the three opioid agonists in the presence of acetylcholine, with or without an antagonist (naloxone).
4. Investigate cardiac responses of the three opioid agonists in the presence of nicotine; for DAMGO and U50488H, experiments were also performed with the antagonists naloxone or norbinaltorphimine (nor-BNI).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments and tools

The instruments and tools used in the Langendorff isolated rat heart perfusion system are listed in the table 2.

Table 2 Instruments and tools for the Langendorff experiment setup

Item	Company
Blood gas monitor	Chiron Diagnostics GmbH, Fernwald, Germany
Filter	5µm pore size, Minisart Sartorius, Göttingen, Germany
Gear Pump	L. Eschweiler & Co., Kiel, Germany
Water circulator	Lauda K20KS, Lauda, Königshafen, Germany
Thermostat E1	P-D Industriegesellschaft mbH, Dresden, Germany
Pressure transducer	DTX-Plus™, Becton Dickinson and Company, Singapore
Saran wrap	Toppits®, Melitta Hauhaltsprodukte GmbH, Minden, Germany
Amplifier	Hugo Sachs Elektronik, March-Hugstetten, Germany
Analog-digital converter	Bedo electronic GmbH; Krefeld, Germany
Data acquisition system	DASYLab®, Datalog GmbH; Mönchengladbach, Germany
Data analysis system	Chart 5™, v.5.4.217, ADInstrument; Castle Hill, Australia

2.1.2 Animals

Adult male Sprague-Dawley rats (weight of 250–300 g) were purchased from Charles River (Wiga, Sulzfeld, Germany). Animal care and experiments were in compliance with the “science-based guidelines for laboratory animal care” of the National Research Council (2003). The study was approved by the local animal care committee (animal experiments register No.T 0159/08, Landesamt für Gesundheit und Soziales Berlin).

2.2 Preparation of isolated hearts

Rats were anaesthetised according to the corresponding protocol and supplemental doses of anaesthetic were given if needed. The animals were decapitated when unresponsive to a standardised noxious stimulus. Following thoracotomy the heart was excised and immersed immediately in cold (4°C) modified Krebs-Henseleit buffer (KHB) solution. The ascending aorta was rapidly cannulated distal to the aortic valve and mounted on a

stainless steel cannula placed in the Langendorff perfusion apparatus. Then the heart was retrogradely perfused with filtered KHB solution according to the Langendorff technique at a constant hydrostatic perfusion pressure of 80 mmHg, monitored by a transducer attached to a side arm of the aortic cannula. An incision was then made in the left atrium, and a flexible, saline-filled balloon made of polyvinyl chloride plastic film (Saran Wrap) was passed through the mitral valve and advanced into the left ventricle for evaluation of isovolumic function. Finally, a thermostatic heart chamber was moved to cover the heart and to maintain a "water-jacketed" heart bath at 37°C throughout the experiment. The composition of modified Krebs-Henseleit solution was: NaCl 114 mM; KCl 3.8 mM; $MgCl_2$ 1.2 mM; $CaCl_2$ 1.25 mM; $NaHCO_3$ 25.0 mM; KH_2PO_4 1.2 mM; glucose 11.5 mM; sodium pyruvate 2 mM; mannitol 16 mM; ethylene-diaminetetraacetic acid (EDTA) 0.05 mM and insulin 5 U/L. The perfusate was bubbled with a gas mixture of 95% O_2 and 5% CO_2, and equilibrated at pH 7.35 to 7.45. Perfusion temperature was kept at 37°C using a thermostatically controlled water circulator. Fig.1 shows a simplified schematic circuit of our Langendorff system.

In addition to hydrostatic perfusion pressure, the coronary flow rate was maintained at 10-15 ml/min, which was recorded by a transit time flowmeter. Left ventricular end-systolic pressure (LVESP) was isovolumetrically measured by a pressure transducer, which was connected with the fluid-filled balloon via a polyethylene catheter. The balloon volume was adjusted to yield a left ventricular end-diastolic pressure (LVEDP) of approximately 5 mmHg. The other indices of haemodynamics, including heart rate (HR) and positive and negative maximum instantaneous pressure changes (dP/dt max, dP/dt min), were derived from the pressure signals. All signals were continuously monitored via an analog-digital converter and DASYLab® data acquisition system. Measurements were recorded and stored during the last minutes of each application. Together with DASYLab®, Chart 5™ was used for the final multi- channel data integration and calculation.

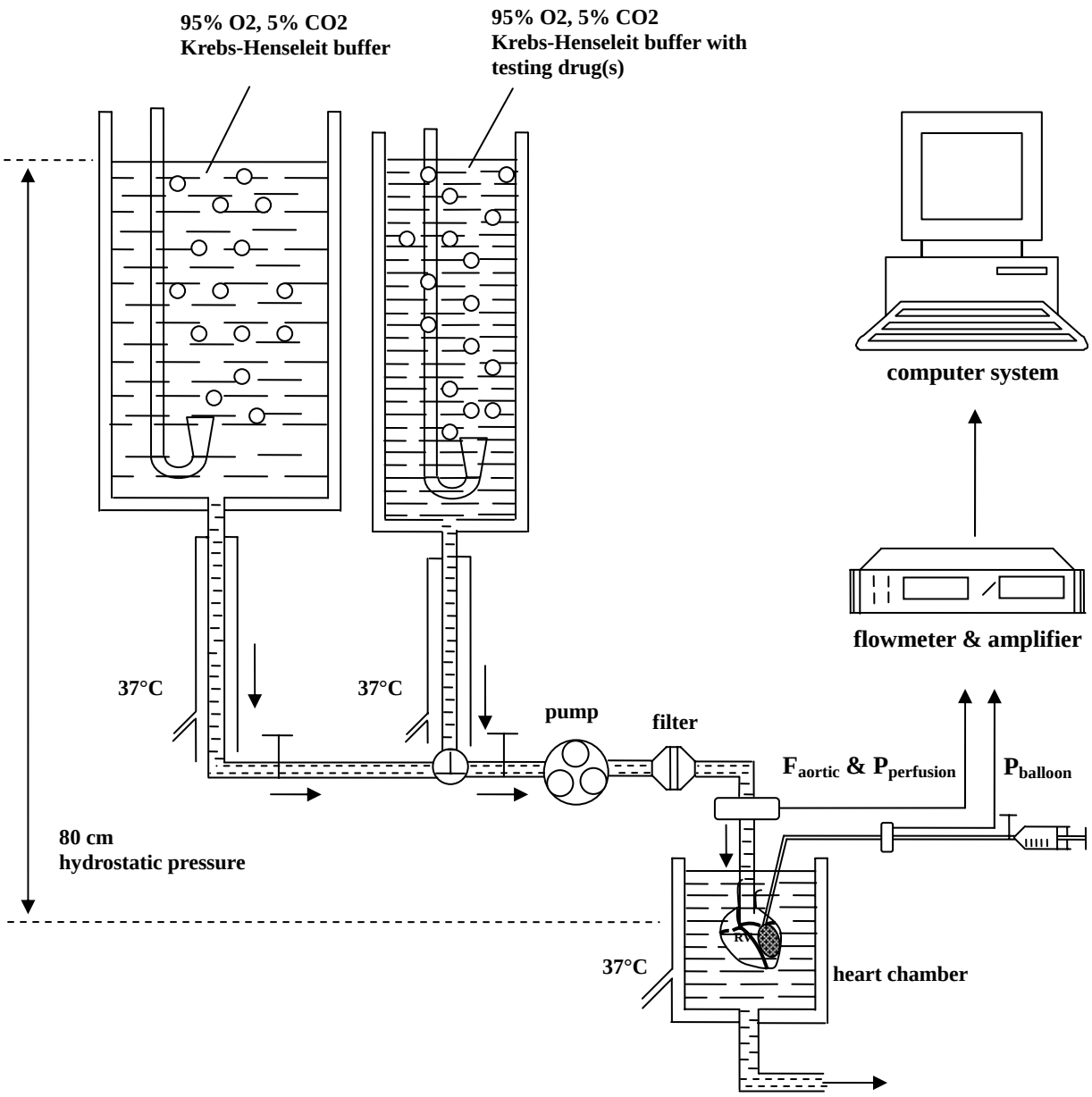

Figure1. Schematic drawing of the Langendorff isolated rat heart system setup

2.3 Protocols

After the preparation, each heart was allowed to equilibrate at least 20 min, by which time the peak developed force had reached a steady state. Hearts were discarded if they met one of following functional criteria during this time: 1) significant rhythm irregularities, 2) unstable contractile function, 3) LVESP below 80 mmHg. Each heart was used for one agonist or antagonist only to avoid drug interaction.

2.3.1 Experimental protocol I

In Protocol I, rats were anaesthetised with 20 mg of ketamine (Ketavat®; 100 mg/ml, Pharmacia & Upjohn GmbH, Erlangen, Germany) injected intraperitoneally. At the same time anticoagulation with 1000 units of heparin (Heparin-Rotexmedica®, Rotexmedica GmbH, Tritau, Germany) was performed.

In preliminary experiments, we initially observed the dose-response reactions of norepinephrine (NE) and acetylcholine (ACh) in the Langendorff setup in order to find the suitable concentration for the following protocols. Briefly, after a baseline period of 20 minutes, either NE or ACh (n=4 each) was added to the perfusate beginning with a concentration of 10^{-9}M, increasing up to 10^{-8}M,10^{-7}M and 10^{-6}M at intervals of 10 minutes each. All haemodynamic parameters were recorded at the end of every 10 min period. Besides, a vehicle group (n=4) of KHB solution was also investigated within the same time. In additional experiments the effects of NE and ACh in selected concentrations were observed over a 1 hour period to determine whether the haemodynamic parameters were stable.

2.3.2 Dose-response of opioid receptor agonists

After baseline measurements, rat hearts were randomly assigned to investigate the dose-response reactions of different opioid receptor agonists. Increasing concentrations of either (D-Ala2, NMe-Phe4, Gly-ol5)-enkephalin (DAMGO), a selective μ-agonist, trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide (U50,488H), a selective κ-agonist, or [D-Pen2, D-Pen5] Enkephalin (DPDPE), a selective δ-agonist, were cumulatively added to 200 ml of the perfusate at 10 minute intervals. The concentration ranges were: 10^{-10}M, 10^{-9}M, 10^{-8}M, and 10^{-7}M for DAMGO, 10^{-8}M, 10^{-7}M, 10^{-6}M, and 10^{-5}M for U50,488H, and 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M for DPDPE. The dose-response curve for each opioid receptor agonist was also obtained in the presence of either NE or ACh, which

was infused for 10 minutes prior to administration of the opioid receptor agonists, with a final concentration of $5x10^{-8}$M and $5x10^{-7}$M, respectively. All haemodynamic parameters were recorded at the end of each 10-minute interval. Figure 2 shows the experimental protocol for this set.

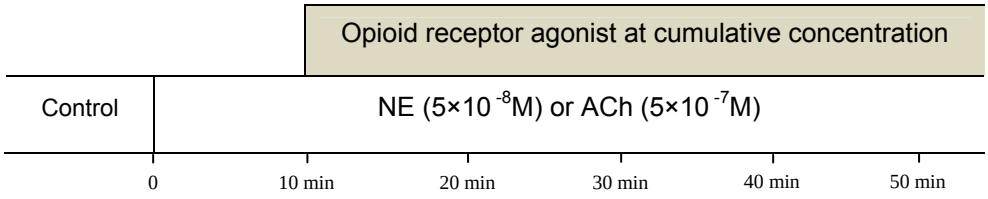

Figure 2. Diagram summarising protocol 4.1.1

2.3.3 Opioid receptor agonist + NE (ACh) + naloxone

In this set of experiments, after the baseline measurements, rats were randomly assigned to either the norepinephrine ($5x10^{-8}$M) or the acetylcholine ($5x10^{-7}$M) group. 10 minutes after addition of NE or ACh, naloxone, a non-specific opioid receptor antagonist was initiated at a concentration of 10^{-5}M. After additional 10 minutes, in the presence of the previous compounds, either DAMGO (10^{-7}M), U50,488H (10^{-5}M), or DPDPE (10^{-6}M) was added to the perfusion (figure 3). The infusion was maintained for 10 min. All haemodynamic parameters were also recorded during a 10 min interval for each drug combination.

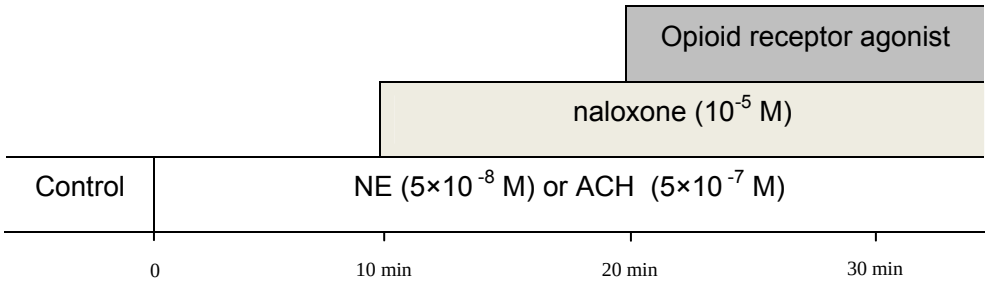

Figure 3. Diagram summarising protocol 4.1.2

2.3.4 Protocol II

In protocol II, rats were anaesthetised with pentobarbital (50 mg/Kg, Narcoren®; Merial GmbH, Hallbergmoos, Germany) injected intraperitoneally. At the same time anticoagulation with 1000 units of heparin was performed. Preparation and perfusion of the hearts was identical to protocol I. After 20 minutes of stabilisation, infusion of either DAMGO 10^{-6}M or U50,488H 10^{-5}M, was started 5 minutes prior to the administration of nicotine (10^{-4}M) for 5 minutes. Because preliminary experiments confirmed a short duration of action for nicotine (1-2 minutes), haemodynamic parameters were recorded continuously for at least 2 minutes as soon as nicotine was added. This enabled us to exactly determine the haemodynamic effects of nicotine.

In the second part, we also tested the interaction of DAMGO or U50,488H with nicotine in the presence of their respective selective antagonists. Naloxone (10^{-5}M) and nor-Binaltorphimine dihydrochloride (nor-BNI, 5×10^{-5}M), as selective μ- and κ-receptor antagonist, respectively, was administrated for 5 minutes. This was followed by exposure to DAMGO or U50,488H for 5 minutes before initiation of the nicotine perfusion. Another additional experiment of nicotine in the presence of either nor-BNI or naloxone alone was also conducted. The schematic model for protocol II is shown in figure 4.

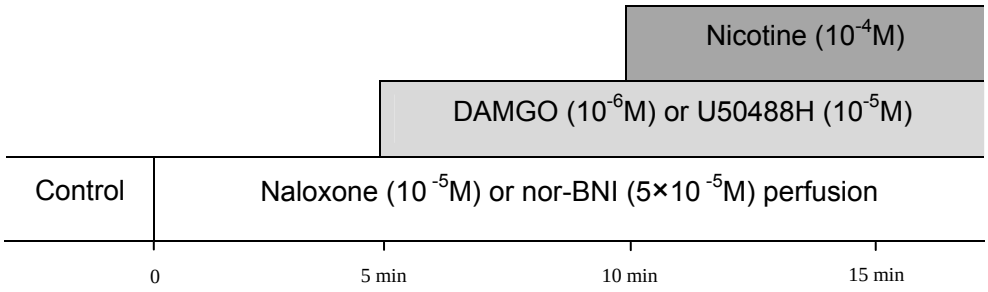

Figure 4. Diagram summarising protocol II

2.4 Reagents and drugs

In this study the following reagents were used: norepinephrine (Arterenol®, Hoechst AG; Frankfurt, Germany), acetylcholine (Sigma-aldrich; Taufkirchen, Germany), DAMGO, U50,488H, DPDPE, naloxone, nor-BNI, and nicotine (Tocris Bioscience, Ellisville, MO, USA). All compounds were dissolved in distilled deionised water as stock solution in advance and stored at -20°C. The concentration of stock solutions was: norepinephrine

100 µg/ml, acetylcholine 1mg/ml, DAMGO 100 µg/ml, U50,488H 2.5 mg/ml, DPDPE 1 mg/ml, naloxone 25 mg/ml, nicotine 100 mg/ml, and nor-BNI 100ug/ml. During the experimental days, the stock solution was diluted with KHB perfusate to the desired concentrations in the volume of 200 ml, which was then used to perfuse the hearts according to the protocol. Other chemicals, unless stated otherwise, were purchased from Sigma-Aldrich.

2.5 Statistical analysis

All tests were performed using Sigma Stat 2.03 software (SPSS Inc., Erkrath, Germany). Results are expressed as the mean±standard deviation. Intra-group comparison was performed by one-way repeated measures ANOVA followed by multiple t-tests versus baseline and Bonferroni-Holm procedure to adjust for multiple comparisons. For inter-group comparisons, one-way ANOVA analysis was conducted followed by Student-Newman-Keuls post-hoc test. For all statistical tests, significance was assumed at $p<0.05$.

3. RESULTS

3.1 NE and ACh dose responses

Dose response results of norepinephrine (NE) and acetylcholine (ACh) can be seen in table 3. In the NE group HR, LVSEP, dp/dt_{max}, and dp/dt_{min} significantly increased ($p<0.05$) when the concentration was higher than 10^{-7}M. In the ACh group, haemodynamic variables decreased significantly when concentration of ACh was higher than 10^{-6}M ($p<0.05$). We chose NE and ACh at concentrations of 5×10^{-8}M and 5×10^{-7}M, respectively, as testing dosage for further experiments.

In time control experiments, during perfusion with Krebs-Henseleit buffer or with the selected concentrations of NE and Ach, haemodynamic variables did not significantly change within a 50 min period (table 4).

Table 3. Results of norepinephrine and acetylcholine dose response

	NE group (n=4)				
	Baseline	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M
HR (sec^{-1})	275±11.5	280±11.5	281±11.4	301±11.0	362±30.9*
LVESP (mmHg)	105±3.0	104±5.2	107±6.7	124±8.0*	136±3.5*
dp/dt_{max} ($mmHg.sec^{-1}$)	2902±190	2877±227	2986±239	3876±312*	5975±233*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1884±41.3	-1818±153	-1884±154	-2479±173*	-3655±147*
	ACh group (n=3)				
	Baseline	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
HR (sec^{-1})	310±1.6	304±2.9	292±10.6	239±12.6*	106±10.9*
LVESP (mmHg)	106±2.3	110±2.8	91.1±11.8	89.6±6.1	71.2±17.5*
dp/dt_{max} ($mmHg.sec^{-1}$)	3132±142	3186±168	2709±169	2334±148	1476±259*
dp/dt_{min} ($mmHg.sec^{-1}$)	-2013±29.1	-2006±46.9	-1483±183*	-1387±175*	-815±211*

NE = norepinephrine, ACh = acetylcholine, HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt min = negative maximum instantaneous pressure changes. Data are mean±SEM. * $p<0.05$ vs. corresponding baseline value.

Table 4. Results of time control observations

	NE group (5×10^{-8}M, n=3)					
	Baseline	10 min	20 min	30 min	40 min	50 min
HR (sec^{-1})	280±26.5	314±13.5	316±18	319±27	323±25.1	321±29.4
LVESP (mmHg)	88.2±6*	115±11.8	112±12.2	106±9.7	106±6.5	102±6.7
dp/dt_{max} ($mmHg.sec^{-1}$)	2244±126*	3095±293	2967±329	3024±320	2966±179	2957±231
dp/dt_{min} ($mmHg.sec^{-1}$)	-1695±158*	-2503±227	-2422±212	-2516±286	-2381±253	-2377±290
	ACh group (5×10^{-7}M, n=3)					
	Baseline	10 min	20 min	30 min	40 min	50 min
HR (sec^{-1})	280±27.2	229±36.5	229±42.9	231±28.5	249±5.4	243±20.7
LVESP (mmHg)	110±7	104±7.1	98.6±13.8	100±18.5	98.1±22.8	95.0±24.4
dp/dt_{max} ($mmHg.sec^{-1}$)	3255±436	2652±447	2538±635	2544±657	2613±709	2444±719
dp/dt_{min} ($mmHg.sec^{-1}$)	-2175±210	-1791±251	-1672±381	-1692±430	-1682±503	-1571±430
	KHB group (n=4)					
	Baseline	10 min	20 min	30 min	40 min	50 min
HR (sec^{-1})	307±41.1	304±37.5	305±33.8	305±41.2	303±37.2	302±36.6
LVESP (mmHg)	93.6±6.1	94.6±6.3	95±7	93.7±5.4	92.6±5.3	90.3±6.6
dp/dt_{max} ($mmHg.sec^{-1}$)	2674±115.2	2678±107	2664±202	2676±191	2639±178	2568±110
dp/dt_{min} ($mmHg.sec^{-1}$)	-1828±124	-1851±99.3	-1871±157	-1790±119	-1800±98.0	-1791±111

NE = norepinephrine, ACh = acetylcholine, HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt_{min} = negative maximum instantaneous pressure changes, KHB = Krebs- Henseleit buffer solution. Data are mean±SEM. * $p<0.05$ vs corresponding 10 min value.

3.2 Cardiovascular responses to opioid-receptor agonists

The results for the dose responses of different opioid agonists are shown in table 5. Response curves expressed as percentage change are shown in figure 5. Although HR, LVESP, dp/dt_{max}, and dp/dt_{min} decreased with increasing concentrations of DAMGO, intragroup differences in the DAMGO group were not significant and the maximum decreases of HR, LVESP, dp/dt_{max} and dp/dt_{min} were 3.1±3.1%, 4.1±1.5%, 5.8±3.8%, and 4.9±2.6%. U50488H decreased HR (14.6±4%) and dp/dt_{max} (29.3±6.5%) significantly at the highest concentration of 10^{-5}M ($p<0.05$), whereas LVESP (18.6±6.8%) and dp/dt_{min} (23.9±8%) were not notably altered. In the group receiving DPDPE, dp/dt_{max} and dp/dt_{min} both showed significant differences compared to baseline values at 10^{-8}, 10^{-7} and 10^{-6}M ($p<0.05$). HR and LVESP, however, were unaltered. The maximum decrease of HR, LVESP, dp/dt_{max}, and dp/dt_{min} obtained with DPDPE was by2.4±0.7%, 14.2±4.4%, 13±3.6%, and 17±5.2%, respectively.

Table 5 Cardiovascular responses to opioid receptor agonists

	DAMGO group (n=7)				
	Baseline	10^{-10}M	10^{-9}M	10^{-8}M	10^{-7}M
HR (sec^{-1})	295±11.2	290±9.4	288±9.5	286±11.1	285±11.2
LVESP (mmHg)	107±5.2	105±6.0	108±5.2	106±4.5	103±4.8
dp/dt_{max} ($mmHg.sec^{-1}$)	3060±173	2968±121	3058±61.5	2950±48	2844±33.2
dp/dt_{min} ($mmHg.sec^{-1}$)	-2105±124	-2048±145	-2103±117	-2082±108	-1993±104
	DPDPE group (n=6)				
	Baseline	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M
HR (sec^{-1})	265±22.3	265±25.1	265±25	267±25.4	271±22.6
LVESP (mmHg)	97.3±5.2	92.1±5.3	89.9±6.4	84.3±6.9	83.7±6.6
dp/dt_{max} ($mmHg.sec^{-1}$)	2420±242	2304±283	2253±310*	2131±306*	2135±287*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1734±140	-1628±146	-1585±171*	-1461±177*	-1457±180*
	U50488H group (n=4)				
	Baseline	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
HR (sec^{-1})	239±12.1	238±11.5	233±10.6	223±14.5	204±9.7*
LVESP (mmHg)	89.8±4.4	88.2±6.8	86.3±7.5	81.6±5.7	72.3±3.7
dp/dt_{max} ($mmHg.sec^{-1}$)	2414±90.6	2381±163	2303±183	2152±117*	1692±113*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1652±114	-1618±136	-1569±156	-1641±117	-1236±91.5

HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt_{min} = negative maximum instantaneous pressure changes. Data are mean±SEM. * $p<0.05$ *vs* corresponding baseline value.

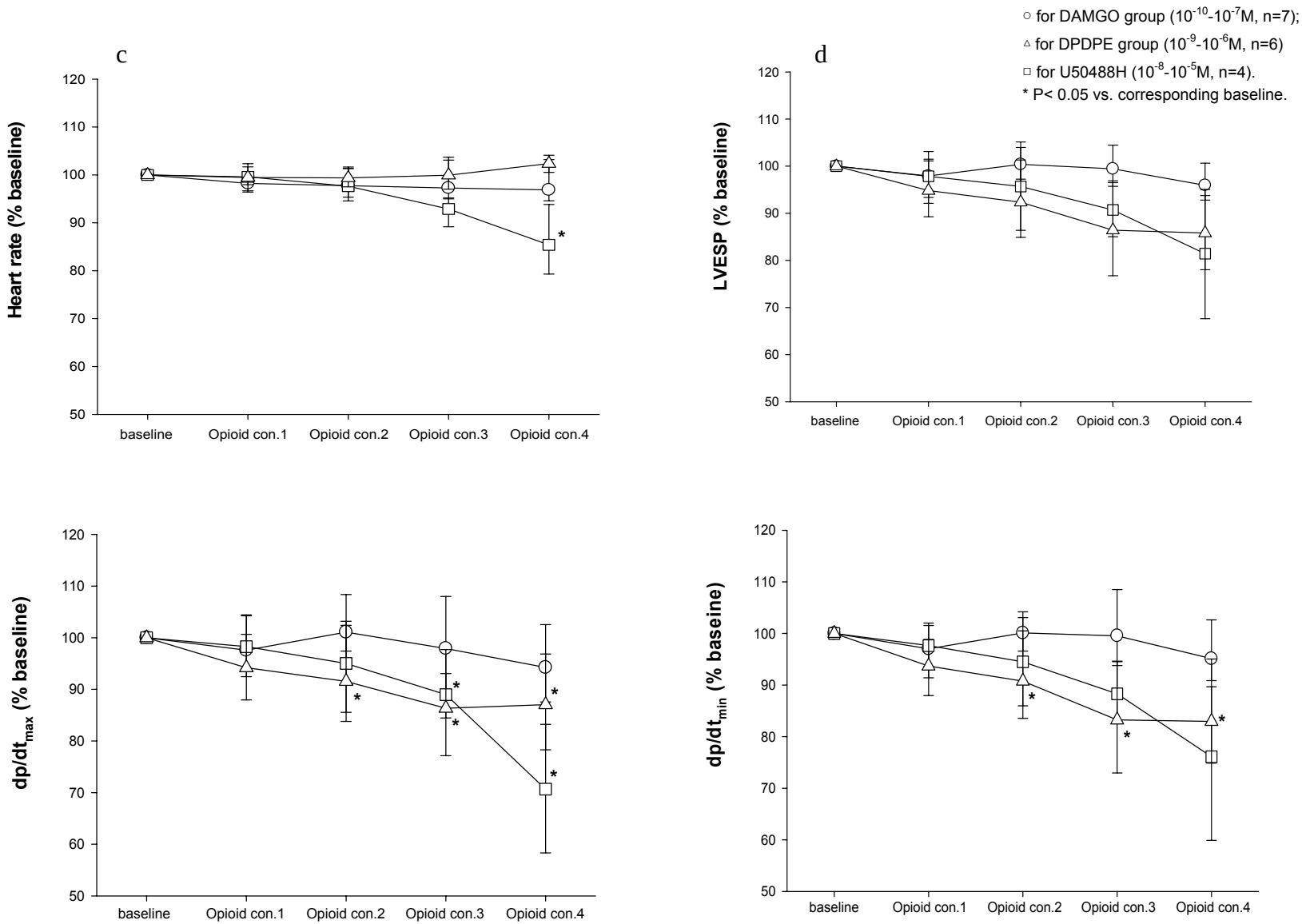

Fig.5. Dose-related effects of three opioid agonists on heart rate, left ventricular end systolic pressure (LVESP), positive and negative maximum instantaneous pressure changes (dp/dt_{max} and dp/dt_{min}) in rat isolated heart. (a) show percentage changes, from pre-drug values, in heart rate,(b) LVESP, (c) dp/dt_{max} and (d) dp/dt_{min}. Values are expressed as mean±SEM.

3.3 Effects of different opioid agonists on the cardiac response to norepinephrine

The results of the cardiac responses to opioid receptor agonists in the presence of NE are shown in table 6 and figure 6. In groups pretreated with NE, the haemodynamic parameters significantly increased after adding NE (5×10^{-8}M) as already shown in preliminary experiments.

Compared with NE alone, addition of DAMGO at increasing concentrations significantly decreased LVESP, dp/dt_{max} at 10^{-9}M - 10^{-7} M ($p<0.05$), and dp/dt_{min} at a concentration of 10^{-8}M and 10^{-7}M ($p<0.05$). The maximum decrease of LVESP, dp/dt_{max} and dp/dt_{min} obtained with DAMGO in the presence of NE was by 20.9±3.8%, 22.5±4.6% and 22±4.6% (all $p<0.05$ vs NE $5x10^{-8}$M), respectively. However, there were no associated changes in HR).

In the U50488H group, LVESP, dp/dt_{max}, as well as dp/dt_{min} significantly decreased at concentrations higher than 10^{-7}M ($p<0.05$). HR also decreased at the highest concentration of 10^{-5}M ($p<0.05$). The maximum decrease of HR, LVESP, dp/dt_{max} and dp/dt_{min} obtained with U50488 in the presence of NE was by 15.6±6.2%, 42.2±5.1%, 52.6±4.3%, and 53.7±5.2%, respectively (all $p<0.05$ vs NE $5x10^{-8}$M).

Analogously, addition of DPDPE at all applied concentrations resulted in a significant decrease in the observed parameters. The maximum decrease of HR, LVESP, dp/dt_{max} and dp/dt_{min} obtained with DPDPE in the presence of NE was by 4.1±1.6%, 32.2±2.3%, 35.7±2.9%, and 38.9±3.7% (all $p<0.01$), respectively, as compared to NE alone.

In further experiments, we investigated whether the depressive cardiovascular effects of opioids are transmitted via opioid receptors. This was performed by adding the non-selective opioid receptor antagonist naloxone at a dose known to cause the maximum antagonistic effect. Naloxone alone at a concentration of 10^{-5}M did not have any effects on cardiovascular performance throughout the experiments. Addition of naloxone antagonised the effects of DAMGO (10^{-7}M) and DPDPE (10^{-6}M) on the haemodynamic response to NE (5×10^{-8}M, table7, figure7). In contrast, effects of U50488H (10^{-5}M) were only partially antagonized: HR and dp/dt_{max} decreased significantly in the presence of naloxone ($p<0.05$).

Table 6. Cardiovascular responses to opioid agonists in the presence of norepinephrine

	Baseline	**NE**	**DAMGO group** (n=6)			
		(5×10^{-8}M)	10^{-10}M	10^{-9}M	10^{-8}M	10^{-7}M
HR (sec^{-1})	268±5.1	298±7.4†	300±7.5	307±8.3	313±7.5	310±8
LVESP (mmHg)	90.7±2.7	120±9†	110±9.7	102±7.5*	101±7.2*	101±8.1*
dp/dt_{max} ($mmHg.sec^{-1}$)	2364±190	3518±461†	3114±479	2914±354*	2901±345*	2706±354*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1666±324	-2572±324†	-2304±317	-2168±246	-2163±234*	-1995±241*
	Baseline	**NE**	**DPDPE group** (n=5)			
		(5×10^{-8}M)	10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M
HR (sec^{-1})	282±15.8	304±15.3†	299±12.9	293±14.9*	292±15.7*	292±14.6*
LVESP (mmHg)	98±1.6	126±7.4†	102 ±6.5*	98.3±5.0*	88.9±3.2*	84.7±3.8*
dp/dt_{max} ($mmHg.sec^{-1}$)	2671±117	3685±211†	2862±88.5*	2715±92.7*	2476±101*	2351±91.6*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1917±80.8	-2765±194†	-2148±146*	-1967±108*	-1969±263*	-1672±97.4*
	Baseline	**NE**	**U50488H group** (n=6)			
		(5×10^{-8}M)	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
HR (sec^{-1})	302±13.3	307±16.6	305±14.6	296±13.5	288±16.1	257±17.3*
LVESP (mmHg)	89.6±3.6	118±7.4†	110±7.2	102±6.8*	94.1±6.2*	67±3.4*
dp/dt_{max} ($mmHg.sec^{-1}$)	2407±100	3277±229†	3033±205	2766±218*	2492±184*	1516±76.0*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1714±59.2	-2552±153†	-2322±143	-2062±148*	-1858±152*	-1158±102*

NE = norepinephrine, HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt_{min} = negative maximum instantaneous pressure changes. Data are mean±SEM. † $p<0.05$ *vs.* corresponding baseline value, * $p<0.05$ *vs.* corresponding NE value.

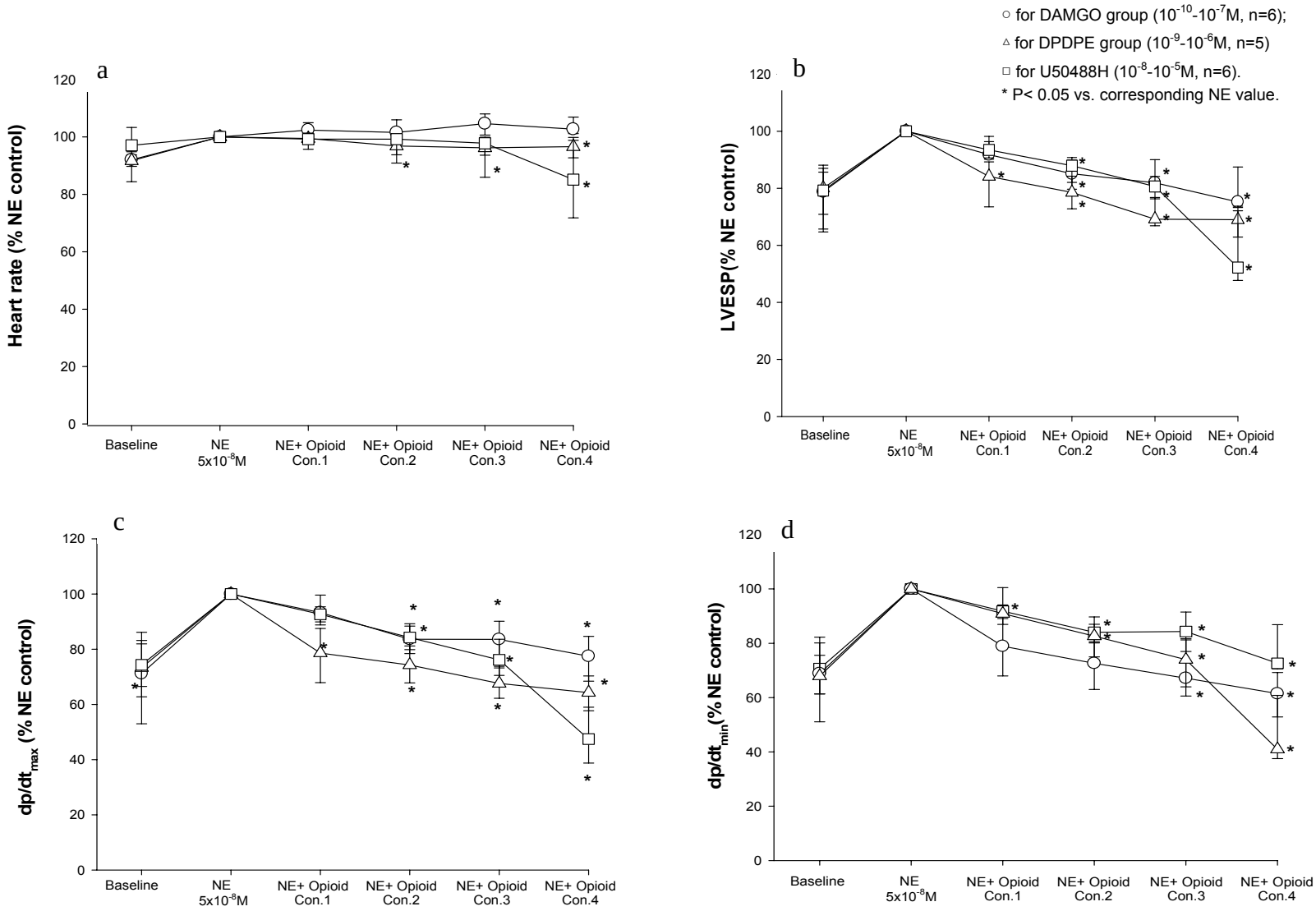

Fig.6. Dose-related effects of three opioid agonists on heart performances in presence of norepinephrine (NE). (a) show percentage changes, from NE treatment values, in heart rate,(b) left ventricular end systolic pressure (LVESP), (c) positive maximum instantaneous pressure changes (dp/dt_{max}) and (d) negative maximum instantaneous pressure changes (dp/dt_{min}). Values are expressed as mean±SEM.

Table 7. Cardiovascular responses to opioid agonists in the presence of naloxone and norepinephrine

	DAMGO group (n=4)			
	Baseline	NE (5×10^{-8}M)	NE+NAL(10^{-5}M)	NE+NAL+DAMGO(10^{-7}M)
HR (sec^{-1})	300±3.4	310±7.5*	301±7.4#	285±11.1#
LVESP (mmHg)	90.4±1.9	107±9.3	108±10.8	97.5±5.4
dp/dt_{max} ($mmHg.sec^{-1}$)	2555±175	3050±213	3043±217	2685±27.4
dp/dt_{min} ($mmHg.sec^{-1}$)	-1662±51.9	-2117±285	-2124±321	-1833±169
	DPDPE group (n=5)			
	Baseline	NE (5×10^{-8}M)	NE+NAL(10^{-5}M)	NE+NAL+DPDPE(10^{-6}M)
HR (sec^{-1})	307±21.2	307±19.2	290±21.9	286±22.2
LVESP (mmHg)	93.9±5.4	106±7.0	108±6.5	95.2±5.6
dp/dt_{max} ($mmHg.sec^{-1}$)	2709±221	3017±190	3016±241	2605±181
dp/dt_{min} ($mmHg.sec^{-1}$)	-1886±184	-2180±163	-2205±165	-1893±132
	U50488H group (n=4)			
	Baseline	NE (5×10^{-8}M)	NE+NAL(10^{-5}M)	NE+NAL+U50488H(10^{-5}M)
HR (sec^{-1})	286±14.4	305±19.4	296±17.9	264±21.7*#‡
LVESP (mmHg)	85.6±4.4	93.6±6.9	88.2±3.9	78.7±6.5#
dp/dt_{max} ($mmHg.sec^{-1}$)	2495±155	2812±255	2589±161	2094±154*#‡
dp/dt_{min} ($mmHg.sec^{-1}$)	-1643±77.7	-1955±181	-1804±101	-1614±234

NE = norepinephrine, HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt_{min} = negative maximum instantaneous pressure changes, NAL = naloxone. Data are mean and standard error of mean. * $p<0.05$ *vs.* corresponding baseline value, # $p<0.05$ *vs* corresponding NE treatment value, ‡, $p<0.05$ vs corresponding NE+NAL treatment value.

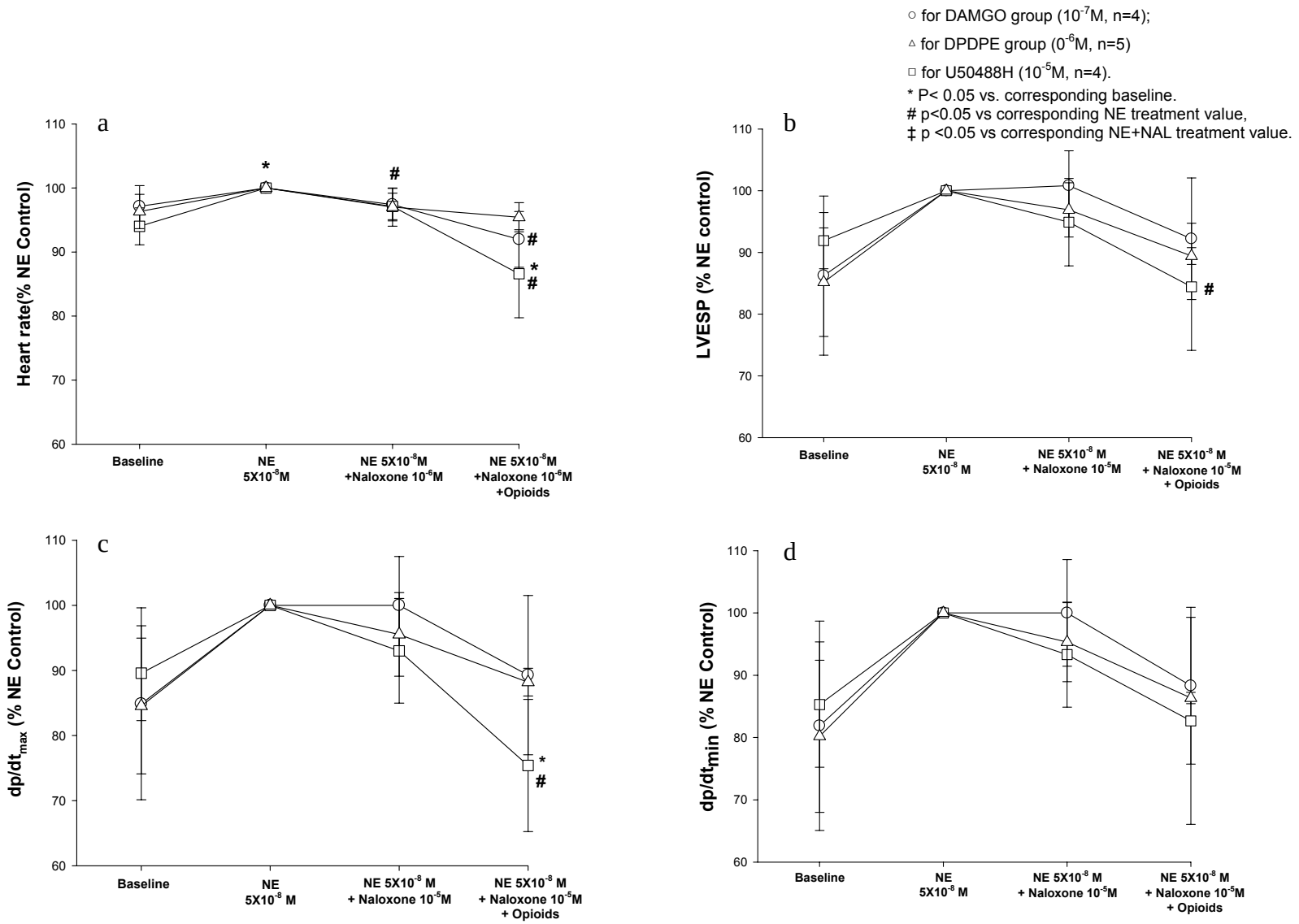

Fig.7. Cardiovascular responses of three opioid agonists in the presence of naloxone(NAL) and norepinephrine (NE). (a) show percentage changes, from NE treatment values, in heart rate,(b) left ventricular end systolic pressure (LVESP), (c) positive maximum instantaneous pressure changes (dp/dt_{max}) and (d) negative maximum instantaneous pressure changes (dp/dt_{min}). Values are expressed as mean±SEM.

3.4 Effects of different opioid agonists on the cardiac response to acetylcholine

In groups pretreated with acetylcholine (ACh) the opioid receptor agonists DAMGO, U50488H and DPDPE also showed similar negative inotropic effects in a dose-related fashion (Table 8, figure8). DAMGO and U50588H significantly decreased the LVESP, dp/dt_{max}, and dp/dt_{min} at the highest concentration used ($p<0.05$). DPDPE decreased the same variables at concentrations higher than 10^{-8}M ($p<0.05$). Only U50488H exerted negative chronotropic effects at 10^{-5}M ($p<0.05$), while heart rate was unchanged with DAMGO and DPDPE.

The maximum decrease of HR, LVESP, dp/dt_{max} and dp/dt_{min} obtained with DAMGO in the presence of ACh was 2.2±1.6%, 20.4±4.4%, 18.8±4.6%, and 21.3±5.5%, respectively. Only HR did not demonstrate significant differences ($p> 0.05$, vs ACh $5x10^{-7}$M).The maximum decrease of HR, LVESP, dp/dt_{max} and dp/dt_{min} obtained with U50488H in the presence of ACh was 22.3±6.6%, 18.8±3.8%, 31.8±3%, and 24.2±3.6%, respectively, which achieved significant difference to those obtained with Ach alone.

The maximum decrease of HR, LVESP, dp/dt_{max} and dp/dt_{min} obtained with DPDPE in the presence of ACh was 4.1±5.0%, 32.6±4.9%, 30.87±6.3%, and 39.2±7.1% respectively. Except for HR, the other three measurements were significantly lower than during ACh alone ($p<0.01$).

Naloxone inhibited the effects of DAMGO (10^{-7}M) and DPDPE (10^{-6}M) on ACh transmission (table 9, figure9). However, the modulation of ACh effects on the heart rate by U50488H (10^{-5}M, $p<0.05$) was not blocked by naloxone.

Table 8. Cardiovascular response to opioid receptor agonists in the presence of acetylcholine

	Baseline	**ACh** (5×10^{-7}M)	**DAMGO**(n=6)			
			10^{-10}M	10^{-9}M	10^{-8}M	10^{-7}M
HR (sec^{-1})	307±13.1	285±13.4	289±12.2	284±10.6	284±10.9	279±12.4
LVESP (mmHg)	106±7.2	93.5±5.1	88.6±6.2	87.3±7.6	80.2±6.6*	74.8±7.4*
dp/dt_{max} ($mmHg.sec^{-1}$)	3008±312	2561±197	2478±232	2429±242	2240±210	2089±235*
dp/dt_{min} ($mmHg.sec^{-1}$)	-2113±200	-1788±147	-1739±169	-1726±192	-1572±175	-1422±204*
	Baseline	**ACh** (5×10^{-7}M)	**DPDPE** (n=5)			
			10^{-9}M	10^{-8}M	10^{-7}M	10^{-6}M
HR (sec^{-1})	271±25	222±32.6	217±33.1	217±31.2	211±34.7	212±35.2
LVESP (mmHg)	91.2±3.5	83.8±5.3	73.8±2.8	71.1±2.8*	65.0±3.9*	57±5.2*
dp/dt_{max} ($mmHg.sec^{-1}$)	2385±161	2049±252	1794±183	1753±184*	1627±232*	1430±253*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1649±105	-1387±188	-1153±103	-1138±110	-1027±137*	-844±154*
	Baseline	**ACh** (5×10^{-7}M)	**U50488H** (n=6)			
			10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
HR (sec^{-1})	228±19.3	203±28.5	197±30.2	195±30.4	191±27.9	162±31.3*
LVESP (mmHg)	100±5.9	101±2.7	102±4.4	104±4.8	101±5.0	82.2±4.8*
dp/dt_{max} ($mmHg.sec^{-1}$)	2612±108	2655±185	2592±194	2657±252	2565±224	1795±76.2*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1769±33.4	-1739±81	-1694±86.5	-1724±87.1	-1693±84.3	-1309±24*

ACh = acetylcholine, HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt_{min} = negative maximum instantaneous pressure changes. Data are mean±SEM.* $p<0.05$ *vs* corresponding ACh baseline value.

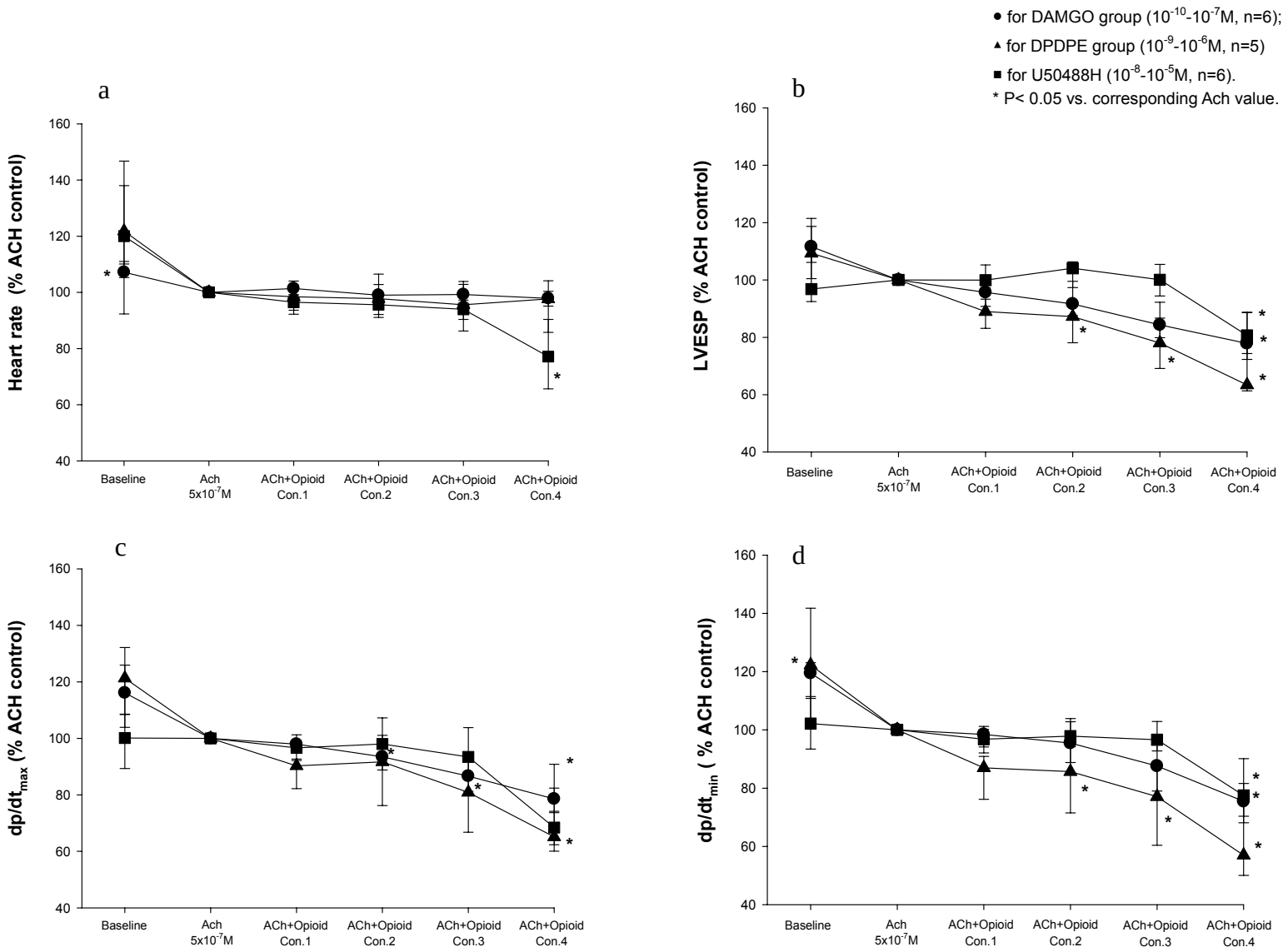

Fig.8. Dose-related effects of three opioid agonists on heart performances in presence of acetylcholine (ACh). (a) show percentage changes, from ACh treatment values, in heart rate,(b) left ventricular end systolic pressure (LVESP), (c) positive maximum instantaneous pressure changes (dp/dt_{max}) and (d) negative maximum instantaneous pressure changes (dp/dt_{min}). Values are expressed as mean±SEM.

Table 9 . Cardiovascular response to opioid agonists in the presence of naloxone and acetylcholine

	DAMGO group (n=4)			
	Baseline	ACh (5×10^{-8}M)	ACh+NAL(10^{-5}M)	ACh +NAL+DAMGO(10^{-7}M)
HR (sec^{-1})	267±10.5	216±19.3	216±18.7	214±20
LVESP (mmHg)	94±4.5	71.3±7.6	68.4±5.4	66.7±5.3
dp/dt_{max} ($mmHg.sec^{-1}$)	2364±143	1719±202	1673±160	1649±159
dp/dt_{min} ($mmHg.sec^{-1}$)	-1756±130	-1151±136	-1110±121	-1065±126
	DPDPE group (n=4)			
	Baseline	ACh (5×10^{-8}M)	ACh +NAL(10^{-5}M)	ACh +NAL+DPDPE(10^{-6}M)
HR (sec^{-1})	320±8.3	263±26.9	250±28.7	242±29
LVESP (mmHg)	88.2±3.3	76.0±8.5	73.8±9.2	71.9±9.4
dp/dt_{max} ($mmHg.sec^{-1}$)	2563±262	2055±354	2015±357	1960±366
dp/dt_{min} ($mmHg.sec^{-1}$)	-1657±101	-1305±212	-1274±232	-1227±245
	U50488H group (n=5)			
	Baseline	ACh (5×10^{-8}M)	ACh +NAL(10^{-5}M)	ACh +NAL+U50488H(10^{-5}M)
HR (sec^{-1})	289±11.6	262±12.4	257±12.7	223±6.8*
LVESP (mmHg)	93.1±1.9	83.1±5.7	80.2±4.9	85.0±4.7
dp/dt_{max} ($mmHg.sec^{-1}$)	2614±152	2301±175	2278±145	2102±126
dp/dt_{min} ($mmHg.sec^{-1}$)	-1867±82	-1512±165	-1511±127	-1597±120

ACh = acetylcholine, HR = heart rate, LVESP = left ventricular end systolic pressure, dp/dt_{max} = positive maximum instantaneous pressure changes, dp/dt_{min} = negative maximum instantaneous pressure changes, NAL = naloxone. Data are mean±SEM. * $p<0.05$ *vs* corresponding ACh+NAL value.

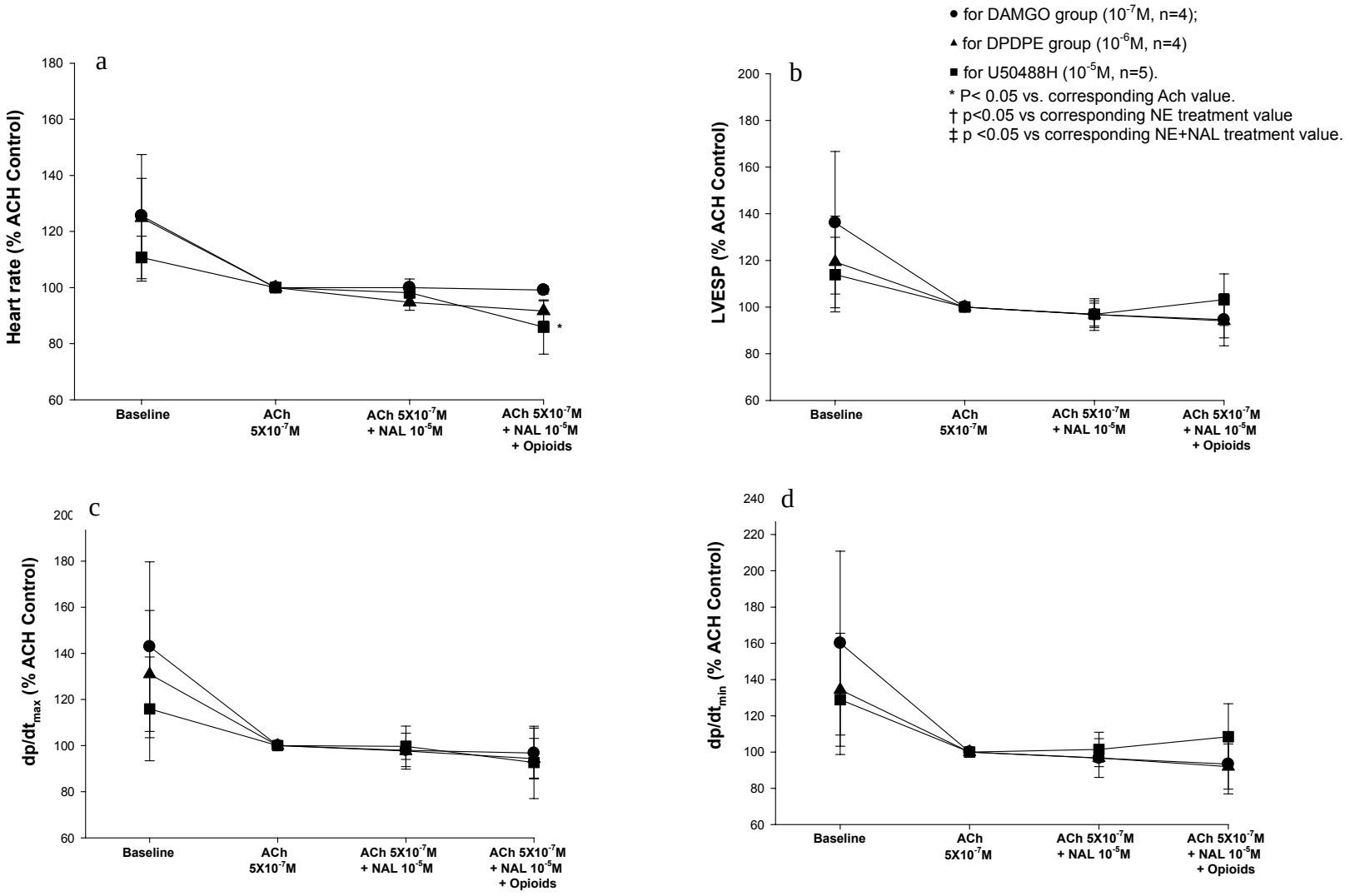

Fig.9. Cardiovascular responses of three opioid agonists in the presence of naloxone(NAL) and acetylcholine (ACh). (a) show percentage changes, from NE treatment values, in heart rate,(b) left ventricular end systolic pressure (LVESP), (c) positive maximum instantaneous pressure changes (dp/dt_{max}) and (d) negative maximum instantaneous pressure changes (dp/dt_{min}). Values are expressed as mean±SEM.

3.5 Effects of nicotine on the response to opioid peptides

In this set of experiments, three types of opioid receptor agonists, DAMGO (10^{-6}M), DPDPE (10^{-6}M), and U50488H (10^{-5}M), were used to investigate the potential interaction between opioid receptor agonists and nicotine. In addition, DAMGO and U50488H were also tested with their respective antagonists, naloxone (10^{-5}M), and nor-BNI (5×10^{-5}M), in presence of nicotine (10^{-4}M) and all results are demonstrated in Tables 10-12 and figures 10 and 11.

Administration of nicotine 10^{-4}M (n=4), an agonist at the nicotinic acetylcholine receptor of intracardiac parasympathetic ganglia, yields the following changes in cardiac hemodynamic variables (expressed as %change to baseline values): 0.02% by heart rate ($p>0.05$), 14.7%, 17.4% and 22.3% by LVESP, dp/dt_{max} and dp/dt_{min}, respectively (all $p<0.05$).

Addition of nicotine (10^{-4}M) also partly reversed the depressive effect of corresponding opiate agonists in DAMGO and DPDPE hearts, as shown in LVESP, dp/dt_{max} and dp/dt_{min} measurements ($p<0.05$), whereas no significant effect of nicotine was seen in the U50488H group ($p>0.05$).

In the DAMGO group, nicotine still exerted significant positive cardiac effects in combination of DAMGO with naloxone ($p<0.05$, fig 26). Paradoxically, κ-opioid antagonist, nor-NBI (5×10^{-5}M) did not block the inhibitory effect of U50488H, and markedly enhanced the depressive effect of U50488H on heart rate ($p<0.05$).

In order to exclude a potential interaction between the antagonists and nicotine, additional experiments were conducted showing that nicotine plus either naloxone or nor-BNI did not significantly alter haemodynamic measurements (data not shown).

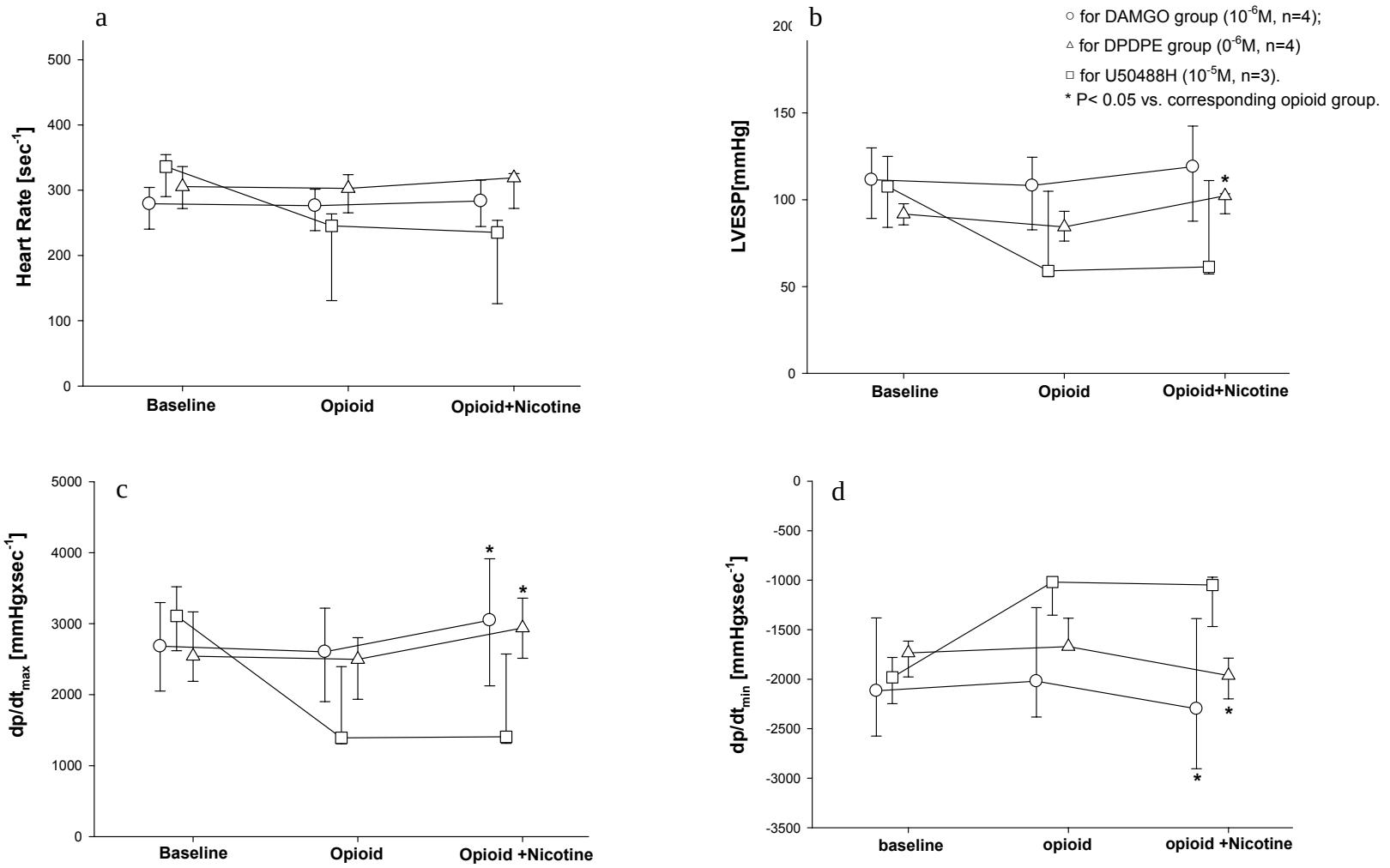

Fig. 10. Consecutive haemodynamic responses to nicotine (10^{-4}M) in the presence of different opioids

LVESP= left ventricular end systolic pressure, dp/dt_{max} = maximum rate of change of left ventricular pressure, dp/dt_{min} = minimum rate of change of left ventricular pressure. (a) shows changes, from pre-drug values, in heart rate and (b) left ventricular end systolic pressure (LVESP), (c) positive maximum instantaneous pressure changes (dp/dt_{max}) and (d) negative maximum instantaneous pressure changes (dp/dt_{min}). Data are expressed as mean±SEM.

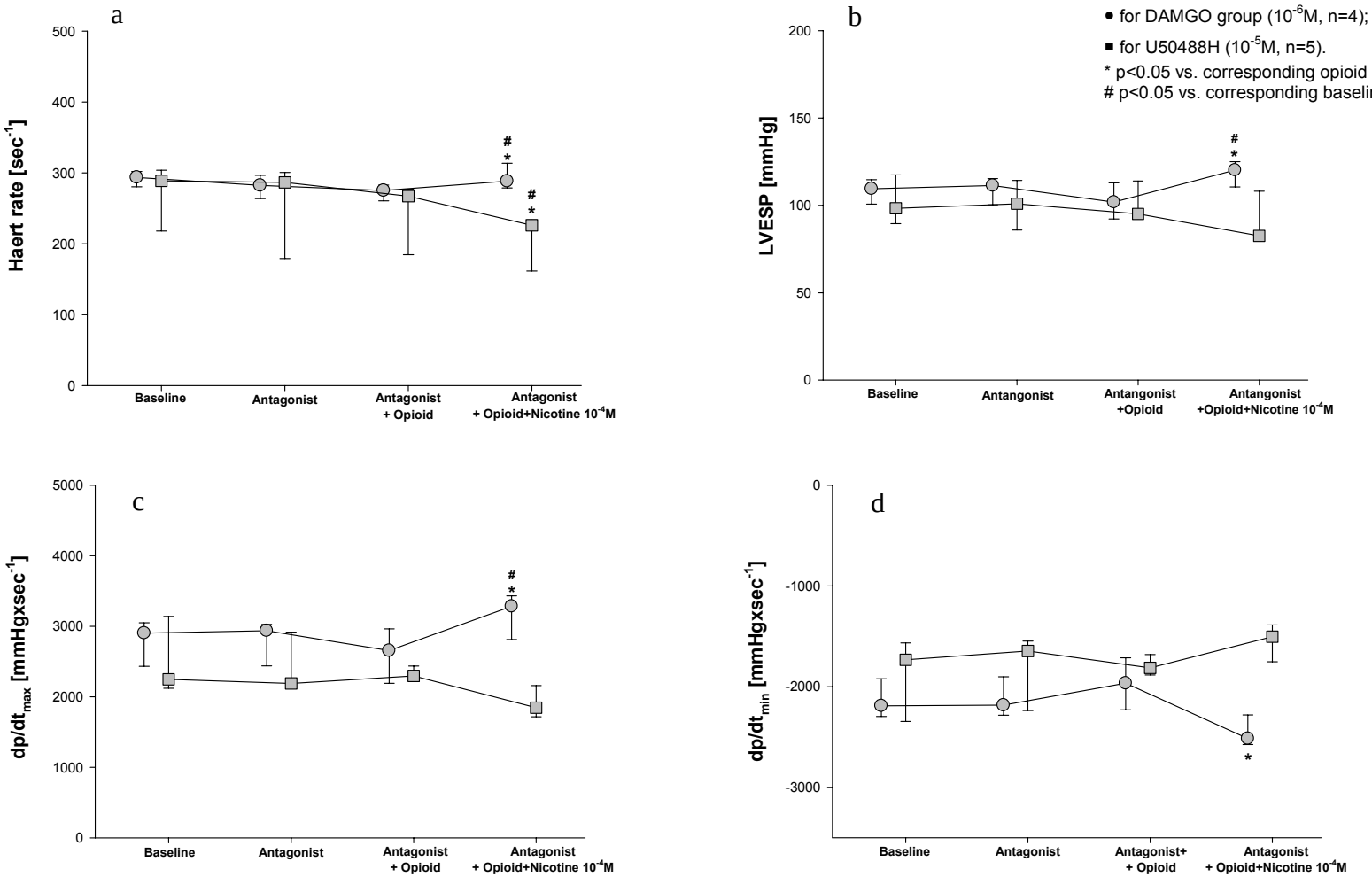

Fig. 11. Consecutive haemodynamic responses to nicotine in the presence of different opioids and antagonists

LVESP= left ventricular end systolic pressure, dp/dt_{max} = maximum rate of change of left ventricular pressure, dp/dt_{min} = minimum rate of change of left ventricular pressure. Antagonist: naloxone (10^{-5}M) to DAMGO (10^{-6}M), Nor-BNI (5×10^{-5}M) to U50488H (10^{-5}M). (a) shows changes, from pre-drug values, in heart rate and (b) left ventricular end systolic pressure (LVESP), (c) positive maximum instantaneous pressure changes (dp/dt_{max}) and (d) negative maximum instantaneous pressure changes (dp/dt_{min}). Data are mean±SEM.

Table 10 . Nicotine effects on the isolated heart (n=4)

	Baseline	Nicotine(10^{-4}M)
HR (sec^{-1})	331±12.6	332±13.6
LVESP (mmHg)	93.0±2.7	106±1.8*
dp/dt_{max} ($mmHg.sec^{-1}$)	2751±244	3213±248*
dp/dt_{min} ($mmHg.sec^{-1}$)	-1859±97	-2266±73*

* P< 0.05 Vs. baseline value, determined by paired t-test. Data are mean±SEM.

Table 11 . Nicotine effects on the isolated heart in the present of three opiate agonists.

		DAMGO group (n=4)	
	Baseline	DAMGO(10^{-6}M)	DAMGO(10^{-6}M)+Nicotine(10^{-4}M)
HR (sec^{-1})	275±16.9	272±17.1	281±18.4
LVESP (mmHg)	110±10.5	105±11.0	116±14.5
dp/dt_{max} ($mmHg.sec^{-1}$)	2677±322	2575±341	3029±463#
dp/dt_{min} ($mmHg.sec^{-1}$)	-2024±637	-1893±590	-2197±795#
		DPDPE group (n=4)	
	Baseline	DPDPE(10^{-6}M)	DPDPE(10^{-6}M) +Nicotine(10^{-4}M)
HR (sec^{-1})	305±16.8	297±15.7	306±16.1
LVESP (mmHg)	91.7±3.4	84.6±4.6	99.2±3.6#
dp/dt_{max} ($mmHg.sec^{-1}$)	2632±260	2412±235	2937±220*#
dp/dt_{min} ($mmHg.sec^{-1}$)	-1776±101	-1580±96	-1983±111#
		U50488H group (n=3)	
	Baseline	U50488H(10^{-5}M)	U50488H(10^{-5}M)+Nicotine(10^{-4}M)
HR (sec^{-1})	327±19.1	213±41.6	205±39.8
LVESP (mmHg)	106±11.9	73.2±15.9*	76.5±17.3
dp/dt_{max} ($mmHg.sec^{-1}$)	3083±260	1698±349*	1765±405*
dp/dt_{min} ($mmHg.sec^{-1}$)	-2003±135	-1122±116*	-1162±155*

Data are expressed as mean±SEM. * P< 0.05 vs.corresponding baseline value, # P< 0.05 vs.corresponding opioid treatment value.

Table 12 . Consecutive haemodynamic responses to nicotine in the presence of opiate agonists and corresponding antagonists

	DAMGO group (n=4)			
	Baseline	Naloxone(10^{-5}M)	Naloxone(10^{-5}M) +DAMGO(10^{-6}M)	Naloxone(10^{-5}M) +DAMGO(10^{-6}M) +nicotine(10^{-4}M)
HR (sec^{-1})	292±5.7	281±8.6	273±5.9*	294±9.5†
LVESP (mmHg)	108±3.8	109±4.1	102±5.4	119±4.0$^{*\#\dagger}$
dp/dt_{max} ($mmHg.sec^{-1}$)	2796±176	2801±172	2603±202	3175±172$^{*\#\dagger}$
dp/dt_{min} ($mmHg.sec^{-1}$)	-2136±104	-2123±104	-1969±134	-2456±83$^{*\#\dagger}$
	U50488H group (n=5)			
	Baseline	Nor-BNI(5×10^{-5}M)	Nor-BNI(5×10^{-5}M) +U50488H(10^{-5}M)	Nor-BNI(5×10^{-5}M) +U50488H(10^{-5}M) +nicotine(10^{-4}M)
HR (sec^{-1})	267±23.3	249±37.6	238±30.4	203±26.3$^{*\#\dagger}$
LVESP (mmHg)	103±7.4	100±7.4	102±6.6	91.8±7.9
dp/dt_{max} ($mmHg.sec^{-1}$)	2554±277	2456±215	2331±53.5	1921±113
dp/dt_{min} ($mmHg.sec^{-1}$)	-1910±212	-1842±191	-1788±45.9	-1556±84.3

Data are expressed as mean±SEM. * P< 0.05 vs.corresponding baseline value, $^{\#}$ P< 0.05 vs.corresponding naloxone treatment value,† p< 0.05 vs. corresponding naloxone+opioid treatment value.

4. DISCUSSION

This study analysed the differential effects of selective opioid receptor agonists and antagonists on cardiac haemodynamic performance in a Langendorff isolated heart perfusion system. The results can be summarised as follows: (1) Increasing doses of DAMGO (μ-agonist) and DPDPE (δ-agonists) showed no or only small dose-dependent depressive effects on functional variables of the isolated heart, whereas U50488H (κ-agonist) at high concentration significantly decreased those variables. (2) In presence of either NE or ACh all opiate peptides showed depressive effects on most of the cardiac variables. (3) Using the unspecific antagonist naloxone these inhibitory agonistic effects could be antagonised in DAMGO and DPDPE groups but not in the U50488H group. (4) Addition of nicotine to the perfusate led to a short but significant increase in all cardiac variables except heart rate. (5) This effect was not influenced by pretreatment with DAMGO (μ-agonist) or DPDPE (δ-agonists) but it was markedly blunted by pretreatment with U50488H (κ-agonist). (6) Addition of the specific κ-antagonist nor-BNI blocked the depressive effects of U50488H and additionally evoked inverse nicotine effects.

4.1 Langendorff model

Opioid peptides and endogenous opioid receptors involved in the regulation of the cardiovascular system have long been investigated and the central neurological signalling pathway of opioid peptides has been well characterised.[15] Besides a central action, with the development of high selective opioid ligands, it was demonstrated recently that stimulation of peripheral opioid receptors can also induce profound functional changes in the cardiovascular system, including alteration of vasopressor responses, cardiac electrophysiological function, as well as the heart's responsiveness to sympathetic and parasympathetic stimulation.[15;16] However, studies have shown conflicting results about the effects of opioid peptides on cardiovascular responses, depending on the animal species, type of anaesthesia, route of administration, and site and dosages of the various opioid agonists. In fact, assessment of opioid myocardial effects *in vivo* may be biased by influencing factors such as general anaesthesia, surgical stress, and a pathologic state. Thus, experimental studies using the Langendorff isolated heart perfusion system, which was first described in 1897, are an appropriate way to precisely assess the myocardial effects of opioids independently of influencing systemic factors. Due to its

simplicity, broad spectrum of variables that can be measured, high reproducibility, and relatively low cost, the Langendorff model has been used worldwide as the gold standard for evaluation of drug action and interaction on the heart.[54] The fact that the isolated heart is a denervated organ is important for this study, because it allows the differentiation between sympathetic and vagal cardiac stimulation. In our experiments, endogenous transmitters such as norepinephrine, acetylcholine, and nicotine were exogenously added to the perfusate in a controlled manner. Another reason to use the Langendorff setup for our experiments was that it allows investigation of the distribution and function of cardiac opioid receptor subtypes. The denervated organ in the Langendorff setup was supposed to help in localising differential opioid receptor action, because the anatomical target is narrowed down to the second parasympathetic neuron and the cardiac sarcolemma. This is important because opioid action is so far assumed to be presynaptic with a localisation on the distal 1st neuron of the vegetative nervous system. Interaction with cholinergic and adrenergic receptors in our setup was therefore restricted to 3 locations: postsynaptic on the proximal 2nd neuron, postsynaptic on the distal end of the 2nd neuron and postsynaptic in the sarcolemma. Furthermore, the setup may allow differentiation between atrial and ventricular effects. For instance, it has been demonstrated that κ- and δ-opioid receptors were mainly located on the ventricular cardiac sarcolemma in rats.[28] It was also shown that only μ-agonists inhibited neurally stimulated contractile response in the guinea pig atrium, but κ- and δ-receptor agonists were effective in this regard in the ventricle,[55;56] implicating heterogeneity of opioid receptor distribution. Therefore, the Langendorff isolated heart preparation provides a valuable intact organ environment to investigate or differentiate the direct actions of opioid peptides on various cardiac variables.

4.2 Depressive effects of opioid agonists

In the first part of the study, results from dose-response experiments demonstrated that: (1) the specific μ-agonist DAMGO, even at the highest concentration of 10^{-7}M, did not produce a significant inhibitory effect on the isolated heart. (2) the specific δ-agonist DPDPE at 10^{-6}M produced a small but significant depressive inotropic effect on rat heart, demonstrated by the changes in dp/dt_{max} and $dp/dt_{min,}$ (3) the specific κ-agonist U50488H at 10^{-5}M caused a pronounced negative chronotropic and inotropic effect, manifested by the significant decrease in heart rate and $dp/dt_{max.}$

Although it was speculated in the past that all subtype selective opiate receptor agonists have effects on the heart and most effects are related to the respective endogenous receptors,[57] no consensus has been established regarding the exact role that opioids play in the regulation of the cardiovascular system.[16;32] In our experiments, using spontaneously beating isolated hearts, we demonstrated that stimulation of δ- and κ-receptor agonists yields depressive responses when directly added into the perfusate, whereas stimulation of μ-receptors did not show any depressive effect. In line with this, a functional study demonstrated that U50488H (10^{-7} to 2×10^{-5}M, κ-agonist), Met-enkephalin, and Leu-enkephalin (10^{-10} to 10^{-6}M, δ-agonists), but not DAMGO (10^{-5}M, μ-agonist), have a negative inotropic action on adult rat left ventricular myocytes.[3]

The different cardiovascular effects of opioid agonists might be related to the different distribution of endogenous cardiac opioid receptors. Opioid receptors and opioid binding have been demonstrated in the cardiac sarcolemma,[3;58;59] and the cardiac autonomic nervous system.[60-63] Several previous studies found only δ- and κ-receptors in adult rat atria and ventricular tissue using binding studies.[4;28;30] More recent investigations demonstrated mRNA expression of all three receptor subtypes in human or rat atrial tissue.[27;64] The absence of major changes in heart rate using DAMGO and concomitant effects on cardiac contractility indices (dp/dt_{max} and dp/dt_{min}) may suggest that DAMGO preferentially acts on the ventricles rather than the right atrium. Localisation of cardiac μ-receptors in the right atrium, however, does not support this hypothesis morphologically.[27;64] Differential effects of increasing dosage of opioids in our study, therefore, may be related to different location of receptor subtypes within these tissues. For example, primarily presynaptic ganglionic action of μ-receptors would explain the missing direct effects of DAMGO when administered alone in the perfusate of the denervated organ.

More importantly, in our experiment we also find the phenomenon that, compared with the inhibitory effects of opioid agonists alone, in the presence of norepinephrine, all the opioid agonists could exert much stronger negative chronotropic and inotropic effects even at very low concentration. Therefore, it could be easily postulated that the adrenergic pathway may be involved in the amplification of inhibitory effects of

opioids, mediated via some potential interaction mechanisms. This point will be further discussed in the section focussing on the effects of opioid agonists in the presence of norepinephrine.

Blockade of opioid action with naloxone reversed the effects of DAMGO and DPDPE seen in combination with NE and ACh (table 7 and 9). U50488H action could not be fully reversed with naloxone (table 7 and 9) but complete antagonism was established when the more specific kappa-antagonist nor-BNI was used (table 12). We believe that naloxone action was not specific enough to block U50488H action. Nevertheless, U50488H may also have acted via a direct mechanism independent of opioid receptors, which has been suggested by some authors. The opioid receptor-independent action may be related to the drug interaction with cardiac sodium and potassium channels,[32;51] a direct contractile effect,[35] or may lead to myocyte shortening mediated by opioid receptor via the pertussis toxin-sensitive Gi/o proteins pathway,[65] which may reflect concentration-dependent activation of different second messengers, rather than interaction of different types of myocardial opioid receptors. The impact of drug concentration on non-opioid action seems to be crucial. As we used U50488H at concentrations higher than DAMGO or DPDPE, discussion about a direct mechanism of U50488H is even more important. Changes in heart rate caused by the three agonists used in our experiments are very consistent with a previous report,[5] in which DAMGO (10^{-3}M) and DPDPE (10^{-3}M), even at their highest concentration, yielded only a small decrease (8% and 11%, respectively) in heart rate, whereas U50488H ($5x10^{-4}$ M) decreased the heart rate by 75%. The average decrease in inotropy with DAMGO, DPDPE and U50488H in maximum concentrations analogous to our experiments was approximately 35%, 29%, and 51%, respectively. The authors could exclude a receptor-mediated effect by using the unspecific opioid antagonist naloxone or the specific kappa-antagonist MR-2266.[5] Pugsley et al[51] demonstrated that U50488H, at a high concentration of 1-30 μM, dose-dependently decreased cardiac contractility, and prolonged the P-Q, QRS and Q-T intervals of the ECG using isolated perfused rat hearts. These depressant effects could not be reversed by the opioid receptor antagonists, naloxone or MR-2266. Moreover, Alarcón et al[52] also showed that in isolated right ventricular papillary muscles of the guinea pig, neither DAMGO nor DPDPE (both at 5×10^{-6} to 5×10^{-5}M) at a concentration very close to the concentrations used in our study could produce

significant effects on the action potential characteristics. Only U50488H (10^{-5}M) caused a concentration-dependent reduction in the maximum rate of depolarisation phase and in the duration of the action potential.

As haemodynamic effects differed between opioid receptor agonists, the question of whether equipotent dosages of these drugs were used also has to be addressed. Other studies focussing on comparisons between the different opioid receptor subtypes in the heart[5;48;66] postulated that U50488H is the most potent agonist when using three opioid receptor agonists at the same concentration. In our study, the range of concentrations used with opioid peptides was based on the results of previous studies,[3;5;15;49;52;66] which demonstrated effective doses of opioids in the range of 10^{-5}-10^{-10}M. We adopted the reported ranges for our setup in preliminary experiments, which confirmed apparent differences in potency. We, therefore, cannot exclude that higher concentrations of DAMGO and DPDPE would have achieved similar results as U50488H.

4.3 Interaction between opioid agonists and norepinephrine

In this study we demonstrated that all opioid receptor agonists significantly attenuated the positive inotropic effect of norepinephrine. Taking into account that the maximum inhibitory effects of DAMGO and DPDPE were blocked by naloxone (table 7), the present study strongly confirms the hypothesis that activation of opioid receptors attenuates the β-adrenergic receptor-mediated positive inotropic and chronotropic effects. Accumulating evidence indicates that these inhibitory effects can be attributed to interaction between opioid receptors and the β-adrenergic receptor. Previous *in vivo* and *in vitro* investigations introduced “cross-talk” between κ- or δ-receptors and β-adrenergic receptor signal transduction pathways.[1;2;37] These investigations demonstrated that pretreatment with naloxone or specific κ-receptor antagonist, norbinaltorphimine (nor-BNI) or preincubation with pertussis toxin, completely blocked the action of opioid agonists.[1;2;36;37] The authors suggested that this “cross-talk” results from inhibition of the G_s-protein and adenylate cyclase of the ß-adrenergic receptor pathway by the pertussis toxin-sensitive G-protein of the opioid pathway.[33;34;67] Based on their findings, the authors further affirmed that the inhibitory effects of opioid receptor stimulation may result from negative modulatory action on β-adrenergic receptor stimulation. Our results of DAMGO and DPDPE were perfectly

in consistent with those findings. Some may argue that the effects may be independent of β-adrenergic receptor action and could be mediated via opioid receptors on the 2nd parasympathetic neuron or in the sarcolemma of the heart muscle cell, because the denervated Langendorff heart should not have any adrenergic supply as the sympathetic 2nd neuron is not intact. Even intact sympathetic neurons (so called SIF-cells), which have been described in atrial ganglia[64;68] should not be able to continuously release adrenergic transmitters in the Langendorff model. Concerning this issue, it should be emphasized that continued ß adrenergic stimulation could be guaranteed in our experiments by uninterrupted norepinephrine perfusion in the Langendorff setup and therefore opioids could well interfere with the adrenergic signalling pathway. Then our results of opioids in the presence of norepinephrine would clearly support this “cross talk” theory.

Furthermore, the maximum changes in haemodynamic variables after using a combination of NE and opioids were strikingly higher than with opioids alone. For instance, in our experiment, NE+DAMGO decreased LVESP by 21%, while DAMGO alone altered LVESP only by 4.1%. DPDPE+NE decreased LVESP by 32%, whereas DPDPE alone altered LVESP only by 14%. Similar trends could also be seen in the studies of Vargish[36] and Clo.[59] Vargish reported that morphine (10^{-4}M) alone maximally decreased the heart rate and cardiac output by about 13.5% and 25%, respectively, while morphine in combination with NE (10^{-9}M) decreased the heart rate by 29.6% and cardiac output by 34.5%.[36] Thus one cannot simply conclude that the depressive action of opioid alone solely depends on the interaction with the adrenergic receptor. One could argue that differences in percentage values were due to differences in the reference value, e.g., opioid alone was compared to baseline value; whereas NE+opioid was compared to the NE value. However, according to the protocol of Vargish et al., NE alone at 10^{-9}M had no haemodynamic effects on isolated perfused hearts.[36] In the study of Clo et al.,[59] the reference point for all measurements was the baseline value. NE increased the heart rate and developed tension up to 80% and 92%, and the maximal decrease in the NE+opioid group was more pronounced than with the opioid alone.[59]

For the sympathetic system, our study and other investigations[1;37] suggest that stimulation of endogenous opioid receptors modulates sympathetic actions. This may be achieved by influencing the postsynaptic intracellular signalling pathway of the ß-

adrenoceptor. Using sympathetic electrical stimulation, others have shown that opioid receptors also play a pivotal role in presynaptic inhibition.[62;69]

4.4 Interaction between opioid agonists and acetylcholine

In this study, preliminary experiments showed that perfusion of isolated rat hearts with ACh at a dose of 5×10^{-7}M results in negative inotropic and chronotropic responses. In combination with 5×10^{-7}M ACh, μ- and δ-receptor agonists induced additional negative inotropic effects, while κ-receptor agonist yielded both negative inotropic and chronotropic effects on isolated hearts. Moreover, naloxone can completely attenuate the inhibitory effect of DAMGO and DPDPE, but not of U50488H on chronotropy.

Among the molecular mechanisms suggested to be involved in the depressive cardiac effects of ACh via muscarinic acetylcholine receptors are: (1) increase in outward current, (2) slowing of the inward current[70] and clamp voltage,[71] (3) coupling to an inhibition of adenylate cyclase via pertussis toxin-sensitive G protein (G_i/G_o)[72;73] and activation of the ACh-regulated potassium channel current.[74] The haemodynamic measurements in our experiments were in line with George et al[75], who observed a negative inotropic effect and increasing cGMP levels at an ACh concentration of 7.4×10^{-8}M. In contrast to these results and our investigations, Ates et al.[76] reported positive inotropic effects and coronary vasoconstriction in Wistar rats when perfused with ACh at a concentration of 10^{-7}M, 5×10^{-7}M and 10^{-6}M.

Only few data regarding the interaction of ACh and opioid receptor agonists in the heart have been published, with most of the studies using direct nerve (sympathetic or vagal) stimulation in a spontaneously beating heart. It has been well documented that opioid receptor agonists inhibit parasympathetic or sympathetic transmission. It is assumed that δ-presynaptic opioid receptors probably exist in dog vagal nerves, while no opioid receptors could be detected in sympathetic nerves.[77] Hatano and Toda[39] showed that fentanyl and morphine attenuate the negative chronotropic effect induced by stimulation of cholinergic nerves on isolated rabbit atria, and naloxone could reverse it, implicating co-existence of cholinergic- and μ-receptors in the sinoatrial node. Weitzell et al.[78] also demonstrated that vagal transmission to the rabbit heart could be inhibited by activation of both μ- and δ-receptors. It is worthwhile emphasizing that nerve stimulation is very helpful in demonstrating ganglionic effects of opioids, i.e. on presynaptic sites of the first neuron (e.g. effects

on the release of ACh from peripheral nerve terminals). In our experiments with denervated hearts, the decrease in heart rate elicited by exogenous ACh was not reversed by opioid agonists. This may be because presynaptic effects of opioids were excluded in our setup with denervated hearts. All opioid agonists, however, exaggerated the depressive effects of ACh on left ventricular developed pressure , as well as on velocity of contraction (dp/dt_{max}) and relaxation (dp/dt_{min}). Here, a major difference between an intact and our denervated model is evident. Our data, however, do not allow to differentiate, whether the influence of opioid agonists on ACh-mediated effects is a combined but unrelated response or an interaction between opioid agonists and ACh receptors.

Chittal et al[47] reported in 1969 that morphine blocked the responses of the isolated rabbit atria to acetylcholine, as well as the stimulant and inhibitory responses to nicotine in normal atria and in atria obtained from reserpinised animals. The author further postulated that morphine may act on the ACh receptors in the atrial tissue directly. However, in this model, the authors also found that morphine completely blocked the action of nicotine but did not influence the positive inotropic and chronotropic effects of adrenaline, which is in obvious contrast to our findings. Therefore it is doubtful whether above conclusions based on experiments on rabbits can be applied to the rat heart used in the present study.

The endogenous opioid peptides have profound effects on the action of autonomic neurotransmitters possibly at both pre- and postsynaptic sites: Recently, Deo and colleagues[79] demonstrated by means of immunohistochemistry that δ-opioid receptors are located presynaptically on the parasympathetic nerve terminals innervating canine atria and the sinoatrial node. Analogous results were obtained by the same group using vagus stimulation.[41] Other authors demonstrated that morphine reduces the atrial response to stimulation of postganglionic cholinergic neurones, suggesting a postganglionic location of μ-opioid receptors.[80] The location of the acetylcholine release-inhibiting κ-receptors is not known. As we used denervated hearts in our study, we can exclude effects on the first neuron. However, the exact functional site of ACh and opioid agonists on the second neuron or sarcolemma remains unclear.

4.5 Effects of opioids on the response to nicotine

Nicotinic acetylcholine receptors (nAChR) play a very important role in the regulation

of the peripheral autonomic nerve system and it was well established that nicotine stimulatory action on sinus node automaticity is caused by the release of catecholamine from adrenergic nerve terminals.[42;43;81] We, therefore, decided to detect, whether opioids can attenuate or enhance the cardiovascular response to nicotine in a consecutive experiment. In addition, we found in the preliminary studies that infusion of nicotine (10^{-4}M) induced a brief cardiovascular response within 1-2 minutes (figure 12). We, therefore, decided to record haemodynamic parameters continuously after nicotine application and to determine the maximum change evoked by nicotine.

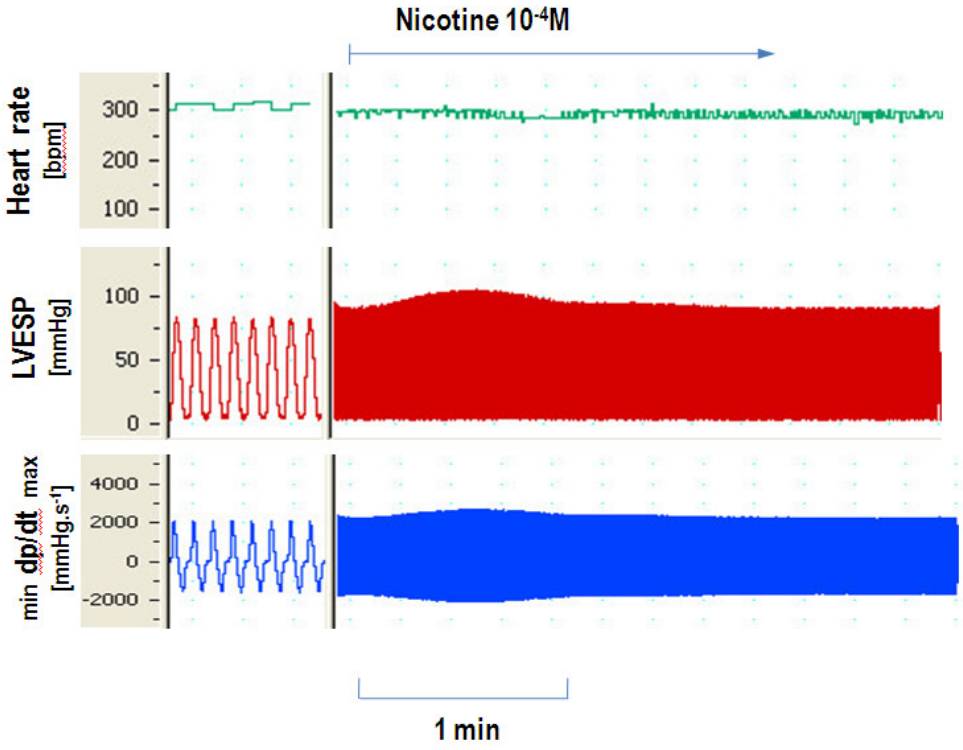

Figure 12. Acute effects of nicotine on the isolated perfused rat heart. Record from top to bottom: spontaneous heart rate, left ventricle pressure (LVESP), rate of the left ventricular pressure changes (dp/dt_{max} and dp/dt_{min})

Although the functional roles of nicotine and related nicotinic acetylcholine receptors (nAChRs) in autonomic regulation have been extensively investigated, the possible interaction between opioids and nicotine in the peripheral cardiovascular system is less clear, and only a few studies have so far investigated this subject. Most of the studies investigating cardiovascular effects of nicotine use animal models other than that we used in this study,[43;82] which may explain the differences in dosage due to differences in the density of receptors among different species. Becker et al[44]

reported a biphasic haemodynamic response in guinea pig hearts after adding nicotine to the perfusate, i.e., a positive response lasting 3-5 min followed by a sustained negative response. Fuder et al.[45] reported a weak release of [^{3}H]noradrenaline from Wistar rat hearts in response to nicotine, while Westfall and Saunders failed to observe an increase in the [^{3}H]noradrenaline overflow from the Sprague-Dawley rat heart following infusion of nicotine.[46] The only study that demonstrated direct chronotropic effects of nicotine on spontaneously beating isolated rat hearts was published by Ji et al.[83] In hearts from female Sprague-Dawley rats, nicotine (10^{-4}M) induced an initial brief decrease followed by a much higher increase in heart rate, which happened at 2 min and 5 min after adding nicotine, and lasted about 8 min. Moreover, they also demonstrated that this biphasic response was separately mediated by α7 and β4 subunits of nAChRs. Moreover, they reported that a second dose of nicotine after a 25 min washout period did not increase the heart rate. For this reason we only applied one dose of nicotine and performed opioid+nicotine and opioid+nicotine+antagonist experiments separately.

In our experiments, the lack of a significant chronotropic effect after nicotine is in disagreement with Ji's investigation,[83] but in parts consistent with the results of others.[45;46] Although we used the same mode of anaesthesia and the same concentration of nicotine as Ji et al. did, we did not observe any negative chronotropic effect and the duration of the peak response in our experiments was significantly shorter as compared to the results of Ji et al. The involvement of different neurotransmitters or receptors as a cause for the differences between study results has to be discussed. One author has reported a very brief negative chronotropic effect lasting only 60 seconds in rat sinoatrial preparations after administration of nicotine, and discussed an involvement of muscarinic receptors.[84] Another possibility could be a decreased density of receptors and/or less affinity of nicotine to receptors in the rat heart, which would make higher concentrations of nicotine necessary compared to other species.[44;84] Nevertheless, this suggests that the rat model is not as sensitive as the rabbit or guinea pig models.

Regarding the effect of DAMGO on the response to nicotine, there is no significant difference in haemodynamic measurements with increasing concentrations of DAMGO, indicating that the μ-agonist DAMGO does not influence the weak sympathoexcitatory effects of nicotine. However, Chittal et al.[47] reported that morphine blocked the stimulatory and inhibitory responses triggered by nicotine, as

well as the negative chronotropic and negative inotropic effects of ACh in rabbit atria. The authors suggested[47] that morphine may act as an agonist at the cholino-receptors, and therefore block the action of acetylcholine, whereas nicotine acts on the parasympathetic ganglia and produces cholinergic effects, which in turn releases catecholamines.[47] This discrepancy may be caused by the difference of species, which plays a pivotal role in the cardiovascular autonomic modulation.[85;86]

It is noteworthy that all three opioid receptor agonists induced similar effects on the heart at the same concentration (10^{-6} M), therefore it can be reasonably speculated that all opioids act via the same or similar mechanism in inhibiting cardiac haemodynamics in the presence of nicotine. As far as we know, no scientific work on the combined effect of κ-opioid agonist and/or antagonist with nicotine in the isolated heart or in similar preparations has been published. Our experiments involving U50488H and nicotine showed interesting results: U50488H counteracted the small sympathoexcitatory response of nicotine when the concentration of U50488H was increased from 10^{-6}M to 10^{-5}M. By adding a specific κ-opioid receptor antagonist, nor-BNI, this effect was not abolished, but significantly enhanced. Moreover, the changes in haemodynamic parameters were markedly different to nicotine or U50488H without antagonist. There are three possible explanations for this unexpected phenomenon: (1) nor-BNI has a direct non-opioid heart depressive function, especially in presence of nicotine; (2) an unknown potential mechanism promotes nor-BNI, or even a locally produced metabolic derivative of nor-BNI, depressed heart performances when it was used in combination with U50488H and/or nicotine. The former possibility is ruled out by a consecutive experiment in 2 rats in which nor-BNI alone failed to produce any effects on isolated hearts in presence of nicotine. Therefore, a non-opioid effect of these compounds was strongly suspected. Studies using different cardiac muscle preparations have suggested that U50488H and related kappa agonists, MR 1452 or Win44441-3 have sodium or potassium channel blocking actions.[6;53] Such opioid receptor-independent cardiovascular effects usually occur with the application of higher concentrations of opioid drugs, e.g., at micro molar level as we used in our experiments. In addition to the possible blockade of sodium and potassium channels, kappa agonists may have other membrane effects which result in reduced calcium fluxes and release of potassium.[32] Moreover, lack of involvement of the κ-agonist receptor in attenuating effects of U50488H was possibly due to the length of drug exposure to the κ- antagonist, nor-BNI, because it has been suggested that nor-BNI

exhibits slow binding kinetics to the agonist,[87;88] thus the 10 minute incubation time used in our experiment may have been too short to allow thorough binding. So, many reasons can account for the unexpected negative chronotropic and inotropic responses after co-administration of high dose κ-opioid agonist and antagonist with nicotine. Taken together, the results of the experiments on the potential interaction between opioids and nicotine do not provide adequate evidence that endogenous opiate peptides or opioid receptors are involved in the modulation of the sympathoexcitatory responses to nicotine.

4.6 Limitations of the study

The advantages and limitations of the Langendorff model have been discussed previoulsly[54;89] and caution must be exercised when interpreting the effects of pharmacological interventions on isolated perfused rat heart. Firstly, in many studies, including our experiments, quite often concentrations of pharmacological agents are used that are much higher than the upper limit of what an intact animal would tolerate. Thus, one must be careful when extrapolating these results to different experimental circumstances or other species. Secondly, in our experiments, different dosage series for each opioid were selected after the appropriate initial experiments. This selection can offer relative broad information regarding the inhibitory effects of opioids in advance but it also complicates a comparison of the effects of identical concentrations.

Moreover, with respect to the non-opioid receptor effect of U50488H, our results and other studies have suggested that this action is intimately connected with the concentration of the opiate peptide and tends to be more pronounced at concentrations at micromolar levels.[32] The detailed mechanisms of non-opioid mediated effects and whether these may also exist at lower doses could not be clarified in our experiment. Finally, relatively short perfusion periods for the κ-opioid antagonist nor-BNI may have influenced the reported results. Nevertheless, with limited information with respect to the potential interactions of specific opioid receptor subtype agonists and nicotinic receptors within the heart, it is important to continue to strive for better understanding and delineation of the co-activation of multiple opioid receptor subtypes and their roles in modulation of cardiovascular responses.

5. CONCLUSION

This study investigated differential effects of opioid receptor agonists in denervated rat hearts using a Langendorff heart setup. The results demonstrate that opioid receptor agonists have depressive effects when applied alone (only δ- and κ-agonist) or in combination with transmitters of the autonomous nervous system. Effects of μ- and δ-agonists were transmitted via opioid receptors. For κ-opioid receptor agonists this cannot be definitely stated. These results in a denervated heart demonstrate that apart from a previously described preganglionic localisation and action, opioid receptors must also be located and act either on the postganglionic neurons within the heart or the sarcolemma of the heart muscle. Further studies are needed to specify the exact localisation and function of these opioid receptors in the heart in more detail.

6. SUMMARY

Direct functional effects of opioid agonists on the isolated perfused rat heart.

By Wei Huang

Objective: In addition to their anaesthetic and analgesic actions, opioids also play an important role as endogenous neurotransmitters by modulating organ function. Despite these findings, the direct local effects of opioids on the cardiovascular system have not been fully understood yet. The present study was designed to test the hypothesis that the cardiac endogenous opioid system, including three opioid agonists, μ-, κ- and δ-agonist and their receptors, participate in the regulation of sympathetically- and parasympathetically-mediated cardiovascular responses, and in particular the nicotine-mediated vegetative neurotransmission.

Materials and Methods: A Langendorff heart perfusion model was established using adult male Sprague-Dawley rats. Then the following protocols were carried out: 1. Investigate the basic cardiovascular responses of three opioid-agonist subtypes: μ-, κ- and δ-agonist (referring to DAMGO, U50488H and DPDPE, specifically). 2. Investigate cardiac responses to the three opioid agonists in presence of norepinephrine (NE), with or without an antagonist (naloxone). 3. Investigate cardiac responses of the three opioid agonists in presence of acetylcholine (ACh), with or without an antagonist (naloxone). 4. Investigate cardiac responses of the three opioid agonists in presence of nicotine; for DAMGO and U50488H, experiments were also performed with the antagonists naloxone or norbinaltorphimine (nor-BNI). The haemodynamic indices, including heart rate (HR), left ventricular end-systolic pressure (LVESP) and end-diastolic pressure (LVEDP), positive and negative maximum instantaneous pressure changes (dP/dt max, dP/dt min) were continuously monitored and digitally recorded.

Results: Different opioid agonists demonstrated similar cardiac dose-response trends. However, at each highest concentration, DAMGO(10^{-7}M) did not yield a significant decrease in all the haemodynamics indices, U50488H(10^{-5}M) significantly decreased HR (14.6±4%) and dp/dt_{max} (29.3±6.5%), while DPDPE (10^{-6}M) significantly decreased dp/dt_{max} (13±3.6%) and dp/dt_{min} (17±5.2%). Compared with NE ($5x10^{-8}$M) alone, except HR in DAMGO group, the maximum decreases of all parameters in the three opioid agonist groups in the presence of NE reached statistical significance ($p<0.05$). Also, addition of naloxone blocked the cardiac effects

of DAMGO and DPDPE in the presence of NE. In contrast, effects of U50488H were only partially antagonized: HR and dp/dt_{max} still decreased significantly in the presence of naloxone ($p<0.05$). In groups pretreated with ACh, the opioid receptor agonists DAMGO, U50488H and DPDPE also demonstrated similar negative inotropic effects in a dose-related fashion. DAMGO and U50588H significantly decreased the LVESP, dp/dt_{max}, and dp/dt_{min} at the highest concentration ($p<0.05$). DPDPE decreased the same variables at concentrations higher than 10^{-8}M ($p<0.05$). Only U50488H exerted negative chronotropic effects at 10^{-5}M ($p<0.05$), while the heart rate was unchanged with DAMGO and DPDPE. Moreover, naloxone inhibited the effects of DAMGO (10^{-7}M) and DPDPE (10^{-6}M) in the presence of ACh. However, the modulation of ACh effects on the heart rate by U50488H was not blocked by naloxone. In the last protocol, administration of nicotine 10^{-4}M (n=4), an agonist at the nicotinic acetylcholine receptor of intracardiac vegetative ganglia, yielded the following changes in cardiac hemodynamic variables (expressed as % change to baseline values): 0.02% of heart rate ($p>0.05$), 14.7%, 17.4% and 22.3% by LVESP, dp/dt_{max} and dp/dt_{min}, respectively (all $p<0.05$). Addition of nicotine (10^{-4}M) also partly reversed the depressive effects of corresponding opiate agonists in DAMGO(10^{-6}M) and DPDPE(10^{-6}M) hearts, as shown in LVESP, dp/dt_{max} and dp/dt_{min} measurements ($p<0.05$), whereas no significant effect of nicotine was seen in the U50488H group ($p>0.05$). In the DAMGO group, nicotine still exerted significant positive cardiac effects in combination of DAMGO with naloxone ($p<0.05$). Paradoxically, the κ-opioid antagonist, nor-NBI (5×10^{-5}M) did not block the inhibitory effect of U50488H, but markedly enhanced the depressive effect of U50488H on heart rate ($p<0.05$).

Conclusion: Our results demonstrate that, in term of cardiac inhibitory effects, opioid receptor agonists are associated with the depression of cardiac function when applied alone (only δ- and κ-agonist) or in combination with transmitters of the autonomous nervous system. Our data also implicate that the effects of μ- and δ-agonists may be transmitted via opioid receptors. However, for κ-opioid receptor agonists, this cannot be definitely stated. These results in a denervated heart demonstrate that apart from a previously described preganglionic localisation and action, opioid receptors must also be located and act either on the postganglionic neurons within the heart or the cardiomyocytes. Further studies are required to further specify the target cells of direct opioid effects on the heart and to clarify the potential mechanisms in more detail.

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List of abbreviations

ACh	acetylcholine
AUC	area under of curve
β-AR	β-adrenergic receptor
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAMGO	D-Ala^2, N-Me-Phe^4, Gly-ol^5] - enkephalin
dp/dt max	positive maximum rate of rise of pressure in the left ventricle
dp/dt min	negative maximum rate of rise of pressure in the left ventricle
DPDPE	[D-Pen^{2,5}] enkephalin
GPCRs	G-protein–coupled receptors
HR	heart rate
IP_3	inositol triphosphate
KHB	Krebs- Henseleit buffer
Leu-ENK	leucine5-enkephalins
LVESP	Left ventricular end-systolic pressure
Met-ENK	methionine5-enkephalin
nAChRs	nicotinic acetylcholine receptor
NAL	naloxone
NE	norepinephrine
N/OFQ	nociceptin/orphanin FQ peptide
nor-BNI	Norbinaltorphimine
ORL1	opioid receptor-like
ORs	opioids receptors
POMC	pro-opiomelanocortin
ppEnk	preproenkephalin
protein kinase C	PKC
PTX	pertussis toxin
U50488H	trans-3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) - cyclohexyl]- benzeneacetamide

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of Publications

Book chapters (in Chinese):

1. As chef translator (into Chinese) : SEPSIS: NEW INSIGHTS, NEW THERAPIES. Mitchell P. Fink, Mervyn Singer. ed. Novartis Foundation, John Wiley & Sons Ltd. London. 2007.
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3. Huangwei. Analysis of prognosis factors in patients requiring long-term mechanical ventilation. 14th European respiratory society annual congress (ERS Glasgow 2004), Glasgow, Scotland. (Abstract no. 1339).

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1. Huangwei, Wan xianyao. Bundle Strategy in Sepsis : Where We Are? *Chinese Journal of Respiratory and Critical Care Medicine.* 2009,8(2):106~109 (ISSN: 1671- 6205)
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Erklärung

I hereby assure on oath that I, Huang Wei, born on April 13th, 1971, in Heilongjiang of the People's Republic of China, independently have written the present dissertation myself, namely " Direct functional effects of opioid agonists on the isolated perfused rat heart", without the illegal help of any third party. The dissertation, does not represent, even in parts, a copy of the works of others. Any sources, literary or otherwise, that I have used are stated in full.

Hiermit versichere ich, Huang Wei, geboren am 04.13.1971 in Heilongjiang in der Volksrepublik China, an Eides statt, dass ich die vorgelegte Dissertation mit dem Thema:"Direct functional effects of opioid agonists on the isolated perfused rat heart" selbst verfasst und ohne die Hilfe Dritter verfasst habe. Sie stellt auch in Teilen keine Kopie anderer Arbeiten dar und die benutzten Hilfsmittel sowie die Literatur sind vollständig angegeben.

Huang Wei

Dalian, den 21.09.2012