EPICARDIAL AUTOFLUORESCENCE NAD(P)H KINETICS IN THE ISCHEMICALLY PRECONDITIONED LANGERDORFF RAT HEART. EFFECTS OF CAPSAICIN. PART 1.

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SUMMARY

The data presented here are preceded by a review of the conventional *ex vivo* model of conditioned ischemic learning. The fluorescence kinetics of indistinct pools of reduced pyridinnucleotides, together with the conventional functional parameters observed in short, long and preconditioning preperfusion conditions, as well as in the common phase of protracted ischemia (30 min) and in the phase of reperfusion monitored over 60 min, demonstrate significant trends in the context of the more recent integrated metabolic observations of heart perfusion after capsaicin pretreatment. The present paper reports the mean kinetic values of the metabolic parameters and those from functional sampling, with emphasis on protracted ischemia following short (15 min) and long (1 h) perfusion with and without short preconditioning ischemic insults in capsaicin-treated and untreated specimens. We also describe some trends observed in single cases, where the phases of preischemic conditioning, protracted ischemia, and reperfusion demonstrated both the expected protection effect and the prevalent damage. The paper examines the significance of this experimental model in the biological and pharmacotoxicological integrated context.

1. INTRODUCTION

1.1. LITERATURE OVERVIEW

Oxygen use and short-term adaptation to its deprivation have been analyzed in a number of experimental models. Cardiovascular observations have mainly focused on ischemic/anoxic and reperfusion damage and protection. Some of the most recent studies have been conducted not only *in vivo and ex vivo* on native, supposedly intact organs and tissues perfused with blood or crystalloid buffered solutions, but also on the cellular and subcellular samples. Different phenotypes and/or molecular dynamics and kinetic behaviours have been observed with cultured (24-48 h) cardiomyocytes vs freshly isolated myocytes and isolated perfused hearts, as well as within immature vs mature isolated cells and hearts from young vs aged rodents and other non human widely used experimental preparations [1-11]. Regional or global ischemia has been obtained *in vivo, in situ* or *in vitro* by occlusion of the coronary flow or by stopping the perfusion. The perfusate was mostly Krebs-Henseleit (KH) bicarbonate buffer, reequilibrated with a 95% O_2 and 5% CO_2 mixture, previously equilibrated with a mixture of 95% N_2 and CO_2 at pH 7.40. The preparations were maintained at constant temperature during observations and measurements also under occlusion, to avoid hypothermia- induced cardioprotection.

The Langerdorff rodent heart preparation is a widely used model because the most advanced metabolic properties have been monitored associated with the evaluation of traditional functional parameters. In the isolated rat heart (r.h.), transient ischemic endogenous preconditioning (IPC) has previously been shown firstly mediated via a subfamily of protein kinase C (PKC) activation and translocation coupled to α_1-adrenoceptor and B_2 associated bradykinin receptors [12-13], or partly through endothelial function and B_1 - not B_2 - receptors [14-15]. IPC is not affected by depletion of endogenous catecholamines resulting from reserpine or 6-hydroxydopamine treatment [16]. Activation of the α-$_1$ adrenergic receptor has been shown to confer protection against the lethal injury from Ca^{2+} preconditioning (via the protein kinase C signaling pathway) [17]. Hearse and Sutherland [18] have more recently observed paradoxical exacerbation of contracture [19-22] followed by enhanced post-anoxic recovery both under ischemic and l-nor-epinephrine preconditioning (PC), and Hearse, Ferrari and Sutherland [23] observed PC, but not paradoxical contracture in blood perfused r.h. during ventricular fibrillation and/or rapid pacing.

In the transient, early energy imbalance of IPC protocols, a small population of α-G, *s* or *i* subunit proteins appears to be involved as coupled to muscarinic M_2 receptors and A_1 adenosine receptors [24-26].

Adenosine does not mediate improvement of functional recovery after PC in globally ischemic, isolated Langerdorff r.h. [27]. Increased adenosine formation through β-adrenergic receptors and noradrenaline release protects ischemic rat heart after hypoxic PC [27, 28-30]. Adenosine mediates persistent adrenergic desensitization in the r.h. via activation of iso-PKCs [31]. Although targeted deletion of the A_3 Adenosine receptor confers resistance against myocardial ischemic/reperfusion injury, A_3ARs are not required for the development of the early phase of IPC [32]. Additional references related to the open selectivity of the nucleoside receptors and transporters are mentioned below [133-136, 145, 155, 161].

Upregulation of cardiac uptake1 carrier and related loss of extra-tissutal norepinephrine increase under ischemia and thereafter - except in the 1st minute - up to more than 20 min through reperfusion [33].

Activation and translocation of iso-PKCs appear to be key events in r.h. ischemic and reperfusion damage, as well as in IPC [34-36]. In the same Langerdorff r.h. preparation, inhibition of some iso-PKC, which limits ischemic injury and eliminates the effect of IPC on stunning during reflow, is not related to PC attenuation of acidification [37-39]. In different cardiomyocytes from transgenic or normal *in* or *ex vivo* heart, not only the protein kinase C ε and δ isoforms, which

have been seen to have opposite effects [40-41], but also protein kinase A has been found to be independently associated to IPC (see also [42-47]).

RNA expression of the Na^+/H^+ exchanger isoform 1 is rapidly regulated in acutely ischemic rat myocardium [48].

Inhibition of Na^+/H^+ exchange adds to the protective effect of IPC [49-51], but the same $[pH]_i$ decrease attenuation does not appear to be tightly coupled to Na^+/H^+ turnover [52]. In reperfusion injury, exchange of accumulated Na^+ with Ca^{2+} is detrimental to function [53-57]. Dietary cariporide, a Na^+/H^+ exchange inhibitor, as well as treatment with an inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger, confer cardioprotection following coronary occlusion and reperfusion [58-60]. Sodium and calcium overloads [61-63] and protection by metabolic uncoupling in reperfusion [64-66] may contribute to the understanding of our present data (see also [67]).

Some observations may be related to methodological conditions: in the case of myocardial stunning, an important functional impairment parameter in the evaluation of hypoxia-ischemia and reperfusion dynamics, the isovolumic preparation, has sometimes been subjected to changes in systolic and diastolic pressure by collapse and reinflation of the left ventricular balloon in order to counteract the no-reflow phenomenon [20-21, 52, 68]. Pacing at 2 Hz at 35 °C showed delay of ischemic contracture [50] instead of exacerbation. In the heart not immersed and overdrive paced (300-

330 bpm), earlier contracture development was found only after repetitive PC [69].

Modulation of stunning by glycolysis, glyconeogenesis, glycogenolysis, and associated balance of proton production and cytosolic (coupled) export, are still debated (see [70-79] and below).

The role of endogenous NO in monophosphoryl lipid A acute cardioprotection in the working isolated perfused r.h. [80] has not been confirmed in the isolated retrogradely perfused isovolumic preparation - either under constant flow or pressure perfusion - as a mediator of early IPC [81], whereas in a feline study NO-peroxynitrite exchange has been confirmed to be cardioprotective [82-83], and in mice cardiac myocyte IPC has been found to contribute integratively by both inducible and constitutive NOSs [84-85], a topic under that is rapidly evolving: NOSs “imported” by rapidly IPC-recruited endothelial progenitor cells mediate a protective myocardial effect [86]. Although oxygen-derived free radicals are not believed to contribute to PC in the r.h. [87], they are held to play a role both in reperfusion injury [88] and in IPC [89], as they affect myocardial stunning [90-91].

Downregulation of the Na^+-creatine cotransporter has been considered an important feature of the failing myocardium [92]. The dual regulation of muscle AMP-activated protein kinase, which inhibits the creatine kinase-phosphocreatine system and is inhibited

by phosphocreatine, while creatine antagonizes this inhibition [93], may be active in the heart.

IPC has been reported to be independent of r.h. mitochondrial F_1F_0-ATPase inhibition [94], whereas other researchers consider its integrity essential, and not only in this rodent assay (i.e.: rat [95]; dog [96]). In the same perfused r.h., mitochondrial vs glycolytic phosphate and redox potential sensitive mechanisms have been shown to be involved in the protection afforded by IPC ([68]. See the Discussion section). The sulphydryl redox potential modulates sarcoplasmic reticulum Ca^{2+} release in PC [97], even though glutathione depletion has not been found to be essential to ventricular reperfusion arrhythmias [98]. The large production of oxygen radicals from ischemic mitochondria in the Langerdorff r.h. has been measured in 1991 [99], and the importance of mitochondrial/cytosolic couplings in acute short-term ischemic/reoxygenation cycles, particularly in IPC transients, indirect pyridine redox potential [100] and sarcoplasmic reticulum Ca^{2+} turnover, have recently been restated in rat heart by Dhalla and Brandes et al. [101-102], after Zucchi et al. [103], as previously observed in ischemic heart failure in the guinea pig [104]. The initial phases of ischemia are associated with a time-dependent positive imbalance in mitochondrial oxyphosphorylation reactions [105-106]. Downregulation of oxygen demand, and altered mechanisms of energy transfer have also been reported in acute hypoxia [107].

PC mechanisms activated by stretching tissues (injection of salts into the myocardium [108]), those elicited by pharmacological substances such as ethanol [109] and general anesthetics (i.e. [110]), or phosphodiesterase-5 and/or -6 inhibitors [111] occurring in endothelia [Cf.: 84, 112-113], or associated with the heat stress to cytoprotection, finally appear to develop through K_{ATP} channels [114-118], and are not related to enhanced action potential duration in a dog model [119]. Use of K channel openers, such as cromakalim and bimakalim, pinacidil and micorantil, and antagonists such as glibenclamide, glyburide and 5-hydroxydecanoate, has confirmed the role of these channels in ischemia and reperfusion phenomena [120-122]. In the r.h., cardioprotection, but not PC, is related to the special K_{IR}, the inward-rectifier potassium channel assayed by dofetilide and terikalant [123]. Nevertheless, in the Langendorff r.h. calcium PC, but not IPC, bypasses the K_{ATP} channel, a model condition that may explain why patients chronically exposed to sulphonylurea hypoglycemics remain protected [124]. Mitochondrial K_{ATP} channels [125], as proven with diazoxide [36, 126-127], have been shown to be an (i.e. one) end-point receptor/effector contributing to triggering and mediation of cardioprotective effects in r.h., not only in acute and chronic ischemia/reperfusion, but also in both early and delayed PC [128] (the same holds true in rabbit ventricular myocytes, e.g.:[129]).

As the cross-talks among K^+ specific channels, NO and interchanged reactive oxygen species, noradrenaline, mostly β [130-132], and adenosine subfamilies of receptors and transporters [31,

133-136] are studied, naloxone sensitive δ1 opioid receptors - not μ or κ, which appear to be related to delayed cardioprotection [137-143; i.e.: 144] -, and other peptidergic extended modulations, continue to be evaluated. Mitochondrial vs cytosolic phosphorylation and redox subcellular control networks are the focus of present research, both in r.h. and cardiac myocytes; the same problems are also being studied, for example, in rabbit [145] and chick cardiomyocytes [146-148]. G-Protein-coupled receptor internalization and primary triggering vs secondary processing signaling pathways, even in the immediate phase of protection against ischemia reperfusion injury - which consists both of irreversible necrosis and apoptosis by induction of phosphatidyl inositol 3-OH, P13-kinase, but not the p42/p44 cascade [149] -, are actually required to act together on mitochondria for IPC cardioprotection [150-162]. However, more integrated approaches extend the analyses to the responsive transcription factors [i.e.: 163].

1.2. THE CAPSAICIN-VANILLOID TOOL

In the same way as reserpine and 6-hydroxydopamine [16] have been used to prevent catechol-dopaminergic and enteraminergic/serotoninergic sympathetic neurotransmission, capsaicin and other vanilloids have been used (after Jáncso and Jáncso-Gabór [164]) to activate by release nonadrenergic, noncholinergic, mostly peptidergic modulators, and produce a long-lasting refractory state referred to as desensitization. A cloned subset

of capsaicin activated cation channel receptors has been associated with thermal and proton sensitive neuronal functions [165], and multiple iso-receptor groups have been characterized [166] by use of specific agonists and antagonists both in newborn [167-168] and adult mammals [169-171]. In the r.h., capsaicin targets and mechanisms of action have mostly been related to specific primary Ad-1 small myelinated centrally signaling afferent fibers that selectively contribute to the short local efferent circuits, which are activated by transient hypoxia and by anoxia/ischemia, underpinning chronic neuro-inflammatory disorders.

These structures release endogenous bradykinin, substance P and other tachykinins, atriopeptin(s) and α-calcitonin-gene-related peptide (CGRP), which has in turn been correlated to oxygen deprivation/redistribution insults and even to IPC adaptation [172-174].

Epoxy eicosatrienoid acid products of cytochrome P450 epoxygenases - like the CYP2J2 human cloned isoform [175-176], contribute to the endogenously activated anandamide reactive cannabinoid receptors on peripheral sensory nerves, showing selectivity to capsaicin-vanilloid receptors accompanied by release of CGRP [177]. While capsaicin induces a reversible stimulus length dependent negative staircase inotropic effect in the rat ventricle, without inhibition of its calcium handling [178-179], its anti-arrhythmic and anti-ischemic activity has been postulated to act by blocking

some K^+ and/or Ca^{2+} channels [180-181], possibly through the release of neuropeptides, especially CGRP. Modulation of coronary circulation [182], an interplay (in guinea pig heart) between NO and CGRP in capsaicin induced increase in coronary flow and heart rate [183], and capsaicin related r.h. PC [184] associated with oxygen radicals and NO contributions (i.e.: [185]) or pacing induced [186], have been reported. (Our preparatory work on cannabinoids vs vanilloids, and a contribution on some O_2-NO-redox dependent structurally covalent post-translational cellular issues are reported in [187, 188]).

1.3. GENERAL AIM OF THE STUDY

We present our first paper, divided into two parts, on autofluorescence, and the second and third contributions on near infrared and NMR spectrometry studies. In this 1st paper, the adult r.h. spontaneously beating Langerdorff preparation was used while submerged and infused at a constant standard temperature and pressure with the widely used crystalloid buffered solution. Three sets of control conditions (short preinfusion, long preperfusion, and a commonly applied preconditioning protocol) were established in a total of 41 hearts, 18 of which were acutely pretreated *in vitro* at one capsaicin saturating dose. All hearts were thereafter subjected to 30 min global ischemia/anoxia followed by at least 60 min oxygenated

reperfusion, while conventional functional parameters were continuously monitored. For the fluorescence observations (2nd part), 3 other control groups and 3 groups of capsaicin-pretreated hearts were monitored in the same previously standardized conditions. All hearts of all animals sacrificed were used in the experiments.

The work first assessed the suitability of the most commonly monitored functional parameters to characterize *ex vivo*, in the preconditioned rat heart, early amelioration, protection or delay in recovery, following the *in vivo* original studies (i.e.: [189]) and the most recent hypotheses (i.e.: [190]) and contributions, as briefly reviewed above.

Noninvasive technique(s) were applied to analyze the kinetics of the interrelations of the most relevant redox markers - pyridine nucleotide fluorescence signals in this first paper. Their unique properties to express mito-cytosolic dynamic equilibria will help - it is our aim and basic hypothesis - to clarify the feedback interrelations among different organ/tissue/cell functional compartments. In particular, the optical techniques applied are held to be sufficiently fast to identify in a *peripheral* network of coupled metabolic vs functional adaptations, memory acquisition and maintenance processes, substrate/oxygen use vs deprivation precursor-product relationships and signaling. Our aim is thus to characterize matching of energy demand with respect to supply and the related damage vs protection features in the *in vitro*

preparation, after having analyzed some of them in our previous studies [191-198]. So, we would like to contribute to a description of those steps which, in the native *in vitro preparation*, are basic to modulation of energy availability vs oxygen deprivation processes associated with repeated short time insults, particularly in the frequency domain control of the metabolic machinery. Last but not least, capsaicin specific mechanisms will help clarify some *peripheral* residual short memory acquired adaptations in a model free of other neurohormonal and vascular factors [i.e.: 187]. The other noninvasive measurements of the metabolic parameters, analyzed by near-infrared and NMR spectrometric techniques, both *in vivo*, as well as *in vitro* capsaicin treatments, will be presented In the next two papers, and their modulation in ischemic and reperfusion injuries and interference on acute adaptation/attenuation throughout early IPC will be more comprehensively elaborated (work in progress).

1.4. TOPICS NOT ANALYZED

The second window of protection, i.e. the delayed effects of preconditioning (for r.h.: [199-203], for mouse heart [204], and for conscious rabbit heart, with different mechanisms shown at 24 vs 72 hours, [205]), the effects of remote and transferable preconditioning ([201, 204-208]), and those of the form of modified reperfusion called post-conditioning ([[209-210] were excluded from the study.

Some preliminary considerations and single heart-data slides have been presented in a local Academy Seminar [211].

2. MATERIAL AND METHODS

2.1. HEART PREPARATION

All experiments met the guidelines of the Canadian Council on Animal Care regarding the care and use of experimental animals, and were approved by the local Animal Committee of the National Research Council of Canada.

Sprague Dawley rats of both sexes, weighing 250 ± 15 (S.D.) g, obtained from Charles River and acclimatized to animal facilities were submitted to 12 hour cycles of artificial light at constant temperature and relative humidity for at least one week prior to use, standard food and water being allowed ad libitum. The rats were anesthesized with sodium pentobarbital (120 mg/kg ip), and the hearts removed as soon as the toe reflex disappeared (within 3 min), immediately immersed in ice-cold buffer and perfused according to Langerdorff at 36.5 ± 0.1 °C in less than 30 sec at a constant pressure of 80 mm Hg. The Krebs-Henseleit (KH) buffer contained (mM) NaCl 118, KCl 4.7, CaCl2 1.75 (free Ca^{2+} ≈ 1.1), $MgSO_4$ 1.2, EDTA 0.5, $NaHCO_3$ 25 and glucose 11, and was equilibrated at pH of 7.4 with a 95% N_2 and 5% CO_2 gas mixture prior to the 95% O_2/5% CO_2 gas mixture. An apical drain was inserted via the mitral valve in the left ventricle to vent the drainage from the thebesian veins, and a

water-filled compliant balloon was placed into the same ventricle. The balloon was connected to a Statham P23Db, or to WPI BLPR5326 (Sarasota, FL, USA) pressure transducers to monitor left ventricular pressure and heart rate. The left ventricular end diastolic pressure was adjusted to the averaged initial % of any maximum systolic pressure of 7.5 mm Hg by inflating the balloon, its volume being kept constant throughout all experiments. Functional parameters were monitored with a Digi Med^{R} Instantaneous Data-capture and Analysis System (model 200, Micro-Med Inc., Louisville, Ky, USA), by sampling at 600 Hz and monitoring the successive 120 sec arithmetic averages.

2.2. FUNCTIONAL PARAMETERS

The first parameter, coronary flow (CF), was followed with an ultrasonic blood flow meter (model T101, Transonic Systems Inc., Ithaca, New York) standardized by repeated collection of the effluent from the heart. Hearts were subjected to periods of global ischemia by clamping the perfusion line to the aortic cannula; reperfusion was achieved by releasing the clamp; the dead volume of fluid up to the aorta was maintained constant and equal to 13.50 ml. Mechanical

function was assessed as frequency (BPM), maximum systolic left ventricular pressure (MSLVP), and end diastolic left ventricular pressure (EDLVP). These parameters were used to obtain: rate pressure product; heart rate times left ventricular developed pressure (systolic minus diastolic pressure) (RPP); and RPP divided by the coronary flow (RPP/CF). The interleaved lengths of unspecified arrhythmia were taken into account. The internal heart temperature was monitored continuously using a thermocouple (model 39641-T Atkins Technology Inc., Gainesville, Florida, USA) placed into the pulmonary artery.

2.3. PROTOCOLS

The three standard sets of assays consisted of *controls* and *in vitro* capsaicin-pretreated preparations.

2.3.1. Short perfusion (SP)

All hearts were observed for 15 min after the start of the perfusion. In treated specimens, after oxygenated KH perfusion and monitoring of all parameters for 5 min, a capsaicin/DMSO (see below) solution was infused through a collateral line at the top of the Langerdorff cannula for 5 min; the bathing fluid external to the heart was then substituted with control oxygenated, 36.5 °C KH Ringer, whose infusion was protracted up to the end of the *first* step of the SP protocol. The *second step* consisted of 30 min global ischemia and the *third step* of 60 min constant pressure oxygenated reperfusion. At the end of the

protocol, the heart was removed from the fluid and weighed or frozen immediately with Wollenberger clamps precooled with liquid nitrogen.

2.3.2. Long Perfusion (LP)

After the *first* 15 min step, perfusion was continued for an additional 45 min before ischemia and reperfusion (*second* and *third* steps unchanged). Long perfusion, from 30 to 60 min, in the isolated r.h. perfused with glucose as the only external substrate, has been shown to correlate with O_2 uptake and decreased mechanical activity [212-213]. Osmotic swelling, a key feature of ischemic/reperfusion injury, is attenuated by activation of volume regulated chloride channels, a candidate for the final step of ischemic preconditioning, which is the subject of debate due to contradictory results obtained in isolated perfused rabbit heart and isolated cardiomyocytes [1-6].

2.3.3. Preconditioning (PC)

After the first step, three cycles of 6 min global ischemia/anoxia, each following the first and the last by 10 min, and the middle by 8 min reperfusion, were repeated before the final long ischemia and reperfusion. The PC protocol replaced the LP, which acted as the most appropriate control.

A series of different conditions (i.e.: insults of varying ischemia and reperfusion times, from 1 to 10 PC), and final ischemia from 15 up to 45 min, which may encompass distinct mechanisms of regulation of iso-PKCs [214], were also assayed in different groups of rats (not included in the presentation) to assess the effect of PC on a roughly 50% recovery as evaluated through RPP. Another set of

hearts was treated with rising concentrations of DMSO, which is known to interfere with myocardial contractility and ischemic transients [215]. Capsaicin from 0.1 to 30 μmolar was also assayed for one up to 10 min perfusion.

2.4. AUTOFLUORESCENCE

The direct fluorimetric technique for recording intracellular oxidation-reduction states (i.e.: [193-194, 217]) was performed using a commercial instrument (Ratiometer and Quantimeter Photon Technology Int. Inc., S. Brunswick, NJ, USA). The OC-4000 optical chopper and the shutter controller were used with a 100 W LPS-220 Xenon lamp power supply and a 710 photon multiplier system, interfaced with Felix software. The 340 nm peak FS10-25, AM28470-03, and 430 nm peak FS10-25, AM28128-01 (Andover Corporation, Salem, NH, USA) excitation and emission filters were used. Even though the flavin-ox 436 - 460 excitation vs the 570 - 580 emission nm peaks was a possible second channel for time-shared observation (i.e.: [194, 217-221], the second interleaved channel was used to monitor the light scattering (at 550 nm), which did not show any coherent optical changes. At the wavelength used, in the glucose-enriched KH perfusate, the surface fluorescence of the intact organ has been confirmed to originate from reduced nicotinamide nucleotides in mitochondria [222], with a contribution from cytosolic exchanges mostly pertaining to modulation by glyceraldehyde 3-

phosphate- and lactate dehydrogenases (i.e.: [223]), and the NADH shuttle system (i.e.: [224]).

A custom bifurcated fiber optic bundle (Ceram Optics, Enfield, CT, USA) that delivered the UV excitation light to the heart and also collected the emitted light was used. The common end of the optic fiber was a stainless steel cylinder 5 mm in external diameter at the polished optical end. This was placed through a hole in the water bath such that the left ventricular wall of the totally submerged heart focused at 4 mm distance, which had been adjusted to the best signal to noise ratio. The end of the fibers and the surface of the ventricle were maintained at a fixed distance with a lucite chamber empty of fluid by adjustment of the maximum energy emission.

Calibration with NADH in a KH solution confirmed the linearity of fluorescence over a wide range of concentrations, including those observed in the heart. After initial monitoring of quenching (less than 10% when observations were performed for 30 sec any 3 min), the fluorescence emission was found to be stable (less than ± 5% variation) in each heart for as long as 3 hours, the maximum allowed for any experiment when the irradiation chopped 3 times/sec. For comparisons with the functional parameters, fluorescence emissions were averaged up to 120 sec intervals (abscissae), and their values (ordinatae) standardized as % between the zero, almost steady, initial level and the maximum value, taken as 100%, reached in the 30 min ischemia.

It should be noted that our approach could not monitor the turnover and magnitude kinetics of heterogeneous ischemic areas in the perfused r.h., which can be evaluated using advanced imaging technologies [225-235].

2.5. STATISTICAL ANALYSIS

Fluorescence data and single cardiac functional parameter kinetics were averaged to achieve coincident steps at 120 sec intervals, and their trends were evaluated in the 6 groups. The absolute values of the four functional parameters of each heart evaluated as independent - CF, ml/min; BPM, Hz or beats/min; MSLVP and EDLVP, mm Hg – were averaged as monitored in the first 5 min of the standard protocols and normalized as 100%. Differences between each percentually transformed variable in control and treated preparations were assessed as averages of the subsequent 2 min kinetic steps using the t test (unpaired, two tailed) applied to each next repeated measurement. All data sets, averages, standard errors of the means (S.E.) and probabilities (P) for each parameter, control vs treated groups, were calculated using the Microsoft Excel 2000 and SPSS 13.0 statistical packages. Additional evaluation were performed with the SPSS 13.0 for Windows full package, and the HTM (Microsoft word editable) & PDF formats, as well as the proprietary SPSS.SPO (editable with SPSS) format. All data and evaluations are presented in the attached files; a few, selected final Figures are included into the Results section.

2.6. PRODUCTS

Capsaicin synthetic analog (N-vanillylnonamide RBI, Natick, MA, USA; m.w. 293.41, lot VPR-396A) was prepared 60x of the corresponding measured CF final 10 μmole, diluted in dimethyl sulfoxide (DMSO) (Merck & Co., Inc., Rahway, NJ, USA), final 0.05%, maintained under nitrogen. All other chemicals were Sigma Chem. Co. (St. Louis, MI, USA) reagents.

3. RESULTS

The means and S.E. of the absolute values of the four functional parameters assumed as independent in the 41 hearts of the 6 groups are presented in Table I. Figures 1 - 4 show the percentually normalized, t test evaluated, related time courses, means and S.E. of control vs capsaicin pretreated SP, LP and PC groups (1^{st} attachment). Figures 5 and 6 show the time cources of the means of the two calculated, dependent parameters from same attachment.

Figure 7 reports the specific kinetics of the single measurable parameter of the 30 min ischemia applied to all 41 hearts.

Table 1

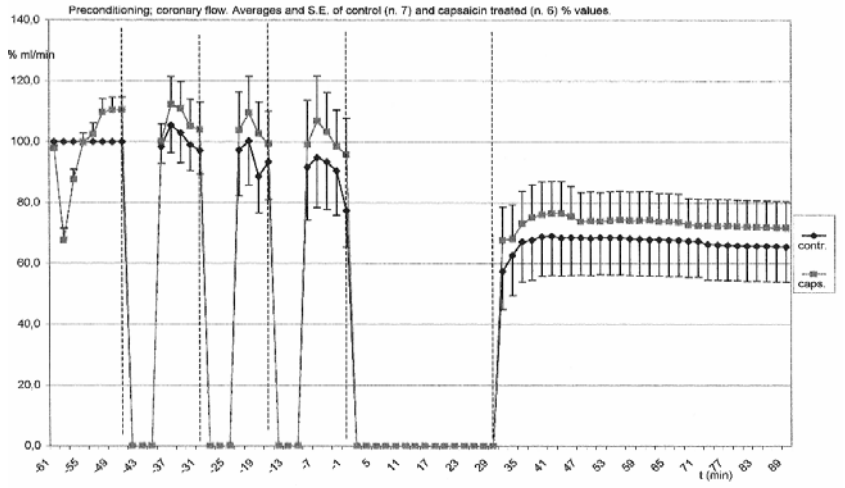
Absolute values of the 4 functional parameters measured in each rat taken as the average of the first 5 min of *ex vivo* KH Langendorff perfusion. Means and S.E. of the 6 groups and P values of the t test (unpaired, two tailed) between control and capsaicin-treated specimens subjected to preconditioning (PC), long perfusion (LP) or short perfusion (SP).

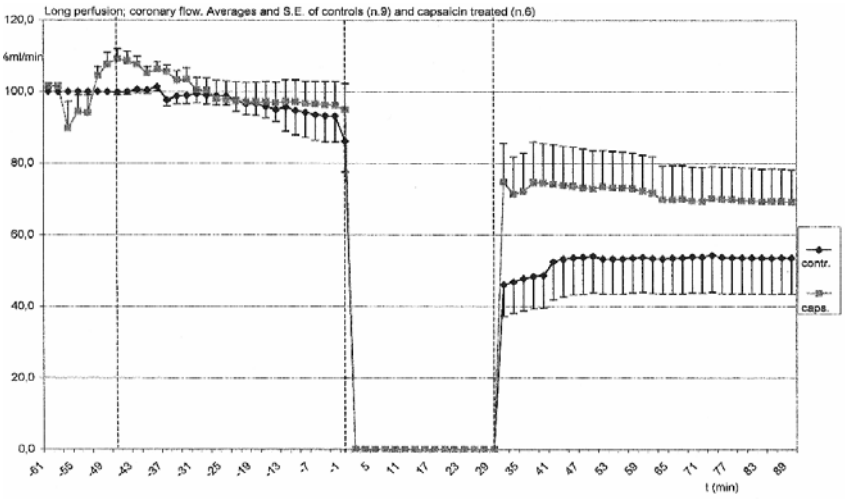
n total	Groups	Coronary Flow (CF; ml/min)				Frequency (BPM; Hz)				Maximum Systolic Pressure (MSLVP; mmHg)				End Diastolic Pressure (EDLVP; mmHg)			
	Preconditioned (PC)	n	Control	n	Treated	n	Control	n	Treated	n	Control	n	Treated	n	Control	n	Treated
2	2	1	16.3	1	19.4	1	245.8	1	246.	1	134.7	1	98.0	1	5.6	1	7.7
4	4	2	16.3	2	21.6	2	237.	2	284.2	2	131.5	2	111.9	2	5.7	2	6.7
6	6	3	17.7	3	20.4	3	231.8	3	313.9	3	116.1	3	82.0	3	6.5	3	9.2
8	8	4	17.	4	18.	4	252.1	4	243.2	4	94.3	4	99.2	4	8.0	4	7.3
10	10	5	13.5	5	15.8	5	267.2	5	273.4	5	114.5	5	94.9	5	6.5	5	7.6
12	12	6	16.3	6	17.7	6	256.8	6	258.7	6	94.7	6	124.7	6	7.9	6	5.1
13	13	7	17.1			7	305.2			7	104.			7	7.2		
	Means		16.31		18.8		255.1		269.9		112.8		101.8		6.8		7.2
	S.E.		0.51		0.84		9.1		10.9		6.2		6.		0.4		1.2
	P		0.024				0.32				0.2				0.46		
	Long Perfusion (LP)	n	Control	n	Treated	n	Control	n	Treated	n	Control	n	Treated	n	Control	n	Treated
15	2	1	12.5	1	19.	1	222.1	1	261.5	1	134.2	1	113.2	1	5.6	1	6.6
17	4	2	13.1	2	20.	2	252.1	2	272.8	2	141.6	2	91.8	2	5.3	2	8.3
19	6	3	13.2	3	15.	3	269.8	3	221.3	3	124.2	3	95.5	3	6.0	3	7.9
21	8	4	13.9	4	21.	4	184.7	4	268.3	4	85.6	4	102.9	4	8.8	4	11.0
23	10	5	13.8	5	14.7	5	238.3	5	222.	5	155.3	5	108.3	5	4.8	5	7.5
25	12	6	12.9	6	16.1	6	247.7	6	284.5	6	121.2	6	89.	6	6.2	6	10.1
26	13	7	12.6			7	250.4			7	88.6			7	8.5		
27	14	8	13.3			8	208.			8	106.5			8	6.9		
28	15	9	13.9			9	267.8			9	101.1			9	7.4		
	Means		13.2		17.6		255.1		255.1		117.8		100.1		6.6		8.5
	S.E.		0.2		1.11		11.		11.		7.95		3.92		0.5		0.7
	P		0.00034				0.26				0.11				0.05		
	Short Perfusion (LP)	n	Control	n	Treated	n	Control	n	Treated	n	Control	n	Treated	n	Control	n	Treated
30	2	1	19.4	1	22.8	1	235.5	1	251.1	1	103.5	1	95.8	1	7.3	1	7.8
32	4	2	21.5	2	23.5	2	219.4	2	286	2	112.8	2	81.6	2	6.7	2	9.2
34	6	3	19.2	3	22.2	3	225.3	3	268.3	3	103.5	3	101.9	3	7.3	3	7.4
36	8	4	18.7	4	19.2	4	201.9	4	237.9	4	109.1	4	104.2	4	6.9	4	7.2
38	10	5	22.3	5	19.7	5	301.7	5	278.1	5	99.3	5	96.7	5	7.5	5	7.7
40	12	6	19.4	6	22.1	6	280.2	6	309.8	6	96.5	6	91.5	6	7.8	6	8.3
41	13	7	19.4			7	273.3			7	102.4			7	7.4		
	Means		19.99		21.58		248.2		271.9		103.9		95.3		7.3		7.9
	S.E.		0.51		0.71		14.		10.4		2.1		3.3		0.1		0.3
	P		0.089				0.21				0.04				0.05		

Figure 1

Coronary flow (CF; ml/min). Control (♦) vs capsaicin-pretreated (■) time courses of the means of the percent values and of their S.E. Data from 1° attachment.

X axis: consecutive measurement at 2 min intervals.
Y axis: means of the percent values and their S.E. (vertical bars).





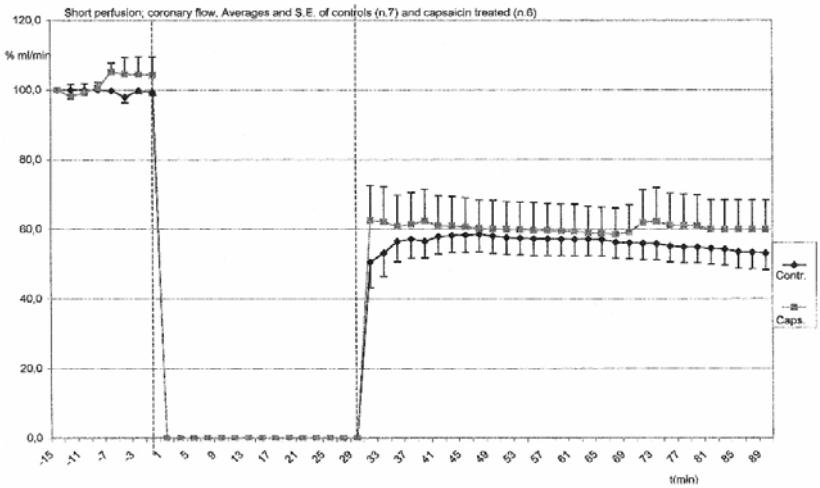
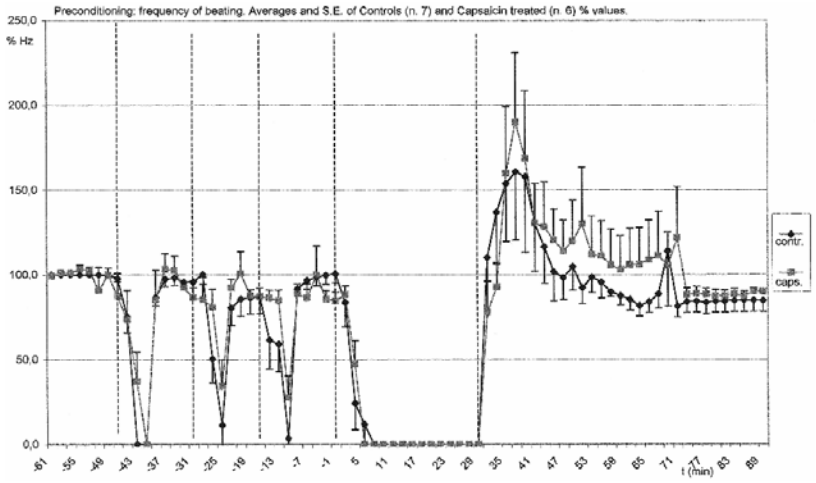
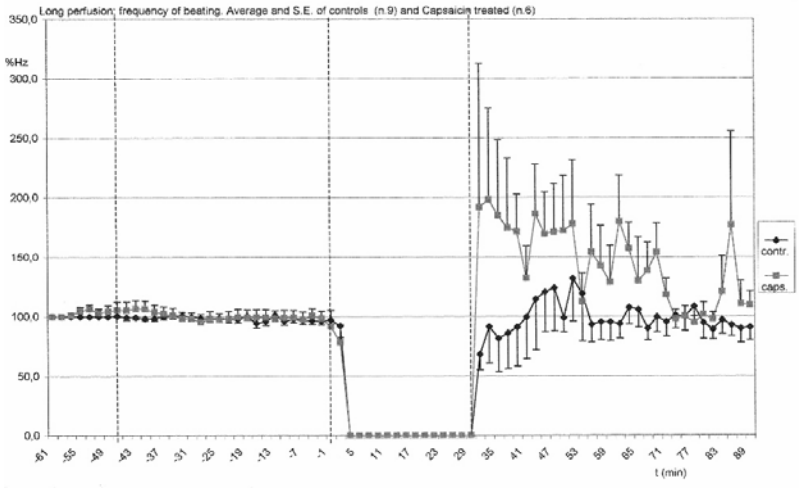


Figure 2

Frequency (BPM; Hz). Control (♦) vs capsaicin-pretreated (■) time courses of the means of the percent values and of their S.E. Data from 1° attachment.

X axis: consecutive measurement at 2 min intervals.
Y axis: means of the percent values and their S.E. (vertical bars).





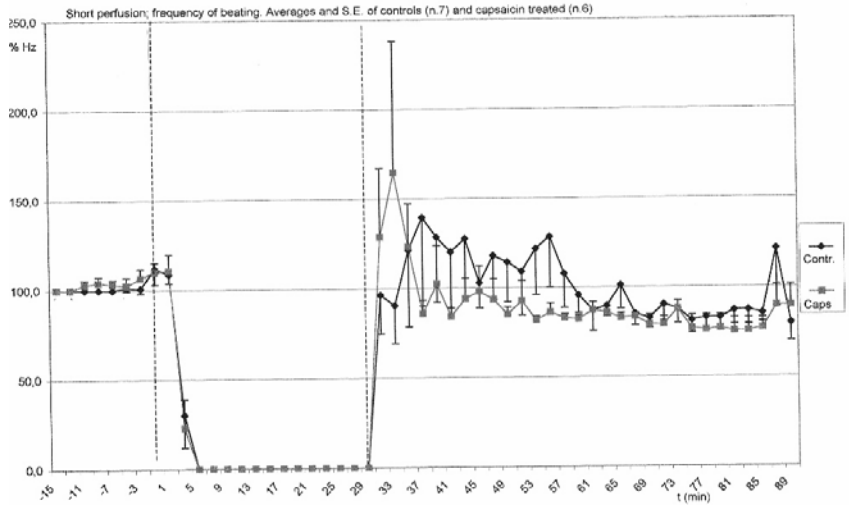


Figure 3

Maximum systolic left ventricular pressure (MSLVP; mmHg). Control (♦) vs capsaicin-pretreated (■) time courses of the means of the percent values and of their S.E.
X axis: consecutive measurement at 2 min intervals.
Y axis: means of the percent values and their S.E. (vertical bars).

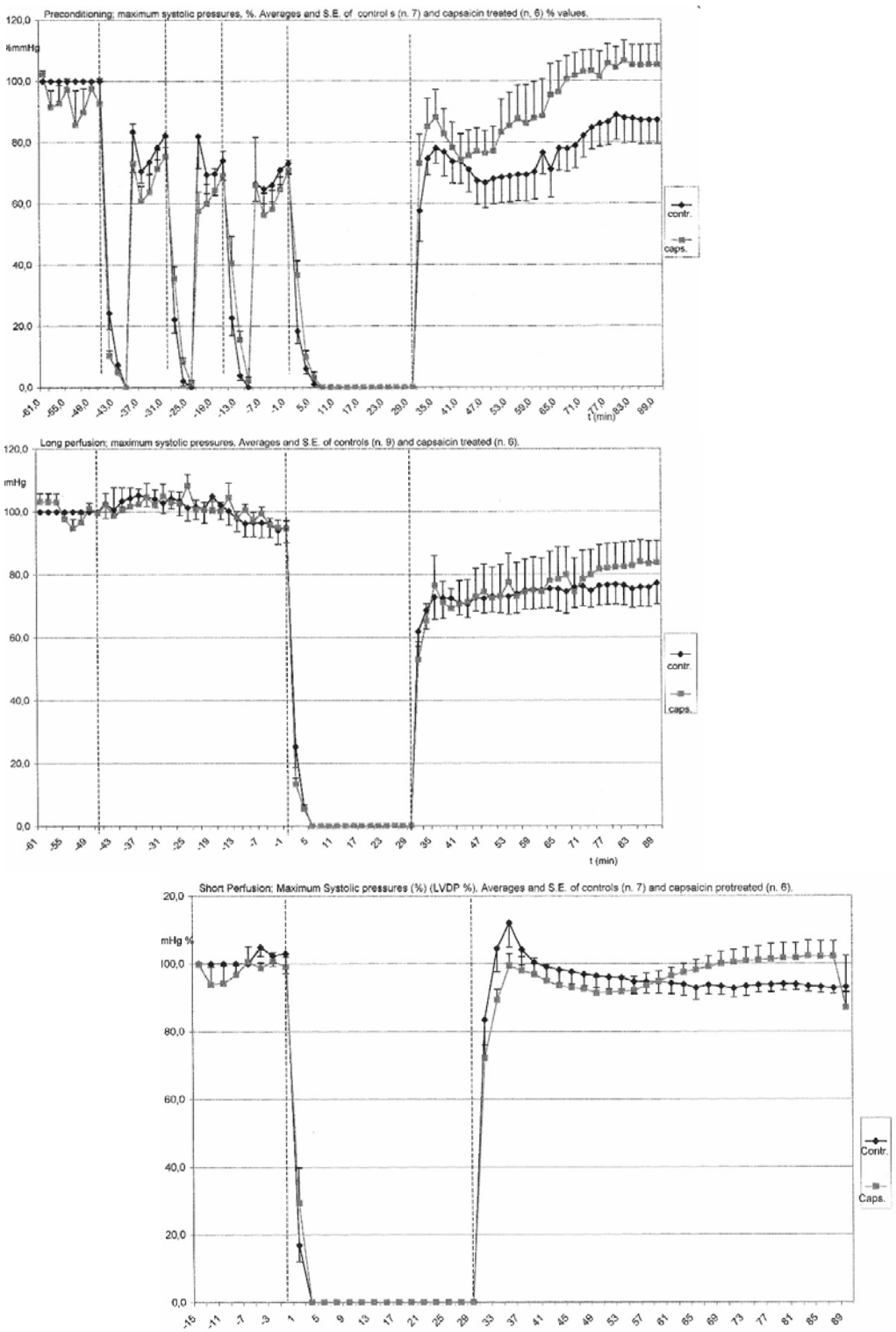
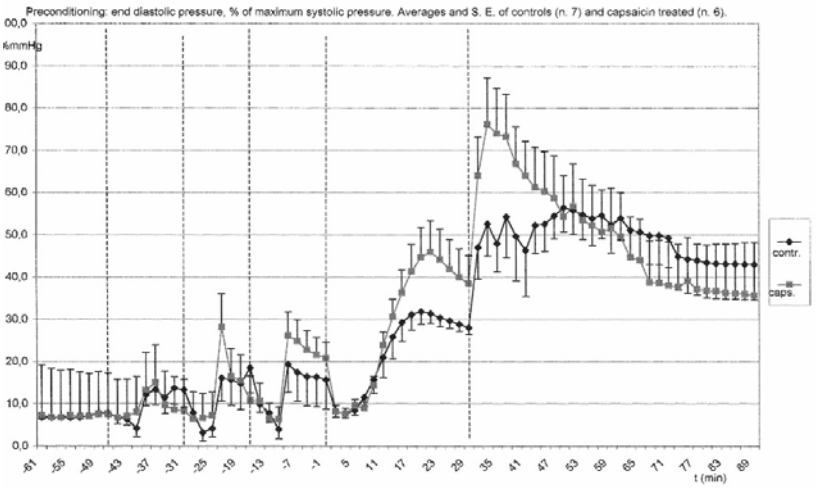
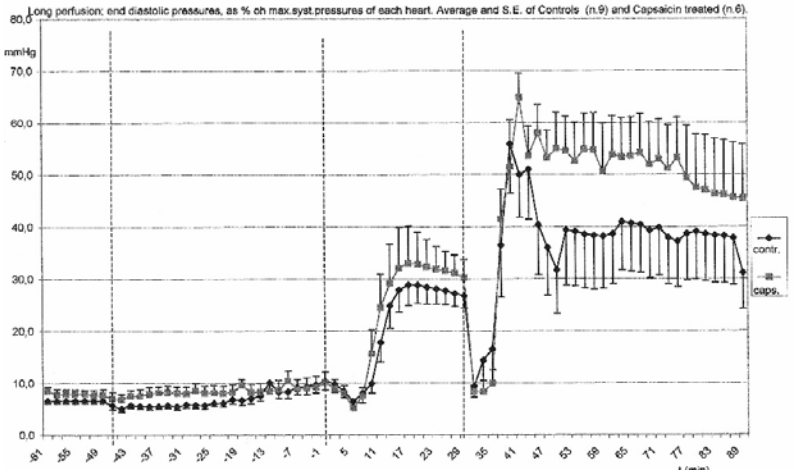


Figure 4

End diastolic left ventricular pressure (EDLVP; mmHg).). Control (♦) vs capsaicin-pretreated (■) time courses of the means of the percent values and of their S.E.
X axis: consecutive measurement at 2 min intervals.
Y axis: means of the percent values and their S.E. (vertical bars).





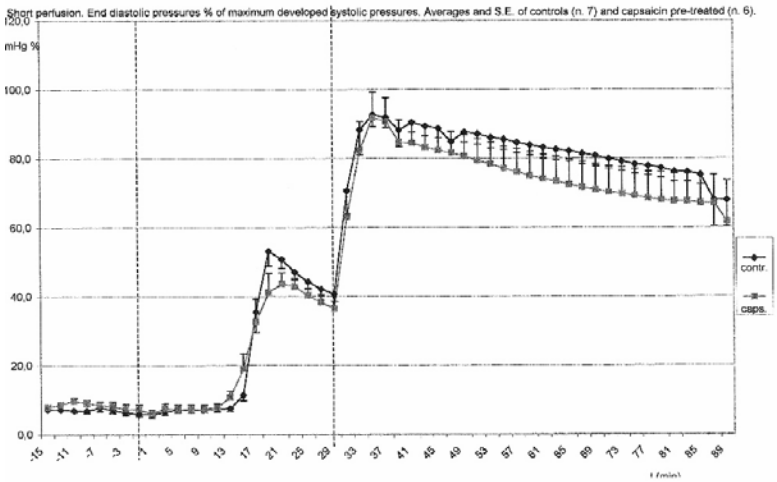
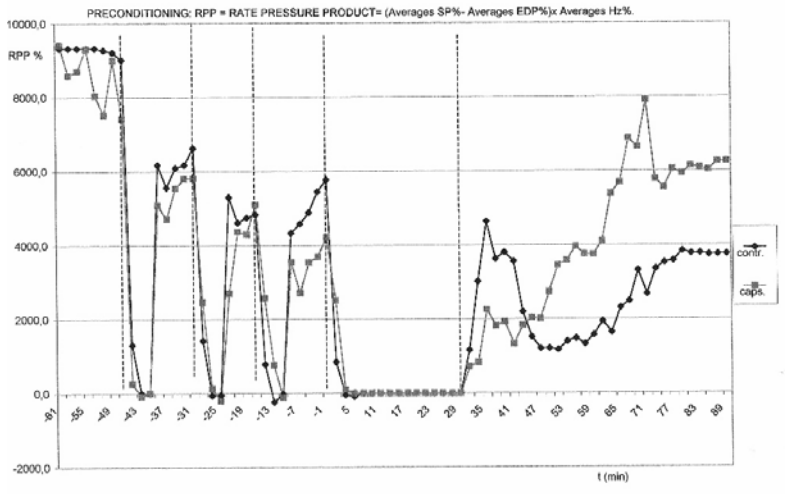
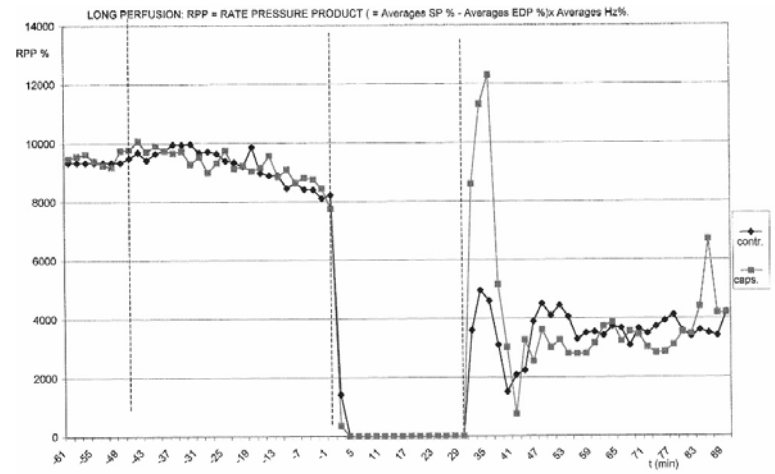


Figure 5

Rate pressure product (RPP). Control (♦) vs capsaicin-pretreated (■) time courses of the means of the percent values.
X axis: consecutive measurements at 2 min intervals.
Y axis: means of the percent values.





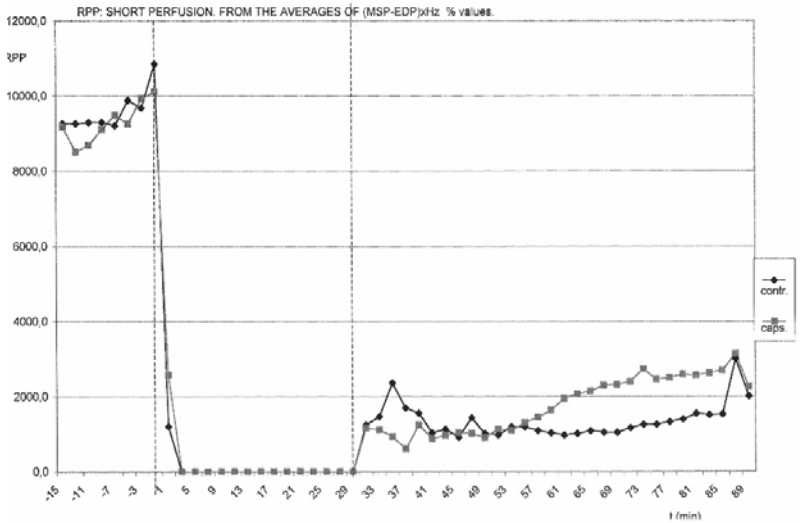


Figure 6

Rate pressure product divided by the coronary flow (RPP/CF). Control (♦) vs capsaicin-pretreated (■) time courses of the means of the percent values.
X axis: consecutive measurements at 2 min intervals.
Y axis: means of the percent values.

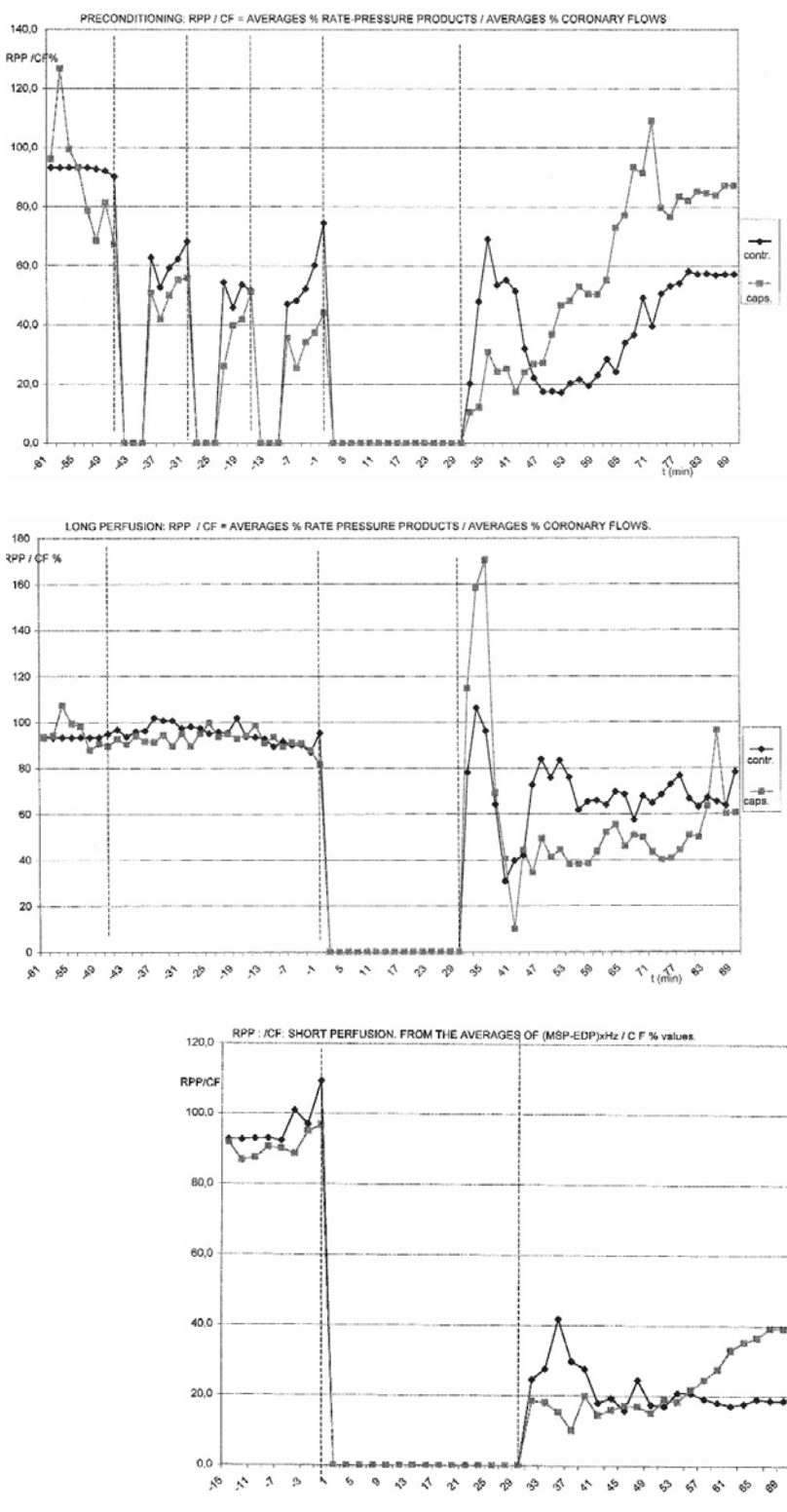
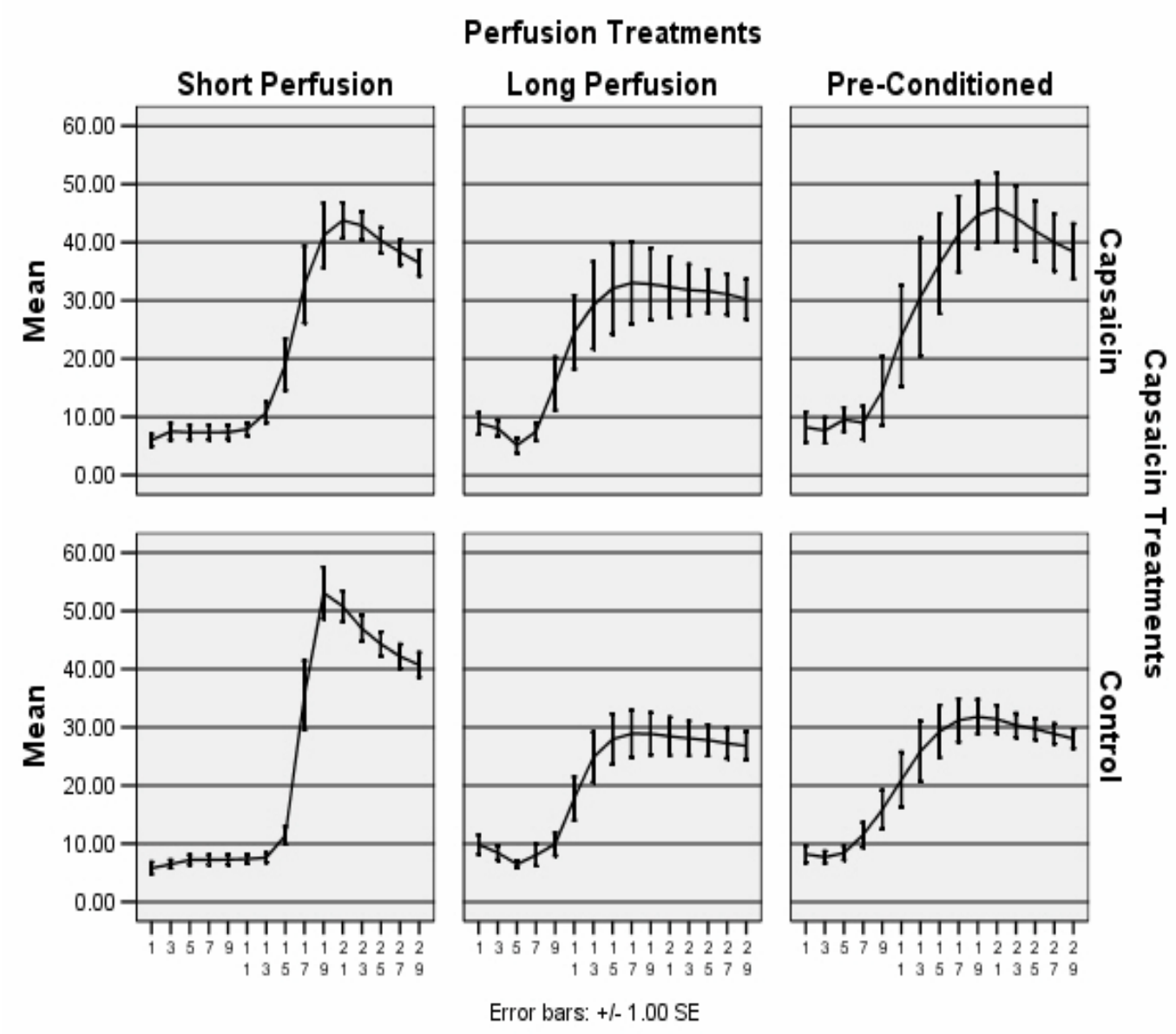


Figure 7

Time course of the sole measurable functional parameter (ischemic contracture) . Kinetic trends in the 6 experimental conditions.

X axis: consecutive measurements at 2 min intervals.
Y axis: means of the percent values and their S.E. (vertical bars).



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