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Myocardial Contractile Response and IP_3, cAMP and cGMP Interrelationships

An experimental study in the perfused working normal and pressure overloaded rat heart. A mini review based on a doctoral thesis

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GENERAL BACKGROUND

In an organism the functions of the cells must be co-ordinated. Signals are transferred and interpreted between cells. The message should be accepted correctly leading to deliberated consequences for each cell but also for the organism as a whole. The organism must economize, *i.e.* it has not one signal protein (with its own amino acid sequence) for each possible message. Hence, the signal component is able to serve different functions. The first example of transforming an extracellular signal (quantitatively minor) to an intracellular consequence (quantitatively major) in an organism was established by Earl Sutherland (118, 119). He showed that certain types of hormonal signals would generate a new intracellular signal in the target cell, cAMP, functioning as a second messenger. The transduction of the hormone signal to an increase in cAMP content in the cell was clarified during the last decade. In order to secure a correct signal transformation three specific functions have to be available. Firstly, the receiving cell needs a discriminator, *i.e.* a receptor, which is a glycoprotein with the ability to specifically recognize a certain hormone. There must also be a transducer (known to be GTP regulated) for transduction of the receptor signal. Finally, a transformer (adenylyl cyclase, phospholipase C) amplifies the hormone signal by generating a large amount of second messenger.

When an agonist interacts with a receptor *via* a non covalent bond (78), the receptor undergoes a conformational change. This change makes the receptor refractory to agonist binding for a certain period. The receptor-agonist complex activates a transducer today known as a GTP-binding regulatory protein, G protein (39). G proteins function essentially as on-off switches for cellular signalling. They consist of three nonidentical protein subunits (α, β and γ) that are noncovalently associated, noted as $G\alpha\beta\gamma$. The α subunit is the biggest and is activated by GTP. The β subunit is smaller and is always bound to the smallest subunit, the γ. Currently, at least 16 α subunits, 5 β subunits and 6 γ subunits have been found (13). In the resting state, GDP is tightly bound to the α subunit.

This is the "off" position of the G-protein switch. By activation of the membrane receptor the agonist-receptor complex interacts with the G protein. This interaction catalyzes the dissociation of GDP from the α subunit (see Fig. 1). The released GDP is quickly phosphorylated to GTP by membrane bound nucleoside diphosphate kinase (2). Subsequent binding of GTP is followed by the dissociation of $G\alpha$ from $\beta\gamma$ which alone or in combination activates the effectors. The switch is now "on". Within short GTP is catalyzed by the GTPase intrinsic to the α subunit to form GDP. The α subunit is now inactivated leading to

Table 1. The G protein subunits, receptor activation, function and possible inhibition

subtype	receptor	effector	toxin
α_S (n forms)	β-adrenergic, Vasopressin, TSH, Dopamine D_1, Adenosine A_2, VIP, Glucagon,	Adenylyl cyclase (+), Ca^{2+} channels (L type, opens),	Cholera toxin enhances the ability of G_S to activate AC. Pertussis toxin inhibits the function of G_i
α_i (n forms)	α_2-adrenergic, Adenosine A_1, Opioid, Dopamine D_2, $GABA_\beta$,	Adenylyl cyclase (-), K^+ channels (opens),	Inhibition by pertussis toxin,
α_o (n forms)	Muscarinic (M_2 and M_4),	Ca^{2+} channels (N,L types, closes),	Inhibition by pertussis toxin,
α_q	α_1-adrenergic, Muscarinic (M_1 and M_3), Histaminic (H_1),	Phospholipase C (+),	

(simplified after (13, 39)).

reassociation with the $\beta\gamma$-subunits returning to the "off" position (38, 39, 40). The activated $G\alpha$ complex mediates stimulation or inhibition of effector enzymes (cyclase or phosphodiesterase) and ion channels (*cf.*: 115). The type of α subunit decides whether the $G\alpha$ complex would be stimulatory ($G_{s\alpha}$) or inhibitory ($G_{i\alpha}$) (23, 38, 123) (see Table 1).

However, the transduction is complex due to a large number of possible combinations. More than 15 different combinations are known between the β and γ subunits (*cf.*: 88). The different $\beta\gamma$ combinations can be recombined with different α subunits resulting in specific cellular responses. Recently, the $\beta\gamma$ subunits *per se* were found to have regulatory functions in certain types of adenylyl cyclases (97, 122). Hence, each receptor can react with several types of

G proteins. Furthermore, each G protein can interact with several types of effector systems. Thus, activation of a G protein can concomitantly generate more than one response in the stimulated cell. At least three factors are decisive: 1. Actual receptor type of the cells of interest; 2. Presence of particular G proteins in the cell and; 3. Type of transformer in the cell. In summary the cell exhibits a high number of signal expressions using a restricted number (less than 50) of signal proteins.

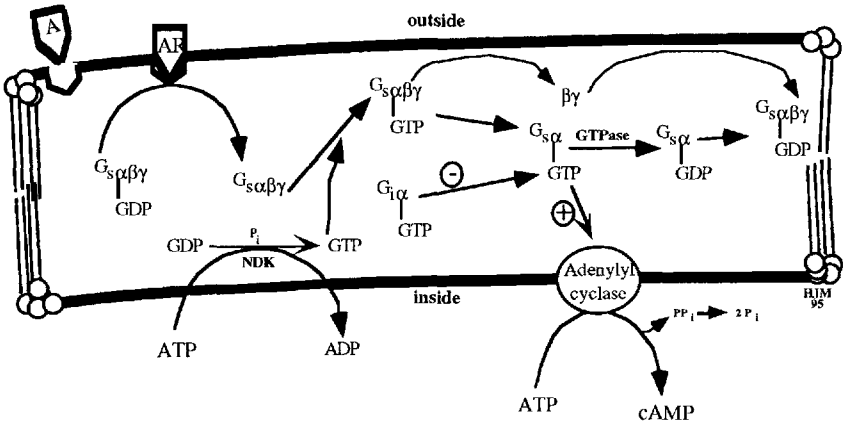

Fig. 1. G-protein related transduction. Note that GDP is tightly bound to the α subunit. A, agonist; R, receptor. NDK, nucleoside diphosphate kinase; PP_i, inorganic pyrophosphate; P_i, orthophosphate.

Inositoltrisphosphate (IP_3) is one of two second messengers formed by hydrolysis of phosphatidyl inositolbisphosphate, the other being diacyl glycerol (DAG) (9). IP_3 released into the cytoplasm mobilizes Ca^{2+} from internal stores, while DAG activates protein kinase C (11). IP_3 could either be phosphorylated or dephosphorylated. IP_3 kinase phosphorylates IP_3 to IP_4 (57, 100). IP_5 and IP_6 have been found in various cells including cardiomyocytes (107) suggesting the existence of IP_4 and IP_5 kinases. IP_3 phosphatase dephosphorylates IP_3 to IP_2. IP_2 phosphatase cleaves IP_2 to IP_1. Inositol monophosphatase hydrolyzes IP_1 to free inositol and orthophosphate (8). The liberated inositol is required to resynthesize phosphatidyl inositol (a phospholipid) in the cell membrane which is subsequently phosphorylated to phosphatidyl inositol bisphosphate. The inositol monophosphatase is inhibited by LiCl (8, 14) which may be the molecular level at which lithium ions act in the central nervous system. Inositol (1,4,5) trisphosphate (IP_3 (1,4,5)) releases calcium from an internal IP_3 sensitive calcium pool in many different cell types including smooth muscle cells (7, 56, 116). IP_3 (1,4,5) exerts its effect by binding to a specific IP_3 receptor located on the endo- or sarcoplasmic reticulum (*cf.*: 11). In some cell types IP_3

(1,4,5) has been proposed to activate second messenger operated Ca^{2+} channels directly (11). The intracellular level of calcium may oscillate when the phosphatidyl inositolbisphosphate pathway was stimulated (10, 11). Several explanations have been given for this amplification of the Ca^{2+} concentration resulting in generation of repetitive calcium spikes (*cf.*: 11). The calcium induced calcium release (36, 37) (probably a central component in the IP_3 induced calcium increase), contributes to the calcium oscillations, *i.e.* the IP_3 sensitive calcium stores respond either to a rise in IP_3 level or the stores release calcium in the presence of a constant level of IP_3 through a process of calcium induced calcium release (*cf.*: 11). Recently this two-pool model was modified to consist of one IP_3 pool only. The characteristic of the pools were suggested to depend on how deep in the cell they were located, *i.e.* at low agonist concentration (where the Ca^{2+} oscillations were seen) only the Ca^{2+} pools near the membrane were influenced but not those situated deeper in the cell. The bulk of the pools thought to be deep within the cytosol are gradually charged by calcium flux from the outside of the cell. The charging up of the deep pools is believed to increase their IP_3 sensitivity resulting in discharging of the stored calcium in an all-or-none fashion upon resting IP_3 level (*cf.*: 11).

Activation of adenylyl cyclase catalyzes cAMP production by ATP hydrolysis. cAMP is metabolized to AMP by phosphodiesterase (in the heart, phosphodiesterase III) (25, 129). cAMP activates certain protein kinase reactions, *i.e.* phosphorylation of proteins including enzymes. cAMP will in general increase sarcoplasmic Ca^{2+} concentration (*cf.*: 54). The cAMP activated protein kinase (*cf.*: 90) will open the Ca^{2+} L channel in the sarcolemma. Through action on the sarcoplasmic reticulum cAMP may also influence intracellular Ca^{2+} distribution (*cf.*: 89, 90).

The contractile apparatus has been extensively studied over the last decades. The contractile proteins consist of myosin and actin chains functioning by forming filaments (1). The myosin molecule contains, *i.a.* two heavy chains (MHC), α and β. The α-MHC has a higher ATPase activity than the β-MHC. There is a strong correlation between the ATPase activity and the muscular velocity of contraction (108). Three different combinations of MHC are possible, where the $\alpha\alpha$ homodimer has the highest ATPase activity and the highest mobility in gel electrophoresis, forming the V_1 band. The $\beta\beta$ homodimer has the lowest ATPase activity and the slowest mobility, the V_3 band. The $\alpha\beta$ heterodimer is in between concerning both ATPase activity and mobility, the V_2 band (*cf.*: 120). The β-MHC is predominant in human ventricular myocardium (41) while α-MHC is predominant in rat myocardium (the V_1 form) (102). In senescent and hypertrophied myocardium a decrease in α-MHC and increase in β-MHC is seen in the rat (76, 102).

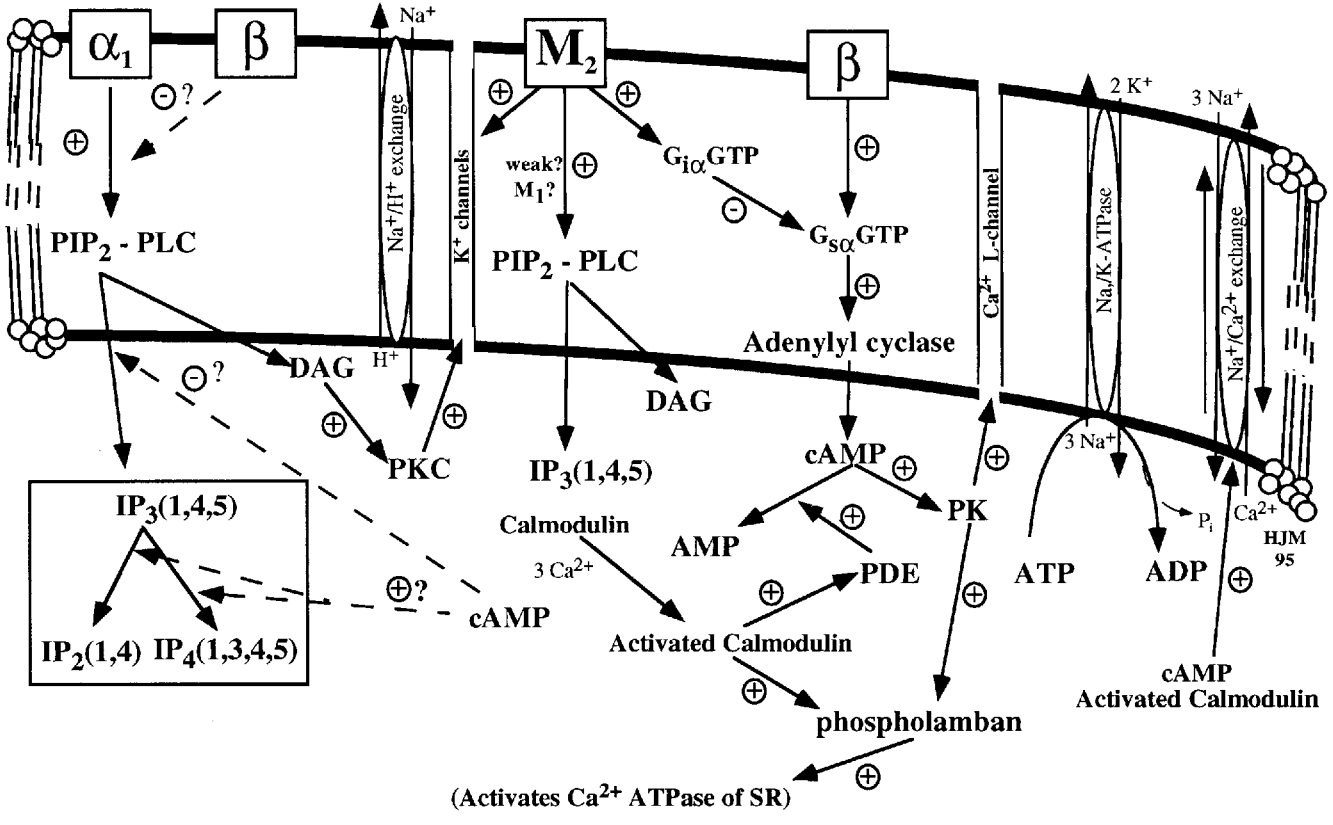

Fig. 2. Simplified scheme of myocardial sarcolemma related receptors and receptor related signalling pathways.
PK, protein kinase; PKC protein kinase C.

The contraction takes place *via* several steps. The actin filaments are attached to the Z line structure in myocardial cells. The myosin molecules form a helix with a globular end. At this end the ATP binding site is situated. When free cytosolic Ca^{2+} increases, which is the trigger of the contraction, Ca^{2+} will bind to troponin C, whereby a conformational change of the troponin complex takes place, such that the actin binding site for the myosin head becomes exposed. ATP binds to the myosin head and hydrolyzes. Then a conformational "bending" of the myosin neck takes place inducing a sliding action (shortening) as the head is attached to actin (*cf.*: 1) see also (89).

Catecholamines have been claimed to play an important role in the development of cardiac hypertrophy (58, 101). Hence, β-adrenergic stimulation was shown to give marked hypertrophy *in vivo*, while α-adrenergic stimulation resulted in a decreased ventricular weight in rats (96). On the other hand, noradrenaline induced hypertrophy both in adult (53) and neonatal rat cardiomyocytes in culture where the latter were inhibited by α_1-blockade but not by nonselective β-blockade (111). Thus, catecholamine-induced hypertrophy in cultured rat myocytes may be an α_1-adrenergic response. On the other hand, acute overloading in an isolated rat heart model increased cAMP content and this was considered to be one of the effectors responsible for acceleration of protein synthesis (79) although the cellular level of cAMP is not regulated *via* the α_1-adrenergic receptor. Enhanced phosphatidyl inositol and phosphatidyl inositolbisphosphate turnover observed in hypertrophy has also been claimed to play a possible role in the development of cardiac hypertrophy (109). Stretch-induced hypertrophy activated inositol phosphate turnover by a direct way that was not inhibited by α_1-adrenergic or muscarinic blockade (44). Thus myocardial hypertrophy could be produced by catecholamine stimulation where differences in experimental conditions seemed to be important for the type of receptor response induced. Moreover, a phosphatidyl inositolbisphosphate turnover independent of receptor mediated activation seems to be involved in myocardial hypertrophy.

INTRODUCTION

The myocardial tissue is under influence of hormones the actions of which are transmitted via receptors (*cf.*: 90) resulting in a series of intracellular responses (39). The receptors interact with each other thereby modifying the ultimate response at the cellular level (74). This includes modulation of receptor affinity and modification of the amplification signal. The cardiomyocytes contain a number of different receptors allowing a variety of interactions involving the ability to act with multiple and highly adaptable responses (77). The adrenergic

receptors are part of this system and consist of α-receptors and β-receptors (3). Many agonists have the potential to bind to various subclasses of receptors albeit with different binding affinities (18). There are also different agonists which bind to the same subclass of receptors which notwithstanding this may evoke a different tissue response (30). This multifaceted behaviour is reflected by modifications in the second messengers or second messenger systems which follow receptor stimulation (77).

Our primary interest to study changes in myocardial second messenger contents originated from the observation that patients suffering an inferior wall infarction often sustain reflex bradycardia also shown experimentally by occlusion of the right coronary artery in cats (128). Moreover, limitation of infarct size upon β-blockade treatment could not be demonstrated in patients with inferior wall infarction (45). We therefore speculated that the high parasympathetic tone already *per se* protected the myocardium to such an extent that additional β-blockade was without a demonstrable effect. The question arose whether a myocardium exposed to a massive amount of noradrenaline (NA) due to the infarction exhibited any benefit from a parallel high parasympathetic tone in form of improved myocardial protection. Moreover, could changes in myocardial second messenger contents be related to a possible improved myocardial protection?

Since Hokin & Hokin (46) observed an increased incorporation of $^{32}P_i$ (orthophosphate) into pancreatic phospholipids by acetylcholine stimulation much effort has been devoted to clarify the nature of the second messenger system utilizing the phospholipid bilayer of the plasma membrane as part of an amplification and transduction system. Several authors have shown that the mediating link between the plasma membrane and the internal calcium stores is IP_3 (8, 29, 116, 117, 131). Hence, there is strong evidence that the hydrolysis of phosphatidyl inositolbisphosphate due to the activation of phospholipase C leads to rapid mobilization of intracellular Ca^{2+} in various cell types (12). However, the role of IP_3 in the intact myocardium is still under debate (15, 82, 86, 130, 141). Both α_1-adrenergic as well as muscarinic receptor stimulation induced increased phosphatidyl inositolbisphosphate hydrolysis (15). These findings may indicate that IP_3 modulates the concerted effects of both receptor types.

Alpha-receptor stimulation leads to an increased activation of phospholipase C-β2 (11), a phosphodiesterase which hydrolyzes phosphatidyl inositolbisphosphate resulting in the formation of IP_3 and DAG (94, 95) (see Fig. 2). The α-receptor stimulation induces an increase in inotropy (31, 112) which in some species is preceded by the release of IP_3 (105). The responsiveness of the myocardial contractile apparatus to Ca^{2+} in isolated rabbit papillary muscles was increased upon α-receptor stimulation (30). However, the link between the α-receptor and the increased Ca^{2+} sensitivity of the

myofilaments is not known. Whether this could be related to IP_3 is still unclear (*cf.*: 103, 125). Besides IP_3, DAG, the second reaction product of phosphatidyl inositolbisphosphate hydrolysis, has been shown to induce certain reactions in the cell (84). DAG activates protein kinase C which may lead to alkalinization of the cell *via* an effect on Na^+/H^+-ATPase (121, 124). In skinned single cells α_1-adrenergic stimulation increased Ca^{2+} sensitivity of the contractile proteins in spite of prevention of the alkalinizing effect upon α_1-adrenergic stimulation (124). IP_3 itself or hydrolysis of phosphatidyl inositolbisphosphate might therefore account for the increase in contractility as a result of increased Ca^{2+} sensitivity seen after α_1-adrenergic agonistic stimulation although this was not found in skinned myocardial cells (85). In skinned myocardial preparations IP_3 induced Ca^{2+} release (87, 130) although not found by Movsesian (82). Several explanations could be possible for this discrepancy. One might be the differences in experimental conditions, *i.e.* whether the study was done on rat myocytes (82), fibres from papillary muscles (87) or atrial muscle fibres from chicks (130).

Muscarinic stimulation also induces an increase in myocardial IP_3 content (17). Muscarinic agonist stimulation results in transduction via G-proteins, in one case leading to an increased catabolism of phosphatidyl inositolbisphosphate very much alike the one described for α_1-adrenergic transduction (39) (see Fig. 2). Besides this system muscarinic stimulation also induces activation of an adenylyl cyclase related G_i-protein (14, 16) where $G_{i\alpha}$ interacts with the β-adrenergic activated $G_{s\alpha}GTP$. In auricular heart muscle preparations and in single chick ventricular cells carbachol (Cch) exerts a negative inotropic effect probably induced by a potassium conductance *via* activation of a K^+-channel (*cf.*: 72). At least two different affinity states of the muscarinic receptor have been discussed. A high affinity (H) state associated with adenylyl cyclase deactivation (G_i-protein related transduction) and a low affinity (L) state associated with the phosphatidyl inositolbisphosphate hydrolysis (phospholipase C related transduction) (72). Conceivably, as demonstrated in ventricular preparations a minor positive inotropic effect was seen at high muscarinic concentrations ($\geq 10^{-6}$ mol L^{-1}) (*cf.*: 103) while a negative inotropic effect was seen at lower concentrations of Cch. Similarly, only 50 % of the concentration of Cch that brought about phosphatidyl inositolbisphosphate hydrolysis was enough to inhibit β-adrenergic induced cAMP-accumulation (14). Moreover, pretreatment with pertussis toxin, which will uncouple pertussis-toxin sensitive G_i proteins from adenylyl cyclase (75) (*cf.*: 39) inhibited the muscarinic agonist induced attenuation of the increase in cAMP due to β-adrenergic stimulation (32), while the phosphatidyl inositolbisphosphate hydrolysis was unhampered as demonstrated in cultured chick heart cells (75). These observations might link IP_3 to positive inotropy also after muscarinic stimulation (55). Muscarinic

receptor stimulation has also been shown to increase Ca^{2+} sensitivity in hyperpermeable rat heart trabeculae (50). Besides possible effects of IP_3, another explanation could be the accelerated dephosphorylation of troponin I, found to be an effect of cGMP upon β-adrenergic induced phosphorylation (33). Cyclic GMP activates cGMP-dependent protein kinase (64) which can modify the activity of cAMP-dependent protein kinase *via* a protein kinase modulator. This results in an attenuation of the cAMP induced response at the protein kinase level (65) (*cf.*: 93). The muscarinic agonist stimulation has been claimed to activate guanylyl cyclase. The role of cGMP in the heart is not yet fully understood (72). In the atria increased cGMP content upon NO treatment was suggested to inhibit phosphofructokinase leading to a decreased lactate level during hypoxia (68, 69). Moreover, cGMP was found to improve EC in hypoxic isolated rat hearts (67). Ventricular cGMP might have a modulating role as described above, *i.e.* the degree of phosphorylation of troponin-I might be an important mechanism regulating the tone and the rate of diastolic filling in the heart (*cf.*: 72). The myocardial content of cGMP is about 1 % of that of cAMP, with minimal changes in whole heart preparations after stimulation. Too low a sensitivity in the methods for determination of cGMP has also been considered (69) and the role of this second messenger in the heart is still spurious.

Several changes which could influence the second messengers have been described in the evolution of hypertrophy. This includes an unchanged number of receptors in the cardiomyocytes with increased size giving fewer receptors per surface area (20), downregulation of β-adrenergic receptors (decrease in number) (138), and low-affinity agonist binding to the remaining receptors suggesting an uncoupling of G_S (43) which may result in a desensitization of the β-adrenergic receptor-coupled pathway of adenylyl cyclase (20, 138). What is more, in cardiac hypertrophy the basic activities of phospholipase C, IP_3-kinase as well as IP_3-phosphatase were increased (109) which may influence the IP_3 level in hypertrophied hearts.

MATERIALS AND METHODS

Perfusion apparatus and perfusion technique

The isolated heart can either be retrogradely perfused through the aorta as originally described by Langendorff (1895)(66) or anterogradely *via* the left atrium (81, 83). All hearts have been anterogradly perfused in this thesis. In the anterogradely perfused heart both aorta and left atrium are cannulated which enables the heart to do external volume work and maintain its physiological role as a pump *in vitro* (working heart model). In this model preload as well as afterload could be controlled. Preload and afterload were kept constant at 10 cmH_2O and 85 cmH_2O, respectively, in all experiments included in this thesis.

The isolated perfused heart has advantages over isolated muscle preparation or muscle tissue slices since oxygen, substrates and hormones were supplied by a capillary network which make the rate of diffusion very high (80). It was noteworthy, however, that whole heart preparations did not permit discrimination of biochemical changes from different cell types within the ventricles (cardiomyocytes, Purkinje fibres and connective tissue). The stability of the heart preparation could be judged by measuring mechanical parameters like heart rate, coronary flow and aortic output.

Perfusion buffer

Krebs-Henseleit buffer was used throughout all experiments (63). Disodium salt of EDTA (0.5 mmol L^{-1}) was included to chelate trace quantities of heavy metals in the perfusate. Glucose (14 mmol L^{-1}) was used as exogenous substrate (133). Ascorbic acid (0.6 mmol L^{-1}) was added in order to avoid oxidation of noradrenaline. The recirculating volume was 100 mL and was kept at

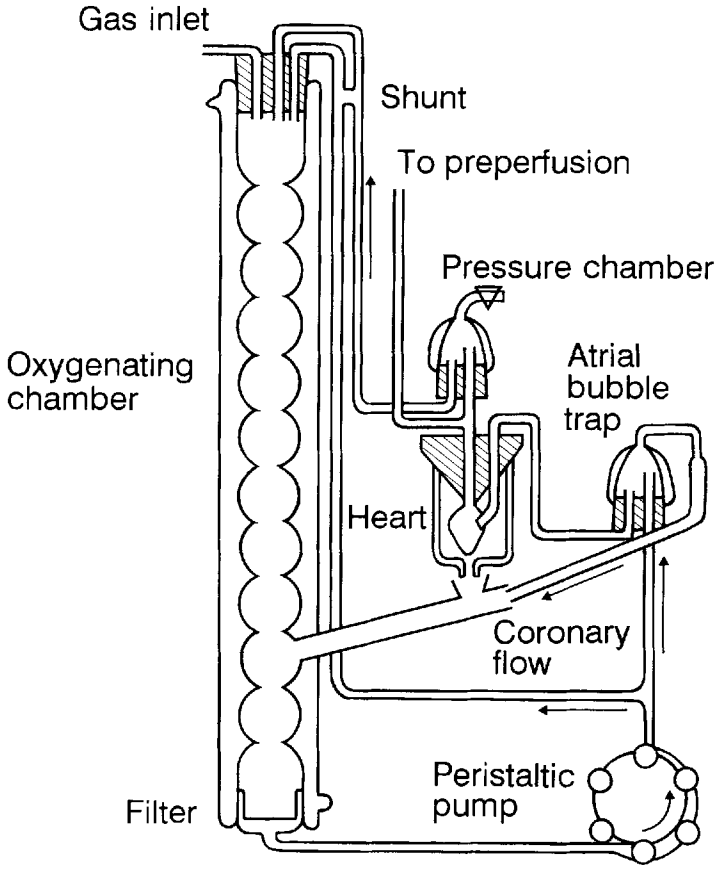

Fig. 3. Perfusion apparatus for isolated working rat heart. See text for description.

37°C by use of a water jacketed oxygenating chamber, atrial bubble trap, aortic pressure chamber and a glass container surrounding the working heart as depicted in Fig. 3. The buffer was

continuously gassed with 95% O_2 and 5% CO_2 equilibrated with water at 37°C maintaining the pH at 7.4 (7.35 - 7.45) (133).

Heart extirpation and preperfusion

After induction of anaesthesia the hearts were exposed through a transverse laparotomy followed by a bilateral thoracotomy. The diaphragm was transsected close to the ribcage. The whole anterior part of the thorax was lifted upwards, after which the pericardium was removed. The heart now exposed was carefully picked up by the fingers (I - III) and excised by a single scissor cut and placed in a beaker with ice-cold saline until the heart stopped beating. A drop of the buffer hung from the aortic cannula allowing fluid interface with the saline in the aorta, thereby avoiding air embolism (prevention of air embolism was a major concern). The aorta was attached to the aortic cannula by a ligature and retrograde perfusion was started. The preperfusion buffer was Krebs-Henseleit bicarbonate buffer identical to the buffer used in the experiment but without addition of ascorbic acid. The preperfusion buffer was discarded after one passage through the heart. The retrograde preperfusion period was 10 min for stabilization and washout of blood (Fig. 4). In the meantime the left atrium was cannulated and secured to the cannula by a suture. The hearts usually started to beat within seconds after initiation of the retrograde preperfusion. During the retrograde preperfusion the pressure in the aortic root was about 75 cm H_2O.

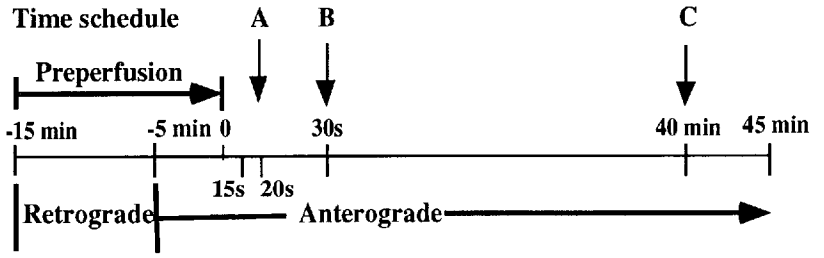

Fig. 4. Perfusion procedure and time schedule for freezing.
0, start of experimental procedure; A, 20 s; B, 30 s (inflecion point); C, 40 min.

Anterograde heart perfusion

The apparatus is shown in Fig. 3. Recirculation was instituted by clamping the tube from the preperfusion reservoir and unclamping the tubes from the atrial bubble trap and from the aortic cannula to the top of the oxygenating chamber. A peristaltic pump carried buffer to the atrial bubble trap where the overflow was returned to the oxygenating chamber by a sidearm. The heart (left ventricle) had to propulse the perfusion fluid to the top of the oxygenating chamber 85 cm above the heart (afterload). The buffer ejected by the heart first entered a pressure chamber filled with 2 ml of air to provide compliance (elasticity) in an otherwise rigid system. The exact volume of air was important for the shape of the aortic pressure curves. The coronary flow was returned to the oxygenating chamber by a separate tube and was measured, like the aortic output at the top of the oxygenating chamber by collecting the perfusate in a graded cylinder at predetermined

intervals. The cardiac output was calculated by adding the coronary flow to the aortic output. Another sidearm from the peristaltic pump provided an overflow to the oxygenating chamber giving optimal oxygenation of the system since a large volume of perfusate per min was exposed to the high oxygen tension in the oxygenating chamber. Since Cch induced bradycardia the Cch perfused hearts were paced to the rate observed during anterograde preperfusion (external pacemaker, Elema-Schönander, Stockholm, Sweden). Left ventricular pressure registrations were made through a 21 gauge cannula introduced into the basal part of the left ventricle. The cannula was connected to a pressure transducer (Abbot Scandinavia AB, Stockholm, Sweden). Ventricular pressure curves and peak rate of contraction during isovolumic period ($_{max}$dP/dt) were displayed on a direct ink writer (Siemens Elema, Stockholm, Sweden). External standards were used for correct measurement of the left ventricular pressure while the derivative was corrected to calibration standards (1000, 2000, 4000 and 10000).

After a 5 min stabilizing period of anterograde preperfusion the agonists (effectors) were added. The hearts were then perfused in one study for 15 s and 45 min an in the other for 20 s and 40 min after which they were freeze-clamped. At 20 s the maximal contractile response was registered in hearts receiving noradrenaline (NA) or noradrenaline plus carbachol (NA plus Cch). In two studies another group of hearts were perfused for 30 s before freeze-clamping. This time point corresponded to an inflection point at the pressure curve (at $_{max}$dP/dt) after NA and NA plus Cch stimulation, respectively. NA, 10^{-6} mol L^{-1}, in the perfusion buffer was known as a supramaximal stimulation of the heart from earlier studies (134). This concentration was therefore used whenever NA was added to the perfusion buffers. In one study different Cch concentrations were tested to find a combination with NA where the heart rate was similar to that of control hearts. Cch, $3x10^{-7}$ mol L^{-1} plus NA, 10^{-6} mol L^{-1} in combination resulted in a heart rate near the control situation. Hence, in the rest of the studies Cch, $3x10^{-7}$ mol L^{-1} was used when hearts were perfused with Cch alone and when the hearts were perfused with the combination of NA and Cch. The concentration of isoproterenol (Iso), 10^{-6} mol L^{-1} was chosen to make possible a comparison with the NA perfused hearts regarding contractility and it was similar to the concentration used in a recent study (107). A similarly high concentration of phenylephrine (Phe), 10^{-6} mol L^{-1} was used in order to secure supramaximal α-receptor stimulation as was the case for NA. Only rather high concentrations of dibutyryl cyclic adenosine monophosphate (db-cAMP) (139) resulted in a clear cut effect on contractility under the given conditions as revealed by a dose-response study (these data are not given in the thesis). The concentration used in the perfusion buffer was $2x10^{-4}$ mol L^{-1}. Furthermore, hearts were perfused without addition of agonists or effectors and freeze-clamped at 15 s and 45 min in one study as well as at initial time (0) and 40 min in the other studies (Fig. 4). These time points corresponded to the time point at which hearts were freeze-clamped after agonistic stimulation or effector perfusion.

Biochemical analyses

At the end of the experiments the hearts were instantly frozen with a Wollenberger clamp precooled in liquid nitrogen. The hearts were kept at -70°C and lyophilized. Biochemical analyses were performed on neutralized perchloric acid extracts of the lyophilized material (51). The extract content of adenine containing nucleotides was determined by high performance liquid

chromatography (34). Energy charge was calculated according to Atkinson (5). The concentration of cyclic nucleotides (cAMP and cGMP) was assessed by a radio-ligand method (Amersham, Buckinghamshire, England) (52). Lactate and creatine phosphate were determined by enzymatic methods (127). Creatine kinase activity in the effluent part of the perfusion fluid was determined according to the Scandinavian Committee of Enzymes and expressed in μkat L^{-1} (126).

IP_3 analysis of myocardial tissue was the result of different separation techniques using tracer IP_3 added to biological specimens (radioactive D-myo-(2-^{3}H)inositol 1,4,5-trisphosphate) including fast performance liquid chromatography (Pharmacia Fine Chemicals, Sweden), Sephadex G 10 chromatography and isotachophoresis in accordance with a previous publication (132). The isotachophoresis apparatus (ITABA, Delta Tachophor (3-5) Stockholm, Sweden) was equipped with a pre-column (35) and a separation capillary, inner diameter of 0.3 mm, connected to a 3-channel recorder (Linseiss L 6414, Germany). In isotachophoresis ion species of the same

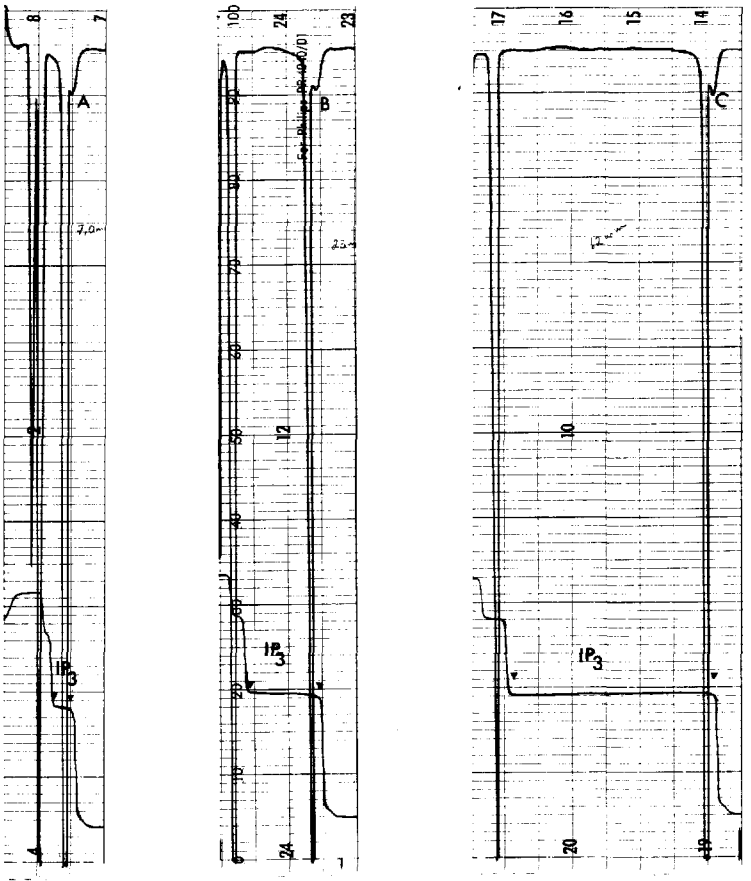

Fig. 5. Conductivity tracing of IP_3. Isotachophoretic runs of myocardial extracts in the absence (A) and presence of exogenous IP_3; enriched with 750 pmol IP_3 (B) and 2250 pmol (C).

sign migrate in an electrical field with all ions moving with the same velocity at equilibrium. Thus

differences in charge of molecules will be separated. IP_4, IP_5 and IP_6 as well as IP_2 and IP_1 will therefore not interfere with the determination of IP_3. However, this method allows only determination of free total IP_3 and does not distinguish between IP_3 isoforms. The migration takes place when an electric field is applied to a system of electrolytes consisting of a leading and a terminating electrolyte and a polarity that directs the movement of ions to be measured towards the leading electrolyte and the detectors. The leading electrolyte for isotachophoretic separation consisted of a mixture of 10 mmol L^{-1} HCl and hydroxypropylmethylcellulose (Dow Chemicals, Midland, USA) which was further purified by ITABA (Stockholm, Sweden). 0.5%/ H_2O q.s. and β-alanine (3-amino propionate, Sigma, Mo, USA) was added to pH 3.88. The terminating electrolyte was 10 mmol L^{-1} succinic acid (Merck, Darmstadt, Germany). Detection was carried out by monitoring conductivity changes. To secure reproducibility known amounts of IP_3 (inositol 1,4,5-trisphosphate, potassium salt from Sigma Company, Mo, USA) were added to the same preparation of heart muscle extract before the FPLC separation and used as internal standards. Figure 5 illustrates isotachoporetic runs of myocardial extracts in the absence (A) and presence of 750 pmol exogenous IP_3 (B) and 2250 pmol exogenous IP_3 (C) as internal standards. This methodology allowed the measurement of IP_3 in the pmolar range with a recovery for IP_3 that was nearly 100 % (132). Since the IP_3 molecule is negatively charged, it has a strong tendency to adhere to glass surfaces. Therefore, HCl-washed glass material was consistently used during the biochemical analysis for IP_3 content. The total imprecision of the method displayed a coefficient of variation of 3 % (132).

Statistics

The statistical methods used are presented separately in each paper. All values are reported as means ± SEM (Papers II - V) and means ± SD (Paper I).

RESULTS

Effects of adrenergic stimulation

Mechanical

By addition of NA an expectedly early and excessive increase in $_{max}$dP/dt was produced. $_{max}$dP/dt reached a maximum of about 100 % above initial value at 20 s followed by a marked decrease reaching its inflection point at 30 s. Hereafter $_{max}$dP/dt decreased slowly approaching control or near control levels at 40 and 45 min. Cardiac output was reduced significantly already after 5 min of perfusion by addition of NA, while coronary flow was relatively stable. However, after 40 min of perfusion coronary flow was significantly decreased although the biological significance may be less obvious (20 ml min^{-1} to 18 ml min^{-1}). HR was significantly increased during the first 15 min after addition of NA. At 30 min HR declined to control value. When NA was added to POH-hearts $_{max}$dP/dt underwent increases and decreases not different from those described in normal hearts. However, the decline in $_{max}$dP/dt after 40 min of

perfusion was different as it was significantly increased at termination of the experiments. Iso perfusion induced similar changes in $_{max}$dP/dt with those seen in normal hearts after NA perfusion. However, the maximal $_{max}$dP/dt was unexpectedly lower than the one found after NA perfusion. AO was significantly decreased by the addition of Iso similar to the changes seen after addition of NA. Phe perfusion induced a slow but significant increase in $_{max}$dP/dt reaching a maximum after 10 min, after which it decreased approaching control level at 30 min. No significant decrease was found in AO during Phe perfusion.

Second messengers

NA perfusion did not influence myocardial IP_3 content during the first 30 s (Fig. 6). After 40 min of perfusion IP_3 was increased by about 56 %. In POH-hearts perfused with NA the myocardial IP_3 content was significantly increased (53 %) already after 20 s of perfusion. After 40 min the IP_3 content was decreased still being higher than POH-hearts after control perfusion ($P<0.05$). Iso- and Phe perfusion in normal hearts produced a significant and similar increase (about 27 %) in IP_3 content after 40 min.

NA addition resulted in a rapid and decisive increase in myocardial cAMP content being highest after 15 s of perfusion (Fig. 6). cAMP was decreased already after 20 s approaching control level at 30 s. After 40 min of perfusion cAMP again reached the same level as seen after 20 s (being slightly higher than the one seen after 45 min). In POH-hearts NA induced a significant increase after 20 s similar to the one seen in normal hearts. In comparison to normal hearts cAMP content was lower after 40 min of perfusion. Myocardial cAMP content was about 2 fold higher than control after 40 min of Iso perfusion. Phe produced only a minor increase in cAMP content (not significant).

None of the used adrenergic agonists induced changes of cGMP content in normal ventricular myocardium. On the other hand, NA produced a significant increase in cGMP when added to the buffer in perfused POH-hearts.

Metabolic effects

The increase in $_{max}$dP/dt by NA at 20 s of perfusion was concomitant with a substantial decrease in ATP content (about 50 %)(Fig. 8). It is noteworthy that the decrease in myocardial ATP was seen already after 15 s although not being significant. The ATP content was further decreased after 40 and 45 min of perfusion. Myocardial ATP content was also decreased and to the same extent by perfusion with Iso, while Phe perfusion resulted in less ATP depression although being significantly decreased compared to control. An interesting dissociation between ATP and CP was observed with ATP being low and CP being high at 40 min of NA perfusion. Contrary to normal hearts NA perfusion in POH-hearts did not show early (20 s) changes in myocardial ATP content. However, after 40 min the ATP content was decreased to a level similar to the one seen in normal hearts.

Effects of muscarinic stimulation

Mechanical

Cch addition induced a minor decrease in $_{max}$dP/dt except when used in the concentration 10^{-5} mol L^{-1}. The hearts stopped beating at this concentration. When paced to a fixed rate $_{max}$dP/dt decreased significantly compared to control value. Normal- and POH-hearts paced to the same rate as during the stabilizing anterograde preperfusion did not deteriorate in contractile performance. HR was decreased with an increased concentration of Cch in the perfusion buffer. Addition of $3x10^{-7}$ mol L^{-1} induced a decrease in HR by about 33 %. CO and CF were stable and not different from control perfused hearts during perfusion with Cch ($3x10^{-7}$ mol L^{-1}).

Second messengers

Cch in the perfusion buffer lead to an increase in myocardial content of IP_3 at an early (20 s) stage while the IP_3 content was within control range after 40 min of perfusion (Fig. 6). Also, a significant increase in IP_3 content was seen already after 20 s of perfusion in POH-hearts. Contrary to normal hearts the content was in the same range in POH-hearts after 40 min of perfusion being significantly higher than in control perfused hearts (Fig. 7).

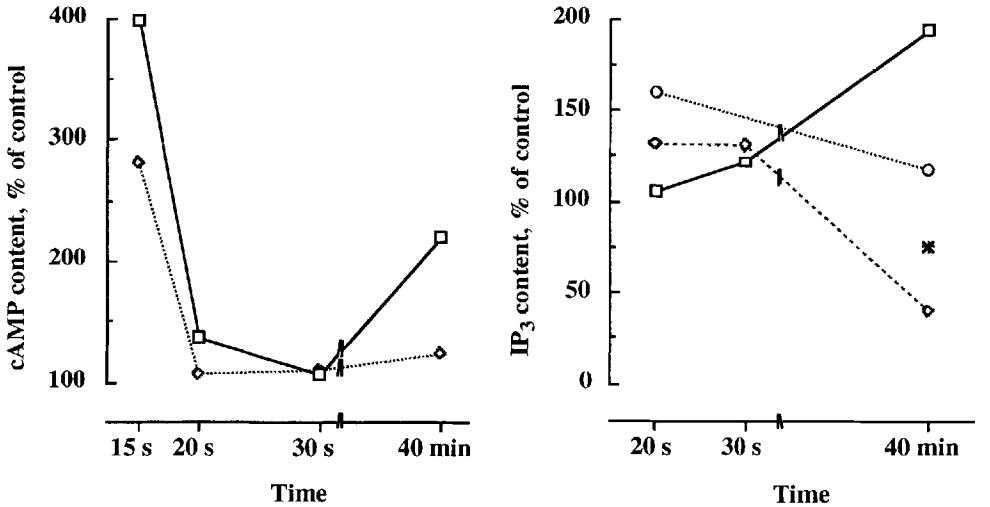

Fig. 6. Myocardial IP_3 and cAMP contents after noradrenaline, carbachol and combined noradrenaline plus carbachol stimulation or perfusion with db-cAMP. NA, noradrenaline; Cch, carbachol. Left panel; **cAMP:** □, NA; ◇, NA+Cch. Right panel; **IP_3:** □ NA; ○, Cch; ◇, NA+Cch; ✱, db-cAMP.

Addition of Cch did not change myocardial ventricular cAMP content when compared to control. cGMP content was initially increased in POH-hearts after addition of Cch being subsequently decreased reaching control level after 40

min of perfusion while cGMP content was unchanged and comparable to control level in normal hearts.

Metabolic effects

No change in ATP content was seen at 15 s after addition of Cch. A decreasing content was seen from 20 s to 45 min, where the decrease was pronounced and significant. By addition of Cch in POH-hearts a significant decrease in ATP content was seen after 40 min compared to that seen at 20 s. The decrease was less than the decrease seen after adrenergic stimulation.

Effects of combined adrenergic and muscarinic stimulation

Mechanical

When hearts were perfused with the combination of NA plus Cch $_{max}$dP/dt was increased 2 fold compared to initial value (Fig. 6). When hearts were initially perfused with NA and thereafter stimulated with increasing concentrations of Cch there was a further increase in maximal $_{max}$dP/dt compared to stimulation with NA alone. If NA and Cch were added together to the perfusion buffer the maximal $_{max}$dP/dt was smaller than after perfusion with NA alone still being about 100 % increased compared to initial value. This was also found in POH-hearts. Furthermore, $_{max}$dP/dt was decreasing slowly as in normal hearts and not different from the hearts perfused with NA alone. However, after 40 min of NA plus Cch perfusion in POH-hearts $_{max}$dP/dt was significantly higher than after control perfusion. HR was decreased when NA was combined with increasing Cch concentrations whereas perfusion with the combination of NA plus Cch seemed to prevent heart stand still. When Cch ($3x10^{-7}$) was added to NA perfused hearts the HR was stable and not different from control perfused hearts.

Second messengers

Concomitant addition of NA plus Cch resulted in an initial increase in ventricular myocardial IP_3 content. The increase was smaller than the increase seen after perfusion with Cch alone but higher than the content seen after NA alone. A conspicuously low value of IP_3 was noted after 40 min of combined stimulation with NA plus Cch (Fig. 7). The combined perfusion with NA plus Cch resulted in a significant increase in IP_3 content in POH-hearts compared to control but the increase was not comparable to the one observed after perfusion with either NA or Cch ($P < 0.05$). IP_3 content was unchanged after 40 min and in the same range as after perfusion with each of the agonists.

The myocardial cAMP content was increased by about 100% after 15 s of perfusion being only about 50 % of the increase seen after stimulation with NA alone. No increase occurred after 20 s. No change was seen at 30 s and 40 min in myocardial cAMP content. Also, cAMP content was unchanged at 20 s and 40 min in POH-hearts perfused with the combination of NA plus Cch. cGMP

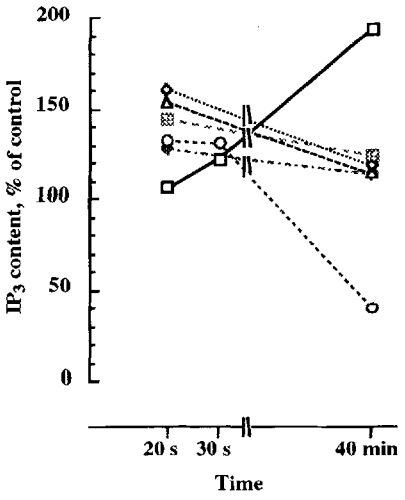

Fig. 7. Myocardial IP_3 content in normal and hypertrophied hearts after stimulation with noradrenaline, carbachol or the combination noradrenaline plus carbachol. Note that time scale is not real. NA, noradrenaline; Cch, carbachol. **Normal:** □, NA; ◇, Cch; ○, NA+Cch; **Hypertrophied:** △, NA; ⊞, Cch; ◆, NA+Cch.

content was unaltered after the addition of NA plus Cch in normal heart. On the contrary, cGMP content was increased early (20 s) and late (40 min) after addition of NA plus Cch in POH-hearts.

Metabolic effects

Only minor changes were seen in myocardial ATP content after 15 s and 20 s of perfusion with the combination NA plus Cch (Fig. 8). The decrease was significant after 30 s but not different from hearts perfused with NA alone. A pronounced, significant decrease was also seen after 40 min where the content was in the same range as after perfusion with NA alone. No early (20 s) changes were observed in POH-hearts (Fig. 8). After 40 min the ATP content was decreased to the same extent as the content seen after perfusion with NA alone .

Effects of dibutyryl cAMP

Mechanical

Addition of db-cAMP to the perfusion buffer resulted in a slowly increasing $_{max}$dP/dt with the highest value just prior to termination of the experiment. AO and CF were unchanged after addition of db-cAMP during the 40 min perfusion time and not different from control.

Second messengers

Ventricular myocardial IP_3 content was decreased after 40 min of db-cAMP perfusion compared to control (Fig. 6). At the same time point myocardial cAMP content was in the same range as the one seen when hearts were perfused

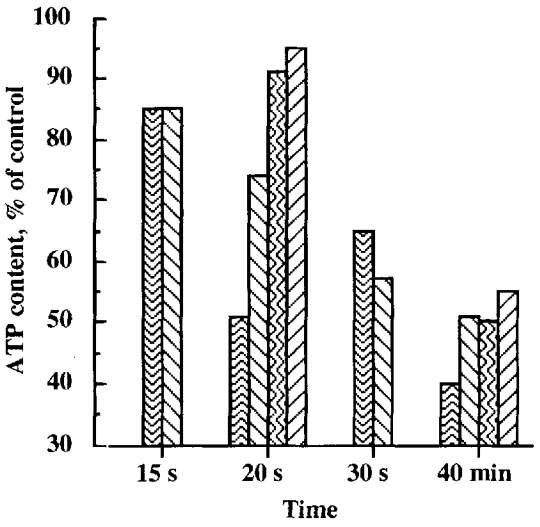

Fig. 8. Myocardial ATP content in normal and hypertrophied hearts stimulated with noradrenaline or the combination of noradrenaline plus carbachol. NA, noradrenaline; Cch, carbachol. **Normal:** ▨, NA; ▧ NA+Cch; **Hypertrophied:** ▨, NA; ▨ NA+Cch.

with NA or Iso.

Contrary to agonistic perfusion db-cAMP induced a decrease in myocardial cGMP content.

Metabolic effects

Forty min of perfusion with db-cAMP induced an ATP turnover well comparable with the metabolic effects seen after NA, NA plus Cch and Iso perfusions.

Discussion

Ventricular IP_3 content in whole heart preparation was increased after muscarinic as well as adrenergic stimulation in agreement with previous studies on cardiomyocytes (98, 141) and myocardial tissue (60, 95, 141). Hence, we may conclude that IP_3 formation is the result of agonist stimulation of both receptor types. The increased IP_3 content after muscarinic stimulation with Cch was seen early after stimulation indicating that the experimental model and the analytical method were sensitive enough to demonstrate rapid changes in IP_3 content. In spite of this, we could not find an expected early (30 s) increase in IP_3 content upon NA stimulation as reported by others using different heart tissue preparations (62, 95, 107, 114, 139). Importantly, all of the mentioned studies were performed with the addition of a β-blocker. The discrepancy in results between the mentioned studies and our raised the question whether concomitant β-adrenergic stimulation (as in our study) could attenuate α_1-adrenergic IP_3 response. Edes (28) reported a decreased phophatidyl

inositolbisphosphate-phospholipase C activity upon Iso stimulation with addition of an α_1-adrenergic blocker in guinea pig hearts leading to a reduction in IP_3 release. The tissue levels of IP_3 isoforms were decreased about 25 % after 3 min of perfusion (28). Furthermore, phosphatidyl inositolbisphosphate turnover decreased over time (28) which could be part of the explanation for the blunted IP_3 response early upon concomitant α_1- and β-adrenergic stimulation as was the case in our study. The inotropic response after α_1-adrenergic stimulation was found to be attenuated upon concomitant β-adrenergic stimulation (92) which could be an indication of decreased phosphatidyl inositolbisphosphate hydrolysis and thereby decreased IP_3 and DAG contents. Moreover, the IP_3 content found after 40 min of perfusion (late) was decreased in non stimulated hearts after circumvention of the receptor-mediated stimulatory step by the use of db-cAMP, *i.e.* cAMP *per se* depressed basal content of IP_3. This observation had some support in the literature where cAMP was found to regulate IP_3 kinase purified from rat brain (110). Furthermore, the agonist stimulated phosphatidyl inositolbisphosphate-phospholipase C was inhibited by cAMP at high levels in another cell system (26) although not found in α_1-stimulated rat hearts when perfused with db-cAMP for up to 20 min (141). Both modes of interaction, *i.e.* the attenuation at the transduction level by concomitant α_1- and β-adrenergic stimulation and the modulation of disappearance rate of IP_3 by db-cAMP, constitute mechanisms of cross-talk between cellular signalling pathways that use different receptors and various second messengers. However, our observation that db-cAMP depressed basal IP_3 content was done only after 40 min of perfusion. The content of cAMP at that time point was increased to the same level as after Iso perfusion. The kinetics of internalisation and hydrolysis of db-cAMP were not studied. Therefore no conclusion can be drawn about time related events involving interaction with the inositol pathway. In contrast to what could be expected from the above mentioned proposals stimulation with both NA and Iso resulted in late (40 min) increases in IP_3 content (60 % and 30 %, respectively). Both agonists had at this time point also produced significant increases in cAMP levels as expected (19, 28). The effect of Iso on α-receptors was small (136) and in agreement with this no changes in IP_3 content were found in rat hearts after 15 min of perfusion when propranolol was added (107). However, absolute specificity for α- or β-receptors was not found of any catecholamine although Iso was found to be relatively specific for the β-receptor (especially at lower concentrations) (*cf.*: 136). NA exhibited a well known α_1-adrenergic effect (15, 112, 141) which may account for the increased IP_3 content at 40 min. Considering the continuous β-adrenergic stimulation achieved by NA the above described (early) modulating effect of concomitant β-adrenergic stimulation on α_1-adrenergic IP_3 production was somehow diminished over time. This could be explained by a sustained β-adrenergic

produced deactivation of phospholipase C eventually leading to a desensitisation of this complex. The same mechanism could apply for factors modulating the disappearance rate of IP_3. Moreover, the pronounced (about 4 fold) increased myocardial cAMP content seen about 5 to 15 s prior to the early blunted IP_3 response might contribute to the difference in early and late attenuation as such an increase in cAMP content could not be expected just prior to the late observations.

An early increase in IP_3 content upon muscarinic stimulation was in accordance with the literature (6, 61, 98). However, the elevated IP_3 content was produced upon stimulation with a low Cch concentration in agreement with a previous publication (60). Muscarinic stimulation also increased inositol mono-, bis-, tris- and tetrakisphosphates in isolated cardiomyocytes from rats (6). $IP_3(1,4,5)$ was increased about 3 fold after 20 s of Cch stimulation while the isoform $IP_3(1,3,4)$ was increased only slightly until 5 min of stimulation after which it increased about 3 fold (6). At 15 min $IP_3(1,4,5)$ and $IP_3(1,3,4)$ were increased by about 100 % and 200 % compared to control values, respectively (6). The experiments were carried out in the presence of LiCl, why the response under physiological conditions could differ. At 40 min IP_3 content was in the control range in spite of continuous stimulation by Cch. This could imply desensitisation of the signalling pathway of the Cch receptor. Increasing concentrations of Cch produced decreased force of contraction in auricular tissue (60) probably due to potassium conductance *via* a K^+-channel (*cf.*: 72). No changes in force of contraction were found in papillary muscles at lower concentrations (60) in analogy with our study. However, at higher concentrations ($\geq 10^{-5}$ mol L^{-1}) papillary muscles even showed significant increase in force of contraction (60) also found by us. Through pretreatment with pertussis toxin which uncoupled G_i from adenylyl cyclase (75) Cch was found to increase inotropy (61), *i.e.* the muscarinic receptor took part in the hydrolysis of phosphatidyl inositolbisphosphate and given specific prerequisites also in the increase of inotropy. The physiological significance of the high Cch concentration needed to induce positive inotropy has been questioned (28). However, the presence of high and low affinity states of the muscarinic receptor (see INTRODUCTION) connected to different signalling pathways, respectively, indicates a physiological role of the two states. As a corollary, IP_3 may under physiological conditions function as one modulator of the total effects induced by muscarinic receptor stimulation.

Through concomitant NA and Cch perfusion we stimulated both α_1- and β-adrenergic as well as muscarinic receptors at the same time. This triple receptor stimulation described previously in a rat papillary experiment (22) might have relevance to the clinical setting (see INTRODUCTION). Concomitant α_1-adrenergic and muscarinic stimulation increased IP_3 content in an additive way

in cardiomyocytes from rat ventricles (15). This may indicate that the two receptors did not compete for a common limited amplification system (15). Early after the triple receptor stimulation myocardial IP_3 content was increased to a level in between the contents seen after NA and Cch alone, respectively. The IP_3 content was unchanged after concomitant α_1- and β-adrenergic stimulation (see above). Therefore, the combined α_1- and β-adrenergic stimulation was less likely involved in the increase in IP_3 seen after the triple receptor stimulation. Consequently, one explanation could be that the increase in IP_3 content arises from the muscarinic receptor signalling pathway. As the increase in IP_3 content was lower by triple receptor stimulation than after muscarinic receptor stimulation alone a possible β-adrenergic induced attenuation of the Cch effect on IP_3 production could not be excluded. However, the attenuated β-adrenergic adenylyl cyclase response upon concomitant Cch stimulation may also contribute to the change in IP_3 content. Hence, as β-adrenergic stimulation decreased IP_3 content *per se* (see above) (28) a low cAMP content would result in increased IP_3 levels. The actual IP_3 increase early after triple stimulation could therefore also be due to combined α_1-adrenergic and muscarinic stimulation. However, the mechanism(s) responsible for the early IP_3 change upon triple stimulation could not be elucidated by the present studies. The nearly 60 % decrease in IP_3 content after 40 min of triple receptor stimulation could again be due to the attenuation by concomitant β-adrenergic stimulation mentioned above. However, the cAMP content at this time point was at or near control level. Hence, if the effect upon myocardial IP_3 content could be related to β-adrenergic stimulation, the mechanism of attenuation should probably be related to the transduction phase. This, however, raised the question why the combined α_1- and β-adrenergic stimulation resulted in increased IP_3 content as well as increased cAMP content at 40 min when the triple stimulation resulted in decreased IP_3 and unchanged cAMP contents. The explanation for those seemingly contradictory results were not clarified in the present studies. However, the attenuation of the β-adrenergic response upon concomitant Cch stimulation (which left myocardial cAMP content at the control level) may also change desensitisation properties in the β-receptor signalling pathway. Hence, beside a possible direct action (of the concomitant α_1- and β-adrenergic stimulation) on the transduction phase an unchanged or increased sensibility of the signalling systems to cAMP could contribute to the decreased IP_3 content after triple stimulation. This idea might have some support in our observations that concomitant Cch stimulation did not depress the increase in the contractile response upon NA stimulation in spite of low cAMP concentrations.

We did not find any direct relation between myocardial IP_3 content and contractility neither after adrenergic nor after muscarinic stimulation nor after the combined stimulation. In this context we want to emphasize that the β-

adrenergic component determined the quality of the response to NA perfusion, *i.e.* the possible α_1-adrenergic contribution to increased contractile response upon concomitant α_1- and β-adrenergic stimulation was difficult to evaluate when no antagonist was used (22, 112). The decreased level of IP_3 late during perfusion with triple stimulation did not change contractility. The increased IP_3 content after 40 min of Phe perfusion, where the contractility was at control level did not exclude an IP_3 related increase in contractility (106). The maximal contractility was found at 10 min in agreement with other studies (106) indicating that α_1-adrenergic induced increase in contractility was a slow process (112). At this time point no biochemical analysis was carried out in our study.

The rapid (more than 2 fold) increase in $_{max}$dP/dt upon NA stimulation was preceded by a 4 fold increase in cAMP at about 5 s prior to the contractile peak in agreement with other publications (19, *cf.*: 27). Despite the continuous β-adrenergic stimulation achieved by NA, the cAMP level was not continuously elevated. Already at 20 s the cAMP level was decreased and at 30 s the cAMP content was at control level, in spite of increased contractility. Activation of protein kinases was delayed in relation to the increased myocardial content in cAMP (*cf.*: 27). A relative receptor refractoriness (*cf.*: 115) may be present contributing to the decreased adenylyl cyclase activity. Furthermore, adenylyl cyclase activity was reduced by activated calmodulin which also activated cyclic nucleotide phosphodiesterase (135). cAMP was elevated above control level late in the perfusion despite the contractility being at the control level. The elevated cAMP content late during perfusion with a contractile response at the control level is puzzling and not previously reported.

Attenuation of the β-adrenergic induced elevation of myocardial cAMP levels upon concomitant muscarinic (Cch) stimulation were in agreement with previous publications in atria (32) and ventricles (71). cAMP content was increased about 2 fold 5 s prior to the maximal response in $_{max}$dP/dt. The increase in cAMP content was about 50 % of that seen after NA stimulation alone. The contractile response was near or at the same level as the response seen after NA alone indicating that cAMP might not be the only determinant for the contraction (91). The muscarinic agonist decreased the activity of adenylyl cyclase resulting in decreased cAMP content through activation of G_i. However, this decreased cAMP content produced the same contractile response indicating a possible sensibilization of the β-adrenergic related signalling pathway upon concomitant Cch stimulation. These reflections would be in line with the one mentioned under IP_3 attenuation (see above). Another suggestion could be an increased α-adrenergic inotropic component of NA upon concomitant Cch stimulation as found in rat papillary muscles (22).

High concentrations of catecholamines can induce myocardial damage as evidenced by, *i.a.* an increased release of CK. Addition of all agonists used in the studies resulted in increased ATP turnover. Early after concomitant NA plus Cch perfusion (20 s) a possible energy sparing effect was noted. This effect could be ascribed to the decreased heart rate upon Cch stimulation. The NA induced enzyme release was prevented by concomitant Cch perfusion. However, energy state did not seem to be directly related to the degree of myocardial enzyme release since energy charge was practically the same regardless of which agonist(s) being included in the perfusion buffer. Cch may therefore have the potential of being cardioprotective in another, hitherto, not fully understood way that may include receptor cross-talk resulting in inhibition of β-receptor signal transduction (see Fig. 2).

The POH-hearts demonstrated aberrant features regarding function and metabolism including an altered membrane phospholipid composition where a 20 % increase in phosphatidyl inositol content was seen (99). In response to pressure overload an increased protein synthesis (79) including protein kinase C was noted (42). However, we did not find any difference between basal myocardial IP_3 contents in POH-hearts compared to hearts from sham-operated rats. IP_3 contents in POH-hearts were increased upon agonistic stimulation (NA, Cch and NA plus Cch, respectively) concordant to what was found in hearts from spontaneously hypertensive rats after NA-stimulation (59). The increased basal phospholipase C activity (109) might contribute to the increased IP_3 content after agonistic stimulation. The elevated IP_3 content after 20 s of NA stimulation may also indicate changes in attenuating properties, *i.e.* if concomitant β-adrenergic stimulation attenuated (28) the α_1-adrenergic induced IP_3 elevation early in the perfusion of normal hearts (see above), this attenuating effect was abolished in POH-hearts. The reason for that is not clear from the present studies. However, hypertension-induced β-receptor downregulation (43, 138) as well as desensitization of the β-receptor-related cellular signalling pathway (20, 43) could contribute to the altered pattern of attenuation. Hence, a decreased β-adrenergic modulation of the α_1-adrenergic response suggested an additive effect on IP_3 content upon combined NA and Cch stimulation as seen in cardiomyocytes from normal rats (15). In contrast, IP_3 content was significantly decreased upon the combined NA and Cch stimulation compared to perfusion with one of the two agonists alone. Late in the perfusion IP_3 content was elevated after agonistic stimulation contrary to what we found in normal hearts. This could be due to changes in attenuating properties as already mentioned, but could also be the result of changes in the inositol phosphate pathways induced by pressure overload, *i.e.* increased production of IP_3 isoforms. This view might have some support in an increased IP_3 kinase activity found in hypertrophic hearts from spontaneously hypertensive rats (59).

Contrary to what we found in normal hearts, myocardial cAMP content late after NA perfusion was significantly decreased in POH-hearts. This decrease which was present in spite of continuous agonist stimulation could be the result of downregulation of the β-receptor as seen in volume overload induced hypertrophy (43). Hence, the number of β-receptors was unchanged, but the density was decreased in POH-hearts (20). In both types of hypertrophy a change at a postreceptor level was found (20, 43). An uncoupling from G_s was suggested while no change was observed in pertussis toxin-mediated labelling, suggestive of unchanged G_i (43), *i.e.* the decreased cAMP content could be related to a decreased adenylyl cyclase activity due to modulation at the transduction phase. The attenuation of the β-adrenergic-produced elevation of myocardial cAMP content upon concomitant muscarinic stimulation was *n.b.* preserved in POH-hearts. Furthermore, the contractile response upon NA plus Cch stimulation (triple stimulation) was not different from the response upon NA stimulation alone. The unchanged increased contractility upon NA stimulation in POH-hearts was not confirmed after Iso stimulation (20). However, these authors measured dP/dt *via* a balloon introduced in the left ventricle which only allowed measurement of isometric tension development.

Previous observations that a myosin isoform shift evoked a decreased contraction velocity which allowed the heart to produce tension at a lower cost (4, 47, 48) may be the reason for normal ATP content in POH-hearts early after agonistic stimulation.

cGMP content increased after activation of guanylyl cyclase by hydrolysis of GTP. cGMP regulates ion channels and cAMP concentrations through its selective action on phosphodiesterases (*cf.*: 21, 137). The role of cGMP as a second messenger in the myocardium remains unclear, *i.e.* none of the agonists used increased myocardial cGMP content above control range in normal hearts in line with previous observations (69). However, cGMP was elevated in POH-hearts upon agonistic stimulation (NA, Cch and the combination of NA plus Cch, respectively) suggesting a possible physiological role for cGMP in hypertrophied ventricles.

CONCLUSIONS

The following conclusions can be drawn from the present experiments in isolated perfused rat hearts.

1 NA-induced myocardial damage, *i.e.* increased CK release, was prevented by muscarinic agonist stimulation by Cch. One important factor could be a decrease in heart rate. Another could be receptor cross-talk resulting in inhibition of the β-receptor signal transduction.

2 NA stimulation induced late increase in IP_3 content while Cch stimulation produced early increase in IP_3 content in isolated normal rat hearts. No direct relation between myocardial IP_3 content and contractility was found neither after adrenergic nor after muscarinic stimulation.

3 Combined adrenergic (α_1- and β-) stimulation concomitant with muscarinic agonist stimulation attenuated the IP_3 response in isolated normal rat hearts.

4 cAMP depressed *per se* basal myocardial IP_3 levels in isolated normal rat hearts.

5 Contractile response correlated initially to changes in myocardial cAMP content. After the initial peaking as well as late in the perfusion no direct relation between myocardial cAMP content and contractile response was found. Concomitant muscarinic and adrenergic stimulation significantly decreased cAMP response with no or only minimal changes in contractile response. cAMP seems important in early regulation of contractility, but other factors induced at a later stage might be important modulators.

6 POH-hearts displayed myocardial IP_3 content not different from sham-operated hearts after control perfusion. NA and Cch alone increased the myocardial IP_3 content significantly and to about the same level. The combination of the two, however, also induced an increased myocardial IP_3 level, although to a lesser extent.

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