The Effect of Acetylcholine, Ischemia, and Anoxia on Rat Heart Purine Cyclic Nucleotides and Contractility

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SUMMARY The effects of acetylcholine, ischemia, and anoxia on myocardial cyclic GMP and cyclic AMP were investigated in isolated rat hearts perfused under either constant flow (14 ml/min) or constant pressure (100 cm H2O) conditions with physiological saline gassed with 85% O2-5% CO2. In paced, constant flow-perfused hearts, cyclic GMP increased from a control of 35 to 85 fmol/mg protein and remained elevated, while dP/dt, an index of contractility, decreased only transiently from 2,859 to 1,750 mm Hg/sec then returned toward control levels during the first 2 minutes of 1 μM acetylcholine. In paced, constant pressure-perfused hearts, the acetylcholine caused a persistent decrease in dP/dt and a transient increase in coronary perfusate flow, followed by a marked decrease in flow. Reduction of coronary perfusion to one-half control flow and ischemia (termination of perfusion) increased cyclic GMP from 43 to 85 and 127 fmol/mg protein, respectively. Anoxia (perfusion with physiological saline gassed with 95% N2-5% CO2) did not produce a significant change in cyclic GMP. Neither atropine nor indomethacin at 10 μM prevented the increase in cyclic GMP produced by ischemia, but the muscarinic blocking agent did block the increase caused by acetylcholine. Cyclic AMP increased from a control of 3.8 to 6.0 pmol/mg protein with anoxia and to 12.8 pmol/mg protein with 1 μM isoproterenol, but was not affected by acetylcholine or ischemia. Myocardial lactate increased significantly only with ischemia.

Ca-free perfusion for 1 minute partially blocked the increase in cyclic GMP caused by ischemia, but did not prevent the acetylcholine-induced increase in the cyclic nucleotide. These results indicate: (1) that there is not always an inverse relationship between myocardial cyclic GMP and contractility and (2) that limitation of coronary perfusion augments cardiac cyclic GMP by a mechanism(s) that is independent of acetylcholine but largely dependent on external Ca2+.

Gardner and Allen, 1976). The enzymes responsible for cyclic nucleotide synthesis, namely adenylate cyclase and guanylate cyclase, are present in the mammalian myocardium (Drummond and Duncan, 1970; Limbird and Leffkowitz, 1975).

Acetylcholine has been shown to produce a rapid concentration-dependent increase in cGMP concentration in the isolated rat heart that correlates with the magnitude of the cholinergic-elicited decrease in myocardial contractility (George et al., 1970, 1973). However, it has been reported that low concentrations of acetylcholine and carbamylcholine (carbachol) depress contractility in isolated atrial preparations without producing a detectable increase in cGMP (Brooker, 1977; Diamond et al., 1977). Acetylcholine has also been reported to increase cGMP in the isolated guinea pig heart without significantly affecting contractility (Watanabe and Besch, 1975). Other studies suggest that the increase in cGMP elicited by agents other than acetylcholine do not correlate with changes in contractility. For example, sodium nitroprusside and sodium azide have been shown to increase cardiac cGMP without affecting contractility (Diamond et al., 1977; Katsuki et al., 1977).

Whereas a great deal of information is available regarding the effect of cholinergic agents on myocardial cGMP, much less is known about the effect of ischemia and anoxia on cardiac purine cyclic...
nucleotides. In dogs, chronic coronary ligation (3-35 days) has been reported to produce an increase in cGMP and a decrease in cAMP in the myocardium that was previously perfused by the ligated arteries (Stewart et al., 1978). Acute ischemia in the in vivo rat heart has been shown to produce an increase in cAMP within seconds (Dobson and Mayer, 1973). Hypoxic perfusion of isolated rat hearts (12% O2-84% N2-4% CO2) resulted in an increase in cAMP and a decrease in myocardial cGMP (Nesher et al., 1977). Anoxic perfusion (95% N2-5% CO2) of isolated rat hearts has been reported to cause a sustained increase in myocardial cGMP and only a transient increase in cAMP (Busuttil et al., 1976; Busuttil et al., 1978). Thus, the above reports might suggest that the effects of ischemia and anoxia on myocardial cyclic nucleotides are unresolved. However, the depressant effects of ischemia and anoxia on cardiac contractility are well recognized (Dobson and Mayer, 1973; Rovetto et al., 1973).

The purpose of this investigation was to study the relationship between myocardial cGMP concentration, contractility, and coronary flow in the myocardium of isolated hearts subjected to either constant pressure or constant flow perfusion and cholinergic stimulation. In addition, the effect of ischemia and anoxia on cardiac contractility and coronary flow in the myocardium was studied and the importance of Ca2+ in cyclic nucleotide formation investigated.

Methods

Heart Preparation

The isolated perfused rat heart preparation used in these studies was similar to one described previously (Dobson et al., 1974). Male Sprague-Dawley rats (250-350 g) were maintained on nonmedicated Purina rat chow ad libitum in rooms with a lighting sequence of 12 hours light and 12 hours dark. The rats were anesthetized with 40 mg/kg sodium pentobarbital (ip) and received 500 U of sodium heparin (ip). After 30-40 minutes, hearts were excised and their aortas immediately slipped onto perfusion cannulas which dripped oxygenated physiological saline solution (PSS) at 7 ml/min. Either constant pressure (100 cm H2O) or constant flow (14 ml/min, unless otherwise indicated, via a Harvard peristaltic pump, frequency of 24/min) retrograde perfusion down the aorta was initiated as soon as each heart was secured. The right atrium was removed and platinum electrodes were placed on the base of the right ventricle in the vicinity of the His bundle so that the heart could be paced at 300 contractions/min with a minimal voltage and pulse duration (1-2 V, 3-5 msec) supplied by a Grass SD9 stimulator. A degassed saline-filled cannula (polyethylene, 1.5 mm i.d., 8.0 cm long) attached to a Statham strain-gauge manometer (P23 Dl) was inserted into the chamber of the left ventricle through the left atrium and mitral valve for recording changes in intraventricular pressure. Maximum developed intraventricular pressure generally ranged from 85 to 125 mm Hg. In some experiments, a fluid-filled latex balloon was attached to the end of the cannula which permitted the myocardium to develop force at a constant ventricular volume. The intraventricular balloon was initially inflated with fluid until left ventricular pressure development was maximal. The balloon volume was held constant during the course of an experiment. The rate of left ventricular pressure development (dP/dt) was derived from the left ventricular pressure signal by resistance-capacitance differentiation (Hewlett-Packard derivative preamplifier, model 8814A). All pressure and dP/dt data were recorded with a multichannel polygraph (Hewlett-Packard series 7700 recorder).

The hearts were equilibrated for 30 minutes and always perfused with nonrecirculated PSS, pH 7.4 at 37°C. The PSS was prepared fresh daily and contained in millimoles per liter: NaCl, 118.4; KCl, 4.69; CaCl2, 2.52; NaHCO3, 25.6; MgSO4, 1.18; KH2PO4, 1.18; and glucose 10. For some experiments, calcium-free PSS was prepared by replacing the Ca2+ with Na2+. After the equilibration period, acetylcholine, carbachol (carbamylcholine chloride), or isoproterenol was administered (0.2-0.5 ml/min) into the aortic perfusion cannula just above the heart via a syringe infusion pump (Harvard, model 940). The resulting perfusion cannula PSS concentration of all agents is that reported herein. Atropine sulfate, propranolol, or phenolamine was delivered to some hearts by the same means of administration 5 minutes prior to and during infusion of either acetylcholine or isoproterenol. Coronary flow was measured in constant pressure-perfused hearts by collecting the venous effluent in a graduated cylinder. In some instances, coronary perfusate was collected every 10 seconds into preweighed 20-ml glass scintillation vials. The flow was determined by weight difference assuming 1 g equivalent to 1 ml of fluid. Myocardial ischemia was produced by termination of perfusion flow. Anoxia was produced by perfusing the hearts with PSS gassed with 95% N2-5% CO2. After the introduction of one of the above experimental interventions, the hearts were frozen while still attached to the aortic cannula by compression into thin wafers (thickness 1-2 mm) with polished clamps precooled in liquid nitrogen.

Analytical Procedures

Tissue Preparation and Extraction.

Frozen PSS and nonventricular tissue were shipped from the frozen myocardial wafers and the tissue was finely powdered in a liquid nitrogen cooled blender as described previously (Dobson, 1978). The powdered samples were transferred to screw cap glass vials and stored (2-30 days) at -80°C. Extraction procedures were initiated by weighing out portions of each sample at -25°C.
For cGMP assays, approximately 300 mg of muscle were transferred to a Duall homogenization tube (glass-glass, size 22, Kontes Glass Co.) Based on sample weight, 5 volumes of ice-cold 0.4 M trichloroacetic acid were added and the mixture was homogenized at 0°C. The homogenate was centrifuged at 2,000 g for 30 minutes at 0°C and the supernatant fluid extracted 5 times with 4 volumes of H₂O-saturated ethyl ether. The extract was subjected to reduced atmospheric pressure (328 mm Hg) at 37°C for 30 seconds to remove most of the remaining ether. The extract was then placed on 0.5 × 5 cm alumina (1 g of aluminum oxide, WoeIm neutral, activity grade 1) column previously equilibrated with 0.06 M Tris, pH 7.5, to remove most of the non-cyclic purine nucleotides (Mao and Guidotti, 1974). The alumina column was first eluted with 10 ml of the above Tris buffer, then with 5 ml of 0.6 M Tris, pH 7.5. Both elutions were collected directly onto a 0.5 × 3.5 cm Bio Rad AG 1-X8 (200-400 mesh, chloride form) column washed previously with 15 volumes of H₂O. The latter column was eluted with 10 ml of 0.02 N HCl, and this eluate, which contained most of the cAMP, was discarded. A second elution with 10 ml of 2 N HCl was collected, lyophilized, and contained primarily cGMP. The column chromatographic procedure was effective in removing the non-cyclic purine nucleotides as judged by chromatographing some samples on thin layer polyethyleneimine cellulose sheets (Trifilo and Dobson, 1976) and comparing them to samples not subjected to the column chromatography. The sample was resuspended in 100 μl of 0.05 M sodium acetate buffer, pH 6.2, and assayed for cGMP. Prior to homogenization of the tissue, tracer amounts of tritiated cGMP (approximately 4,200 dpm or 50 fmol) were added to the powdered hearts to check for contamination of cGMP in the pellet quantified in a γ counter (Searle model 1195). The limit of sensitivity of the assay was 0.02 pmol/assay tube. Fifty percent displacement of Bₐ occurred upon the addition of 0.4 pmol of cGMP per tube. The possibility of other purines cross-reacting in this assay was unlikely because the concentration of contaminating purines necessary to cause a detectable decrease in Bₐ in the assay was 10²--10³-fold greater (2.5 nmol of either cAMP or guanosine monophosphate, 250 nmol of either guanosine triphosphate or guanosine, and 500 nmol of either adenosine triphosphate or adenosine) than that present in the partially purified extracts. All assays were performed in duplicate and the error between duplicates was generally <1.5%. When the same sample was assayed at different dilutions or in different assays performed on separate days, the agreement was ≥96%. The addition of 0.1 mg (0.025 U) phosphodiesterase to tissue extracts before column chromatography removed 98% of the assayable cGMP. The concentration of cGMP is expressed as fmol/mg protein.

The concentration of cAMP was measured in tissue extracts by methods described previously (Dobson et al., 1976) based on the activation of skeletal muscle cAMP-dependent protein kinase.

Both in isolated and intact heart preparations of different species, this cAMP assay has been reported to give consistent results (Dobson and Mayer, 1973; Dobson et al., 1976; Dobson, 1978). The concentration of cAMP is expressed as pmol/mg protein. The lactate concentration in tissue samples plus acid immediately homogenized at 0°C. The homogenate was centrifuged at 5,000 g for 20 minutes and the resulting supernatant was brought to pH 8-9 with 3.2 M KHC₇O₄. After most of the evolved CO₂ had dissipated, the KCIO₃ precipitate was removed by centrifugation and the extract assayed for lactate.
extracts was determined enzymatically by the method of Passonneau (1974). The concentration of lactate is expressed as nmol/mg tissue wet weight. Protein was measured by the method of Lowry et al. (1951), and bovine serum albumin was used as a standard. The protein content of the hearts ranged from 8.82 to 9.98 mg protein/100 mg tissue wet weight.

Materials

Acetylcholine chloride, carbachol (carbamylcholine chloride), L-isoproterenol chloride, atropine sulfate, dl-propranolol, 2-amino-2-methyl-1-propranol, and beef heart lactate dehydrogenase were purchased from Sigma Chemical Co. Phentolamine (Regitine-HCl) was generously supplied by Ciba-Geigy Corp. All salts, acids, solvents, hydrazine hydrate and sodium heparin were certified grade from Fisher Scientific Co. Cyclic and non-cyclic purine nucleotides, nucleosides, nicotinamide-adenine dinucleotide, l-lactate, beef heart phosphodiesterase, and enzymes used for [γ-32P]ATP synthesis (Glynn and Chappell, 1964, a substrate for cAMP assay) were from Boehringer Mannheim Corporation. Crystallized bovine albumin was obtained from Pentex Biochemicals. Woelm neutral alumnum oxide was obtained from ICN Pharmaceuticals, Inc. Carrier-free (32P) inorganic phosphate was purchased from New England Nuclear. 3H-cAMP (specific activity 19 Ci/mmol) was from Amersham-Searle. 125I-Sc-cGMP (specific activity of approximately 600 Ci/mmol), rabbit cyclic GMP antiserum, sheep anti-rabbit serum, and normal rabbit serum were obtained from Collaborative Research.

Statistical Methods

An analysis of variance was performed for paired and unpaired observations (Sokal and Rohlf, 1969). A probability of <0.05 was accepted as indicating a significant difference.

Results

Acetylcholine, cGMP, and Contractility

Acetylcholine produced an increase in myocardial cGMP concentration that was not always associated with a decrease in the rate of left ventricular pressure development.

Constant Flow Perfusion

Acetylcholine infusion in 1 minute produced a dose-dependent increase in myocardial cGMP (Fig. 1). cGMP increased from a control of 40.1 ± 2.9 to 55.6 ± 2.7 fmol/mg protein with 0.1 μM acetylcholine. The increase in cGMP produced by acetylcholine was essentially the same whether hearts were paced (300/min) or un-paced. When compared to acetylcholine, carbachol was equipotent and efficacious as judged from a dose-response relationship obtained for this compound (data not shown).

The rate of left ventricular pressure development (dP/dt, an index of contractility) decreased from a control of 2,570 ± 60 to 2,100 ± 100 and 1,610 ± 110 mm Hg/sec after 1 minute of 0.1 and 1 μM acetylcholine perfusion, respectively, in paced hearts (Fig. 1). In paced and un-paced hearts, the decreases in dP/dt caused by 1 minute of either 0.01, 0.1, or 1 μM acetylcholine were similar in magnitude (Fig. 2). After 2 minutes of acetylcholine, the dP/dt declined further in un-paced hearts but returned toward control levels in paced hearts. In the paced hearts, the return of dP/dt toward control persisted for an additional 8 minutes of the acetylcholine infusion. In the un-paced hearts the rate of contraction decreased from a control of 270 ± 11 to 128 ± 12, 90 ± 10, 60 ± 13, and 18 ± 10 contractions per minute with 0.3, 0.5, 1, and 2 minutes, respectively, of 1 μM acetylcholine. Propranolol, a β-adrenergic blocking agent, at 10 μM did not prevent the restoration of contractility observed in paced hearts in which acetylcholine produced only a transient decrease in dP/dt. This concentration of propranolol prevented a 2- to 3-fold increase in dP/dt produced by 0.2–1 μM isoproterenol. Atropine, a muscarinic blocking agent, at 10 μM prevented the decrease in dP/dt and contraction frequency elicited by acetylcholine. Propranolol or atropine alone decreased dP/dt by 9–10% and 1–4%, respectively. During the 2-minutes infusion period, acetylcholine was without affect on contractility observed in paced hearts in which acetylcholine produced only a transient decrease in dP/dt. This concentration of propranolol prevented a 2- to 3-fold increase in dP/dt produced by 0.2–1 μM isoproterenol. Atropine, a muscarinic blocking agent, at 10 μM prevented the decrease in dP/dt and contraction frequency elicited by acetylcholine. Propranolol or atropine alone decreased dP/dt by 9–10% and 1–4%, respectively. During the 2-minutes infusion period, acetylcholine was without affect on
FIGURE 2. The time-course of acetylcholine effects on myocardial contractility in paced and unpaced rat hearts perfused at 14 ml/min with oxygenated (95% O2-5% CO2) PSS. At zero time, acetylcholine infusion directly into the PSS entering the aorta was initiated resulting in the following concentrations: 0.01 μM (○), 0.1 μM (□), or 1 μM (△). dP/dt was recorded at the times indicated. Values represent the mean of five experiments. Asterisks denote a significant difference from zero time. See legend of Figure 1 for further explanation.

left ventricular end-diastolic pressure in paced hearts. However, in unpaced hearts, the cholinergic agent caused a 2–4 mm Hg increase in left ventricular end-diastolic pressure. In experiments in which a fluid-filled balloon cannula was used to hold left ventricular volume constant, acetylcholine produced decreases in dP/dt that were similar to data presented in Figures 1 and 2 for paced and unpaced hearts.

Constant Pressure vs. Constant Flow Perfusion

Since previous reports (George et al., 1970, 1973; Watanabe and Besch, 1975) have suggested various relationships between cGMP and contractile state with acetylcholine perfusion in constant flow- and constant pressure-perfused hearts, it was important to compare these perfusion situations. When paced hearts, were used, a submaximal concentration of acetylcholine was employed to maximize any differences that might occur. Infusion of acetylcholine initially at 1 μM produced within 1 minute a 3.5-fold increase in myocardial cGMP in constant pressure-perfused hearts compared to only a 1.7-fold increase in the cyclic nucleotide in constant flow-perfused hearts (Fig. 3). It should be noted that the concentration of acetylcholine delivered to the myocardium of the constant flow-perfused hearts remained constant at 1 μM, whereas in the constant pressure-perfused hearts the concentration of acetylcholine delivered to the myocardium varied and was inversely related to changes in coronary flow that occurred during the course of the experiment. Since the protein contents of constant pressure- and constant flow-perfused hearts were 9.69 ± 0.29 and 9.46 ± 0.28 mg/100 mg heart (wet weight), respectively, the constant flow-perfused hearts were not significantly more edematous than the constant pressure-perfused hearts. In both the constant pressure- and constant flow-perfused hearts, the cGMP concentration remained elevated with the continual infusion of acetylcholine for 10 minutes.

The rate of left ventricular pressure development in paced constant pressure-perfused hearts decreased and remained depressed during a 10-minute acetylcholine infusion period in a manner similar to that observed in unpaced constant flow-perfused hearts (Fig. 2). This contractile response was observed when either a left ventricular pressure cannula or balloon cannula was used to obtain dP/dt.

In the paced constant pressure-perfused hearts, acetylcholine infusion (1 μM initially) caused a transient increase (not due to the infusion pump) in coronary perfusate flow from 6.8 ± 0.2 to 8.2 ± 0.3 ml/min within the first 10 seconds of the infusion followed immediately by a pronounced decrease in coronary flow (Fig. 3). After 30 seconds of the acetylcholine infusion (1 μM initially), the coronary flow decreased to 3.1 ± 0.4 ml/min.

FIGURE 3. The effect of 1 μM acetylcholine on the formation of myocardial cGMP in rat hearts perfused under either constant pressure or constant flow conditions. All hearts were paced. Continuous acetylcholine infusion was initiated at zero time and carried out for the times indicated as outlined in Figure 1. Coronary flow of the constant pressure-perfused hearts was determined at the times indicated as described in Methods. While the concentration of acetylcholine was initially 1 μM in the constant pressure perfused hearts the concentration to which the myocardium was actually exposed varied after zero time inversely with coronary flow. Values represent the mean of four experiments. See the legend of Figure 1 for further explanation.
Ischemia, Anoxia, and cGMP

Since coronary flow decreased in constant pressure-perfused hearts during acetylcholine infusion, it seemed possible that, besides the enhanced concentration of acetylcholine delivered to the myocardium, coronary flow might be an additional determinant of myocardial cGMP concentration. To determine whether coronary perfusate flow itself was important, paced hearts initially equilibrated at a perfusion rate of 14 ml/min for 30 min, were then subjected to perfusion flows of either 0, 7, 14, or 30 ml/min for 10 minutes. Reduction of coronary perfusion from 14 to 7 and 0 (ischemia) ml/min produced a significant increase in myocardial cGMP from 43 to 85 and 127 fmol/mg protein, respectively, whereas no change in the concentration of the cyclic nucleotide resulted from an increase in flow to 30 ml/min (Fig. 4). Upon initiation of myocardial ischemia by termination of coronary perfusion (zero flow), the elevation of cGMP became significant within 2 minutes and maximal after 10 minutes (Fig. 5). Within the first 4 minutes of ischemia, cGMP increased more than 2-fold from 38 to 90 fmol/mg protein.

Since ischemia produced a marked increase in myocardial cGMP, paced hearts were made anoxic for 10 minutes by perfusing the hearts at 14 ml/min with PSS gassed with 95% N\textsubscript{2}-5% CO\textsubscript{2} to determine whether a lack of O\textsubscript{2} delivery would cause an elevation in cGMP. Anoxia, unlike ischemia and acetylcholine, did not affect myocardial cGMP (Fig. 6). The myocardial cGMP concentration determined after 0.5, 1, 2, and 4 minutes of anoxia was also not significantly different from the value in oxygenated myocardium (PSS at 14 ml/min, gassed with 95% \textsubscript{O}2-5% \textsubscript{CO}2). The concentration of myocardial lactate in control, acetylcholine stimulated (1 \textmu m, 1 minute, constant flow perfused) ischemic (10 minutes) and anoxic (10 minutes) hearts was 1.67 ± 0.13, 1.44 ± 0.18, 4.90 ± 0.24 (significantly different from control), and 1.74 ± 0.16 nmol/mg tissue (weight wet), respectively.

Continual infusion of atropine (10 \textmu m) for 5 minutes before the paced hearts were either subjected to 10 \textmu m acetylcholine or rendered ischemic prevented the acetylcholine-produced increase in cGMP but did not appreciably attenuate the increase in cGMP caused by ischemia (Fig. 7). The atropine also did not affect the early increase in cGMP that occurred after 2 minutes of ischemia. Indomethacin at 10 \textmu m in the perfusion PSS did not
influence the ischemia-induced increase in myocardial cGMP.

cGMP, cAMP, Ischemia, Anoxia, and Calcium

Myocardial cAMP increased significantly from 3.8 ± 0.5 to 6.0 ± 0.4 pmol/mg protein in paced anoxic hearts but did not differ from control in paced ischemic or acetylcholine (1 μM)-stimulated hearts (Fig. 6). Isoproterenol (1 μM) infusion for 1 minute significantly increased cAMP from a control of 3.8 ± 0.8 to 12.8 ± 1.2 pmol/mg protein in paced constant flow-perfused hearts. The isoproterenol did not influence the cGMP concentration in these hearts.

Since Ca²⁺ appears to play an important role in tissue cGMP metabolism (Schultz et al., 1973), the effect of this cation on the acetylcholine and ischemia elicited increase in cGMP was determined. Constant flow perfusion of paced hearts with Ca²⁺-free PSS for 1 minute before the termination of coronary flow decreased the ischemia-induced increase in cGMP but did not affect the increase in the cyclic nucleotide caused by 1 μM acetylcholine (Fig. 8). Upon the initiation of Ca²⁺-free perfusion, the hearts ceased contracting within 15 seconds. Perfusion of hearts with Ca²⁺-free PSS for 20 minutes prevented the acetylcholine-elicited increase in myocardial cGMP. Twenty minutes of Ca²⁺-free perfusion prior to the initiation of ischemia atten-
uated but did not completely prevent the ischemia-produced increase in cGMP.

Discussion

Acetylcholine, cGMP, and Contractility

Constant Flow Perfusion

Acetylcholine administration to isolated constant flow-perfused hearts, either paced or un-paced, produced a dose-dependent increase in myocardial cGMP concentration. Carbachol, a choline ester resistant to hydrolysis, produced similar dose-response results indicating that the acetylcholine probably did not undergo significant destruction when freshly prepared and infused into the aortic cannula of perfused hearts. There was a correlation between the increase in myocardial cGMP and the decrease in contractility (dP/dt) after acetylcholine administration for 1 minute in paced and un-paced hearts (Figs. 1 and 2). These results are in agreement with similar studies performed by George et al. (1970, 1973). However, in paced hearts, acetylcholine caused only a transient dose-dependent decrease in dP/dt during the first 2 minutes of infusion (Fig. 2) while the myocardial GMP concentration remained elevated (Fig. 3). Thus, there was not always an inverse relationship between cGMP concentration and contractility. Alterations in left ventricular chamber volume probably are not involved, since left ventricular end-diastolic pressure did not change with acetylcholine infusion in paced hearts. The return of contractility toward control following the transient decrease probably was not due to an acetylcholine-induced release of catecholamines (Haeusler et al., 1968) because propranolol, at a concentration that prevented the positive inotropic response of isoproterenol, did not prevent the restoration of contractility toward control levels. Therefore, the rapid and transient decrease in contractility observed with acetylcholine stimulation in paced hearts may indicate: (1) that the decrease in contractility is not related to an increase in myocardial cyclic GMP or (2) that there is an inverse relationship between cGMP and contractility, the prolonged effect of the cyclic nucleotide is masked by a compensatory mechanism.

Other investigators have reported a lack of association between an increase in cGMP and a decrease in contractility (Watanabe and Besch, 1975; Brooker, 1977; Diamond et al., 1977). Using paced isolated perfused guinea pig hearts, Watanabe and Besch (1975) reported that a 2-minute infusion of acetylcholine produced a dose-dependent increase in cGMP but had only a slight negative inotropic effect that did not show a marked concentration dependence. In isolated guinea pig and cat atrial preparations, Brooker (1977) and Diamond et al. (1977), respectively, have shown that low concentrations of acetylcholine attenuate atrial twitch tension without causing a detectable increase in cGMP. However, the disagreement between experimental results may be due to differences in species used or differences in experimental techniques employed. The effects of acetylcholine may be mediated in part by changes in the intracellular concentrations of cGMP. However, it is possible that the acetylcholine-induced reduction of the slow inward Ca2+ current (Ten Eick et al., 1976) may represent another pathway independent of cGMP that is important in the manifestation of the effects of this choline ester.

Constant Pressure vs. Constant Flow Perfusion

Acetylcholine produced approximately a 2-fold increase in myocardial cGMP in constant pressure-perfused hearts as compared to hearts perfused with constant flow (Fig. 3). In the constant pressure-perfused hearts, an increase in coronary flow occurred within the first 10 seconds of acetylcholine infusion, which was expected (Folkow et al., 1949). However, after the initial increase, coronary flow precipitously decreased over the next 10–30 seconds and remained at approximately one-third the control level (Fig. 3). It is likely that an acetylcholine-elicted release of catecholamines could have played an important role in the decrease in coronary flow, since phentolamine, an α-adrenergic blocking agent, substantially attenuated the coronary flow reduction. The catecholamine release presumably was small in magnitude, localized in the vicinity of the coronary resistance vessels, and mediated by a α-adrenergic vasoconstriction, since total myocardial cAMP concentration was not significantly influenced with acetylcholine administration (Fig. 6). The decrease in coronary flow may have also been associated with a decrease in myocardial oxygen consumption (Eckenhoff et al., 1947) fostered by the acetylcholine-induced decrease in contractility. A direct vasoconstricting effect of acetylcholine on the coronary vessels (De La Lande et al., 1974) also could have contributed to the decrease in coronary flow. Thus, the decrease in coronary flow observed could have caused a partial degree of ischemia which, in turn, could have been a factor contributing to the marked cGMP elevation in constant pressure-perfused hearts. Since acetylcholine was delivered into the aortic cannula so as to achieve a desired concentration based on the control coronary flow rate with a constant flow infusion pump, a reduction in coronary flow elicited by acetylcholine would only increase the effective concentration of the agent administered. Presumably, this was another factor responsible for producing what appeared to be an enhanced increase in myocardial cGMP at a given concentration of acetylcholine in the constant pressure-perfused hearts. This suggests that there may be problems inherent in studies such as the one reported here and others (George et al., 1973) in which acetylcholine infusions into constant pressure-perfused
hearts have been used to investigate the relationship between myocardial cGMP and contractility with acetylcholine stimulation.

**Myocardial cGMP Values**

The values for myocardial cGMP concentration reported here are approximately one-tenth those reported by Busuttil et al. (1978), George et al. (1970, 1973), and Keely et al. (1977) and one-third those reported by Watanabe and Besch (1975). The reason for the discrepancy is not readily apparent, but the column chromatographic steps to which the tissue extracts were subjected and the high specificity of the cGMP antiserum used in the present study, as well as species and/or other differences in experimental technique, may be responsible.

**Ischemia, Anoxia, and cGMP**

Reduction of coronary flow in paced constant flow-perfused hearts to levels below 14 ml/min produced an increase in myocardial cGMP (Figs. 4 and 5). Anoxia (perfusion with PSS gassed with 95% N2–5% CO2) was also employed to limit O2 delivery to the myocardium. However, this intervention was without effect on cGMP (Fig. 6). Therefore, the ischemia elicited increase in cardiac cGMP is probably not due to a reduction in myocardial oxygen tension per se. These results are in agreement with the ischemic dog myocardial studies of Steward et al. (1978), but do not agree with the anoxic rat heart studies of Busuttil et al. (1978, 1976). The latter reported that isolated rat hearts perfused with a modified Tyrode’s solution (containing 5 mM Ca2+ and 5 mM glucose) gassed with 95% N2–5% CO2 increased myocardial cGMP approximately 1.6-fold with 1–5 minutes of anoxic perfusion. These results are not directly comparable to those of the present study because the hearts were perfused at constant pressure. Thus, it is possible that coronary flow may have been limiting in their experiments and this in turn could have contributed to the observed increase in cGMP.

It is well known that the effects of ischemia and anoxia on cardiac metabolism are different (Dobson and Mayer, 1973; Rovetto et al., 1973). In the present studies, the concentration of myocardial lactate markedly increased in the ischemic hearts as compared to the anoxic hearts where flow was not limited. Presumably, the lactate formed in the anoxic hearts was continually released and washed into the coronary perfusate. The washout of metabolites, lactate being a good example (Rovetto et al., 1973), may also partially explain why cGMP did not accumulate in the anoxic hearts as it did in the ischemic hearts. Anoxic hearts were continually perfused with PSS containing glucose, whereas the ischemic hearts were not perfused. Consequently, the ischemic hearts were not continuously supplied with exogenous glucose. Recently, Busuttil et al. (1976) reported that in oxygenated isolated hearts, the absence of exogenous glucose was associated with an elevation of myocardial cGMP. Therefore, the availability of exogenous oxidizable substrates may be another important determinant of cGMP formation.

Atropine completely blocked an acetylcholine-induced increase in cGMP that was similar in magnitude to the increase in the cyclic nucleotide produced by ischemia. However, atropine did not prevent the ischemia-elicited increase in cGMP (Fig. 7). This indicates that the ischemia-elicited increase in cGMP was probably not due to the release of myocardial acetylcholine. It has been reported that the release of prostaglandins from the heart is enhanced following either myocardial ischemia or anoxia (Block et al., 1974; Berger et al., 1976) and that prostaglandins are capable of facilitating cGMP formation (Kadowitz et al., 1975). However, indomethacin, an inhibitor of prostaglandin synthesis, did not prevent the increase in myocardial cGMP elicited by ischemia, suggesting that the prostaglandins were not involved in the present study. In addition, ATP has been shown to inhibit both a soluble and particulate guanylate cyclase in a cell-free preparation from rat heart (Limbird and Lefkowitz, 1975). It is feasible that the marked decrease in ATP that occurs with myocardial ischemia (Imai et al., 1964) may relieve the inhibitory effect of ATP on guanylate cyclase and thereby promote cGMP synthesis.

**cGMP, cAMP, Ischemia, Anoxia, and Calcium**

Anoxia elicited a slight increase in myocardial cAMP (Fig. 6) that was smaller than the increase in the cyclic nucleotide caused by 1 μM isoproterenol. In contrast to the increase in cGMP caused by 10 minutes of ischemia, cAMP was not influenced by the ischemic conditions. Perhaps in the ischemic heart both the fall in myocardial ATP concentration (substrate for cAMP formation) and an augmentation of cAMP phosphodiesterase activity caused by an elevated concentration of cGMP (Beavo et al., 1971) do not permit cAMP to increase significantly. Previously, ischemia has been shown to increase cAMP in the heart (Wollenberger et al., 1969; Dobson and Mayer 1973). However, the hearts were intact and catecholamine release probably was an important factor in the augmentation of myocardial cAMP.

The removal of Ca2+ from the PSS for 1 minute partially inhibited the ischemia-induced increase in cGMP but did not prevent the acetylcholine-induced increase in the cyclic nucleotide (Fig. 8). Twenty minutes of Ca2+-free perfusion was required to prevent the increase in cGMP caused by acetylcholine. Since the hearts perfused with Ca2+-free PSS ceased contracting within 15 seconds, the Ca2+ involved in excitation-contraction coupling probably does not play a direct role in the elevation of cardiac cGMP caused by acetylcholine. In the present studies the requirement of Ca2+ for the acetylcholine-stimulated increase in cyclic GMP agrees...
with the studies of Schultz et al. (1973). These investigators reported that acetylcholine failed to increase the cGMP of rat ductus deferens when the preparation was incubated in the absence of Ca\(^{2+}\) for 30 minutes. The role of Ca\(^{2+}\) in the ischemia-induced elevation of myocardial cGMP may involve the formation and or disappearance of some metabolites(s) but awaits further investigation.

In summary, the results indicate that (1) the acetylcholine-produced increase in cGMP is not necessarily associated with a decrease in contractility—whether or not there is a correlation depends on the techniques and heart preparation employed; (2) ischemia but not anoxia increases myocardial cGMP; (3) the acetylcholine-induced increase in cGMP is blocked by atropine and does not depend directly on the presence of external Ca\(^{2+}\); (4) the ischemia-elicited increase in cardiac cGMP is not prevented by atropine, but is largely dependent on external Ca\(^{2+}\).

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