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Cyclic GMP-dependent protein kinase activation in the absence of negative inotropic effects in the rat ventricle

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1 It has been suggested that activation of cyclic GMP-dependent protein kinase (PKG) is a necessary step in the chain of events leading to the production of negative inotropy by muscarinic receptor agonists in mammalian ventricles, and that some cyclic GMP-elevating agents, such as sodium nitroprusside (SNP), fail to exert a negative inotropic effect because they elevate cyclic GMP levels in a pool that does not activate the kinase. This hypothesis was tested in the present study by monitoring the effects of carbachol, SNP and atrial natriuretic peptide (ANP) on contractility, cyclic GMP content and PKG activity in rat intact ventricular preparations and freshly isolated ventricular cardiomyocytes.

2 The presence of PKG in both the intact vehicle and in isolated ventricular cardiomyocytes was confirmed by MonoQ anion exchange chromatography and Western blotting. The elution profile indicated that the conditions of the PKG assay were selective for measuring PKG activity.

3 Carbachol induced a marked negative inotropic effect in intact, perfused hearts and ventricular strips in the presence of isoproterenol. The negative inotropic effect of carbachol was not associated with significant changes in cyclic GMP content or PKG activity in intact ventricular tissue, or in PKG activity in isolated cardiomyocytes.

4 SNP and ANP significantly increased cyclic GMP levels and activated PKG in intact ventricular preparations. Both drugs also activated PKG in isolated cardiomyocytes. However, neither drug had any negative inotropic effect in isoprenaline-stimulated perfused hearts and ANP did not change the contractility of isoprenaline-stimulated isolated cardiomyocytes.

5 The results of this study demonstrate that the negative inotropic effects of muscarinic receptor agonists can occur in the absence of significant activation of PKG. Conversely, marked increases in ventricular cyclic GMP content and PKG activity caused by SNP or ANP were not accompanied by a negative inotropic effect.

6 These results suggest that increases in cyclic GMP levels and activation of PKG do not play important roles in the regulation of rat ventricular contractility by muscarinic receptor agonists.

Keywords: Atrial natriuretic peptide; carbachol; cyclic GMP; cyclic AMP; cyclic GMP-dependent protein kinase; cardiomyocyte; contractility; muscarinic receptor agonist; sodium nitroprusside; ventricle

Introduction

In the early 1970s, it was suggested that cyclic GMP plays a role in mediating the negative inotropic effects of muscarinic receptor agonists in the heart (Goldberg *et al*., 1975). This hypothesis was initially based on reports that cyclic GMP was elevated in cardiac preparations treated with concentrations of muscarinic receptor agonists known to depress contractility (George *et al*., 1970, 1973) and was supported by reports that exogenous cyclic GMP analogues could mimic some of the mechanical effects of muscarinic receptor agonists (Nawrath, 1976). If, in fact, cyclic GMP elevation mediates the negative inotropic effects of muscarinic receptor agonists in the heart, then any agent which can increase cyclic GMP in the heart should cause a decrease in cardiac contractility. However, an early study designed to test this hypothesis failed to provide support for this relationship (Diamond *et al*., 1977). In that study, it was found that a low concentration of acetylcholine (ACh; 0.05 μM) exerted a negative inotropic effect in cat atrial strips with no apparent increase in cyclic GMP levels, and a marked (17-fold) increase in cyclic GMP caused by the nitrovasodilator, sodium nitroprusside (SNP), was not accompanied by a negative inotropic effect. These results argued against an important role for cyclic GMP as a mediator of the negative inotropic effects of muscarinic receptor agonists, at least in the cat atrial appendage. In confirmation of these results, Lincoln & Keely (1980, 1981) found that SNP failed to decrease contractility in isolated Langendorff-perfused rat hearts, even though it elevated cyclic GMP levels in the hearts to a greater extent than did negative inotropic concentrations of ACh. However, Lincoln and Keely carried the study a step further in that they also measured a putative downstream receptor for cyclic GMP, the cyclic GMP-dependent protein kinase (PKG). They found that concentrations of ACh which decreased cardiac contractility also increased cyclic GMP levels and activated PKG, while SNP failed to activate PKG activity or to alter contractility, even though it markedly elevated cyclic GMP. Thus, it was suggested that activation of PKG might be an essential step in the chain of events leading to inhibition of the contractile force by muscarinic receptor agonists and that SNP fails to activate PKG, and therefore fails to affect cardiac contractility, because it elevates cyclic GMP in a pool which does not have access to PKG.

The studies of Lincoln & Keely (1980, 1981) are the only reports in the literature describing direct measurements of PKG activity in agonist-treated hearts. These studies represent some of the best evidence for compartmentalisation of cyclic GMP or its kinase in biological tissues, and they provide an explanation for some of the results in the literature which do not appear to be consistent with the proposed role for cyclic GMP as a mediator of the negative inotropic effects of muscarinic agonists. Nevertheless, due largely to the difficulty of measuring activation of PKG in cardiac tissues, these results

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have not yet been confirmed. Recently, improvements in the PKG assay have been described, based on the development of a novel, relatively specific substrate for PKG, BPDEtide (Jiang *et al*., 1992). Using this improved assay method, we have attempted to confirm the results of Lincoln & Keely (1980, 1981). The hypothesis that PKG activation is important in mediating the negative inotropic effects of cyclic GMP-elevating agents in the mammalian ventricle was tested by monitoring the effects of carbachol, SNP and atrial natriuretic peptide (ANP) on contractile activity, cyclic nucleotide levels and PKG activity in rat ventricular preparations. To ensure that PKG activity measured in intact ventricular tissue reflected the activity occurring in the cardiac muscle cells themselves, hormonal activation of PKG was also measured under similar conditions in freshly isolated rat ventricular cardiomyocytes.

Methods

Measurement of contractile properties of rat cardiac preparations

Preparation and measurement of contractility of Langendorff-perfused rat hearts After administration of sodium pentobarbital (80 mg kg^{-1}, i.p.) and heparin (2200 iu kg^{-1}, i.p.), hearts from male Wistar rats (200–300 g) were perfused through the aorta in a retrograde direction via a peristaltic pump with aerated (5% CO_2 in O_2) Chenoweth-Koelle buffer (35°C) of the following composition (mM): NaCl (120), KCl (5.6), $CaCl_2$ (2.18), $MgCl_2$ (2.1), $NaHCO_3$ (19), L-arginine (0.1), and D-glucose (10) at 10 ml min^{-1} (19 ml min^{-1} with heart unattached to perfusion apparatus). Hearts were paced at 255 beats per min by means of two platinum electrodes placed on either side of the ventricles (5 ms pulse, $\approx$2 V, Grass SD9 square wave stimulator). Maximal left ventricular pressure (LVP) and rates of rise and fall of left ventricular pressure (positive and negative dP/dt) were measured by a water-filled latex balloon attached to a pressure transducer (P23AA, Statham-Gould Instruments, Cleveland, Ohio) (diastolic pressure, 0–4 mm Hg) and analysed using a microcomputer (software designed by Roland Burton, University of British Columbia, Vancouver, Canada).

After at least 20 min, hearts were perfused with 1 nM isoprenaline (EC_{60}, data not shown) for 2–3 min and then subjected to washout (perfusion 1). After contractililty returned to baseline, hearts were perfused with isoprenaline alone for 2 min, or isoprenaline in combination with carbachol or SNP for 2 min, or ANP for 7 min with the last 2 min including isoprenaline (perfusion 2). In each heart, changes in the isoprenaline-mediated positive inotropic effect due to carbachol, SNP, or ANP were tested by comparing the relative effect of isoprenaline on contractility in perfusion 1 to the relative effect of isoprenaline plus carbachol, SNP, or ANP in perfusion 2. Times for perfusion of hearts with carbachol and SNP were based on a previous demonstration of significant increases in cardiomyocyte cyclic GMP levels by both agents within 2 min (MacDonell *et al*., 1995). The protocol for pre-perfusion of hearts with ANP was used in an effort to allow a reasonable time for significant accumulation of cyclic GMP prior to isoprenaline exposure (Cramb *et al*., 1987). Glassware was foil-wrapped and room lighting was dimmed during SNP treatment. At the completion of drug treatments, hearts were freeze-clamped, atrial tissue was trimmed away and the ventricular tissue was stored at −70°C until biochemical assays were performed.

Preparation and measurement of contractility in rat right ventricular strips The right ventricles of Wistar rat hearts (as described above) were cut into 4–6 strips and one end of each was impaled by two platinum electrodes and the other end was attached to a Grass FT03 force-displacement transducer. Tissues were mounted in 20 ml water-jacketed baths containing Chenoweth-Koelle buffer (35°C, aerated with 5% CO_2 in O_2), placed under 1 *g* resting tension, and stimulated to contract with electrical pulses (5 ms, 1 Hz, $\leqslant$10 V). Contractile activity was recorded on a polygraph (Grass 7 D). The tissues equilibrated for 60 min, after which maximal twitch tension was evaluated in the presence or absence of drugs. The effects of carbachol (10 μM), SNP (100 μM) or ANP (100 nM) on maximal twitch tension in the presence of isoprenaline were determined. Tissues were exposed to isoprenaline (30 nM) until twitch tension reached steady-state (2–3 min), at which time carbachol, SNP, or ANP was added to the bath and tension was monitored for up to 6 min. At least one strip from each ventricle was exposed to only isoprenaline and was used as a control. In separate experiments, the direct effects of carbachol (100 μM), SNP (100 μM) or ANP (100 nM) on maximal twitch tension were assessed for up to 9 min. At least one strip from each ventricle was not exposed to drugs and was used as a control.

Preparation of isolated rat ventricular cardiomyocytes Ventricular cardiomyocytes were isolated from rats using an isolation procedure modified from the protocol of Kryski *et al*. (1985) as described in MacDonell *et al*. (1995). After isolation, cells were then suspended in modified Kreb's-HEPES buffer for use in contractility or PKG studies.

Measurement of contractility of single isolated rat ventricular cardiomyocytes Contractility of isolated cells was measured as previously described (MacDonell *et al*., 1995) with the following modifications: the superfusion rate of buffer with and without drugs was 3 ml min^{-1}, amino acids were omitted from the buffer and the digitized output was evaluated with the computer program Felix 1.01 (Photon Technology International, Inc.). The treatment protocol for isolated ventricular cardiomyocytes was as follows: following an initial control period, an electrically stimulated cell was perfused with buffer containing 1 nM isoprenaline for 6 min while parameters of contractility were monitored. The buffer was then changed to control solution until contractility returned to baseline ($\approx$10 min). The cell was then perfused with isoprenaline and 100 nM ANP for at least the same period of time. After washout, treatment with isoprenaline alone was repeated to establish reproducibility of the isoprenaline effect.

Cyclic GMP and cyclic AMP estimation

Frozen and crushed ventricular tissue from agonist-treated Langendorff-perfused rat heart (75–100 mg) was placed in a liquid N_2-chilled teflon capsule (1 ml capacity) (Hansen Industries Ltd, Richmond, BC, Canada) with a chilled metal pestle and pulverized in a Vari-Mix III dental amalgam mixer (10 s at medium speed, 5 s at high speed). After extraction with trichloroacetic acid, as described by Diamond & Chu (1985), cyclic AMP and cyclic GMP levels were measured using commercially available scintillation proximity radioimmunoassays (Amersham). Ventricular cyclic nucleotide content was calculated as fmol cyclic GMP or pmol cyclic AMP mg^{-1} protein.

Preparation of rat ventricular tissue and cardiomyocyte extracts

Frozen and crushed ventricular tissue from agonist-treated Langendorff-perfused rat heart ($\approx$150 mg) was pulverised in a Vari-Mix III dental amalgam mixer as described above. Ice-cold homogenisation buffer (5 volumes) was added to the capsule and the mixture was agitated in the Vari-Mix (10 s at high speed). The composition of the buffer was as follows: 10 mM HEPES (pH 7.4), 1 mM EDTA, 10 mM DTT, 1 mM 3-isobutyl-1-methylxanthine, 125 mM KCl, 1 mM benzamidine, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, 1 μg ml^{-1} apoprotinin and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged in a Heraeus Contifuge 28RS centrifuge (30 000 *g* for 5 min at 4°C). The supernatant

(soluble fraction) was placed on ice and immediately assayed in duplicate for kinase activity as described below. As each sample was individually homogenized, centrifuged and immediately assayed for kinase activity, no more than 7 min passed from the initiation of homogenization to initiation of soluble kinase activity measurement.

The particulate fraction was prepared from the pellet remaining after removal of the soluble fraction. The pellet was resuspended in five volumes of homogenization buffer which included 0.1% (w/v) Triton X-100 detergent as a solubilising agent. The mixture was placed on ice and gently vortexed three times for 10 s each every 10 min. After 30 min, the mixture was centrifuged as before and the supernatant (particulate fraction) was assayed in duplicate as described below.

Isolated cardiomyocytes prepared from a single rat heart were evenly divided into two 50 ml conical tubes containing 10 ml of modified Kreb's-HEPES buffer (approximately 5×10^6 cells). One suspension was treated with 1 nM isoprenaline for 2 or 6 min and the other suspension was treated with 1 nM isoprenaline and 10 μM carbachol for 2 min, 10 μM to 100 μM SNP for 6 min or 100 nM ANP for 6 min. The cells were then rapidly pelleted in a Dynac centrifuge (speed at no.70 for 10 s), the buffer removed, and the cell pellet frozen in liquid N_2 and stored at -70°C until required for preparation of subcellular fractions. Preparation of the soluble and particulate fractions from cell pellets was as described above.

Cyclic GMP- and cyclic AMP-dependent protein kinase assay

A phosphocellulose paper assay was used to measure the phosphotransferase activity of PKG and cyclic AMP-dependent protein kinase (PKA) in rat ventricular tissue and cardiomyocytes using a method modified from that described by Jiang *et al*. (1992). PKG activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P] ATP to BPDEtide (RKISASEFDRPLR) (Colbran *et al*., 1992). PKG activity was measured in a total volume of 70 μl containing 150 μM BPDEtide, 10 mM HEPES, 4 mM magnesium acetate, 150 μM ATP (2.5 μCi per tube for assay of soluble and particulate fractions of rat ventricular tissue and cardiomyocytes, 1 μCi per tube for assay of MonoQ fractions), 5 μM PKA synthetic inhibitor (PKI), 35 mM β-glycerophosphate, 100 ng ml^{-1} microcystin-LR, 0.5 mM EGTA, and 5 μM heparin in the presence and absence of 5 μM cyclic GMP. No substrate blanks were determined for each crude extract in the presence and absence of added cyclic GMP to correct for phosphorylation of endogenous substrates. The reaction was initiated by adding 20 μl of sample ($\approx$100 μg soluble or 10 μg particulate fraction protein of ventricular tissue or cardiomyocytes, or MonoQ column eluate) to 50 μl reaction cocktail. The reaction proceeded for 4 min at 4°C and was stopped by spotting 50 μl of the reaction mixture onto 2×2 cm squares of phosphocellulose paper (Whatman P81) pre-coated with 1 mM ATP and 10 mM KH_2PO_4. The paper was washed in 0.5% *o*-phosphoric acid for four times for 10 min each. Bound radioactivity was counted in a Beckman LS 6000TA liquid scintillation counter. The assay conditions allowed for the estimation of PKG activity using less than 0.05% of ATP and BPDEtide. The assay was linear with respect to time (up to 8 min) and with respect to activation by exogenous cyclic GMP. PKG activity was expressed as pmols or fmols of phosphate incorporated into substrate min^{-1} mg^{-1} protein or μl^{-1} eluate. The extent of PKG activation was assessed by calculating the activity ratio, which is the ratio of kinase activity in the absence of added cyclic GMP to activity in the presence of sufficient cyclic GMP to maximally activate the enzyme.

PKA activity in eluates from the MonoQ chromatography of soluble fractions of ventricular tissue and soluble and particulate fractions of cardiomyocytes was measured using the same assay conditions as the PKG assay except that PKI was excluded and samples were assayed in the presence and absence of 5 μM cyclic AMP, rather than cyclic GMP.

Chromatographic separation of protein kinases in rat ventricular extracts

Cyclic nucleotide protein kinase activity was analysed in soluble fractions of rat ventricular tissue and soluble and particulate fractions of rat ventricular cardiomyocytes chromatographically separated using a MonoQ anion exchange column (HR5/5, Pharmacia) in a fast protein liquid chromotography system (Pharmacia LKB Biotech, Uppsala, Sweden). All procedures were carried out at 4°C. The column, pre-equilibrated with buffer A (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM DTT, 0.2 mm mesh filtered), was loaded with up to 22 mg of sample protein at a flow rate of 0.5 ml min^{-1}. After the column was washed with 5 ml of buffer A, buffer B (buffer A plus 400 mM NaCl) was applied to the column in combination with buffer A so as to develop a linear gradient of NaCl (0–400 mM). Eluate was collected at a rate of 0.5 ml min^{-1} in 40×0.5 ml fractions which were assayed for PKG and PKA activities. Selected fractions were immunoblotted to determine the presence of PKG.

Immunoblotting of chromatographic eluate

A Bio-Rad Protein II electrophoresis unit was used to perform the separation of proteins by sodium dodecyl sulphate polyacryamide gel electrophoresis (SDS–PAGE), using a modification of the method described by Hei *et al*. (1993). Briefly, samples of commercially available purified kinases (bovine lung Iα PKG, 0.1 mg ml^{-1}; porcine heart PKA, 0.1 mg ml^{-1}; PKA catalytic subunit, 0.05 mg ml^{-1}) and molecular mass pre-stained standards were boiled for 3 min with digestion buffer (final concentrations: 2% w/v SDS, 120 mM Tris-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.004% bromophenol blue). Slab gels (11% SDS) were cast according to the method of Laemmli (1970). Proteins were electrophoretically separated overnight by a constant current (10 mA per gel) and were transferred from the separating gel onto a nitrocellulose membrane using a Hoefer TE 50 Transphor (Hoefer Scientific Instruments) unit (constant current of 250 mA for 3 h at 4°C). The membrane was treated for 2 h in blocking buffer, which consisted of 3% (w/v) skim milk powder in TTBS (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 0.05% Tween 20) was then probed with a polyclonal, affinity-purified antibody raised against a peptide sequence (CDEPPPDDNSGWDIDF) derived from the carboxyl terminus of the Iα isoform of PKG (gift of Dr Steven L. Pelech, Kinetek Biotechnology Corporation, Vancouver, B.C.). The membrane was exposed to the primary antibody (1/200 diluted in antibody buffer which was TTBS and 0.05% sodium azide) overnight at room temperature and was then incubated for 2 h with the secondary antibody (1/2000 diluted goat anti-rabbit IgG alkaline phosphatase conjugate). Immunologically recognized proteins were detected by a colour reaction due to the interaction of alkaline phosphatase and its substrates, nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate. The colour reaction proceeded for approximately 5 min.

Preparations of drug solutions

Stock solutions of carbachol, isoprenaline and SNP were prepared in distilled water on the day of use. Ascorbic acid (1 mg ml^{-1}) was present in stock solutions of isoprenaline. Solutions of SNP were protected from light. Solutions of ANP were prepared in either 0.05 M acetic acid or distilled water and were stored in aliquots at -20°C until required for use. Preliminary experiments demonstrated that the diluents had no effect on contractility, cyclic GMP levels or PKG activity in the cardiac preparations used.

Protein determination

Protein in TCA-precipitated ventricular preparations (from cyclic nucleotide studies) was measured using the method of

Lowry *et al.* (1951) as modified by Markwell *et al.* (1981). Protein in the soluble and particulate fractions of samples used in PKG assays and in samples applied to MonoQ columns was assayed using a commercially available assay (Bio-Rad), based on the method of Bradford (1976). The Lowry method was not used for these preparations because of interference by components of the homogenization buffer, such as HEPES (Peterson, 1979).

Statistical analysis

Results are expressed as means ± s.e.mean. Effects of carbachol, SNP or ANP on the contractility of rat intact hearts or right ventricular strips, in the presence of isoprenaline, were compared to isoprenaline alone at the same time point using one-way ANOVA followed by Bonferroni's test or the nonparametric Dunn's test, as appropriate. Effects of carbachol, SNP or ANP, in the absence of isoprenaline, on the contractility of rat right ventricular strips were compared to controls (untreated) at the same time point using one-way ANOVA followed by Bonferroni's test. Cell shortening (as a percentage of diastolic length) of isolated ventricular cardiomyocytes was evaluated by one-way repeated measures ANOVA followed by Student–Newman–Keuls (SNK) multiple comparison procedure or the nonparametric Friedman repeated measures analysis of variance on ranks, as appropriate.

Cyclic nucleotide levels, PKG activity ratios or absolute PKG activity levels in rat ventricle treated with isoprenaline plus carbachol, SNP or ANP were compared to ratios or levels in the presence of isoprenaline alone using one-way ANOVA followed by Bonferroni's or Dunn's test. Differences in PKC activity in isolated cardiomyocytes were tested using the paired Student's *t* test.

Results were considered significant when $P<0.05$. SigmaStat for Windows Version 1.0 (Jandel Scientific, San Mateo, CA, U.S.A.) software was used for statistical analyses. Values of *n* in contractility studies indicate number of hearts, ventricular strips or single cells, and in cyclic nucleotide and protein kinase studies indicate number of hearts, ventricular strips or cell suspensions.

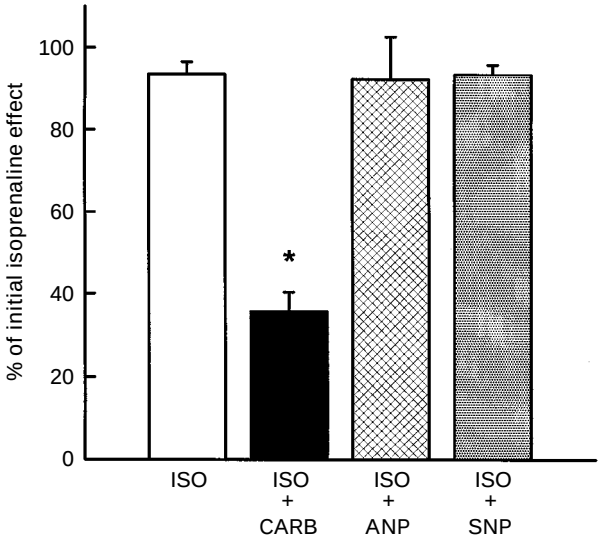

Figure 1 Effect of carbachol, ANP and SNP on Langendorff-perfused rat ventricular contractility in the presence of isoprenaline. Rat hearts were perfused with 1 nM isoprenaline for 2 min (□; $n=15$), isoprenaline plus 10 μM carbachol for 2 min (■; $n=10$), 100 nM ANP for 7 min of which the final 2 min included isoprenaline (▨; $n=5$), or isoprenaline plus 10 μM SNP for 2 min (grey bar; $n=11$). Effect of treatment was expressed as percent of negative dP/dt after an initial 2 min exposure to isoprenaline. Asterisk (*) indicates significant difference from isoprenaline alone, one-way ANOVA, Dunn's test.

Materials

BIOTRAK® cyclic AMP or cyclic GMP scintillation proximity assay kits were obtained from Amersham International (Little Chalfont, Buckinghamshire). PKG substrate (BPDEtide) and ANP (rat, 1–28 amino acids) were obtained from Bachem California (Torrance, CA, U.S.A.). A catalytic subunit of porcine heart cyclic PKA and bovine lung Iα cyclic GMP-dependent protein kinase holoenzyme were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, U.S.A.). Materials used in Western blot experiments were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.) except pre-stained molecular mass standards and antibodies to C terminus of type I PKG which were obtained from Kinetek Biotechnology Corp. (Vancouver, BC, Canada). BSA (fraction V) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P] Adenosine 5′-triphosphate was obtained from DuPont NEN Research Products (Boston, MA, U.S.A.). Scintiverse scintillation fluid was obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Carbachol, porcine PKA holoenzyme and sodium nitroprusside were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Collagenase (type II, CLS II) was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.).

Results

Effects of carbachol, SNP and ANP on contractility of rat hearts in the presence of isoprenaline

A major objective of this study was to confirm the studies of Lincoln & Keely (1980, 1981). Therefore, initial experiments were performed using a similar preparation, namely the paced, Langendorff-perfused rat heart. Hearts were stimulated with isoprenaline during exposure to carbachol, SNP or ANP because muscarinic receptor agonists produce a negative inotropic effect in mammalian ventricles primarily in the presence of cyclic AMP-elevating agents (for a review, see Loffelholz & Pappano, 1985).

The average contractile parameters of Langendorff-perfused hearts prior to exposure to drugs were as follows: negative dP/dt = −1206 ± 56 mm Hg s^{-1}, positive dP/dt = 2243 ± 124 mm Hg s^{-1}, maximal LVP = 101.3 ± 4.1 mm Hg ($n=41$). After the first exposure to 1 nM isoprenaline for 2 min (perfusion 1), contractility was as follows: negative dP/dt = −2150 ± 81 mm Hg s^{-1}, positive dP/dt = 3378 ± 172 mm Hg s^{-1}, maximal LVP = 137.5 ± 5.2 mm Hg ($n=41$). As illustrated in Figure 1, negative dP/dt after the second exposure to isoprenaline alone was slightly less than after the first exposure. This decrease was greatly magnified if the second exposure to isoprenaline was co-incident with perfusion with 10 μM carbachol. Within 2 min, carbachol inhibited isoprenaline-stimulated negative dP/dt to values less than those seen prior to exposure to drugs. In contrast, changes in negative dP/dt in the presence of isoprenaline plus 100 nM ANP or 10 μM SNP after 2 min were not significantly different from isoprenaline alone. Similarly, positive dP/dt and maximal LVP were significantly reduced by 10 μM carbachol, in the presence of isoprenaline, to 50 ± 4% and 58 ± 4% of the response in the initial treatment with isoprenaline alone, respectively. Neither 10 μM SNP nor 100 nM ANP significantly effected isoprenaline-stimulated positive dP/dt (as a percentage of initial isoprenaline effect, isoprenaline alone = 94 ± 3%, isoprenaline plus SNP = 92 ± 2%, isoprenaline plus ANP = 93 ± 10%). Stimulation of maximal LVP by isoprenaline was also unchanged by 10 μM SNP or 100 nM ANP (as a percentage of initial isoprenaline effect, isoprenaline alone = 91 ± 2%, isoprenaline plus SNP = 86 ± 2%, isoprenaline plus ANP = 88 ± 5%). These results demonstrate that 10 μM carbachol elicits a marked negative inotropic effect in the β-adrenoceptor-stimulated ventricle but 10 μM SNP or 100 nM ANP do not affect contractility under similar conditions.

Effects of carbachol, SNP and ANP on contractility of rat right ventricular strips in the presence of isoprenaline

Small negative inotropic effects of SNP or ANP have been reported in some ventricular preparations, such as ferret ventricular strips (Smith *et al*., 1991). To test whether or not small negative inotropic effects by SNP or ANP were occurring in the intact rat heart study described above but were not detected due to sensitivity limitations, the effects of carbachol, SNP or ANP on the contractility of electrically stimulated rat right ventricular strips were examined. This preparation exhibits very stable basal and isoprenaline-stimulated contractility, in terms of amplitude of twitch tension, so it was reasoned that small mechanical effects would be detected, if present.

Isoprenaline (30 nM) increased baseline twitch tension from 1.98 ± 0.18 g to 2.81 ± 0.25 g ($n=23$). As shown in Figure 2, a significant inhibitory effect of 10 μM carbachol was apparent within 1 min. After 3 min, tension had fallen to $8.0 \pm 0.03\%$ ($n=6$) above baseline from $47 \pm 0.14\%$ above baseline in the presence of isoprenaline alone ($n=7$). Neither ANP (100 nM) nor SNP (100 μM) changed the isoprenaline-stimulated amplitude of twitch tension of rat ventricular strips during 6 min of treatment (Figure 2). These results complement the findings of a β-adrenoceptor-dependent negative inotropic effect of carbachol and the absence of such an effect by SNP and ANP in the perfused rat heart.

Direct effects of carbachol, SNP and ANP on contractility of rat right ventricular strips

The effects of carbachol (100 μM), SNP (100 μM) and ANP (100 nM) on maximal twitch tension of electrically stimulated rat right ventricular strips in the absence of a cyclic AMP-elevating agent were examined. As shown in Figure 3, no changes in baseline twitch tension were observed in the presence of SNP or ANP over the course of 9 min. Carbachol caused a small positive inotropic effect between 1.5 and 7.5 min of incubation. It was considered that some inotropic changes might only be apparent at lower concentrations of agonists and, therefore, concentration–response relations were evaluated at a range of lower concentrations. The twitch tension of strips in the presence of cumulative concentrations of carbachol (0.1–10 μM), SNP (0.1–10 μM) and ANP (1–100 nM) was not significantly different from the contractility of control strips assessed at the same point in time (Figure 4). The slopes of the lines reflect the gradual decline in baseline contractility over time, independent of exposure to drugs. These results demonstrate a lack of direct negative inotropic effects of carbachol, SNP and ANP on a temporal and concentration-dependent basis in the rat right ventricle.

Effect of atrial natriuretic peptide on contractility of isoprenaline-stimulated single, isolated cardiomyocytes

A previous study demonstrated that carbachol (1–10 μM), but not SNP (10–100 μM), mediated a negative inotropic effect in

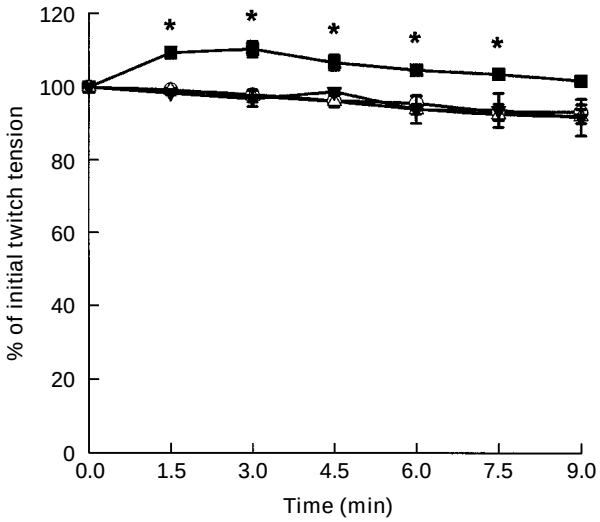

Figure 3 Temporal effects of 100 μM carbachol (■; $n=3$), 100 nM ANP (▼; $n=3$), and 100 μM SNP (△; $n=7$) on rat right ventricular strip twitch tension. Twitch amplitude was expressed as percentage of tension prior to exposure to drugs. Asterisks (*) indicate significant difference from control (○; $n=4$) at same time point, one-way ANOVA, Bonferroni adjustment.

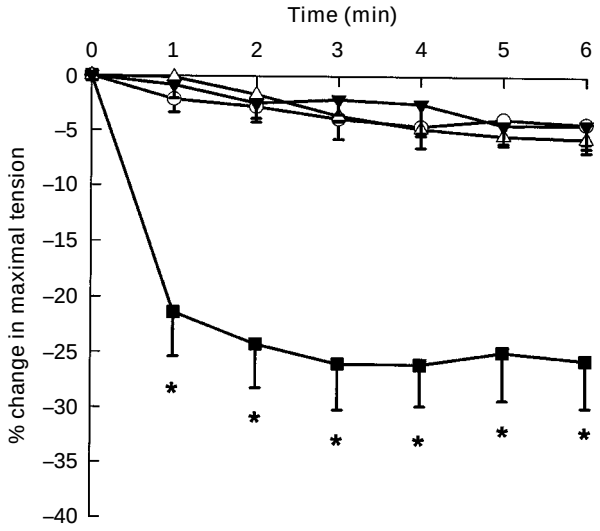

Figure 2 Effect of 10 mM carbachol (■; $n=6$), 100 nM ANP (▼; $n=4$), or 100 μM SNP (△; $n=6$) on rat right ventricular strip contractility in the presence of 30 nM isoprenaline. Twitch tension was expressed as percent change in maximal tension elicited by isoproterenol prior to exposure to carbachol, ANP or SNP. Asterisks (*) indicate significant differences from 30 nM isoprenaline alone (○; $n=7$) at the same time point, one-way ANOVA, Bonferroni adjustment.

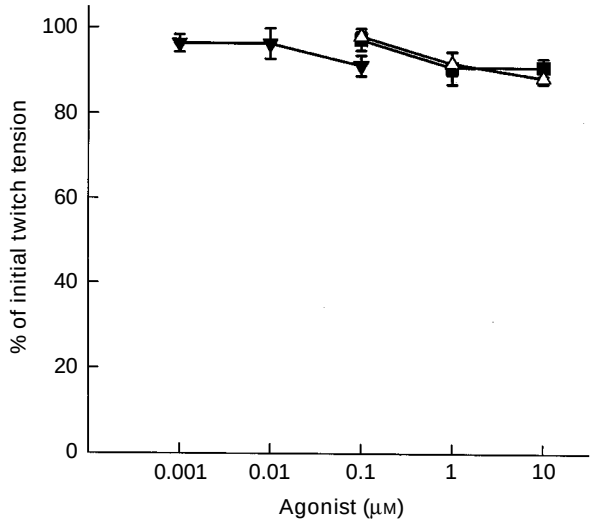

Figure 4 Lack of effects of 0.1–10 μM carbachol (■; $n=3$), 1–100 nM ANP (▼; $n=3$), or 0.1–10 μM SNP (▽; $n=4$) on amplitude of rat right ventricular strip twitch tension. Drugs were added to the baths every 4–5 min in increasing concentrations. Accordingly, tension was measured at 5, 10 and 15 min after the initiation of the drug treatments and was expressed as percentage of tension prior to exposure to drugs. Control tension was $96.3 \pm 0.8\%$ at 5 min, $94.3 \pm 2.1\%$ at 10 min and $96.2 \pm 3.4\%$ at 15 min ($n=4$).

isolated rat ventricular cardiomyocytes (MacDonell *et al.*, 1995). We sought to determine the effects of ANP under similar conditions. The amplitude of cell shortening, measured at 2, 4 and 6 min, was significantly elevated above control in the presence of 1 nM isoprenaline alone and in the presence of isoprenaline plus 100 nM ANP. Similarly to results from intact rat hearts and isolated ventricular strips, superfusion with 100 nM ANP did not affect the contractile response of single cardiomyocytes to 1 nM isoprenaline (Figure 5).

Effects of carbachol, ANP or SNP on cyclic GMP levels in rat ventricle

Cyclic GMP levels were measured in ventricular tissue from agonist-treated, Langendorff-perfused hearts which had been frozen after assessment of contractility. Carbachol (10 μM), in the presence of isoprenaline, did not significantly increase total tissue cyclic GMP levels after 2 min when compared to levels in the presence of isoprenaline alone (Figure 6). Conversely, both 100 nM ANP and 10 μM SNP, in the presence of isoprenaline, significantly increased cyclic GMP levels after a 2 min perfusion by approximately threefold above levels in the presence of isoprenaline alone. Isoprenaline alone (1 nM) did not significantly increase cyclic GMP levels above those found in untreated tissue (untreated = 290 ± 30 fmol cyclic GMP mg^{-1} protein, $n = 5$; isoprenaline = 403 ± 47 fmol cyclic GMP mg^{-1} protein, $n = 10$). Based on these findings in whole tissue preparations, cyclic GMP elevation is not correlated with the mediation of a negative inotropic effect in the presence of carbachol, SNP or ANP in the ventricle.

Effects of isoprenaline, carbachol, ANP and SNP on cyclic AMP levels in rat ventricle

Ventricular tissue in which cyclic GMP levels had been measured was also assessed for total tissue cyclic AMP content. After perfusion of Langendorff-mode rat hearts with 1 nM isoprenaline for 2 min, total ventricular tissue cyclic AMP levels were significantly increased above control (control = 12.0 ± 2.6 pmol cyclic AMP mg^{-1} protein, $n = 5$; isoprenaline = 18.8 ± 0.7 pmol cyclic AMP mg^{-1} protein, $n = 10$). As shown in Figure 7, neither 10 μM carbachol, 100 nM ANP nor 10 mM SNP altered the ability of isoprenaline to increase cyclic AMP levels in the rat ventricle.

Chromatography of cyclic GMP-dependent protein kinase in rat ventricle and ventricular cardiomyocytes

Cyclic nucleotide-dependent protein kinase activities in the soluble fraction of ventricular tissue and isolated cardiomyocytes were resolved (Figure 8a,b). In order to demonstrate PKG activity, fractions were assayed in the presence of PKI, a specific peptide inhibitor of PKA, either in the presence or absence of 5 μM cyclic GMP. Cyclic GMP-dependent activity eluted as a single distinct peak, with a small early shoulder of activity, at the same point in the elution profiles (fractions 27–32) of the soluble fractions of ventricular tissue and cardio-

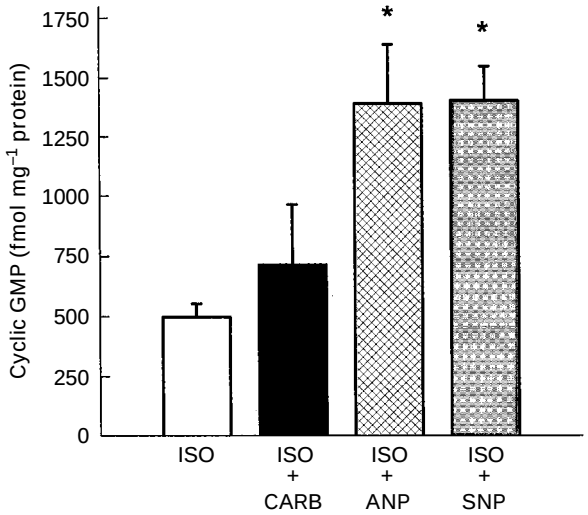

Figure 6 Effects of 10 μM carbachol (■; $n = 6$), 100 nM ANP (▨; $n = 5$) or 10 μM SNP (grey bar; $n = 6$) in the presence of 1 nM isoprenaline on cyclic GMP levels in ventricular tissue of Langendorff-perfused rat hearts. Treatment protocol was as described for Figure 1. Asterisks (*) indicate significant differences from isoprenaline alone (□; $n = 10$), one-way ANOVA, Dunn's method.

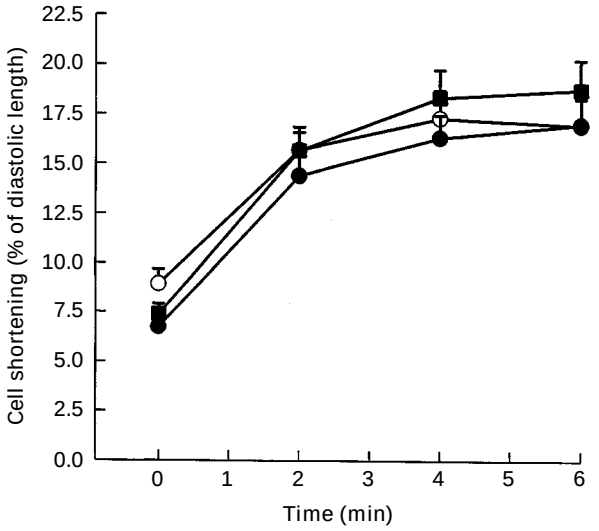

Figure 5 Lack of effect of 100 nM ANP on amplitude of 1 nM isoprenaline-stimulated isolated rat cardiomyocyte cell shortening ($n = 6$). Single, electrically stimulated myocytes were perfused with 1 nM isoprenaline (○) for ⩾6 min, washed with control buffer, treated with ANP plus isoprenaline (■), washed and treated again with isoprenaline alone (●). Cell shortening in the presence of drugs at 2, 4 and 6 min was significantly different from corresponding controls (zero time) (one-way ANOVA, Bonferroni's adjustment). Cell shortening at 2, 4 and 6 min did not differ between treatment groups, repeated measures one-way ANOVA, SNK or Friedman's test.

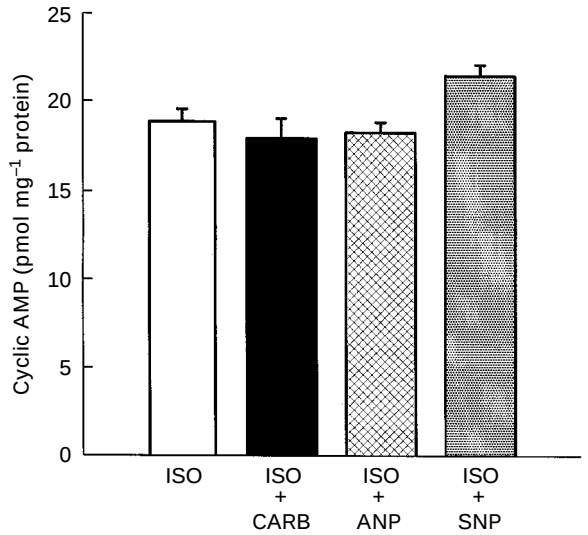

Figure 7 Effects of 10 μM carbachol (■; $n = 6$), 100 nM ANP (▨; $n = 5$) or 10 μM SNP (grey bar; $n = 6$) in the presence of 1 nM isoprenaline on cyclic AMP levels in ventricular tissue of Langendorff-perfused rat hearts. Cyclic AMP was measured in the same samples as shown in Figure 5. No differences from 1 nM isoprenaline (□; $n = 10$) were found, one-way ANOVA, Dunn's method.

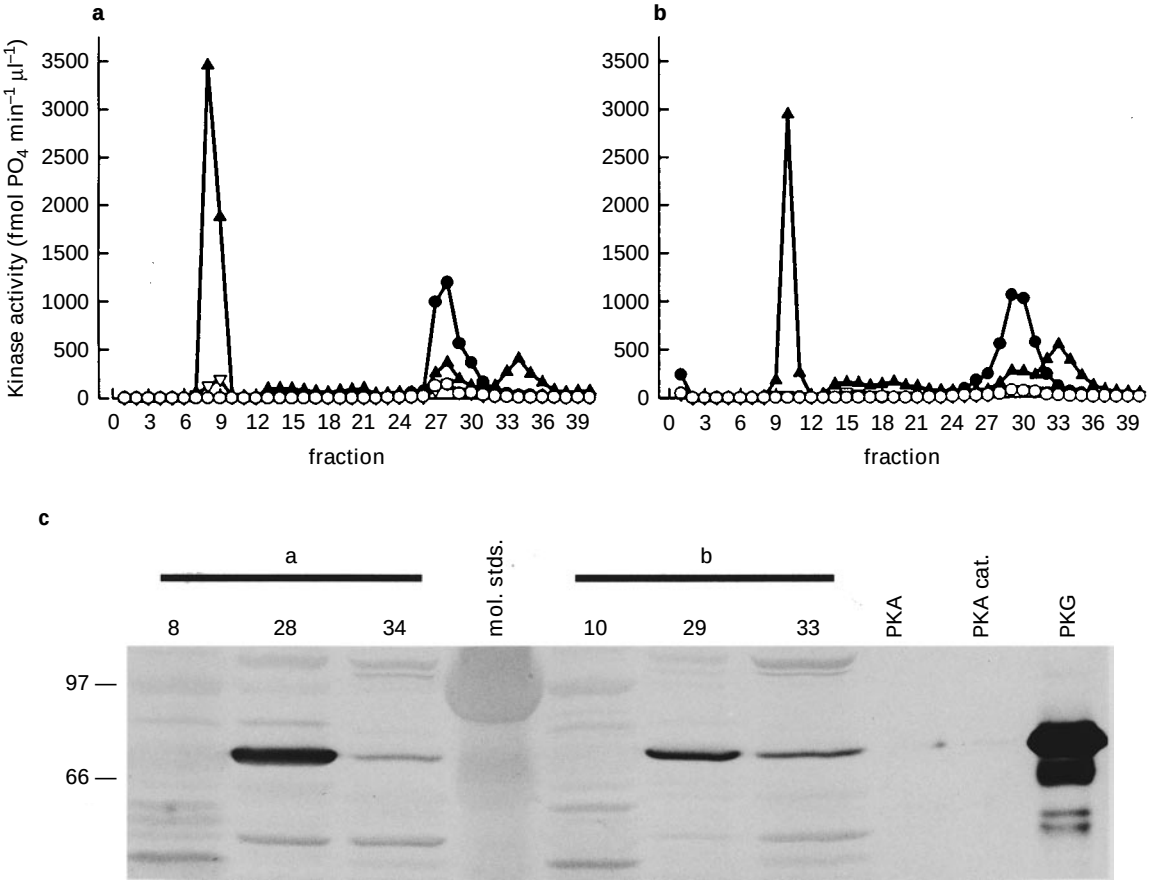

Figure 8 Fractions from MonoQ chromatography (0–400 mM NaCl gradient elution) of rat ventricular (a) and isolated rat ventricular cardiomyocyte (b) soluble extracts were assayed for cyclic GMP- and cyclic AMP-dependent kinase activity. Total amount of protein loaded onto the column was 14.5 mg (a) and 22 mg (b) soluble protein. Assay incubations were conducted at 30°C for 10 min. PKG activity was measured in the presence (▲) and absence (▽) of 5 μM cyclic GMP, in the presence of 5 μM PKI. PKA activity was measured in the presence (●) and absence (○) of 5 μM cyclic AMP, in the absence of PKI. Profiles are representative of cyclic GMP-dependent kinase activity in four ventricular preparations and three cellular preparations and cyclic AMP-dependent kinase activity in three ventricular preparations and two cellular preparations. (c) Fractions from MonoQ chromatography of rat ventricular tissue (Figure 8a, fractions 8, 28, 34) and cardiomyocytes (Figure 8b, fractions 10, 29, 33) were resolved by SDS–PAGE for Western blotting with an antibody raised to the C-terminus of type Iα PKG as described in Methods. Commercially obtained PKG and PKA holoenzymes and PKA catalytic subunit (PKA cat.) were included as controls. Positions of proteins of known molecular mass (mol. stds.) are indicated by their molecular mass values (kDa) on the left of the figure.

myocytes. This was also observed in the particulate fraction of isolated cardiomyocytes (data not shown). Cyclic nucleotide-independent kinase activity was negligible under conditions used to assay PKG.

PKA activity was detected in MonoQ fractions in the presence and absence of 5 μM cyclic AMP, in the absence of PKI. Two distinct peaks of cyclic AMP-dependent activity were seen in the MonoQ fractions (Figure 8a,b). The absence of these peaks in the presence of PKI (plus cyclic GMP) suggests that they correspond to type I and II PKA. Furthermore, the identification of the first peak as type I PKA is corroborated by its elution at relatively low ionic strength (Lincoln, 1983). Another cyclic AMP-dependent peak, which corresponds to the PKG peak, presumably represents activation of PKG by 5 μM cyclic AMP. This peak was both cyclic AMP- and cyclic GMP-dependent, although cyclic GMP was a considerably more effective activator. Cross-activation by cyclic AMP and cyclic GMP, greater sensitivity to cyclic GMP as an activator, and insensitivity to PKI all serve to identify this peak as PKG.

The chromatographic profiles demonstrate several points: the assay conditions used to detect PKG-dependent phosphorylation were very selective for measuring PKG activity (no peaks of activity in fractions 7–10 or 31–35 in the presence of PKI, ± cyclic GMP). Also, very little cyclic nucleotide-independent protein kinase activity was detected using standard assay conditions (± cyclic GMP, + PKI) and the profiles show that PKG can be partially activated by 5 μM cyclic AMP.

Western blotting of cyclic GMP-dependent protein kinase

Western blots of peak PKG and PKA activities were performed using a polyclonal antibody raised against the C terminus of type Iα PKG to confirm the identify of PKG in the MonoQ elution profiles. As shown in Figure 8c, the strongest immunoreactivity was seen in the presence of commercially obtained PKG at the molecular mass appropriate for authentic PKG; M_r values for type Iα and Iβ PKG monomer are 78 and 80, respectively (Wolfe *et al*., 1989). Specificity of the antibody for PKG was confirmed by the absence of binding to purified PKA holoenzyme or catalytic subunits. Marked immunoreactivity was detected at a similar molecular mass as purified PKG in lanes which corresponded to PKG peaks in the MonoQ profiles (fraction 28, Figure 8a; fraction 29, Figure 8b). Bands at a similar molecular mass as purified PKG stained less intensely in lanes corresponding to type II PKA, although considering the absence of PKG-like immunoreactivity in the presence of type I PKA (fraction 8, Figure 8a and fraction 10, Figure 8b) and commercially purified PKA, this probably reflects an overlap of PKG into the nearby type II PKA fractions in the MonoQ chromatography. Therefore, Western blotting

for PKG immunoreactivity resulted in the identification of PKG protein in MonoQ fractions which showed PKG activity. This confirms the presence of PKG in the intact ventricle as well as the isolated cardiomyocyte and also serves as a verification of the conditions of the assay in terms of specificity for PKG.

Effect of carbachol, SNP and ANP on cyclic GMP-dependent protein kinase activity in rat ventricle

The activity ratio of PKG was assessed in the soluble fraction of ventricular tissue from agonist-treated, Langendorff-perfused rat heart. When compared to isoprenaline alone, 10 μM carbachol in the presence of 1 nM isoprenaline did not have a significant effect on PKG activity after 2 min (Figure 9). In contrast, the PKG activity ratio significantly increased by approximately fivefold after a 2 min exposure to either 100 nM ANP plus isoprenaline or 10 μM SNP plus isoprenaline, when compared to isoprenaline alone. Isoprenaline (1 nM) had no significant effect on the ventricular soluble PKG activity ratio in comparison to control (control $=0.05\pm0.02$, $n=5$; isoprenaline for 2 min $=0.13\pm0.04$, $n=10$). The absence of PKG activation by carbachol and the marked activation by ANP and SNP correlated well with changes in total tissue cyclic GMP levels in ventricular tissue (Figure 6). A comparison of these findings with the results of contractility experiments, represented in Figures 1 and 2, illustrates a dissociation between PKG activation and agonist-mediated negative inotropy in the rat isoprenaline-stimulated intact ventricle (carbachol mediated a negative inotropic effect with no significant activation of PKG, whereas marked activation of PKG by ANP and SNP was not associated with a negative inotropic effect).

Effect of carbachol, SNP and ANP on cyclic GMP-dependent protein kinase activity in isolated ventricular cardiomyocytes

It was important to assess the effects of carbachol, ANP and SNP on PKG activity in isolated ventricular cardiomyocytes as the relative contribution of different cell types to the total mass of the heart and the content and activation state of PKG in different cell types may lead to erroneous extrapolations of PKG activity from the intact ventricle to cardiomyocyte. As shown in Figure 10, carbachol (10 μM for 2 min) did not significantly increase PKG activity over paired control levels, similar to results from the intact ventricle. While significant PKG activation by 10 μM SNP for 6 min was not demonstrated in this study, significant activation of PKG did occur in the presence of 100 μM SNP for 6 min. ANP (100 nM for 6 min) increased the soluble PKG activity ratio by approximately threefold.

Discussion

The primary objective of the present study was to confirm the reports of Lincoln & Keely (1980, 1981) which concluded that the negative inotropic effects of cyclic GMP-elevating agents in the heart were mediated via activation of PKG. This was based on the findings that the negative inotropic effects of ACh were accompanied by increases in cyclic GMP and activation of PKG, whereas SNP, which also elevated cyclic GMP, did not activate PKG and had no effect on contractility. In the present study, we confirmed the observation that a cholinergic agonist (carbachol), but not SNP, exerted a negative inotropic effect in rat ventricular preparations. However, in contrast to the results of Lincoln and Keely, we found that SNP, but not carbachol, significantly activated PKG in intact ventricular tissues and isolated cardiomyocytes. Furthermore, ANP, which elevates cyclic GMP by a mechanism distinct from that of carbachol and SNP, significantly activated PKG in intact hearts and isolated cardiomyocytes, but had no negative inotropic effect in either preparation. Thus, we were unable to confirm the reports of Lincoln & Keely (1980, 1981). The results of the present study argue against an important role for cyclic GMP or PKG in the negative inotropic effects of cholinergic agonists in the rat heart.

The difference between the results of the present study and those of Lincoln & Keely (1980, 1981) may reside in the methods used to measure PKG activation. In our opinion, improvements in the PKG assay since 1981 have substantially improved its specificity and reliability. A key difference in the present study is the use of a novel, relatively PKG-specific substrate, BPDEtide (Jiang *et al*., 1992). Interference by cyclic

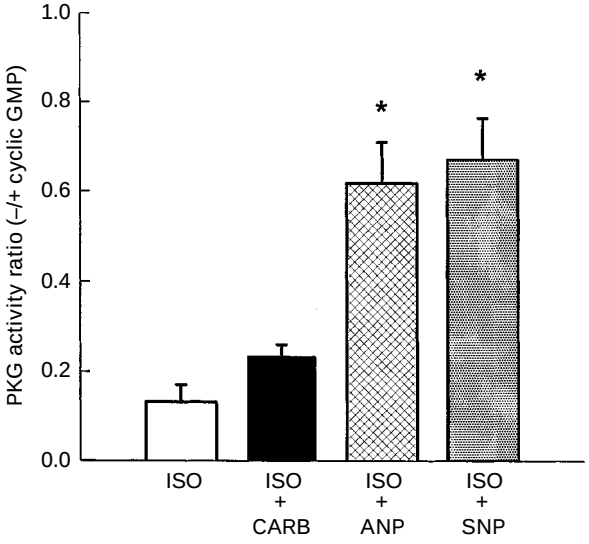

Figure 9 Effect of 10 μM carbachol (■; $n=5$), 100 nM ANP (▨; $n=5$) and 10 μM SNP (grey bar; $n=6$), in the presence of 1 nM isoprenaline, on soluble cyclic GMP-dependent protein kinase activity ratio in rat ventricular tissue. Treatment protocol was as described for Figure 1. PKG activity was expressed as the ratio of phosphorylation of substrate min^{-1} mg^{-1} protein at 4°C in the absence and presence of 5 μM cyclic GMP. Asterisks (*) indicate significant difference from 1 nM isoprenaline alone (□; $n=10$), one-way ANOVA, Bonferroni's method.

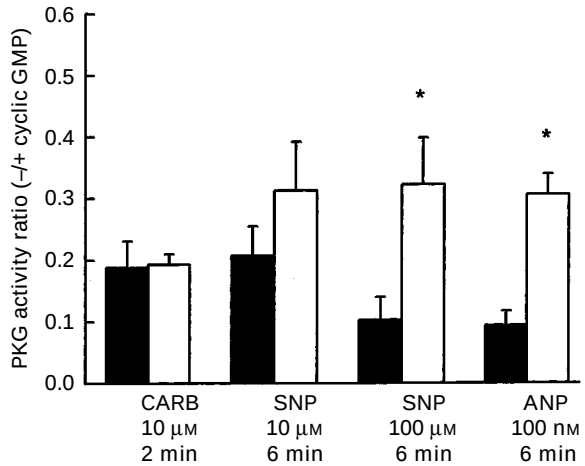

Figure 10 Effect of 1 nM isoprenaline for 2 or 6 min (■) or 1 nM isoprenaline plus either 10 μM carbachol for 2 min ($n=6$), 100 nM ANP for 6 min ($n=5$), or 10–100 μM SNP for 6 min ($n=5$–6) (□) on soluble cyclic GMP-dependent protein kinase activity in isolated rat ventricular cardiomyocytes. PKG activity was expressed as the ratio of phosphorylation of substrate min^{-1} mg^{-1} protein at 4°C in the absence and presence of 5 μM cyclic GMP. Asterisks (8) indicate significant difference from isoprenaline alone, paired Student's t test.

nucleotide-independent protein kinase activity was eliminated using BPDEtide in the assay as demonstrated by the absence of cyclic nucleotide-independent peaks in MonoQ elution profiles and the detection of low control PKG activity ratios in crude extracts. Activity ratios of untreated and isoproterenol-treated ventricular tissues in the present study ($\leqslant 0.20$) were 30–50% lower than the activity ratios of control intact tissues, including the ventricle, when assayed with histone H2B as a substrate (Lincoln & Keely, 1981; Fiscus *et al*., 1984, 1985) and were comparable to basal activity ratios in porcine coronary arteries assayed with BPDEtide (Jiang *et al*., 1992). Additionally, measurement of agonist-mediated PKG activation was conducted in the present study at 4°C in rapidly processed samples using brief incubation times. As discussed by Fiscus *et al*. (1984), higher assay temperatures such as those used in the earlier studies of Lincoln & Keely (1980, 1981), would likely result in rapid dissociation of cyclic GMP from PKG such that PKG activity ratios would not reflect the activation state of the enzyme in the tissue prior to processing.

The present study demonstrates, for the first time, PKG enzymatic activity in isolated cardiomyocytes. Total soluble PKG recovered from chromatography of intact ventricular tissue was comparable to total soluble PKG in isolated cells, as calculated on the basis of activity per mg of loaded protein. Cardiac PKG activity levels are low in general but it is apparent that cardiomyocytes contribute significantly to total cardiac PKG content. In contrast, immunological techniques have detected PKG immunoreactivity almost exclusively in smooth muscle cells of cardiac tissue with comparatively little immunoreactivity in cardiomyocytes (Joyce *et al*., 1984; Ecker *et al*., 1989). The reason for the difference between the immunocytochemical studies and the biochemical results described herein is not clear but may be related to differences in the immunoreactivity of PKG between smooth muscles and cardiomyocytes. Contamination of the cardiomyocyte preparation with PKG-rich cells, such as smooth muscle cells, could be postulated to account for the apparent cardiomyocyte-specific PKG activity in the present study. However, contamination was minimal because repeated gravity sedimentation of isolated cardiomyocytes removed essentially all non-cardiomyocyte cells.

The results of the present study agree with those of others who have found NO donors to be ineffective in reducing contractility in a variety of cardiac preparations. For example, SNP and other NO-releasing agents have been reported to have no effect on twitch tension in canine and rabbit ventricular tissue (Endoh & Yamashita, 1981; Inui *et al*., 1982; Rodger & Shahid, 1984), on maximal ventricular pressure or force in rat and guinea pig perfused hearts (Lincoln & Keely, 1980, 1981; Grocott-Mason *et al*., 1994), on the force and time course of contraction in rat, guinea pig and human cardiac preparations (Nawrath *et al*., 1995) and on maximal cell shortening in guinea pig isolated ventricular cells (Stein *et al*., 1993). On the other hand, negative inotropic effects of SNP have been reported in the literature (e.g. Smith *et al*., 1991; Brady *et al*., 1993), although these are generally of small magnitude compared to the responses commonly observed with muscarinic agonists. The reasons for the discrepancies in the published reports are not clear at the present time. It has been suggested that qualitative differences in the response to nitric oxide donors such as SNP may exist at different concentrations of the drugs. Kodja *et al*. (1996) have reported that low concentrations of NO donors *increase* contractile force in rat ventricular cardiomyocytes (by increasing cyclic AMP levels) whereas higher concentrations of the agents *decrease* contractile force (by increasing cyclic GMP levels). Arguing against this possibility in the present study is the finding that SNP had no positive or negative inotropic effects in ventricular strips over a concentration range of 0.1–100 μM. Furthermore, 10 μM SNP had no demonstrable effect on ventricular cyclic AMP levels in this or previous studies from our laboratory (MacDonell *et al*., 1995). In order to explain the lack of effect seen with any concentration of SNP in our study, it would have to be argued that a negative inotropic effect, due to activation of PKG, is balanced at each concentration by an equal and opposite positive inotropic effect, with the end result being an absence of contractile effect at any concentration. There is no evidence to support this possibility in the present study. In addition, ANP, a peptide with no structural similarity to SNP, and which elevates cyclic GMP by stimulating particulate guanylyl cyclase in a nitric oxice-independent manner (Anand-Srivastava & Trachte, 1993), was without effects on ventricular and cardiomyocyte contractility, although it activated PKG to a similar extent as did SNP. It seems unlikely that such a dissimilar compound would activate PKG as well as activate a counter-regulatory process in a manner identical to SNP.

Interpretation of previous reports which compared cyclic GMP content with ventricular contractility in the presence of ANP is hampered by the fact that no attempts were made to measure cyclic GMP in the same preparations as were used for contractility studies. In the presence of 400 nM ANP, only a 3% decline in twitch tension and indices of relaxation time in ferret papillary muscle was observed by Smith *et al*. (1991) while that concentration of ANP stimulated a fourfold increase in cyclic GMP in separate tissue samples. Neyes & Vetter (1989) reported that shortening amplitude of rat cardiomyocytes declined from approximately 5.5% to 3.7% of resting cell length in the presence of 100 nM ANP and, in separate experiments, 100 nM ANP increased cyclic GMP content by about twofold. However, the two phenomena appeared to be functionally unrelated, as much higher concentrations of ANP were required to elevate cyclic GMP levels (100 nM ANP) than were necessary to diminish cell shortening ($EC_{50} \approx 70$ pM). Based on these reports and the data in the present study, changes in cyclic GMP content caused by SNP or ANP in ventricular preparations do not appear to be well correlated with changes in contractile activity of the preparations.

A number of recent studies have used other approaches to investigate the role of PKG in the negative inotropic effects of cyclic GMP-elevating agents. For example, Méry *et al*. (1991) observed an inhibition of cyclic AMP-stimulated L-type calcium current (I_{Ca}) when rat ventricular cardiomyocytes were infused with catalytically active PKG fragments. They suggested that the mechanism of action of agents which elevate ventricular cyclic GMP, including muscarinic receptor agonists, involves activation of PKG. While the authors took care to infuse concentrations of PKG believed to reflect the average concentration of endogenous cellular PKG, high concentrations at the site of infusion may have occurred and resulted in phosphorylation of the calcium channel in a manner which did not reflect physiological conditions. Also, the catalytically active fragment of PKG may function dissimilarly to the endogenous kinase as it is free from regulation by endogenous cyclic GMP and is not specificaly localised within the cell. Localisation of PKG has been suggested to be of importance in regulating smooth muscle and neutrophil function (Cornwell *et al*., 1991; Wyatt *et al*., 1993; Pryzwansky *et al*., 1995). Interpretation of the studies on calcium currents is also complicated by the fact that no contractility measurements were made in those studies. It is possible that the reported changes in I_{Ca} might not occur in intact preparations or, if they do occur, that they might not be sufficient to cause a negative inotropic effect. Some evidence for this possibility has been provided in a recent report by Talukder & Endoh (1997). In that study, inhibition of L-type calcium channels by verapamil was able to diminish the positive inotropic effects of phenylephrine, endothelin and angiotensin in rabbit ventricular myocardium, but did not alter the positive inotropic response to isoprenaline. The implication is that inhibition of I_{Ca} might not be sufficient to overcome the positive inotropic effects of β-adrenoceptor agonists. It is important to note that in our studies, where contractility, cyclic GMP levels and PKG activity were measured in the same preparations, large increases in cyclic GMP levels and marked activation of PKG by SNP and ANP were not able to overcome the positive inotropic effects of isoprenaline. Carbachol,

on the other hand, was able to block the effects of isoprenaline in concentrations that had little or no effect on cyclic GMP or PKG.

Inhibition of β-adrenoceptor agonist-mediated cyclic AMP elevation, due to inhibition of adenylyl cyclase activity by the guanine nucleotide binding protein, G_i, has been suggested to account for the negative inotropic effects of muscarinic receptor agonists in some studies (Inui *et al*., 1982; Katano & Endoh, 1993). However, as noted above, no changes in isoprenaline-mediated cyclic AMP elevation were observed in the present study in the presence of a concentration of carbachol which markedly decreased ventricular contractility. This lack of a cyclic AMP-lowering effect by a cholinergic agonist is in agreement with previous observations in a variety of mammalian ventricular preparations (Watanabe & Besch, 1975; MacLeod & Diamond, 1986; Schmeid & Korth, 1990; Gupta *et al*., 1994; MacDonell *et al*., 1995). Thus, it appears that the lowering of cyclic AMP levels by muscarinic receptor agonists is not always necessary for their negative inotropic effects.

A small positive inotropic effect, in the absence of β-adrenoceptor stimulation, was observed in the present study in rat ventricular strips exposed to a high concentration of carbachol (Figure 3). It has been previously suggested that the positive inotropic effects of agonists acting at α-adrenoceptors and muscarinic cholinoceptors may be due to G-protein coupled activation of the β isozyme of phospholipase C, leading to the production of the second messengers, inositol trisphosphate and diacylglycerol (see e.g. Brown & Martinson, 1992). No attempt was made in the present study to investigate this possibility as the scope of the study was restricted to mechanisms involved in negative inotropy.

In summary, the results of the current study show that activation of PKG by cyclic GMP-elevating agents such as SNP and ANP does not mediate a negative inotropic effect in the rat ventricle. Furthermore, the negative inotropic effects of muscarinic receptor agonists in the rat ventricle are not necessarily accompanied by PKG activation. These results suggest that elevation of cyclic GMP and activation of PKG do not play important roles in the negative inotropic response to cholinergic agonists in rat ventricular preparations.

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References

ANAND-SRIVASTAVA, M.B. & TRACHTE, G.J. (1993). Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol. Rev*., **45,** 445–497.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72,** 248–254.

BRADY, A.J.B., WARREN, J.B., POOLE-WILSON, P.A., WILLIAMS, T.J. & HARDING, S.E. (1993). Nitric oxide attenuates cardiac myocyte contraction. *Am. J. Physiol*., **265** (*Heart Circ. Physiol*., **34),** H176–H182.

BROWN, J.H. & MARTINSON, E.A. (1992). Phosphoinositide-generated second messengers in cardiac signal transduction. *Trends Cardiovasc. Med*., **2,** 209–214.

COLBRAN, J.L., FRANCIS, S.H., LEACH, A.B., THOMAS, M.K., JIANG, H., MCALLISTER, L.M. & CORBIN, J.D. (1992). A phenylalanine in peptide substrates provides for selectivity between cGMP- and cAMP-dependent protein kinases. *J. Biol. Chem*., **267,** 9589–9594.

CORNWELL, T.L., PRYZWANSKY, K.B., WYATT, T.A. & LINCOLN, T.M. (1991). Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells. *Mol. Pharmacol*., **40,** 923–931.

CRAMB, G., BANKS, R., RUGG, E.L. & AITON, J.F. (1987). Actions of atrial natriuretic peptide (ANP) on cyclic nucleotide concentrations and phosphatidylinositol turnover in ventricular myocytes. *Biochem. Biophys. Res. Commun*., **148,** 962–970.

DIAMOND, J. & CHU, E.B. (1985). A novel cyclic GMP-lowering agent, LY83583, blocks carbachol-induced cyclic GMP elevation in rabbit atrial strips without blocking the negative inotropic effects of carbachol. *Can. J. Physiol. Pharmacol*., **63,** 908–911.

DIAMOND, J., TEN EICK, R.E. & TRAPANI, A.J. (1977). Are increases in cyclic GMP levels responsible for the negative inotropic effects of acetylcholine in the heart? *Biochem. Biophys. Res. Commun*., **79,** 912–918.

ECKER, T., GOBEL, C., HULLIN, R., RETTIG, R., SEITZ, G. & HOFFMAN, F. (1989). Decreased cardiac concentration of cGMP kinase in hypertensive animals: an index for cardiac vascularization? *Circ. Res*., **65,** 1361–1369.

ENDOH, M. & YAMASHITA, S. (1981). Differential responses to carbachol, sodium nitroprusside and 8-bromo-guanosine 3′,5′-monophosphate of canine atrial and ventricular muscle. *Br. J. Pharmacol*., **73,** 393–399.

FISCUS, R.R., RAPOPORT, R.M., WALDMAN, S.A. & MURAD, F. (1985). Atriopeptin II elevates cyclic GMP, activates cyclic GMP-dependent protein kinase and causes relaxation in rat thoracic aorta. *Biochim. Biophys. Acta*, **846,** 179–184.

FISCUS, R.R., TORPHY, T. & MAYER, S.E. (1984). Cyclic GMP-dependent protein kinase activation in canine tracheal smooth muscle by methacholine and sodium nitroprusside. *Biochim. Biophys. Acta*, **805,** 382–392.

GEORGE, W.J., POLSON, J.B., O'TOOLE, A.G. & GOLDENBERG, N.D. (1970). Elevation of guanosine 3′,5′-cyclic phosphate in rat heart after perfusion with acetylcholine. *Proc. Natl Acad. Sci. U.S.A*., **67,** 305–312.

GEORGE, W.J., WILKERSON, R.D. & KADOWITZ, P.J. (1973). Influence of acetylcholine on contractile force and cyclic nucleotide levels in the isolated perfused rat heart. *J. Pharmacol. Exp. Ther*., **184,** 228–235.

GOLDBERG, N.D., HADDOX, M.K., NICHOL, S.E., GLASS, D.B., SANFORD, C.H., KUEHL, F.A. & ESTENSEN, E. (1975). Biologic regulation through opposing influences of cyclic GMP and cyclic AMP: the yin-yang hypothesis. *Adv. Cyclic Nucleotide Res*., **5,** 307–330.

GROCOTT-MASON, R., FORT, S., LEWIS, M.J. & SHAH, A.M. (1994). Myocardial relaxant effect of exogenous nitric oxide in isolated ejecting hearts. *Am. J. Physiol*., **266** (*Heart Circ. Physiol*. **35),** H1699–H1705.

GUPTA, R.C., NEUMANN, J., BOKNIK, P. & WATANABE, A.M. (1994). M_2-specific muscarinic cholinergic receptor-mediated inhibition of cardiac regulatory protein phosphorylation. *Am. J. Physiol*., **266** (*Heart Circ. Physiol*. **35),** H1138–H1144.

HEI, Y.-J., MCNEILL, J.H., SANGHERA, J.S., DIAMOND, J., BRYER-ASH, M. & PELECH, S.L. (1993). Characterization of insulin-stimulated seryl/threonyl protein kinases in rat skeletal muscle. *J. Biol. Chem*., **268,** 13203–13213.

INUI, J., BRODDE, O.-E. & SCHÜMANN, H.J. (1982). Influence of acetylcholine on the positive inotropic effect evoked by α- or β-adrenoceptor stimulation in the rabbit heart. *Naunyn-Schmiedeberg's Arch. Pharmacol*., **320,** 152–159.

JIANG, H., COLBRAN, J.L., FRANCIS, S.H. & CORBIN, J.D. (1992). Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. *J. Biol. Chem*., **267,** 1015–1019.

JOYCE, N.C., DECAMILLI, P. & BOYLES, J. (1984). Pericytes, like vascular smooth muscle cells, are immunocytochemically positive for cyclic GMP-dependent protein kinase. *Microvasc. Res*., **28,** 206–219.

KATANO, Y. & ENDOH, M. (1993). Cyclic AMP metabolism in intact rat ventricular cardiac myocytes: Interaction of carbachol with isoproterenol and 3-isobutyl-1-methylxanthine. *Mol. Cell. Biochem*., **119,** 195–201.

KOJDA, G., KOTTENBURG, K., NIX, P., SCHÜLTER, K.D., PIPER, H.M. & NOAK, E. (1996). Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. *Circ. Res.*, **78,** 91–101.

KRYSKI, A., KENNO, K.A. & SEVERSON, D.L. (1985). Stimulation of lipolysis in rat heart myocytes by isoproterenol. *Am. J. Physiol.*, **248,** H208–H216.

LAEMMLI, R. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680–685.

LINCOLN, T.M. (1983). cGMP-dependent protein kinase. *Meth. Enzymol.*, **99,** 62–71.

LINCOLN, T.M. & KEELY, S.L. (1980). Effects of acetylcholine and nitroprusside on cyclic GMP-dependent protein kinase in the perfused heart. *J. Cyclic Nuc. Res.*, **6,** 83–91.

LINCOLN, T.M. & KEELY, S.L. (1981). Regulation of cardiac cyclic GMP-dependent protein kinase. *Biochim. Biophys. Acta*, **676,** 230–244.

LOFFELHOLZ, K. & PAPPANO, A.J. (1985). The parasympathetic neuroeffector junction of the heart. *Pharmacol. Rev.*, **37,** 1–24.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

MACDONELL, K.L., TIBBITS, G.F. & DIAMOND, J. (1995). cGMP elevation does not mediate muscarinic agonist-induced negative inotropy in rat ventricular cardiomyocytes. *Am. J. Physiol.*, **269** (*Heart Circ. Physiol.*, **38),** H1905–H1912.

MACLEOD, K.M. & DIAMOND, J. (1986). Effects of the cyclic GMP lowering agent LY83583 on the interactions of carbachol with forskolin in rabbit isolated cardiac preparations. *J. Pharmacol. Exp. Ther.*, **238,** 313–318.

MARKWELL, M.A.K., HAAS, S.M., TOLBERT, N.E. & BIEBER, L.L. (1981). Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.*, **72,** 296–303.

MÉRY, P.F., LOHMANN, S.M., WALTER, U. & FISCHMEISTER, R. (1991). Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 1197–1201.

NAWRATH, H. (1976). Cyclic AMP and cyclic GMP may play opposing roles in influencing force of contraction in mammalian myocardium. *Nature*, **262,** 509–511.

NAWRATH, H., BAUMNER, D., RUPP, J. & OELERT, H. (1995). The ineffectiveness of the NO-cyclic GMP signaling pathway on the atrial myocardium. *Br. J. Pharmacol.*, **116,** 3061–3067.

NEYES, L. & VETTER, H. (1989). Action of atrial natriuretic peptide and angiotensin II on the myocardium: studies in isolated rat ventricular cardiomyocytes. *Biochem. Biophys. Res. Commun.*, **163,** 1435–1443.

PETERSON, G.L. (1979). Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.*, **100,** 201–220.

PRYZWANSKY, K.B., WYATT, T.A. & LINCOLN, T.M. (1995). Cyclic GMP-dependent protein kinase is targeted to intermediate filaments and phosphorylates vimentin in A23187-stimulated human neutrophils. *Blood*, **85,** 222–230.

RODGER, I.W. & SHAHID, M. (1984). Forskolin, cyclic nucleotides and positive inotropism in isolated papillary muscles of the rabbit. *Br. J. Pharmacol.*, **81,** 151–159.

SCHMEID, R. & KORTH, M. (1990). Muscarinic receptor stimulation and cyclic AMP-dependent effects in guinea pig ventricular myocardium. *Br. J. Pharmacol.*, **99,** 401–417.

SMITH, J.A., SHAH, A.M. & LEWIS, M.J. (1991). Factors released from endocardium of the ferret and pig modulate myocardial contraction. *J. Physiol.*, **439,** 1–14.

STEIN, B., DRÖGEMÜLLER, A., MÜLSCH, A., SCHMITZ, W. & SCHOLZ, H. (1993). Ca^{2+}-dependent constitutive nitric oxide synthase is not involved in the cyclic GMP-increasing effects of carbachol in ventricular cardiomyocytes. *J. Pharmacol. Exp. Ther.*, **266,** 919–925.

TALUKDER, M.A. & ENDOH, M. (1997). Pharmacological differentiation of synergistic contribution of L-type Ca^{2+} channels and Na^{+}/H^{+} exchange to the positive inotropic effect of phenylephrine, endothelin-3 and angiotensin II in rabbit ventricular myocardium. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **355,** 87–96.

WATANABE, A.M. & BESCH, H.R. (1975). Interaction between cyclic adenosine monophosphate and cyclic guanosine monophosphate in guinea ventricular myocardium. *Circ. Res.*, **37,** 309–317.

WOLFE, L., CORBIN, J.D. & FRANCIS, S.H. (1989). Characterization of a novel isozyme of cGMP-dependent protein kinase from bovine aorta. *J. Biol. Chem.*, **264,** 7734–7741.

WYATT, T.A., LINCOLN, T.M. & PRYZWANSKY, K.B. (1993). Regulation of human neutrophil degranulation by LY-83583 and L-arginine: role of cGMP-dependent protein kinase. *Am. J. Physiol.*, **265,** (*Cell Physiol.*, **34),** C201–C211.

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