Enhanced Inositol Trisphosphate Response to α_1-Adrenergic Stimulation in Cardiac Myocytes Exposed to Hypoxia

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Abstract

Myocardial ischemia elicits an enhanced responsivity to α_1-adrenergic stimulation and a reversible increase in α_1-adrenergic receptor number. In adult cardiac myocytes, α_1-adrenergic receptor number increases two- to threefold after 10 min of hypoxia, an increase similar to that seen during ischemia in vivo. To determine whether this increase in α_1-adrenergic receptor number leads to an enhanced synthesis of inositol trisphosphate, the intracellular second messenger for the α_1-adrenergic receptor, the mass of inositol trisphosphate was quantified by a novel procedure developed in our laboratory that circumvents problems associated with using labeled precursors. The peak increases in inositol trisphosphate levels of three- to fourfold were measured after 30 s of norepinephrine stimulation and exhibited a 50% effective concentration (EC_{50}) of 7.9×10^{-8} M. Hypoxia produced a marked leftward shift in the dose-response curve for the production of inositol trisphosphate in response to norepinephrine stimulation ($EC_{50} = 1.2 \times 10^{-8}$ M). Hypoxia also induced a 100-fold reduction in the concentration of norepinephrine required to elicit a threshold increase in inositol trisphosphate (10^{-9} M), compared with control normoxic myocytes (10^{-7} M). Thus, hypoxia, which increases α_1-adrenergic receptor density, also leads to an enhanced production of inositol trisphosphate and could account for the enhanced α_1-adrenergic responsivity in the ischemic heart in vivo, which is known to facilitate arrhythmogenesis.

Introduction

Myocardial ischemia results in an increase in responsivity to α_1-adrenergic stimulation, which contributes to the development of malignant ventricular arrhythmias (1). These lethal arrhythmias result from both reentrant and nonreentrant mechanisms (2, 3). The nonreentrant mechanisms are largely unknown, but may involve the development of delayed afterdepolarizations resulting in triggered rhythms secondary to an increase in intracellular calcium. Blockade of α_1-adrenergic receptors has been shown to inhibit the production of delayed afterdepolarizations under hypoxic conditions in vitro (4) and the accumulation of intracellular calcium after reperfusion in vivo (5). Therefore, it is likely that enhanced α_1-adrenergic input increases intracellular calcium, resulting in the occurrence of cardiac arrhythmias due to a nonreentrant mechanism. This increase in α_1-adrenergic responsivity during early ischemia is associated with a twofold reversible increase in α_1-adrenergic receptors (6). The mechanisms responsible for the increase in α_1-adrenergic receptors has recently been explored with the use of isolated adult canine myocytes exposed to hypoxia as an in vitro model of myocardial ischemia. Acute hypoxia produced a two- to threefold reversible increase in cell surface α_1-adrenergic receptors secondary to the sarcolemmal accumulation of long-chain acylcarnitines (7). A critical question is whether these newly exposed receptors are linked to an intracellular second messenger that could contribute to the increase in intracellular calcium.

The intracellular second messengers mediating α_1-adrenergic stimulation in many tissue types are inositol (1, 4, 5) trisphosphate (IP_3)[1] (8) and diacylglycerol produced through cleavage of phosphatidylinositol (4,5)-bisphosphate by phospholipase C. IP_3 increases the concentration of cytosolic Ca^{2+} (9), whereas diacylglycerol activates protein kinase C (10), which may also increase cytosolic Ca^{2+} by phosphorylating sarcolemmal calcium channels (11). However, the evidence demonstrating that IP_3 can act as the intracellular second messenger for the α_1-adrenergic receptor in cardiac tissue is circumstantial. Preincubation of cardiac tissue with [^{3}H]*myo*-inositol and subsequent measurements of label appearing in the IP_3, inositol bisphosphate (IP_2), or inositol monophosphate (IP_1) fractions have indicated that breakdown of phosphatidylinositols after α_1-adrenergic stimulation does occur (12, 13), although only after prolonged stimulation and in the presence of pharmacologic interventions. This study was performed to determine first whether α_1-adrenergic stimulation elicits an increase in the production of IP_3 in adult cardiac myocytes under normoxic conditions using a method developed in our laboratory and based in part on that of Rittenhouse and Sasson (14) to measure directly the mass of inositol phosphates. The second objective was to assess whether this response was altered by hypoxia.

Methods

The isolated adult canine myocyte preparation has been characterized extensively with the use of biochemical, electrophysiologic, and histo-

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Received for publication 9 September 1988 and in revised form 30 November 1988.

J. Clin. Invest.

0021-9738/89/04/1409/05 $2.00
Volume 83, April 1989, 1409–1413

1. *Abbreviations used in this paper:* IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol (1, 4, 5) trisphosphate.

logic endpoints (7). All measurements indicate that the cells are intact and have physiologic characteristics similar to those measured in intact cardiac tissue. The isolated adult myocytes (900 μl, ~ 500,000 cells) in Hepes buffer (115 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 500 μM Ca^{2+}, 35 mM sucrose, 10 mM glucose, 10 mM Hepes, 4 mM taurine, pH 7.2), were incubated in the presence of 100 μl of norepinephrine or vehicle (Hepes plus 1 mg/ml ascorbic acid, pH 7.2). After a specified time interval, all incubations were terminated by addition of an equal volume of ice-cold 15% (wt/vol) TCA, vortexed and placed on ice for 10 min. At this stage, [^{3}H]inositol phosphates (~ 10,000 dpm) were added to assess subsequent recovery. The precipitate was removed by centrifugation and the supernatant was decanted and placed in siliconized 13 × 100 mm glass tubes. TCA was removed with 2 ml of H_2O-saturated diethyl ether. The extraction was repeated four times and residual ether was removed by heating the samples to 60°C for 1 h.

Ion-exchange chromatography. Samples were diluted to 10 ml with Tris buffer (0.01 M, pH 8.5) and titrated to pH 8.5 with 1.0 N $NaHCO_3$. Anion exchange resin (AG 1-X8, formate form, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA) was extensively washed with Tris buffer (pH 8.5) to remove fines, and loaded onto 10-cm columns, 1.5 cm i.d., (Bio-Rad Laboratories) such that the resin occupied 1 cm of the column. The resin was washed with 20 ml of Tris buffer (pH 8.5) before the samples were loaded onto separate columns. After loading the samples, the resin was washed with a further 30 ml of Tris buffer (pH 8.5) to remove all inositol. Subsequent elutions involved 8 ml of 0.05 M Na_2SO_4, 0.075 M Na_2SO_4, 0.19 M Na_2SO_4, and 0.30 M Na_2SO_4 in Tris buffer (pH 8.5) to elute sequentially IP_1, IP_2, IP_3, and inositol tetrakisphosphate. Each sample was collected in siliconized 13 × 100 mm glass tubes. The recovery of added [^{3}H]*myo*-inositol 1-phosphate; 1,4-bisphosphate; 1,4,5-trisphosphate; and 1,3,4,5-tetrakisphosphate (~ 10,000 dpm each; Amersham Corp., Arlington Heights, IL) was assessed by determining the amount of radiolabel from each inositol phosphate to elute from the column in each of the sodium sulfate fractions.

Dephosphorylation of inositol phosphates. Samples were concentrated prior to dephosphorylation by lyophilization to dryness and resuspended in 1 ml of Tris buffer (pH 8.5). To dephosphorylate the samples, 5 mM $MgCl_2$ and 35–50 U alkaline phosphatase (type VII S; Sigma Chemical Co., St. Louis, MO) were added to each tube. Samples were incubated overnight at 37°C and then boiled for 3 min to terminate the reaction. The extent of dephosphorylation was assessed using 10^4 dpm of [^{3}H]*myo*-inositol (1,4,5)-trisphosphate (1.0 Ci/mmol) with subsequent ion-exchange chromatography as described above to determine the amount of radioactivity in the individual inositol fractions. After dephosphorylation, 1 nmol of *chiro*-inositol was added to each sample to serve as an internal standard for gas chromatographic analysis.

Desalting inositol fractions. Samples were desalted using an extensively washed mixed-bed anion-cation exchange resin (MSZ 501, 20–50 mesh, Bio-Rad Laboratories). Inositol was eluted with 8 ml of distilled water. The eluate was collected in 13 × 100 mm siliconized glass tubes.

Derivatization and gas chromatography. After desalting, samples were lyophilized to dryness under vacuum followed by derivatization to the hexatrimethylsilyl derivatives. This was achieved by the addition of 25 μl of anhydrous pyridine (Aldrich Chemical Co., Milwaukee, WI) and 25 μl of bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Pierce Chemical Co., Rockford, IL) to each dried residue. Samples were vortexed and transferred to 0.3-ml glass reactivials (Pierce Chemical Co.). Reactivials were capped and placed in a heating block at 60°C overnight. Separation of *chiro*- and *myo*-inositol was achieved by gas chromatography using a gas chromatograph model 3700 (Varian Associates, Inc., Palo Alto, CA). The derivatized sample (1.0 μl) was injected onto a column of cross-linked dimethyl silicone (Ultra 1, Hewlett-Packard Co., Palo Alto, CA) (25 m long, 0.20 mm i.d., 0.11 μm film thickness). Gas chromatographic conditions were as follows: injection temperature was 170°C, detection temperature was 240°C, column temperature was 110–210°C at 25°C/min. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injections were made in the splitless mode (splitter flow = 150 ml/min) with a 30-s period between sample injection and opening of the splitter solenoid valve. Products were detected by flame ionization at 240°C. *Myo*- and *chiro*-inositol peaks were separated by ~ 3 min and quantified by integration of peak area using an integrator (model 4270, Varian Associates, Inc.). *Chiro*- and *myo*-inositol from tissue samples were identified by comigration with authentic standards. The detector response ratio for *chiro*- and *myo*-inositol derivatives was determined by analysis of equal quantities of *chiro*- and *myo*-inositol derivatives. Derivatized samples from standard or tissue preparations were injected onto the column and the amount of *myo*-inositol from a particular inositol phosphate fraction was determined from the ratio of *chiro*- to *myo*-inositol peak areas corrected for the detector response ratio. Protein was measured by the method of Lowry (15). This gas chromatographic method can detect as little as 50 fmol of inositol per injection, corresponding to an assay sensitivity of 1 pmol IP_3 per mg of cellular protein.

Results

Recovery of [^{3}H]IP_3. The recovery of added [^{3}H]*myo*-inositol phosphates (~ 10,000 dpm) after anion-exchange chromatography was > 95% after their respective elutions with a stepwise sodium sulfate gradient in Tris buffer (16). Recovery of [^{3}H]*myo*-(1,4,5)-inositol trisphosphate ([^{3}H]IP_3) through the concentration step before dephosphorylation was 100% (16). Subsequent dephosphorylation by alkaline phosphatase at 37°C for 10 h resulted in complete dephosphorylation of [^{3}H]IP_3.

The greatest loss occurred during desalting (30%). However, because *chiro*-inositol was added to the samples as an internal standard before this step, this loss of *myo*-inositol only represents a loss of signal to noise and does not enter into the calculation of inositol mass. Complete derivatization of both *chiro*- and *myo*-inositol standards (10 nmol of each) was achieved after 5 h of incubation at 60°C. The detector response ratio to *chiro*- and *myo*-inositol at the gas chromatographic settings described in Methods was found to be 1.51. Excellent linearity was also found between the detector response and increasing amounts of *myo*-inositol (r = 0.999).

Inositol trisphosphate response. Initial studies were aimed at assessing the inositol phosphate response of isolated adult canine myocytes to stimulation by norepinephrine. The levels of IP_3, IP_2, and IP_1 after norepinephrine stimulation are shown in Fig. 1. An initial increase in IP_3 was apparent as early

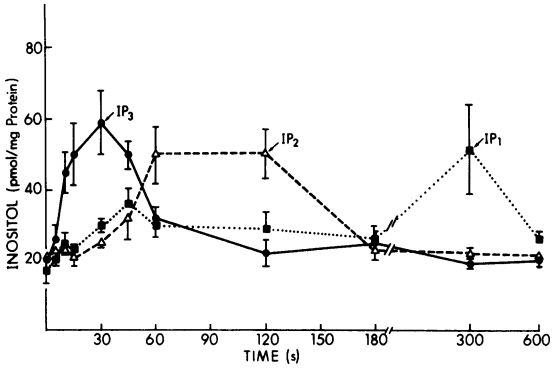

Figure 1. Time course of production of IP_1, IP_2, and IP_3 after norepinephrine (10^{-5} M) stimulation of adult canine myocytes (mean±SEM of three separate experiments). Myocytes were incubated in Hepes buffer (500 μM Ca^{2+}, pH 7.2) in the presence or absence of norepinephrine (10^{-5} M) for selected time intervals at 37°C and the mass of IP_3, IP_2, and IP_1 was determined as in Methods.

as 5 s after stimulation. IP_3 levels were maximal after 30 s of stimulation, followed by a rapid return to control levels by 60 s. Peak production of IP_2 was seen between 1 and 2 min, whereas elevated IP_1 was apparent after 5 min of stimulation. Although the level of IP_3 returned to control by 2 min, this does not necessarily indicate that the physiologic response to IP_3 terminates as well. Many, but not all, agonists that stimulate IP_3 production elicit a transient increase in IP_3 (similar to that presented in this manuscript) during continuous agonist exposure. This desensitization of the agonist response with respect to IP_3 is not likely to be analogous to the in vivo situation, in which receptors are transiently exposed to an agonist that undergoes rapid presynaptic reuptake and rapidly diffuses from the synaptic cleft. Even during continuous exposure to norepinephrine, the physiologic α_1-adrenergic response is likely to outlast greatly the transient increase in IP_3. This prolongation of the α_1-adrenergic response could result from either altered intracellular calcium concentrations (secondary to IP_3) or from protein phosphorylation (secondary to diacylglycerol activation of protein kinase C). This sequence of events would be highly analogous to the β-adrenergic response, in which a transient accumulation of cAMP is magnified and maintained by cAMP-dependent protein kinase. The sequential production of IP_3, IP_2, and IP_1 was observed after stimulation of myocytes with norepinephrine, suggesting a sequential hydrolysis of IP_3 to IP_2 and to IP_1. However, a contribution from sequential hydrolysis of phosphatidylinositol bisphosphate, phosphatidylinositol phosphate, and phosphatidylinositol cannot be ruled out. The question as to how much phosphatidylinositol bisphosphate, phosphatidylinositol phosphate, and phosphatidylinositol is hydrolyzed cannot be answered by these data, as the rate of dephosphorylation (or activity of the phosphatases) is unknown. These measurements indicate the amount of IP_3, IP_2, and IP_1 that accumulates, but not the extent of phosphatidylinositol hydrolysis.

Inositol trisphosphate exists in two isomeric forms: a physiologically active form, inositol 1,4,5-trisphosphate, and a relatively inactive form, inositol 1,3,4-trisphosphate. Analysis of the mass of the isomers of IP_3, (1,3,4)IP_3, and (1,4,5)IP_3 after separation by a standard HPLC procedure (17) indicated that both isomers were present in low levels before stimulation (8 pmol/mg protein for (1,3,4)IP_3 and 12 pmol/mg protein for (1,4,5)IP_3). In contrast, stimulation with norepinephrine for 30 s resulted in a selective increase in the (1,4,5)IP_3 isomer (48 pmol/mg protein), indicating production of the physiologically active isomer, which can mobilize intracellular calcium in several cell systems (9).

To determine whether the norepinephrine-induced increase in IP_3 was mediated specifically through the α_1-adrenergic receptor, myocytes were stimulated with norepinephrine for 30 s in the absence or presence of specific α_1-, α_2-, and β-adrenergic antagonists (Fig. 2). As with the time course experiment, norepinephrine produced a threefold increase in IP_3 levels. The stimulation of IP_3 by norepinephrine was completely inhibited by the specific α_1-adrenergic antagonist BE-2254; yohimbine (α_2-adrenergic antagonist) or propranolol (β_1- and β_2-adrenergic antagonist) had no significant effect, thereby conferring an α_1-adrenergic specificity to the IP_3 response.

The dose-response curve for IP_3 production was then assessed by determination of the level of IP_3 produced after 30 s of stimulation by selected concentrations of norepinephrine

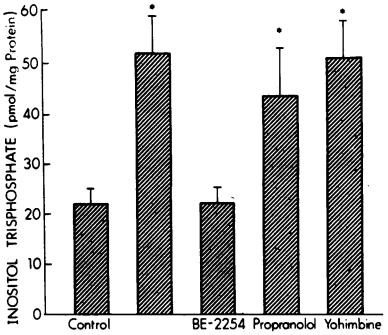

Figure 2. The effect of α_1-, β-, and α_2-adrenergic blockade on norepinephrine-stimulated IP_3 content in isolated adult canine myocytes. Myocytes were incubated in Hepes buffer at 37°C in the absence (control) or presence of norepinephrine (10^{-5}) plus either BE-2254, an α_1-adrenergic antagonist (10^{-7} M); *l*-propranolol, a β_1- and β_2-adrenergic antagonist (10^{-7} M); or yohimbine, an α_2-adrenergic antagonist (10^{-7} M). After 30 s, the incubations were terminated by addition of TCA and the mass of IP_3 was determined. Values are the mean±SEM of four separate experiments. Statistical significance was assessed by analysis of variance followed by Dunnett's test for comparing treated groups with control. $^*P < 0.05$ compared with control.

(Fig. 3, *inset*). Maximum IP_3 levels were again equivalent to a three- to fourfold stimulation over control values and the 50% effective concentration (EC_{50}) for the IP_3 response (the con-

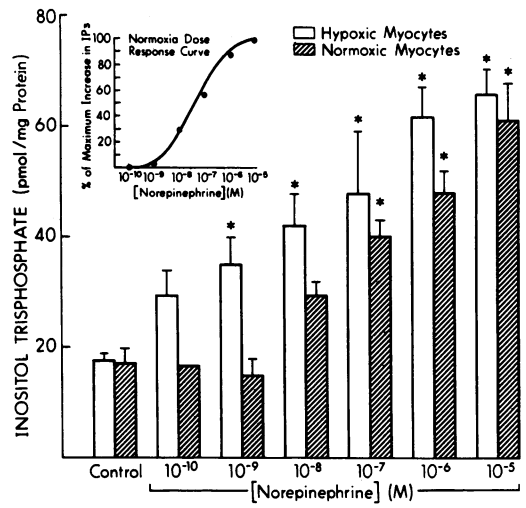

Figure 3. IP_3 content after 30 s of stimulation with selected concentrations of norepinephrine in control normoxic myocytes and myocytes exposed to 10 min of hypoxia ($Po_2 < 15$ mmHg) at 37°C. The dose-response curve for IP_3 production after norepinephrine stimulation in normoxic myocytes is shown in the *inset;* results are expressed as a percentage of the maximum response. Incubations were carried out at 37°C in the absence or presence of norepinephrine (10^{-10}–10^{-5} M) for 30 s, after which the mass of IP_3 was determined. Hypoxia was produced by degassing Hepes buffer (pH = 7.2) followed by equilibration with prepurified N_2 for 3 h. Myocytes were introduced into an air-tight vial under N_2, which was blown continuously over the myocyte suspension. Values for IP_3 production from normoxic and hypoxic myocytes given above are the mean±SEM from four separate experiments. Statistical significance ($P < 0.05$) of the difference between each norepinephrine-stimulated value and the corresponding hypoxic or normoxic control value was assessed by analysis of variance followed by Duncan's test, and is denoted by an asterisk. The EC_{50} for each curve was obtained by normalization of the data and calculation of the norepinephrine concentration giving half the maximum response. There was a significant difference between the normoxic and hypoxic dose-response curves ($P < 0.001$), as determined by analysis of covariance.

centration of norepinephrine giving half-maximal response) was determined as $7.9\pm0.9 \times 10^{-8}$ M. This EC_{50} value for norepinephrine stimulation of IP_3 is considerably lower than the EC_{50} for norepinephrine displacement of specific [^{3}H]prazosin binding ($1.6\pm0.7 \times 10^{-6}$ M) on adult canine myocytes (7). This finding indicates that a maximal IP_3 response can be elicited by occupancy of a small fraction of the α_1-adrenergic receptors on adult canine myocytes. This phenomenon could be explained by a vast excess of α_1-adrenergic receptors or "spare receptors" on the myocytes. Alternatively, there may be several subtypes of α_1-adrenergic receptors on these cells, all of which would be detected by [^{3}H]prazosin binding but only some of which may be functionally coupled to IP_3 production.

We have characterized previously the biochemical and morphologic response of adult canine myocytes to selected intervals of hypoxia (7). Hypoxia of 20 min or less at 37°C is associated with reversible alterations, as assessed by ATP and creatine phosphate content, a lack of intracellular enzyme release, and a lack of membrane disruption assessed using electron microscopy. More prolonged periods of hypoxia (> 20 min) are associated with release of intracellular enzymes and gross morphologic disruption indicative of irreversible cellular damage. Hypoxic intervals of 10 min result in a two- to threefold reversible increase in α_1-adrenergic receptor number (7). To determine whether this increase in α_1-adrenergic receptors is coupled to an increase in the IP_3 response, myocytes were exposed to hypoxia (Po_2 < 15 mmHg, 37°C) for 10 min and then stimulated with selected concentrations of norepinephrine, followed by measurement of the content of IP_3 (Fig. 3). An initial time course of IP_3 production after norepinephrine (10^{-5} M) stimulation of hypoxic myocytes verified that the peak increase again occurred at 30 s (data not shown). Hypoxic myocytes demonstrated a dramatic increase in the production of IP_3 compared with production in normoxic myocytes after stimulation with submaximal concentrations of norepinephrine. The EC_{50} for norepinephrine stimulation in hypoxic cells was $1.2\pm0.9 \times 10^{-8}$ M, sixfold lower than that obtained from the dose-response curve in normoxic cells. Most importantly, the threshold concentration of norepinephrine required to produce a significant increase in IP_3 was 10^{-9} M in hypoxic myocytes and 100-fold higher (10^{-7} M) in normoxic myocytes. However, there was no significant difference between the maximum IP_3 level produced when hypoxic and normoxic myocytes were exposed to the highest concentration of norepinephrine (10^{-5} M). Therefore, at submaximal concentrations of norepinephrine, a greater IP_3 response is induced in hypoxic compared with normoxic myocytes. This difference is particularly evident in the physiologic range of norepinephrine concentrations (10^{-10} to 10^{-8} M). Indeed, exposure of the myocytes to hypoxia has shifted the IP_3 response to α_1-adrenergic stimulation into the physiologic range of norepinephrine concentrations.

Discussion

These data indicate that the increase in α_1-adrenergic receptor number in response to acute hypoxia is concurrent with a greater IP_3 response after stimulation by norepinephrine. In many systems, there are more receptors present than are required for a maximum response and can be referred to as spare but equivalent receptors. In these systems, increased receptor number can produce a leftward shift in the dose-response curve with no change in maximum response, similar to results found in this study. Thus, regulation of receptor number can provide a mechanism for control of cell responsiveness. Although increased exposure of α_1-adrenergic receptors probably accounts for at least a portion of the increased IP_3 response, a number of other factors may also be operative. The increased fluidity of the sarcolemmal membrane during hypoxia, facilitated by the sarcolemmal accumulation of long-chain acylcarnitines (7, 18), could enhance coupling between the α_1-adrenergic receptor and phospholipase C or an activator of phospholipase C, possibly a guanine nucleotide–binding protein (G protein). However, direct identification of the G protein involved has not been reported. If coupling of α_1-adrenergic receptors to IP_3 production relies on the collision coupling of receptor, G protein, and phospholipase C, increased membrane fluidity could enhance this coupling. Alternatively, enhanced IP_3 production may result from a component of the coupling system remaining in an active configuration for a longer period of time. The dramatic difference between the IP_3 response in hypoxic compared with normoxic myocytes suggests that mechanisms other than the increase in α_1-adrenergic receptor number may contribute to the increased IP_3 response.

These results and findings in vivo demonstrate that the α_1-adrenergic system in hypoxic or ischemic cardiac tissue is more responsive than that in normoxic cardiac tissue. Lindemann et al. (19) have shown that α_1-adrenergic stimulation leads to an increase in [^{32}P]phosphate incorporation into a 15-kD sarcolemmal protein that has previously been identified as a substrate for protein kinase C (11). Phosphorylation was associated with restoration of contractions in hearts depolarized with elevated extracellular K^+, which was probably mediated by the slow inward current carried by Ca^2. A recent preliminary study indicates that norepinephrine only stimulates protein kinase C in depolarized cardiac tissue (20) and the authors speculate that α_1-adrenergic stimulation of protein kinase C may depend on membrane potential or the degree of calcium loading. Both IP_3 production and activation of protein kinase C can result in an increase in the intracellular concentration of calcium. The increased α_1-adrenergic responsivity in the heart during ischemia probably results in an increase in the synthesis of IP_3 and possibly activation of protein kinase C. By increasing the intracellular concentration of calcium, α_1-adrenergic stimulation may act as a secondary positive inotropic system in the failing heart, independent of adenylate cyclase. However, increased intracellular calcium may contribute to the development of triggered rhythms. Activation of this α_1-adrenergic mechanism leading to enhanced production of IP_3 could be an important mediator of malignant cardiac arrhythmias leading to sudden cardiac death.

Acknowledgments

We would like to express our appreciation to Ava Ysaguirre for preparing the manuscript, Dr. Leona Rubin for the HPLC analysis, Evelyn Kanter and Beverly Kekec for preparing the myocytes, and Dr. Kathryn Yamada for discussions and advice throughout this project.

This work was supported by National Institutes of Health (NIH) grants HL-17646, Specialized Center of Research in Ischemic Heart Disease, HL-28995, and HL-36773 to Peter B. Corr; and NIH grant GM-37846 to Alex S. Evers. Guy P. Heathers was supported by a Research Fellowship from the American Heart Association, Missouri Heart Affiliate.

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