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IMMUNE CYTOKINE INHIBITION OF BETA-ADRENERGIC AGONIST STIMULATED CYCLIC AMP GENERATION IN CARDIAC MYOCYTES

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SUMMARY: We hypothesize that reversible depression of cardiac function in cardiac allograft rejection and lymphocytic myocarditis reflects down modulation of the beta-adrenergic receptor system by a soluble product of activated immune cells. Thus, exposure of cultured cardiac myocytes to mixed lymphocyte culture or activated splenocyte supernatants produces 70% inhibition of isoproterenol-stimulated cAMP concentrations (K_i = 5% supernatant) in the absence of gross cellular injury or control media effects. This cAMP suppressive factor is not dialyzable and is ammonium sulfate precipitable. Beta-adrenergic receptor density, binding constant and affinity states are unaffected. These results demonstrate the existence of a cytokine inhibitor of cAMP accumulation that may mediate, in part, depression of cardiac contractility observed when immune cells invade the myocardium.

Cardiac allograft rejection and lymphocytic myocarditis associated with idiopathic dilated congestive cardiomyopathy are clinically prevalent heart diseases characterized by reversible cardiac failure and a myocardial mononuclear cell infiltrate. Typically, cardiac dysfunction is disproportionately more severe than is the extent of myocardial infiltration and necrosis and is ameliorated by immunosuppressive therapy (1). The

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Abbreviations: cAMP = cyclic adenosine monophosphate; CYP = cyanopindolol; G_s = stimulatory guanine nucleotide binding protein; G_i = inhibitory guanine nucleotide binding protein; MLC = mixed lymphocyte culture; S.D. = standard deviation; TCA = trichloroacetic acid.

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reversible pathophysiology of these clinical states is therefore most consistent with a deleterious effect on cardiac function exerted by the non-cytotoxic efferent limb of the cellular immune response. Because adrenergic stimulation is crucial to the maintenance and augmentation of cardiac contractility (2), we hypothesized that the reversible decrement in contractility in these clinical syndromes may reflect inhibition of the cardiac responsiveness to beta-adrenergic stimulation and that this effect is mediated by a soluble product of activated immune cells.

To examine this potential immunomodulation of cardiac function, we established an in vitro model system based upon the culture of monolayers of spontaneously contracting neonatal rat cardiac myocytes. The myocytes were exposed to various cell-free supernatants from activated lymphocytes and macrophages, the predominant cell types in myocarditis and the rejecting allograft. Results demonstrate the production of a cytokine that is a potent inhibitor of cardiac myocyte cyclic adenosine monophosphate (cAMP) generation in response to beta-adrenergic agonists. Negative inotropic changes observed in the clinical setting of invasion of the myocardium by activated immune cells may thus be mediated by a prolonged, functional desensitization of the beta-adrenergic receptor.

MATERIAL AND METHODS

Myocyte Cell Culture. Neonatal rat cardiac myocytes were isolated and cultured using a modification of the technique of Harary and Farley (3). Briefly, hearts were removed aseptically from one-day old Sprague-Dawley rats and rinsed of blood, minced, and subjected to six rounds of digestion in phosphate buffered saline (PBS) without divalent cations containing collagenase 200 u/ml (Gibco), 2% v/v fetal bovine serum (Hyclone) and 5.5 mM dextrose. Collected cell suspensions were pooled, filtered (#80 Nitex cloth), and intact myocytes separated from fibroblasts and dead myocytes by centrifugation through a step gradient of Percoll (Pharmacia). Cells collected from the Percoll layer interface were washed and resuspended in Ham's F-12 nutrient mixture supplemented with 10% v/v fetal bovine serum, 25 mM HEPES, 50 u/ml penicillin, and 50 ug/ml streptomycin. Myocyte yield averaged 1.5 X 10^6 cells/animal with greater than 99% viability and less than 5% contamination with non-myocyte cell elements. Cells were suspended at a density of 5.0 X 10^5/ml in culture medium and plated (150 ul/well) in 96 well Primaria (Falcon) or vinyl (Costar) tissue culture plates.

Immune Cell Culture. Rat bidirectional primary mixed lymphocyte cultures (MLC) were established using splenocytes harvested from Lewis strain and outbred Sprague-Dawley adult rats (4). Pulp cells teased from splenic capsules into iced Hank's Balanced Salt Solution (Gibco) were subjected to ammonium chloride erythrocyte lysis (5), washed and suspended at 5.0 X 10^6 cells/ml in culture medium identical to that used for myocytes. Ten ml of cell suspension from both strains were added to T75 culture flasks and incubated at 37°C in 5% CO_2 and humidified air. Cellular activation was confirmed by documenting accelerated proliferation in MLC compared to control syngenic splenocyte cultures using [^{3}H]thymidine incorporation rates, and by confirmation and quantitation of interleukin-1 and interleukin-2 activities in MLC supernatants utilizing the D10.G4.1 (6) and CTLL (7) T-cell line proliferation assays, respectively.

Activated macrophage supernatant was obtained from cultures of macrophages isolated from rat peritoneum plated in 75 cm^2 T flasks (8). Following a 24-hour incubation in medium containing 5 ug/ml lipopolysaccharide, macrophage monolayers were exhaustively washed and incubated in medium identical to myocyte culture medium for 48 hours. Activated splenocyte supernatant was obtained from 24 hour cultures of adult rat splenocytes suspended at 5.0 X 10^6 cells/ml in culture medium containing 5 ug/ml concanavalin A (Sigma). Lectin was removed by adsorption to Sephadex G25 (Pharmacia) (9) and all supernatants were centrifuged and sterile filtered prior to use.

cAMP Assay. Following incubation of myocytes under control and experimental conditions, culture medium was aspirated and replaced with 200 ul assay buffer at 37°C consisting of Dulbecco's PBS, 5.5 mM dextrose, 25 mM HEPES, 1 mM ascorbic acid, and stated concentrations of isoproterenol. After incubation for 10 minutes at 37°C, samples were deproteinized with perchloric acid (final [PCA] = 0.6 M) and cooled to 4°. Aliquots were then neutralized with $KHCO_3$, and analyzed for cAMP content by radioimmunoassay (10). The ED_{50} of isoproterenol was 3 X 10^{-8} M.

Beta-Adrenergic Receptor Binding Assay. Myocyte beta-adrenergic receptors were assayed by quantitating binding of the radioligand [^{125}I]cyanopindolol, ([^{125}I]CYP, SRA 2200 Ci/mmole, New England Nuclear) to intact cells in monolayer culture. Briefly, myocytes established in culture in vinyl 96-well plates received 200 ul/well assay buffer at 4°, consisting of Dulbecco's PBS, 25 mM HEPES, 5.5 mM dextrose, 1 mM ascorbic acid, and 0.1% BSA with [^{125}I]CYP concentrations ranging from 2 to 300 pM in triplicate wells. Following 16 hr incubation at 4°C (required for equilibrium binding), wells were washed exhaustively with 4°C Dulbecco's PBS, an aliquot of buffer and wash counted for determination of free [^{125}I]CYP concentration, and individual wells counted for quantitation of bound [^{125}I]CYP. Parallel assays with buffer containing 1 uM pindolol were performed to determine nonspecific binding which was less than 5% at K_D. Binding affinity constant (K_D = 6.3 pM) and receptor density (B_{max} = 275 fmole/mg; approximately 5 X 10^4 receptors/cell) were calculated after Scatchard transformation (11) and were similar to published results in myocyte cell suspensions (12). For competition binding studies, 50 pM [^{125}I]CYP was used with isoproterenol concentrations varying from 10^{-10} to 10^{-4} M in half-log increments.

General Assays of Cell Integrity. Total cellular protein in culture wells was determined by Bradford assay (13) following solubilization with 0.1 M NaOH. Protein synthesis was assessed by the rate of incorporation of [^{3}H]leucine into 10% TCA precipitable material using a modification of standard methods (14). Cellular ATP concentration was determined using the luciferin-luciferase fluorometric assay (15).

RESULTS

Initial experiments were carried out on neonatal rat cardiac myocyte cultures established for 48 hours following cell plating, at which time cells exhibited spontaneous, regular and synchronous beating. Following exhaustive washing, culture medium was changed and cells were incubated in the presence of 1:4 dilutions of cell-free supernatants from various cell cultures. Following a further 72 hour incubation and subsequent washing, intracellular cAMP was assayed under basal and isoproterenol-stimulated conditions. Mixed lymphocyte culture, lectin activated splenocyte, and activated peritoneal macrophage culture supernatants all exhibited potent inhibition of isoproterenol-stimulated increases in myocyte cAMP concentration without significantly changing basal concentrations (Table I, lines 2, 3 and 4). Control

myocytes contained 8 pmol cAMP/mg protein under basal conditions, with isoproterenol (10^{-7} M) stimulation to 143 pmole/mg protein. In immune supernatant exposed cells, cAMP production after isoproterenol stimulation was markedly inhibited, with only 50 pmol cAMP/mg protein present. In contrast, control supernatants from subconfluent, confluent and splenocyte supernatant activated fibroblast cultures all failed to demonstrate suppression of agonist-stimulated increases in cAMP concentration (Table I, lines 5 to 7).

Inhibitory activity of the immune cell supernatants was further examined as a function of supernatant concentration by measuring basal and isoproterenol-stimulated myocyte cAMP concentrations following incubation with various dilutions of an MLC supernatant (Figure 1). As before, under control conditions, cAMP was stimulated 15-fold from 10 to 145 pmole/mg protein following incubation with 10^{-7} M isoproterenol. In MLC supernatant exposed myocytes there was a dose-dependent inhibition of isoproterenol-stimulated increases of cAMP concentration with a maximal inhibition of 60% at a supernatant concentration of 20%. Half-maximal inhibition occurred at 5% supernatant. Baseline myocyte cAMP concentration (approximately 10 pmole/ mg protein) was not altered by prior MLC supernatant exposure. This suppressive bioactivity was reproducibly present in multiple preparations of both MLC (n = 3) as well as lectin-stimulated splenocyte (n = 12) and activated macrophage (n = 2) culture supernatants.

TABLE I

Immune Cell Supernatant Inhibition of Isoproterenol Stimulated cAMP Concentrations in Neonatal Rat Cardiac Myocytes

	[cAMP] (pmole/mg)		
Culture Supernatant	basal	stimulated	% inhibition
Control	8.0	143	-
MLC	8.5	49	72%
ConA Splenocyte	8.0	52	70%
LPS Macrophage	12.0	53	69%
Fibroblast Confluent	-*	146	0
Non-confluent	-*	164	0
Activated	-*	166	0

Myocytes were exposed to cell free supernatants from activated immune cell or fibroblast cultures for 72 hours and cAMP concentrations quantitated under basal and 10^{-7} M isoproterenol stimulated conditions. Numbers represent means of triplicate determinations for a typical experiment and were reproducible with multiple supernatant (n = 15) and myocyte (n > 100) preparations.

*not determined

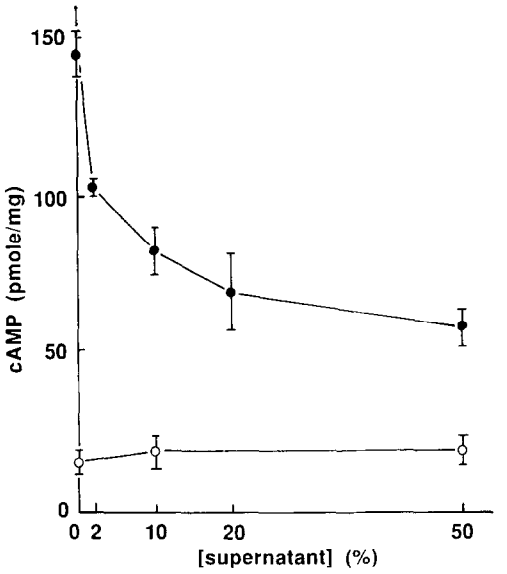

FIGURE 1 - Activated Immune Cell Supernatant Induced Inhibition of Beta-Arenergic Agonist Stimulation of Myocyte Cyclic AMP. Cardiac myocyte intracellular [cAMP] was determined following 72 hr culture in the presence of 0 to 50% activated splenocyte supernatant. Isoproterenol (10^{-7}M) stimulated increases in [cAMP] (●—●) was markedly inhibited in supernatant exposed cells with a K_I of 5% supernatant and a maximal inhibition of 60%. Basal [cAMP] (o—o) was unaffected by prior supernatant exposure. Points represent quadruplicate means ± S.D.s.

Cyclic AMP suppressive bioactivity was maintained following both saturated ammonium sulfate precipitation and dialysis (6,000 to 8,000 MW cutoff membranes, Spectrum). Absence of supernatant-induced nonspecific cellular toxicity was confirmed by lack of microscopic evidence of cytotoxicity, or significant changes in myocyte total ATP concentration, total protein content, or protein synthetic rates as assessed by [^{3}H]leucine incorporation into TCA precipitable material (Table II). These results indicate that the activated immune cell supernatant inhibition of isoproterenol stimulation of cAMP does not reflect gross cellular injury.

Because isoproterenol is known to stimulate intracellular cAMP production via binding to the beta-adrenergic receptor (16), the diminished isoproterenol-stimulated cAMP response in immune cell supernatant-exposed myocytes could reflect a diminution in cell surface beta-receptor density. Equilibrium binding studies of control and MLC supernatant exposed myocytes using the beta-antagonist [^{125}I]CYP revealed that the sarcolemma receptor density (B_{max}) and low affinity state equilibrium dissociation constant (K_D) were unchanged by prior supernatant exposure under conditions described above that result in the inhibition of cAMP stimulation (Figure 2A). Thus, B_{max} was 282 fmole/mg (approximately 5 X 10^4 receptors/cell) and K_D was 7.3 pM for

TABLE II

Lack of Immune Cell Supernatant Induced Changes in Neonatal Rat Cardiac Myocyte ATP Concentration, Protein Content, and Protein Synthetic Rate

Culture Supernatant	ATP (nmole/mg)	Protein (ug/well)	[^{3}H]Leu Incorp (CPM/mg.hr)
Control	6.9 ± 0.75	11.15 ± 0.32	13,680 ± 1,275
Activated Splenocyte	7.3 ± 1.06	11.36 ± 0.85	12,490 ± 1,250

Myocytes were exposed to control medium or activated splenocyte supernatant for 72 hours after which cellular integrity was assessed microscopically and total cellular [ATP], [protein], and protein synthetic rates [assessed by incorporation of [^{3}H]leucine (1 uCi/ml) into TCA precipitable material during a one hour pulse] were quantitated. Numbers represent means and S.D.s of quadruplicate determinations.

immune supernatant exposed myocytes compared to 275 fmole/mg and 6.3 pM for control cells (Figure 2B).

Additionally, because there are two affinity states for adrenergic receptors, the higher of which binds agonists preferentially and is coupled through the stimulatory nucleotide regulatory protein G_s to adenylate cyclase (16), an alternative hypothesis to explain diminished cAMP production in response to isoproterenol is an increase in the binding constant of this high affinity receptor state, or a decrease in the ratio of the number of

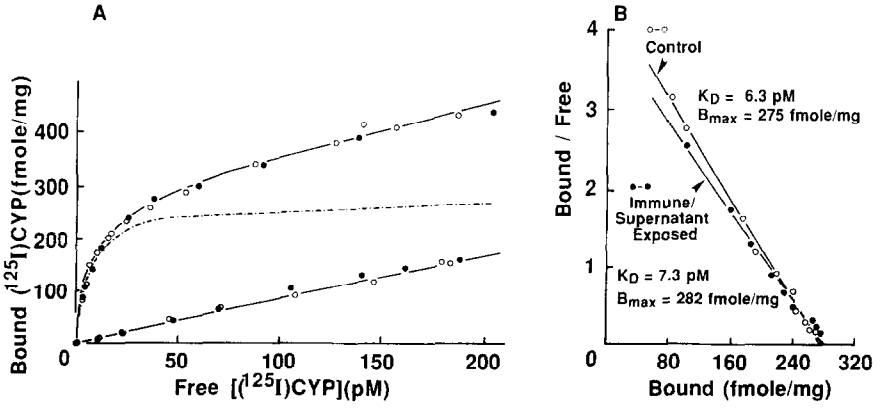

FIGURE 2 - Equilibrium Binding of [^{125}I]CYP to Intact Cardiac Myocytes in Monolayer Cultures. A) Total and nonspecific binding (___) and specific binding (. . .) of [^{125}I]CYP to intact myocytes was quantitated after 72 hr culture in the presence of 20% activated immune cell supernatant (•) and control medium (o). Points represent means of triplicate determinations and S.D.s were all less than 5% of means.
B) Scatchard transformation of binding data for control (o) and immune supernatant exposed (•) cells.

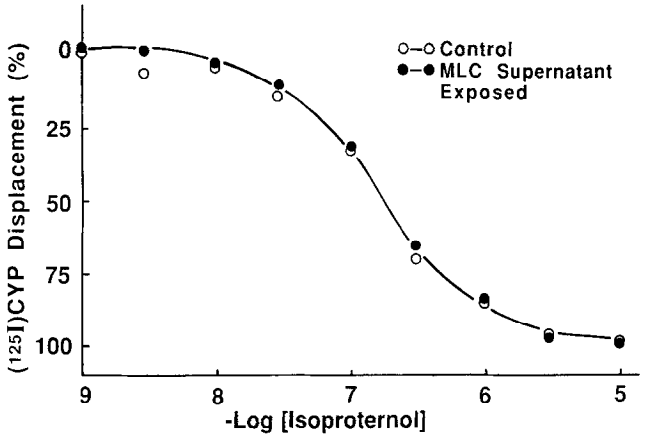

FIGURE 3 - Equilibrium Isoproterenol Competition Binding to Intact Cardiac Myocytes in Monolayer Cultures. Isoproterenol displacement of [^{125}I]CYP binding to intact myocytes was determined after 72 hr culture in the presence of 20% activated immune cell supernatant (●) and control medium (o). Points represent means of triplicate determinations. S.D.s were less than 5% of means.

high to low affinity receptors. Competition binding studies with isoproterenol and [^{125}I]CYP demonstrated no change in the proportion of sarcolemma high affinity receptors. IC_{50} for isoproterenol was 2 X 10^{-7} M in both control and MLC supernatant exposed cells, with displacement profiles that superimpose (Figure 3). Thus, inhibition of isoproterenol stimulated accumulation of cAMP induced by immune cell supernatants is not due to diminished sarcolemma expression of total or high affinity receptors.

DISCUSSION

We have described inhibition of beta-adrenergic agonist stimulated increases in the intracellular concentration of cAMP in cardiac myocytes by a soluble product of activated immune cells. This represents the first description of immunomodulation of cAMP in non-dividing cells and has profound direct implications for several prevalent myocardial disease states. In particular, since depressions of intracellular cAMP in the cardiac myocyte produce decrements in the contractile state of the heart (17), our observations relate to cardiac allograft rejection and idiopathic cardiomyopathy associated with lymphocytic myocarditis, conditions in which reversible cardiac failure is prominent. Characterization and identification of the bioactive immune cell product, dissection of the mechanism by which agonist-stimulated cAMP concentrations are depressed, and investigation of whether this phenomenon generalizes to other cells and other hormonally regulated metabolic systems are of obvious interest.

Intracellular cAMP concentration reflects a balance of synthesis by adenylate cyclase and metabolism by adenylate kinase and cyclic nucelotide phosphodiesterases. Numerous cell surface receptor-ligand interactions are known modulators of adenylate cyclase, and most are coupled to the enzyme via guanine nucleotide binding proteins which either activate (G_s) or inhibit (G_i) enzymatic activity in response to ligand binding (18). In the case of the beta-adrenergic receptor, binding of agonist stimulates adenylate cyclase activity to increase intracellular cAMP concentration through the stimulatory GTP binding protein G_s (19). Thus, the inhibition of agonist-stimulated rises in cAMP concentration in the absence of a change in receptor density or affinity described here could be a consequence of accelerated cAMP hydrolysis or of uncoupling of the receptor-G_s protein-adenylate cyclase system. In addition, immune factor induced changes in the amount of G_i, its GTP binding avidity or affinity for cyclase could provide an explanation for our findings. This cAMP suppressive effect may well have relevance in other systems if direct effects on adenylate cyclase or on GTP binding proteins are responsible.

The identity of the factor(s) responsible for inhibiting beta agonist-induced increases in myocyte cAMP concentration is unknown but its lack of dialyzability and ammonium sulfate precipitability strongly suggest that it is a protein. Because peritoneal macrophage supernatant possesses bioactivity similar to that of the MLC and splenocyte supernatants, the cell source is likely to be the macrophage. Indeed, initial fractionation steps have indicated that there are two distinct cAMP inhibitory activities with cationic properties, one of which co-migrates with activity in the D10.G4 T cell proliferation assay used to detect the macrokine interleukin-1.

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