Mechanism of Cytokine Inhibition of 
β-Adrenergic Agonist Stimulation of 
Cyclic AMP in Rat Cardiac Myocytes

Impairment of Signal Transduction

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George F. Schreiner, and Louis G. Lange

Studies conducted in our laboratory have demonstrated that activated immune cells produce a soluble inhibitor(s) of cardiac myocyte contractile and cyclic AMP (cAMP) responses to β-adrenergic stimulation. To examine the mechanism of this effect, metabolic assays were conducted on cultured rat cardiac myocytes incubated in the presence and absence of supernatants harvested from rat activated splenocyte cultures. Intracellular cAMP accumulation in response to isoproterenol was inhibited by up to 74% in a dose-dependent fashion by conditioned media containing soluble cytokines from activated immune cells. By use of myocyte cultures in which contaminating nonmyocyte proliferation was inhibited by nonlethal irradiation, this phenomenon was shown to be independent of mitogenic effects. Isobutylmethylxanthine, a phosphodiesterase inhibitor, did not ablate cytokine-induced inhibition of cAMP accumulation. Parameters of β-adrenergic receptor binding and affinity were also unaffected. cAMP suppression was maintained after cholera toxin stimulation of cAMP production via stimulatory G protein ADP-ribosylation. cAMP inhibition was not apparent when cells were stimulated with forskolin, a direct adenylate cyclase activator. Importantly, pertussis toxin treatment significantly ablated cytokine-induced cAMP inhibition. Thus, interference with agonist-occupied β-adrenergic receptor coupling to adenylate cyclase to produce cAMP and subsequent contractile responses is induced by a factor(s) elaborated by activated immune cells. This interference occurs at the level of signal transduction across the membrane, can be overridden by pertussis toxin, and may involve changes in the coupling of the stimulatory/inhibitory G proteins to adenylate cyclase. These results demonstrate a novel mechanism of cytokine-induced myocyte dysfunction and may have important pathophysiological ramifications in immune-mediated myocardial diseases. (Circulation Research 1990;67:753–763)

A substantial subset of patients with inflammatory myocardial diseases experience reversible cardiac pathophysiology, although the pathogenesis of cardiac failure in these disease states is unclear. For example, in some groups of patients with idiopathic dilated congestive cardiomyopathy associated with lymphocytic myocarditis, cardiac failure is reversible with immunosuppressive therapy, although the histological appearance of endomyocardial biopsy specimens does not necessarily predict severity of ventricular dysfunction or clinical course and response to therapy. Likewise, serious declines in cardiac performance occur during mild and moderate cardiac allograft rejection; however, degrees of such decrements do not necessarily correlate with the appearance of endomyocardial biopsies or with cardiac myocyte cell death. Moreover, reversibility of cardiac failure with institution of immunosuppressive therapy is often achievable.

As cardiac allografting has become a viable therapeutic alternative for patients with New York Heart Association class IV disease and as the need has continued for the identification of effective and spe-
cific immunosuppressive regimens, understanding the pathogenesis of cardiac failure in myocardial states has assumed greater importance. The observation that invasion of the myocardium by leukocytes could be associated with reversible cardiac failure, coupled with the knowledge that immune cell cytokines could exert potential metabolic effects on other nonimmune cell types, led us to devise an in vitro system to study potential pathophysiological effects of immune cell–derived products on cardiac myocytes.

Thus, recent studies from our laboratory have established a model of cardiac inflammation consisting of cultured cardiac myocytes coincubated with activated immune cell–conditioned media, which contain soluble mediators, or cytokines. We have demonstrated a potential biochemical correlate of the immunomodulation of cardiac function relevant to cardiac allograft rejection and idiopathic dilated congestive cardiomyopathy associated with lymphocytic myocarditis. In essence, immunologically derived cytokines can produce an uncoupling of the β-adrenergic receptor from adenylate cyclase, which results in marked inhibition both of β-adrenergic agonist–stimulated cyclic AMP (cAMP) production and of contractility in the context of unaltered general cell metabolism. Cytokines, released in situ by macrophages and lymphocytes recruited to areas of inflammation, can therefore, at least theoretically, modulate cardiac function in the disease states mentioned.

In an effort to probe the molecular mechanisms responsible for this uncoupling effect, we have undertaken in the current report a systematic examination of the effect of these cytokines on the β-adrenergic receptor–adenylate cyclase axis. Findings of impaired signal transduction from agonist-occupied receptors to adenylate cyclase represent an extension of our knowledge about adrenergic receptor physiology, as modulated by cytokines, and may bear significantly on the cardiac dysfunction seen in disease states in which immune cells invade the myocardium.

**Materials and Methods**

**Cardiac Myocyte Monolayer Cultures**

Neonatal rat cardiac myocytes were isolated and cultured using a modification of the technique of Harary and Farley, as previously described. Briefly, ventricles from 1–2-day-old Sprague-Dawley rats were minced and digested with collagenase in divalent cation-free isotonic buffer consisting of (mM) NaCl 136, KCl 2.7, NaH2PO4 8, and dextrose 5.5, supplemented with 2% vol/vol fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah). The resulting cell suspension was enriched in intact myocytes by centrifugation through a Percoll (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) step gradient (densities 1.070 and 1.095), effecting purification to greater than 95%. Cells from the Percoll interface were suspended in control medium (Ham's F-12 nutrient medium with 10% vol/vol fetal bovine serum, 25 mM HEPE, 50 units/ml penicillin, and 50 μg/ml streptomycin) at a density of 7×107/ml and, unless otherwise stated, gamma-irradiated with 2,000 R to inhibit proliferating nonmyocyte growth. Myocytes were then plated in 96-well or 60 mm Falcon Primaria (Becton-Dickinson, Rutherford, N.J.) or 96-well vinyl (Costar Corp., Cambridge, Mass.) tissue culture plates and incubated at 37° C in 5% CO2 and humidified air. Experiments were performed after the establishment of cultures for 48 hours, at which time syncytium formation and spontaneous beating were exhibited.

**Immune Cell Cytokine-Containing Conditioned Media**

Activated splenocyte–conditioned media (ASCM) were obtained as previously described either from 24-hour cultures of adult rat splenocytes suspended in medium containing concanavalin A (5 μg/ml) or from 96-hour cultures of rat bidirectional mixed lymphocyte cultures, using splenocytes harvested from Lewis strain and outbred Sprague-Dawley rats. Lectin was removed by batch adsorption with Sephadex G25 (Pharmacia). Cellular activation in mixed lymphocyte cultures was confirmed by documenting accelerated proliferation with these cultures as compared with syngeneic splenocyte cultures using [3H]thymidine incorporation rates. Supernatants were clarified by centrifugation (2000g for 10 minutes), sterile-filtered (0.2 μm), and stored in aliquots at −20° C. Interleukin-1, interleukin-2, and tumor necrosis factor activities were confirmed using the D10.G4.1 proliferation, CTLL proliferation, and L929 fibrosarcoma cytotoxicity assays, respectively. Typical supernatants contained these cytokines in respective concentration ranges of 20–40, 35–60, and 10–20 units/ml.

**cAMP Assay**

After incubation with control culture medium or cytokines, myocytes were stimulated with indicated agents, and cAMP accumulation was determined. Where noted, pertussis toxin was added to cells 24 hours before stimulation, and cholera toxin was added 1 hour before stimulation. After exposure to control medium or cytokines with or without these toxins, medium was aspirated, and cells were washed once with phosphate-buffered saline (PBS). Monolayers were then incubated at 37° C in 100 μM cAMP assay buffer containing PBS with (mM) dextrose 5.5, HEPE 25, and ascorbic acid 1. Where indicated, 3×10−7 M isoproterenol, 1 mM isobutylmethylxanthine, or 3×10−5 M forskolin (Sigma Chemical Co., St. Louis) was included. After 10 minutes, stimulation was stopped, and cAMP was liberated by the addition of perchloric acid to a final concentration of 0.6 M. Aliquots of supernatant were neutralized with KHCO3, and cAMP was determined by radioimmunoassay. Values were normalized to cellular protein concentration as determined below.
**β-Adrenergic Receptor Binding Assay**

After incubation under control and experimental conditions for stated periods, intact myocyte β-adrenergic receptors were assayed by quantitated binding of the radioligand [125I]cyanopindolol (ICYP; specific radioactivity 2,200 Ci/mmol, New England Nuclear, Boston). Culture medium was aspirated and replaced with cold (4°C) assay buffer, consisting of PBS, 10 mM HEPES, 5.5 mM dextrose, 1 mM ascorbic acid, and 0.1% bovine serum albumin, with stated ICYP concentrations in triplicate wells. After a 16-hour incubation at 4°C (determined in preliminary experiments to be required for equilibrium binding), aliquots of buffer were retrieved for determination of free ICYP concentration. Wells were then exhaustively and rapidly washed with 4°C PBS and dried. Individual vinyl culture wells were then counted for quantitation of bound ICYP. Parallel wells received assay buffer containing 1 μM pindolol to determine nonspecific binding, which was less than 5% at the Kd concentration. Kd and receptor density were determined by Scatchard transformation of binding data. For competition binding studies, 50 pM ICYP was included in assay buffer with varying agonist concentrations.

**Membrane Preparation**

After incubation of myocytes with immune cell medium or cytokines, cells were washed with PBS, scraped, and Dounce-homogenized (30–40 strokes) in (mM) potassium phosphate 20 (pH 7.5), EDTA 1, sucrose 250, and MgCl2 5. Homogenates were centrifuged at 500g for 10 minutes to remove nuclei and then at 100,000g for 60 minutes. The resulting crude membrane pellets were resuspended in the above buffer without sucrose and stored at −70°C until use. Typical membrane yields were 50–75 μg/7×10⁶ myocytes plated in 60-mm tissue culture dishes.

**Adenylate Cyclase Assay**

Membrane adenylate cyclase activity was assayed as described.14 Membranes (20–30 μg/sample) were incubated at 37°C for 10 minutes in 20 mM Tris (pH 7.6), 26 mM KCl, 1 mM ATP, 1 mg/ml creatine phosphokinase, 6.4 mg/ml creatine phosphate, 5 mM MgCl2, 0.1% bovine serum albumin, and 1 mM isobutylmethylxanthine, with or without 100 μM forskolin, in a total volume of 100 μl. The reaction was stopped by boiling for 3 minutes. Samples were diluted in 20 mM 2-[N-morpholino]ethanesulfonic acid (MES) buffer (pH 6.1) and centrifuged at 1,500g for 20 minutes to separate precipitated proteins. cAMP generation and membrane protein were determined as described. Enzyme activity was expressed in picomoles of cAMP generated per milligram protein per minute.

**ADP-Ribosylation of Pertussis and Cholera Toxin Substrates**

The GTP-binding proteins were ADP-ribosylated using pertussis toxin (List Biological Laboratories, Inc., Campbell, Calif.), cholera toxin (Sigma), and [adenylate-32P]nicotinamide adenine dinucleotide (NAD) (specific radioactivity 800 Ci/mmol, New England Nuclear) as described.15–17 In summary, pertussis (50 μg/ml) and cholera (1 mg/ml) toxins were activated by incubation at 37°C for 10 minutes with 10 mM dithiothreitol in 100 mM Tris (pH 8.0) and 1 mM EDTA. Pertussis toxin–catalyzed ADP-ribosylation was performed by the addition of 50 μg membrane protein to a reaction mixture (total volume, 100 μl) containing 100 mM Tris (pH 8.0), [adenylate-32P]NAD (15–20 μCi/sample), 10 μM NAD, 1 mM ATP, 5 mM MgCl2, 0.2 mM GTP, 1 mM EDTA, 15 mM thymidine, and either a no-toxin control or 10 μg/ml activated pertussis toxin. Incubation at 37°C for 30 minutes was stopped by the addition of 1 ml cold 10% trichloroacetic acid. Samples were centrifuged at 1,500g for 20 minutes. The precipitates were then washed four times with ether, solubilized and boiled 3 minutes in reducing buffer with 1 mM β-mercaptoethanol, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% polyacrylamide). Gels were stained with Coomasie brilliant blue, destained, dried, and autoradiographed. Incorporation of [32P]NAD was demonstrated to be linear with protein concentration by optical densitometry. Cholera toxin–catalyzed ADP-ribosylation was performed similarly with the use of 200 μg/ml cholera toxin, 100 mM potassium phosphate (pH 6.0) buffer instead of Tris, and the deletion of thymidine in the reaction mixture.

**Proliferation Assay**

Cardiac cells plated in 96-well plates were pulsed with [3H]thymidine (0.4 μCi/well) during incubation with control and experimental media for indicated periods. DNA was harvested from cell wells using a PhD cell harvester (Cambridge Technology, Inc., Watertown, Mass.) with water washes through glass fiber filters (Whatman Inc., Clifton, N.J.). Filters were dried and counted for [3H] in 3 ml scintillant.

**Protein Assay**

Total cellular and membrane protein concentrations were determined by the method of Bradford18 after solubilization with 0.1N NaOH.

**Results**

**Cytokine Inhibition of Isoproterenol-Stimulated Cardiac Myocyte cAMP Concentration**

Myocytes were exposed to varying dilutions of ASCM for 72 hours, after which time cellular cAMP content was determined under basal conditions and after half-maximal stimulatory concentrations of isoproterenol (3×10⁻⁷ M). In the absence of ASCM, cAMP content increased from a basal level of 18 to 134 pmol/mg protein after stimulation with isoproterenol. As ASCM concentration increased, there was a progressive inhibition of isoproterenol-stimulated myocyte cAMP concentration (Figure 1),...
such that isoproterenol-stimulated cAMP concentration was only 34 pmol/mg protein at 20% ASCM. This represented a 74% inhibition from control levels. Half-maximal and maximal suppression of cAMP occurred at 2% and 20% ASCM, respectively. Basal cAMP levels were not affected significantly with supernatant exposure. Although we have noted biological variation in the suppression of isoproterenol-stimulated cAMP, ranging from 50% to 80% of control values, the observations of ASCM-induced cAMP inhibition have been replicated at least 100 times over the course of 3 years in our laboratory. In addition, the use of indomethacin did not affect cAMP inhibition, suggesting that cyclooxygenase inhibition was not involved in this effect.

**Time Course and Reversibility**

The duration of supernatant exposure required for manifestation of myocyte cAMP suppression was examined by incubating myocytes in control medium or 25% ASCM for various time periods before isoproterenol stimulation and cAMP determination. At appropriate time points, cells were washed with medium, and cAMP concentration was determined in the isoproterenol-stimulated (3\times10^{-7} M) state (n=3 at each time). For cardiac myocytes incubated with control medium alone (Figure 2A, control), cAMP content was essentially unchanged over the time periods examined, averaging approximately 150 pmol/mg protein. In contrast, cardiac myocytes exposed to ASCM showed progressively less isoproterenol-stimulated cAMP as time of exposure increased (Figure 2A, ASCM). Thus, after 24, 48, 72, and 96 hours of exposure, there was 7%, 24%, 69%, and 61% less cAMP present, respectively. Inhibition was thus manifest by 48 hours of exposure, with maximal inhibition seen at 72 hours.

Related experiments indicated that this effect is reversible upon continued culture after removal of ASCM (Figure 2B). For example, cardiac myocytes previously exposed to 20% ASCM for 72 hours displayed a 53% inhibition of isoproterenol-stimulated cAMP response. After removal of the ASCM, thorough washing, and continued culture in control medium, inhibition of stimulated cAMP was reduced to 9% by 96 hours after cytokine removal. These data demonstrate that the uncoupling of cAMP responses from \(\beta\)-adrenergic stimulation is reversible with a time course that is quite similar to that required for the production of inhibition.

**Exclusion of a Mitogenic Influence on Immune Cytokine-Induced cAMP Suppression**

Because numerous secretory products of activated immune cells are known to be mitogenic or antimitogenic,19 experiments were conducted to determine whether myocyte cAMP suppression was independent of these effects. Although contaminating fibroblasts, smooth muscle cells, and other non-cardiac cells comprise less than 5% of cells plated in
TABLE 1. Inhibition of Cardiac Myocyte Agonist–Mediated Cyclic AMP Increases by Immune Cell Culture Supernatants is Independent of Mitogenic Effects

<table>
<thead>
<tr>
<th>Medium supplement</th>
<th>- XRT</th>
<th>+ XRT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]Tdr (cpm/well)</td>
<td>[cAMP] (pmol/mg)</td>
</tr>
<tr>
<td>None</td>
<td>9,617±922</td>
<td>131±5</td>
</tr>
<tr>
<td>Supernatant</td>
<td>13,057±1,695</td>
<td>105±9</td>
</tr>
<tr>
<td>5%</td>
<td>9,263±2,912</td>
<td>81±9</td>
</tr>
<tr>
<td>20%</td>
<td>6,158±974</td>
<td>50±10</td>
</tr>
<tr>
<td>50%</td>
<td>6,380±370</td>
<td>81±9</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Cardiac myocytes were cultured after receiving 2,000 rads of gamma irradiation (+ XRT) or no irradiation (- XRT) and then exposed to varying dilutions of immune cell supernatants for 72 hours. Cells assayed for proliferation were cultured in the presence of 0.4 μCi/well [3H]thymidine ([3H]Tdr), and DNA was harvested and counted after 72 hours of supernatant exposure. Other cells were stimulated with isoproterenol (10⁻⁷ M) for 10 minutes after 72 hours of supernatant exposure and then assayed for cyclic AMP concentration ([cAMP]).

our system,⁸ these dividing cell types might be expected to respond to mitogenic factors. Indeed, we noted that in both conditioned and control medium proliferation of these cells occurred as determined by [3H]thymidine incorporation (Table 1, column 2).

To minimize the effects of proliferating nonmyocyte elements, nonlethal doses of gamma irradiation (2,000 rads) before cell plating were used. Such irradiation inhibited the proliferation of contaminating nonmyocytes by greater than 90% (Table 1, column 2 versus column 5) without affecting the ability of myocytes to generate cAMP in response to β-adrenergic stimulation (Table 1, column 6). Thus, [3H]thymidine DNA incorporation was reduced from 9,617 to 955 cpm/well under control conditions by gamma irradiation, whereas stimulation with 10⁻⁷ M isoproterenol still increased irradiated myocyte cAMP concentration from basal levels of approximately 10 pmol/mg protein to a stimulated level of 112 pmol/mg protein. Importantly, activated immune cell–conditioned media produced myocyte cAMP suppression in these irradiated cells, with nearly identical potency to that seen in nonirradiated cells (Table 1, column 4 versus column 7). Thus, when the contribution of nonmyocytes to the cell population under study was minimized by inhibiting their proliferation, supernatant-induced myocyte cAMP suppression was still manifest.

Effect of Phosphodiesterase Inhibition on Isoproterenol-Stimulated cAMP

The addition of a phosphodiesterase inhibitor during isoproterenol stimulation did not affect inhibition induced by ASCM exposure. After incubation with varying dilutions of ASCM for 72 hours, myocytes were stimulated with 3×10⁻⁷ M isoproterenol in the presence of 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor (Table 2). These conditions in control cardiac myocytes allowed cAMP to accumulate to 6,380 pmol/mg, a level 80-fold above that stimulated by isoproterenol alone. Nevertheless, increasing concentrations of ASCM produced progressive inhibition of this cAMP accumulation. The concentration of cAMP rose to only 4,450 and 3,570 pmol/mg at 20% and 50% ASCM, respectively. These data suggest that cytokine alteration of phosphodiesterase activity does not account for the observed inhibition of cAMP accumulation.

Baseline β-Adrenergic Receptor Antagonist Ligand Binding and Affinity in Monolayer Cardiomyocytes

Cardiac β-adrenergic receptors were assayed using equilibrium binding of the radioligand antagonist ICYP to intact viable beating monolayer cells. Nonlethally irradiated myocytes in monolayers within vinyl tissue culture microwells were incubated with various concentrations of ICYP at 4°C to experimentally determined equilibrium to determine low-affinity antagonist-binding affinity (Kd) and receptor density. Myocytes exhibited 286 fmol receptors/mg protein, equivalent to approximately 20,000 receptors/cell (Figure 3). The Kd for ICYP binding was 14 pM. Both receptor density and Kd values were in excellent agreement with findings on nonirradiated myocytes in our laboratory⁸ and others.⁰

One mechanism by which cells are known to down-regulate adrenergic responses is by agonist-mediated receptor internalization. This effectively sequesters receptors from the membrane transducer stimulatory nucleotide regulatory protein (Gα) and from effector adenylate cyclase.²¹ Irradiated myo-

TABLE 2. Effect of Phosphodiesterase Inhibition on Activated Splenocyte–Conditioned Medium–Induced Isoproterenol-Stimulated Cyclic AMP Inhibition in Myocytes

<table>
<thead>
<tr>
<th>ASCM (%)</th>
<th>[cAMP] (pmol/mg protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6,380±370</td>
<td>...</td>
</tr>
<tr>
<td>10</td>
<td>4,560±220</td>
<td>29</td>
</tr>
<tr>
<td>20</td>
<td>4,450±850</td>
<td>30</td>
</tr>
<tr>
<td>50</td>
<td>3,570±160</td>
<td>44</td>
</tr>
</tbody>
</table>

Values for cyclic AMP concentration ([cAMP]) are mean±SEM. Myocytes were exposed to varying concentrations of activated splenocyte–conditioned medium (ASCM) for 72 hours and then stimulated with isoproterenol (3×10⁻⁷ M) in the presence of isobutylmethylxanthine (1 mM) for 10 minutes at 37°C. [cAMP] was then assayed and normalized to cellular protein (n=3 for each value).
cytes demonstrated the capacity for this agonist-induced receptor internalization. As shown in Figure 3, the ICYP binding capacity of myocytes preexposed to 1 μM isoproterenol for 30 minutes exhibited 45% fewer receptors than control cells, or 184 versus 286 fmol/mg. As expected, the $K_d$ for ICYP binding (24 pM) was not significantly altered in these desensitized cells.

**Effect of ASCM on $\beta$-Adrenergic Receptor Density and Antagonist Affinity**

A means by which immune cell–conditioned media could produce impaired myocyte cAMP increases in response to $\beta$-adrenergic agonist stimulation is by diminishing myocyte receptor expression. To examine this hypothesis, receptor binding assays were performed on cells incubated in control medium or various concentrations of ASCM under conditions that produced impaired metabolic and contractile responses. Supernatant exposure produced no significant alteration in receptor expression (Table 3). Cells exhibited 275–298 fmol receptors/mg protein when exposed to control medium and up to 50% ASCM. Further, $K_d$ was not significantly altered (from 6.3 to 7.3 pM). Thus, impaired responses of cAMP concentration in immune factor–exposed myocytes did not appear secondary to decreases in $\beta$-receptor expression.

**$\beta$-Adrenergic Receptor Agonist Binding**

$\beta$-Adrenergic receptor subtypes are distinguished by the relative binding affinity of agonists. First, intact monolayer cardiac cell agonist binding was assayed by competitive displacement of ICYP. The $IC_{50}$ for isoproterenol binding was $10^{-7}$ M; that for both epinephrine and norepinephrine was approximately $5 \times 10^{-6}$ M (Figure 4). Because $\beta_1$-receptors exhibit rank-order potency of isoproterenol greater than that of epinephrine and norepinephrine, the identification of the $\beta_1$-subtype receptor on these cells was confirmed.

Isoproterenol binding to intact cells was not altered by prior exposure to medium conditioned by

**TABLE 3. Effect of Activated Splenocyte–Conditioned Medium on $\beta$-Adrenergic Receptor Density and Antagonist Affinity**

<table>
<thead>
<tr>
<th>ASCM (%)</th>
<th>$B_{\text{max}}$ (fmol/mg)</th>
<th>$K_d$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>275</td>
<td>6.3</td>
</tr>
<tr>
<td>5</td>
<td>297</td>
<td>6.3</td>
</tr>
<tr>
<td>20</td>
<td>283</td>
<td>7.3</td>
</tr>
<tr>
<td>50</td>
<td>298</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Myocytes were exposed for 72 hours to varying concentrations of activated splenocyte–conditioned medium (ASCM) and the equilibrium binding of varying concentrations of $[^{125}]$cyanoepin dololo to cell monolayers measured after a 16-hour incubation at 4°C. Kinetics were calculated after Scatchard analysis. These results are representative of five separate similar experiments using 20% ASCM. $B_{\text{max}}$, receptor density; $K_d$, binding affinity.
spleenic cells (Figure 5). The IC₅₀ was 10⁻⁷ M isoproterenol in both control and ASCM-exposed cells, and maximal radioligand displacement was unaltered. Since isoproterenol is a hydrophilic ligand that does not enter cells,²³ this latter information constitutes further evidence that sarclemma receptor density was not altered by immune-factor exposure. No differences were found in the Kᵦ values of epinephrine and norepinephrine (data not shown). These results indicate that the observed impaired cAMP increases are not secondary to changes in β-adrenergic receptor expression.

Cholera Toxin ADP-Ribosylation

Gₛ was evaluated using cholera toxin–catalyzed ADP-ribosylation of the α-subunit of Gₛ to assess the functional and structural effects of immune cell cytokines. Preliminary studies indicated that in our system maximal cAMP-stimulating effects of cholera toxin occurred with 1 hour of exposure. Accordingly, control medium or cholera toxin (300 ng/ml) was added to myocytes during the last hour of ASCM or control medium exposure, and then myocyte cAMP content was quantitated with or without exposure to isoproterenol. As seen in Figure 6Aa, in control cells, cholera toxin exposure raised cAMP from 13 to 157 pmol/mg. Prior treatment with ASCM inhibited this response by 45% and 57% at 20% and 50% ASCM, respectively. These degrees of ASCM-induced inhibition were virtually unchanged from that observed with isoproterenol-stimulated cAMP concentration in the absence of cholera toxin. Thus, cholera toxin was unable to typically stimulate cAMP accumulation in cells exposed to ASCM.

Isoproterenol stimulation after cholera toxin exposure raised cAMP to 407 pmol/mg in control cells, and ASCM strikingly inhibited this response to 112 and 57 pmol/mg at 20% and 50% ASCM, representing 69% and 84% inhibitions, respectively (Figure 6B). Demonstration of the cholera toxin [³²P]ADP-ribosylation substrates by autoradiography after sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that the intensity of the labeling of the polypeptide substrates was not appreciably changed by previous exposure of cells to ASCM (figure not shown). Thus, ASCM exposure impairs the ability of Gₛ to activate adenylate cyclase, at least as transduced by cholera toxin.

Cytokine Effect on Forskolin-Stimulated Adenylate Cyclase

Forskolin, a diterpene whose major action appears to involve direct activation of adenylate cyclase,²⁴ was used to evaluate adenylate cyclase in intact myocytes after exposure to varying concentrations of ASCM (Table 4). After a 10-minute incubation with a half-maximal stimulatory concentration of forskolin (3 × 10⁻³ M), the cAMP concentration was 162 pmol/mg in control cells. In cells exposed to 20% and 50% ASCM, cAMP concentrations actually were higher
TABLE 4. Forskolin-Stimulated Cyclic AMP Concentration in Cytokine-Exposed Myocytes

<table>
<thead>
<tr>
<th>ASCM (%)</th>
<th>cAMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>162±4</td>
</tr>
<tr>
<td>10</td>
<td>227±24</td>
</tr>
<tr>
<td>20</td>
<td>300±9</td>
</tr>
<tr>
<td>50</td>
<td>284±7</td>
</tr>
</tbody>
</table>

Values are mean±SEM of three samples. Myocytes were exposed to varying concentrations of activated splenocyte-conditioned medium (ASCM) for 72 hours at 37°C, then washed once with phosphate-buffered saline, and incubated with 3×10−5 M forskolin for 10 minutes at 37°C. Cellular cyclic AMP (cAMP) concentration was then assayed and normalized to cellular protein.

than in control cells, 300 and 284 pmol/mg, respectively.

Adenylate cyclase activity was additionally measured in membranes of similarly treated cells in the presence and absence of forskolin (Table 5). Forskolin (100 μM) stimulated adenylate cyclase activity in control membranes to 419 pmol cAMP/mg/min. With 25% ASCM preexposure, forskolin-stimulated activity was 766 pmol cAMP/mg/min. These data demonstrate that inhibition of the catalytic subunit of adenylate cyclase is not produced by exposure to ASCM under conditions that we have demonstrated and can result in decreased intracellular accumulation of cAMP and an impaired ability of cholera toxin to stimulate cAMP production.

Pertussis Toxin ADP-Ribosylation

Pertussis toxin was used to evaluate the potential role of the inhibitory guanine nucleotide binding protein (Gi) in producing the cytokine effects on intracellular cAMP concentration (Figure 7A). When added at a concentration of 200 ng/ml during the last 24 hours of cytokine exposure, pertussis toxin did not significantly alter basal cAMP concentration. In contrast, pertussis toxin virtually ablated the inhibition of isoproterenol-stimulated cAMP concentration. Under these conditions, non-pertussis toxin-exposed cells, when stimulated with isoproterenol, had their cAMP response significantly inhibited from 85 pmol/mg to 26 pmol/mg (70% inhibition) by 50% ASCM. However, with pertussis toxin exposure, isoproterenol-stimulated cAMP concentration was

79 pmol/mg in control cells and 69 pmol/mg in 50% ASCM-exposed cells. Thus, pertussis toxin diminished the amount of ASCM-induced inhibition from 70% to 13%. There was no demonstrable change detected in the concentration of the pertussis toxin substrates, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography.

TABLE 5. Forskolin-Stimulated Membrane Adenylate Cyclase Activity in Cytokine-Exposed Myocytes

<table>
<thead>
<tr>
<th>ASCM (%)</th>
<th>Adenylate cyclase (pmol cAMP/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal Forskolin-stimulated</td>
</tr>
<tr>
<td>0</td>
<td>34 419</td>
</tr>
<tr>
<td>25</td>
<td>30 766</td>
</tr>
</tbody>
</table>

Myocytes were exposed to control medium or 25% activated splenocyte-conditioned medium (ASCM) for 72 hours at 37°C in 60-mM tissue culture plates, then scraped, and homogenized. A crude membrane fraction was then assayed for adenylate cyclase activity in the presence and absence of forskolin (100 μM). cAMP, cyclic AMP.
phy of the 41,000-Da pertussis toxin ADP-ribosylation substrate in membrane preparations (Figure 7B). From these results, one can conclude that pertussis toxin can overcome the impaired ability to stimulate cAMP accumulation in ASCM-exposed cells.

**Discussion**

Our present findings demonstrate that immune cell-derived cytokines can uncouple β-adrenergic receptor occupancy from producing cAMP by interfering with signal transduction across the sarcolemma, though the molecular mechanism for this effect may be quite complex. The ability of pertussis toxin treatment to increase the levels of cAMP via its known effect of modifying Gβ suggests that the inhibitory limb of the signal transduction system is intact or that pertussis toxin can override an ASCM effect on Gβ. Explanations for the involvement of Gβ could include 1) the ASCM-induced production of a ligand that binds to a Gβ-coupled receptor, 2) changes in the mass of Gβ, 3) changes in the structure of Gβ, such as phosphorylation or production of an isoenzyme of Gβ, or 4) membrane effects to enhance the inhibitory capacity of Gβ. Only explanation 2 would seem doubtful, given the present data (Figure 7B). Gβ could also be involved, as judged from the cholera toxin experiments. The inability of cholera toxin to stimulate adenylate cyclase in ASCM-exposed cells (Figure 6) suggests that the basis of impaired cAMP production might also involve an impaired interaction of Gβ with adenylate cyclase. Whether this occurs through a direct effect on the α-subunit of Gβ or on the composition of the sarcolemmal membrane is unknown. In any event, our data demonstrate that ASCM-induced inhibition occurs via a process that affects signal transduction and that is sensitive to pertussis toxin.

Because of the known relations between cAMP accumulation and cell proliferation and because many cytokines are known to be mitogenic, especially upon fibroblasts and other nonmyocyte cells, we excluded other bases for the observed inhibition of cAMP accumulation, such as the influence of proliferating nonmyocyte elements. Nonlethal irradiation of myocyte preparations at the initiation of culture permitted the maintenance of a very pure population of myocytes for up to 7–10 days. These irradiated myocytes expressed normal β-adrenergic receptor density and the capacity to down-regulate by internalization (Figure 3), as well as normal functional ability to generate cAMP and the ability to manifest cytokine-induced inhibition of β-adrenergic agonist-stimulated cAMP accumulation (Table 1).

Phosphodiesterase inhibition during β-adrenergic stimulation did not abolish the cytokine-induced inhibition of cAMP accumulation (Table 2). This indicated that a stimulation of cAMP degradation by enhanced phosphodiesterase activity could not explain the observed cAMP suppression.

Parameters of β-adrenergic receptor binding and affinity (Table 3 and Figure 5) were not affected by immune cell supernatants. Prior binding studies using intact myocytes have generally been performed only on cells that have been liberated from a culture substratum by scraping, which is likely to at least partially damage the integrity of the myocyte sarcolemma and potentially alter receptor physiology. The modified technique used in these studies utilized intact monolayer cells and avoided this complication. No demonstration of cytokine-induced differences in β-adrenergic receptor characteristics was noted. β-Adrenergic receptor assays, using the binding of the radioligand antagonist ICYP to intact monolayer cells, revealed that total myocyte receptor density was not altered by supernatant exposure. Supernatant-exposed cells (Table 3) did not exhibit altered sarcolemma receptor density or affinity for antagonist binding (Kd, approximately 6–7 pM). Agonist binding, assayed by competitive displacement of ICYP, demonstrated rank-order potency characteristic of β1-subtype receptors (Figure 4). Isoproterenol binding was identical in supernatant-exposed and control myocytes with respect to potency and maximal radioligand displacement (Figure 5). Thus, cytokine-induced alterations in the β1-adrenergic receptors are not responsible for the observed inhibition of cAMP formation.

The effects of cytokines on adenylate cyclase are less clear. Forskolin-stimulated cAMP levels and adenylate cyclase activities are not inhibited. Similar experiments confirm this finding and also suggest a trend toward an increase in forskolin-stimulated adenylate cyclase activity, although data are not conclusive. Alterations in Gβ-cyclase coupling, intrinsic changes of the functional activity of adenylate cyclase, and/or an upregulation of the quantity of enzyme present are all possible explanations for this effect.

Demonstration of this cytokine-induced alteration of signal transduction has new implications, both with respect to cytokine biology and the β-adrenergic receptor system. Although intensive effort has uncovered many actions for cytokines, understanding the secondary intracellular metabolic effects or mechanisms of action is far from complete. Our results suggest that cytokines may alter signal transduction across the membrane and that such effects may take some time to occur (e.g., 72 hours). Other metabolic events may therefore be required to alter the functional activity of a pertussis toxin substrate, for example, Gβ. Although we could not detect a difference in the concentration of the pertussis toxin substrate via [32P]ADP-ribosylation, this technique has well-known limitations, and transcriptional and/or translational regulatory changes could certainly be occurring within this time frame. Cytokine effects upon the adrenergic system may also involve intermediary factors. In addition, the ability of the β-adrenergic–adenylate cyclase system to interact with soluble effectors from immune system cells might implicate the immune system in potentially modulat-
ing a wide array of physiological processes under the control of cAMP.

Medium conditioned by mitogenically activated splenocytes contains numerous cytokines released by lymphocytes or macrophages. We have studied the effect of a complex immune-conditioned medium because this would most accurately reflect the actual conditions experienced by an inflamed myocardium in vivo in which both lymphocytes and macrophages participate in the cellular infiltrate. We have established that leukocytes can reversibly modulate signal transduction of adrenergic stimulation of the myocardium. Assignment of these effects to identifiable cytokines has recently been confirmed in our laboratory.\(^{25}\) cAMP-suppressive activity comigrated with interleukin-1 and tumor necrosis factor activities during cation exchange chromatography. Both human recombinant interleukin-1 and tumor necrosis factor produced dose-dependent inhibition of \(\beta\)-adrenergic agonist-stimulated cAMP accumulation. Synergistic interactions between these cytokines as well as the existence of a third factor are under active study.

Immunologically mediated cardiac disease has received scant attention in the basic science literature despite increasing clinical evidence that cardiac myocyte function may be reversibly inhibited by immune factors. A similarity of pathophysiological end points in such syndromes suggests that our present findings might provide a partial cellular and molecular understanding of immunological events that produce reversible decrements in cardiac function in both allograft rejection and lymphocytic myocarditis. A hallmark of a subset of patients with these diseases is the reversibility of heart failure upon treatment with immunosuppressive therapy.\(^1,4,26\) In this regard, we have found that there is a marked blunting of cardiac myocyte contractility correlated with the marked cytokine-induced inhibition of \(\beta\)-adrenergic agonist-stimulated cAMP accumulation.\(^8\)

In summary, we have shown that immune cell cytokines inhibit cardiac myocyte \(\beta\)-adrenergic agonist-stimulated cAMP accumulation by disrupting signal transduction across the sarcolemma. This effect is overridden by pertussis but not cholera toxin and does not affect \(\beta\)-adrenergic receptor expression or adenylate cyclase activity. This uncoupling appears to represent a novel biological mechanism by which endogenous agents, that is, immune cell cytokines, might modulate cardiac function, particularly in diseases where immune cells invade the myocardium.

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References


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