Apoptotic Signaling through the β-Adrenergic Receptor

A NEW Gs EFFECTOR PATHWAY*

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Stimulation of β-adrenergic receptor normally results in signaling by the heterotrimeric G protein Gs, leading to the activation of adenyl cyclase, production of cAMP, and activation of cAMP-dependent protein kinase (PKA). Here we report that cell death of thymocytes can be induced after stimulation of β-adrenergic receptor, or by addition of exogenous cAMP. Apoptotic cell death in both cases was observed with the appearance of terminal deoxynucleotidyl transferase-mediated UTP end labeling reactivity and the activation of caspase-3 in S49 T cells. Using thymocytes deficient in either Goα or PKA, we find that engagement of β-adrenergic receptors initiated a Goα-dependent, PKA-independent pathway leading to apoptosis. This alternative pathway involves Src family tyrosine kinase Lck. Furthermore, we show that Lck protein kinase activity can be directly stimulated by purified Goα. Our data reveal a new signaling pathway for Goα, distinct from the classical PKA pathway, that accounts for the apoptotic action of β-adrenergic receptors.

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The mechanism of β-adrenergic receptor signaling was partly determined by pioneering studies utilizing S49 mouse lymphoma T cells. Variant cell lines deficient in responses to agents that elevate intracellular cAMP levels or exogenous cAMP proved to be instrumental in deciphering the components that mediated cAMP production and function (5, 6). The mutant cell lines were identified by their ability to be resistant to cAMP treatment, which normally lead to growth arrest and cytotoxicity. One of the resistant cell lines, kin−, displayed a complete lack of PKA activity, as well as binding of cAMP (7, 8). The β-adrenergic receptor agonist isoproterenol was also used to select for cell lines (such as cyc− and UNC) that were defective in Goα and were not capable of generating cAMP (9, 10). The purification of Goα was assayed by reconstitution with membranes prepared from cyc− cells since these cells contained normal levels of β-adrenergic receptors and adenyl cyclase activity, but lacked Goα (11, 12).

The effects of cAMP and isoproterenol upon S49 cell viability suggested that activation of β-adrenergic receptors leads to cytolytic signaling possibly through a PKA-dependent mechanism. Here we have examined β-adrenergic receptor initiation of cell death in thymocytes. Using mutant cell lines deficient for particular gene activities, we provide genetic evidence that β-adrenergic receptor initiates an apoptotic pathway in thymocytes that is not dependent upon PKA. Significantly, we further demonstrate that this novel PKA-independent pathway requires the action of a Src family tyrosine kinase, Lck. A potential mechanism of activation is proposed based upon the ability of purified Goα to regulate the kinase activity of purified Lck protein. This mechanism may help to explain physiological effects of β-adrenergic receptors and other Gs-coupled receptors that give an apoptotic cell outcome.

EXPERIMENTAL PROCEDURES

Cell Culture—S49 mouse lymphoma T cells (and their mutant derivatives cyc−, kin−, UNC, and H21a cells) were obtained from the Cell Culture Facility at the University of California at San Francisco, and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) supplemented with 10% heat-inactivated horse serum. The Jurkat and Lck-deficient T cells were obtained from American Type Culture Collection and maintained in RPMI 1640 medium with 10% fetal bovine serum.

In Situ Detection of Apoptotic Cells (TUNEL Assay)—In situ detection of apoptotic cells was performed by using TUNEL assay (terminal deoxynucleotidyl transferase-mediated UTP end labeling), as described previously (13). Cells treated with or without 1 mM cAMP (adenosine 3′,5′-cyclic monophosphate; Ncyc, O2-dibutyryl, and sodium salt were from Calbiochem), or 100 μM isoproterenol (Sigma) for 24 h were plated on microscope slides (Fisher) at 500 rpm for 2 min in cytosin machine (Shandon). The air dried cell samples were fixed with 4% paraformaldehyde solution for 30 min at room temperature and permeabilized with 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice. The slides were rinsed with phosphate-buffered saline several times and the samples

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were then processed for TUNEL using the in Situ Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals), following the manufacturer's instructions. Samples were rinsed with phosphate-buffered saline for 3 times, mounted, and analyzed under a fluorescence microscope.

Viability Assay (MTT Assay)—Cells were plated in each well of the 96-well plates and treated in triplicate with 1 mM cAMP, 1, 10, 100, and 1000 μM isoproterenol, 100, 300, or 1000 μM terbutaline, or untreated for 24, 48, or 72 h. The addition of inhibitors or other compounds was carried out concomitantly with treatment. Viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) in triplicate in 96-well plates using a microplate reader (Bio-Rad). Viability was expressed as the ratio of the signal obtained from treated cells and the signal from untreated control cells.

Immunoblotting—Preparation of cell extracts, immunoprecipitation, and Western blot were performed as described (13). Cells treated with or without isoproterenol and cAMP were harvested from 60-mm plates. Pellets were then resuspended in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 2 mM/ml aprotinin, 1 μg/ml leupeptin, 25 μg/ml phenylmethylsulfonyl fluoride, 10 mM ATP, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM magnesium chloride, 1 mM sodium pyruvate, and non-essential amino acids (Life Technologies, Inc.) and 10% fetal calf serum (Hyclone). The culture was grown at room temperature until the absorbance at 600 nm was ~1.4. Then the culture was split into 2 liters and G protein expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (Research Products International) for 18 h at room temperature. The bacterial pellet was resuspended into lysis buffer (50 mM Hepes, pH 7.4, 5 mM KCl, 5 mM MgCl₂, 2 mM MnCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 mM ATP, 0.1 mM of [γ-32P]ATP). After 30 min at 30 °C, samples were separated by 10% SDS-PAGE. Gels were then exposed for autoradiography.

Protein Purification—Recombinant Ga₃ was purified from Escherichia coli as described (16). The pQE-Gα₃ plasmid (from Drs. T. Kozasa and A.G. Gilman) was transformed into BL21. One liter of bacterial culture was grown at room temperature until the absorbance at 600 nm was ~1.4. Then the culture was split into 2 liters and G protein expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (Research Products International) for 18 h at room temperature. The bacterial pellet was resuspended into lysis buffer (50 mM Hepes, pH 8.0, 3 mM MgCl₂, 20 mM β-mercaptoethanol, 0.7% CHAPS, 20 mM GDP, and protease inhibitor mixture tablet (Roche Molecular Biochemicals)) on ice.

Flow Cytometric Analysis—Events within a pre-defined forward- and side-scatter gate were collected and stored in list mode files using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed using CellQuest Software.

Immunocomplex Kinase Assay—An immunocomplex kinase assay was performed as described previously (14, 15). Membrane extracts were made after stimulation with isoproterenol (100 μM for 1 min). Lck immunoprecipitation was carried out with an antibody to Lck (Santa Cruz). Lck kinase assay was done with 5 μg of acid-denatured enolase as substrate. The kinase buffer included 50 mM HEPES (pH 7.4), 5 mM MgCl₂, 5 mM MnCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μM ATP, 10 μM of [γ-32P]ATP). After 30 min at 30 °C, samples were separated by 7% SDS-PAGE. Gels were then exposed for autoradiography.

Isolation of Thymocytes—Single-cell suspension of thymocytes were prepared by mechanical disruption of thymic lobes in Hank’s buffered saline solution (Life Technologies, Inc., Grand Island, NY) supplemented with 2% fetal calf serum (HyClone, Logan, UT) and passage through a 100-μm nylon cell strainer. Cells were resuspended at 2 × 10⁷/ml and stained with phycoerythrin-conjugated anti-CD8α and fluorescein isothiocyanate-conjugated anti-CD4 (Caltag Laboratories, Burlingame, CA). Double positive thymocytes were sorted and resuspended in Dulbecco’s modified Eagle’s medium supplemented with 50 μM 2-mercaptoethanol, penicillin, and streptomycin, 1-glutamine, sodium pyruvate, and non-essential amino acids (Life Technologies, Inc.) and 10% fetal calf serum (HyClone).

The blots were washed again and processed with Supersignal Chemiluminescence (Pierce) and visualized under fluorescence microscopy. The phase-contrast (a, c, and e) images correspond to the identical fields as TUNEL immunofluorescence microscopy (b, d, and f), respectively. TUNEL-positive cells were identified in both ISO (d) and cAMP (f)-treated samples but not in untreated control (b) samples. B, quantitation of cell viability by MTT assay. Cells were treated with either ISO (1, 10, 100, and 300 μM) or cAMP (1 mM) for 24, 48, or 72 h. Cell viability was measured by MTT assay and expressed as percentage of the untreated controls. Data represent mean ± S.D. of four experiments.

C, caspase-3 (CPP32) activity was analyzed during ISO and cAMP treatment in wild-type S49 cells by monitoring cleavage to p10 and p20 subunits. Cells were left untreated (Control) or treated with ISO (100 μM) or cAMP (1 mM) for 18 h. Total cell lysates were run on 15% SDS-PAGE gel and subjected to Western blotting using antibodies against the p20 subunit of caspase-3 (anti-CM1) and the p10 subunit of caspase-3 (anti-CPP32), or anti-actin antibody as a loading control.

FIG.1

A. TUNEL staining of apoptotic S49 cells when treated with β-adrenergic receptor agonist ISO and cAMP. Cells were left untreated (a and b), treated with 100 μM ISO (c and d), or 1 mM cAMP (e and f) for 24 h. DNA fragmentation was detected by TUNEL staining (b, d, and f) and visualized under fluorescence microscopy. The phase-contrast (a, c, and e) images correspond to the identical fields as TUNEL immunofluorescence microscopy (b, d, and f), respectively. TUNEL-positive cells were identified in both ISO (d) and cAMP (f)-treated samples but not in untreated control (b) samples. B, quantitation of cell viability by MTT assay. Cells were treated with either ISO (1, 10, 100, and 300 μM) or cAMP (1 mM) for 24, 48, or 72 h. Cell viability was measured by MTT assay and expressed as percentage of the untreated controls. Data represent mean ± S.D. of four experiments. C, caspase-3 (CPP32) activity was analyzed during ISO and cAMP treatment in wild-type S49 cells by monitoring cleavage to p10 and p20 subunits. Cells were left untreated (Control) or treated with ISO (100 μM) or cAMP (1 mM) for 18 h. Total cell lysates were run on 15% SDS-PAGE gel and subjected to Western blotting using antibodies against the p20 subunit of caspase-3 (anti-CM1) and the p10 subunit of caspase-3 (anti-CPP32), or anti-actin antibody as a loading control.
was added into the supernatant after pre-equilibration of the resin with lysis buffer. The mixture was gently agitated overnight at 4 °C and packed into a C16/20 column (Amersham Pharmacia Biotech), washed with lysis buffer plus 100 mM NaCl, and eluted with lysis buffer plus a linear gradient of imidazole (10–500 mM). Ga was in the 200 mM imidazole fraction. Ga elutions were pooled for further chromatography on hydroxylapatite column. Ga elute fractions were changed to HPHT buffer (10 mM Tris, pH 8.0, 1 mM dithiothreitol, 10 mM K2HPO4) and purified by using a Bio-Rad ChT-II cartridge with a linear gradient of phosphate (20–500 mM). Ga was activated with GTPγS at 30 °C for an hour. Control experiments showed no effect of GTPγS (up to 1 mM) alone on Lck kinase activity. Purified Lck was from Upstate Biotechnology. Recombinant Csk was purified from E. coli as described (18). Phosphorylation of Lck by Csk and removal of Csk by chromatography was done as described (17). Protein concentration, purity, and identity were analyzed by silver stain and Western blot.

**Kinase Assays**—An in vitro kinase assay was performed as described previously (19, 20). Purified Lck was phosphorylated using purified Csk as described previously (17). Lck protein (10 ng) in kinase buffer (30 mM Hapes, pH 7.4, 5 mM MgCl2, 5 mM MnCl2) was combined with 2 μg of Src substrate peptide (KVKRIGEGTGTYGVVKK). The appropriate amount of purified G-protein subunits was added and kinase buffer was used to bring the total reaction volume to 20 μl. [γ-32P]ATP (10 μCi; 3,000 Ci/mmol) was added and the mixture incubated at 30 °C for 15 min. The reaction was stopped by adding Laemmli sample buffer. After 90 °C for 5 min, the substrate peptide was separated on 20% SDS-PAGE gel, dried, autoradiographed, and quantified with a PhosphorImager. Bands were cut out of the gel and counted in a scintillation counter.

**RESULTS**

Apoptosis of S49 T Cells by Isoproterenol and cAMP—Even though cytolysis of S49 cells by exogenous cAMP and isoproterenol was observed over two decades ago, the mechanism leading to this form of cell death has not been thoroughly investigated. To study the nature of cell death imposed by exogenous cAMP and isoproterenol, we used TUNEL assay to detect DNA fragmentation, a hallmark of apoptosis. As shown in Fig. 1A, apoptotic cells stained with green fluorescence were readily identified as TUNEL-positive (Fig. 1A, panel d) among S49 cells treated for 24 h with isoproterenol (Fig. 1A, panel a). A similar profile of TUNEL positive cells was observed 24 h after treatment with cAMP (Fig. 1A, panel f). To quantitate the effects of isoproterenol and cAMP on S49 cell death, uptake of MTT was used as an independent measure of cell viability. Wild type S49 cells displayed a loss of viability after treatment with isoproterenol or exogenous cAMP in a time- and dosage-dependent manner (Fig. 1B). S49 cells were treated with 1, 10, 100, or 300 μM isoproterenol and then assayed after 24, 48, and 72 h. The selection of concentrations of isoproterenol and cAMP used here was based on previous studies with S49 cells (5, 6). Treatment with cAMP (1 mM) induced ~30% cell death after 24 h, ~80% after 48 h, and ~90% after 72 h, compared with untreated cells. Increas-
ing concentrations of isoproterenol induced an increased level of S49 cell death in a time-dependent manner. After 3 days of treatment with 100 \(\mu M\) isoproterenol, only \(-20\%\) of the cells remained alive (Fig. 1B).

The mode of cell death in S49 cells was further verified by an increase in caspase activity following cAMP and isoproterenol treatment. Activation of caspases is required for the execution of apoptosis. Caspases exist as proenzymes that require proteolytic cleavage for their activation and have been divided into two main groups, initiator and effector caspases (21). Caspase-3, one of the major downstream effector caspases, is cleaved at consensus sequences into p20 and p10 subunits upon activation (22).

Using polyclonal antibodies against the cleaved peptides (p10 and p20) of caspase-3 (CPP32) in immunoblotting analysis, we observed that both p20 and p10 subunits were produced when S49 cells were treated with isoproterenol or cAMP for 18 h (Fig. 1C). Pretreatment of S49 cells with the \(\beta\)-adrenergic receptor antagonist propranolol blocked isoproterenol-induced caspase-3 cleavage (data not shown). Together, these results demonstrate that stimulation of \(\beta\)-adrenergic receptors and exposure to exogenous cAMP can trigger apoptosis in S49 cells.

PKA Is Required for cAMP but Not Isoproterenol-induced Apoptosis—To understand how \(\beta\)-adrenergic receptors and exogenous cAMP initiate apoptosis, we have employed well characterized mutant S49 cell lines that are deficient in specific signaling activities. \(\kappa^\text{in}\) cells which lack PKA activity were selected as a result of the sensitivity of S49 cells to cytosis by cAMP (7, 8). A \(\gamma^\text{cyt}^\text{a}\) mutant variant, which lacks Gs, was also isolated as a result of treatment of viable cells in the presence of isoproterenol (9).

To investigate the role of PKA in cAMP and \(\beta\)-adrenergic receptor-induced cytosis, we employed the \(\kappa^\text{in}\) cells. With 1 \(\mu M\) cAMP, even after 3 days of treatment, nearly all \(\kappa^\text{in}\) cells were still viable, as measured by three different assays: TUNEL immunoreactivity (Fig. 2A), MTT cell viability (Fig. 2B), and caspase-3 cleavage (Fig. 2C). Therefore, the absence of PKA activity in S49 cells prevented apoptosis initiated by exogenous cAMP. Thus, PKA activity controls cAMP-induced death signaling.

In contrast, isoproterenol still induced apoptosis in \(\kappa^\text{in}\) cells as effectively as observed in the parental S49 cells. Apoptosis of \(\kappa^\text{in}\) cells was verified by TUNEL, MTT measurements, and caspase-3 cleavage (Fig. 2). As shown in Fig. 2A (panel d), TUNEL positive cells (as indicated by green fluorescence) could still be clearly identified when \(\kappa^\text{in}\) cells were treated with isoproterenol. The dose and time dependence of isoproterenol-induced cell death of \(\kappa^\text{in}\) cells, as monitored by MTT assay, were similar to those of wild-type S49 cells (compare Fig. 2B with Fig. 1B). Furthermore, addition of adenylyl cyclase inhibitors (2',5'-dideoxyadenosine and MDL-12, 330A) did not block isoproterenol-induced apoptosis (data not shown). Pertussis toxin, an inhibitor of Gi proteins, had no effect on isoproterenol or cAMP-initiated apoptosis (data not shown). Stimulation of endogenous Gi-coupled somatostatin receptors did not induce apoptosis in S49 cells (data not shown). The induction of caspase-3 cleavage products by isoproterenol treatment of \(\kappa^\text{in}\) cells (Fig. 2C) further indicated that isoproterenol caused cell death in the absence of PKA activity.

To confirm these findings and to rule out the possibility that the effects of isoproterenol are due to its oxidative degradation, we tested another \(\beta\)-adrenergic receptor agonist, terbutaline, which is resistant to oxidative degradation (10). As shown in Fig. 2D, increasing concentrations of terbutaline (100 \(\mu M\), 300 \(\mu M\), or 1 \(mM\)) induced an increased level of S49 cell death in a time-dependent manner. The concentrations of terbutaline used here were similar to those used in previous studies (10).

The response of \(\kappa^\text{in}\) cells to terbutaline was similar to that of the parental S49 cells (Fig. 2E). In the absence of PKA, terbutaline still induced cell death, similar to the treatment with isoproterenol. These results strongly suggest that \(\beta\)-adrenergic receptors could use a PKA-independent route leading to apoptosis.

Ga \(_{\alpha}\) Is Required for \(\beta\)-Adrenergic Receptor-induced Apoptosis—We next investigated the role of Ga in \(\beta\)-adrenergic receptor-induced apoptotic signaling by examining a S49 cell line lacking Ga \(_{\alpha}\) (\(\gamma^\text{cyt}^\text{a}\)). Exogenous cAMP was found to induce apoptosis in \(\gamma^\text{cyt}^\text{a}\) cells, similar to wild-type S49 cells (Fig. 3A). However, in contrast with wild-type S49 cells, \(\gamma^\text{cyt}^\text{a}\) cells were resistant to isoproterenol-induced cell death. Treatment with 1 or 10 \(\mu M\) isoproterenol for 3 days did not induce an apoptotic response (Fig. 3A). 100 or 300 \(\mu M\) isoproterenol caused cell death, which was significantly delayed and reduced compared with the data from wild-type S49 cells. In \(\gamma^\text{cyt}^\text{a}\) cells, 100 \(\mu M\) isoproterenol, after 2 days of treatment, induced only \(-5\%\) cell death (Fig. 3A), compared with \(-60\%\) in wild-type S49 cells (Fig. 1B). After 3 days of treatment with 100 \(\mu M\) isoproterenol, a significant (\(-70\%\)) fraction of \(\gamma^\text{cyt}^\text{a}\) cells still remained alive, compared with \(-20\%\) of wild-type S49 cells.

Thus, the \(\beta\)-adrenergic receptor-initiated apoptotic pathway
requires Gαs. The small fraction of dead cells after 3 days of isoproterenol treatment may indicate a Gαs-independent signaling pathway(s) (23–25). Similar results were obtained with two other Gαs mutated S49 cells, UNC and H21a (10, 26) (data not shown). Furthermore, these results were also confirmed with the β-adrenergic receptor agonist terbutaline (Fig. 3B). After 3 days of treatment, 100 or 300 μM terbutaline did not induce cell death, similar to isoproterenol treatment. Taken together with the above data, these results demonstrate that β-adrenergic receptors use Gαs to initiate a PKA-independent pathway that results in apoptosis.

β-Adrenergic Receptor-induced Apoptosis of Murine CD4+CD8+ Thymocytes—To demonstrate the physiological relevance and the generality of the findings in S49 cells, we next assessed the effects of isoproterenol and exogenous cAMP on primary thymocytes isolated from mice. Primary double positive (CD4+CD8+) immature thymocytes were isolated and analyzed for TUNEL reactivity with a FACScan flow cytometer. As shown in Fig. 4A, both isoproterenol and exogenous cAMP treatment caused an increase in the percentage of TUNEL-positive cells in CD4+CD8+ thymocytes. After treatment for 40 h, 10 μM isoproterenol induced ~18% cell death and 100 μM isoproterenol caused 55% cell death (Fig. 4A). Primary thymocytes were also very sensitive to cAMP treatment (Fig. 4A). These results were consistent with the effects of isoproterenol and cAMP on S49 cells.

Involvement of Tyrosine Kinase in β-Adrenergic Receptor-induced Apoptosis—In mature T cells, apoptosis is frequently the result of induction of FasL or TNF-α ligands, which act through their cognate death receptors, Fas or p55 TNF receptor. Signaling from these death receptors activate caspases through adapter proteins, leading to apoptosis (27). We have investigated the possible involvement of these death receptors in β-adrenergic or exogenous cAMP initiated apoptotic signaling pathways. We did not detect any changes of FasL or TNF-α mRNA levels after treatment of thymocytes with either isoproterenol or cAMP (data not shown). Furthermore, isoproterenol or cAMP still induced apoptosis in thymocytes isolated from Fas-deficient lpr mice (data not shown). A neutralizing anti-TNF-α monoclonal antibody (MP6-XT22) did not block isoproterenol or cAMP initiated apoptosis of thymocytes (data not shown). Thus, neither FasL/Fas nor TNF-α/TNFR appeared to be essential for β-adrenergic receptor or cAMP-initiated apoptotic pathways in thymocytes.

Most developing thymocytes are destined to undergo apoptosis during selection. During negative selection, a strong signal through ligation of the T cell antigen receptor induces cell death, which is most likely independent of Fas or TNF signaling (28, 29). Since T cell antigen receptor signaling is initiated by the activation of Src family tyrosine kinases (30), we explored whether inhibition of Src tyrosine kinases would attenuate isoproterenol-initiated apoptosis. We therefore employed PP1, a specific inhibitor for the Src family tyrosine kinases (31).
Pretreatment of primary thymocytes with 5 μM PP1 reduced the isoproterenol-induced apoptosis by ~50% (Fig. 4B), while it had no effect on exogenous cAMP-induced apoptosis (Fig. 4B). PP1 treatment also reduced isoproterenol-induced apoptosis in S49 cells and tyrosine phosphorylation of cellular proteins as assayed by Western blot with an anti-phosphotyrosine antibody (data not shown). On the other hand, pretreatment with a PKA inhibitor (KT5720) blocked exogenous cAMP initiated apoptosis (by ~80%), but not apoptosis initiated by isoproterenol (Fig. 4C). Thus, in primary thymocytes isolated from mice, β-adrenergic receptor and exogenous cAMP use distinct apoptotic pathways. Cell death induced by β-adrenergic receptor appeared to require the action of Src family tyrosine kinases, but not PKA.

Since Lck is the major Src family tyrosine kinase in T cells, we analyzed the role of Lck tyrosine kinase in β-adrenergic receptor-initiated apoptosis. Lck-deficient Jurkat T cells have been established (32). Treatment of wild-type Jurkat T cells with 100 μM isoproterenol resulted in ~60% cell death (Fig. 5A). Significantly, Lck-deficient Jurkat cells were resistant to isoproterenol-induced apoptosis, which was delayed and reduced compared with wild-type Jurkat cells (Fig. 5B). The PKA inhibitor KT5720 did not have any effect on isoproterenol-induced apoptosis in wild-type and Lck-deficient Jurkat cells (Fig. 5, A and B). A residual apoptotic effect of isoproterenol in Lck-deficient cells may indicate a functional compensation of other Src family tyrosine kinases in T cells, or the effect of a Lck-independent pathway. Furthermore, isoproterenol induced similar cAMP production in wild-type and Lck-deficient Jurkat cells (data not shown). Together with the above tyrosine kinase inhibitor data, these results suggest that Src family tyrosine kinases represent a potential signaling component of the β-adrenergic receptor-initiated apoptotic pathway in T cells.

Ga₃ Directly Stimulates the Tyrosine Kinase Activity of Lck—Since the Src family tyrosine kinase Lck partly mediates β-adrenergic receptor initiated apoptosis in T cells, we next examined the activation of Lck by β-adrenergic receptors. As shown in Fig. 6A, isoproterenol stimulation of β-adrenergic receptors in S49 cells could increase the kinase activity of Lck, as assayed by both its autophosphorylation and phosphorylation of an exogenous substrate enolase. This activation could be blocked by pretreatment with the β-adrenergic receptor antagonist propranolol (Fig. 6A). β-Adrenergic receptor activation of Lck is Ga₃-dependent (assayed in cys⁻ cells), but PKA-independent (assayed in kin⁻ cells) (data not shown). Transfection of activated Ga₃-Q227L into Jurkat cells increased Lck kinase activity (Fig. 6B). Furthermore, Lck was co-immunoprecipitated with Ga₃ in these Ga₃-Q227L transfected cells (Fig. 6C). Moreover, the association of endogenous Lck with Ga₃ was observed...
after cholera toxin treatment to activate endogenous Gαs (Fig. 6D).

Since the cAMP-PKA pathway is not required for β-adrenergic receptor-initiated Lck-dependent apoptosis, we tested whether Gαs directly stimulates the kinase activity of Lck. We used purified recombinant Gαs and Lck proteins, and performed an in vitro reconstitution assay. We found that increasing concentrations of Gαs-GTPγS augmented the specific activity of Lck (Fig. 6E). The concentrations of Gαs-GTPγS purified from E. coli (0.4 to 300 nM) used here for this assay are similar to those for stimulation of adenyl cyclase (33). A downregulated form of purified Lck was used, which was phosphorylated by its negative regulatory tyrosine kinase, Csk. This form of Lck exhibited a low specific activity of ~1 nmol/mg/min (17). The increase in Lck activity by Gαs was unlikely due to a contaminating phosphatase, since addition of vanadate did not affect this increase (data not shown). In addition, heat-inactivated Gαs-GTPγS had no effect on Lck activity (Fig. 6E). An increase in Lck activity required G protein activation, since Gαs-GDP did not display stimulatory effect (Fig. 6E). These data demonstrate that Gαs can directly stimulate the kinase activity of Lck, indicating that the Src family tyrosine kinase can act as a direct effector of Gαs.

**DISCUSSION**

G proteins transduce receptor signals to initiate diverse physiological processes, including programmed cell death. Apoptosis is a fundamental cellular process that is essential for embryonic development and tissue homeostasis. However, the contribution of heterotrimeric G proteins to this process has not been extensively characterized. Recently, overexpression of some G proteins (such as Gαs, Gα12, or Gα13) has been shown to lead to apoptosis in heterologous cells (34, 35), and some forms of amyloid-induced cell death are mediated through an interaction with G proteins (36, 37). Here our data have shown that β-adrenergic receptors use a Gαs-coupled mechanism to induce apoptosis in thymocytes through a novel pathway that feeds into the apoptotic machinery. We have also observed that the Gαs-coupled prostaglandin E2 receptor induces apoptosis in S49 cells requiring Gαs, but not PKA.2 We found that β-adrenergic receptors engage the Src family tyrosine kinase Lck to signal apoptosis. These observations were supported by the ability of Lck to act as a direct effector of Gαs. It has been reported that β2-adrenergic receptors, when expressed in HEK-293 cells, use β-arrestin to recruit active c-Src to the membrane (38). Whether Gαs and β-arrestin operate independently or in similar pathways is a subject for future investigation. Although the biochemical pathways whereby PKA and Src family tyrosine kinases lead to gene induction and then caspase activation are not clear, both PKA and Src family tyrosine kinases have been shown to be involved in apoptotic pathways initiated by a variety of receptors (39–46). Our findings that Gαs could directly activate Src family tyrosine kinases may help to explain the physiological effects of β-adrenergic receptors and other Gαs-coupled receptors that use PKA-independent pathways.

Stimulation of β-adrenergic receptors in S49 cells clearly increases cAMP and activates PKA (2). It has been assumed that an apoptotic outcome by Gαs would also involve activation of PKA. Our analyses of S49 cells and primary thymocytes indicate that there are alternative mechanisms which trigger apoptosis in these cells. The experimental evidence indicated that β-adrenergic receptors use novel tyrosine kinase-dependent signaling pathways, apart from the classical PKA pathway. It is possible that due to receptor desensitization, the amplification and/or transient elevation of the cAMP stimulated by β-adrenergic receptors may not be high or long enough to trigger apoptotic responses. Alternative signaling by β-adrenergic receptors may result in different consequences through the magnitude and duration of signaling by effector molecules.

The involvement of Src family tyrosine kinases in Gαs-coupled β-adrenergic receptor initiated apoptosis in T cells has implications not only for adrenergic signaling, but also for other G protein-coupled receptors in other cell contexts. Thrombin induces apoptosis in cultured neurons and astrocytes through activation of its receptor and the small G protein RhoA, together with an unidentified tyrosine kinase activity (47). In cultured cardiac myocytes, β-adrenergic receptor stimulation by isoproterenol leads to apoptosis (48, 49). Transgenic mice that overexpress Gαs in the myocardium develop dilated cardiomyopathy due to increased apoptosis (50), and myocytes cultured from Gαs-overexpressing mice underwent rapid apoptosis when exposed to isoproterenol (51). Other Gαs-coupled receptors, such as receptors for follicle-stimulating hormone, could induce apoptosis (52). It will be of interest to investigate the role of Src family tyrosine kinases in these biological responses.

Several other biological processes mediated by Gαs have been reported that could not be accounted by the classical PKA pathway. Inhibition of magnesium uptake in S49 cells by isoproterenol or prostaglandin E1 had previously been shown to be Gαs-dependent, but cAMP and PKA-independent (53). Furthermore, Gαs represses adipogenesis of 3T3-L1 mouse embryonic fibroblasts (54). This process is suggested to be independent of adenyl cyclase and cAMP (54–56). Very recently, it was shown that Gαs represses adipogenesis through a tyrosine kinase Syk (57). PKA independent regulation of L-type calcium channels by Gαs had been reported (58). Moreover, in differentiating wing epithelial cells of Drosophila, activation of Gαs leads to formation of wing blisters (59). This pathway was genetically demonstrated to be independent of PKA (59). These reports, together with our observations, clearly indicate that Gαs can signal through alternate transduction pathways.

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