G protein (heterotrimeric guanine nucleotide-binding protein)-coupled receptors (GPCRs) activate numerous cellular signals through the combined actions of G proteins, GPCR kinases, and arrestins. Although arrestins have traditionally been thought of as mediating GPCR desensitization, they have now been shown to play important roles in the internalization, trafficking, and signaling of many GPCRs. We demonstrate that in cells devoid of arrestins, the stimulation of numerous GPCRs including the N-formyl peptide receptor (FPR) initiates rapid cell rounding, annexin V positivity, and caspase activation followed by cell death. The apoptotic response is initiated by G protein signaling and involves activation of phosphoinositide 3-kinase, mitogen-activated protein kinases, and c-Src resulting in cytochrome c release from mitochondria and ultimately caspase 9 and caspase 3 activation. Reconstitution with either arrestin-2 or arrestin-3 is completely sufficient to prevent FPR-mediated apoptosis. Surprisingly, a non-desensitizing and non-internalizing mutant of the FPR is unable to initiate apoptosis, indicating that receptor phosphorylation and internalization, but not solely chronic activation due to a lack of desensitization, are critical determinants for the induction of apoptosis by the FPR.

We further demonstrate that this response is not unique to the FPR with numerous additional GPCRs, including the V2 vasopressin, angiotensin II (type 1A), and CXCR2 receptors, capable of initiating apoptosis upon stimulation, whereas GPCRs such as the β2-adrenergic receptor and CXCR4 are not capable of initiating apoptotic signaling. These data demonstrate for the first time that arrestins play a critical and completely unexpected role in the suppression GPCR-mediated apoptosis, which we show is a common consequence of GPCR-mediated cellular activation in the absence of arrestins.

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**Arrestins Block G Protein-coupled Receptor-mediated Apoptosis**

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G protein (heterotrimeric guanine nucleotide-binding protein)-coupled receptors (GPCRs) represent the largest family of signaling molecules in the human genome (1), responding to light, odors, peptides, proteins, lipids, ions, neurotransmitters, and hormones. Following ligand binding, GPCRs bind and activate specific G proteins, which in turn activate downstream effectors resulting in the generation of second messengers (2). Activated GPCRs rapidly undergo phosphorylation by G protein-coupled receptor kinases on serine and/or threonine residues located most often in the carboxyl-terminal domain of the protein. Receptor phosphorylation leads to the binding of arrestins (in non-visual cells, arrestin-2 and/or arrestin-3), resulting in desensitization of cellular activation through G proteins. However, in recent years, arrestins have been shown to regulate additional cellular signaling pathways by serving as multifunctional scaffold/adapter proteins (3). Through binding interactions with clathrin and the adapter AP-2, arrestin can mediate clathrin-dependent internalization of many GPCRs. In addition, arrestins can initiate the recruitment and activation of numerous kinases including c-Src family kinases (4) and mitogen-activated protein kinases (MAPKs), such as extracellular-regulated kinase 2 (ERK2) (5) and c-Jun NH2-terminal kinase 3 (JNK3) (6). The simultaneous direct binding of both the first and third MAPK components ( Raf and ERK2 or apoptosis signal-regulating kinase 1 and JNK3) suggests that arrestins serve to increase the specificity of MAPK signaling as well as recruit the resulting kinase activity to the sites of activated GPCRs.

Because of the diversity among GPCRs, it is perhaps not surprising that arrestins mediate different functions with distinct GPCRs despite the fact that virtually all of the GPCRs appear to bind arrestins as a consequence of receptor phosphorylation. Although many GPCRs, exemplified by the β2-adrenergic receptor, internalize in an arrestin-dependent manner (7), a growing number of GPCRs (e.g. protease-activated receptor 1 (8) and the N-formyl peptide receptor (FPR) (9)) are capable of undergoing internalization in an arrestin-independent manner. In the case of the FPR, a chemoattractant leukocyte GPCR (10, 11), numerous lines of evidence including the use of dominant negative arrestin constructs (arrestin-2-2319–4189) (12) and partially phosphorylated receptor mutants (13), which internalize in the absence of demonstrable arrestin binding, had suggested that receptor internalization does not require arrestin binding despite the observation that the FPR

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DIRECTLY BINDS ARRESTINS AND COLocalizes with Them during Receptor Internalization (14–18).

To investigate the roles of arrestins in GPCR function further, we recently expressed the FPR in mouse embryonic fibroblasts (MEFs) that are deficient in both arrestin-2 and arrestin-3. Our results demonstrated that FPR internalization was unaffected by the absence of arrestins, indicating that the FPR does not require arrestin to internalize (9). However, further studies revealed that FPR recycling required arrestin, the first demonstration that arrestin is required for the proper trafficking of a GPCR back to the cell surface following internalization. In this report, we provide the first evidence that GPCR activation in the absence of arrestins initiates rapid apoptosis. This response is dependent on both receptor-mediated signaling as well as receptor phosphorylation and probably internalization. We conclude that arrestins play a critical, previously unknown, role in preventing apoptosis initiated by active GPCRs.

MATERIALS AND METHODS

Reagents—Formyl-Nle-Leu-Phε-Nle-Tyr-Lys-fluorescein, formyl-Met-Leu-Phε, isoproterenol, angiotensin II, stromal-derived factor 1α, and Dulbecco’s modified Eagle’s medium (DMEM) were from Sigma. [Arg₈]Vasopressin and IL-8 were from R&D Systems SB2242190, SB202474, PD98059, U0126, SP600125, pertussis toxin, wortmannin, PP2, tyrphostin AG1478, Ro-31-8220, GF109203X, fluphenazine, pan-caspase inhibitor Z-VD-fmk, caspase 2 inhibitor Z-VAD-fmk, caspase 3 inhibitor Z-DEVD-fmk, caspase 8 inhibitor Z-LEHD-fmk, and caspase 9 inhibitor Z-LEHD-fmk were from Calbiochem. LY294002 was from Cell Signaling Technology. Staurosporine and Mitotracker Red (chloro-methyl X-rosamine) were from Molecular Probes. DNA constructs expressing β₂-adrenergic receptor and arrestin-2/3-green fluorescent protein (GFP), AT₁R, and V2 vasopressin receptor were generous gifts from Drs. Jeffrey Benovic, Laszlo Hurany, and Jurgen Wess, respectively. Mouse embryonic fibroblasts with and without β-arrestins were established as described previously (7) and generously provided by Dr. Robert Lefkowitz. Stable clones expressing the FPR were generated and cultured as described previously (9).

Cell Rounding and Cell Detachment Measurements—Cells grown on 12-mm round glass coverslips at 50% confluence were incubated with serum-free DMEM for 30 min before stimulation with the ligand at 37 °C as indicated and fixed for 15 min with chilled 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The cellular morphology then was evaluated by phase-contrast microscopy. The criteria for round cells were spherical refractile cells with no visible extensions. A minimum of 300 cells (in at least five random fields) was evaluated in each experiment. (See Fig. 1, a–c.) The percentage of cells rounded versus well adhered cells was measured as described previously (9).

Annexin V/Propidium Iodide (PI) Staining—Cells grown on 12-mm round glass coverslips at 50% confluence were incubated with serum-free DMEM for 30 min before stimulation with the ligand and at 37 °C as indicated. The cells were stained with annexin V-FITC (1:25, Caltag Laboratories) and PI (1 μg/ml, Sigma) in annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) and incubated for 10–15 min at room temperature in the dark and fixed for 15 min with chilled 2% PFA in PBS. The coverslips were then rinsed twice with annexin V binding buffer and analyzed on Zeiss LSM 510 confocal system. Annexin V-FITC and PI were excited at 488 and 546 nm, respectively. The cells showing visible annexin V staining (with no PI staining) were categorized as apoptotic cells. Similarly, cells showing both annexin V and PI staining were categorized as late apoptotic cells.

Activated Caspase 3 and Cytochrome c Staining—For activated (cleaved) caspase 3 analysis, 2 × 10⁵ cells seeded onto 12-mm glass coverslips were treated as indicated and fixed for 15 min with chilled 2% PFA in PBS. The coverslips were then rinsed twice with annexin V binding buffer and incubated for 30 min with 0.5% Triton X-100 in PBS with 3% bovine serum albumin. After washing once, cells were incubated with activated caspase 3 antibody (Cell Signaling Technology) diluted in 3% normal goat serum for 2 h at room temperature in a humidified chamber. Cells were washed in PBS and then incubated with Alexa-568-conjugated secondary antibody (Molecular Probes) diluted in 3% normal goat serum. Cells were rinsed three times in PBS and mounted in Vectashield (Vector Laboratories). To calculate the percentage of activated caspase 3-positive cells, a minimum of 300 cells/experiment was counted on a Zeiss Axiovert microscope. For mitochondrion and cytochrome c staining, cells were first stained with 25 nm Mitotracker Red for 20 min at 37 °C, fixed for 15 min with chilled 2% PFA in PBS, and subsequently permeabilized with 0.5% Triton X-100 in PBS with 3% bovine serum albumin for 60 min. Cells were then incubated with anti-cytochrome c antibody (BD Biosciences) for 2 h. Cells were washed with PBS and incubated with FITC-conjugated secondary antibody diluted in 3% normal goat serum. Cells were rinsed, mounted in Vectashield, and visualized on a Zeiss LSM 510 confocal system. Caspase 3 staining was carried out as described by the manufacturer (Serologicals Inc.) and quantitated by flow cytometry.

Inhibitor Studies—Cells seeded onto 12-mm glass coverslips were pretreated with inhibitors for the concentration and time period as indicated in the figure legend. In the presence of inhibitors, the cells were treated with serum-free DMEM with or without ligand for 30 min and fixed with chilled 2% PFA. The percentage of rounded cells was calculated as described above.

RESULTS

GPCR Activation Initiates Cell Death in Arrestin⁻/⁻ Cells—Because arrestins have become central players in the regulation of multiple kinase pathways (19–21), many of which are known to regulate cellular growth and proliferation, we sought to examine the relationship between GPCR activation and arrestin-mediated functions with respect to cell proliferation. The development and maintenance of complex organisms and tissues is dependent upon physiologically regulated cell death or apoptosis (22). Dysregulated apoptosis is a crucial component in numerous disease states including cancer, (auto)immune disorders, and neurodegenerative disorders (23). Additionally, defects in visual arrestin (arrestin-1) are known to mediate retinal degeneration through necrotic and apoptotic mechanisms in organisms ranging from Drosophila to humans (24–26). Finally, arrestin-2/3 double knock-out mice do not survive until birth, indicating a critical role in embryonic development (27). Thus, to examine whether, in the absence of arrestins, GPCR stimulation would affect the proliferative capacity of cells, MEF cells derived from knock-out mice that lack arrestin-2 (arr²⁻/⁻), arrestin-3 (arr³⁻/⁻), or both arrestins (arr²/³⁻/⁻) as well as two wild-type littermate controls (WT1 and WT2) stably expressing with the FPR were evaluated (9). Stimulation of FPR-transduced arr²/³⁻/⁻ MEF (arr²/³⁻/⁻ MEF-FPR) cells with agonist resulted in the rounding of cells followed by detachment of a significant fraction (30–40%) of the rounded cells (Fig. 1a, bottom right panel). This was not observed in stimulated FPR-expressing WT cells (Fig. 1a, upper right panel). Quantitation of the cell rounding revealed that the response was rapid with >50% of the cells having rounded at 1 h and with ~80% of the cells having rounded by 4–5 h (Fig. 1b). To address whether this response was specifically because of the absence of either arrestin-2 or arrestin-3, we examined cell rounding of stimulated FPR-expressing arr²⁻/⁻ and arr³⁻/⁻ cells individually. Neither of these cell lines displayed agonist-promoted cell rounding after 5 h of stimulation. These results indicate that the presence of either arrestin-2 or arrestin-3 is sufficient to suppress the cell rounding induced by FPR stimulation.

To examine whether the cell rounding was a manifestation of programmed/apoptotic cell death, we stimulated both WT and
Arr2/3−/− MEF cells expressing the FPR with agonist and assessed apoptotic events by annexin-V FITC/PI staining (Fig. 2a). Agonist-stimulated arr2/3−/− MEF-FPR cells underwent a significant increase in the percentage of rounded cells by 1–2 h, whereas the kinetics of nuclear PI staining (indicating cell membrane breakdown) were delayed by 2–3 h. Unstimulated arr2/3−/− MEF-FPR cells or stimulated and unstimulated WT MEF-FPR cells, which remained fully adherent, were completely viable (PI-negative). Annexin V staining (in the absence of PI staining), a reporter of phosphatidylserine accumulation on the outer leaflet of the plasma membrane, an early event in many mechanisms of apoptosis, was consistently elevated in the stimulated arr2/3−/− MEF-FPR cells as compared with the wild-type cells (Fig. 2b). The number of annexin V-positive/PI-negative cells remained constant between 10 and 15% of the cells from 1 to 24 h as the number of PI-positive cells increased from ~10 to 80% in the same time period (Fig. 2c).

Because one of the hallmarks of apoptotic signaling is the activation of caspases (28), we evaluated the activity of cellular caspases using a pan-fluorescent caspase inhibitor, CaspaTag, that binds only to proteolytically activated caspases. CaspaTag staining increased over time only in the arrestin-deficient cells (Fig. 2d) in a manner that paralleled the increase in PI staining (cf. Fig. 2, c and d). To confirm that caspase 3 in particular was activated, we used an antibody that recognizes only the active-cleaved form of caspase 3, the terminal caspase in most apoptotic pathways. Only stimulated arr2/3−/− MEF-FPR cells demonstrated a stimulation-specific increase in the level of active caspase 3-positive cells (Fig. 2e). Furthermore, although a significant fraction of the adherent rounded cells were caspase 3-positive, a smaller but significant fraction of the remaining adherent spread cells was also caspase 3-positive (not shown). The observation that spread cells were caspase 3-positive suggests that the cellular activation of caspasases precedes the rounding and detachment of cells from the substratum and subsequent death, indicating that cell death is not induced by de-adhesion, a process that has been termed anoikia. The involvement of caspasases in this process is specific for and defines apoptosis (28).

Activation of Multiple Downstream Effectors Is Required for Apoptosis—The observation that the activation of caspasases preceded the rounding and detachment of cells from the matrix, observed to begin between 60 and 90 min, suggested that relatively rapid signaling events initiated by the FPR were responsible for this phenomenon. To investigate these mechanisms further, we employed a panel of signal transduction inhibitors to examine the effects of blocking specific pathways resulting from FPR activation and leading to apoptosis (Fig. 4). Of the inhibitors tested, p38, MEK, and JNK MAPK inhibitors as well as inhibitors of Gαq protein (pertussis toxin), phosphoinositide 3-kinase (wortmannin and LY294002), and Src kinases (PP2) completely blocked the cell rounding and cell death induced by stimulation of the FPR. Protein kinase C inhibitors (staurosporine, Ro-31-8220, and GF109203X) and the calmodulin inhibitor fluphenazine had no inhibitory effect on the level of FPR-induced cell rounding in the arr2/3−/− MEF cells, although all four induced a lower extent of cell rounding in the WT cells, consistent with known properties of these compounds. In addition, the epidermal growth factor receptor kinase inhibitor AG1478 had no effect on cell rounding, indicat-
ing that transactivation of epidermal growth factor receptor was not involved. Finally, as suggested by the activation of caspases prior to cell rounding (Fig. 3), the irreversible pan-caspase inhibitor Z-VAD-fmk was completely effective in blocking FPR-induced cell rounding, detachment, and death, confirming that caspase activation represents a critical event in the GPCR-mediated apoptosis of arrestin-deficient cells.

**GPCR-induced Apoptosis Is Executed through the Mitochondrial Pathway**—The caspase family of proteases are differentially involved in both the initiation phases (31) of apoptosis as well as the propagation and effector phases of the response (32). Whereas caspase 8 has been shown to be required for Fas-induced death, multiple classical apoptotic signals are propagated through the mitochondrial release of cytochrome c and the activation of caspase 9 (31). Additionally, caspase 2 has recently been described to function as an initiator caspase (33). All initiator caspase activation leads to the eventual proteolytic activation of the effector caspase 3, which cleaves large numbers of cellular proteins leading to apoptosis. We tested the effects of specific caspase inhibitors for caspases 2, 3, 8, and 9 on FPR-induced apoptosis. Inhibitors of caspases 9 and 3 but not 2 or 8 inhibited FPR-induced cell rounding to the same extent as the pan-caspase inhibitor, suggesting that the mitochondrial route of caspase activation was involved (Fig. 5a). To test this finding, we examined the cellular distribution of cytochrome c upon FPR stimulation (Fig. 5b). Whereas cytochrome c is confined completely to mitochondria in unstimulated arr2/3Δ/Δ MEF-FPR cells and stimulated arr2/3Δ/Δ MEF-FPR cells, agonist stimulation of arr2/3Δ/Δ MEF-FPR cells results in the redistribution of cytochrome c to the cytosol. In addition, the structure of the mitochondria as determined by Mitotracker Red staining is significantly degraded in the stimulated arrestin-deficient cells, suggesting that GPCR activation leads to compromised mitochondrial integrity.

**Multiple GPCRs Are Capable of Initiating Apoptosis**—Finally, to determine whether the activation of other GPCRs in arr2/3Δ/Δ MEF cells also resulted in the initiation of apoptotic signaling leading to cell detachment and death, we expressed the β2-adrenergic, angiotensin II (type 1A), and vasopressin V2 receptors as well as the chemokine receptors for IL-8 (CXCR2) and stromal-derived factor-1α (CXCR4) in WT and arrestin-deficient MEFs. Each of these receptors was stimulated with the appropriate ligand and assessed for apoptotic events as evidenced by cell rounding. As with the FPR, stimulation of the IL-8 and angiotensin receptors in arr2/3Δ/Δ MEF cells induced cell rounding of ~80% of the cells, whereas stimulation of the vasopressin receptor induced rounding in ~60% of the cells (Fig. 6a). However, stimulation of neither the β2-adrenergic
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Neural crest cells, which give rise to many peripheral tissues and organs, are derived from the intermediate mesoderm. In this context, we have previously shown that the expression of arrestin-2 and -3 protects neural crest cells from apoptosis induced by serum deprivation (35). Given the diverse roles of arrestins in modulating GPCR signaling, we hypothesized that arrestins may play a role in the regulation of neural crest cell survival. To test this, we examined the effect of arrestin expression on apoptosis induced by serum deprivation in neural crest cell-derived cultures.

RESULTS

A. Arrestins Protect Neural Crest Cells from Serum Deprivation

We first examined the effect of arrestin expression on apoptosis induced by serum deprivation in neural crest cell-derived cultures. We used a cell line derived from a neural crest cell culture that exhibits spontaneous apoptosis in the absence of serum. This cell line was transduced with a retroviral vector expressing arrestin-2 or arrestin-3, and the effect of arrestin expression on apoptosis was measured by quantifying the number of cells that undergo apoptosis following serum deprivation.

B. Arrestions Block GPCR-mediated Apoptosis

Next, we investigated the effect of arrestins on apoptosis induced by GPCR activation. We used a cell line expressing an GPCR that stimulates apoptosis through a specific intracellular signaling pathway. We then transduced this cell line with arrestin-2 or arrestin-3, and measured the effect of arrestin expression on apoptosis induced by GPCR activation.

DISCUSSION

In this report, we have described a previously unknown function of arrestins 2 and 3, namely the suppression of apoptotic signaling following GPCR activation. In the absence of arrestins, FPR stimulation and phosphorylation result in the activation of numerous downstream effectors as well as the internalization of the receptor. This signaling leads to the release of cytochrome c from mitochondria, resulting in the initiation of apoptotic signaling. Arrestins, by virtue of their ubiquitous expression and interactions with essentially every GPCR, as well as growth factor receptors (e.g. insulin-like growth factor receptor (37) and transforming growth factor-β receptor (38)), thus are poised to regulate cellular proliferation and apoptosis of virtually every type of cell. By acting as a bridge or scaffold to multiple intracellular signaling cascades, arrestins can influence GPCR localization as well as the activation and localization of downstream signaling pathways including Src and MAPKs. In addition, arrestins have recently been shown to effect insulin-like growth factor-mediated recovery following serum deprivation through the activation of Akt, suggesting a direct role in proliferative signaling through insulin-like growth factor receptors (39).

In contrast to this proliferative signaling, we have described a distinct and unique function for arrestins, namely the suppression of apoptotic signaling initiated by GPCRs. At this point, there are many possible mechanisms by which GPCR-mediated signaling in the absence of arrestins may initiate apoptosis. Our results demonstrate the involvement of a diverse array of signaling components including G proteins, phosphoinositide 3-kinase, Src, MAPKs, and caspases. It is possible that the localization of certain signaling events in the cells is altered in the absence of arrestin as has been documented for the ERK and JNK, supporting our findings that these kinases play a role. A specific role for JNK (JNK1 and JNK2) in UV-induced apoptosis of MEF cells has been described, which involves mitochondrial-specific pathways (40). Because the arrestin-JNK interaction is specific to arrestin-3 and JNK3 (which is absent in MEF cells) (6, 40), it likely does not represent the route of stimulation resulting in apoptosis described here, which notably can be rescued by arrestin-2 in addition to arrestin-3. However, the observation that FPR phosphorylation and internalization but not chronic stimulation alone are required for the initiation of apoptotic signaling suggests that aberrantly localized signaling may be at fault...
and that one critical role of arrestin is to secure the appropriately localized series of signaling events.

Contrary to the observations described here using mammalian cells, in the Drosophila eye, visual arrestin can participate in apoptotic signaling under circumstances where the receptor phosphatase (rdgC) or an eye-specific phospholipase C (norpA) is absent (41, 42). Under these circumstances, a stable internalized rhodopsin-arrestin complex is formed that results in the initiation of G protein-independent apoptosis. Alternatively, apoptosis initiated by chronic stimulation of rhodopsin signaling in wild-type photoreceptor cells is largely G protein-dependent. Inhibition of clathrin-mediated processes (internalization and trafficking) in Drosophila can rescue this rhodopsin-initiated apoptosis, suggesting that rhodopsin-arrestin complex internalization may be required for this pathway in WT photoreceptor cells. It should be noted, however, that mammalian rhodopsins do not interact with clathrin and are not known to undergo internalization/trafficking following activation and arrestin binding (24). Although our results also demonstrate a requirement for GPCR internalization in mammalian cell apoptosis, this occurs only in the absence of arrestins and additionally requires G protein signaling to numerous intracellular effectors. This differs from apoptosis of the Drosophila norpA mutant, which requires arrestin; therefore, rhodopsin internalization yet occurs independent of G protein signaling. The mechanism we describe also differs from the necrosis of arrestin-deficient photoreceptor cells, which are G protein-dependent yet does not require rhodopsin internalization. Thus, our results describe an entirely novel pathway for GPCR-mediated apoptosis that occurs in mammalian cells not expressing arrestins.

Whether and under what physiological circumstances arrestins regulate cellular proliferation and apoptosis remain to be determined. With the recent description of cellular arrestin levels being depleted by ubiquitination as a result of the stimulation of tyrosine kinase receptors (43), it is intriguing to speculate that the balance of cellular proliferation and apoptosis can be regulated in such a manner. Not only does such a mechanism suggest novel pathways in development and disease, it also implies that the specific manipulation of arrestins may provide a therapeutic means to induce apoptosis of cancerous cells.

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