Endosomes: A legitimate platform for the signaling train

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Although long regarded as a conduit for the degradation or recycling of cell surface receptors, the endosomal system is also an essential site of signal transduction. Activated receptors accumulate in endosomes, and certain signaling components are exclusively localized to endosomes. Receptors can continue to transmit signals from endosomes that are different from those that arise from the plasma membrane, resulting in distinct physiological responses. Endosomal signaling is widespread in metazoans and plants, where it transmits signals for diverse receptor families that regulate essential processes including growth, differentiation and survival. Receptor signaling at endosomal membranes is tightly regulated by mechanisms that control agonist availability, receptor coupling to signaling machinery, and the subcellular localization of signaling components. Drugs that target mechanisms that initiate and terminate receptor signaling at the plasma membrane are widespread and effective treatments for disease. Selective disruption of receptor signaling in endosomes, which can be accomplished by targeting endosomal-specific signaling pathways or by selective delivery of drugs to the endosomal network, may provide novel therapies for disease.

signal transduction | trafficking | endocytosis | receptors

Cell surface receptors allow cells to detect and respond to signals from the external environment. The binding of an extracellular ligand to a cell surface receptor initiates a cascade of signals that begins at the plasma membrane. Given the importance of this process, signaling at the plasma membrane has been intensively studied, and many drugs target signaling by cell surface receptors. However, upon activation, many receptors enter the endosomal system, a large, dynamic tubulovesicular network extending throughout the cytoplasm. Trafficking of a ligand-receptor complex within this system provides a mechanism to either terminate signaling through degradation of the receptor in lysosomes and proteasomes, or to sustain signaling through recycling of the receptor back to the cell surface where it can rebind extracellular ligands. Although the endocytic system has traditionally been viewed as a conduit that transports receptors to a degradative or recycling fate, endosomes are also a site at which receptor signaling can be initiated, sustained, and terminated. Activated receptors accumulate in endosomes, and certain essential signaling components are confined to endosomes. Different signals can arise from receptors at endosomal and plasma membranes, resulting in distinct physiological responses. Moreover, different mechanisms regulate signaling of receptors at endosomal and plasma membranes. These disparate mechanisms of signaling and regulation raise the possibility of novel therapies based on targeting endosomal rather than plasma membrane signaling. In this article, we review the mechanisms of receptor signaling from endosomes and summarize how this signaling is regulated. We discuss the physiological relevance of endosomal signaling and speculate on whether drugs that target endosomal signaling could be new therapies for disease.

Diverse Receptor Families Signal From Endosomes. (Summarized in Table S1.)

Receptor Tyrosine Kinases (RTKs). The view that RTK signaling occurs solely at the plasma membrane was challenged when subcellular fractionation and coimmunoprecipitation studies revealed that epidermal growth factor (EGF) induced accumulation of activated EGF receptor (EGFR) and its downstream signaling factors (SOS, Grb2, SHC) in early endosomes of liver parenchymal cells (1). A similar analysis of insulin-treated adipocytes revealed that internalized insulin receptors were more highly phosphorylated than those at the plasma membrane and that insulin receptor substrate (IRS-1) was associated with internal membranes where IRS-1 phosphorylation paralleled that of the insulin receptor (2). Insulin also preferentially activates PI3K in internal rather than plasma membranes (3) and causes accumulation of mitogen-activated protein kinase (MAPK) signaling components in endosomes isolated from fibroblasts by using insulin-coated magnetic microbeads (4). Nerve growth factor (NGF) similarly causes accumulation of NGF, activated TrkA receptor, phospholipase C-γ1 (PLC-γ1), and components of MAPK and PI3K signaling pathways in endosomes of pheochromocytoma PC12 cells (5) and nociceptive neurons (6).

Although these studies indicated that endosomes contain signaling machinery, it was less clear if signals could arise from endosomes themselves. Initial studies that addressed this issue focused on EGF-induced activation of MAPK and PI3K/Akt signaling pathways, which regulate cell proliferation and survival. Disruption of EGFR endocytosis, by expression of a mutant of the endocytic protein dynamin, suppressed EGF-induced activation of ERK 1/2 and PI3K, suggesting that EGFR internalization is required for the full spectrum of EGF signaling (7). However, a subsequent study reported that trafficking of the activated downstream kinase MEK, rather than of activated EGFR, from the plasma membrane is the critical step of endosomal signaling (8). To further establish—without the use of endocytic inhibitors—whether activated EGFR per se can signal from endosomes, a pharmacological approach was used to selectively activate EGFR in endosomes (9). Treatment of cells with the EGFR tyrosine kinase inhibitor AG-1478 blocked activation of EGFR at the plasma membrane but allowed endocytosis to proceed. Withdrawal of AG-1478 caused activation of endosomal EGFR, induced recruitment of signaling factors (SHC, Grb2, p85 subunit of PI3K) to endosomes, and led to the activation of ERK1/2 and Akt. This endosome-specific signaling of activated EGFR was sufficient to promote cell survival by the PI3K/Akt pathway. Thus, signaling pathways can originate from EGFR activated within endosomes. However, AG-1478 can attenuate EGFR internalization (10, 11), which may limit its usefulness in studying endosome-specific EGFR signaling. Signaling of the insulin receptor

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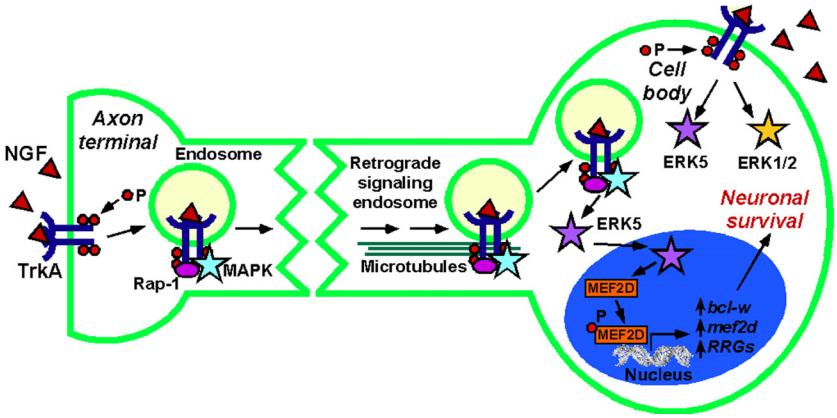

Fig. 1. Signaling endosomes transport NGF signals from axon terminals to the cell body of neurons, resulting in activation of ERK5 in the cell body and neuronal survival. In contrast, TrkA activated directly at the cell body activates both ERK5 and ERK1/2. P, phosphate.

in endosomes was demonstrated by using a peroxovanadium compound (bpV-(phen)) that activates insulin receptor kinase by inhibiting receptor-associated phosphotyrosine phosphatases, together with colchicine, which inhibits receptor recycling (12). Treatment of rats with bpV(phen) and colchicine allowed selective activation of the insulin receptor kinase in hepatic endosomes, which was accompanied by phosphorylation of IRS-1, thus demonstrating the signaling potential of the endosomal insulin receptor. Endocytosis mediates the full biological activity of other RTKs including the platelet-derived growth factor receptor (13) and vascular endothelial growth factor receptor-2 (14).

Given that receptors can signal at the cell surface and in endosomes, what is the relevance of endosomal signaling? Insight into this question is provided by consideration of NGF signaling in the nervous system. For growth factors released from target tissues to promote survival of innervating neurons, signals that arise from axon terminals must be sufficiently sustained and robust to travel long distances (>1,000-fold soma diameter) to the soma. The identification of NGF, activated TrkA, and signaling components in endosomes led to the hypothesis that NGF signals are transmitted in an axon to the cell body by retrograde transport of "signaling endosomes" (5) (Fig. 1). NGF-containing endosomes are retrogradely transported at ≈1.3 μs/s in axons of nociceptive neurons (15). In PC12 cells, NGF causes assembly of a stable endosomal signaling complex comprising TrkA, MAPK, and Rap-1 (Ras GTPase that causes sustained activation of MEK/MAPK) (16). Disruption of endosomes inhibits sustained activation of Rap-1 and MAPK, suggesting that NGF-TrkA signals in endosomes by Rap-1 to cause persistent MAPK activation. Thus, endosomes can provide a platform for sustained and robust signaling that can be transported to distant sites.

Do receptors in endosomes transmit signals that are distinct from those originating from receptors at the plasma membrane? Studies of the TrkA receptor provide evidence for endosome-specific signaling of NGF. Whereas NGF signaling in endosomes causes sustained MAPK activation, NGF-activated TrkA at the plasma membrane activates Ras transiently (16). In highly differentiated cells, such as neurons, the site of receptor activation can influence the nature of the endosomal signal. NGF-induced activation of TrkA in axon terminals of dorsal root ganglia neurons leads to retrograde transport of signaling endosomes to the cell body, where TrkA activates ERK5 (17). ERK5 translocates to the nucleus to activate CREB and enhance neuronal survival. In contrast, TrkA activated directly at the cell body signals through both ERK1/2 and ERK5 pathways (Fig. 1). Addition of neurotrophins to distal axons, but not cell bodies, also enhances activation of the transcription factor MEF2D by a Trk-dependent ERK5 pathway (18). Together, ERK5 and MEF2D increase expression of the antiapoptotic protein bcl-w, MEF2D, and other retrograde response genes. Thus, retrograde signaling from endosomes has a different outcome from that of direct stimulation at the soma and is required to activate an ERK5/MEF2D transcriptional response that enables neurons to survive in the presence of target-derived neurotrophins.

G Protein-Coupled Receptors (GPCRs). GPCRs, or 7 transmembrane receptors, are the largest family of cell surface receptors. They participate in physiological control and disease and are the targets of many drugs. GPCRs signal at the plasma membrane by coupling to heterotrimeric G proteins. Although GPCRs are rapidly uncoupled from G proteins at the plasma membrane by receptor desensitization, these "desensitized" receptors can continue to signal at the plasma membrane and in endosomes by G protein-independent mechanisms. Arrestins are critically important for desensitization, endocytosis, and G protein-independent signaling of GPCRs (19). Arrestins were discovered as inhibitors of GPCR signaling; β-arrestin (βarr) 1 and 2 were identified as inhibitors of β_2 adrenergic receptors (β_2AR) but were subsequently found to regulate many GPCRs. βarrs interact with agonist-occupied, G protein-coupled receptor kinase (GRK)-phosphorylated GPCRs. This interaction sterically uncouples receptors from G proteins to mediate desensitization and couples receptors to clathrin and AP2 to mediate endocytosis. However, βarrs also recruit diverse signaling proteins to activated receptors at plasma and endosomal membranes and are essential mediators of signaling.

The MAPK cascades [ERK, c-Jun amino-terminal kinase (JNK), p38] are the most thoroughly characterized βarr-dependent signaling pathways (19, 20) (Fig. 2). The first evidence that βarrs are active participants in signaling was the observation that dominant negative mutants of βarr inhibited β_2AR-induced activation of ERK1/2 (21). Subsequently, βarrs were found to couple β_2AR to c-Src and mediate ERK1/2 activation (22). βarrs similarly participate in ERK1/2 signaling by other GPCRs, including neurokinin-1 receptor (NK_1R), protease-activated receptor 2 (PAR_2), angiotensin II type 1A receptor ($AT_{1A}R$), and vasopressin V2 receptor (V_2R) (23–26). These observations led to the view that βarrs are scaffolds that couple activated GPCRs with MAPK signaling complexes or "signalosomes". βarrs thereby mediate a second wave of GPCR signaling that is distinct from G protein-dependent signaling at the plasma membrane. The importance of this mechanism depends on the affinity with which GPCRs interact with βarrs, which varies depending on the extent of GPCR phosphorylation by GRKs. "Class A" GPCRs (e.g., β_2AR, α_{1b}AR) have few phosphorylation sites, and transiently interact with βarr1 and βarr2, mostly at the plasma membrane, with a higher affinity for βarr2. "Class B" GPCRs (e.g., $AT_{1A}R$, V_2R, NK_1R, PAR_2) are phosphorylated at multiple sites and interact with both βarr1 and 2 with high affinity for prolonged periods at plasma and endosomal membranes. "Class C" GPCRs (e.g., bradykinin B_2 receptor) internalize with βarrs into endosomes followed by rapid dissociation of βarr upon agonist removal (27). The extent of βarr-induced MAPK signaling depends on the

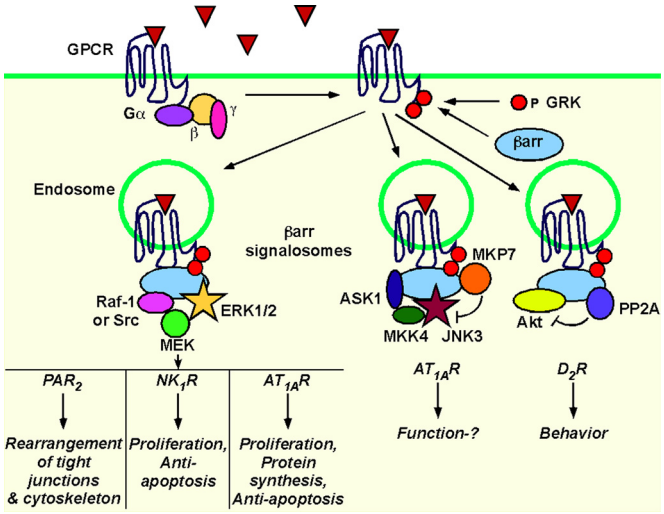

Fig. 2. βarrs recruit signaling complexes to endosomes. Complexes can include activators and inhibitors of signaling. Overlapping symbols specify direct interaction with βarr.

affinity of the receptor for βarrs, which depends on the receptor structure and on which of the seven mammalian GRKs phosphorylate the receptor. Thus, activation of $AT_{1A}R$ and V_2R causes greater phosphorylation of βarr-bound ERK1/2 than activation of $\alpha_{1b}AR$ and β_2AR, suggesting that the class B receptors signal more robustly through this pathway (26). Phosphorylation of the $AT_{1A}R$ by GRK5 and 6 is required for βarr-mediated ERK activation, whereas phosphorylation by GRK2 and 3 inhibits this signaling pathway (28). Different receptor agonists can lead to differential stimulation of GRKs with distinct outcomes. The chemokine receptor CCR7 has two endogenous agonists, CCL19 and CCL21. Whereas both agonists activate G proteins and induce recruitment of βarr2 and βarr2-dependent ERK activation, only CCL19 causes redistribution of βarr2 to endosomes and CCR7 desensitization (29). CCL19 induces GRK3- and 6-dependent phosphorylation of CCR7, whereas CCL21 activates GRK6 only, which may explain the different functional effects of these agonists.

By recruiting receptors and MAPK to endosomes, βarrs can determine the subcellular location and function of activated ERKs. As is the case for RTKs, receptors in endosomes may activate signals that differ from those originating from G proteins at the plasma membrane, resulting in distinct physiological responses. These distinct mechanisms of signaling have been evaluated by disrupting βarr or G proteins, by studying mutant receptors that are unable to interact with βarrs, or by using agonists that selectively activate particular pathways. PAR_2 and $AT_{1A}R$ coupling to Gαq activates conventional isoforms of PKC and stimulates rapid Ras-mediated activation of the Raf-1/MEK1/ERK1/2 module; activated ERK1/2 translocate to the nucleus to regulate proliferation and transcription (23, 30). PAR_2 and $AT_{1A}R$ also activate ERK1/2 by βarr-dependent mechanisms. PAR_2 activation induces assembly of a signaling complex comprising PAR_2/βarr/Raf-1/MEK1/ERK1/2, which retains ERK activity in the cytosol rather than the nucleus (23). A PAR_2 mutant that was unable to interact with βarrs failed to promote formation of this complex or cause cytosolic retention of activated ERK1/2, which instead translocated to the nucleus to promote proliferation. βarr-dependent mechanisms mediate delayed and sustained activation of ERK1/2 that accumulates in endosomes with $AT_{1A}R$ and βarrs (30). V_2R activation also results in βarr-dependent activation of ERK1/2, which are mostly retained in the cytosol (26). Substance P (SP) also induces the formation of a signaling complex comprising SP/NK_1R/βarr/Src/MEK1/ERK1/2 (24). When βarr1 is fused to the NK_1R C terminus, the receptor is constitutively associated with a c-Raf/MEK1/2/ERK1/2 complex in endosomes, leading to robust activation of cytosolic but not nuclear ERK1/2 (31).

βarrs similarly participate in activation of the JNK MAPK cascade, a regulator of stress-induced apoptosis, cell survival, and morphogenesis. Stimulation of $AT_{1A}R$ promotes assembly of a signaling complex in endosomes comprising βarr2, the upstream kinases MAP kinase kinase (MKK4), and apoptosis signaling kinase (ASK1), and active JNK3 (32, 33). Whereas JNK3 and ASK1 directly interact with βarr2, MKK4, although part of the complex, interacts indirectly with βarr2. As is the case with ERK1/2, βarr2 retains activated JNK3 in the cytosol. Notably, the complex includes MAP kinase phosphatase 7 (MKP7), which interacts with βarr2 and can dephosphorylate JNK3 (34) (Fig. 2). Thus, the complex contains machinery to both initiate and terminate JNK3 activation. The p38 MAPK mediates transcriptional responses to stress and inflammation. βarrs are necessary for p38-dependent signaling of the chemokine receptor CXCR4 (35) and the κ-opioid receptor (36).

βarrs also control PI3K, a regulator of cell growth, movement, and apoptosis. Activation of PAR_2 promotes interaction of βarrs and PI3K, which inhibits PI3K catalytic activity (37, 38). This mechanism opposes PAR_2-induced stimulation of PI3K, which is mediated by Gαq. The result of these opposing mechanisms depends on the level of βarr expression, with PI3K inhibition predominating in cells that highly express βarrs.

In keeping with inhibition of PI3K, βarrs can also inhibit Akt, a downstream target of PI3K that controls transcription, apoptosis, and the cell-cycle (Fig. 2). Phosphatidylinositol-dependent kinase 1 and target of rapamycin complex 2 kinase phosphorylate and activate Akt, whereas protein phosphatase 2A (PP2A) dephosphorylates and inactivates Akt. Dopamine 2 receptor (D_2R) stimulation induces formation of a βarr2/Akt/PP2A complex, identified in striatal extracts by pull-down assays (39, 40). Whether this complex forms at the plasma membrane or endosomes is unknown. Sustained stimulation of D_2R in the mouse striatum inactivates Akt by a βarr2-dependent mechanism (39, 40). This mechanism is another example, along with regulation of JNK3, of βarr recruiting both activators and inhibitors (PP2A, MKP7) to signaling complexes. Ghrelin, a regulator of food intake and metabolism, also activates Akt, here in a biphasic fashion with an early Gi/*o*-dependent pathway and a late βarr-dependent pathway involving recruitment of Src and Akt (41).

Despite the focus on βarr-mediated signaling in endosomes, G proteins can also signal from endosomes. In the mating pheromone response pathway of *Saccharomyces cerevisiae*, the GPCR Ste2 transduces signals to secreted α-factor, which were thought to depend on the plasma membrane bound Gβγ subunits. However, Gα subunits translocate to endosomes to stimulate PI3K activity (42). Gβγ subunits can also mediate signals from endosomes in mammalian cells (43). Gβγ interact with Rab11a, lysophosphatidic acid promotes association of Gβγ, PI3K, and Akt with Rab11a-positive endosomes in HEK cells. Disruption of these associations attenuates effects of lysophosphatidic acid on cell survival and proliferation, suggesting that endosomal signaling of G proteins is functionally important.

Toll-Like Receptors (TLRs). Endosomes are a platform for signaling of TLRs, major mediators of innate immunity. TLR9 binding to its ligand CpG oligodeoxynucleotide (CpG-A) induces IFN by activating the transcription factor IRF-7 via the adaptor protein myeloid differentiation primary response gene 88 (MyD88) (44). However, this response only occurs in the plasmacytoid subset of dendritic cells. The reason for this cell-type specificity has been attributed to the ability of plasmacytoid dendritic cells to retain the TLR9-bound CpG-A and MyD88-IRF-7 complex in endosomes for long periods, which is required for a robust IFN response. In conventional dendritic cells, CpG-A is rapidly degraded in lysosomes. Inducing endosomal retention of CpG-A in conventional dendritic cells by using a cationic lipid activates the TLR9-MyD88-IRF-7 pathway, causing IFN production. Endosomes also play a vital role in the antiviral responses triggered by double-stranded RNA binding to TLR3 (45). Stimulation of dendritic cells with double-stranded RNA induces redistribution of TLR3 from the endoplasmic reticulum to endosomes. TLR3 and c-Src accumulate in endosomes containing double-stranded RNA, and c-Src is essential for antiviral signaling. Endosomes are a site for the coordinated activation of signaling pathways by TLR4, a receptor for lipopolysaccharide from bacterial cell walls (46). TLR4 activates two pathways: the Toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP)-MyD88 pathway that induces cytokines, and the Toll-receptor-associated molecule (TRAM)–Toll-receptor-associated activator of interferon (TRIF) pathway that induces IFN. Inhibiting TLR4 endocytosis disrupts the TRAM–TRIF pathway, and localization of TRAM to endosomes is necessary for TLR4 signaling (46). Thus, TIRAP-MyD88 signaling is initiated by TLR4 at the plasma membrane, whereas TRAM–TRIF signaling is initiated by endocytosed TLR4. The switch between the two pathways may be caused by depletion of phosphatidylinositol-4,5-bisphosphate from the membrane during endocytosis, which releases the TIRAP-MyD88 complex from TLR4, thereby enabling TLR4 to interact with TRAM–TRIF in endosomes (Fig. 3).

Other Mechanisms of Endosomal Signaling. Although many signal-transduction cascades are propagated by phosphorylation, signaling can also require proteolysis, which may activate a substrate or allow a product to translocate to a different cellular location to exert its effect. Proteases are essential for Notch signaling, a regulator of development. Notch receptors exist in the plasma membrane as heterodimers composed of the Notch extracellular domain and the membrane-anchored intracellular domain. Ligand binding results in cleavage of the membrane-anchored intracellular domain at an extracellular site by metalloproteases (Fig. S1). Notch then undergoes intramembrane cleavage of the membrane-anchored intracellular domain by γ-secretase to liberate the Notch intracellular domain, which translocates to the nucleus to regulate gene transcription (Fig. S1). Although the role of endocytosis in Notch cleavage and signaling is poorly understood, observations of *Drosophila melanogaster* mutants with defects in the endocytic pathway indicate that entry of Notch into early endosomes is required for efficient γ-secretase-mediated cleavage of Notch and Notch signaling (47). Alterations in Notch trafficking in endosomes may underlie developmental abnormalities that are related to defects in Notch signaling.

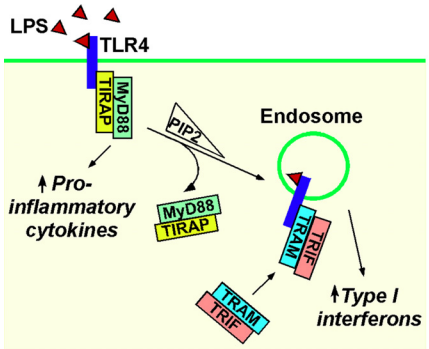

Fig. 3. Endosomes are key to TLR signaling. The figure depicts the requirement of TLR4 internalization to endosomes for the exchange of the TIRAP-MyD88 signaling complex with the TRAM–TRIF signaling complex.

Although mostly studied in metazoans, endosomes are a site for receptor signaling in plants. Increasing the endosomal localization of the steroid receptor BRI1 in *Arabidopsis thaliana* by overexpression enhances transcriptional signaling and genomic responses, suggesting that in plants, as in animal cells, endosomes play an essential role in receptor signaling (48).

Endosomal Signaling Is Tightly Controlled. Receptor signaling at the plasma membrane is precisely regulated by mechanisms that control agonist availability, receptor coupling to signal-transduction machinery, and subcellular distribution of signaling components. Defects in these mechanisms can cause disease, and drugs that target these mechanisms have powerful effects. Considerably less is known about the mechanisms that regulate receptor signaling from endosomes.

Endosomal Proteolysis Attenuates Signaling. The mechanisms that terminate endosomal signaling are not fully understood, and it is uncertain whether endosomal signaling ceases before receptor degradation or recycling. The EGFR is substantially phosphorylated in endosomes but is dephosphorylated and deactivated before trafficking to lysosomes (49). EGFR dephosphorylation coincides with a loss of EGF from endosomes, suggesting that ligand dissociation from the internalized receptor attenuates EGFR signaling. This conclusion is supported by the observations that cathepsin B in soluble endosome extracts degrades EGF and that cathepsin B inhibition enhances EGFR phosphorylation in endosomes (50). The importance of ligand dissociation in terminating EGFR signaling is further illustrated by comparing signaling of TGF-α and EGF. Whereas both agonists bind EGFR with equal affinity at the plasma membrane where the pH is neutral, TGF-α more readily dissociates from EGFR at the acidic pH of endosomes and exhibits diminished EGFR mitogenic signaling (51). Thus, EGFR signaling in endosomes is regulated by the rate of ligand dissociation in the acidic endosomal environment and subsequent degradation.

Ligand dissociation from GPCRs and subsequent degradation by endosomal peptidases also controls trafficking and signaling of neuropeptide receptors (Fig. 4). Endothelin-converting enzyme 1 (ECE-1) is a membrane-associated metalloendopeptidase that shuttles between plasma and endosomal membranes (52). ECE-1 rapidly degrades SP, calcitonin gene-related peptide (CGRP) and somatostatin in endosomes to disrupt the peptide-receptor-βarr complex, allowing βarrs to return to the cytoplasm and receptors, freed from βarrs, to recycle and resensitize (52–54). This mechanism promotes recycling and resensitization of receptors for SP (NK_1R), CGRP, and somatostatin (somatostatin receptor 2A). For these class B GPCRs, dissociation from βarrs in endosomes is necessary for recycling and resensitization. ECE-1 does not regulate resensitization of the class B $AT_{1A}R$ because angiotensin II is not an ECE-1 substrate. Similarly, ECE-1 does not regulate recycling and resensitization of the bradykinin B_2 receptor, which transiently interacts with βarrs and rapidly recycles and resensitizes. Neuropeptide degradation in endosomes also regulates βarr-mediated MAPK signaling. Inhibition of ECE-1 or treatment with the vacuolar H^+-ATPAse inhibitor bafilomycin A_1 causes retention of the SP/NK_1R/βarr/MEK1/ERK1/2 complex in endosomes and sustained ERK1/2 activation (55).

Deubiquitinating Proteases (DUBs) Control Endosomal Trafficking and Perhaps Signaling. Attachment of ubiquitin to lysines of target proteins is a critical determinant of

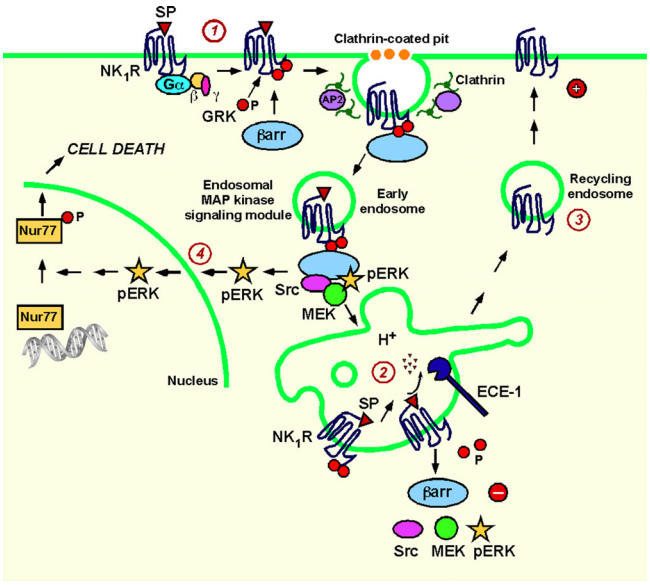

Fig. 4. Endosomal ECE-1 regulates SP-induced ERK activation and cell death. (**1**) SP binding to the NK_1R leads to recruitment of βarr to the receptor, assembly of a MAPK signalosome, and ERK1/2 activation. (**2**) Degradation of SP by ECE-1 in acidified endosomes disrupts the SP/NK_1R/βarr/MAPK signalosome. (**3**) NK_1R recycles to the plasma membrane for resensitization. (**4**) Inhibiting ECE-1 activity causes sustained ERK1/2 activation and SP-induced cell death.

their subcellular distribution and function. Agonist-induced ubiquitination of RTKs and GPCRs at the plasma membrane and possibly in endosomes controls receptor trafficking throughout the endosomal system. For some receptors, the ubiquitin E3 ligase that mediates agonist-stimulated ubiquitination is known. c-Cbl ubiquitinates the EGFR (56) and PAR_2 (57), AIP4 ubiquitinates CXCR4 (58) and δ-opioid receptor (59), and Nedd4 ubiquitinates the β_2AR (60). β_2AR agonists also promote interaction between βarr2 and the E3 ligase Mdm2, which ubiquitinates βarr2 (61). The importance of ubiquitination for trafficking varies between receptors. Ubiquitination is necessary for endocytosis of the yeast GPCRs Ste2p and Ste3p (62, 63). However, ubiquitination-defective mutants of β_2AR, CXCR4 and PAR_2 internalize normally but are instead retained in early endosomes, escaping lysosomal trafficking and degradation (57, 61, 64). Similarly, ubiquitination is not necessary for EGFR endocytosis because mutation of lysine residues in the EGFR kinase domain does not impair internalization (65) and EGFR internalization is unaltered in fibroblasts lacking c-Cbl (66). Mdm2-mediated ubiquitination of βarr is required for endocytosis of the β_2AR and perhaps other GPCRs (61, 67) and is necessary for high-affinity interactions of βarr and GPCRs that determine receptor endocytosis and downstream signal transduction (68).

Before receptors are incorporated into the intralumenal vesicles of multivesicular bodies en route to lysosomes, they are deubiquitinated, which maintains levels of free ubiquitin. Two endosomal DUBs, associated molecule with the Src homology 3 (SH3) domain of signal transducing adapter molecule (STAM) (AMSH) and ubiquitin-specific protease Y (UBPY or USP8), control EGFR deubiquitination and postendocytic trafficking. AMSH and UBPY interact directly with STAM through a common binding site within its SH3 domain (69, 70). Whereas c-Cbl promotes lysosomal degradation of the EGFR (56), AMSH opposes c-Cbl action and promotes EGFR recycling (71), and UBPY is required for lysosomal sorting and degradation of EGFR (72–74). It is unclear whether AMSH and UBPY act in opposition or in a coordinated fashion (75). AMSH and UBPY also deubiquitinate δ-opioid receptor and PAR_2 and are required for lysosomal trafficking and degradation of these receptors (59, 76). USP33 and USP20 deubiquitinate the β_2AR, which inhibits receptor degradation and promotes recycling from late endosomes (77).

Lysosomal degradation irrevocably terminates receptor signaling, and disruption of this process would be expected to prolong signaling of receptors at the plasma membrane or in endosomes. Fusing the C-tail of the recycling NK_1R to PAR_1, which normally traffics to lysosomes, generates a receptor that recycles and continues to signal at the plasma membrane (78). Ubiquitination-defective PAR_2 mutants also recycle and resensitize at the cell surface (57). DUBs regulate recycling and resensitization of the β_2AR. Knockdown of both USP33 and USP20 inhibits β_2AR recycling and resensitization of cAMP responses but increases agonist-induced β_2AR ubiquitination, lysosomal trafficking and degradation of the receptor (77). Little is known about the role of endosomal DUBs in controlling signaling of endocytosed receptors. However, disruption of AMSH and UBPY does not affect the association of PAR_2 with βarrs in endosomes and does not influence the duration or magnitude of PAR_2-induced activation of ERK1/2 (76). In contrast, the balance of βarr ubiquitination and deubiquitination regulates the association of βarr with GPCRs and βarr-dependent ERK1/2 activation (79). Whereas the E3 ligase Mdm2 mediates agonist-induced ubiquitination of βarr2, the DUB USP33 interacts with and deubiquitinates βarr2. Overexpression of Mdm2 or knockdown of USP33 stabilizes the endosomal interaction of βarr2 with β_2AR (transiently interacts with βarrs), leading to prolonged and enhanced ERK1/2 activation. Conversely, overexpression of USP33 destabilizes the interaction of βarr2 with V_2R (stably interacts with βarrs in endosomes), which attenuates ERK1/2 activation. Thus, βarr ubiquitination and deubiquitination regulate stability of the βarr MAPK signalosome to control the duration of ERK1/2 signaling.

The Mechanism of Endocytosis Specifies the Outcome of Endosomal Signals. Differences in the mechanism of endocytosis of Trk and EGFR explain the quandary that whereas Trk promotes neuronal differentiation and survival, other growth factors do not (80). NGF induces endocytosis of Trk in PC12 cells by a mechanism involving the Rho GTPase Rac and the trafficking protein Pincher, termed "macroendocytosis". This results in accumulation of Trk in immature multivesicular bodies containing Rab5 but lacking the late endosome protein Rab7. In contrast, EGF stimulates clathrin-dependent endocytosis of EGFR into Rab5 endosomes, with rapid exchange of Rab7 for Rab5 and transition to late endosomes and lysosomes. Whereas NGF/Trk induce sustained ERK1/2 activation, EGF/EGFR transiently activate ERK1/2 because EGFR is rapidly degraded. Thus, endosomes provide a specialized NGF/TrkA platform for sustained signaling required for neuronal survival.

Receptor Transit Through the Endosomal Network Refines Signals. The importance of endosomal transit for signaling is illustrated by the finding that disruption of trafficking of c-Met (hepatocyte growth factor receptor) from peripheral to perinuclear endosomes inhibits nu-

clear accumulation of the transcription factor STAT3 (81). Similarly, redirecting endosomes containing EGFR between peripheral and perinuclear locations affects EGFR degradation, MAPK activation and transcription (82).

Physiological Outcomes of Endosomal Signaling. The signals that emanate from diverse families of receptors in endosomes control essential processes of growth, differentiation, survival, inflammation, and immunity. However, most information about endosomal signaling derives from studies of cell lines that often overexpress receptors and signaling components at supraphysiological levels. Although such studies provide important mechanistic insights, the physiological outcomes of endosomal signaling in functionally important cells or in intact animals are not fully understood.

The importance of endosomes to the physiological outcomes of RTK signaling is illustrated by neurotrophin signaling to sensory nerves (Fig. 1). Neurotrophins from innervated tissues stimulate endocytosis of TrkA in axon terminals, and endosomes convey signals to the nucleus to induce transcriptional events that promote neuronal growth. Disruption of this process enhances lysosomal degradation of TrkA, inhibits NGF-induced ERK activity in endosomes, and attenuates effects of NGF on gene expression and neurite outgrowth (83).

βarrs couple GPCRs to multiple signaling pathways and may therefore mediate many physiological responses (Fig. 2). For some receptors, βarrs retain activated ERK1/2 in endosomes or the cytosol, thereby restricting nuclear translocation and effects on transcription and proliferation. The precise downstream targets of βarr-activated ERK1/2 are not fully characterized. However, PAR_2 controls tight junction assembly and paracellular permeability of colonocytes by βarr-dependent activation of ERK1/2, with implications for intestinal inflammatory diseases characterized by bacterial and macromolecule translocation from the lumen (84). A PAR_2/βarr/ERK1/2 complex is enriched in pseudopodia of migrating cells and is required for cytoskeletal reorganization, pseudopodia extension, and chemotaxis (85). This mechanism may mediate the migration of breast cancer cells induced by the release of trypsin and autocrine activation of PAR_2 (86). A SP/NK_1R/βarr/Src/ERK1/2 complex is required for the proliferative and antiapoptotic actions of the SP (24). Inhibition of ECE-1 stabilizes this complex and results in markedly sustained ERK1/2 signaling, where activated ERK1/2 translocates to the nucleus and activates the death receptor Nur77, causing neurodegeneration (55).

The physiological consequences of βarr signaling have been characterized in βarr-deficient mice (87). βarr2-deficient mice fail to develop antinociceptive tolerance to morphine due to diminished βarr-mediated desensitization of the μ-opioid receptor (88). However, other effects of morphine (for example, induction of constipation) are diminished in these mice, suggesting a role for βarr2 in μ-opioid receptor signaling in enteric neurons by mechanisms that remain to be explored (87). βarr2 deficiency impedes formation of the βarr2/Akt/PP2A complex, disrupts the effects of dopamine on Akt activity, and abrogates the behavioral effects of dopaminergic drugs without affecting G protein signaling (39, 40). Given the importance of dopamine in locomotion, reward, and affect, and its involvement in Parkinson's disease, Huntington's disease, and schizophrenia, this mechanism of βarr2-dependent signaling could be of considerable importance. Although studies of βarr2-deficient mice illustrate the physiological importance of βarr2 for opioid and dopamine signaling, it remains to be determined whether this role depends on formation of endosomal signaling complexes.

The use of agonists that selectively activate G protein- or βarr-dependent signaling (biased agonists) has provided further insight into the physiological importance of βarr signaling. An analogue of angiotensin II, SII-angiotensin, is a specific agonist of the βarr-dependent pathway of AT_1R signaling and does not activate G protein-dependent signaling. This analogue induces proliferation, protein synthesis, and antiapoptotic signals in vascular smooth muscle cells by βarr and ERK1/2-dependent processes, demonstrating the functional relevance of this pathway (89–91).

βarrs also regulate signaling in primary cilia, hair-like extensions of cells that detect environmental stimuli (92). The GPCR Smoothened is a component of the Hedgehog signaling pathway that is essential for development, stem cell function, and cancer. Translocation of Smoothened to cilia is necessary for regulation of gene transcription. βarrs couple Smoothened to the kinesin motor protein Kif3A and thereby promote the translocation of this complex to primary cilia where Smoothened regulates transcription. Because other GPCRs are found in cilia (93, 94), βarrs may control the location and activity of several signaling pathways in this location.

Endosomal mechanisms also regulate signaling of TLRs (Fig. 3) and Notch (Fig. S1), and may therefore be essential for innate immunity and development.

Targeting Endosomal Signaling: New Opportunities for Therapy? Many drugs target receptor signaling at the cell surface. Given the importance of signals that originate from receptors in endosomes, which are sometimes quite distinct from those that derive from cell surface receptors, therapies specifically directed to endosomal signals will likely offer a novel and important pharmacological approach to disease. The concept that receptors can signal differently at the cell surface and in endosomes emphasizes the importance of screening multiple pathways during drug discovery. Specific inhibition of endosomal signaling may be achieved by targeting drugs to disrupt only endosomal signaling pathways or by designing drugs that are selectively delivered to endosomes. Both strategies have been successful.

The observation that therapeutic actions of lithium depend on disrupting βarr function illustrates the feasibility of targeting βarr. Lithium is used to treat certain psychiatric disorders, including schizophrenia, bipolar disorder, and depression. Lithium disrupts the ability of βarr2 to assemble the Akt/PP2A complex, which mediates some of its therapeutic actions (95). Importantly, lithium does not disrupt other actions of βarr2 at the plasma membrane and in endosomes, including βarr2 interaction with receptors, clathrin, and Raf-1, βarr-mediated desensitization of receptors, and βarr-dependent activation of ERK1/2. Whereas chronic administration of lithium to WT mice has effects on tail suspension and dark–light emergence behaviors, it is completely without effect in mice deficient in βarr2. Thus, lithium specifically targets βarr-dependent interactions of Akt/PP2A to exert its effects on behavior. Given that distinct domains of βarr interact with various proteins at plasma and endosomal membranes, it should be possible to target specific domains to influence particular actions of βarrs.

The differential effects of drugs on G protein- and βarr-mediated signaling can explain some of their beneficial and detrimental effects. Deletion of βarr2 enhances morphine analgesia due to diminished desensitization of the μ-opioid receptor but reduces the detrimental side effect of constipation (87, 88). Deletion of βarr1 does not affect the beneficial actions of nicotinic acid on lowering circulating triglycerides and raising high-density lipoproteins but attenuates the side effect of flushing, which is associated with burning and itching of the skin (96).

Drugs that target mechanisms that regulate endosomal signaling may also be useful. Endosomal ECE-1 is a target for disorders of inflammation and pain. By degrading SP and CGRP in acidified endosomes, ECE-1 promotes recycling and

resensitization of receptors that mediate neurogenic inflammation and pain (52, 53). An ECE-1 inhibitor prevents resensitization of SP-induced plasma extravasation, providing evidence for an antiinflammatory effect of ECE-1 inhibitors (53, 97). Thus, in the case of βarrs and ECE-1, therapies that specifically target GPCR signaling in endosomes without affecting signaling at the plasma membrane are a viable and novel pharmacological strategy.

Although most drug development focuses on drug interaction with active sites of target proteins, strategies that target drugs to specific subcellular regions can be effective. Endosomal β-secretase is critical for formation of β-amyloid protein and is a therapeutic target for Alzheimer's disease. By synthesizing a β-secretase inhibitor coupled to a sterol moiety, an inhibitor was developed that concentrated the inhibitor at the endosomal membrane (98). This "endosomally targeted" inhibitor was more effective than the free inhibitor. Similar strategies could be used to target other drugs to the endosomal system.

Concluding Remarks and Future Directions. Endocytosis was originally viewed as a mechanism that delivered receptors to degradatory or recycling pathways. It is now clear that diverse families of receptors cells can signal from the endosomal network to control essential cellular responses. These endosomal signals differ from those originating from receptors at the plasma membrane, both mechanistically and temporally, and endosomal signaling is tightly regulated by mechanisms that are not fully understood.

A major challenge is to understand the physiological relevance of endosomal signaling: Why do receptors signal from endosomes? and Do endosomes transmit unique and functionally important signals? Studies of model systems, typically cell lines overexpressing receptors, have provided a wealth of information about the potential mechanisms of receptor signaling in endosomes, but whether they faithfully replicate signaling in complex, highly differentiated cells such as neurons is not completely clear. Difficulties in studying endosomal signaling in cells in primary culture or in intact animals include detection of signaling molecules, which are often expressed at low levels, and discrimination between plasma membrane and endosomal signaling events. Promising approaches include studies of mice expressing fluorescently tagged receptors (99) or lacking key endosomal signaling proteins (87), siRNA knockdown of signaling intermediates in neurons (18), use of innovative methods to isolate endosomal signaling complexes (4, 55), and studies of agonists that selectively activate endosomal rather than plasma membrane signaling pathways (89–91).

The knowledge that receptors can signal in endosomes by mechanisms that are distinct from those at the plasma membrane raises the possibility of developing drugs that specifically target endosomal signaling. This strategy may offer improved selectivity with fewer side effects than targeting more proximal steps of receptor signaling. Indeed, drugs that either target endosomal signaling events (53, 95–97) or that are specifically delivered to endosomes (98) have powerful effects. The challenge will be to design drugs that target endosomal signaling relevant to disease. Given the success of drugs that target signals generated at the plasma membrane, this challenge is likely to be worthwhile.

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