G Protein-Coupled Receptors in Gastrointestinal Physiology II. Regulation of neuropeptide receptors in enteric neurons*

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McConalogue, Karen, and Nigel W. Bunnett. G Protein-Coupled Receptors in Gastrointestinal Physiology. II. Regulation of neuropeptide receptors in enteric neurons. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G792–G796, 1998.—Neuropeptides exert their diverse biological effects by interacting with G protein-coupled receptors (GPCRs). In this review we address the question, What regulates the ability of a target cell, in particular a neuron, to respond to a neuropeptide? Available evidence from studies of many GPCRs in reconstituted systems and transfected cell lines indicates that much of this regulation occurs at the level of the receptor and serves to alter the capacity of the receptor to bind ligands with high affinity and to couple to heterotrimeric G proteins. Although some of the knowledge gained from these studies is applicable to the regulation of neuropeptide receptors on neurons, at present there are far more questions than answers.

neurotransmission; desensitization; resensitization; G protein receptor kinases; arrestins

NEUROPEPTIDES REGULATE multiple cell types in the gastrointestinal tract and participate in the control of all aspects of digestive function in health and disease states. They exert their diverse biological effects by interacting with members of the large family of G protein-coupled receptors (GPCRs). Communication between a peptidergic nerve and a target cell requires release of the neuropeptide into the interstitial fluid after stimulation of a reflex pathway, diffusion of the peptide to the target cell (which may include another neuron or an epithelial, endothelial, smooth muscle, or immune cell), interaction between the neuropeptide and the cell surface GPCR, and finally, appropriate coupling of the activated receptor to heterotrimeric G proteins that transduce the signal, triggering the cells to secrete, contract, or divide. At each of these steps there is ample opportunity for regulation and integration of the signal.

What regulates the ability of a target cell, in particular a neuron, to respond to a neuropeptide? Assuming that the neuropeptide is appropriately released from a nerve and is at a sufficiently high concentration in the extracellular fluid, a simplistic view is that signaling requires interaction of the neuropeptide with a GPCR at the plasma membrane that is in a high-affinity state and appropriately coupled to the machinery of signal transduction. But what determines whether the receptor is appropriately located at the plasma membrane in the vicinity of a peptidergic nerve fiber? What governs the ability of a GPCR to bind to a neuropeptide? And how is the interaction between the agonist-occupied receptor and the heterotrimeric G proteins regulated?

In an attempt to answer these questions, we discuss some of the events that alter receptor function after neuropeptide binding and signaling. Biological responses to GPCR agonists are often transient and desensitize in response to repeated stimulation. With time between stimuli, responses recover or resensitize. This regulation principally occurs at the level of the receptor and serves to alter its capacity to bind ligands with high affinity and to couple with heterotrimeric G proteins (see Ref. 4 for review). Much of the available information on GPCR desensitization and resensitization derives from studies in reconstituted systems and transfected cell lines of two receptors: rhodopsin and the β2-adrenergic receptor. Although these observations are undoubtedly applicable to other GPCRs, surprisingly little is known about the regulation of signaling by neuropeptide receptors in highly differentiated, functionally important cells, such as neurons, which naturally express the receptors and are of great importance in the digestive tract. We briefly summarize some of the available information about desensitization and resensitization of neuropeptide receptors and speculate how this regulation may affect gastrointestinal function.

AGONISTS INDUCE CELLULAR RESPONSES IN NEURONS THAT RAPIDLY DESENSITIZE AND GRADUALLY RESENSITIZE

Exposure of cells to neuropeptides usually renders them unresponsive to subsequent challenges by the same agonist several minutes later, a phenomenon known as homologous desensitization. Responses resensitize when the interval between agonist challenges increases (minutes to hours). These phenomena occur in transfected cell lines that overexpress the receptor of interest and in highly differentiated cells, such as enteric neurons, that naturally express the receptors. For example, the neuropeptide substance P induces Ca2+ mobilization by activating the neurokinin-1 recep-
tor in guinea pig myenteric neurons (Bunnett, unpublished observations). This response desensitizes when the interval between repetitive substance P challenge is 10 min, but fully recovers after only 30 min. The desensitization and resensitization of responsiveness to substance P correlates with a loss and recovery of high-affinity binding sites for substance P at the plasma membrane of the soma, suggesting that alterations in the ability of neurons to bind substance P contribute to altered cellular responsiveness (10).

The loss and recovery of function that accompany GPCR activation are likely to be of considerable biological importance in the gastrointestinal tract. Desensitization terminates the signal and may thereby contribute to the transient nature of secretory and motility changes that accompany feeding. The importance of desensitization is illustrated by the discovery of point mutations in certain peptide receptors that result in constitutive activation even in the absence of agonist and that cause certain heritable diseases (21). Receptor resensitization allows cells to recover or maintain their ability to respond to stimulation with time. How could receptor-mediated events such as peristalsis be sustained unless neurons quickly recovered after activation?

In view of the biological importance of desensitization of GPCRs in neurons, it is perhaps surprising that we know so little about the mechanisms of these processes in the nervous system. However, from studies of many GPCRs in reconstituted systems and cell lines, the current view is that rapid desensitization involves phosphorylation of the agonist-bound receptor, followed by uncoupling from G proteins and consequent termination of the signal. A second, slightly slower phase may involve agonist-induced receptor endocytosis, which could contribute to desensitization by removing receptors from the plasma membrane, where they are able to bind hydrophilic peptides in the extracellular fluid. However, GPCR endocytosis also contributes to resensitization, and recent evidence suggests a role for endocytosis in certain aspects of signal transduction.

AGONIST-INDUCED RECEPTOR PHOSPHORYLATION UNCOUPLES GPCRS FROM HETEROTRIMERIC G PROTEINS AND MEDIATES DESSENSITIZATION

A family of serine and threonine kinases, the G protein receptor kinases (GRKs), and their functional cofactors, the arrestins, play a critically important role in desensitization of GPCRs (4). There are six GRKs and three arrestins that have been identified by molecular cloning, as well as several splice variants. GRK-2 and GRK-3 (or β-adrenergic receptor kinase-1 and -2), and β-arrestin-1 and β-arrestin-2 are of particular importance in desensitization of GPCRs for neurotransmitters. GRK-2 and GRK-3 phosphorylate many receptors that are normally expressed by enteric neurons, including adrenergic, muscarinic, dopaminergic, and peptidergic receptors that couple to various G proteins \( (G_\alpha, G_\beta, G_{q/11}\alpha) \). GRK overexpression enhances desensitization, and overexpression of a dominant negative GRK mutant or use of neutralizing GRK antibodies attenuates desensitization. Recently, neutralizing antibodies to GRKs have been used to confirm the importance of this mechanism for desensitizing adrenergic receptors in sensory neurons (16). β-Arrestin-1 and β-arrestin-2 serve as functional cofactors for GRKs and thereby mediate desensitization of many GPCRs, including some from the enteric nervous system. β-Arrestins bind to GRK-phosphorylated receptors to interdict their interactions with G proteins and thereby terminate signaling. Overexpression of β-arrestin and GRK-2 amplifies GPCR desensitization, whereas neutralizing antibodies to β-arrestin-2 attenuate desensitization. Some of these methods may also be applied to the study of regulation of receptors in neurons.

GRKs and β-arrestins are widely expressed in the central and peripheral nervous systems and may thus regulate many GPCRs. GRK-2 and GRK-3 are highly expressed in brain, where they are localized in neuronal cell bodies and presynaptic and postsynaptic locations, with the latter predominating (1). This localization suggests that they are likely to function in the desensitization of many receptors, especially those activated at synapses. β-Arrestin-1 and β-arrestin-2 are also highly expressed in neuronal tissues. Ultrastructural studies reveal β-arrestin-2 immunoreactivity in multivesicular bodies of neurons, suggesting that β-arrestins interact with endocytosed receptors (2). GRK-2 and GRK-3 and β-arrestin-1 and -2 are also present in the gastrointestinal tract (Bunnett, unpublished observations). Most enteric neurons of the rat ileum express GRK-2 and GRK-3 and β-arrestin-1 and -2, which suggests that these proteins participate in the regulation of many receptors in the digestive tract.

AGONISTS ALTER THE SUBCELLULAR DISTRIBUTION OF GPCRS AND ASSOCIATED REGULATORY PROTEINS IN NEURONS

The ability of cells to respond to GPCR agonists requires the appropriate subcellular localization of the receptor and depends on the subcellular distribution of other proteins that are involved in signal transduction and receptor regulation (15). How could a neuron respond to a hydrophilic neuropeptide unless the receptor was present at the cell surface in the vicinity of the peptidergic nerve fiber, and in close proximity to heterotrimeric G proteins and second messenger systems? How could GRKs and β-arrestins, which are cytosolic proteins, interact with GPCRs to terminate signaling unless they were translocated to the cell surface upon agonist stimulation? Clearly the responsiveness of target cells is critically dependent on the subcellular distribution of receptors and associated regulatory proteins.

Agonists cause rapid endocytosis of many GPCRs, including those expressed by enteric neurons such as adrenergic, muscarinic, and peptidergic receptors (4). For example, substance P stimulates endocytosis of the neurokinin-1 receptor in neurons of the enteric and central nervous systems (10, 12, 13). Substance P and its receptor internalize into early endosomes by a clathrin-mediated mechanism, and subsequent endo-
somal acidification permits dissociation of the ligand and its receptor and sorting into different pathways: substance P is degraded in lysosomes, and the neurokinin-1 receptor recycles (Figs. 1 and 2). Certain agonists of the μ-opioid receptor also induce endocytosis in enteric neurons (22). Peptide agonists and the alkaloid agonist etorphine induce rapid internalization of the receptor into endosomes in neurons. Remarkably, morphine, a high-affinity alkaloid agonist, does not cause internalization but partly blocks etorphine-induced endocytosis. These observations, which indicate differential receptor regulation by different agonists, may be of considerable importance for understanding the pharmacological and therapeutic properties of opioid analogs.

The mechanism of agonist-induced endocytosis of GPCRs has been a topic of intense research, but unfortunately there are few underlying themes that are common for most receptors. The predominant route of endocytosis is via clathrin, but some receptors internalize through caveolin (19). Clathrin-mediated endocytosis requires interaction of specific receptor domains with components of the endocytic machinery (23). Endocytic domains have also been identified for many GPCRs by mutational analysis, but a common endocytic motif has not been discovered.

The trigger for endocytosis is yet to be determined, but agonist-induced phosphorylation of receptors may induce endocytosis of certain receptors. For example, GRK-2-induced phosphorylation of the β2-adrenergic and muscarinic m2 receptors is important for agonist-induced endocytosis (14, 20, 24). β-Arrestins also contribute to endocytosis of the β2-adrenergic receptor (7–9). β-Arrestin binds to clathrin with high affinity, and β2-adrenergic receptor colocalizes with β-arrestin and clathrin in the first-formed endosomes. Thus β-arrestins may serve as adaptor molecules that recruit cellular proteins, which facilitate endocytosis of several GPCRs or directly mediate endocytosis themselves. Dynamin may interact with arrestins to enhance the progression of the receptor to endosomes. Dynamin is important for internalization of the β2-adrenergic receptor, since overexpression of a dynamin dominant negative mutant inhibits endocytosis.

β-Arrestins and β-arrestins are cytosolic or vesicular proteins that must be targeted to the plasma membrane to mediate desensitization of cell surface receptors. GRK-2 and GRK-3 rapidly translocate from the cytosol to the plasma membrane after agonist stimulation (4). They contain a 125-residue extension at the COOH terminus that interacts with prenylated βγ-subunits of heterotrimeric G proteins. Free βγ-subunits are only found in the plasma membrane at sites of receptor activation, so this is an extremely precise mechanism for targeting these kinases to activated receptors. β-Arrestins undergo a similar alteration in subcellular distribution after receptor activation. This has recently been documented using β-arrestin tagged with the green fluorescent protein derived from jellyfish (3). Agonists of many GPCRs induce redistribution of fluorescent β-arrestin from the cytosol to the plasma membrane and then into endosomes containing the receptors.

Agonist-induced trafficking of some of these proteins has also been examined in myenteric neurons express-
tor is blocked by inhibition of endocytosis. These findings imply that processing of the internalized receptor is necessary for resensitization. Processing may involve dissociation of the ligand in acidified endosomes, dephosphorylation of the receptor, dissociation of arrestins, and recycling of the receptor to the plasma membrane.

Detailed studies of the phosphorylation state of receptors in endosomes and their association with agonists and arrestins are hampered by difficulties in endosomal purification.

Does endocytosis have other functions besides receptor regulation? Could endosomes containing ligand-bound, activated receptors serve as signaling platforms that convey signals deep within the cell? This may be the case with the nerve growth factor receptor TrkA, since endosomes containing TrkA and nerve growth factor receptor continue to signal and may carry information from nerve terminals to the distant cell body by axoplasmic transport (11). Recent evidence suggests that certain aspects of GPCR signaling may also require receptor endocytosis. Thus stimulation of the mitogen-activated protein kinases extracellular signal-related kinase (Erk-1) and Erk-2 by agonists of the β2-adrenergic receptor is suppressed by expression of dominant negative mutants of β-arrestin and dynamin, which block internalization of this receptor (6). It is tempting to speculate that axonal transport of endosomes containing activated receptors may convey long-term signals to the soma of neurons. However, it is not known whether the receptors are appropriately coupled to heterotrimeric G proteins in endosomes, and the possibility that receptors in endosomes still signal requires further study.

**RECEPTORS MAY BE DIFFERENTIALLY REGULATED IN THE SOMA AND PROCESSES**

Neurons are highly polarized cells with distinct functional domains. Is it possible that receptors are differentially localized and regulated in neurons and, if so, what would be the consequences of such regulation to neuronal function?

GPCRs are not uniformly distributed on neurons. For example, in guinea pig myenteric neurons the neurokinin-1 receptor is selectively localized to the plasma membrane of the soma and dendrites but not axon terminals (18), whereas neurokinin-2 receptors are preferentially located at varicosities (17). It is not known how this selective localization is achieved, and the functional implications remain to be defined. There is also evidence for differential trafficking of receptors in different regions of neurons. Whereas the neurokinin-1 receptor recycles in the soma of myenteric neurons, there is no detectable recycling in neurites in the same time period (10). One possible explanation for this difference is that endosomes in the neurites are not sufficiently acidic to cause dissociation of the neurokinin-1 receptor and substance P, which is required for receptor recycling and ligand degradation. Another possibility is that retrograde transport to the soma is required for dissociation of the receptor-ligand complex. Finally, retrograde transport of the neurokinin-1

![Fig. 2. Confocal images showing colocalization of fluorescent substance P (red, B and E), the neurokinin-1 receptor (green, A), and β-arrestins (green, D) in myenteric neurons. Neurons were incubated with cyanine-3-labeled substance P for 2 h at 4°C, washed, and warmed to 37°C for 5 min. Proteins were localized by immunofluorescence using secondary antibodies conjugated to fluorescein. C and F: superimpositions of images in A and B, or D and E, respectively, where yellow denotes colocalization.](http://www.physiology.org/ajpgi.org)
receptor and substance P may convey a signal to the cell body, as appears to be the case with neurotensin in the central nervous system (5).

CONCLUSIONS

The mechanisms that regulate the capacity of neurons to respond to neurotransmitters such as neuropeptides determine whether neurons can participate in functionally important reflexes, such as peristalsis and nociception. Receptor desensitization prevents the uncontrolled stimulation of neurons, and resensitization allows neurons to maintain their ability to respond to agonists over time. These regulatory mechanisms are also important from a disease and therapeutic considerations. Defects in the attenuation mechanisms result in the uncontrolled stimulation of neurons, which may lead to disease. In addition, many effective drugs exert their effects through signaling pathways that involve G proteins. Discovery of new ways in which these signaling pathways are regulated may lead to new therapies. Many of the experiments performed thus far have used supraphysiological concentrations of agonists, and much of the information available derives from studies of reconstituted systems and transfected cell lines expressing abnormally large numbers of receptors. Far less is known of receptor regulation by physiological concentrations of agonists in highly differentiated cells, such as neurons, that naturally express GPCRs. Although some of the knowledge gained from studies of reconstituted systems and transfected cell lines is applicable to regulation of neuropeptide receptors in neurons, at present there are many unanswered questions.

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