Exploring Hormonal Responses in Vivo with Engineered Receptors, Gene Expression, and GenMAPP

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G protein–coupled receptors (GPCRs) are the largest family of cell-surface receptors (~750 human genes) and play a central role in the control of a variety of physiological responses. Because they orchestrate the intracellular biochemical signals with the ion channels that create electrical signals, GPCRs are particularly important in electrically active tissues, such as the brain and the heart (Figure 1). Prolonged signaling results in changes in gene expression, new levels of responsiveness, and occasionally, pathological states.

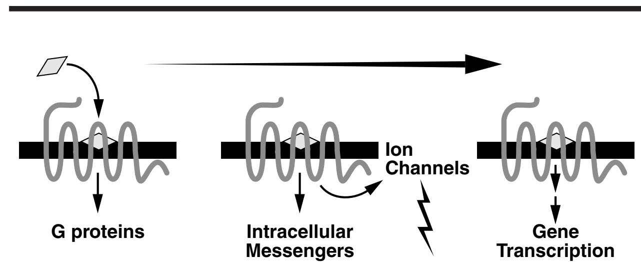

Figure 1. *G proteins control electrical tissues by activating intracellular second messengers and cell-surface ion channels.*

We have devised several new methods to control G protein signals in vivo and have used new techniques to monitor signaling events on a genomic level. We have three primary avenues of research. To take control of signaling at the receptor level, we are engineering new receptors. To take control of signaling at the G protein level, we use the tetracycline transactivator (tet) system to express constitutively active G proteins. To analyze changes in gene expression profiles that result from the major G protein signaling pathways, we developed a new bioinformatics program, GenMAPP (Gene Microarray Pathway Profiler).

New Receptors

To control G protein signaling in vivo, we modified GPCRs to respond exclusively to synthetic small-molecule agonists and not to their natural agonists. These engineered receptors are designated RASSLs (receptors activated solely by a synthetic ligand). By changing specific amino acids of the κ opioid receptor, we greatly decreased receptor activation by endogenous ligands, while maintaining activation by small-molecule drugs. Our modified versions of the human κ opioid receptor, Ro1 and Ro2 (RASSL, opioid, 1 and 2), contain δ opioid receptor sequences in the second extracellular loop and signal through a G_i pathway. These modifications reduce binding of the endogenous agonist (dynorphin) to the native receptor by >99.5%, while maintaining normal binding and activation by the small-molecule drug spiradoline. Ro1 activation increases G_i signaling and subsequent proliferation of Rat-1a cells.

Recently, we used the heart as a model in vivo system for our RASSLs because of the heart's known sensitivity to G_i signaling. Acetylcholine, which signals through the G_i-coupled M2-muscarinic receptor in the heart, was identified by Otto Loewi in 1920 as the first neurotransmitter ("vagusstoff"). Like Loewi, we use the heart as a model system to study the physiological effects of neurotransmitters. Short-term G_i-signaling events in the heart include inhibition of adenylyl cyclase and activation of a membrane potassium channel, resulting in a decreased heart rate (bradycardia). In addition, aberrant long-term G_i signaling has been implicated in human heart failure. In a recent study, we used the tet system to inducibly express Ro1 in the mouse heart. In less than 1 minute, activation of the receptor by spiradoline caused a

bradycardia that lasted for several hours and was subsequently desensitized for greater than 24 hours (Figure 2). These studies demonstrated that a RASSL can control G protein signaling in vivo and that the speed of that signaling event is unique among conditional signaling systems.

When these RASSLs are vastly overexpressed in the heart, the mice develop a lethal cardiomyopathy. Although the G_i signaling pathway is upregulated in human cardiomyopathy, previous studies suggested this upregulation was compensatory rather than causal. We have shown that induced expression of Ro1 for 8 weeks in adult mice causes pathologic, physiologic, and gene-expression changes similar to those in human cardiomyopathy and an eventual mortality rate greater than 90%. Ro1 also causes ventricular conduction delay, which is associated with a poor prognosis in humans. Suppression of Ro1 expression after 8 weeks protected mice from further mortality and allowed partial improvement in cardiac function. DNA-microarray analysis of over 6000 genes from hearts expressing Ro1 identified a pattern of gene expression that indicated hyperactive G_i signaling. DNA-microarray analysis also identified known markers of cardiomyopathy, as well as hundreds of novel potential diagnostic markers and therapeutic targets for this syndrome. With our system, ventricular conduction delay and cardiomyopathy can be induced and reversed in adult mice, providing an unprecedented opportunity to dissect the role of G_i signaling in pathogenesis.

In addition to controlling Ro1 expression in the heart, we have induced Ro1 expression in mouse liver, salivary gland, and brain. These results demonstrate that a conditional signaling system based on a human opioid receptor can be expressed in multiple mouse tissues. Activation of Ro1 in multiple tissues has both experimental and therapeutic implications. For example, in cells that proliferate in response to a G_i signal, Ro1 activation could cause targeted proliferation of that population. Thus, Ro1 might be used to amplify a population of transfected neural stem cells for therapeutic purposes. Another possible use of Ro1 is to activate G_i signaling directly in the nervous system, where G protein signaling is critical for olfaction, taste transduction, addiction, weight control, memory, and locomotion. Although naturally occurring κ opioid receptors are expressed at high levels in the brain, knockout mice lacking the κ opioid receptor are essentially normal and provide an ideal background for using κ opioid–based RASSLs in the brain. Thus, Ro1 could be used to dissect the neural circuitry involved in complex physiologic processes. A list of studies currently under way with RASSLs is summarized in a recent review (Scearce-Levie et al., 2001).

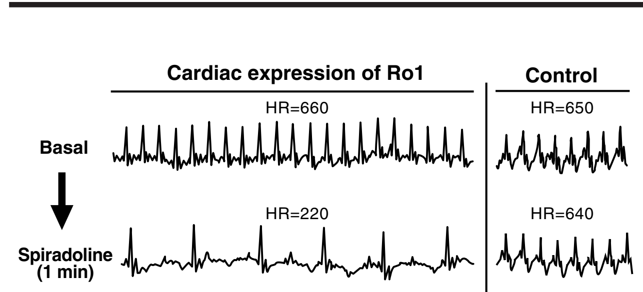

Figure 2. *Spiradoline-mediated bradycardia occurs immediately. Less than 1 minute after injection of spiradoline (1 × 10^{-5} mol/kg), the mouse expressing Ro1 in the heart has a heart rate (HR) one-third of baseline. Electrocardiographic tracings during the bradycardia reveal a relative sinus bradycardia and atrioventricular block. Spiradoline (1 × 10^{-5} mol/kg) had no effect on the control mouse.*

Constitutively Active G Proteins Under Control of the Tet System

We have expressed constitutively active versions of G proteins under the temporal-spatial control of the tet system in mice. Constitutively "active" mutants of each Gα subunit are locked in the GTP-bound state and do not bind or "soak up" Gβγ. This allows the investigation of different physiological functions of each Gα subunit in whole cells or whole tissues of transgenic mice. The apparent identification of all the Gα subunit genes raises the possibility of a comprehensive functional comparison of the signaling pathways activated by each major G protein. The classic biochemical effects of G_i, G_s, and G_q are to inhibit adenylyl cyclase, stimulate adenylyl cyclase, and stimulate phospholipase C, respectively. The cellular effects of G_{12} and G_{13} result from the modulation of the low-molecular-weight G proteins, such as Ras, Rho, and Rac. We have focused our initial studies on Gα subunits of G_i, G_q, G_{12}, and G_{13}. Several different

G protein–specific phenotypes have resulted, including a distinct hypertrophic cardiomyopathy model, which provide an alternative strategy for identifying the principal responses to G protein signals in vivo.

Expression Profiles of GPCR Signals in Vivo

A key advantage of DNA microarrays for signaling studies is the ability to monitor many biochemical responses, known and unknown, with a single assay. Many current biochemical assays require highly specialized technical skills and expensive radioactive reagents. Many of these assays also reflect our historical knowledge (such as for cAMP) rather than the relative biological importance of the pathway. DNA microarrays allow us to study biologically important signals in vivo, even before a biochemical assay is established. We anticipate that DNA microarrays will provide valuable new insights into a wide variety of signaling events.

We use DNA microarrays developed by Affymetrix (Santa Clara, CA) to simultaneously compare the relative expression levels of thousands of genes in a given tissue sample. Oligonucleotides corresponding to the sequences of known genes are synthesized directly onto 260,000 distinct square regions of a 1.2-cm^2 piece of glass. Total mRNA is isolated from the sample of interest, amplified, and labeled with fluorescent nucleotides. After the labeled RNA (now called cRNA) is hybridized to the oligonucleotides on the DNA microarray, a laser scans for the regions of fluorescence. Since each region contains oligonucleotides representing a defined gene, the pattern of fluorescence can be used to quantitate the relative levels of specific cellular mRNAs. The basic method for using DNA microarrays to monitor gene expression is illustrated in Figure 3.

The first DNA microarrays for mouse genes (murine 6K array) consisted of a set of four chips, each containing 65,000 different oligonucleotides, which could assess the expression levels of over 6000 genes. We have recently begun using the murine 12K array, which contains 260,000 oligonucleotides and can assess the expression levels of 12,000 genes. Our data

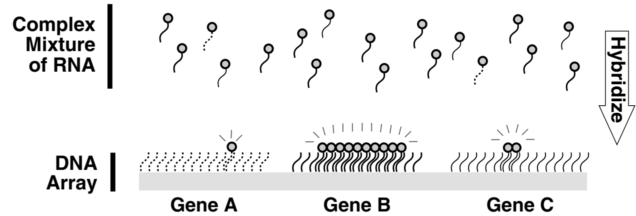

Figure 3. *Monitoring gene expression with a DNA microarray. A single murine 12K DNA microarray can assess the expression level of 12,000 genes. These arrays have up to 260,000 oligonucleotides synthesized on a 1.2-cm^2 chip. Each gene has 20 perfect-match and 20 mismatch control oligonucleotides.*

confirm previous reports that this method has excellent sensitivity and is quantitative over more than three orders of magnitude. The required 10–40 mg of tissue can easily be harvested from a mouse organ while allowing for concurrent histopathological analysis.

During the past 2 years, we have processed approximately 200 DNA microarrays, each assessing the levels of 6000–12,000 genes. In these experiments, the initial focus was to examine the genomic response to a cardiomyopathy induced by hyperactive G_i signaling. The gene-expression "fingerprint" of each G protein signaling pathway will aid in identifying the downstream effects of that pathway. Furthermore, the gene expression patterns should help to link our mouse models with the equivalent human diseases.

The greatest challenge in these experiments is to interpret the massive amounts of data that result from monitoring thousands of genes. A single experiment may yield 700 pages of data. To help meet this challenge, we have developed a software program called GenMAPP. GenMAPP is a stand-alone, public domain computer program designed for viewing and analyzing gene expression data on MAPPs representing biological pathways or any other functional grouping of genes. A MAPP is a special file format produced with the graphics tools in GenMAPP that depicts the biological relationship between genes or gene products. When a MAPP is linked to an expression data set, GenMAPP automatically and dynamically color-codes the genes on the MAPP according to criteria supplied by the user (Figure 4). For example, the user can tell GenMAPP to color-code all

genes upregulated in an experiment red and color-code all genes downregulated in an experiment blue. MAPPs are independent of the data; MAPPs from any source can be used to view any expression data set, promoting the exchange of information. Also, clicking on a gene on a MAPP reveals annotations for that gene from the GenBank and SWISS-PROT public databases, including hyperlinks. The GenMAPP program and accessory files can be downloaded free of charge from the GenMAPP web site (www.GenMAPP.org). Version 1.0 beta of GenMAPP was released in April 2001 with 35 MAPPs containing over 1000 genes. As of October 2001, we had over 300 MAPPs with over 3000 genes. More than 20 laboratories have committed to contributing other pathways that cover areas of specific expertise. Since GenMAPP is publicly distributed, it is being used for presenting data for publication, for teaching bioinformatics classes, and by other public organizations involved in genomics. Furthermore, some groups are able to construct pathways that contain proprietary information for internal use until these data can be made public. We anticipate that GenMAPP will provide a valuable new means of analyzing and distributing genomic-scale gene profiling data.

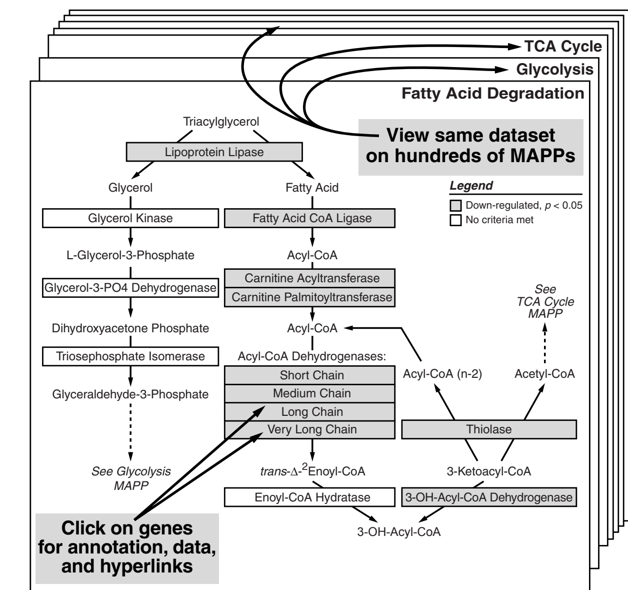

Figure 4. *Fatty acid degradation pathway adapted from a view in GenMAPP. Each box represents a gene. This MAPP is color-coded (black-and-white in this report) with gene expression data from a mouse model of dilated cardiomyopathy. Grey, fold change of less than –1.2 ($p < 0.05$) in the disease group (nine mice) versus a control group (eight mice). White, no criteria met or gene not on the array. TCA, tricarboxylic acid. For the interactive output of this MAPP and other MAPPs from GenMAPP, see www.GenMAPP.org.*

Overall, the year has provided advances in several areas: the use of specifically designed receptors (RASSLs) to control signaling in vivo, the establishment of a new model of dilated cardiomyopathy induced by the G_i signaling pathway, the use of DNA microarrays to obtain phenotypic data on mice, and the development of bioinformatics tools to analyze these data. Further information is available on our web site (www.ConklinLab.org).

Selected References

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