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Barak et al.(10) **Patent No.:** **US 7,541,151 B2**
(45) **Date of Patent:** ***Jun. 2, 2009**(54) **SINGLE-CELL BIOSENSOR FOR THE MEASUREMENT OF GPCR LIGANDS IN A TEST SAMPLE**(75) Inventors: **Lawrence S. Barak**, Durham, NC (US); **Michael A. Shetzline**, Chapel Hill, NC (US); **Robert H. Oakley**, Durham, NC (US); **Marc G. Caron**, Hillsborough, NC (US)(73) Assignee: **Duke University**, Durham, NC (US)

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(51) **Int. Cl.****G01N 33/566** (2006.01)**G01N 21/76** (2006.01)**G01N 33/53** (2006.01)(52) **U.S. Cl.** **435/7.1; 435/7.2; 435/7.21; 436/172; 436/501; 530/350; 536/23.4**(58) **Field of Classification Search** None
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**3,654,090 A 4/1972 Schuurs et al.
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The present invention is related to the detection of GPCR ligands in a test sample by using a single cell biosensor expressing a GPCR. Preferably, the test sample is derived from a biological or environmental sample. This invention may be used to detect the presence of a disease or to detect the presence of a harmful agent in the environment. Included in the present invention is an array of biosensors that detect ligands of various GPCRs.

35 Claims, 19 Drawing Sheets

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FIG. 1A

Human G Protein Coupled Receptor Family
(Receptors known as of January, 1999)

CLASS	LIGAND	NUMBER	TISSUE	PHYSIOLOGY	THERAPEUTICS
•Class I					
Rhodopsin like					
•Amine					
•Acetylcholine (muscarinic & nicotinic)		5	Brain, Nerves, Heart	Neurotransmitter	Acuity, Alzheimer's
•Adrenoceptors					
•Alpha Adrenoceptors		6	Brain, Kidney, Lung	Gluconeogenesis	Diabetes, Cardiovascular
•Beta Adrenoceptors		3	Kidney, Heart	Muscle Contraction	Cardiovascular, Respiratory
•Dopamine		5	Brain, Kidney, GI	Neurotransmitter	Cardiovascular, Parkinson's
•Histamine		2	Vascular, Heart, Brain	Vascular Permeability	Anti-inflammatory, Ulcers
•Serotonin (5-HT)		16	Most Tissues	Neurotransmitter	Depression, Insomnia, Analgesic
•Peptide					
•Angiotensin		2	Vascular, Liver, Kidney	Vasoconstriction	Cardiovascular, Endocrine
•Bradykinin		1	Liver, Blood	Vasodilation,	Anti-inflammatory, Asthma
•C5a anaphylatoxin		1	Blood	Immune System	Anti-inflammatory
•Fmet-leu-phe		3	Blood	Chemoattractant	Anti-inflammatory
•Interleukin-8		1	Blood	Chemoattractant	Anti-inflammatory
•Chemokine		6	Blood	Chemoattractant	Anti-inflammatory
•Orexin		2	Brain	Fat Metabolism	Obesity
•Noiceptin		1	Brain	Bronchodilator, Pain	Airway Diseases, Anesthetic
•CCK (Gastrin)		2	Gastrointestinal	Motility, Fat Absorption	Gastrointestinal, Obesity, Parkinson's
•Endothelin		2	Heart, Bronchus, Brain	Muscle Contraction	Cardiovascular, Respiratory
•Melanocortin		5	Kidney, Brain	Metabolic Regulation	Anti-inflammatory, Analgesics
•Neuropeptide Y		5	Nerves, Intestine, Blood	Neurotransmitter	Behavior, Memory, Cardiovascular
•Neurotensin		1	Brain,	CNS	Cardiovascular, Analgesic
•Opioid		3	Brain,	CNS	Depression, Analgesic
•Somatostatin		5	Brain, Gastrointestinal	Neurotransmitter	Oncology, Alzheimer's

FIG. 1B

•Tachykinin (Substance P, NKA ₁)	3	Brain Nerves	Neurohormone	Depression, Analgesic
•Thrombin	3	Platelets, Blood Vessels	Coagulation	Anti-coagulant, Anti-inflammatory
•Vasopressin-like	4	Arteries, Heart, Bladder	Water Balance	Anti-diuretic, Diabetic Complications
•Galanin	1	Brain, Pancreas	Neurotransmitter	Analgesics, Alzheimer's
•Hormone protein				
•Follicle stimulating hormone	1	Ovary, Testis	Endocrine	Infertility
•Lutropin-choriogonadotropic	1	Ovary, Testis	Endocrine	Infertility
•Thyrotropin	1	Thyroid	Endocrine	Thyroidism, Metabolism
•(Rhodopsin				
•Opsin	5	Eye	Photoreception	Ophthalmic Diseases
•Olfactory	4(~1000)	Nose	Smell	Olfactory Diseases
•Prostanoid				
•Prostaglandin	5	Arterial, Gastrointestinal	Vasodilation, Pain	Cardiovascular, Analgesic
•Lysophosphatidic Acid	2	Vessels, Heart, Lung	Inflammation	Cancer, Anti-inflammatory
•Sphingosine-1-phosphate	2	Most Cells	Cell proliferation	Cancer
•Leukotriene	1	White Blood Cells, Bronchus	Inflammation	Asthma, Rheumatoid Arthritis
•Prostacyclin	1	Arterial, Gastrointestinal	Platelet Regulation	Cardiovascular
•Thromboxane	1	Arterial, Bronchus	Vasoconstriction	Cardiovascular, Respiratory
•Nucleotide-like				
•Adenosine	4	Vascular, Bronchus	Multiple Effects	Cardiovascular, Respiratory
•Purinoceptors	4	Vascular, Platelets	Relaxes Muscle	Cardiovascular, Respiratory
•Cannabis	2	Brain	Sensory Perception	Analgesics, Memory
•Platelet activating factor	1	Most Peripheral Tissues	Inflammation	Anti-inflammatory, Anti-asthmatic
•Gonadotropin-releasing hormone like				
•Gonadotropin-releasing hormone	1	Reproductive Organs, Pituitary	Reproduction	Prostate Cancer, Endometriosis
•Thyrotropin-releasing hormone	1	Pituitary, Brain	Thyroid Regulation	Metabolic Regulation
•Growth hormone-inhibiting factor	1	Gastrointestinal	Neuroendocrine	Oncology, Alzheimer's
•Melatonin	1	Brain, Eye, Pituitary	Neuroendocrine	Regulation of Circadian Cycle

FIG. 1C

<p>● Class II Secretin like</p>	<p>• Secretin</p>	1	Gastrointestinal, Heart	Digestion	Obesity, Gastrointestinal
	• Calcitonin	1	Bone, Brain	Calcium Resorption	Osteoporosis
	• Corticotropin releasing factor/urocortin	1	Adrenal, Vascular, Brain	Neuroendocrine	Stress, Mood, Obesity
	• Gastric inhibitory peptide (GIP)	1	Adrenals, Fat Cells	Sugar/Fat Metabolism	Diabetes, Obesity
	• Glucagon	1	Liver, Fat Cells, Heart	Gluconeogenesis	Cardiovascular
	• Glucagon-like Peptide 1 (GLP-1)	1	Pancreas, Stomach, Lung	Gluconeogenesis	Cardiovascular, Diabetes, Obesity
	• Growth hormone-releasing hormone	1	Brain	Neuroendocrine	Growth Regulation
	• Parathyroid hormone	1	Bone, Kidney	Calcium Regulation	Osteoporosis
	• PACAP	1	Brain, Pancreas, Adrenals	Metabolism	Metabolic Regulation
	• Vasoactive intestinal polypeptide (VIP)	1	Gastrointestinal	Motility	Gastrointestinal
<p>● Class III</p>	• Metabotropic Glutamate	7	Brain	Sensory Perception	Hearing, Vision
	• GABA _B	1	Brain	Neurotransmitter	Mood Disorders
	• Extracellular Calcium Sensing	1	Parathyroid, Kidney, GI Tract	Calcium Regulation	Cataracts, GI Tumors

FIG. 2**G Protein-Coupled Receptors that Translocate Arrestins**

Adrenergic Receptors	Serotonergic Receptors	Others
alpha 1B	5HT1A	FMLP f-Met-Leu-Phe Receptor
alpha 2A	5HT2A	AT1AR Angiotensin Receptor
alpha 2B	Chemokine Receptors	CRFR Corticotropin Releasing Factor
alpha 2C	CXCR2	ETAR Endothelin A Receptor
beta1	CXCR4	NK-1 Substance P Receptor
beta2	Edg Receptors	NTR Neurotensin Receptor
Dopaminergic Receptors	Edg1	OAMB Octopamine Receptor (Drosophila)
D1A	Edg2	OXY Oxytocin Receptor
D1B	Edg3	TRHR Thyroid Releasing Hormone Receptor
D2	Edg5	V2 Vasopressin Receptor
D3	GI Receptors	
D4	CCK- A and B	
Muscarinic Receptors	glucagon	
m1	secretin	
m2	VIP	
m3	Prostaglandin Receptors	
m4	EP3	
Opioid Receptors		
Mor1	EP4	
Delta		

FIG. 3A

homo sapiens muscarinic receptor 1

Amino acid sequence

Accession NM_000738

```
MNTSAPPAVSPNITVLAPGKGPWQVAFIGITTGLLSLATVTGNL
LVLISFKVNTLKTVMNYFLLSLACADLIIGTFSMNLYTTYLLMGHWALGTLACD
ALDYVASNASVMNLLLSIFDRYFSVTRPLSYRAKRTPRRAALMIGLAWLVSFVLWAPA
ILFWQYLVGERTMLAGQCYIQFLSQPIITFGTAMAAFYLPVTVMCTLYWRIYRETENR
ARELAALQGSSETPGKGGGSSSSSERSQPGAEGSPETPPGRCCRCRAPRLQAYSWKE
EEEEDEGSMESLTSSEGEFPGSEVVIKMPMVDPEAQAPTKQPPRSSPNTVKRPTKKGR
DRAGKQKPRGKEQLAKRKTFSLVKEKKAARTLSAILLAFILTWTPYNIMVLVSTFCK
DCVPETLWELGYWLCYVNSTINPMCYALCNKAFRDTFRLLLLCRWDKRRWRKIPKRPG
SVHRTPSRQC
SEQ ID NO: 1
```

homo sapiens muscarinic receptor 1 (gene CHRM1, CDS 1..1383)

Nucleic acid sequence

```
1 atgaacactt cagccccacc tgctgtcagc cccaacatca ccgtcctggc accaggaaag
61 ggtccctggc aagtggcctt cattgggatc accacgggcc tctgtcgtc agccacagtg
121 acaggcaacc tgctggtact catctcttcc aaggtcaaca cggagctcaa gacagtcaat
181 aactacttcc tgctgagcct ggcctgtgct gacctcatca tccgtacctt ctccatgaac
241 ctctatacca cgtacctgct catgggccac tgggctctgg gcacgctggc ttgtgacctc
301 tggctggccc tggactatgt ggccagcaat gcctccgtca tgaatctgct gctcatcagc
361 tttgaccgct acttctccgt gactcggccc ctgagctacc gtgccaagcg cacaccccgc
421 cgggcagctc tgatgatcgg cctggcctgg ctggtttcc tttgtgctctg ggccccagcc
481 atcctcttct ggcagtacct ggtagggggag cggacgatgc tagctgggca gtgctacatc
541 cagttcctct cccagcccat catcacctt ggacagcca tggctgcctt ctacctcctt
601 gtcacagtca tgtgcacgct ctactggcgc atctaccggg agacagagaa cggagcacgg
661 gagctggcag cccttcaggg ctccgagacg ccaggcaaag ggggtggcag cagcagcagc
721 tcagagaggt ctcagccagg ggctgagggc tcaccagaga ctctccagg ccgctgctgt
781 cgctgctgcc gggccccag gctgctgcag gcctacagct ggaaggaaga agaggaagag
841 gacgaaggct ccatggagtc cctcacatcc tcagagggag aggagcctgg ctccgaagtg
901 gtgatcaaga tgccaatggt ggaccccgag gcacaggccc ccaccaagca gccccacgg
961 agtccccaa atacagtcaa gaggccgact aagaaagggc gtgatcgagc tggcaagggc
1021 cagaagcccc gtggaaagga gcagctggcc aagcgggaaga ccttctcgct ggtcaaggag
1081 aagaaggcgg ctcggacctt gactgcatc ctctggcct tcatcctcac ctggacaccg
1141 tacaacatca tgggtgctggt gtccaccttc tgcaaggact gtgttcccga gacctgtgg
1201 gagctgggct actggctgtg ctacgtcaac agcaccatca accccatgtg ctacgcactc
1261 tgcaacaaag ccttccggga caccttctgc ctgctgctgc tttgccgctg ggacaagaga
1321 cgctggcgca agatcccaaa gcgcctggc tccgtgcacc gcaactccctc ccgccaatgc
1381 tga
SEQ ID NO: 2
```

FIG. 3B

homo sapiens muscarinic receptor 2

Amino acid sequence

Accession NM_000739

MNNSTNSSNNSLALTSFYKTFEVVFIVLVAGSLSLVTIIGNILV

MVSIVKVRHLQTVNNYFLFSLACADLIIGVFSMNLTYTVIGYWPLGPVVCDLWLAL

DYVVSNASVMNLLIISFDRYFCVTKPLTYPVKRRTKMGMMIAAAWVLSFILWAPAIL

FWQFIVGVRTVEDGECYIQFFSNAAVTFGTAAIAFYLPVIIMTVLYWHISRASKSRIK

KDKKEPVANQDPVSPSLVQGRIVKPNNNMPSDDGLEHNKIQNGKAPRDPVTENCVQ

GEEKESSNDSTSVSAVASNMRRDEITQDENTVSTSLGHSKDENSQKTCIRIGTKTPKS

DSCTPTNTTVEVVGSSGQNGDEKQNI VARKIVKMTKQPAKKKPPPSREKKVTRTILAI

LLAFIITWAPYNVMVLINTFCAPCIPNTVWTIGYWLICYINSTINPACYALCNATFKKT

FKHLLMCHYKNIGATR

SEQ ID NO: 3

homo sapiens muscarinic receptor 2 (gene CHRM2, CDS 1..1401)

Nucleic acid sequence

```
1 atgaataact caacaactc ctctaacaat agcctggctc ttacaagtcc ttataagaca
61 tttgaagtgg tgtttattgt cctggtggct ggatccctca gtttggtgac cattatcggg
121 aacatcctag tcatggtttc cattaagtc aaccgccacc tccagaccgt caacaattac
181 tttttattca gcttggcctg tgctgacctt atcataggtg ttttctccat gaacttgtac
241 accctctaca ctgtgattgg ttactggcct ttgggacctg tgggtgtgta cctttggcta
301 gccctggact atgtggctag caatgcctca gttatgaatc tgctcatcat cagctttgac
361 aggtacttct gtgtcacaaa acctctgacc taccagtcag agcggaccac aaaaatggca
421 ggtatgatga ttgcagctgc ctgggtcctc tctttcatcc tctgggctcc agccattctc
481 ttctggcagt tcattgtagg ggtgagaact gtggaggatg gggagtgtca cattcagttt
541 ttttccaatg ctgctgtcac ctttggtaag gctattgcag ccttctatit gccagtgatc
601 atcatgactg tgctatattg gcacatatcc cgagccagca agagcaggat aaagaaggac
661 aagaaggagc ctggttgcaa ccaagacccc gtttctccaa gtctggtaca aggaaggata
721 gtgaagccaa acaataacaa catgcccagc agtgacgatg gcctggagca caacaaaatc
781 cagaatggca aagccccagc ggatcctgtg actgaaaact gtgttcaggg agaggagaag
841 gagagctcca atgactccac ctcagtcagt gctgttgccct ctaatatgag agatgatgaa
901 ataaccagg atgaaaacac agtttccact tccctgggcc attccaaaga tgagaactct
961 aagcaaacat gcatcagaat tggcaccaag accccaaaaa gtgactcatg taccccaact
1021 aataccaccg tggaggtagt ggggtcttca ggtcagaatg gagatgaaaa gcagaatatt
1081 gtagcccgca agattgtgaa gatgactaag cagcctgcaa aaaagaagcc tctccttcc
1141 cgggaaaaga aagtcaccag gacaatcttg gctattctgt tggctttcat catcacttgg
1201 gcccataca atgtcatggt gctcattaac accttttgtg caccttgcac ccccaacact
1261 gtgtggacaa ttggttactg gctttgttac atcaacagca ctatcaaccc tgctgtctat
1321 gcactttgca atgccacctt caagaagacc tttaaacacc ttctcatgtg tcattataag
1381 aacataggcg ctacaaggta a
```

SEQ ID NO: 4

FIG. 3C

homo sapiens muscarinic receptor 3

Amino acid sequence

Accession NM_000740

```
MTLHNNSTTSPLFPNIISSSWIHSPSDAGLPPGTVTHFGSYNVS  
AAGNFSSPDGTTDDPLGGHTVWQVVFIAFLTGILALVTIIGNILVIVSFKVNKQLKTV  
NNYFLLSLACADLIIGVISMNLFTTYIIMNRWALGNLACDLWLALDYVASNASVMNLL  
VISFDRYFSITRPLTYRAKRTTKRAGVMIGLAWVISFVLWAPALFWQYFVGKRTVPP  
GECFIQFLSEPTITFGTAIAAFYMPVTIMTILYWRIYKETEKRTELQASGTEAE  
TENFVHPTGSSRSCSSYELQQQSMKRSNRRKYGRCHFWFTTKSWKPSSEQMDQDHSS  
DSWNNNDAAASLENSASSDEEDIGSETRAIYSIVLKLPGHSTILNSTKLPSSDNLQVP  
EELGMVDLERKADKLQAQKSVDDGGSPKSFSLKLPQLESVDTAKTSDVNSSVGKS  
TATLPLSFKEATLAKRFALKTRSQITKRKRMSLVKEKAAQTLAAILLAFIITWTPYN  
IMVLVNTFCDSCIPKTFWNLGYWLCYINSTVNPVCYALCNKTFRTTFKMLLLCQDKK  
KRRKQQYQQRQSVIFHKRAPEQAL  
SEQ ID NO: 5
```

homo sapiens muscarinic receptor 3 (gene CHRM3, CDS 1..1773)

Nucleic acid sequence

```
1 atgaccttgc acaataacag tacaacctcg cctttgtttc caaacatcag ctccctcctgg  
61 atacacagcc cctccgatgc agggctgccc cggggaaccg tcactcattt cggcagctac  
121 aatgtttctc gagcagctgg caatttctcc tctccagacg gtaccaccga tgaccctctg  
181 ggaggtcata cgcctctggca agtggctctc atcgctttct taacgggcat cctggccttg  
241 gtgaccatca tcggcaacat cctggtaatt gtgtcattta aggtcaacaa gcagctgaag  
301 acgggtcaaca actacttctc ctttaagcctg gcctgtgccc atctgattat cggggtcatt  
361 tcaatgaatc tgtttacgac ctacatcatc atgaatcgat gggccttagg gaacttggcc  
421 tgtgacctct ggcttgccat tgactacgta gccagcaatg cctctgttat gaatcttctg  
481 gtcacagata ttgacagata cttttccatc acgaggccgc tcacgtaccg agccaaacga  
541 acaacaaaga gagccggtgt gatgatcggc ctggcttggg tcactctcctt tgccttttg  
601 gctcctgcca tcttgttctg gcaatacttt gttggaaaga gaactgtgcc tccgggagag  
661 tgcttcatc agttcctcag tgagcccacc attacttttg gcacagccat cgctgctttt  
721 tatatgcctg tcaccattat gactatttta tactggagga tctataagga aactgaaaag  
781 cgtaccaaag agcttgctgg cctgcaagcc tctgggacag aggcagagac agaaaacttt  
841 gtccacccca cgggcagttc tcgaagctgc agcagttacg aactcaaca gcaaagcatg  
901 aaacgctcca acaggaggaa gtatggccgc tgccacttct ggttcacaac caagagctgg  
961 aaaccagct cggagcagat ggaccaagac cacagcagca gtgacagtgt gaacaacaat  
1021 gatgctgctg cctccctgga gaactccgcc tcctccgacg aggaggacat tggctccgag  
1081 acgagagcca tctactccat cgtgctcaag ctccggggtc acagcaccat cctcaactcc  
1141 accaagttac cctcatcgga caacctgcag gtgcctgagg aggagctggg gatggtggac  
1201 ttggagagga aagccgacaa gctgcagccc cagaagagcg tggacgatgg aggcagtttt  
1261 caaaaaagct tctccaagct tcccattccag cttagagtcag ccgtggacac agctaagact  
1321 tctgacgtca actcctcagt gggtaagagc acggccactc tacctctgtc cttcaaggaa  
1381 gccactctgg ccaagagggt tgctctgaag accagaagtc agatcactaa gcggaaaagg  
1441 atgtccctgg tcaaggagaa gaaagcggcc cagaccctca gtgcgatott gcttgccctc  
1501 atcatcactt ggaccccata caacatcatg gttctggtga acaccttttg tgacagctgc  
1561 atacccaaaa ccttttgtaa tctgggtctc tggctgtgct acatcaacag caccgtgaac  
1621 cccgtgtgct atgctctgtg caacaaaaca ttcagaacca ctttcaagat gctgctgctg  
1681 tgccagtggt acaaaaaaaaa gagggcgaag cagcagttacc agcagagaca gtcggtcatt  
1741 tttcacaagc gcgcaccgga gcaggccttg tag  
SEQ ID NO: 6
```

FIG. 3D

homo sapiens muscarinic receptor 4

Amino acid sequence

Accession NM_000741

MANFTPVNGSSG^uQSVRLVTSSSHNRYETVEMVFIATVTGSLSL
VTVVGNILVMLS^uIKVNRQLQTVNNYFLFSLACADLIIGAFSMNLYTVYIIKGYWPLGA
VVCDLWLALDYVVSNASVMNLLIIISFD^uRYFCVTKPLTYPARRTTKMAGLMIAAAWVLS
FVLWAPAILFWQFVVGKRTVPDNHCFIQFLSNPAVTFGTAAIAAFYLPVVIMTVLYIHI
SLASRSRVHKHRPEGPKEKKAKTLAFLKSP^uLMKQSVKKPRPGGRPGGLRNGKLEEAPP
PALPPPPRPVADKDTSN^uESSSGSATQNTKERPATELSTTEATTPAMPAPPLQPRALNP
ASRWSKIQIVTKQTGN^uECVTAIEIVPATPAGMRPAANVARKFASIARNQVRKKRQMAA
RERKVRTIFAILLAFIL^uTWTPYNVMVLVNTFCQSCIPD^uTVWSIGYWL^uCYVNSTINPA
CYALCNATFKK^uTFRHLLLCQYRNIG^uTAR

SEQ ID NO: 7

homo sapiens muscarinic receptor 4 (gene CHRM4, CDS 1..1437)

Nucleic acid sequence

1 atggccaact tcacacctgt caatggcagc tggggcaatc agtccgtgcg cctgggtcacg
61 tcatcatccc acaatcgcta tgagacgggtg gaaatgggtct tcattgccac agtgacagggc
121 tccctgagcc tggtgactgt cgtgggcaac atcctgggtga tgctgtccat caagggtcaac
181 aggcagctgc agacagtcaa caactacttc ctcttcagcc tggcgtgtgc tgatctcatc
241 ataggcgctt tctccatgaa cctctacacc gtgtacatca tcaagggcta ctggcccctg
301 ggcgcccgtg tctgcgacct gtggctggcc ctggactacg tggtgagcaa cgcctccgtc
361 atgaaccttc tcatcatcag ctttgaccgc tacttctgcg tcaccaagcc tctcacctac
421 cctgcccggc gcaccaccaa gatggcagggc ctcatgattg ctgctgcctg ggtactgtoc
481 ttcgtgctct gggcgctgc catcttgctt tggcagtttg tggtgggtaa gcggacgggtg
541 cccgacaacc actgcttcat ccagtctctg tccaaccag cagtgcctt tggcacagcc
601 attgctgctt tctacctgcc tgtggtcate atgacgggtg tgtacatcca catctccctg
661 gccagtcgca gccgagtcca caagcaccgg cccgagggcc cgaaggagaa gaaagccaag
721 acgctggcct tcctcaagag cccactaatg aagcagagcg tcaagaagcc ccgcccggga
781 ggccgcccgg gaggactgcg caatggcaag ctggaggagg ccccccgcc agcgtgcca
841 ccgccaccgc gccccgtggc tgataaggac acttccaatg agtccagctc aggcagtgcc
901 acccagaaca ccaaggaacg cccagccaca gagctgtcca ccacagaggc caccactccc
961 gccatgcccg cccctcccct gcagccgagg gccctcaacc cagcctccag atgggtccaag
1021 atccagattg tgacgaagca gacaggcaat gagtgtgtga cagccattga gattgtgctt
1081 gccacgcccg ctggcatgcg ccctgcccgc aacgtggccc gcaagtctgc cagcatcgct
1141 cgcaaccagg tgcgcaagaa gcggcagatg gcggcccggg agcgcaaagt gacacgaacg
1201 atctttgcca ttctgctagc cttcatcctc acctggacgc cctacaacgt catgggtcctg
1261 gtgaacacct tctgccagag ctgcatccct gacacgggtg ggtccattgg ctactggctc
1321 tgctacgtca acagcaccat caaccctgcc tgctatgctc tgtgcaacgc cacctttaa
1381 aagaccttcc ggcacctgct gctgtgccag tatcggaaca tcggcactgc caggtag

SEQ ID NO: 8

FIG. 3E

homo sapiens acetylcholinesterase (YT blood group)

Amino acid sequence

Accession XM_036148

MRPPQCLLHTPSLASPLLLLLLLWLLGGGVGAEGREDAELLVTVR
 GGRLRGIRLKTGGPVSFAFLGIPFAEPPMGPRRFLPPEPKQPWSGVVDATTFQSVCYQ
 YVDTLYPGFEGTEMWNPNRELSDECLYLNWVTPYPRPTSPTPVLVWIYGGGFYSGASS
 LDVYDGRFLVQAERTVLSVMNYRVGAFGLALPGSREAPGNVGLLDQRLALQWVQENV
 AAFGGDPTSVTLFGESAGAASVGMHLLSPPSRGLFHRAVLQSGAPNGPWATVGMGEAR
 RRATQLAHLVGCPPGGTGGNDTELVACLRTRPAQVLVNHEWHVLPQESVFRFSFVFPV
 DGDFLSDTPEALINAGDFHGLQVLVGVVKEGSYFLVYGAPGFSKDNESLISRAEFLA
 GVRVGVQVSDLAEEAVVLHYTDWLHPEDPARLREALSDVVDHNVVCPVAQLAGRLA
 AQGARVYAYVFEHRASLWPLWGMVPHGYEIEFIFGIPLDPSRNYTAEKIFAQRLM
 RYWANFARTGDPNEPRDPKAPQWPPYTAGAQQYVSLDLRPLEVRRGLRAQACAFWNR
 LPKLLSATDLDLDEAERQWKAEFHRWSSYVHVKNFQDHYSKQDRCSDL
 SEQ ID NO: 9

homo sapiens acetylcholinesterase (YT blood group)

(gene ACHE, CDS 95..1939)

Nucleic acid sequence

1	cagcctgcgc	cggggaacat	cggccgcctc	cagctcccgg	cgcgggcccgg	cccggcccgg
61	ctcggccgcc	tcagacgccg	cctgcctcgc	agccatgagg	ccccgcagc	gtctgctgca
121	cacgccttcc	ctggcttccc	cactccttct	cctcctctc	tggctcctgg	gtggaggagc
181	gggggctgag	ggccgggagg	atgcagagct	gctgggtgacg	gtgctgctggg	gccggcttgc
241	gggcattcgc	ctgaagacc	cggggggccc	tgtctctgct	ttcctgggca	tcccctttgc
301	ggagccaccc	atgggacccc	gtcgttttct	gccaccggag	cccaagcagc	cttggctcagg
361	ggtggttagac	gctacaacct	tccagagtgt	ctgctaccaa	tatgtggaca	ccctataccc
421	aggttttgag	ggcaccgaga	tgtggaacc	caaccgtgag	ctgagcgagg	actgcctgta
481	cctcaacgtg	tggacaccc	accccggcc	tacatcccc	accctgtcc	tctgtctggat
541	ctatgggggt	ggcttctaca	gtggggcctc	ctccttggac	gtgtacgatg	gccgcttctt
601	ggtacaggcc	gagaggactg	tgctggtgct	catgaactac	cgggtgggag	cttctggctt
661	cctggccctg	ccggggagcc	gagaggcccc	gggcaatgtg	ggtctcctgg	atcagaggct
721	ggccctgcag	tgggtgcagg	agaacgtggc	agccttcggg	ggtgaccgca	catcagtgac
781	gctggttggg	gagagcgagg	gagccgcctc	ggtgggcatg	cacctgctgt	ccccgcccag
841	ccggggcctg	ttccacaggg	ccgtgctgca	gagcggtgcc	cccaatggac	cctggggccac
901	ggtgggcatg	ggagaggccc	gtcgcagggc	cacgcagctg	gcccaccttg	tgggctgtcc
961	tccaggcggc	actggtggga	atgacacaga	gctggtagcc	tgcttcgga	cacgaccagg
1021	gcaggctcctg	gtgaaccacg	aatggcacgt	gctgcctcaa	gaaagcgtct	tccggttctc
1081	cttctgtgct	gtggttagatg	gagacttctc	cagtgaacc	ccagaggccc	tcatcaaccg
1141	gggagacttc	cacggcctgc	aggtgctggt	gggtgtggtg	aaggatgagg	gctcgtatct
1201	tctgggtttac	ggggccccag	gcttcagcaa	agacaacgag	tctctcatca	gccgggcccga
1261	gttcctggcc	gggggtgctg	tccgggttcc	ccaggtaagt	gacctggcag	ccgaggctgt
1321	ggtcctgcat	tacacagact	ggctgcatcc	cgaggaccgc	gcacgcctga	gggaggccct
1381	gagcgatgtg	gtgggcgacc	acaatgtcgt	gtgcccctg	gcccagctgg	ctggggcact
1441	ggctgcccag	ggtgcccggg	tctacgccta	cgtctttgaa	caccgtgctt	ccacgctctc
1501	ctggcccctg	tggatggggg	tgccccacgg	ctacgagatc	gagttcatct	ttgggatccc
1561	cctggacccc	tctcgaaact	acacggcaga	ggagaaaatc	ttcgcccagc	gactgatgcg
1621	atactgggcc	aactttgccc	gcacagggga	tcccattgag	ccccgagacc	ccaaggcccc
1681	acaatggccc	ccgtacacgg	cgggggctca	gcagtagctt	agtctggacc	tggggcctgt
1741	ggaggtcctg	cgggggctgc	gcgcccaggc	ctgcgcctc	tggaaaccgt	tccctcccaa
1801	attgctcagc	gccaccgaca	cgctcgacga	ggcggagcgc	cagtggaagg	ccaggtcca
1861	ccgctggagc	tcctacatgg	tgcactggaa	gaaccagttc	gaccactaca	gcaagcagga
1921	tcgctgctca	gacctgtgac	cccggcggga	cccccatgtc	ctccgctccg	cccggccccc
1981	tagctgtata	tactatctat	ttcagggctg	ggctataaca	cagacgagcc	ccagactctg
2041	cccatcccca	ccccaccctg	acgtcccccg	gggctcccgg	tctctgcat	gtctcaggct
2101	gagctccctc	ccccgcgggtg	ccttcgcccc	tctgggctgc	caataaactg	ttacag

SEQ ID NO: 10

FIG. 3F

Human cholecystokinin A receptor

Amino acid sequence

Accession L13605

MDVVDSLIVNGSNITPPCELGLENETLFCILDQPRPSKEWQPAVQ
ILLYSLIFLLSVLGNLTVITVLRNKRMRVTNIFLLSLAVSDLMLCLFCMPFNLI PN
LLKDFIFGSAVCKTTTTYFMGTSVSVSTFNLVAISLERYGAICKPLQSRVWQTKSHALK
VIAATWCLSFTIMTPYPIYSNLVPFTKNNNQANMCRFLLENDVMQOSWHTFLLLIIF
LIPGIVMMVAYGLISLELYQGIKFEASQKKSARKERKPSTTSSGKYEDSDGCYLQKTRP
PRKLELRQLSTGSSSRANRIRSNSSAANLMAKKRVIRMLIVIVVLFFLCWMPIFSANA
WRAYDTASAERRLSGTPI SFILLLSYTSNCVNP I IYCFMKNRFRLLGFMATFPCCPNPG
PPGARGEVGEHEEGGTTGASLSRFSYSHMSASVPPQ

SEQ ID No: 11

Human cholecystokinin A receptor (gene CCKAR, CDS 72..1358)

Nucleic acid sequence

1 cattagagga atgagccggg agtgagcaat tcaccagctc tccagcactt ggtggaaagc
61 agcaggcaag gatggatgtg gttgacagcc ttcttgtgaa tggagcaac atcactcctc
121 cctgtgaact cgggctcgaa aatgagacgc ttttctgctt ggatcagccc cgtccttcca
181 aagagtggca gccagcgggtg cagattctct tgtactcctt gatattcctg ctcagcgtgc
241 tgggaaacac gctggtcac accgtgctga ttcggaacaa gcggatgcgg acgggtcacca
301 acatcttctt cctctccctg gctgtcagcg acctcatgct ctgtctcttc tgcattgccg
361 tcaacctcat cccaatctg ctcaaggatt tcatcttcgg gagcgcggtt tgcaagacca
421 ccacctactt catgggcacc tctgtgagtg tatctacctt taatctggta gccatatctc
481 tagagagata tgggtgcgatt tgcaaacctt tacagtcccg ggtctggcag acaaaatccc
541 atgctttgaa ggtgattgct gctacctggg gcctttcctt taccatcatg actccgtacc
601 ccatttatag caacttgggtg ccttttacca aaaataacaa ccagaccgcg aatattgtgcc
661 gctttctact gccaaatgat gttatgcagc agtcctggca cacattcctg ttactcatcc
721 tctttcttat tcttgggaatt gtgatgatgg tggcatatgg attaactctt ttggaactct
781 accaggggat aaaatttgag gctagccaga agaagtctgc taaagaaagg aaacctagca
841 ccaccagcag cggcaaatat gaggacagcg atgggtgta cctgcaaaag accaggcccc
901 cgaggaagct ggagctccgg cagctgtcca cgggcagcag cagcagggcc aaccgcatcc
961 ggagtaacag ctccgcagcc aacctgatgg ccaagaaaag ggtgatccgc atgctcatcg
1021 tcatcgtggt cctcttcttc ttgtgctgga tgcccatctt cagcgccaac gcctggcggg
1081 cctacgacac cgcctccgca gagcgcgccc tctcaggaac cccatttcc ttcatcctcc
1141 tcctgtccta cacctcctcc tgcgtcaacc ccatcatcta ctgcttcatg aacaaacgct
1201 tccgctcgg ctctcatggcc accttcccct gctgccccaa tcttgggtccc ccaggggcca
1261 ggggagaggt gggggaggag gaggaaggcg ggaccacagg agcctctctg tccaggttct
1321 cgtacagcca tatgagtgcc tcggtgccac cccagtgaga tgtcccctga ccctccaccg
1381 cagaaggaag gca

SEQ ID No: 12

FIG. 3G

Homo sapiens cholecystokinin B receptor

Amino acid sequence

Accession NM_000731

MELLKLNRSVQGTGPGPGASLCRPGAPLLNSSSVGNLSCEPPRI
 RGAGTRELELAIRITLYAVIFLMSVGGNMLIIIVVLGLSRRLRRTVTNAFLLSLAVSDLL
 LAVACMPFTLLPNLMGTFIFGTVICKAVSYLMGVSVSVSTLSLVAIALERYSAICRPL
 QARVWQTRSHAARVIVATWLLSGLLMVYPVYTVVQPVGPRVLQCVHRWPSARVRQTW
 SVLLLLLFFIPGVVMAVAYGLISRELYLGLRFDGSDSDSQSRVRNQGLPGAVHQN
 GRCRPETGAVGEDSDGCYVQLPRSRPALELTALTAPGPGSGSRPTQAKLLAKKRVRM
 LLVIVVLFFLCWLFPVYSANTWRAFDGPGAHRLSGAPISFIHLLSYASACVMPLVYCF
 MHRFRQACLETCARCCPRPPRARPRALPDEDPPTPSIASLSRLSYTTTISTLGPG
 SEQ ID No: 13

Homo sapiens cholecystokinin B receptor (gene CCKBR, CDS 1..1344)

Nucleic acid sequence

1 atggagctgc tcaagctgaa cgggagcgtg caggggaaccg gaccogggcc gggggcttcc
 61 ctgtgccgcc cgggggcgcc tctcctcaac agcagcagtg tgggcaacct cagctgcgag
 121 cccctcgcga ttcgcgagc cgggacacga gaattggagc tggccattag aatcactctt
 181 tacgcagtga tcttctgat gagecgttga gaaatagc tcatcatcgt ggtcctggga
 241 ctgagccgcc gcctgaggac tgtcaccaat gccttctctc tctcactggc agtcagcgac
 301 ctctgtctgg ctgtggcttg catgcccttc accctcctgc ccaatctcat gggcacattc
 361 atctttggca cegtcatctg caaggcgtt tcctacctca tgggggtgtc tgtgagtgtg
 421 tccacgctaa gcctcgtggc catcgactg gagcggta ca gcgccatctg ccgaccactg
 481 caggcacgag tgtggcagac gcgctcccac gcggctcgcg tgattgtagc cagctggctg
 541 ctgtccggac tactcatggt gccctacccc gtgtacactg tctgtgcaacc agtggggcct
 601 cgtgtgctgc agtgcgtgca tgcgtggccc agtgcgcggg tccgccagac ctgggtccgta
 661 ctgctgcttc tgccttggtt ctcatcccc ggtgtggtta tggccgtggc ctacgggctt
 721 atctctcgcg agctctactt agggcttgc tttgacggcg acagtgacag cgacagccaa
 781 agcaggggtcc gaaaccaagg cgggctgcca ggggctgttc accagaacgg gcgttgccgg
 841 cctgagactg gcgcggttgg cgaagacagc gatggctgct acgtgcaact tccacgttcc
 901 cggcctgccc tggagctgac ggcgctgacg gctccagggc cgggatccgg ctcccggccc
 961 acccaggcca agctgctggc taagaagcgc gtggtgcaaa tgttgctggt gatcgttgtg
 1021 cttttttttc tgtgttggtt gccagtttat agtgccaaca cgtggcgcgc ctttgatggc
 1081 ccgggtgac accgagcact ctcggtgct cctatctcct tcattcactt gctgagctac
 1141 gcctcggcct gtgtcaacc cctggtctac tgcttcatgc accgtcgctt tcgccaggcc
 1201 tgcctggaaa cttgcgctcg ctgctgcccc cggcctccac gagctcgccc cagggtctt
 1261 cccgatgagg accctccac tcctccatt gcttcgctgt ccaggcttag ctacaccacc
 1321 atcagcacac tgggcccctgg ctgaggagta gaggggccgt gggggttgag gcagggcaaa
 1381 tgacatgcac tgacccttcc agacatagaa aacacaaacc acaactgaca caggaaacca
 1441 acacccaaag catggactaa cccaacgac aggaaaaggt agcttacctg acacaagagg
 1501 aataagaatg gagcagtaca tgggaaagga ggcattgcctc tgatatggga ctgagcctgg
 1561 cccatagaaa catgacactg accttgaga gacacagcgt ccctagcagt gaactatttc
 1621 tacacagtgg gaactctgac aagggtgac ctgcctctca cacacataga ttaatggcac
 1681 tgattgtttt agagactatg gagcctggca caggactgac tctgggatgc tctagtttg
 1741 acctcacagt gacccttccc aatcagcact gaaaatacca tcaggcctaa tctcatacct
 1801 ctgaccaaca ggctgttctg cactgaaaag gttcttcat ccttccagt taaggaccgt
 1861 ggccctgccc tctccttctt tccaaaactg ttcaagaaat aataaattgt ttggcttctt
 1921 cctgaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aggaattcc
 SEQ ID No: 14

FIG. 4A

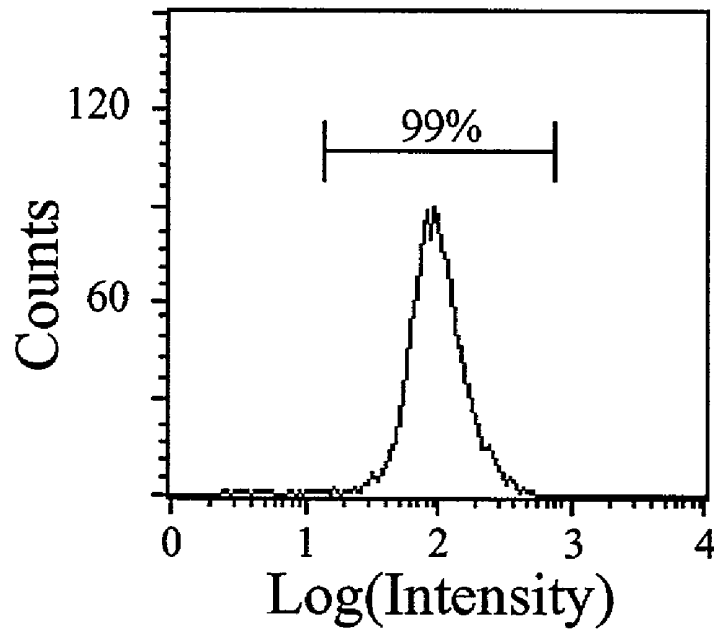
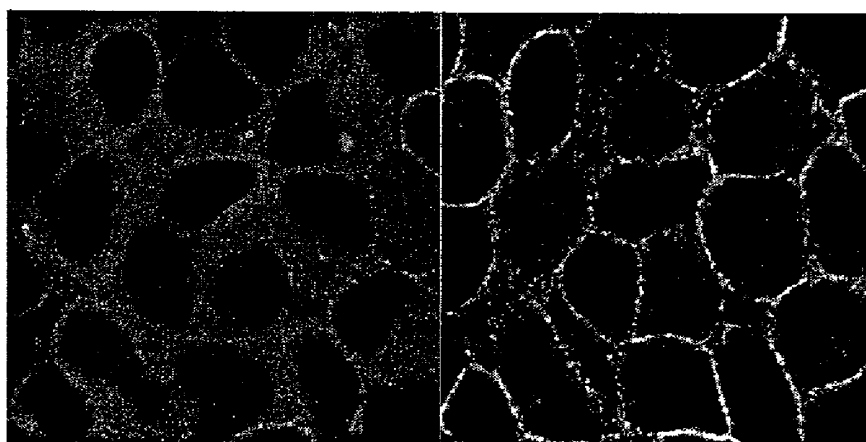


FIG. 4B



- Gastrin-17

+ Gastrin-17

FIG 5A

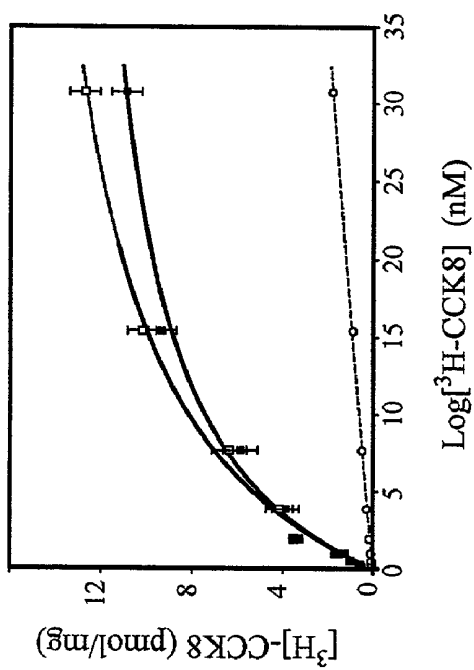


FIG. 5B

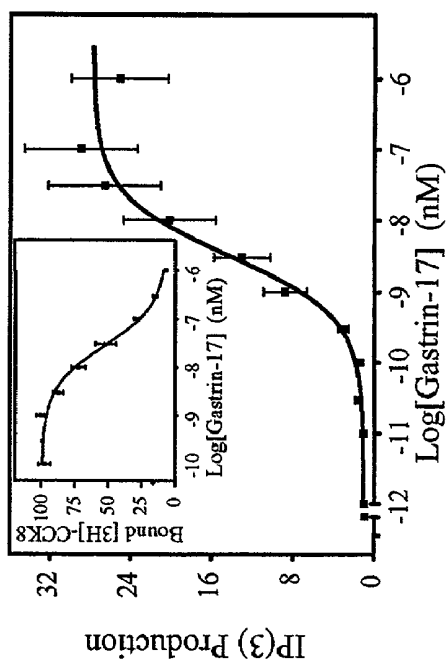


FIG 5C

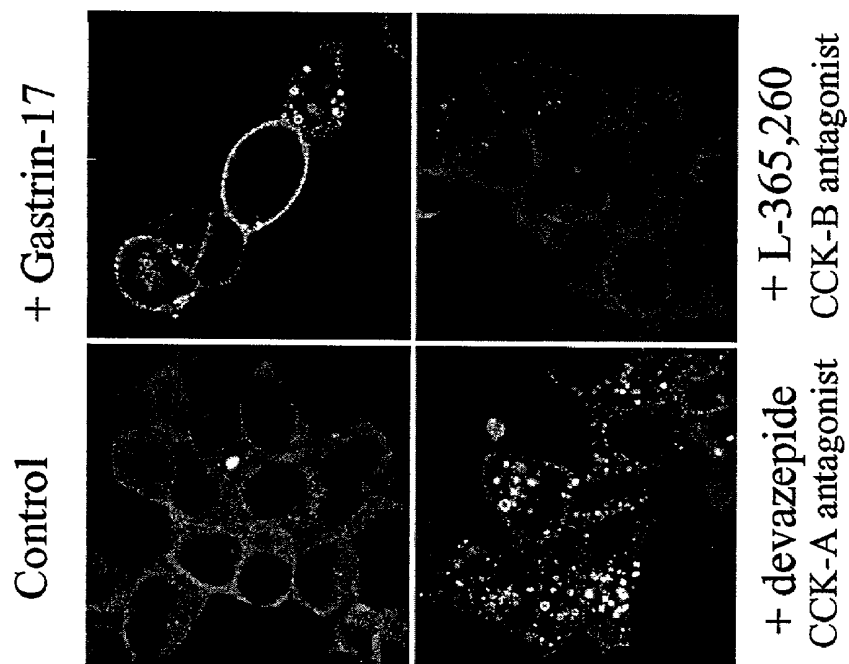


FIG. 6A

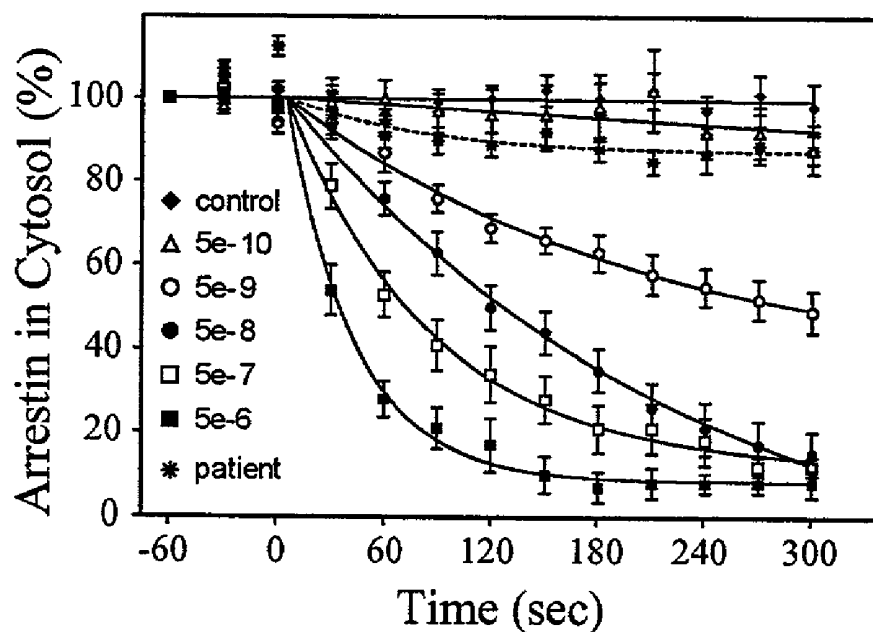


FIG. 6B

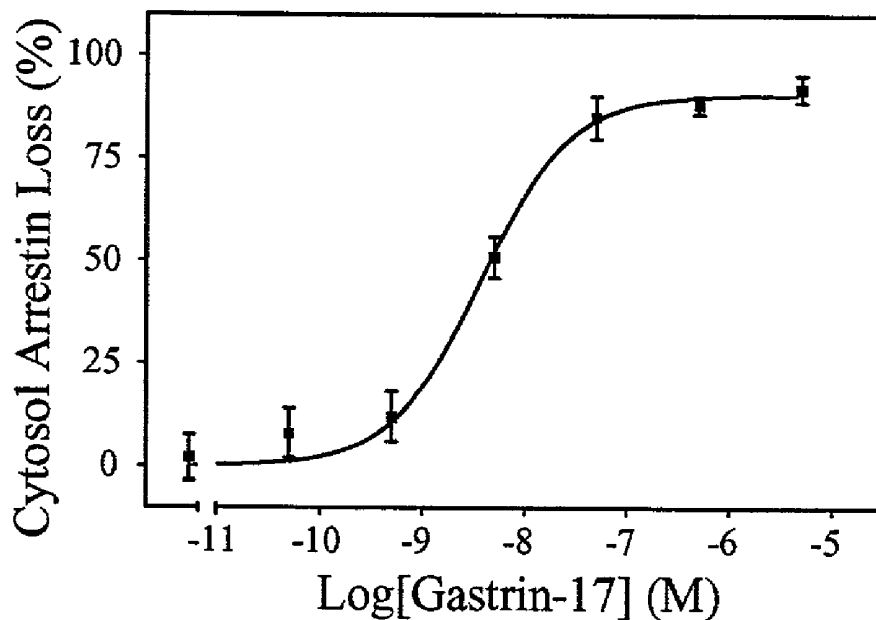


FIG. 6C

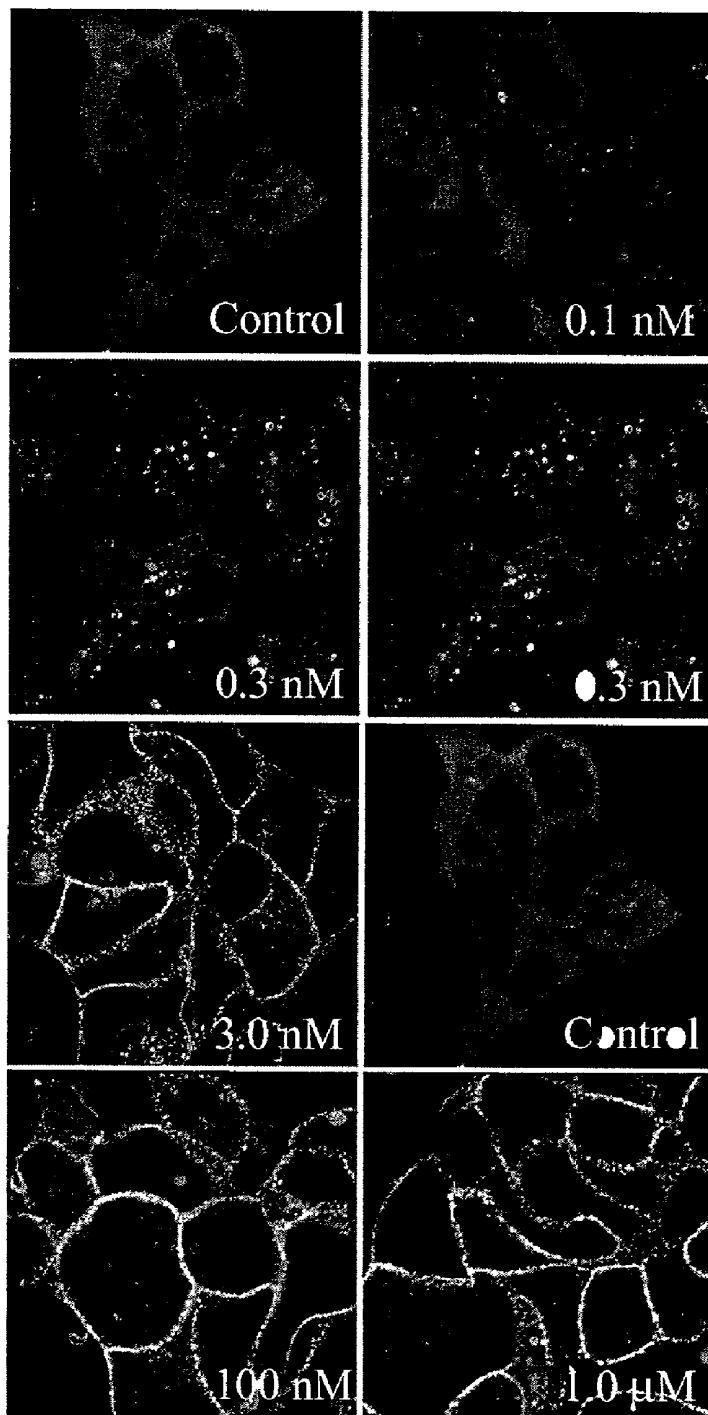


FIG. 7A

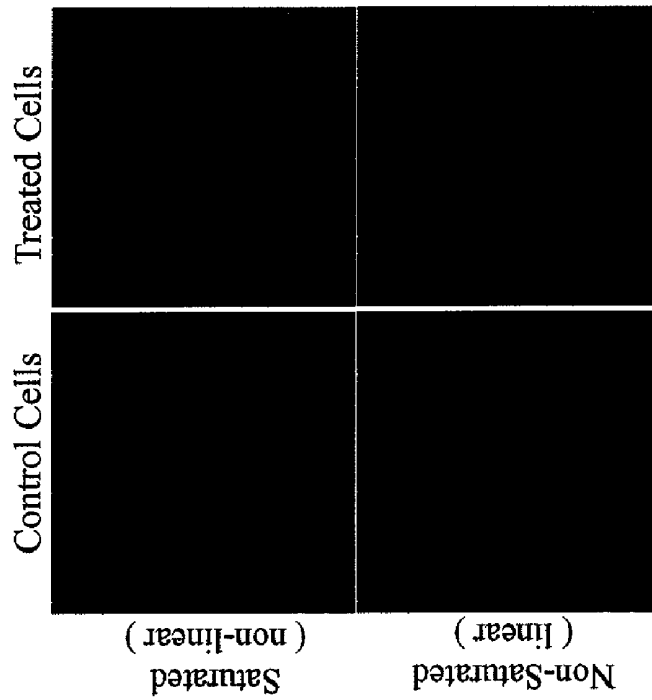


FIG. 7B

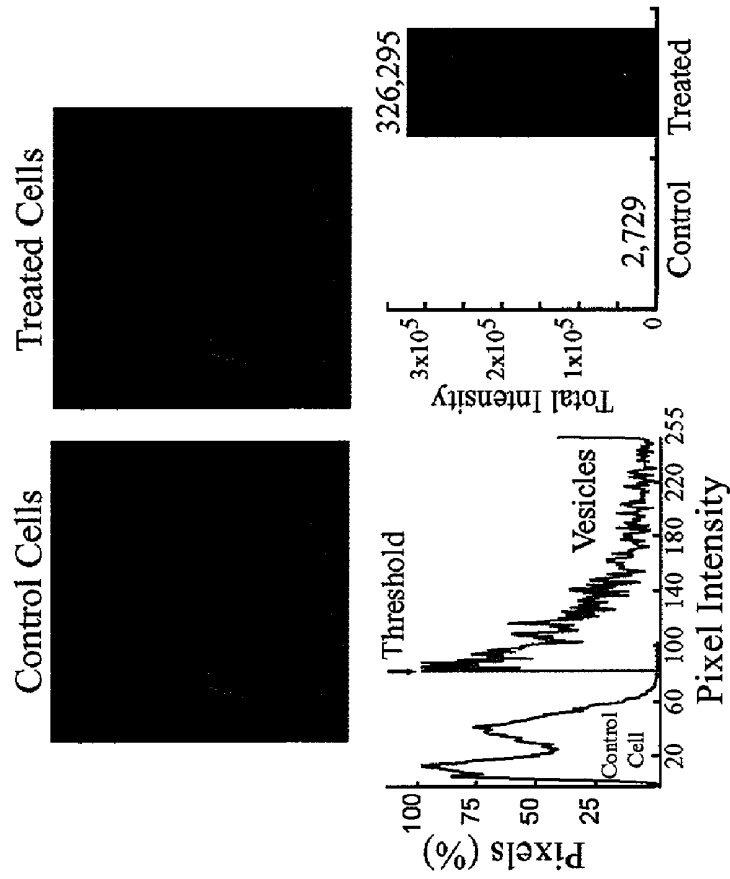


Fig. 8A

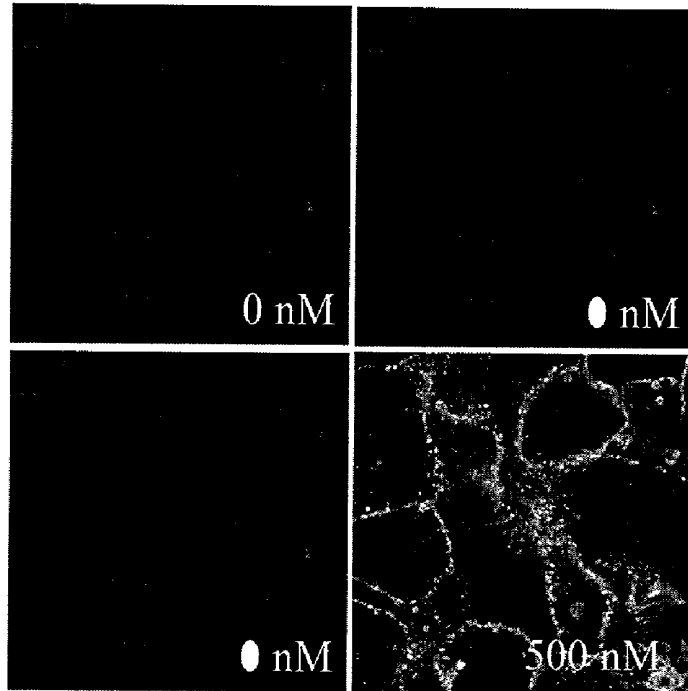


Fig. 8B

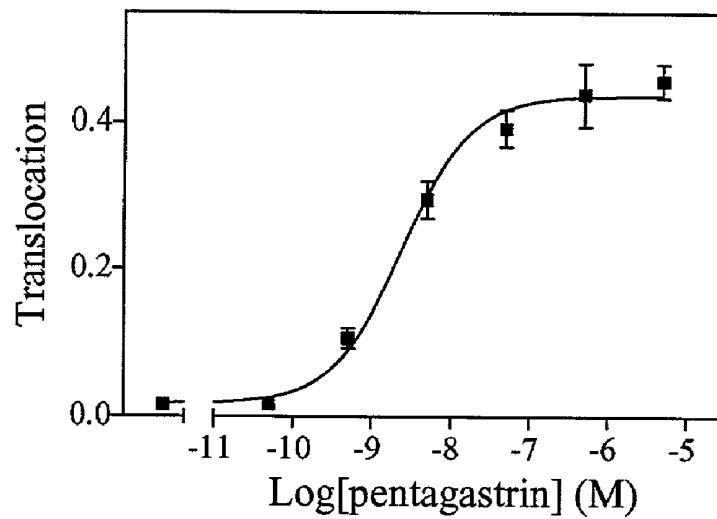


FIG. 9A

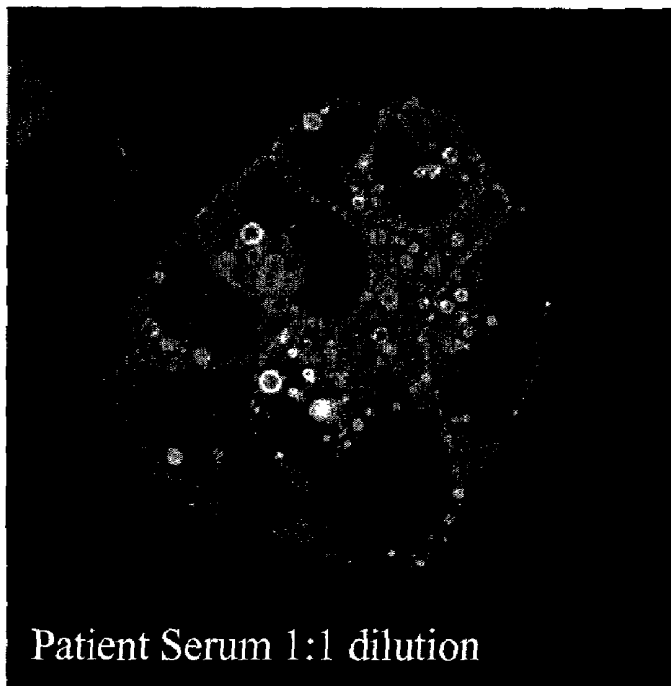


FIG. 9B

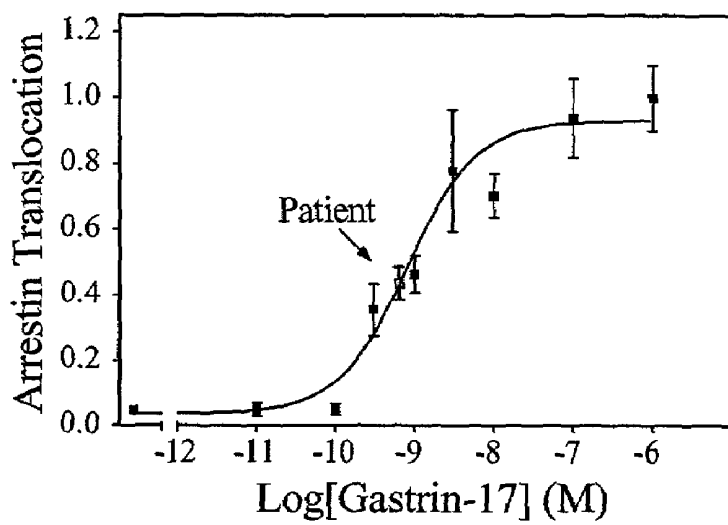
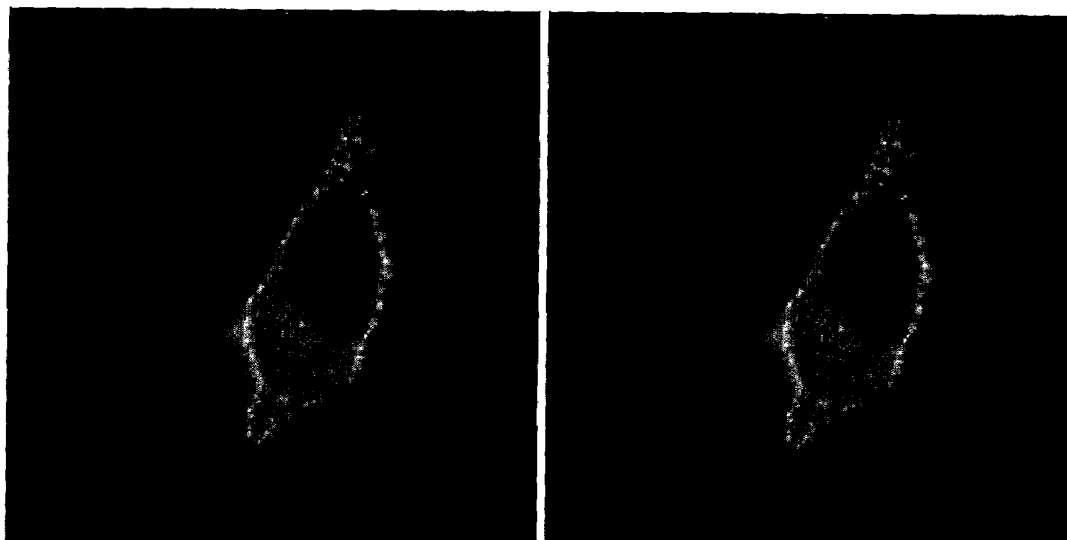


FIG. 10

Human Muscarinic Receptor type 1 / V2 Tail Conjugate



5-10 minutes

30-60 minutes

+ Acetylcholine Chloride

SINGLE-CELL BIOSENSOR FOR THE MEASUREMENT OF GPCR LIGANDS IN A TEST SAMPLE

This application claims priority under 35 U.S.C. § 119(e) to U.S. Ser. No. 60/295,945 filed Jun. 5, 2001, and is a continuation-in-part claiming priority under 35 U.S.C. § 120 to U.S. Ser. No. 09/631,468 filed Aug. 3, 2000, now abandoned which is a continuation of U.S. Ser. No. 09/233,530 filed on Jan. 20, 1999, now U.S. Pat. No. 6,110,693, which is a continuation of U.S. Ser. No. 08/869,568 filed on Jun. 5, 1997, now U.S. Pat. No. 5,891,646, the contents of which are hereby incorporated by reference in their entireties.

This work was supported by National Institutes of Health Grants DK 02544, HL 61365, and NS 19576, and therefore the government may have certain rights to the invention.

FIELD OF THE INVENTION

The present invention relates to methods of analyzing the presence and concentration of a GPCR ligand in test samples, including biological and environmental samples. Preferably, the present invention relates to the detection of multiple GPCR ligands in a test sample, wherein the test sample may be heterogeneous. The present invention provides improved methods of disease diagnosis, as well as detection of harmful chemicals, such as insecticides, neurotoxins, and chemicals used in bioterrorism.

BACKGROUND

G protein-coupled receptors (GPCRs) are cell surface proteins that translate hormone or ligand binding into intracellular signals. GPCRs are found in all animals, insects, and plants. GPCR signaling plays a pivotal role in regulating various physiological functions including phototransduction, olfaction, neurotransmission, vascular tone, cardiac output, digestion, pain, and fluid and electrolyte balance. Although they are involved in various physiological functions, GPCRs share a number of common structural features. They contain seven membrane domains bridged by alternating intracellular and extracellular loops and an intracellular carboxyl-terminal tail of variable length.

GPCRs have been implicated in a number of disease states, including, but not limited to cardiac indications such as angina pectoris, essential hypertension, myocardial infarction, supraventricular and ventricular arrhythmias, congestive heart failure, atherosclerosis, renal failure, diabetes, respiratory indications such as asthma, chronic bronchitis, bronchospasm, emphysema, airway obstruction, upper respiratory indications such as rhinitis, seasonal allergies, inflammatory disease, inflammation in response to injury, rheumatoid arthritis, chronic inflammatory bowel disease, glaucoma, hypergastrinemia, gastrointestinal indications such as acid/peptic disorder, erosive esophagitis, gastrointestinal hypersecretion, mastocytosis, gastrointestinal reflux, peptic ulcer, Zollinger-Ellison syndrome, pain, obesity, bulimia nervosa, depression, obsessive-compulsive disorder, organ malformations (for example, cardiac malformations), neurodegenerative diseases such as Parkinson's Disease and Alzheimer's Disease, multiple sclerosis, Epstein-Barr infection and cancer.

The magnitude of the physiological responses controlled by GPCRs is linked to the balance between GPCR signaling and signal termination. The signaling of GPCRs is controlled by a family of intracellular proteins called arrestins. Arrestins bind activated GPCRs, including those that have been ago-

nist-activated and especially those that have been phosphorylated by G protein-coupled receptor kinases (GRKs).

The abnormal regulation of hormones that bind to G protein-coupled receptors underlies the pathogenesis of many diseases. The ability to measure serum and tissue levels of these regulators, while clinically and scientifically desirable, is presently limited to very specialized biochemical and immunochemical assays.

Altered concentrations of a GPCR ligand in a biological sample may be indicative of a disease state. Altered concentrations of a GPCR ligand in an environmental sample may indicate the presence of harmful chemicals. There is a need for highly sensitive and specific methods for the quantitative detection of GPCR ligands in a heterogeneous sample, as well as methods for the detection of the multiple bioactive isoforms of a GPCR ligand in a heterogeneous sample. Sensitive, rapid methods of analyzing the presence of GPCR ligands in heterogeneous samples, both biological and environmental, would improve disease diagnosis and the detection of harmful compounds in the environment.

SUMMARY

A first aspect of the present invention is a method of detecting a GPCR ligand in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample or an environmental sample. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined. The cellular distribution of the GPCR or the arrestin in the presence of the test sample may be compared to the cellular distribution of the GPCR or arrestin in the absence of the test sample.

In one aspect of the present invention, the GPCR or the arrestin is detectably labeled, another endogenous molecule is detectably labeled, or another exogenous molecule is detectably labeled. The distribution of the detectably labeled molecules represents the cellular distribution of the GPCR or the arrestin proteins.

In a further aspect, the cellular distribution of the GPCR or arrestin is determined at different time points after exposure to the test sample. The cellular distribution of the GPCR or arrestin is determined after exposure to different concentrations of the test sample. The cellular distribution of the detectably labeled molecules may be quantified.

In an additional aspect, the concentration of the ligand in the test sample is quantified by comparing the cellular distribution of the GPCR or arrestin in the presence of the test sample to the cellular distribution of the GPCR or arrestin in the presence of a known concentration of the ligand.

The biological sample provided as the test sample may be serum, tissue, blood, urine, or derived therefrom.

In a further aspect, the GPCR is CCK-B or CCK-A. The ligand may be gastrin, preprogastrin, cleaved preprogastrin, gastrin-34, gastrin-17, pentagastrin, progastrin, glycine-extended gastrin-17, glycine-extended gastrin-34, gastrin-71, gastrin-6, hG17, a compound with an amidated tetrapeptide of the sequence Trp-Met-Asp-Phe-NH₂ (SEQ ID NO: 15), or another bioactive isoform of gastrin.

In a further aspect, the GPCR is a muscarinic receptor. The ligand may be acetylcholine.

In an additional aspect, the labeled molecule may be localized in the cytosol, plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes. An increase in the local concentration of the labeled molecule results in an increase in

the local signal intensity. The signal intensity of the labeled molecule in the plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes may be increased with respect to the level of signal intensity in the cytosol. The local signal intensity may be increased in the presence of increased concentration of ligand in the test sample.

In a further aspect, the concentration of the ligand in the test sample indicates a disease state. The concentration of the ligand in the test sample may indicate the presence of a compound in the test sample that alters the ligand concentration. The concentration of the ligand in the test sample indicates the presence of a compound in the test sample that modifies acetylcholine. The concentration of the ligand in the test sample indicates the presence of a compound that inhibits an acetylcholinesterase.

The detectable molecule may be a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group. The molecule may be detectably labeled due to its interaction with another molecule, which may be detectably labeled.

The present invention relates to a method of monitoring a GPCR ligand in a mammal. The test sample is a biological sample derived from the mammal. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined. The concentration of the ligand in the test sample may be quantified by comparing the cellular distribution of the GPCR or arrestin in the presence of the test sample to the cellular distribution of the GPCR or arrestin in the presence of a known concentration of the ligand. This method may be used to monitor a clinical condition, which may indicate the presence of a disease state, or may indicate that the subject has a disorder or is at risk for developing a disorder. The clinical condition monitored may be gastrointestinal cancer, hypergastrinemia, atrophic gastritis, gastric ulcers, malignant tumors, or other GPCR-related disease. The mammal may be on prolonged acid suppressive medications.

In a further aspect of the present invention, the provided cell may express a protein that increases the internalization of the GPCR. The GPCR may itself be modified, resulting in an increased concentration of the labeled molecule at the plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes. The provided cell may express a G protein-coupled receptor kinase (GRK).

One aspect of the present invention is a single cell biosensor. This biosensor includes a cell which overexpresses arrestin and at least one GPCR, wherein the GPCR, the arrestin, or the cell is detectably labeled for monitoring internalization of the GPCR. A further aspect of the present invention is a method of detecting a GPCR ligand in a test sample, wherein the test sample is a biological sample, an environmental sample, or a sample derived therefrom. In this method, the single cell biosensor is provided, the biosensor is exposed to the test sample, and the cellular distribution of the GPCR or arrestin in the presence of the test sample is determined. In the single cell biosensor, the GPCR may be a CCK-A, a CCK-B, or a muscarinic receptor. The arrestin may be conjugated to a Green Fluorescent Protein. The biosensor may have increased sensitivity due to longer incubation time, increased concentration of test sample, GPCR mutation, or GPCR antibodies.

The present invention is related to a method of altering GPCR internalization, comprising providing to the cells an effective amount of an antagonist of CCK-B.

In the methods of the present invention, the cellular distribution may be visualized by flow cytometry or fluorescence confocal microscopy. A computer may analyze an image of the cellular distribution and the distribution may be quantified. The test sample to be analyzed may comprise a ligand of the GPCR, or an antagonist of the GPCR.

The present invention is related to a method of detecting a compound which modulates a GPCR ligand in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample or an environmental sample. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined. The cellular distribution of the GPCR or the arrestin in the presence of the test sample may indicate the presence of a compound which modulates a GPCR ligand.

The present invention is related to a method of detecting a compound which modulates a GPCR ligand in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample or an environmental sample. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined.

The present invention is related to a method of continuous screening of GPCR ligands in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample or an environmental sample. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined. Then, the cell is replaced with another cell comprising a GPCR and an arrestin.

A further aspect of the present invention is a method of detecting an inhibitor of acetylcholinesterase in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample or an environmental sample. Preferably, a cell is provided that expresses a muscarinic receptor and an arrestin. A mixture, containing a test sample, acetylcholinesterase, and an agonist of the muscarinic receptor, is provided. The agonist is sensitive to acetylcholinesterase. The cell is exposed to the mixture. The cellular distribution of the muscarinic receptor or arrestin in the presence of the test sample is determined. The agonist may be acetylcholine.

In an aspect of the present invention, the test sample may contain acetylcholine and acetylcholinesterase. The test sample may contain an agonist. The ligand may have been identified, and multiple bioactive isoforms of the GPCR ligand in the test sample may be detected.

In a further aspect of the invention, the test sample may be derived from a mammal with hypergastrinemia. The gastrin concentration in the test sample may be less than 10 nM. The test sample may be heterogeneous.

In an aspect of the present invention, the cellular distribution may be determined after 15-30 minutes of exposure to the test sample. The cellular distribution may be determined after 1 hour of exposure to the test sample. The cell may be exposed to the test sample at a temperature of approximately 37° C.

The present invention is related to a method of detecting a compound that modulates GPCR internalization in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample

or an environmental sample. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined.

In a further aspect, the present invention is related to a method of detecting a compound that modulates GPCR internalization in a test sample. The test sample is a biological sample, an environmental sample, or a sample derived from a biological sample or an environmental sample. Preferably, a cell is provided that includes at least one GPCR and an arrestin. The cell is exposed to an agonist. The test sample is provided and the cell is exposed to the test sample. The cellular distribution of the GPCR or arrestin in the presence of the test sample is determined.

A further aspect of the present invention is a bioarray containing at least one single cell biosensor. The bioarray may detect multiple GPCR ligands.

BRIEF DESCRIPTION OF DRAWINGS

The objects and advantages of the invention will be understood by reading the following detailed description in conjunction with the drawings in which:

FIG. 1 is an illustrative, non-limiting list of known GPCRs with which the present invention may be used is contained in FIG. 1. The receptors are grouped according to classical divisions based on structural similarities and ligands. FIGS. 1A to 1B includes a non-limiting list of the known Class I GPCRs. FIG. 1C is a non-limiting list of the known Class II and Class III GPCRs.

FIG. 2 lists some of the over 40 different GPCRs that may associate with arrestin and subsequently internalize. This may be visualized using expressed GPCRs and fusion proteins between arrestin and a green fluorescent protein.

FIG. 3A illustrates the amino acid and nucleic acid sequences of the *homo sapiens* muscarinic receptor 1, Accession NM_000738. FIG. 3B illustrates the amino acid and nucleic acid sequences of the *homo sapiens* muscarinic receptor 2, Accession NM_000739. FIG. 3C illustrates the amino acid and nucleic acid sequences of the *homo sapiens* muscarinic receptor 3, Accession NM_000740. FIG. 3D illustrates the amino acid and nucleic acid sequences of the *homo sapiens* muscarinic receptor 4, Accession NM_000741. FIG. 3E illustrates the amino acid and nucleic acid sequences of the *homo sapiens* acetylcholinesterase (YT blood group), Accession XM_036148. FIG. 3F illustrates the amino acid and nucleic acid sequences of the human cholecystokinin A receptor, Accession L13605. FIG. 3G illustrates the amino acid and nucleic acid sequences of the *homo sapiens* cholecystokinin B receptor, Accession NM_000731. Amino acid sequences are listed in the amino-terminal to carboxy-terminal orientation. Nucleic acid sequences are listed in the 5'→3' orientation.

FIG. 4 illustrates the uniformity of arrestin-GFP and CCK-B receptor expression in cells by flow cytometry and arrestin-GFP translocation. FIG. 4A shows the relative expression of arrestin-GFP in cells belonging to Clone A. FIG. 4B shows fluorescence images of a field of cells from Clone A before (left panel) and after treatment (right panel) with 10 nM hG17 for 5 minutes at room temperature.

FIG. 5 shows the characterization of ligand binding and second messenger response in a cell line expressing arrestin-GFP and the CCK-B receptor. As shown in FIG. 5A, cells from Clone A were incubated with increasing concentrations of [³H]CCK-8 in order to determine the average CCK-B receptor expression per cell and the receptor affinity for [³H]

CCK-8. FIG. 5B shows that Clone A cells were exposed to increasing concentrations of hG17 peptide in order to evaluate the IP3 second messenger response. The inset shows the competitive displacement of [³H]CCK8 by hG17 from this cell line. FIG. 5C shows the fluorescence images of cells from Clone A that were treated with vehicle (upper left panel), or treated for one hour with 10 nM of the agonist hG17 (upper right panel), or with 10 nM hG17 plus 1 μM of the CCK-A antagonist devazepide (L-364,718, lower left panel); or with 10 nM hG17 plus 1 μM, of the CCK-B antagonist (lower right panel).

FIG. 6 illustrates the dose response to hG17 at five minutes in an HEK-293 cell line containing arrestin-GFP and the CCK-B receptor. FIG. 6A is a representative experiment depicting the arrestin-GFP translocation of Clone A cells that were exposed to various concentrations of hG17 for 5 minutes. In FIG. 6B, the fractional amount of arrestin-GFP lost after 5 minutes from the cell cytosol was used to generate a sigmoid dose response curve for the increasing concentrations of hG17 shown in the graph in A. FIG. 6C illustrates images from an experiment demonstrating the response of Clone A cells exposed to hG17 for one hour at 37° C.

FIG. 7 shows the calculation of the Fluorescence Signal from the Distribution of βarrestin2-GFP. FIG. 7A shows the distribution of βarrestin2-GFP fluorescence in cells stably expressing the βarrestin2-GFP fusion protein and receptor was visualized before and after a 30-minute agonist treatment. In FIG. 7B, a histogram of the pixel count versus pixel intensity (green curve in the graph at the lower left) was generated using a representative control cell.

FIG. 8 shows the dose response to pentagastrin in an HEK-293 cell line containing arrestin-GFP and the CCK-B receptor. FIG. 8A shows images from a representative experiment depicting the response of Clone A cells that were exposed to pentagastrin at 37° C. for two hours. The graph in FIG. 8B depicts the increase in the normalized sum of pixel intensity (TI/TF) above a threshold value (Methods) for images obtained at each concentration of ligand.

FIG. 9 shows the dose response at one hour in a clonal cell line containing arrestin-GFP and the CCK-B receptor. FIG. 9A illustrates the representative image of Clone A cells incubated for one hour with a 1:1 dilution of serum that was obtained from a patient with hypergastrinemia. FIG. 9B shows the dose response curve to hG17 of Clone A cells computed from the imaged translocation data obtained at one hour and analyzed as in FIG. 7. Between 9 and 16 separate images were analyzed for each hG17 concentration and the patient's serum (arrow).

FIG. 10 illustrates the Internalization of muscarinic receptor in present of acetylcholine. HEK-293 cells expressing arrestin-GFP and the human muscarinic receptor type 1 conjugated with the Vasopressin carboxyl-terminal tail were exposed to micromolar concentrations of acetylcholine. Arrestin-GFP was observed at the membrane edge or in vesicles, in response to acetylcholine.

DETAILED DESCRIPTION

In accordance with the present invention there may be employed conventional molecular biology, microbiology, immunology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (3rd edition, 2001); "Current Protocols in Molecular Biology" Volumes I-IV [Ausubel, R. M., ed. (2002 and updated bimonthly)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current

Protocols in Immunology” Volumes I-IV [Coligan, J. E., ed. (2002 and updated bimonthly)]; “Oligonucleotide Synthesis” (M. J. Gait ed. 1984); “Nucleic Acid Hybridization” [B. D. Hames & S. J. Higgins eds. (1985)]; “Transcription And Translation” [B. D. Hames & S. J. Higgins, eds. (1984)]; “Culture of Animal Cells, 4th edition” [R. I. Freshney, ed. (2000)]; “Immobilized Cells And Enzymes” [IRL Press, (1986)]; B. Perbal, “A Practical Guide To Molecular Cloning” (1988); *Using Antibodies: A Laboratory Manual Portable Protocol No. 1*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1998); *Using Antibodies: A Laboratory Manual*, Harlow, Ed and Lane, David (Cold Spring Harbor Press, 1999); “G Protein-Coupled Receptors” [T. Haga, et al., eds. (1999)].

Unless otherwise stated, the following terms used in the specification and claims have the meanings given below:

“hG17” is the human gastrin-17 amino acid peptide. It may be produced in a human, another organism, such as *E. coli*, yeast, mouse, or it may be synthesized chemically.

“RIA”, or radioimmunoassay, is an antibody-based method of detecting a particular compound in a sample. Presently, serum gastrin measurements are performed by RIA using antibodies directed against one or more distinct gastrin isoforms.

A “neurotoxin” is any compound that has the ability to damage or destroy nerve tissues. Of particular relevance to the present invention are compounds which inhibit acetylcholinesterase. Normally, acetylcholinesterase breaks down acetylcholine, a natural ligand of the muscarinic receptor. Nerve toxins which inhibit acetylcholinesterase prevent the normal degradation of acetylcholine. The present invention can be used to detect the presence of nerve toxins which inhibit acetylcholinesterase by detecting the acetylcholine concentration in a sample. Compounds that inhibit acetylcholinesterase include organophosphate insecticides such as diazinon and the neurotoxin sarin.

“Acetylcholine” is a neurotransmitter and functions at least at neuromuscular synapses, which are synapses between neurons and cardiac, smooth, and skeletal muscle, as well as at a variety of neuron-neuron synapses in the central and peripheral nervous systems. It is synthesized in nerve terminals from acetylCoA and choline, in a reaction catalyzed by the enzyme choline acetyltransferase.

A “muscarinic receptor” is a GPCR which is located at least in many brain neurons, sympathetic neurons, smooth muscle, gland cells and heart cells. Muscarinic receptor is meant to include muscarinic acetylcholine receptor, muscarinic cholinergic receptor, other references for muscarinic receptors, including sub-types 1, 2, 3, 4, and other sub-types known to those of skill in the art. The term muscarinic receptor includes, but is not limited to, muscarinic receptor sequences of *homo sapiens*, eukaryota, metazoa, chordata, craniata, vertebrate, euteleostomi, mammalia, eutheria, primates, catarrhini, homimidae, homo, and others. Acetylcholine is an agonist of the muscarinic receptor.

“Acetylcholinesterase” is the enzyme which degrades acetylcholine into acetate and choline. This enzyme is clustered at high concentrations in the synaptic cleft.

An “acetylcholinesterase inhibitor” is a compound that inhibits the activity of acetylcholinesterase. Compounds that inhibit acetylcholinesterase include organophosphate insecticides such as diazinon and the neurotoxin sarin.

“Insecticides” include compounds which are nerve toxins. Insecticides, including organophosphate insecticides such as diazinon, may be acetylcholinesterase inhibitors.

A “bioassay” is the use of a physiological response to assay for a biologically active compound.

A “biosensor” utilizes a biological process or component to detect the presence of compound. The single-cell biosensors of the present invention are cells which include a GPCR and an arrestin. By exposing the cells to a heterogeneous sample and monitoring the GPCR or arrestin response to the sample, they are useful for the detection of a GPCR ligand in a heterogeneous sample.

“ZE syndrome” is Zollinger-Ellison syndrome, which is caused by a gastrin producing tumor.

“Biological sample” is intended to include tissues, cells and/or biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject; wherein said sample can be blood, serum, a urine sample, a fecal sample, a tumor sample, a cellular wash, an oral sample, sputum, biological fluid, a tissue extract, freshly harvested cells, or cells which have been incubated in tissue culture. The biological sample may be selected from the group consisting of whole blood, serum, plasma, saliva, urine, sweat, ascitic fluid, peritoneal fluid, synovial fluid, amniotic fluid, cerebrospinal fluid, skin biopsy, and the like. The biological sample may include serum, whole blood, plasma, lymph and ovarian follicular fluid as well as other circulatory fluid and saliva, mucus secretion, and respiratory fluid or fractionated portions thereof. The sample may be extracted, untreated, treated, diluted or concentrated from a patient.

“Biologically active” and “bioactive” are used interchangeably herein to refer to a compound, compound fragment, or compound isoform which has biological activity. Preferably, biologically active or bioactive is used to describe a GPCR ligand, or ligand isoforms, which have the ability to bind a GPCR.

A “replicon” is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A “vector” is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A “DNA molecule” refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An “origin of replication” refers to those DNA sequences that participate in DNA synthesis.

A DNA “coding sequence” is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers,

polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A “promoter sequence” is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain “TATA” boxes and “CAT” boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An “expression control sequence” is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is “under the control” of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A “signal sequence” can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term “oligonucleotide,” as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term “primer” as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be “substantially” complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient

complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms “restriction endonucleases” and “restriction enzymes” refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been “transformed” by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A “clone” is a population of cells derived from a single cell or common ancestor by mitosis. A “cell line” is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Two DNA sequences are “substantially homologous” when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. For example, stringent conditions may include hybridization 6×SSC or 6×SSPE at 68° C. for 1 hour to 3 days. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al, “Molecular Cloning: A Laboratory Manual” (3rd edition, 2001), supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.

It should be appreciated that also within the scope of the present invention are DNA sequences having the same amino acid sequence as SEQ ID NO:1, 3, 5, 7, 9, 11, and 13, but which are degenerate to SEQ ID NO:1, 3, 5, 7, 9, 11, and 13. By “degenerate to” is meant that a different three-letter codon is used to specify a particular amino acid.

“Arrestin” means all types of naturally occurring and engineered variants of arrestin, including, but not limited to, visual arrestin (sometimes referred to as Arrestin 1), β arrestin 1 (sometimes referred to as Arrestin 2), and β arrestin 2 (sometimes referred to as Arrestin 3).

“ β ARK1” is a GRK termed β -adrenergic receptor kinase 1, also called GRK2.

“ β AR” is a GPCR termed a β -adrenergic receptor.

“Gastrin receptors” are GPCRs, preferably CCK-A and CCK-B, that bind gastrin. CCK-A and CCK-B, the cholecystokinin A and B receptors, are GPCRs that bind gastrin, cholecystokinin, and similar ligands.

“Internalization” of a GPCR is the intracellular translocation of a GPCR. Internalization includes the translocation of a GPCR to clathrin-coated pits, endocytic vesicles, and endosomes.

“Carboxyl-terminal tail” means the carboxyl-terminal tail of a GPCR. The carboxyl-terminal tail of many GPCRs begins shortly after the conserved NPXXY motif that marks the end of the seventh transmembrane domain (i.e. what follows the NPXXY motif is the carboxyl-terminal tail of the GPCR). The carboxyl-terminal tail may be relatively long

(approximately tens to hundreds of amino acids), relatively short (approximately tens of amino acids), or virtually non-existent (less than approximately ten amino acids). As used herein, "carboxyl-terminal tail" shall mean all three variants (whether relatively long, relatively short, or virtually non-existent).

"Class A receptors" preferably do not translocate arrestin to endocytic vesicles or endosomes in HEK-293 cells.

"Class B receptors" preferably do translocate arrestin to endocytic vesicles or endosomes in HEK-293 cells.

"DACs" mean any desensitization active compounds. Desensitization active compounds are any compounds that influence the GPCR desensitization mechanism by either stimulating or inhibiting the process. DACs influence the GPCR desensitization pathway by acting on any cellular component of the process, as well as any cellular structure implicated in the process, including but not limited to, arrestins, GRKs, GPCRs, PI3K, AP-2 protein, clathrin, protein phosphatases, and the like. DACs may include, but are not limited to, compounds that inhibit arrestin translocating to a GPCR, compounds that inhibit arrestin binding to a GPCR, compounds that stimulate arrestin translocating to a GPCR, compounds that stimulate arrestin binding to a GPCR, compounds that inhibit GRK phosphorylation of a GPCR, compounds that stimulate GRK phosphorylation of a GPCR, compounds that inhibit protein phosphatase dephosphorylation of a GPCR, compounds that stimulate protein phosphatase dephosphorylation of a GPCR, compounds that regulate the release of arrestin from a GPCR, antagonists of a GPCR, inverse agonists and the like. DACs preferably inhibit or stimulate the GPCR desensitization process without binding to the same ligand binding site of the GPCR as traditional agonists and antagonists of the GPCR. DACs act independently of the GPCR, i.e., they do not have high specificity for one particular GPCR or one particular type of GPCRs.

"Detectable molecule" means any molecule capable of detection by spectroscopic, photochemical, biochemical, immunochemical, electrical, radioactive, and optical means, including but not limited to, fluorescence, phosphorescence, and bioluminescence and radioactive decay. Detectable molecules include, but are not limited to, GFP, luciferase, β -galactosidase, rhodamine-conjugated antibody, and the like. Detectable molecules include radioisotopes, epitope tags, affinity labels, enzymes, fluorescent groups, chemiluminescent groups, and the like. Detectable molecules include molecules which are directly or indirectly detected as a function of their interaction with other molecule(s).

"GFP" means Green Fluorescent Protein which refers to various naturally occurring forms of GFP which may be isolated from natural sources or genetically engineered, as well as artificially modified GFPs. GFPs are well known in the art. See, for example, U.S. Pat. Nos. 5,625,048; 5,777,079; and 6,066,476. It is well understood in the art that GFP is readily interchangeable with other fluorescent proteins, isolated from natural sources or genetically engineered, including but not limited to, yellow fluorescent proteins (YFP), red fluorescent proteins (RFP), cyan fluorescent proteins (CFP), blue fluorescent proteins, luciferin, UV excitable fluorescent proteins, or any wave-length in between. As used herein, "GFP" shall mean all fluorescent proteins known in the art.

"Unknown or Orphan Receptor" means a GPCR whose function and/or ligands are unknown.

"NPXXY motif" means a conserved amino acid motif that marks the end of the seventh transmembrane domain. The conserved amino acid motif begins with asparagine and proline followed by two unspecified amino acids and then a

tyrosine. The two unspecified amino acids may vary among GPCRs but the overall NPXXY motif is conserved.

In referring to a polypeptide, "downstream" means toward a carboxyl-terminus of an amino acid sequence, with respect to the amino-terminus. In referring to a polynucleotide, "downstream" means in the 3' direction.

In referring to a polypeptide, "upstream" means toward an amino-terminus of an amino acid sequence, with respect to the carboxyl-terminus. In referring to a polynucleotide, "upstream" means in the 5' direction.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β -turns in the protein's structure.

Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein. Heterologous DNA may include, but is not limited to, DNA from a heterologous species ("foreign DNA"), as described in U.S. Pat. No. 6,331,415, which is incorporated by reference herein.

A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

"Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine (A) and thymine (T) are complementary nucleobases which pair through the formation of hydrogen bonds.

The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 6 \times SSC and 68 $^{\circ}$ C. for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are

RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20° C. below the predicted or determined T_m with washes of higher stringency, if desired. These conditions are described in Protocol 10 of Sambrook et al, "Molecular Cloning: A Laboratory Manual" (3rd edition, 2001).

By "animal" is meant any member of the animal kingdom including vertebrates (e.g., frogs, salamanders, chickens, or horses) and invertebrates (e.g., worms, etc.). "Animal" is also meant to include "mammals." Preferred mammals include livestock animals (e.g., ungulates, such as cattle, buffalo, horses, sheep, pigs and goats), as well as rodents (e.g., mice, hamsters, rats and guinea pigs), canines, felines, primates, lupine, camelid, cervidae, rodent, avian and ichthyces.

"Antagonist(s)" include all agents that interfere with wild-type and/or modified GPCR binding to an agonist, wild-type and/or modified GPCR desensitization, wild-type and/or modified GPCR binding arrestin, wild-type and/or modified GPCR endosomal localization, internalization, and the like, including agents that affect the wild-type and/or modified GPCRs as well as agents that affect other proteins involved in wild-type and/or modified GPCR signaling, desensitization, endosomal localization, resensitization, and the like.

"GPCR" means G protein-coupled receptor and includes GPCRs naturally occurring in nature, as well as GPCRs which have been modified. Such modified GPCRs are described in U.S. Ser. No. 09/993,844 filed on Nov. 5, 2001 and U.S. Ser. No. 10/054,616 filed on Jan. 22, 2002 which is incorporated herein by reference in its entirety.

"Abnormal GPCR desensitization" and "abnormal desensitization" mean that the GPCR desensitization pathway is disrupted such that the balance between active receptor and desensitized receptor is altered with respect to wild-type conditions. There may be more active receptor than normal or there may be more desensitized receptor than wild-type conditions. Abnormal GPCR desensitization may be the result of a GPCR that is constitutively active or constitutively desensitized, leading to an increase above normal in the signaling of that receptor or a decrease below normal in the signaling of that receptor.

"Concurrent administration," "administration in combination," "simultaneous administration," or "administered simultaneously" mean that the compounds are administered at the same point in time or sufficiently close in time that the results observed are essentially the same as if the two or more compounds were administered at the same point in time.

"Conserved abnormality" means an abnormality in the GPCR pathway, including but not limited to, abnormalities in GPCRs, GRKs, arrestin, AP-2 protein, clathrin, protein phosphatase and the like, that may cause abnormal GPCR signaling. This abnormal GPCR signaling may contribute to a GPCR-related disease.

"Desensitized GPCR" means a GPCR that presently does not have ability to respond to agonist and activate conventional G protein signaling. Desensitized GPCRs of the present invention do not properly respond to agonist, are phosphorylated, bind arrestin, constitutively localize in clathrin-coated pits, and/or constitutively localize to endocytic vesicles or endosomes.

"Desensitization pathway" means any cellular component of the desensitization process, as well as any cellular structure implicated in the desensitization process and subsequent processes, including but not limited to, arrestins, GRKs, GPCRs, AP-2 protein, clathrin, protein phosphatases, and the like. In the methods of assaying of the present invention, the polypep-

tides may be detected, for example, in the cytoplasm, at a cell membrane, in clathrin-coated pits, in endocytic vesicles, endosomes, any stages in between, and the like.

"GPCR signaling" means GPCR induced activation of G proteins. This may result in, for example, cAMP production.

"G protein-coupled receptor kinase" (GRK) includes any kinase that has the ability to phosphorylate a GPCR.

"G protein-coupled receptor phosphatase" includes any phosphatase that has the ability to dephosphorylate a GPCR.

"*Homo sapien* GPCR" means a naturally occurring GPCR in a *Homo sapien*.

"Inverse agonist" means a compound which, upon binding to the GPCR, inhibits the basal intrinsic activity of the GPCR. An inverse agonist is a type of antagonist.

An "isolated" or "purified" nucleic acid molecule or protein, biologically active portion thereof, or antibody is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5 and 3 ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For purposes of the invention, "isolated" when used to refer to nucleic acid molecules, excludes isolated chromosomes. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of another protein. When the protein or biologically active portion thereof is recombinantly produced, preferably, culture medium represents less than about 30%, 20%, 10%, or 5% of the volume of the protein preparation. When protein is produced by chemical synthesis, preferably the protein preparations have less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein chemicals.

"Modified GRK" means a GRK modified such that it alters desensitization.

"Naturally occurring GPCR" means a GPCR that is present in nature.

"Odorant ligand" means a ligand compound that, upon binding to a receptor, leads to the perception of an odor including a synthetic compound and/or recombinantly produced compound including agonist and antagonist molecules.

"Odorant receptor" means a receptor protein normally found on the surface of olfactory neurons which, when activated (normally by binding an odorant ligand) leads to the perception of an odor.

"Sensitized GPCR" means a GPCR that presently has ability to respond to agonist and activate conventional G protein signaling.

GPCRs and Desensitization

The present invention is generally directed to the detection of a GPCR ligand present in a heterogeneous solution that contains one or more GPCR ligands. For example, such solutions could include serum, blood, another biological sample, or an environmental sample.

G protein-coupled receptors (GPCRs) regulate a wide variety of physiological processes and are important targets for clinical drug discovery. GPCRs function in vivo as sensitive plasma-membrane sensors that sample the extracellular envi-

ronment for biologically active molecules. They transduce the binding event across the plasma membrane by interacting with one or more of the numerous classes of intracellular G proteins.

The exposure of a GPCR to agonist produces rapid attenuation of its signaling ability that involves uncoupling of the receptor from its cognate heterotrimeric G-protein. The cellular mechanism mediating agonist-specific or homologous desensitization is a two-step process in which agonist-occupied receptors are phosphorylated by a G protein-coupled receptor kinases (GRKs) and then bind an arrestin protein.

It has been discovered that after agonists bind GPCRs, G-protein coupled receptor kinases (GRKs) phosphorylate intracellular domains of GPCRs. After phosphorylation, an arrestin protein associates with the GRK-phosphorylated receptor and uncouples the receptor from its cognate G protein. The interaction of the arrestin with the phosphorylated GPCR terminates GPCR signaling and produces a non-signaling, desensitized receptor.

The arrestin bound to the desensitized GPCR targets the GPCR to clathrin-coated pits for endocytosis (i.e., internalization) by functioning as an adaptor protein, which links the GPCR to components of the endocytic machinery, such as adaptor protein-2 (AP-2) and clathrin. The internalized GPCRs are dephosphorylated and are recycled back to the cell surface desensitized. The stability of the interaction of arrestin with the GPCR is one factor which dictates the rate of GPCR dephosphorylation, recycling, and resensitization. The involvement of GPCR phosphorylation and dephosphorylation in the desensitization process has been exemplified in U.S. Ser. No. 09/993,844, filed Nov. 5, 2001, the disclosure of which is hereby incorporated by reference in its entirety.

The abnormal regulation of hormones that bind to G protein-coupled receptors underlies the pathogenesis of many diseases. The ability to measure serum and tissue levels of these regulators, while clinically and scientifically desirable, is presently limited to very specialized biochemical and immunochemical assays. The present invention provides generalized methods that evaluate a process common to GPCR activity, providing useful methods for the screening and diagnosis of GPCR-based disease. Additionally, the present invention provides a method of screening a sample, biological, environmental, or the like, for compounds which alter GPCR ligands.

The present inventors have harnessed this desensitization process common among GPCRs to develop a method of detecting the presence of a GPCR ligand in a biological or environmental solution. The present invention is related to methods of detecting the concentration, presence, absence, or altered concentration of GPCR agonists, ligands, antagonists, or related compounds in a biological sample, environmental sample such as water or soil, or other solution.

In one embodiment of the present invention, a biosensor is employed. The biosensor is a host cell or cells that include a GPCR and arrestin. In the presence of agonist, the arrestin binds the GPCR, and the GPCR is internalized. Preferably, this process is visualized by the detection of the arrestin or the GPCR. The biosensor may be used to detect the presence of an agonist in a test sample, such as a biological or environmental sample. The detection of agonists by biosensors of the present invention is useful in disease diagnosis, as well as in the detection of dangerous compounds in the environment.

The present inventors determined that the host cells including a GPCR and arrestin could be used to detect various ligands of a GPCR in a test sample. The biosensors of the present invention are useful for the detection of multiple bioactive isoforms of a ligand in a test sample. In U.S. Ser. No.

09/993,844 filed on Nov. 5, 2001, U.S. Ser. No. 10/054,616 filed on Jan. 22, 2002, and U.S. Ser. No. 10/101,235 filed on Mar. 19, 2002, which are hereby incorporated by reference in their entirety, cells expressing GPCRs and arrestin-GFP were used for the identification of a ligand, or antagonist, in a solution. In the present invention, the present inventors determined that cells expressing GPCRs and arrestin-GFP were useful for the detection of all bioactive isoforms of a ligand in the test sample, not just one ligand isoform. The present invention is useful for determining the concentration of all bioactive isoforms, not just one, of a ligand in a test sample.

In a preferred embodiment of the present invention, the test samples are heterogeneous. They may include various proteins and compounds. They may include multiple isoforms of a GPCR ligand.

The methods of the present invention present a number of advantages over current methods of detecting GPCR ligands in a test sample. The present methods are highly sensitive and specific. In the methods of the present invention, the GPCRs detect the various bioactive ligand species in the sample, as opposed to other antibody-based methods, such as RIA, which detect only the ligand species with the reactive epitope. Additionally, the present method broadly applies to all GPCRs and is easily adapted for the various GPCRs.

A particular strength of the bioassay of the present invention is the virtual elimination of all false positive results. In a standard RIA any epitope capable of interacting with antisera could produce a positive reading. In contrast, the bioassay of the present invention, employing a GPCR, measures bioactivity rather than immunoreactivity. A ligand-receptor interaction that results in arrestin translocation is biologically relevant regardless of the immunological properties of the ligand. The ability to determine the degree of biological activity in the absence of radioactivity in a serum or tissue sample is a much needed laboratory addition for identifying disease pathology or predicting potential complications arising from abnormal hormone concentrations.

As opposed to other methods, the methods of the present invention are not subject to cross-reactivity with other compounds in the sample. The methods of the present invention are specific for the detection of GPCR ligands which are biologically active and do not cross-react with compounds in the sample which are not biologically active. Additionally, as opposed to the methods of the present inventions, other methods of detection do not have the ability to detect all of the bioactive isoforms of a ligand in a test sample.

Detecting Gastrin

A number of disease conditions are associated with abnormal regulation of GPCR ligand concentration. The present invention provides a method of detecting the presence, absence, concentration, or change in concentration of a GPCR ligand. Using such methods, the present invention provides methods of diagnosing a disease or a disease-causative state.

Clinical assays are often hampered by an inability to diagnose disease when it exists, a false negative result, or inappropriately indicating pathology, a false positive result. The methods of the present invention are resistant to false negative results because the methods involve the detection of all bioactive isoforms of the GPCR ligands. For example, the present inventors have determined that the methods of the present invention detected all bioactive isoforms of gastrin rather than just immunoactive forms of gastrin, and the methods are resistant to false negative results. This is particularly evident from the robust response observed in response to pentagastrin, a potent receptor agonist that is not detectable

by the immunological assays. The experimental sensitivity for detecting endogenous ligand using hG17 as a standard was approximately 100-200 μM (200-400 pg/ml of hG17), a range approximating the upper limit of normal as defined by RIA (<200 pg/ml , approximately 50 to 90 pM). Various strategies to increase the sensitivity include blocking receptor recycling to allow for more internalization, sample concentration, or receptor modification by mutagenesis to increase affinity.

One embodiment of the present invention is the diagnosis of hypergastrinemia by analyzing the location of the CCK-B GPCR after exposure to gastrin in a test sample. Cells are provided that express the CCK-B GPCR and arrestin. The GPCR or arrestin may be detectably labeled. These cells are exposed to a test sample, and subsequent changes in the location of the GPCR or arrestin are analyzed. Such analyses may be quantitative and may indicate the concentration of gastrin, or biologically-active isoforms, in a test sample. As discussed below, the gastrin concentration may be indicative of a disease condition, such as hypergastrinemia.

Gastrin is a ligand which binds a GPCR, and is the major hormonal regulator of gastric acid secretion. Two major forms of gastrin are secreted (Gastrin-34 and Gastrin-17), however, all gastrins have an amidated tetrapeptide (Trp-Met-Asp-Phe-NH₂) at the carboxyl terminus, which imparts full biological activity. The vast majority of gastrin is produced in endocrine cells of the gastric antrum. Progastrin is known to be expressed in a number of mammalian tissues: the gastric antrum, jejunum, ileum, colon, and pancreas of the gastrointestinal tract; the ovaries, testicles, and spermatozoa of the genital tract; the cerebellum, vagus nerve, hypothalamus, pituitary, and adrenal medulla of the neuroendocrine tissue; and the bronchial mucosa of the respiratory tract, although it may be expressed in other tissues as well.

Gastrin is a member of the cholecystokinin (CCK) family of gastrointestinal (GI) peptides, hormones that bind to CCK-A and CCK-B receptors, GPCRs found in the GI tract and brain. The cloning and characterization of the CCK-B receptor as a typical heptahelical G protein-coupled receptor (GPCR) has provided a valuable tool in the study of gastrin. The human CCK-B receptor has a nanomolar affinity for gastrin and cholecystokinin. The circulating levels of CCK are beyond detection by conventional radioimmunoassay (RIA), but the major biologically active forms of gastrin, gastrin-17 and gastrin-34 that are secreted into the blood are immunologically detectable by RIA. Presently, serum gastrin measurements are performed by radioimmunoassay using antibodies directed against one or more distinct gastrin isoforms. Occasionally, antisera may show cross-reactivity to gastrin precursors or other serum proteins that vary in their biological potency or have no biological consequence related to CCK-B receptor signaling. Alternatively, patients have presented with symptoms of hypergastrinemia, and/or known gastrin-secreting tumors where the RIA determinations of serum gastrin were normal. This has led to the hypothesis that certain tumors may produce non-RIA detectable gastrin variants.

The two major biologically active forms of gastrin, 17 and 34 amino acids in length, are produced by enzymatic digestion of preprogastrin and secreted into the blood by gastric antral G cells. Gastrin primarily regulates the release of stomach acid and the growth of GI mucosa, and its oversecretion is associated with enterochromaffin cell hyperplasia and tumors.

In the endoplasmic reticulum, the signal peptide of preprogastrin is cleaved resulting in progastrin. Further enzymatic modification of progastrin in the Golgi generates products

which are packaged into secretory granules. A number of secretory granule products are derived from preprogastrin: progastrin, glycine-extended gastrin-17, glycine-extended gastrin-34, gastrin-71, gastrin-34, gastrin-17, and gastrin-6.

Hypergastrinemia is associated with GI malignancies and consequently serum gastrin levels are routinely measured in clinical practice. Hypergastrinemia may occur in pathophysiologic states and serum gastrin levels can also become elevated in patients on prolonged acid suppressive medications. Presently, serum gastrin measurements are performed by radioimmunoassay (RIA), using antibodies directed against one or more distinct gastrin isoforms. Occasionally, antisera may show cross-reactivity to gastrin precursors or other serum proteins that vary in their biological potency or have no biological consequence related to CCK-B receptor signaling. Alternatively, patients have presented with symptoms of hypergastrinemia, and/or known gastrin-secreting tumors where the RIA determinations of serum gastrin were normal. This has led to the hypothesis that certain tumors may elaborate non-RIA detectable gastrin variants.

Detecting Acetylcholine

In another embodiment of the present invention, a number of chemical/biological agents of interest to the military and civilian communities may be sensed readily by the described sensors. The present invention may be used to detect biological agents, toxins, neurotoxins, nerve gases, and the like. The ability to rapidly and accurately detect and quantify biologically relevant molecules with high sensitivity is a central issue for medical technology, national security, public safety, environmental safety and civilian and military medical diagnostics.

Such a biosensor for the detection of agents, such as bioterrorism agents, in the environment provides a number of advantages over present detection methods. First, the assay is sensitive and, since based on the biological activity of the ligand, detects the presence of any bioactive variants of the ligand of interest. Secondly, the assay is quantitative and can detect altered or minimal concentrations of the ligand in the sample. The assay also can be monitored in a continuous fashion. Additionally, sample preparation is quite straightforward: a sample need only be suspended in an aqueous solution for detection.

A number of neurotoxins including sarin and organophosphate insecticides, for example diazinon, inhibit acetylcholinesterase, an enzyme which inactivates the neurotransmitter acetylcholine. In vivo, compounds which decrease acetylcholine esterase activity result in an increase in the concentration of acetylcholine in the synaptic cleft, producing excessive nerve excitation. Levels of acetylcholine in a test sample can be used to monitor acetylcholinesterase activity, and detect the presence of acetylcholinesterase inhibitors. One aspect of the present invention is the use of a single cell biosensor expressing the muscarinic receptor as a method of detecting the presence of acetylcholine esterase inhibitors in the environmental or a biological sample.

Another embodiment of the invention pertains to field-testing of environmental conditions. Automated sensing of environmental conditions, including the presence of natural chemicals, industrial wastes, and biological/chemical warfare agents is possible using an embodiment of the invention. Uploading of test results via radio transmission may provide remote sensing capabilities, and may provide response capabilities through human or central computer directed action. Response instructions may then be downloaded either to the sensing site or to another strategic response position. Such a system may be useful, for example, in determining the pres-

ence of toxins in a public water supply, and the subsequent centralized-directed cessation of water flow from the supply pool.

Described above are embodiments of the present invention employing the CCK-B or the muscarinic GPCRs. Additionally, the present invention encompasses biosensors employing any GPCR. By fluorescence microscopy, GPCR association with arrestin and subsequent internalization at least 40 different GPCRs using fusion proteins between arrestin and a green fluorescent protein is possible (FIG. 2). Each of these cells, as well as other like cells, is a useful biosensor of the present invention.

Methods of the Present Invention

The present invention provides highly specific, sensitive, generalized, and quantitative methods of analyzing the presence of GPCR-binding compounds in samples.

One embodiment of the present invention is a method of detecting a GPCR ligand in a test sample. Most preferably, this method comprises the steps of (a) providing a cell including a GPCR and an arrestin; (b) exposing the cell to the test sample; and (c) determining the cellular distribution of the GPCR or arrestin in the presence of the test sample, wherein the test sample is, or is derived from, a biological sample or an environmental sample.

In a preferred embodiment, the cellular distribution of the GPCR or arrestin in the presence of the test sample is compared to the cellular distribution of the GPCR or arrestin in the absence of the test sample. Different concentrations of the test sample may be analyzed. The cellular distribution may be determined at different time points after exposure to the test sample.

In one embodiment of the present invention, the GPCR or arrestin is detectably labeled, other endogenous molecules are detectably labeled, or exogenous molecules are detectably labeled. The distribution of the detectably labeled molecules represents the cellular distribution of the GPCR or arrestin. The distribution of the GPCR or arrestin may indicate the extent to which the GPCR is internalized. The cellular distribution of the detectably labeled molecule may be quantified.

In one embodiment of the present invention, a sample may include a known concentration of the ligand. By comparing the cellular distribution of the detectably labeled molecule in the presence of the test sample to the distribution in the presence of known concentrations of ligand, the concentration of ligand in the test sample may be determined.

The test sample may be a biological sample or an environmental sample. The biological sample may be or may be derived from serum, tissue, blood, or urine.

In one embodiment, the GPCR is CCK-B or CCK-A. The ligand may be gastrin, preprogastrin, cleaved preprogastrin, gastrin-34, gastrin-17, pentagastrin, progastrin, glycine-extended gastrin-17, glycine-extended gastrin-34, gastrin-71, or gastrin-6. In one embodiment, the GPCR is a muscarinic receptor. The ligand may be acetylcholine.

In a preferred embodiment, the labeled molecule is localized in the cytosol, clathrin-coated pits, the plasma membrane, endocytic vesicles, or endosomes. An increase in the local concentration of the labeled molecule results in a local increase in signal intensity. The signal intensity in the plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes may be greater than the signal intensity in the cytosol. The local signal intensity may be increased or decreased in the presence of increased or decreased amounts of a compound, such as a ligand, agonist, or antagonist.

In a preferred embodiment, the concentration of a ligand in the test sample indicates a disease state. The concentration of

the ligand in the test sample may indicate the presence of a compound in the test sample that alters the ligand concentration. The ligand concentration may indicate the presence of a compound in the test sample that modifies acetylcholine or inhibits acetylcholinesterase.

In a preferred embodiment, the detectable molecule is a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group. In one embodiment, a molecule may be detectably labeled due to its interaction with another molecule which is detectably labeled.

One embodiment of the present invention is a method of monitoring a GPCR ligand in mammals, wherein the analysis of the ligand concentration in a test sample is based on the binding of the ligand to the GPCR. This method may be used to monitor a clinical condition and/or may indicate the presence of a disease state. The clinical condition may indicate that the subject has a disorder or is at risk for developing a disorder. The clinical condition may be gastrointestinal cancer, hypergastrinemia, atrophic gastritis, gastric ulcers, or malignant tumors.

Arrestin coupled to a detectable molecule may be detected and monitored as it functions in the GPCR pathway. The location of the arrestin may be detected, for example, evenly distributed in the cell cytoplasm, concentrated at a cell membrane, concentrated in clathrin-coated pits, localized in endocytic vesicles or endosomes, and the like. The proximity of arrestin to a GPCR may be monitored, as well as the proximity to any other cell structure.

Preferably, the arrestin, the GPCR, and/or the arrestin/GPCR complex may be detected in endocytic vesicles or endosomes. The arrestin, the GPCR, and/or the arrestin/GPCR complex thus may be detected in endocytic vesicles or endosomes absence of agonist. The association of arrestin with a GPCR in endocytic vesicles or endosomes may give a strong, readily recognizable signal that persists for extended periods of time. Under magnification of 40 \times objective lens, the signal may be doughnut-like in appearance. The signal resulting from the compartmentalization of arrestin and GPCR colocalized in endocytic vesicles or endosomes is typically easy to detect. Similarly, blocking this association is easy to detect. Examples of detection methods are described herein. Such methods include, for example, polarization microscopy, BRET, FRET, evanescent wave excitation microscopy, and standard or confocal microscopy.

One embodiment of the present invention is a method of measuring the gastrin concentration in a test sample. By employing cell lines permanently expressing the gastrin receptor (CCK-B) and a fusion protein consisting of β -arrestin 2 and green fluorescent protein (GFP), the present inventors have constructed a single cell biosensor for the measurement of serum gastrin. The quantitative redistribution of arrestin-GFP in response to agonist-activated gastrin receptors was measured by analysis of cell images obtained by fluorescence confocal microscopy, and provided a sensitive and specific determination of receptor activation. Such a single cell biosensor is a practical means to measure the bioactive serum concentration of gastrin, allowing the diagnosis of hypergastrinemia.

In one embodiment, the presence of GPCR ligand in the test sample indicates the presence of a disease, that a subject has a disorder, or is at risk for getting a disorder. Alternatively, the absence, altered concentration, or other alteration of the GPCR ligand may indicate the presence of a disease, that a subject has a disorder, or is at risk for getting a disorder.

In one embodiment, the analysis of the GPCR ligand indicates the presence, absence, enhancement, inhibition, or other

alteration of a compound that alters the GPCR ligand. The analysis may indicate the presence, absence, altered concentration, or other alteration of the ligand. The compound that alters the GPCR ligand may be an enzyme, an inhibitor, an activator, a small molecule, or other compound that directly affects the GPCR ligand. The compound that alters the GPCR ligand may be an enzyme, an inhibitor, an activator, a small molecule, or other compound that indirectly affects the GPCR ligand.

In a specific embodiment, the GPCR is the muscarinic receptor and the method of determining the concentration of acetylcholine in a test sample. By employing cell lines transiently transfected with the muscarinic receptor and a fusion protein consisting of β -arrestin 2 and green fluorescent protein, the present inventors have constructed a single cell biosensor for the measurement of acetylcholine in a sample.

Acetylcholine, the ligand of the muscarinic receptor, is altered by acetylcholinesterase. In the presence of acetylcholinesterase, the concentration of acetylcholine in a test sample is decreased. A decrease in the amount of acetylcholine in a test sample decreased the amount of internalization of the muscarinic receptor, as visualized by the decreased internalization of the arrestin-GFP conjugate.

An additional embodiment of the present invention is related to methods of increasing the sensitivity of the above methods. In one aspect, as described in U.S. Ser. No. 09/993,844 filed on Nov. 5, 2001, which is hereby incorporated by reference in its entirety, the GPCR itself may be modified in its C-terminal tail such that it has enhanced phosphorylation sites. The sensitivity of the assay may also be increased with GRK over-expression. The biosensor may be exposed to the test sample for longer periods of time, or at increased concentrations, in order to increase the signal.

Expression of the Proteins

Another feature of this invention is the expression of the DNA sequences encoding a GPCR and/or arrestin in a cell to form a biosensor, as disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host.

Such operative linking of a DNA sequence to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids colEI, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage λ , e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Any of a wide variety of expression control sequences—sequences that control the expression of a DNA sequence operatively linked to it—may be used in these vectors to express the DNA sequences. Such useful expression control sequences include, for example, the early or late promoters of

SV40, CMV, vaccinia, polyoma or adenovirus, the lac system, the trp system, the TAC system, the TRC system, the LTR system, the major operator and promoter regions of phage λ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast α -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, plant cells, nematode cells, and animal cells, such as HEK-293, CHO, R11, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture. However, mammalian cells are preferred for creating the biosensors of the invention.

It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences on fermentation or in large scale animal culture.

As mentioned above, a DNA sequence encoding a modified GPCR can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the GPCR amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature*, 292:756 (1981); Nambair et al., *Science*, 223:1299 (1984); Jay et al., *J. Biol. Chem.*, 259:6311 (1984).

Synthetic DNA sequences allow convenient construction of genes which will express GPCR analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native or modified GPCR genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis.

A general method for site-specific incorporation of unnatural amino acids into proteins is described in Christopher J.

Noren, Spencer J. Anthony-Cahill, Michael C. Griffith, Peter G. Schultz, *Science*, 244:182-188 (April 1989). This method may be used to create analogs with unnatural amino acids.

Conjugates

The cells used in the methods of assaying of the present invention may comprise a conjugate of an arrestin protein and a detectable molecule, a conjugate of a GPCR and a detectable molecule, a conjugate of any member of a GPCR/arrestin complex and a detectable molecule, a conjugate of a detectable molecule and a molecule that interacts with any member of a GPCR/arrestin complex, and the like. The detectable molecule allows detection of molecules interacting with the detectable molecule, as well as the molecule itself.

All forms of arrestin, naturally occurring and engineered variants, including but not limited to, visual arrestin, β -arrestin 1 and β -arrestin 2, may be used in the present invention. GPCRs may interact to a detectable level with all forms of arrestin.

Detectable molecules that may be used include, but are not limited to, molecules that are detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, radioactive, and optical means, including but not limited to bioluminescence, phosphorescence, and fluorescence. These detectable molecules should be a biologically compatible molecule and should not compromise the biological function of the molecule and must not compromise the ability of the detectable molecule to be detected. Preferred detectable molecules are optically detectable molecules, including optically detectable proteins, such that they may be excited chemically, mechanically, electrically, or radioactively to emit fluorescence, phosphorescence, or bioluminescence. More preferred detectable molecules are inherently fluorescent molecules, such as fluorescent proteins, including, for example, Green Fluorescent Protein (GFP). The detectable molecule may be conjugated to the arrestin protein by methods as described in Barak et al. (U.S. Pat. Nos. 5,891,646 and 6,110,693). The detectable molecule may be conjugated at the front-end, at the back-end, or in the middle.

The GPCRs may also be conjugated with a detectable molecule. Preferably, the carboxyl-terminus of the GPCR is conjugated with a detectable molecule. If the GPCR is conjugated with a detectable molecule, proximity of the GPCR with the arrestin may be readily detected. In addition, if the GPCR is conjugated with a detectable molecule, compartmentalization of the GPCR with the arrestin may be readily confirmed. The detectable molecule used to conjugate with the GPCRs may include those as described above, including, for example, optically detectable molecules, such that they may be excited chemically, mechanically, electrically, or radioactively to emit fluorescence, phosphorescence, or bioluminescence. Preferred optically detectable molecules may be detected by immunofluorescence, luminescence, fluorescence, and phosphorescence.

For example, the GPCRs may be antibody labeled with an antibody conjugated to an immunofluorescence molecule or the GPCRs may be conjugated with a luminescent donor. In particular, the GPCRs may be conjugated with, for example, luciferase, for example, *Renilla* luciferase, or a rhodamine-conjugated antibody, for example, rhodamine-conjugated anti-HA mouse monoclonal antibody. Preferably, the carboxyl-terminal tail of the GPCR may be conjugated with a luminescent donor, for example, luciferase. The GPCR, preferably the carboxyl-terminal tail, also may be conjugated with GFP as described in L. S. Barak et al. Internal Trafficking

and Surface Mobility of a Functionally Intact β 2-Adrenergic Receptor-Green Fluorescent Protein Conjugate, *Mol. Pharm.* (1997) 51, 177-184.

Cell Types and Substrates

The cells of the present invention express at least one GPCR, and arrestin, wherein at least one of the molecules is detectably labeled. Cells useful in the present invention include eukaryotic and prokaryotic cells, including, but not limited to, bacterial cells, yeast cells, fungal cells, insect cells, nematode cells, plant cells, and animal cells. Suitable animal cells include, but are not limited to, HEK cells, HeLa cells, COS cells, U2OS cells and various primary mammalian cells. An animal model expressing a conjugate of an arrestin and a detectable molecule throughout its tissues or within a particular organ or tissue type, may also be used in the present invention.

A substrate may have deposited thereon a plurality of cells of the present invention. The substrate may be any suitable biologically substrate, including but not limited to, glass, plastic, ceramic, semiconductor, silica, fiber optic, diamond, biocompatible monomer, or biocompatible polymer materials.

Methods of Detection

Methods of detecting the intracellular location of the detectably labeled arrestin, the intracellular location of a detectably labeled GPCR, or interaction of the detectably labeled arrestin, or other member of GPCR/arrestin complex with a GPCR or any other cell structure, including for example, the concentration of arrestin or GPCR at a cell membrane, colocalization of arrestin with GPCR in endosomes, and concentration of arrestin or GPCR in clathrin-coated pits, and the like, will vary dependent upon the detectable molecule(s) used.

One skilled in the art readily will be able to devise detection methods suitable for the detectable molecule(s) used. For optically detectable molecules, any optical method may be used where a change in the fluorescence, bioluminescence, or phosphorescence may be measured due to a redistribution or reorientation of emitted light. Such methods include, for example, polarization microscopy, BRET, FRET, evanescent wave excitation microscopy, and standard or confocal microscopy.

In a preferred embodiment arrestin may be conjugated to GFP and the arrestin-GFP conjugate may be detected by confocal microscopy. In another preferred embodiment, arrestin may conjugated to a GFP and the GPCR may be conjugated to an immunofluorescent molecule, and the conjugates may be detected by confocal microscopy. In an additional preferred embodiment, arrestin may conjugated to a GFP and the carboxy-terminus of the GPCR may be conjugated to a luciferase and the conjugates may be detected by bioluminescence resonance emission technology. In a further preferred embodiment arrestin may be conjugated to a luciferase and GPCR may be conjugated to a GFP, and the conjugates may be detected by bioluminescence resonance emission technology. The methods of the present invention are directed to detecting GPCR activity. The methods of the present invention allow enhanced monitoring of the GPCR pathway in real time.

In a preferred embodiment, the localization pattern of the detectable molecule is determined. In a further preferred embodiment, alterations of the localization pattern of the detectable molecule may be determined. The localization pattern may indicated cellular localization of the detectable molecule. Certain methods of detection are described in U.S. Ser. No. 10/095,620, filed Mar. 12, 2002, which claims pri-

ority to U.S. Provisional Patent Application No. 60/275,339, filed Mar. 13, 2001, the contents of which are incorporated by reference in their entirety.

Molecules may also be detected by their interaction with another detectably labeled molecule, such as an antibody.

Test Kits

The present invention includes test kits for analysis of test samples. Most preferably, the test kits would be useful for determining the GPCR ligand concentration in a biological or environmental sample. Even more preferably, the test kit would include a host cell expressing a GPCR and arrestin, and method of determining ligand concentration in sample.

EXAMPLES

The invention will be further explained by the following illustrative examples which are intended to be non-limiting.

Example 1

Materials & Methods

Human Embryonic Kidney Cells (HEK-293) were obtained from the American Type Culture Collection (ATCC, Manassas, Va.). Media, fetal bovine serum (FBS), and antibiotics were purchased from MediaTech (Herndon, Va.) and Gibco Invitrogen Corp (Carlsbad, Calif.). 96 well glass plates were obtained from Whatman (Clifton, N. J.) and binding resins from BioRad Laboratories (Hercules, Calif.). The CCK-A receptor antagonist devazepide and a specific CCK-B receptor antagonist were used. Pentagastrin was obtained from Sigma.

Membrane Preparation/Binding—All steps were performed at 4° C. Membrane binding was performed in triplicate as described in Shetzline et al., *J. Biol. Chem.* 273:6756-6752 (1998). Membrane fractions were assayed immediately or stored at -80° C. Saturation or competition binding was performed with [³H]CCK-8 polypeptide (Peninsula Labs, San Carlos, Calif.), with non-specific binding determined in the presence of 1 μM hG17. Competitive binding was performed using hG17. Data were analyzed using Graph Pad-Prism.

Cloning of the Human Gastrin Receptor: The human gastrin/CCK-B receptor cDNA was amplified from a human brain cDNA library (Clontech) using two oligonucleotide primers matching the 5' and 3' ends of the coding region, a sense oligonucleotide (5'-GCGCCC GCTAGCACCGCCATGGAGCTGCTAAAGCTGAACCG-G) (SEQ ID NO: 17) with a NheI restriction site, and an antisense oligonucleotide (5'-GCGCCC GGTACCTCAGCCAGGGCCAGTGTGCTGAT) (SEQ ID NO: 18) with a KpnI restriction site. The 1.4 kb amplified CCK-B receptor DNA band was subcloned into the pcDNA3.1-ZEO(-) expression vector (Invitrogen) at NheI and KpnI and verified on an ABI 377 fluorescent scanner.

Cell Culture and Transfection: Transient transfection in HEK-293 cells was done as described in Walker et al., *J. Biol. Chem.* 274:31515-23.

Inositol Phosphate Determination—HEK 293 cells expressing the CCK-B receptors were plated into 12-well plates coated with 25 μg/ml Poly-D-Lysine (Sigma Cat# P-6407, St. Louis, Mo.) and incubated overnight at 37° C. in MEM containing 10% FBS (fetal bovine serum). They were next placed for 24 hours in labeling media (1 μCi/0.5 mL/well of [³H]inositol in 5% FBS/MEM/Gentamicin), washed with MEM, 20 mM HEPES, pH 7.40, 20 mM LiCl for 5 minutes at

37° C., and treated with agonist. The reactions were stopped by addition of 500 μL of ice-cold 0.8 M HClO₄, the cells gently agitated at 4° C. for 30-60 minutes and the cell lysate then added to polypropylene tubes. 200 μL of neutralizing solution (0.72 M KOH/0.6 M KCO₃) was added to each tube and the tubes remained at 4° C. until analysis. BioRad AG-1X8 Resin (200-400 mesh) columns (BioRad Econo-Pac) were prepared with 1 ml of 50% slurry each in order to assay IP3 activity. Each column was washed twice with 10 ml of 18 megaohm deionized water, 800 μL of lysate was added, and after 5-10 minutes the columns were again washed twice with 10 mL of water. Samples were eluted into scintillation vials containing 15 mL of Lefko-Fluor (Research Products International Mt. Prospect, Il) using 3.5 mL of a 1 M ammonium formate/0.1 M formic acid solution. Fifty μL lysate samples corresponding to each fraction were also counted to determine total radioactivity uptake per sample.

Fluorescence Confocal Microscopy and Data Analysis:

The measurements of arrestin-GFP translocation for quantitative determinations of dose responses or of patient serum levels were done in the following manner. Cells permanently expressing the human CCK-B receptor and arrestin-GFP were seeded at 20,000 cells per well in 96 well, glass bottomed plates in 200 μL of MEM supplemented with 10% fetal bovine serum. 100 μL was removed and replaced by media containing a known concentration of hG17 peptide, pentagastrin, or a known volume of patient serum. Fluorescence cell images were obtained with a Zeiss LSM-510 confocal microscope. The dynamic translocation of arrestin-GFP over 5 minutes was analyzed as described. Static cell images obtained after one-two hour incubations were analyzed using the computer program IP LABS (Scanalytics, Fairfax, Va. 22031).

The analysis of translocation proceeded as follows. An average and standard deviation of pixel intensity was determined for images of untreated cells containing arrestin-GFP. An intensity corresponding to 3 standard deviations above the mean for these cells was set as the threshold to define translocation. To determine the subset of pixels representing translocated arrestin-GFP, only pixels that had at least one neighbor above this threshold were counted. This second restriction was set to eliminate noise. The measured amount of translocation, TI, in an image was then calculated to be sum of the intensities from pixels representative of translocation. The number of cells in an image could vary, but the total amount of fluorescence obtained from an image was independent of the distribution of chromophore and remained constant over time. To correct for the variation in the number of cells contained in different images, the calculated translocation for an image was normalized by the total image fluorescence, TF (i.e. sum of intensities for all pixels in the image). The computed translocation was defined as TI/TF. The mean intensity of the untreated cells was set to fall within the bottom 10% of the dynamic range of the microscope imaging system in order to avoid clipping the signal from areas with large amounts of receptor/arrestin-GFP complexes. Data are presented as mean±SEM.

Evaluation of the Signal to Noise (See FIG. 7) In order to evaluate the signal/noise ratio, the following two assumptions were made about the experimental system; (1) the sum of the intensities over all pixels, is independent of time and redistribution of βarrestin2-GFP and (2) the intensity distribution of cell fluorescence is gaussian and is given by the normalized probability distribution:

$$P(I) = \frac{2}{\sigma \cdot \sqrt{\pi} \cdot [1 + \operatorname{erf}(I_o/\sigma)]^{1/2}} \exp\left(-\frac{(I - I_o)^2}{\sigma^2}\right); \int_0^\infty P(I) dI = 1.$$

I is the intensity, I_o is the mean intensity, σ is the standard deviation, $\exp(z)$ is the exponential function, and $\operatorname{erf}(z)$ is the error function

$$\operatorname{erf}(z) = \frac{2}{\sqrt{\pi}} \cdot \int_0^z \exp(-z^2) \cdot dz.$$

The threshold for measuring the signal from translocated β arrestin2-GFP is to be set to $I_o + j$ where $j = \beta \times \sigma$. The mean square deviation of intensity above this threshold in the absence of agonist is:

$$\langle \sigma^2 \rangle_j = \langle (I - (I_o + j))^2 \rangle_j = \int_{I_o + j}^\infty P(I) (I - (I_o + j))^2 dI,$$

where

$$\sigma_N = \sqrt{\langle \sigma^2 \rangle_j} = \frac{\frac{\sigma}{\sqrt{2}} \left[(1 - \operatorname{erf}(\beta))(1 + 2\beta^2) - \frac{2\beta}{\sqrt{\pi}} \exp(-\beta^2) \right]^{1/2}}{[1 + \operatorname{erf}(I_o/\sigma)]^{1/2}}$$

A mean intensity of translocated β -arrestin2-GFP, I_f , can be determined over the subset of pixels, N_a that exceed the threshold value of intensity $I_o + j$. I_f results from an intensity contribution from translocated β -arrestin2-GFP and from untranslocated protein. It is related to the average intensity I_o before translocation by:

$$I_f = \eta \cdot I_o \cdot \frac{N_b}{N_a} + (1 - \eta) \cdot I_o,$$

N_b is the number of pixels that image the cells prior to translocation, I_o as defined above is their mean intensity, and

η is the fraction of translocated receptors. The ratio

$$\frac{N_b}{N_a}$$

represents the magnitude of the change in volume occupied by the β arrestin-GFP after translocation. The signal to noise ratio for a typical individual pixel that exceeds the threshold value can now be calculated as:

$$S/N = \frac{I_f - (I_o + \beta \cdot \sigma)}{\sigma_N} = \frac{\eta \cdot I_o \cdot \frac{N_b}{N_a} + (1 - \eta) \cdot I_o - I_o - \beta \cdot \sigma}{\sigma_N},$$

which simplifies to:

$$S/N = \frac{\sqrt{2} \cdot [1 + \operatorname{erf}(I_o/\sigma)]^{1/2}}{\left[(1 - \operatorname{erf}(\beta))(1 + 2\beta^2) - \frac{2\beta}{\sqrt{\pi}} \exp(-\beta^2) \right]^{1/2}} \cdot \left\{ \eta \cdot \frac{I_o}{\sigma} \cdot \left[\frac{N_b}{N_a} - 1 \right] - \beta \right\}$$

and

$$\eta \cdot \frac{I_o}{\sigma} \cdot \left[\frac{N_b}{N_a} - 1 \right] - \beta > 0$$

15

For a homogeneous line of cells described by a narrow gaussian distribution of intensity the ratio of the mean intensity to its standard deviation

20

$$\frac{I_o}{\sigma}$$

25 can be chosen greater than 4 by appropriately adjusting the range of the imaging system. The term $\sqrt{2} \cdot [1 + \operatorname{erf}(I_o/\sigma)]^{1/2}$ is then approximately equal to 2. The fraction of translocated receptors represented by the term η varies between 0 (no translocation) and 1 (100% translocation) whereas the ratio of the volumes (areas) due to redistribution of the arrestin-GFP

30

$$\frac{N_b}{N_a}$$

35

may vary between 5-100 depending upon the imaging system and the identity of the cellular compartment containing the translocated arrestins (for example, membrane, coated pits, or endosomes). The term in the denominator

40

$$\left[(1 - \operatorname{erf}(\beta))(1 + 2\beta^2) - \frac{2\beta}{\sqrt{\pi}} \exp(-\beta^2) \right]^{1/2}$$

45

equals 0.014 and 0.00070 for the threshold intensity set to $b=2$ and $b=3$ standard deviations above the mean intensity respectively. Therefore the signal to noise, S/N , can easily exceed 10^3 - 10^4 for very homogeneous populations of cells with even minimal amounts of translocation

50

55

$$\eta > \frac{\beta}{\frac{I_o}{\sigma} \cdot \left[\frac{N_b}{N_a} - 1 \right]} > \frac{3}{4 \cdot 10} \cong 0.1.$$

60 Even though an inhomogeneous fluorescence distribution or background fluorescence will cause the intensity profile to depart from this ideal case, the analysis indicates that translocation by simply considering intensity changes can be a very sensitive method for evaluating receptor behavior. Moreover, the use of simple pattern recognition algorithms could provide even greater discrimination of translocated arrestin and be useful for cell populations that are not homogenous such as in transient transfections.

65

Patient serum collection and serum determination by RIA: The Institutional Review Board approved this study at Duke University Medical Center. Patient serum was obtained from patients scheduled for serum gastrin analysis as requested by their primary care provider. Patients signed informed consent and an additional sample of serum was drawn for use in this study. Conventional RIA determinations were performed at Mayo Medical Laboratories, Rochester, Minn.

Example 2

Cells Expressing the CCK-B Receptor and Arrestin-GFP Respond to the Presence of Synthetic Human Gastrin-17 Peptide (hG17): Determination of the Uniformity of Arrestin-GFP and CCK-B Receptor Expression in Cells by Flow Cytometry and Arrestin-GFP Translocation

To simplify quantitative analysis of arrestin-GFP redistribution, cells which respond to ligand in an identical manner were established. In order to achieve a large degree of uniformity in receptor and arrestin expression among the entire cell population, an HEK-293 cell line permanently expressing the CCK-B receptor and arrestin-GFP was established. The degree of homogeneity of arrestin expression within the clone (Clone A) used in this study was determined by flow cytometry (FIG. 4A) and confirmed by fluorescence (FIG. 4B, Left Panel). The general ability of cells to respond to the presence of synthetic human gastrin-17 peptide (hG17) by redistributing arrestin-GFP is shown in FIG. 4B (Right Panel).

In FIG. 4A, the relative expression of arrestin-GFP in cells belonging to Clone A was determined using a Becton Dickinson FACScan flow cytometer. The x-axis is logarithmic in the relative cell intensity and the y-axis indicates the number of cells (Counts) at that intensity. Ninety-nine percent of the cell population was within the bounds of the bar seen above the intensity profile. FIG. 4B shows fluorescence images of a field of cells from Clone A before (left panel) and after treatment (right panel) with 10 nM hG17 for 5 minutes at room temperature.

Example 3

Characterization of Ligand Binding and Second Messenger Response

Clone A was further characterized by measuring the binding of the gastrin peptide agonists [³H]-CCK8 and hG17, and determining the hG17-mediated second messenger response. Saturation binding with [³H]-CCK8 showed that the cells expressed (14±1) pmol CCK-B receptor/mg cell protein and had an affinity for [³H]-CCK8 of (9.0±1.6) nM (FIG. 5A). Human gastrin-17 stimulation of inositol phosphate (IP3) production yielded an EC₅₀=(3.2±0.7) nM (FIG. 5B). The inset in FIG. 5B shows the competitive displacement of [³H]-CCK8 by hG17. The EC₅₀ was (28±5) nM and the K_d of hG17 for the CCK-B receptor was (17±3.5) nM.

FIG. 4 shows that 10 nM hG17 produced a measurable arrestin-GFP redistribution to the plasma membrane even after five minutes. After 30-60 minutes of exposure to hG17, endosomes containing arrestin-GFP became visible (FIG. 5C Upper Right Panel). This translocation was blocked completely by addition of 10 μM of the specific CCK-B receptor antagonist (FIG. 5C Lower Left Panel), but was not blocked

by 10 μM of the closely related CCK-A receptor antagonist devazepide (FIG. 5C Lower Right Panel). The data in FIG. 5C confirm that the CCK-B receptor is a class B GPCR, since it promotes arrestin internalization into endosomes.

FIG. 5A shows that cells from Clone A were incubated with increasing concentrations of [³H]CCK-8 in order to determine the average CCK-B receptor expression per cell and the receptor affinity for [³H]CCK-8. Total binding of [³H]CCK-8, □; specific binding of [³H]CCK-8, ■; non-specific binding in the presence of excess (1 μM) unlabeled hG17, ○. In FIG. 5B, Clone A cells were exposed to increasing concentrations of hG17 peptide in order to evaluate the IP3 second messenger response. The inset shows the competitive displacement of [³H]CCK8 by hG17 from this cell line. Data are presented as mean±SEM. FIG. 5C shows the fluorescence images of cells from Clone A that were treated with vehicle (upper left panel), or treated for one hour with 10 nM of the agonist hG17 (upper right panel), or with 10 nM hG17 plus 1 μM of the CCK-A antagonist devazepide (L-364,718, lower left panel); or with 10 nM hG17 plus 1 μM, of the CCK-B antagonist (lower right panel).

Example 4

Dose Response to hG17 and Analysis of Serum Samples from a Patient with Hypergastrinemia

Using increasing concentrations of hG17, the time-dependent loss of cytoplasmic arrestin-GFP was measured in order to determine if the pharmacology of arrestin redistribution correlated with that of IP3 production. Sequential fluorescence images of ligand-treated cells grown in 96 well plates were obtained in 30-second intervals over 5 minutes by confocal microscopy and analyzed by measuring the loss of cytosolic fluorescence. The time and dose dependence of arrestin redistribution for increasing concentrations of hG17 is plotted in FIG. 6A. From this data a dose response curve was calculated which resulted in an EC₅₀ for translocation of 4.2±1.5 nM (FIG. 6B), in agreement with the IP3 results.

Serum samples from a patient with hypergastrinemia were evaluated in addition to hG17 using this 5 minutes assay paradigm (FIG. 6A). Arrestin translocated in response to the serum, but the measured response occurred in a range approximating the lower limits of assay sensitivity for hG17. In order to increase assay sensitivity at concentrations near or below 1 nM agonist, arrestin-GFP redistribution in response to hG17 after 1-2 hours of incubation at 37° C. was directly measured. FIG. 6C shows representative cell fields that were exposed to increasing concentrations of hG17. Vesicles are readily apparent at concentrations below 10 nM hG17.

FIG. 6A illustrates a representative experiment depicting the response of Clone A cells that were exposed to various concentrations of hG17 for 5 minutes. Fluorescence images were obtained every 30 seconds and analyzed for arrestin-GFP translocation. Nine to eleven cells were analyzed for each time point for each curve. The graph shows the fractional amount of arrestin-GFP remaining in the cytosol as a function of time. FIG. 6B shows that the fractional amount of arrestin-GFP lost after 5 minutes from the cell cytosol was used to generate a sigmoid dose response curve for the increasing concentrations of hG17 shown in the graph in A. Data are presented as mean±SEM. FIG. 6C shows images from an

experiment demonstrating the response of Clone A cells exposed to hG17 for one hour at 37° C.

Example 5

Calculation of the Fluorescence Signal from the Distribution of β arrestin2-GFP

The methodology for these measurements of Example 4 is described in FIG. 7. The signal over background obtained by simply measuring fluorescent intensities was quite large due to the concentration of translocated arrestins in small volumes, as shown in FIG. 7B where a 120-fold increase was observed.

FIG. 7A shows the distribution of β arrestin-GFP fluorescence in cells stably expressing the β arrestin2-GFP fusion protein. The receptor was visualized before and after a 30-minute agonist treatment. Image intensity was acquired using 8 bits per pixel, yielding a grayscale range of intensities from 0-255. Relative intensities greater than 255 were clipped and set equal to 255. The upper and lower panels show the same field of cells. The cells appear brighter in the upper panels because the average pixel intensity in the upper images was selected to fall midway between 0-255. In the lower panels the mean pixel intensity falls within the bottom 12% of the dynamic range. The selection of a higher mean intensity caused many of the pixels representing translocated β arrestin2-GFP in the right upper panel to be clipped at a relative brightness of 255. This resulted in an underestimate of the amount of agonist-induced translocation, which is avoided in the lower images due to the selection of a smaller mean intensity. FIG. 7B illustrates a histogram of the pixel count versus pixel intensity (green curve in the graph at the lower left) was generated using a representative control cell. The first minimal intensity peak represents background and the second peak is green fluorescent protein. The mean cell intensity plus three standard deviations (>99th percentile) was selected as a threshold to separate the fluorescence signal of the untranslocated cytosolic β arrestin, from the β arrestin that translocated with the receptor into vesicles. Pixels with intensities above this baseline (magenta curve corresponds to vesicles in the treated cells) are indicated by the magenta color overlay in both the control and treated images (upper left and right panels). Note the correspondence between the magenta-colored pixels in the upper right image of FIG. 7B and the β arrestin-GFP-containing endocytic vesicles in the lower right image of FIG. 7A. Comparison of the total pixel intensity from pixels above the baseline for the two images is depicted in the lower right graph and shows a 120-fold increase in the fluorescence signal in the treated cells. Image data were analyzed by the computer program IP labs.

Example 6

Detection of Immunologically Undetectable Gastrin Receptor Agonists in serum: Dose Response to Pentagastrin

There have been numerous reports of clinical hypergastrinemia in the absence of elevated serum gastrin levels. Consequently, RIA detection of serum gastrin may miss a group of patients with immunologically undetectable gastrin receptor agonists. Therefore the effects on translocation of the CCK-B receptor agonist pentagastrin, which is not detectable by conventional RIA, were evaluated. As illustrated in FIG. 8, the response of the biosensor to increasing amounts of pentagastrin produced a dose-response with an EC₅₀ for transloca-

tion of 2.4±1.9 nM, similar to the reported K_d of 1 nM for pentagastrin binding to the CCK-B receptor and 3.9 nM for polyphosphoinositide turnover.

FIG. 8A shows images from a representative experiment depicting the response of Clone A cells that were exposed to pentagastrin at 37° C. for two hours. Fluorescence images were analyzed for arrestin-GFP translocation as described in Methods. The graph in FIG. 8B depicts the increase in the normalized sum of pixel intensity (TI/TF) above a threshold value (Methods) for images obtained at each concentration of ligand. Data are representative of two experiments, each with eight to ten separate images and are presented as mean±SEM.

Example 7

Detection of Gastrin from Patient with Hypergastrinemia: Dose Response at One Hour

The ability of the biosensor to respond to various synthetic gastrin isoforms suggested its potential to measure the multitude of bioactive forms of gastrin contained in human serum. The upper panels in FIG. 9A show the cellular response to a patient serum sample (documented hypergastrinemia by RIA of 5000 pg/ml, range of 3,400 to 6,600 pg/ml) after one hour of incubation. Arrestin-GFP was seen at the plasma membrane and in vesicles. The amount of translocated arrestin-GFP was compared to an hG17 standard curve generated from the data represented by the images of FIG. 6C. The EC₅₀ of the hG17 dose response curve was (0.80±0.25) nM (FIGS. 6B and 6C), and the patient's bioactive gastrin serum concentration was determined to be 0.63 nM±0.16 nM (see arrow FIG. 9B).

Depicted in FIG. 9A is a representative image of Clone A cells incubated for one hour with a 1:1 dilution of serum that was obtained from a patient with hypergastrinemia. FIG. 9B shows a dose response curve to hG17 of Clone A cells computed from the imaged translocation data obtained at one hour and analyzed as in FIG. 7. Between 9 and 16 separate images were analyzed for each hG17 concentration and the patient's serum (arrow). Data are presented as mean±SEM.

The present inventors demonstrated that the agonist-mediated arrestin interaction with the CCK-B receptor mirrored the pharmacology of CCK-B receptor signaling using a biosensor consisting of a cell permanently expressing CCK-B receptors and β arrestin2-GFP (arrestin-GFP). Moreover, this biosensor was used to determine the serum gastrin concentration of a patient with hypergastrinemia. All bioactive gastrin isoforms, including those not identified with conventional RIA, were detected with methods of the present invention.

These data show that the CCK-B receptor underwent agonist-mediated arrestin regulation. The IP₃ receptor signaling was activated at the same gastrin and pentagastrin concentrations that produced arrestin translocation. The residues in gastrin that produce CCK-B receptor conformations capable of desensitization likely reside in the gastrin terminal pentapeptide.

After the CCK-B receptor bound agonist and arrestin initiated receptor internalization, the CCK-B receptor moved into the cell via arrestin-mediated clathrin-coated vesicular pathway. The CCK-B receptor was shown to be a "Class B" GPCR and subsequent receptor endocytosis may initiate secondary (or intracellular) signaling events, for example MAP kinase activation.

This pharmacology was exploited to construct a single cell biosensor to measure serum concentrations of gastrin. Given the large number of GPCRs that desensitize by arrestin, bio-

sensors have been similarly constructed for other GPCR ligands; additional biosensors will be constructed. The novelty of this biological approach is that combinations of G protein-coupled receptors and fluorescent proteins form some of the most sensitive biosensor arrays developed and provide a mechanism to detect thousands of natural and synthetic compounds.

An area where synthetic compounds are clinically useful is in cancer chemotherapy. In particular, heptagastrin conjugated to an ellipticine moiety was used to kill tumors expressing the CCK-B receptor. The toxicity of this agent was shown to require receptor endocytosis, which based on our findings, was most likely arrestin dependent. This agent was administered to mice in concentrations well within the measurable range of our biosensor. The present invention has broad applications for the evaluation of newly designed drugs where the pharmacology is unknown and where serum or tissue levels need to be determined.

GPCRs signal the presence of bioactive substances. The present inventors exploited the common biochemical paradigm for terminating GPCR signaling and determined that GPCRs can also be used to detect those substances for clinical diagnosis.

Example 7

Muscarinic Receptor is Internalized in the Presence of Acetylcholine

HEK-293 cells expressing the human muscarinic receptor type 1 conjugated with the vasopressin carboxyl tail were exposed to micromolar concentrations of acetylcholine. The translocation of arrestin-GFP was determined. Arrestin-GFP was observed at the membrane edge or in vesicles, as shown in FIG. 10.

The biosensor expressing the muscarinic receptor and arrestin-GFP was useful for the detection of acetylcholine in a sample, as indicated by the agonist-induced internalization of arrestin-GFP.

Example 8

Acetylcholinesterase Inhibits Acetylcholine Induced Internalization of Muscarinic Receptor

HEK-293 cells were incubated in Minimal Essential Media containing 10% Fetal Bovine Serum (FBS). The cells had been transiently transfected with cDNA to induce the expression of arrestin-GFP and the human muscarinic receptor type 1 conjugated with the vasopressin carboxyl tail. In the presence of 10% FBS, no arrestin-GFP translocation was observed after the cells were exposed for up to 30 minutes to concentrations of acetylcholine in the range of 10-100 micromolar. However, millimolar amounts of acetylcholine did produce arrestin-GFP translocation. When cells were exposed to 10-20 micromolar concentrations of acetylcholine in the absence of serum, arrestin-GFP translocated readily to the cell membrane. Acetylcholinesterase, an enzyme known to degrade acetylcholine, is a common component of serum, including FBS. The acetylcholinesterase broke down the acetylcholine, the ligand of the muscarinic receptor, thereby preventing acetylcholine-induced internalization of the muscarinic receptor and arrestin. Exceeding large amounts of acetylcholine (millimolar) in the presence of acetylcholinesterase were able to produce only a transient amount of arrestin internalization. In contrast, a much smaller concentration of acetylcholine (10-20 micromolar) was able to produce a

robust response when serum was absent from the media. This suggests that arrestin translocation can be used to assay serum for inhibitors of acetylcholinesterase, as a potent inhibitor such as an organophosphate compound would produce effects similar to removing the serum and all its constituent ingredients entirely from the environment of the test cell containing arrestin-GFP and the acetylcholine-exposed muscarinic receptor.

Example 9

Use of Muscarinic Acetyl Choline Receptor to Screen for Acetylcholinesterase Inhibitors

A sample containing the putative inhibitor is extracted into an appropriate solvent, in one instance this may be an aqueous buffer. The extract either is diluted or combined with a buffer containing acetylcholinesterase protein and an agonist to the muscarinic receptor such as acetylcholine chloride sensitive to the acetylcholinesterase. This mixture containing the agonist, acetylcholinesterase, and the putative inhibitor is allowed to incubate for a given period of time between zero and a few hours, between 5 and 60 minutes is most practical, and then placed in contact with a cell containing the muscarinic receptor with its natural tail or the tail interchanged with a high affinity tail such as from the vasopressin receptor and arrestin-GFP. If a putative inhibitor of acetylcholinesterase is present, the acetylcholine chloride will not be broken down and translocation of the arrestin-GFP to the plasma membrane or endosomes will occur due to the activation of the receptor by the acetylcholine. If an inhibitor of the acetylcholinesterase is not present, the acetylcholine will be degraded and a lesser amount or no amount of arrestin-GFP translocation will occur. This assay can be used to assess commonly used inhibitors of the acetylcholinesterase enzyme in the environment such as the organophosphate insecticides, for example diazinon (EPA Completes Risk Assessment and Announces Risk Reduction Agreement for the Pesticide Diazinon. On Dec. 5, 2000, EPA released its revised risk assessment and announced an agreement with registrants to phase out/eliminate certain uses of the organophosphate pesticide diazinon.), and more potent inhibitors not commonly found such as derivatives the neurotoxin sarin. A particular use of such an assay system could be the continuous monitoring of a municipal water system for insecticides and like compounds by continuously adding aliquots of water, pre-mixed with an acetylcholine chloride like agonist and acetylcholinesterase, to chambers with cells containing arrestin-GFP and the muscarinic receptor, and observing the cells for loss of inhibition of arrestin-GFP translocation. The assays can be performed on a high throughput basis by instruments that are commercially available for this purpose. Another use of this assay would be to assess a person's physiological exposure to compounds that inhibit acetylcholinesterase by measuring the presence of these compounds in serum or tissue. For example a drop of blood could directly be placed in a well containing a cell exposed to acetylcholine chloride and possessing, arrestin-GFP and the muscarinic acetylcholine receptor. Human blood or serum normally contains sufficient acetylcholinesterase to rapidly degrade acetylcholine. Thus, a loss of inhibition of the ability of the cell to translocate arrestin-GFP would be indicative of the presence of an acetylcholinesterase inhibitor.

While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material,

and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The following documents, as well as any documents referenced in the foregoing text, should be considered as incorporated by reference in their entirety.

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Phe	Ser	Ser	Pro	Asp	Gly	Thr	Thr	Asp	Asp	Pro	Leu	Gly	Gly	His	Thr
50						55					60				
Val	Trp	Gln	Val	Val	Phe	Ile	Ala	Phe	Leu	Thr	Gly	Ile	Leu	Ala	Leu
65					70					75					80
Val	Thr	Ile	Ile	Gly	Asn	Ile	Leu	Val	Ile	Val	Ser	Phe	Lys	Val	Asn
				85					90					95	
Lys	Gln	Leu	Lys	Thr	Val	Asn	Asn	Tyr	Phe	Leu	Leu	Ser	Leu	Ala	Cys
			100					105						110	
Ala	Asp	Leu	Ile	Ile	Gly	Val	Ile	Ser	Met	Asn	Leu	Phe	Thr	Thr	Tyr
	115						120						125		
Ile	Ile	Met	Asn	Arg	Trp	Ala	Leu	Gly	Asn	Leu	Ala	Cys	Asp	Leu	Trp
	130					135						140			
Leu	Ala	Ile	Asp	Tyr	Val	Ala	Ser	Asn	Ala	Ser	Val	Met	Asn	Leu	Leu
	145				150					155					160
Val	Ile	Ser	Phe	Asp	Arg	Tyr	Phe	Ser	Ile	Thr	Arg	Pro	Leu	Thr	Tyr
				165					170					175	
Arg	Ala	Lys	Arg	Thr	Thr	Lys	Arg	Ala	Gly	Val	Met	Ile	Gly	Leu	Ala
			180					185						190	
Trp	Val	Ile	Ser	Phe	Val	Leu	Trp	Ala	Pro	Ala	Ile	Leu	Phe	Trp	Gln
		195					200						205		
Tyr	Phe	Val	Gly	Lys	Arg	Thr	Val	Pro	Pro	Gly	Glu	Cys	Phe	Ile	Gln
	210					215						220			
Phe	Leu	Ser	Glu	Pro	Thr	Ile	Thr	Phe	Gly	Thr	Ala	Ile	Ala	Ala	Phe
	225				230					235					240
Tyr	Met	Pro	Val	Thr	Ile	Met	Thr	Ile	Leu	Tyr	Trp	Arg	Ile	Tyr	Lys
				245					250					255	
Glu	Thr	Glu	Lys	Arg	Thr	Lys	Glu	Leu	Ala	Gly	Leu	Gln	Ala	Ser	Gly
			260					265						270	
Thr	Glu	Ala	Glu	Thr	Glu	Asn	Phe	Val	His	Pro	Thr	Gly	Ser	Ser	Arg
		275					280						285		
Ser	Cys	Ser	Ser	Tyr	Glu	Leu	Gln	Gln	Gln	Ser	Met	Lys	Arg	Ser	Asn
	290					295					300				
Arg	Arg	Lys	Tyr	Gly	Arg	Cys	His	Phe	Trp	Phe	Thr	Thr	Lys	Ser	Trp
	305				310					315					320
Lys	Pro	Ser	Ser	Glu	Gln	Met	Asp	Gln	Asp	His	Ser	Ser	Ser	Asp	Ser
				325					330					335	
Trp	Asn	Asn	Asn	Asp	Ala	Ala	Ala	Ser	Leu	Glu	Asn	Ser	Ala	Ser	Ser
				340				345						350	
Asp	Glu	Glu	Asp	Ile	Gly	Ser	Glu	Thr	Arg	Ala	Ile	Tyr	Ser	Ile	Val
		355					360					365			
Leu	Lys	Leu	Pro	Gly	His	Ser	Thr	Ile	Leu	Asn	Ser	Thr	Lys	Leu	Pro
	370					375						380			
Ser	Ser	Asp	Asn	Leu	Gln	Val	Pro	Glu	Glu	Glu	Leu	Gly	Met	Val	Asp
	385				390					395					400
Leu	Glu	Arg	Lys	Ala	Asp	Lys	Leu	Gln	Ala	Gln	Lys	Ser	Val	Asp	Asp
				405					410					415	
Gly	Gly	Ser	Phe	Pro	Lys	Ser	Phe	Ser	Lys	Leu	Pro	Ile	Gln	Leu	Glu
			420					425						430	
Ser	Ala	Val	Asp	Thr	Ala	Lys	Thr	Ser	Asp	Val	Asn	Ser	Ser	Val	Gly
		435					440						445		
Lys	Ser	Thr	Ala	Thr	Leu	Pro	Leu	Ser	Phe	Lys	Glu	Ala	Thr	Leu	Ala
	450					455							460		

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Lys Arg Phe Ala Leu Lys Thr Arg Ser Gln Ile Thr Lys Arg Lys Arg
 465 470 475 480
 Met Ser Leu Val Lys Glu Lys Lys Ala Ala Gln Thr Leu Ser Ala Ile
 485 490 495
 Leu Leu Ala Phe Ile Ile Thr Trp Thr Pro Tyr Asn Ile Met Val Leu
 500 505 510
 Val Asn Thr Phe Cys Asp Ser Cys Ile Pro Lys Thr Phe Trp Asn Leu
 515 520 525
 Gly Tyr Trp Leu Cys Tyr Ile Asn Ser Thr Val Asn Pro Val Cys Tyr
 530 535 540
 Ala Leu Cys Asn Lys Thr Phe Arg Thr Thr Phe Lys Met Leu Leu Leu
 545 550 555 560
 Cys Gln Cys Asp Lys Lys Lys Arg Arg Lys Gln Gln Tyr Gln Gln Arg
 565 570 575
 Gln Ser Val Ile Phe His Lys Arg Ala Pro Glu Gln Ala Leu
 580 585 590

<210> SEQ ID NO 6
 <211> LENGTH: 1773
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

atgaccttgc acaataacag tacaacctcg cctttgtttc caaacatcag ctctcctcgg 60
 atacacagcc cctccgatgc agggctgccc cegggaaaccg tcaactcattt cggcagctac 120
 aatgtttctc gagcagctgg caattttctcc tctccagacg gtaccaccga tgaccctctg 180
 ggaggtcata ccgtctggga agtgggtcttc atcgctttct taaegggeat cctggccttg 240
 gtgaccatca tcggcaacat cctggtaatt gtgtcattta aggtcaacaa gcagctgaag 300
 acgggtcaaca actacttctc ctttaagcctg gcctgtgccg atctgattat cggggtcatt 360
 tcaatgaatc tgtttacgac ctacatcatc atgaatcgat gggccttagg gaacttggcc 420
 tgtgacctct ggcttgccat tgactacgta gccagcaatg cctctgttat gaatcttctg 480
 gtcacagctc ttgacagata cttttccatc acgaggccgc tcacgtaccg agccaaaacga 540
 acaacaaaga gagccgggtg gatgatcggg ctggcttggg tcatctcctt tgccttttgg 600
 gctcctgcca tcttgttctg gcaatacttt gttggaaga gaactgtgcc tccgggagag 660
 tgcttcattc agttctcag tgagcccacc attacttttg gcacagccat cgtgctttt 720
 tataatgctg tcaccattat gactatttta tactggagga tctataagga aactgaaaag 780
 cgtaccaaag agcttgctgg cctgcaagcc tctgggacag aggcagagac agaaaacttt 840
 gtccacccca cgggcagttc tcgaagctgc agcagttacg aacttcaaca gcaaagcatg 900
 aaacgctcca acaggaggaa gtatggccgc tgccacttct gggtcacaac caagagctgg 960
 aaaccagctc ccgagcagat ggaccaagac cacagcagca gtgacagttg gaacaacaat 1020
 gatgctgctg cctcccctga gaactccgcc tcctccgacg aggaggacat tggctccgag 1080
 acgagagcca tctactccat cgtgctcaag cttccgggtc acagcaccat cctcaactcc 1140
 accaagttac cctcatcgga caacctgcag gtgcctgagg aggagctggg gatggtggac 1200
 ttggagagga aagccgacaa gctgcaggcc cagaagagcg tggacgatgg aggcagtttt 1260
 ccaaaaagct tctccaagct tcccatccag ctagagtcag ccgtggacac agctaagact 1320
 tctgacgtca actcctcagt gggtaagagc acggccaactc tacctctgtc cttcaaggaa 1380

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gccactctgg ccaagaggtt tgctctgaag accagaagtc agatcactaa gcggaaaagg 1440
atgtccctgg tcaaggagaa gaaagcggcc cagaccctca gtgcgatctt gcttgccctc 1500
atcatcactt ggaccaccata caacatcatg gttctgggtga acaccttttg tgacagctgc 1560
atacccaaaa ccttttgtaa tctgggctac tggtctgtgt acatcaacag caccgtgaac 1620
cccgtgtgct atgctctgtg caacaaaaca ttcagaacca ctttcaagat gctgctgctg 1680
tgccagtgtg acaaaaaaaaa gaggcgcaag cagcagtacc agcagagaca gtcggtcatt 1740
tttcacaagc gcgcaccoga gcaggccttg tag 1773
    
```

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<210> SEQ ID NO 7
<211> LENGTH: 478
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
    
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<400> SEQUENCE: 7

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Met Ala Asn Phe Thr Pro Val Asn Gly Ser Ser Gly Asn Gln Ser Val
 1           5           10           15
Arg Leu Val Thr Ser Ser Ser His Asn Arg Tyr Glu Thr Val Glu Met
 20           25           30
Val Phe Ile Ala Thr Val Thr Gly Ser Leu Ser Leu Val Thr Val Val
 35           40           45
Gly Asn Ile Leu Val Met Leu Ser Ile Lys Val Asn Arg Gln Leu Gln
 50           55           60
Thr Val Asn Asn Tyr Phe Leu Phe Ser Leu Ala Cys Ala Asp Leu Ile
 65           70           75           80
Ile Gly Ala Phe Ser Met Asn Leu Tyr Thr Val Tyr Ile Ile Lys Gly
 85           90           95
Tyr Trp Pro Leu Gly Ala Val Val Cys Asp Leu Trp Leu Ala Leu Asp
100           105           110
Tyr Val Val Ser Asn Ala Ser Val Met Asn Leu Leu Ile Ile Ser Phe
115           120           125
Asp Arg Tyr Phe Cys Val Thr Lys Pro Leu Thr Tyr Pro Ala Arg Arg
130           135           140
Thr Thr Lys Met Ala Gly Leu Met Ile Ala Ala Ala Trp Val Leu Ser
145           150           155           160
Phe Val Leu Trp Ala Pro Ala Ile Leu Phe Trp Gln Phe Val Val Gly
165           170           175
Lys Arg Thr Val Pro Asp Asn His Cys Phe Ile Gln Phe Leu Ser Asn
180           185           190
Pro Ala Val Thr Phe Gly Thr Ala Ile Ala Ala Phe Tyr Leu Pro Val
195           200           205
Val Ile Met Thr Val Leu Tyr Ile His Ile Ser Leu Ala Ser Arg Ser
210           215           220
Arg Val His Lys His Arg Pro Glu Gly Pro Lys Glu Lys Lys Ala Lys
225           230           235           240
Thr Leu Ala Phe Leu Lys Ser Pro Leu Met Lys Gln Ser Val Lys Lys
245           250           255
Pro Arg Pro Gly Gly Arg Pro Gly Gly Leu Arg Asn Gly Lys Leu Glu
260           265           270
Glu Ala Pro Pro Pro Ala Leu Pro Pro Pro Pro Arg Pro Val Ala Asp
275           280           285
Lys Asp Thr Ser Asn Glu Ser Ser Ser Gly Ser Ala Thr Gln Asn Thr
290           295           300
    
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Lys Glu Arg Pro Ala Thr Glu Leu Ser Thr Thr Glu Ala Thr Thr Pro
 305 310 315 320
 Ala Met Pro Ala Pro Pro Leu Gln Pro Arg Ala Leu Asn Pro Ala Ser
 325 330 335
 Arg Trp Ser Lys Ile Gln Ile Val Thr Lys Gln Thr Gly Asn Glu Cys
 340 345 350
 Val Thr Ala Ile Glu Ile Val Pro Ala Thr Pro Ala Gly Met Arg Pro
 355 360 365
 Ala Ala Asn Val Ala Arg Lys Phe Ala Ser Ile Ala Arg Asn Gln Val
 370 375 380
 Arg Lys Lys Arg Gln Met Ala Ala Arg Glu Arg Lys Val Thr Arg Thr
 385 390 395 400
 Ile Phe Ala Ile Leu Leu Ala Phe Ile Leu Thr Trp Thr Pro Tyr Asn
 405 410 415
 Val Met Val Leu Val Asn Thr Phe Cys Gln Ser Cys Ile Pro Asp Thr
 420 425 430
 Val Trp Ser Ile Gly Tyr Trp Leu Cys Tyr Val Asn Ser Thr Ile Asn
 435 440 445
 Pro Ala Cys Tyr Ala Leu Cys Asn Ala Thr Phe Lys Lys Thr Phe Arg
 450 455 460
 His Leu Leu Leu Cys Gln Tyr Arg Asn Ile Gly Thr Ala Arg
 465 470 475

<210> SEQ ID NO 8
 <211> LENGTH: 1437
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

atggccaact tcacacctgt caatggcagc tggggcaatc agtccgtgcg cctgggtcacg 60
 tcatcatccc acaatgcgta tgagacgggtg gaaatggtct tcattgccac agtgacaggc 120
 tccctgagcc tggtgactgt cgtgggcaac atcctgggtga tgctgtccat caaggtcaac 180
 aggcagctgc agacagtcaa caactacttc ctcttcagcc tggcgtgtgc tgatctcatc 240
 ataggcgctt tctccatgaa cctctacacc gtgtacatca tcaagggcta ctggcccctg 300
 ggcgccgtgg tctgcgacct gtggctggcc ctggactacg tggtagcaa cgctccgtc 360
 atgaaccttc tcatcatcag ctttgaccgc tacttctgcg tcaaccaagc tctcacctac 420
 cctgcccggc gcaccaccaa gatggcaggc ctcatgattg ctgctgcctg ggtactgtcc 480
 ttcgtgtctt gggcgctgct catcttgttc tggcagtttg tggtaggtaa gcggacgggtg 540
 cccgacaacc actgcttcat ccagttcctg tccaaccag cagtgcactt tggcacagcc 600
 attgtgctt tctacctgcc tgtggctcgc atgacgggtgc tgtacatcca catctccctg 660
 gccagtcgca gccgagtcca caagcaccgg cccgagggcc cgaaggagaa gaaagccaag 720
 acgctggcct tctcaagag cccactaatg aagcagagcg tcaagaagcc ccgcccggga 780
 ggcgcccggg gaggactgcg caatggcaag ctggaggagg cccccccgcc agcgtgcca 840
 ccgccaccgc gcccccgtgg tgataaggac acttccaatg agtccagctc aggcagtgcc 900
 acccagaaca ccaaggaacg cccagccaca gagctgtcca ccacagaggc caccactccc 960
 gccatgccg cccctcccct gcagccggcg gcctcaacc cagcctccag atggtecaag 1020
 atccagattg tgacgaagca gacaggcaat gactgtgtga cagccattga gattgtgcct 1080
 gccacgccgg ctggcatgcg ccctgcggcc aacgtggccc gcaagttcgc cagcatcgct 1140

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cgcaaccagg tgcgcaagaa gcggcagatg gcggcccggg agcgcaaagt gacacgaacg 1200
atctttgccca tctgtctagc cttcatcctc acctggacgc cctacaacgt catggtcctg 1260
gtgaacacct tctgccagag ctgcacccct gacacgggtg ggtccattgg ctactggctc 1320
tgctacgtca acagcaccat caaccctgcc tgctatgctc tgtgcaacgc cacctttaa 1380
aagaccttcc ggcacctgct gctgtgccag tatcggaaca tcggcaactgc caggtag 1437

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<210> SEQ ID NO 9

<211> LENGTH: 614

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

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Met Arg Pro Pro Gln Cys Leu Leu His Thr Pro Ser Leu Ala Ser Pro
 1          5          10          15
Leu Leu Leu Leu Leu Leu Trp Leu Leu Gly Gly Gly Val Gly Ala Glu
 20          25          30
Gly Arg Glu Asp Ala Glu Leu Leu Val Thr Val Arg Gly Gly Arg Leu
 35          40          45
Arg Gly Ile Arg Leu Lys Thr Pro Gly Gly Pro Val Ser Ala Phe Leu
 50          55          60
Gly Ile Pro Phe Ala Glu Pro Pro Met Gly Pro Arg Arg Phe Leu Pro
 65          70          75          80
Pro Glu Pro Lys Gln Pro Trp Ser Gly Val Val Asp Ala Thr Thr Phe
 85          90          95
Gln Ser Val Cys Tyr Gln Tyr Val Asp Thr Leu Tyr Pro Gly Phe Glu
100          105          110
Gly Thr Glu Met Trp Asn Pro Asn Arg Glu Leu Ser Glu Asp Cys Leu
115          120          125
Tyr Leu Asn Val Trp Thr Pro Tyr Pro Arg Pro Thr Ser Pro Thr Pro
130          135          140
Val Leu Val Trp Ile Tyr Gly Gly Gly Phe Tyr Ser Gly Ala Ser Ser
145          150          155          160
Leu Asp Val Tyr Asp Gly Arg Phe Leu Val Gln Ala Glu Arg Thr Val
165          170          175
Leu Val Ser Met Asn Tyr Arg Val Gly Ala Phe Gly Phe Leu Ala Leu
180          185          190
Pro Gly Ser Arg Glu Ala Pro Gly Asn Val Gly Leu Leu Asp Gln Arg
195          200          205
Leu Ala Leu Gln Trp Val Gln Glu Asn Val Ala Ala Phe Gly Gly Asp
210          215          220
Pro Thr Ser Val Thr Leu Phe Gly Glu Ser Ala Gly Ala Ala Ser Val
225          230          235          240
Gly Met His Leu Leu Ser Pro Pro Ser Arg Gly Leu Phe His Arg Ala
245          250          255
Val Leu Gln Ser Gly Ala Pro Asn Gly Pro Trp Ala Thr Val Gly Met
260          265          270
Gly Glu Ala Arg Arg Arg Ala Thr Gln Leu Ala His Leu Val Gly Cys
275          280          285
Pro Pro Gly Gly Thr Gly Gly Asn Asp Thr Glu Leu Val Ala Cys Leu
290          295          300
Arg Thr Arg Pro Ala Gln Val Leu Val Asn His Glu Trp His Val Leu
305          310          315          320
Pro Gln Glu Ser Val Phe Arg Phe Ser Phe Val Pro Val Val Asp Gly

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	325		330		335										
Asp	Phe	Leu	Ser	Asp	Thr	Pro	Glu	Ala	Leu	Ile	Asn	Ala	Gly	Asp	Phe
			340					345					350		
His	Gly	Leu	Gln	Val	Leu	Val	Gly	Val	Val	Lys	Asp	Glu	Gly	Ser	Tyr
		355					360					365			
Phe	Leu	Val	Tyr	Gly	Ala	Pro	Gly	Phe	Ser	Lys	Asp	Asn	Glu	Ser	Leu
	370					375					380				
Ile	Ser	Arg	Ala	Glu	Phe	Leu	Ala	Gly	Val	Arg	Val	Gly	Val	Pro	Gln
385				390						395					400
Val	Ser	Asp	Leu	Ala	Ala	Glu	Ala	Val	Val	Leu	His	Tyr	Thr	Asp	Trp
			405					410						415	
Leu	His	Pro	Glu	Asp	Pro	Ala	Arg	Leu	Arg	Glu	Ala	Leu	Ser	Asp	Val
			420					425						430	
Val	Gly	Asp	His	Asn	Val	Val	Cys	Pro	Val	Ala	Gln	Leu	Ala	Gly	Arg
		435					440					445			
Leu	Ala	Ala	Gln	Gly	Ala	Arg	Val	Tyr	Ala	Tyr	Val	Phe	Glu	His	Arg
	450					455					460				
Ala	Ser	Thr	Leu	Ser	Trp	Pro	Leu	Trp	Met	Gly	Val	Pro	His	Gly	Tyr
465					470					475					480
Glu	Ile	Glu	Phe	Ile	Phe	Gly	Ile	Pro	Leu	Asp	Pro	Ser	Arg	Asn	Tyr
			485					490						495	
Thr	Ala	Glu	Glu	Lys	Ile	Phe	Ala	Gln	Arg	Leu	Met	Arg	Tyr	Trp	Ala
			500					505						510	
Asn	Phe	Ala	Arg	Thr	Gly	Asp	Pro	Asn	Glu	Pro	Arg	Asp	Pro	Lys	Ala
		515					520					525			
Pro	Gln	Trp	Pro	Pro	Tyr	Thr	Ala	Gly	Ala	Gln	Gln	Tyr	Val	Ser	Leu
		530				535						540			
Asp	Leu	Arg	Pro	Leu	Glu	Val	Arg	Arg	Gly	Leu	Arg	Ala	Gln	Ala	Cys
545					550					555					560
Ala	Phe	Trp	Asn	Arg	Phe	Leu	Pro	Lys	Leu	Leu	Ser	Ala	Thr	Asp	Thr
			565					570						575	
Leu	Asp	Glu	Ala	Glu	Arg	Gln	Trp	Lys	Ala	Glu	Phe	His	Arg	Trp	Ser
			580					585						590	
Ser	Tyr	Met	Val	His	Trp	Lys	Asn	Gln	Phe	Asp	His	Tyr	Ser	Lys	Gln
		595					600							605	
Asp	Arg	Cys	Ser	Asp	Leu										
	610														

<210> SEQ ID NO 10
 <211> LENGTH: 2156
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

cagcctgcgc	cggggaacat	eggcgcctc	cagctcccgg	cgcgcccgg	cccggcccgg	60
ctcgccgcc	tcagagcgcg	cctgccctgc	agccatgagg	ccccgcagt	gtctgtgca	120
cacgccttcc	ctggcttccc	cactccttct	cctcctcctc	tggtcctgg	gtggaggagt	180
gggggctgag	ggccgggagg	atgcagagct	gctggtgacg	gtgcgtgggg	gcccgtgag	240
gggcattcgc	ctgaagaccc	ccgggggccc	tgtctctgct	ttcctgggca	tccccttgc	300
ggagccaccc	atgggacccc	gtcgcttctc	gccaccggag	cccaagcagc	cttggtcagg	360
ggtggtagac	gctacaacct	tccagagtgt	ctgctaccaa	tatgtggaca	ccctataccc	420
aggttttgag	ggcaccgaga	tggtgaaccc	caaccgtgag	ctgagcgagg	actgcctgta	480

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cctcaacgtg tggacacat acccccggcc tacatcccc acccctgtcc tcgtctggat 540
ctatgggggt ggctttaca gtggggcctc ctccctggac gtgtacgatg gcegettett 600
ggtacaggcc gagaggactg tgetggtgtc catgaactac cgggtgggag cctttggctt 660
cctggccctg ceggggagcc gagaggcccc gggcaatgtg ggtctectgg atcagaggct 720
ggccctgcag tgggtgcagg agaacgtggc agccttcggg ggtgaccga catcagtgac 780
gctgtttggg gagagcgcgg gagccgcctc ggtgggcatg cacctgctgt ccccgcccag 840
ccggggcctg ttccacaggg ccgtgctgca gagcggtgcc cccaatggac cctggggccac 900
ggtgggcatg ggagaggccc gtcgcagggc caccgagctg gcccacettg tgggctgtcc 960
tccaggcgcc actggtggga atgacacaga gctggtagcc tgccttcgga cagcaccagc 1020
gcaggtcctg gtaaacacg aatggcaagt gctgcctcaa gaaagcgtct tccggttctc 1080
ctctgtcct gtggtagatg gagacttctc cagtgcaccc ccagaggccc tcatcaacgc 1140
gggagacttc cacggcctgc aggtgctggt ggggtgtggt aaggatgagg gctcgtattt 1200
tctggtttac ggggccccag gcttcagcaa agacaacgag tctctcatca gccgggcccga 1260
gttctggcc ggggtgcggg tcggggttcc ccaggtaatg gacctggcag ccgaggctgt 1320
ggtcctgcat tacacagact ggctgcatcc cgaggaccgc gcaagcctga gggaggccct 1380
gagcgatgtg gtggggcagc acaatgtcgt gtgcccctg gcccagctgg ctgggcgact 1440
ggctgcccag ggtgcccggg tctacgccta cgtctttgaa caccgtgctt ccacgctctc 1500
ctggcccctg tggatggggg tgccccacgg ctacgagatc gagttcatct ttgggatccc 1560
cctggacccc tctcgaact acacggcaga ggagaaaatc ttcgcccagc gactgatgcg 1620
atactgggcc aactttgcc gcacagggga tcccaatgag ccccgagacc ccaaggcccc 1680
acaatggccc ccgtacacgg cgggggctca gcagtagctt agtctggacc tgcggccgct 1740
ggaggtgcgg cgggggctgc gcgcccagcc ctgcgccttc tggaaccgct tcctcccaa 1800
attgctcagc gccaccgaca cgctcgacga ggcggagcgc cagtgggaagg ccgagtteca 1860
ccgctggagc tcctacatgg tgcactggaa gaaccagtcc gacctacta gcaagcagga 1920
tcgctgctca gacctgtgac cccggcgggg cccccatgct ctcgctccg cccggcccc 1980
tagctgtata tactatttat ttcagggctg ggctataaca cagacgagcc ccagactctg 2040
cccateccca cccaccccc agctcccccg gggctccccg tcctctgcat gtctcaggct 2100
gagctccctc ccccgcggtg ccttcgcccc tctgggctgc caataaactg ttacag 2156

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<210> SEQ ID NO 11
<211> LENGTH: 428
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 11

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Met Asp Val Val Asp Ser Leu Leu Val Asn Gly Ser Asn Ile Thr Pro
 1           5           10           15
Pro Cys Glu Leu Gly Leu Glu Asn Glu Thr Leu Phe Cys Leu Asp Gln
 20           25           30
Pro Arg Pro Ser Lys Glu Trp Gln Pro Ala Val Gln Ile Leu Leu Tyr
 35           40           45
Ser Leu Ile Phe Leu Leu Ser Val Leu Gly Asn Thr Leu Val Ile Thr
 50           55           60
Val Leu Ile Arg Asn Lys Arg Met Arg Thr Val Thr Asn Ile Phe Leu
 65           70           75           80

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Leu Ser Leu Ala Val Ser Asp Leu Met Leu Cys Leu Phe Cys Met Pro
 85 90 95
 Phe Asn Leu Ile Pro Asn Leu Leu Lys Asp Phe Ile Phe Gly Ser Ala
 100 105 110
 Val Cys Lys Thr Thr Thr Tyr Phe Met Gly Thr Ser Val Ser Val Ser
 115 120 125
 Thr Phe Asn Leu Val Ala Ile Ser Leu Glu Arg Tyr Gly Ala Ile Cys
 130 135 140
 Lys Pro Leu Gln Ser Arg Val Trp Gln Thr Lys Ser His Ala Leu Lys
 145 150 155 160
 Val Ile Ala Ala Thr Trp Cys Leu Ser Phe Thr Ile Met Thr Pro Tyr
 165 170 175
 Pro Ile Tyr Ser Asn Leu Val Pro Phe Thr Lys Asn Asn Asn Gln Thr
 180 185 190
 Ala Asn Met Cys Arg Phe Leu Leu Pro Asn Asp Val Met Gln Gln Ser
 195 200 205
 Trp His Thr Phe Leu Leu Leu Ile Leu Phe Leu Ile Pro Gly Ile Val
 210 215 220
 Met Met Val Ala Tyr Gly Leu Ile Ser Leu Glu Leu Tyr Gln Gly Ile
 225 230 235 240
 Lys Phe Glu Ala Ser Gln Lys Lys Ser Ala Lys Glu Arg Lys Pro Ser
 245 250 255
 Thr Thr Ser Ser Gly Lys Tyr Glu Asp Ser Asp Gly Cys Tyr Leu Gln
 260 265 270
 Lys Thr Arg Pro Pro Arg Lys Leu Glu Leu Arg Gln Leu Ser Thr Gly
 275 280 285
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 Leu Phe Phe Leu Cys Trp Met Pro Ile Phe Ser Ala Asn Ala Trp Arg
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 Ile Tyr Cys Phe Met Asn Lys Arg Phe Arg Leu Gly Phe Met Ala Thr
 370 375 380
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Thr Arg Glu Leu Glu Leu Ala Ile Arg Ile Thr Leu Tyr Ala Val Ile
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Phe Leu Met Ser Val Gly Gly Asn Met Leu Ile Ile Val Val Leu Gly
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100          105          110
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Val Ile Val Val Leu Phe Phe Leu Cys Trp Leu Pro Val Tyr Ser Ala
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Val Asn Pro Leu Val Tyr Cys Phe Met His Arg Arg Phe Arg Gln Ala
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Cys Leu Glu Thr Cys Ala Arg Cys Cys Pro Arg Pro Pro Arg Ala Arg
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<210> SEQ ID NO 16
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<222> LOCATION: 3, 4
<223> OTHER INFORMATION: Xaa = Any Amino Acid

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<400> SEQUENCE: 16
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<210> SEQ ID NO 17
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42

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What is claimed is:

1. A method of detecting the presence or absence of a G protein-coupled receptor (GPCR) ligand in a test sample, comprising the steps of:

- (a) providing a cell comprising a GPCR and a detectably labeled arrestin, wherein the cellular distribution of the detectably labeled arrestin changes in response to activation of the GPCR;
- (b) determining the cellular distribution of the detectably labeled arrestin in the absence of a test sample;
- (c) exposing the cell to the test sample;
- (d) determining the cellular distribution of the detectably labeled arrestin in the presence of the test sample; and
- (e) comparing the cellular distribution of the detectably labeled arrestin in the presence of the test sample to the cellular distribution of the detectably labeled arrestin in the absence of the test sample,

wherein a change in the cellular distribution of the detectably labeled arrestin in the presence of the test sample in response to a GPCR ligand as compared to the cellular distribution of the detectably labeled arrestin in the absence of the test sample indicates the presence of a GPCR ligand in the test sample.

2. The method of claim 1, wherein the cellular distribution of the detectably labeled arrestin is determined at different time points after exposure to the test sample.

3. The method of claim 1, wherein the cellular distribution of the detectably labeled arrestin is determined after exposure to different concentrations of the test sample.

4. The method of claim 1, wherein the cellular distribution of the detectably labeled arrestin is quantified.

5. The method claim 4, wherein a concentration of the GPCR ligand in the test sample is quantified by comparing localization of the detectably labeled arrestin in plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes as compared to cytosol for the detectably labeled arrestin in the presence of the test sample to localization of the detectably labeled arrestin in plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes as compared to cytosol for the detectably labeled arrestin in the presence of a known concentration of the ligand.

6. The method of claim 5, wherein when a change in the concentration of the GPCR ligand in the test sample is observed over a period of time as a result of a compound that alters the concentration of the ligand indicates the presence of a compound in the test sample that alters the concentration of the ligand.

7. The method of claim 1, wherein the test sample is or is derived from serum, tissue, blood, or urine.

8. The method of claim 1 wherein the GPCR is CCK-B (SEQ ID NO: 13) or CCK-A (SEQ ID NO: 11).

9. The method of claim 8, wherein the test sample was derived from a patient with hypergastrinemia.

10. The method of claim 1, wherein the ligand is gastrin, preprogastrin, cleaved preprogastrin, gastrin-34, gastrin-17, pentagastrin, progastrin, glycine-extended gastrin-17, glycine-extended gastrin-34, gastrin-71, gastrin-6, hG17, a compound with an amidated tetrapeptide of the sequence Trp-Met-Asp-Phe-NH₂ (SEQ ID NO: 15), or an isoform of gastrin capable of binding to a gastrin receptor.

11. The method of claim 10, wherein the gastrin concentration is less than 10 nM.

12. The method of claim 1, wherein the GPCR is a muscarinic receptor.

13. The method of claim 1, wherein the ligand is acetylcholine.

14. The method of claim 1, wherein the labeled arrestin is localized in the cytosol, plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes.

15. The method of claim 1, wherein an increase in a local concentration of the detectably labeled arrestin in plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes as compared to cytosol results in an increase in a local signal intensity.

16. The method of claim 15, wherein the local signals intensity is increased in the presence of increased concentration of ligand in the test sample.

17. The method of claim 1, wherein a signal intensity of the labeled arrestin in the plasma membrane, clathrin-coated pits, endocytic vesicles, or endosomes is increased as compared to a level of signal intensity in the cytosol.

18. The method of claim 1, wherein the detectable label is a radioisotope, an epitope tag, an affinity label, an enzyme, a fluorescent group, or a chemiluminescent group.

19. The method of claim 1, wherein the cellular distribution is visualized by flow cytometry.

20. The method of claim 1, wherein the cellular distribution is visualized by fluorescence confocal microscopy.

21. The method of claim 1, wherein a computer analyzes an image of the cellular distribution.

22. The method of claim 1, wherein the distribution of the detectably labeled arrestin is quantified.

23. The method of claim 1, wherein the test sample comprises a ligand of the GPCR.

24. The method of claim 1, wherein the test sample comprises an antagonist of the GPCR.

25. The method of claim 1, wherein the test sample further comprises acetylcholine and acetylcholinesterase.

26. The method of claim 1, wherein the test sample further comprises an agonist of the GPCR.

27. The method of claim 1, wherein the ligand has been identified.

28. The method of claim 1, wherein the test sample is heterogeneous.

29. The method of claim 1, wherein the cellular distribution is determined after 15-30 minutes of exposure to the test sample.

30. The method of claim 1, wherein the cellular distribution is determined after 1 hour of exposure to the test sample.

31. The method of claim 1, wherein the cell is exposed to the test sample at a temperature of 37° C.

32. A method of detecting the presence or absence of a G protein-coupled receptor (GPCR) ligand in a test sample, comprising the steps of:

- (a) providing a single cell biosensor comprising a cell which overexpresses a detectably labeled arrestin and at least one GPCR, wherein the cellular distribution of the detectably labeled arrestin changes in response to activation of the GPCR;
- (b) determining the cellular distribution of the detectably labeled arrestin in the absence of a test sample;
- (c) exposing the biosensor to the test sample;
- (d) determining the cellular distribution of the detectably labeled arrestin in the presence of the test sample; and
- (e) comparing the cellular distribution of the detectably labeled arrestin in the presence of the test sample to the cellular distribution of the detectably labeled arrestin in the absence of the test sample,

wherein a change in the cellular distribution of the detectably labeled arrestin in the presence of the test sample in response to a GPCR ligand as compared to the cellular distribution of the detectably labeled arrestin in the absence of the test sample indicates the presence of a GPCR ligand in the test sample.

33. A method of continuous screening for the presence or absence of G protein-coupled receptor (GPCR) ligands in a test sample, comprising the steps of:

- (a) providing a cell comprising a GPCR and a detectably labeled arrestin, wherein the cellular distribution of the detectably labeled arrestin changes in response to activation of the GPCR;
- (b) determining the cellular distribution of the detectably labeled arrestin in the absence of a test sample;
- (c) exposing the cell to the test sample;
- (d) determining the cellular distribution of the detectably labeled arrestin in the presence of the test sample;

(e) comparing the cellular distribution of the detectably labeled arrestin in the presence of the test sample in response to a GPCR ligand to the cellular distribution of the detectably labeled arrestin in the absence of the test sample, wherein a change in the cellular distribution of the detectably labeled arrestin in the presence of the test sample as compared to the cellular distribution of the detectably labeled arrestin in the absence of the test sample indicates the presence of a GPCR ligand in the test sample; and

(f) replacing the cell with another cell comprising a GPCR and a detectably labeled arrestin and repeating steps (b)-(e) thereby continuously screening for the presence or absence of G protein-coupled receptor (GPCR) ligands in a test sample.

34. A method of detecting the presence or absence of a compound that modulates G protein-coupled receptor (GPCR) internalization in a test sample, comprising the steps of:

- (a) providing a cell comprising a GPCR and a detectably labeled arrestin, wherein the cellular distribution of the detectably labeled arrestin changes in response to activation of the GPCR and wherein when the GPCR is activated and internalized the GPCR and the detectably labeled arrestin form a detectably labeled arrestin/GPCR complex;
- (b) determining the cellular distribution of the detectably labeled arrestin in the absence of a test sample;
- (c) exposing the cell to an agonist of the GPCR,
- (d) exposing the cell to the test sample;
- (e) determining the cellular distribution of the detectably labeled arrestin/GPCR complex in the presence of the test sample; and
- (f) comparing the cellular distribution of the detectably labeled arrestin/GPCR complex in the presence of the test sample to the cellular distribution of the detectably labeled arrestin in the absence of the test sample, wherein a change in the cellular distribution of the detectably labeled arrestin/GPCR complex in the presence of the test sample as compared to the cellular distribution of the detectably labeled arrestin/GPCR complex in the absence of the test sample indicates the presence of a compound that modulates GPCR internalization in the test sample.

35. A method of detecting the presence or absence of a compound that modulates G protein-coupled receptor (GPCR) internalization in a test sample, comprising the steps of:

- (a) providing a cell comprising a GPCR and a detectably labeled arrestin, wherein the cellular distribution of the detectably labeled arrestin changes in response to activation of the GPCR and wherein when the GPCR is activated and internalized the GPCR and the detectably labeled arrestin form a detectably labeled arrestin/GPCR complex;
- (b) determining the cellular distribution of the detectably labeled arrestin in the presence of an agonist of the GPCR and the absence of a test sample;
- (c) exposing the cell to the test sample;
- (d) determining the cellular distribution of the detectably labeled arrestin/GPCR complex in the presence of the test sample; and
- (e) comparing the cellular distribution of the detectably labeled arrestin/GPCR complex in the presence of the test sample to the cellular distribution of the detectably labeled arrestin in the absence of the test sample,

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wherein a change in the cellular distribution of the detectably labeled arrestin/GPCR complex in the presence of the test sample as compared to the cellular distribution of the detectably labeled arrestin/GPCR complex in the

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absence of the test sample indicates the presence of a compound that modulates GPCR internalization in the test sample.

* * * * *

专利名称(译)	用于测量测试样品中GPCR配体的单细胞生物传感器		
公开(公告)号	US7541151	公开(公告)日	2009-06-02
申请号	US10/161916	申请日	2002-06-04
[标]申请(专利权)人(译)	巴拉克LAWRENCE小号 SHETZLINE迈克尔 OAKLEY罗伯特· 卡朗MARC摹		
申请(专利权)人(译)	巴拉克LAWRENCE小号 SHETZLINE迈克尔 OAKLEY罗伯特· 卡朗MARC摹		
当前申请(专利权)人(译)	杜克大学		
[标]发明人	BARAK LAWRENCE S SHETZLINE MICHAEL A OAKLEY ROBERT H CARON MARC G		
发明人	BARAK, LAWRENCE S. SHETZLINE, MICHAEL A. OAKLEY, ROBERT H. CARON, MARC G.		
IPC分类号	G01N33/566 G01N21/76 G01N33/53 G01N33/68 C12N15/09 C12Q1/00 C12Q1/02 C12Q1/44 G01N33/49 G01N33/50 G01N33/74		
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优先权	60/295945 2001-06-05 US		
其他公开文献	US20090075314A1		
外部链接	Espacenet USPTO		

摘要(译)

本发明涉及通过使用表达GPCR的单细胞生物传感器检测测试样品中的GPCR配体。优选地，测试样品衍生自生物或环境样品。本发明可用于检测疾病的存在或检测环境中有害物质的存在。本发明包括检测各种GPCR配体的生物传感器阵列。

FIG. 1A

Human G Protein-Coupled Receptor Family
(Receptor known as of January, 1999)

CLASS	LIGAND	NUMBER	TISSUE	PHYSIOLOGY	THERAPEUTICS
*Class I	*Arenine				
	*Asphobolide (muscarinic & acetylcholinergic)	5	Brain, Nerve, Heart	Neurotransmitter	Anxiety, Alzheimer's
	*Atrial natriuretic peptide	6	Brain, Kidney, Lung	Glucocorticoid	Diabetes, Cardiovascular
	*Beta adrenoreceptors	3	Kidney, Heart	Muscle Contraction	Cardiovascular, Respiratory
	*Dopamine receptors	5	Brain, Kidney, GI	Neurotransmitter	Cardiovascular, Parkinson's
	*Histamine receptors	2	Vascular, Heart, Brain	Vascular Permeability	Anti-inflammatory, Ulcers
	*Opioid (OP) receptors	16	Most Tissues	Neurotransmitter	Depression, Insomnia, Analgesic
	*Pituitary growth hormone releasing hormone	2	Vascular, Liver, Kidney	Vasodilation	Cardiovascular, Endocrine
	*Pituitary growth hormone releasing hormone	1	Liver, Blood	Vasodilation	Anti-inflammatory, Asthma
	*Calcitonin receptor	1	Blood	Immune System	Anti-inflammatory
	*Pituitary growth hormone releasing hormone	3	Blood	Chemotactant	Anti-inflammatory
	*Histamine receptors	1	Blood	Chemotactant	Anti-inflammatory
	*Chemokine receptors	6	Blood	Chemotactant	Anti-inflammatory
	*Opioid receptors	2	Brain	Pit. Melanin	Obesity
	*Opioid receptors	1	Brain	Bronchodilator, Pain	Airway Disease, Anesthetic
	*Opioid receptors	2	Gastrointestinal	Motility, Fat Absorption	Gastrointestinal, Obesity, Parkinson's
	*CCX (Gastrin)	2	Heart, Bronchus, Brain	Muscle Contraction	Cardiovascular, Respiratory
	*Fractalkin	1	Kidney, Brain	Metabolic Regulation	Anti-inflammatory, Analgesic
	*Melanocortin	5	Nerve, Intestine, Blood	Neurotransmitter	Behavior, Memory, Cardiovascular
	*Neuropeptide Y	1	Brain,	CNS	Cardiovascular, Analgesic
	*Neurotensin	3	Brain,	CNS	Depression, Analgesic
	*Opioid	3	Brain,	CNS	Depression, Analgesic
	*Somatostatin	5	Brain, Gastrointestinal	Neurotransmitter	Oncology, Alzheimer's