Detect and process extracellular physiologic and pathophysiologic molecules

Structure of Ligand determines structure of receptor and Mechanism of Action: Hydrophobic vs. Hydrophilic

Hydrophobic: Nuclear Hormone Receptors
Cytoplasmic/Nuclear Localization
Complex with hsp90 and others to hold binding site open for the ligand
Nuclear Hormone Receptors:
Four Mechanisms used by the Estrogen Receptor

The retinoic acid receptor, in the absence of a hormone, is a repressor and it collects a series of repressor proteins like a magnet. Addition of the ligand reverses the polarity of the magnet, causing the repressors to fly off and the coactivators to bind, resulting in chromatin modification to activate transcription. This is how the hormonal or physiological signals affect the recruitment of cofactors that modify the chromatin in target genes. Every cell has receptors and therefore can respond, but each cell responds in its own unique way, and thus the same hormone can have a different effect on a neuron versus an epithelial skin cell versus a bone cell.

Endocrine Receptors
1. High-affinity, hormonal ligands
2. Feedback regulation
3. Endocrine sensor

Adopted Orphan Receptors
1. Low-affinity, dietary lipids
2. Feedforward regulation
3. Lipid sensor

Orphan Receptors
1. Competence factor
2. Ligands unknown
3. Regulation unknown

GR glucocorticoid
MR mineralocorticoid
PR progesterone
AR androgen
ERα estrogen
ERβ estrogen
RARα,β,γ retinoic acid
TRα,β thyroid hormone
VDR vitamin D, LCA, farnesone
RXRα,β nuclear hormone
PPARγ nuclear hormone

48 HUMAN NR GENES

Hydrophilic Ligand/Receptor System
Plasma Membrane is Permeability Barrier
WHAT ARE THE CHARACTERISTIC PROPERTIES OF A LIGAND/RECEPTOR INTERACTION?

How do you define a receptor, how do you find a receptor, how do you study receptors?

MEASURE BINDING

Receptor-preparation:
Intact Cells:
Cultured cells, tissue slices, perfused organs, whole organism

Ligand-preparation:
Detection

Avoid artifacts of labeling
(e.g. denaturation, crosslinking)

How to Label Ligands

Protein Ligands

Protein Labeling:
Radioisotopes
Fluorescent dyes
Epitope tags
Others (biotin)

$^{125}$I-Protein
$[^3]$H]Protein
$[^35]$S]methionine
BINDING Assays

Variables:
Concentration, Time, Temperature

A. Saturation Kinetics

"Hot + Cold"

Look out for bad data and beware of people who only show delta!

Alternative to Chemical Modification of Ligand
Immunoreceptor vs Radioreceptor Assays

For immunoreceptor assay, nonspecific is calculated by regression analysis.

100% Reversible Binding to Receptor

time after binding

Off rates are critical
must not loose signal during washing

Binding vs Cell Association:
Inhibit uptake at 4°C

BINDING Assays
Quantitative Analysis:
How many binding sites? How tight is the binding?

Rigorous analysis depends on system being in equilibrium

\[ \text{R} + \text{L} \rightleftharpoons \text{RL} \]

\( R \) = unbound receptors
\( L \) = unbound ligand
\( RL \) = ligand/receptor complex
\( R_t \) = total # of receptors (binding sites) = \( R + RL \)
**Classic Scatchard Analysis**

\[ R = \text{unbound receptors} \quad L = \text{unbound ligand} \quad RL = \text{ligand/receptor complex} \]

\[ R_t = \text{total # of receptors (binding sites)} = R + RL \]

\[ \text{bound} = \frac{[RL]}{[L]} \]

\[ \text{slope} = -\frac{1}{K_d} \]

\[ \text{intercept} = R_t \]

**Complications!**

*Error propagation*

*Cannot always trust your eyes!*

**Simple Straight Line:**

Single Class of Binding Sites
**Direct Plot:**

- Positive co-op.
- Negative co-op.

**Scatchard Plot:**

- Fewer high affinity
- More abundant lower affinity

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**Non-linear Scatchard Plots:**

- Multiple Classes of Binding Sites

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**Old Fashion Graphical Analysis:**

- Not good for quantitative determinations

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Use Non-linear Regression Analysis
Good Packaged Programs Available
E.g., GraphPad Prism

Plot Graph and Calculate!
http://www.graphpad.com/
http://www.graphpad.com/curvefit/introduction9e.htm

Sources of Nonlinearity
Multiple Classes of Binding Sites
Complex Classes of Binding Sites - Negative and Positive Cooperativity
- ensemble effect

Sources of Nonlinearity
Multiple Classes of Binding Sites
Complex Classes of Binding Sites - Negative and Positive Cooperativity

Poor Data:
Nonequilibrium Conditions
Instability of Ligand or Receptor Site
Ligand Problems: Bad modifications, Dilution of ligand (endogenous ligand)

[inadequate ligand concentration range]
Biological Relevance: LDL and Glass Beads
You can differentiate to some extent effects on binding and response, e.g.:

R = H (epinephrine); $K_d = 5 \times 10^{-6} M$
R = C$_2$ (isoproterenol); $K_d = 0.4 \times 10^{-6}$ (higher affinity)
both epi. and isopro. stimulate cyclase
(called ‘Agonists’)

R=C-C-C-C-phenyl-OH $K_d = 0.06 \times 10^{-6} M$

Bind Tightly!
Don’t Activate Cyclase!

Antagonists
(vs Agonists and Inverse Agonists)
R $\rightarrow$ R’(active)
### How to isolate receptors?

A) Need an assay to follow purification
- radioligand binding, immunoochemical detect. (Ligand blot)
- Filter binding in reconstituted liposomes

#### Tricks to help purify:

1) Affinity Labeling (crosslinking)
2) Ligand affinity chromatography:
   a) nonionic detergent solubilize protein - detergent micelles
   b) run on ligand-crosslinked column
   c) elute with competitor or altered salt or pH
3) Immunoaffinity chromatography
4) Clone by functional expression (e.g., cellular response)

### Examples:

**SR-AI** (innate immunity pattern recognition receptor)
- Ligand affinity, ion exchange chromatography, preparative SDS-PAGE -> monoclonal antibody
- Immunoaffinity -> 240,000-fold purification from lung

**SR-BI** – epitope tag, express, immunoaffinity – gel

**βAR** – ¹²⁵I-cyanopindolol (CYP) binding assay; alprenolol-sepharose in digitonin, reconstitution requires proper lipids

### Purified BINDING ACTIVITY, but how do you know that you’ve actually purified the biologically relevant receptor?

#### Number of indirect and some direct tests

**Indirect:**
1) exhibits binding affinity and specificity seen in cells
2) protein exhibits cell type and tissue type distribution of activity
3) expressed on cell surface - proteolysis, surface labeling reagents
4) glycoprotein

**Direct:**
5) regulation makes sense
Purified BINDING ACTIVITY, but how do you know that you've actually purified the biologically relevant receptor?

Direct:
2) Express cloned gene in cells or tissues and measure bio activity
3) Inhibit activity in appropriate system (blocking antibody, siRNA, KO animal)

STRUCTURE OF THE ß-ADRENERGIC RECEPTOR

Heptahelical (7-Transmembrane) domain receptor
7-TM or G-Protein Coupled Receptors (GPCR)

- Large family (>2000) - ~5% of worm genome coding sequences, perhaps 3% of mammals
- \( \alpha_2 \)-AR, \( \beta_1 \) and \( \beta_2 \)-AR, dopamine receptors, muscarinic acetyl choline receptors (4 subclasses), sight (Rhodopsin), taste, smell, angiotensin receptor (mas oncogene), serotonin receptor, a- and \( \alpha \)-factor yeast receptors, LH receptor, PAR (protease activated receptor)
- Many intronless genes!

all operate using similar signal transduction systems (G-proteins)

Major Drug Targets for Pharmaceutical Industry...

<table>
<thead>
<tr>
<th>Family of structurally and functionally similar proteins provides special opportunity to explore structure/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>method: insert cDNA into expression vector, transfec into L cells because they are BAR-ive, look for binding and correct pharmacologic specificity, affinity, etc.-G-protein and cyclase required to work!</td>
</tr>
</tbody>
</table>

For example:
- Delete N- or C-terminus -> binding ok
- Delete 5-6 loop (C3=i3) -> binding ok
- Conclusion -> not needed for binding
For example:
Co-express SR(1-5, aa1-262)
+SR(6-7, aa263-end) -> still works!
Conclusion -> self assemble

Delete 6-7 loop (E4) or
helices 6&7 -> No binding!
Conclusion -> ?????????
What can you do?

Similar structures permit
testing of chimeras of
GPCRs for structural
determinants of ligand
specificity and downstream
target (G-protein) specificity

α2-AR -> decrease in
adenylate cyclase activity
β2-AR -> increase in adenylate
cyclase

different ligand specificities

Major determinant of specific
binding in 7th membrane
spanning domain

Combined Use of Chemistry (Pharmacology - alter structure
of the ligand) and Molecular Biology (mutagenesis- modify
putative ligand binding sites) to define Ligand/Receptor
Interactions - ‘complementary mutations’

α2-AR from outside of cell binding
to norepinephrine
Side chain interactions within the transmembrane region of the β₂-adrenergic receptor with agonist isoproterenol (A) and antagonists alprenolol (B), as derived from mutagenesis and/or modeling studies. For clarity of pictorial representation, the interaction of Phe-289 is not shown.

Scheme 3. Schematic drawing of the interaction of ATP with the P2Y1 receptor as derived from mutagenesis and modeling studies. For reasons of clarity the interaction with Arg310(TM7) is not shown.
Desensitization: tendency of biological responses to wane over time despite continuous presence of stimulus of constant intensity.

Homologous desensitization: β-adrenergic receptor kinase (ßARK) phosphorylates receptor and lowers activity somewhat, after phosphorylation, ß-arrestin binds and really drops activity. NB at high ligand occupancy

Heterologous desensitization: general reduction on stimulation of other receptors, protein kinase A (PKA) plays a role here, PKA is about 6x slower than ßARK at phosphorylating the receptor also, ßARK mediated desensitization is t1/2<15 sec while PKA is 3.5 min (>14 fold) see Roth et al, PNAS 88:6201-6204 (91), arrestin independent.
beta-Arrestin, a promiscuous mediator of receptor endocytosis. (A) Binding of ligand to its GPCR activates heterotrimeric G proteins, causing dissociation of the alpha subunit from the beta/gamma dimer (not shown). The betagamma dimer, anchored to the inner surface of the plasma membrane by the prenylated gamma subunit, facilitates translocation of GFL to the plasma membrane. This enables GFL to phosphorylate the serine/threonine residue in the GPCR carboxyl terminus, resulting in beta-arrestin translocation to the GPCR. beta-Arrestin binds to adaptor proteins in clathrin-coated pits, which are then either recycled or degraded with concomitant activation of G protein-independent signaling pathways. (B) On binding to its ligand, the Fz4 receptor induces translocation of Dvl2 to the plasma membrane. Phosphorylation of Dvl2 by protein kinase C (PKC) leads to binding of beta-arrestin 2. Binding of Wnt5A to the cysteine-rich domain (CRD) in the extracellular amino terminus of Fz4 leads to endocytosis of the Fz4 complex. (C) Transforming growth factor-beta type II receptor (TbetaR-II) binds to, and its intracellular kinase domain phosphorylates, the intracellular carboxyl terminus of TbetaR-III. This leads to translocation of beta-arrestin 2 to the plasma membrane, followed by endocytosis of the TbetaR-II/TbetaR-III complex. This process does not require binding of TGF-beta to its receptor, hence the TbetaR-II extracellular binding site is depicted as empty.

end