

**ADVANCED CELL BIOLOGY//
MOLECULAR BIOLOGY OF CELLS**
01-146:470 + 16-148:514

•Monday 11.23.2009 (8:40-10:00)

•SEC-118

• Class web page:

<http://lifesci.rutgers.edu/~denhardt/course/cellmolbiol.htm>

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•Office hours, Friday 3:30pm-5:00 pm

Lodish • Berk • Kaiser • Krieger • Scott • Bretscher • Ploegh • Matsudaira

Molecular Cell Biology
6th Edition

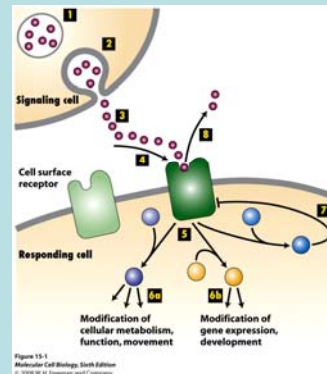
Chapter 15:

Cell signaling I: Signal transduction and short-term cellular responses

- 15.1 From extracellular signal to cellular response.
- 15.2 Studying cell-surface receptors.
- 15.3 Highly conserved components of intracellular signal-transduction pathways.
- 15.4 General elements of G protein-coupled receptor systems.
- 15.6 G protein-coupled receptors that activate or inhibit adenylyl cyclase.
- 15.7 G protein-coupled receptors that activate phospholipase C.

**From Extracellular signals
to cellular responses**

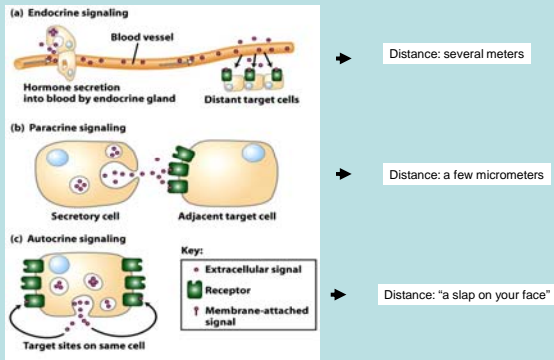
**From extracellular signal to cellular response, or
why signal transduction is a must**



- 1) Synthesis and packaging of secreted molecules (ligands).
- 2) Exocytosis
- 3) Transport of ligand to target cell
- 4) Binding of ligand to receptor
- 5) Activation of intracellular signaling pathways.
- 6a) Short term modifications
- 6b) Long term modifications
- 7) Signaling inhibition ("feedback loop")
- 8) Removal of ligand

Figure 15-1
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Close range, or far reaching signaling



Attached ligands can also induce signaling

Signaling by plasma membrane-attached proteins

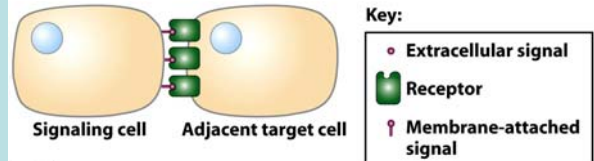


Figure 15-24
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Ligands attached on one cell can trigger signaling in an adjacent target cell (adherence molecules such as integrins)

Ligand-Receptor interactions: Harmonic "groove"

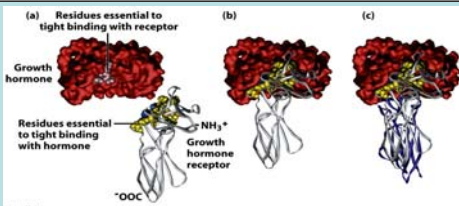
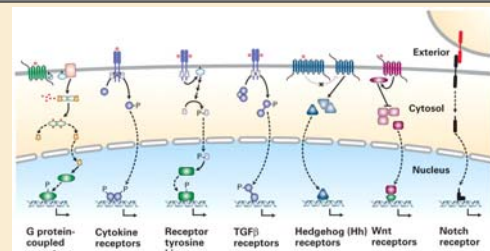


Figure 15-3
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- Essential residues on the ligand and the receptor determine a specific binding.
- In this example only 8/28 aa of the hormone that are found at the binding interface of the hormone to the receptor are contributing 85% of the binding energy (pink in the cartoon). Similarly, while several aa of the receptor binding interface are important (yellow), 2 tryptophan (trp) residues (blue) contribute most of the energy for binding growth hormones.
 - Binding of growth hormone to 1 receptor molecule is followed by binding of a 2 receptor (c; purple) to the opposing side of the hormone; this involves the same set of aa on the second receptor (yellow and blue in the cartoon), but different residues on the hormone.
 - Hormone-induced receptor dimerization is a common mechanism for activation of receptors and the start of signal transduction.

Studying cell surface receptors



Specific signaling mechanisms exist downstream of distinct receptor families, but they are all activated by ligand binding to the receptor and their activation mainly leads to gene transcription.

Dissociation constants (Kd) determine the affinities of specific ligand-receptor interactions



Ligand (L) binding to a receptor (R) depends on the rate constant for formation (k_{on}) of a receptor-ligand complex (RL) as well as its rate constant of dissociation (k_{off}). At equilibrium, the rate of formation of the receptor-ligand complex is equal to the rate of its dissociation and follows the equilibrium-binding equation:

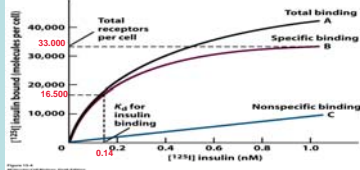
$$K_d = \frac{[R][L]}{[RL]}$$

[R] and [L] is the concentration of free receptor respectively, free ligand and [RL] is the concentration of the receptor-ligand complex.

The dissociation constant, K_d is thus a measure of the affinity of the receptor for its ligand.

For a simple binding reaction, $K_d = k_{off}/k_{on}$. The lower the K_d , (tight binding) the lower the K_d .

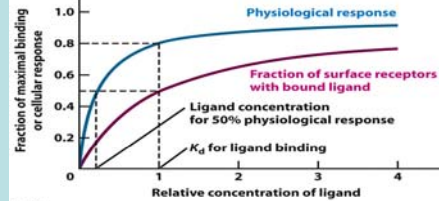
Another way to measure K_d is to determine the concentration of ligand at which 50% of the receptors have a ligand bound.



The constant of dissociation (K_d) identifies a receptor. The K_d can be determined in a binding assay in which, a suspension of cells is incubated with increasing concentrations of radioactively- (^{125}I) labeled agonist such as insulin (Ins) that specifically binds to its high affinity receptor, insulin receptor (InsR). Cells are separated from unbound agonist by centrifugation and the amount of radioactivity is measured. Curve A represents insulin specifically bound to high-affinity InsR as well as Ins nonspecifically bound with low affinity to other receptors on the cell surface. The contribution of non-specific binding (curve C) is measured by repeating the same binding assay, but in presence of a 100-fold excess of unlabelled Ins (saturation of all specific InsR). The specific binding of Ins to InsR (curve B) is the difference between curve A and C.

In this example, the number of high-affinity InsR per cell is 33,000 (max of curve B). The K_d is the concentration of Ins required to bind 50% of InsR (-16,500 receptors/cell). Thus the K_d of InsR in this experiment $\sim 1.4 \times 10^{-10}$ or, 0.14 nM.

Physiologic response to receptor engagement: Less is more

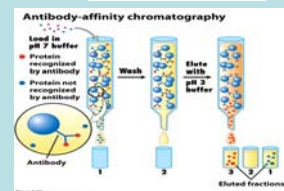


Not all receptors need to be occupied for maximal physiologic response. For signaling pathways that exhibit this behavior, plots of physiological response to distinct ligand concentrations and of ligand binding to the receptor differ.

As shown in this example, 80% of the maximal physiological response is induced at the K_d value for this particular receptor (concentration of ligand at which 50% of the receptors are occupied). Only ~18% of the receptors need to be occupied to induce 50% of the maximal physiological response.

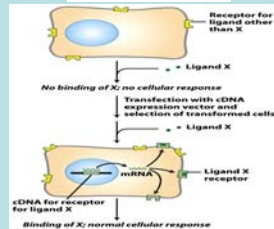
Techniques to generate soluble receptors

Receptor purification by affinity chromatography



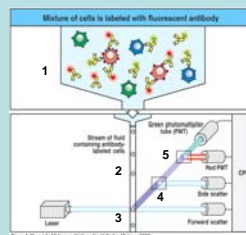
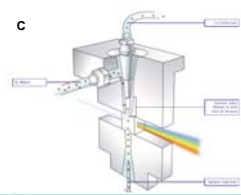
In an antibody-affinity chromatography, a mixture of proteins is passed through a column packed with beads to which an antibody that specifically recognizes a receptor of interest is bound. Only proteins (receptors) with high affinities for the antibody are retained by the column; all nonbinding proteins flow through. After the column is washed, the bound receptor is eluted with an acidic solution, or some other solution that disrupts the receptor-antibody complexes and collected for further characterization.

Receptor gene cloning



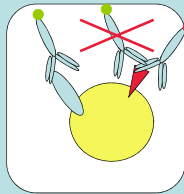
Functional expression assay can identify a cDNA encoding a cell-surface receptor. Target cells lacking receptors for a particular ligand (X) are stably transfected with a cDNA expression vector encoding the receptor (vector also contains selection marker). The transfected cell will respond to ligand X, if the receptor is expressed at the cell surface, and providing that the transfected cell constitutively expresses all the relevant intracellular signaling proteins required to transduce a signal downstream of high-affinity receptors for ligand X.

Principle of fluorescence-activated cell sorting (FACS), or flow cytometric analysis



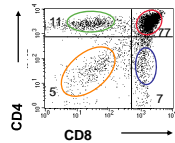
Fluorescence-activated cell sorter (FACS) separates cells that are labeled differentially with a fluorescent antibody. Step 1: Mixture of cells is incubated with distinct antibodies that are differentially labeled with distinct fluorescent dyes. Step 2: The concentrated cell suspension is mixed with sheath fluid so that the cells pass single-file through a laser light beam (3). Step 3: Both the light scattered by each cell (4; forward and side scatter) and the fluorescence light emitted (5) are measured and recorded. The size and shape of each cell can be deduced from the scattered light, while the expression of a particular receptor can be measured by the source of the fluorescent light. C) In addition to expression analysis, particular cell populations can be isolated and recovered for further experimentation. (cf. chapter 9.5 figure 9-28)

Receptor expression on distinct cell populations can be measured by antibody staining and flow cytometric analysis



Cells are stained with a fluorescently-labeled antibody that only recognizes a specific surface receptor.

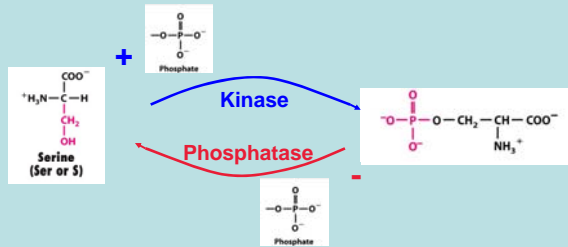
T lymphocyte development in the thymus



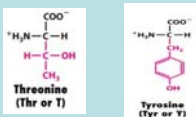
The distinct stages of $\alpha\beta$ thymocytes can be identified by FACS analysis in respect to the expression of the CD4 and CD8 coreceptors. Percentages of the distinct cell populations are indicated in the respective quadrants (CD4 SP: upper left, CD4CD8 DP: upper right, CD8 SP: lower right, CD4CD8 DN: lower left) (Cf. chapter 24.5 for T cell development).

Ubiquitous components of intracellular signaling pathways

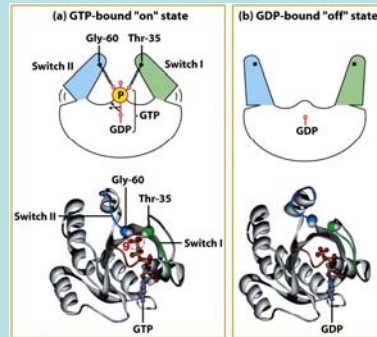
Phosphorylation is king of signal transduction



Kinases replace hydroxyl residues with phosphate residues. Phosphatases do the opposite.



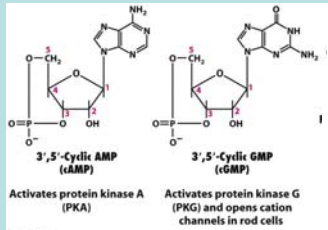
"on-off" switch controls G protein activation



G proteins switch between an inactive state, where GDP is bound, to an active state where GTP is bound. A) In the active "on" state, conserved glycine and threonine residues that are located in the switch II and switch I domains, respectively bind the terminal γ phosphate of GTP. The GDP-GTP switch triggers a change in the conformation of the G protein, which allows it to activate downstream signaling components. B) Similarly, the release of the γ phosphate by GTPase-catalysed hydrolysis causes switch I and II to relax into a different conformation; the inactive "off" state.

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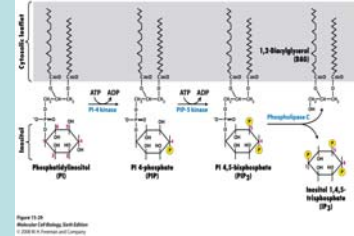
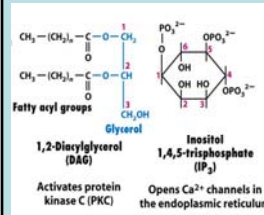
Phosphorylated nucleotide derivatives serve as second messengers



Highly diffusible molecules such as cAMP or cGMP are widely used to transduce signals even if the target is localized in intracellular stores.

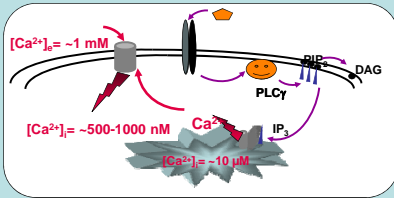
Figure 15-9
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Lipid derivatives serve as second messengers.



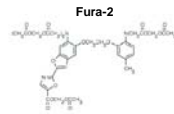
Lipid derivatives act as second messengers at the membrane (for example, DAG) or such as IP₃ translocate to particular organelles across the cytosol. DAG and IP₃ are generated by the cleavage of membrane localized phosphoinositide phosphate (PIP₂). DAG remains within the cytosolic leaflet of the membrane, while IP₃ is released into the cytosol and activates the release of calcium, Ca²⁺ from storing organelles.

Intracellular calcium concentrations, [Ca²⁺]_i, are tightly regulated.

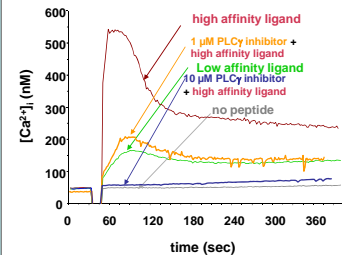


In a resting cell $[Ca^{2+}]_i \approx 50 \text{ nM}$, while $\sim 1 \text{ mM}$ Ca^{2+} is found in the extracellular environment ($[Ca^{2+}]_o$). Distinct organelles, such as the Golgi, the nucleus or specialized Ca^{2+} stores (calciosomes) contain up to $10 \mu\text{M}$ Ca^{2+} . Inositol trisphosphate (IP₃) diffuses through the cytosol and binds to IP₃ receptors (IP₃R) that are localized on the surface of Ca^{2+} -storing organelles. The engagement of IP₃R triggers the opening of Ca^{2+} -channels on the surface of the storing organelles and a burst of Ca^{2+} is released into the cytosol. This in turn activates cell membrane localized Ca^{2+} -operated- Ca^{2+} -channels and the entry of more Ca^{2+} from the extracellular milieu, resulting in 10-20 fold increase of $[Ca^{2+}]_i$. Ca^{2+} -homeostasis is restored by ligand-receptor complex dissociation, PLCy inactivation and the repumping of Ca^{2+} into the storing organelles.

Measurement of intracellular Ca²⁺ transients

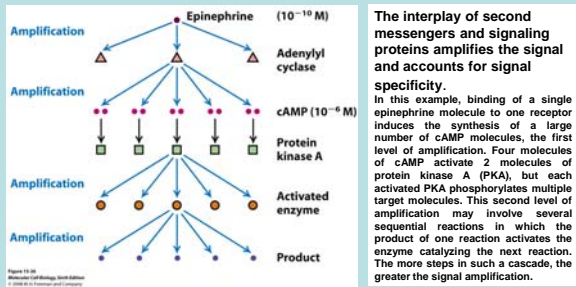


Fluorescent Ca^{2+} -binding dyes, such as the EGTA-derived compound Fura-2 measure changes in $[Ca^{2+}]_i$. Before agonist stimulation, cells are preincubated with fura-2 and the baseline of fluorescence (corresponding to homeostatic $[Ca^{2+}]_i$) is established. The addition of an agonist induces Ca^{2+} release and increases the amount of Ca^{2+} bound to fura-2, hence a measured increase in fluorescence. A rapid decrease in fluorescence indicates repumping of Ca^{2+} into intracellular storage organelles, as well as the extracellular environment. Exact $[Ca^{2+}]_i$ concentrations can be calculated from the measured fluorescence. (Grynkiewicz, G., Poenie, M. & Tsien, R.Y. 1985. J. Biol. Chem., 260: 3440 - 3450).



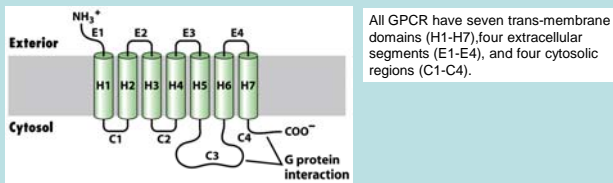
High affinity ligands induce more potent Ca^{2+} transients as compared to low affinity ligands. The phospholipase PLCy controls the Ca^{2+} transients.

Signal transduction = signal amplification

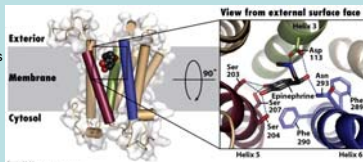


General elements of G protein-coupled receptor (GPCR) systems

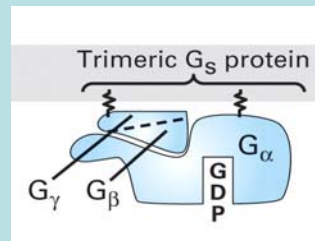
GPCR are hepta-(membrane)spanning molecules



Specific residues of a ligand, such as Epinephrine engage particular residues of the GPCR (β 2-adrenergic receptor in the case of epinephrine).

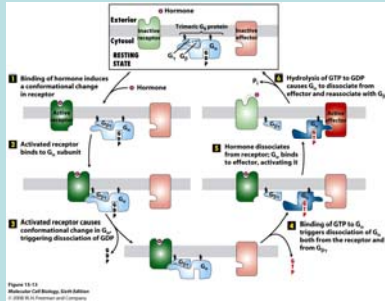


GPCR activate trimeric G-proteins



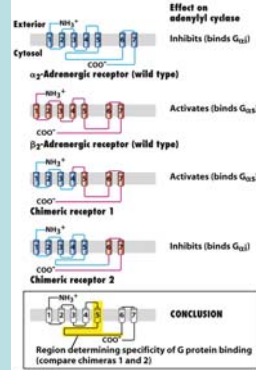
The G_α and $G_\beta\gamma$ subunits of trimeric G proteins are tethered to the membrane by covalently attached lipid molecules (wiggly black lines). In the inactive "off" state G_α binds GDP (similar to a small G protein), upon activation of the receptor associated to the trimeric G protein, GDP is exchanged for GTP and G_α undergoes a conformational change. The $G_\beta\gamma$ subunits are regulatory (in some instances they bind the receptor).

GPCR associate to trimeric G proteins



Intracellular trimeric G proteins transduce the signal generated by engagement of GPCR. Trimeric G proteins are composed of the catalytic subunit $G\alpha$ that binds GTP or GDP as well as the regulatory subunits, $G\beta$ and $G\gamma$.

The long C3 loop of GPCRs interacts with G proteins



Expression of chimeric constructs of GPCRs has characterized their C3 loop as critical in binding downstream G proteins.

Xenopus oocytes that normally do not express adrenergic receptors, were microinjected with mRNA encoding α_2 -adrenergic, β_2 -adrenergic or chimeric $\alpha\beta$ -receptors. The adenylyl cyclase activity of each oocyte was measured in response to epinephrine, which determined whether the expressed receptor was binding to the stimulatory, $G_{\alpha s}$ or inhibitory, $G_{\alpha i}$ type of oocyte G proteins. By comparing chimeras 1 (interacts with $G_{\alpha s}$) and 2 (interacts with $G_{\alpha i}$), it was possible to determine that the C3 loop (yellow) between the α helices 5 and 6 determines the specificity of the binding G protein.

Distinct GPCR > distinct G proteins > distinct effectors

G_{α} CLASS	ASSOCIATED EFFECTOR	2ND MESSENGER	RECEPTOR EXAMPLES
$G_{\alpha s}$	Adenylyl cyclase	cAMP (increased)	β -Adrenergic (epinephrine) receptor; receptors for glucagon, serotonin, vasopressin
$G_{\alpha i}$	Adenylyl cyclase K ⁺ channel ($G_{\alpha i}$ activates effector)	cAMP (decreased) Change in membrane potential	α_2 -Adrenergic receptor Muscarinic acetylcholine receptor
$G_{\alpha olf}$	Adenylyl cyclase	cAMP (increased)	Olfactory receptors in nose
$G_{\alpha 12}$	Phospholipase C	IP_3 , DAG (increased)	α_1 -Adrenergic receptor
$G_{\alpha 13}$	Phospholipase C	IP_3 , DAG (increased)	Acetylcholine receptor in endothelial cells
$G_{\alpha t}$	cGMP phosphodiesterase	cGMP (decreased)	Rhodopsin (light receptor) in rod cells

*A given G_{α} subclass may be associated with more than one effector protein. To date, only one major G_{α} has been identified, but multiple G_{α} and G_{β} proteins have been described. Effector proteins commonly are regulated by G_{α} , but in some cases by G_{β} or the combined action of G_{α} and G_{β} .

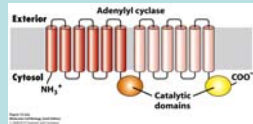
IP_3 = inositol 1,4,5-trisphosphate; DAG = 1,2-diaclyglycerol.
sources: See L. Birbaumer, 1992, Cell 71:1069; Z. Farfel et al., 1999, New Eng. J. Med. 340:1012; and K. Pierce et al., 2002, Nature Rev. Mol. Cell Biol. 3:639.

Table 15-1
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Response-specificity upon GPCR engagement is not only determined by the type of ligand and the type of GPCR, but also the class of G proteins.

GPCRs that activate or inhibit Adenylyl Cyclase

Dual regulation of Adenylyl Cyclase by GPCR/G proteins



The two similar cytosolic catalytic domains of Adenylyl cyclase are separated by six transmembrane spanning regions.

Activation (synthesis of cAMP) as well as inhibition of adenylyl cyclase occurs via the binding of a G α subunit of either a stimulatory or an inhibitory G protein.

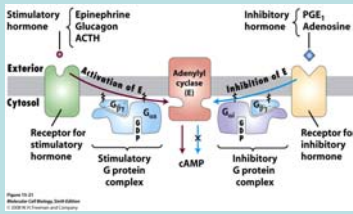
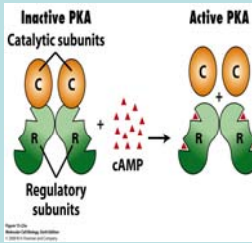


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cAMP binds to the regulatory subunits of PKA and activates the kinase



Binding of cAMP to the two regulatory subunits of Protein kinase A (PKA), induces conformational changes in the regulatory subunits that trigger the release of the two catalytic subunits of the kinase.

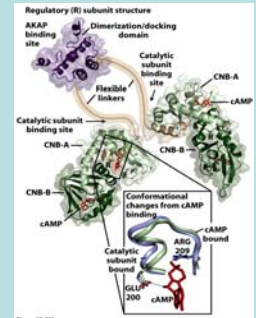


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Scaffold proteins localize PKA to the cell membrane

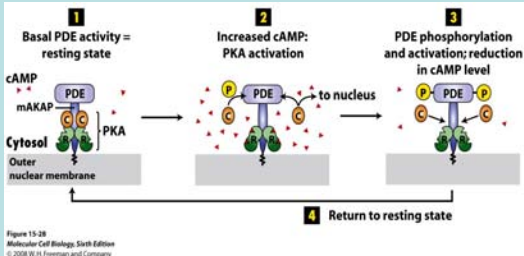


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The anchoring protein, AKAP maintains PKA and its target in a particular cellular localization. PKA and cAMP phosphodiesterase (PDE) are maintained in close proximity to each other and attached to the nuclear membrane of heart muscle by AKAP. cAMP levels control the activity of PKA and PDE downregulates the levels of cAMP, providing a feedback loop.

Cell specific responses induced by ubiquitous cAMP

TABLE 15-2 Cellular Responses to Hormone-Induced Rise in cAMP in Various Tissues*

TISSUE	HORMONE INDUCING RISE IN cAMP	CELLULAR RESPONSE
Adipose	Epinephrine; ACTH; glucagon	Increase in hydrolysis of triglyceride; decrease in amino acid uptake
Liver	Epinephrine; norepinephrine; glucagon	Increase in conversion of glycogen to glucose; inhibition of glycogen synthesis; increase in amino acid uptake; increase in gluconeogenesis (synthesis of glucose from amino acids)
Ovarian follicle	FSH; LH	Increase in synthesis of estrogen, progesterone
Adrenal cortex	ACTH	Increase in synthesis of aldosterone, cortisol
Cardiac muscle	Epinephrine	Increase in contraction rate
Thyroid gland	TSH	Secretion of thyroxine
Bone	Parathyroid hormone	Increase in resorption of calcium from bone
Skeletal muscle	Epinephrine	Conversion of glycogen to glucose
Intestine	Epinephrine	Fluid secretion
Kidney	Vasopressin	Resorption of water
Blood platelets	Prostaglandin I	Inhibition of aggregation and secretion

*Nearly all the effects of cAMP are mediated through protein kinase A (PKA), which is activated by binding of cAMP.

SOURCE: E. W. Sutherland, 1972, Science 177:401.

Table 15-2 Molecular Cell Biology, Sixth Edition

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cAMP regulates glycogen metabolism in liver and muscle cells

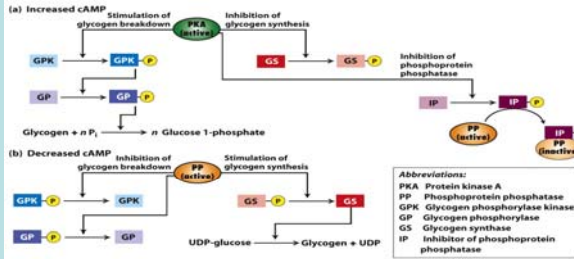


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- A) An increase in cytosolic cAMP activates PKA, which inhibits glycogen synthesis directly and promotes glycogen degradation via a protein kinase cascade. At high cAMP, PKA also phosphorylates an inhibitor of phosphoprotein phosphatase (PP). Binding of the phosphorylated inhibitor to PP prevents this phosphatase from dephosphorylating the activated enzymes in the kinase cascade or the inactive glycogen synthase.
- B) A decrease in cAMP inactivates PKA, leading to release of the active form of PP. The action of this enzyme promotes glycogen synthesis and inhibits glycogen degradation.

GPCRs that activate phospholipase C (PLC)

Intracellular Ca²⁺ acts as second messenger

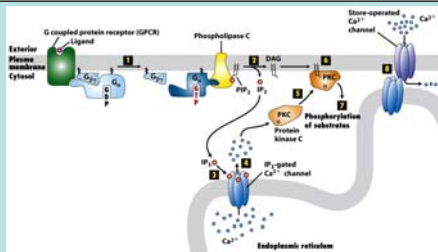


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Both IP₃ and DAG act as second messengers. 6) DAG activates signaling molecules such as protein kinase C (PKC), while 3) IP₃ binds to an IP₃ receptor that is expressed on the cytosolic leaflets of organelle membranes and 4) induces calcium channels to open and release Ca²⁺. 5) Ca²⁺ released into the cytosolic compartment will 7) activate Ca²⁺-dependent proteins such as PKC. The depletion of Ca²⁺ from its storage organelles will induce a channel operated entry from extracellular Ca²⁺ that will replenish the organelles.

NO/cGMP control arterial smooth muscle relaxation

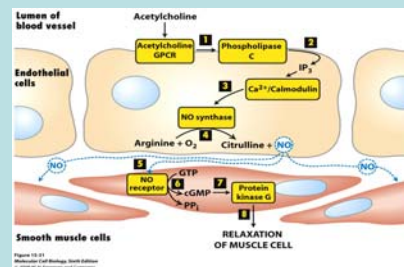


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1-4) Nitric oxide (NO) is synthesized in endothelial cells in response to acetylcholine and the subsequent elevation of Ca²⁺. 5-6) NO diffuses to nearby smooth muscle cells and triggers cGMP production via its binding to NO receptors. 7-8) cGMP activates protein kinase G (PKG) and induces the relaxation of the smooth muscles.

Ca²⁺ as a regulator of cell responses

TABLE 15-3 Cellular Responses to Hormone-Induced Rise in Cytosolic Ca²⁺ in Various Tissues*

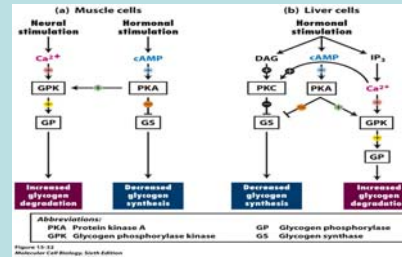
TISSUE	HORMONE INDUCING RISE IN Ca ²⁺	CELLULAR RESPONSE
Pancreas (acinar cells)	Acetylcholine	Secretion of digestive enzymes, such as amylase and trypsinogen
Parotid (salivary) gland	Acetylcholine	Secretion of amylase
Vascular or stomach smooth muscle	Acetylcholine	Contraction
Liver	Vasopressin	Conversion of glycogen to glucose
Blood platelets	Thrombin	Aggregation, shape change, secretion of hormones
Mast cells	Antigen	Histamine secretion
Fibroblasts	Peptide growth factors (e.g., bombesin and PDGF)	DNA synthesis, cell division

*Hormone stimulation leads to production of inositol 1,4,5-trisphosphate (IP₃), a second messenger that promotes release of Ca²⁺ stored in the endoplasmic reticulum.

SOURCE: M. J. Berridge, 1987, *Ann. Rev. Biochem.* 56:159; M. J. Berridge and R. F. Irvine, 1984, *Nature* 312:315.

Table 15-3
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Interaction of signaling pathways specify cell responses



The interplay of activation and inhibition signaling pathways eventually account for the specificity in cell responses. A) Neuronal stimulation of striated muscle cells or epinephrine binding to β -adrenergic receptors on their surfaces leads to increased cytosolic Ca²⁺ or cAMP, respectively. The key regulatory enzyme glycogen phosphorylase kinase (GPK) is activated by Ca²⁺ ions and by PKA. B) In liver cells, hormonal stimulation of β -adrenergic receptors leads to increased cytosolic cAMP as well as DAG and IP₃. IP₃ increases [Ca²⁺]_i and glycogen degradation, while DAG activates PKC that will block glycogen synthase in synergy to PKA.