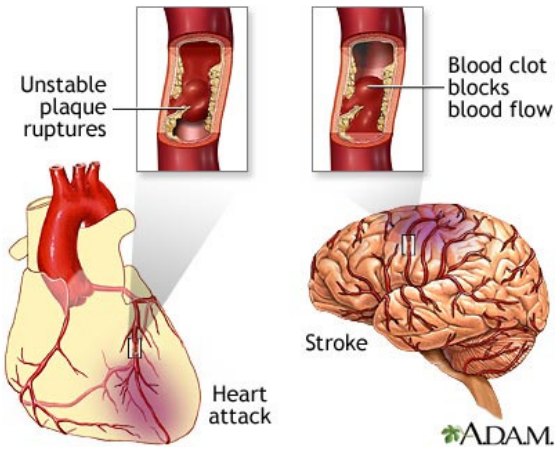
Death of Heart & Neuronal Cell:

Ischemia/Reperfusion Injury Excitotoxicity

MUDr. Jan Plášteník, Ph.D.

Ischemic damage to heart or brain is the major cause of death worldwide



http://www.nlm.nih.gov/medlineplus/ency/imagepages/19314.htm

Distinguish:

- **HYPOXIA (ANOXIA)**
  - Lack of oxygen
- **ISCHEMIA**
  - Lack of oxygen
  - Blood flow restricted
  - → block in supply of nutrients
  - → block in removal of metabolic waste products

Metabolic changes due to hypoxia/ischemia:

Lack of energy

- Healthy myocardium:
  - Oxidizes both glucose (32 ATP/mol) and fatty acids (106 ATP/mol palmitate)
  - 95% ATP from oxidative phosphorylation
  - 60-70% ATP used for contraction
  - 30-40% ATP used for ion pumps
- Hypoxia/ischemia:
  - Only anaerobic glycolysis available, it provides 2 ATP/mol from delivered glucose, or 3 ATP/mol from glycogen (ultimate source)
  - …contractile dysfunction must develop

Metabolic changes due to hypoxia/ischemia:

Catabolism of ATP

$$ATP \rightarrow ADP + Pi \qquad ADP + Pi \rightarrow ATP$$

$$\text{Creatine-phosphate} + ADP \leftrightarrow \text{Creatine} + ATP$$

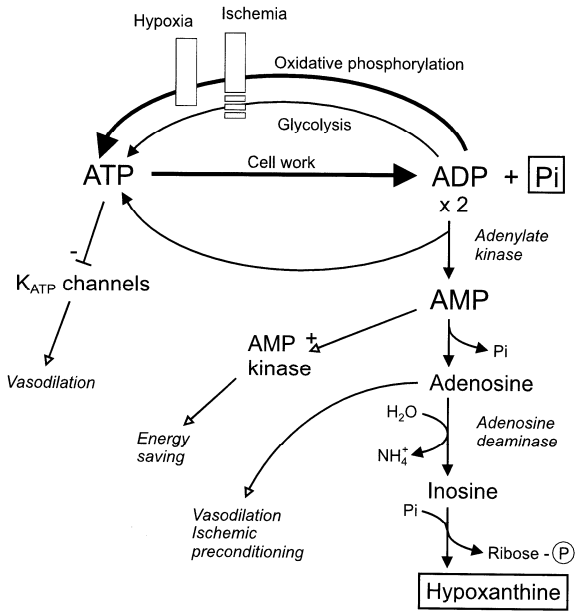
- Energy pool of the heart:
  - ATP ~ 5 µmol/g wet wt
  - Creatine phosphate ~ 8 µmol/g wet wt
- Energy consumption:
  - ~ 0.5 µmol ATP/g wet wt and second
- Adenylate kinase reaction:

$$ADP + ADP \leftrightarrow ATP + AMP$$

AMP-dependent protein kinase

- Stimulates:
  - Glucose entry
  - Glycolysis
  - Autophagy
  - Mitochondrial biogenesis
- Inhibits:
  - Protein synthesis
  - Fatty acid synthesis

…energy saving in ischemia

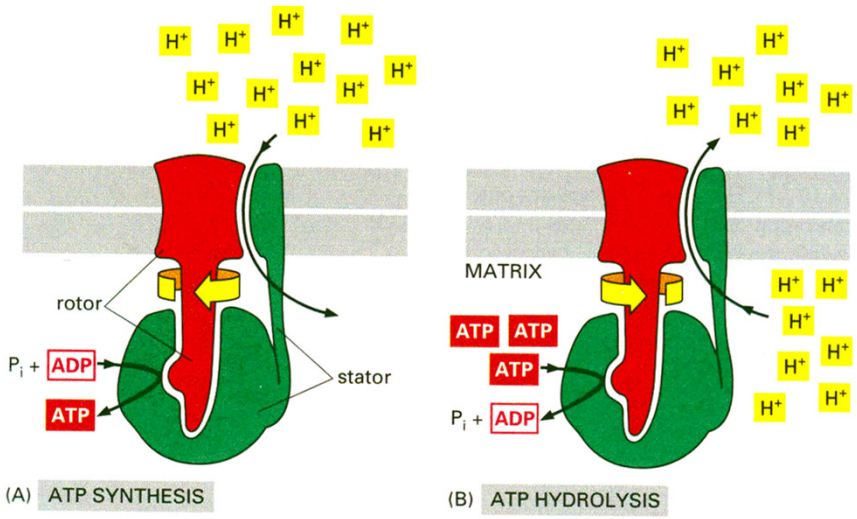


Adenosine

- Produced in ischemia by:
  - Cellular 5´-endonucleases from AMP
  - Membrane phosphohydrolases from ATP/ADP
- Acts on membrane receptors:
  - A_1R, $A_{2A}R$, $A_{2B}R$, A_3R
- Beneficial effects in ischemia:
  - Decreases heart rate (…saves energy)
  - Vasodilation of coronary arteries
  - Inhibition of inflammatory resposne to hypoxia
  - Ischemic preconditioning

Metabolic changes due to hypoxia/ischemia:

ATP synthase works in reverse mode!



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Inhibiting factor 1 (IF-1)

- Ubiquitous mitochondrial polypeptide
- Partial inhibitor of ATP synthase/hydrolase
- pH optimum 6.8…cytoprotective in ischemia
- Inhibited by cAMP-dependent phosphorylation
- Role in metabolic reprogramming (balance glycolysis/oxidative phosphorylation)
- Regulation of ATP synthesis in response to demand

Metabolic changes due to hypoxia/ischemia:

Myoglobin and NO

- Myoglobin - only short-time oxygen storage
- Removes excess of NO:

$Mb(FeII)\text{-}O_2 + NO \rightarrow Mb(FeIII) + NO_3^-$

- In hypoxia switch to NO production:

$Mb(FeII) + NO_2^- + H^+ \rightarrow Mb(FeIII) + NO + OH^-$

- Effects of NO in cardiac ischemia:
  - Vasodilation of coronary arteries
  - Decreased contractility
  - Decreased mito ATP (and ROS) production

Cardio-protective

Metabolic changes due to hypoxia/ischemia:

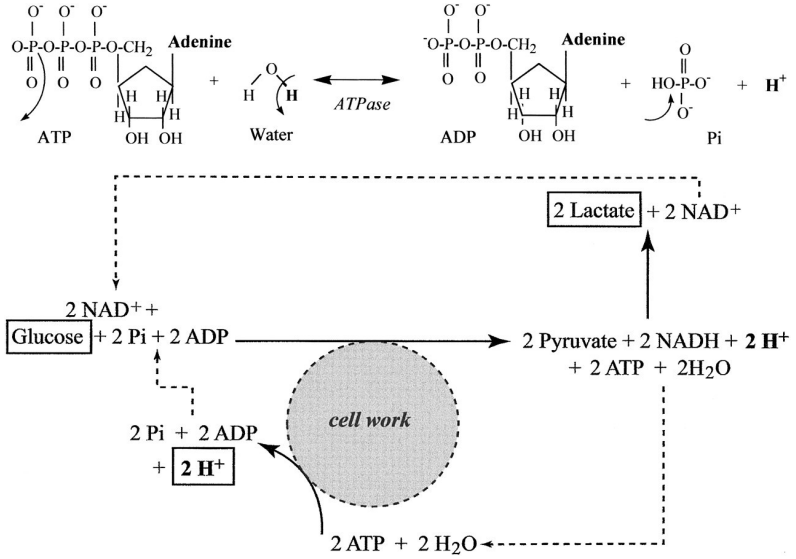
„Lactic acidosis“ is not caused by lactate !

- Lactate is formed as anion
- Glycolysis from glucose to lactate is pH neutral
- H^+ producing:
  - Hexokinase + $1H^+$
  - Phosphofructokinase + $1H^+$
  - Glyceraldehyde-3-phosphate dehydrogenase + $2H^+$
- H^+ consuming:
  - Pyruvate kinase – $2H^+$
  - Lactate dehydrogenase – $2H^+$

$CH_3COCOO^- + NADH + H^+ \longleftrightarrow CH_3CHOHCOO^- + NAD^+$

Pyruvate — Lactate dehydrogenase — Lactate

Protons accumulate due to non-mitochondrial ATP turnover!



Robergs et al. Am J Physiol Regul Integr Comp Physiol 287: R502-R516, 2004

Metabolic changes due to hypoxia/ischemia: **Acidosis in ischemia is caused by:**

- Non-mitochondrial ATP turnover
- Accumulation of carbon dioxide
- Accumulation of phosphate from degradation of AMP
- Exhaustion/inhibition of homeostatic mechanisms:
  - Buffering with inorganic phosphate and proteins
  - Transport of lactate and H^+ across the cell membrane

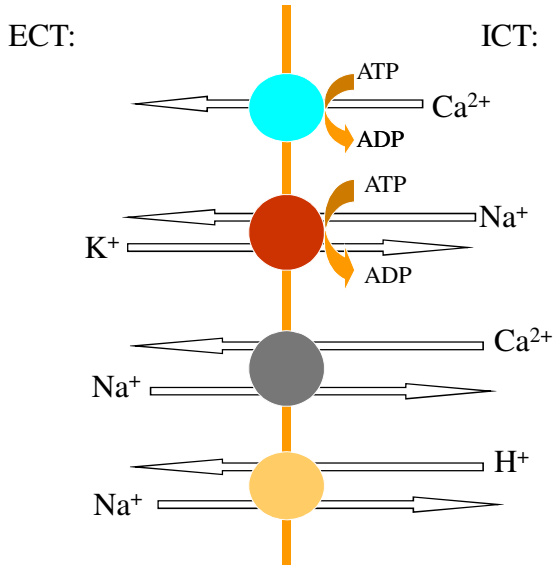
Metabolic changes due to hypoxia/ischemia:

Disruption of ion gradients

- Physiological gradients:

	ICT:	ECT:
H^+:	70 µM (pH 7.2)	40 µM (pH 7.4)
Na^+:	5-15 mM	145 mM
K^+:	140 mM	5 mM
Ca^{2+}:	10^{-4} mM	2.5 mM

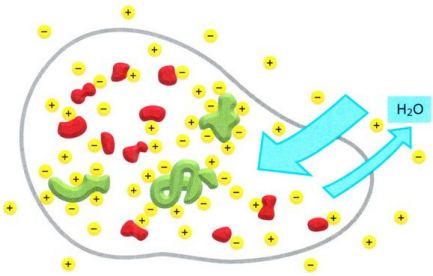
The ion gradients are kept by continuous action of membrane ion pumps:



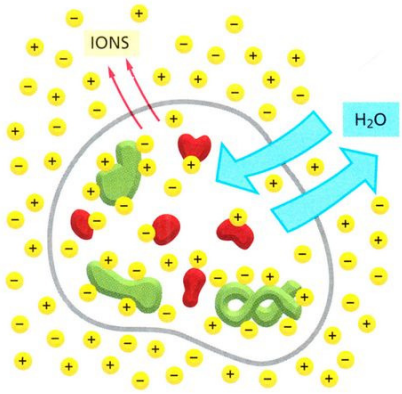
The primary reason for necessity of ion pumping:

The Donnan effect

The problem:



The solution:



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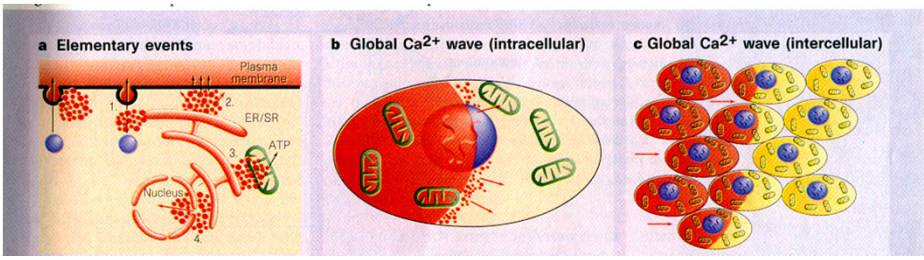
Calcium in the cell:

- Second messenger involved in virtually aspect of cell life
- In cytosol only 0.1-0.2 μM, about 1 μM is a signal
- Source of the signal is:
  - outside:
    - ligand-operated Ca^{2+} channels
    - voltage-operated Ca^{2+} channels
  - ER stores:
    - PI3 receptor/channel
    - ryanodine receptor/channel
      - cell membrane potential-dependent (striated muscle)
      - Ca^{2+} -dependent (heart, CNS)



- **Information in Ca^{2+} signal is encoded by its**
  - **LOCALISATION**
  - **FREQUENCY**
  - **AMPLITUDE**

(Berridge et al., Nature 1998, 395: 645-648)

(Patho)biochemical effects of intracellular calcium elevation:

- Activation of enzymes:
  - Ca/calmodulin-dependent protein kinases
  - Some phosphatases (calcineurin)
  - Endothelial and neuronal NO synthases
  - Calpains (Ca-activated non-lysosomal proteases)
  - Phospholipases (→ membrane disruption, synthesis of prostanoids)
  - Endonucleases (→ DNA fragmentation in apoptosis)
- Muscle contraction
- Release of hormones and neurotransmitters
- Regulation of gene expression
- Assembly of cytoskeleton (excessive Ca → membrane blebbing)
- Mitochondria:
  - Regulation of respiration
  - Induction of mitochondrial permeability transition (MPT)

Metabolic changes due to hypoxia/ischemia:

Disruption of ion gradients

H^+ ⇑

Acidosis

Na^+ ⇑

Failure of Na/K ATP-ase

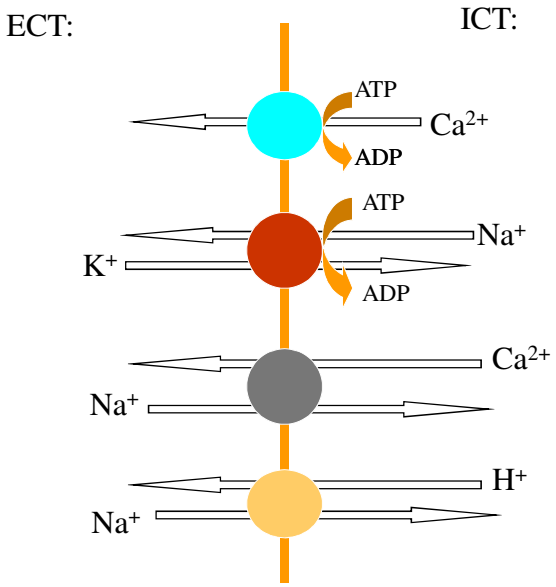
Membrane Na/H exchanger

Ca^{2+} ⇑

Failure of ATP-dependent pumping to ECT (PMCA) and ER (SERCA)

Membrane Na/Ca exchange impaired

Ion gradients are disrupted in ischemia:



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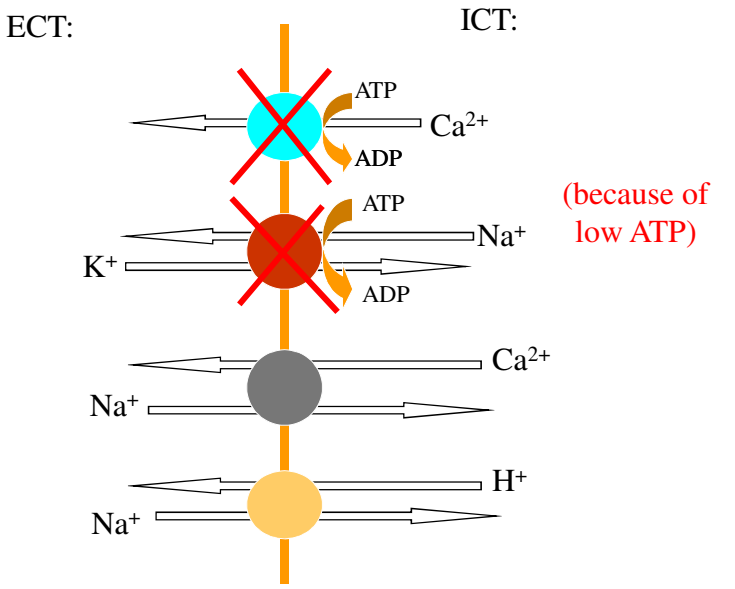
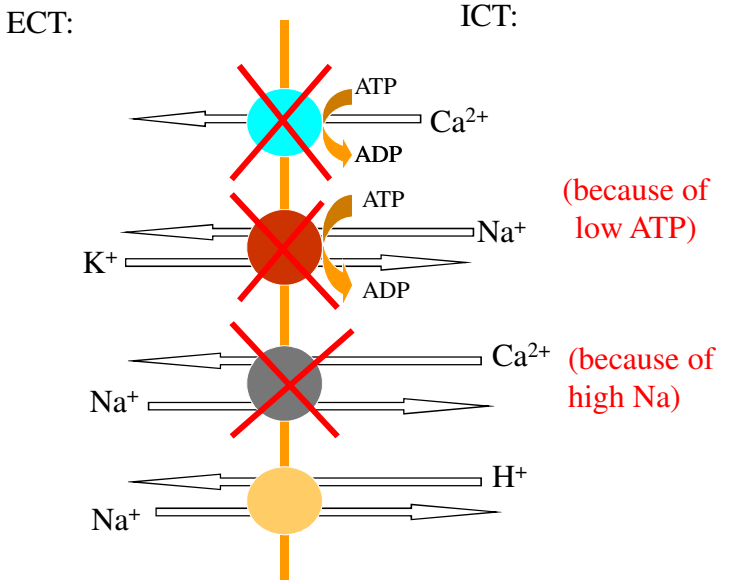


(because of low ATP)

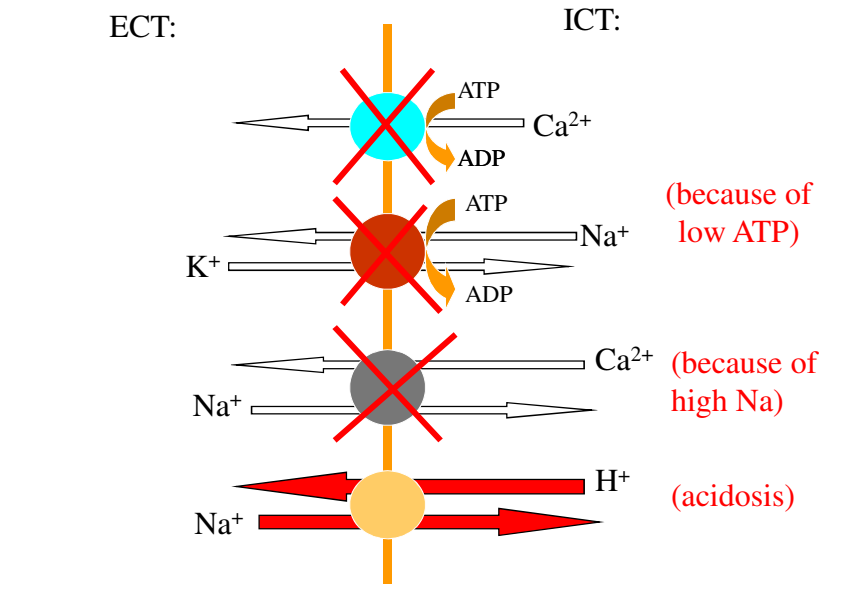
Ion gradients are disrupted in ischemia:



(because of low ATP)

(because of high Na)

Ion gradients are disrupted in ischemia:

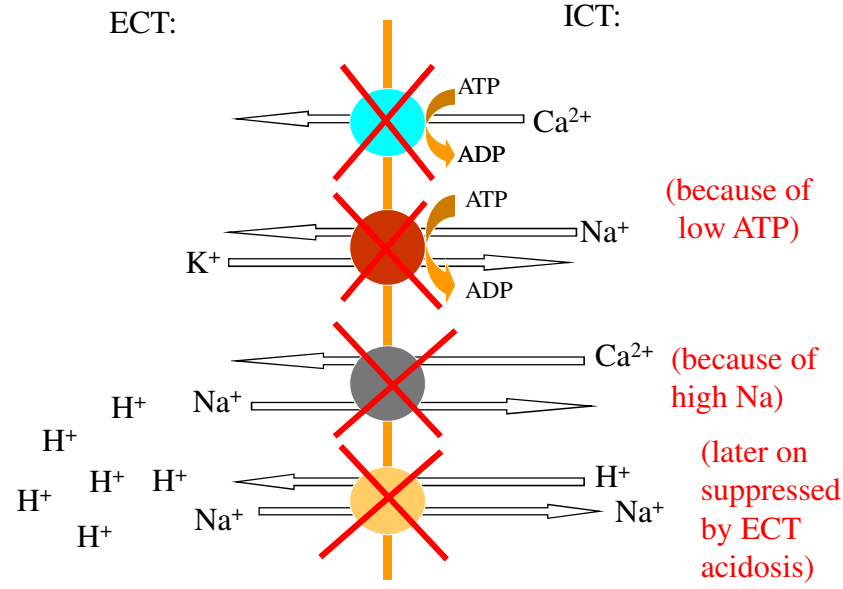


(because of low ATP)

(because of high Na)

(acidosis)

Ion gradients are disrupted in ischemia:



(because of low ATP)

(because of high Na)

(later on suppressed by ECT acidosis)

Metabolic changes due to hypoxia/ischemia:

Disruption of ion gradients

$H^+ \Uparrow$, $Na^+ \Uparrow$, $Ca^{2+} \Uparrow$

Consequences:

Effects of Ca^{2+} are limited by acidosis

Increased intracellular osmolarity leads to swelling, osmotic rupture and cells death

Reoxygenation injury

Reoxygenation injury

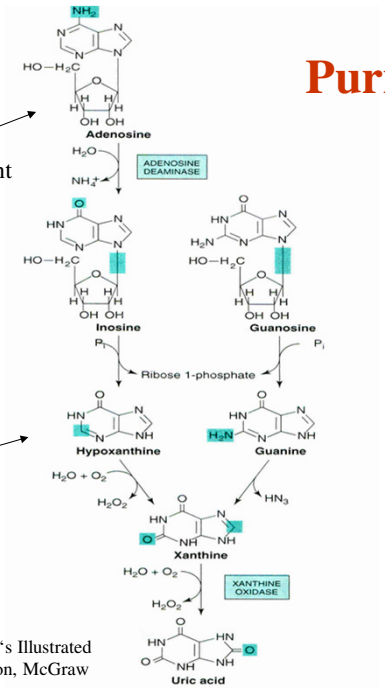
- Additional insult to the ischemic tissue after restoration of blood flow
- Important especially after short ischemia
- If tissue damaged irreversibly by ischemia, reperfusion adds little to the damage, but can still release toxins from dead tissue (HMGB1 and other DAMPs) to the circulation
  - Multiple organ dysfunction syndrome (MODS)
  - Systemic inflammatory response syndrome (SIRS)
- Burst of oxygen radicals, NO and eicosanoids

Reoxygenation injury: Where relevant?

- Intestine: thrombosis, torsions and strangulated hernias
- Heart stroke: thrombolytic therapy, angioplasty
  - … arrhythmias, myocardial stunning
- Perinatal asphyxia
- Hemorrhagic shock after restoration of blood volume
- Carbon monoxide poisoning treated with oxygen
- Sleep apnoea
- Storage of organs for transplantation

Purine degradation

Vasodilating agent



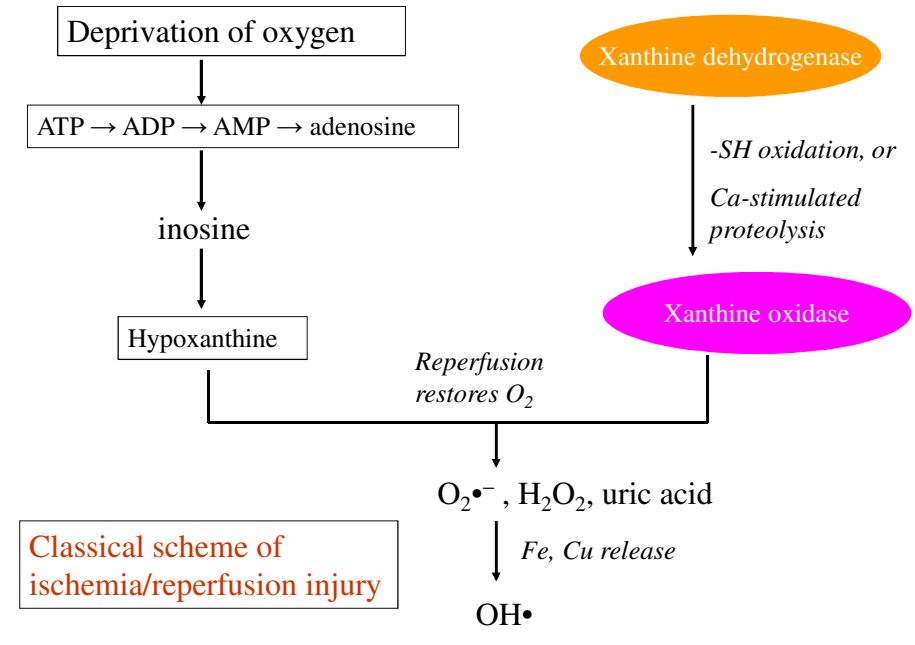
Accumulates in ischemia

Scheme from the Harper's Illustrated Biochemistry, 27th edition, McGraw Hill Co., 2006

Xanthine oxidase/dehydrogenase:

D form (dehydrogenase): NAD-dependent, physiologically prevails

O form (oxidase): oxygen-dependent, generates ROS



Modified from: J.M.McCord Fed. Proc. 46, 2402, 1987

- Xanthine oxidase model explains well the ischemia/reperfusion injury of cat intestine
- But human heart does not contain any xanthine oxidase
  - Enzyme present only in the liver, intestine and lactating mammary gland
  - Conversion to O form in human intestine is slow
- Other sources of ROS more important:
  - **Leukocytes**
    - … no-reflow phenomenon (obturation of capillaries with white blood cells
  - **Mitochondria**
    - … more leaky after a period of ischemia
    - … mitochondrial permeability transition (MPT)

Ca^{2+} uptake into mitochondria

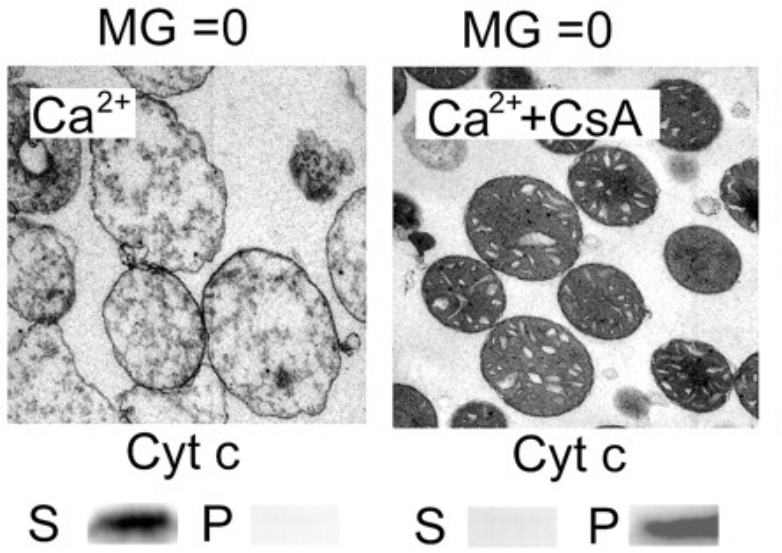
- Ca^{2+} uniporter: facilitated diffusion down the electrochemical gradient
- Metabolic regulation: Rate-limiting dehydrogenases are sensitive to Ca^{2+}:
  - Pyruvate dehydrogenase
  - Isocitrate dehydrogenase
  - 2-oxoglutarate dehydrogenase

In this way heart mito respond to the cellular need of ATP (not ATP depletion!)

- Sequestration/buffering of cytosolic calcium under certain condition

Mitochondrial Permeability Transition Pore (MPT)

- Opening of a "megachannel" in the inner mitochondrial membrane
- Permeable for any molecule < 1500 Da
- Collaps of the inner membrane potential, dissipation of proton gradient, uncoupling or inhibition of respiration
- Swelling of mitochondria



Speer O et al. J. Biol. Chem. 2003;278:34757-34763

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"Megachannel" (MPT) opening is

- Triggered by: matrix Ca^{2+}
- Stimulated by:
  - Oxidants
  - Depolarisation
  - Inorganic phosphate
- Inhibited by:
  - Protons (low matrix pH)
  - Magnesium ions
  - ATP and ADP
  - Cyclosporin A

Function of MPT:

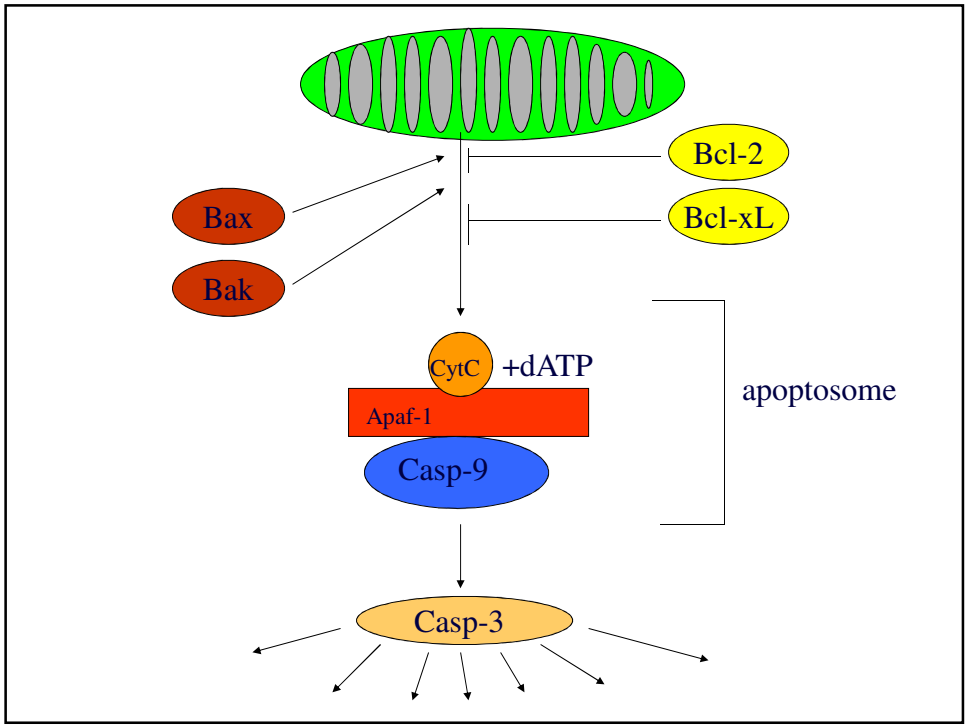
- Physiologic (reversible) MPT opening:
  - Energetically "cheap" efflux of Ca^{2+} from mitochondria?
  - Calcium signalling:

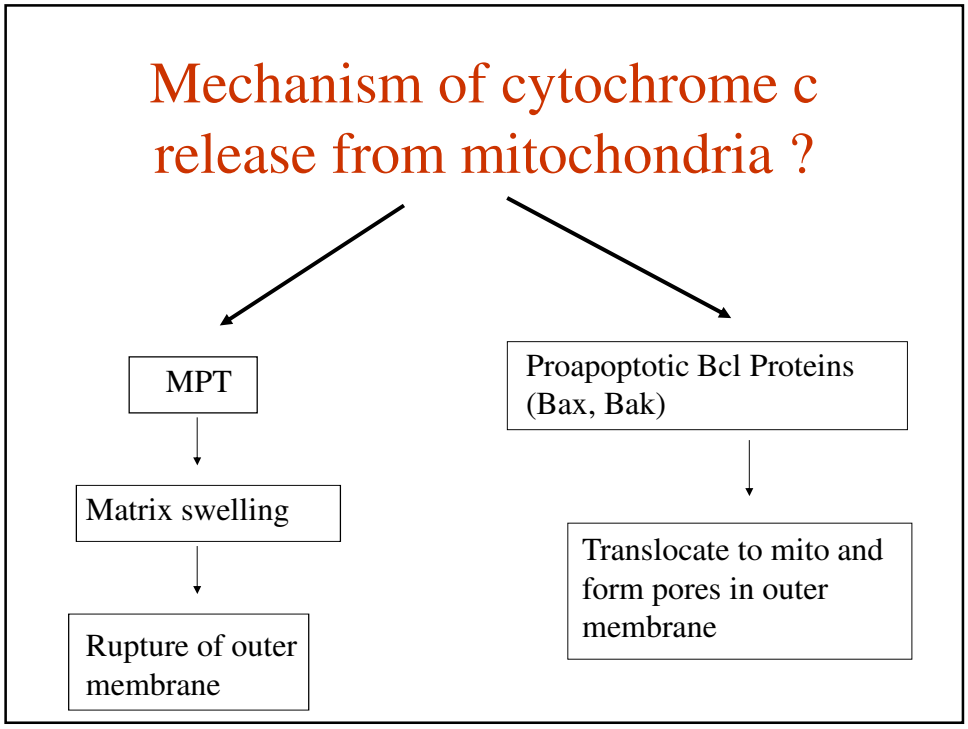
    Ca^{2+}-induced calcium release

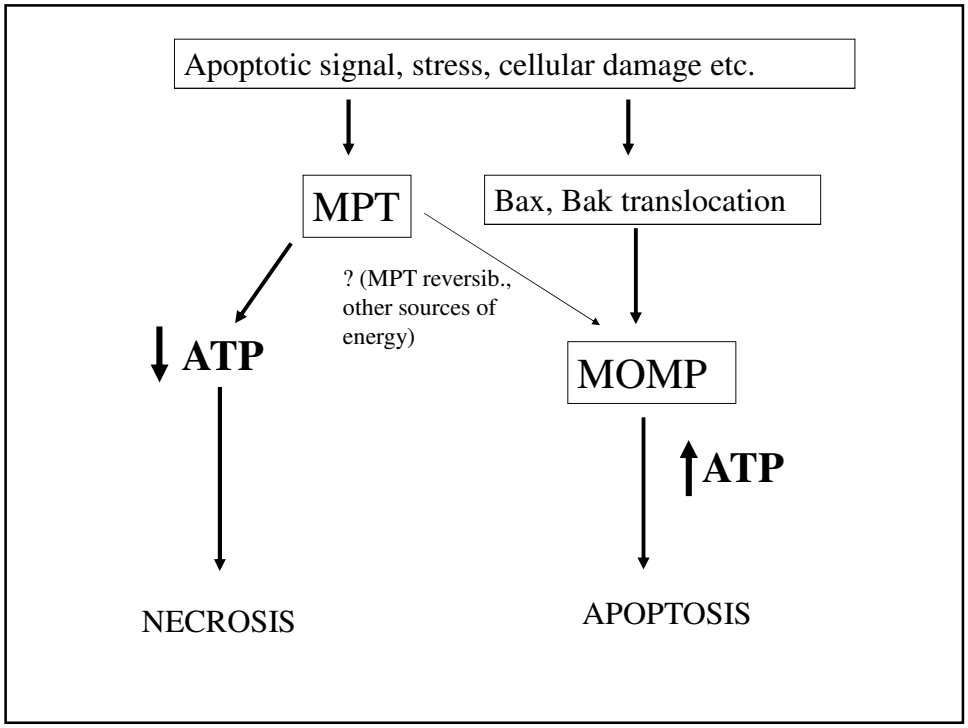
    ....mitochondria as a "Ca^{2+} signalling storing memory device"
- Pathologic (irreversible):
  - Cell death (necrosis and apoptosis)
  - Mechanism how old mitochondria are marked for autophagy ?

MPT and apoptosis:

Common misconception ?



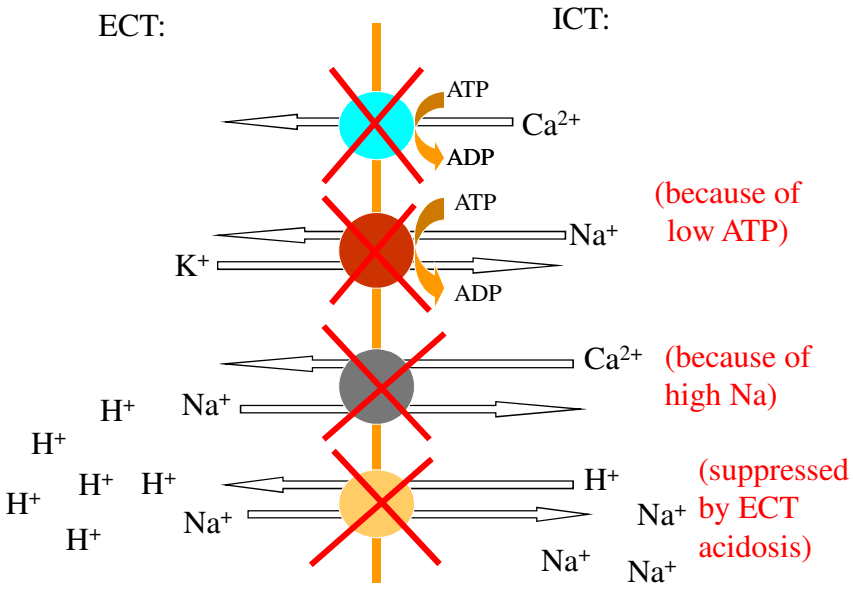

Mechanism of cytochrome c release from mitochondria ?
MPT
Matrix swelling
Rupture of outer membrane
Proapoptotic Bcl Proteins (Bax, Bak)
Translocate to mito and form pores in outer membrane


Apoptotic signal, stress, cellular damage etc.
MPT
Bax, Bak translocation
? (MPT reversib., other sources of energy)
ATP
MOMP
ATP
NECROSIS
APOPTOSIS

MPT:
The critical event in reoxygenation injury of the heart

- Ischemia: low pH protects from MPT
- Reperfusion: the MPT opening in myocardium is favored - coincidence of:
  - Burst of oxidative stress
  - Depletion of adenine nucleotides
  - High level of Pi (from ATP degradation)
  - High level of Ca^{2+}
  - Rapid normalization of pH

Upon reperfusion acidosis is normalized first:



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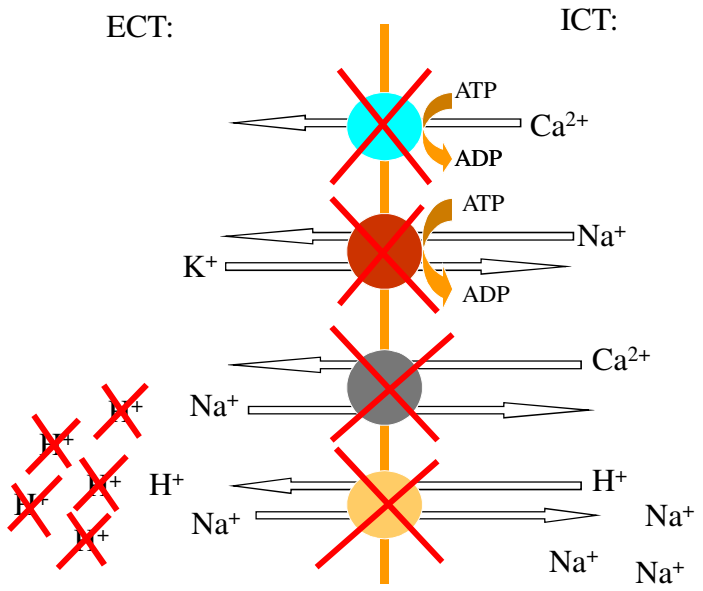

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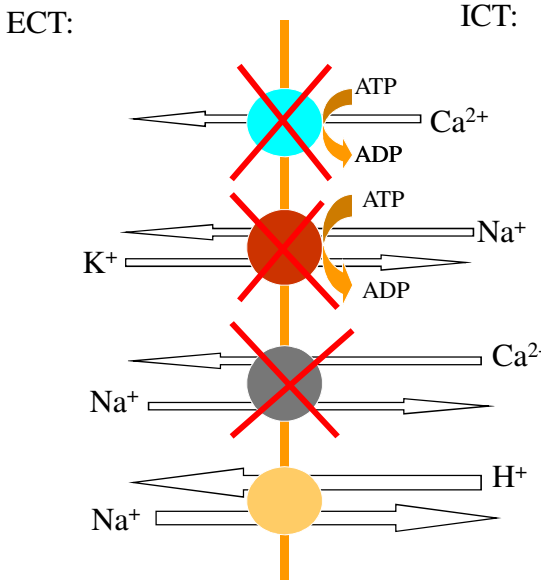
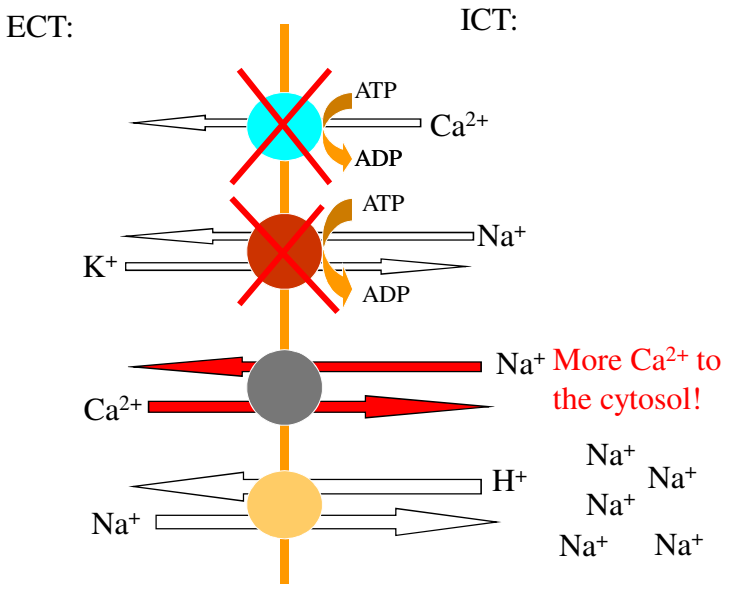


The Na/Ca exchanger can start to work in reverse:



MPT:
The critical event in reoxygenation injury of the heart

- Favored at reperfusion by high Ca^{2+}, high phosphate, still low ATP/ADP + normal pH
- Mitochondria following MPT:
  - Degrade ATP (reverse action of ATP-synthase)
  - Produce more ROS (lack of cytochrome c)
  - Spill Ca^{2+}
- …MPT stimulated in further mitochondria
- … …Necrotic cell death

Poly(ADP-ribose)polymerase (PARP-1)

- Chromatin-bound enzyme, activated by damage to DNA (single-strand breaks)
  - By ROS
  - By Ca-activated endonucleases
- Splits NAD^+, adds poly(ADP-ribose) on nuclear proteins

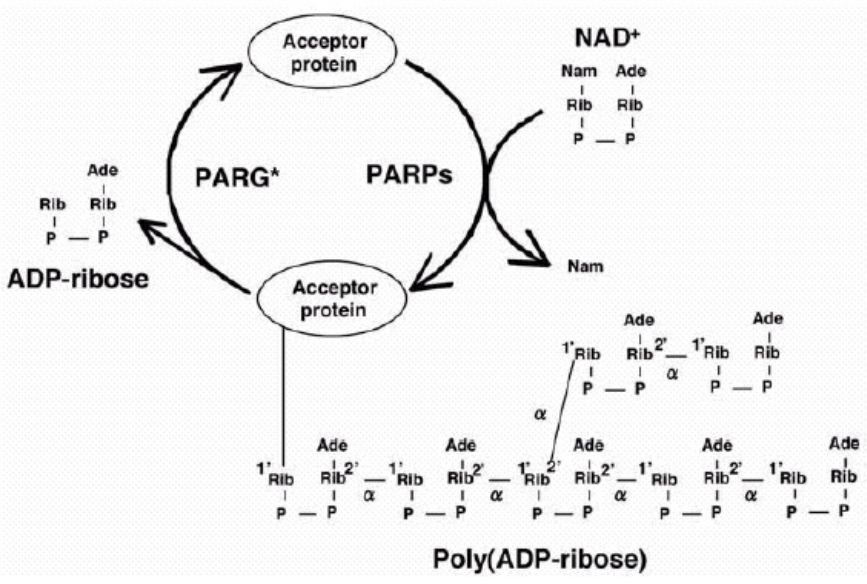


Figure from:Miwa M et al:Roles of Poly(ADP-Ribose) Metabolism in the Regulation of Centrosome Duplication and in the Maintenance of Neuronal Integrity, DNA Surveillance and Repair, Madame Curie Bioscience Database, Landes Biosciences

Poly(ADP-ribose)polymerase (PARP-1)

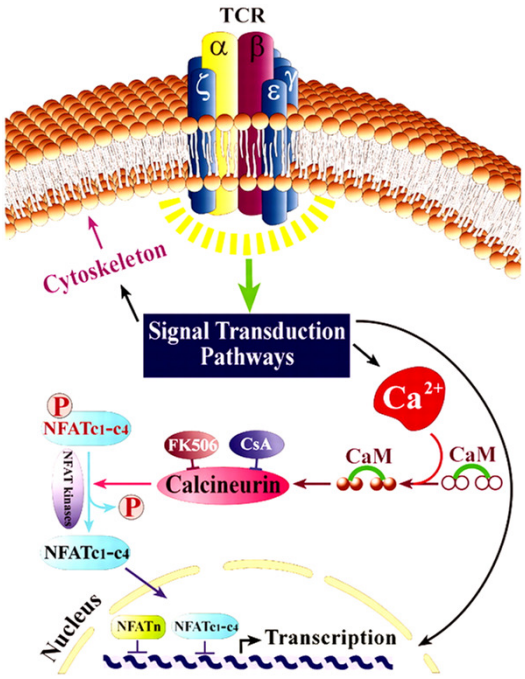
- Chromatin-bound enzyme, activated by damage to DNA
- Splits NAD^+, adds poly(ADP-ribose) on nuclear proteins
- Facilitates DNA repair and promotes survival
- Cleaved (inactivated) by caspases in apoptosis
- But if DNA damage excessive, it depletes cellular NAD^+
- Parthanatos – form of regulated cell death:

Poly(ADP-ribose)
↓
Translocation of mitochondrial apoptosis-inducing factor (AIF) to the nucleus
↓
DNA fragmentation

Prevention of ischemia/reperfusion injury?

- THERAPEUTIC HYPOTHERMIA
- MPT inhibitors
  - Cyclosporin A:
    - Binds cyclophilin D (part of the MPT pore) – but not specific.
    - Effect in heart ischemia?
    - Currently Phase II/III clinical trial for brain trauma.
  - TRO40303: failed Phase II clinical trial for myocardial infarction.
- Antioxidants?
- PARP-1 inhibitors used for treatment of cancer, others in clinical trials

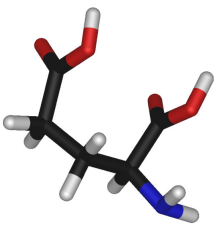
Immunosuppressive effect of cyclosporin A <u>is not</u> caused by MPT inhibition

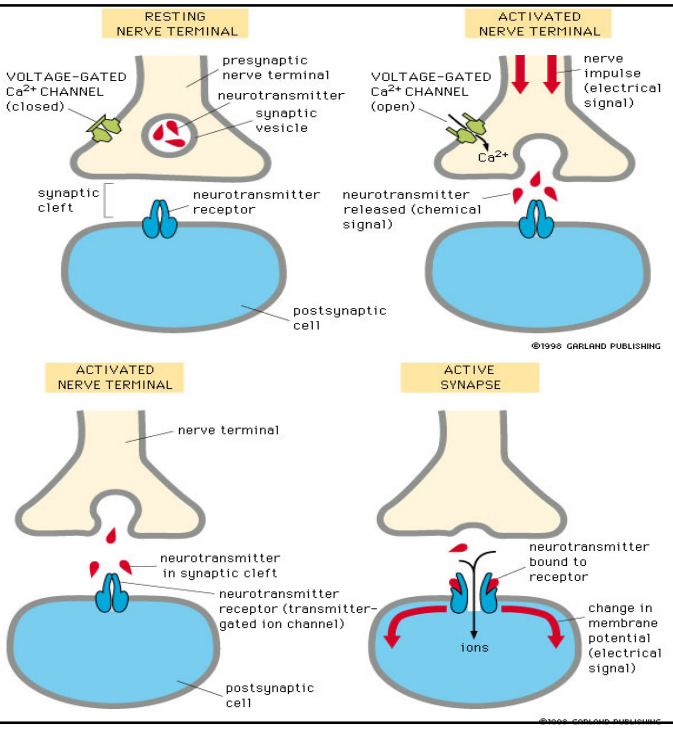


Katzav S, Blood 2004, 103 (7), 2443-51.

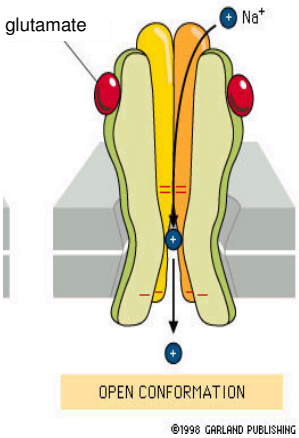
Excitotoxicity

Most of the excitatory synapses in human brain use **glutamate**

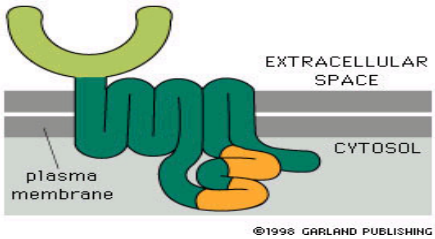


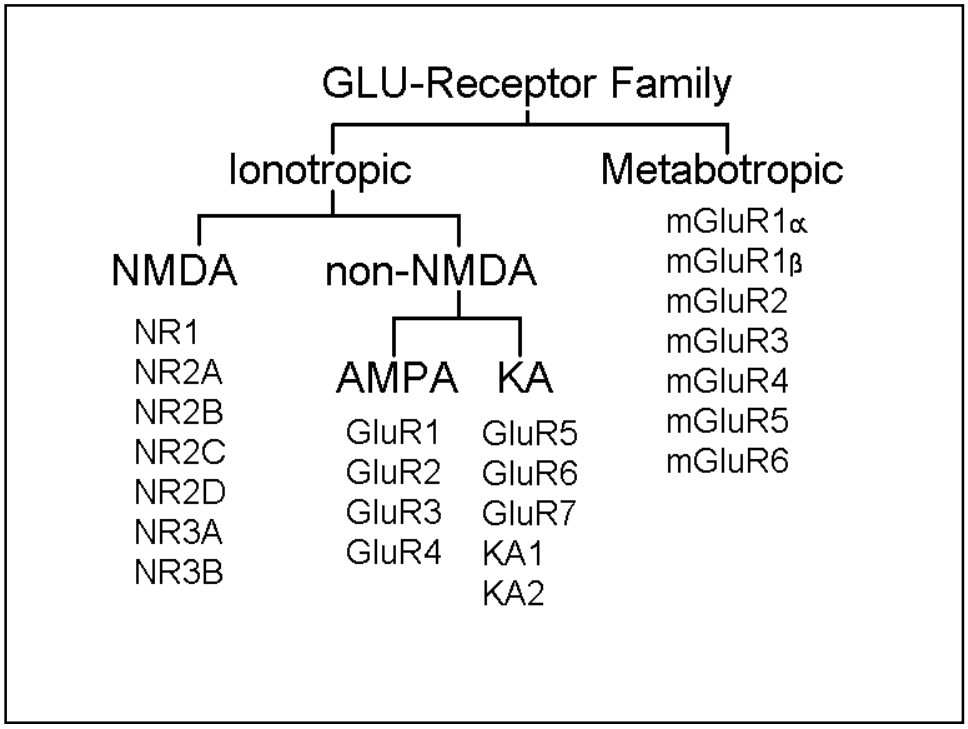


Ionotropic glutamate receptors:
Ligand-gated ion channels

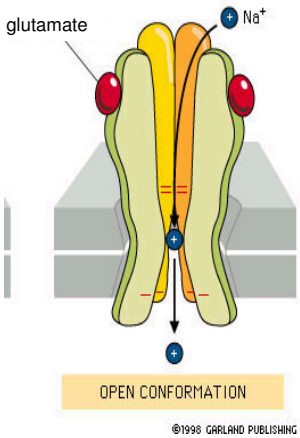


Metabotropic glutamate receptors:
„Seven-spanning“
Coupled to G proteins





Ionotropic glutamate receptors: Ligand-gated ion channels



Non-NMDA receptors (AMPA, kainate):

Rapid synaptic transmission, pass Na^+

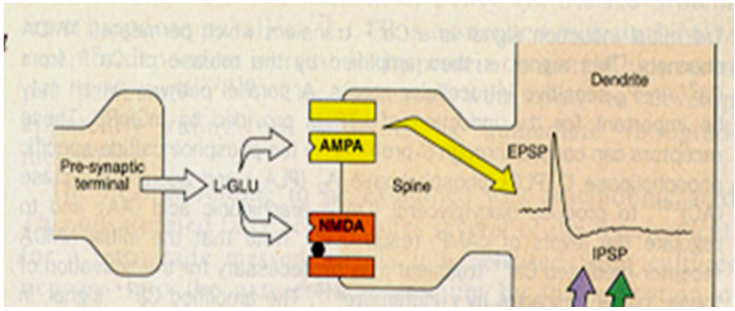
NMDA receptors:

Long-lasting changes in synaptic efficacy,

pass Na^+ and Ca^{2+}

Glutamate receptors and induction of LTP (long-term potentiation)

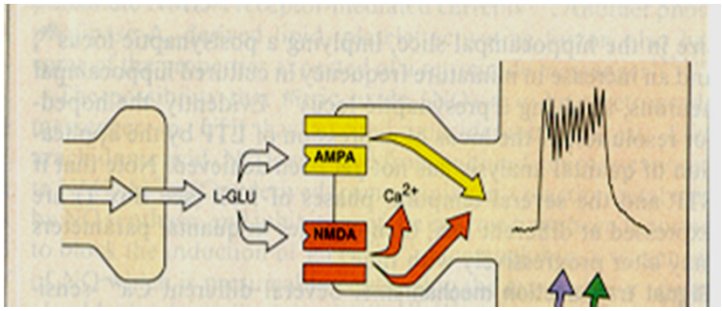
1. Low-frequency: only AMPA receptors active



Bliss & Collingridge, Nature 1993, 361: 31-39)

Glutamate receptors and induction of LTP (long-term potentiation)

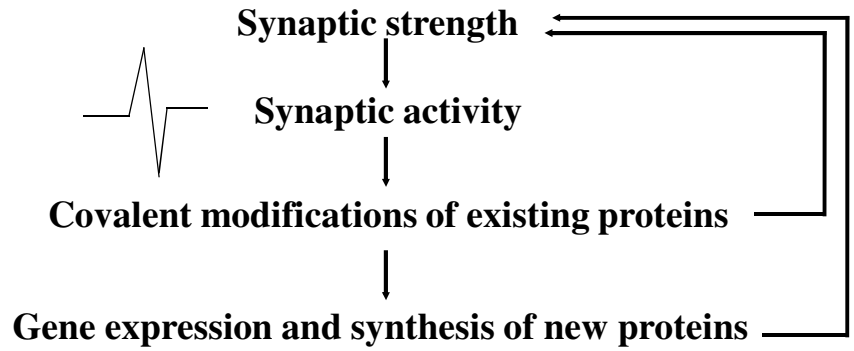
2. High-frequency: both AMPA and NMDA receptors active



...NMDA receptor: "coincidence detector"

Bliss & Collingridge, Nature 1993, 361: 31-39)

Synaptic plasticity:



(Finkbeiner & Greenberg, J. Neurobiol. 1998, 37: 171-189).

Excitotoxicity in brain ischemia/reperfusion

- Energy depletion → collapse of ionic gradients → Ca^{2+} ⇑ → excesive release of glutamate
- Flow of Na^{+} and Ca^{2+} to postsynaptic neurones
- Na^{+} ⇑: osmotic swelling, necrosis
- Ca^{2+} ⇑:
  - → Mitochondrial ROS ⇑
  - → Nitric oxide ⇑
  - → Ca^{2+} -stimulated proteases ⇑
  - → Ca^{2+} -stimulated phospholipases ⇑
    - → eicosanoids ⇑

Damage perpetuates

Delayed cell death (apoptotic or necrotic)

Glutamate transporters depend on ion gradients across cell membrane

Membrane depolarization → glutamate transport can be reversed !

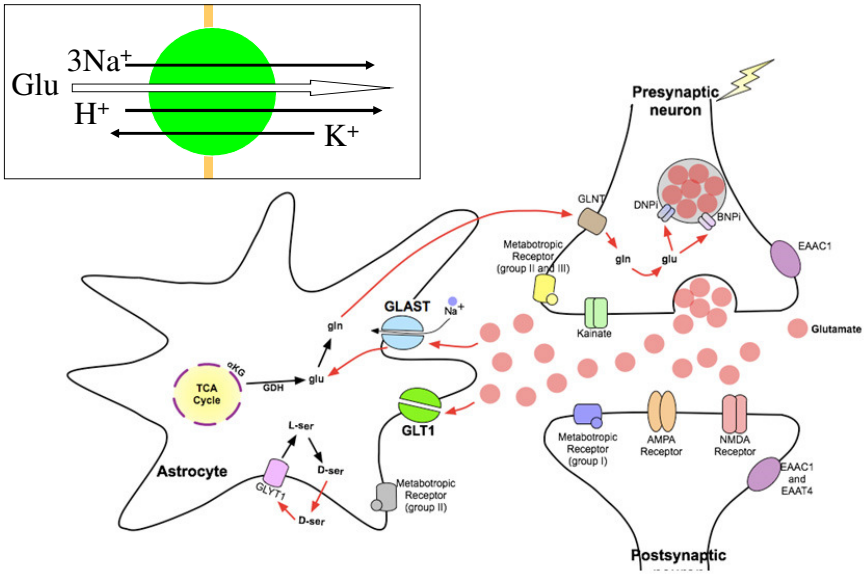


Figure from: Liu Y-P et al: A possible therapeutic strategy for CNS repair: To blockade calcium regulation of glutamate aspartate transporter regulation in astrocytes (http://research.ncku.edu.tw/re/articles/e/20081128/3.html)

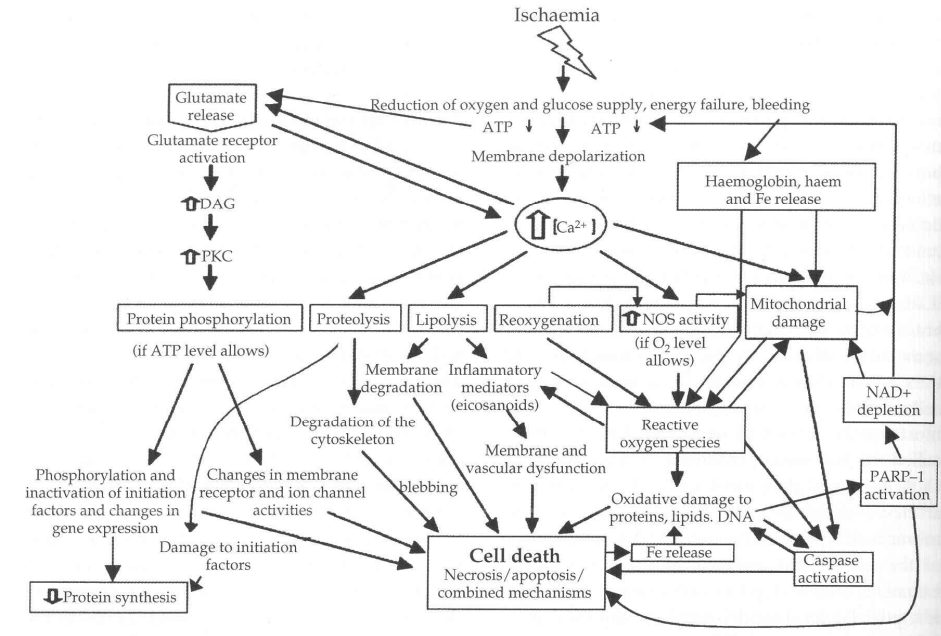


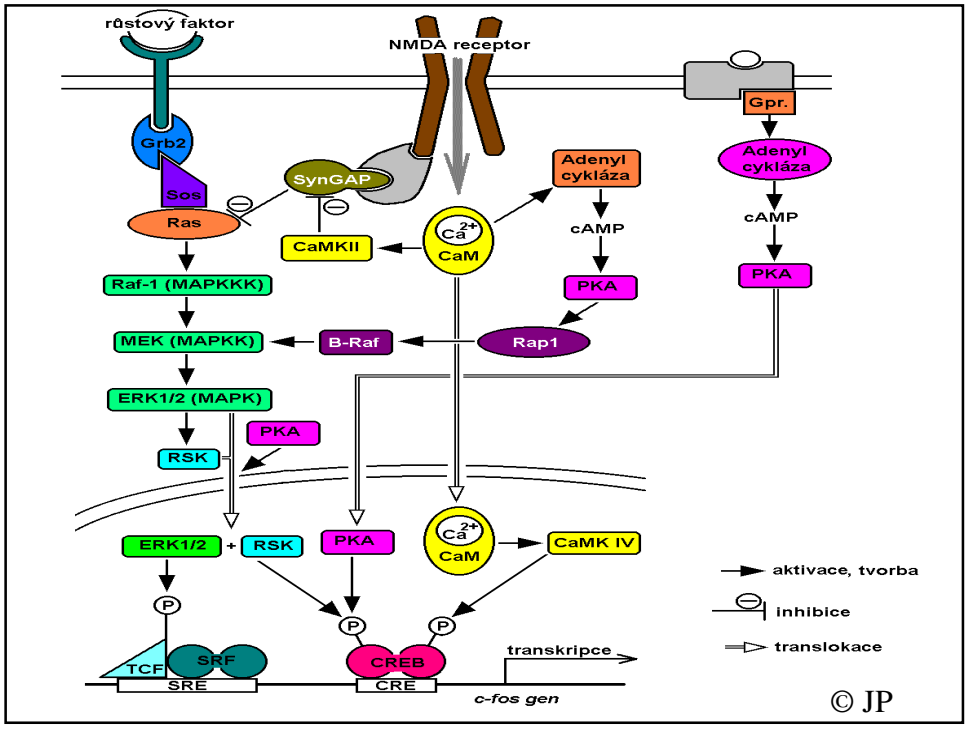
Fig. from: B. Halliwell & J.M.C. Gutteridge: Free Radicals in Biology and Medicine, 4th edition, Oxford University Press 2007

Excitotoxicity in chronic neurodegeneration

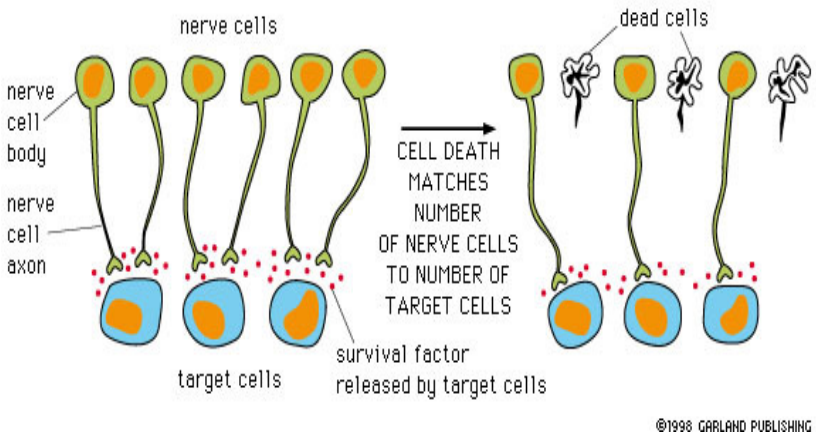
- Concept of **‚slow excitotoxicity‘**:
  - Even normal stimulation with glutamate can be toxic to the neuron if its energy production is impaired
  - If formulated in this way, it can be implicated in pathogenesis of many chronic neurodegenerative diseases

Too little glutamate is harmful as well

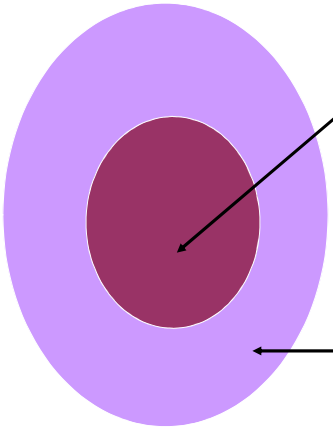
- Certain level of neuronal activity is necessary for neuronal survival
- Cultivation of neurones in vitro: additions of glutamate/KCl in certain (early) phase of culture development prevent apoptosis
- NMDA receptor connected to the growth factor signaling (Ras/MAPK etc.)
- Overlap between survival (antiapoptotic) pathways and pathways of synaptic plasticity



Ontogenesis of nervous system



Brain infarction



Core lesion (anoxia):

Rapid necrotic death, too much glutamate

Penumbra (hypoxia):

Delayed cell death, rather apoptotic

Too little glutamate because of lost afferentation?

Is it why all clinical tests with NMDAR inhibitors failed?

Young et al., Nature Med., 1999, 5: 448-453 :

- **Rats kept in an enriched environment:**
  - Stimulation of neurogenesis and reduction of spontaneous apoptotic cell death in hippocampus (by 45 %)
  - Higher expression of neurotrophic growth factors GDNF and BDNF in hippocampus
  - Protection against kainate-induced seizures and excitotoxicity