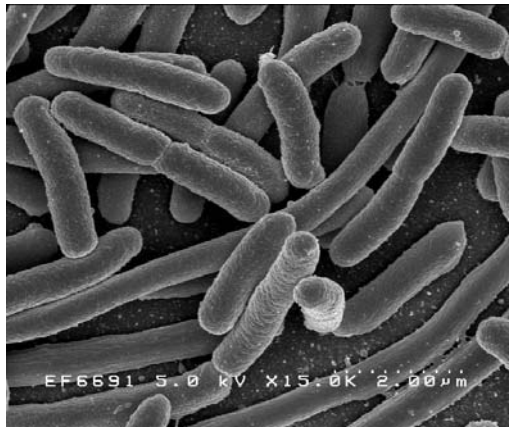
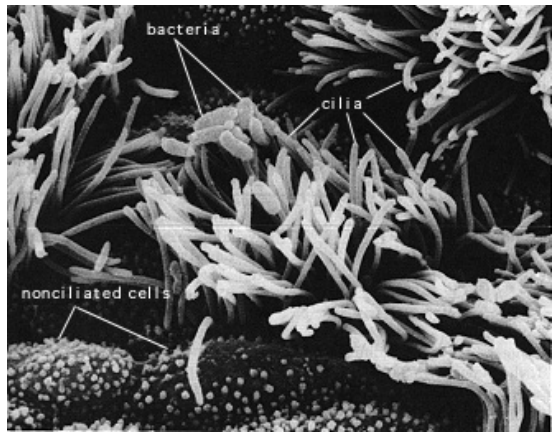
Moscow-Bavarian Joint Advanced Student School 2007:

Properties of Channels Formed by Bacterial Porins and Toxins

RTX toxins

HlyA of *E.coli* & CyaA of *Bordetella pertussis*



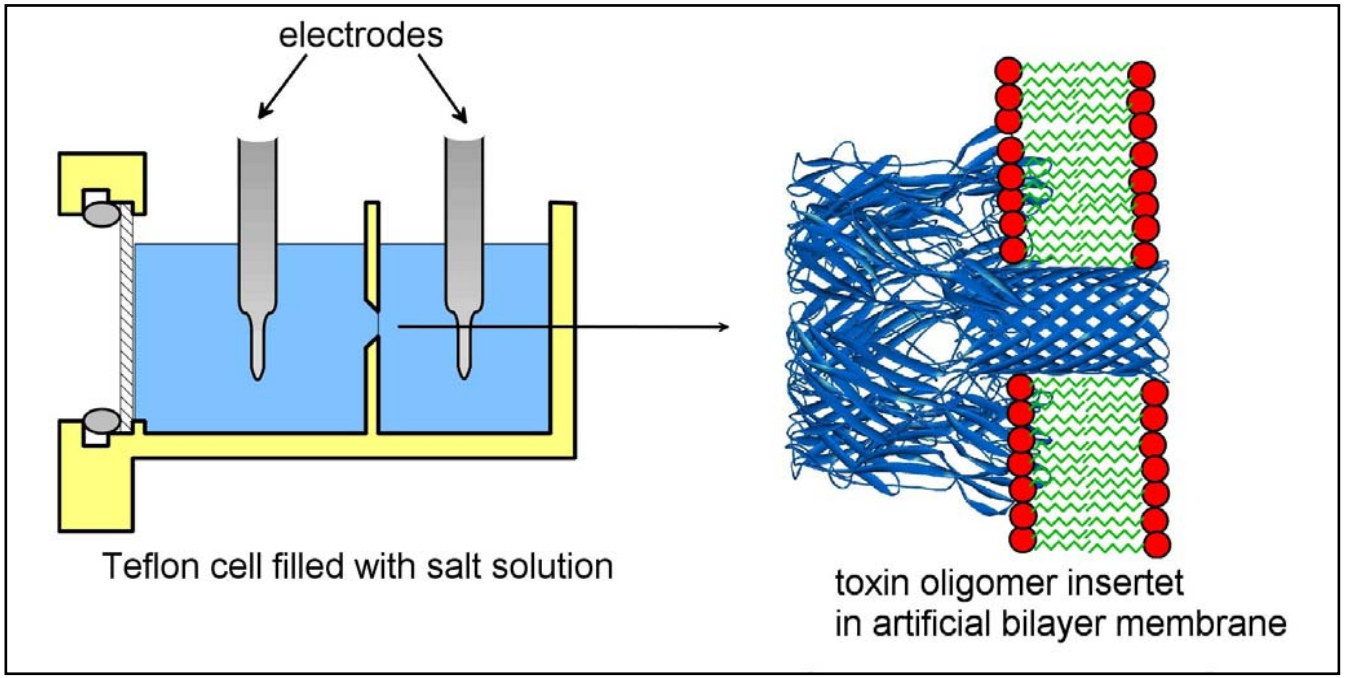


Florian Rohleder – Student of Biology – 16.03.2007

Introduction What is it all about?

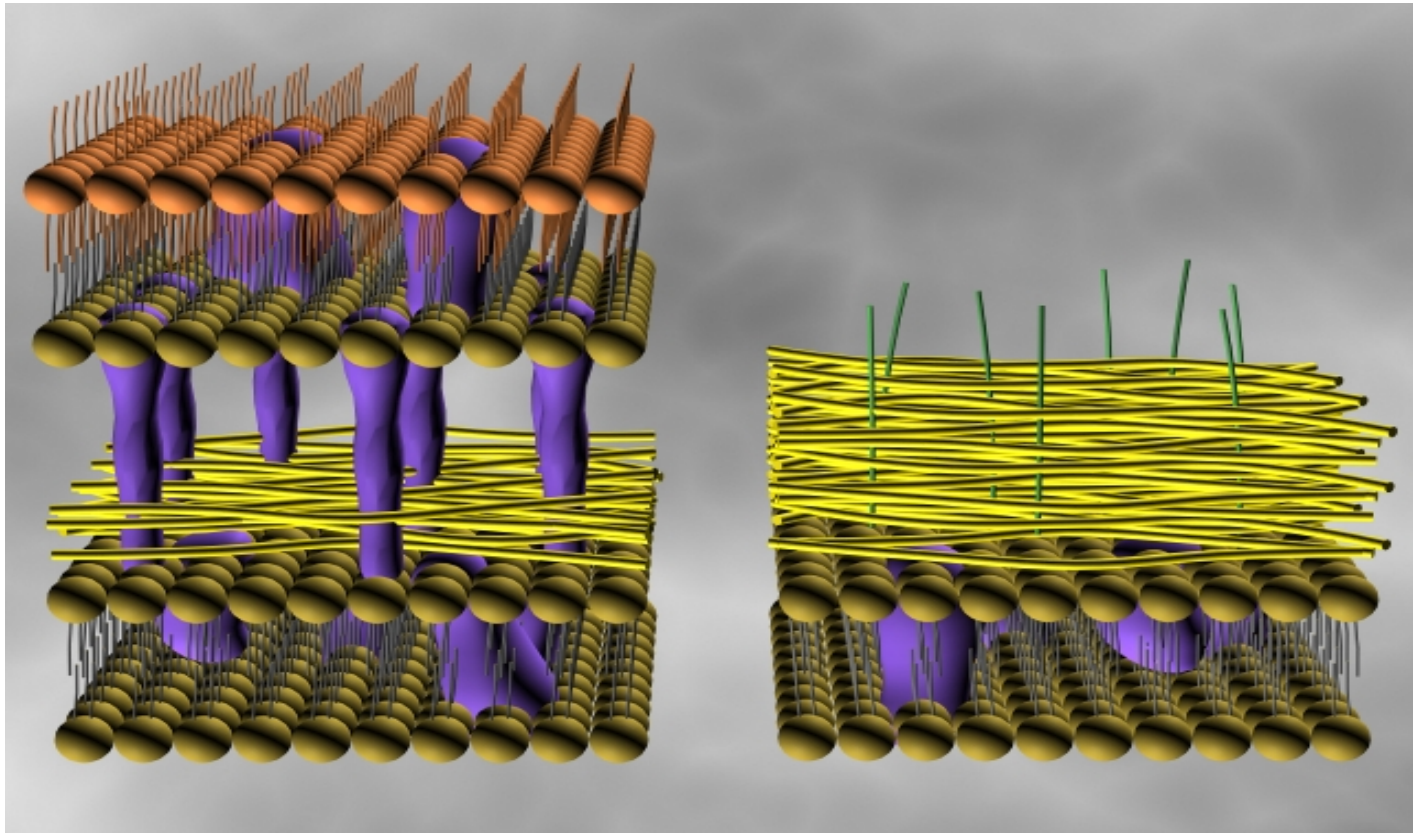
- cytolysins represent important virulence factors of many pathogenic bacteria
- most well-known bacterial cytolysins are pore-forming proteins
  - transmembrane pores are cytotoxic and may cause osmotic cell lysis
- bacterial pore-forming cytolysins represent a heterogeneous group of exotoxins
- pore-forming cytolysins of Gram-positive bacteria
  - N-terminal signal peptide that is cleaved during Sec-dependent transport across the cytoplasmatic membrane
  - active *per se* and do not require any activation
- those of Gram-negative bacteria
  - synthesized as inactive protoxins / activation by modification or proteolytic procession
  - secretion across inner and outer membranes by complex systems
- **RTX toxins** represent the largest family of bacterial pore-forming cytolysins

Introduction Lipid Bilayer Membrane and Toxins



- membranes of pure lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserin) inactive targets
- asolectin (mixture of many different lipids isolated from soy beans) active targets
- reason for „lipid specifity“ is not clear

Introduction Bacterial Cellwalls



gram negative gram positive

RTX Toxins General Characteristics I

1.

- synthesized as inactive proteins
- molecular mass typically around 100 - 120 kDa
- C-terminal half includes tandem array of glycine and aspartate-rich nonapeptide repeats
  - UXGGXG(N/D)DX (U = large hydrophobic, X = arbitrary)
- `RTX toxins´ stands for repeats in toxin
- number of repeats varies between 10 and 40

2.

- post-transcriptional activation by modification
- e.g. acylation of specific internal lysine residues

RTX Toxins General Characteristics II

3.

- no cleavable N-terminal signal peptide / secretion not *sec* dependent
- secretion via type-I pathway
  - direct translocation across inner and outer membran in one step
  - specific, highly conserved export system (ABC exporter)
- target signal for export located within C-terminal ~60 amino acids / not processed during secretion

4.

- activity is Ca^{2+} dependent
- Ca^{2+} binds in unknown stoichiometry to signature repeat domain

RTX Toxins General Characteristics III

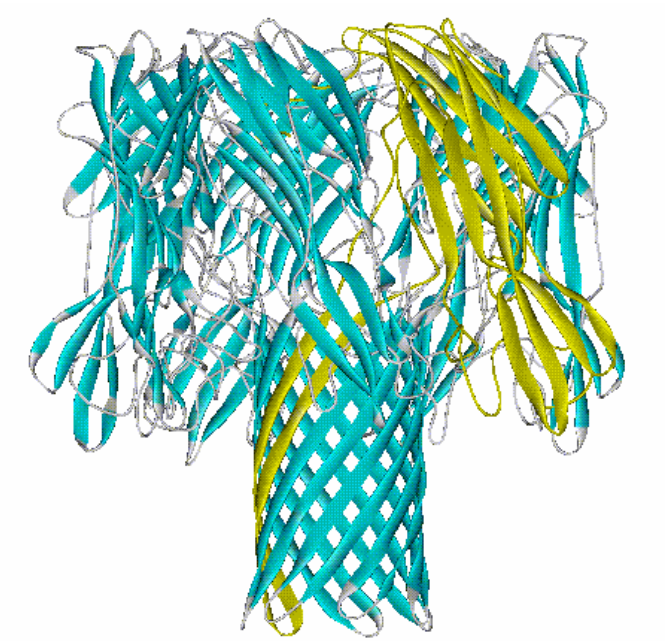
5.

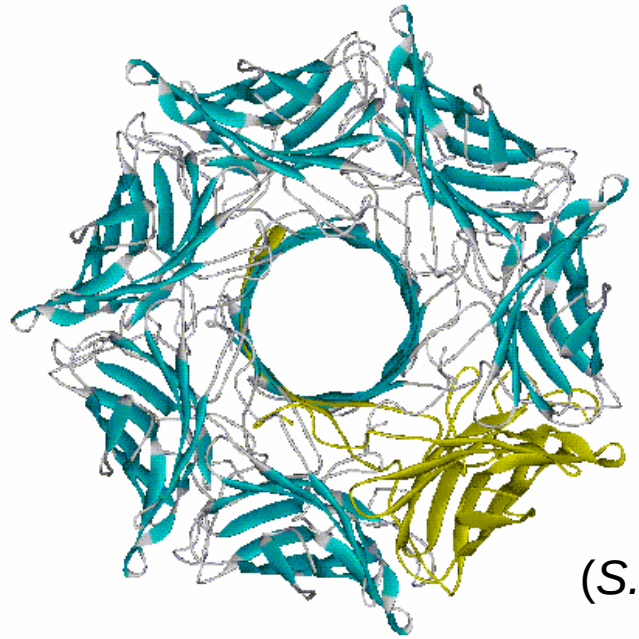
- form transient, cation-selective pores of different sizes in lipid membranes

6.

- genes specifically required for synthesis, activation and secretion are clustered
- the operon typically contains four contiguous genes in the order C-A-B-D
  - A: structural gene of the toxin protein
  - C: activator protein
  - B+D: ABC protein and MFP component of ABC exporter
  - (outer membrane component encoded somewhere else)

HlyA Introduction

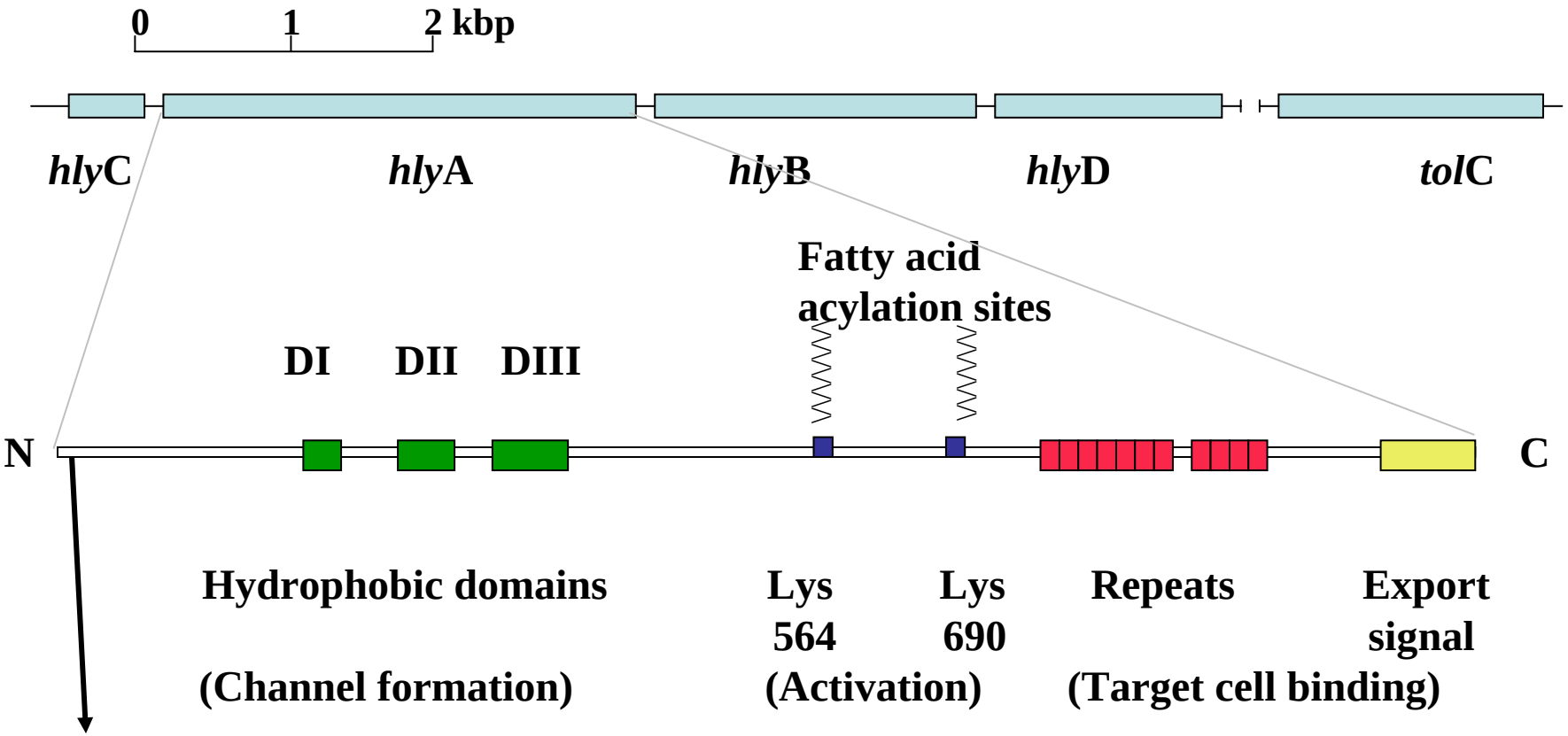




(*S.aureus*)

- one of the best characterized members of RTX toxins
- lyses erythrocytes from many cell types / kills immune cells involved in first-line defence mechanisms
- secondary reactions triggered by passive influx of Ca^{2+}

HlyA Organisation of Operon and Protein

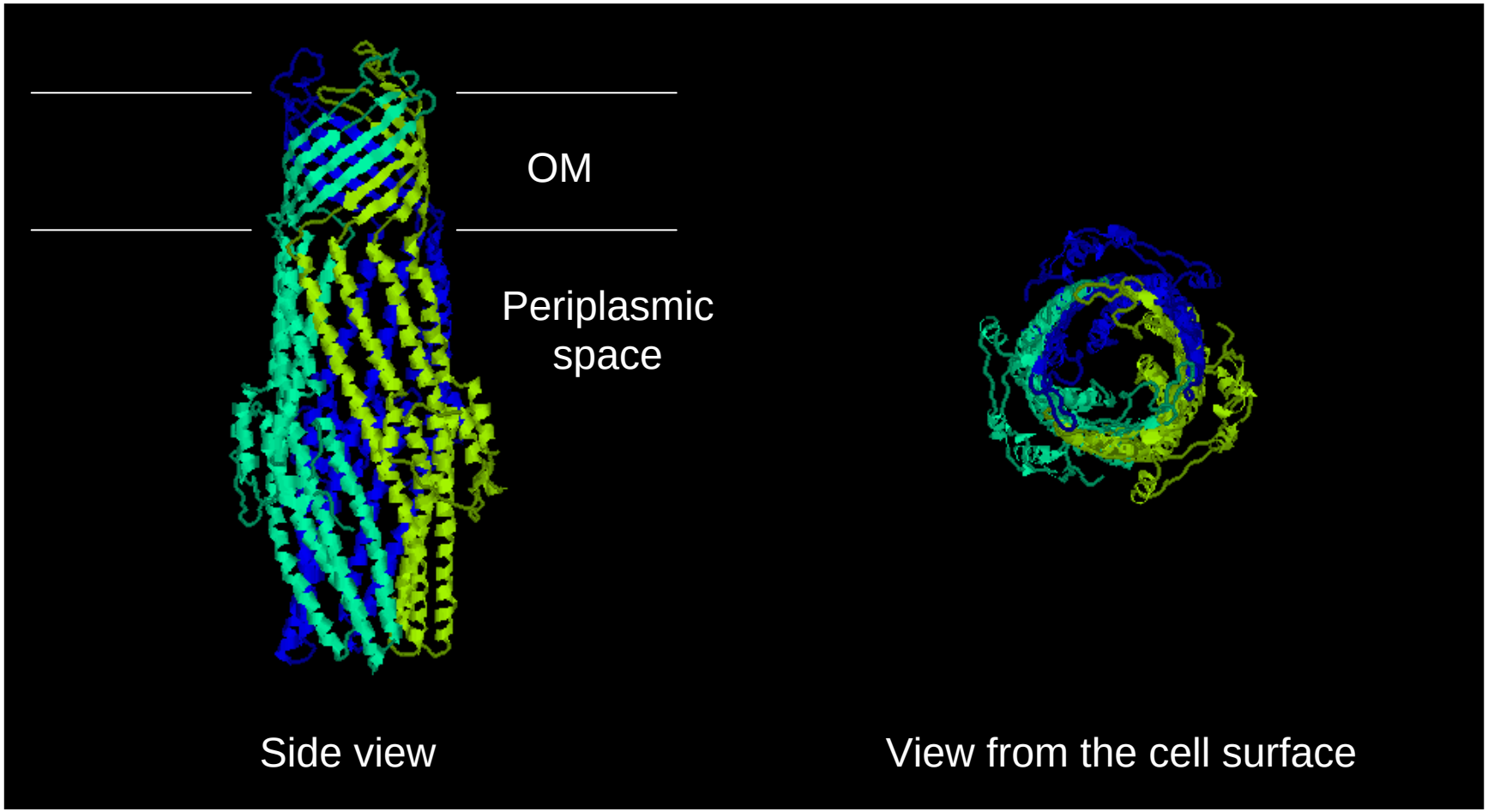


CyaA (ACT) contains an enzymatic component (AC) at the N-terminus

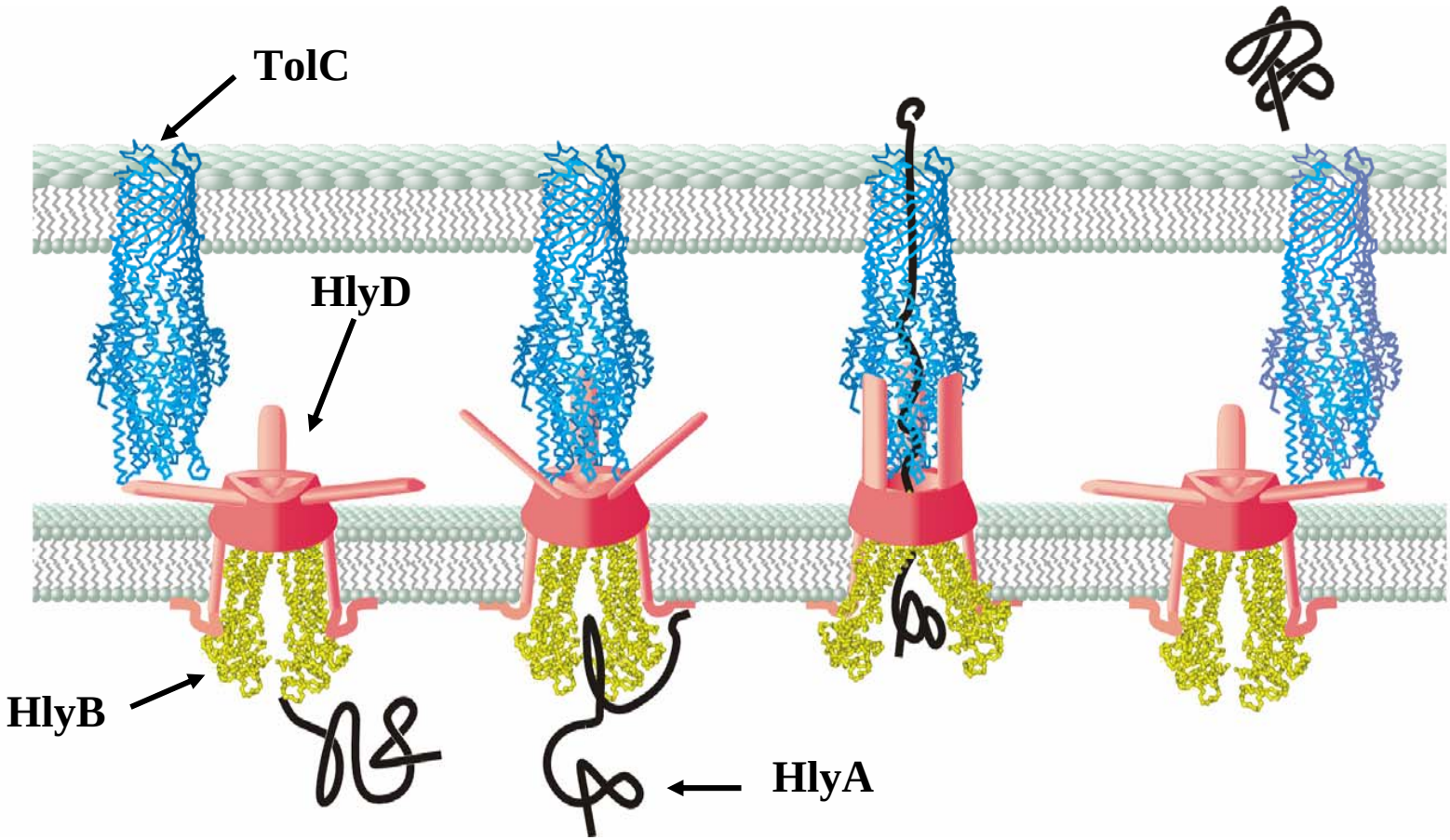
Repeats: (L/I/F)-X-G-G-X-G-(N/D)-D-X

in variable number for different toxins

HlyA Structure of TolC and Possibly also of CyaE

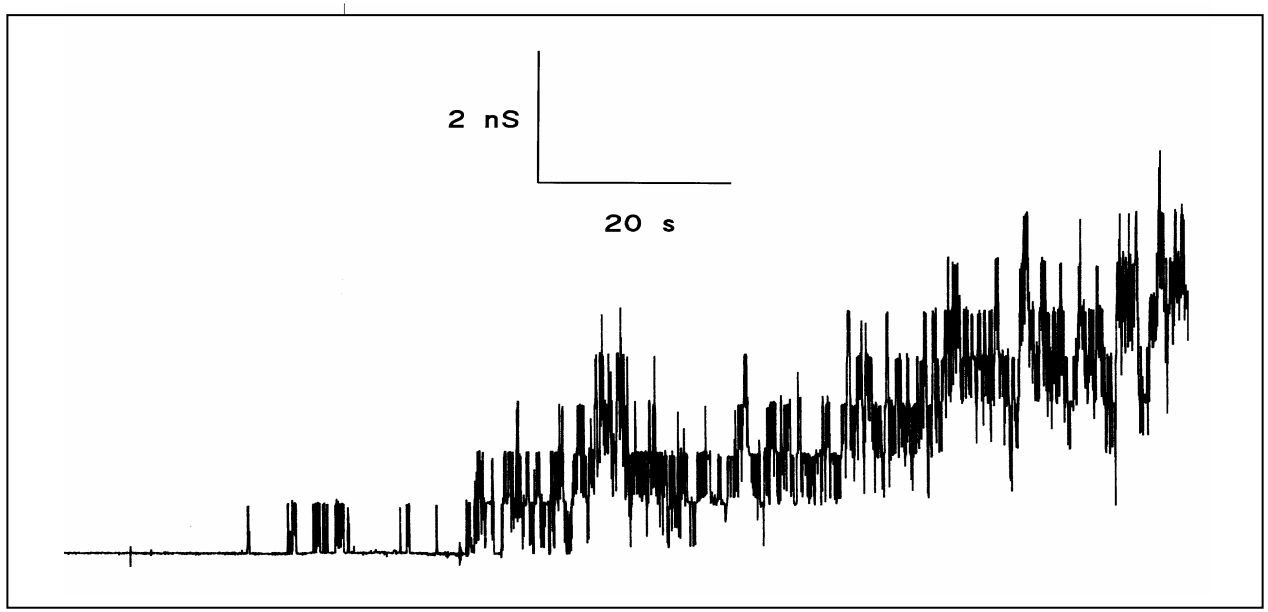


HlyA Possible Export Mechanism



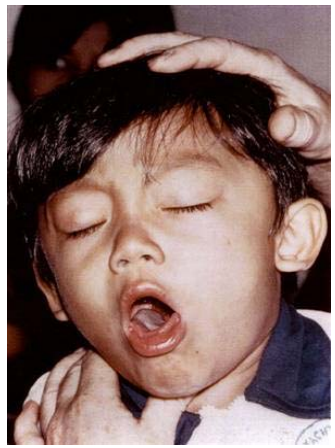
TolC is involved in type 1 export of RTX toxins

HlyA Single-channel Analysis



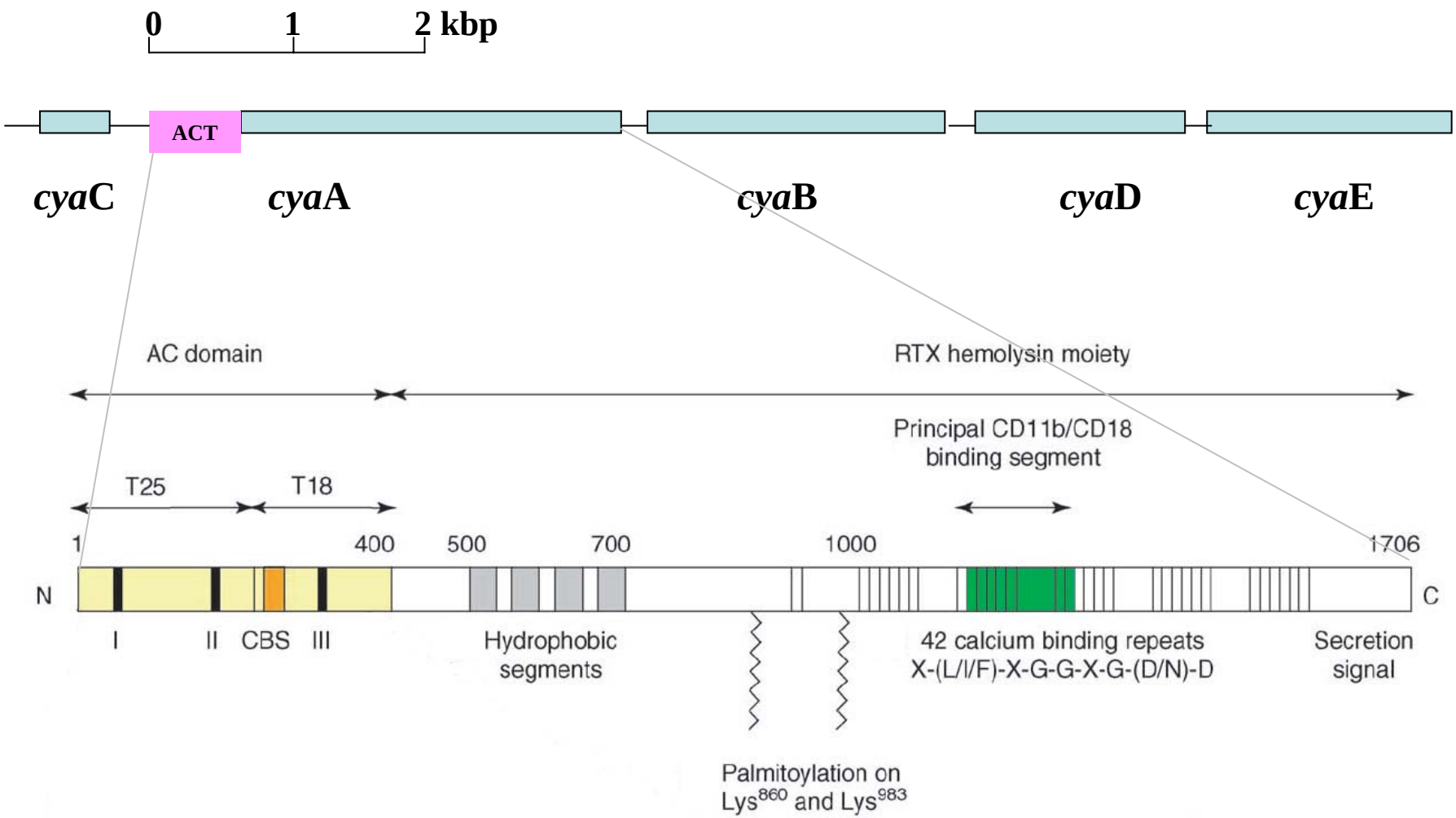
Single-channel recording of an asolectin/n-decane membrane in the presence of HlyA of *Escherichia coli*. The aqueous phase contained 150 mM KCl (pH 6) and 100 ng/ml HlyA. The applied membrane potential was 20 mV; T = 20°C. The toxin forms transient channels with a lifetime of about 2 to 5 s.

CyaA Introduction

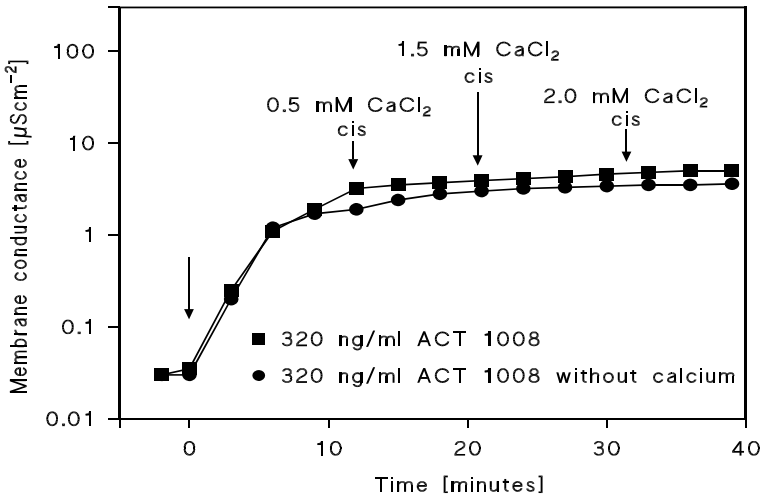


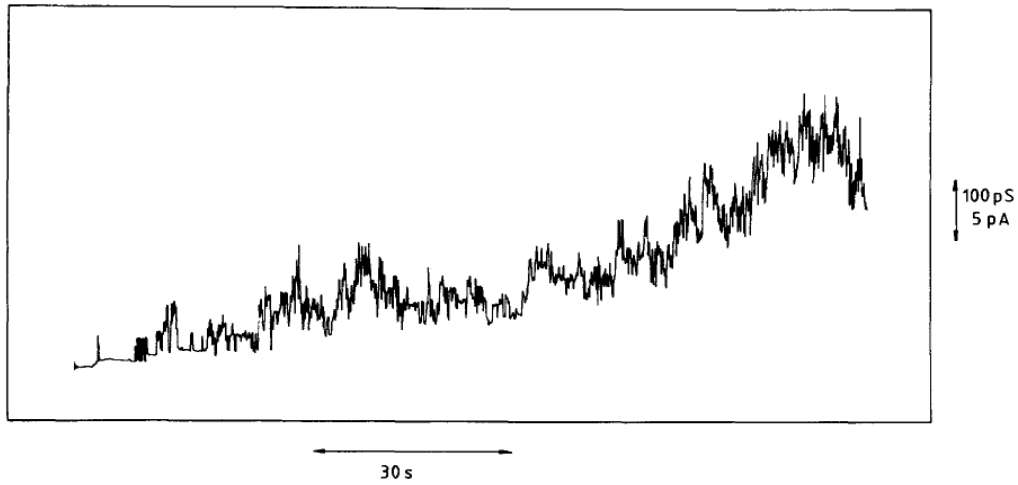
- key virulence factor of the wooping cough agent *Bordetella pertussis*
- infects the respiratory tract / strictly human pathogen
- protein of 177kDa
- fusion of a cytolysin with a adenylate cyclase enzyme
- member of RTX toxin family of bacterial pore-forming toxins
- targets phagocytes expressing the $\alpha_M\beta_2$ integrin (CD11b/CD18)
- AC domain is delivered into cytosol and catalyzes uncontrolled conversion of cellular ATP to cAMP

CyaA Organisation of Operon and Protein



CyaA Mutants and Related Effects I

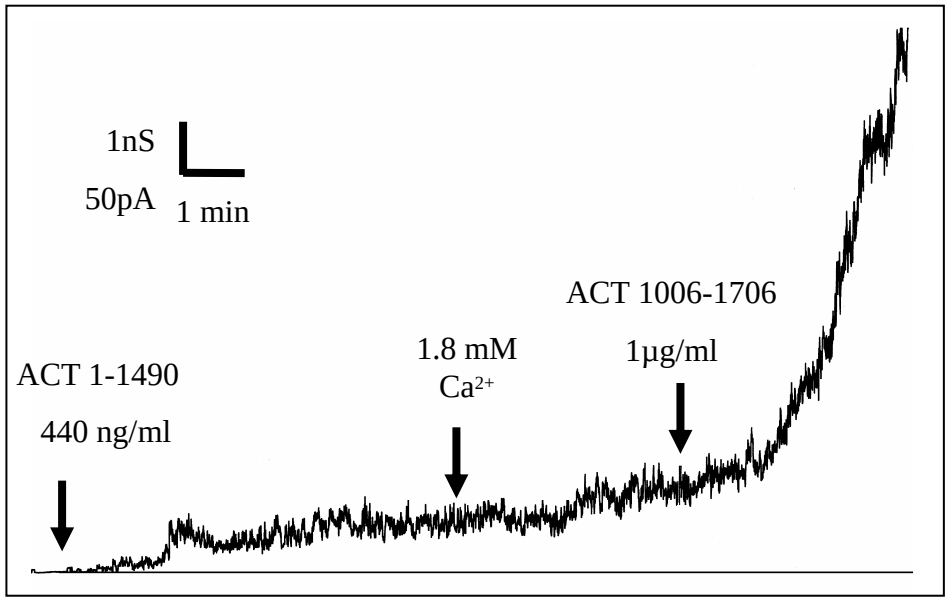


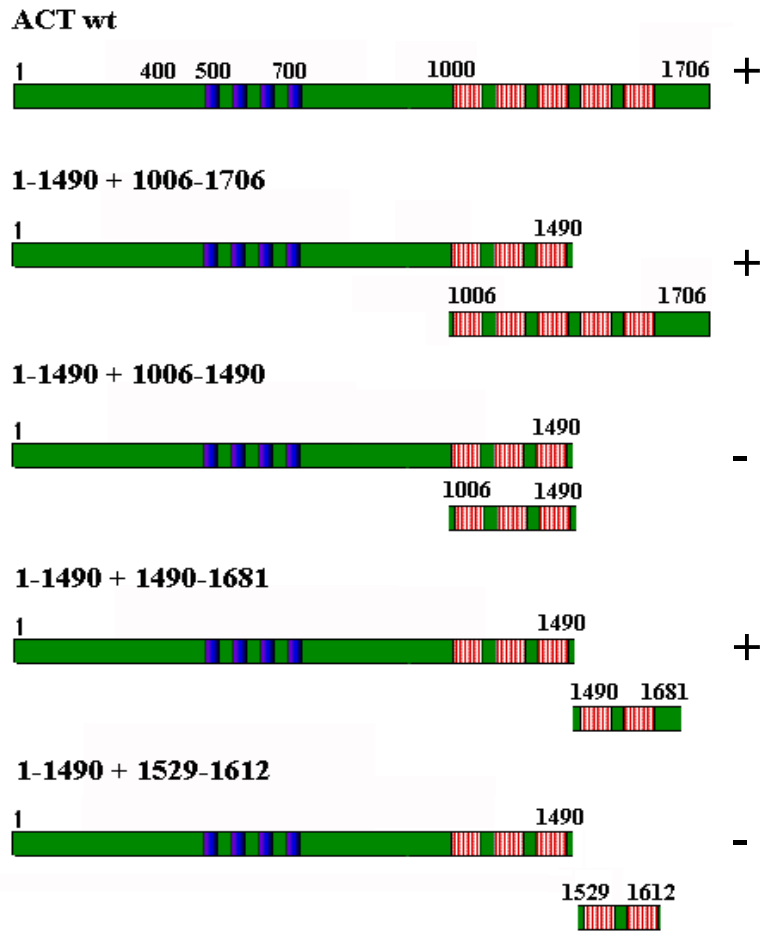


ΔC698: last 698 amino acids removed including repeat region

- *in vivo* nonhemolytic / *in vitro* indistinguishable from wild type
- repeats are required for recognition and binding to target cell but not for formation or insertion into lipid bilayer membrane
- no Ca^{2+} required for activity in lipid bilayer membranes

CyaA Mutants and Related Effects II





Only combinations of ACT 1-1490 together with ACT 1006-1706 and 1490-1681 are able to raise the membrane conductance in a calcium dependent manner. The flanking region 1628-1681 is the elicitor for calcium binding and mediates the signal to the adjacent repeats.

CyaA Mutants and Related Effects III

ΔC843: deletion of repeat region and putative activation site (between residues 913 and 1000)

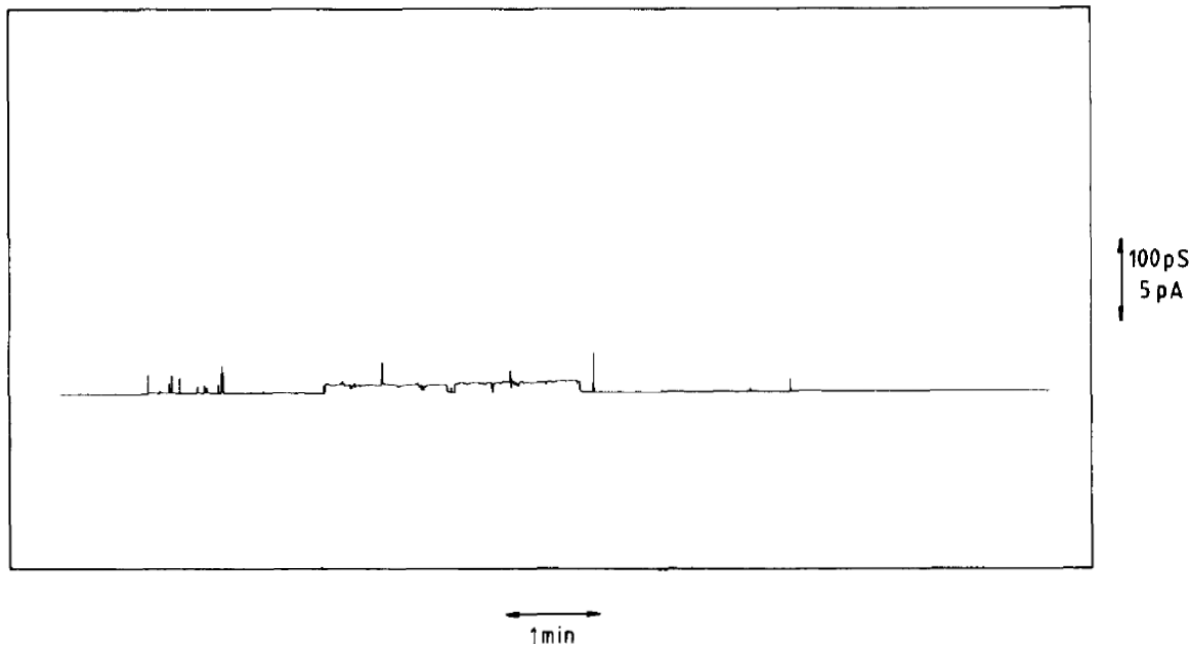
- together with CyaC very low channel-forming activity
- number of channels similar to that of nonactivated CyaA
- no CyaC-mediated activating modification

ΔAC: deletion of catalytic adenylate cyclase domain

- no influence on the CyaA-induced hemeolysis
- no difference in channel-formation compared with WT

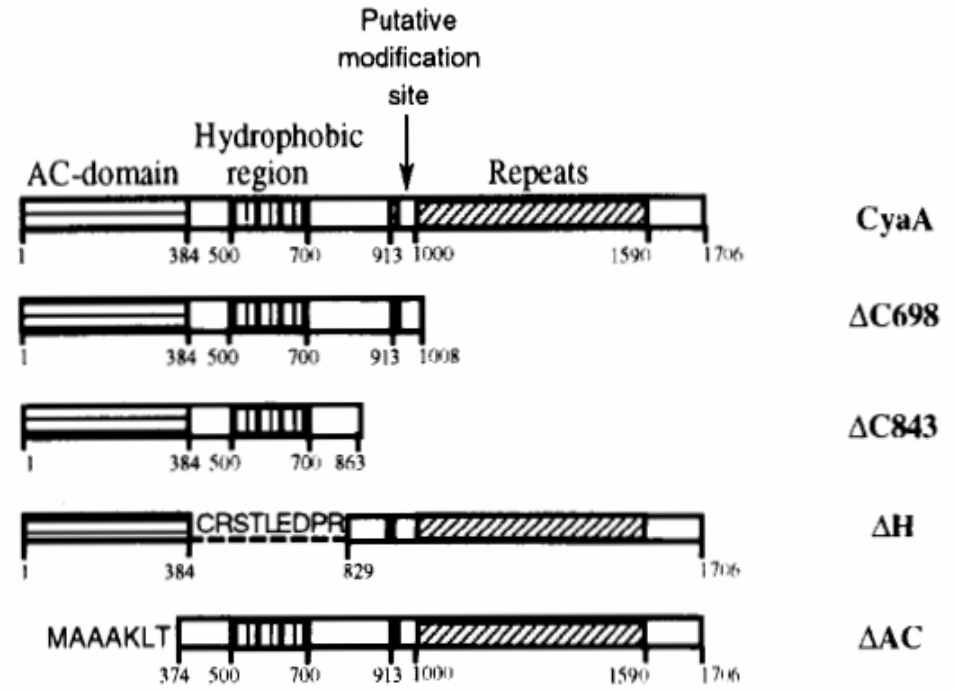
CyaA Mutants and Related Effects IV

ΔH:
deletion of the hydrophobic region between residues 500 and 700



- replacement of hydrophobic region by a linker
- not able to increase conductance of lipid bilayer membrane even at very high protein concentrations (10 µg/ml)
- no insertion

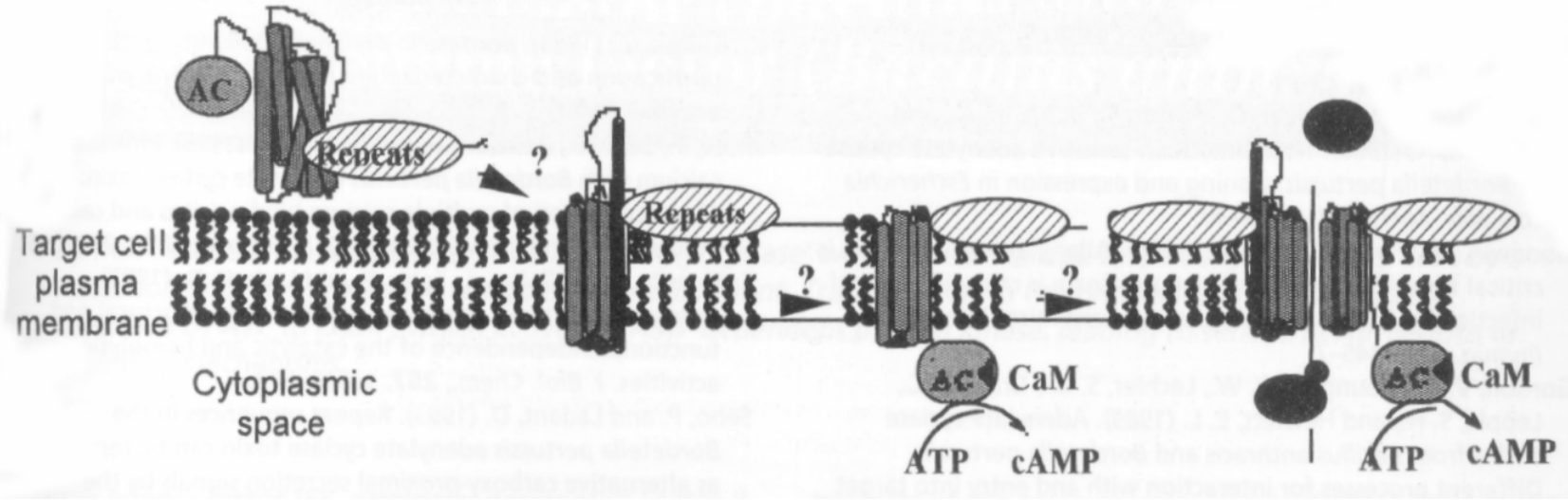
CyaA Mutants and Related Effects V



CyaA mutant	Deletion	G	Membrane activity
		pS	
Wild-type (*B. pertussis*)		27	++++
Wild-type (*E. coli*)		28	+++
Non-activated wild-type (*E. coli*)		28	+
ΔC698	1008–1706	25	+++
ΔC843	863–1706	26	+
ΔAC	1–373	25	+++
ΔH	385–828	ND	

repeats: recognition and binding of target cell (Ca^{2+} dependent)
PMS: channel activity
HR: necessary for integration into membrane
AC: adenylate cyclase domain has no influence on hemolytic activity and channel formation

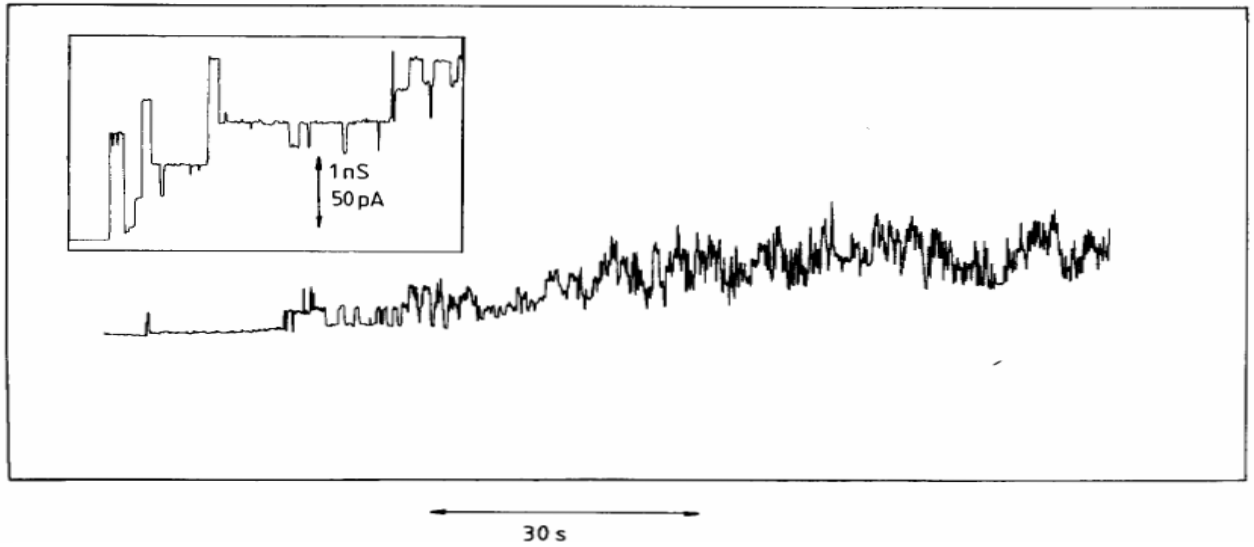
CyaA Properties and Effect



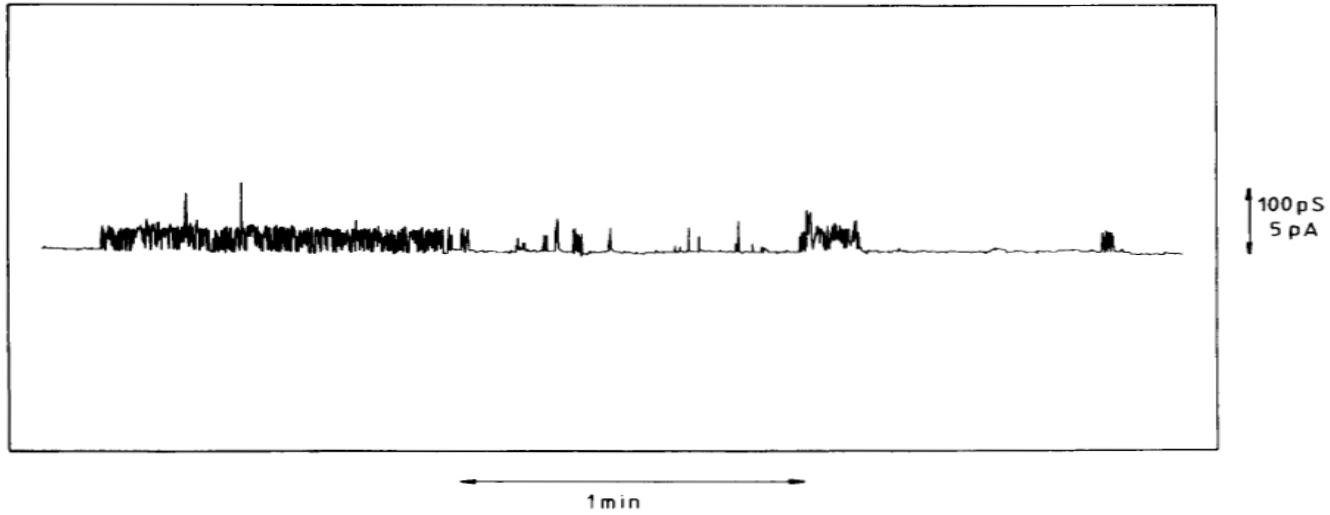
- monomer of 177kDa
- organized in different domains
- intoxication of target cell is fast and direct; no endocytosis
- hemolytic activity and intoxication calcium- and calmodulin-dependent
- current model of ACT intoxication of target cells

CyaA Single-Channel Analysis I

100 ng/ml CyaA

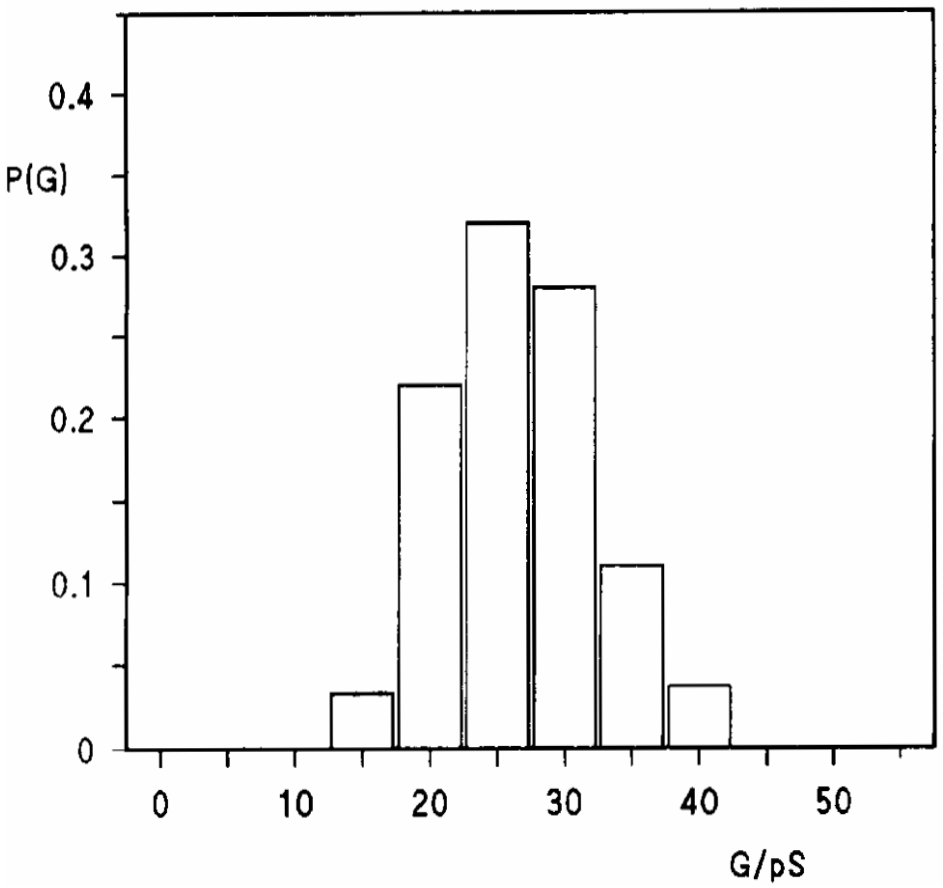


10 ng/ml CyaA



(1 M KCl, 50 mV)

CyaA Single-Channel Analysis II



histogram of the probatility for the occurence of a given conductivity unit observed with membranes formed of asolectin/n-decane in the presence of 10-100ng/ml CyaA purified from *B. pertussis*

single-channel conductance: 27 pS
(1 M KCl, 50 mV, 25°C, 452 single channel events in 10 membranes)
> HlyA 1500 pS <

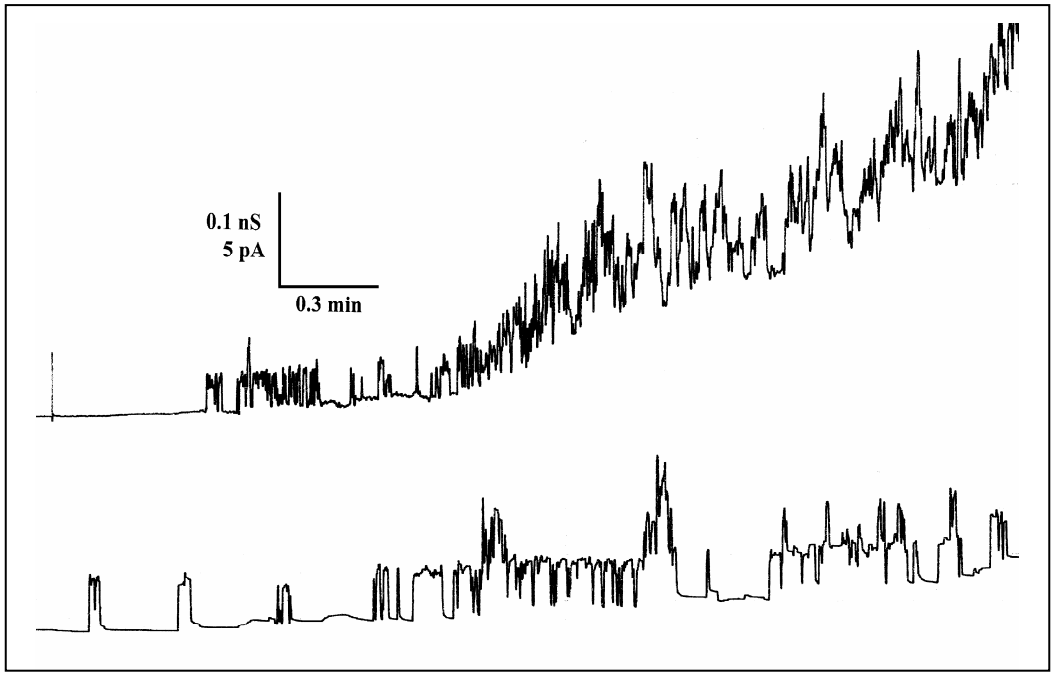
CyaA Cation Selectivity I

Salt	c	G	
		CyaA	HlyA
	M	pS	
LiCl	1.0	15	700
NaCl	1.0	18	1200
KCl	0.10	4.8	310
	0.3	11	720
	1.0	27	1500
	3.0	48	3900
RbCl	1.0	29	1700
KCH_3COO (pH 7)	1.0	25	1400
Tris-HCl (pH 6)	1.0	ND	240
$CaCl_2$	0.15	8.5	
	1.0	24	

CyaA Cation Selectivity II

- zero-current membrane potential measurements
- asolectin membrans formed in 50 mM KCl
- toxin added to aqueous phase
- incorporation of 100 – 1000 channels
- addition of KCl to one side (10-fold salt gradient)
- more diluted side becomes postive
- preferential movement of potassium through CyaA channel

CyaA Calcium Dependency I

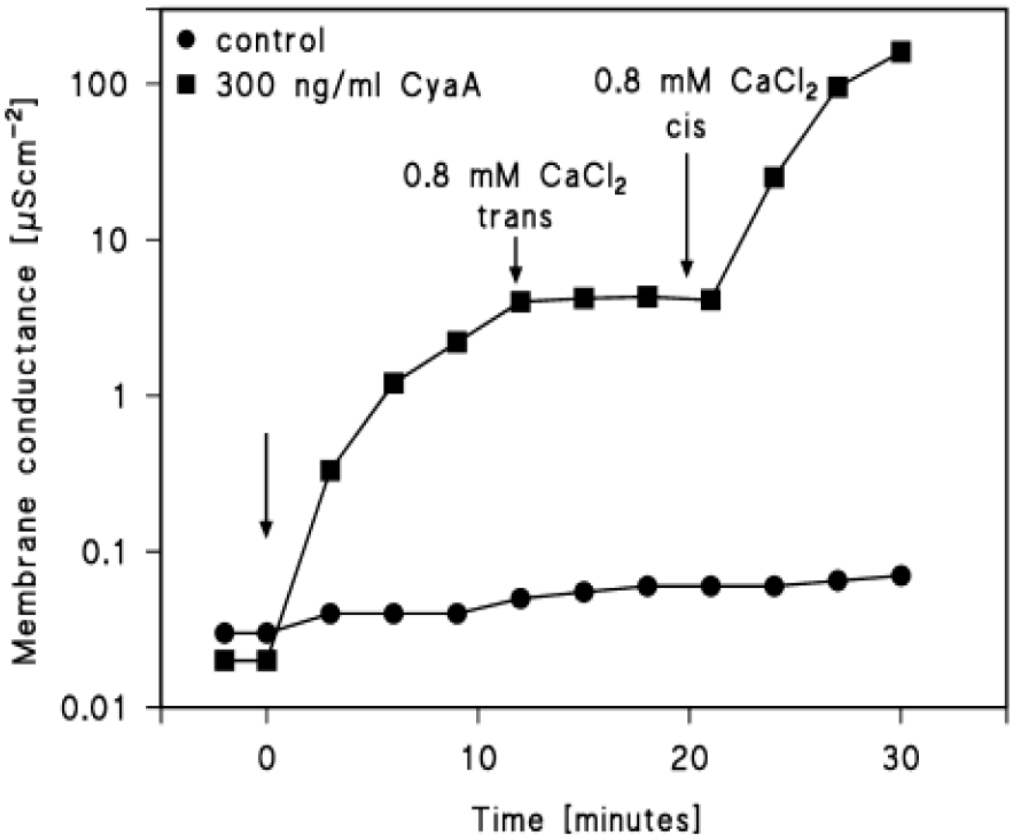


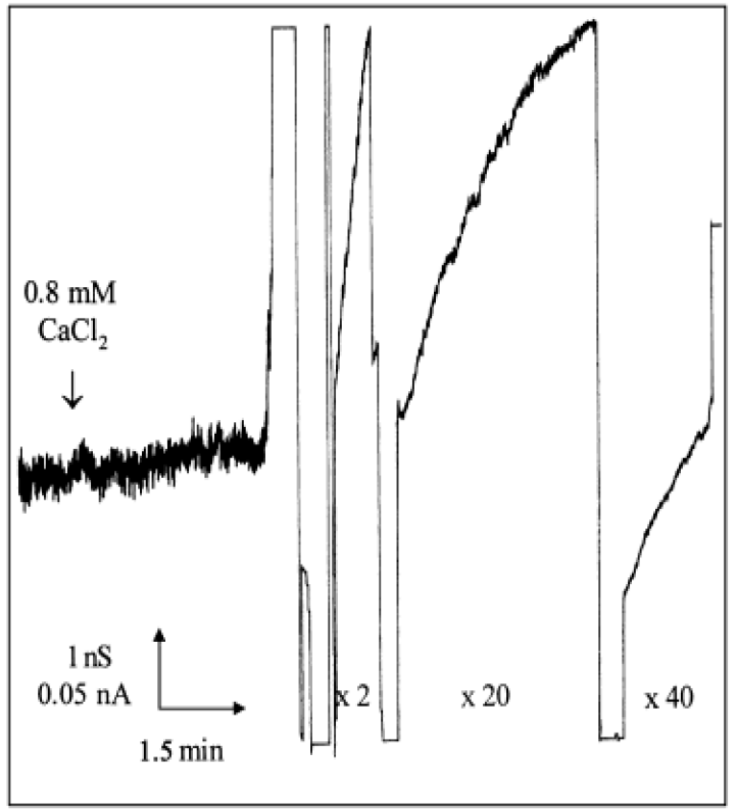
Calcium has no effect on the single-channel conductance of ACT

(1 M KCl, pH 7, 100 ng/ml CyA, 50 mV, 20°C)

The dramatic calcium-mediated increase of the conductance is caused by the generation of new channels and not by the change of the single-channel conductance of the ACT-channels.

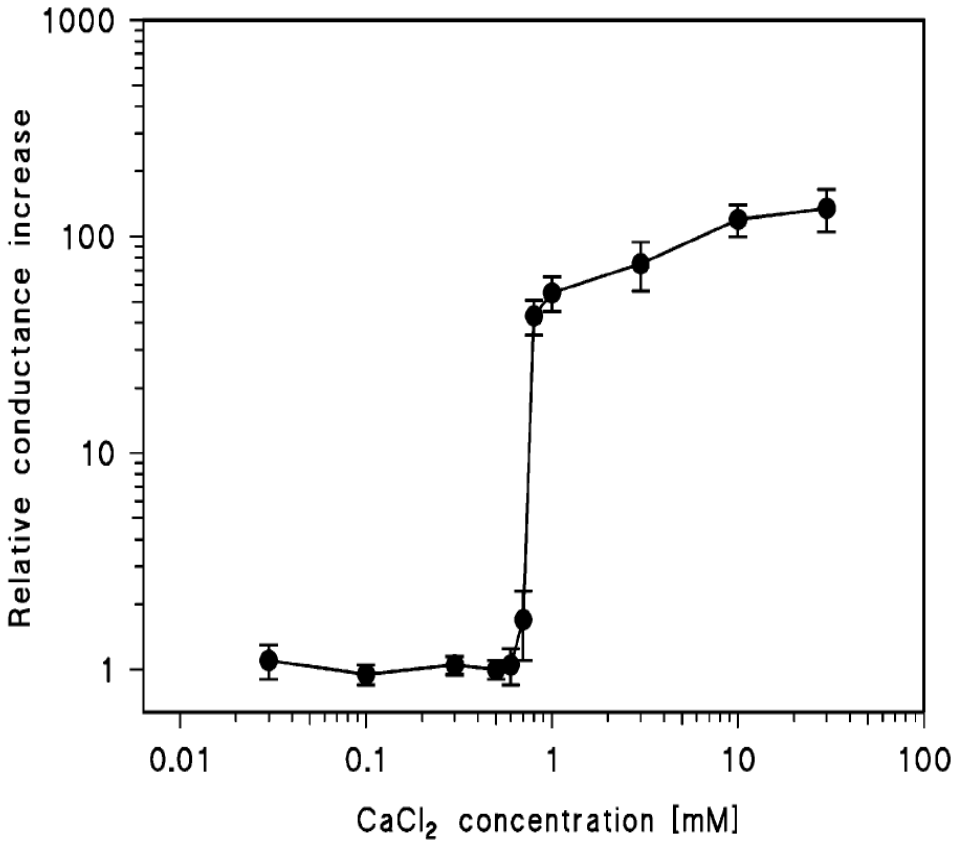
CyaA Calcium Dependency II





Addition of 0.8 mM Ca^{2+} on *cis* side causes a steep increase of the conductance

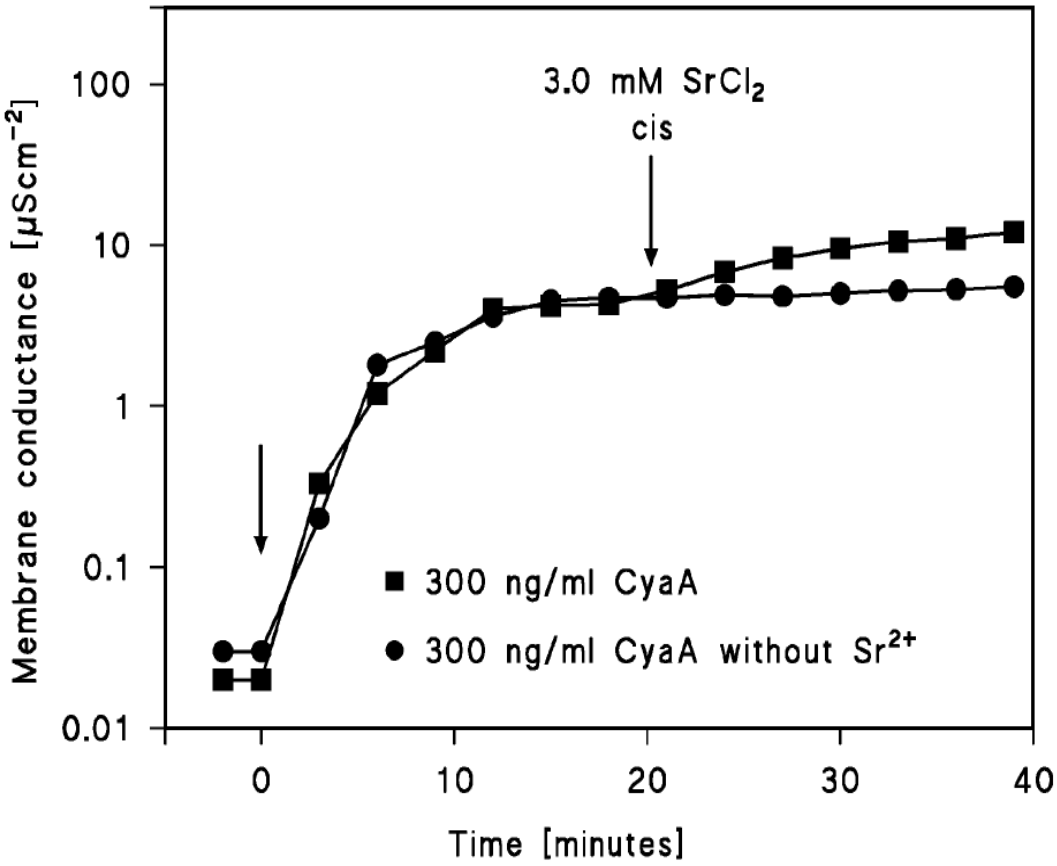
CyaA Calcium Dependency III



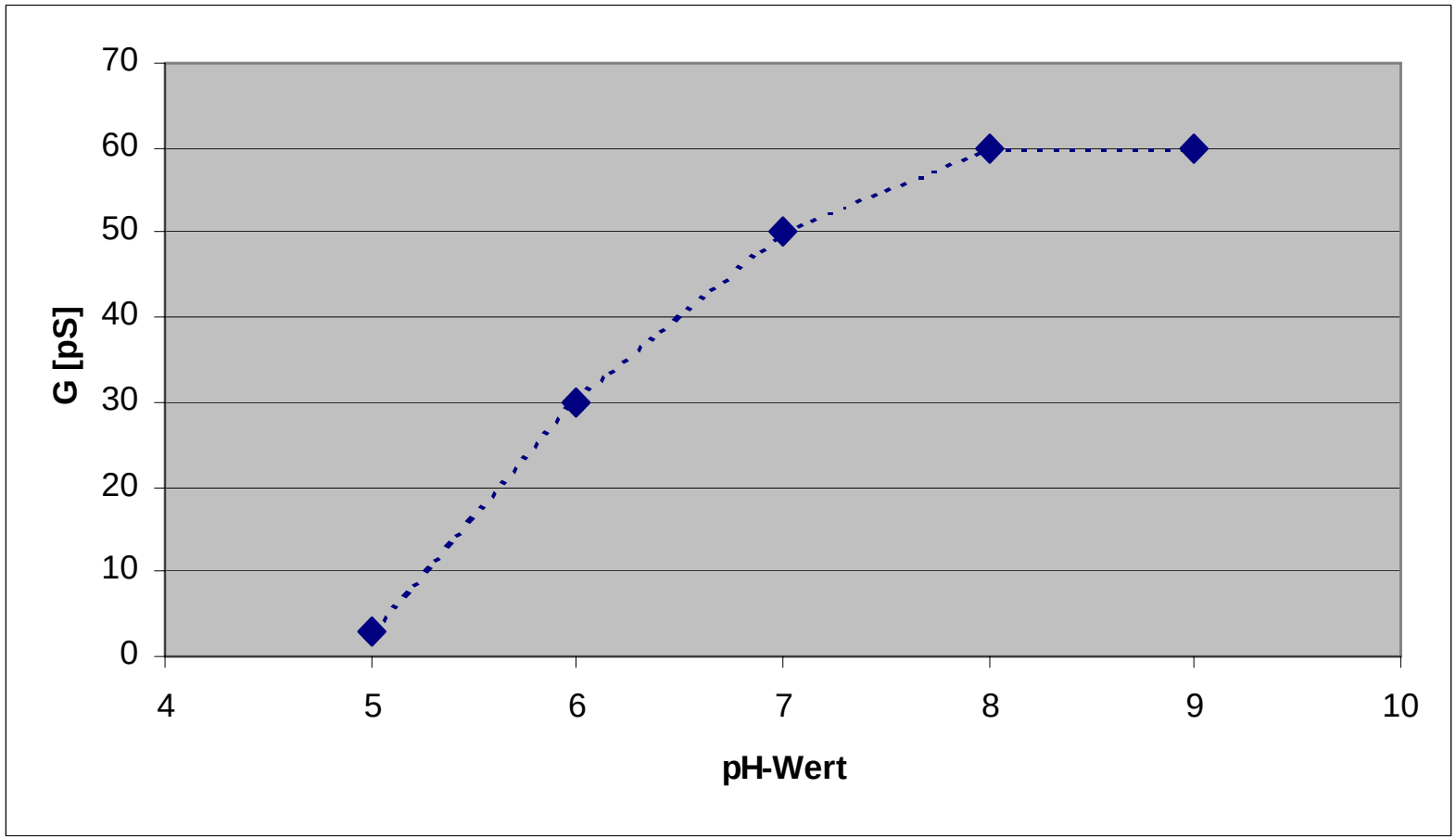
- 2 affinity classes of calcium binding sites
  - high-affinity
  - low-affinity
- extremely cooperative function of the calcium concentration
  - < 0.8 mM insignificant conductance enhancement
  - 15% increase in Ca^{2+} concentration leads to 50-fold increase of membrane activity

CyaA Effects of Other Divalent Cations

- 20 mM of Mg^{2+} or Ba^{2+}: no effect on membrane conductance
- Sr^{2+}: very small increase starting with 3 mM



CyaA pH-Dependence of Channel Conductance



(1 M KCl, 50 mV at the cis-side)

CyaA Size of Channel

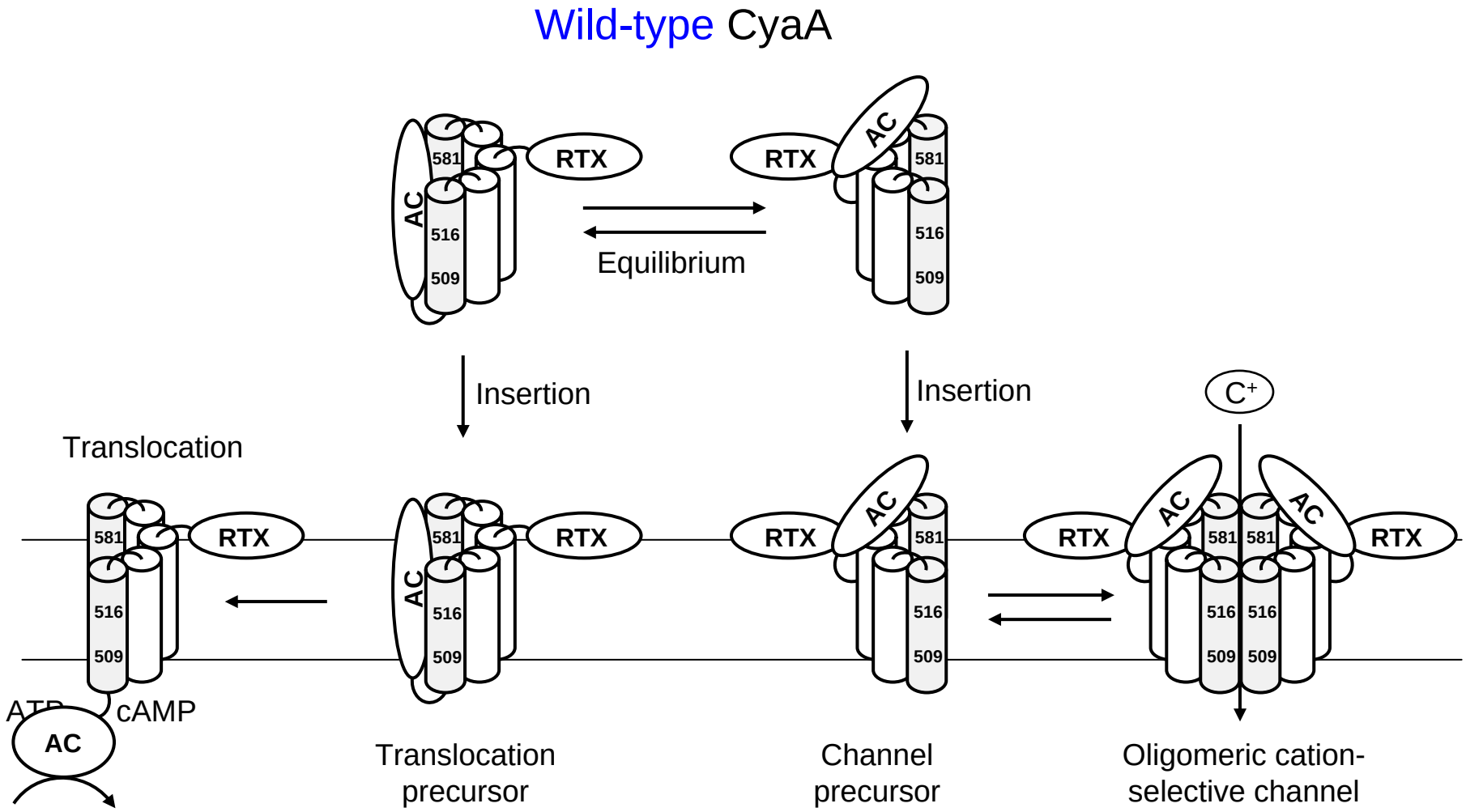
- single-channel conductance considerably lower than HlyA
- cross-section of CyaA smaller
- for small channels precise estimations of radii difficult
  - conductance is not proportional to cross-section
- ions partially dehydrated and interact with channel walls
- observed CyaA-mediated conductance similar to potassium channels of nerve and muscle tissues
  - diameter of less than 0.6 – 0.8 nm
- consistent with no detectable channels in Tris-HCl
- osmotical protection by addition of small sugars to external medium (e.g. sucrose, mannitol or arabinose)
- too small to allow passage of even a fully unfolded polypeptide chain
  - alternative model for AC delivery into cell

CyaA Translocation of AC Domain and Channel Formation I

Toxin	Results for J774A.1 cells			
	cAMP intoxication ($C_{10\_pmol\_cAMP}$ [ng/ml])[b]		Cell lysis (CL_{50} [ng/ml])[c]	
	AC^{+}[e]	AC^{-}[f]	AC^{+}[e]	AC^{-}[f]
CyaA	15 ± 2	ND	171 ± 11	2,812 ± 457
CyaA-E509K	24 ± 7	ND	112 ± 7	536 ± 47
CyaA-E509K+E516K	956 ± 117	ND	367 ± 91	485 ± 96
CyaA-E581K	61 ± 7	ND	97 ± 6	643 ± 56
CyaA-E570Q	12 ± 2	ND	169 ± 12	>5,000
CyaA-E581P	20 ± 4	ND	273 ± 47	>5,000
proCyaA[g]	493 ± 107	Not determined	6,273 ± 766	Not determined

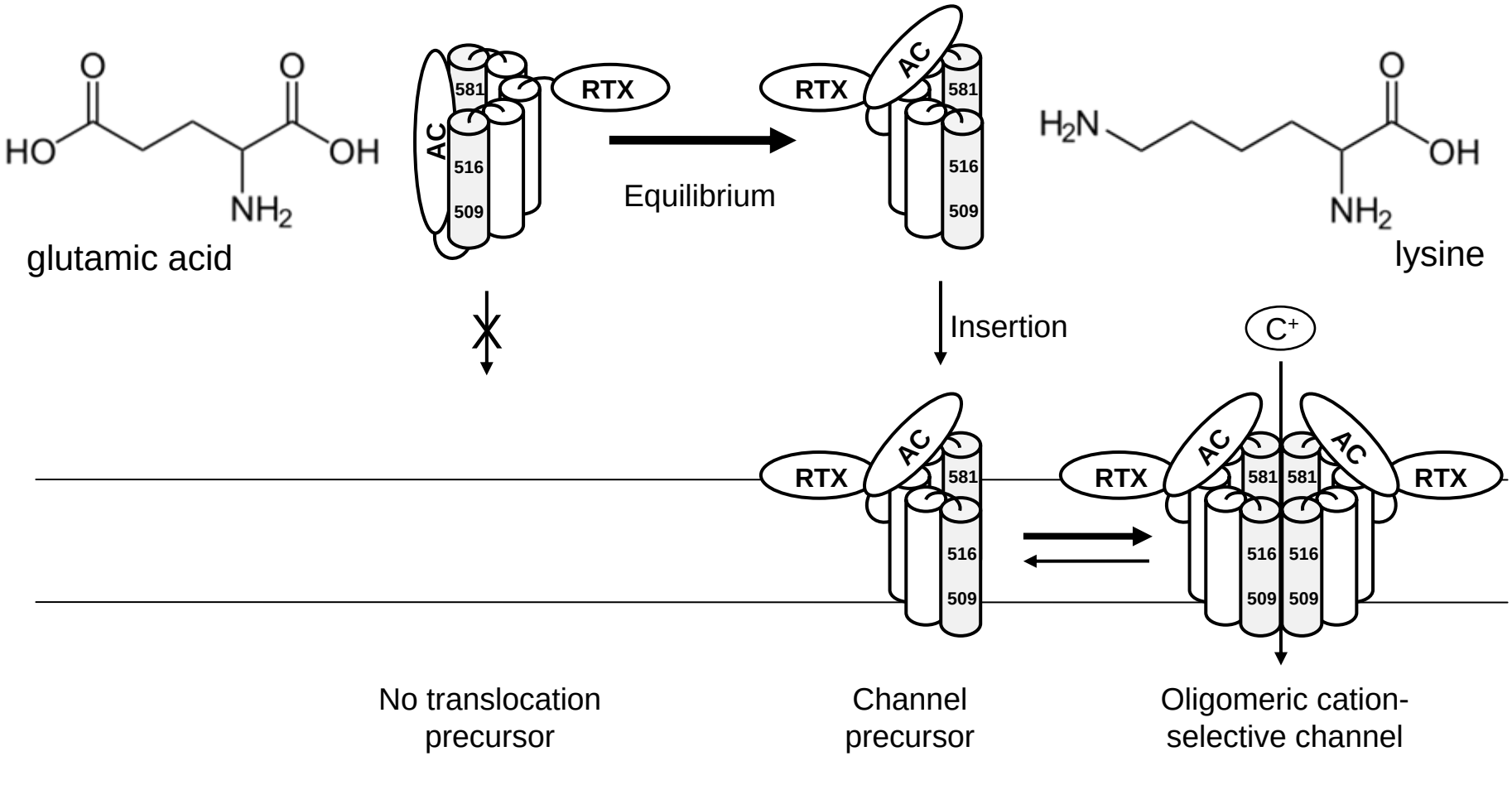
hydrophobic region (500-700) is also responsible for AC translocation

CyaA Translocation of AC Domain and Channel Formation II



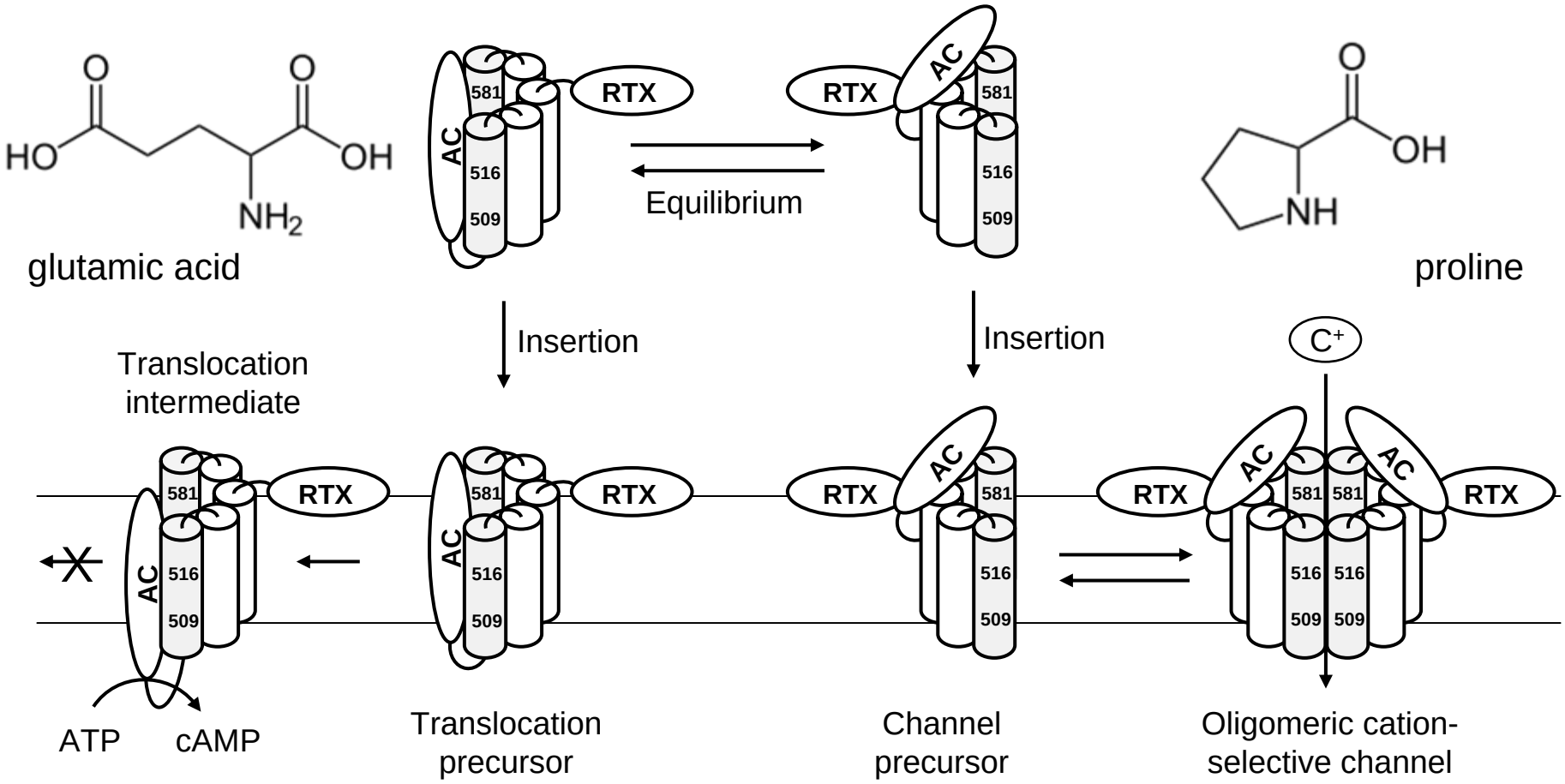
CyaA Translocation of AC Domain and Channel Formation III

CyaA-E509K+E516K or CyaA-E581K

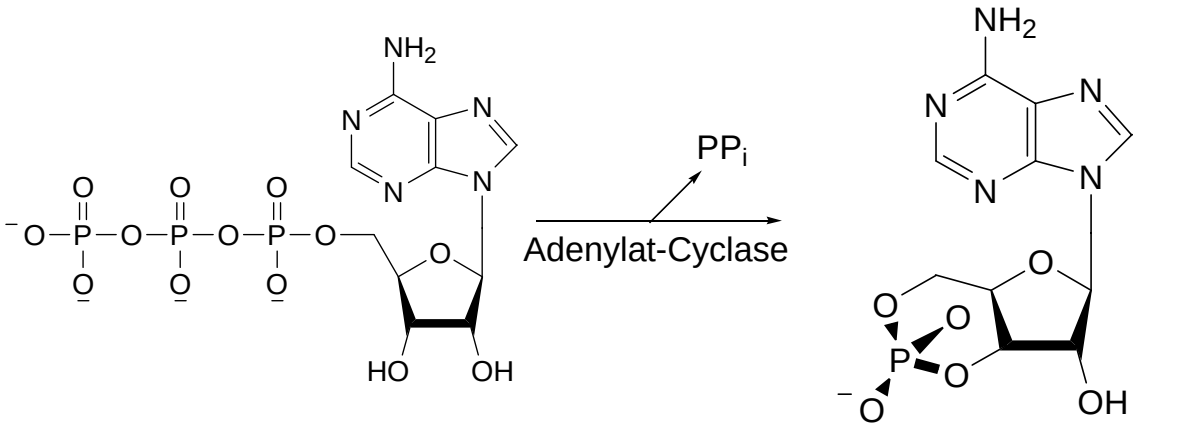


CyaA Translocation of AC Domain and Channel Formation IV

CyaA-E509P, CyaA-E516P or CyaA-E581P



CyaA Adenylate Cyclase I

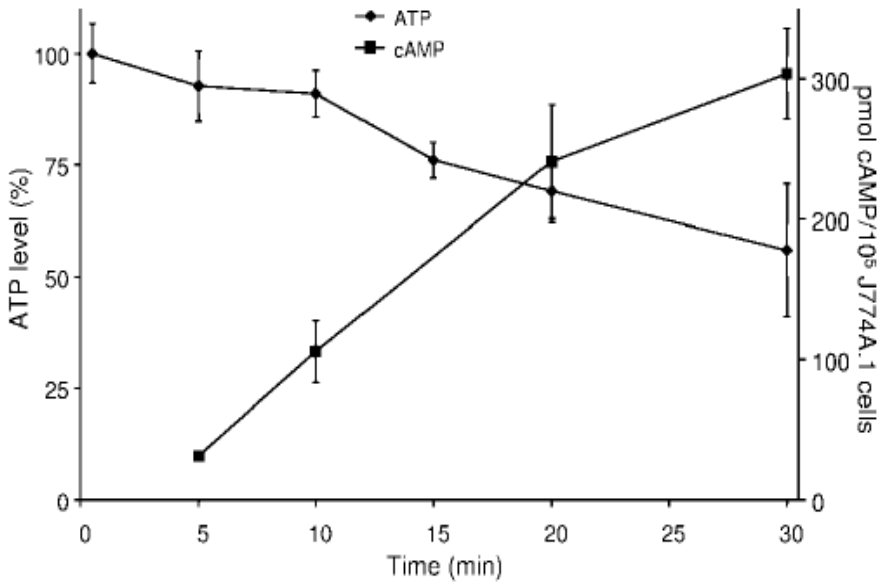


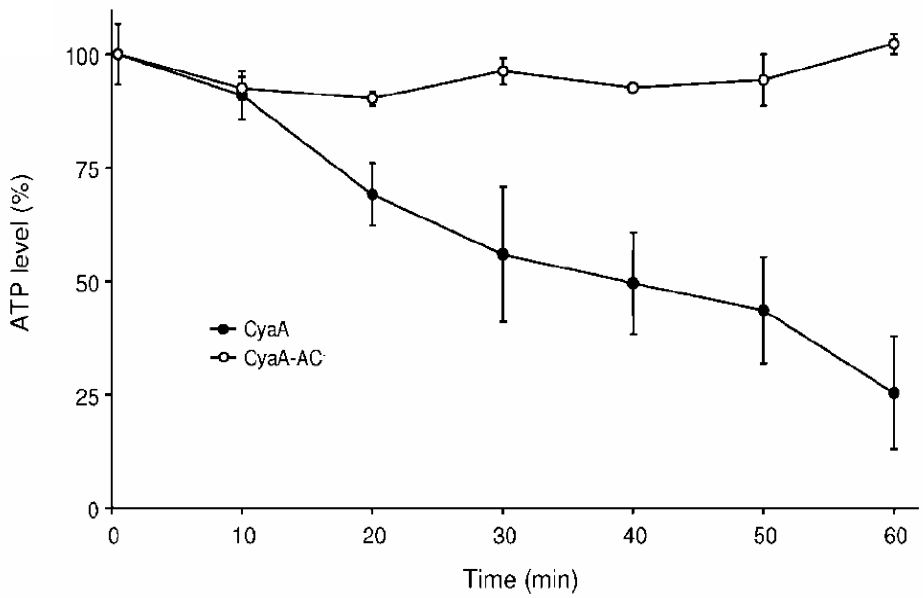
ATP

3´,5´-cyclisches AMP
cAMP
(second messenger)

- k_{cat} ~ 2000s^{-1}
- 4 discrete regions bind to calcium-loaded eukaryotic calmodulin
- W242 of AC plays a crucial role and makes extensive contacts with the calcium-induced hydrophobic pocket of CaM

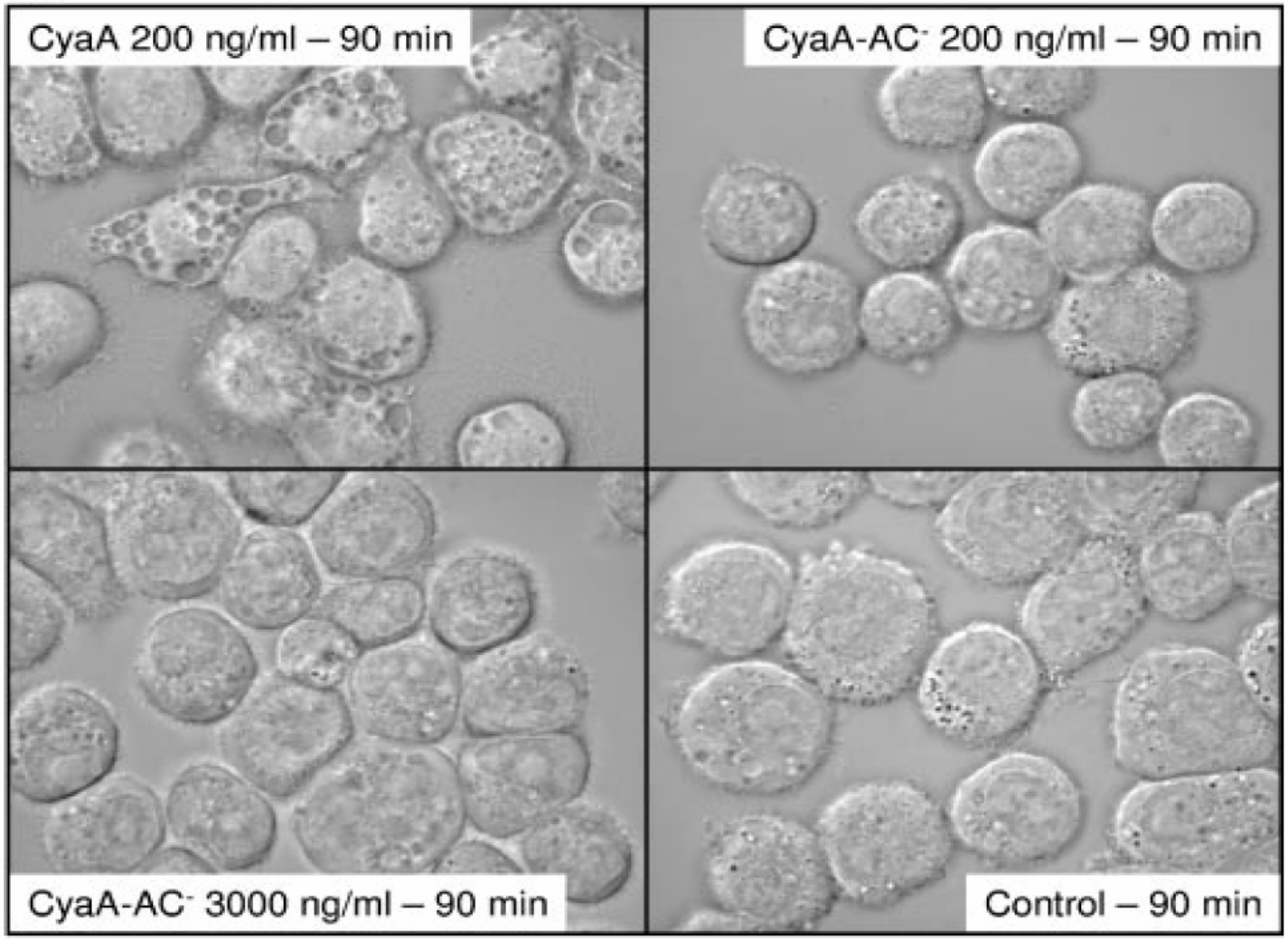
CyaA Adenylate Cyclase II



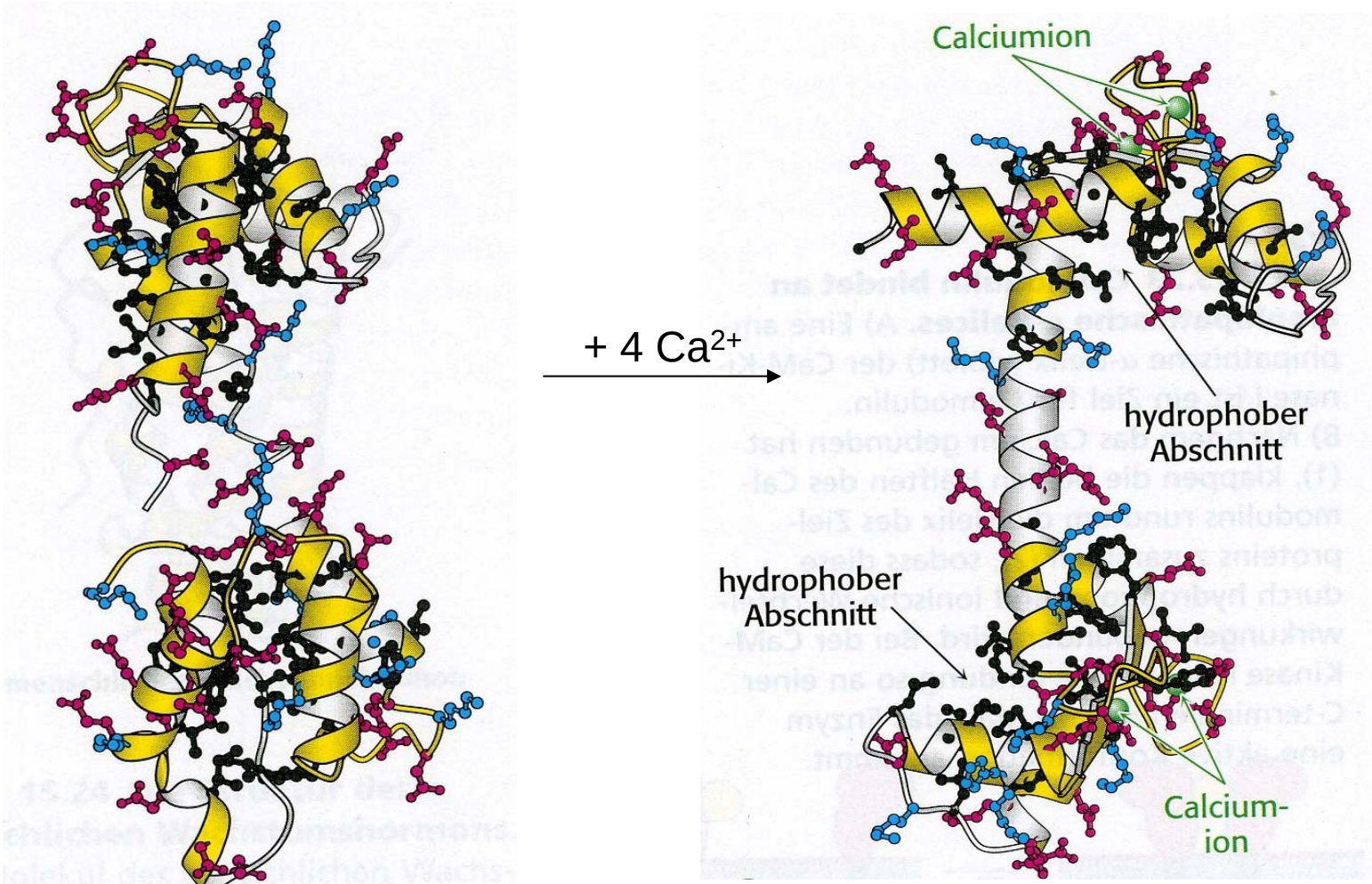


- increase of cAMP level accompanied by decrease of ATP
- permeabilization of cell membrane by CyaA could be expected to activate ATP-consuming membrane transporters for ion homeostasis?
- no measurable decrease of ATP level with CyaA-AC$^-$

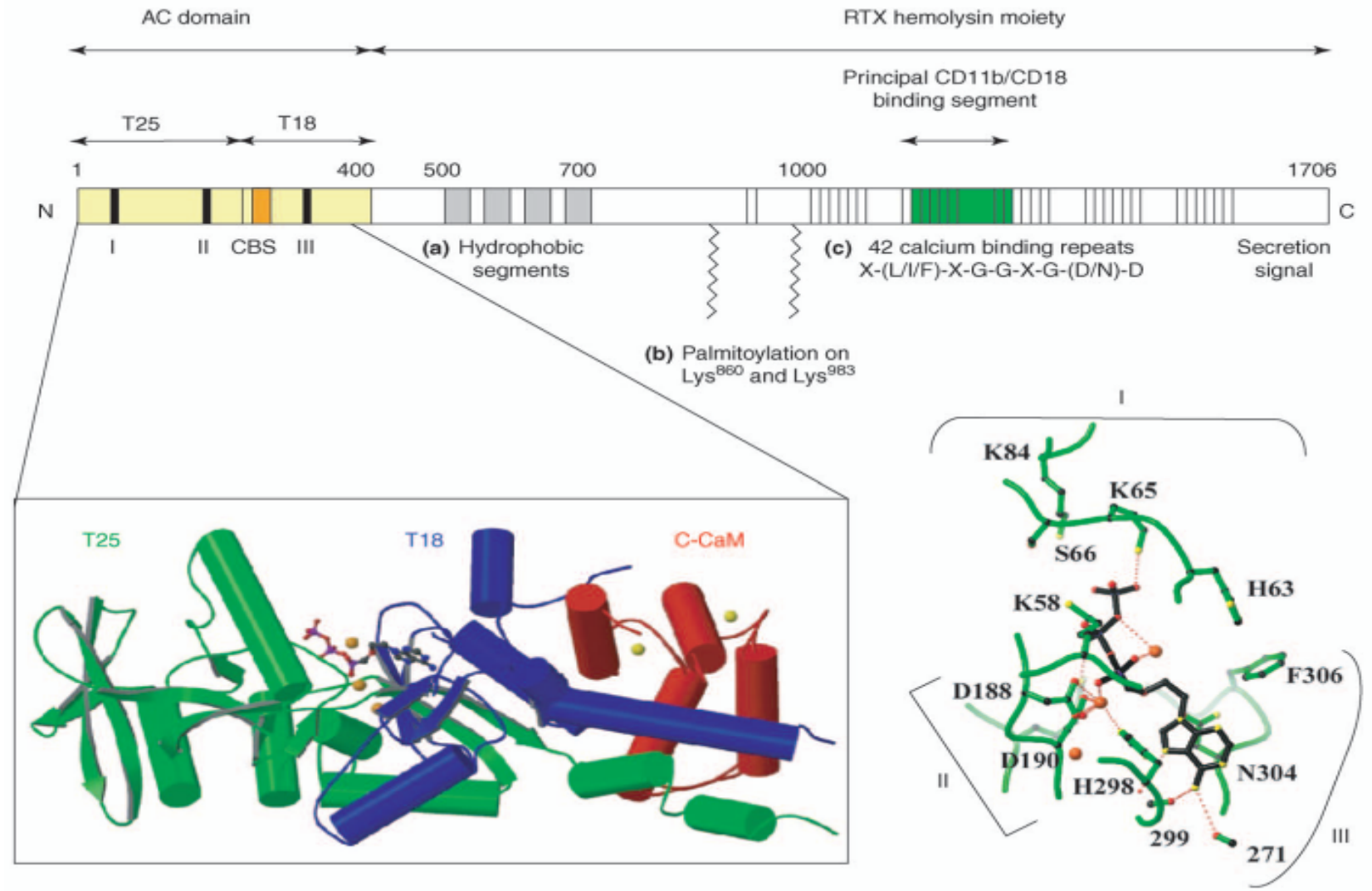
CyaA Adenylate Cyclase III



CyaA Conformational Change of Calmodulin



CyaA Structure of AC Domain



References

- Department of Biotechnology, University Würzburg
- Freer, A. (1999) The Comprehensive Sourcebook of Bacterial Protein Toxins (2nd Edition); Academic Press
- Benz et al. (1994) Adenylate Cyclase Toxin (CyaA) of *Bordetella pertussis*, J. Biol. Chem. 269, 27231-27239
- Knapp et al. (2003) Channel Formation in Model Membranes by the Adenylate Cyclase Toxin of *Bordetella pertussis*: Effect of Calcium, Biochemistry, 42, 8077-8084
- Vojtova, J. et al. (2006) *Bordetella* adenylate cyclase toxin: a swift saboteur of host defense, Curr. Opin. Microbiol., 9: 1-7
- Basler, M. (2006) Pore-Forming and Enzymatic Activities of *Bordetella pertussis* Adenylate Cyclase Toxin Synergize in Promoting Lysis of Monocytes, Infect. Immun. 74 , 2207-2214
- Fiser, R. (2006) Third Activity of *Bodetella* Adenylate Cyclase (AC) Toxin-Hemolysin, J. Biol. Chem. 282, 2808 - 2820
- Some pictures are taken from the internet (Google)

The End

Thank you for your attention.