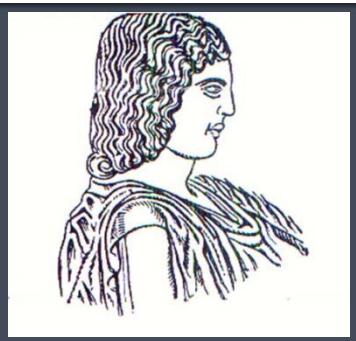
Session 39
Email: i.politis@aua.gr

Biologically - active peptides from bovine milk and their effect on human health

I. Politis
Agricultural University of Athens
i.politis@aua.gr



Biological properties of milk peptides

- Opioid
- Antihypertensive
- Antithrombotic
- Immunoregulatory

Enhancement or Suppression ?

Immunoregulatory peptides

Origin of immunoregulatory peptides derived from caseins in bovine milk

Protein	Peptide	Immune effect
αs1-casein	f(194-199)	↑ antibody formation, ↑ phagocytosis
	f(1-23)	↑ phagocytic response
	f(90-96)	↑ lymphocyte proliferation, ↑ NK cell activity, ↑neutrophil chemotaxis
	f(90-95)	
	f(23-34	ACE inhibitor, ↑ phagocytosis, protective response to *Klebsiella pneumoniae* infection
β-casein	f(193-209)	↑ proliferating responses in lymphocytes
	f(63-68)	↑ antibody formation, ↑ phagocytosis
	f(191-193)	↑ antibody formation, ↑ phagocytosis, ↑ antigen dependent T cell proliferation
κ-casein	f(106-169)	↑ lymphocyte proliferation
	f(17-21)	↑ antibody formation and↑ phagocytosis *in vitro*
	f(38-39)	↑ lymphocyte proliferation

The β – casein story (I)

Hydrolytic products

Inhibit proliferation of splenic lymphocytes

Inhibit proliferation of Peyer's patch cells

Inhibit proliferation of human lymphocytes

The β – casein story (II)

Two critical peptides

Pro – Gly – Pro – Ile – Pro – Asn (63-68)

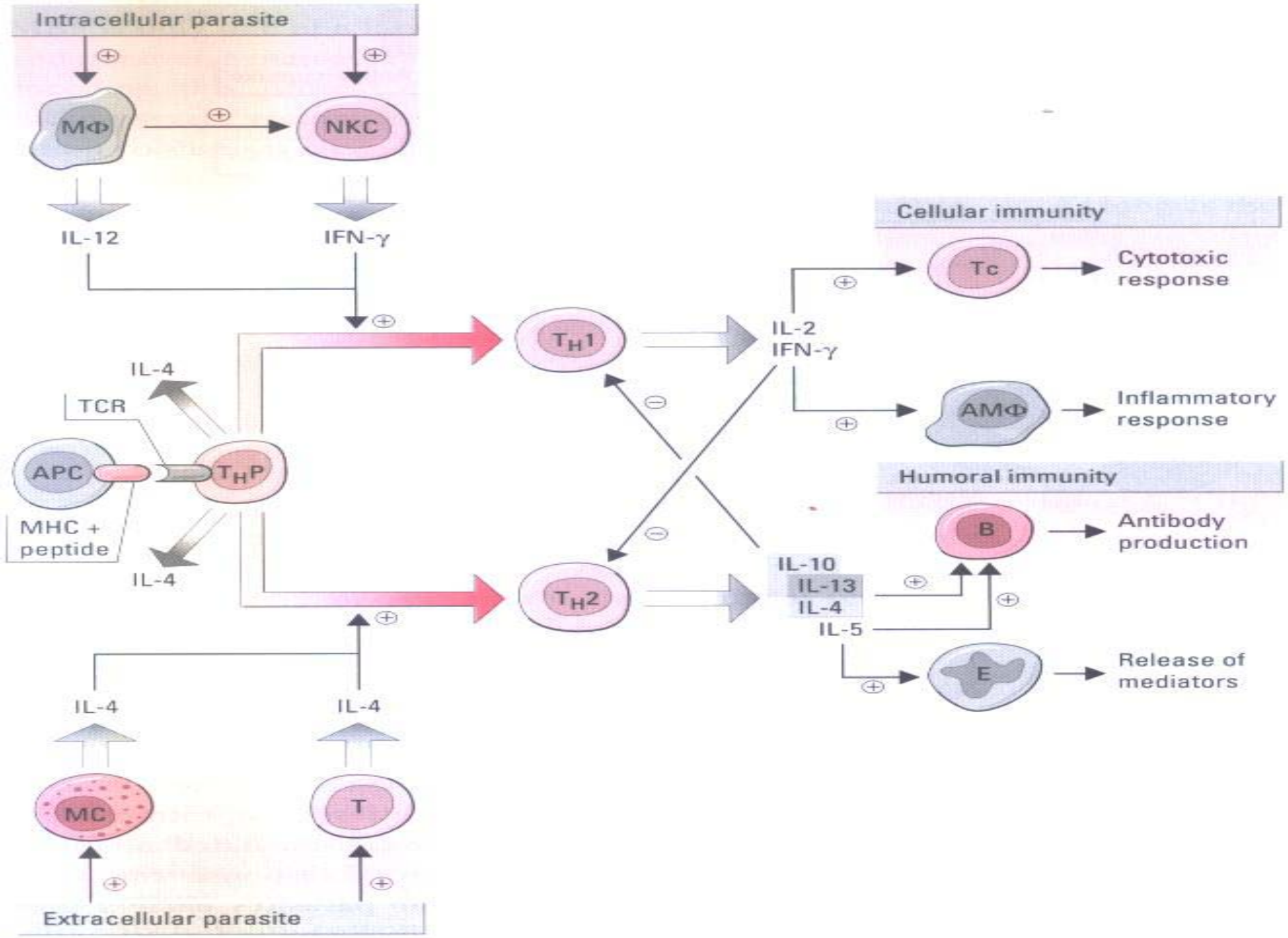
Leu –Leu – Tyr (191-193)

- Promote antibody production
- Enhance phagocytosis and T-cell proliferation

Experimental part

Effect of bovine milk protein

peptides on the function of T-cells


Intracellular parasite
MΦ
NKC
IL-12
IFN-γ
IL-4
TCR
APC
THP
MHC + peptide
IL-4
IL-4
IL-4
MC
T
Extracellular parasite
TH1
TH2
IL-2
IFN-γ
IL-10
IL-13
IL-4
IL-5
Cellular immunity
Tc
Cytotoxic response
AMΦ
Inflammatory response
Humoral immunity
B
Antibody production
E
Release of mediators

Methodology

- **Peptides tested**

  Low molecular weight peptide fraction

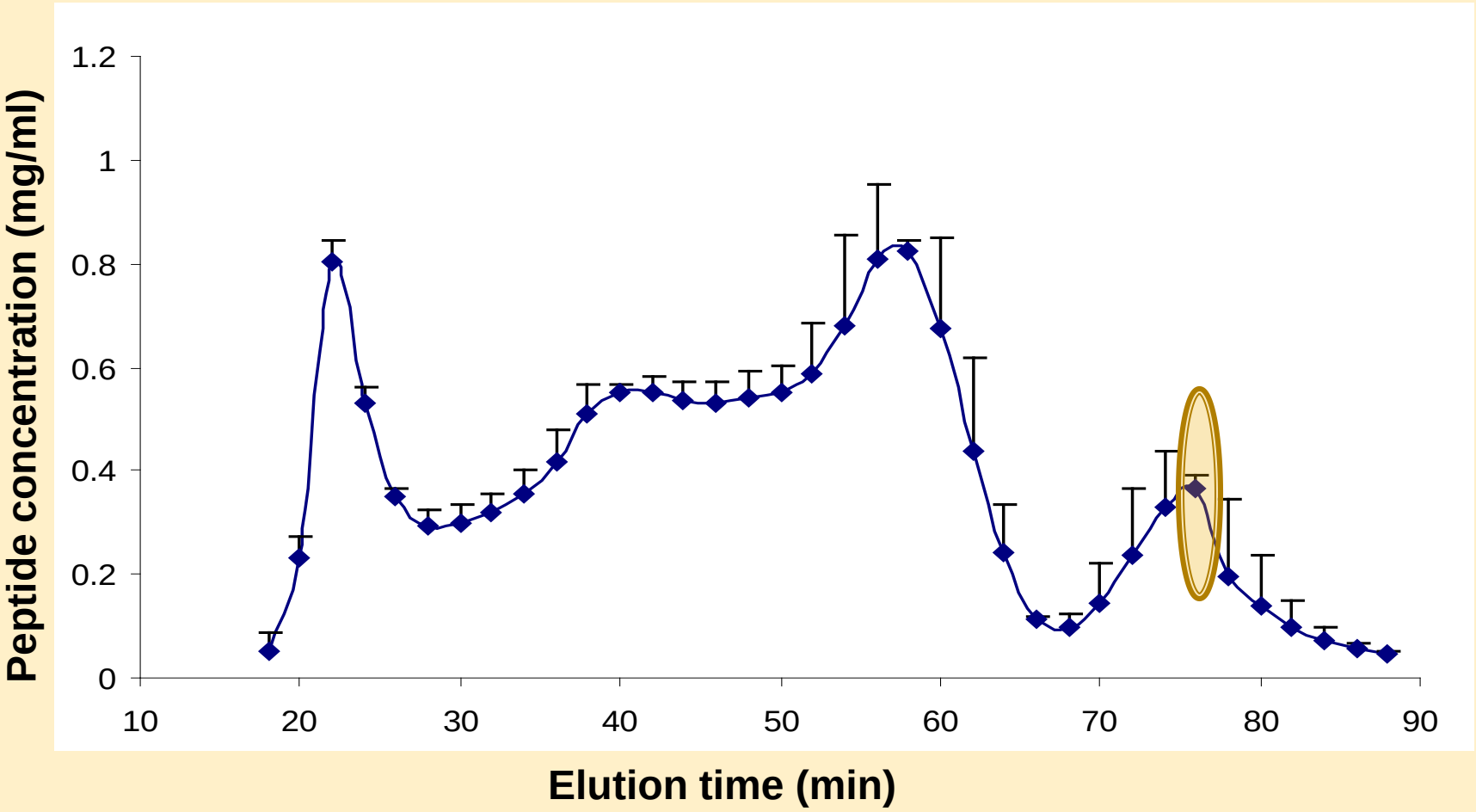
  Leu – Leu –Tyr (tripeptide)

- **Target cells**

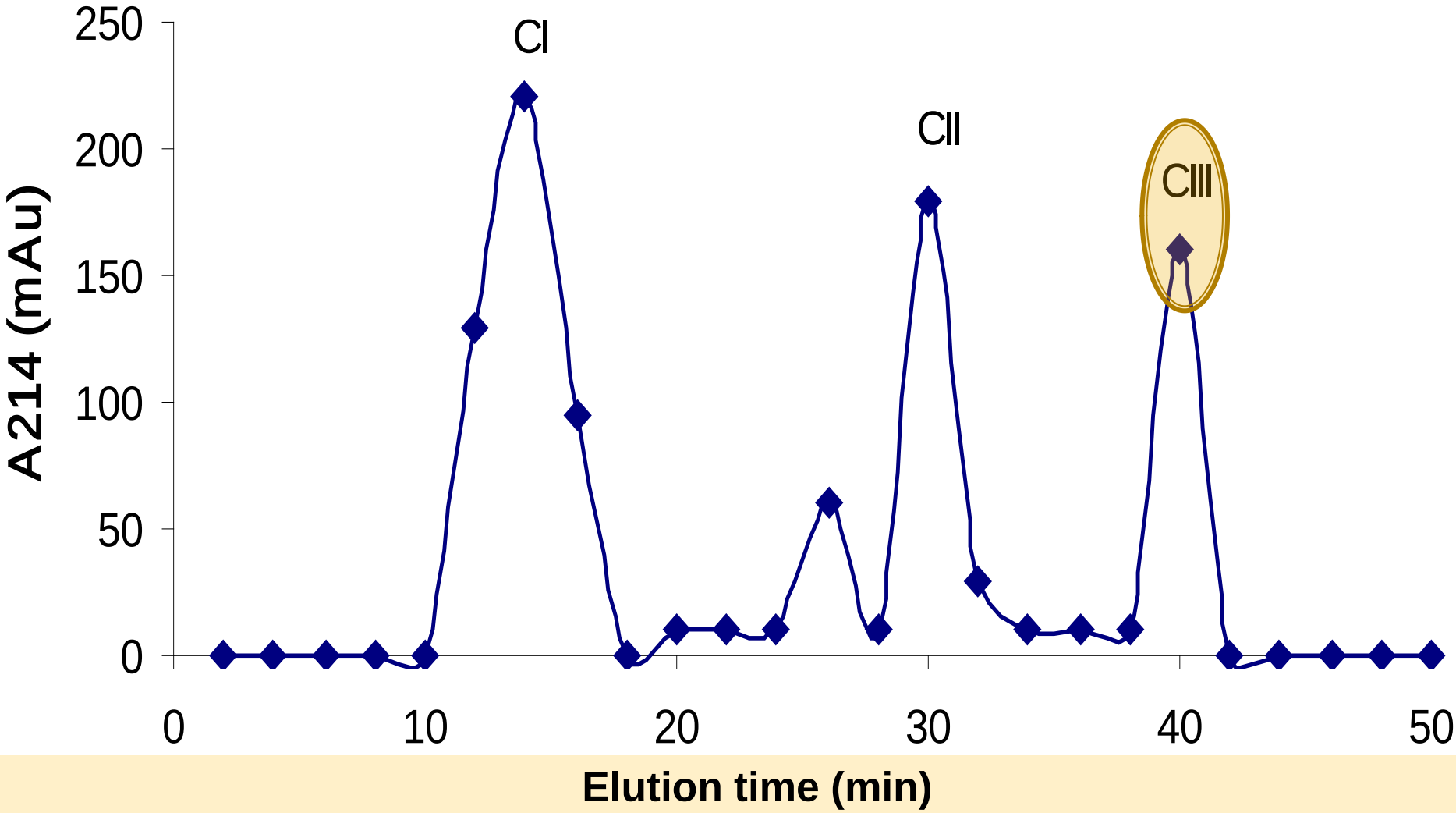
  T-cells

- **Parameters**
  - IL-2
  - IFN-γ
  - IL-4

Liquid chromatography
Sephadex G-25



Size-exclusion HPLC



In vitro effect of various milk protein peptides on IL-2 secretion by stimulated porcine T-cells.

Data are expressed as pgx1000/ml/10^6 cells (six independent experiments)

IL-2 (pg x 1000/ml/10^6 cells)		
Treatment	Weaning (w)	w + 8 weeks
Control	30.2 ± 10.3	32.6 ± 9.7
Fraction III	12.4 ± 4.7*	39.3 ± 6.8
Tripeptide	25.2 ± 6.3	34.2 ± 6.5

In vitro effect of various milk protein peptides on IFN-γ secretion by stimulated porcine T-cells.

Data are expressed as pg/ml/10^6 cells (six independent experiments)

IFN-γ (pg/ml/10^6 cells)		
Treatment	Weaning (w)	w + 8 weeks
Control	21.8 ± 6.7	24.6 ± 7.8
Fraction III	10.3 ± 4.7*	34.3 ± 5.8*
Tripeptide	18.3 ± 5.9	28.2 ± 4.7

In vitro effect of various milk protein peptides on IL-4 secretion by stimulated porcine T-cells.

Data are expressed as pg/ml/10^6 cells (six independent experiments)

IL-4 (pg/ml/10^6 cells)		
Treatment	Weaning (w)	w + 8 weeks
Control	40.3 ± 8.5	38.3 ± 7.6
Fraction III	18.5 ± 5.6*	45.3 ± 8.4
Tripeptide	35.2 ± 7.4	40.9 ± 7.6

Conclusions

Low molecular weight (LMW) peptide fraction down-regulated IL-2, IL-4 and IFN-γ production by T-cells at WEANING

LMW peptide fraction showed a tendency to upregulate IL-2, IL-4 and IFN-γ production by T-cells at 8 weeks after weaning

Leu – Leu –Tyr had no effect on cytokine production by T-cells

Experimental part

Effects of two milk peptides on the function of porcine macrophages and neutrophils

Methodology

- Peptides tested

  Pro – Gly – Pro – Ile – Pro – Asn (hexapeptide)

  Leu – Leu –Tyr (tripeptide)

- Target cells

  Macrophages, Neutrophils

- Parameters
  - Chemotaxic index
  - U-PA system
  - Superoxide anion

Effect of two peptides (10μM) on chemotactic responsiveness of porcine neutrophils

Chemotactic Index		
Treatment	Weaning (w)	w + 4 weeks
Control	2.4 ± 0.5	2.9 ± 0.5
Tripeptide	1.5* ± 0.5	2.7 ± 0.6
Hexapeptide	1.4* ± 0.6	3.5 ± 0.7

Effect of two peptides (10μM) on membrane-bound u-PA by porcine macrophages

u-PA (ΔA/h)		
Treatment	Weaning (w)	w + 4 weeks
Control	0.32 ± 0.04	0.47 ± 0.05
Tripeptide	0.16* ± 0.03	0.44 ± 0.07
Hexapeptide	0.22* ± 0.04	0.43 ± 0.07

Effect of two peptides (10μM) on membrane-bound u-PA by porcine neutrophiles

u-PA (ΔA/h)		
Treatment	Weaning (w)	w + 4 weeks
Control	0.47 ± 0.06	0.57 ± 0.07
Tripeptide	0.23* ± 0.04	0.55 ± 0.08
Hexapeptide	0.34* ± 0.04	0.60 ± 0.09

Effect of two peptides (10μM) on superoxide anion (SA) production by porcine neutrophils

SA (nm/10^6 cells)		
Treatment	Weaning (w)	w + 4 weeks
Control	2.0 ± 0.03	3.6 ± 0.05
Tripeptide	1.5* ± 0.02	4.9* ± 0.04
Hexapeptide	1.9 ± 0.04	5.0* ± 0.05

Effect of two peptides (10μM) on superoxide anion (SA) production by porcine macrophages

SA (nm/10^6 cells)		
Treatment	Weaning (w)	w + 4 weeks
Control	2.5 ± 0.04	3.5 ± 0.03
Tripeptide	1.2* ± 0.03	4.6* ± 0.04
Hexapeptide	1.6* ± 0.03	4.7* ± 0.04

Conclusions (I)

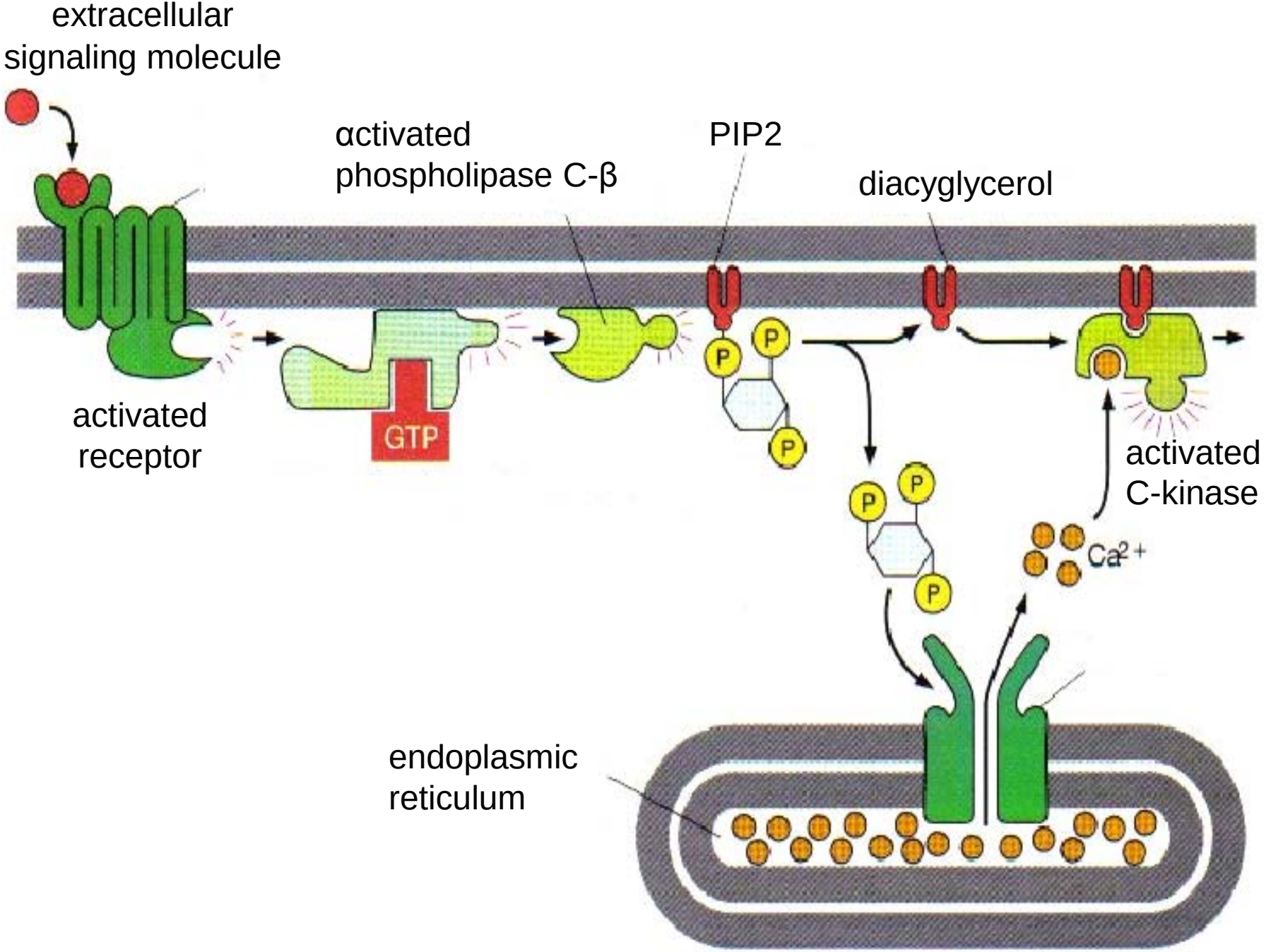
Tripeptide and Hexapeptide down-regulated various immune functions at WEANING

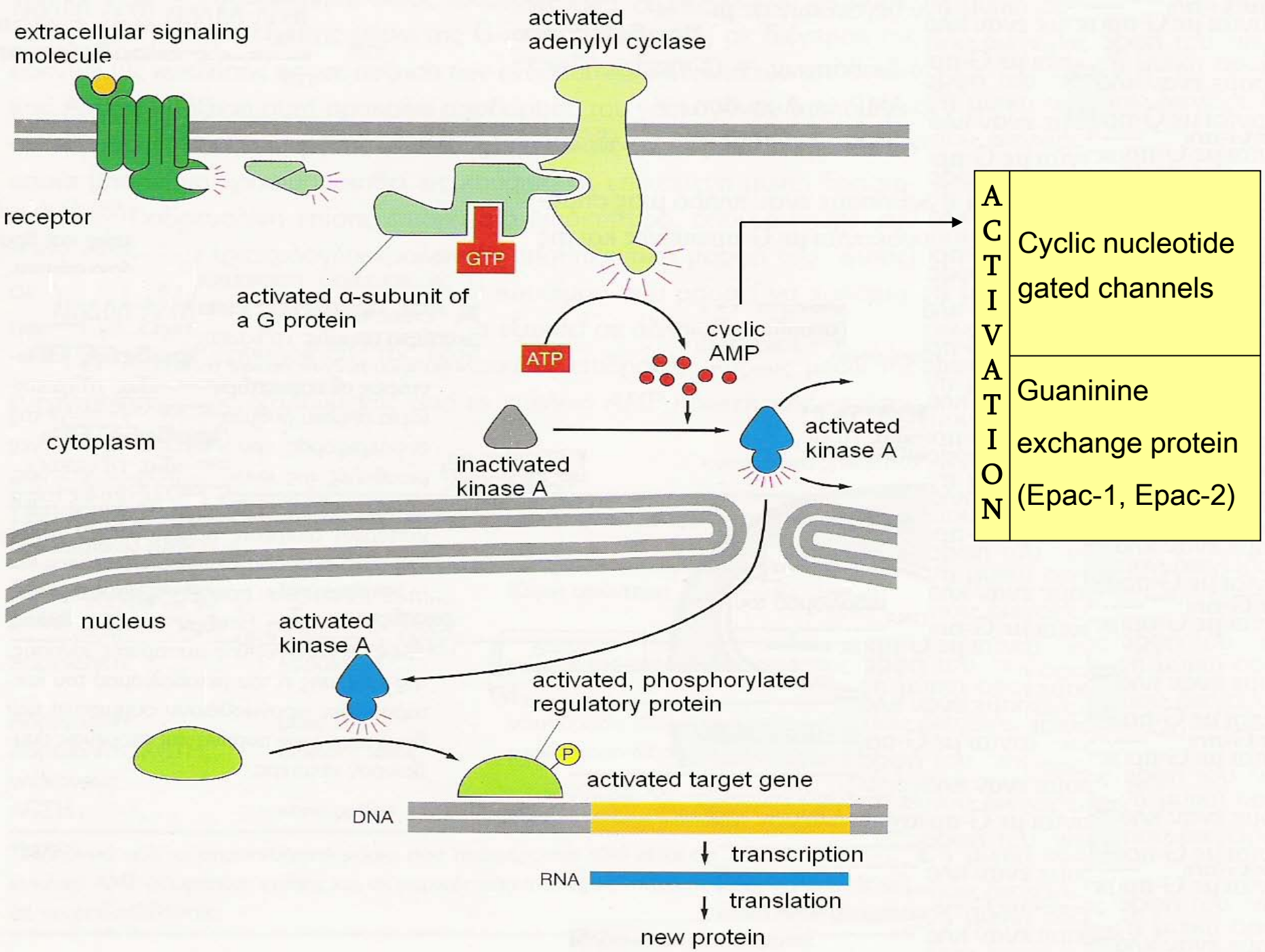
- Chemotactic activity, u-PA production
- Superoxide production

Conclusions (II)

Tripeptide and Hexapeptide increased superoxide production and had no effect on various immune functions at FOUR WEEKS AFTER WEANING

Milk peptides - Mechanism of action


extracellular
signaling molecule
activated
receptor
GTP
activated
phospholipase C-β
PIP2
diacyglycerol
activated
C-kinase
Ca2+
endoplasmic
reticulum


extracellular signaling molecule
activated adenylyl cyclase
receptor
GTP
activated α-subunit of a G protein
ATP
cyclic AMP
cytoplasm
inactivated kinase A
activated kinase A
nucleus
activated kinase A
activated, phosphorylated regulatory protein
P
activated target gene
DNA
transcription
RNA
translation
new protein
ACTIVATION
Cyclic nucleotide gated channels
Guaninine exchange protein (Epac-1, Epac-2)

Effect of various cAMP analogues (10μM) on membrane-bound u-PA

u-PA (ΔA/h)		
Treatment	Macrophages	Neutrophils
Control	$0.159^{a} \pm 0.018$	$0.241^{a} \pm 0.036$
PKA/Epac-1 activator (10μM)	$0.082^{b} \pm 0.010$	$0.166^{b} \pm 0.016$
PKA activator (10 μM)	$0.091^{b} \pm 0.017$	$0.176^{b} \pm 0.016$
Epac-1 activator (10 μM)	$0.164^{a} \pm 0.025$	$0.237^{a} \pm 0.030$

Effect of various cAMP analogues (10μM) on superoxide anion (SA) production

SA (nm/10^6 cells)		
Treatment	Macrophages	Neutrophils
Control	$1.203^{a} \pm 0.095$	$2.035^{a} \pm 0.211$
PKA/Epac-1 activator (10μM)	$0.593^{d} \pm 0.068$	$1.476^{b} \pm 0.109$
PKA activator (10 μM)	$0.774^{b} \pm 0.064$	$1.631^{b} \pm 0.088$
Epac-1 activator (10 μM)	$0.676^{c} \pm 0.027$	$1.509^{b} \pm 0.156$

Effect of PKA inhibitors on membrane-bound u-PA

u-PA (ΔA/h)		
Treatment	Macrophages	Neutrophils
PMA	$0.204^{a} \pm 0.031$	$0.297^{a} \pm 0.029$
PMA+tripeptide	$0.087^{b} \pm 0.019$	$0.190^{b} \pm 0.020$
PMA+tripeptide+H89 (10μM)	$0.205^{a} \pm 0.035$	$0.302^{a} \pm 0.034$
PMA+tripeptide+KT5720 (10μM)	$0.206^{a} \pm 0.039$	$0.305^{a} \pm 0.054$
PMA+tripeptide +PKI_{14-22} (10μM)	$0.205^{a} \pm 0.038$	$0.298^{a} \pm 0.032$
PMA+tripeptide+HA1004 (10μM)	$0.205^{a} \pm 0.036$	$0.294^{a} \pm 0.039$

Effect of PKA inhibitors on superoxide anion (SA) production

SA (nm/10^6 cells)		
Treatment	Macrophages	Neutrophils
PMA	$1.464^{a} \pm 0.148$	$2.361^{a} \pm 0.233$
PMA+tripeptide	$0.593^{c} \pm 0.061$	$1.556^{c} \pm 0.119$
PMA+tripeptide+H89 (10μM)	$1.172^{b} \pm 0.090$	$1.893^{b} \pm 0.092$
PMA+tripeptide+KT5720 (10μM)	$1.151^{b} \pm 0.058$	$1.917^{b} \pm 0.138$
PMA+tripeptide +PKI_{14-22} (10μM)	$1.155^{b} \pm 0.096$	$1.955^{b} \pm 0.136$
PMA+tripeptide+HA1004 (10μM)	$1.139^{b} \pm 0.073$	$1.972^{b} \pm 0.188$

Conclusions

1. Activation of PKA, but not Epac-1, is responsible for the downregulation of the u-PA system
2. Activation of PKA and/or Epac-1 is responsible for the downregulation of the SA system

Effect of two protein kinases on IL-2, IL-4 and IFN-γ production by stimulated T-cells.

Data are expressed as pg/ml/10^6 cells (six independent experiments)

Treatment	IL-2 (x1000)	IL-4	IFN-γ
Control	32.2 ± 8.3[a]	43.3 ± 7.9[a]	24.7 ± 5.6[a]
Fraction III	14.7 ± 5.2[b]	20.0 ± 4.7 [b]	9.8 ± 4.3 [b]
Fraction III +H7	0.8 ± 0.8[c]	1 ± 0.8[c]	0.6 ± 0.7 [c]
Fraction III +HA1004	12.5 ± 6.3[b]	23.5 ± 5.7 [b]	9.6 ± 4.4 [b]

Conclusions

Suppresion of T-cell activation is related to inhibition of PKC rather than of PKA

Antihypertensive peptides

Hypertension
Summary of facts

- Affects 25% of the population
- 22 billion $ per annum (US alone)
- Increase of 5 mmHg in BP → 16% increase in the risk of CVD

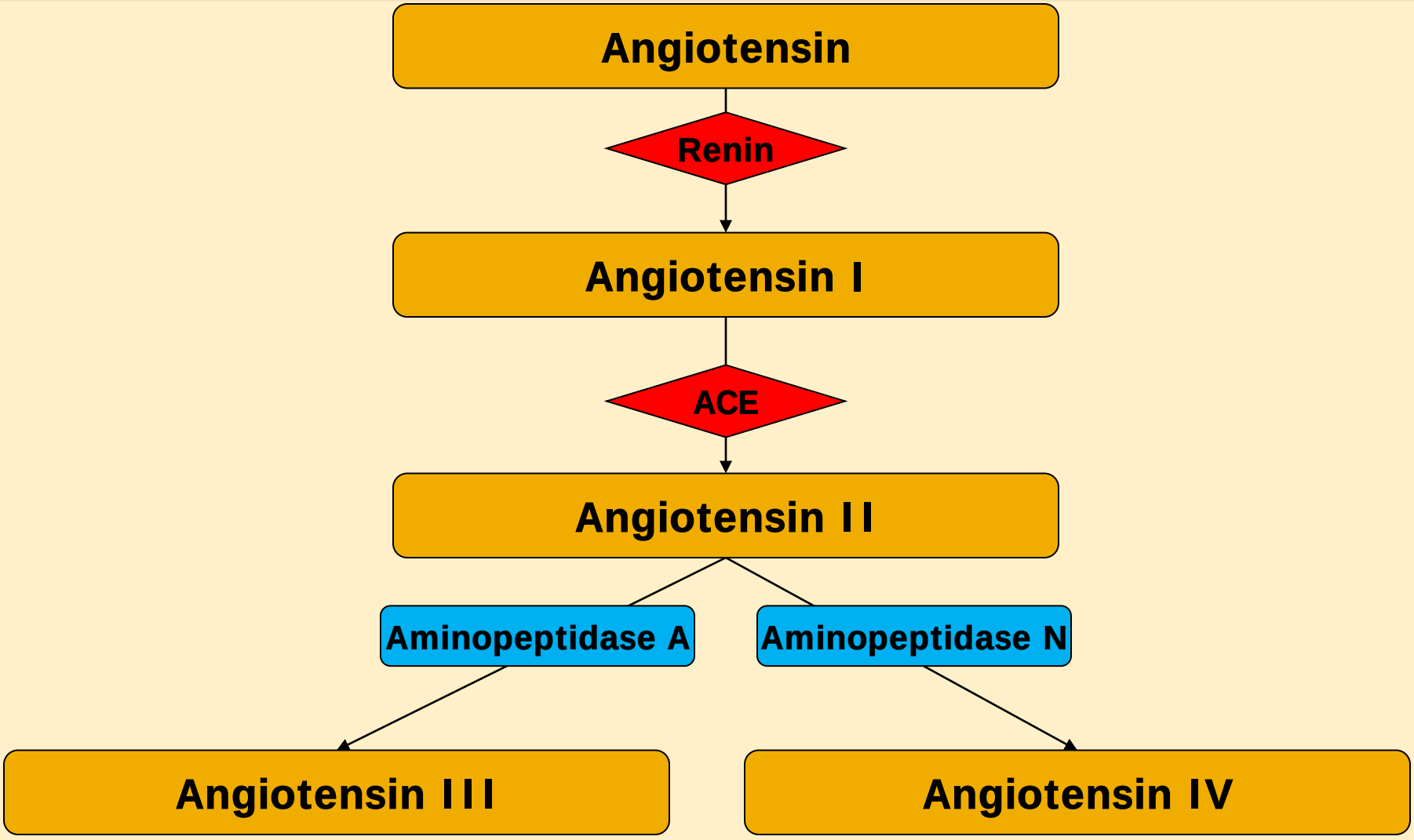
BP Guidelines

	SBP (mmHg)	DBP (mmHg)
Normal	< 120	< 80
Pre-hypertension	< 139	< 90
Stage 1 hypertension	< 159	< 99
Stage 2 hypertension	≥ 160	≥ 100

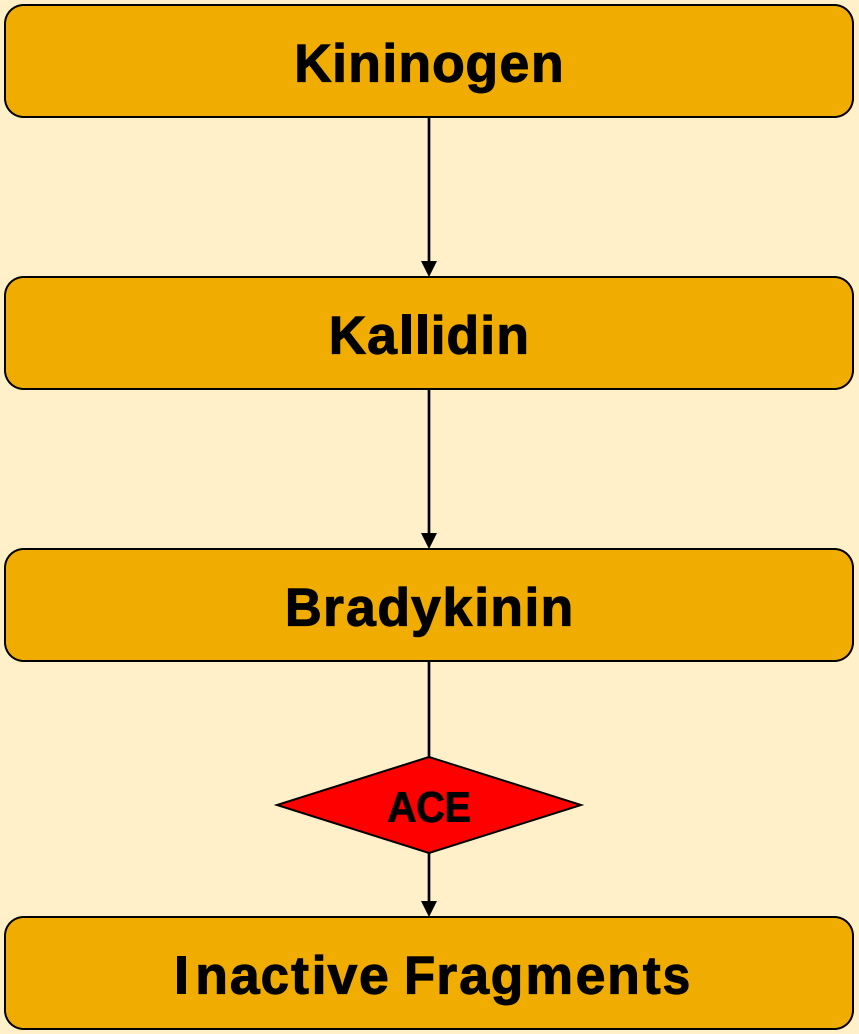
Mechanisms implicated in BP control

- Renin- Angiotensin system
- Kinin-bradykinin system

Basic steps of the renin – angiotensin metabolic pathway



Basic steps of the kinin – bradykinin metabolic pathway



Potent casokinin and lactokinin sequences

Protein	Peptide fragment	Primary sequence	ACE IC_{50} (μmol/L)
Casokinins			
αs1-casein	f(25-27)	VAP	2.0
αs2-casein	f(174-179)	FALPQY	4.3
β-casein	f(74-76)	IPP	5.0
κ-kasein	f(185-190)	VTSTAV	52.0
Lactokinins			
α-lactoglobulin	f(104-108)	WLAHK	77.0
β-lactoglobulin	f(142-148)	ALPMHIR	42.6
BSA	f(208-216)	ALKAWSVAR	3.0

FitzGerald et al. (2004)

Bovine casein-derived peptides displaying hypotensive effects in spontaneously hypertensive rats (I)

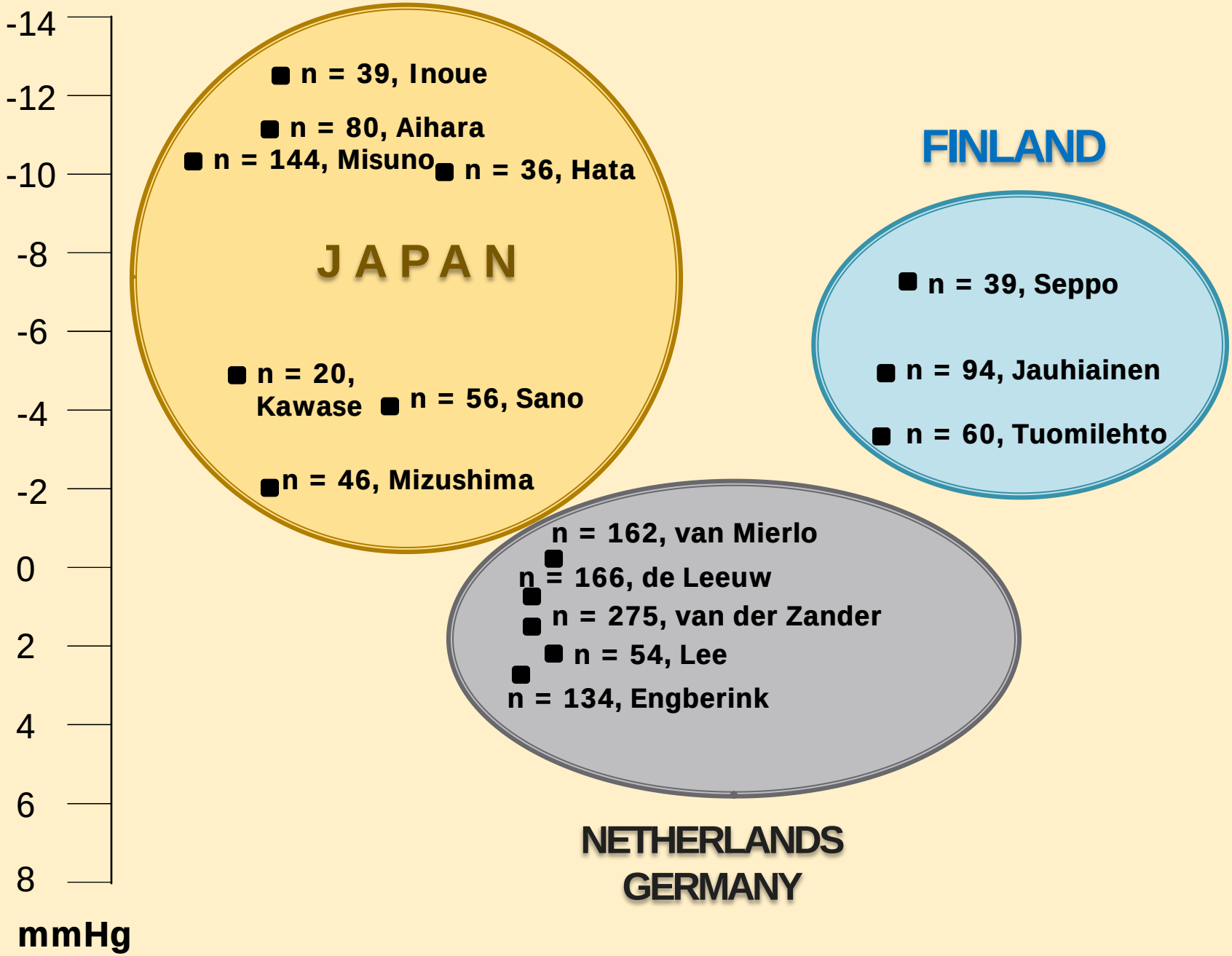
Peptide	Sequence	IC_{50} (μmol/L)	Maximum decrease in SBP (mmHg)
αs1-casein			
f(1-9)	RPKHPIKHQ	13	-9.3
f(23-34)	FFVAPFPEVFGK	77	-34.0
f(104-109)	YVKVPQL	22	-13.0
f(146-147)	YP	720	-32.1
f(194-199)	TTMPLW	16	-14.0
αs2-casein			
f(189-192)	AMPKPW	580	-5.0
f(190-197)	MKPWIQPK	300	-3.0
f(198-202)	TKVIP	400	-9.0

FitzGerald et al. (2004)

Bovine casein-derived peptides displaying hypotensive effects in spontaneously hypertensive rats (II)

Peptide	Sequence	IC_{50} (μmol/L)	Maximum decrease in SBP (mmHg)
β-casein			
f(59-61)	VYP	288	-21.0
f(59-64)	VYPFPG	221	-22.0
f(60-68)	YPFPGPIPN	15	-7.0
f(74-76)	IPP	5	-28.3
f(80-90)	TPVVVPPFLQP	749	-8.0
f(84-86)	VPP	9	-32.1
f(140-143)	LQSW	500	-2.0
f(169-174)	KVLPVP	5	-32.2
f(169-175)	KVLPVPQ	1000	-31.5
f(177-183)	AVPYPQR	15	-10.0

FitzGerald et al. (2004)



Summary of human studies

European Food Safety Authority

EFSA Journal 2009; 7(9):1259

SCIENTIFIC OPINION

Scientific Opinion on the substantiation of health claims related to isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) and maintenance of normal blood pressure (ID 615, 661, 1831, 1832, 2891), and maintenance of the elastic properties of the arteries (ID 1832) pursuant to Article 13(1) of Regulation (EC) No 1924/2006[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2]

European Food Safety Authority (EFSA), Parma, Italy

EFSA conclusions

- Evidence is insufficient to establish a cause-effect relationship between consumptions of tripeptides VPP and IPP and:
  - Maintenance of normal blood pressure
  - Elastic properties of arteries

CONCLUSIONS

- Antihypertensive effect of milk peptides is still debatable
- Results concerning bioavailability are not convincing
- Mechanism of action unknown