Regulation of membrane transporters by kinases

der Fakultät für Biologie
der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

von

Manzar Banoo Shojaeifard
aus Shiraz / Iran

vorgelegte

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ABBREVIATIONS

List of Abbreviations:

A	Gain
ADC	Analoge-Digital Converter
AgCl	Silver-Chloride
AGAT	L-Arginine:glycine amintransferase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
cAMP	cyclic adenosine monophosphate
Ca^{2+}	Calcium ion
CaLB	Calcium Lipid-binding
cDNA	complementary deoxyribonucleic acid
CE	Current Electorde
CK	Creatine Kinase
CIC-K	kidney chloride channel
Cl^{-}	Chloide ion
C_M	Membrane Capacitance
Cr	Creatine
Crn	Cretinine
CreaT/CrT	Creatine Transporter
cRNA	complementary ribonucleic acid
DAC	Digital –Analoge Converter
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotidetriphosphate
DTNB	5,5’-dithio-bis[-2-nitrobenzoic acid]
e_o	output voltage
ECaC1	epithelial calcium channel type 1
EDTA	ethylene diamine tetra-acetate
EGTA	ethyleneglycol-bis (ß-aminoethyl)-N, N, N‘, N‘- tetraacetic acid

ABBREVIATIONS

EK^+	equilibrium potential for the ion K^+
eIF	eukaryotic initiation factor
ENaC	epithelial Na^+ channel
FBA	feedback amplifier
FKBPs	Family of intracellular receptors termed FK506 binding protein
FRAP	FKBP-RAP-associated protein
GAMT	S-adenosyl-L-methionine:n-guanidinoacetate methyltransferase
h	human
H^+	Hydrogen ion
HEPES	N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)
HEK293	human embryonic kidney cell line
IC50	concentration at which a 50% inhibition is reached
IGF-1	insulin-like growth factor 1
IFN	interferon
IL	interleukin
I-V	current-voltage relation
K^+	Potasium ion
kDa	Kilodalton
Kv	voltage-gated potassium channel
Ly6E	Antigen on cell surface of YAC1
mRNA	messenger ribonucleic acid
mM	Millimolar
mTOR	Mammalian target of rapamycin

ABBREVIATIONS

mV	milliVolt
Na^+	Sodium ion
NaPi	sodium-dependent phosphate transporter
NHE3	Na^+/H^+ exchanger type 3
NHERF	Na^+/H^+ exchanger type 3 regulating factor
NKCC	sodium potassium chloride cotransporter
NMDG	N-methyl-D-glucamine
PCR	polymerase chain reaction
PCr	PhosphorylCreatine
PE	Potential Electrode
pHi	intracellular pH
PI 3-Kinase	Phosphatidylinositol 3-Kinase
Pka	apparent pK
PKA	protein kinase A; cAMP-dependent protein kinase
PKB (Akt)	protein kinase B; oncogene from Akt mouse
PKC	protein kinase C
PT	proximal tubule
PTH	parathyroid hormone
Ra	access Resistance
RAP	Rapamycin
RAPT	Rapamycin target
R_M	Membrane Resistance
RNA	ribonucleic acid
RyR/CRC	Ryanodine receptor/Calcium release channel

ABBREVIATIONS

SEM	standard error of the mean
SEP	Sirolimus effector protein
SGK	serum and glucocorticoid inducible kinase
SLC	Solute Carrier
TcR	T cell receptor
TEA	Tetraethylammonium
TEVC	two-electrode voltage clamp
TOR	target of rapamycin
µA	Microampere
µCi	microcurie (1 Ci = 37 x 109 Bq)
µM	micromolar
V_C/E	Clamp Potential /Command Potential
VE	Voltage Electrode
Vm	membrane potential
YAC	Murine T cell lymphoma

INTRODUCTION

In the name of God

I- Electophysiology

Electrophysiology is the Science and branch of Physiology that pertains to the flow of ions in biological tissues and, in particular, to the electrical recording techniques that enable the measurement of this flow and the potential changes related to them. In almost all cases, electrophysiological techniques record the voltage maintained across a cell membrane (i.e. the electrical potential difference between the inside and outside of a cell) or the ion currents that flow across a cell membrane (i.e. the movement of ions from the inside of the cell to the outside or vice versa). The technical goal of the electrophysiology is simple: to record the voltage across a cell's membrane, the current flowing across that membrane. There are two major divisions of electrophysiological technique: intracellular recording & extracellular recording. Within these two divisions are many variations. Extracellular recording includes single unit recording, field potential recording, single channel recording and amperometry. Intracellular recording techniques include voltage clamp and current clamp.

I-A
Voltage Clamp

I-A. 1 Motivation

The function of a cell is governed to a large extend by transport across the cell membrane that is mediated by specific membrane proteins. To learn about functional characteristics of Transport proteins, electrophysiological techniques have turned out to be a powerful method. Particularly the combination of electrophysiology with molecular biology allows to obtain fundamental information on structure, function and regulation of transport proteins. This can be achieved by expression of genetically modified proteins in *Xenopus* oocytes and functional characterization by electrophysiological methods. [Hille 2003, Schwarz & Rettinger 2003].

I-A. 2 Background

I-A. 2. a *Theoretical background of voltage clamp*

The most powerful electrophysiological method for basic research is the voltage-clamp technique, which allows at a given membrane potential the measurement and analysis of currents across the cell membrane that are mediated by specialised channels and carriers. The voltage-clamp technique was the basis for the two milestones in modern electrophysiology: the Hodgkin-Huxley description of excitability and the demonstration of single-channel events by [Sakmann, B. & Neher, E. 1995].

I-A. 2. b *The ideal voltage clamp*

The ideal voltage clamp (Figure 1) consists of a voltage source providing the clamp potential V_C, the model membrane (membrane resistance R_M and capacitance C_M in parallel), a switch and an ampere meter for measuring membrane current I_M.

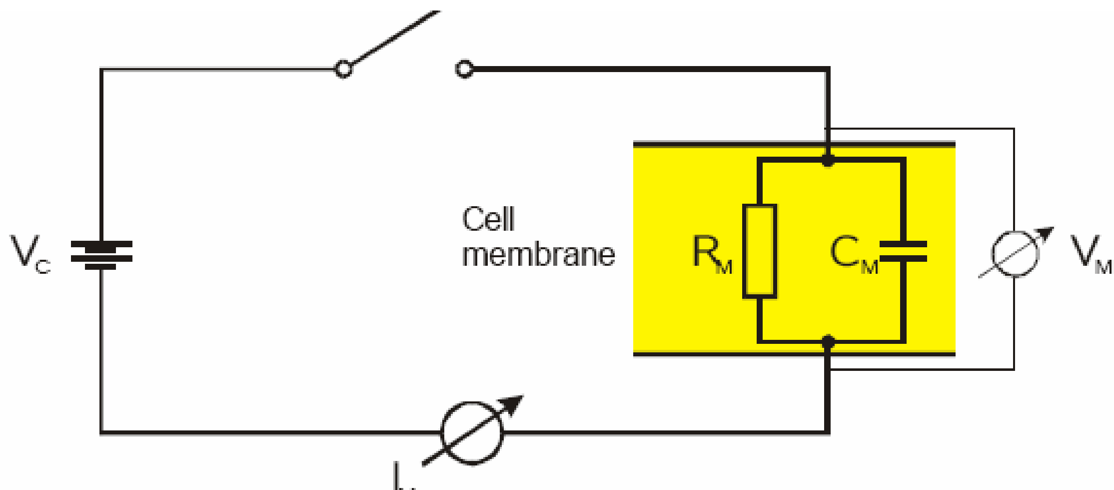


Figure 1: The ideal Voltage Clamp

This circuit is "ideal" since wires, ampere meter and battery are assumed to have negligible intrinsic resistance. Therefore, after the switch is closed, the model membrane reaches the potential of the battery as soon as the capacitance is charged ($V_M = V_C$).

I-A. 2. c ***The Real Voltage Clamp***

The main difference between the ideal and the real voltage clamp is that the connection between the electronic circuit and the cell (in which currents are carried by ions) cannot be treated with negligible resistance. In many cases glass micro-electrodes are used to penetrate the cell membrane for intracellular access. These electrodes have tip resistances in the range of MΩ, similar to the input resistance of large cells such as frog oocytes. Therefore, one has to add the electrode resistance to the "ideal" circuit of Figure 1 ending up with Figure 2.

$$V_M = V_C \frac{R_M}{R_M + R_E}$$

This means that only a fraction

$$R_M / (R_M + R_E)$$

of the clamp potential V_C reaches the cell membrane, and only for the case that this fraction is not significantly different from unity (i.e. if $R_M >> R_E$), the cell is voltage-clamped to the command potential ($V_M = V_C$).

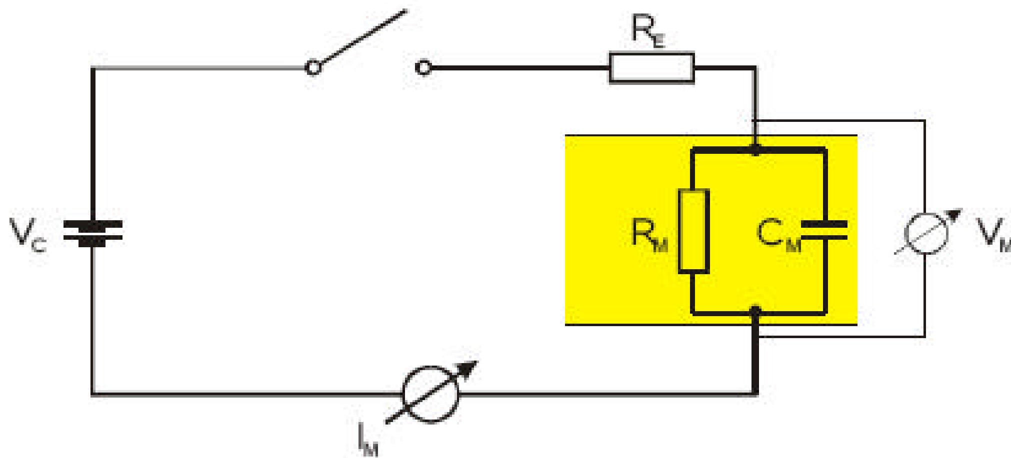


figure 2: The Real Voltage- Clamp

I-A. 2. d *The voltage clamp with two electrodes (TEVC)*

For large cells with low input resistances ($R_M < R_E$), it is obvious that performing voltage clamp with one electrode is not possible. Therefore, one needs a second electrode serving for independent determination of the actual membrane potential. The voltage source is then adjusted in a way that the membrane potential matches exactly the command potential. Figure 3 gives a graphical representation of this.

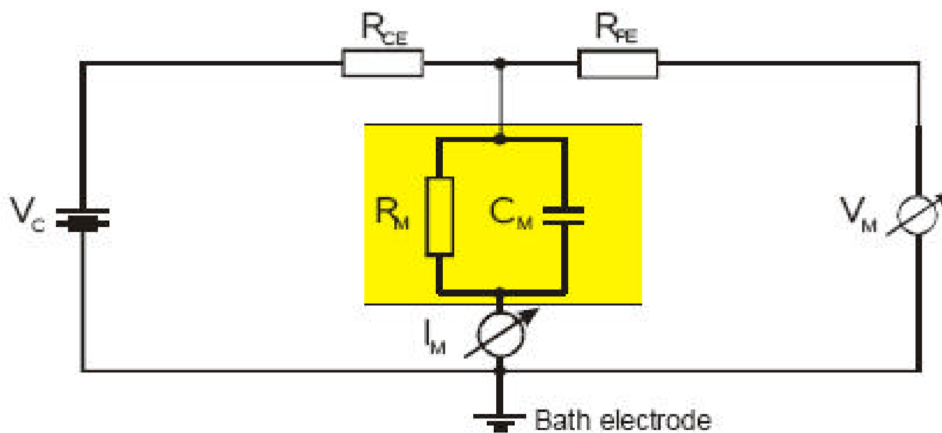


Figure 3: Voltage-Clamp circuit with a current electrode (CE) and a voltage electrode(VE).

In order to clamp the cell membrane to a certain potential V_M, it is necessary to apply a clamp potential that is large enough to compensate for the voltage drop at the electrode resistance R_{CE} which is quantitatively described by:

INTRODUCTION

$$V_C = V_M \frac{R_M + R_{CE}}{R_M}$$

Since the membrane resistance R_M as well as the electrode resistance R_{CE} can change during an experiment, it is necessary to compare the membrane potential V_M measured via the potential electrode PE continuously with the command potential, and to re-adjust the clamp potential V_C. Instead of doing this manually, it is possible to use electronic devices which allow for an exact and rapid communication between command potential and measured membrane potential. The central part of such an electronic set-up is the "operational amplifier" (op-amp) which can be used in various ways.

Figure 4: Schematic drawing of an op-amp (left) and an voltage follower (op-amp with unity gain, right)

The main characteristic of an op-amp (Figure 4) is its ability to amplify a difference (e_+ - e_-) between its two inputs by a factor A (gain)

$$e_0 = A\,(e_+ - e_-) \quad \text{with output voltage } e_0.$$

When the negative input is connected to the output, the op-amp works as a voltage follower meaning that the output signal equals the signal at the negative input.

$$e_0 = A\,(e_+ - e_-) = A\,(e_+ - e_0) \longrightarrow e_0 = \frac{A}{A+1}\, e_+ \sim e_+ \qquad \text{with } A = 10^4 - 10^6$$

These two op-amp varients can be used to complete the two–electrode voltage–clamp circuit as shown in figure 5 which form the basis of the commerically available amplifiers.

The voltage follower is used to uncouple the sensitive signal of the voltage electrode from the following devices such as oscilloscope or pen recorder, and to serve as a high reistance input in order to minimize the current flow through the voltage electrode. The second op-amp is used as a negative feedback amplifier with high gain. The positive input is connected to the command potential, the negative input to the signal delivered by the voltage follower.
These two input signals define the potential at the output, and hence allow the cell to be clamped fast and accurately to the command potential. The current flow from the feedback

amplifier is identical to the membrane current and can be measured either at the output of the op-amp or at the grounded bath electrode.
Very often two bath electrodes are used, one current-passing grounded electrode and one bath electrode serving as a reference electrode for the intracellular voltage electrode (virtual ground). The use of two bath-electrodes has the advantage that only the grounded electrode passes large currents and the non-grounded bath electrode cannot polarise due to current flow.[Salonkidis, Petrus and schwarz, wolfgang & Rettinger 2003]

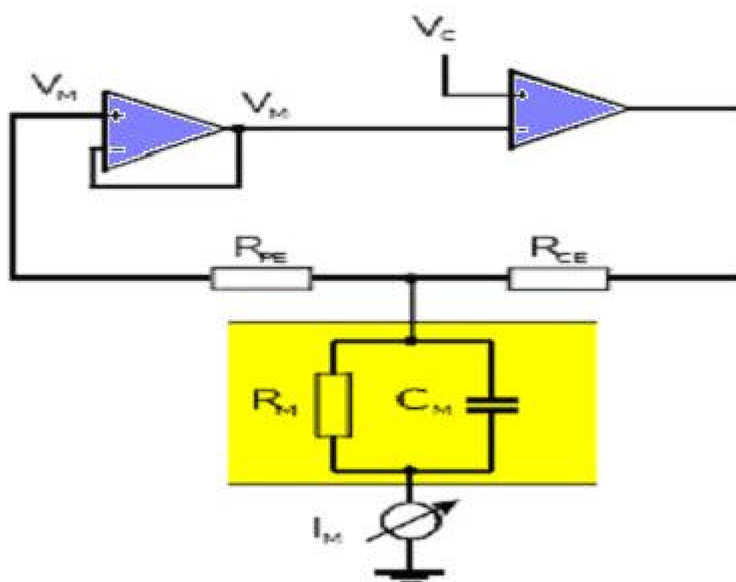


Figure 5: Two-electrode voltage-clamp circuit using op-amps for measuring membrane potential and performing voltage clamp by a negative feedback circuit

I-B

Control of membrane potential:

The accuracy with which the membrane voltage is controlled depends on having sufficiently high gain in the clamping amplifier. This can be seen by considering the schematic voltage clamp circuit of Fig. 6, as discussed by [Moore 1971]. The membrane potential, *V*m, is measured by the voltage follower, which has very high input impedance and so draws negligible input current. The clamping amplifier, of gain A, compares *V*m with the command potential E, and passes current through the access resistance *R*a (which might consist of an electrode and the cytoplasmic resistance) to control *V*m. The output of the clamping amplifier, *V*o, is given by

$$V_O = eA = A(E - V_m)$$

This output is divided between the access resistance and the membrane (for the moment we will assume that *R*s, the series resistance, is zero), so for a current *I*

$$Vo = Vm + RaI$$

Substituting for *Vo* and rearranging gives

$$V_m = E\frac{A}{1+A} - \frac{R_a I}{I+A}$$

Thus, as the gain *A* is increased, the membrane potential approaches the command potential more closely, and the effect of the access resistance is reduced.

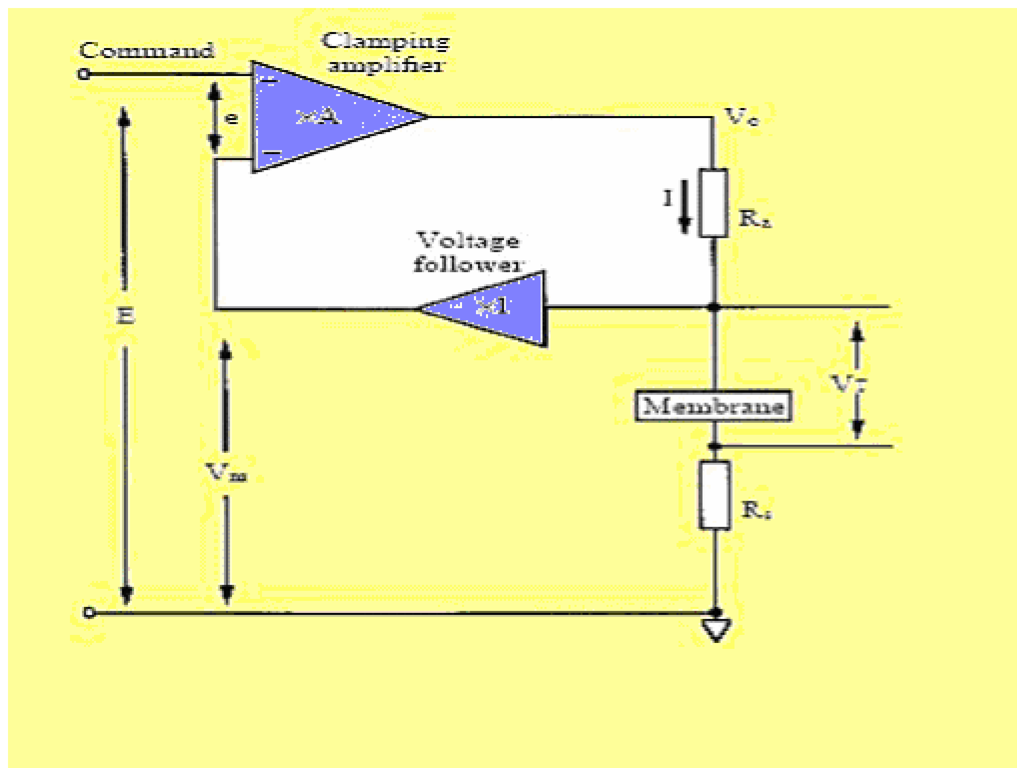
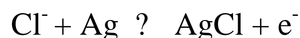


Fig. 6. Simplified schematic voltage clamp circuit [after Moore, 1971]

I-C
Microelectrodes & Micropipettes:

Electrodes convert ionic current in solution into electron current in wires, they are made of materials that can participate in a reversible reaction with one of the ions in the solution .The most frequently used electrod material in electrophysiology is a silver (Ag) wire coated with a composite of Ag and silver-chloride (AgCl). For this electrode, Chloride ions (Cl^-) react with the Ag to produce AgCl plus an electron(e^-), or an electron reacts with AgCl to produce Ag plus Cl^-. Thus, the current carried by chloride ions in the solution is converted into electrons according to the following reversible reaction:



$Cl^- + Ag$? $AgCl + e^-$

The electrical potential at one of these electrodes is equal to the standard electrochemical potential for Ag/AgCl plus RT/F ln(aCl^-), where R is the gas constant (8.314 V C K^{-1} mol^{-1}), T is the absolute temperature on the Kelvin Scale, F is Faraday's constant (9.648 x 10^4 C mol^{-1}), and aCl^- is the activity (i.e.,the effective concentration) of Cl^- in solution near the electrode solution interface.
The potential difference between a pair of these electrodes should be zero if they are immersed in the connected compartments with equal chloride concentrations. These concentrations must be kept constant the course of an experiment or an offset voltage will occure. An offset may be encountered when a Ag/AgCl electrode is used as a bath electrode and the bath Cl^- concentration is changed. To prevent this type of voltage offset, many investigators connect their bath electrode through an agar bridge that maintains a constant cl^- concentration in the immiate vicinity of the Ag/AgCl electrode.
The eletrolyte solution provide the fluide connection from the cell to the electrode. Thus the glass "electrodes" one pull arenot electrodes at all but simple conduits of proper geometry to establish this fluid bridge. In recognition of this, microelectrodes are often referred to as "pipettes" or "micropipettes."
The composition of the pipette solution depend on the type of measurment being made. Standard intracellular microelectrodeses that are used to penetrate the cell membrane must be filled with a highly concentrated electrolyte solution, usually 2-4 M salt. The high electrolyte concentration reduces the electrode resistance. This minimized the rectifying current flow, lowers voltage noise and provides a wider recording bandwidth. The concentrated pipette solution is important because it negates the development of the liquid junction potential, thus preventing an erroneous measurment of the resting potential.
However, there is a disadvantage in using the conentrated filling solution since it can enter the cell and produce a hyperosmotic load that would cause the cell to swell and alter its normal anion and cation content. While pipettes with very small tip diameters can minimize or prevent concentrated solution from entering the cell, they do so at the expense of noise, diminishing current passing ability, and limiting recording bandwidth.These limitations result from the high resistance of small-tip pipettes. [Axon Guide,1993].

I-D
Xenopus oocytes as expression model for heterologous membrane proteins

One of the first and still most widely used assay system for quantifying an authentic protein biosynthetic process is the fully grown oocyte of the South African clawed frog, *Xenopus laevis*. The value of *Xenopus laevis* first became apparent in 1971, when Gurdon and co-workers discovered that the oocyte constitutes an efficient system for translating foreign messenger RNA [Gurdon et al., 1971].
The *Xenopus* oocyte is a cell specialized for the production and storage of proteins for later use during embryogenesis and developmentally divided into 6 stages [Dumont,1972]. In addition, the complex architecture of the frog oocyte includes the subcellular systems involved in the export and import of proteins. Therefore, the mRNA-microinjected oocyte is an appropriate system in which to study the synthesis of specific polypeptides, as well as the storage of particular proteins in various subcellular organelles and the export of others into the extracellular space. Moreover, the subcellular compartmentalization, as well as the structure and biochemical, physiological, and biological properties of the synthesized protein, may be examined from exogenous proteins in the injected oocyte [reviewed in Wagner et al., 2000].

INTRODUCTION

For experimental studies oocytes of stages V-VI are used with a diameter of some 1.3 mm allowing easy preparation. The developmental stages V-VI are characterized by the occurence of 2 poles i.e. the vegetable (light) and the animal (dark) poles. While the nucleus resides in the animal pole [Nieuwkoop, 1977], more mRNA is present in the vegetable pole [Capco and Jeffery, 1982]. The main ion conductance in *Xenopus* oocytes is a Ca^{2+}-dependent Cl conductance governing the resting membrane potential close to the Cl^- reversal potential of -40 mV, [Dascal, 1987].
The primary advantage of using *Xenopus* oocytes for the expression of transporters is the ability to perform detailed electrophysiological recording using an *in vivo* system. In the simplest arrangement, the membrane is penetrated with a single microelectrode and the membrane potential is measured. The oocyte can easily be penetrated with two microelectrodes. This arrangement allows the use of one of the two classical methods: current clamp or voltage clamp. Most electrophysiological studies on oocytes were performed using the two-electrode voltage-clamp. The large size of the oocytes also permits extracellular recording of currents flowing through the cell membrane at various locations using a vibrating probe. The patch clamp method has been successfully applied in devitellinized oocytes for the study of single channels [Hamill et al., 1981].
Whole-cell voltage clamping of oocytes involves two electrodes inserted into the oocyte. The large size of the oocyte (about 1 mm in diameter and 0.5 to 1 µl in volume for stage V-VI oocytes) make this feasible, and is both the major advantage and disadvantage of the system. The advantage is that it is possible to insert multiple electrodes and injection needles into the same oocyte. Therefore, modulators of channel function can be injected inside the cell while recording, so that a rapid and direct response to an intracellular signal can be observed. The disadvantage is that the large size results in an extremely large membrane capacitance (about 150-200 nF), which causes a slow clamp setting time following voltage shifts. This makes it difficult to obtain any data during the first 1 to 2 msec of a hyper- or depolarization, the time during which rapidly activating voltage sensitive channels such as the cardiac sodium channel open. The large capacitance is not a serious problem in examining slow responses or ligand-gated responses in the absence of voltage shifts [Stuhmer, 1992]. Despite their advantages, several precautions should be taken into consideration.
Firstly, the expression of endogenous carriers may interfere with the exogenously expressed proteins in various ways. For instance, it has been observed that injection of heterologous membrane proteins at high level can induce endogenous channels. [Tzounopoulos et al.,1995].
Secondly, due to the fact that Xenopus laevis is a poikilothermic animal, its oocytes are best kept at lower temprature & most experiments are carried out at room teperature. Hence, tempreture sensitive processes i.e. protein trafficking or kinethics may be altered.
Finally, since Xenopus oocytes may have different signaling pathways, precatuion should be taken when studying the regulation of expressed proteins. It has been revealed that the PTH receptor regulates the internalization of NaPi, mediated by the PKA & PKC pathway. However, in Napi-3 expressing Xenopus oocytes PKC-mediated PTH regulation can not be observed [Wagner et al.,1996]. Instead, coupling to the PKA pathway leads to the alteration of PKA-regulated ion channels [Waldegger et al.,1996].

In summary, the *Xenopus* oocyte system has the advantage that channels, receptors and transporters can rapidly be expressed and analyzed both biochemically and electrophysiologically in an *in vivo* situation. The system can be used quite effectively as an assay for the functional cloning of channels that have only been identified by their electrophysiological properties. Once cDNA clones have been isolated, oocytes are an excellent system for correlating structure with function using a combination of molecular biological and electrophysiological techniques.

I-E
Role of foreign RNA:

Foreign RNA injected into *Xenopus* oocytes can be translated into proteins [(Gurdon et al., 1971] and since the first use of oocytes for the expression of receptors and ion channels [Gundersen et al., 1983; Miledi et al.,1983], oocytes from *Xenopus laevis* have become a popular expression system for ion channels, receptors, and transporters. Ion channels expressed in oocytes can be electrophysiologically investigated by the voltage clamp technique [Marmont, 1949; Cole, 1949; Hodgkin et al., 1949]. For oocytes the two-microelectrode voltage clamp is the simplest approach for whole-cell recordings [Stühmer and Parekh, 1995]. The principle is depicted in Fig. 7.

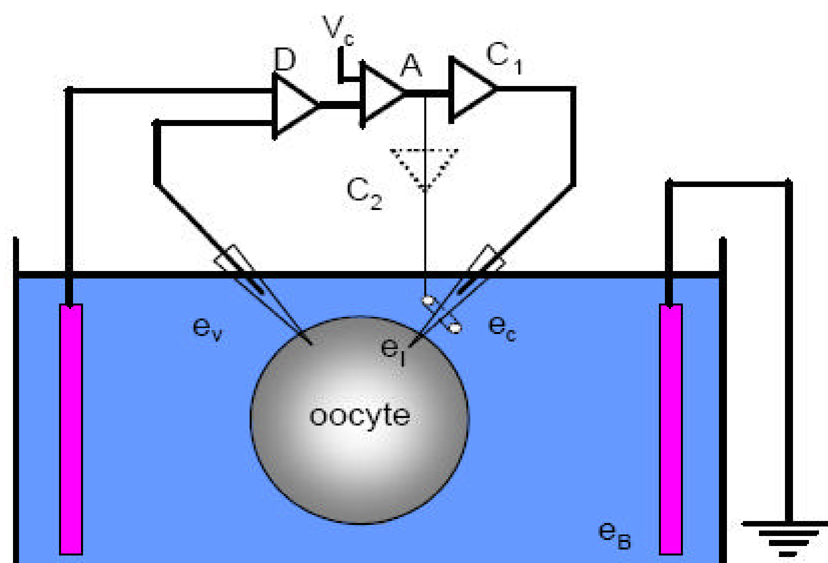


Fig. 7: The two-electrode voltage-clamp (adapted from Baumgartner et al., 1999). The voltage recording electrode eV monitors the membrane potential; this is compared with a command voltage Vc, and the magnified difference is applied to a current injection electrode, eI. A bath electrode eB serves as the return path for the injected current

The membrane of the oocyte is penetrated by two microelectrodes, one for voltage sensing and one for current injection. The membrane potential as measured by the voltage-sensing electrode is compared with a command voltage, and the difference is brought to zero by a control amplifier. The injected current is monitored to provide a measure of the total membrane current.

I-F
Transporters :

are the gatekeepers for all cells and organelles, controlling uptake and efflux of crucial compounds such as sugars, amino acids, nucleotides, inorganic ions and drugs. Transporters can be divided into passive and active transporters (Fig. 8). Passive transporters, also known as facilitated transporters, Allow passage of solutes (e.g., glucose, amino acids, urea) across membranes down their electrochemical gradients.

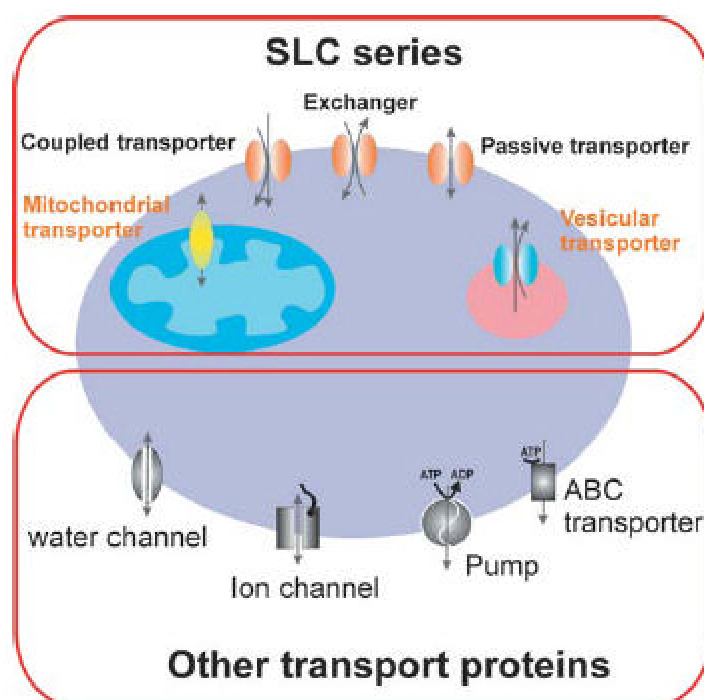


Fig. 8: Cartoon showing a cell with solute carrier (SLC)- and non-SLC-transporters expressed in the plasma membrane or in intracellular compartment membranes. Note that the non-SLC transporters can also be expressed in intracellular compartments

Active transporters create ion/solute gradients across membranes, utilizing diverse energy-coupling mechanisms. These active transporters are classified as primary- or secondary-active transporters according to the directness of coupling to cellular energy (e.g., ATP hydrolysis). Ion pumps hydrolyze ATP to pump ions such as Na^+, K^+, H^+, Ca^{2+} and Cu^{2+} out of cells or into organelles [Cox DW, Moore SD. 2002; Dunbar LA, Capllan MJ. 2000; Muller V, Gruber G. 2003]. These pumps also generate and maintain electrochemical ion gradients across membranes, and thus are called active transporters. Such ion gradients are used in turn by secondary-active, ion-coupled transporters to drive uphill transport of nutrients across biological membranes.

I-F. 1 SLC (Solute Carrier) series:

The SLC series includes genes encoding passive transporters, ion coupled transporters and exchangers (see Fig. 8). A transporter has been assigned to a specific SLC family if it has at least 20–25% amino acid sequence identity to other members of that family. The list of

currently approved SLC human gene symbols is shown in Table 1. The table comprises 43 different SLC transporter families of the SLC series and the number of members in each family.

Table 1 List of currently approved solute carrier (SLC) families. The total numbers of members in each family are shown on the right

	The HUGO Solute Carrier Family Series	Total
SLC1	The high-affinity glutamate and neutral amino acid transporter family	7
SLC2	The facilitative GLUT transporter family	14
SLC3	The heavy subunits of the heteromeric amino acid transporters	2
SLC4	The bicarbonate transporter family	10
SLC5	The sodium glucose cotransporter family	8
SLC6	The sodium-and chloride-dependent neurotransmitter transporter family	16
SLC7	The cationic amino acid transporter/glycoprotein-associated amino-acid transporter family	14
SLC8	TheNa^+/Ca^{2+} exchanger family	3
SLC9	The Na^+/H^+ exchanger family	8
SLC10	The sodium bile salt cotransport family	6
SLC11	The proton coupled metal ion transporter family	2
SLC12	The electroneutral cation-Cl cotransporter family	9
SLC13	The human Na^+-sulfate/carboxylate cotransporter family	5
SLC14	The urea transporter family	2
SLC15	The proton oligopeptide cotransporter family	4
SLC16	The monocarboxylate transporter family	14
SLC17	The vesicular glutamate transporter family	8

SLC18	The vesicular amine transporter family	3
SLC19	The folate/thiamine transporter family	3
SLC20	The type-III Na^+ -phosphate cotransporter family	2
SLC21/ SLCO	The organic anion transporting family	11
SLC22	The organic cation/anion/zwitterion transporter family	18
SLC23	The Na^+ -dependent ascorbic acid transporter family	4
SLC24	The Na^+/(Ca^{2+} - K^+) exchanger family	5
SLC25	The mitochondrial carrier family	27
SLC26	The multifunctional anion exchanger family	
SLC 27	The fatty acid transport protein family	6
SLC28	The Na^+-coupled nucleoside transport family	3
SLC29	The facilitative nucleoside transporter family	4
SLC30	The zinc efflux family	6
SLC31	The copper transporter family	2
SLC32	The vesicular inhibitory amino acid transporter family	1
SLC33	The acetyl-CoA transporter family	3
SLC34	The type-II Na^+ -phosphate cotransporter family	3
SLC35	The nucleoside-sugar transporter family	17
SLC36	The proton-coupled amino acid transporter family	4
SLC37	The sugar-phosphate/phosphate exchanger family	4

SLC38	The System A and N, sodium-coupled neutral amino acid transporter family	6
SLC39	The metal ion transporter family	14
SLC40	The basolateral iron transporter family	1
SLC41	The MgtE-like magnesium transporter family	3
SLC42	The Rh ammonium transporter family (pending)	3
SLC43	The Na^+-independent, system-L-like amino acid transporter family	2
Total		298

In general the genes are named using the root symbol SLC, followed by a numeral (e.g., SLC1, solute carrier family 1), the letter A (which acts as a divider between the numerals) and finally the number of the individual transporter (e.g., SLC3A1). These general rules of SLC gene nomenclature have been elaborated further for a couple of families. Family SLC35, the letter between SLC35 and the family member number has been exploited to specify specific subfamilies, called A, B, C, D and E [Ishida N., Kawakita M., 2003].
A least 5% (>2,000) of all human genes are transporter-related, consistent with the biological significance of transporters and their roles in cell homeostasis. The SLC families represent a considerable portion of these genes: about 300 different SLC human transporter genes exist. [Hediger, Mathias A., et al., 2003].

I-F. 2 Creatine Transporter CreaT (SLC6A8):

The creatine transporter CreaT (SLC6A8) is an electrogenic transporter belonging to the superfamily of Na^+, Cl^- coupled transporters accomplishing the transport of various neurotransmitters (e.g., dopamine, GABA, serotonin, and norepinephrine) and amino acids (e.g., glycine) [Dodd, J.R., Christie. D.L., 2001; Sora, I., et al., 1994;. Nash, S.R., et al. 1994]. It is most closely related to the cell volume sensitive transporters for the organic osmolytes betaine [Takenaka, M., et al., 1995] and taurine [Uchida, S., et al., 1992]. DNA sequencing approaches have shown that Cr transporters are composed of 611–636 amino acid residues. All Cr transporters lack a hydrophobic NH2-terminal signal sequence and display 12 putative transmembrane domains like other members of the Na^+-dependent neurotransmitter transporter family. Consequently, the NH2 and COOH termini of the polypeptide chain are probably both directed toward the cytosol.

By classical biochemical means, a saturable uptake mechanism for Cr was identified in rat and mouse skeletal muscle [Daly et al.,1980; Fitch et al.,1966; Odoom et al.,1996], human fibroblasts, human uterine and calf aortic smooth muscle cells [Daly, et al.,1980], human red blood cells [Syllm et al., 1985], In addition to the saturable component of Cr uptake, kinetic analysis often revealed a second component, displaying a *K*m for Cr of =1.3 mM or being not saturable [Daly, et al.,1980, Loike et al., 1985; Syllm et al., 1985]. In the light of a Cr concentration in the serum of 25–50 mM [Delanghe et al., 1989], this latter component seems to be irrelevant for Cr uptake in vivo and may represent passive diffusion of Cr across the plasma membrane.
Creatine taken up by SLC6A8 can reversibly bind phosphate and replenish ATP at short term energy depletion [Speer, O., et al., 2004]. The BBB supplies creatine to the brain for an energy storing system, and creatine transporter localized at the brain capillary endothelial cells (BCECs) is involved in BBB creatine transport. The creatine transport at the BBB is saturated by plasma creatine, since the plasma level of creatine is 50-100 m M in the human and 200m M in the mouse. In the human brain, the creatine level is about 180-fold higher than that in plasma.
A genetic defect of SLC6A8 leads to mental retardation with seizures [Hahn, K.A., et al., 2002]. Moreover, decreasing SLC6A8 abundance has been observed in the failing heart [Neubauer, S., et al., 1999]. SLC6A8 activity was found to be inhibited by cyclosporine A [Tran, T.T. et al., 2000] and regulated by extracellular and cytosolic creatine levels [Loike, J.D., et al., 1988]. Moreover, the expression of the creatine transporter is increased by growth hormone [Omerovic, E., et al., 2003].
The creatine transporter has been suggested to be phosphorylated by Src [Wang, W., et al., 2002]. In a thorough investigation of the Cr transporter activity in cultured mouse G8 myoblasts,. [Odoom JE, et al., 1996] showed that Cr uptake is stimulated by isoproterenol, norepinephrine, but not by the a_1-adrenergic receptor agonist methoxamine. The Cr transporter activity may be controlled predominantly by $ß_2$-adrenergic receptors that have cAMP as their intracellular signal.
The highest amounts of Cr transporter mRNA seem to be expressed in kidney, heart, and skeletal muscle; somewhat lower amounts in brain, small and large intestine, vas deferens, seminal vesicles, epididymis, testis, ovary, oviduct, uterus, prostate, and adrenal gland; and only very low amounts or no Cr transporter mRNA at all in placenta, liver, lung, spleen, pancreas, and thymus.
In fact, analysis of the Cr transporter cDNA sequence revealed consensus phosphorylation sites for cAMP-dependent protein kinase (PKA) and for protein kinase C (PKC) (Nash SR., et al., 1994; Sora I., et al, 1994].
Further mechanisms mediating the regulation of SLC6A8 activity have, however, remained elusive. Among the candidate signaling molecules involved in the regulation of SLC6A8 is the serum and glucocorticoid inducible kinase SGK1. The kinase was originally cloned as a glucocorticoid sensitive gene from rat tumor cells [Firestone, G.L., et al., 2003], and later as a human cell volume sensitive gene upregulated by osmotic and isotonic cell shrinkage [Waldegger, S., et al., 1997].

I-F. 3 Sodium-Phosphate Cotransporter NaPi-2b (SLC34A2):

Members of this family were originally classified as the type II Na^+/Pi cotransporters based on their molecular homology and similarity of functional properties [for review see Murer and Biber, 1996; Murer et al., 2000].

More recently, this family has been subclassified into type IIa and type IIb [Hilfiker et al., 1998] based on the presence of unique structural motifs in the C-terminal of the protein.
Type IIa are found exclusively in the mammalian renal cortex, whereas type IIb are ubiquitously distributed, but not expressed in the mammalian kidney. In non-mammalian species (e.g., flounder), type IIb is expressed in both kidney and small intestine [Werner et al., 1994; Kohl et al., 1996].
The Na^+-coupled electrogenic phosphate transporter NaPi IIb (SLC34A2) accomplishes the intestinal absorption of phosphate [Werner, A., Kinne, R.K., 2001]. Moreover, it is expressed in further epithelia such as epididymis [Xu, Y., et al., 2003] and liver [Frei, P., et al., 2005]. SLC34A2 transport activity is critically important for the maintenance of phosphate balance and thus bone mineralization and energy metabolism.
Accordingly, SLC34A2 activity is under tight hormonal control. Intestinal phosphate transport is stimulated by 1,25 $(OH)_2$ D_3 [Glorieu, F.H., et al., 1980], glucocorticoids [Yeh, J.K., J.F. Aloia, 1987], estrogens [Xu, H., et al., 2003], insulin like growth factor IGF1 [Veldman, C.M., et al., 1997] and epidermal growth factor [Xu, H., et al., 2005]. It is inhibited by fibroblast growth factor 23 [Miyamoto, K., et al., 2005].
Moreover, its expression and/or activity is influenced by phosphate depletion [Capuano, P. , et al., 2005], and acid base balance [Stauber, A., et al., 2005]. The signaling of SLC34A2 regulation is, however, still incompletely understood. Recent observations disclosed the ability of the serum and glucocorticoid inducible kinase SGK1 to stimulate SLC34A2 [Palmada, M., et al., 2004].
Most recent experiments indicated that the SGK1 dependent regulation of the creatine transporter SLC6A8 [Shojaiefard, M., et al., 2005] involves the mammalian target of rapamycin (mTOR), another transport regulating kinase [Shojaiefard, M., et al., 2006]. Thus, mTOR could similarly be considered a candidate kinase in the regulation of phosphate transport. mTOR is stimulated by growth factors [Martin, D.E., et al., 2005], insulin [Ueno, M., et al., 2005] and leptin [Mezey, E., et al., 2005]. mTOR plays a pivotal role in the regulation of cell proliferation and cell growth [Martin D.E., et al., 2005]. The kinase is inhibited during hypoxia [Wouters, B.G., et al., 2005]. mTOR stimulates cellular amino acid uptake [Peyrollier, K., et al., 2000] and protein synthesis [Wouters, B.G., et al., 2005]. mTOR inhibition leads to cell shrinkage [Fumarola, C., et al., 2005], and compromises energy metabolism, lactate production and formation of reactive oxygen species [Christians, U., 2004]. The kinase influences apoptosis [Yu C., et al., 2005] and is a pharmacological target in the treatment of cancer [Easton, J.B., et al., 2004] and diabetes [Ueno, M., et al., 2005].
With respect to basic transport properties, the turnover rate of the type II system is a function of the concentration of the two substrates and membrane potential. Under physiological conditions, Pi can exist in both monovalent and divalent forms in the physiological pH range p*K*a [$HPO_3{}^{2-}$/$HPO_4{}^{-}$] ~ 6.8). Therefore the observed pH-dependence of Pi reabsorption could be attributed to a Pi titration effect and/or an interaction of protons with the type II cotransporter. However, it has been experimentally difficult to identify which, if any, Pi species is preferentially transported and to unequivocally demonstrate interactions of protons with the cotransporter itself.

I-G Substrates:

I-G. 1 Creatine (Cr)

Cr (from greek *kreas*, flesh). In recent years, a series of fascinating new discoveries have been made. For instance, Cr analogs have proven to be potent anticancer agents that act synergistically with currently used chemotherapeutics. Cyclocreatine, one of the Cr analogs, as well as phosphorylcreatine (PCr) protect tissues from ischemic damage and may therefore have an impact on organ transplantation. Circumstantial evidence suggests a link between disturbances in Cr metabolism and muscle diseases as well as neurological disorders, and beneficial effects of oral Cr supplementation in such diseases have in fact been reported. Oral Cr ingestion has also been shown to increase athletic performance, and it therefore comes as no surprise that Cr is currently used by many athletes as a performance-boosting supplement. Some data suggest that Cr and creatinine (Crn) may act as precursors of food mutagens and uremic toxins. Finally, the recent identification, purification, and cloning of many of the enzymes involved in Cr metabolism have just opened the door to awide variety of biochemical, physiological, as well as clinical investigations and applications. [Wyss Markus & Kaddurah-Daouk, 2000].

An important aspect of Cr biosynthesis to add is that in humans, the daily utilization of methyl groups in the GAMT reaction approximately equals the daily intake of "labile" methyl groups (Met + choline) on a normal, equilibrated diet [Mudd SH., et al., 1980].

The highest levels of Cr and PCr are found in skeletal muscle, heart, spermatozoa, and photoreceptor cells of the retina. Intermediate levels are found in brain, brown adipose tissue, intestine, seminal vesicles, seminal vesicle fluid, endothelial cells, and macrophages, and only low levels are found in lung, spleen, kidney, liver, white adipose tissue, blood cells, and serum [Berlet HH., et al., 1976; Christensen M., et al., 1994; Delanghe, J., et al., 1989; Kushmerick MJ., et al., 1992.; Lee HJ., et al., 1988 ; Loike JD., et al., 1992; Loike JD., et al., 1986; Navon G., et al., 1985; Peters JP., and Van Slyke DD., 1946; Wallimann T., et al., 1986a; Wallimann T., et al., 1986b; Indischbauer A., et al., 1994; Yanokura M., and Tsukada K., 1982; Windischbauer A., 1982].

Phosphorylcreatine (PCr) and Cr, relative to ATP and ADP, are smaller and less negatively charged molecules and can build up to much higher concentrations in most CK-containing cells and tissues, thereby allowing for a higher intracellular flux of high-energy phosphates. The main route of Cr biosynthesis in mammals involves formation of guanidinoacetate in the kidney, its transport through the blood, and its methylation to Cr in the liver (Fig. 9). Cr exported from the liver and transported through the blood may then be taken up by the CrT requiring tissues.

Cr uptake into CK-containing tissues, e.g., skeletal muscle, heart, brain, or kidney, is effected by a specific, saturable, Na^+- and Cl^- -dependent Cr transporter. The expression and/or specific activity of the Cr transporter seems to be influenced by dietary and hormonal factors.

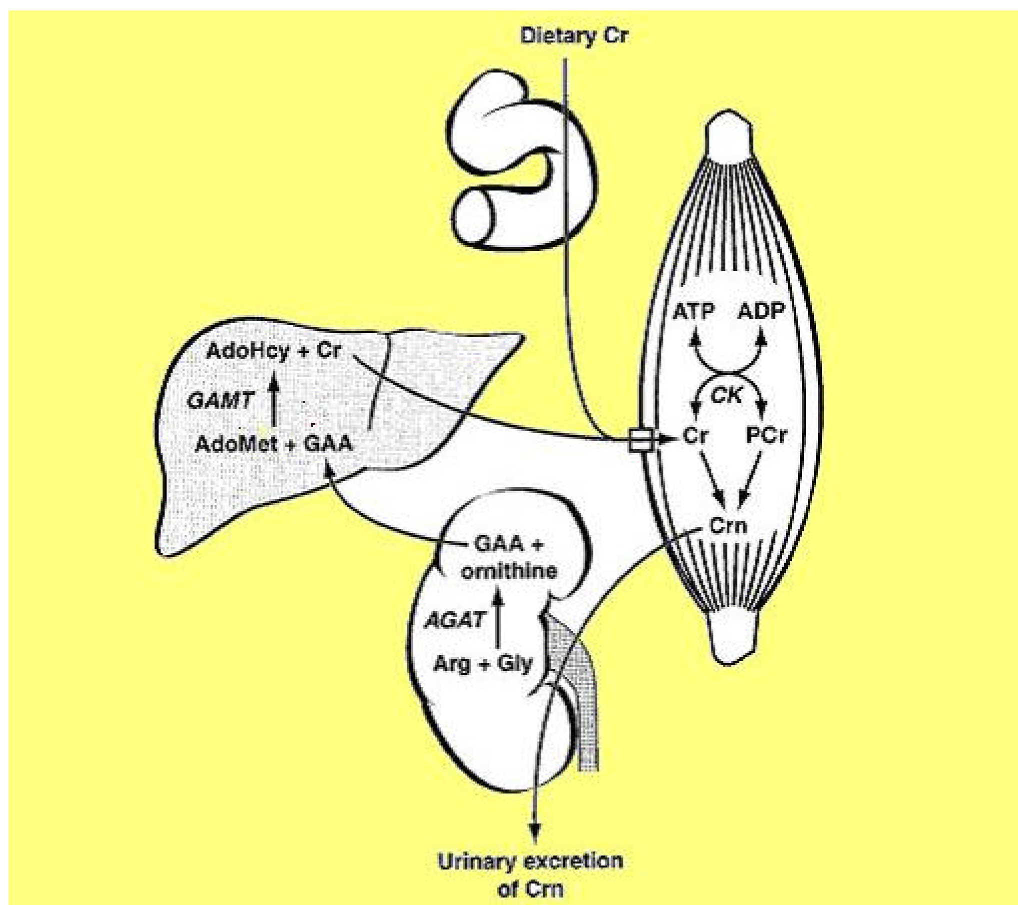


FIG. 9: Major routes of Cr metabolism in the mammalian body.
The most part (up to 94%) of Cr is found in muscular tissues. Because muscle has virtually no Cr-synthesizing capacity, Cr has to be taken up from the blood against a large concentration gradient by a saturable, Na1- and Cl2-dependent Cr transporter that spans the plasma membrane (h). The daily demand for Cr is met either by intestinal absorption of dietary Cr or by de novo Cr biosynthesis. The first step of Cr biosynthesis probably occurs mainly in the kidney, whereas the liver is likely to be the principal organ accomplishing the subsequent methylation of guanidinoacetic acid (GAA) to Cr .It must be stressed that the detailed contribution of different bodily tissues (pancreas, kidney, liver, testis) to total Cr synthesis is still rather unclear and may vary between species (see text). The muscular Cr and PCr are nonenzymatically converted at an almost steady rate (~ 20% total Cr per day) to creatinine (Crn), which diffuses out of the cells and is excreted by the kidneys into the urine.

Na^+-dependent Cr uptake is decreased by extracellular [Cr] >1 µM, with 50% inhibition being observed at 20–30 µM, i.e., in the range of the physiological plasma concentration of Cr. In media containing 5 mM Cr, transport of Cr is decreased by 50% within 3–6 h, and maximal inhibition (70–80%) is observed within 24 h. Upregulation of Cr transport upon withdrawal of extracellular Cr seems to occur more slowly.
Excessive concentrations (5 mM) of guanidinoacetate and GPA also reduce Cr transport significantly, whereas D- and L-ornithine, Crn, Gly, and PCr are ineffective. Because the downregulation of the Cr transporter activity by extracellular Cr is slowed by cycloheximide, an inhibitor of protein synthesis, it has been hypothesized that Cr transport, like Na^+ -dependent system A amino acid transport [Handlogten ME., and Kilberg MS., 1984], is

controlled by regulatory proteins. However, no conclusive evidence for or against this hypothesis is currently available. It also remains to be clarified how extracellular [Cr] is transformed into an intracellular signal.
Insulin increases Na^+-K^+-ATPase activity which, indirectly, may stimulate Cr transporter activity. it seems noteworthy that guanidinoacetate, and to a lower extent Arg and Cr, were seen to stimulate insulin secretion in the isolated perfused rat pancreas [Alsever RN., 1970]. As to be expected from the Na^+ dependence of the Cr transporter, Cr uptake is diminished in deenergized cells and is also depressed by the Na^+-K^+-ATPase inhibitors ouabain and digoxin [Bennett SE., et al., 1994; Gonda O., and Quastel JH., 1962; Ku C-p., and Passow H., 1980; Loike JD., et al., 1986; Odoom JE., et al., 1996].

Finally, in erythrocytes from uremic patients, the Na^+-dependent component of Cr influx is 3.3 times higher than in normal human erythrocytes. This finding may be due, by analogy, to the known occurrence of inhibitors of Na^+-K^+-ATPase in uremic plasma [Stokes GS., et al., 1990; Tao Q-F., et al., 1996]. Obviously, cells may respond to decreased Na^+-K^+-ATPase activity, which in turn likely decreases Cr transporter activity, by compensatory upregulation of Na^+-K^+-ATPase [Hosoi R., 997] and/or Cr transporter expression.

I-G. 2 Sodium-Phosphate:

The reabsorption of inorganic phosphate (Pi) from the glomerular filtrate occurs principally along the renal proximal tubule and is an essential means of controlling phosphate homeostasis. Up to 80% of Pi resorption is mediated by a secondary-active, electrogenic Na^+coupled cotransport system located in the proximal tubule apical brush border membrane [for review see Murer et al., 1999]. This system was identified through expression cloning [Magnanin et al.,1993] and comprises a unique family of proteins expressed in a number of mammalian and non-mammalian species.

from recent voltage clamp studies of renal type IIa Na^+ /Pi cotransporters heterologously expressed in *Xenopus laevis* oocytes, [Hartmann et al., 1995] found that lowering external pH shifted the apparent affinity for Na^+ ($Km_{Na}{}^+$). Moreover, in the pH range 7.4–6.8, preferential transport of divalent Pi has been recently confirmed by simultaneous electrophysiological recording and tracer flux measurements on two type II Na^+/Pi cotransporter isoforms [Forster et al., 1999a].

I-H Protein Kinases:

I-H. 1 SGK & Isomers:

Webster and co-workers (1993) were the first to observe an increase in mRNA encoding a kinase in a rat mammary tumor cell line after 30 min serum and glucocorticoid treatment. This kinase was then named serum and glucocorticoid induced kinase (SGK). SGK belongs to the serine/threonine kinase gene family. Its catalytic domain has significant sequence homology (45-55% identity) with rac protein kinase, the PKC family, ribosomal protein S6 kinase, cyclic AMP dependent protein kinase A (PKA) (Webster et al., 1993a and b) as well as protein kinase B (Kobayashi et al., 1999a).

Human SGK was subsequently identified in a human hepatoma cell line which is regulated by cell shrinkage (Waldegger et al., 1997) through p38/mitogen activated protein kinase (MAP) kinase (Waldegger et al., 2000b). The human isoform has been cloned as a cell volume-sensitive gene up-regulated by both hypertonic and isotonic cell shrinkage [Bell LM., et al., 2000; Waldegger S., et al., 1997; Waldegger S., et al., 2000] Since the discovery of the two isoforms sgk2 and sgk3 [Kobayashi T., et al., 1999] the originally cloned kinase has been labelled sgk1. SGK3 is also known as cytokine-independent survival kinase (CISK) since it is involved in cell survival through its Phox homology (PX) domain (Xu et al., 2001). SGK1 shares 80% homology with its isoforms SGK2 and SGK3 [Kobayashi T., et al., 1999a] and 60% homology with the protein kinase B [Alessi DR., et al., 1996]. Despite the high sequence similarity between all types of SGK, several differences are apparently present. SGK2 is only present at significant levels in liver, kidney and pancreas and only at lower levels in the brain, whereas SGK1 and SGK3 are ubiquitously expressed in all tissues examined. Furthermore, SGK1 is to a greater extent regulated by insulin-like growth factor 1 (IGF-1) whereas activation of SGK2 and SGK3 by H_2O_2 are only partially inhibited by inhibitors of phosphatidylinositide 3-kinase (PI3 -kinase) (Kobayashi et al., 1999a).
Besides glucocorticoids [Brennan FE., et al., 2000; Webster MK., et al., 1993a; Webster MK.,1993b] mineralocorticoids [Brennan FE., et al., 2000*6*; Chen SY., et al., 1999; Cowling RT., Birnboim HC., 2000; Naray-Fejes-Toth A., Fejes-Toth G., 2000; Naray-Fejes-Toth A,1999; Pearce D., et al., 2000; Shigaev A., et al., 2000; Verrey F., et al., 2000] also up-regulate sgk1 transcription strongly. The up-regulation by aldosterone and the expression of sgk1 in renal distal tubule and collecting duct [Lang F., et al., 2000; Loffing J., et al., 2001] implies a role for sgk1 in the regulation of renal epithelial Na^+ transport. Co-expression of sgk1 with the epithelial Na^+ channel (ENaC) in Xenopus oocytes indeed markedly up-regulates Na^+ channel activity [Alvarez de la Rosa D., et al., 1999; Böhmer C., et al., 2000; Lang F., et al., 2000; Naray-Fejes-Toth A., et al., 1999; Wagner CA., 2001].
The activation of SGK1, SGK2 and SGK3 is mediated through PI3-kinase and 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PDK2 (Kobayashi and Cohen, 1999b; Park et al., 1999).
SGK1 is involved in many physiological and pathological processes such as membrane transport (Wagner et al., 2001; Wang et al., 2001; Gamper et al., 2002a, b), cell growth and survival (Xu et al.,2001). In addition, SGK has been shown to facilitate memory consolidation of spatial learning in rat (Tsai et al.,2002).Cerebral expression of SGK1 is increased by ischemia [Nishida, Y., Waldegger, S., et al., 2004]. Cerebral SGK1 expression has further been shown to be enhanced by enriched environment [Lee, E.H., Waldegger, S., et al., 2003] and was correlated with learning abilities of rats [Tsai, K.J. et al., 2002]. Moderate concentrations of glucocorticoid enhance memory consolidation in a variety of learning tasks in animals. *Sgk* is known as a primary glucocorticoid-induced gene. These results bridge the relationships among glucocorticoid, *sgk* expression, and memory consolidation.

I-H. 2 protein kinase B(PKB/Akt)

Protein kinase B is the prototype of a family of kinases that includes three known members, Akt-1/PKBa, Akt-2/PKBß and Akt-3/PKB? [Jones et al., 1991; Coffer and Woodget, 1991]. All three isoforms SGK1-3 and protein kinase B are activated via a signaling cascade involving phosphatidylinositol-3 kinase and phosphoinositide dependent kinase PDK1 [Kobayashi T., et al., 1999a; Alessi DR., et al., 1996; Kobayashi T., et al., 1999b; Park J., et al.,1999] The activation of SGK1 requires phosphorylation at Ser422, the activation of PKB phosphorylation at Thr308 and Ser473.

Replacement of those amino acids by aspartate leads to the respective constitutively active kinases, ^{S422D}SGK1 [Kobayashi T., et al., 1999a] and ^{T308D,S473D}PKB [Alessi DR., et al., 1996]. Destruction of the catalytic subunit by replacement of the lysine at position 127 with asparagine leads to the inactive mutant ^{K127N}SGK1 [Kobayashi T., et al., 1999a].
PKB has been known to be a key mediator of the insulin signaling pathway. Overexpression of PKB not only leads to an increased glycogen and protein synthesis but also to an enhancement of glucose and amino acids uptake (Barthel et al., 1997; Hajduch et al., 1998). Similar to SGK, PKB is capable of phosphorylating several proteins containing certain sequence motifs, for instance glycogen synthase kinase 3 beta (GSK3b), B-RAF and the fork head transcription factor 1 (FKHRL1). (Barthel et al., 1997, 1999; Hajduch et al., 1998).

I-H. 3 Neuronal cell expressed developmentally downregulated 4-2(Nedd4-2).

Nedd4 (neuronal precursor cell-expressed, developmentally down-regulated 4) was identified originally as a developmentally regulated mouse gene highly expressed in early embryonic central nervous system [Kumar et al., 1992]. Further analysis revealed that the expression of Nedd4 is not restricted to the embryonic central nervous system and that it is expressed at varying levels in different tissues including lung, kidney, and colon [Staub et al., 1996, 1997; Kumar et al., 1997]. At the time that Nedd4 was first cloned in 1992, the only identifiable structure in the protein was a Ca^{2+}/lipid-binding (CaLB) domain (C2 domain) and three to four repeats of approximately 40 amino acids. These repeats are now known as WW domains [Sudol, 1996].The Ca^{2+} /lipid-binding (CaLB) domain (C2 domain) is located towards the amino-terminus of Nedd4. The C2 domain was first identified in protein kinase C and is responsible for Ca^{2+}-dependent binding of membrane phospholipids [Knopf et al., 1986]. The function of the C2 domain in the Nedd4 protein is not well understood, but it might mediate the redistribution of Nedd4 from the cytosol to the plasma membrane in response to fluctuations in intracellular Ca2+ concentration [Plant et al., 1997].The ubiquitin-protein ligase domain is situated at the COOH-termini of the Nedd4 protein. It is a large domain (approximately 350 residues) and was first characterized in the human ubiquitin-protein ligase E6-AP, and hence is often referred to as the HECT (homologous to E6-AP COOH-terminus) domain [Scheffner et al., 1993, 1995].The characteristic feature of the Nedd4 family of proteins is the organization of the C2, WW and ubiquitin-protein ligase domains. Two closely related Nedd4 isoforms (or paralogues) exist: Nedd4-1 [also named Nedd4, KIAA0093, or RPF1] and Nedd4-2 (also known as KIAA0439, LdI-1, Nedd4La, Nedd18, or Nedd4-L) [Kamynina et al., 2001].Nedd4-1 is composed of one C2 domain, a HECT domain, and three to four WW domains. [Kamynina et al., 2001].Nedd4-2 contains four WW domains and a HECT domain (Fig. 10). Only human and *Xenopus laevis* Nedd4-2 comprise a C2 domain, whereas such a domain appears to be lacking in mouse Nedd4-2. Again, there is evidence for alternative splicing of this isoform as well [Chen et al., 2001; Kamynina et al., 2001], and there may be isoforms that contain, and others that do not contain, a C2 domain.The ubiquitin ligase neuronal cell expressed developmentally downregulated 4-2 (Need4-2) [Debonneville C., E., et al., 2001; Snyder, PM., 2002] ubiquitinates the channel protein, thereby inducing the subsequent clearance of the channel protein from the cell membrane [Debonneville C., E., et al., 2001; Snyder, PM., 2002] Nedd4-2 is phosphorylated and inactivated by the serum- and glucocorticoid-dependent kinase 1 (SGK1).

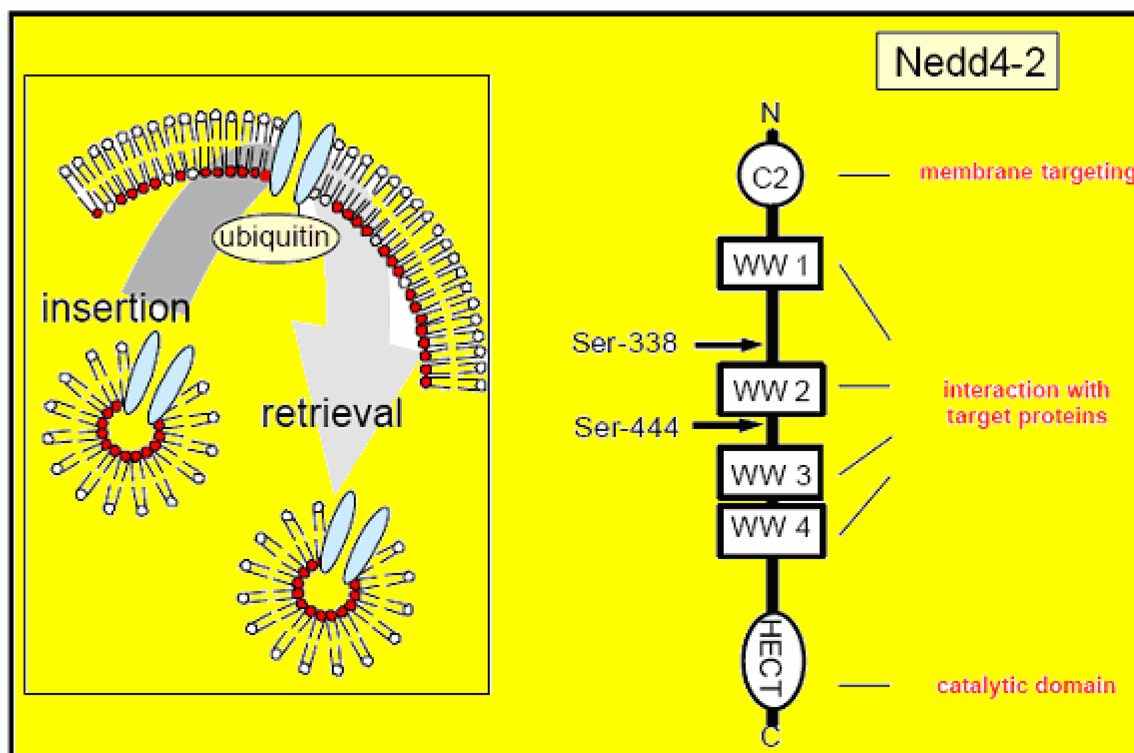


Fig. 10. Domain organization of Nedd4-2 with the consensus phosphorylation sites of SGK1, depicting the C2 domain, four WW domains and the ubiquitin protein ligase HECT domain (right panel). The binding of Nedd4-2 to targets proteins (proline-rich proteins) is shown, resulting in ubiquitination, internalization, andsubsequent degradation of these proteins (left panel). Abbreviations are: C2, Ca2+-dependent lipid binding; WW domains, named after a pair of conserved tryptophans, are highly compact (35-45 residues) modular domains and serve protein-protein interaction; HECT, a domain homologous to the E6-AP-COOHterminal domain is the catalytic portion of this protein.

I-H. 4 Rapamycin ($C_{51}H_{79}NO_{13}$)

Rapamycin (RAP), a lipophilic macrolide, was identified more than twenty years ago during antibiotic screening at Ayerst Research Laboratories. Produced by a strain of *Streptomyces hygroscopicus* isolated from a soil sample obtained from the Vai Atore region of Easter Island (Rapa Nui) [Vezina C., et al., 1975] RAP is a white crystalline solid (m.p. 183–185°C), virtually insoluble in water but readily soluble in ethanol, methanol, dimethylsulfoxide, and other organic solvents [Sehgal SN., et al., 1975].
Although lacking antibacterial activity, RAP is a potent inhibitor of yeast growth and a moderate growth inhibitor of filamentous fungi [Sehgal SN., et al., 1975] The first demonstration of RAP's immunosuppressive activity was obtained from studies showing its inhibitory effects upon production of humoral IgE as well as its preventative effects in two animal models of human autoimmune disease, experimental autoimmune encephalitis and adjuvant arthritis [Martel RR., et al., 1977].

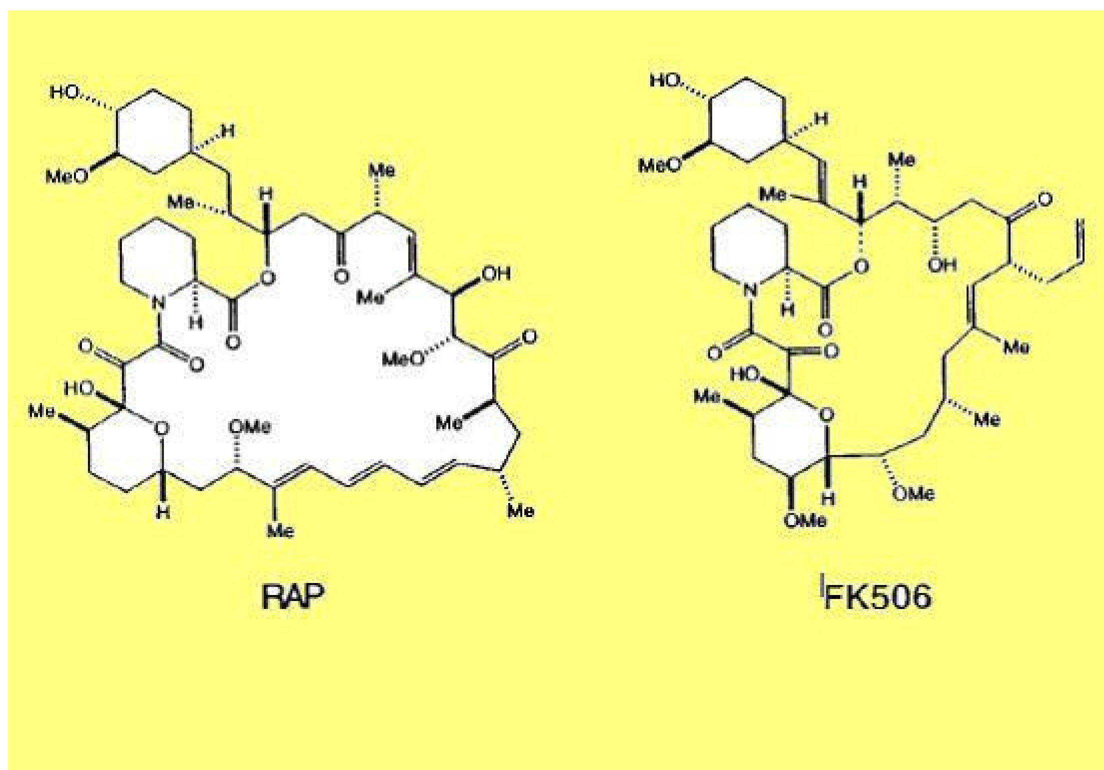


Figure 11: The chemical structures of rapamycin (*left*) and FK506 (*right*).

FK506 ($C_{44}H_{69}NO_{12}$, Figure 11) was identified in a strain of *Streptomyces tsukabaensis* isolated from a soil sample taken from the Tsukuba region of Northern Japan during a screen for natural products that inhibit IL-2 production [Kino R., et al., 1987a]. FK506 was shown to be a powerful immunosuppressive agent as measured by its inhibitory effects in several immune function assays, including alloantigen-induced proliferation of lymphocytes, generation of cytotoxic T lymphocytes, expression of IL-2 receptors, and the production of T lymphocyte–derived lymphokines such as IL-2, IL-3, and IFN-? [KinoT., et al., 1987b]. RAP and FK506 bind to the same family of intracellular receptors, termed FK506 binding proteins (FKBPs). Structural studies have shown that FK506 has two domains—a domain bound by FKBP and an effector domain that, together with FKBP, forms a composite surface that interacts with CaN [Clardy J., et al., 1995; Griffith JP., et al., 1995].
Like FK506, RAP also has two domains—an effector domain forming a composite surface with FKBP that interacts with the mammalian target of RAP, mTOR (see below), as well as a binding domain that mediates the interaction with FKBP. The FKBP-binding domain is conserved in FK506 and RAP, providing a chemical basis for the mutual antagonism exerted by the two molecules in intact cells [Dumont F., et al., 1990a; Bierer B., et al., 1990].
There is no evidence linking the physiological function of the FKBPs to their inhibitory functions in the presence of RAP. FKBP12 and FKBP12.6, which associate with mTOR in the presence of RAP, normally interact with the calcium release channel (CRC)/ryanodine receptors (RyR) of the terminal cisternae of skeletal muscle and heart muscle sarcoplasmic reticulum, respectively, and they are required for proper channel function [Jayaraman T., et al., 1992; Timerman A., et al., 1993; Timerman A., et al., 1995; Brillantes A-M., et al., 1994; Mayrleitner M, et al., 1994].

Although the immunosuppressive activity of RAP is commonly attributed to its antiproliferative effect on lymphoid cells, this drug affects a number of other cellular functions that may contribute to its ability to suppress immune responses in vivo. Studies performed with the murine T cell lymphoma, YAC-1,have shown that RAP inhibits IL-1-stimulated production of interferon (IFN)-? as well as IFN-?-induced expression of Ly6E antigen on the cell surface [Dumont F, et al., 1994a; Altmeyer A, et al., 1993; Dumont F, Kastner C. 1994b]. Both of these actions are antagonized by FK506, suggesting that they are dependent upon the formation of FKBP12 · RAP complexes in YAC-1 cells. Furthermore, YAC-1 somatic mutants selected for resistance to the growth-inhibitory effect of RAP were correspondingly resistant to the suppressive actions of RAP on cytokine-driven production of IFN-? and expression of Ly6E [Dumont FJ, et al., 1994a]. As mentioned previously, the resistant phenotype of these YAC-1 clones is apparently explained by a mutational event(s) that leads to a decreased affinity of mTOR for the FKBP12 · RAP complex.
These results suggest that,in addition to its cell-cycle regulatory function, mTOR participates in the signal transduction pathways that mediate IL-1- and IFN?-stimulated responses in YAC-1 cells.[Abraham Robert T. And Wiederrecht Gregory J 1996].

I-H. 5 The Mammalian Target of Rapamycin (mTOR):

The mammalian target of rapamycin (mTOR) is a kinase coupling cell growth and proliferation to stimulation by, growth factors, nutrient availability, and energy status [Hidayat, S., et al., 2003; Meijer, A.J., et al., 2004; Nakajo, T., et al., 2005; Proud, C.G., et al., 2004; Sarbassov, d.D., et al., 2005; Tokunaga, C., et al., 2006].
mTOR activity is stimulated by growth factors [Sarbassov, d.D., et al., 2005], insulin [Cho, H.J., et al., 2004; Hiratani, K., et al., 2005; Mussig, K., et al., 2005; Ueno, M., et al., 2005], and leptin [Mezey, E., et al., 2005]. The activity of mTOR is inhibited by hypoxia [Wouters, B.G., et al., 2005]. mTOR dependent cellular functions include cellular uptake of amino acids [Liu, X.M., et al., 2005; Peyrollier, K., et al., 2000] and protein synthesis [Wouters B.G., et al , 2005; Ijichi, C., et al., 2003]. Inhibitors of mTOR decrease cell volume [Fumarola, C., et al., 2005], lactate production, energy metabolism, and formation of reactive oxygen species [Christians, U., et al., 2004] and influence the machinery leading to apoptosis [Hamanaka, Y., et al., 2005; Yu, C., et al., 2005].
Thus, inhibitors of mTOR are considered for the treatment of cancer [Houghton, P.J., 2004]and diabetes [Ueno, M., et al., 2005].
The primary target of rapamycin in cells is the FKBP12-rapamycin-associated protein (FRAP), also known as the target of rapamycin (TOR) (Heitman et al., 1991; Brown J.B., Easton, et al. 1994]. Five groups, working independently, identified the mammalian protein target of the FKBP12 _ RAP complex. Homologous high molecular weight proteins were isolated from various mammalian sources including bovine brain (FKBP-RAP-associated protein, FRAP) [Brown EJ., et al., 1994], rat brain (RAP and FKBP12 target, RAFT; mammalian target of RAP, mTOR) [Sabatini DM., et al., 1994; Sabers C., 1994], and human lymphocyte(Sirolimus effector protein, SEP; RAP target, RAPT) [Chen Y., et al., 1994; Chiu MI., et al.,1994]. In keeping with the precedent yeast nomenclature, we refer to the protein as mTOR.

Two observations helped to confirm that mTOR mediates the inhibitory effects of RAP in mammalian cells.
First, two structural analogs of RAP (16-keto-RAP and 25, 26 iso-RAP) were identified that bind with high affinity to FKBP12 but which are 100-fold less potent inhibitors of G1 progression in MG-63 osteosarcoma cells. The FKBP12 complexes with 16-keto-RAP and 25, 26 iso-RAP complexes bind mTOR poorly, if at all [Brown EJ., et al., 1994].
Second, little or no mTOR is bound by the FKBP12 _ RAP complex in extracts prepared from mutant murine T cell (YAC) lines selected for RAP resistance [Sabers C., et al., 1994; Dumont F., et al., 1994]. Extracts prepared from a RAP-sensitive revertant derived from one of the RAP-resistant T cell lines show wild-type levels of mTOR bound to the FKBP12 _ RAP complex [Sabers C., et al., 1994]. Thus, sensitivity of T cells to RAP correlates with binding of mTOR to the FKBP12 _ RAP complex.
The mRNA of mTOR is ubiquitously expressed in human tissues with the highest levels found in testis and significant expression found in skeletal muscle [Brown EJ., et al., 1994; Chiu MI., et al., 1994]. Both human and rat mTOR are slightly more similar to yeast TOR2 (46% identity) than to TOR1 (44% identity), with the greatest similarity (65% identity) found in the C-terminal 600 amino acids of the three proteins. This region of mTOR contains the lipid kinase motif (amino acids 2186–2359) found in the yeast TORs. There are other regions, particularly in the amino terminal portions of the proteins, where there is little or no homology between the yeast TORs and TOR. Thus, although it is unclear whether mTOR is the functional equivalent of yeast TOR1 or TOR2, the high degree of amino acid sequence identity in the lipid kinase domain suggests that these proteins have similar enzymatic activities.
To date, in vitro kinase assays have failed to detect lipid kinase activity in mTOR although a serine autophosphorylation activity has been reported [Zheng X-F., et al., 1995].
Studies of the mechanism of action of RAP are beginning to uncover a previously unrecognized signal transduction pathway that may play a general role in the control of growth of hematopoietic cells (see Figure 12 for summary). The FKBP12 _ RAP complex binds to and inhibits the function of a newly defined target protein termed mTOR. The remarkable degree of sequence identity between mTOR and its yeast homologs TOR1 and TOR2 suggests that the cellcycle regulatory function of mTOR has been highly conserved in eukaryotes. Sequence similarities in the putative catalytic domain of mTOR indicate that the RAP target protein is evolutionarily related to the phosphoinositide kinases PI 3-kinase and PI 4-kinase.
The most recent addition to this expanding family of signal transducers is the product of the human *AT* gene, which is mutated in the autosomal recessive disorder ataxia telangiectasia [Savitsky K., 1995]. Mutations in AT result in neurologic, immunologic, and cell-cycle abnormalities, as well as radiationsensitivity and a predisposition to cancer. These findings hint that members of the family of PI 3-kinase-like enzymes, including mTOR, are involved in a broad range of physiologic processes linked to control of the cell-cycle.
The growth-arrest state induced by RAP in T lymphocytes and other hematopoietic cells suggests that this protein executes a biochemical function(s) required for progression from G1- to S-phase. In spite of the sequence homology to PI 3-kinase, mTOR possesses no detectable kinase activity toward phosphoinositides or other lipid substrates [Zheng X-F., et al ., 1995].

However, recombinant mTOR phosphorylates itself on serine residues [Zheng X-F, et al., 1995], suggesting that mTOR, ATM, and related proteins may constitute a novel family of protein serine-threonine kinases. Although the proximate substrate(s) for mTOR remains obscure, the connection to components of the machinery that control translation of mRNA is becoming increasingly compelling. The next few years should see some exciting advances in our understanding of the functions of mTOR and other members of this protein family.
It is anticipated that these advances will seed the development of novel strategies for immunosuppression, and for the treatment of leukemias, lymphomas, and other cancers.

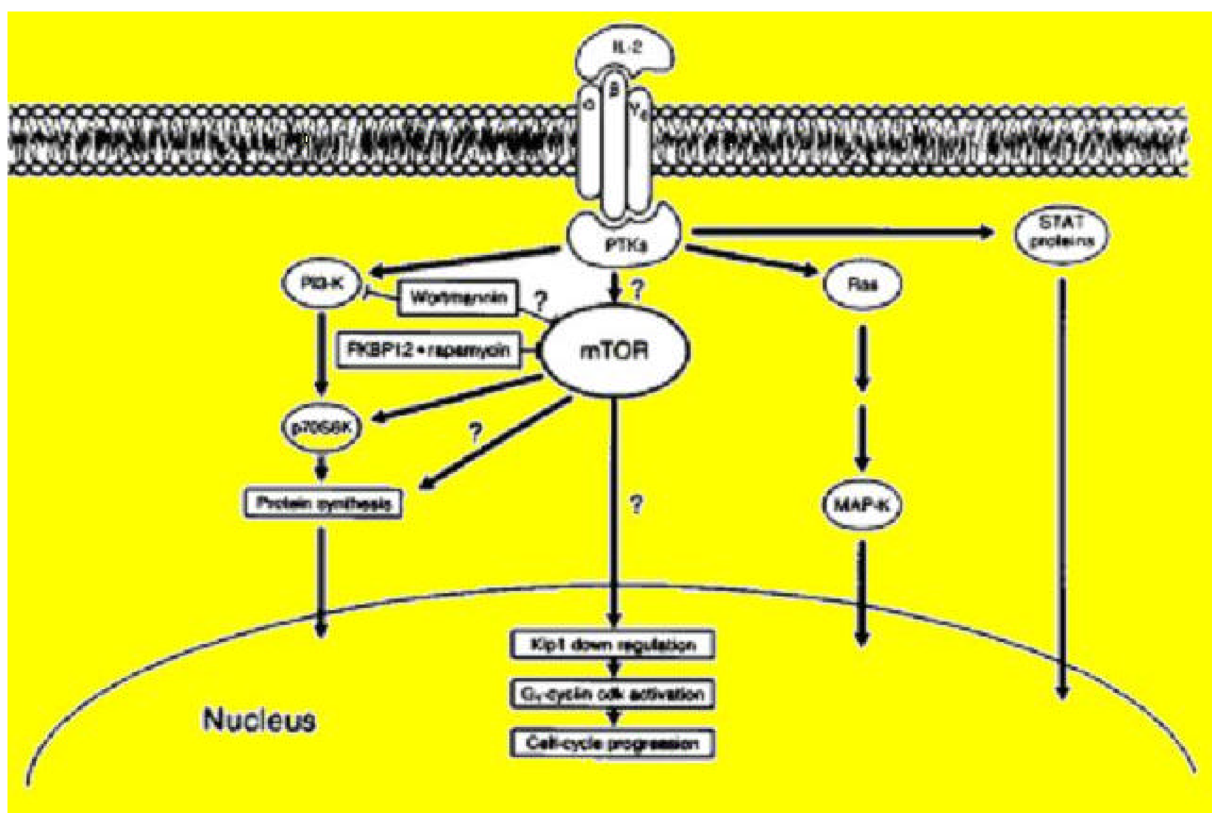


Figure 12: Schematic representation of IL-2 receptor-coupled signaling pathways. The model proposes that mTOR functions as an inducible transducer of regulatory signals for p70S6K activation, eukaryotic initiation factor 4E-dependent protein synthesis, and Kip1 downregulation. Interaction with FKBP12 _ RAP disrupts mTOR-dependent functions. Wortmannin, an irreversible inhibitor of certain PI 3-kinase family members, also interferes with a subset of mTOR-dependent responses, including p70S6K activation.

MATHERIALS
&
METHODS

II- Table 2: List of Laboratory Equipment

Equipment:	Address:
Acquisition software	ADInstruments, Castle Hill, Australia
Autoclave	Technoklav50, Tecnomara, Fernwald
Balance	Mettler AE 163, Mettler Waagen, Gießen
DMZ-Universal Puller	Zeitz - Instrumente, Augsburg
Geneclamp 500	World Percision Instruments, New Haven, Conn, USA
Incubator Type B5070	Heraeus, Hanau
Lamp KL 200	Leica, Bensheim
Lamp KL 1500 LCD	Zeiss, German
Microcentrifuge	Micro12 - 24 Bachofer, Reutlingen
Microinjector	World Precision Instruments, SaraSota, Florida, USA
Power Lab/400 (Maclab)	ADInstruments, Australia
Refrigerator UF 85-300s	Colora Messtechnik, Lorch
Steriomicroscope MS5	Leica, Bensheim
Surgery set (scissors, Foreceps, ..)	Fine Science Tools, Heidelberg, German
Precision forceps (size 3 & 5)	Dumont, Basel, Switzerland
Vaccum	Erlab, type Captair 5000 C
Vortex Genie 2	Bender & Hobein AG, Zürich, Schwitzerland

III- Table 3: List of Materials and Chemicals

Material and Chemicals:	Address:
Ampicillin	Sigma - Aldrich, Deisenhofen
3-Aminobenzoic acid ethyl ester (or :Ethyl 3-Aminobenzoate methane sulfonate salt)	Sigma - Aldrich Inc, GmbH
Borosilicate glass (Glass Replacement 3.5 NANOLTR)	Clarck Electromedical Instruments. Or (World Precision Instruments, Sarasota, Florida, USA)
Borosilicate glass Capillaries GC 150 TF-7.5 (1.5mm O. D. x 1.17mm I. D.)	Harvard Apparatus LTD, UK
Collagenase Type: CLS II	Biochrom AG, Berlin
Creatine hydrate, minimum 99%	Sigma - Aldrich, Deisenhofen
Diethylpyrocarbonat (DEPC)	Sigma - Aldrich, Deisenhofen
Dimethylsulfoxid (DMSO)	Sigma - Aldrich, Deisenhofen
Discofix	Braun, Brisil
Di Sodium hydrogen Phosphate dihydrate ($H Na_2O_4P. 2H_2O$)	Merck, Germany
Micropipet	Eppendorf, Hamburg, Germany
Micropipet tip & eppendorf	Eppendorf, Hamburg, Germany
Parafilm	labratory Film, Chicago, USA
Petri Dish	Becton Dickinson Labward, Ireland
Rapamycin from Streptomyces hygroscopicus	Sigma - Aldrich, Deisenhofen
Sodium dihydrogen Phosphate Monohydrate ($Na H_2PO_4 . H_2O$)	Merck , Germany
Syring perfusion	Becton Dickinson Labward, Ireland
Syring Needle (18G 11 / 2)	Becton Dickinson Labward, Ireland

IV- Solutions used for enzymatic defolliculation, storage and measurments of *Xenopus* oocytes

Table 4: List of Solutions

substances	ND96	Storage ND96	OR2
$CaCl_2$ (mM)	1.8	1.8	-
Gentamycin (µg/ml)	-	50	-
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (mM)	5	5	5
KCl (mM)	2	2	2
$MgCl_2$ (mM)	1	1	1
NaCl (mM)	96	96	82.5
Na_2PO_4 (mM)	-	-	1
Na -Pyruvate (mM)	-	2.5	-
Theophyline (mM)	-	0.5	-
PH	7.4	7.4	7.8

For the measurements of CrT, 50 µM Creatine monohydrate and for Napi-2b, 1mM Sodium Phosphate were added to ND96. The final solutions were titrated to PH 7.4 with NaOH or HCl. Also Rapamycin 50 nM was added to storage ND96 for incubation of cells.

V- AIMS OF THE PRESENT STUDY

The motivation of this study was to resolve the potential role of Serum and Glucocorticoid Inducible Kinase (SGK) isoforms, Protein kinase B (PKB_{mut}), mammalian target of rapamycin (mTOR), Rapamycin (RAP) in the regulation of creatine transporter CreaT (SLC6A8) & in Na^+-coupled electrogenic phosphate transporter NaPi IIb (SLC34A2). Therefore for this purpose two carrier proteins mediating transporter of creatine and phosphate have been investigated.

V- A. Experimental setup :

Defolliculated oocytes of the Xenopus laevis are placed in perfusing chamber (**1**) mounted under a microscope and connected to a stopcock for changing the solutions. The cell is impaled with two microelectrodes. Current-Voltage (IV) dependencies of the membrane currents of Xenopus oocytes are determined by conventional two-microelectrode voltage clamp technique with the Voltage–clamp amplifier, like GeneClamp500 (Axon Instruments)(**2**). The set up is under control of a personal computer (**3**) with powerLab/400 (16-bit DAC & ADC, 10V max. Out & input respectively, (ADInstrument) (**4**). From a constant holding potential of -60 mV (close to the resting potential). For control of the quality of voltage clamp, the time courses of Vc and Vm are monitored on an oscilloscope **(5)**. For convenience, membrane potential and holding potential at -60 mV are continuously recorded by a software for data acquisition (ADInstrument) or by a pen recorder **(6)**. (Schwarz & Rettinger 2003).

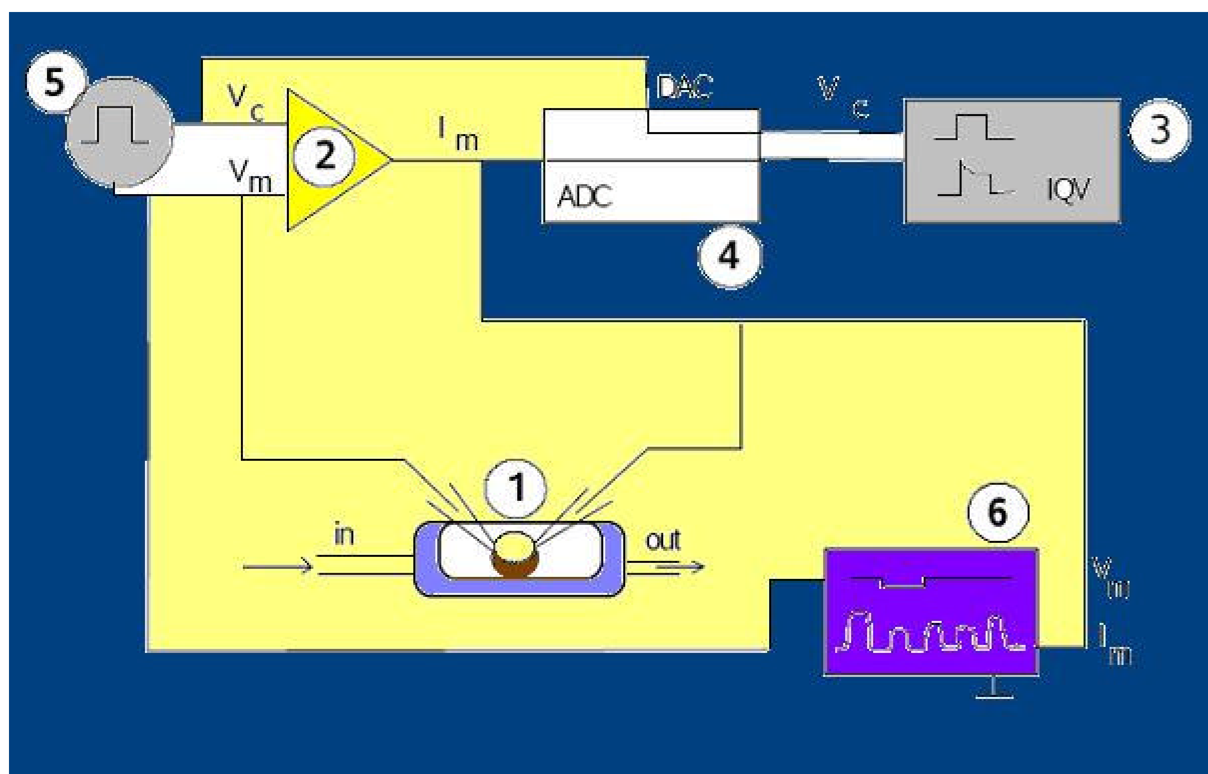


Figure13: Schematic diagram of experimental set-up (see text).

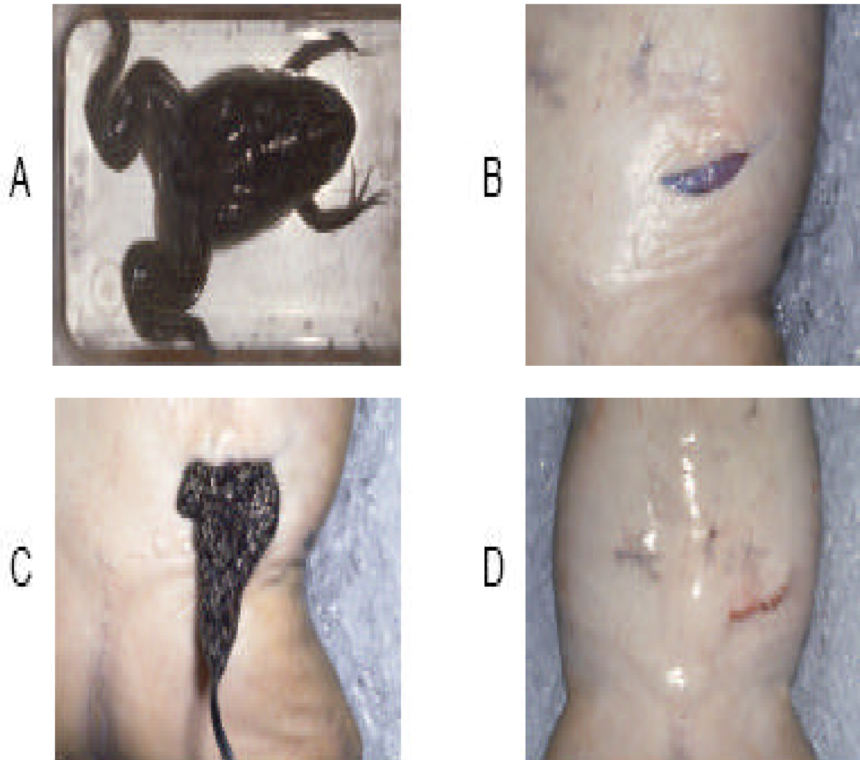
V-A. 1-Preparation of oocytes

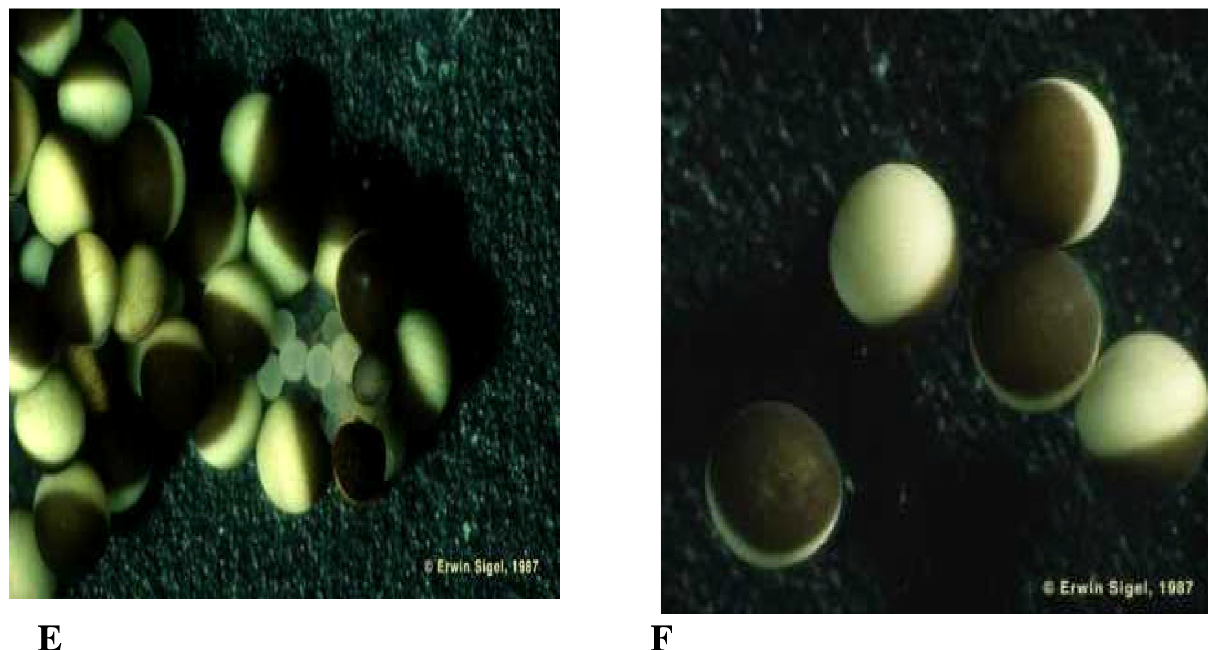
An adult female Xenopus laevis frog was submersed in lL water consisting of the anaesthetic agent 3-aminobenzoic acid ethyl ester (0.1%) for about 15-30 minutes (Fig. 14A). After the frog was fully anaesthetized it was placed on ice for surgery. A small abdominal incistion (1cm) was carried out and small segments of ovary were removed (Fig. 14B,C). Subsequently the wound was closed with a reabosrbable suture (Fig. 14D).

The frog was then kept wet and but not under water until reflexes were fully recovered to avoid of drowing and hypothermia.

The ovarial sacs were manually seperated with fine tweezers to groups of 10-20 oocytes, put into 15ml tube then enzymatically defolliculated by treatment with an OR-2 solution containnig 1-2mg/ml collagenase A for 1-1.5 h hours at room temperature (Fig. 14E) with gentle agitation. Defolliculation of the oocytes was stopped by washing several times with ND96. This step also removes all detritus permiting the ease of oocyte sorting.

Oocytes were then sorted using a self-made apparatus (Fig. 14F). Only large oocytes (stage V of VI) showing evenly coloured poles and a sharp border between both poles were selected and stored overnight in a ND96 storage solution. (complemented after sterilisation with 5 mM pyruvate, 50 µg/ml gentamycin and 0.5 mM theophylline) at 15 °C. Gentamycin helps preventing infections and theophylline inhibits the further maturation of the oocytes.





E **F**

Fig. 14: Steps of the oocytes preparation and injection.
The frog is anesthetized in 1 liter of 3-aminobenzoic acid ethyl ester (1%) in tap water at near room temperature. (B) The frog is placed on its back during operation. An incision about 1 cm long is made in the skin. (C) A small portion of the ovary is pulled out with forceps and removed with a pair of scissors. (D) The peritoneum and the muscle tissue are sewn up and then the skin closed off using cat gut. (E) The clump of oocytes is immediately transferred to a petri dish containing modified Barth medium with antibiotic. (F) Oocytes of stage V and VI are separated with a platinum wire loop.

V-A. 2 -cRNA injection

After sorting overnight, oocytes were then injected using cRNA microcapillaries (filled with the required cRNA) mounted in a micromanipulator controlled microinjector (Fig.14G). Precaution should be taken that cRNA wasnot contaminated with RNAases and that the injection capillary wasnot clogged with small particles. To aviod those problems several procedures were carried out such as using only sterile pipettes, gloves and DEPC treated water for dilution of cRNA.

Glass capillaries with a long shank were pulled using a normal puller. The tip was manually broken under the microscope (diameter of about 10-20µm), backfilled with Paraffin oil to seal the pipette from air and loaded into the capillary with cRNA by suction (1-2 µl). Oocytes were then placed into a 35 mm petridish with a polypropylene mesh gluted to the bottom to fix the oocytes & injected with a given volume of cRNA was injected into oocytes one by one according to the experimental requirements. (usually25- 50nl).

After injection, oocytes were kept in ND96 storage solution in an incubator at 15°c. To avoid sticking of oocytes to the petri-dish or to other oocytes, the dish was gently shaken. At least every days the storage solution was exchanged and damaged oocytes were removed to maximise the survival of the oocytes.

G

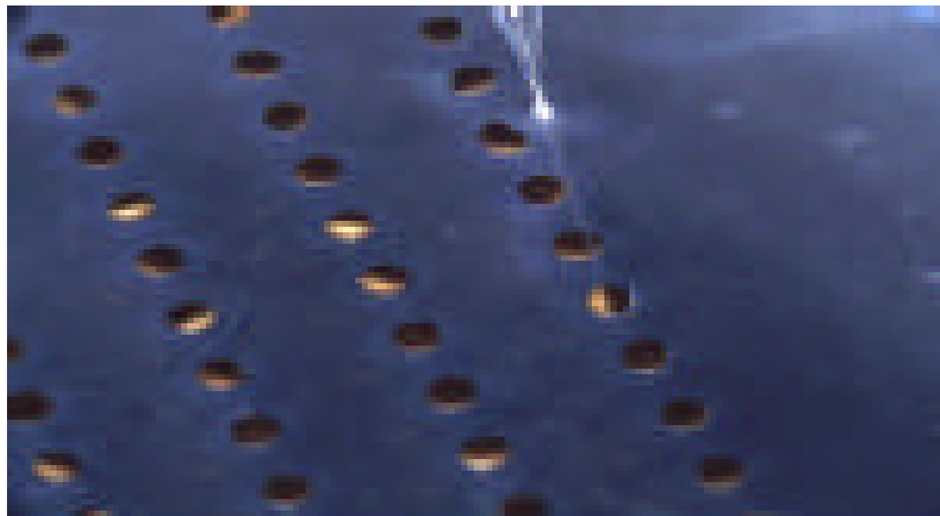


Fig. 14: Steps of the oocytes preparation and injection
(G*) For injection, the oocytes are aligned relative to the tip of the needle.*

V-A. 3 - Preparation of microelectrodes& clamping amplifier

Glass micropipettes are pulled from the filament containing capillaries of Borosilicate glass using a horizontal or vertical puller. The pipettes are filled with 3M KCl by using a syringe. An inserted silver wire covered with AgCl serves as connection to the electronics. To remove the bubbles from the tip, a negative pressure can be applied from the cut end of the pipette. The microelectrodes are inserted into electrode holders mounted to micromanipulators. The resistance of microelectrodes is measured with an ohm-meter built-in to the amplifier Fig. 7&13(**2**).

Two-electrode voltage-clamp (TEVC) was used to measure whole cell currents in *Xenopus laevis* oocytes expressing ion transport or channels. As shown in Fig. 7&13 oocytes were impaled by two glass electrodes. One was to record membrane potential while the other was to deliver currents. Both were made from pulled glass capillaries with a thin filament which were filled with 3 mM KCl and subsequently inserted with an Ag/AgCl electrode. The bath-grounding electrode was made of 3% agar and 3 M KCl or was pulled as the other two electrodes. The membrane potential electrode was connected to a feedback amplifer comparing the signal with the voltage clamp command. The difference of these signals, which was highly amplified, was applied as a current through the electrode across the cell membrane and to the bath-grounding electrode. The gain of the clamping amplifier is set low and the voltage clamp switched on, and the preamplifier records differentially between these two inputs with a gain of 10. Because of the x10 gain of the preamplifier, the command voltage must be 10 times the desired voltage step. The command summing amplifier is followed by a variable low-pass filter, which may be used to reduce the risetime of the command voltage step. This often makes it easier to obtain a fast voltage clamp step while avoiding oscillation. [Halliwell J.V. & et al., 1987]. The whole set up was grounded and shielded with a Faraday cage [Ohlemeyer, C. Meyer, J.W. 1992].

V-B. Methods:

V-B.1 Constructs

For generation of cRNA, constructs were used encoding wild type bovine CreaT [[Dodd, J.R. et al., 2001], wild type NaPi IIb (SLC34A2) [Hilfiker, H. et al., 1998], wild type mammalian target of rapamycin mTOR [Hudson, C.C., et al., 2002], wild type human SGK1 [Waldegger, S., et al., 1997], constitutively active human ^{S422D}SGK1 [Kobayashi T, et al., 1999a], inactive human^{K127N}SGK1 [Kobayashi T, et al., 1999a], wild type human SGK2, wild type human SGK3 [Kobayashi T, et al., 1999b],constitutively active human ^{S419D}SGK3 [Böhmer C, et al., 2005], inactive ^{K191N}SGK3 [Böhmer C, et al., 2005],and the constitutively active human ^{T308D,S473D}PKB [Alessi DR, et al., 1996], as describedpreviously [22]. ^{S422D}SGK1, ^{K127N}SGK1, SGK2, SGK3, and ^{T308D,S473D} PKB were kindly provided by Sir Philip Cohen. and NaPi IIb by Jo¨rg Biber and Heini Murer.

V-B.2 Electophysiology

Xenopus oocytes was prepared as previously described [Wagner, C.A., et al., 2000]. 7.5 ng of the respective kinase cRNAs 7.5 ng of the respective kinase cRNAs also, fifteen nanograms of mTOR cRNA were injected on the firstday and 15 ng SLC6A8 /or SLC34A2 cRNA on the second day after preparation of the Xenopus oocytes. Where indicated, 7.5 ng cRNA encoding SGK1, ^{S422D}SGK1, ^{K127N}SGK1, SGK2, SGK3 , ^{S419D}SGK3, inactive ^{K191N}SGK3 ,and the constitutively active human ^{T308D,S473D}PKB was injected separately or together with TOR. All experiments were performed at room temperature 5–6 days after the second injection. Two-electrode voltage-clamp recordings were performed at a holding potential of -60 mV. The data were filtered at 10 Hz and recorded with Digidata A/D–D/A converter and Chart V.4.2 software for data acquisition and analysis (Axon Instruments).

The control solution (superfusate / ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.4. Creatine monohydrate or Phosphate was added to the solutions at the indicated concentrations. The final solutions were titrated to pH 7.4 using NaOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

V-B.3 Data Statistic Analysis:

Data are provided as means ±SEM, n represents the number of oocytes investigated. All expriments were repeated with at least 3 batches of oocytes from different frogs in all repetitions qualitatively similar data were obtained. All data were tested for significance using the student t-test, and only results with $P < 0.05$ were considered statistically significant.

RESULTS

Results:

I-Stimulation of the creatine transporter SLC6A8:
A: by the protein kinases SGK1 and SGK3

SGK1 increased creatine induced current in SLC6A8 expressing Xenopus oocytes

In Xenopus oocytes expressing human SLC6A8 but not in water injected oocytes creatine induced an inward current (I_{CREA}), which was significantly increased by additional coexpression of wild type SGK1 (Fig. 15). The coexpression of SGK1 increased I_{CREA} approximately twofolds.

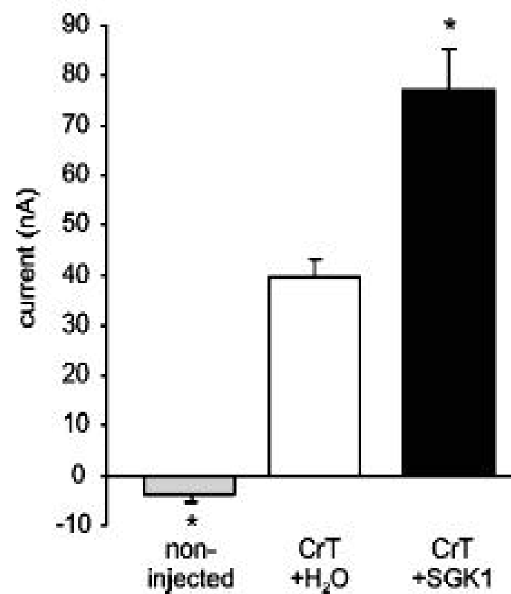


Fig. 15. SGK1 stimulated electrogenic creatine transport in SLC6A8 expressing Xenopus oocytes.
In Xenopus oocytes expressing SLC6A8 (open bar) but not in water injected oocytes (grey bar), creatine induced inward currents. Coexpression of the wild type SGK1 together with SLC6A8 (closed bar) increased the current approximately twofold. Arithmetic means ± SEM, (*) statistically significant difference to current in Xenopus oocytes expressing SLC6A8 alone (open bar).

SGK1 increased the maximal current of SLC6A8

The effect of wild type SGK1 was mimicked by constitutively active ^{S422D}SGK1 (Fig. 16). Coexpression of ^{S422D}SGK1 significantly increased I_{CREA} at any concentration tested without appreciably affecting the creatine concentration required for half maximal current (Fig. 15). Thus, it appears safe to conclude that SGK1 increases the maximal transport rate of the creatine transporter.

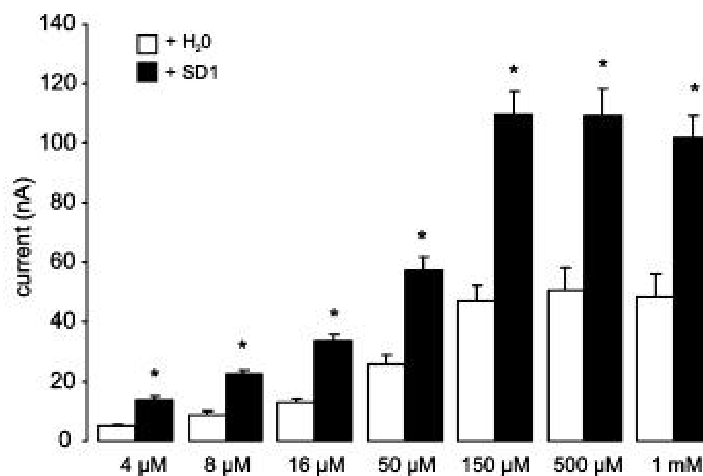


Fig. 16: SGK1 enhanced the maximal transport rate of SLC6A8.
In Xenopus oocytes expressing SLC6A8 the creatine induced inward current increased with substrate concentration varied from 4 µM to 1 mM. Approximately halfmaximal currents were observed at 50 µM creatine concentration. Coexpression of the constitutively active SGK1 increased the current at any concentration tested without appreciably altering the concentration required for halfmaximal current. Arithmetic means ± SEM, (*) statistically significant difference between currents in Xenopus oocytes expressing SLC6A8 together with constitutively active ^{S422D}SGK1 (closed bars) and respective currents in Xenopus oocytes expressing SLC6A8 alone (open bars).

The effect of SGK1 on SLC6A8 required intact catalytic activity .

The activation of SLC6A8 by SGK1 depended on intact activity of the kinase as coexpression of the catalytically inactive SGK1 mutant ^{K127N}SGK1 did not stimulate SLC6A8 mediated current. Fig. 17 demonstrates that coexpression of ^{K127N}SGK1 did not significantly alter I_{CREA} at any of the concentrations tested (50 µM–1 mM). For comparison, coexpression of the constitutively active ^{S422D}SGK1 at each concentration tested increased I_{CREA} approximately twofold (Fig. 17).

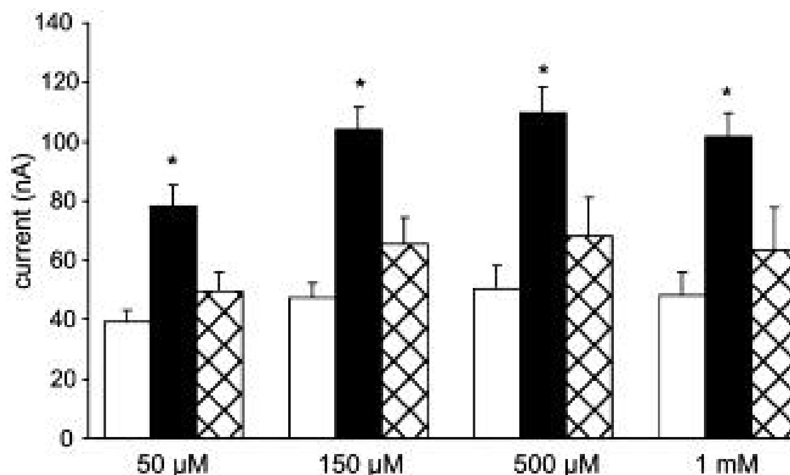


Fig. 17: The effect of SGK1 required functional kinase activity.
The creatine induced inward currents in Xenopus oocytes expressing SLC6A8 were stimulated by coexpression of the constitutively active serum and glucocorticoid inducible kinase ^{S422D}SGK1 (closed bars) but not by coexpression of the inactive mutant ^{K127N}SGK1 (crossed bars). Creatine was added at the concentrations 50 µM, 150 µM, 500 µM, and 1 mM, as indicated. Arithmetic means ± SEM, (*) statistically significant difference to currents in Xenopus oocytes expressing SLC6A8 alone (open bars).

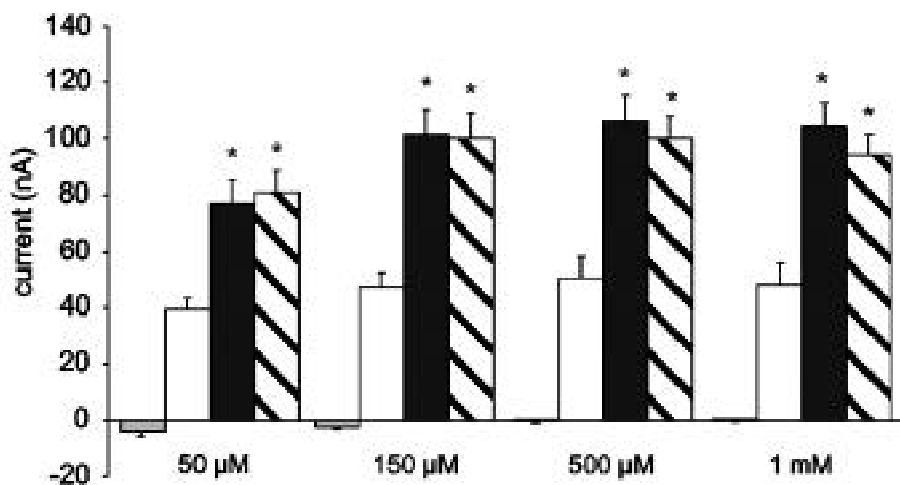


Fig. 18: SGK3 mimicked the stimulating effect of SGK1.
Coexpression of the wild type SGK isoform 3 was similarly capable to enhance the creatine induced currents as coexpression of wild type SGK1 with SLC6A8. In Xenopus oocytes expressing SLC6A8 together with SGK1 (closed bars) or SGK3 (hatched bars), the creatine induced current was about twofold the current in oocytes expressing SLC6A8 alone (open bars). No current was observed in water injected oocytes (grey bars). Creatine was added at the concentrations 50 µM, 150 µM, 500 µM, and 1 mM, as indicated. Arithmetic means ± SEM, (*) statistically significant difference to current in Xenopus oocytes expressing SLC6A8 alone.

SGK3 but not SGK2 or PKB similarly stimulated SLC6A8 mediated currents

Similar to SGK1, its isoform SGK3 enhanced I_{CREA}. As illustrated in Fig. 18, the effect of SGK3 was similar to to an approximately twofold increase of I_{CREA}. Similar to SGK1, the effect of SGK3 required its catalytic activity. The inactive mutant ^{K191N}SGK3 failed to increase I_{CREA}, while constitutively active ^{S419D}SGK3 was similarly effective as wild type SGK3. In contrast to SGK3, neither SGK2 nor constitutively active PKB significantly increased I_{CREA}. Thus, SGK1 and SGK3 but not SGK2 or PKB were able to increase creatine transport via SLC6A8 (Fig. 19).

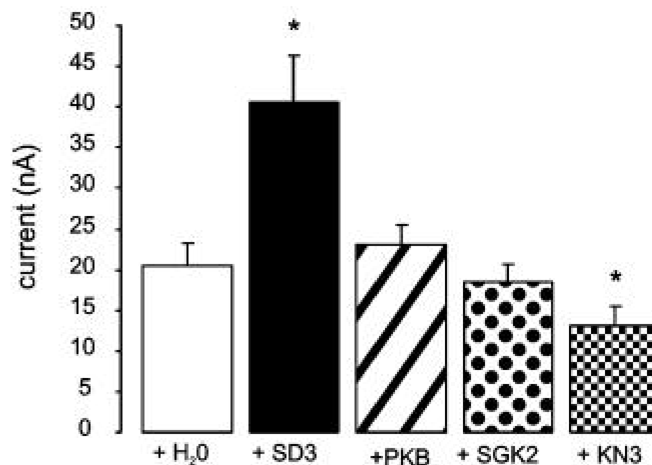


Fig. 19: Active SGK3 but not inactive SGK3, wild type SGK2 or active PKB mimicked the stimulating effect of SGK1.
Similar to wild type SGK3, the constitutively active ^{S419D} SGK3 isoform 3 (SD3) activated SLC6A8 while the inactive mutant ^{K191N}SGK3 (KN3) was unable to upregulate SLC6A8 currents. Moreover, neither wild type SGK2 nor constitutively active ^{T308D,S473D}PKB (PKB) significantly modulated creatine induced currents. As compared to the creatine induced current in Xenopus oocytes expressing SLC6A8 alone (open bar), the creatine induced currents were significantly enhanced in Xenopus oocytes expressing SLC6A8 together with ^{S419D}SGK3 (closed bar), were significantly decreased in Xenopus oocytes expressing SLC6A8 together with ^{K191N}SGK3 (squared bar) and were not significantly modified in Xenopus oocytes expressing SLC6A8 together with SGK2 (dotted bar) or ^{T308D,S473D}PKB (hatched bar). Arithmetic means ± SEM, (*) statistically significant difference to current in Xenopus oocytes expressing SLC6A8 alone (open bar).

I-Stimulation of the creatine transporter SLC6A8:
B: by the protein kinase mTOR

mTOR increased creatine-induced current in SLC6A8 expressing Xenopus oocytes

In Xenopus oocytes expressing bovine SLC6A8 but not in water injected oocytes, creatine induced an inward current (I_{CREA}), which was significantly increased by additional coexpression of the mammalian target of rapamycin mTOR (Fig. 20). The coexpression of mTOR increased I_{CREA} approximately two-fold.

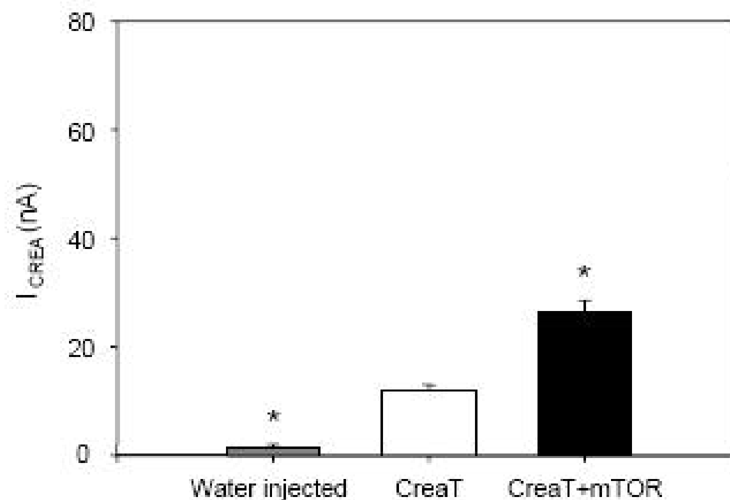


Fig. 20: Coexpression of mTOR stimulates electrogenic creatine transport in SLC6A8 expressing Xenopus oocytes.
*In Xenopus oocytes expressing SLC6A8 (open bar, n = 19) but not in water injected oocytes (grey bar, n = 7), creatine (50 µM) induced inward currents (I_{CREA}). Coexpression of the mammalian target of rapamycin mTOR together with SLC6A8 (closed bar, n = 21) increased ICREA approximately two-fold. Arithmetic means ± SEM, *indicates statistically significant difference to current in Xenopus oocytes expressing SLC6A8 alone.*

mTOR increased the maximal current of SLC6A8

Coexpression of mTOR significantly increased I_{CREA} at any concentration tested without appreciably affecting the creatine concentration required for half-maximal current (Fig. 21). Thus, it appears safe to conclude that mTOR increases the maximal transport rate of the creatine transporter.

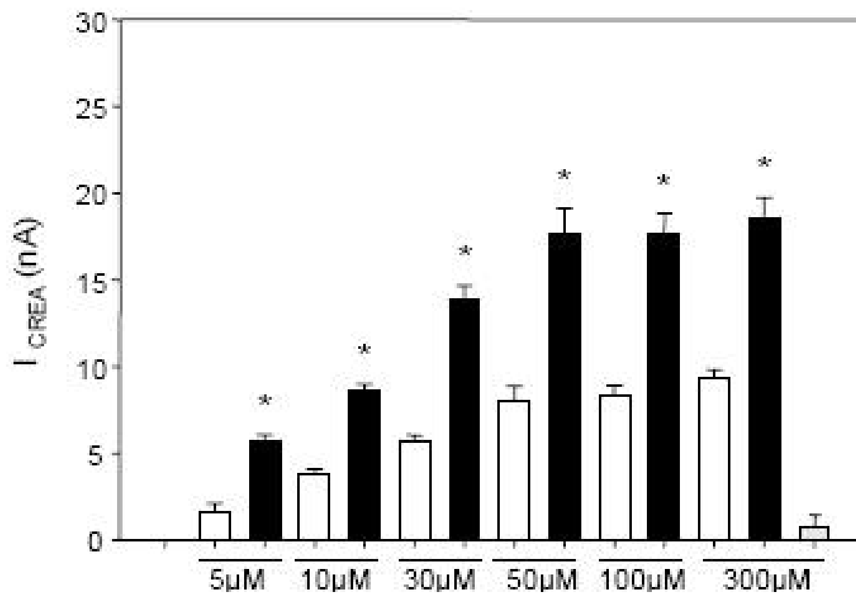


Fig. 21: mTOR enhances the maximal transport rate of SLC6A8.
*Creatine induced inward current (I_{CREA}) in Xenopus oocytes expressing SLC6A8 at different substrate concentrations (5, 10, 30, 50, 100, and 300 µM). Coexpression of mammalian target of rapamycin mTOR increased I_{CREA} at each concentration tested without appreciably altering the concentration required for half-maximal current. Arithmetic means ± SEM, *indicates statistically significant difference between I_{CREA} in Xenopus oocytes expressing SLC6A8 together with mTOR (closed bars, n = 9) and respective I_{CREA} in Xenopus oocytes expressing SLC6A8 alone (open bars, n = 6).*

The effect of mTOR was reversed by rapamycin

The activation of SLC6A8 by mTOR was abolished by the mTOR inhibitor rapamycin (50 nM). The effect of rapamycin was not immediate. While a 1 h preincubation of rapamycin was not effective, a preincubation of rapamycin for 32 h resulted in significant inhibition of I_{CREA} and abrogated the stimulating effect of mTOR coexpression on I_{CREA} (Fig. 22).

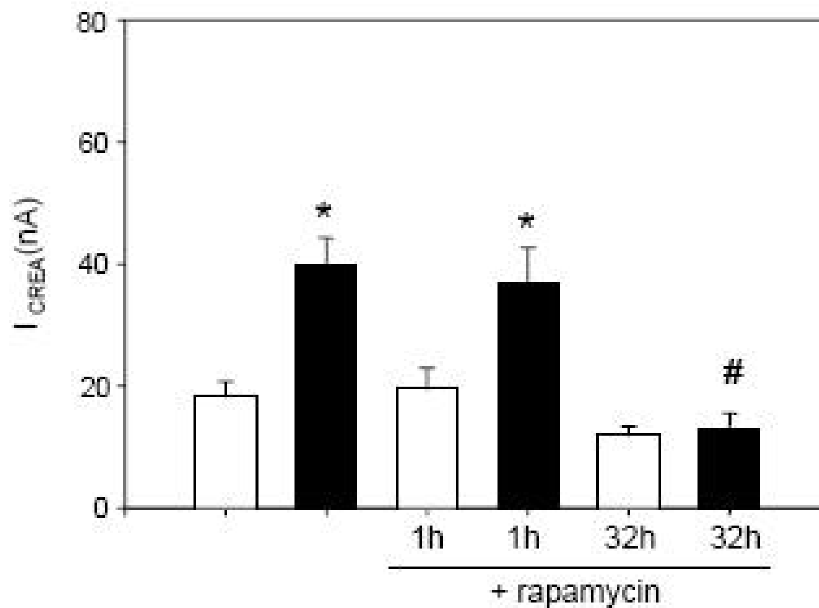


Fig. 22: The effect of mTOR is reversed by rapamycin.
*The stimulating effect of coexpressed mammalian target of rapamycin (mTOR) on creatine-induced inward currents (I_{CREA}) in Xenopus oocytes expressing SLC6A8 was reversed by preincubation with rapamycin (50 nM) for 32 h, but not for 1 h. Arithmetic means ±SEM of I_{CREA} in Xenopus oocytes expressing SLC6A8 without (open bars) and with mTOR (closed bars). The Xenopus oocytes were left untreated (n = 21), treated for 1 h (n = 17) or for 32 h (n = 8) with 50 nM rapamycin. *indicates statistically significant difference between Xenopus oocytes expressing mTOR together with SLC6A8 and Xenopus oocytes expressing SLC6A8 alone, $^{\#}$indicates significant difference between presence and absence of rapamycin.*

The effect of mTOR was mimicked by SGK1 and reversed by inactive mutant ^{K127N}SGK1

Similar to coexpression of mTOR, coexpression of the serum and glucocorticoid-inducible kinase SGK1 enhanced I_{CREA}. Coexpression of SGK1 and mTOR increased I_{CREA} to a similar extent as coexpression of either kinase alone (Fig. 23), suggesting that the kinases may be effective through overlapping mechanisms. Coexpression of the inactive mutant ^{K127N}SGK1 did not stimulate I_{CREA} and blunted the stimulating effect of mTOR coexpression on I_{CREA} (Fig. 23). Thus, ^{K127N}SGK1 apparently exerts a dominant negative effect on the stimulation of I_{CREA} by mTOR further pointing to overlapping mechanisms.

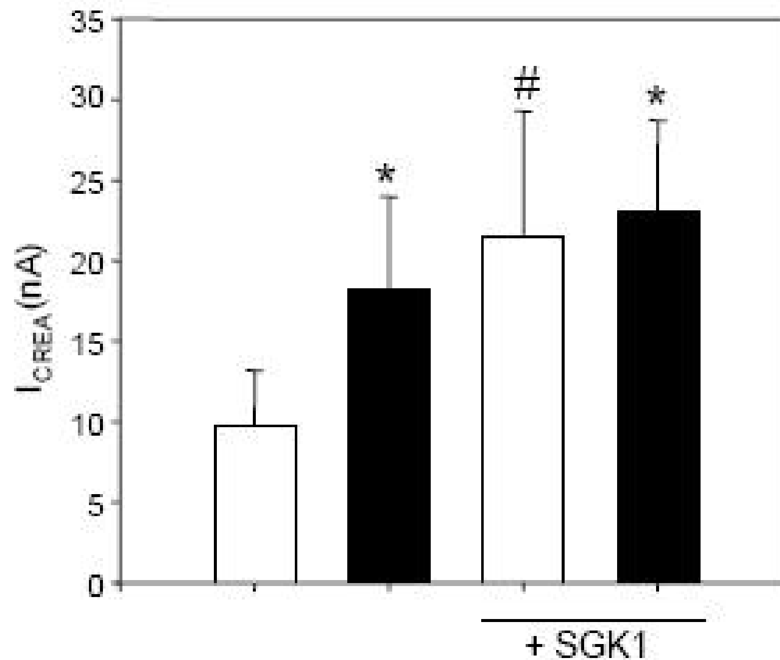


Fig. 23: The effect of mTOR is mimicked by SGK1.
*Coexpression of the serum and glucocorticoid-inducible kinase SGK1 was similarly capable to enhance the creatine-induced currents (I_{CREA}) as coexpression of mTOR with SLC6A8. Arithmetic means ± SEM of I_{CREA} in Xenopus oocytes expressing SLC6A8 without (open bars) and with mTOR (closed bars). The currents were determined without (left bars, n = 27 and 19, respectively) or with (right bars, n = 24 and 9, respectively) additional expression of wild type SGK1. *indicates statistically significant difference between Xenopus oocytes expressing mTOR together with SLC6A8 and Xenopus oocytes expressing SLC6A8 alone, #indicates significant difference between the respective values with and without additional expression of SGK1.*

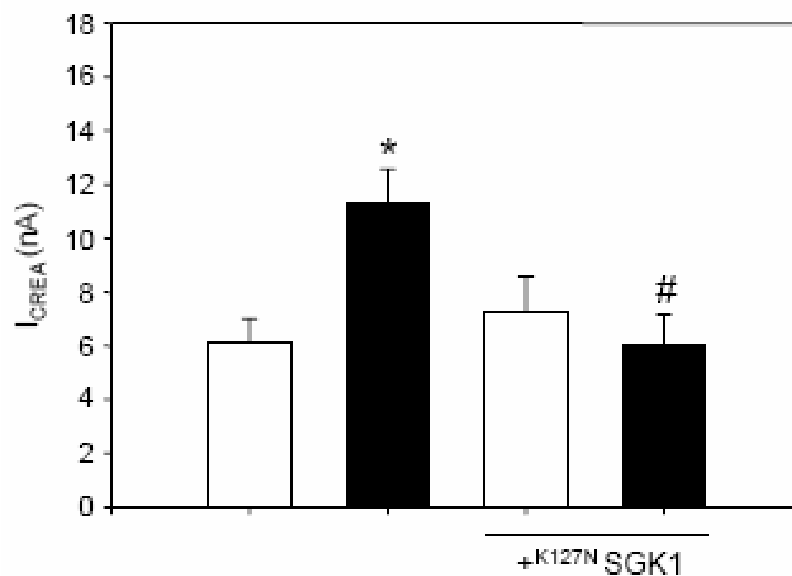


Fig. 24: The effect of mTOR is reversed by inactive ^{K119N}SGK1.
*Coexpression of the inactive mutant serum and glucocorticoid-inducible kinase ^{K119N}SGK1 did not stimulate the creatine-induced currents (I_{CREA}) in SLC6A8 expressing Xenopus oocytes and blunted the stimulation of I_{CREA} by coexpression of the mammalian target of rapamycin mTOR. Arithmetic means ± SEM of I_{CREA} in Xenopus oocytes expressing SLC6A8 without (open bars) and with mTOR (closed bars). The currents were determined without (left bars, n = 7 and 6, respectively) or with (right bars, n = 5 and 6, respectively) additional expression of inactive mutant ^{K127N}SGK1. *indicates statistically significant difference between Xenopus oocytes expressing mTOR together with SLC6A8 and Xenopus oocytes expressing SLC6A8 alone, $^{\#}$indicates significant difference between the respective values with and without additional expression of ^{K127N}SGK1.*

II- Stimulation of the intestinal phosphate transporter SLC34A2:
by the protein kinase mTOR

In *Xenopus* oocytes expressing SLC34A2 but not in water injected oocytes phosphate induced an inward current (Ip), which was significantly increased by additional coexpression of the mammalian target of rapamycin mTOR (Fig. 25). The coexpression of mTOR increased Ip approximately twofold.

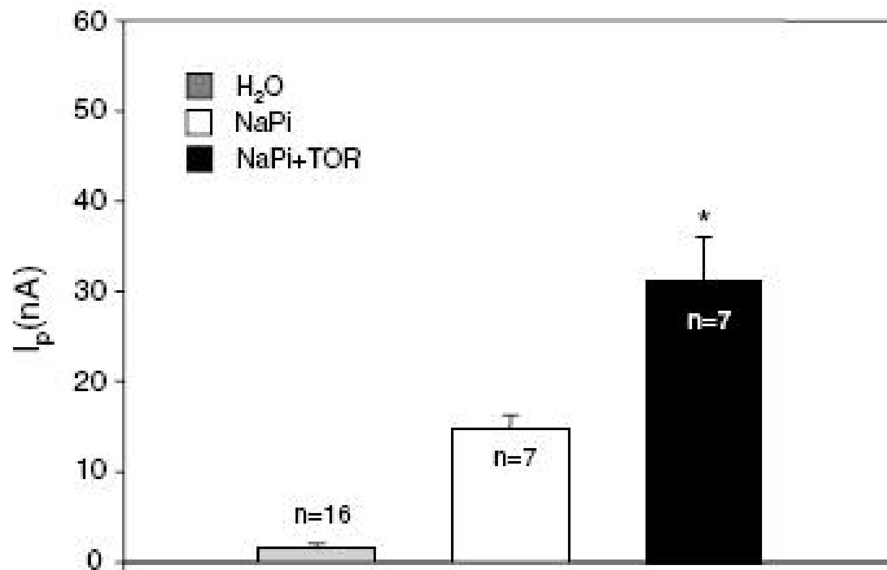


Fig. 25: Coexpression of mTOR stimulates electrogenic phosphate transport in SLC34A2 expressing Xenopus oocytes.
*In Xenopus oocytes expressing SLC34A2 (NaPi, open bar) but not in water injected oocytes (H2O, grey bar), phosphate (1 mM) induced inward currents (Ip). Coexpression of the mammalian target of rapamycin mTOR (TOR) together with SLC34A2 (closed bar) increased Ip approximately twofold. Arithmetic means ±SEM. *Indicates statistically significant difference to the respective value in the absence of mTOR, n = number of experiments*

The activation of SLC34A2 by mTOR was abolished by the mTOR inhibitor rapamycin (50 nM). Preincubation of rapamycin for 24 hours did not significantly modify Ip in the absence of TOR but virtually abrogated the stimulating effect of mTOR coexpression on Ip (Fig. 26).

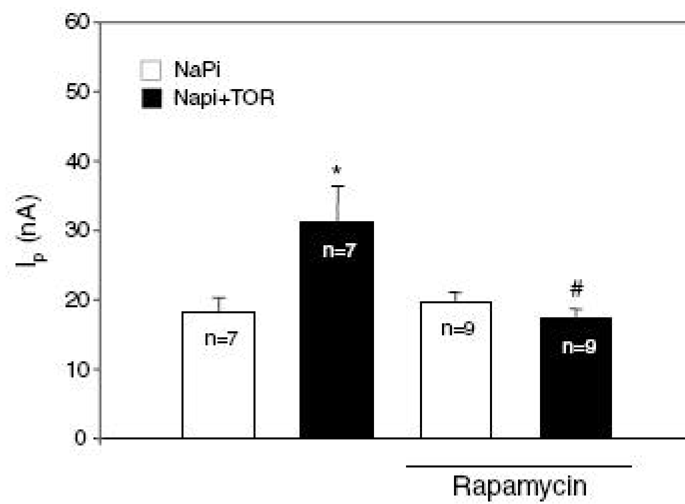


Fig. 26: The effect of mTOR is reversed by rapamycin.
*The stimulating effect of coexpressed mammalian target of rapamycin (mTOR) on phosphate induced inward currents (Ip) in Xenopus oocytes expressing SLC34A2 was reversed by preincubation with rapamycin. Arithmetic means ± SEM of Ip in Xenopus oocytes expressing SLC34A2 (NaPi) without (open bars) and with mTOR (closed bars). The Xenopus oocytes were left untreated or treated for 24 hours with 50 nM rapamycin. *Indicates statistically significant difference to respective value in the absence of mTOR, #indicates significant difference to the respective value without addition of rapamycin, n = number of experiments*

Similar to coexpression of mTOR, coexpression of the serum and glucocorticoid inducible kinase SGK1 enhanced Ip (Fig. 27). The effect of SGK1 and mTOR was further mimicked by the constitutively active ^{S422D}SGK1. In *Xenopus* oocytes expressing ^{S422D}SGK1, the additional expression of mTOR was not followed by an additive significant increase of Ip in SLC34A2 expressing *Xenopus* oocytes, pointing to at least partially overlapping mechanisms in the regulation of SLC34A2 by SGK1 and mTOR (Fig. 27).

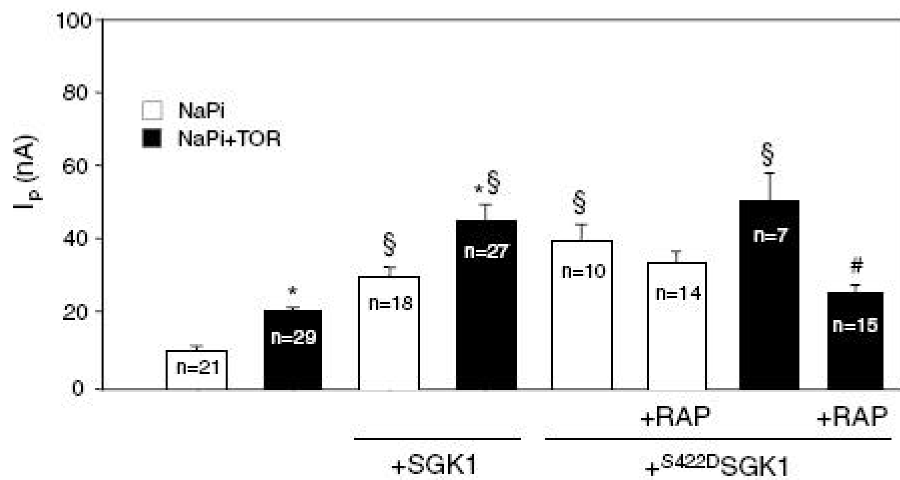


Fig. 27: The effect of mTOR is mimicked by SGK1
*Coexpression of the serum and glucocorticoid inducible kinase SGK1 was similarly capable to enhance the phosphate induced currents (Ip) as coexpression of mTOR with SLC34A2. Arithmetic means ± SEM of Ip in Xenopus oocytes expressing SLC34A2 without (open bars) and with mTOR (closed bars). The currents were determined without (left bars) or with (right bars) additional expression of wild type SGK1 or constitutively active ^{S422D}SGK1. The effect of ^{S422D}SGK1 is reversed by rapamycin (+ Rap). *Indicates statistically significant difference to respective value in the absence of mTOR, §indicates significant difference to the respective value without additional expression of SGK1 or ^{S422D}SGK1, $^{\#}$indicates significant difference to the respective value without addition of rapamycin, n = number of experiments.*

In contrast to coexpression of SLC34A2 with SGK1 or ^{S422D}SGK1, coexpression of SLC34A2 with the inactive mutant ^{K127N}SGK1 significantly decreased Ip and significantly blunted the effect of mTOR coexpression on Ip (Fig. 28). Thus, ^{K127N}SGK1 apparently exerts a dominant negative effect on the stimulation of Ip by mTOR further pointing to overlapping mechanisms in the stimulation of SLC34A2 by mTOR and SGK1. Accordingly, in *Xenopus* oocytes coexpressing SLC34A2 with the inactive mutant ^{K127N}SGK1, rapamycin did not significantly modify Ip (Fig. 28).

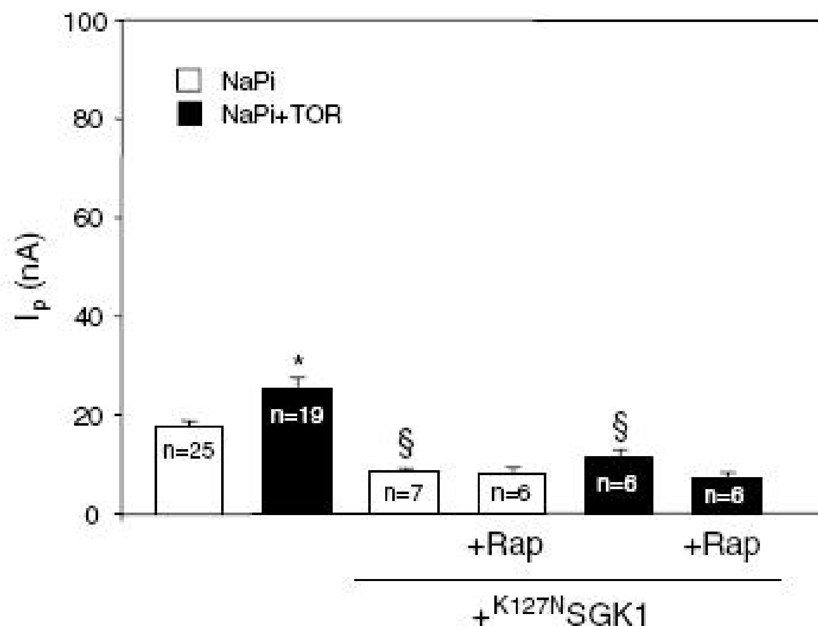


Fig. 28: The effect of mTOR is reversed by inactive ^{K119N}SGK1.
*In contrast to wild type and constitutively active serum and glucocorticoid inducible kinase SGK1, coexpression of the inactive mutant kinase ^{K119N}SGK1 did not stimulate the phosphate induced currents (Ip) in SLC34A2 expressing Xenopus oocytes and blunted the stimulation of Ip by coexpression of the mammalian target of rapamycin mTOR. Arithmetic means ± SEM of Ip in Xenopus oocytes expressing SLC34A2 without (open bars) and with mTOR (closed bars). The currents were determined without (left bars) or with (right bars) additional expression of inactive mutant ^{K127N}SGK1. In the presence of ^{K127N}SGK1 rapamycin (+ Rap) does not significantly modify Ip. *Indicates statistically significant difference to respective value in the absence of mTOR, §indicates significant difference to the respective value without additional expression of ^{K127N}SGK1, n = number of experiments..*

DISCUSSION

Discussion:

I- Stimulation of the creatine transporter SLC6A8:
A: by the protein kinases SGK1 and SGK3

The present research confirms the exquisitely high affinity of the electrogenic creatine transporter CreaT (SLC6A8) which reaches half maximal transport rates at concentrations well below 100 µM [Guimbal, C., Kilimann, M., 1993]. More importantly, the present paper demonstrates that the maximal transport rate of SLC6A8 is pregulated by the serum and glucocorticoid inducible kinase isoforms SGK1 and SGK3. SGK1 has previously been shown to regulate several carriers [Lang, F., et al., 2003] including the Na^+/H^+ exchanger NHE3 [Yun, C.C., 2003], the dicarboxylate transporter NaDCT1 [Boehmer, C., et al., 2004], the glucose transporter SGLT1[Dieter M., et al., 2004] and the amino acid transporters SN1 [Boehmer, C., et al., 2003], EAAT1 [Boehmer, C., et al., 2003], EAAT3 [Schniepp, R., et al., 2004], EAAT4 [Boehmer, C., et al., 2004], and EAAT5 [Boehmer, C,. et al., 2005].
The kinase could directly phosphorylate its target proteins thereby influencing its tertiary structure and kinetic properties, as shown for the cardiac sodium channel SCN5A [Boehmer, C., et al., 2003], the renal epithelial K^+ channels ROMK [Palmada, M., et al., 2003], and the renal epithelial Na^+ channel EnaC [Diakov, A., Korbmacher, C. 2004]. Unlike those channels, the creatine transporter SLC6A8 does not contain any SGK phosphorylation motif in its intracellular sequence. Thus, a direct phosphorylation of SLC6A8 by SGK is unlikely.
Another mechanism involved in SGK dependent transport regulation is the phosphorylation of the ubiquitin ligase Nedd4-2 [Pearce, D., 2003; Verrey, F., et al., 2003]. Nedd4-2 ubiquitinates its target proteins thus preparing them for the clearance from the cell membrane. SGK1 phosphorylates Nedd4-2 and thus decreases the affinity of the ubiquitin ligase to its target proteins [Pearce, D., 2003; Verrey, F., et al., 2003]. Beyond regulation by ubiquitination, the SGK1 and SGK3 dependent regulation of SLC6A8 may be accomplished by an additional, hitherto unknown protein that itself targets the creatine transporter.
Given the mental retardation observed in individuals with defective SLC6A8 [Hahn, K.A., et al., 2002], impaired SLC6A8 function could well contribute to the compromised learning ability of rats expressing low levels of SGK1 [Tsai, K.J., 2003]. Moreover, as SGK1 activity is stimulated by insulin like growth factor IGF1 [Kobayashi, T., et al., 1999], SGK1 could participate in the upregulation of SLC6A8 by growth hormone [Omerovic, E., et al., 2003]. Finally, given the exquisite cell volume sensitivity of SGK1 expression [Waldegger, S., et al., 1997] and the close relation of SLC6A8 to the osmolyte transporters BGT and TAUT [Sora, I. et al.,1994, Nash, S.R., et al.,1994], SGK1 could well participate in the adaptation of osmolyte transporters to the osmotic cell shrinkage.
In conclusion, the serum and glucocorticoid inducible kinase isoforms SGK1 and SGK3 are potent regulators of SLC6A8. The kinases might tune the creatine uptake under both physiological and pathophysiological conditions.

I- Stimulation of the creatine transporter SLC6A8:
B: by the protein kinase mTOR

The demonstrates that the mammalian target of rapamycin mTOR stimulates the creatine transporter CreaT (SLC6A8). The kinase increases the maximal transport rate without affecting the high substrate affinity[Guimbal, C., Kilimann, M.W., 1993] of the carrier.
The effect of mTOR is similar to the effect of the serum and glucocorticoid-inducible kinase [Firestone, G.L., et al., 2003], which has most recently been shown to stimulate SLC6A8 [Shojaiefard, M., et al., 2005]. The ubiquitously expressed [Waldegger, S., et al.,1997] kinase is sensitive to diverse cell stressors and a wide variety of hormones [Lang, F., et al., 2001]. The present observations do not allow any conclusion as to how mTOR and SGK1 interact in the regulation of CreaT. Possibilities include that mTOR is upstream of SGK1 and exerts its stimulating effects on transport through activation of SGK1. As an alternative possibility both kinases converge to the same activating mechanism. Whatever the mechanism, mTOR may regulate further SGK1 sensitive transport mechanisms. SGK1 has been shown to modify the activity and cell membrane abundance of a wide variety of ion channels and transporters [Lang, F. et al., 2003] including the Na^+-coupled glucose transporter SGLT1 [Dieter, M., et al., 2004], the amino acid transporters SN1 [Boehmer, C., et al., 2003], EAAT1 [Boehmer, C., et al., 2003], EAAT3 [Schniepp, R., et al., 2004], EAAT4 [Boehmer, C., et al., 2004], and EAAT5 [Boehmer, C., et al., 2005].
In view of the influence of mTOR on cell volume [Fumaro C., et al., 2005], and the close relation of SLC6A8 to the osmolyte trans porters BGT and TAUT [Nash, S.R., et al., 1994], mTOR may well participate in the adaptation of osmolyte transporters to osmotic cell shrinkage.
The effect of TOR may participate in the regulation of creatine transport in diverse physiological and pathophysiological conditions. Decreasing SLC6A8 abundance has been observed in the failing heart [Neubauer, S., et al., 1999] and defective SLC6A8 leads to mental retardation and epilepsy [Hahn, K.A., et al., 2002, Salomons, G.S., et al., 2003].
In conclusion, the creatine transporter SLC6A8 is stimulated by the mammalian target of rapamycin mTOR. The kinases might participate in the adjustment of cellular creatine content to nutrient and energy supply.

II- Stimulation of the intestinal phosphate transporter SLC34A2: by the protein kinase mTOR

This study demonstrates that the mammalian target of rapamycin mTOR stimulates the Na^+ coupled phosphate transporter NaPi IIb (SLC34A2) and thus discloses a novel regulator of this important carrier. The carrieris under the control of several hormones, which are at least in part effective through a signaling involving mTOR. SLC34A2 is critically important for the regulation of intestinal phosphate absorption and its regulation is of paramount importance for the fine tuning of phosphate balance [Werner, A., Kinne, R.K., 2001]. Phosphate is the major intracellular anion and is required for a myriad of cellular functions including energy metabolism [Knochel, J.P., 1992]. Besides compromising bone mineralization, phosphate depletion leads to cardiac insufficiency, skeletal muscle injury, anemia, and deranged function of platelets, leukocytes, liver, kidney, and brain [Knochel, J.P., 1992]. The function of the epididymal SLC34A2 is less well defined [Xu, Y., et al.,. 2003]. However, it may serve to fine tune the luminal phosphate concentration. Luminal phosphate is presumably required to provide the sperms with phosphate for their energy metabolism but its concentration must not exceed the solubility of calcium phosphate.
The effect of mTOR is mimicked by the serum and glucocorticoid inducible kinase [Werner, A., Kinne, R.K., 2001]. The kinase has been shown to regulate several channels and transporters [Lang, F., et al., 2003].
The present observations demonstrate overlapping activity of SGK1 and mTOR in the regulation of SLC34A2. Accordingly, coexpression of the inactive and thus presumably transdominant inhibitory mutant ^{K127N}SGK1 in SLC34A2 expressing oocytes virtually abrogated the stimulating effect of mTOR on Ip and abolished the rapamycin sensitivity of Ip irrespective of the additional coexpression of mTOR. In theory, mTOR may be upstream of SGK1 and may exert its stimulating effects on transport through activation of SGK1. Conversely,SGK1 may be effective through mTOR. Alternatively, both kinases could be effective through the same mechanism.
In conclusion, the phosphate transporter SLC34A2 is stimulated by the mammalian target of rapamycin mTOR. The kinase thus participates in the regulation of intestinal, epididymal, and hepatic phosphate transport.

Summery:

Creatine binds phosphate thus serving energy storage. Cellular creatine uptake is ccomplished by the Na^+,Cl^-, creatine transporter CreaT (SLC6A8). The present study explored the regulation of SLC6A8 by the serum and glucocorticoid inducible kinase SGK1, a kinase upregulated during ischemia. In Xenopus oocytes expressing SLC6A8 but not in water injected oocytes creatine induced a current which was significantly enhanced by coexpression of wild type SGK1 and constitutively active ^{S422D}SGK1, but not inactive ^{K127N}SGK1. Kinetic analysis revealed that ^{S422D}SGK1 enhanced maximal current without significantly altering affinity. The effect of SGK1 was mimicked by the constitutively active isoform ^{S419D}SGK3 but not by inactive ^{K119N}SGK3, wild type isoform SGK2 or constitutively active related kinase ^{T308D,S473D}PKB.
In conclusion, the kinases SGK1 and SGK3 increase SLC6A8 activity by increasing the maximal transport rate of the carrier. Deranged SGK1 and/or SGK3 dependent regulation of SLC6A8 may affect energy storage particularly in skeletal muscle, heart, and neurons.

Cellular accumulation of creatine is accomplished by the Na^+, Cl^-, and creatine transporter CreaT (SLC6A8). The mammalian target of rapamycin (mTOR) is a kinase stimulating cellular nutrient uptake. The present experiments explored whether SLC6A8 is regulated by mTOR. In Xenopus oocytes expressing SLC6A8 but not in water injected oocytes, creatine-induced a current which was significantly enhanced by coexpression of mTOR. Kinetic analysis revealed that mTOR enhanced maximal current without significantly altering affinity. Preincubation of the oocytes for 32 h with rapamycin (50 nM) decreased the creatine-induced current and abrogated its stimulation by mTOR. The effect of mTOR on CreaT was blunted by additional coexpression of the inactive mutant of the serum and glucocorticoid inducible kinase ^{K119N}SGK1 and mimicked by coexpression of wild type SGK1.
In conclusion, mTOR stimulates the creatine transporter SLC6A8 through mechanisms at least partially shared by the serum and glucocorticoid-inducible kinase SGK1.

Adequate phosphate homeostasis is of critical importance for a wide variety of functions including bone mineralization and energy metabolism. Phosphate balance is a function of intestinal absorption and renal elimination, which are both under tight hormonal control.
Intestinal phosphate absorption is accomplished by the Na^+, phosphate cotransporter NaPi IIb (SLC34A2). Signaling mechanisms mediating hormonal regulation of SLC34A2 are incompletely understood. The mammalian target of rapamycin (mTOR) is a kinase regulating a variety of nutrient transporters. The present experiments explored whether mTOR regulates the activity of SLC34A2. In Xenopus oocytes expressing SLC34A2 but not in water injected oocytes phosphate (1 mM) induced a current (Ip) which was significantly enhanced by coexpression of mTOR. Preincubation of the oocytes for 24 h with rapamycin (50 nM) did not significantly affect Ip in the absence of mTOR but virtually abolished the increase of Ip following coexpression of mTOR. The wild type serum and glucocorticoid inducible kinase SGK1 and the constitutively active ^{S422DS}GK1 similarly stimulated Ip, an effect again reversed by rapamycin. Coexpression of the inactive mutant of the serum and glucocorticoid inducible kinase ^{K119N}SGK1 significantly decreased Ip and abrogated the stimulating effect of mTOR on Ip.
In conclusion, mTOR and SGK1 cooperate in the stimulation of the intestinal phosphate transporter SLC34A2.

zusammen:

Creatine bindet Phosphat, das folglich Energienaufbewahrung dient. Zellulares creatine Auffassungsvermögen wird vom Na^+, Cl^-, creatine Transporter CreaT (SLC6A8) vollendet. Das anwesende Studium hat die Regelung von SLC6A8 durch das Serum und den glucocorticoid inducible kinase SGK1 erforscht, ein kinase upregulated während ischemia. In Xenopus oocytes ausdrückt den SLC6A8 aber nicht in Wasser hat oocytes creatine hat veranlasst eine Strömung eingespritzt die war bedeutend erhöht durch coexpression wilden Typs SGK1 und constitutively tätigen ^{S422D}SGK1, aber nicht untätig ^{K127N}SGK1. Kinetische Analyse hat offenbart, dass ^{S422D}GK1 maximale Strömung ohne bedeutende Verändernverwandtschaft erhöht hat. Die Wirkung von SGK1 wurde vom constitutively tätigem isoform ^{S419}DSGK3 aber nicht durch untätigen ^{K119N}SGK3, wilden Typ isoform SGK2 oder constitutively tätig verwandt kinase ^{T308D,S473D}PKB nachgeahmt.
Zum Schluss vermehren die kinases SGK1 und SGK3 SLC6A8 Tätigkeit durch Vermehren der maximalen Transportsrate vom Boten. Gestört SGK1 bzw. SGK3 abhängige Regelung von SLC6A8 kann Energienaufbewahrung besonders in skelettartigem Muskel, Herzen, und Neuronen beeinflussen.
Zellulare Ansammlung von creatine wird vom $Na^{+,}$ Cl^- vollendet, und creatine Transporter CreaT (SLC6A8). Das Säugetierziel von rapamycin (mTOR) ist ein kinase, der zellulares Nährstoffauffassungsvermögen anregt. Die anwesenden Versuche haben erforscht, ob SLC6A8 von mTOR geregelt wird. In Xenopus oocytes, ausdrückt den SLC6A8 aber nicht in Wasser oocytes eingespritzt hat, creatine eine Strömung hat veranlasst, die bedeutend erhöht durch coexpression von mTOR war. Kinetische Analyse hat jenen mTOR hat erhöht maximale Strömung ohne bedeutende Verändernverwandtschaft offenbart.
Preincubation des oocytes für 32 h mit rapamycin (50 nM) hat die creatineveranlasste Strömung abgenommen und hat seine Anregung durch mTOR annulliert. Die Wirkung von mTOR auf CreaT wurde von zusätzlichem coexpression vom untätigen Mutanten vom Serum und dem glucocorticoid inducible kinase ^{K119N}SGK1 abgestumpft und hat durch coexpression wilden Typs SGK1 nachgeahmt.
Zum Schluss regt mTOR den creatine Transporter SLC6A8 durch Mechanismen an, die wenigstens teilweise vom Serum und dem glucocorticoid dem inducible kinase SGK1 geteilt werden. Angemessene Phosphathomöostase ist von kritischer Wichtigkeit für eine Vielfalt der Funktionen einschließlich Knochens mineralization und Energienstoffwechsels. Phosphatgleichgewicht ist eine Funktion von Darmaufnahme und Nierenbeseitigung, die beide unter dichter hormonaler Steuerung sind. Darmphosphataufnahme wird vom Na^+ vollendet, Phosphat cotransporter NaPi IIb (SLC34A2). Signalisierenmechanismen, die hormonale Regelung von SLC34A2 unvollständig vermitteln, sind verstanden. Das Säugetierziel von rapamycin (mTOR) ist ein kinase, der verschiedene Nährstoffetransporter regelt. Die anwesenden Versuche haben erforscht, ob mTOR die Tätigkeit von SLC34A2 regelt. In Xenopus oocytes ausdrückt den SLC34A2 aber nicht in
Wasser hat oocytes Phosphat (1 mM) hat veranlasst eine Strömung (Ip) eingespritzt die bedeutend erhöht durch coexpression von mTOR war. Preincubation des oocytes für 24 h mit rapamycin (50 nM) machte nicht bedeutend beeinflusst Ip in der Abwesenheit von mTOR aber hat praktisch die Zunahme von Ip coexpression von mTOR abgeschafft folgend. Das wilde Typserum und glucocorticoid inducible kinase SGK1 und der constitutively tätig ^{S422D}SGK1 hat ähnlich Ip, eine Wirkung angeregt, die wieder von rapamycin umgekehrt worden sind. Coexpression vom untätigen Mutanten vom Serum und dem glucocorticoid inducible kinase ^{K119N}SGK1 bedeutend abgenommen Ip und hat die Anregungswirkung von mTOR auf Ip annulliert.
Zum Schluss arbeiten mTOR und SGK1 in der Anregung vom Darmphosphat Transporter SLC34A2 zusammen.

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LIST OF PUBLICATION:

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1990: Central roles of a $_2$-noradrenergic activity in TENS induced analgesia
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1991: Comparing the effects of central a $_1$ & a $_2$ adrenoceptors in TENS induced analgesia.
The 10th Iranian Congress of physiology & pharmacology, Ahvaz University of Medical Sciences

2001: Effect of ICV injection of GLP-1 and its related peptides on sertonine metabolism & on level of amino acids in the rat hypothalamus.
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10th Congress of Biology, Biology dept. Shiraz University of Medical Sciences
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