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Cooperation between potassium channels and gap junctions: interaction between Kv1.1 channel and Pannexin 1

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"There is a motive force greater than steam, electricity and atomic energy : the willpower."

Albert Einstein

Abstract

The beta 3 subunit of voltage gated potassium channels has been recently identified as a modulatory macromolecule of pannexin 1. Our interest has focused on the possible interaction between the alpha subunit of Kv1.1 channel and pannexin 1. Through voltage clamp studies we have analyzed the electrical activity of the single channels and their behavior when they were coexpressed. With our results we have demonstrated that pannexin 1 was less susceptible to its inhibitors, like probenecid and DTT, when it was coexpressed with Kv1.1 channel, on the contrary pannexin 1 did not seem to influence the activity of Kv1,1 channel. Through immunocytochemistry on HEK-hBK1 cells expressing in stable way Kv1.1 channel we have observed the colocalization of the two channels but through coimmunoprecipitation we proved the lack of a physical interaction between these proteins, therefore the interaction should be functional. Moreover previous studies has reported an involvement of pannexin 1 in apoptosis at elevated concentrations of extracellular potassium. So we wanted to estimate the cell death in presence and absence of pannexin 1 inhibitors, using like control SH-SY5Y cells, being a cell line that does not express Kv1.1 channel. With our results we have observed a decrease in the cell death when HEK-hBK1 cells were treated with 1 mM probenecid in presence of 140 mM KCl, suggesting that this behavior was the consequence of pannexin 1 inhibition, therefore in these conditions Kv1.1 channel did not influence in some way its activity; on the contrary the treatment with 10 mM DTT did not produce any beneficial effects in HEK-hBK1 cells. These findings confirmed that Kv1.1 channel influenced the sensibility of pannexin 1 to its inhibitors only when redox potential was altered and then in presence of reducing agents, or when a depolarization of the membrane was induced like in oocytes, but this phenomenon didn't occur in other conditions. Probably this indirect interaction is mediated by other proteins, such as calmodulin and kinase proteins or by lipids of the

membrane such as PIP2; it could represent a regulatory mechanism that replaces or enhances that exercised by beta 3 subunit on pannexin 1, in order to control the 'potassium buffering' and then the cellular excitability and survival, both in pathological and physiological conditions.

Contents

Glossary

Br^-	bromide ion
Ca_{2+}	calcium ion
Cl^-	chloride ion
Cs^+	cesium ion
H^+	proton
K^+	potassium ion
Li^+	litium ion
$MgCl_2$	magnesium chloride
$NO3^-$	nitrate
$NaHCO_3$	sodium carbonate
Na^+	sodium ion
Rb^+	rubidium ion
$\mathbf{\Delta E}$	equilibrum potential for ions
$\mu\mathbf{A}$	microamper
$\mu\mathbf{F}$	microfaraday
$\mu\mathbf{M}$	micromolar
$\mu\mathbf{g}$	microgram
$\mu\mathbf{l}$	microlitre
Ala	Alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
A°	amstrong

b.p	base pairs
BSA	bovin serum albumin
C	capacity
$CaCl_2$	calcium chloride
CaMKII	calcium calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
$CaNO_3$	calcium nitrate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CK1	casein protein kinase
Cx	connexin
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DMEM	modified eagle medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
Eag	ether-à-go-go potassium channel
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulus
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GΩ	gigaohm
G.Js	gap junctions

GFP	fluorescent green protein
Gln	glutamine
Glu	glutamic acid
HEK-293	normal human embryonic kidney cell line
HEK-hBk1	normal human embryonic kidney cell line stably expressing Kv1.1 channel
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERG	human channel ether-à-go-go-related
His	histidine
Ic	current intensity
IgG	immunoglobulin
KATP	ATP-dependent potassium channel
Kb	kilobase)
KchAP	K channel-associated protein
KCl	potassium chloride
KCNQ	M-type potassium channel
KcsA	potassium channel from streptomycens lividans
kDa	kilodalton (1000 dalton; 1 dalton=1,650 × 10-27)
kHz	kiloherz
Kv1.1	voltage-gated potassium channel, subfamily A, member 1
Kv1.2	voltage-gated potassium channel, subfamily A, member 2
Kv1.3	voltage-gated potassium channel subfamily A, member 3

Kv1.5	voltage-gated potassium channel subfamily A, member 5
Kv1.6	voltage-gated potassium channel subfamily A , member 6
Kv3.4	voltage-gated potassium channel subfamily C, member 4
L.B	standard medium used to grow bacteria
LTP	long-term potentiations
Lys	lysine
M	molar
MΩ	megaohm
M-type currents	voltage-dependent potassium current that persists at slightly depolarized membrane potentials
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
mg	milligram
MgSO4	magnesium sulphate
mM	millimolar
mm	millimetre
mRNA	messenger RNA
ms	millisecond
nA	nanoamper
NaCl	sodium chloride
ng	nanogram
nl	nanolitre
nm	nanometre
NMR	nuclear magnetic resonance

p.m	molecular weight
P2X7	purinoreceptor for ATP
Panx	pannexin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	domain that binds to a short region of the C-terminus of other specific proteins
pF	picofaraday
PIP2	Phosphatidylinositol 4,5-bisphosphate
PK	kinase protein
PMSF	phenylmethylsulfonyl fluorid
Q	electric charge
R	resistence
RNA	ribonucleic acid
Rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SEM	standard error
Ser	serine
SH-SY5Y	human derived neuroblastoma cell line
SNC	central nervous system
SNP	peripheral nervous system
Src	tyrosine kinase
STP	short-term potentiations
TBST	tween phosphate buffered saline
TEA	tetraethylammonium
Tes	2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid

TM	transmembrane
TRICT	tetramethyl rhodamine isothiocyanate
Trp	tryptophan
Tyr	tyrosine
U.V	ultraviolet radiation
ZO-1	zonula occludens

Chapter 1

Introduction

Gap junctions and ion channels play an important role in the cellular homeostasis and in the synchronization of electrical signals. A functional interaction between potassium channels and gap junctions has been already documented (GJ Christ, Drug News Perspective, 13 (1): 28-36, 2000; Kotsias et al, J Biol States, 203 (3): 143 -50, 2005), but more recent researchs have shown that also a direct interaction between ion channels and gap junctions occurs (Chanson et al, Prog Biophys Mol Biol, 94 (1-2) : 233-244, 2007). In particular i want to focus on a recent study that described the existence of a physical interaction between the mouse $\beta 3$ subunit of voltage-dependent potassium channel and the C-terminal region of mouse pannexin 1 (Bunse et al ., FEBS Journal, 276, 6258-6270, 2009) , presenting the beta 3 subunit as new modulator of pannexin 1. In fact this investigation reported that the electrical activity's inhibition of pannexin 1 by reducing agents and blockers (like probenecid) is compromised when it is coexpressed with the Kv$\beta 3$ subunit, confirming the idea that the latter controls the pannexin activity. This is the first example of functional modulation and physical interaction between pannexin and potassium channels. The study of this cooperation has a great importance because it could clarify the physiological pathways that underlie the electrical synapse and explain the functions of this interaction. Before discussing in detail the work i've done i'm going to do a brief introduction about the electrical communication, the gap junctions and the

potassium channels, in order to better understand their relationship.

1.1 Electrical Cell communication: the synapse

Today it is demonstrated that there are basically two different intercellular communication mechanisms, one that involves the release of secreted molecules such as hormones and neurotransmitters that interact with receptors on neighboring cells and the other is based on the formation of channels that permit a direct contact between the cells with the passage of ions, metabolites and second messengers, allowing the electrical coupling. Synapses are structures through which two neurons communicate (one is called 'presynaptic' and the other that receives the signal is called 'postsynaptic'). Interneuronal communication has been discussed for years, some neuroscientist was in favor of electrical transmission, in which the action potential in the presynaptic cell induces a current flow to the post-synaptic cell, therefore there is no an actual anatomical separation between the two cells but, small molecules and small ions pass directly from the cytoplasm of one cell to another through gap junctions (G.Js) and ion channels, the passage of the potential is direct and faster; while other scientists were in favor of chemical transmission, in which between the presynaptic membrane and the post-synaptic membrane there is an inter-synaptic space, then can't exist a direct electrical communication and transmission of signals occurs because a chemical substance released by the presynaptic neuron interacts with the receptor channel in the post-synaptic membrane activating a series of intracellular responses (release of cytosolic calcium, phosphorylation and activation of PK, CaMKII etc), allowing the propagation of the nerve impulse. Examples of neurotransmitters are acetylcholine, GABA, monoamines, dopamine, that bind their corresponding receptors causing the opening and the entry of ions. These events cause a depolarization with the further opening of additional voltage-gated channels. Anyway has been proved the existence of both chemical and electrical transmission and it is assumed that in vertebrates one

could be complementary to the other (see fig. 1.1). For several years it has been believed that the chemical synapses were only present in the brain of vertebrates. The presence of electrical synapses has been shown for the first time in the axon of the crayfish motor nerve, in these synapses the contact between two neurons is by G.Js (Furshpan&Potter, Nature 180:342-43, 1957; Watanabe, Jpn. J. Physiol. 8:305-18 , 1958; B. Litch et al. Journal of Neurocytology, 18 (6), 749-761; WJHeitler et al. Journal of Neurocytology, Vol 20, No 2, 109-123, 1991). Electrical synapses function as 'low pass filter', that transmit low frequency stimuli; the transmission power is more rapid than in chemical synapse, moreover the electrical synapse can be two-directional. This type of communication for many years was considered a feature of the structures of invertebrates, but in reality it plays a key role also in the development of the vertebrate CNS, when chemical synapses are still immature and their number is limited. It has been observed that the electrical synapses is linked to certain events during the development of the CNS, such as cell differentiation, migration and the formation of neuronal circuits. This coupling decreases with the differentiation proceeding. The electrical synapses are abundantly present in the brains of mammals, Cx36 is widely expressed in central nervous system where it plays a key role in coupling neuronal electrical, and probably other proteins are involved like connexin 43 and pannexins. In the CNS electrical synapses seem to occur in most regions of the brain, as in the lower olivary nucleus, in the interneurons of the cerebellum, in the the reticular nucleus of the thalamus, in the retina, in the hippocampus, in the neocortex, in the olfactory bulb and also between the motoneurons synapses. This type of synapse could also allow the passage of small signaling molecules between cells, previous experiments of 'dye coupling' support this idea (Hatton GI., 1998, Cell Biol. Int 22:765-80; Roerig B, Feller MB, 2000, Brain Res Rev 32:86 -114, BW Connors et al. Annu. Rev. Neurosci. 2004, 27:393-418). Several studies have also demonstrated the existence of mixed synapses (chemical and electrical), in which gap junctions play an important role (for example in motoneurons). Mixed synapses are considered a single functional unit (Michelson and Wong, J. Physiol. 477, 35-45, 1994; Bernard, J. Neurophysiol. 77, 3134-3144,1997, Gibson et al. Nature

402:75-79, 1999; Mann-Metzer and Yarom, J Neurosci.19(9):3298-306, 1999; Fukuda and Kosaka, Neurosci. 20(4):1519-28, 2000). Several studies have suggested that mixed synapses are usefuls to synchronize the 'inflammation' of neurons (Bernardo, J. Neurophysiol. 77, 3134-3144, 1997; Galarreta and Hestrin, Nature 402, 72-75, 1999, Tamas et al., Nat Neurosci 3:366–37, 2000) and myocytes in the myocardium, or that they represent a combination to facilitate the excitation of the post-synaptic fiber; it is like electrical transmission prepares the chemical nerve excitation, (Galarreta and Hestrin, Nat. Rev. Neuro. 2, 425-433, 2001). In addition, mixed synapses are implicated in neuronal ritmogenesis (Galarreta and Hestrin, Nat. Rev. Neuro. 2, 425-433, 2001), providing a mechanism of correction and adjustment of the excitation. Important studies have been performed on the pyloric neurons, in which the two types of communication presented opposite effects in the transmission of the signal from lateral pyloric neuron to the constrictor neuron , in fact when the lateral pyloric neuron (PL) explodes, the electrical transmission promotes the activity of the pyloric constrictor (PY) in which the chemical component acts by killing it (through dopamine) (A. Mamiya et al., The Journal of Neuroscience, October 22, 2003 23 (29):9557-9564). In motoneurons about 3-5% of the axo-somatic and axo-dendritic synapses are mixed and about 30-100% are excitatory synapses.So it is clear that the mixed synapse have a significant influence on the activity of post-synaptic neurons (in the CNS and PNS) and that the chemical and electrical transmissions don't work alternatively, but rather they cooperate. In vitro experiments have shown that the conditions of the medium in which cells growth can modulate the formation of synapses. In fact, when the nerve-cells were cultured and juxtaposed in vitro they formed inappropriate electrical synapses, presumably because of the contact conditions between cells, instead when they were grown separately they formed chemical synapses. The G. Js in the CNS have been found also in oligodendrocytes and astrocytes (Massa and Mugnaini, Neuroscience, 7 : 523-538, 1982; 1982; Giaume et al. Neuron 6, 133-143, 1991; Altevogt et al., Neurosci 22:6458-6470, 2002) and play an important role both during embryogenesis and in the adult brain, in fact transgenic studies have shown the presence of electrical synapses in the rodent adult brain like in motoneu-

rons of the rat (Jerash et al. Neurobiology, Vol 93, pp. 4235-4239, April 1996; Galarreta and Hestrin, Nature 402, 72-75, 1999; Beierlein et al. Nat. Neurosci. 3, 904-910, 2000; Blatow et al., Neuron, Vol. 38, 79-88, 2003), these data confirms again that these two types of communication are complementary. In conclusion the mixed synapses are widely present in most regions of the CNS of mammals, the abundance of these synapses suggests that they play an important role in the transmission of electrical signals but the mechanism by which they function are still unclear.

1.2 Gap junctions

The junctions between cells are of various types, generally consist of: occluding junctions (tight junctions), their main components are the occludins and claudins that seal the gaps between adjacent epithelial cells; adherens junctions, that are formed by cadherins and catenins and create adhesion between cells; the desmosomes, that serve to connect the cells through a filament structure and also connect the cytoskeleton filaments of two adjacent cells; finally the gap junctions, they constitute transmembrane channels that permit the communication between the cytoplasm in adjacent cells through the passage of small ions, small metabolites ($<$ 1kDa) and second messengers, (WR Loewenstein, Phy-siol. Rev 61, 829-913, 1981) . The term 'gap' means 'regular separation'. In fact in two adjacent cells their cytoplasmatic membrane is separated by a regular space of 2 or 3 nm. Gap junctions are multicellular channels large about 16-20 A °.These cylinders have been identified using staining with lanthanum hydroxide (Revel and Karnovsky, J.Cell Biol. 33: C7-C12, 1967). Each cell contributes to the adjacent channel with a hemichannel that consists of six protein subunits, that are called connexins (Beyer et al., J.Membr.Biol. 116, 187-194, 1990, Willecke et al., Eur.J.Cell.Biol. 56, 1-7, 1991; Bennett et al., Neuron 6, 305-320, 1991, Bruzzone et al., BioEssay 18, 709-718, 1996). The hemichannels are bound with the help of other proteins, the cadherins; when they consist of identical connexins are called 'homomeric' , if are formed by different connexins are called 'heteromeric'. Moreover the gap junction is called homotypic when consists

of two identical hemichannels, it is called heterotypic when consists of different hemichannels. So we can have four possibilities: G. J homotypic / homomeric, G. J heterotypic / heteromeric, G. J homotypic/heteromeric, G. J heterotypic / homomeric (see fig. 1.2). The closing and opening of these channels is influenced by change in voltage, pH and intra-extracellular ion concentration, by the interaction with macromolecules, by phosphorylation reactions (because the conformation of the protein changes), moreover their expression and permeability is controlled by hormons like estrogens in the uterus. The mechanism by which hormones control the permeability of these channels is still unclear, probably this modulation occurs through cyclic AMP. Neurotransmitters may also modulate the permeability of GJS, such as dopamine with an inhibitory action, and acetylcholine, that also reduces the permeability of gap junctions, but how this happens is not yet clear. In both cases the inhibition is reversible. The modulation of the gap junction conductance is of two types: short-term and long-term. The short term modulation is due to changes in permeability in the range of time between seconds and minutes, is too fast that can't cause changes in the turnover of these proteins, it is possible that it is due to alterations of the basic properties of the channels , like variations in pH, in intracellular calcium, in neurotransmitter signaling. Surely this fast modulation permits the cell to respond rapidly to specific stimuli, this phenomenon is called 'functional plasticity'. The long term modulation occur during a larger time, is due to changes in properties of gap junctions or changes in their number. It can be caused by interaction with extracellular messengers, in this case the turnover of these proteins changes. For example, this type of modulation occurs during development (Neyton et al., J. exp. Biol. 124, 93-114 (1986)). Communication mediated by gap junctions is required for many biological processes, including cell growth and differentiation, embryonic morphogenesis, metabolic homeostasis, muscle contraction and secretion, synchronization of electrical activity. Their pore size is about 1.5-2 nm and permit the passage of ions and small molecules of 1-1.5 kDa (metabolites, second messengers, etc.), but gap junctions are not selective pore, cations and anions can cross the channel, the passage is free also for dyes such as LY and DAPI (Cao et al, Journal of Cell

Science 111, 31-43 (1998)). The channel formed by Cx43 and Cx40 is permeable to various ions such as Cs^+, Rb^+, K^+, Na^+, Li^+, H^+, Br^-, Cl^-, $NO3^-$, acetate, glutamate, but with different affinity for these ions, this affinity depends on the composition and the internal charge. The positive ions cross the pore that is partially hydrated and anions move in the opposite direction to the flow and can be complexed with cations (Wang et al, J.Gen.Physiol, 109, 491-507, 1997; Beblo et al, J . Gen.Physiol, 109, 509-522, 1997). The channel is also permeable to divalent cation, such as Mg^{++}, Ba^{++}, (Fireck Ludwick et al, J Mol Cell Cardiol, 27, 1633-1643, 1995, Matsuda et al, Prog Biophys Mol Biol. 2010 Sep; 103 (1):102-1) and Ca^{++}. The closure is often triggered by phosphorylation of the subunits constituting the channel, closure can be observed also by increasing the concentration of intracellular calcium. On cell membrane gap junctions are often present in the form of plaques or agglomerations of many channels (Wang et al, Journal of Cell Science 108, 3501-3508, 1995). The junctions are easily detectable with the use of dyes such fluorescein. Connexins form a family of membrane proteins that are different for their molecular weight and aminoacid sequence; according to the nomenclature they are indicated with the abbreviation Cx followed by a number indicating the molecular weight. All connexins have four transmembrane regions (TM1-4) in alpha helix structure, two extracellular loops (E 1-2), one intracellular loop (L), and two cytoplasmic terminal parts, the one shorter is placed before TM1 region and is the amino terminal part, the one longer is placed after TM4 region and is the carboxyl terminal part. Connexins are divided into three groups α, β and γ, according to the structure of their gene. The two extracellular loops E1 and E2 and the transmembrane regions are the portions most conserved, for this reason show high degree of homology. For example all connexins have the repetition of three cysteine residues in the extracellular loops. Instead the intracellular loop L and the C-terminal part are not conserved regions, this confer specificity to the protein, in fact these portions are needed for the recognition between two cellular connexons. Furthermore, by varying the length of these regions varies also the molecular weight. The loop E1 moreover should be the voltage sensitive region.Twenty different connexins have been identified in the genome of mice

and rats and three pannexins (Bruzzone et al., Eur J Biochem 238(1):1-27, 1996, Condorelli et al., J. Neurosci. 10, 1202-1208, 1998, Sohl et al., FEBS Lett 428:27-31, 1998). Connexins have been found both in vertebrates and in chordates.

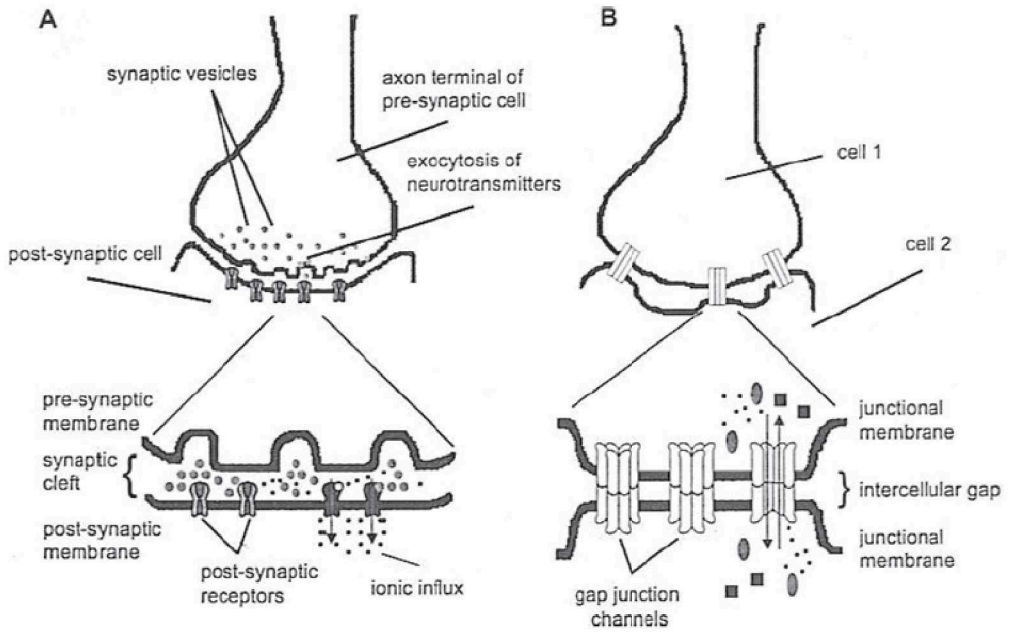


Figure 1.1: chemical (A) and electrical (B) (Zygon®, Volume 44, Issue 4, pages 807–824, December 2009)

The specificity of connexins to certain tissues is related to their function, the same connexins can be expressed in different tissues and a specific tissue may coexpress different connexins, allowing the phenomenon of redundancy or compensatory expression that could be helpful in pathological conditions and is important to maintain cellular homeostasis. It is possible that this redundancy exist also between pannexins and / or between pannexins and connexins. The anomalous variation in the number of gap junctions results in pathological states, but this variation can also have a physiological significance. In mammals the G .Js in the uterus are highly expressed in pregnant females, this phenomenon contributes to improve the synchronization of contractions during the labour. The increase in the number is possibly caused

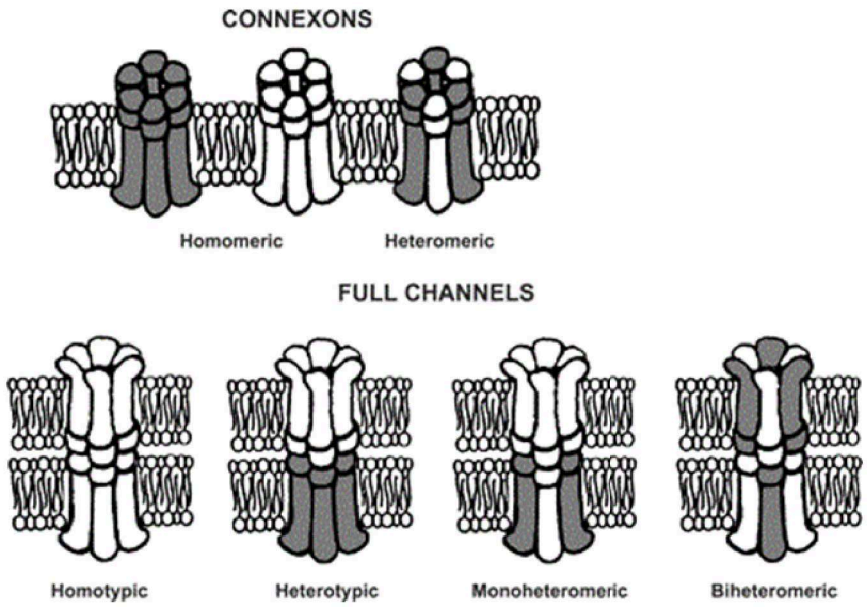


Figure 1.2: homomeric and heteromeric gap junctions (Cardiovasc Res (2004) 62 (2):276-286).

by hormonal stimulation. After delivery, the gap junction number decreases again, because these proteins are removed by endocytosis and degraded as all proteins in the proteasome. The compatibility code of connexins allows only selective interactions between connexons. In some cases, the connexins can form only homotypic channels , in other cases can form heterotypic channels. For example, Cx46 may form channels with at least 5-6 connexins (Elfgang et al., J Cell Biol. May;129(3):805-17, 1995; White et al., Kidney Int. , Oct;48(4):1148-57, 1995), instead Cx31 can only produce homotypic combinations. In addition, the connexin composition of gap junctions influence the permeability of the channel. For example, the heteromeric gap junction formed by Cx32 and Cx26 is not permeable to cGMP and cAMP, instead the homomeric channel formed by Cx32 is permeable to both signal molecules. The compatibility is strongly linked to the second extracellular domain E2 (White et al., J. Cell Biol. 125: 879-892, 1994). For example Cx43 functionally interacts with Cx46 but not with the Cx50, but swapping the domain E2 of Cx50 with that of Cx46 the capacity to form channels with

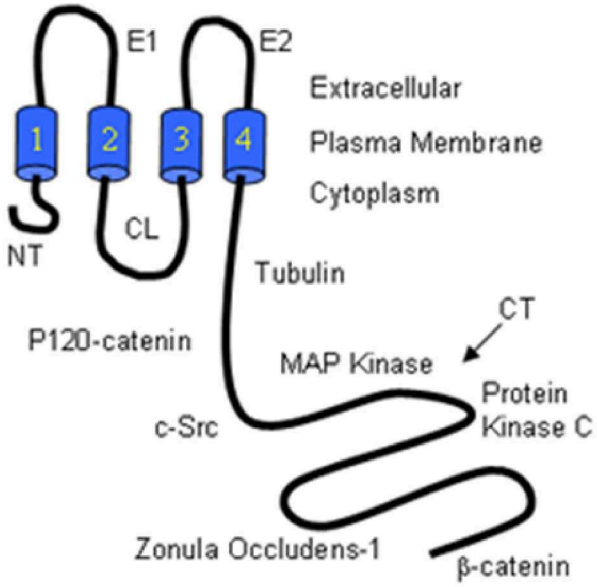


Figure 1.3: model of a connexin with cellular partners

Cx43 is moved to the new chimeric macromolecule. This ability of connexins to discriminate between one and the other is very important because it may represent a mechanism to limit rather than promote cellular communication, this is necessary in certain physiological situations. It represent infact a basic mechanism for the 'compartmentalization' within the same tissue, so that the cells communicate in the area of the same group but not between different groups. This event mainly occurs during embryonic development. The gap junctions, however, have further partners of interaction, infact connexins can bind calmodulin, kinase proteins and the zonula occludens (ZO-1), a membrane protein especially present in tight junctions of epithelial and endothelial cells; this protein plays a role in the connexin targeting to the plasma membrane, it mediates also their interaction with the cytoskeleton and perhaps also influences the interaction between different connexins. The GJS can bind also other components of tight junctions, Cx26 for example directly interacts with the occludin (Nusrat et al., Biol Chem. 22;275(38):29816-22, 2000) and has binding sites for calmodulin. These interactions must be still

explored to understand the function and effects on gap junctions.

1.3 Pannexins, a new family of gap junction proteins

Pannexins (from the greek "pan," neuter of the adjective "pas," which means "all," "whole," "entire" to reflect their broad expression in both protostomes and deuterostomes) have recently been discovered, they form a second family of proteins belonging to the gap junctions. Like connexins they are transmembrane porins and consist of two hexameric hemichannels. They are structurally similar to connexins, they have four transmembrane portions, two extracellular loops, an intracellular loop and the N-and C- terminal portions facing on the cytoplasm (see fig. 1.4). But pannexins are genetically different, they derive from innexins, a group of proteins that form intercellular channels in invertebrates (Panchin, Journal of Experimental Biology 208, 1415-1419, 2005; Barbe et al, Physiology 21: 103 -114, 2006.). There is infact some sequence similarity between pannexins and innexins from shellfish, viruses and insects (see fig. 1.4 A). Also in poly DNA virus they have been recently identified and are called vinnexins. Pannexins don't have aminoacid sequence similarity with connexins but contain two conserved residues of cysteine in the extracellular loop. Today we know only three pannexins : pannexin 1, pannexin 2 and pannexin 3 (panx1,panx2,panx3), their functions are still unclear. **Pannexin 1** is more expressed in the central nervous system but is also present in other tissues, pannexin 2 is expressed only in the CNS, are both localized mainly in the interneurons of the hippocampus and in Purkinje cells, pannexin 1 alone or in combination with pannexin 2 induces formation of active intercellular channels, instead homomeric hemichannel of pannexin 2 is not active (plausibly the formation of this channel is involved in the compartmentalization process); the expression of pannexin 3 has been identified in the brain but mainly in the skin, in osteoblasts and in fibroblasts with pannexin 1 (Bruzzone et al., PNAS, 2003 November 11, 100 (23): 13644-13649; Baranova et al., Genomics 83, 706-716, 2004, Bench et al.

Curr. Biol. 10, R473-R474, 2000; Bench, The Journal of Experimental Biology 208, 1415 - 1419, 2005). Pannexin 1 forms mechano-sensitive channels in fact, electrophysiological studies have shown that pannexon is active after 'stretching' (see fig. 1.4 C); it is probably also voltage-sensitive like channels formed by connexins whose opening and closing is dependent on changes in electrical potential (Bao et al. FEBS Letters 572 (2004) 65-68; Yang Qu et al. PNAS, January 22, 2002, vol. 99, no. 2, 697-702). Pannexin 1 molecular weight changes from 37 kDa to 48 kDa, according to the degree of glycosylation. Pannexins infact can exist in different isoforms due to post-translational modifications such as phosphorylation and glycosylation that occur during their synthesis and are important for their formation (Boasso et al, The Journal of Biological Chemistry, 282 (43), 31733 -31743, 2007, Jiang et al, Investigative Ophthalmology & Visual Science, Vol 34, No. 13, 3558-3565.., 1993;. Shearer et al, Investigative Ophthalmology Visual Science &, April 2008, Vol 49, No..4,1553-1562). The post-translational modifications (PMT) can affect the folding of proteins and are important for their function, it is interesting that some proteins with different structure and sequence, but with similar PMT show similar function. Pannexins unlike connexins are highly glycosylated (from mannose), but Pannexin 1 exist also in a non-glycosylated form (Peñuela et al, Journal of Science 120, 3772-3783, 2007; Peñuela et al, MBoC, Vol 20, Issue 20, 4313 -4,323, 2009); glycosylation occurs in predicted aminoacid sites as in the arginine of extracellular loop in position 254, that is important to address the protein on the cytoplasmic membrane (Boasso et al, The Journal of Biological Chemistry, 282 (43) , 31733-31743, 2007), but the reaction may also occur in the C-terminal part (Peñuela et al, Journal of Science 120, 3772-3783, 2007; Peñuela et al, Molecular Cell Biology, Vol.20, 4313-4323 , 2009). Glycosylation is important for the cellular localization of pannexins and also for their interaction and assembly. In fact when these proteins exist in non-glycosylated form they have intracellular distribution because fail to reach the membrane. N-glycosylation occurs on arginine residues and O-glycosylation occurs on serine or threonine residues present mainly in the cytoplasmic portions. In pannexins happens mainly N-glycosylation (see fig. 1.4 B). Panx1 moreover facilitates the location of panx 2 on the cell

surface and also colocalizes with panx3, so panx1 should be important for the functionality of the other two pannexins. The function of pannexins is not yet clear. They could have an important role in the synchronous activity in the brain, for example pannexins are also expressed in certain types of interneurons, it is interesting to note that the residual gamma activity in the knockout animal can be completely wiped out with carbenoxolone, which is a potent pannexin blocker (Pais I et al., J Neurophysiol 89: 2046–2054, 2003). A relationship between schizophrenic symptoms and disruption of neural synchrony has been suggested by a recent paper that reported a lower frequency of gamma-band oscillations in schizophrenic patient (Ray A et al., Eur J Neurosci 21: 3277–3290). Moreover it has been demonstrated that the function of the nucleus accumbens may be disturbed in schizophrenia; pannexin 1 is distributed also in this nucleus (O'Donnell et al., Ann NY Acad Sci 877: 157–175, 1999) .Thus it is probable that all neuronal gap-junction proteins are candidate genes in the familiar forms of schizophrenia. Pannexins can work like hemichannels (Huang et al.Neuroscience 104 (15):6436-6441, 2007), because past studies through oocyte voltage clamp have reported that, first injection of synthetic RNA for rat Panx1 results in the development of nonselective, voltage-activated currents in the nonjunctional plasma membrane, indicating that this pannexin can assemble homomeric hemichannels; second, Panx1 hemichannels are permeable to small molecules; third, Panx1 shows the ability to form functionally competent intercellular channels that can be closed by commonly used gap-junction blockers (Bruzzone et al., J Neurochem 92: 1033–1043, 2005; Bruzzone et al., Proc Natl Acad Sci USA 100: 13644–13649, 2003; Locovei et al., FEBS Lett 572: 65–68, 2004). Moreover previous studies have shown that pannexins have a role in the ATP release and in the calcium homeostasis; for example the hemichannel activity is important in erythrocytes where in response to low oxygen it releases ATP, and in astrocytes where hemichannels are formed only by pannexin 1 and not by connexins (Bao et al. FEBS Lett, 572, 65 - 68, 2004; Locovei et al, PNAS, 2006 May 16, 103 (20): 7655-7659; Iglesias et al., The Journal of Neuroscience, May 27, 2009, 29 (21):7092-7097). Pannexon activity may be required in many cellular pathways that are still unclear or unknown, an

example is the involvement in ILβ1 release and in caspase-1 cascade, that are mechanisms induced by activation of ATP-gated P2X7 receptor expressed on macrophages; recent studies have shown that pannexin 1 is associated with this receptor, moreover that its blocking inhibits the release of ILβ1 and that when it is overexpressed it works like a non-selective hemichannel stimulating the cascade of caspases (Pellegrin et al. The EMBO Journal (2006) 25, 5071-5082). Panx 1 is also involved in the calcium wave or indirectly by ATP releasing and cooperation with P2Y receptor and through IP3 diffusion, or directly with calcium diffusion between cells (Barbe et al, Physiology, 21:. 103 -114, 2006;. Abeele V. et al, The Journal of Cell Biology, Vol 174, No. 4, 535-546, 2006). Anyway further studies are required to clarify the cellular pathways in which pannexins are involved.

1.4 Mechanisms of regulation in gap junctions: pannexins and connexins

The opening of gap junctions is certainly regulated but further analysis are necessary to clarify the mechanism of this regulation. For example intracellular calcium appears to play a role in the modulation of gap junctions (as in Cx32) through calmodulin, that interacts with the C-terminal part of connexins (Torok et al, Biochem J. 326, 479-483, 1997.) and possibly with the C-terminal region of pannexins. Also the extracellular ATP might have a regulatory action, infact previous studies reported that ATP has an allosteric effect on pannexin 1, inhibiting its permeability (Qiu et al, Cell Physiol, 296: 250-255, 2009). Another factor that regulates the activity of gap junctions is the temperature. A recent research documented that hemichannels formed by Cx26 increases its conductivity after heating, this temperature dependence may have a physiological significance in thermoregulation, in fact, Cx26 is expressed in the skin (Steffens et al. BBA, Vol 1778, 1206 -1212, 2008). It is interesting to note that Panx1 is also expressed in the skin. Many studies have also shown that cytoplasmic acidification affects the activity of gap junctions and that can cause alterations on phosphorylation

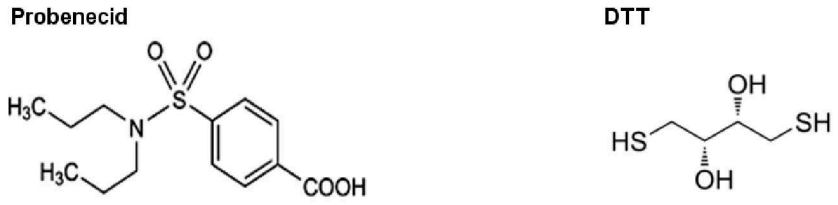
sites in the C-terminal part with a consequent conformational change and closing of the channel (Alan F. Lau, Sci STKE, 2005 (291). In particular, studies performed on Cx43 show that acidification leads to protonation of the histidine residues in Cx43L2 sequence (in the intracellular loop) facilitating the interaction with Cx43CT (the C-terminal part), moreover this acidification increases the formation of alpha-helices, in particular in 119-144 domain that is important for dimerization between Cx43CT and Cx43CL (Alan F Lau Sci STKE;. 2005 (291), J. Hirst-Jensen et al, The Journal of Biological Chemistry Vol 282, No. 8, pp 5801-5813, 2007; Sorgen et al, Biophys J, Vol 87, 574-581, 2004). This is a hypothetical model of 'ball-and-chain' inactivation (Duffy et al, J Biol Chem, Vol 277, No. 39, 36706-36714, 2002, Duffy et al, Circ Res 94, 215-222, 2004) where the low pH facilitates the process of dimerization that is also regulated by phosphorylation (Sorgen et al, Biophys J, Vol 87, 574-581, 2004; Hirst-Jensen et al, The Journal of Biological Chemistry Vol 282, No. 8, pp 5801-5813, 2007). Pannexins could have a similar mechanism of dimerization. Acidification inhibits activity of gap junctions also because reduces their coupling (Rorig et al., January 1, 1996 The Journal of Physiology, 490, 31-49). In addition, pure lipids can play a role in the modulation of pannexins, in particular in their conformation, in fact, cholesterol is involved in the remodeling of gap junctions (Biswas et al, Molecular Vision 2009, 15:1492-1508) and can stimulate or inhibit their assembly and their permeability depending on the concentration of cholesterol (Lars Bastians et al, Cardiovasc Res 33, 272-283, 1997, Meyer et al, J Cell Sci, 96:231-238, 1990). Lipids may be also involved in the formation of plaques, where gap junctions shows an increased activity (Ghosh et al , Bioelectrochemistry, 68, 150-157, 2006); studies on the clustering of pannexins must still be done.

The reversible phosphorylation by kinases is another mechanism of modulation of a large number of proteins, including Panx1. For example, the permeability of Cx43 is affected by phosphorylation in Ser 368, resulting in a conformational change in the C-terminal part (Bao et al., J Biol Chem, 279 (19), 20058-20066, 2004). NetPhosK program identifies many sites of phosphorylation in the sequence of Panx1 and particularly in the C-terminus,

for example, it reports that Ser 328 could be phosphorylated by PKA and DNAPK, Ser 343 by PKC, Thr 383 by PKC, Ser 405 by CKII etc. C-terminal part is the portion that mainly interact with other macromolecules such as zonula occludens ZO-1, calmodulin and kinase proteins, the phosphorylation itself can modulate these interactions (The Journal of Biological Che- mistry, Sorgen et al, 279 (52), 54695-54701, 2004;. The Journal of Biological Chemistry, Singh et al, 280 (34), 30416-30421, 2005, Toyofuku et al., J Biol Chem, 276 (3), 1780-1788, 2001). Modulation mechanisms of pannexins are little known, so further studies on their phosphorylation, glycosylation and possible interactions with calmodulin, ZO and other macromolecules should be performed. Finally, it is also interesting to consider the pharmacological modulation of pannexins, in particular carbenoxolone (anti-ulcer medication), FFA (NSAIDs) and the probenecid (drug used for gout) have already been identified as blockers of pannexin 1, also reducing agents like DTT (1,4-dithio-D-threitol) and TCEP (Tris(2-carboxyethyl)phosphine) cause its inhibition, as shown by the analysis of electrical activity (Bruzzone et al, Journal of Neurochemistry, 2005, 92, 1033-1043; Silverman et al, Am J Physiol Cell Physiol 295:761-767, 2008; But Weihong, The Journal of Pharmacology and Experimental Therapeutics, Vol 328, No. 2, 2008; Bunse et al., FEBS Journal 276 (2009) 6258). Previous patch and voltage clamp studies in oocytes show that the channels costituted of pannexins are activated by extracellular potassium and have a large conductance of up to 500 ps when perfused with 150 mM KCl (Li Bao et al, FEBS Letters 572 (2004) 65-68). How potassium chloride actives pannexon in vivo is still unclear, the hypothesis is that the ion binds the extracellular domain directly or through an auxiliary molecule, causing the activation, moreover if the pannexin activation by potassium chloride occurs also in vivo is not yet known (Silverman et al, The Journal Of Biological Chemistry Vol 284, No. 27, pp. 18143-18151, July 3, 2009). Inhibitors such as carbenoxolone and probenecid significantly inhibit the electrical activity in a dose-dependent manner (Silverman et al, The Journal of Biological Chemistry, Vol.284, N 27 , 2009). Carbenoxolone abolishes the electrical activity of pannexins with much lower doses than those requests to inhibit connexins, probenecid seems to be enough specific

for pannexins rather than connexins. The mechanism by which probenecid abolishes the activity is not clear, may interact with the hydrophilic parts of pannexin or access to the channel through the lipid layer of membrane considering that it is lipophilic. Moreover it induces depolarization of the plasma membrane, interferes with mitochondrial oxidative phosphorylation and ATP production (Masereeuw British Journal of Pharmacology (2000) 131, 57 – 62). At a concentration of 1 mM there is almost total inhibition of electrical activity (Silverman et al, Am J Physiol Cell Physiol 295: C761-C767, 2008; But Weihong, The Journal of Pharmacology and Experimental Therapeutics, 328:409-418, , Vol 328, No. 2., 2009). Recent researchs have shown that the susceptibility of pannexin 1 to reducing agents and to their inhibitors is reduced when coexpressed with the Kvβ3 subunit (Bunse et al. FEBS Journal, 276, 6258-6270, 2009) and that a physical interaction occurs between both proteins. These data are interesting because the same phenomenon could occur during hypoxic conditions such as ischemia. However, pharmacological characterization of gap junctions and in particular of pannexins requires further evaluations, as well as their functional and structural characterization.

Chemical structure of Probenecid and DTT:



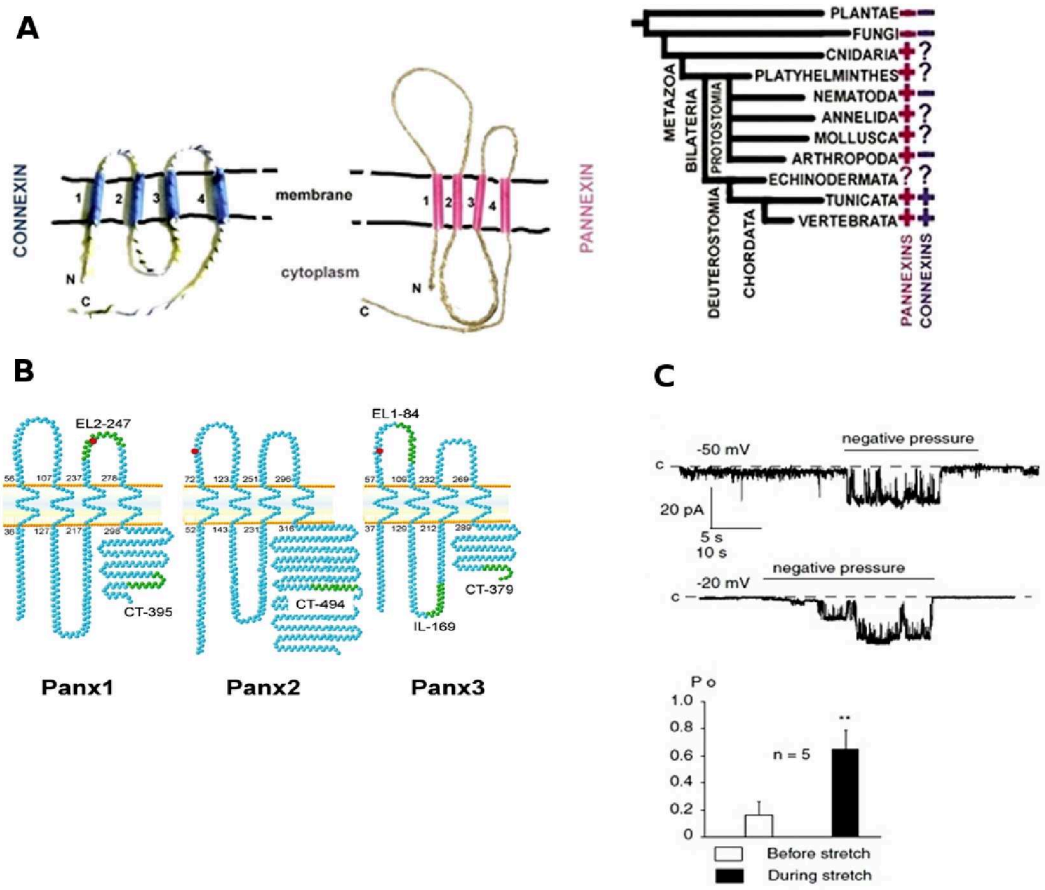


Figure 1.4: A: topography of pannexins and connexins (The Journal of Experimental Biology 208, 1415-141, 2005); B :glycosylation sites of pannexins (Journal of Cell Science 120, 3772-3783, 2007); C: mechanical activation of pannexin channels (FEBS Letters 572 (2004) 65–6).

1.5 Action Potential

Electricity plays an important role in biology, when solutes such as phosphate, aminoacids and inorganic ions are transported across cell membranes the movement of their charges produces an electrical current that generates a membrane voltage difference. In general, electrical signals are mainly generated by the flow of inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl^- that pass through large transmembrane proteins called ion channels. To generate an action potential in a neuron are required millions of ions in one millisecond, so to fill this request without using millions of proteins is necessary that each single channel presents high adaptability and high selectivity. A cell can be compared to a RC circuit (resistor-capacitor) based on the resistance and on the presence of a dynamic element, the condensator that stores and releases electrical charges (see fig. 1.5). The membrane is in fact able to conduct ionic current, and has both resistive and capacitive elements. The resistance depends on the cell ability to transport ions, it is inversely proportional to the amount of water present in the cell (for example the lipidic bilayer is a poor conductor, unlike water, and opposes a resistance to the passage of ions); seeing that the flow is given by the opening of ion channels, the higher the number of open channels the greater the conductance and less the resistance. The capacity of the membrane depends on its hydrophobic nature because the lipidic bilayer is a poor conductor. When the charges are separated by the lipidic bilayer generates a potential difference whose magnitude depends on the characteristics of the capacitor. The membrane capacity thus influences the transition phase during which the action potential changes. At the opening of the 'circuit' the current is predominantly capacitive, then when the potential reaches the stationary phase, the current becomes resistive. In fact, the flow of capacitive current occurs only at the beginning and at the end of the potential change, but, during the fixed phase of the potential, ionic current can be considered pure. Extracellular fluids have high concentration of sodium and chloride instead intracellular fluid contains mostly potassium and organic anions, this difference of ionic distribution generates also a difference in electric charge between the membrane sides, the inte-

rior side will be negatively charged and the exterior side will be positively charged. The capacity of the membrane, as mentioned before, maintains this charge separation generating the potential difference. The amount of charge Q is proportional to the difference in potential and capacity:

$$Q = C \times V$$

The movement of charges from one side to the other generates the electrical power that is proportional to the capacity and to the speed at which the voltage changes over time:

$$IC = C \times \frac{dV}{dt}$$

So we can express the variation of the voltage as a function of current and time during which the current is passed:

$$dV = \frac{I_c \times dt}{C}$$

Seeing that the capacity increases with the size of the cell, to determine a change of membrane potential in a large neuron respect to a smaller neuron will be necessary more charges, and therefore more current, so cells of smaller dimensions are more excitable. Nervous and muscle tissues are for example excitable tissues, when cells do not conduct impulses they are at rest. Typical values of resistence for a cell move between 1 MΩ and 100 MΩ, current values move normally from fractions to hundreds nA. The rest membrane potential remains around -70mV, this value varies depending on the type of cells (nerve, skeletal or smooth muscle cells), the resting potential of neurons for example is - 65mV. During this resting phase anion and sodium channels are closed, but potassium channels remain open. The resting potential is generated by an unequal distribution of ionic species between intracellular and extracellular fluid; sodium and chloride are in fact about 40 times more concentrated outside the cell and potassium 50 times more concentrated inside the cell. If the cell was permeable only to one ion it will reach the electro-chemical equilibrium.

Intracellular concentration

Sodium : 12 mM
Potassium: 150 mM
chloride : 4 mM

Extracellular concentration

Sodium: 145 mM
Potassium : 4 mM
chloride: 118 mM

The Nernst equation allows to calculate the potential difference at the electro-chemical equilibrium of a single ion:

$$\Delta E_x = \frac{RT}{zF} \log \frac{[X]_e}{[X]_t}$$

where:

- ΔE_x = equilibrium potential for ion X
- R= gas constant
- T = absolute temperature (Kelvin)
- z = valence of the ion
- F = Faraday constant (96,500 coulombs / gram equivalent of charge)
- $[X]_i$ = intracellular concentration of the ion X
- $[X]_E$ = extracellular concentration of the ion X

The Goldman equation instead gives a quantitative description of membrane potential, it can be considered as an approximation of Nernst equation but takes in consideration the permeability to different ions:

$$Vm = \frac{RT}{zF} \log \frac{[Na^+]ePNa + [K^+]ePk + [Cl^-]iPCl}{[Na^+]ePNa + [K^+]ePK + [Cl^-]ePCl}$$

With the help of these formulas we can obtain the contribution to the potential of ionic diffusion when the cell is at rest phase. An action potential is a electrical fluctuation that travels along the surface of the cellular plasma membrane, it occurs when a cell is "activated", so when it carries out a pulse. The Hodgkin-Huxley model is a mathematical equation that describes the process of depolarization in the neuron, is based on the analogy of the neuron with an electrical circuit. The findings of Hodgkin and Huxley scientists about cell depolarization, the membrane potential inversion and their dependency on external ion concentrations, led them to believe that there was an ionic species responsible for this behavior. Thus it born the idea of the 'sodium current', according this idea the membrane depolarization causes first a rapid rise and then a slow descent of the membrane permeability to sodium ions (Na^+) followed by a slow rise in permeability to potassium ions (K^+). The experiments carried out between 1938 and 1940 by Cole and Curtis, and then by Hodgkin and Katz, showed that the action potential of squid giant axon is due to an abrupt change in the Na^+ flow crossing the membrane. The hypothesis of Hodgkin and Huxley was as follows: the membrane depolarization opens Na^+ channels, allowing a massive entry of Na^+ into the cell and thus producing the action potential upswing, followed by the downturn of the action potential caused by the closure of Na^+ channels that reduces the influx of this ion, and the opening of K^+ channels that leads to greater release of K^+ from the cell. To test this hypothesis it was necessary to measure the changes of the conductances of Na^+ and K^+ channels when membrane potential E changes. Cole invented a method to measure these changes called "at voltage block" (voltage clamp), which allowed Hodgkin and Huxley to perform their experiments. The block voltage circuit consists in connecting with two electrodes the ends of a battery to the fluids that contact the axon membrane sides. In this way the membrane potential is controlled by the experimenter, which may impose variations. The amperometer records the current that the battery must deliver to maintain a constant membrane potential, this current repre-

sent the current flowing through the membrane. Analyzing the conductance in correspondence to depolarizing pulses of different intensity and duration, Hodgkin and Huxley obtained the empirical equations that allow to predict with good accuracy the size and shape of action potential, refractory period and other characteristics of the nerve impulse (Hodgkin AL, Huxley, AF, andKatz, B., Measurement of current-voltage relations in the membrane of the giant axon of Loligo J. Physiol. 116: 424-448, 1952). The complexity of the model does not allow the mathematical resolution (a significantly simplified model is that of Fitzhugh-Nagumo), however, it allowed to explore and understand the electrical circuit of a neuron. The model describes three types of channels: sodium channels, potassium channels and leak channels that are represented mainly by chloride channels.

The equations are as follows:

$$\begin{cases} C\frac{dV}{dt} = g_{Na}m^3h(V - V_{Na}) - g_K m^4(V - V_K) - g_L(V - V_L) + I_a \\ \frac{dm}{dt} = \alpha m(V)(1 - m) - \beta_m(V)m \end{cases}$$

dove:

- C is the capacity of the membrane
- I_a is the external electrical impulse
- g_{Na} and g_K are the conductance of sodium and potassium ions
- V is the equilibrium potentials
- gL is the leak conductance of linear channels

The phases of an action potential in general can be described like this:

- An adequate stimulus causes the opening of sodium channels. Sodium enters the cell and establishes a local depolarization

- If the local value of the depolarization reaches or exceeds a certain value (threshold potential, with an average of about - 50 mV) many voltage-gated sodium channels open. The potential is the minimum threshold value for the opening of these channels. If the threshold is not reached these voltage-gated sodium channels remain closed, without generating an action potential.

- The entry of sodium causes decrease in the cell membrane potential, which assumes positive values (on average up to +30+60 mV), so occurs an inversion of charge between the membrane sides.

- The voltage-gated channels remain open for about 1 millisecond, then close again.

- Once reached the action potential and after the closing of voltage-gated sodium channels, the cell begins the repolarization phase, the membrane potential returns to its resting negative value. The voltage-dependent potassium channels open only to values of around +30/+60mV. The potassium ions begin to go out from the cell for reasons of chemical and electrical gradient because there is an excess of positive charges in the intracellular side of the membrane; moreover during this phase the permeability to chloride increases and calcium channels open (plateau phase), then calcium gets in the cell and when the entry of calcium is equal to the exit of potassium occurs the plateau. Later also calcium channels close and potassium continues to get out from the cell.

The flow of potassium towards the extracellular space restores the original excess of positive ions on the external surface. The potassium channels may remain open even when the cell has reached the resting potential: in this way a further amount of potassium comes out and for a short period of time the cell can be hyperpolarized reaching values more negative like -80/-90mV; at this stage it is impossible creating another depolarization.

- Finally the action of Na^+/K^+ pumps that bring out sodium ions and the closure of all voltage-gated channels restore the state of normality.

The action potential works on 'all or nothing law', it means that it occurs maximally or not at all and only if the depolarization reachs the threshold value.The refractory period is the amount of time it takes for an excitable membrane to be ready for a second stimulus once it returns to its resting state following excitation. The absolute refractory period is the interval during which a second action potential absolutely cannot be initiated, no matter how large a stimulus is applied. The relative refractory period is the interval immediately following during which initiation of a second action potential is inhibited but not impossible.

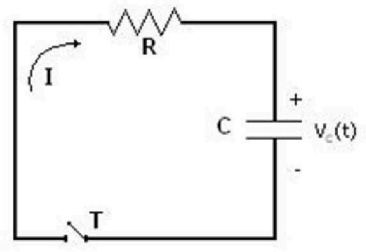


Figure 1.5: RC circuit

1.6 Potassium channels

Ion channels are divided into: voltage-gated channels that open in response to changes in membrane potential, ligand-gated channels that open upon interaction with a chemical mediator, and mechanical channels that only open after mechanical movements of the membrane. The potassium channels are widely distributed, they transport potassium across the cellular membrane, they are classified in :

- Calcium-dependent channels that open in the presence of calcium.
- Channels 'inwardly rectifying' in which the ion current is directed toward the intracellular space.

- Channels 'tandem pore domain', that are constitutively open or have high activity, maintaining the negative membrane potential; they are similar to leak channels represented mainly by chloride channels, that are independent of the voltage and are always open, participating in the natural permeability system of the cell.

- Voltage-gated channels that open and close in response to changes in membrane voltage.

Potassium channels represent a large family of channels, including about 70 different genes, of which about 40 genes encode for voltage-gated potassium channels such as eukaryotic Shaker channels that are homologous to Shaker channels from Drosophila melanogaster. Structurally, Shaker potassium channel consists of 6 transmembrane alpha helices segments (S1-S2-S3-S5-S5-S6) and the N and C-terminal cytoplasmic portions (see fig. 1.6, 1.7 and 1.8). The C-terminal portion is the part that mainly interacts with the cytoplasmic macromolecules, for example in voltage-gated channels it contains the PDZ binding domain that binds 'like PSD-95' protein, this interaction is important for the clustering. In addition, the C-terminal region belongs to the recent class of proteins called 'intrinsically disordered', or rather proteins that lack a defined tertiary structure under physiological native conditions. In fact, the C-terminal sequence has some unusual features for a folded protein ,it is rich in hydrophilic aminoacids, glutamine repeats and presents depletion of hydrophobic aminoacids. These features suggest that the primary sequence is unstructured and therefore belongs to the 'intrinsically disordered protein' family, thus with highly flexible structure that facilitates the different conformations required for the binding to enzymes or other proteins (Magidovich et al. Bioinformatics, Vol . 22 no. 13 2006, pages 1546-1550). The S1 and S4 segments are voltage sensitive, in particular S4 region contains residues of lysine and arginine that are positively charged and are involved in the voltage-sensitive function, infact their movement during the depolarization leads to the channel opening. The domain of TM S5-S6 P-core is the part involved in the selectivity, in the inactivation and in the tetramerization. All potassium channels are tetrameric with each monomer

placed around the central pore that presents structural differences between the various types of channels. The S4 segment contains cysteine residues, for example in Shaker channels has been studied the function of cysteine in position 361; these cysteine residues with the formation of disulfide bridges are important for the activation of the channel, infact the removal of these aminoacids leads to a mutant that is susceptible to oxidation, the latter occurs in absence of C-type inactivation, impeding the channel opening. So the formation of disulfide bonds is part of the activation process and contributes to the structural changes (Aziz et al., The Journal of Biological Chemistry, Vol 277, No. 45, Issue of November 8, pp. 42719-42725, 2002). Studies on 'voltage gating' were performed in KvAP voltage-dependent channel (of Aeropyrum pernix). This channel is structurally different from Shaker channels, however, presents some aminoacidic sequenc's similarity; its 3D structure is already known, it has been used to study the interactions between the subunit S1 and S4, and other regions of the channel like the pore, obtaining various models of interaction (Shrivastava et al. Biophysical Journal Volume 87 October 2004 2255-2270). All potassium channels present in the pore region TM five aminoacids highly conserved , TVGYG in KcsA (bacterial channel), this domain is homologous to the eukaryotic domain (see fig. 1.12 and 1.14) and it is involved in the selectivity. Normally the potassium ion is solvated by water molecules, in particular, is surrounded by two groups of four oxygen atoms, the selective filter of the channel is designed precisely to mimic the structure of water around the potassium ion, in fact, the aminoacids involved in the selectivity expose oxygen groups for the ion solvation. The structure of the pore fails to adapt to the sodium ion, perhaps for the size, so the sodium ion can not cross it. In fact, the hypothesis is that the constriction of the channel is not sufficient to accommodate the dehydrated sodium ion that is too small and therefore the energy barrier to transfer the ion is too high.

1.7 Mechanisms of inactivation and destabilization in potassium channels

Potassium channels must be able to open and close rapidly in response to biological signals, this process is called 'gating' and is due to conformational changes of the channel protein. However, usually differences in the structure of the pore domain correspond to different gating mechanisms, for example the bacterial KcsA channel gating depends on the pH , the gating of KvAP (Aero-pyrum pernix) and eukaryotic Kv channels depends on voltage. A channel moves from closed state to open state in less than 10 microseconds, a channel never takes the shape of 'half open'. There are actually three states: closed, open and inactive. In the closed state the channel does not work but it can be activated after a stimulus, usually channels close when the membrane is at rest condition; instead in the inactive state the channel can not be open from any pulse. The inactivation follows a period of prolonged activation, for example, is a phenomenon that occurs during the refractory period and it is reversible. One of the most important mechanisms of inactivation of these channels is the N-type Inactivation , also called A-type Inactivation or Fast Inactivation, in which a subunit of the N-terminal portion (called 'ball') blocks physically the passage of ions. Alternatively, the inactivation may be mediated by the beta subunit, that is an auxiliary subunit of the channel, it blocks the channel with its N-terminal portion in a mechanism similar to N-type inactivation (see fig. 1.8 , 1.9 and 1.10). In mammals there are three beta subunits, the $\beta 1$, $\beta 2$ and $\beta 3$. They are highly conserved. During the depolarization the channel opens transiently, then falls in the inactive state due to a conformational change and then opens again during repolarization. The model of 'ball-and-chain' inactivation has been studied on potassium channels expressed in Drosophila melanogaster (encoded by the Shaker gene) and on channels expressed in oocytes of Xenopus frog. Experiments of deletions and insertions have shown that the first 83 aminoacids of the N-terminal portion are involved in the channel inactivation. In fact when some of the first 20 aminoacids were changed the channel lost the capacity of inactivation, but when deletions were induced

in the aminoacidic portion 20-83 the channel showed hyperinactivation, on the contrary insertions reduced it. Moreover intracellular trypsin removed the inactivation, suggesting that in the 'ball' were present sites for trypsin cleavage (see fig. 1.11).

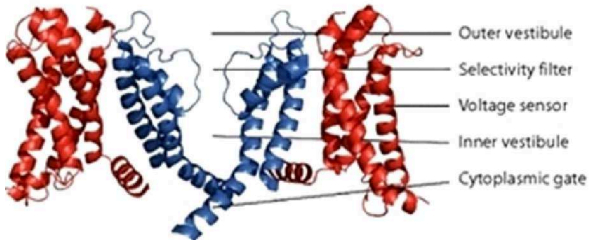


Figure 1.6: functional voltage-gated potassium channel domains (The Journal Of Biological Chemistry, Vol. 283, No. 37, pp. 25105–25109, 2009).

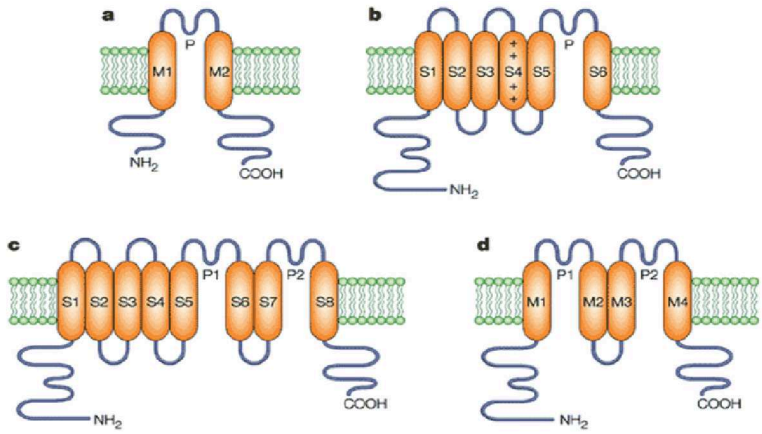


Figure 1.7: structure of potassium channel in various species: a: KcsA ; b: Shaker ; c: TOK1,motivi trovati nei funghi; d: Paramecium bursaria, (Nature Reviews Neuroscience 3, 115-121 , 2002).

Patch clamp studies have also shown that the ' ball-and -chain' inactivation is not voltage-dependent, infact only during activation there is a movement of charges, but any electrical component seems to be associated to the inactivation. The latter is instead removed by proteolytic agents and the increase of extracellular potassium concentration causes a total recovery of activity (Antz et al., News Physiol. Ski. Volume 13, August 1998), but

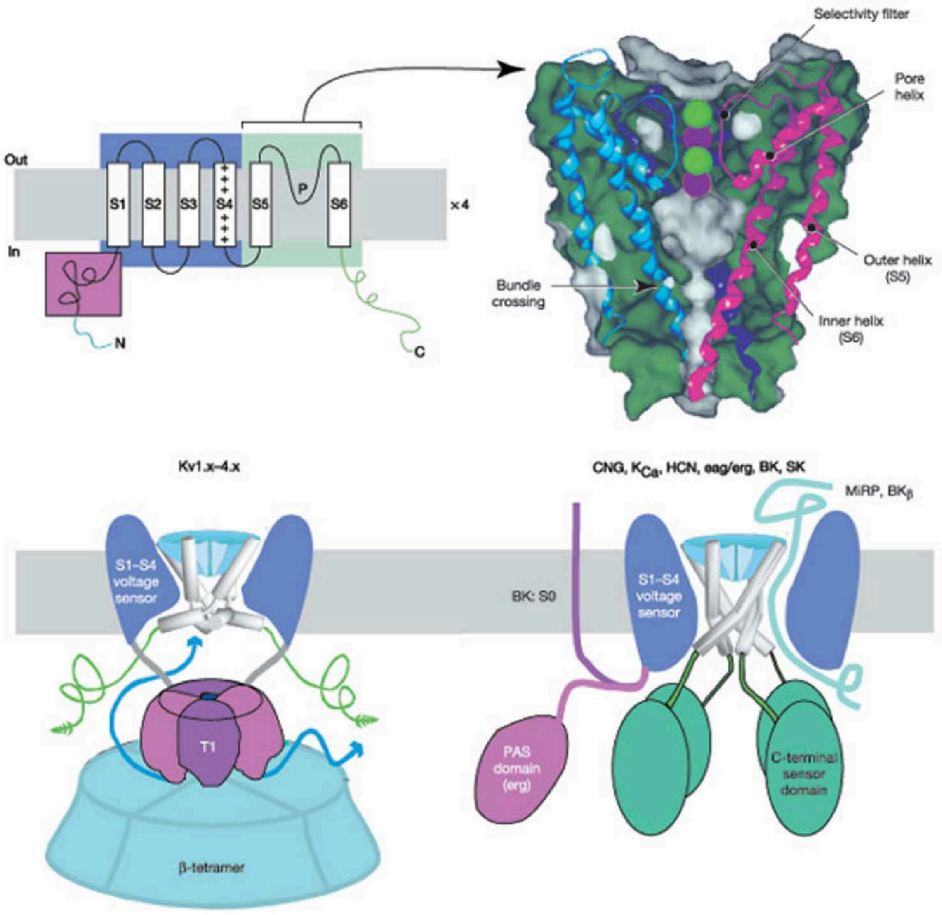


Figure 1.8: the tetrameric 6TM architecture of the K^+ channel family (Nature 419, 35-42, 5 September 2002)

also an excess of extracellular potassium can inactivate the channel; moreover inactivation can be mimicked by blockers such as tetraethylammonium (TEA) and 4-aminopyridine. Voltage gated potassium channels infact are very sensitive to TEA inhibition, this molecule binds the extracellular tyrosine residue in position 449 in each subunit of the tetramer, the consequent inactivation is also temperature-dependent, suggesting that this interaction is not purely hydrophobic (Heginbotham L; Neuron. 1992 Mar;8(3):483-91).

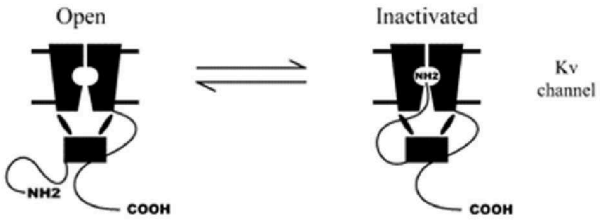


Figure 1.9: 'ball and chain' inactivation (Bioinformatoc, Vol. 22 no. 13 2006, pages 1546–1550).

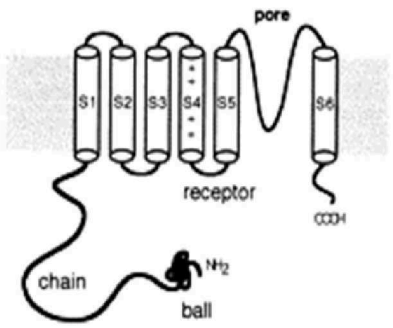


Figure 1.10: NH2-terminal ball (News in Physiological Sciences, Vol. 13, No. 4, 177-182, 1998)

In addition to the N -type inactivation and the inactivation mediated by beta subunit, there is also an additional blocking mechanism called C-type inactivation that is due to a transition of the selective filter and represent a slower inactivation. This type of inactivation has been studied in KcsA, the potassium channel from Streptomycens lividans. This channel is used

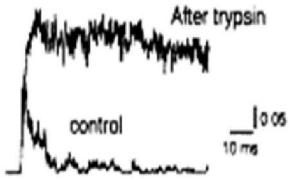


Figure 1.11: removal of trypsin inactivation (News in Physiological Sciences, Vol. 13, No. 4, 177-182, 1998)

as model to explore the molecular basis of selectivity and ion permeation, because it presents a simplified structure,it is a tetramer, each unit of the tetramer is composed of two transmembrane subunits M1 and M2 connected by an extracellular loop, that have sequence and structure homologies with S5 and S6 subunit of the eukaryotic channel (see fig. 1.7 and 1.14); it is permeable to potassium, rubidium, cesium, and is not very permeable to sodium and lithium. Moreover past studies have shown that mutations of some residues in TM domain of KcsA makes it similar to the Shaker channel, it gets high affinity binding to agitoxin (AgTx2) that blocks Shaker channels, on the other hand chimeric channels obtained by the replacement of segments S5 -P- S6 of Shaker channels with M1-P- M2 segments of KcsA are functional channels, suggesting that the structure of the pore domains is similar in the two proteins. The structure of crystallized KcsA was the first to be identified and this has allowed to understand some of the workings mechanism of potassium channels.

In KcsA does not occur the N-type inactivation. The C-type inactivation is governed by the interaction between the residues W67, D80 and E71 (see 1.13), because previous studies have shown that the change of glutamate in position 71 with valine causes the lack of inactivation, the mutant infact has high conductance even in the presence of sodium (Cuello et al. Nature. 2010 July 8, 466 (7303): 203-208; Choi etc. al. Biophys J. 2004 April; 86 (4): 2137 - 2144.). Moreover this channel undergoes the destabilization in the presence of high concentrations of extracellular cations, not only in the presence of sodium ions to which the channel is not permeable, but also in the presence

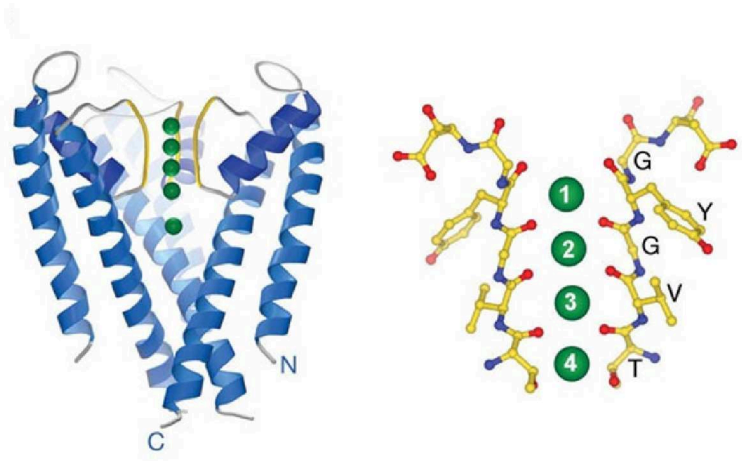


Figure 1.12: KcsA structure and selectivity filter (Nature 414, 37-42(1), 2001)

of potassium, in fact the high extracellular concentration of this ion leads to the dissociation of the tetramer, to the affinity reduction for the ion and thus to the inactivation of the channel. Anyway similar process of destabilization could occur in the eukaryotic channels. As well as in KcsA, in eukaryotic voltage-gated channels the aminoacids that are involved in C-type inactivation are adjiacent to the highly conserved region of the pore that is critical for the integrity of selectivity; this type of inactivation is voltage-dependent. The interaction between certain aminoacid sites present in the pore leads to a constriction of the channel inactivating it. The channel goes into a state of low opening probability that is sensitive to extracellular potassium concentration, when the external potassium increases the channel passes in this reversible inactive state (Morales et al., Nature Structural Molecular Bio-logy &, Volume 13, Number 4 April 2006, Kiss et al. Biophysical Journal Volume 74 April 1998 1840-1849). Recent studies of patch clamp in giant liposomes showed that the ball peptide, that is the 'ball' domain of N-terminal portion of Shaker channels involved in the ball-and-chain mechanism, is able to give 'Fast Inactivation' in KcsA channel. In fact, when the synthesized ball peptide is placed in the bath solution the electrical activity reduces (Molina et al., The Journal of Biological Chemestry Vol 283, No. 26, pp. 18076-18085, June 27, 2008).

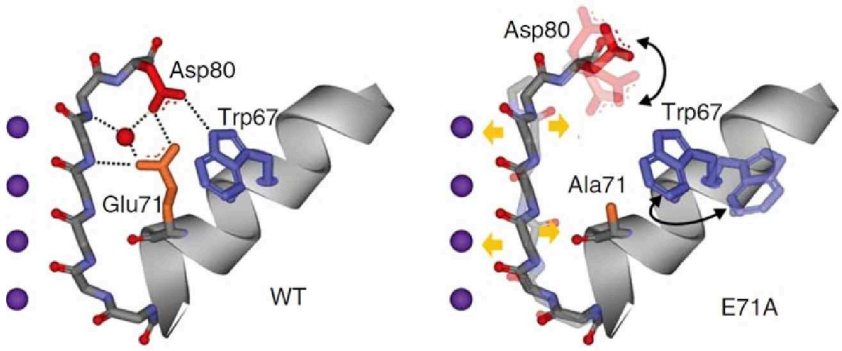


Figure 1.13: aminoacidic interaction in KcsA C-type-inactivation (Nature Structural & Molecular Biology, Volume 13, Number 4, 2006)

1.8 Eukaryotic voltage gated potassium channels

Eukaryotic voltage-gated channels are formed by tetrameric transmembrane glycoproteins, called also 'alpha subunits'. They form aqueous pores for the passage of potassium that open and close in response to changes in voltage. These channels are involved in the maintaining of the resting potential, in repolarization, and in the temporal control of action potential repetitions in neurons; they also participate in electrical activity of smooth muscle and myocardial cells (Koh, SDET al. delayed rectifier potassium currents of Contribution to the electrical activity of murine colonic smooth muscle, American Journal of Physiology 515, 475-487, 1999) and in electrolyte homeostasis of epithelium. The first potassium channel was cloned from Drosophila Melanogaster fly (Papazian et al., Science (Wash DC) 237:749–753, 1987). Thereafter began the identification of this very large family of ion channels,the voltage-dependent potassium channels. Initially, four genes of Kv were cloned in Drosophila: Shaker, Shab, Shal and Shaw. These genes had different isoforms by differential splicing. The first channels cloned into mammals were associated with these isoforms, for this reason the KV1 subfamily was called Shaker . The various isoforms are encoded by different genes allowing a differential expression in different cell types. Currently 11 human

Kv channel subfamilies are known with in total 38 members. The current nomenclature names the channels in according to their structure: Kvx.y, where "K" refers to potassium channel, "v" refers to its voltage dependence, "x" is the number that would refer to the gene family, and "y" numbers the channels of each subfamily in the order as they have been cloned.

The **Kv1.1 channel** belongs to the eukaryotic voltage-gated channels' family A (shaker related), it is encoded by the gene KCNA1 and is highly expressed in the auditory system which has an important role in the transmission of action potentials, it is also prevalent in many parts of the CNS, in neurons and in glia, in myocardium and the interstitial cells of Cajal (smooth muscle cells of the gastrointestinal tract). Like all Shaker channels it forms a tetramer, in wihich each alpha subunit consists of 6 transmembrane segments (S1-S2-S3-S5-S5-S6) and the N and C-terminal cytoplasmic portions. Although this channel as well as the majority of ion channels and gap junctions, can aggregate into clusters with highest activity and can form heteromeric channels with other proteins of subfamily A, like with Kv1.4 (Zhu J. et al., J Biol Chem. 2003 Jul 11;278(28):25558-67). Many studies on mouse L-cells (fibroblasts) transfected with rat Kv1.1 channel cDNA have shown that the immunopurified Kv1.1 channel has a molecular weight ranging from 55 to 59 kDa according to the degree of glycosylation , the non-glycosylated form has a molecular weight of 55 kDa, however,it exists mostly in the glycosylated form as well as pannexins; the glycosylation sites are mainly on the extracellular side, like arginine in 207 position, and they bind especially sialic acid; Shaker channels have infact around 120-220 negatively charged sialic acid residues. Previous voltage clamp studies had reported that the elimination of glycosylation does not alter neither channel function and stability, nor its assembly (Deal et al., The Journal of Neuroscience, 14 (3), 1666-1676, March 1994). But later further analysis has shown that on the contrary glycosylation can affect the channel function, stabilizing its open state and destabilizing its inactive state. Infact channels with deficient glycosylation have a slower kinetics of activation. The hypothesis is that the reduction of sialic acid reduces the negative charges of the protein, this reduction is perceived by the voltage sensor on S4 subunit so that a

higher depolarization is required to activate the channel (Thornhill et al, The Journal of Biological Chemistry, Vol 271, No. 32, Issue of August 9, pp. 19093-19098, 1996). Moreover in the non-glycosylated channel the C-type inactivation kinetics and the recovery from this inactivation are slower, the transaction from active to inactive state is also slower and the gating properties are altered, because the lack of negative charges decreases the sensitivity to extracellular cations. So the not-glycosylated channel becomes less sensitive and responds less to signals; possibly the glycosylation affects the conformation of the channel (Watanabe et al. J Physiol (2003), 550.1, pp. 51-66) and then its operation. It's interesting that the channel obtained from protein extracts of mouse brain instead presented a molecular weight of 80 kDa, this could have a functional significance and suggest that there are several isoforms of the same channel according to their function and the tissue in which they are expressed (Deal et al., The Journal of Neuroscience, 14 (3), 1666-1676, March 1994). Kv channels as well as gap junctions are regulated also by phosphorylation and thus they have binding sites for kinase proteins; for example kinase C inhibits the conductance of Kv channels. In Kv1.5 and Kv1.6 channels the threonine in position 318 is the site of PKC phosphorylation, in rat Kv1.1 channel instead the phosphorylation occurs on serine in position 442, which is located in the S6 region. However, the effects of this protein kinases on the voltage-gated potassium channels are different for different molecular species of Kv channels, for example PKC protein causes the increase of current amplitude in human Kv3.4 channel unlike than in Kv1.1 channel. These opposite effects can be explained by the structure differences between the various Kv channels (Boland et al. Am J Physiol Cell Physiol 277: C100-C110, 1999). Also tyrosine kinase SRC binds potassium channels, through the SH3 domain; Kv1.5 channel contains proline-rich sequence between residues 65 and 82 that bind this protein kinase. Patch clamp studies have shown that when this channel is coexpressed with SRC protein, the currents are suppressed (Holmes et al. SCIENCE, VOL. 274, 20 DECEMBER 1996). Also in Kv1.1 channel the phosphorylation inhibits its activity; the reaction is mediated by protein kinase C that phosphorylates serine 322 and threonine 318 (that are residues conserved in almost

all shaker channels)(Boland et al., Am J Physiol Cell Physiol 277: C100-C110, 1999). There are also other proteins that interact with Kv channels and influence their behavior. For example a cytoplasmic protein KChAP (K +-channel associated protein) that transiently binds Kv channels promots its surface expression . This protein belongs to a family of proteins that bind transcription factors, but it is unique in its binding to Kv channels. KchAP acts as a chaperone for Kv2.1 and Kv4.3 but it has been reported that it interacts also with other Kv channels (Kuryshev et al., Am J Physiol Cell Physiol281: C290–C299, 2001) and that it increases the amplitude of the current without altering the activity in Kv1.3/2.1/2.2/4.3 but has no such effect on Kv1.1/1.2/1.4/1.5/1.6/3.1 channels (Kuryshev and al., Am J Physiol Cell Physiol, vol. 278 no. 5C931-C941, 2000). A further control on potassium channels is mediated by membrane phospholipids, such as PIP2, that is involved in the opening of many potassium channels, for example KCNQ channels that are positively regulated by PIP2 (Ann R. Rittenhouse, June 18, 2008, JGP vol. 132 no.1 5-8). Finally the voltage gated potassium channel's activity can be pharmacologically manipulated, infact they are inhibited by various molecules such as TEA, 4-aminopyridine,dendrotoxin and aminotriptylin (a tricyclic antidepressant drug). α-Dendrotoxina is the most used agent to study Kv1.1 channel, because it blocks specifically Kv1.1 and Kv1.2. Understanding these regulation mechanisms is very important because their effect can alter the electrical and chemical communication in response to specific stimulus and can influence the channel interactions with other proteins. However, further studies are still required for a complete characterization of these complex proteins.

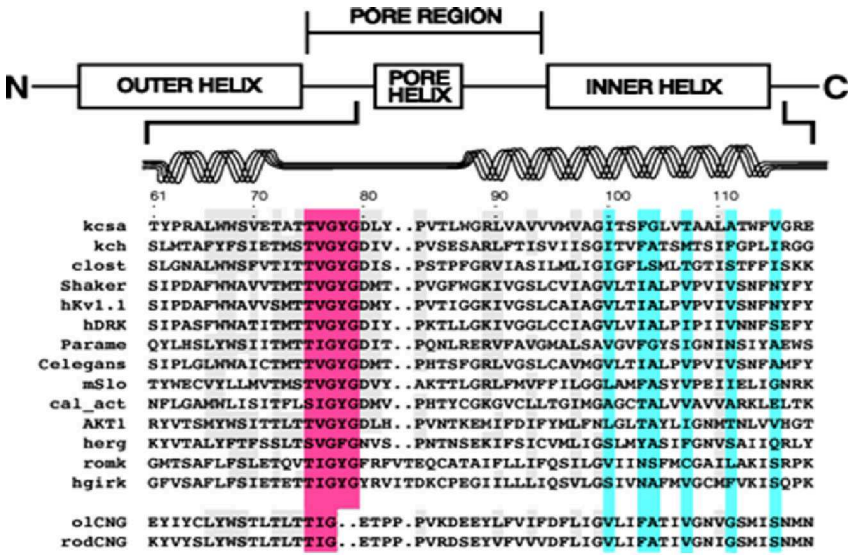


Figure 1.14: sequence homologies in the 'selectivity domain' between various species (Science: Vol. 280 no. 5360 pp. 69-77, 1998).

1.9 Potassium Channelopathies

Ion channels are essential for many physiological functions including neuronal signalling, muscle contraction, cardiac pacemaking, hormone secretion, cell volume regulation and cell proliferation. So it is not surprising that ion channels are implicated in numerous diseases. Most of them results from mutations in genes encoding channel proteins. Some are autoimmune diseases in which the body produces antibodies to its own channel molecules.The study of ion channels diseases usually consists of two stages. First, the chromosome locus of the disease and the protein coded by that gene must be identified. Then the function of mutant channel expressed in special cells as HEK (human embryonic kidney cells) or Xenopus oocytes is studied with electrophysiological techniques. Potassium channel functions are critical for the electrical synchronization in muscles, in the heart and in the nervous system,for this reason channelopathies consist mainly of muscle, cardiac and neural disorders. Most of the neurological potassium channelopathies have been linked to dysfunction of a voltage sensitive potassium channels (figures

1.15 and 1.16 report the tissue expression and desease involvement of ion channels).

Muscle disorders

Episodic ataxia with myokymia syndrome is a disorder characterized by brief episodes of incoordination (ataxia) with continuous muscle movement (myokymia), it is characterized by episodic failure of cerebellar neuron excitation and hyperexcitability of the peripheral motoneurons (Dyke et al., Neurol. Sci. 25: 109–118, 1975). Disease pathogenesis might be explained by a reduced repolarizing effect of the delayed rectifier potassium channels leading to larger action potentials and prolongation of transmitter release. Alterations in Kv1.1 channel are implicated in the development of this desease (D'Adamo et al., FASEB J. 13, 1335–1345, 1999; Adelman et al., Neuron 15, 1449–1454, 1995). Based on immunohistochemistry in rodents, KVα1.1 is enriched in the juxtaparanodal region of the axons of motor neurones (Zhou et al., Neurosci , 18: 7200–15.1998). In the brain, KVα1.1 is widely distributed, but especially prominent around the initial segments of many axons, including those of pyramidal neurones in the hippocampus and Purkinje cells of the cerebellum (Veh et al. Eur J Neurosci, 7: 2189–205, 1995). They are therefore strategically positioned to regulate the firing rates of these neurones. However, they are also present in dendrites and presynaptic terminals, so they may play more extensive roles in regulating signalling (Wang et al., J Neurosci, 14: 4588–99, 1994). Several missense mutations have been detected in the gene that encodes Kv1.1 channel, most of which have been expressed in Xenopus oocytes. Some mutants did not yield significant currents, others showed change in function leading to enhanced deactivation and C-type inactivation, to the shift of voltage dependence of activation and to a slower kinetics of activation. Coexpression of the mutants with wild type mimicking in vivo conditions revealed current reduction between 26 and 100%, indicating a dominant negative effect interaction (Adelman et al., Neuron 15: 1449–1454, 1995). Similar effects have been observed when these mutations were expressed in mammalian cells (BRETSCHNEIDER et al., J. Neurosci.

11: 2403–2412, 1999). These mutant channels in general showed decreased mean open duration compared with the wild type, a faster kinetics and an increased inactivation. It is for these alterations that the nerve cells can't repolarize efficiently. Acquired neuromyotonia (Isaacs' syndrome) is a clinical condition characterized functionally by hyperexcitability of peripheral nerves manifesting as continuous muscle fibre activity. This leads to the characteristic clinical manifestations of myokymia (continuous undulating muscle twitching caused by spontaneous motor nerve discharges), that are muscle cramps, impaired muscle relaxation, stiffness, increased sweating and occasionally muscle weakness. Serum levels of creatine kinase may be elevated, and some patients may also have central nervous system symptoms including insomnia, mood changes and hallucinations. The association of neuromyotonia with these CNS manifestations has been designated Morvan's syndrome. The continuous muscle fibre activity, which characterizes this syndrome, is generated by hyperexcitability of the terminal arborizations of motor nerves. This hyperexcitability is the result of impaired function of delayed rectifier K^+ channels that are ordinarily responsible for neuronal repolarization following action potential firing. In particular recent data reported that patients with acquired neuromyotonia harbour antibodies directed against voltage gated K^+ channels, in particular directed against KCNA6, KCNA2 and KCNA channel.. These antibodies reduce the K^+ current conducted by these channels and thus lead to a prolongation of the nerve action potential (Shillito et al., Ann Neurol 1995, 38:714–22; Sonoda et al., Muscle Nerve 1996, 19:1439–46). Moreover mutations have now been described in the KCNA1 (Kv1.1) potassium channel on chromosome 12p in patients with this disorder (Browne DL et al., Nature Genet 1994, 8:136–40).

Cardiac disorders

Long QT syndrome is a disorder characterized by sensorineural deafness and prolongation of cardiac repolarization that manifests on the surface ECG as a prolonged QT interval. Mutations in 4 cardiac voltage-gated potassium channels are associated with this desease: KCNA8 (KCNQ1, KVLQT1) that

is the one most commonly responsible, KCNE1, KCNE2 (minK) and KCNH (HERG) (Chouabe et al., EMBO J. 16, 5472–5479, 1997; Shalaby et al., Circulation 96, 1733–1736, 1997, Chouabe et al., Cardiovasc. Res.45, 971–980. 2000; Schmitt et al., EMBO J. 19, 332–340. 2000; Zhou et al., J. Biol. Chem. 273, 21061–21066, 1998). KCNQ1 encodes the alpha subunit of potassium channel that are involved mainly in the M-type current propagation (slowly activating and deactivating, and noninactivating voltage-dependent K^+ current widely distributed in the peripheral nervous system and in the CNS). There are about 30 mutations in the KCNQ1 gene that could cause arrythmia and long QT syndrome. The mutations have two different effects: producing not-functional channels or altering channel kinetics. The mutations generally reduces outward repolarizing potassium current, which prolongs cardiac action potential. These mutations in KCNQ1 often result in the generation of premature stop codons and hence protein truncation, this can cause sudden arrhythmias (Splawski I, N Engl J Med 1997, 336:1562–7; Neyroud N et al., Nature Genet 1997; 15:186–9.) KCNE1 codes for the beta subunit that coassembles with the KCNQ1 alpha subunit to form the slowly activating cardiac potassium channel, this α/β channels participate to the cardiomyocyte repolarization. Most of the LQT1-causing point mutations discovered in KCNQ channels are located near the pore region (S5-P-S6) and appear to have loss-of-function effects (Splawski I, N Engl J Med 1997, 336:1562–7; Neyroud N et al., Nature Genet 1997; 15:186–9). HERG encodes an inwardly rectifying potassium channel that mediates too the repolarization of ventricular action potentials, HERG channels have an inactivation mechanism that attenuates efflux during depolarization and they inactivate much more rapidly than they activate; the result is that most HERG channels are closed during the plateau phase of cardiac action potential. An increased incidence of cardiac sudden death has been observed in patients that lack HERG currents, infact the diminished magnitude of potassium inwardly rectifying current causes the prolonged LQ interval observed in affected individuals.

Benign neonatal convulsion

This neurological desease is characterized by brief and frequent generalized seizures, typically commencing within the first week of life and disappearing spontaneoulsy within a few months. Seizure symptoms include tonic movements, shallow breathing, ocular signs and automatisms. Seven mutations have been described in the voltage-sensitive potassium channel gene KCNQ2 (Biervert et al., Science 279: 403–406, 1998; Leach et al., Nature Genet. 18: 25–29, 1998; Lerche et al., Ann Neurol. 1999 Sep, 46(3):305-12.) and one in KCNQ3 (Charlier et al., Nature Genet. 18: 53–54, 1998) that alter the structure of the pore region and/or COOH-terminal cytoplasmic domain leading to potential loss of function, gain of function, or dominant negative effects . KCNQ encoded proteins (Kv7) which contribute to the slow component of delayed rectifier K^+ currents, IKs, in cardiac muscles (Sanguinetti et al., Nature, Nov 7, 384(6604):80-3, 1996) and contribute to the M-type currents in neuronal cells (Brown DA; Nature, 1980 Feb 14, 283(5748):673-6; Selyanko AA, et al., J Neurosci. 1999 Sep 15, 19(18):7742-56). Usually their missense mutations are associated with a variable reduction (20–95%) in current magnitude (Schroeder B. et al., Nature; 396:687–90, 1998) and are responsible for neonatal convulsions. One of the KCNQ2 mutants leading to a truncated channel protein has been expressed in Xenopus laevis oocytes but did not yield any detectable current. This suggests that homotetramers are nonfunctional. Coexpression with wild type at a ratio of 1:1 revealed a 50% current reduction without any support for a dominant negative effect of the mutation, indicating that haploinsufficiency is the decisive mechanism for disease pathogenesis (Biervert et al., Science 279: 403–406). Further studies reported that mutation in S4 region of KCNQ2 decreases the M-type current, prominently in neurons, such as GABAergic interneurons, this results in a resting membrane potential more depolarized with a consequent neuronal hyperexcitability and neonatal convulsions (Maria Virginia Soldovieri et al., The Journal of Neuroscience, May 2, 2007m 27(18):4919–4928). Also other potassium channels are involved in neural synchronization, for example mutations in KVCN2 channel (Kv8.2) influence too epilepsy susceptibility and its progression (Benjamin S. Jorgea et al., PNAS, March 29, 2011,vol. 108 ,no. 13, 5443–5448). Kvcn2 belongs to a group of potassium channel

modulatory subunits that are electrically silent and cannot form functional homotetramers. These silent subunits modulate the properties of Kv2 (Kv2.1 and Kv2.2) with which form heteromeric channels and Kv3 channels.

Epilepsy

Epilepsy is a common chronic neurological disorder characterized by seizures. These seizures are transient signs and/or symptoms of abnormal, excessive or hyper synchronous neuronal activity in the brain. Mutations in KCNQ2 and KCNQ3 are responsible for epileptic seizures, these channels can coassemble to form the M-type channel, which plays an important role in the regulation of the firing rate of neurons and its activation causes a delayed membrane hyperpolarization. Dysfunctions of these channels lead neurones to become slightly depolarized and to fire multiple action potentials rhythmically after receiving excitatory inputs. For example mutations in the pore reduce the K^+ current by affecting ion conductance, whereas mutations in the C-terminus affect the assembly of alpha-subunits to heteromeric channels (H.Lerche, Current Pharmaceutical Design, 2005, Vol. 11, No. 00; Biervert et al., Science 279, 406–409. 1998; Lerche et al., Ann. Neurol. 46, 305-312 1999) .Then the inhibition of these channels' activity appears to be crucial in epilepsy development, infact KCNQ blockers, such as linopirdine, increases neuronal excitability as evidenced by their ability to increase neurotransmitter release and enhance cognition in a variety of animal models (Zazek et al., Drugs, 2:1097-1104, 1993) inducing epilepsy, so this channel represents a target for the treatment of this pathology. Also the absence of KCNA channel's activity is responsible for abnormal neuronal activity. Mice lacking the entire voltage-gated potassium channel α-subunit, Kv1.1, display frequent spontaneous seizures throughout adult life. The loss of Kv1.1 from its normal localization in axons and terminals of the CA3 region of hippocampus results in increased excitability in the CA3 recurrent axon collateral system, perhaps contributing to the limbic and tonic–clonic components of the observed epileptic phenotype (Sharon L. Smar et al., Neuron, Vol. 20, 809–819, April, 1998).

Hypokalaemic periodic paralysis

Hypokalaemic period paralysis (HOPP) is an autosomal dominant disorder in which affected individuals experience attacks of weakness lasting hours to days. The distinguishing features of this form of periodic paralysis is the hypokalaemia that accompanies the episodic paralysis and the absence of myotonia. Attacks may be precipitated by stress, rest after exercise and events that lower serum potassium such as carbohydrate ingestion, insulin or diuretic use. Muscle fibres from patients with HOPP exhibit an abnormally depolarized resting membrane potential with further depolarization (and paralysis) induced by insulin and hypokalaemia. Recent evidence suggests that defective function of the skeletal muscle ATP sensitive potassium channel (KATP) may contribute to the pathogenesis of this disorder (Links T. et al., Clin Sci1993, 85:319–25; Ruff RL., Neurology1999; 53:1556–63). Using macropatch clamp recordings from muscle fibres of patients with HOPP it has been demonstrated a reduced resting outward K^+ current through the KATP channel as well as a further inhibition of this current during hypokalaemia or the administration of insulin (Tricarico et al., J Clin Invest 1999, 103:675–81).

Bartter's syndrome

Bartter's syndrome is an autosomal recessive form of severe intravascular volume depletion due to renal salt-wasting associated with alkalosis (a condition of excess base in body fluids), with reduced potassium (hypokalemic alkalosis), hypercalciuria (the presence of excess calcium in the urine) and increased production of the hormone aldosterone. Mutations in the KCNJ1 gene coding for an inwardly rectifying potassium channel (Kir 1.1) lead to loss of function that is associated with the disease. Potassium channel in the kidney probably plays the major role in K^+ homeostasis during this disorder.

Hyperinsulinemic hypoglycemia

ATP-sensitive potassium channel plays a role in secretion and muscle contraction by coupling metabolic activity to membrane potential. In pancreatic beta cells, ATP-potassium channels are crucial for the regulation of glucose-induced insulin secretion (Sharma et al., Kidney Int. 57, 803–808, 2000). Alteration of these channels causes hyperinsulinemic hypoglycemia, that is a disorder of glucose metabolism with an unregulated secretion of insulin and great hypoglycemia. Previous studied have moreover discovered that mutations in Kir6.2 (inwardly rectifying potassium channel) are present in individuals affected by this pathology (Thomas et al. Hum. Mol. Gen. 5, 1809–1812, 1996).

Schizophrenia

Abnormal function of calcium-activated K^+ channels has been noted in platelets of patients with Alzheimer's disease, although its relevance to the pathology is not clear (de Silva et al., Lancet 352:1590–1593, 1998). The CAG triplet repeat in KCNN3 gene encoding a small conductance calcium-activated K^+ channel (hKCa3) mapped to chromosome 1q21, has been reported to be associated with schizophrenia (Chandy et al., Mol Psychiatry 3:32–37, 1998), although subsequent investigations to confirm these findings have shown mixed results (Austin et al., Mol Psychiatry 4:261–266, 1999). However potassium channels play surely an important role also in this pathology.

Gene	Accession ID	Gene Locus	Potassium Channel Type/Disease	Tissue Expression
KCNA1	GDB: 127903 L02750	12p13	RBK1, HUK1, MBK1, AEMK, Kv1.1, *Shaker* homolog 1, *Shaker*, episodic ataxia 1 (with myokymia)	Brain, nerve, heart, skeletal muscle, retina, pancreatic islet
KCNA1B		3q26.1	Kvβ1.1, Kvβ1.3 (splice product), β-subunit	
KCNA2	GDB: 128062 X17622	12pter-qter	HK4, Kv1.2, *Shaker* homolog 2	Brain, nerve, heart, pancreatic islet
KCNA2B		1p36.3	Kvβ1.2, β-subunit	
KCNA3	GDB: 128079 L23499	1p13.3	Hs.1750, MK3, HLK3, HPCN3, Kv1.3, *Shaker* homolog 3	Skeletal muscle, lymphocytes (brain, lung, thymus, spleen)
KCNA4	GDB: 126730 M60450 M55514	11p14	Hs.89647, Hs.1854, HK1, HPCN2, Kv1.4, *Shaker* homolog 4	Brain, nerve, heart, fetal skeletal muscle, pancreatic islet
KCNA4L	GDB: 386059	11q14	*Shaker* homolog type 4-like	
KCNA5	GDB: 127904 M83254 M60451	12p13.3-13.2 12p13 12p13.33-13.31	Hs.89509, HK2, HPCNI, Kv1.5 *Shaker* homolog 5	Brain, heart, kidney, lung, skeletal muscle, pancreatic islet
KCNA6	GDB: 128080 X17622	12p13	HBK2, Kv1.6, *Shaker* homolog 6	Brain, pancreatic islet
KCNA7	GDB: 127905	19q13.3	HAK6, Kv1.7 *Shaker* homolog 7	
KCNA8			see *KCNQ1*	
KCNA9			see *KCNQ1*	
KCNA10	GDB: 5885822		*Shaker* homolog type 10, cGMP activated	
KCNB1	GDB: 128081	20q13.2	Kv2.1, *Shab* homolog 1	Brain, heart, kidney, retina, skeletal muscle
KCNB2			Kv2.2, *Shab* homolog 2	Brain, heart, retina
KCNC1	GDB: 128082 S56770 M96747	11p15.1	Kv3.1, *Shaw* homolog 1	Brain, skeletal muscle, spleen, lymphocytes
KCNC2	GDB: 127906	19q13.3-13.4	Kv3.2, *Shaw* homolog 2	Brain
KCNC3	GDB: 127907	19q13.3	Kv3.3, *Shaw* homolog 3	Brain, liver
KCNC4	GDB: 127908	1p21	Kv3.4, HKSHIIIC, *Shaw* homolog 4	Brain, skeletal muscle
KCND1	GDB: 128083		Kv4.1, *Shal* homolog 1	Brain
KCND2	GDB: 134771		RK5, Kv4.2, *Shal* homolog 2	Brain, heart, aorta
KCND3	GDB: 134772		Kv4.3, KSHIVB, *Shal* homolog 3	

Figure 1.15: gene names, accession numbers, protein names, and tissue expression of classical voltage-gated potassium channels (PHYSIOLOGICAL REVIEWS, Vol. 79, No. 4, October 1999)

Channel type	Gene	Channelopathy and phenotype
K^+ channel	*KCNA1*	episodic ataxia type 1 (EA-1)
	KCNH1 (hEAG)	oncogenic potential
	KCNJ11	persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI)
	KCNQ2/3	benign familial neonatal convulsions (BFNC)
	KCNQ4	hereditary hearing loss (DFNA2)
	SUR1	persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI)
Na^+ channel	*SCNN1(ENaC)*	Liddle's syndrome (heriditary hypertension, pseudohypoaldosteronism type 1)
	SCN4A	hyperkalaemic periodic paralysis, hypokalaemic periodic paralysis, paramytonia congenita
	SCN1B	generalized epilepsy with febrile seizures type 1
Ca^{2+} channel	*CACNA1A*	episodic ataxia type 2, familial hemiplegic
	CACNA1	migraine, spinocerebellar ataxia type 6
	SRYR1	hypokalaemic periodic paralysis
	CAT-L	malignant hyperthermia, central core disease
	CAT-L	expressed in advanced prostate cancer
Glycine receptor	*GLRA1*	hyperplexia
Acetylcholine receptor	*CHRNA1*	congenital myasthenia
	CHRNA4	autosomal dominant nocturnal frontal lobe epilepsy
CNG channels	*CNGA3*	achromatopsia-2
	CNGB3	achromatopsia-3
Cl^- channel	*CLCN1*	myotonia congenita (dominant or recessive)
	CLCN5	Dent's disease (proteinuria and hypercalciuria)
	CLCN7	osteopetrosis

Figure 1.16: ion channel deseases (Embo reports, vol 2, no 7, 568-573, 2001)

1.10 Potassium channels and gap junctions involved in the same physiological and pathological events

It 's widely demonstrated that potassium channels play a key role in the generation of action potentials, together with gap junctions they operate participating in the electric transportation. The studying of the cooperation between GJS and voltage-gated potassium channel and the characterizion of its functional and structural aspects could provide interesting informations about mixed synapses mechanisms that are still unclear and other cellular pathways. For example, in astrocytes has been shown that electrical activity is given by the potassium channels and gap junctions, in fact, when both channels are inhibited the conductance is almost completely abolished (Louis

et al. Neurochem Int 2008 June; 52 (7): 1365-1372); this demonstrates that electrical activity in astrocytes depends almost exclusively on these channels. The collaboration between these proteins is evident in numerous phenomena, such as in the control of K^+ homeostasis and in the mechanism of 'potassium spatial buffering' (Kamasawa et al. Neuroscience 136 (2005) 65-86), particularly in astrocytes and oligodendrocytes; infact gap junctions are involved with voltage-gated potassium channels in the paranodal potassium distribution during transmission of the pulse in the nodes of Ranvier (see fig. 1.17). Gap junctions appear to be implicated in the potassium uptake in astrocyte mithocondria with KATP channels as well as in the cytoplasmatic membrane (Kozoriz MG. Et al., J Biol Chem. 2010 Oct 8, 285 (41):31107-19). So it's clear that both channels are essential for the control of ion homeostasis. But it is also interesting that G.JS and potassium channels are both related with many pathological conditions, and the idea that gap junctions can also be involved in the potassium channelopathies is not to discard. Potassium channel openers have neuroprotective action during hypoxia, infact the potassium outflow obstacles apoptosis, probably because the hyperpolarization of the cell reduces the excitability of the membrane; in the other hand also potassium channel blockers like TEA reduces apoptosis after an ischemic insult, perhaps because localized high concentrations of TEA reduces glutamate receptor activation (Wei et al. Stroke 2003, 34, 1281-1286). These data support the idea that strategies aimed at maintaining cellular K^+ homeostasis may be of therapeutic benefit in the treatment of cerebral ischemia. Even inhibitors of gap junctions like heptanol play a role as protectors during hypoxia, because they hinder the coupling between junctions and thus the transmission of 'ischemic insults' between the cells (Rodriguez-Sinovas A, Am J Physiol Heart Circ Physiol. 2006 Feb, 290 (2): H648-56); moreover also gap junctions are involved in potassium homeostasis. Potassium channels are obviously important in the control of neuronal impulses (as described above), deletions of Kv1.1 channels in axons lead to an increased excitability by inducing epilepsy (Sharon L. Smar et al., Neuron, Vol. 20, 809–819, 1998) as well as mutations in KCNQ channels, where instead gap junctions inhibitors such as carbenoxelone have an anticonvulsant effect possibly due

to a reduction in transmission and excitability of fast oscillations (Neman et al. Histol Histopathol (2005) 20: 253-259). Various types of gap junctional blocking agents beyond carnenoxelone reduce the duration of evoked seizure-like primary afterdischarges (PADs), such as 1-octanol, sodium propionate and sodium valproate (Shokrollah et al., J Neurophysiol 88:1893-1902, 2002). Another class of potassium channels, the channels activated by calcium, plays a role with gap junctions in arterial vasodilation during obesity conditions, both are overexpressed and activated with a consequent increase of cell hyperpolarization and vasodilation (Chadha et al., The Journal of Pharmacology and Experimental Therapeutics, 335:284-293, Vol 335, No. 2, 2010). And more the interaction between potassium channels and gap junctions could be involved in pain where KCNQ represents a target for the dolor therapy (Mastronardi et al., J. Int. Med. Res. 16:338-348, 1988), in heart attack, in hyperinsulinemia, in erectile dysfunction that involves in particular ATP potassium channels and connexin 43 (George J. Christ, Journal of Andrology, Vol. 23, No. 5, 2002; Pointis G; Int J Biochem Cell Biol. 2006, 38(10):1642-6), in cell proliferation and in cancer. Infact past researchs have shown that pannexin 1 has a suppressive action on cancer, the rat C6 cells (or glioma cells) when transfected with DNA of pannexin 1 show a reduced cell proliferation and a low mobility, therefore the loss of this protein may participate in the development of glioma (Lai et al. Cancer Res 2007 67: (4). February 15, 2007). Pannexin 1 is also implicated in apoptosis, as mentioned before, infact it is involved in ATP release and activation of caspases, perhaps PANX1 is a target of caspases themselves, the latters bind tetrapeptides with a final aspartic acid residue important for their activation; possible sites of 'cleavage' in pannexin 1 are in the intracellular loop on residues 164-167 and in C-terminal part on residues 376-379, but the mechanism is still unclear. Patch clamp studies have shown that in Jurkat cells (immortalized cells of T lymphocytes) pannexons are basically inactive and conduct current only during apoptosis (Chekeni et al, Nature. 14 October 2010, 467 (7317): 863-867). As well as potassium channels have been studied extensively in relation to cancer, apoptosis is in fact accompanied by an increase in potassium current with a decrease of cytoplasmic potassium; the

relationship that exists between intracellular potassium levels and apoptosis is still poorly understood, presumably the decrease in potassium leads to activation of molecules involved in apoptosis, although the data obtained are conflicting, infact in some cells the activation of K^+ channels would seem to inhibit apoptosis, in other cell types would seem to activate it. May be both the excessive loss or increase in intracellular potassium is toxic. However the loss of potassium in a wide variety of cells activates the apoptotic process. Potassium channels play an important role in both normal and pathological cell proliferation, inhibitors of voltage-dependent channels such as HERG channels, Eag channels, Kv10.1, Kv1.3 and Kv11.1 channels reduce cell proliferation, in fact they are overexpressed in cancer cells. In particular, a potential mechanism of carcinogenesis may be explained in the following way: the overexpression of HERG and eag K^+ channels leads to an increase of intracellular calcium and the overexpression of voltage-gated K channels leads to an increase in intracellular potassium with hyperpolarization, the increase of both ions activates the transition from G1 to S phase of the cell cycle, HERG and eag channels also participate in the release of VEGF during hypoxia by promoting angiogenesis (see fig. 1.18). Therefore, ionic channels with gap junctions can be considered as modulators of tumor progression and as new biomarkers (Asher et al. World Journal of Surgical Oncology 2010, 8:113; Pardo; Physiology 19:285-292, 2004), further investigations in this field could provide some interesting results . Until now more evidence exists on the modulation and the interaction between ATP-dependent potassium channels (KATP) and gap junctions, these K^+ channels infact reduce the permeability of GJS in astrocytes, may be in response to the increase of intracellular calcium, on the contrary their inhibition by drugs such as tolbutamide prevents the inactivation of gap junctions by their blockers such as octanol and glycyrrhetinic acid. Gap junction inhibition mediated by these potassium channels, however, is not only due to the increase in intracellular calcium because also when there is a decrease of intracellular calcium the KATP-channel-dependent-inhibition of gap junctions occurs, so this evidence suggests that other mechanisms are involved; ATP itself can directly regulate the opening of G.JS. The cooperation between these channels and

G.JS is also amply demonstrated in myocytes (Granda et al, FEBS Lett, Volume 427, Issue 1, 1 May 1998, Pages 41-45; Velasco et al. J. Neurochem., Vol 74, No. 3, 2000). Another interesting phenomenon where are essential ATP-dependent potassium channels and gap junctions is the secretion of insulin in pancreatic β cells. The synchronization of electrical activity between these cells is mediated by connexins and is crucial for glucose-dependent insulin secretion; KATP channels are also widely expressed in these cells, depolarization and increase of intracellular calcium caused by they closure it is essential for insulin secretion. Studies in mice have shown that about 30% of KATP channels is enough to maintain the membrane potential and glucose-dependent insulin secretion, but the dependence on glucose is lost in isolated cells, as well as the inhibition of gap junctions causes glucose sensitivity loss. This proves that the coupling between these channels mediated by gap junctions is critical for the regulation of insulin secretion (Rochelau et al., PLoS Biology, February 2006, Volume 4, Issue 2, E26), among other things, KATP channels affect the opening of gap junctions (as mentioned before). These are some of the phenomena in which ATP-dependent potassium channels and gap junctions work together and it is clear that their cooperation is widely documented. A few informations exist instead about the interaction between potassium voltage-gated channels (Kv) and gap junctions, for this reason further analysis are still required to clarify their relationship and in particular their role in mixed synapses. Anyway all these current findings could suggest that since gap junctions and potassium channels are simultaneously involved in many physiological and pathological conditions their possible reciprocal modulation may play a key role in several deasese; thus they can represent important drug receptors. A new pharmacological approach may have as focus just their interaction, thus considering both channels as a functional 'single unit, so that they could be examined as single pharmacological target when implicated in the same pathology.

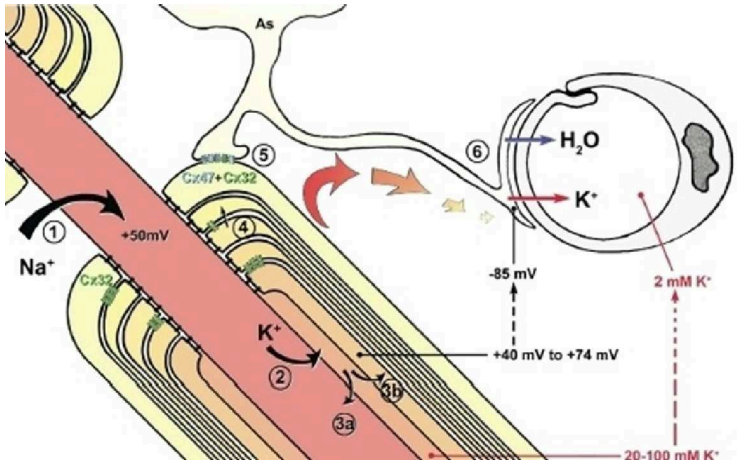


Figure 1.17: 'potassium siphoning' mechanism in glia cells (Neuroscience. 2005;136(1):65-86).

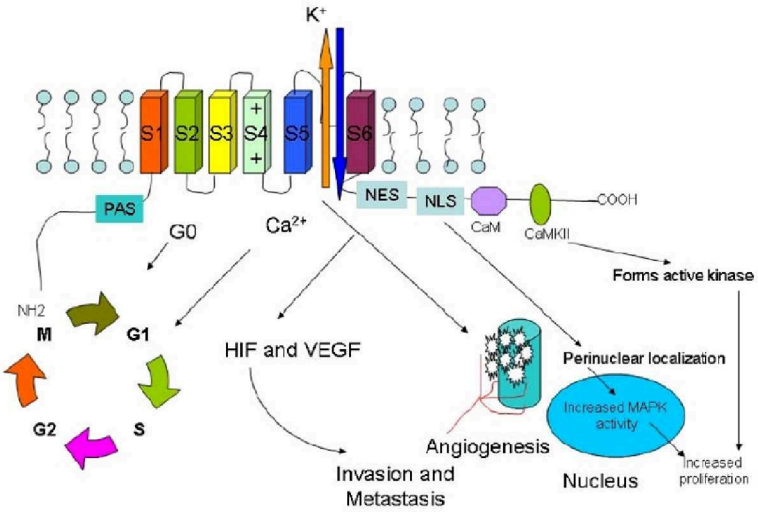


Figure 1.18: probable mechanism of cancerogenesis mediated by potassium channels (World Journal of Surgical Oncology 2010, 8:113).

1.11 Comparison of aminoacid sequence between beta 3 auxiliary subunit and Kv1.1 channel of mouse and human

The beta subunit of voltage-gated potassium channel is an auxiliary subunit that is involved in the inactivation. So far we know beta 1, beta 2 and beta 3 subunits, that are homologous one to each other. In fact, beta 3 subunit of mouse has about 66% of aminoacid sequence homology with the beta 1 and and about 71% with beta 2. According to a recent study already mentioned the Kv1.1 beta 3 subunit interacts with C-terminal portion of pannexin 1 modulating its activity in the presence of inhibitors and reducing agents; in particular, the last 58 aminoacids of the $\beta 3$ subunit bind pannexin (Bunse et al. FEBS Journal, 276, 6258-6270, 2009). Both Kv1.1 channel and $\beta 3$ subunit of mouse exhibits high homology with the corresponding human proteins, about 98% identity for the Kv channel and 93% for the beta subunit, as well as the mouse and human pannexin 1 are homologous one to each other. So studies performed using pannexin 1 and Kv1.1 of both species could be comparable. Aligning the full sequence of human Kv1.1 channel with beta 3 subunit through Clustal W and the last 58 aminoacid sequence of $\beta 3$ subunit with the full sequence of Kv1.1 channels through Blast, some similarities were found, to be more precise were found respectively the presence of many aminoacids with similar or identical chemical and physical properties, (as shown by Clustal W multiple alignment), and an identity of 27% (as shown by Blast multiple alignment). Although the sequence homology is not high, certain aminoacids may be involved in the same type of interactions. Homologies were found mainly in the C-terminus of the Kv1.1 channel, that is the portion of the protein most involved in the interactions with other macromolecules. In this portion are present residues of serine, valine, leucine, glutamic acid, methionine, isoleucine and asparagine perfectly aligned to the beta 3 subunit; it is also interesting the presence of a domain of six aminoacids that is repeated in both macromolecules: LLGVSS in the last 58 aminoacidic of beta 3 subunit and LLHVSS in C-terminal part

of Kv1.1 channel. It is unknown whether this domain has a specific function and if its repetition in both proteins have a particular meaning, but seen that the sequence similarity is often accompanied by structure similarity and thus function homology (QSAR), it is possible the homologous regions present in both proteins are involved together in the same type of interactions; may be this domain or other regions of Kv1.1 channel are able to interact directly with the C-terminal portion of pannexin 1 without the intervention of $\beta 3$ subunit or interact with other macromelcules that mediate the interaction with pannexin 1. However, further investigations are needed to determine whether this phenomenon really happens; for this reason the purpose of my work was also understanding whether exists a direct interaction between the two channels. Below I have reported the results of aminoacidic sequence alignments, performed with Clustal W and Blast:

Sequence overlap between mouse Kv1.1 (sbjct) and human Kv1.1 (query):

```
Score = 1013 bits (2620),  Expect = 0.0, Method: Compositional matrix adjust.
Identities = 487/495 (98%), Positives = 490/495 (99%), Gaps = 0/495 (0%)


Query  1    MTVMSGENVDEASAAPGHPQDGSYPRQADHDDHECCERVVINISGLRFETQLKTLAQFPN  60
            MTVMSGEN DEAS APGHPQDGSYPRQADHDDHECCERVVINISGLRFETQLKTLAQFPN
Sbjct  1    MTVMSGENADEASTAPGHPQDGSYPRQADHDDHECCERVVINISGLRFETQLKTLAQFPN  60

Query  61   TLLGNPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEIKFY  120
            TLLGNPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEIKFY
Sbjct  61   TLLGNPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEIKFY  120

Query  121  ELGEEAMEKFREDEGFIKEEERPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVILIS  180
            ELGEEAMEKFREDEGFIKEEERPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVILIS
Sbjct  121  ELGEEAMEKFREDEGFIKEEERPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVILIS  180

Query  181  IVIFCLETLPELKDDKDFTGTVHRIDNTTVIYNSNIFTDPFFIVETLCIIWFSFELVVRF  240
            IVIFCLETLPELKDDKDFTGT+HRIDNTTVIY SNIFTDPFFIVETLCIIWFSFELVVRF
Sbjct  181  IVIFCLETLPELKDDKDFTGTIHRIDNTTVIYTSNIFTDPFFIVETLCIIWFSFELVVRF  240

Query  241  FACPSKTDFFKNIMNFIDIVAIIPYFITLGTEIAEQEGNQKGEQATSLAILRVIRLVRVF  300
            FACPSKTDFFKNIMNFIDIVAIIPYFITLGTEIAEQEGNQKGEQATSLAILRVIRLVRVF
Sbjct  241  FACPSKTDFFKNIMNFIDIVAIIPYFITLGTEIAEQEGNQKGEQATSLAILRVIRLVRVF  300

Query  301  RIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAEEAESHFSSIP  360
            RIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAEEAESHFSSIP
Sbjct  301  RIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAEEAESHFSSIP  360

Query  361  DAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETE  420
            DAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETE
Sbjct  361  DAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETE  420

Query  421  GEEQAQLLHVSSPNLASDSDLSRRSSSTMSKSEYMEIEEDMNNSIAHYRQVNIRTANCTT  480
            GEEQAQLLHVSSPNLASDSDLSRRSSST+SKSEYMEIEEDMNNSIAHYRQ NIRT NCTT
Sbjct  421  GEEQAQLLHVSSPNLASDSDLSRRSSSTISKSEYMEIEEDMNNSIAHYRQANIRTGNCTT  480

Query  481  ANQNCVNKSKLLTDV  495
            A+QNCVNKSKLLTDV
Sbjct  481  ADQNCVNKSKLLTDV  49
```

Sequence overlap between human β3 subunit (sbjct)e mouse β3 subunit (query):

```
>lcl|38219 unnamed protein product
Length=404

 Score =  716 bits (1847),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 376/404 (93%), Positives = 391/404 (97%), Gaps = 0/404 (0%)

Query  1    MQVSIACTEQNLRSRSSEDRLCGPRPGPGGGNGGFVGGGHGNPPGGGGPSSKSRAAVVPR  60
            MQVSIACTEQNLRSRSSEDRLCGPRPGPGGGNGGP GGGHGNPPGGGG   K+RAA+VPR
Sbjct  1    MQVSIACTEQNLRSRSSEDRLCGPRPGPGGGNGGPAGGGHGNPPGGGGSGPKARAALVPR  60

Query  61   PPAPAGALRESTGRGTGMKYRNLGKSGLRVSCLGLGTWVTFGSQISDETAEDLLTVAYEH  120
            PPAPAGALRESTGRGTGMKYRNLGKSGLRVSCLGLGTWVTFGSQISDETAED+LTVAYEH
Sbjct  61   PPAPAGALRESTGRGTGMKYRNLGKSGLRVSCLGLGTWVTFGSQISDETAEDVLTVAYEH  120

Query  121  GVNLFDTAEVYAAGKAERTLGNILKSKGWRRSSYVITTKIFWGGQAETERGLSRKHIIEG  180
            GVNLFDTAEVYAAGKAERTLGNILKSKGWRRSSYVITTKIFWGGQAETERGLSRKHIIEG
Sbjct  121  GVNLFDTAEVYAAGKAERTLGNILKSKGWRRSSYVITTKIFWGGQAETERGLSRKHIIEG  180

Query  181  LQGSLDRLQLEYVDIVFANRSDPNSPMEEIVRAMTYVINQGLALYWGTSRWSAAEIMEAY  240
            L+GSL+RLQL YVDIVFANRSDPN PMEEIVRAMTYVINQGLALYWGTSRW AAEIMEAY
Sbjct  181  LRGSLERLQLGYVDIVFANRSDPNCPMEEIVRAMTYVINQGLALYWGTSRWGAAEIMEAY  240

Query  241  SMARQFNLIPPVCEQAENHFFQREKVEMQLPELYHKIGVGSVTWSPLACGLITSKYDGRV  300
            SMARQFNLIPPVCEQAE+H FQREKVEMQLPELYHKIGVGSVTW PLACGLITSKYDGRV
Sbjct  241  SMARQFNLIPPVCEQAEHHLFQREKVEMQLPELYHKIGVGSVTWYPLACGLITSKYDGRV  300

Query  301  PDTCKATVKGYQWLKEKVQSEEGKKQQARVMDLLPTARQLGCTVAQLAIAWCLRSEGVSS  360
            PDTC+A++KGYQWLK+KVQSE+GKKQQA+VMDLLP A QLGCTVAQLAIAWCLRSEGVSS
Sbjct  301  PDTCRASIKGYQWLKDKVQSEDGKKQQAKVMDLLPVAHQLGCTVAQLAIAWCLRSEGVSS  360

Query  361  VLLGVSSAEQLMEHLGSLQVLSQLTPQTVVEIDALLGNKSHSKK  404
            VLLGVSSAEQL+EHLG+LQVLSQLTPQTV+EID LLGNK HSKK
Sbjct  361  VLLGVSSAEQLIEHLGALQVLSQLTPQTVMEIDGLLGNKPHSKK  404
```

Sequence overlap between last 58 aminoacids of mouse and human beta 3 subunits:

```
Score =  110 bits (274),  Expect = 2e-30, Method: Compositional matrix adjust.
 Identities = 55/60 (92%), Positives = 58/60 (97%), Gaps = 0/60 (0%)

Query  1   AQLAIAWCLRSEGVSSVLLGVSSAEQLMEHLGSLQVLSQLTPQTVVEIDALLGNKSHSKK  60
           AQLAIAWCLRSEGVSSVLLGVSSAEQL+EHLG+LQVLSQLTPQTV+EID LLGNK HSKK
Sbjct  1   AQLAIAWCLRSEGVSSVLLGVSSAEQLIEHLGALQVLSQLTPQTVMEIDGLLGNKPHSKK  60
```

Sequence overlap between human pannexin 1 (query) and mouse pannexin 1(sbjct):

```
Score =  715 bits (1846),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 370/427 (87%), Positives = 402/427 (94%), Gaps = 6/427 (1%)

Query  1    MAIAHLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEISI  60
            MAIAHLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEISI
Sbjct  1    MAIAHLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEISI  60

Query  61   GTQISCFSPSSFSWRQAAFVDSYCWAAVQQKSSLQSESGNLPLWLHKFFPYILLLFAILL  120
            GTQISCFSPSSFSWRQAAFVDSYCWAAVQQK+SLQSESGNLPLWLHKFFPYILLLFAILL
Sbjct  61   GTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGNLPLWLHKFFPYILLLFAILL  120

Query  121  YLPALFWRFSAAPHLCSDLKFIMEELDKVYNRAIKAAKSARDLDLRDGP-GPPGVTENVG  179
            YLP LFWRF+AAPH+CSDLKFIMEELDKVYNRAIKAAKSARDLD+RDG     PGVTEN+G
Sbjct  121  YLPPLFWRFAAAPHICSDLKFIMEELDKVYNRAIKAAKSARDLDMRDGACSVPGVTENLG  180

Query  180  QSLWEISESHFKYPIVEQYLKTKKNSSHLIMKYISCRLVTFVVILLACIYLSYYFSLSSL  239
            QSLWE+SESHFKYPIVEQYLKTKKNS++LI+KYISCRL+T  ++ILLACIYL YYFSLSSL
Sbjct  181  QSLWEVSESHFKYPIVEQYLKTKKNSNNLIIKYISCRLLTLIIILLACIYLGYYFSLSSL  240

Query  240  SDEFLCSIKSGVLKNDSTIPDRFQCKLIAVGIFQLLSLINLIVYALLIPVVVYTFFIPFR  299
            SDEF+CSIKSG+L+NDST+PD+FQCKLIAVGIFQLLS+INL+VY  LL PVVVYT F+PFR
Sbjct  241  SDEFVCSIKSGILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAPVVVYTLFVPFR  300

Query  300  QKTDILKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISELKSYKCLKVLENIKSNGQG  359
            QKTD+LKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISE+KSYKCLKVLENIKS+GQG
Sbjct  301  QKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYKCLKVLENIKSSGQG  360

Query  360  IDPMLLLTNLGMIKMDIIDGKIPTSLQTKGEDQGSQRVEFKDLDLSSEAAANNGEKNSRQ  419
            IDPMLLLTNLGMIKMD++DGK  P S + + E+QG+Q   E +D    SE  ANNGEKN+RQ
Sbjct  361  IDPMLLLTNLGMIKMDVVDGKTPMSAEMR-EEQGNQTAELQD----SETKANNGEKNARQ  415

Query  420  RLLNPSC  426
            RLL+ SC
Sbjct  416  RLLDSSC  422
```

Sequence overlap between last 58 aminoacids of mouse beta 3 subunit (query) and full mouse Kv1.1 channel (sbjct):

```
lcl|64123 unnamed protein product
Length=495

 Score = 11.9 bits (19),  Expect = 5.6, Method: Composition-based stats.
 Identities = 5/6 (83%), Positives = 5/6 (83%), Gaps = 0/6 (0%)

Query  18   LLGVSS  23
            LL VSS
Sbjct  427  LLHVSS  432
```

Sequence overlap between last 58 aminoacids of human beta 3 subunit (query) and full human Kv1.1 channel (sbjct):

```
Score = 12.3 bits (20),  Expect = 3.6, Method: Composition-based stats.
 Identities = 10/37 (27%), Positives = 17/37 (46%), Gaps = 0/37 (0%)

Query  18   LLGVSSAEQLIEHLGALQVLSQLTPQTVMEIDGLLGN  54
            LL VSS     +   + +  S ++    MEI+  + N
Sbjct  427  LLHVSSPNLASDSDLSRRSSSTMSKSEYMEIEEDMNN  463
```

CLUSTAL 2.1 multiple sequence alignment between mouse Kv1.1 channel full aminoacidic sequence and last 58 aminoacids of mouse beta 3 subunit:

```
kv              MTVMSGENADEASTAPGHPQDGSYPRQADHDDHECCERVVINISGLRFETQLKTLAQFPN 60
beta3           ------------------------------------------------------------

kv              TLLGNPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEIKFY 120
beta3           ------------------------------------------------------------

kv              ELGEEAMEKFREDEGFIKEEERPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVILIS 180
beta3           ------------------------------------------------------------

kv              IVIFCLETLPELKDDKDFTGTIHRIDNTTVIYTSNIFIDPFFIVETLCIIWFSFELVVRF 240
beta3           ---------------------------------------------LAIAW--------- 5
                                                              *.* *

kv              FACPSKTDFFKNIMNFIDIVAIIPYFITLGTEIAEQEGNQKGEQATSLAILRVIRLVRVF 300
beta3           --CLRSE----------GVSSVL-----LGVSSAEQLMEHLGS-------LQVLSQLTPQ 41
                  *  .           .: :::     **.. ***  :: *.       *:*:  :

kv              RIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAEEAESHFSSIP 360
beta3           TVVEID------ALLGN--KSHSKK---------------------------------- 58
                 :.::.       :**:  *:  ::

kv              DAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETE 420
beta3           ------------------------------------------------------------

kv              GEEQAQLLHVSSPNLASDSDLSRRSSSTISKSEYMEIEEDMNNSIAHYRQANIRTGNCTT 480
beta3           ------------------------------------------------------------

kv              ADQNCVNKSKLLTD 494
beta3           --------------
```

CLUSTAL 2.1 multiple sequence alignment between mouse Kv1.1 channel full aminoacidic sequence and mouse beta 3 subunit full aminoacidic sequence:

```
kv              -MTVMSGENADEASTAPGHPQDGSYPRQADHDDHECCERVVINISG-LRFETQLKTLAQF 58
beta3           MQVSIACTEQNLRSRSSEDRLCGPRPGPGGGGNGGPVGGGHGNPPGGGGPSSKSRAAVVPR 60
                  . ::  : :  * :. .   *. *  .. :.           .*    ...  ::.

kv              PNTLLGNPKKRMRYFDPLRNEYFFDR-NRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEI 117
beta3           PPAPAGALRESTGRGTGMKYRNLGKSGLRVSCLGLGTWVTFGSQISDETAEDLLTVAYEH 120
                * :  *  ::       :: . : .   * *  .:  :   *.::   .   *  .: *

kv              KFYELGEEAMEKFREDEGFIKEEERPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVI 177
beta3           GVNLFDTAEVYAAGKAERTLGN---ILKSKGWRRSSYVITTKIFWGGQA----------- 166
                 .  :.   :    : *  : :    * .* ::*. :::      .* *

kv              LISIVIFCLETLPELKDDKDFTGTIHRIDNTTVIYTSNIFTDPFFIVETLCIIWFSFELV 237
beta3           ---------ETERGLSRKHIIEGLQGSLDRLQLEYVDIVFAN----------------- 199
                         **   *. .: : *    :*.  : *.. :*::

kv              VRFFACPSKTDFFKNIMNFIDIVAIIPYFITLGTEIAEQEGNQKGEQATSLAILRVIRLV 297
beta3           --------RSDPNS---PMEEIVRAMTYVINQGLALYWG-----TSRWSAAEIMEAYSMA 243
                        ::*  .    : :**  :.*.*. *  :          .: ::  *:..  :.

kv              RVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAEEAESHFS 357
beta3           RQFNLIPPVCEQAENHFFQR--EKVEMQLPELYHKIGVGSVIWSPLACGLITSKYDGRVP 301
                * *.::    ..   ::: :  :    :*  * . : :* : :*. .    :.: :.:..

kv              SIPDAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHR 417
beta3           --------DTCKATVKGYQWLKEKVQSEEGKKQQARVMDLLPTARQLGCTVAQLAIAWCL 353
                        . . *. **  :   . . :   . . : .:*. *  :   *:::   :

kv              ETEGEEQAQLLHVSSPNLASDSDLSRRSSSTISKSEYMEIEEDMNNSIAHYRQANIRTGN 477
beta3           RSEG-VSSVLLGVSSAEQLMEHLGSLQVLSQLTPQTVVEIDALLGNKSHSKK-------- 404
                .:**  .: ** ***.:   :   * :  * :: .  :**:  :.*.    :

kv              CTTADQNCVNKSKLLTD 494
beta3           ----------------
```

1.12 Analysis of electrical currents in ion channels

The electrical activity of the channels can now be studied mainly through the technique of patch clamp and voltage clamp. The patch clamp is a recent development of the classic voltage clamp. It can be used for cell cultures, or even on single isolated cells, on thin slices of brain tissue and on liposomes. The patch clamp consists of supporting on the cell membrane a thin electrode capillary of the diameter of about 1 micron. By applying a slight suction, the cell membrane is slightly "sucked" inside the electrode. This creates a "seal" that isolates the portion affected by the rest of the membrane (see fig. 1.19). Electronically blocking the potential difference across the portion of "sealed" membrane, physiologists can measure the current passing through the ion channels in the zone of the seal, without interference from the other

membrane current. The voltage clamp instead allows to measure the current through the entire membrane and not in an isolated portion, however, the voltage of the membrane is kept blocked at any predetermined value of the investigator and he is well placed in conditions to measure the ionic currents flow through the cell membrane without interference or annoying "background noise". In 1971 Gurdon and colleagues showed that oocytes extracted from African Xenopus were able to synthesize hemoglobin after injection of the corresponding messenger RNA. In fact, the oocytes are suitable for the expression of proteins from RNA, as they contain enzymes, organelles and macromolecules that the cell accumulates after fertilization and for this reason can be used for the translation of RNA to protein. Frog oocytes besides having this ability to translate exogenous RNA into proteins, have also others advantages:

- Low cost of animal maintaining;
- They can be easily harvested for their large diameter (1-1.2 mm);
- The injection of RNA is easily done thanks to their diameter;
- They can live for long time outside the body of the frog;
- Express a low number of endogenous carriers as they are independent of external nutrients;
- Multiple RNA species can be simultaneously injected to study complex protein;
- The standard electrophysiological techniques are easily applicable;

Electrophysiological studies through the use of frog oocytes could be various, as the characterization of carrier permeability, their pharmacological characterization, the analysis of mutant permeability studying the effect of post-translational modifications, the study of modulation between different channels. The most common method for expressing exogenous proteins in Xenopus oocytes consist of injecting messenger RNA in their cytoplasm. The

expression can also be achieved by injecting cDNA into the nucleus, but this requires visual localization of the nucleus with the risk of damaging the nuclear membrane. Therefore is more efficient the approach that uses injection of RNA, that I have also used in my work; later i will explain each step and technical details. The 'voltage clamp' or 'block of voltage' is a technique that allows to control and maintain the potential inducing the desired cell membrane potential difference and measuring the electrical changes in correspondence to given stimulus. The basis of voltage clamp can be understood considering the cell membrane as an RC circuit where the current passing through the circuit 'lm' is the result of the sum of ion currents 'Ii' and capacity:

$$Im = Ii + C\frac{dV}{dt}$$

In oocytes, voltage clamp is performed with two AgCl microelectrodes (voltage electrode and current electrode), infact the oocyte membrane resistance is low because of the large cell size and using a single microelectrode is not possible. In these microelectrodes are inserted glass microcapillary with a diameter of about 1-3 mm , they are filled with an electrolyte solution that mimics the extracellular environment and are placed within the cell maintaining a low resistance below 1 Mega ohm, not to ruin the membrane and to allow the current passage, (the circuit of 'two-electrode voltage clamp' is described in figure 1.20). Therefore this technique allows to know when the channels open and close and to obtain a measure of current flow at the given transmembrane depolarization, but this technique has some disadvantages that should be taken in consideration during the interpretion of the results; the oocyte has a number of resistances caused by a limited conductivity of the cytoplasm, that are in the order of 0.5 - 1 kΩ, leading to a reduction in potential, then a current of 10 μA will lead to a voltage error of 10 mV (Voltage = Current x Resistance intensity). In addition, the composition of the cytoplasm can not be controlled and this leads to significant variability of measures in different oocytes if the properties of the channel to be tested depend on intracellular contents . The oocyte has some leak currents

, mainly due to a high espression of calcium-dependent chloride channels that open with the increase of intracellular calcium, so that their opening activates the inward current, which inevitably confuses endogenous measures. It is impossible to manipulate the cytoplasmic calcium concentration to study its effects on expressed channels. The current leak, however, can be reduced by using appropriate solutions, for example buffer containing EDTA that chelates calcium, or by using inhibitors of chloride channels as a niflumic acid or subtracting from the current recorded in oocytes expressing the channel of interest the leak current recorded in oocytes at zero expression of the channel (used as a control). Studying voltage-gated channels activity by step protocols, the capacitive current must be subtracted during the register. Despite these drawbacks, the technique of oocyte voltage clamp is one of the most used electrophysiology techniques that has many advantages in comparison with other analysis systems, for its simplicity and for the multiplicity of studies that allows to perform.

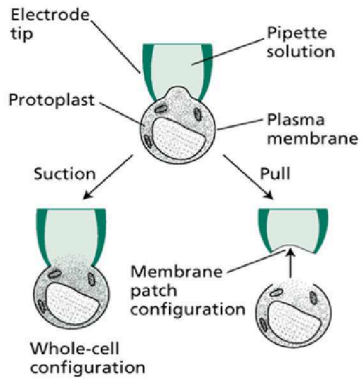


Figure 1.19: patch clamp seal (Plant Physiology, Fifth Edition, Lincoln Taiz and Eduardo Zeiger)

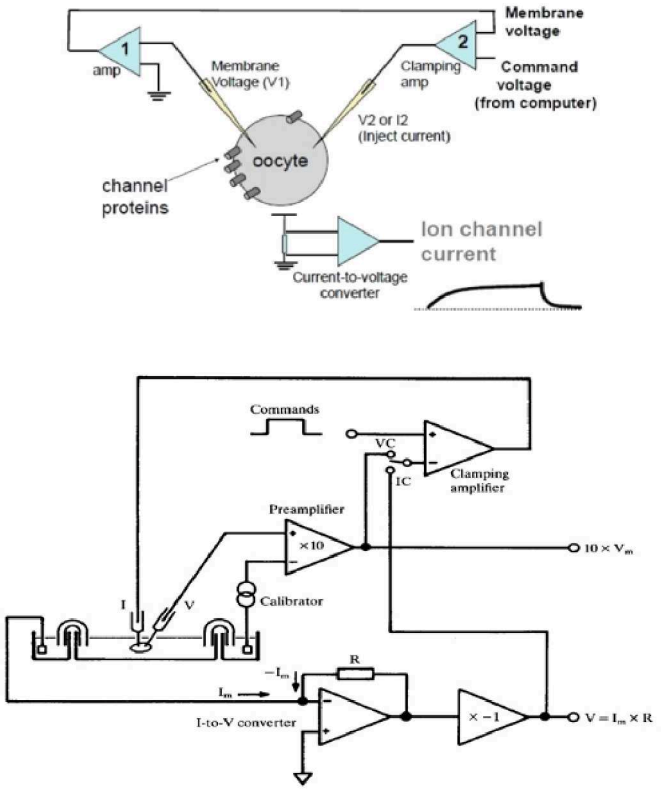


Figure 1.20: circuit of 'two electrod voltage clamp': A voltage recording electrode monitors membrane potential; this is compared with a command voltage, and the magnified difference is applied to a current injection electrode. A bath electrode serves as the return path for the injected current. The voltage clamp amplifier drives two current sources. One provides current q to the current-injection microelectrode, the other provides a current to the extracellular compensation electrode.The voltage clamp operates by negative feedback. The membrane potential amplifier measures membrane voltage and sends output to the feedback amplifier; this subtracts the membrane voltage from the command voltage, which it receives from the signal generator. This signal is amplified and output is sent into the cell via the current electrode (James V. Halliwell, Voltage clamp techniques ,Microelectrode Techniques The Plymouth Workshop Handbook , 1952).

Chapter 2

Objectives and methods

2.1 Objectives and methods

In relation to recent investigations already mentioned that showed the existence of physical and functional interactions between potassium channels and gap junctions, in particular between pannexin 1 and beta 3 subunit of Kv channel (Bunse et al. FEBS Journal, 276, 6258-6270, 2009), we hypothesized that pannexin 1 might be able to physically interact with other domains of the Kv1.1 channel without the intervention of the beta subunit, or that could exist only a functional link between the two proteins may be with a consequent reciprocal modulation; if the phenomenon occurs this interaction may play a key role in pathological conditions such as hypoxia, epilepsy and other channelopathies, in which the membrane potential and the ionic homeostasis are altered. Therefore we wanted to investigate about this possible physical and/or functional interaction and better understand its effects, mechanisms and functions. We used as models of study mouse pannexin 1 and human Kv1.1, the latter presents a sequence homology of 98% with the corresponding mouse channel. Thus the main objectives we set were the electrical analysis in oocytes, the coimmunoprecipitation inducing transient expression of pannexin 1 in HEK-HBK1 that stably expresses HBK1 potassium channel (human Kv1.1) to test the interaction between these proteins and then the purification of pannexin 1 to do a functional and structural characterization

of the protein; this last step of work has not yet been completed. Below I will discuss in detail the work carried out so far. The techniques used were as follows:

A. To measure the electrical activity of Kv1.1 channel 1 and pannexin 1 individually and when coexpressed in oocytes of Xenopus Leavis frog:

   - Midiprep of pcDNA3.1 plamisd (Invitrogen) containing mouse pannexin 1 gene
   - Midiprep of PCI-neo plasmid (Promega) containing human Kv1.1 channel gene
   - Linearization of amplified plasmids
   - Purification of obtained DNA
   - Reverse transcription to RNA
   - Precipitation of RNA
   - Extraction of oocytes from Xenopus Leavis frog and defolliculation
   - Injection of RNA in oocytes
   - Two electrode voltage clamp

B. To induce the expression of pannexin 1 in HEK-HBK1 expressing human Kv1.1 channel and to assess whether there is a direct physical interaction between the two channels:

   - Midiprep of pBMN-I-GFP plamisd (addgene) containing mouse pannexin 1 gene
   - Cell transfection
   - Immunocytochemistry
   - Coimmunoprecipitation
   - Cell death's valuation in presence of pannexin 1 inhibitors when pannexin 1 is coexpressed or not with Kv1.1 channel

All genetic constructs used in this work (except the PCI-neo) were prepared by GENEWIZ company (of New Jersey) with a final concentration of 5 μg, the company also was responsible for the synthesis of mouse pannexin 1 gene (NM_019482.2, GI: 86262133). PCI-neo construct containing the human Kv1.1 channel gene was kindly offered by the laboratory of professor Antonio Ferrer, institute of Molecular and Cell Biology, University Miguel Hernandez of Elche.

2.2 Midiprep of pcDNA3-1 and PCI-neo plasmids containing the DNA inserts of pannexin 1 and Kv1.1 channel

For the midi of the two constructs was used E. coli XLBlue strain. The transformation was performed as follows: the first step was the incubation of the mixture containing 200 ng of plasmid DNA and 100 μl of cells on ice for 30 minutes, with subsequent thermal shock by placing the mixture at 42°C for 45 seconds and then immediately on ice for 2 minutes. After that, 900 μl of preheated sterile Soc medium were added to the mixture and the latter was incubated for 30 minutes at 37°C in a shaking speed of 220 rpm; then 200 μl were plated on an agar dish previously treated with 50 μl of ampicillin 50mg/ml. After incubation at 37°C over night a colony was placed in 5 ml of LB Broth (Conda Pronadisa) supplemented with 5g NaCl/l and 5 μl of ampicillin 50mg/ml, for 8 hours at 37 ° C and shaking at 220 rpm. Then the bacterial culture was diluted in 100 ml of LB Broth supplemented with 5g NaCl/l and 100 μl of the same antibiotic for 12-16 hours at 37°C in shacking. The pellet was collected by centrifugation at 6000 rpm for 15 minutes at 4°C, the cloned DNA was extracted from the bacterial pellet using the Qiagen kit 'Plasmid Midi Kit' and following the directions provided by the manual. The DNA was then precipitated with isopropanol, washed with 70% ethanol, suspended in MQ sterile water and finally quantified by NanoDrop. The obtained yield was 1.4 μg / μl for the Kv1.1 and 1.3 μg / μl for pannexin 1. Then 15 μl of the DNA at final concentration of 100ng/μl were

mixed with 1,5 μl of relative primers prepared at final concentration of 5 μM; for pcDNa3-1 plasmid were used the T7 promoter primer (provided by the sequencing company) and two forward primers (5' to 3') designed by mouse pannexin 1 sequence (CGGACATTCTCAAAGTGTAT; CTGTTTGCCAT-ACTCCTG); for pCI-neo plasmid were used the T7 promoter primer and one primer forward (5' to 3') designed by human Kv1.1 sequence; samples were send to the genetic laboratory of Secugen company (Madrid), that checked and confirmed the correctness of the insert's nucleotidic sequence.

SOB medium:

2%	tryptone
0,5%	yeast extract
0,05%	NaCl

At final volume with dH_2O, following sterilization in autoclave and addition of 10 ml of 1M $MgCl_2$ and 10 ml of 1M $MgSO_4$ previously filtrated

SOC medium:

0,4%	sterile filtrated glucose

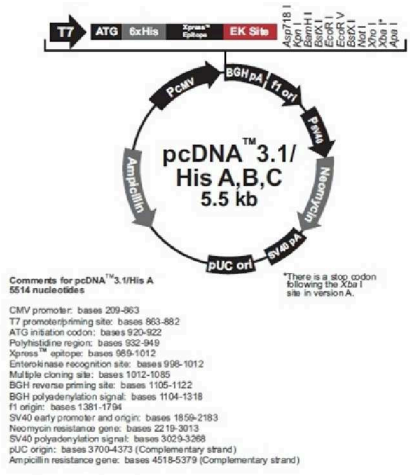
At final volume with SOB medium, preparation fresh immediately before use.

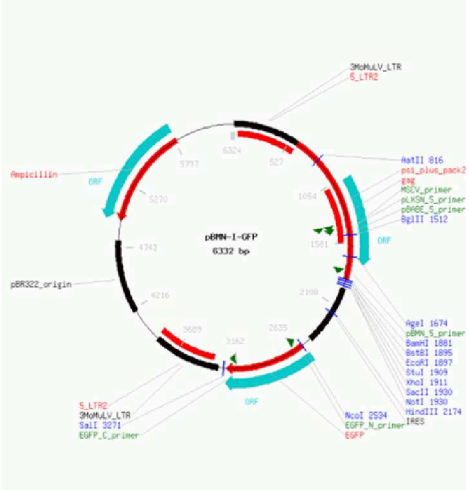
2.3 Linearization of plasmids

30 μg of plasmid DNA were digested using Fast Digest Fermentas kit; pcDNA3.1 construct containing pannexin 1 DNA was digested by XhoI enzyme because the cloning region was between BamHI and XhoI restriction sites; PCI-neo construct containing Kv1.1 DNA was digested by BAMH1 enzyme, it was not possible to use ECORI because the sequence of the human Kv1.1 channel contains portions homologous to the sequence of this restriction enzyme.

The DNA was incubated with the proper enzyme and the reaction buffer as specified in the manual for 15-30 minutes at 37 ° C. After it was performed the analysis on agarose gel to confirm the linearization (see Fig. 2.1). The agarose gel was prepared at 8% concentration with 1,5 μl of 'red nucleic acid gel Gel Stains' (Biotin); the electrophoresis was performed at 100 V in Tris acetic acid-EDTA buffer (TAE 1X), using 1Kb DNA Ladder as marker (Invitrogen).

Map of vectors:





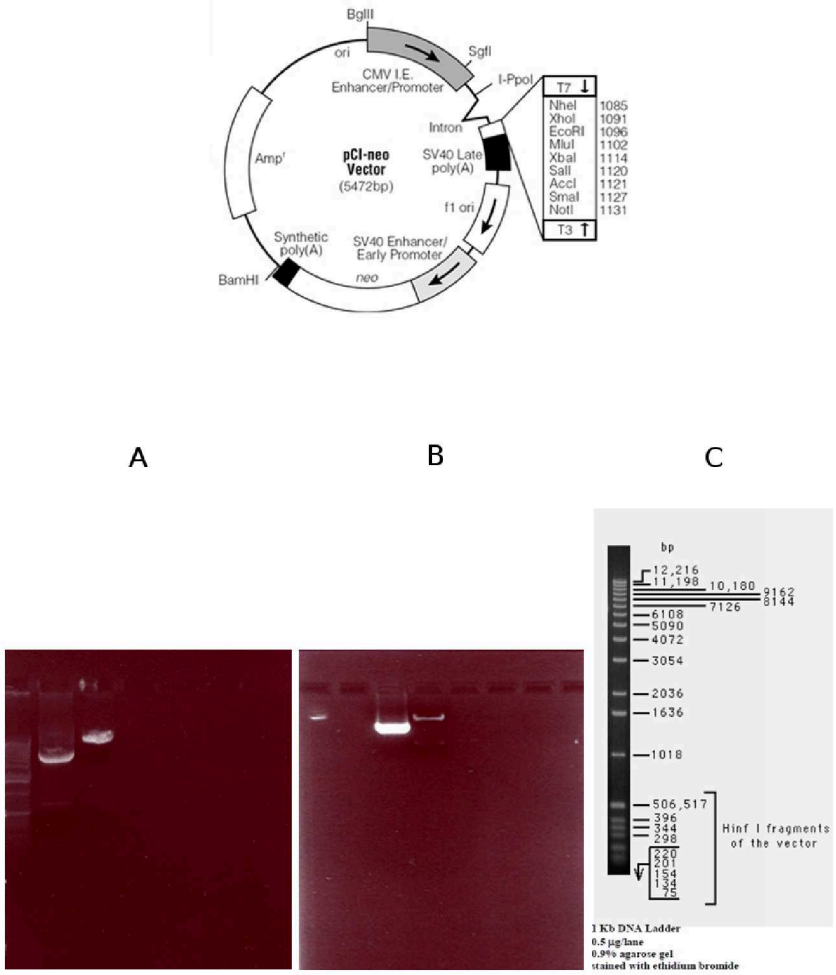


Figure 2.1: A : pCI-neo-Kv1.1 digestion; B: pcDNA3.1- Panx1 digestion; C : 1 Kb DNA ladder; both genetic constructs have a size of about 6,7/6,8 Kb (pCI-neo 5,4 Kb, Kv1.1 cDNA 1488 bp; pcDNA3.1 5,5 Kb; Panx1 cDNA 1297 bp); the digested construct is linear for this reason it runs slower than the undigested construct.

2.4 Purification of digested DNA

The digested DNA was purified using the kit roche 'High Pure PCR Product Purification Kit' according to the instructions in the manual, in order to eliminate the restriction enzyme and other components of the buffers required for digestion. Purified DNA was analyzed on 8% agarose gel (see fig. 2.2) The

obtained yield is often around 200 ng / μl, for example in the first experiment it was obtained 208.3 ng/μl for pannexin 1 DNA and 242.5 ng/μl for Kv1.1 channel DNA, the quantification was performed with the NanoDrop.

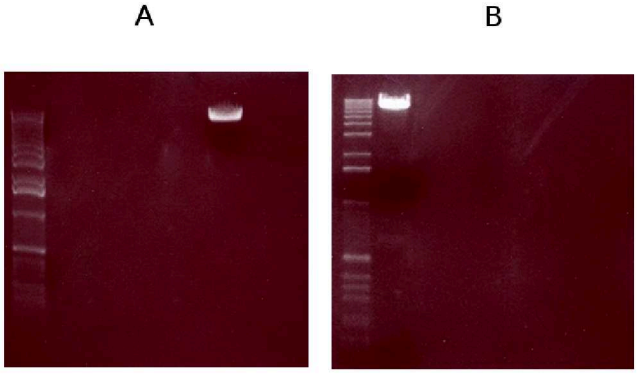


Figure 2.2: Purified digested DNA, A: pannexin 1, B: Kv1.1 channel

2.5 Retrotrascription of purified DNA to RNA

For the retrotrascription it was used Ambion 'mMESSAGE mMACHINE T7 Ultra Kit', using T7 RNA polymerase enzyme because in both insert sequence is present the promoter T7. The protocol was executed according of the handbook indications. 1,5 μg of DNA was used; once mixed all together DNA, buffer of reaction, mixture of ribonucleotids NTP/CAP and enzyme for a final total volume of 20 μl, the mix was incubated at 37 °C for 1 hour and 30 minutes. Then the reaction was quickly stopped with 10 μl of 5 M ammonium acetate and the RNA was precipitated and extracted in two ways:

- with lithium chloride: 50 μl of 7,5 M lithium chloride was added to the reaction and incubated for 30 minutes at -20°C, successively it was centrifugated at the maximum speed for 15 minutes, the pellet obtained was washed with ethanol 70%, resuspended in sterile DEPC water and quantified with nanodrop.

- with phenol/chloroform: at the stopped reaction was added first sterile

DEPC water, then phenol and chloroform, the mixture was centrifugated at the maximum speed in order to collect the superior phase, it was inserted newly chloroform and it was collected again the superior phase by centrifugation, quickly after it was added 1 volume of isopropanol in order tp throw down the RNA, and it was incubated at -20°C over night. Successively the pellet obtained for centrifugation was washed with ethanol 70% and resuspended in sterile DEPC water, finally it was quantified with nanodrop.

The better yield was obtained through the precipitation with lithium chloride. The amount of RNA and the values of purity in these experiments were the following ones:

- for pannexin 1: 799 ng/μl; 260/280 = 1,89; 260/230 = 2,37
- for Kv1.1 channel: 504 ng/μl; 260/280 = 2,02; 260/230 = 2,32

The RNA was then visualized in 8% agarose gel in order to verify that it was not degraded (see fig. 2.3).

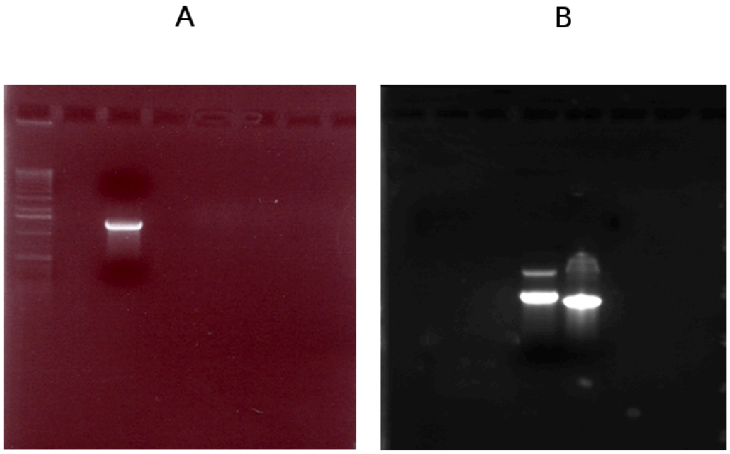


Figure 2.3: A: Kv1.1 RNA; B: pannexin 1 RNA (RNA preparation performed in duplicate)

2.6 Oocyte extraction from Xenopus Laevis frog

Oocytes were obtained from adult Xenopus leavis frog; the frog was anesthetized with 2% tricaine (3 - AMINOBENZOIC ACID ETHYL ESTER METHANESULFONATE) and ice, after the surgery were given 3 stitchs respective in the muscular wall and the skin. The defulliculation was executed placing oocytes in free calcium barth's medium (88 mM NaCl, 1 mM KCl, 2,4 mM $NaHCO_3$, 0,82 mM $MgSO_4$x7H_2O, 10 mM Hepes, pH 7,5) with collagenase 20 mg/ml for approximately 1 hour at room temperature, successively oocytes were placed first in free calcium barth's medium in order to wash them and to eliminate collagenase and after in calcium barth's medium (with 0,33 mM of $CaNO_3$x4H_2O) for 1-3 hours at 18°C, in order to restore the oocyte with extracellular calcium, finally oocytes in good conditions and with uniform pigmentation was selected through the optic microscope.

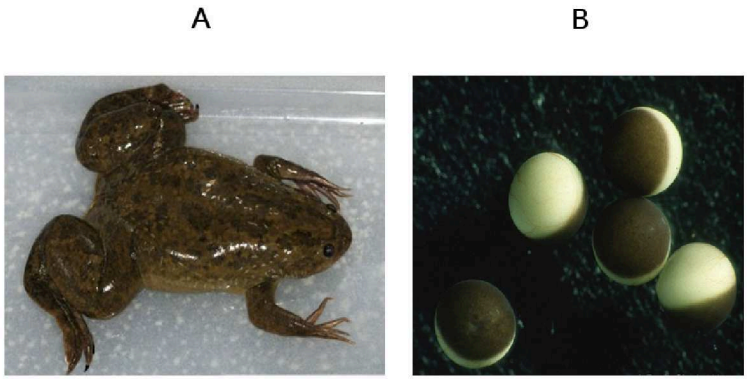


Figure 2.4: A: Xenopus Leavis frog; B: frog oocytes

2.7 Injection of RNA in oocytes

Injection of pannexin 1 RNA:
in order to verify what RNA amount would given the best result, three various amounts were initially injected, then 5, 10, 20 and 40 ng of RNA at oocyte,

after the injection they were incubated for 24, 36, 48, 60 and 72 hours at 18°C.

Injection of Kv1.1 channel RNA:

oocytes were injected with 5 and 10 ng of RNA , according to what was described in literature (Goat ET to, June 15, 2001 The Journal of Biological Chemistry, 276,21070-21076) for 24-48-36-60-72 incubation hours at 18°C.

Injection with pannexin 1 and Kv1.1 RNA:

once the optimal expression condition of proteins was tested, respectively 5 and 10 ng of panx1 RNA were mixed with 5 and 10 ng of Kv1.1 RNA , the mixtures were injected in each oocyte, using like control the cells injected with only RNA of pannexin and only RNA of the Kv1.1; the incubation was made for 24-36-48-60-72 hours before to record the electrical activity.

Injections have been made through the microinjector Drummond Nanoject, using a microcapillary previously filled up of oil, with an injecting volume of 50 nl at oocyte on the vegetable pole.

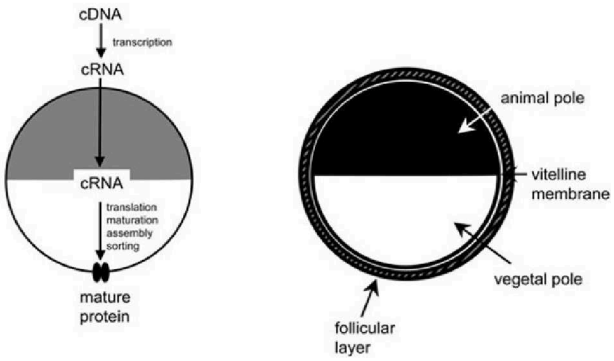


Figure 2.5: frog oocyte morphology (Heterologous expression of C. elegans ion channels in Xenopus oocytes, Laura Bianchi)

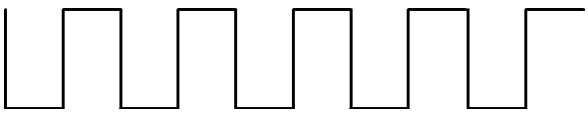
2.8 Two microelectrods voltage clamp (TEVC)

Electrical activity was analyzed using 'two voltage clamp' (the TEC 10CD, npi electronic, Tamm Germany), microcapillaries was prepared from Corning 7052 glass (Garner Glass, Clairemont CA USA) with a P-97 puller (Sutter Instruments, Novato CA USA) and filled up of a solution containing 1M KCl and 10mM Tes; oocytes were continuously perfused in a perfusion chamber. In order to measure the electrical activity of pannexin 1 the potential was reduced in a 'square' manner till the level in which the channel opens; the conductance of the membrane was analyzed at room temperature maintaining the membrane potential at -50 mV, value at what channels are normally closed, applying -10 mV depolarization impulses at 5 impulses per minute and with 10 total intervals per minute, with a duration of 6 second ones. Therefore during the registry membrane potential varied from -50 mV to -40 mV- at intervals of 6 second ones. The resistance of voltage electrodes was maintained less than 0,5 MΩ, the currents were measured at a frequency of 13-20 kHz and filtered at 1kHz by a differential amplifier; moreover only oocytes that had a basal membrane potential between -30 and -70 mV and a basal current around 0 μA were analyzed. During the registry was executed 1 minute of perfusion with Barth's medium (88 mM NaCl, 1 mM KCl, 2,4 mM $Na - HCO_3$, 0,82 mM $MgSO_4$x7H_2O, 0,33 mM $CaNO_3$ x 4H_2O, 10 mM Hepes, pH 7,5), 5-7 minutes of perfusion with a solution containing high potassium chloride concentration(140 mM KCl, 5 mM HEPES, pH 7,5) followed by 4-6 minutes of perfusion with Barth's medium in order to wash the oocyte. The total duration of each registry was of 12 minutes. All perfusion solutions were prepared with MQ water. Initially were analyzed not-injected oocytes as control and injected oocytes with panx1 RNA at different concentrations and various times of incubation as explained before (5-10-20-40 ng/ovocita, for 24-36-48-60-72 hours of incubation), in order to verify the activation of oocytes expressing panx1 channel when perfused with 140mM KCl, as reported in literature (Silverman et al, The Journal Of Biological Chemistry Vol. 284, Not. 27, pp. 18143-18151, July 3, 2009). After many recordings with the described protocol to confirm the expression of panx1,

subsequently the same protocol was used to record the electrical activity in presence of probenecid and DTT, that are a pannexin inhibitors. How i have already mentioned recent studies reported that the inhibition by probenecid is concentration depending and at 1mM occurs almost the total inhibition of electrical activity (Silverman et al, Am J Physiol Cell Physiol 295:C761-C767, 2008; Weihong But, The Journal of Pharmacology and Experimental Therapeutics, 328:409–418,, Vol. 328, No. 2., 2009), while with DTT a partial inhibition occurs. In the present work we have made reference to these recent data. The perfusion in presence of probenecid and DTT was performed in the follow way: oocyte was perfused with barth's medium for 1 minute, immediately after was perfused with KCl 140 mM for 5-7 minutes and then perfused again with barth' s medium for 4 minutes, once ended the first record a second record was carried out on the same oocyte, the latter was first incubated with barth' s medium containing 1 mM probenecid or 10 mM DTT for 3-5 minutes (before beginning the register),then to perform the recording it was perfused with the same solution containing the inhibitors for 1 minute , subsequently again perfused for 5-7 minutes with KCl 140 mM and finally washed with barth' s medium for 4-6 minutes. This procedure of perfusion with probenecid and DTT was formulated after many tests and was that gave the better and mainly reproducible results.

Probenecid was bought from Sigma (cod. P8761), it was made a stock with a concentration of 100 mM in water MQ adding NaOH up till the entire dissolution, with a final pH of about 9. The stock was normally preparated at the moment; DTT was too preparated at the moment, in MQ water with a final pH of 7.

'Square' diagram of applied depolarization pulses (5 impulses/minute and 10 intervals):



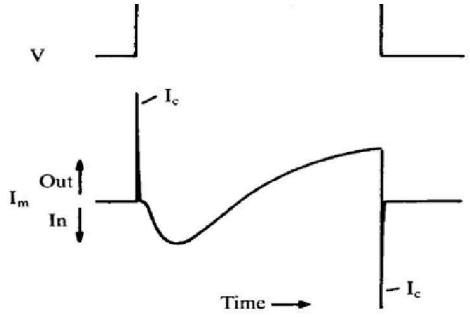
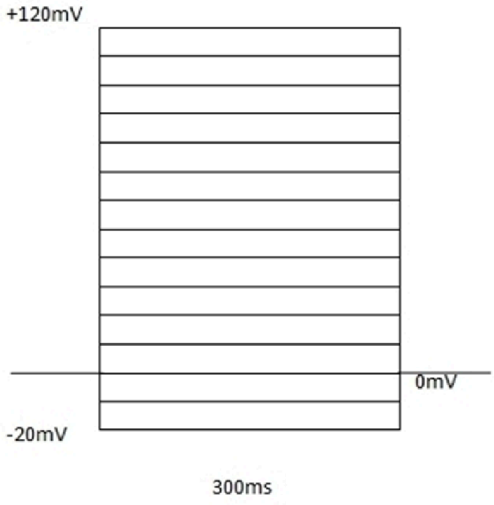


Figure 2.6: currents in a voltage clamped cell when the potential is reduced in 'square fashion' (James V. Halliwell, Voltage clamp techniques ,Microelectrode Techniques The Plymouth Workshop Handbook , 1952).

The electrical activity of Kv1.1 channel was measured with the square protocol and also with a step protocol, blocking the membrane potential at -80 mV, applying depolarization steps from -20 mV to + 120 mV with -10 mV voltage increments of the duration of 300 ms each one. The current was measured at a frequency of 13-20 kHz and filtered at 1kHz. The resistance of voltage electrode was maintained less than 0,5 MΩ, moreover was manually eliminated the capacitive current, monitoring the parameters of amplifier EPC9 on the oscilloscope window, in order to avoid the initial peaks of current at the abrupt variations of potential. Oocytes were perfused constantly with a solution containing 30mM KCl (Hepes 10mM, KCl 30mM, $CaCl_2$ 0,3mM, $MgCl_2$ 1mM, NaCl 70mM, pH 7,4). Using this protocol were analyzed also oocytes expressing pannexin 1; each oocyte was analyzed a single time at registry, that is the same cell never was reused for a successive registry unlike than in square type analysis. Finally in order to estimate the electrical activity of Kv1.1 channel and pannexin 1 when coexpressed, both protocols were used as soon as described for every condition, that is for injected oocytes with RNA of panx1, RNA of Kv1.1 and RNA of both channels. The minimal concentration of KCl necessary to activate the pannexon is of 20 mM. During the perfusion were administered the inhibitors of pannexin 1, probenecid at the concentration of 1 mM and DTT at the concentration of 10 mM, and the inhibitor of the Kv channel, tetraethylammonium (TEA), at the concentration of 10 mM (Zhang ET to, J Neurophysiol 85:362 - 373,

2001). During the step type analysis probenecid, DTT and TEA were added directly in the perfusion buffer containing 30 mM KCl, without any previous incubation of the oocyte with the inhibitors, as well during square type analysis TEA was added directly in the perfusion buffer containing 140 mM KCl without any previous incubation of the oocyte with the inhibitor, unlike probenecid and DTT. All data were acquired and analyzed with the software Pulse (PULSE 8,09, Heka Electronic, Lambrecht Germany). Numerous recordings were performed in order to verify the expression times and the RNA concentration that gave the better response and to do an accurate statistical valuation of the obtained results, considering also that sometimes oocyte have high current of leak or are not in ideal conditions. The current of leak was calculated through the measures obtained from oocytes at null expression of the channels (not injected).
Step protocol:



2.9 Transient expression of mouse pannexin 1 in HEK-hBK-1 cell line

We used HEK-hBK-1 cell line, that consists of human embryonic kidney cells (derived from HEK-293 cells) expressing in stable way hBK-1 potassium channel, or human Kv1.1. To transfect this cell line it was used pBMN-I-GFP plasmid (addgene), to be able to monitorate pannexin 1 expression through the expression of the GFP (green fluorescent protein of victoria aequorea jellyfish), that it is strongly fluorescent with the maximum emission at 507 nm. However for some experiments we also used HEK-293 cell line and SH-SY5Y cells, a human derived neuroblastoma cell line used in our experiments as control because it doesn't express Kv1.1 channel, infact they are undifferentiated cells that express mainly 'inwardly rectifying' potassium channels, some voltage gated potassium channels like Kv3.1 but not Kv1.1; moreover they express pannexin 1.

2.10 Midiprep of pBMN-I-GFP plasmid containing the DNA insert of pannexin 1

The midiprep of the plasmid was executed using E.Coli XLBlue strain with the protocol followed for the other two cloned vectors (see paragraph 2.1), but in this case, once bacteria were transformed and cultivated in agar plate, the latter was incubated for 2-3 days at 37°C, because with this plasmid the bacterial growth was very slow. Moreover, even if this vector was retroviral and LTR sequence could give genetic recombination, the used strain had allowed to clone correctly the vector. We used 'Plasmid Midi Kit' (qiagen) to extract the DNA, as already described in detail for the midiprep of pcDNA3-1 and PCI-neo plasmid (see paragraph 2.1), obtaining a DNA yield of approximately 1 μg. Then 15 μl of the DNA at final concentration of 100ng/μl were mixed with 1,5 μl of relative primers prepared at final concentration of 5 μM; we used 5' to 3' pBMN_5 primer (5'GCTTGGATACACGCCGC) and two 5' to 3' primers designed by mouse pannexin 1

sequence, one reverse (GGAACTCGTCCGAGAGTGAA) and one forward (CGGACATTCTCAAAGTGTAT). The samples were send to the genetic laboratory of Secugen company (Madrid), that checked and confirmed the correctness of the insert nucleotidic sequence.

2.11 Maintenance of cell lines

HEK-hBk1 cells were maintained in 'cellbind flask' of 25 cm^2 (corning) in DMEM-F12 (GIBCO) supplemented with 10% of inactivated fetal bovine serum, 1% of glutamine/penicillin/streptomycin and 1.5% of geneticin. The medium was stored at 4 °C at dark. It was changed every 2 days, once riched the confluence of 90% cells were trypsnized with trypsin-EDTA 0.25% (Sigma) for two minutes at 37°C and successively trypsin was inactivated adding 5 ml of complete medium. The cells were plated on 6 well and 24 well sterile plates at the desired concentration for being successively transfected. HEK-293 cells were maintained in DMEM (+ 4,5 g/l glucose-pyruvate) supplemented with 1% penicillin/streptomycin and 10% FBS. SH-SY5Y cells were maintained in MEM (+ L-Glutamine), supplemented with 1% penicillin/streptomycin , 1% glutamine, 1% no-essential aminoacids, 10% FBS.

2.12 Cell Transfection

Cells were transfected with p-BMN-I-GFP plasmid (containing mouse pannexin 1 gene) using lipofectamine 2000 (invitrogen). DNA and the lipofectamine were diluted separately in DMEM-F12 without FBS and incubated for 5 minutes at room temperature, successively the diluted DNA was added to lipofectamine incubating the mix for 20 minutes at room temperature in order to allow the formation of the lipofectamine-DNA complex; 100 μl a well were added in 24 multiwell plate and 500 μl a well were added in 6 multiwell plate. After 4-6 hours the medium was always changed. Various transfections were carried out in order to test the right concentrations of DNA and lipofectamine and the right number of plated cells to obtain the

better expression of pannexin 1. The expression was estimated through western blot and analysis of GFP fluorescence in confocal microscope. The same condition of trasfections were used for HEK-293 and SH-SY5Y cells.

2.13 Immunocytochemistry

To execute immunocytochemistry cells were plated on 18 mm crystals placed in 24 multiwell plate, previously coated with with poly-L-lysine (sigma) for 30 minutes at 37°C and successively washed with sterile PBS 1X. Various concentrations of cells were tested, 20000 cells/well, 40000 cells/well, 60000 cells/well and 80000 cells/well, transfection were executed the day after and it was tried in presence or in absence of antibiotics in the culture medium. DNA concentrations used were of 400ng/well, 800ng/well and 1μg/well with respectively 0,8 μl, 1,6 μl and 2 μl of lipofectamine. Immunocytochemistry was executed after 24, 48 and 72 hours from trasfection .Cells were fixed in 4% paraformaldehyde supplemented with 4% sucrose for 20 minutes at room temperature and later were washed with PBS 1X pH 7.4. Permeabilization was carried out in PBS 1X pH7.4 supplemented with 3% FBS, 2% BSA, 0.1% triton, for 15 minutes, the blocking was executed with the same solution without triton, for 30 minutes. Rabbit polyclonal anti Kv1.1 (abcam) was incubated for 1 hour and 30 minutes at room temperature or over night at 4°C, in a solution containing PBS 1X, 1,5% FBS and 1% BSA; the concentration of antibody was used at 1:500. Incubation with antirabbit rhodamine conjugated (millipore) was performed in the dark for 1 hour and 30 minutes, its concentration was used at 1:500. Finally cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) at the concentration of 1:10000 in PBS 1X for 10 minutes to mark the nucleus. For negative control cells were incubated with secondary antibody in absence of primary antibody or in presence of a different primary antibody. All washings were performed in PBS 1X. Crystals were mounted with mowiol on appropriate slides and stored at 4° C. Samples were analyzed with confocal microscope 'Carl Laser Zeiss Scanning Confocal Microscope' using the program LSM 5 Pascal; some sample was also analyzed with Eclipse 80I Fluorescence Microscope (Nikon)

using Qimaging QcapturePro software to take pictures in cell nucleus. GFP presents the greatest excitation at 490 nm with emission at 507 nm (green), rhodamine presents greatest absorption at 550 nm and emission at 570 nm (red), therefore the laser argon at 488 nm and the filterset for FITC (fluorescein isothiocyanate, emission at 505-530 nm) or for GFP (emission at 505-600 nm) were used to visualize the GFP, the laser at 543 nm and the filterset for TRITC (Tetramethyl Rhodamine Isothiocyanate, emission at 560 nm) were used for rhodamine visualization. Dapi presents greatest absorbtion at 358 nm (ultraviolet) and greatest emission at 461 nm (dark blue), therefore for nucleus visualization cells were excited with ultraviolet light and detected by blue/cyan filter. To ensure that the GFP did not alter the localization of pannexin 1 it was performed the immunocytochemistry on HEK-293 cells and HEK-hBK1 cells, they were transfected with pBMN-I-GFP plasmid containing pannexin 1 gene, then transfected cells were incubated with the monoclonal antibody for pannexin 1 (anti pannexin 1 monoclonal rabbit antibody, Epitomics) at the concentration of 1:500 and with the secondary antibody (goat anti rabbit IgG rhodamine conjugated, Millipore) at the concentration of 1:500, in order to show both GFP and rhodamine. Moreover some sample of cells marked only with DAPI was analyzed to appraise the toxicity of the lipofectamine and the antimitotic effect of pannexin 1 (reported in literature), confronting between them transfceted cells, not-transfected cells and cells treated only with lipofectamine.

2.14 Valuation of cell death

The experiment was conducted in 24 multiwell plates, in which were plated 80000 cells per well, after 24 hours they were transfected with p-BMN-I-GFP plasmid containing pannexin 1 gene, using the same quantities and conditions of transfection than for immunocytochemistry (paragraph 2.12 and 2.13). After 48 hours of transfection treatments were performed, each condition was performed in duplicate or triplicate, as follows:

- with 140 mM potassium chloride (supplemented in the culture medium)

- with 1 mM probenecid pretreatment for 10 minutes, change of medium and subsequent incubation with 140 mM potassium chloride and 1 mM probenecid
- with 140 mM potassium chloride and 10 mM dithiothreitol
- with 140 mM potassium chloride and 10 mM tetraethylammonium
- with 140 mM potassium chloride, 1 mM probenecid and 10 mM tetraethylammonium
- with 140 mM potassium chloride, 10 mM dithiothreitol and 10 mM tetraethylammonium

Cells were incubated at 37 °C under these conditions for 1 hour. After treatments cells were trypsinized with trypsin-EDTA (100 μl/well) for 2 minutes, trypsin was then neutralized with 400 μl of culture medium and cells were centrifuged. The obtained pellet was suspended in 300 μl of PBS 1X and was incubated with 300 μl of 0.4% trypan blue for 5-10 minutes at 37°C. Immediately after the count of viable and not-viable cells was made using the hemocytometer. Some sample moreover was analyzed in the fluorescence microscope to acquire the images of trypan blue stained cells. For this experiment HEK-hBK1 and SH-SY5Y cells were used; trypan blue was prepared in PBS 1X and stored at room temperature. Stock solutions of probenecid , DTT and tetraethylammonium were prepared just before their use.

2.15 Preparation of protein extracts

For the preparation of protein extracts cells were plated in 6 multiwell plates (previously treated with poly-L-lysine) at various concentrations till to obtain the right confluence of 80-90% in the day of the transfection, therefore 450000 cells/well, 350000 cells/well and 250000 cells/well were plated to be transfected after 2 days. Transfection were carried out using various DNA concentrations and lipofectamine, it was tested 1 μg of DNA with 4 μl of lipofectamine, 1,5 μg of DNA with 4 μl of lipofectamine, 2 μg of DNA with

6μl of lipofectamine and 3 μg of DNA with 8 μl of lipofectamine. After 48-60 hours cellular lysis was made with Ripa buffer containing 50 mM Hepes, 140 mM NaCl, 10% glycerol, 1% Triton, 1 mM EDTA, 2 mM EGTA, sodium deoxycholate 0.5%, pH 7.4. At the moment of lysis were added protease inhibitors with a proportion of 1:100 (PMSF 2 mM, sodium orthovanade 1 mM, iodoacetamide 20 mM and inhibitor of trypsin 10 μg/ml). Lysis was executed on ice with 150-200 μl/well of buffer RIPA, once collected the lysate in eppendorf, it was left in agitation for 10 minutes at 4°C and at last it was centrifugated at 4°C and 13000 rpm for 10 minutes in order to eliminate not soluble parts. Supernatant was harvest and frozen at -20°C for successive analysis. Transfected and not-transfected cell lysates were used for the revelation of pannexin 1 and Kv1.1 channel by western blot and following coimmunoprecipitation.

2.16 Quantification of proteins

The protein extracted were quantified with the BCA kit (BioRad). Samples with increasing concentration of BSA (0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml) were prepared in RIPA buffer and used as standards, as blank it was used RIPA buffer only. According to the manual 20 μl of reagent S were mixed with 1 ml of reagent A, then at 15 μl of each sample (including the extracts to be quantified) were added 75 μl of the mixture prepared with S and A reagents and 600 μl of reagent B. All samples were incubated at room temperature for 20 minutes. The absorbance was read at 750 nm. Obtaining the calibration line on the standards' absorbance readings and using Lambert Beer equation ($A = \epsilon \times c \times d$), the concentration of proteins was calculated. The total yield of protein extracts was about 0.7/1 mg/ml when extracts were prepared from a flask of 25 cm^2 and about 0.4/0.5 mg/ml when the extracts were obtained from 2 wells of the 6 multiwell plate (used for transfection).

2.17 Western Blot

Samples were prepared with laemmli sample buffer 2x (126 ml Tris/Cl (pH 6,8), 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0,004% bromophenol blue) at a proportion 1:1, heated at 100°C for 5 minutes and loaded in a 10% and 8% SDS-Page acrylamide gel (10-20 μg of proteins were loaded for each well). The gel was prepared as reported below:

Running gel 10%:
Tris-HCl 1,5 M pH 8.8 1,25 ml
dH_2O 2,025 ml
Acrylamide 30%/bis-acrilamide 0,8% (Protogel) 2 ml
SDS 10% 50 μl
Temed 3,5 μl
ammonium persulfate 32,5 μl

Running gel 8%:
Tris-HCl 1,5 M pH 8.8 1,25 ml
dH_2O 2,32 ml
Acrylamide 30%/bis-acrilamide 0,8% (Protogel) 1,85 ml
SDS 10% 50 μl
Temed 3,5 μl
ammonium persulfate 32,5 μl

Stacking gel 4%:
Tris-HCl 0,5 M pH 6,8 0,625 ml
dH_2O 1,525 ml
Acrylamide 30%/bis-acrilamide 0,8% (Protogel) 0,325 ml
SDS 10% 25 μl
Temed 2,5 μl
ammonium persulfate 17,5 μl

As marker was employed 'the Spectra™ Multicolor Broad Range Protein Ladder' Fermentas. Electrophoresis was carried out at 100 Volt in Tris-Glicina-SDS 1X buffer; transfer on nitrocellulose membrane (biorad) was

executed at 100 Volt for 2 hours on ice in buffer of transfer (14,4 g/l glycine, 3,03 g/l Tris base, 200 ml/l methanol) that was prepared immediately before the use. Membrane was incubated at least for 1 hour in Tris buffered saline solution with 0,05% Tween, pH 8 (sigma), supplemented with 3% of BSA (for pannexin 1 detection) and 0,5% of BSA or 5% of dried milk (for Kv1.1 detection) ; later it was incubated overnight at 4°C with rabbit polyclonal anti Kv1.1 (abcam) and rabbit monoclonal anti pannexin 1 (epitomics); for anti Kv1.1 antibody the concentration was experienced at 1:5000, 1:3000, 1: 1000, 1:500 ; for anti Panx1 antibody the concentration was tested at 1: 3000 and 1: 5000. After washings membrane was incubated with goat-anti-rabbit-IGg-peroxidase (sigma) at the concentrations of 1:1000 or 1:2000 for 1 hour and 30 minutes; chemiluminescence revelation was performed with 'ECL Plus Western Blotting Detection Reagents' kit, GE Healthcare, for 3-5 minute in the dark and finally photographic plate was impressed or membrane was visualized through 'ChemLite 200 FA, Luminant Image analysis System' (Avegene) and the Xview software.

2.18 Coimmunoprecipitation

G protein is a bacterial cell wall protein original from group G Streptococcus (21,6 kDa, 31-34 kDa on SDS-PAGE gel), like Protein A, Protein G binds to most mammalian immunoglobulins primarily through their Fc regions and provides a good capacity binding for IgG from rabbit, mouse, rat, cow, goat; for this it is suitable to immunoprecipitate proteins and to identify their interaction . The coimmunoprecipitation was performed as follows: once the lysate was prepared from transfected cells, 300 μl of the extract were incubated with 25 ul of Protein G Agarose (Thermo Scientific), previously equilibrated with RIPA buffer, for 30 minutes at 4°C, in order to remove nonspecific interactions. After centrifugation at 14000 rpm for 10 minutes at 4 ° C the obtained supernatant was incubated with rabbit anti-pannexin 1 antibody at the concentration of 1:100, over night at 4°C. Once made a further centrifugation at 14000 rpm in order to remove any precipitate, the supernatant was incubated with 50 μl of G protein (previously equilibrated

in 500 μl of RIPA buffer) for 2-3 hours at 4°C. Subsequently, the resin was precipitated by centrifugation at 2000 rpm for 5 minutes, it was washed 2-4 times with RIPA buffer and suspended in Laemmli sample buffer in a 1:1 ratio. The obtained sample was heated at 90°C for 15 minutes, centrifuged at 2000 rpm for 3-4 minutes and finally the supernatant was loaded into a 10% or 8% acrylamide gel for analysis by western blot (see paragraph 2.17). As control experiments were performed a coimmunoprecipitation without any antibody, a coimmunoprecipitation using only the secondary antibody and a coimmunoprecipitation using a protein extract from SH-SY5Y cell line (that doesn't express Kv1.1 channel). Once carried out the coimmunoprecipitation the western blot membrane has been revealed with the anti-Kv1.1 antibody, then the same membrane has been stripped in order to eliminate the union of the antibody with its antigen, through incubation for 30 minutes at 50^{o}C with reducing buffer (100 mM, β-mercaptoethanol, 2% SDS, 62,5 mM Tris-HCl pH 6,7); after two washings with TBS-0.05% Tween 20, it was blocked again in milk or BSA and incubated with the anti-Panx1 antibody. In order to reduce the contamination from IgG the coimmunoprecipitated has been loaded on a acrylamide gel without previous heating at 90 °C and using laemmli sample buffer lacking in reducing agents (2-mercaptoethanol).

Chapter 3

Results

3.1 Expression of pannexin 1 in frog oocytes and electrical activity in presence of potassium chloride

The RNA of mouse pannexin 1 was injected in oocytes of the Xenopus Leavis frog at different concentrations taking as reference the data reported in literature: 5 ng, 10 ng, 20 ng and 40 ng with incubation for 24 h, 36 h, 48 h, 60 h and 72 h. Using the square protocol already described, with 5ng RNA was detected a certain activity only after 48-60 hours, in fact at 24 and 36 hours the activity was similar to that of the control (see 3.1), with 10 and 20 ng there was a good activity already at 24 hours until 72 hours, but we have not found an increase in activity corresponding to the two increasing concentrations of RNA and incubation times (see fig. 3.2); with 40 ng of RNA instead oocyte showed high electrical current up to -10/-12 μA and they tended to break during the register, probably because of overexpression of the channel with a result in toxic effect (see fig 3.3). Considering the evidences described above we decided to use for our studies the concentration of 10 ng of RNA. Carefully assessing many registers we have done, we have found that the electrical activity in oocyte at good condition expressing pannexin 1 and perfused with 140 mM KCl was maintained in the range

of about -6.5 / -7.5 μA (inward electrical current), sometimes it reached -8 μA; on the contrary the electrical activity observed in not-injected oocyte and perfused with 140 mm KCl was in the range of -1/-3 μA (see fig. 3.1), we considered this value as an index of leak current. So pannexin channels presented a mean activity of -4/-5 μA current. With the step IV protocol described above, perfonding oocytes with 30 mM KCl the activity was rather low, like up to +1/+1,5 μA (outward electrical current, see fig. 3.4). The significant activation of pannexin 1 then occurs at higher concentrations of KCl, in contrast to the Kv1.1 channel. As pannexin inhibitors were used 1 mM and 2 mM probenecid and 10 mM DTT (as reported in literature). At 1 mM probenecid the activity reduction observed was of about -2/-3 μA using square protocol and approximately of 500-600 nA using IV step type analysis, presenting in this case not-injected like activity; at 2 mM probenecid there was a total activity inhibition; finally with DTT the reduction was partial, of about 1-2 μA with square protocol (see fig. 3.5 and 3.6). Not injected oocytes did not provide substantial changes in activity when perfused with probenecid and DTT (see fig. 3.7)

3.2 Expression of human Kv1.1 channel in frog oocytes and electrical activity in presence of potassium chloride

Human Kv1.1 channel RNA was injected into oocytes at two different concentrations, 5 and 10 ng oocyte, and incubated for 24, 36, 48, 60, 72 hours. With both concentrations, 5 and with 10 ng, from 48 hours of incubation up to 72 hours oocytes had a good activity. Not increased activity was observed at higher concentration of injected RNA. With square protocol oocytes expressing Kv1.1 channel and perfused with 140 mM KCl presented a mean inward electrical current of -7/-8 μA (including leak current), similar to pannexin 1 activity. Instead, using step IV protocol, oocytes espressing the channel showed a mean activity ranged from -0.5/-1 μA at the first depolarization step to +4/+7 μA (outward electrical current) at the last depolarization step,

when perfused with 30 mM KCl. However, some oocyte under the same conditions had a maximum current of 9 μA , perhaps due to a greater expression. In the presence of 10 mM TEA was observed an average reduction of about -3/-4 μA with the square type analysis, and a reduction of about 4/5 μA with the IV type analysis. The control did not present reduction (see fig. 3.8 and 3.9).

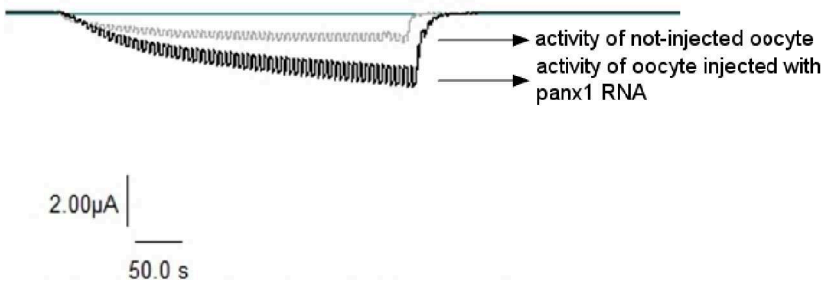


Figure 3.1: voltage-dependent activity in oocyte injected with 5 ng of pannexin 1 RNA (-2,7 μA) and in not-injected oocyte (-1,09 μA), after 24 hours of incubation, perfusion with 140 mM KCl; clamped potential at -50 mV, depolarizating pulse of -10 mV.

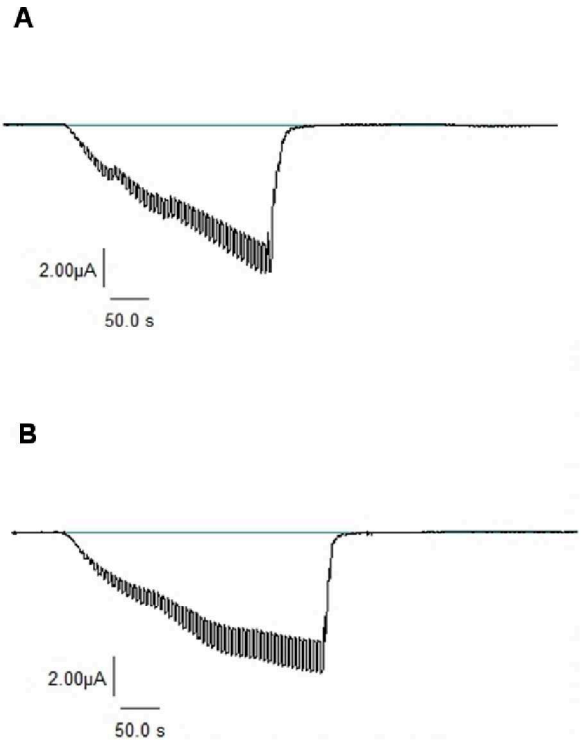


Figure 3.2: activity of injected oocytes with 10 ng of pannexin 1 RNA at 24 hours of incubation (A) and at 48 hours of incubation (B); (square register, perfusion with 140 mM KCl; -50 mV clamped potential, depolarizating pulse of -10 mV) A : -7,5 μA; B: -7 μA; oocytes injected with 20 ng of RNA presented similar activity

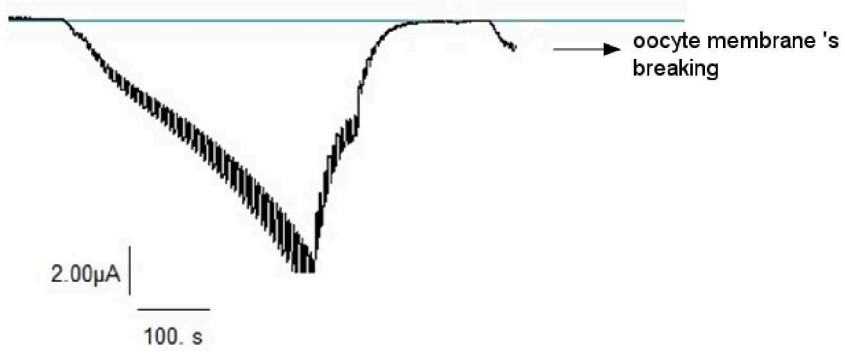


Figure 3.3: high activity up till -10.24 μA in oocyte injected with 40 ng of pannexin 1 RNA at 24 hours of incubation (square register, perfusion with 140 mM KCl; -50 mV clamped potential, depolarizating pulse of -10mV).

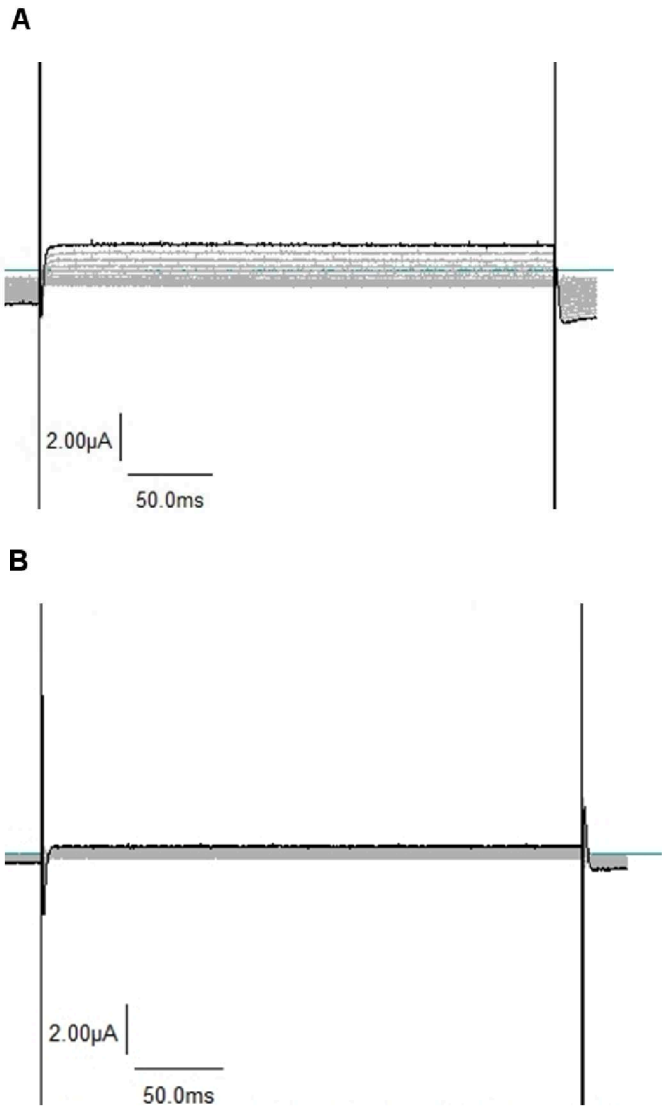


Figure 3.4: IV registry with depolarizating pulses from -20 mV a +120 mV in oocytes expressing pannexin 1 and perfused with 30 mM KCl (activity from -720 nA to +1,18 μA) (A), and in not-injected oocytes (maximal activity up to +392 nA) (B).

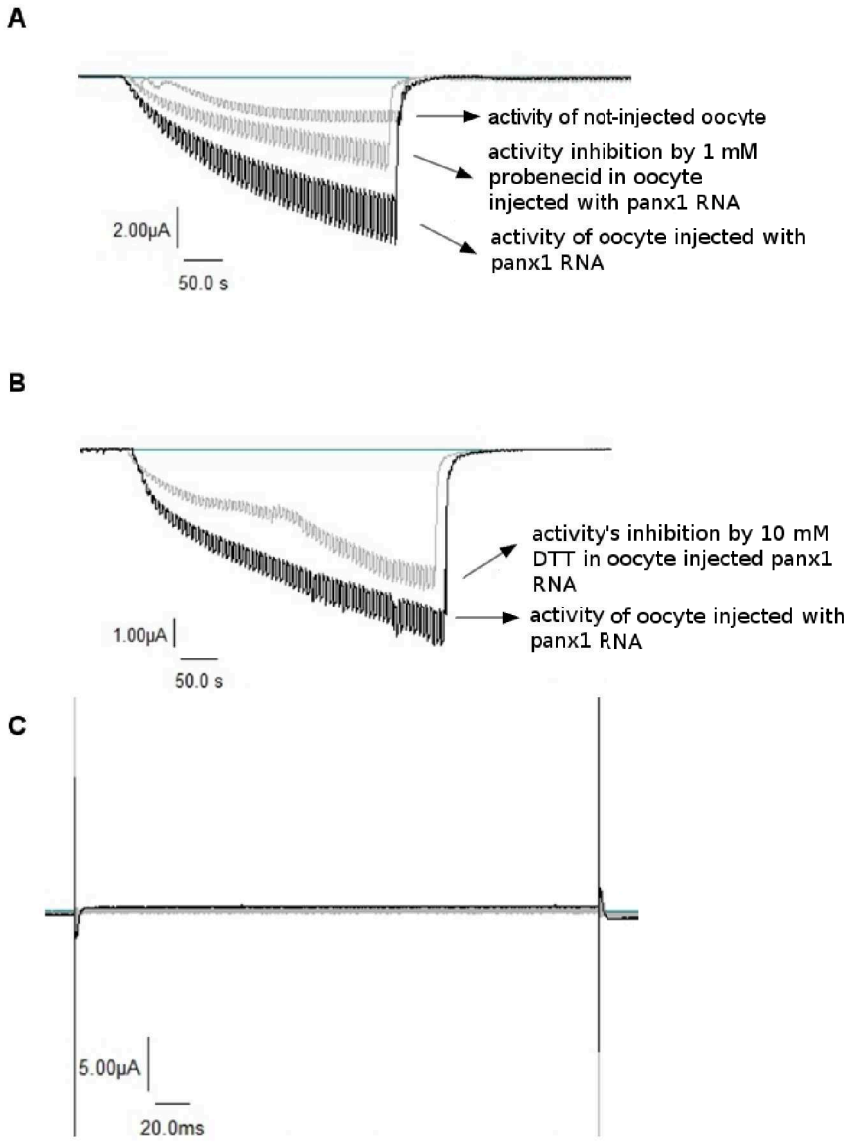


Figure 3.5: electrical activity reduction in oocytes expressing pannexin 1 when perfused with 1 mM probenecid and 10 mM DTT (A: activity overlap of not-injected oocyte, oocyte expressing pannexin 1 and subsequently perfused with 1 mM probenecid; B: activity reduction after perfusion with DTT; C: activity reduction after perfusion with probenecid in IV step type's analysis).

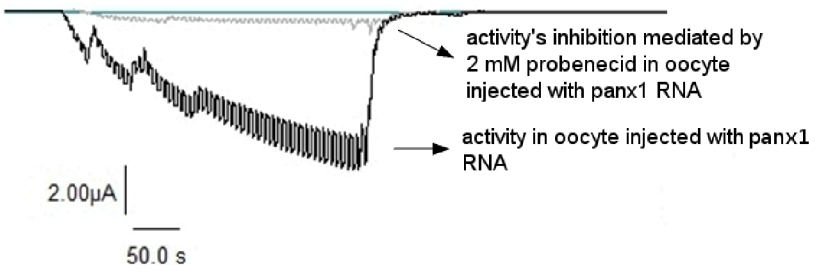


Figure 3.6: electrical activity reduction in oocytes expressing pannexin 1 when perfused with 2 mM probenecid

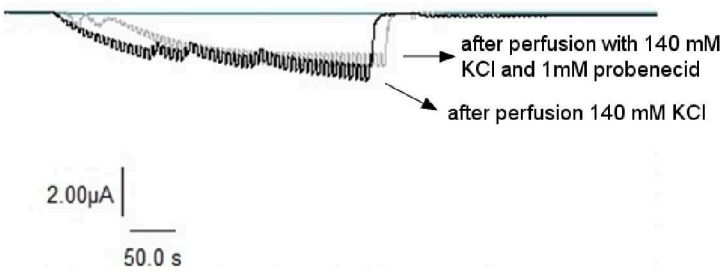


Figure 3.7: activity of not-injected oocyte when perfused with and without 1 mM probenecid

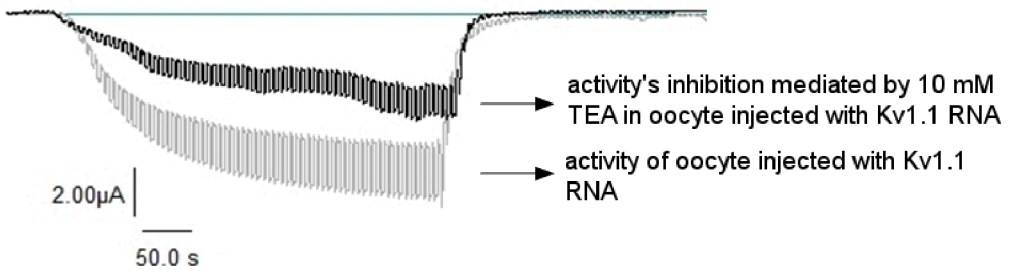


Figure 3.8: activity in oocytes expressing Kv1.1 channel and inhibition by TEA 10 mM; square type analysis, activity decreases from -7,8 μA to -4,3 μA.

3.3 Coexpression of Kv1.1 channels and pannexin1 in frog oocytes and electrical activity in presence of potassium chloride

Seen that oocytes injected with 5 ng of pannexin 1 RNA showed a later expression of protein , 10 ng of each RNA (pannexin 1 and Kv1.1) were injected. Oocytes that expressed both proteins showed the same activity of oocytes that expressed the channels individually (see fig. 3.10). With square type analysis the mean electrical activity was maintained around -7/-8 μA in presence of 140 mM KCl (including leak current). With the IV step type analysis, electricity always reached average value of +5/+8 μA at last depolarization step (see fig. 3.10). With these data we concluded that at the used conditions didn't occur a functional interaction between the two channels because there is no significant change in electrical activity. We saw a different result in the presence of probenecid and DTT. As already explained probenecid and DTT inhibit the activity of pannexin 1, in our experiments when both channels were coexpressed this inhibition was lost or greatly reduced (see fig. 3.11). In contrast, in presence of tetraetylammonium that is a potassium channel inhibitor, we didn't obtain substantial variation when both proteins are coexpressed, or rather a reduction occurred in the same

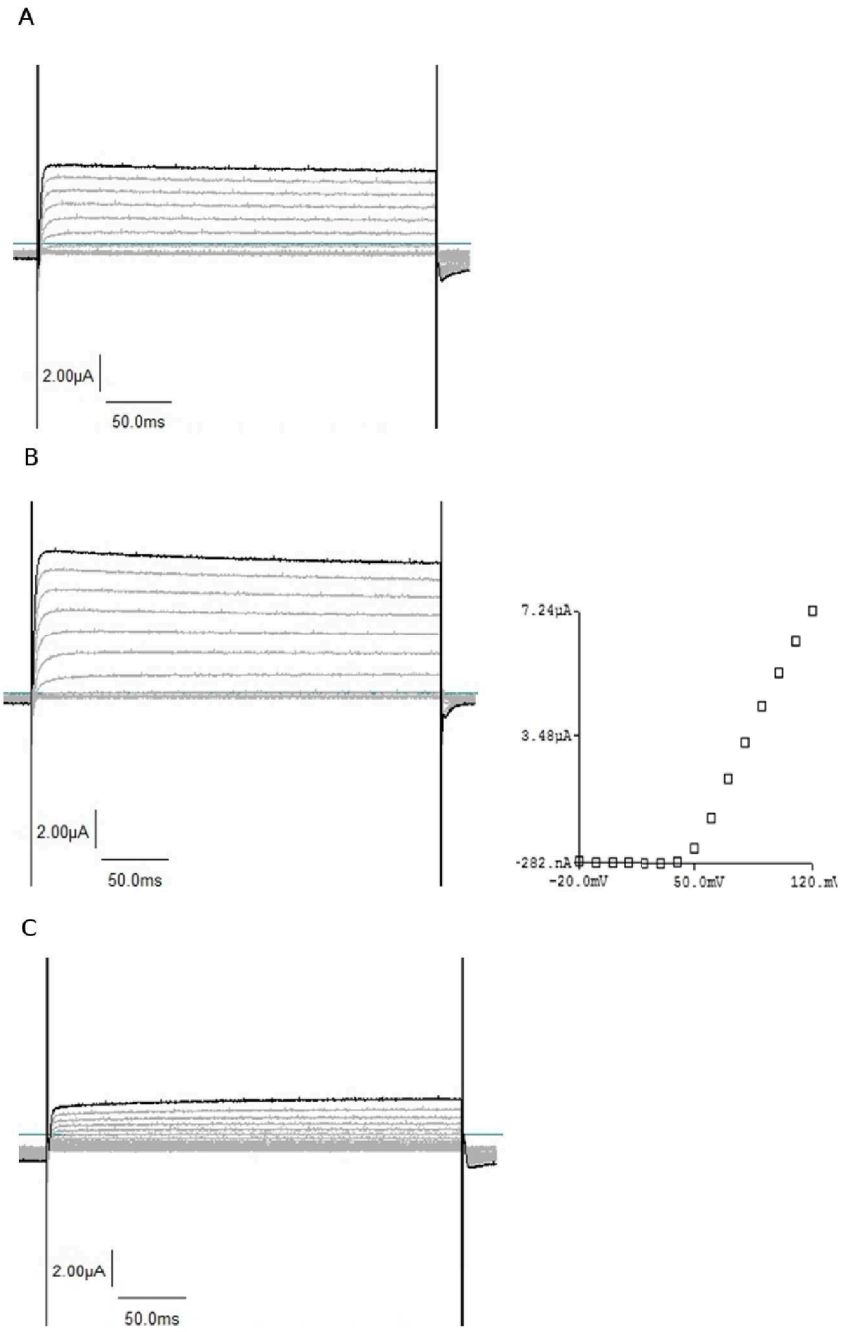


Figure 3.9: activity of Kv1.1 channel and inhibition with TEA 10 mM; A and B: IV type analysis in oocyte expressing Kv1.1 channel, current up to +4,2 μA (A) and up to +7,24 μA (B); C: activity inhibition of Kv1.1 channel by TEA, current up to +1,8 μA.

way than in oocytes expressing only Kv1.1 channel (see fig. 3.12 and 3.13). In summary Kv1.1 channel influenced pannexin 1 inactivation but pannexin 1 didnd't alter in any manner the behavior of Kv1.1 channel. Moreover using at the same time inhibitors of both channels, TEA and DTT or TEA and probenecid, no change in reduction was observed in comparison to the reduction induced by TEA only, suggesting that TEA inhibition didn't restore pannexin inhibition (see fig. 3.12).

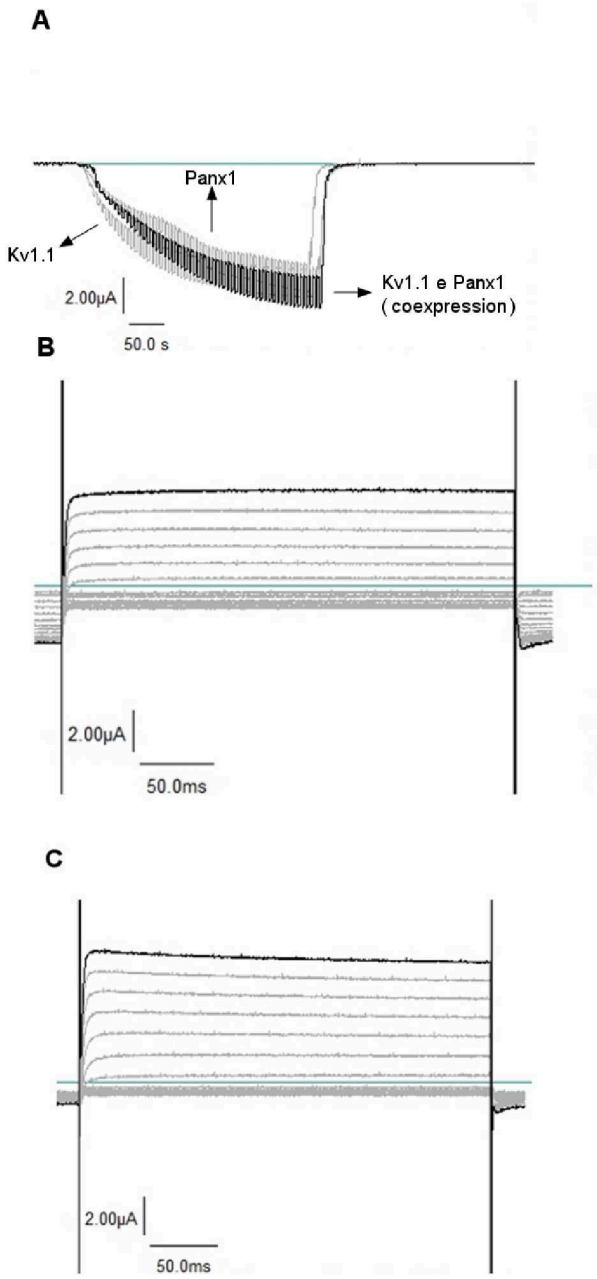


Figure 3.10: coexpression of pannexin 1 and Kv1.1 channel; A: electrical activity overlap of three oocytes expressing respectively Kv1.1, Panx 1 and coexpressing both channels , mean values of activity moves from -7 μA to -7,9 μA during square type registry; B e C: IV analysis in oocytes that express both proteins, normally activity moves in a mean range of $+5/+8$ μA, in the registries reported above activity arrives up to $+4{,}5$ μA (B) and up to $+6{,}7$ μA (C).

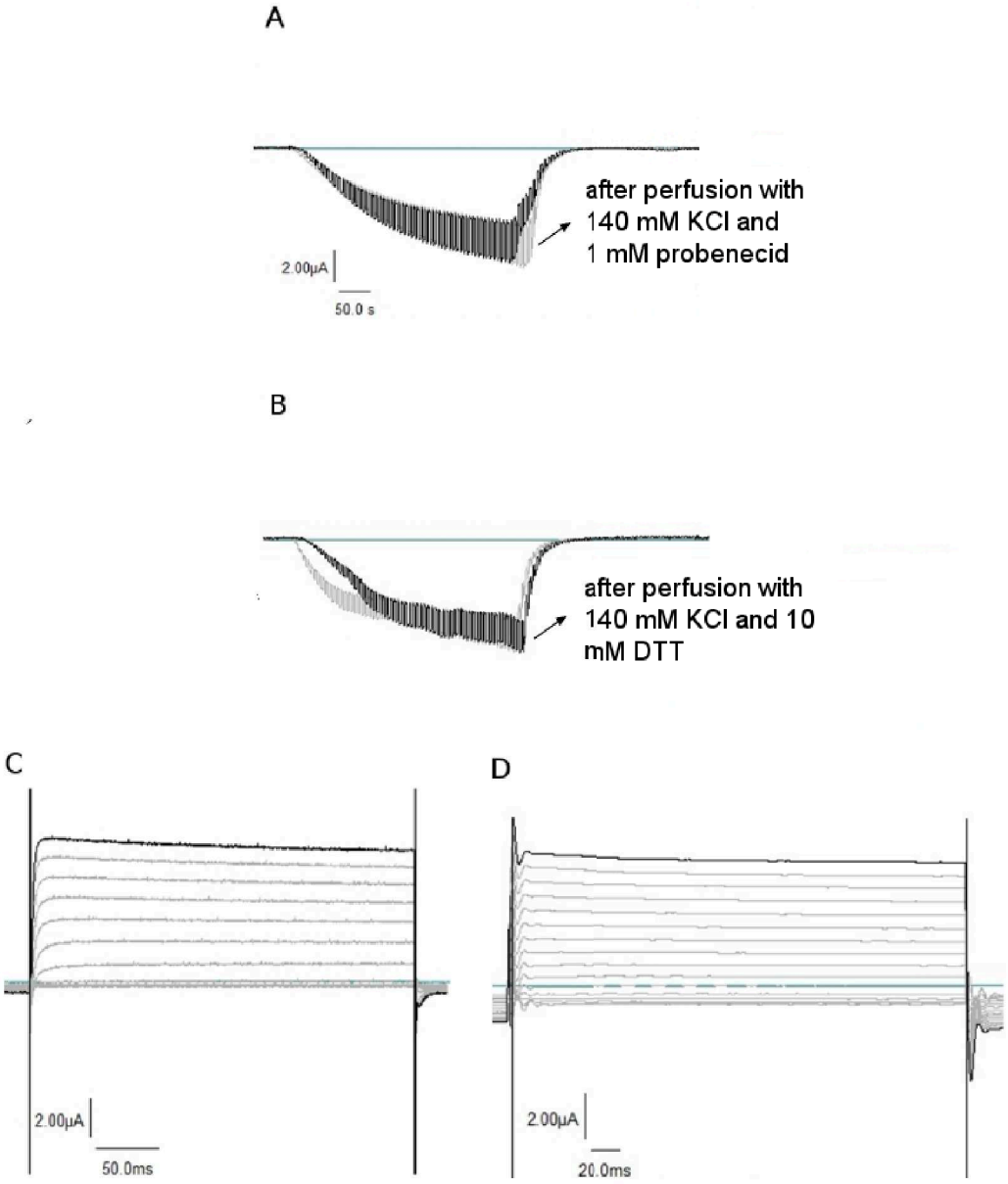


Figure 3.11: electrical activity during coexpression of both channels, A and B: square registry before and after perfusion with 1 mM probenecid (A) and 10 mM DTT (B), (activity of about -7 μA); C and D: IV registry during perfusion with 1 mM probenecid (C) and 10 mM DTT (D), (activity up to $+7$ μA).

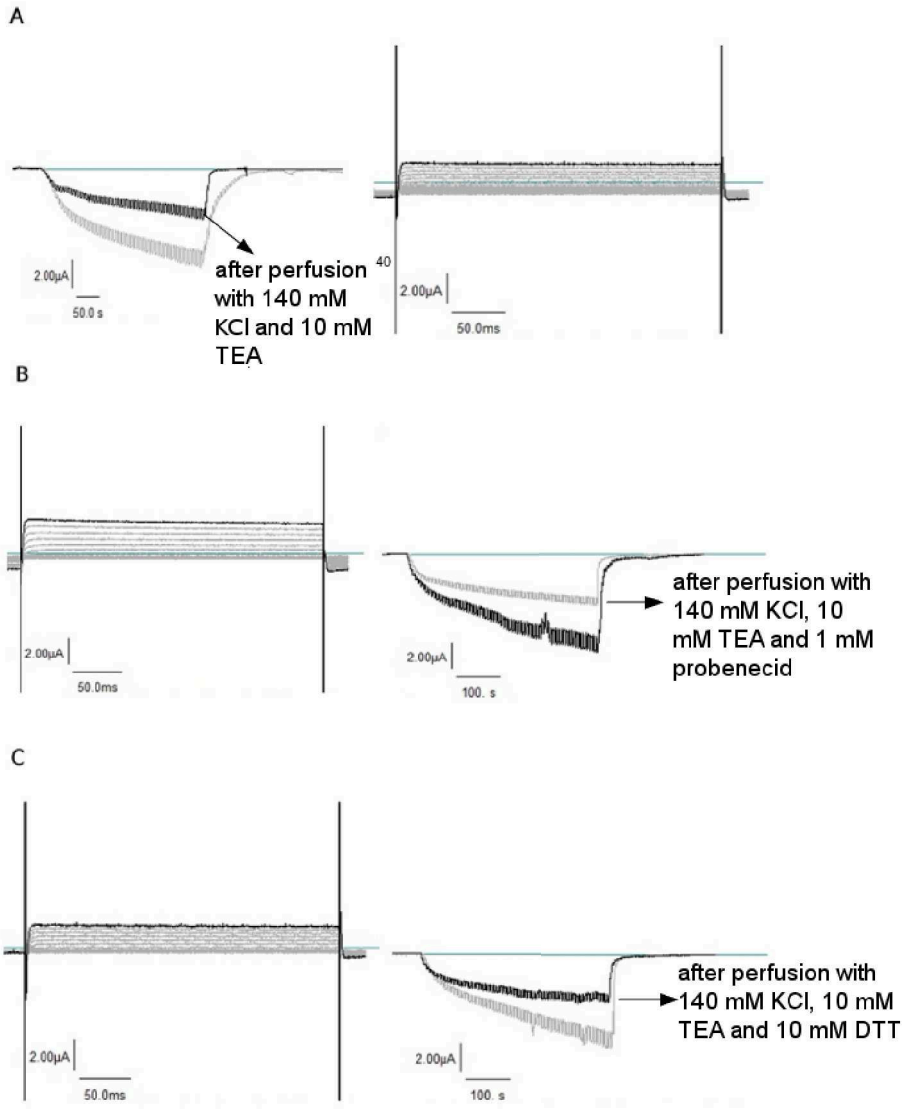


Figure 3.12: activity in oocytes coexpressing Kv1.1 and Panx1 during perfusion with 10 mM TEA (A), 10 mM TEA and 1 mM probenecid (B), 10 mM TEA and 10 mM DTT (C); A: square analysis (left side) where activity changes from -7,4 μA to -3,9 μA and IV step analysis (right side) where activity arrives up to $+1{,}5$ μA; B: IV step analysis, activity up to $+2{,}3$ μA (left) and square analysis, activity changes from -7 μA to -3,6 μA (right); C: activity up to $+1{,}8$ μA (left, IV step analysis) and reduction from -6,5 μA to -3,5 μA (right,square analysis).

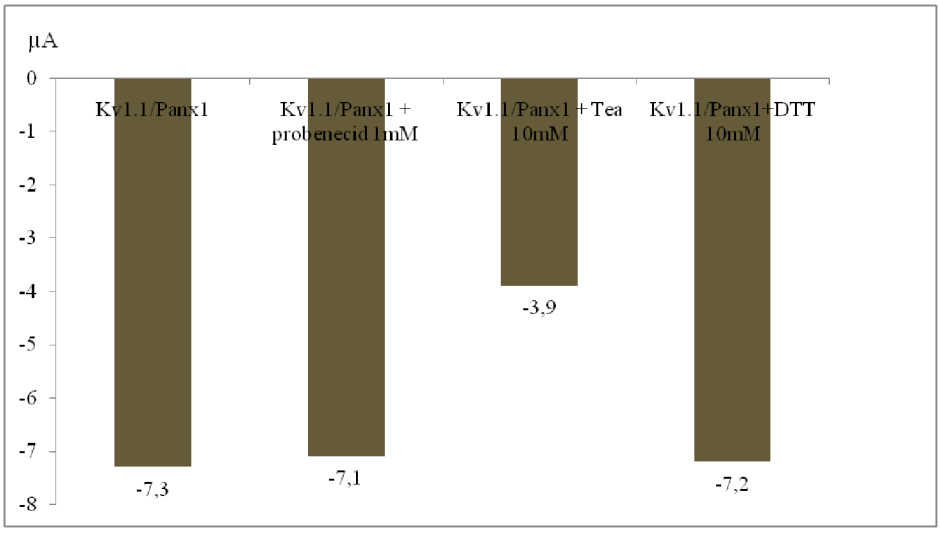


Figure 3.13: variation of Kv1.1 and panx1 electrical activty in oocytes, during perfusion with 140 mM KCl, clamped potential at -50 mV and depolarizating pulse of -10 mV (square protocol).
Kv1.1/Panx1 : -7,3 μA, SEM: +/- 0,05;
Kv1.1/Panx1 + probenecid 1 mM : -7,1 μA, SEM: +/- 0.03;
Kv1.1/Panx1 + TEA 10 mM : -3,9 μA, SEM: +/- 0,05;
Kv1.1/Panx1+ DTT 10 mM : -7,2 μA, SEM: +/- 0.06.

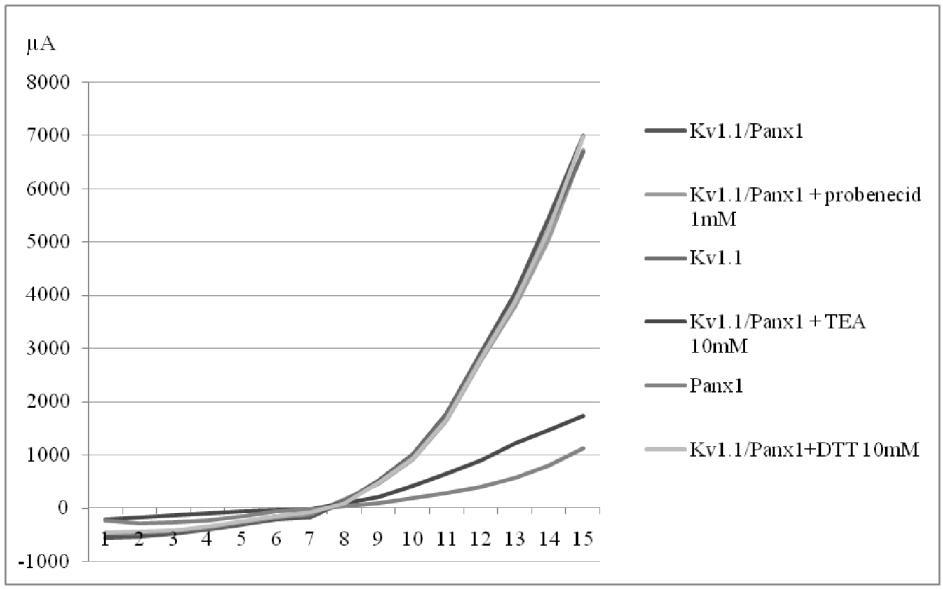


Figure 3.14: electrical currents summary of Kv1.1 and panx1 channels recorded during perfusion with 30 mM KCl, clamped potential at -80 mV and depolarizating step from -20 mV to +120 mV (IV step analysis).
Kv1.1/Panx1 + probenecid 1 mM : +6,99 μA, SEM: +/- 0,05
Kv1.1/Panx1+DTT 10 mM: +6,98 μA, SEM: +/- 0,06
Kv1.1/Panx1 : +6,73 μA, SEM: +/- 0,04
Kv1.1: +6,68 μA, SEM: +/- 0,04
Kv1.1/Panx1 + TEA 10 mM : +1,7 μA, SEM: +/- 0,07
Panx1 : +1,12 μA, SEM: +/- 0,05

3.4 Analysis of transient expression of mouse pannexin 1 in HEK-hBk1 cells by immunocytochemistry

Expression of mouse pannexin 1 in HEK-hBk1 cell line was revealed by western blot and immunocytochemistry with fluorescence microscopic analysis for the green fluorescence emitted by GFP (green fluorescent protein). For immunocytochemistry the best expression was observed when higher number of cells were transfected (80,000 cells/well), waiting 48 hours for protein expression and using 0,8/0,9 μg of DNA and 1.6 μl of lipofectamine per well. We didn't see a significant difference in expression after 72 hours of transfec-

tion (see fig. 3.15). The Kv1.1 channel is visualized through red fluorescence emitted by rhodamine conjugated secondary antibody, the concentration of 1:500 for both primary and secondary antibody was enough (see fig. 3.16 and 3.17). In some fields colocalization of both proteins was well observed (see fig. 3.18). The negative control didn't give staining (see fig. 3.17), not-transfected cells showed a basal endogenous expression of human pannexin 1. The images were acquired in Zeiss confocal microscope with 40x zoom lens.

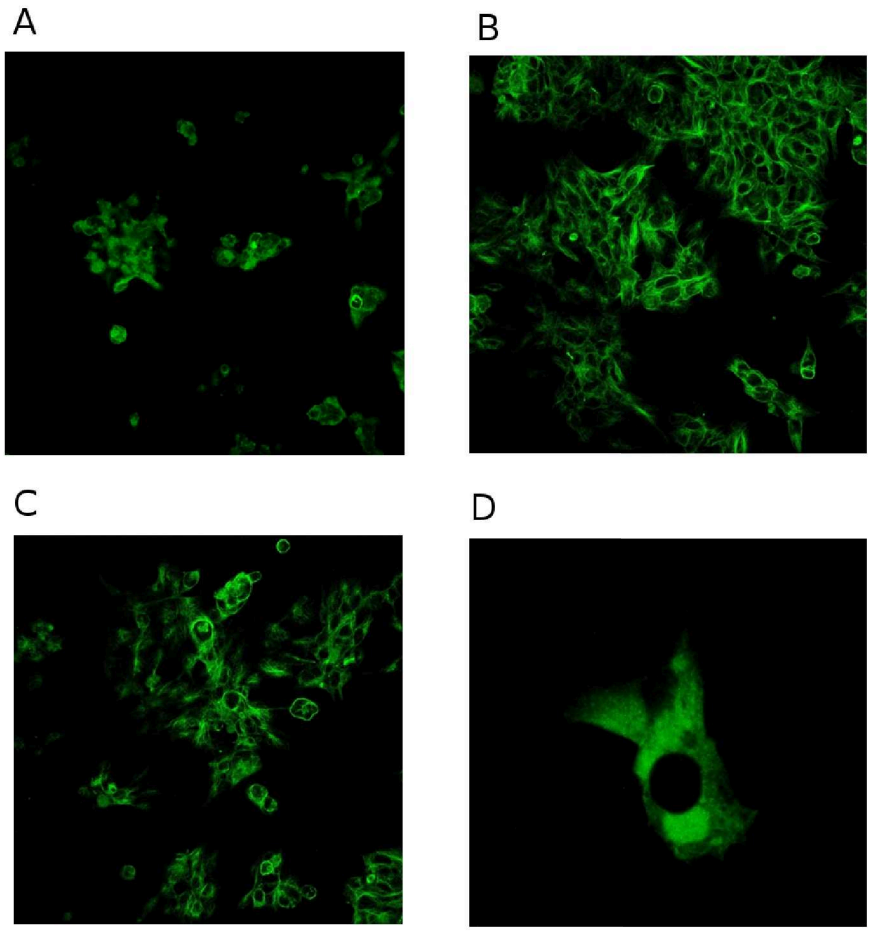


Figure 3.15: GFP expression at 24 hours of post-transfection (A), at 48 hours of post-transfection (B), at 72 hours of post-transfection (C); GFP visualized at 60x zoom (D)

However, the fluorescence emitted by GFP was not always high, about

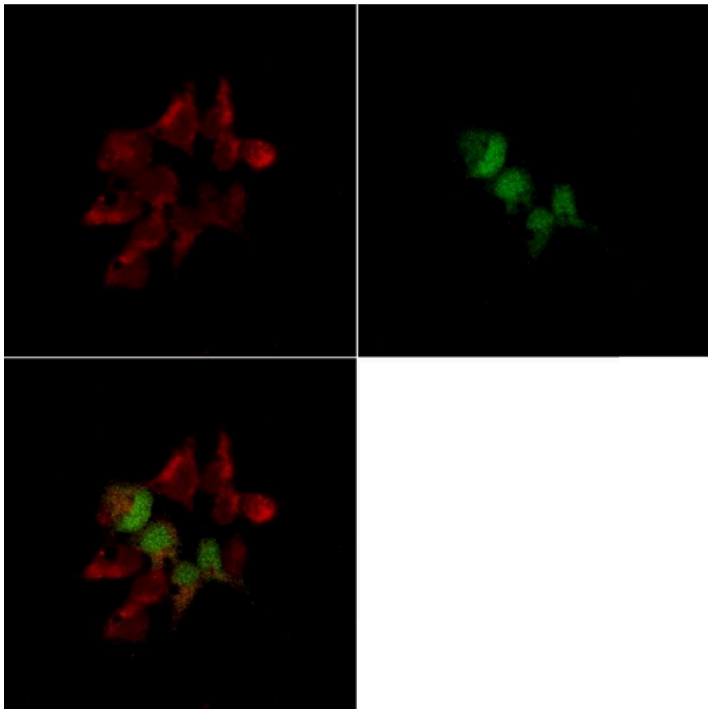


Figure 3.16: expression of Kv1.1 channel (red) and pannexin 1 (green)

this matter it must be kept in mind that immunocytochemistry was performed at a cell confluence of 50% to 70% but the efficiency of transfection with lipofectamine is higher when cells are transfected at a confluence of 90%, moreover hBK1 cells tend to grow in clusters, one above the other, and this could make harder the clear visualization of GFP, that was identified in the microscope by its intrinsic fluorescence and not using an antibody, all these aspects may explain its lower intensity. Immunocytochemistry performed on HEK-293 cells and hBK1 cells to assess the localization of GFP showed that GFP did not alter the actual location of pannexin 1, as shown in Figures 3.19, 3.20, 3.21 and 3.22. During the evaluation of GFP expression we noticed that transfected cells with DNA of pannexin 1 had a lower growth in comparison to not-transfected cells. Previous studies have indeed shown that pannexin 1 has antimitotic effect and suppressive action on tumoral growth in glioma (Charles PK Lai et al. Cancer Res 2007; 67:1545-1554). We then compared the growth of transfected cells with the growth of not-transfected cells and the cells treated with lipofectamine only, through the observation of cells in optic microscope and the display of DAPI stained nucleus in Nikon fluorescence microscopy (see fig. 3.23).

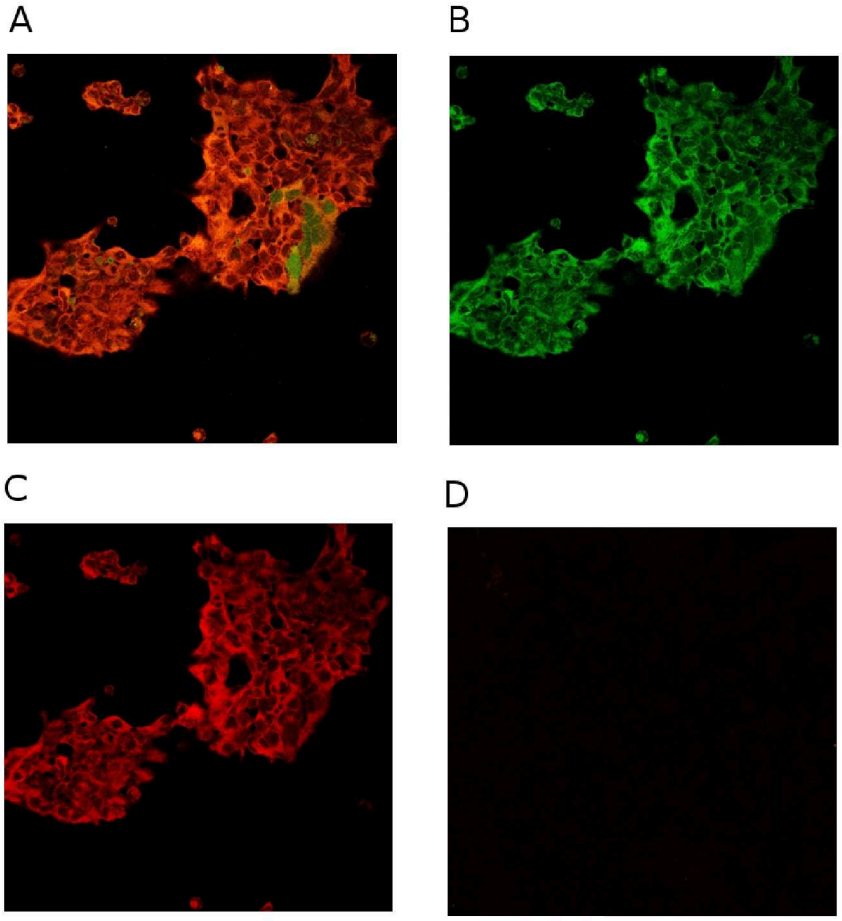


Figure 3.17: expression of Kv1.1 channel (red) and pannexin 1 (green) (A, B, C) the orange fluorescence indicates the colocalization of GFP and Kv1.1 channel; negative control (D)

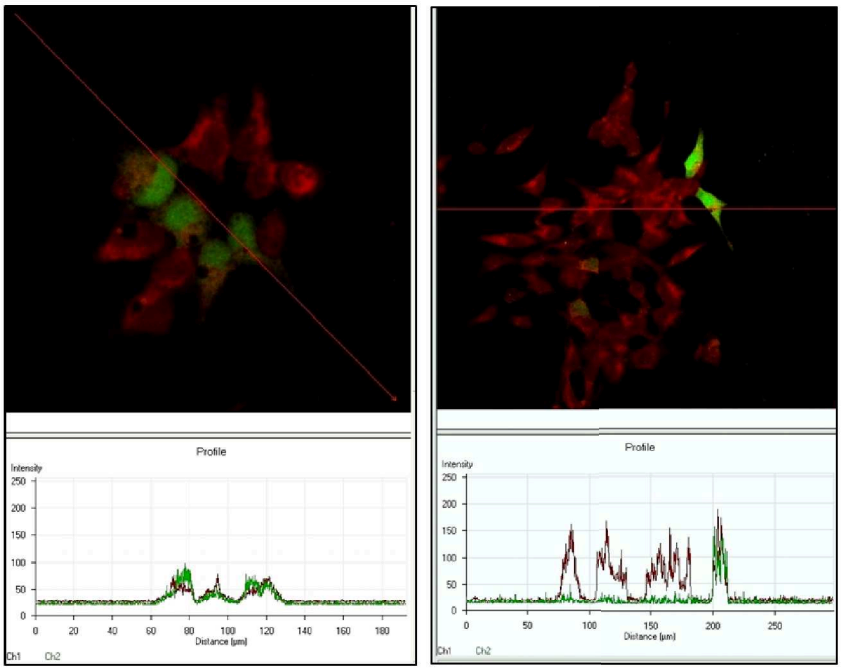


Figure 3.18: colocalization of Kv1.1 channel with pannexin 1

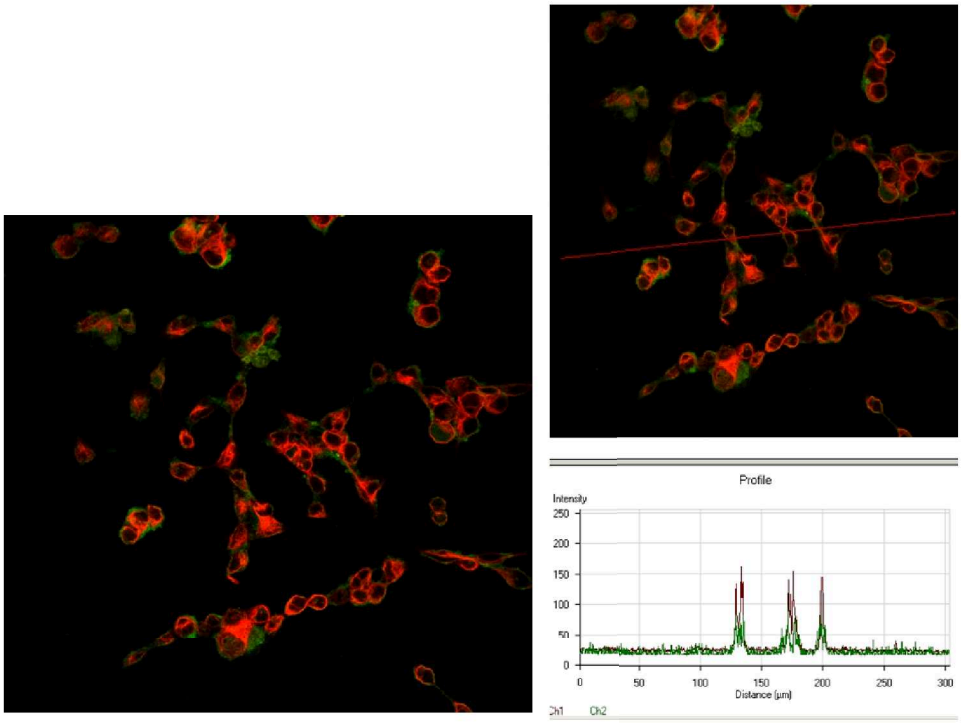


Figure 3.19: GFP (green) and pannexin 1 (red) localization in HEK-293 cells transfected with pBMN-I-GFP plasmid containing Panx 1 cDNA; the orange fluorescence indicates the colocalization of GFP and pannexin1.

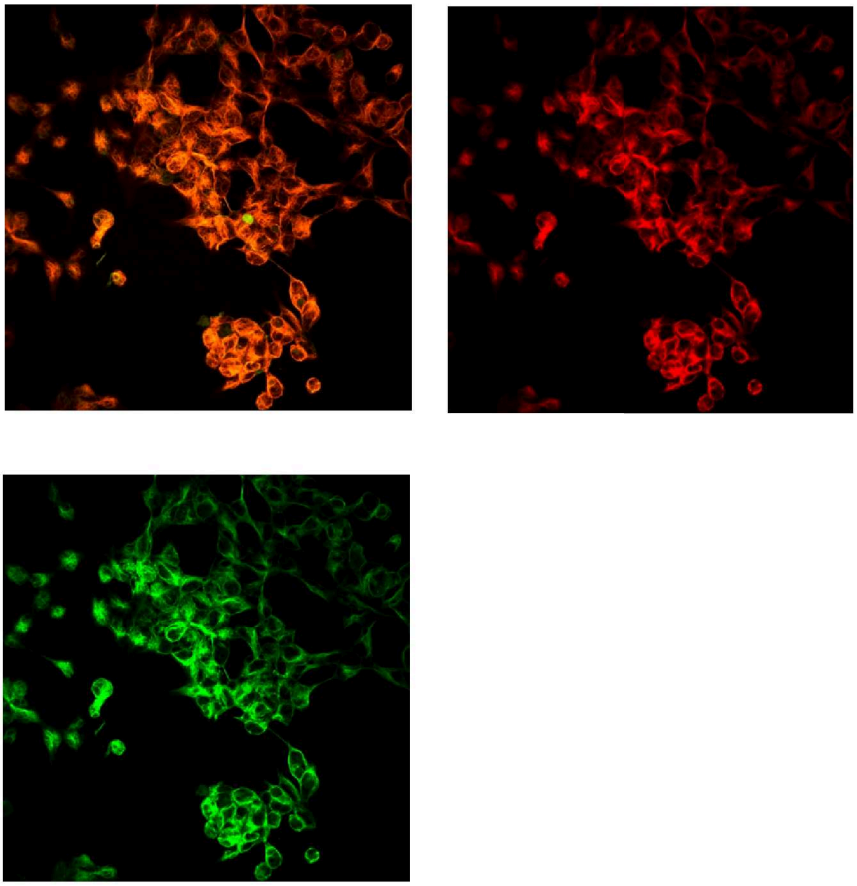


Figure 3.20: colocalization of pannexin 1 and GFP in HEK-293 cells.

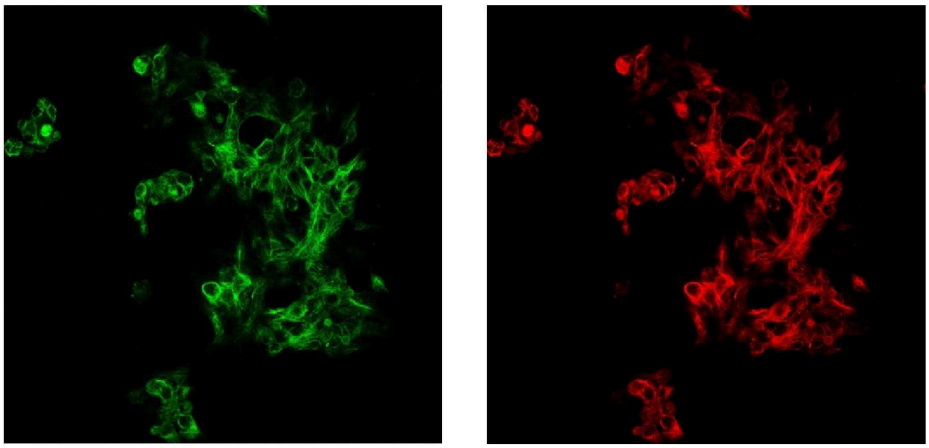


Figure 3.21: GFP (green) and pannexin 1 (red) localization in hBK1 cells transfected with pBMN-I-GFP plasmid containing panx1 cDNA.

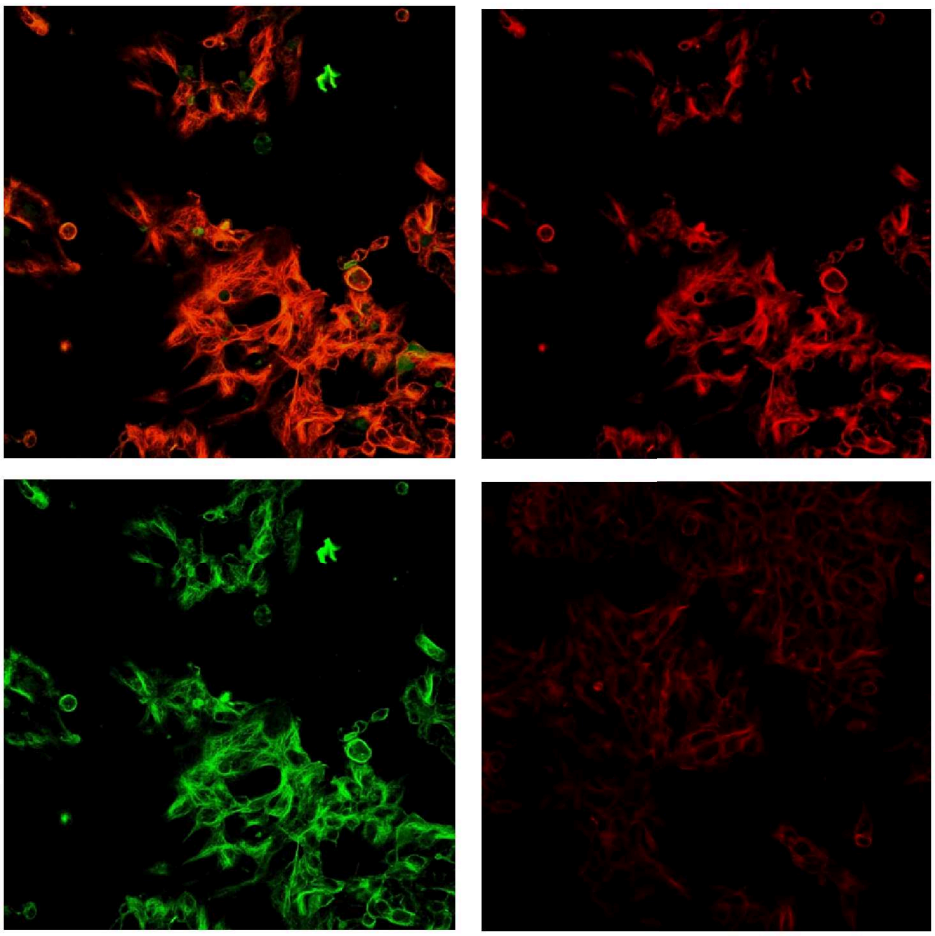


Figure 3.22: A,B,C : colocalization of pannexin 1 (red) and GFP (green) in HEK-hBK-1 cells; D: endogenous expression of pannexin 1 in HEK-hBK1 cells.

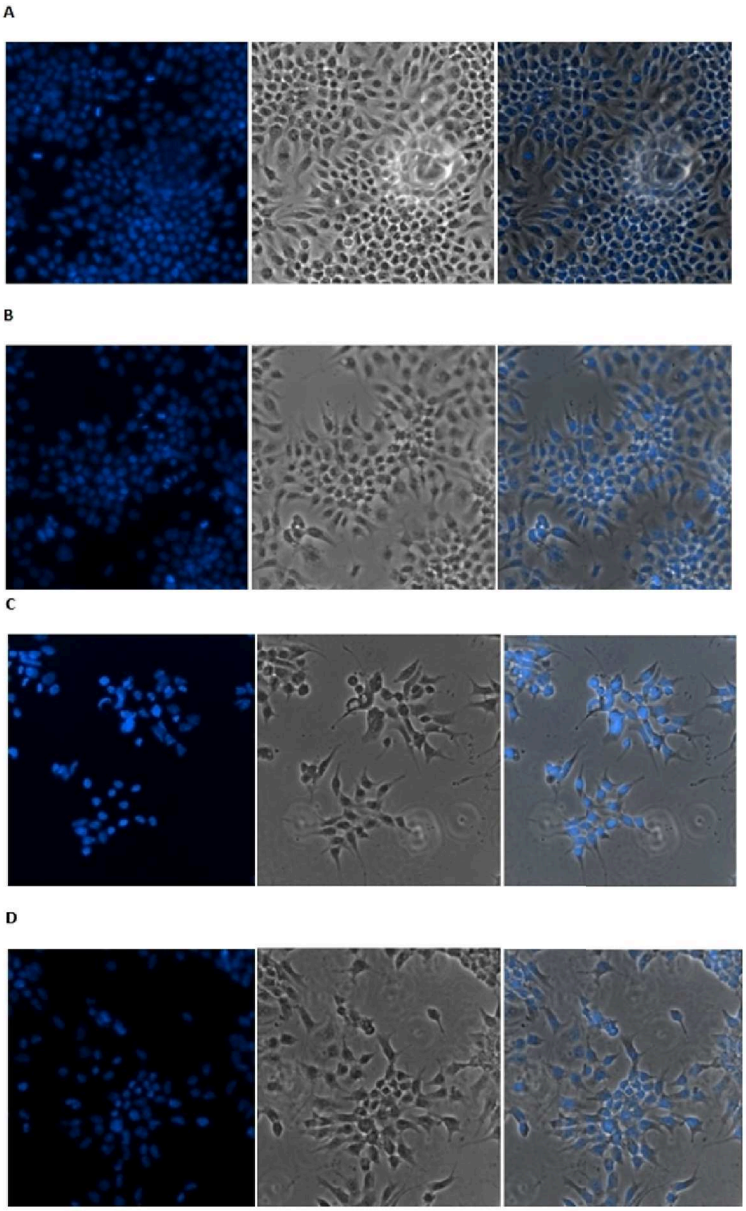


Figure 3.23: staining of nucleus with DAPI, images acquired in fluorescence microscope, 20x magnitude, A: not-transfected cells, B: cells treated only with lipofectamine, C e D: cells transfected with Panx1 cDNA

3.5 Valuation of cell death during treatment with potassium chloride and inhibitors of Kv1.1 channel 1 and pannexin 1

Evaluation of cell death by trypan blue, after the treatments already described on hBK1 cell transfected with pannexin 1 cDNA (Chapter 2, paragraph 2.14), showed that in cells treated with 140 mM potassium chloride there was a reduction in cell survival of about 40%, when was made cotreatment with 10 mM tetraethylammonium the same reduction in cell survival was observed, or rather inhibitor of potassium channels showed no beneficial effects, but when probenecid was administered individually or with TEA a marked improvement in cell survival was observed (see fig. 3.24 and 3.26). Moreover not-transfected cells showed a lower cell death after treatment with 140 mM KCl. So these data reported that pannexin 1 is mainly involved in cell death after treatment with potassium chloride and that in this condition Kv1.1 channel doesn't influence its inhibition by probenecid. A different result we saw with DTT 10mM, infact when this pannexin 1 inhibitor was singly administered or administered with TEA 10 mM, no beneficial effects occurred on hBK1 cells' death, on the contrary when DTT was given to SH-SYS5 cells (transfected too with pannexin 1 cDNA), cell death was lightly reduced, in according to the partial inhibition of pannexin 1 mediated by DTT (see fig. 3.25). These data therefore suggeste that in these conditions Kv1.1 channel interacts with pannexin 1 only in presence of reducing agents so when redox potential changes making pannexin less sensitive to the inhibition and then increasing cell death induced by KCl; moreover the potassium channel's inhibition by TEA doesn't influence its interaction with pannexin.

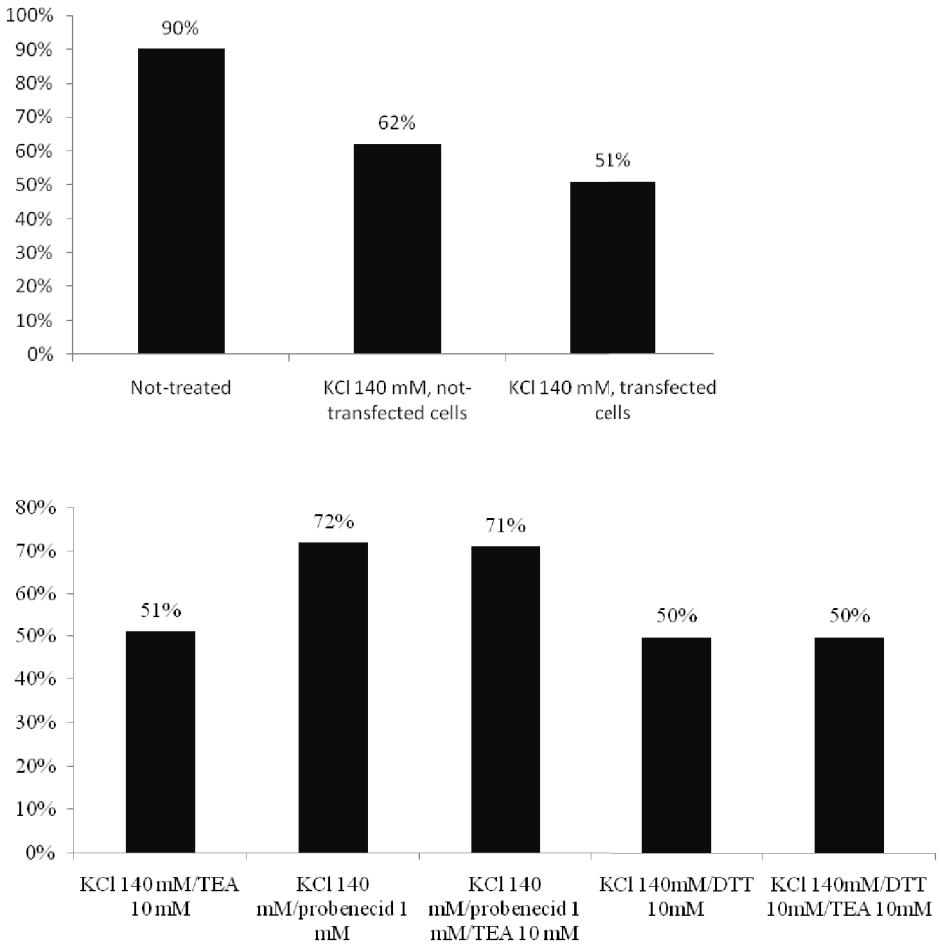


Figure 3.24: valuation of hBK1 cell's viability after 1 hour of treatment with KCl 140 mM, probenecid 1 mM, DTT 10 mM and tetraethylammonium 10 mM; cells were counted in hemocytometer after trypan blue staining; all treatments were done in triplicate, $P < 0.05$.

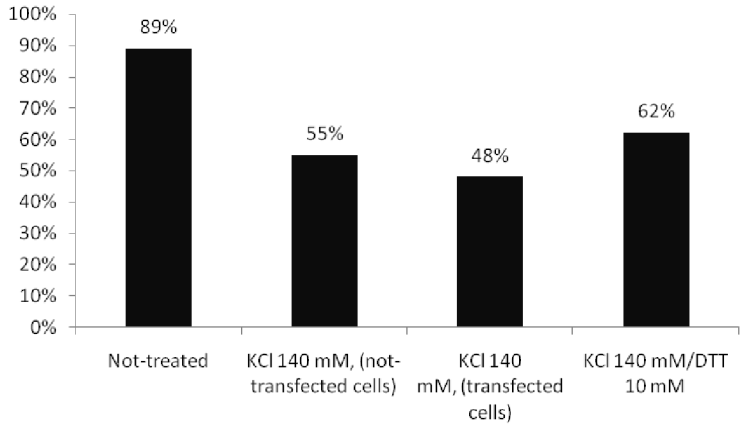


Figure 3.25: valuation of SH-SY5Y cell's viability after 1 hour of treatment with KCl 140 mM, DTT 10 mM; cells were previously transfected with panx1 DNA; cells were counted in hemocytometer after trypan blue staining; all treatments were done in triplicate, $P < 0.06$.

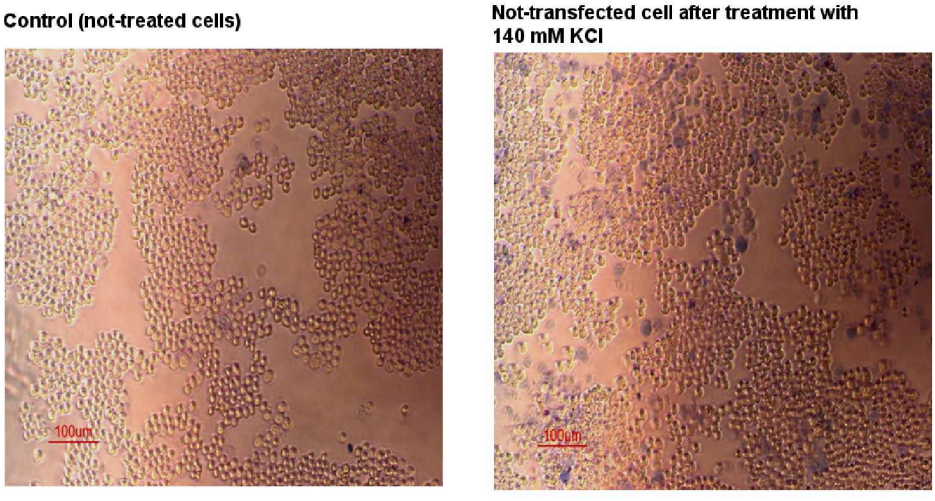
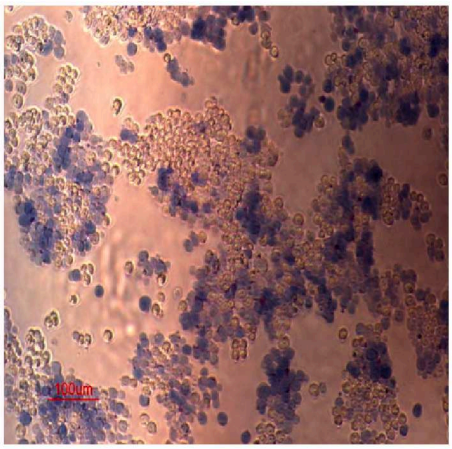
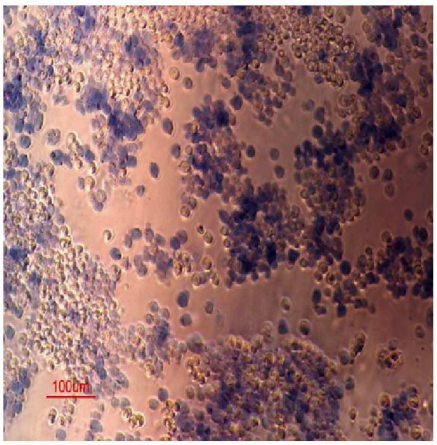


Figure 3.26

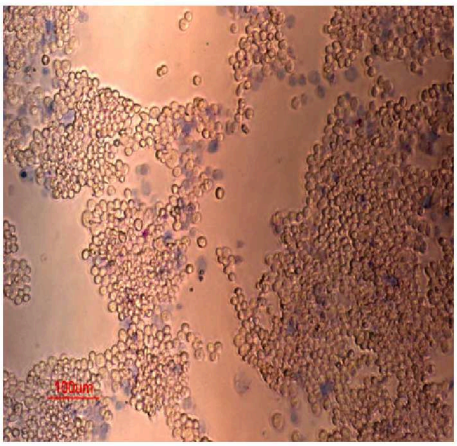
140 mM KCl



140 mM KCl/TEA 10 mM



140 mM KCl/probenecid 1 mM



140 mM KCl/probenecid 1mM/TEA 10 mM

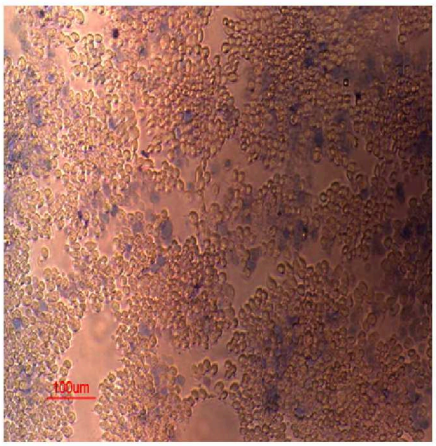


Figure 3.26

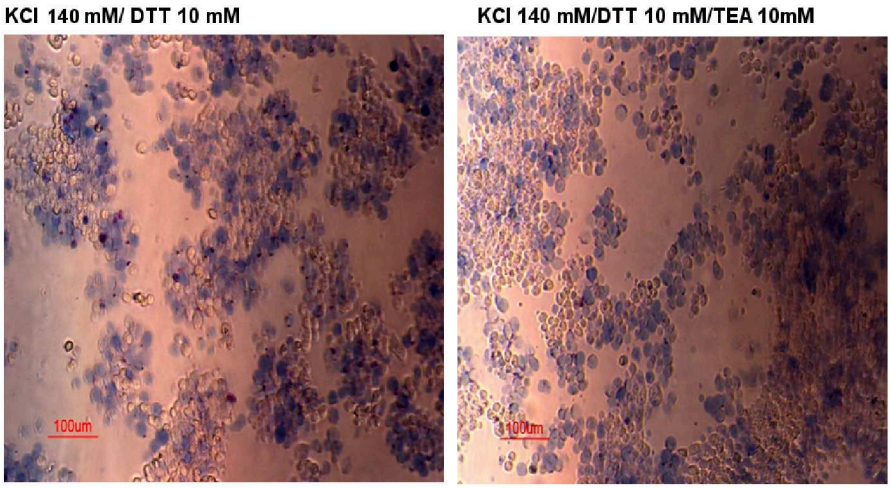


Figure 3.26: trypan blue stained hBK1 cells (images acquired with fluorescence microscope, 10 x magnitude), after treatment with 140 mM KCl, 10 mM TEA, 1 mM probenecid and 10 mM DTT for 1 hour.

3.6 Transient expression analysis of mouse pannexin 1 in HEK-hBk1 cells by western blot

For the analysis of protein expression by western blot technique, 350000 cells/well were plated to obtain the 90% confluence. The best expression was when cells were transfected with 3 μg of DNA and 8 μl of lipofectamine per well (see fig. 3.27), the sufficient concentration of anti-pannexin 1 antibody to detect the protein was of 1:5000, for the detection of Kv1.1 channel expression it has been used a final antibody concentration of 1:500. Cells SH-SY5Y have not shown the expression of Kv1.1 but only the expression of pannexin 1 (see fig. 3.28). However HEK-hBk1 cells show a good basal expression of human pannexin 1 (see fig. 3.27).

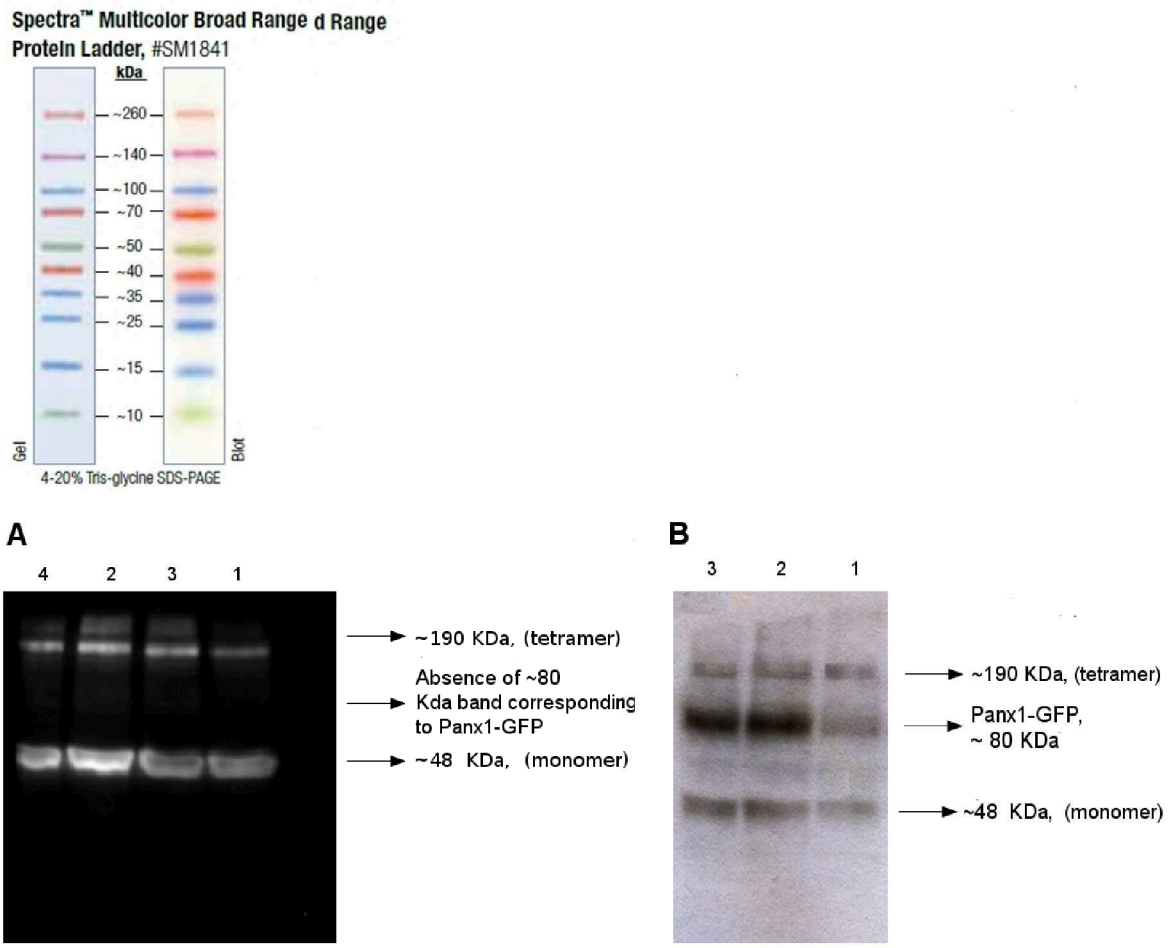


Figure 3.27: A e B : western blot with monoclonal anti-pannexin 1 on protein extracts of hBK1 cells transfected with pannexin 1 cDNA (B) and not-transfected (A); A: lane 1, 2 : 15 μg of protein extract, lane 3, 4: 30 μg of protein extract, membrane revelation through ECL kit and analysis in ChemLite 200 FA instrument; B: the western shows the expression of panx1-GFP, lane 1: 1 μg of DNA with 4 μl of lipofectamine/well, lane 2,3: 3 μg of DNA with 8 μl of lipofectamine/well, membrane revelation with ECL kit and photographic film impression; western blot was performed using 10% acrylamide gel.

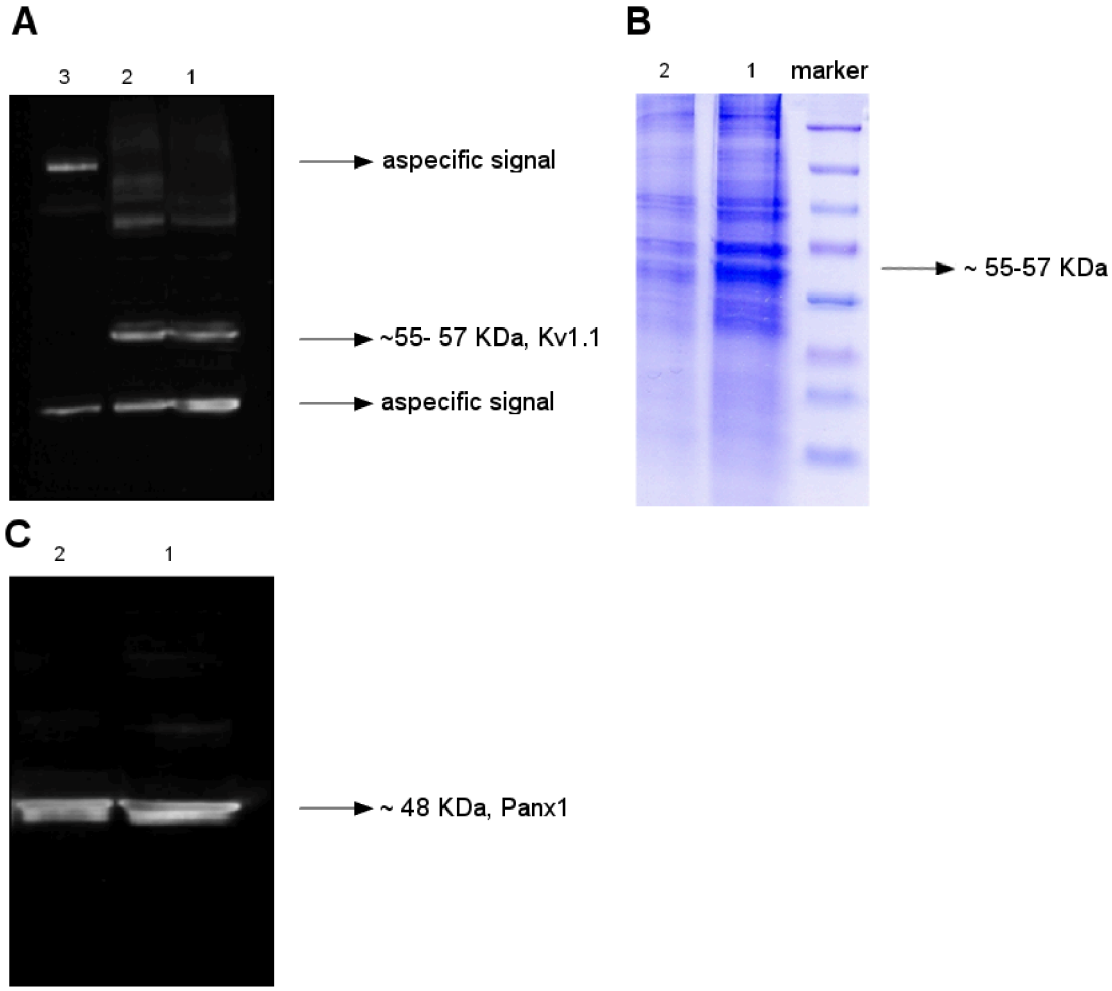


Figure 3.28: A: western blot detected with anti-Kv1.1 (8% acrilamide gel); lane 1: HEK-293 protein extract, lane 2: cellule HEK-hBK1 protein extract, lane 3: SH-SY5Y protein extract; B: SDS-Page analysis on not-transfected hBK1 cell extract (10% acrylamide gel); lane 1: 15 μg, lane 2: 8 μg; C: western blot to detect pannexin 1 in SH-SY5Y cell extract (lane 1: SH-SY5Y cell lysate, lane 2: hBK1 cell lysate).

3.7 Coimmunoprecipitation

The coimmunoprecipitation of pannexin 1 and Kv1.1 channel proved that these proteins do not directly interact during in vitro coimmunoprecipitation assays (see fig. 3.29 and 3.30). Infact performing first the coimmunoprecipitation assay with anti-Panx1 on HEK-hBK1 cell lysate, we observed on anti Kv1.1 western blot some signal from the coimmunoprecipitate sample corresponding to the predicted molecular weight of Kv1.1 channel (see fig. 3.29 A); but later using as control experiments the coimmunoprecipitation performed with anti-Panx1 on SH-SY5Y cell lysate and the coimmunoprecipitation perfomed without any primary antibody on HEK-hBK1 cell lysate, we saw that the signals previously revealed by western blot from the coimmunoprecipitate sample represented only IgG contamination (see fig. 3.29 B and C). Finally, reducing this IgG contamination (as previously explained in paragraph 2.18) we observed on anti Kv1.1 western blot no band corresponding to the predicted Kv1.1 channel molecular weight from the coimmunoprecipitate sample (see fig. 3.30 A). For this reason we concluded that no physical interaction should occur between the two channels. Perhaps they interact with common elements in a 'protein complex', leading to their functional interaction that then depends on the chemical bonds within the protein complexes.

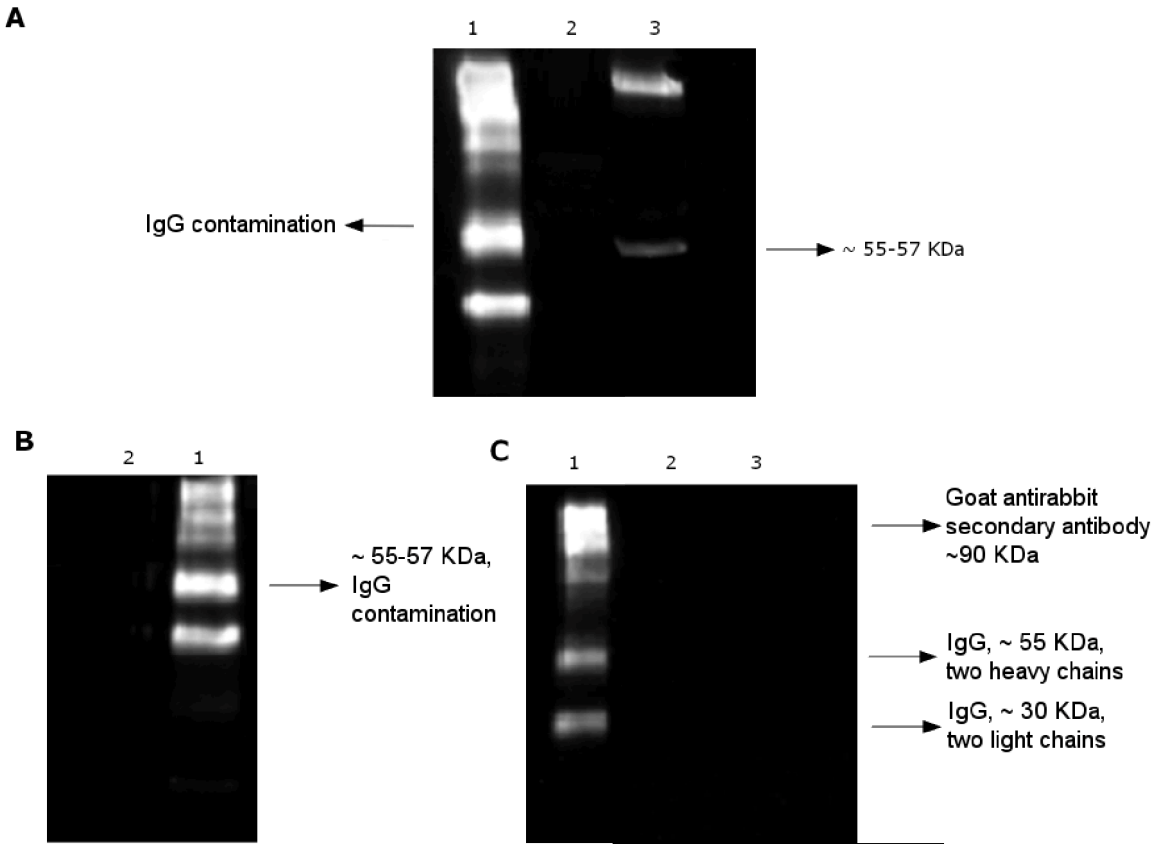


Figure 3.29: A: coimmunoprecipitation of pannexin 1 and Kv1.1 channel, carried out with anti-Panx1 and detection of western blot with anti-Kv1.1; lane 1: coimmunoprecipitate, lane 2: coimmunoprecipitation performed without primary antibody, lane 3: protein extract of hBk1 cells (8% acrylamide gel); B: coimmunoprecipitation executed with anti-Panx1 using SH-SY5Y protein extract, western detected with anti-Kv1.1; lane 1: coimmunoprecipitate, lane 2: protein extract (10% acrylamide gel); C: coimmunoprecipitation made with anti-Panx1 and detection of the western blot without primary antibody; lane 1: coimmunoprecipitate, lane 2,3: hBk1 lysate; (the western shows contamination from IgG in the coimmunoprecipitate).

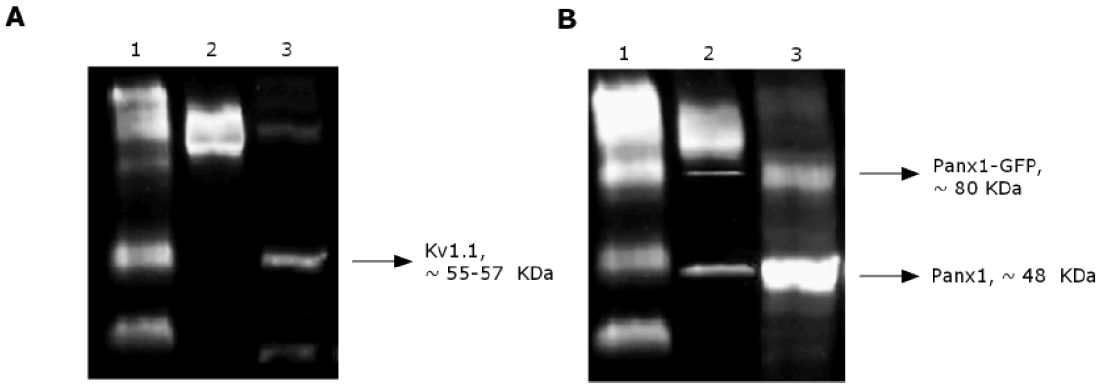


Figure 3.30: coimmunoprecipitation of pannexin 1 and Kv1.1 channel performed with anti-Panx1 using hBk1 cell protein extract; A: western blot detected with anti-Kv1.1; lane 1: coimmunoprecipitate contaminated by IgG, lane 2: coimmunoprecipitate with reduced contamination from IgG, lane 3: lysate (8% acrylamide gel); B: western blot detected with anti-Panx1; lane 1: coimmunoprecipitate contaminated by IgG, lane 2: coimmunoprecipitate with reduced contamination from IgG, lane 3: lysate; the membrane incubated with anti-Kv1.1 has been stripped and successively incubated with anti-Panx1; the westerns show the presence of pannexin 1 in the coimmunoprecipitate but the absence of Kv1.1 channel (lane 2).

Chapter 4

Discussion and Conclusions

4.1 Functional interaction between pannexin 1 and Kv1.1 channel

Considering the results obtained during voltage clamp analysis we found that the susceptibility of pannexin 1 to its inhibitors,probenecid and DTT, was compromised when it was coexpressed with the alpha subunit of Kv1.1 potassium channel infact oocytes dind't show any electrical activity's reduction, but pannexin 1 didn't influence the behavior of Kv1.1 channel in presence of its blockers such as TEA. Moreover Kv1.1 channel inhibition by TEA didn't restore pannexin 1's susceptibility to its blockers suggesting that the modulation of pannexin 1 dind't depend on the activation/inactivation of Kv1.1 channel. This phenomenon was the result of a functional interaction between the proteins because during in vitro coimmunoprecipitation assays they didn't interact; moreover this interaction occurred only when membrane potential and redox potential changed and could represent a cellular mechanism involved in mixed synapse functions and in channelopathies. Then beside the modulation mediated by $\beta 3$ subunit, also an another type of cooperation must exist, in which Kv1.1 channel is involved in some way. The mechanism by which probenecid blocks pannexin 1 activity is not yet clear, it could interact with some domain of the protein causing a conformational change and therefore the closure or it may indirectly inhibit its activity. Pre-

vious studies have shown that this molecule interferes with mitochondrial respiratory chain, reduces intracellular levels of ATP and also causes depolarization of the plasma membrane (Masereeuw et al. British Journal of Pharmacology (2000) 131, 57 - 62). Depletion of ATP, in turn, alters intracellular calcium homeostasis because calcium/magnesium dependent ATPase in absence of ATP stops to work, changing mitochondrial calcium levels with the production of free radicals. Formation of free radicals and alteration of respiratory chain also produces a modification in intracellular pH that becomes acid (A Lemarie and S Grimm, Oncogene , 30, 3985-4003 , 2011; Daniel K. Et al., Am J Physiol Cell Physiol 286:C940-C951, 2004). Some of these effects occur also during hypoxia. Probenecid might cause moreover a disturbance in the membrane because it is highly lipophilic and can remain in the phospholipid bilayer, it alters also mithocondrial membrane permeability to protons and affects the state 3 and 4 of oxidative phosphorylation. Therefore we can suppose that the inactivation of pannexin 1 by probenecid is also consequence of the desorders caused by the molecule. Hovewer it's interesting that when pannexin 1 was coexpressed with Kv1.1 potassium channel in oocytes it was not subject to the inhibition by probenecid; may be the modulation of pannexin 1 by Kv1.1 channel is mediated by other proteins, like kinases, calmodulin and G protein or also by membrane phospholipids such as PIP2; so pannexin 1 and Kv1.1 channel might interact with common elements in a 'protein complex' and this process leads to their functional interaction.

4.2 Mechanisms of potassium's cellular redistribution

Any deviation of extracellular K^+ concentration can affect neural activity. Elevated extracellular potassium occurs during seizure activity, ischemia, and spreading depression, with consequent effects on neuronal excitability and ultimately on cell viability. To limit increased extracellular potassium neural cells, in particular astrocytes, are equipped with a variety of K^+ uptake

mechanisms,including the Na^+/K^+ ATPase, voltage activated K^+ channels and gap junctions. Furthermore, local elevations in extracellular potassium shift the K^+ equilibrium potential (EK) to more positive values relative to the membrane potential (Vm), thus driving K^+ ion into these cells along the electrochemical gradient. Spatial redistribution of K^+ is believed to be enhanced by gap junction, that can work both as membrane hemichannels and coupling channels between cells (Kozoriz et al., The Journal of Neuroscience, August 2, 2006 , 26(31):8023– 8024). Therefore the modulation of pannexin by Kv1.1 channel could be explained as a regulatory mechanism of potassium buffering to control extracellular potassium concentration and neuronal excitability. Moreover potassium plays an important role in respiratory chain in which it takes part in the K^+/H^+ antiporter flow and in the passive flow; previous studied have reported that the potassium uptake K^+ regulates cellular respiration at two structures, one directly in mitochondria, and the second indirectly through control of ADP production at the cell membrane (M.Blond et al., Biochem. J. (1965) 97, 523). Therefore an increased entry of potassium and its redistribution could be also a mechanism to repair the oxidative phosphorylation by stimulating electron transport and oxygen consumption in pathological conditions like hypoxia during that the mithocondrial chain is inhibited and the intracellular potassium level is reduced (McDonald, T. F. et al., Electrogenic sodium pumping, Science (Wash., D. C.), 172: 570-572, 1971; McDonald, T. F., and D. P. MacLeod, J. Physiol. (Lond.). 229: 559-582, 1973; Kristin Heerlein et al., American Journal of Respiratory Cell and Molecular Biology. Vol. 32, pp. 44-51, 2005). In our experiments also DTT partially inhibited pannexin 1 and this inhibition failed when pannexin was coexpressed with Kv1.1 channel. DTT is a reducing agent because contains thiol groups, so it alters the redox potential and the pH (like during hypoxia), probably this alteration cause the pannexin closure; previous studies infact reported that intracellular acidification induces the C-terminal region to interact with other region of the connexin, this intracellular 'dimerization' causes the obstruction of the channel (Morley et al., Biophysical Journal Volume 70 March 1996 1294-1302). May be Kv1.1 channel obstacles pannexin 1 inactivation by DTT to regulate

the 'potassium buffering' , as already explained above. This control could be a compensatory mechanism to restore the normal functionality of the cells. Further attentive analysis occur to clarify these mechanisms, therefore our findings suggest that the influence of potassium channel on pannexin 1 might affect the cellular response to altered electrical and chemical conditions and that this is the result of their functional interaction.

4.3 A new perspective to define channelopathies

As described in paragraph 1.9, chapter 1, potassium channels' activity is critical in various muscle, cardiac and neural disorders. Mutations in voltage-gated KCNQ channels that lead to the suppression of M currents are present in epilepsy, benign neonatal convulsion and QT syndrome as well as mutations in Kv1.1 channel lead to a decreased function causing episodic ataxia, epilepsy and neuromyotonia, in which the nerve cells can't repolarize sufficiently. These alterations result in a resting potential membrane more depolarized with the consequent neuronal hyperexcitability. Since now these pathologies have been explained as the direct response to potassium channel dysfunctions, but our demonstration of the functional interaction between voltage-gated potassium channels and gap junctions may open a new fascinating field of research about the channelopathies, where the modulation of connexins and pannexins mediated by potassium channels can play a key role. Otherwise gap junctions are widely expressed in central and peripheral nervous system, in myocardium, in kidney, participating to the synchronization control of electrical impulse. Blockers of gap junctions have been reported as antoconvulsivant agents (Neman et al. Histol Histopathol (2005) 20: 253-259), where on the contrary dysfunctions of voltage-gated potassium channels with a reduction in their activity causing seizures (Schroeder B. et al., Nature; 396:687–90, 1998; Biervert et al., Science 279: 403–406). Considerings our findings it could be hypothesized that beyond the alterations of potassium channels a further desease process exists, in which the failure in gap junctions' inhibition mediated by Kv channels leads to an increased activity with a consequent convulsivant effect. In the atrium, as in ven-

tricle, gap junctions allow action potentials to be propagated from one cell to the next, facilitating the coordination between the cells and participating to the calcium wave. Therefore it's not surprising that they have a role also in arrhythmia, where their inhibition contributes to generate the pathology (Nakagami et a.l, Cardiovascular Research (2008) 79, 70–7). Moreover uncoupling of connexins have been reported to provide a substrated for ventricular arrhythmias, due to a slower conduction (Berlian Idriansyah Idris et al., J Kardiol Indones. 2011;32:53-60). Potassium channel alterations are crucial in this pathology, where the loss of their function triggers the desease events too (Splawski I, N Engl J Med 1997; 336:1562–7; Neyroud N et al., Nature Genet 1997; 15:186–9.). For this reasons also in arrhythmia a further mechanism involving the interaction between potassium channels and gap junctions could participate to the disorder development, where the positive modulation of connexins and pannexins mediated by voltage-gated potassium channels could be critical and in this case could have a beneficial effect. Similar events might happen also in the other channelopathies where alterations in potassium's current are crucial, like in schizophrenia where also pannexins have been reported as a possible candidate for this desease because of its presence in the nucleus accubens (O'Donnell et al., Ann NY Acad Sci 877: 157–175, 1999), and in ataxia where for example mutation in connexin 47 leads to spastic ataxia (Wolf NI et al., Neurogenetics. 2007 Jan;8(1):39-44. Epub 2006 Sep 13.) Therefore targeting of modulatory subunits of gap junctions may offer a new therapeutic approach for fine-tuning neuronal excitability. Several surveys should be conducted towards this direction, in order to discover what proteins more belonging to gap junctions family could be modulated by Kv channels and to understand the effects of their interaction in various channelopathies; the studying of its function in disease models with the classical electrophysiological techniques could provide useful informations, using for example mutants or trying to break the functional interaction (once molecules involved in this mechanism will be identified) and then extending these studies in vivo.

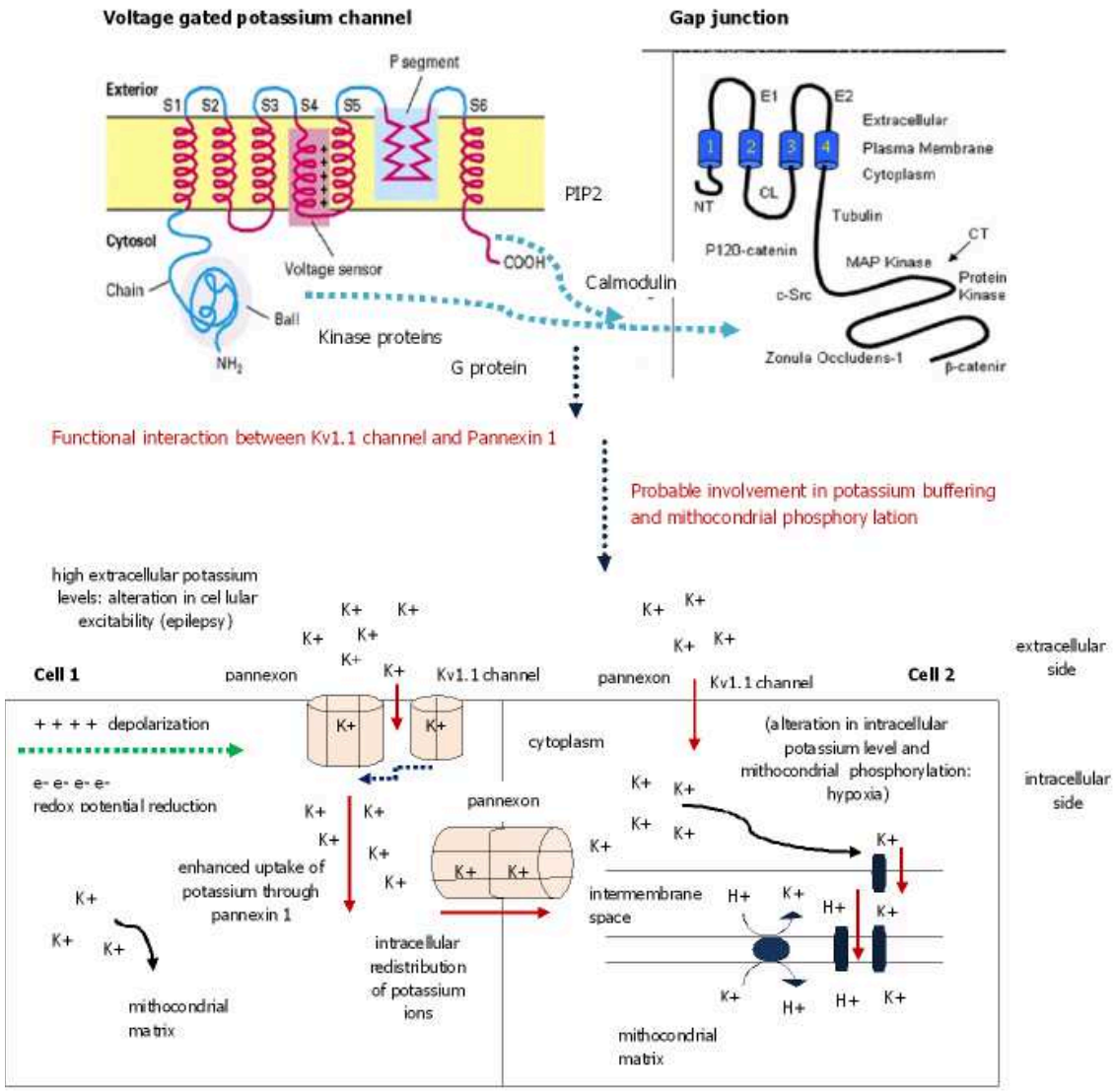
4.4 Kv1.1 channel's influence on pannexin 1 in HEK-hBk1 cell death mediated by high extracellular potassium chloride

The opening of pannexon with release of ATP has been described as a required event during apoptosis (Yan Qu et al., The Journal of Immunology, 2011 Jun 1; 186 (11) :6553-61), in this case, therefore, the failure to close pannexin 1 hemichannel when coexpressed with potassium channel Kv1.1 should facilitate cell death. A possible pannexin's involvement in apoptosis is supported by recent studies that described the opening of gap junctions during ischemic conditions (Thompson RJ et al. Science 312, 924-927, 2006). Moreover pannexon plays a role in the activation of caspases, or through a direct interaction or because its opening indirectly triggers their 'cascade', perhaps because the ATP released from pannexon interacts with P2X7 receptor that in turn activates caspases. Furthermore these evidences support the antimitotic effect's discover of pannexin 1 already documented (Charles P K Lai et al., 2007). High extracellular potassium causes also apoptosis, infact potassium seems to stimulate the maturation of caspase 1 (Silverman et al, 2009; Chekeni et al, 2010). Perhaps the potassium effect on cell death varies depending on many factors, like the type of insult, the type of tissue where it is expressed, the surrounding environment, or perhaps under conditions in which oxidative phosphorylation is impaired the entry of potassium helps to restore the respiratory chain at least initially, but a prolonged and extreme entry of the ion becomes toxic. In fact, as reported in recent researchs, both the excessive outflow and uptake of potassium is toxic for the cell, suggesting that the maintaining of the proper potassium homeostasis is of benefit in the treatment of cell death during stress conditions like ischemia (Wei et al. Stroke 2003; 34 ; 1281-1286). Our results showed that when HEK-hBK1 cells transfected with cDNA of pannexin 1 were treated with 140 mM KCl they underwent an higher cell death than in not-transfected cells; moreover after treatment with probenecid there was a significant reduction in cell death. This data support the idea that the recovery in cell viability is due to the

pannexin inhibition and that in this case Kv1.1 channel should not influence pannexin inactivation. On the other hand DTT treatment didn't produce reduction in cell death, differently than in SH-SY5Y cell line (that doesn't express Kv1.1 channel) where DTT caused a partial improvement in cell viability; in conclusion these evidences suggest that in HEK-hBK1 cells Kv1.1 channel should obstacle pannexin inhibition by DTT but shouldn't obstacle its inhibition by probenecid. These findings confirm the idea that the functional interaction between pannexin 1 and Kv1.1 channel occurs only when the redox potential changes, hence with reducing agents, and when a membrane depolarization is induced, as in oocytes, but the same thing does not happen in other conditions; this phenomenon could replace or support that mediated by beta 3 subunit on pannexin 1 (Bunse et al., FEBS Journal 276 (2009), 6258-6270) and could furthermore affect the course of hypoxic events and cell death.

In summary, the regulation of gap junctions by potassium channels takes a significant meaning because it could allow to better understand the molecular basis of mixed synapses also during certain conditions of cellular sufference, becoming just this modulation a possible new drug target, in particular in pathologies in which the potassium homeostasis alteration is crucial, like channelopathies where electrical neuronal activity plays a key role or hypoxia where also redox potential and intracellular pH are modified and gap junctions are involved in cell death. However the modulation of pannexins by voltage-gated potassium channels and its effects could change according to the tissue where they are expressed and its condition.

Hypotized mechanism of cellular potassium redistribution depending on the interaction between voltage gated potassium channels and gap junctions:



4.5 Conclusion and future perspectives

In conclusion we have demonstrated that a functional interaction exists between Kv1.1 alpha subunit and pannexin 1, but not physical interaction occurs; the potassium channel infact modulates the sensibility of pannexin 1 to its inhibitors, but pannexin 1 does not alter the behavior of Kv1.1 channel in presence of its blockers. The data moreover suggest that this interaction happens only when redox potential changes (therefore when the intracellular pH is modified) and when a depolarization of the membrane is induced. This is the first demonstration of the functional interaction between these two proteins and surely it represents an important control modality on cellular homeostasis. So it's clear as the study of the relationship between gap junctions and potassium channels opens suggestive fields of research for the examination of electrical communication's molecular basis. One of the future directions should be addressed towards the structural characterization of pannexin 1 that is little still known and towards its more detailed functional characterization, in order to better understand its behavior and its interactions; therefore it will be necessary obtaining the purified protein to perform studies of fluorescence, dichroism and studies of liposome patch clamp, in order to describe the secondary and tertiary structure and the pure electrical activity. Moreover further investigations must be done to clarify the exact intracellular process through which these channels interact; in fine the functions and the effects of this cooperation should be defined, both in physiological and pathological conditions, such as channeloptahies and hypoxia, valuating its possible involvement in the control of cellular potassium redistribution and neuronal excitability. The understanding of all these mechanisms is clearly of great meaning as it would allow to gain new therapeutic strategies.

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