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Four nontraditional nano-openers for Maxi-K ion channels

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Dear reader! The format of this scientific communication is somewhat unusual. It resembles a comic strip. Pictures in it carry the main semantic load, and the text is secondary. Those who like to think and speculate for the author will love it. And the rest... let others seek help from the first. So together they will come to a discussion of the problem or they can just flip through the journal pages further.

I want to discuss the problem of mutual interest – so-called potassium channel openers. Opener, in general, it is device for opening somethings. For instance, a bottle opener but we are interested in ion channel openers. These are compounds capable of activating potassium channels in smooth muscle (SM) cells and this way to cause vasodilatation. Why is it important? At first, SM, as a functional unit of all hollow organs, represent itself the final common pathway for majority widely distributed and very dangerous diseases including diabetes vascular disease, angina pectoris and arterial hypertension. Second, potassium channels play an extremely important role in the membrane potential regulation in both endothelial (EC) and SM cells and, this way, they closely involved in contractile force regulation and responsible for adequate SM relaxation. If the vascular cells retain the ability to relax in accordance with the metabolic demands of functional systems, then you do not have to worry about the adequate delivery of oxygen and nutrients to the working organs and tissues.

And final reason – a growing number of diseases associated with ion channel malfunction – Ion Channels diseases or Channelopathies. Ion Channels became now a novel and favourite target in modern pharmacology and pharmacy (Fig. 1.).

Potassium channels family plays an extremely important role in the membrane potential regulation in both endothelial (EC) and SM cells and, this way, they closely involved in contractile force regulation. This ion family is rather large but one of the most important type of potassium channels family is, high conductance Ca^{2+}-dependent potassium channel (BK_{Ca} or Maxi-K). Fig. 2.

At the molecular level, BK_{Ca} channels are composed of pore-forming α-subunits which are coded by the gene Slo 1 (KNCMA1) and regulatory β_1-subunits. This channel has an interesting feature – it is under dual (or even triple if considering stretch) control: membrane potential and intracellular calcium concentration. This is a very important channel, up to 80 % of the total outward current passes through it.

It is generally accepted that impaired potassium channels function is the reason of many pathological conditions triggered or accompanied with vascular vasoconstriction. At the same time, despite intensive research, only a few K^+ channel openers are in everyday use in clinical medicine. That is why the task of discovering a new and effective

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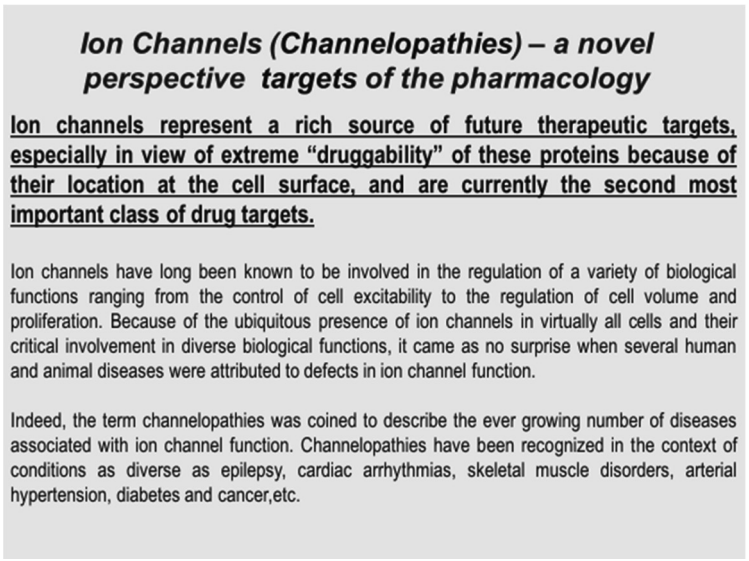


Fig. 1

type of potassium channel openers is now becoming more challenging especially given the wide occurrence of cardiovascular diseases.

The type of channel can be identified by different method: electrophysiological (by the parameters of its conductivity, activation and inactivation), analytical methods (Western blot and PCR analysis, by the expression of its proteins) or pharmacologically, using appropriate blockers and/or activators. Fig. 3.

Below you will see a few pictures showing the methods used in our experiments. Fig. 4–6.

I think that ion channel openers can be divided into 2 groups – direct and indirect. Direct action is understandable. Indirect, it is the action when the channel opens, if we remove some obstacles to its activation. The first such opener was liposomes containing the antioxidant quercetin and protein kinase C blocker in their membranes (Fig. 7).

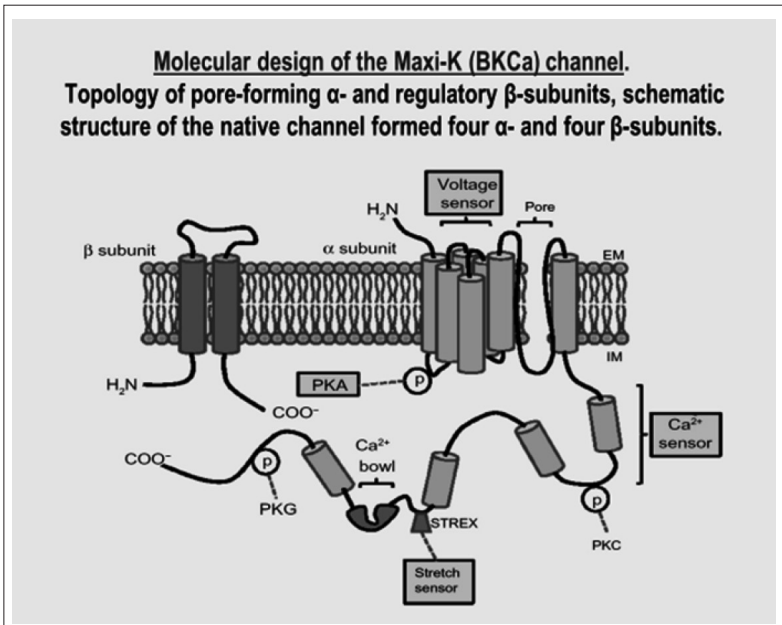


Fig. 2

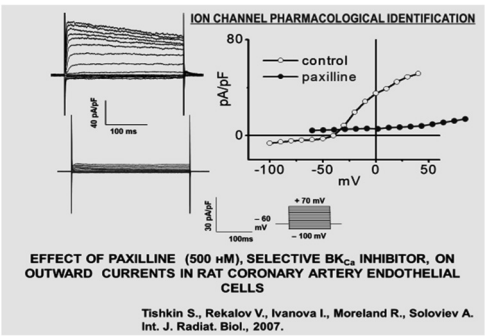


Fig. 3

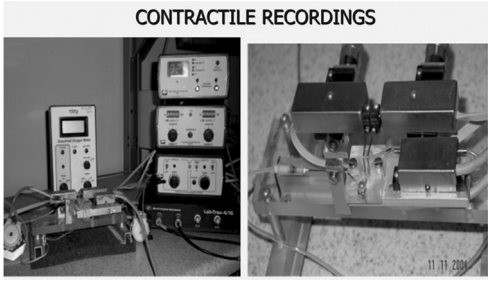


Fig. 4

The impetus for this research was given by the Chernobyl disaster because there was a task to minimize vascular disorders produced by ionized irradiation.

Not everyone knows that the most common health problems in surviving Ukrainian population that was exposed to radiation, as a result of the Chernobyl nuclear accident, are diseases of cardiovascular system and hypertension especially. In those times it was a surprising discovery for all of us. Additionally, radiation therapy is commonly employed as a primary and adjuvant therapeutic modality in patients with neoplasm, and despite many advances in technology, normal tissues including the blood vessels are often affected. This manifested as a blunting vasorelaxation, coronary spasm and cardiac arrhythmias development that seriously complicates the course and the treatment of the main disease. At last, radiation represents itself a good model for oxidative stress which, in turn, involved in pathogenesis number of diseases including arterial hypertension.

It was established that the main target for radiation was endothelium of blood vessels. γ-irradiation induced BK_{Ca} suppression in both EC and SM cells which was evident 9 days postirradiation and progressively increased over 30

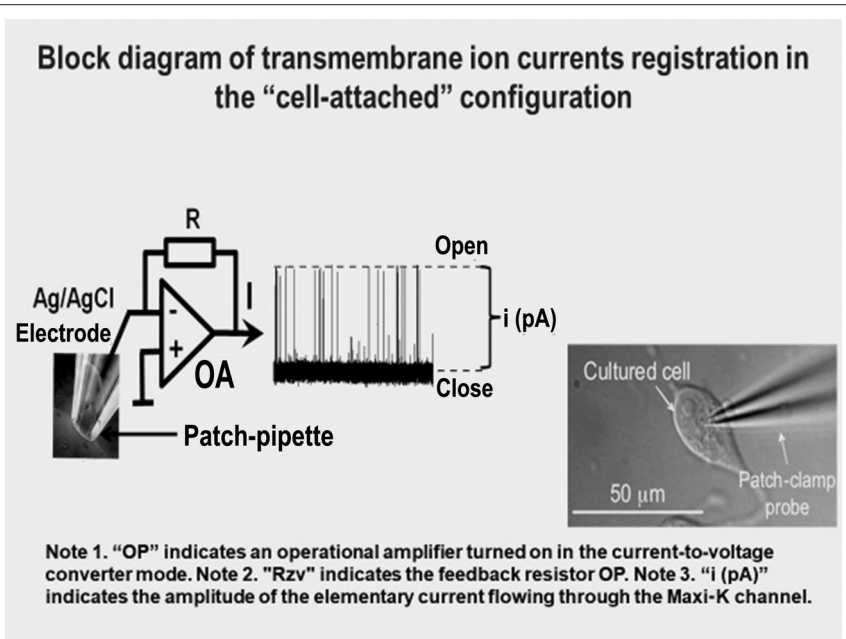


Fig. 5

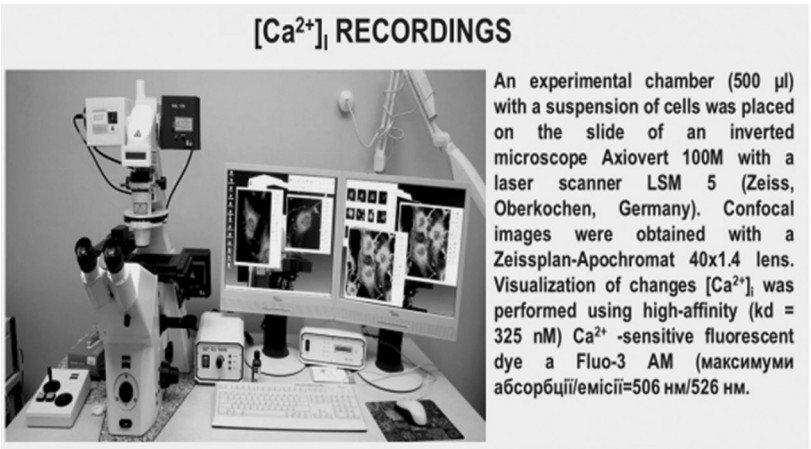


Fig. 6

days of experimental period. Thus, the vasorelaxant force of these SMCs and outward currents were diminished following irradiation and it was surprising discovery when we first saw that quercetin filled liposomes effectively restored outward potassium currents and normalize the contractile force of vascular SM cells damaged following gamma irradiation. Fig. 8–11.

Please, pay your special attention to the values of LD_{50} on the top of Fig. 11. What a huge difference in toxicity between free and liposomal quercetin!

Please note also that the constituents of PCL-Q, i. e., free quercetin (Q) and «empty» liposomes (PCL), being taken separately, showed a decreased ability to recover BK_{Ca} function as compared with combined composition. The protective effects of PCL-Q can be explained by its antioxidant and membrane repairing properties as well as its ability to inhibit protein kinase C activity. Thus, the lipid encapsulation of flavonoid, PCL-Q, appears to be a potential medication in the case of ionizing irradiation accident, and for the patients with neoplasm who have to receive external radiotherapy as well.

Later, the effects of quercetin-loaded liposomes (PCL-Q) and their constituents, that is, free quercetin (Q) and «empty» phosphatidylcholine vesicles (PCL), were studied also using single maxi-K channel activity in isolated

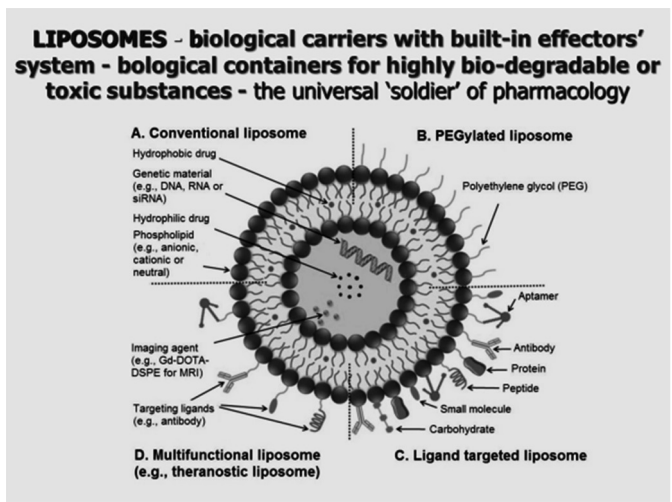


Fig. 7

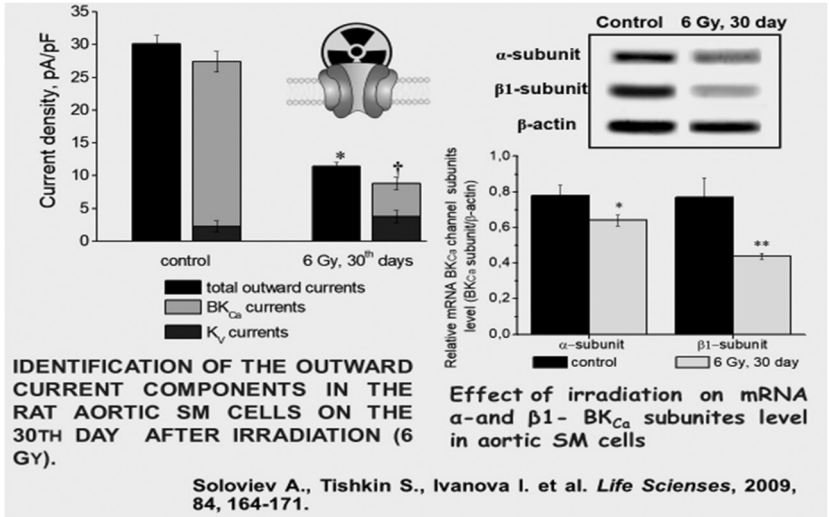


Fig. 8

mouse ileal myocytes before and after H_2O_2-induced oxidative stress in the cell-attached configuration. Fig. 12.

Bath application of PCL-Q (100 μg/ml of lipid and 3 μg/ml of quercetin) increased single Maxi-K channel activity more than three-fold, from (0.010 ± 0.003) to (0.034 ± 0.004) NPo (where N is the maximal number of active channels in a patch and Po is an open channel probability), whereas single-channel conductance increased non-significantly from 138 to 146 pS. In the presence of PCL-Q multiple simultaneous channel openings were observed, with up to eight active channels in the membrane patch. Surprisingly, «empty» PCL (100 μg/ml) also produced some channel activation, although it was less potent compared to PCL-Q, they increased NPo from (0.010 ± 0.003) to (0.019 ± 0.003) and did not affect single-channel conductance (139 pS).

Application of PCL-Q restored macroscopic Maxi-K currents suppressed by H_2O_2- induced oxidative stress in ileal mice SM cells. Additionally, PCL-Q effectively restored BK function in diabetic rats.

We conclude that PCL-Q can activate Maxi-K channels in ileal myocytes

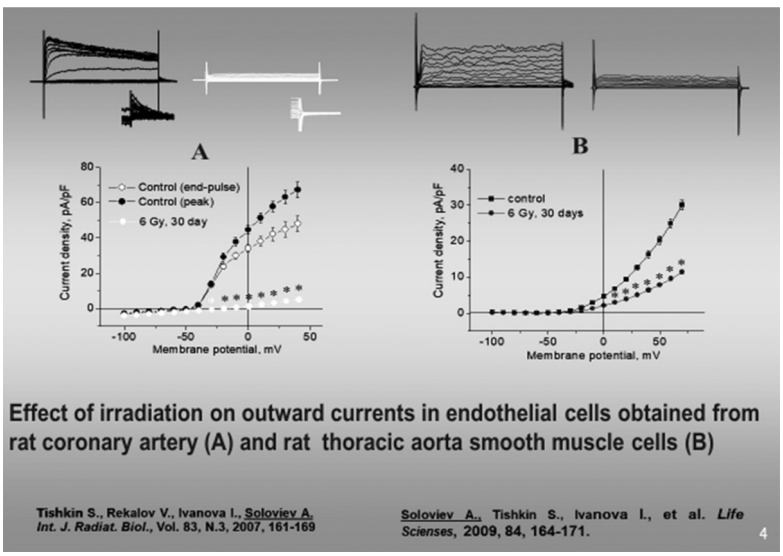


Fig. 9

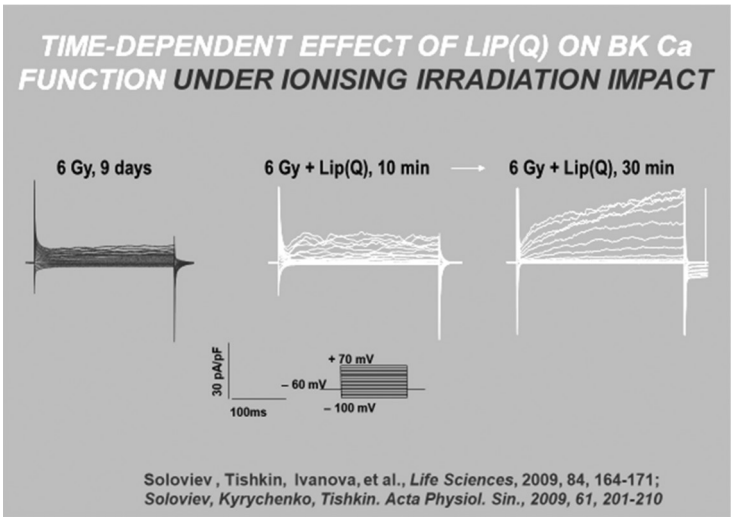


Fig. 10

mainly by increasing channel open probability, as well as maintain Maxi-K-mediated whole-cell current under the conditions of oxidative stress. While fusion of the 'pure' liposomes with the plasma membrane may indirectly activate Maxi-K channels by altering channel's phospholipids environment. The additional potentiating action of quercetin may be due to its better bioavailability in liposomal form.

The next Fig. 13–23 show one of the most surprising discovery in my life – ostensibly biologically «inert» gold nanoparticles opens ion channels! It is becoming clear now that the term inert does not apply to nanogold. According to the Royal Chemical Society gold nanoparticles can be conjugated with biofunctional molecules by means (A) hydrophobic interactions, (B) electrostatic forces, (C) covalent cross coupling, (D) dative covalent binding, (E) oligonucleotide hybridization, and (F) photolabile linkages.

Agents only work when they are bound. This well-known expression can be questioned or the concept of «bound» can be revised.

We studied colloidal gold nanoparticles (AuNPs) of ~5 nm core size and

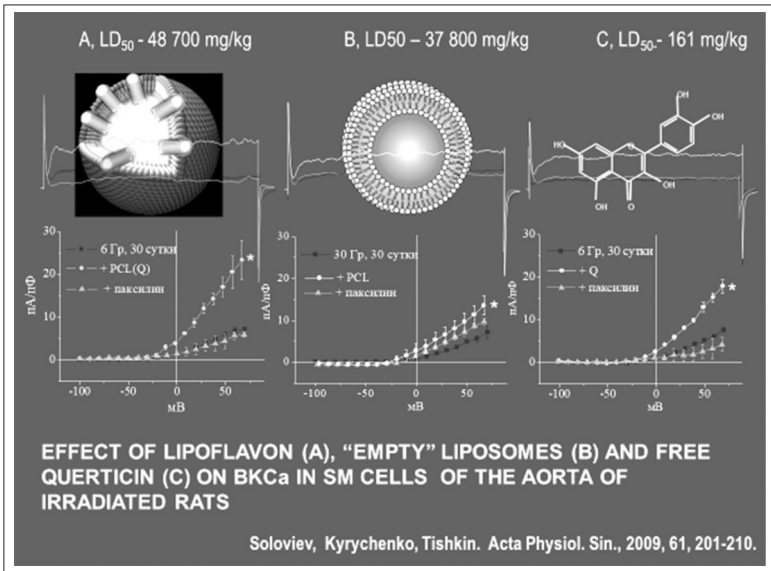


Fig. 11

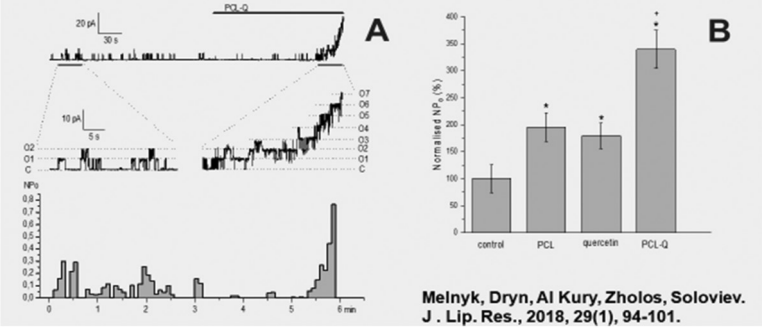


Fig. 12

Zeta-potential of –35 mV, having absorption maximum and plasmon resonance in the range of 510–570 nm.

When externally applied to the organ bath, AuNPs (10^{-6} – 3 · 10^{-4} M) led to decrease in amplitude of norepinephrine-induced contractions in a concentration-dependent and endothelium-independent manner in SM from rat thoracic aorta. Being added to the bath solution in concentration of 10^{-4} M, AuNPs significantly increased whole cell peak outward potassium current (OPC). External irradiation using a 5 mW/532 nm green laser, to facilitate plasmon resonance, led to an increment in the AuNPs-induced macroscopic OPC.

Paxilline (500 nM), when added to the external bathing solution, significantly decreased AuNPs-induced increment of OPC in SM cells (Fig. 17).

Single channel recordings provided a direct confirmation of BK_{Ca} activation by AuNPs at the single-channel level (Fig. 20, 21).

Application of AuNPs to the bath potentiated BK_{Ca} activity with a delay of 1–2 min, as was seen initially by more frequent channel openings followed by the progressive appearance of additional open levels corresponding to multiple openings of channels with identical single-channel amplitudes. Eventually, after 10–15 min in the presence of AuNPs and especially when combined with the green laser illumination, there was a massive increase in

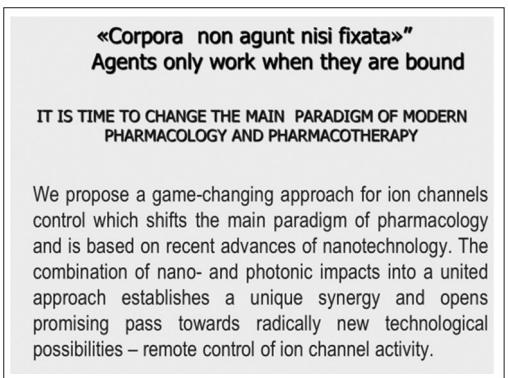


Fig. 13

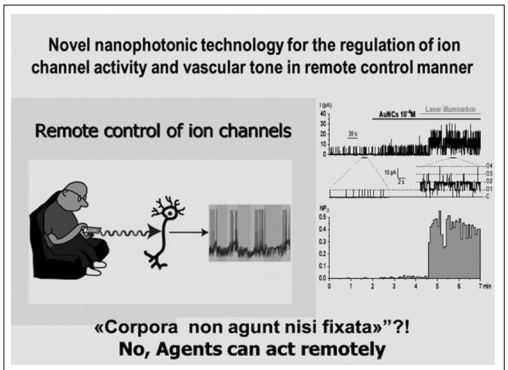
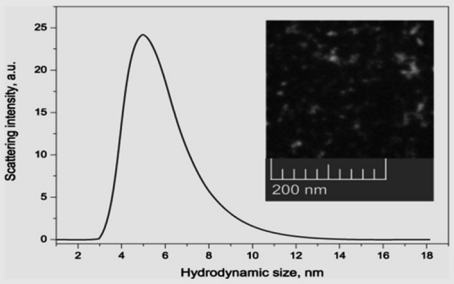
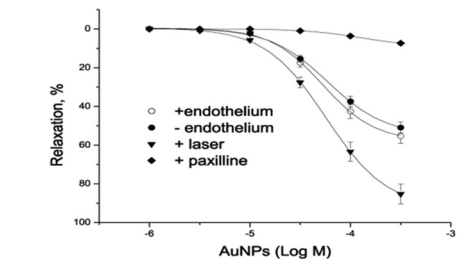


Fig. 14



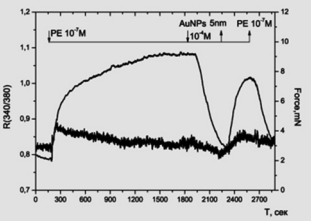
Distribution of colloidal ascorbate-stabilized gold nanoparticle by the hydrodynamic size. Inset: SEM microphotograph of Au nanoparticles.

Fig. 15



Cumulative concentration-relaxation curves for AuNPs ≈ 5nm core size and for AuNPs combined with 5 W/532 nm green laser irradiation (AuNPs+laser) obtained in intact and endothelium denuded rat thoracic aorta rings preactivated with 10^{-6} M norepinephrine before and after paxilline (500 nM) action.

Fig. 17



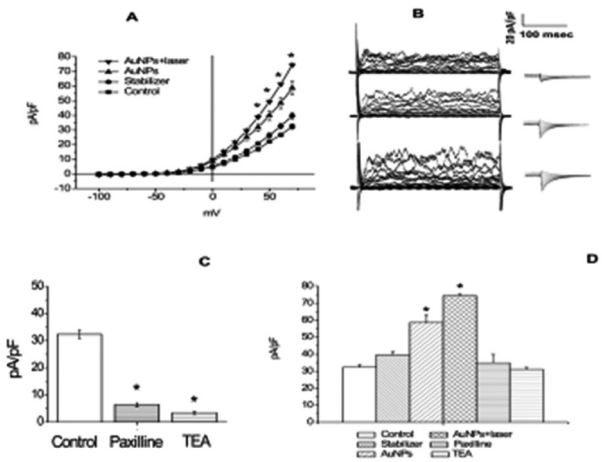
Relaxant effect of AuNPs (10^{-4} M) on NE- induced contraction and intracellular Ca^{2+} concentration ($R_{340/380}$) in intact ring of rat thoracic aorta.

Fig. 16

channel activity with N10 channels evident. When irradiated by laser, AuNPs significantly increased the amplitude of maximal AuNPs induced relaxation while the sensitivity of SM to AuNPs was without changes. In conclusion, plasmonic AuNPs possess the ability to activate BK_{Ca} channel opening in vascular SM.

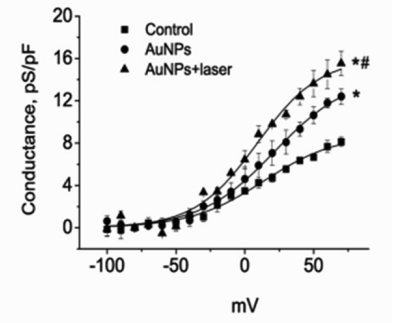
Laser irradiation facilitates this effect due to local plasmon resonance that, in turn, further increases BK_{Ca} channel activity causing SM relaxation.

A plasmon and plasmon resonance are the most plausible explanation of this phenomenon. Plasmon it is density wave in an electron gas (Fig. 22). Plasmons are collective oscillations of the free electron gas density. Plasmons exist mainly in metals, where electrons are weakly bound to the atoms and free to roam. It is analogous to a



I -V relationships (A) and original traces (B) of outward currents recorded in isolated rat thoracic aorta smooth muscle cells in control and after treatment with AuNPs (10^{-4}M) and AuNPs combined with 5W/532 nm green laser irradiation. C, D – effects of potassium channels blockers (paxilline, 500 nM and tetraethylammonium, 10 mM) on outward potassium channel in non-treated (C) and treated with AuNPs (D) aortic SM cells. Currents were elicited by 10 mV step pulses of 300 ms duration between -100 and +70 mV from a holding potential of -60 mV.

Fig. 18



Volage for half-maximal activation ($V_{1/2}$) = 8.5, 14 and 16 mV, respectively, i.e. conductance increases not typical for BK_{Ca}. This is due to the changes of channel conductance (e.g. increase of unitary current amplitude or single channel open probability).

Steady-state activation curves for the I_k in isolated thoracic aorta SM cells in control (squares), following AuNPs (10^{-4} M, circles) application and under AuNPs action combined with 5 mW/532 nm green laser irradiation (triangles) measured with 300 ms step protocol. Currents in each cell were leak subtracted, converted into conductance densities (pS/pF), averaged and plotted against membrane potential (mV). The data obtained were fitted with the standard Boltzmann function with mean parameters described in the text. Resulting current-voltage (I-V curves) were obtained by measuring the current at the end of the pulse.

Fig. 19

Schematic representation of the cell-attached configuration illustrating that AuNPs had no direct access to ion channels in a membrane patch insulated by the glass pipette rim.

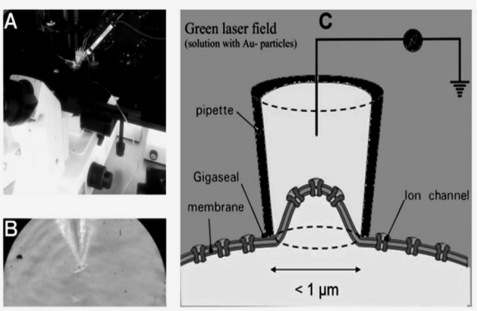
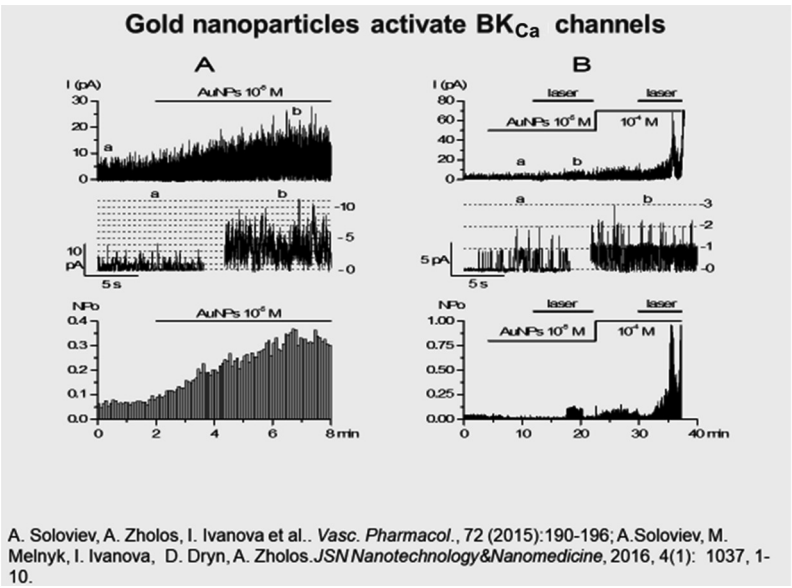


Fig. 20

sound wave, which is a density wave in a gas consisting of molecules. The electrons in a metal can wobble like a piece of jelly, pulled back by the attraction of the positive metal ions that they leave behind. In contrast to the single electron wave function, a plasmon is a collective wave where billions of electrons oscillate in sync. It is likely that AuNPs effect may be related to the surface plasmon and associated with it local electromagnetic field interaction (which, in turn, may be increased by green laser irra-



A. Soloviev, A. Zholos, I. Ivanova et al.. *Vasc. Pharmacol.*, 72 (2015):190-196; A.Soloviev, M. Melnyk, I. Ivanova, D. Dryn, A. Zholos. *JSN Nanotechnology&Nanomedicine*, 2016, 4(1): 1037, 1-10.

Fig. 21

diation) with ion channel voltage sensor. AuNPs itself cannot cross glass pipette but plasmon resonance energy can be transduced within membrane plane by at least 1 μm distance.

In summary, it is clear that plasmonic AuNPs are able to open K^+-channels and relax SM in both endothelium-dependent and independent manner. One plausible explanation for AuNPs action is the interaction of the channel voltage sensors with local electric field on AuNPs surface. In case of laser irradiation this local electrical field enhanced due to local plasmon resonance which, in turn, increases IK and SM relaxation. In the absence of external irradiation such plasmon may be excited by natural tissue chemoluminescence in the presence of nonlinear effects on AuNPs surface.

Fig. 23–25 demonstrate the strange behaviour of gold nanoparticles.

Nitric oxide does not need to be represented. Its role in the functioning of our body can hardly be overestimated. It's application for therapy hampered by a very short life span. That's why we have created a molecular construct, with a quantum dosed release of nitric oxide molecules, which ensures the constancy of its effective concentration in a pharmacological target (Fig. 26–29). Then we studied the action of a novel liposomal nitric oxide carrier on BK_{Ca} channels expressed in vascular SM cells isolated from the rat main pulmonary artery. The liposomal form of NO, Lip(NO), increased whole-cell outward potassium currents in a dose-dependent manner while shifting the activation curve negatively by about 50 mV with respect to unstimulated cells with the EC_{50} value of (0.55 ± 0.17) mM.

At the single channel level, Lip(NO) increased the probability of the open state (Po) of Maxi-K channels from (0.0020 ± 0.0008) to (0.74 ± 0.02) with half maximal activation occurring at (4.91 ± 0.01) μM, while sub-maximal activation was achieved at 10^{-5} M Lip(NO). **Interesting**, channel activation was mainly due to significant decrease in the mean closed dwell time (about 500-fold), rather than an increase in the mean open dwell time, which was comparatively modest (about twofold). There was also a slight

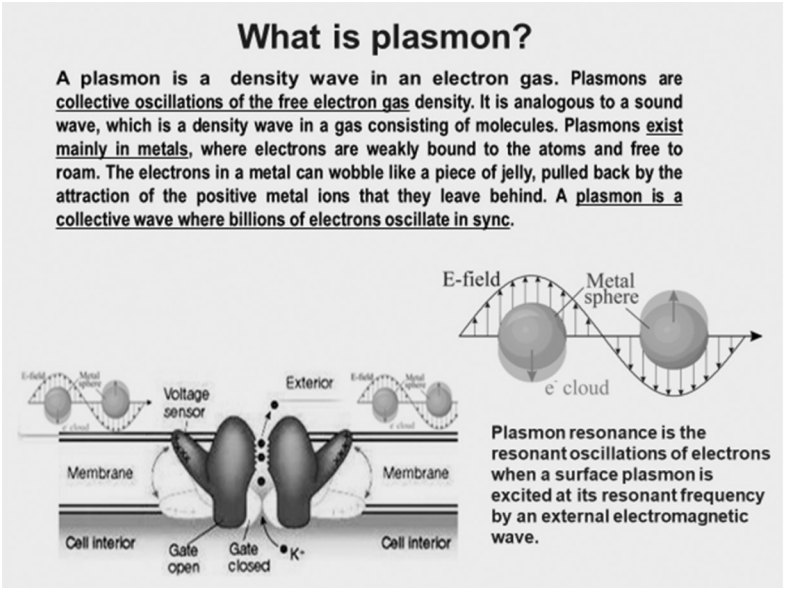
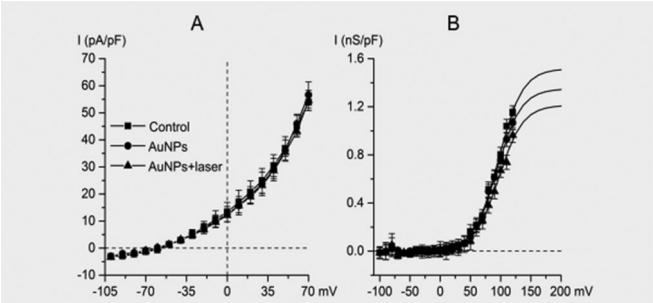


Fig. 22



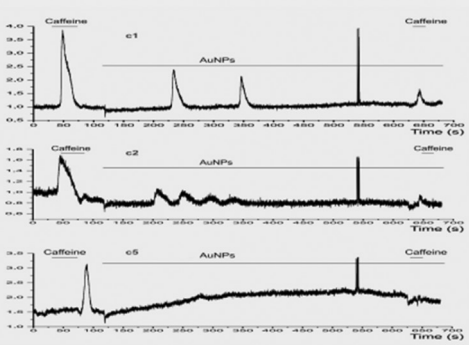
Effects of AuNPs alone and in combination with green laser irradiation on whole-cell outward potassium currents in isolated rat pulmonary artery smooth muscle cells under conditions of strong intracellular Ca^{2+} buffering (10 mM BAPTA/4.6 mM Ca^{2+} mixture was used in the pipette solution to "clamp" $[Ca^{2+}]_i$ at 100 nM).
A, mean I-V relationships of whole-cell outward potassium currents measured in control, after addition of AuNPs alone (10^{-4} M) and AuNPs acting in combination with 5 mW/532 nm laser irradiation, as indicated.
B, corresponding steady-state activation curves. Currents in each cell were leak subtracted, converted into conductance densities at each test potential (nS/pF), averaged and plotted against membrane potential. The data points were fitted with the Boltzmann function.

Fig. 23

decrease in the amplitude of the elementary Maxi-K currents (approximately 15 %) accompanied by an increase in current noise, which might indicate some non-specific effects of Lip(NO) on the plasma membrane itself and/or on the phospholipids environment of the channels. In conclusion, the activating action of Lip(NO) on the Maxi-K channel is due to the destabilization of the closed conformation of the channel protein, which causes its more frequent openings and, accordingly, increases the probability of channel transition to its open state.

Potassium conductance is known to be altered in essential hypertension. An overproduction of reactive oxygen species and related high level of protein kinase C (PKC) activity are common features for arterial hypertension of different genesis. Total outward currents carried mainly through BK_{Ca} are significantly decreased in SHR and the Ach-induced relaxant responses were blunted.

The RT-PCR analysis showed that PKC-δ-isoform mRNA expression is tenfold increased in vascular SM from SHR. PKC inhibitor, chelerythrine (100 nM), restored BK_{Ca} activity and ACh-induced NO-dependent relaxation in SHR but these compounds are known as rather toxic.



Spatio-temporal patterns of AuNPs-induced $[Ca^{2+}]_i$ oscillations after control caffeine application recorded in 3 different representative cells to illustrate similarities (e.g. oscillatory nature) and variability (e.g. amplitude and frequency of $[Ca^{2+}]_i$ rises) of the responses. For each cell (A-C), the time course plot of the normalized fluo-3 fluorescence intensity was averaged within the total optical slice of the SMC, where Ca^{2+} waves induced by 10^{-4} M AuNPs) occurred.

Fig. 24

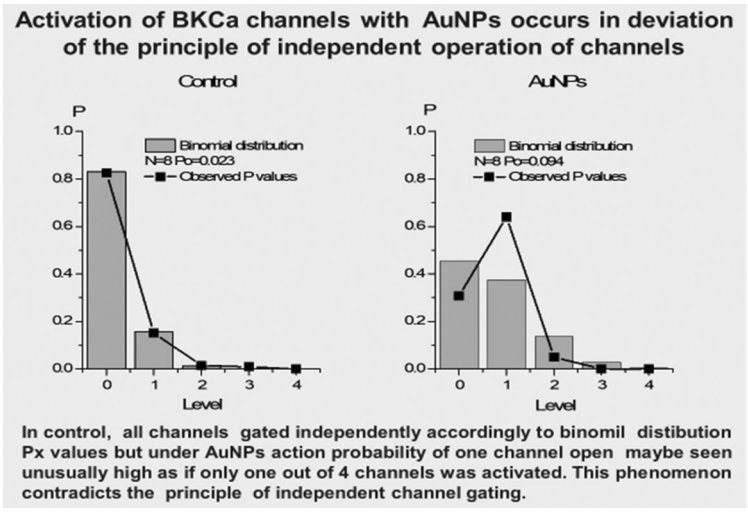


Fig. 25

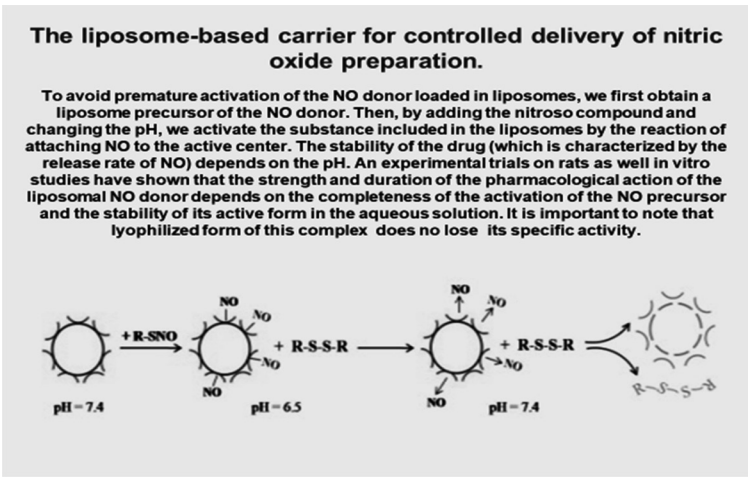


Fig. 26

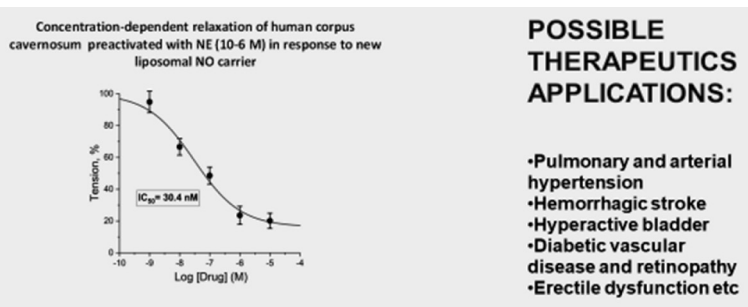


Fig. 27

That's why we have decided to try a more specific and safe method RNAi - RNA interference (RNAi) is a regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules as triggers to direct homology-dependent control of gene activity.

Whereas most of us think of RNA as a messenger molecule that carries DNA information into a form suitable for protein synthesis. In fact, RNA can exist in multiple other forms, some of which are double stranded. Of these double-stranded forms, some can

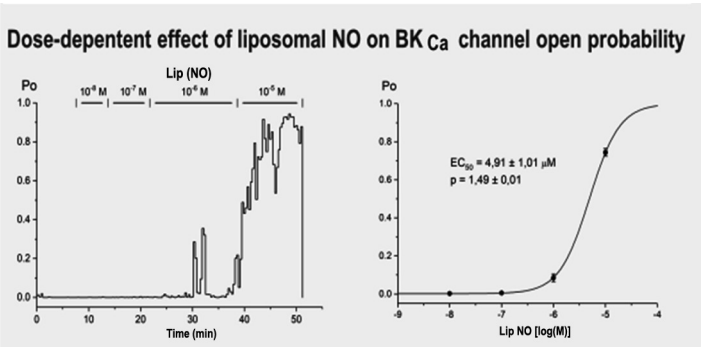


Fig. 28

directly interfere with the relay of messages from DNA out to the translation machinery, and effectively block synthesis of specific proteins.

RNAi is known as a method of post-transcriptional suppression on gene expression (silencing) in which double-stranded siRNA induces degradation of homologous messenger RNA (mRNA). Let me remind again that mRNA is a molecule in cells that carries codes from DNA in the nucleus to the sites of protein synthesis in the cytoplasm (the ribosomes). The essence of the mechanism of RNAi is that when a short double-stranded RNA is introduced into cells, it is capable of causing specific degradation of the mRNA with which it has homology.

In general, the RNA interference mechanism looks like this Fig. 30–31. **Now point the camera of your smartphone at the QR code in the Fig. 30.** First, dsRNA is cut by a specific enzyme (DICER) into short fragments measuring of 19 to 21 pairs of nucleotides (if more, it stimulates interferon synthesis, if shorter – loss of specificity). Then these short dsRNAs join an RNAase complex, RISK (RNA-induced silencing complex). Namely, in this complex, the dsRNA is disintegrated (unwinded/untwisted) and becomes single-stranded. Then, the short single-stranded RNAS, due to its complimentary, interacts with a strictly defined mRNA (copy of the target genes of interest), which is the signal for the cutting of the latter by the enzymes of the RISK complex. The short fragments of the mRNA

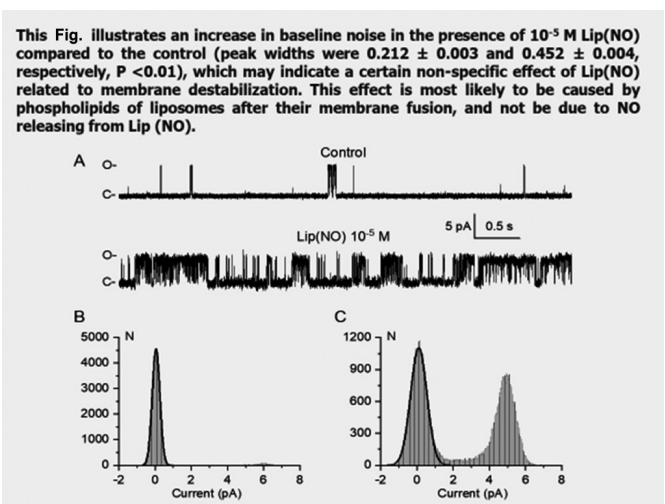


Fig. 29

The Nobel Prize in Physiology and Medicine 2006
""for their discovery of RNA interference - gene silencing by double-stranded RNA"

Andrew Z. Fire

RISC - RNA- induced silencing complex. Function - degradation of target mRNA which reduces the levels of transcript available to be translated by

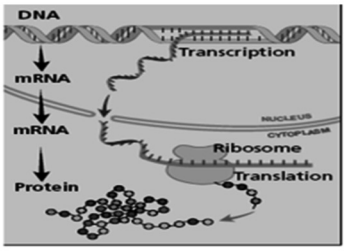
Craig C. Mello

DICER - an enzyme that cleaves double-stranded RNA (dsRNA) in short double-stranded RNA fragments called small interfering RNA. These fragments are

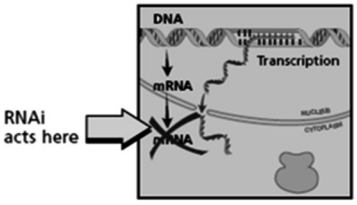
Fig. 30

What is RNA interference (RNAi)?

RNAs can be coding and non-coding molecules, i.e, those that are not translated into protein. DNA contains genetic code. Synthesis of coding mRNA take place on the DNA matrix i.e. RNA molecule is a trancript of DNA fragment and it is sense transcript. Opposite meaning - antisense RNA. In case when both RNAs are present in the cell simultaneously the silencing is happened.

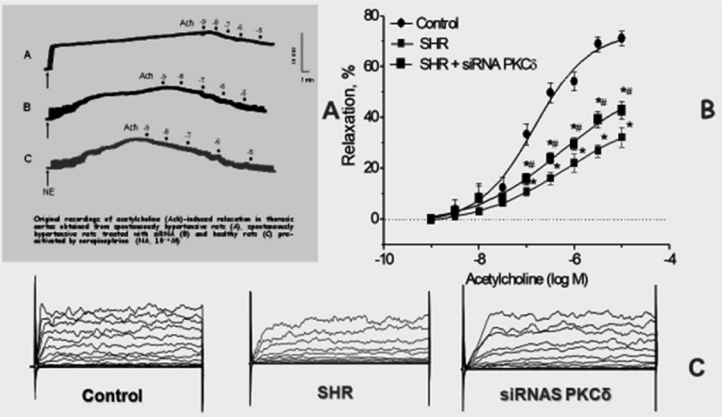


For reference - the length of siRNA is about 10 nm



In RNA interference, RNA in double-stranded form breaks down the mRNA for a specific gene, thus stopping production of protein.

Fig. 31



ORIGINAL TRACES (A) AND "DOSE-EFFECT" CURVES (B) FOR ENDOTHELIUM-DEPENDENT Ach-INDUCED RELAXATION AND BK_{Ca} FUNCTION (C) IN RAT THORACIC AORTA SM CELLS FOLLOWING PKC-δ siRNAs ADMINISTRATION

Novokhatska T., et al. Eur J Pharmacol, 2013, 718, 401-407

Fig. 32

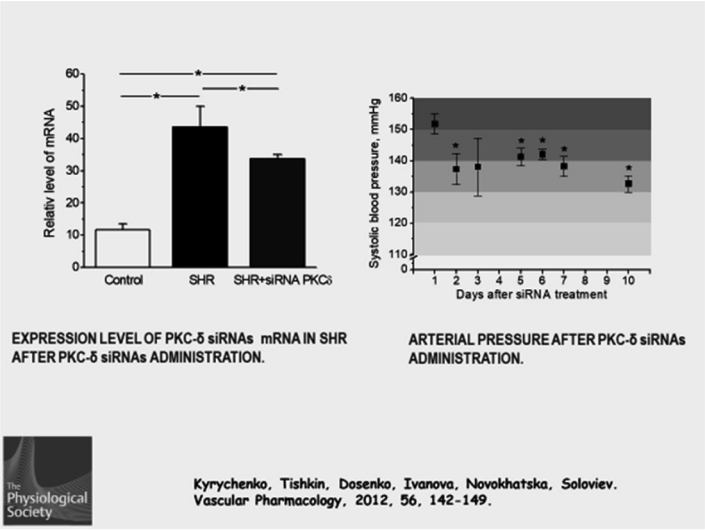


Fig. 33

as a result of this are no longer capable of providing a full protein synthesis.

PKC targeted siRNAs-plasmid delivery system administered intravenously led to an increment in amplitude of ACh-relaxation and restored the currents carried through BK_{Ca}. Arterial blood pressure in SHR was normalized following siRNAs-plasmid complex single administration for 2 weeks. It was great success. It is likely that RNAi phenomenon is a good approach to inactivate PKC gene encoding function and to normalize arterial blood pressure and vascular functions damaged in SHR (See the latest Fig. 32–33).

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