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Research Article

Non-drug Remote Control of Maxi-K Channels in Smooth Muscle Cells with Nano-Photonics Stimuli Using Plasmonic Gold Nanoparticles

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Abstract

In excitable cells, potassium channels control not only resting potential, but also cell excitability, and thus underline normal function and various pathological conditions. Therefore, there are continuing efforts towards developing K^+ channel openers for the treatment of disease states associated with abnormal cellular activity. The aim of this study was to shed light on the intrinsic mechanisms of Maxi-K (BK_{Ca}) channels activation by plasmonic gold nanoparticles (AuNPs) in smooth muscle (SM) cells obtained from different SM tissues. Maxi-K currents in myocytes isolated from rat pulmonary artery and mouse ileum were recorded in the whole-cell and cell-attached configurations of the patch-clamp techniques. We compared the effects of AuNPs on Maxi-K channel activity under weak or strong intracellular Ca^{2+} buffering (Ca^{2+} "clamp" at 100 nM). With weak Ca^{2+} buffering, Maxi-K current density was increased by AuNPs applied at 10^{-4} M, and further increased by green laser irradiation (GLI, 5 mW, 532 nm). The potentiating effect of AuNPs alone was due to an increase in maximal conductance (Gmax) by about 50% without any shift of the activation curve of K^+ conductance. GLI in the presence of AuNPs (AuNPs/GLI) produced little additional effect increasing the maximal K^+ conductance, but instead shifted the potential of half-maximal activation ($V_{1/2}$) value negatively by about 10 mV. Under conditions of strong intracellular Ca^{2+} buffering, no effect of AuNPs alone or AuNPs/GLI on Maxi-K currents was observed. At the single-channel level, the effects of AuNPs/GLI were due to a significant increase in channel open probability, while single channel conductance remained unchanged. Under these conditions, there was deviation from independence of channel gating, suggesting that plasmon resonance may not similarly affect all channels present in the membrane patch. This type of regulation is fundamentally different from other common types of drug action. We conclude that AuNPs activate Maxi-K channels expressed in different SM tissues via both Gmax increase and a negative $V_{1/2}$ shift. Moreover, the potentiating effect of AuNPs/GLI is clearly calcium-dependent, as it could be completely abolished by "clamping" the intracellular Ca^{2+} concentration.

ABBREVIATIONS

AuNPs: Gold Nanoparticles; SM: Smooth Muscle; BK_{Ca} or Maxi-K Channels: Large-Conductance Calcium-Activated Potassium Channels; Gmax: Maximal Conductance; $V_{1/2}$: The Potential of Half-Maximal Activation; GLI: Green Laser Irradiation

INTRODUCTION

We have previously reported potent activation of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca} or Maxi-K channels) in thoracic rat aorta smooth muscle (SM) cells by AuNPs of ~ 5 nm core size having plasmon resonance of 532 nm. Green laser irradiation (GLI) further facilitated this effect and caused SM relaxation [1,2]. It was surprising discovery while underlying mechanisms remained completely unknown.

It is well established that dysfunction of potassium channels is closely linked to a number of pathological conditions triggered or accompanied with vascular disorders, e.g. excessive vasoconstriction. Despite intense research in this area, only a few K^+ channel openers have been introduced into clinical practice. That is why the task of discovering new and efficient openers of potassium channel is now becoming more and more challenging, especially given the wide occurrences of cardiovascular diseases.

Different types of nanomaterials are known to be widely used in medicine and biology as biosensors, biomaterials, drug-delivery systems etc. [3], but not yet as drugs. The properties of nanoparticles (NPs) can substantially differ from those demonstrated by their bulk forms. This way, their novel physical and chemical properties are often evident in some unexpected biological or even cytotoxic effects [4]. Considering the high

reaction ability of NPs to interact with cell proteins, combined with their extremely small size and an increased ability to penetrate into cellular structures, it becomes clear that understanding of how NPs interact with cell membrane is essential for both theoretical and clinical medicine and applied biology.

In the clinical context, it is well appreciated that the proper SM cells contractility is extremely important for almost all aspects of human (patho) physiology, since SM represent the main effector element of hollow organs, including blood vessels. Thus, vascular SM in particular plays a key role in both health and disease due to its involvement in regulation of adequate blood and oxygen supply to all tissues in the body.

Some of the most relevant and widely occurring cardiovascular diseases, such as arterial hypertension [5,6], radiation-induced vascular abnormalities [7] and diabetic vascular disease [8] have been linked to the overproduction of reactive oxygen species (ROS), related protein kinase C activation and dysfunction of ion channels (channelopathies). The mechanisms involved in pathogenesis include impaired vasodilator potential, decreased endothelium-dependent vasorelaxation [9,10] and suppression of ionic currents through potassium channels [7,11-13]. Acting together, all these factors promote vascular hypercontractility and decreased tissue blood perfusion.

SM contractility is closely regulated by SM cells' membrane potential, which, in turn, is determined predominantly by K^+ membrane conductance. Potassium permeability plays an important role in the regulation of SM membrane potential and vascular tone control. The main types of K^+ channels, which have been identified in SM cells are as follows: voltage-activated K^+ channels (Kv), which are encoded by the Kv gene family, inward rectifiers (Kir) encoded by the Kir2.0 gene, ATP-sensitive K^+ channels (K_{ATP}), which are encoded by Kir6.0 and sulphonylurea receptor genes, and large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}, Maxi-K) which are encoded by the *Slo1* (KNCMA1) gene. Maxi-K channels are composed of the pore-forming α–subunits and regulatory β_1-subunits. The presence of the β_1-subunit confers a higher Ca^{2+} sensitivity of Maxi-K channel, which makes this channel an efficient modulator of SM function in health and disease, i.e. Maxi-K have a dual mode of regulation as they are both Ca^{2+} and voltage sensitive.

There is currently strong evidence that Maxi-K channels play an important role in both essential arterial hypertension [13] and radiation-induced vascular hypertension [12], i.e. decrease in outward current density in both endothelial and SM cells combined with diminished BK_{Ca} mRNA expression led to vascular hypercontractility and hypertension development [11].

It is important to note that currently there is a lack of evidence of any direct effects of NPs on effector and regulatory elements of the vascular system, i.e. SM cells and endothelium.

The aim of this study was to shed light on some intrinsic mechanisms involved in Maxi-K channels activation by AuNPs in SM cells, especially in connection with their synergistic activation by both voltage and intracellular calcium, and to compare the effects obtained from different vascular and gastrointestinal tract tissues.

MATERIALS AND METHODS

Preparation of Au nanoparticles

Colloidal gold nanoparticles (AuNPs) were synthesized as described before [2] and in accordance to "Colloidal solution of gold nanoparticles and amino acids and method for producing of gold nanoparticles in colloidal solutions with presence of amino acids". UA Patent 102589 (priority data 13.09.2011). Briefly, colloidal gold nanoparticles (AuNPs) having plasmon resonance of 532 nm were synthesized via reduction of sodium tetrachloroaurate by sodium ascorbate in aqueous solutions at room temperature. Then prepared colloids were neutralized to pH 6-7 by acetic acid. A dynamic light scattering study of colloidal Au solutions (200 mg/L, i.e. ~10^{-3} M or $6.02x10^{20}$ particles/L) showed that the average hydrodynamic size of gold nanoparticles was around 5 nm.

The surface of Au nanoparticles stabilized by ascorbate anions is supposed to be negatively charged (Zeta-potential of -35 mV). The colloidal AuNPs solution was stable for several months and did not show any appreciable temporal changes in the size and size distribution of Au nanoparticles.

Ethical approval and animals

Mammalian animal studies were performed using rats and mice in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committees. All experiments were performed on 6-8 weeks male Wistar rats (weight 250–300 g) and BALb/c male mice housed under controlled environmental conditions (21°C, 12 h-12 h light-dark cycle) and free access to water and standard rodent chow.

Isolation of smooth muscle cells

Briefly, the rats were anesthetized with ketamine (37.5 mg/kg b.w., IP) and xylazine (10 mg/kg b.w. IP) and thoracic aortas were dissected. BALB/c male mice (25-30 g) were killed using cervical dislocation. Smooth muscle cells were isolated from tissues by enzymatic treatment, as described below.

a) Rat pulmonary artery: Vascular rings were cut into small pieces (1.0 x1.5 mm) in Ca^{2+}-free modified Krebs solution containing (in mM) 120 NaCl, 12 glucose, 10 HEPES, 6 KCl, (pH 7.4). The pieces were digested at 36.5 C for 22 min in 2 ml Ca^{2+}-free modified Krebs solution containing papain (1 mg/ml), dithiothreitol (1 mg/ml), bovine serum albumin (1 mg/ml), then removed and digested at 36.5 °C for 15 min in 2 ml Ca^{2+}-free modified Krebs solution containing collagenase type 1A (1.5 mg/ml), dithiothreitol (1 mg/ml), bovine serum albumin (1 mg/ml) and after this washed three times with Ca^{2+}-free modified Krebs solution to stop enzymatic digestion.

b) Mouse ileum: The longitudinal smooth muscles of the ileum was rapidly removed and placed in modified Krebs solution containing (in mM) 120 NaCl, 12 glucose, 10 HEPES, 6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$ (pH 7.4). Segments of smooth muscle tissue were cut into small pieces (1 mm length) in Ca^{2+}-free modified Krebs solution containing (in mM) 120 NaCl, 12 glucose, 10 HEPES, 6 KCl, pH 7.4 with NaOH. The pieces were digested at 36.5 °C for

18 min in 2 ml Ca^{2+} free modified Krebs solution containing collagenase type 1A (1 mg/ml), soybean trypsin inhibitor II-S (1 mg/ml), bovine serum albumin (1.5 mg/ml) and then washed three times with Ca^{2+} free modified Krebs solution to stop enzymatic digestion.

The enzyme-treated pieces of tissue were gently triturated with glass Pasteur pipette in Ca^{2+} free modified Krebs solution at room temperature (22-25 °C) to release single cells. Cells were placed on glass coverslips and were used in experiments within 6 hours after isolation.

Patch-clamp studies

Figure (1A,B) shows experimental setup for single channel recordings under green laser illumination (5 mW, 532 nm) with an area of the light spot of about 1.5 mm^2.

The whole-cell patch clamp technique was used to study integral outward potassium currents under voltage clamp mode. Voltage steps were applied and data acquired using an Axopatch 200B patch-clamp amplifier and Digidata 1200B interface (Molecular Devices, Sunnyvale, CA, USA) interfaced to a PC running the pClamp software (version 6.02, Molecular Devices, USA). Membrane currents were filtered at 2 kHz and digitized at 10 kHz. The reference electrode was an Ag-AgCl plug connected to the bath via a salt bridge. For each cell, current amplitudes were normalized by its membrane capacitance and current density was expressed in pA/pF. The membrane capacitance of each cell was estimated by integrating the capacitive current generated by a 10 mV hyperpolarizing pulse after electronic cancellation of pipette-patch capacitance using the Clampfit software (version 6.02, Molecular Devices, USA). All electrophysiological experiments were carried out at room temperature (about 20°C).

Patch pipettes were made from borosilicate glass (Clark Electromedical Instruments, Pangbourne Reading, England) and backfilled with intracellular solution (in mM):: KCl 130, MgATP 1, creatine 5, glucose 10, EGTA 0.3, HEPES 10 and pH was set to 7.4 by adding KOH. Maxi-K calcium-sensitive component was turned off with intracellular solution containing (mM): KCl 80, $CaCl_2$ 4.6, MgATP 1, creatine 5, glucose 5, BAPTA 10, HEPES 10 and pH was set to 7.4 with KOH. Pipettes had resistances of 4 – 5.5 MΩ. The standard Krebs-HEPES external solution contained (in mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, and glucose 7.8 at pH 7.4.

Maxi-K single-channel currents in isolated myocytes were recorded at room temperature in the cell-attached configuration using borosilicate patch pipettes of 3-4 MΩ resistance. Currents were filtered at 1 kHz and digitized at 10 kHz. For illustration purposes, single-channel traces were additionally filtered at 100 Hz and re-sampled at 500 Hz. In these experiments, patch pipettes were filled with the normal Krebs-HEPES solution, while the bath contained hyper-K^+ (130 mM) solution in order to bring the cell resting potential to about 0 mV. Thus, membrane potentials refer to the holding potential with the opposite sign, while current polarity was conventionally reversed in all illustrations.

Single-channel events were identified on the basis of the 50% threshold-crossing criteria. Channel activity was expressed as NPo, where N is the number of active channels in each membrane patch and Po is channel open probability. Mean NPo values were

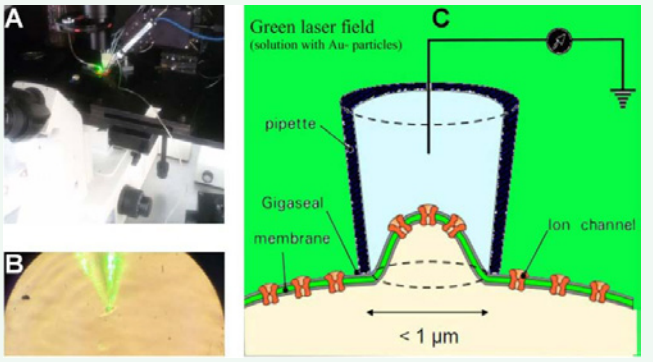

Figure 1 A schematic of study illustrating patch-clamp recording of BK_{Ca} channel activation by green laser-induced plasmon resonance in an isolated smooth muscle myocyte.

calculated in 5 s segments after performing the "event detection" search in the pClamp 9 software (Molecular Devices, Sunnyvale, CA, USA).

Measurement of contractile force

Segments of thoracic aorta and pulmonary artery were obtained as described above, cleaned of both connective and adipose tissue, and cut into 1 to 2 mm width rings. All preliminary procedures were performed at room temperature in a nominally Ca^{2+} - free physiological salt solution. Then, vessel rings were mounted isometrically in a tissue bath between a stationary stainless steel hook and an isometric force transducer (AE 801, SensoNor, A/S, Norten, Norway) coupled to an AD converter Lab-Trax-4/16 (World Precision Instruments, Inc., Sarasota, USA). The rings were equilibrated for 1 hour at a resting tension of 5 mN (pulmonary artery) or 10 mN (aorta). Experiments were performed at 370 C in modified Krebs bicarbonate buffer solution of the following composition (in mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.3, NaH_2PO4 1.38, $CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 10, D-glucose 7.8 at pH 7.4. Following the equilibration period, the aortic rings were exposed several times to norepinephrine (NE, 10-6 M) until reproducible contractile responses were obtained.

Chemicals

Collagenase type IA, pronase E type XXV, papain, trypsin inhibitor II-S, bovine serum albumin, norepinephrine, as well all the constituents of the Krebs and pipette solutions were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Statistics and analysis

The data are shown as means ± standard error of the means (S.E.M.), and *n* indicates the number of cells, preparations or animals tested. Data analysis was performed using Origin 8.0 (OriginLab Corporation, Northampton, MA, USA) software. Half-maximally effective concentration (EC_{50}) values were expressed as pD_2 ($-log\ EC_{50}$). Concentration-response curves were fitted by the Hill equation ($F=F_{max}/[1+exp(logEC_{50}-logC)^h]$, were F and F_{max} are actual and maximal relaxation (constriction) responses, h is the Hill coefficient, C is actual concentration) to determine the maximal response and the EC_{50} value. Multiple comparisons were made using one-way analysis of variance (ANOVA) and if any

significant difference was found, the Tukey's multiple comparison post hoc tests were performed. Differences were considered to be statistically significant when *P* was less than 0.05.

RESULTS AND DISCUSSION

Effects of AuNPs and AuNPs/GLI on vascular tone

It is important to note that in our previous studies [1,2] we have tested a number of AuNPs, which differed both in their size and stabilizer used but only AuNPs with core size of ≈5 nm stabilized with ascorbate demonstrated clearly the ability to relax thoracic aorta SM in a remote control manner [1,2].

Figure (2) shows the concentration-relaxation curves in response to AuNPs for rat thoracic aorta SM and rat pulmonary artery preconstricted with norepinephrine (NE, 10^{-6} M). It is clear that AuNPs at concentrations ranged from 10^{-6} to 3 x 10^{-4} M possess the ability to decrease the amplitude of the NE-induced contraction in both aortic and pulmonary artery rings significantly in a dose-dependent manner (pD_2 = 4.2 ± 0.3, E_{max} = 55 ± 4% and pD_2 = 4.2 ± 0.2, E_{max} = 54 ± 4%, respectively), i.e. AuNPs demonstrated very similar and clearly evident vasorelaxant activity in both types of vessels. Endothelial disruption was without any significant effect on AuNPs-induced SM relaxation. It is important to note that stabilizer *per se* had no effect on the tone of thoracic aorta and pulmonary artery SM preconstricted with NE (data not shown).

To facilitate AuNPs plasmon resonance, we illuminated tissue organ bath using a 5 mW/532 nm green laser during the entire period of SM incubation with AuNPs. Under these conditions, in rat aortic rings AuNPs produced a significantly larger relaxation, with maximal relaxation increasing from 55 ± 4% to 85 ± 5% (*n*=10, *P*<0.05). At the same time, the sensitivity of SM to AuNPs remained unchanged, with the mean pD_2 value of 4.2 ± 0.01 (*n*=10, *P*>0.05). Similar results, when AuNPs were illuminated by green laser, were obtained in pulmonary artery rings: E_{max} = 89 ± 5 % (*n*=8, *P*<0.05), pD_2 = 4.2 ± 0.1 (*n*=8, *P*>0.05), respectively.

It is important to note that GLI intensity was such that it did not produce any significant change of the temperature of Krebs solution in the organ bath.

Effects of AuNPs and AuNPs/GLI on integral BK_{Ca} currents in vascular and visceral smooth muscles

We have next examined the possibility that AuNPs- and AuNPs/GIL-induced vasorelaxation could be accounted for by an increase in outward potassium currents leading to membrane hyperpolarisation. Figure (3A) shows current-voltage (I-V) relation curves measured in isoalted pulmonary artery SM cells in control and in the presence of AuNPs. At maximum depolarization of +70 mV the net outward K^+ (I_K) current density was 49.7 ± 4.9 pA/pF (n=10). Application of AuNPs (10^{-4} M) produced a significant enhancemnet of I_K density (to 130.2 ± 5.7 pA/pF). GLI in the presence of AuNPs further increased I_K density to 205.3 ± 7.6 pA/pF (n=10, *P* < 0.05)

For voltage-gated ion channels, such as BK_{Ca} channels studied here, their voltage dependence is typically described by Boltzmann relation. Plots of conductance versus membrane potential (the so called activation curves) account for variations

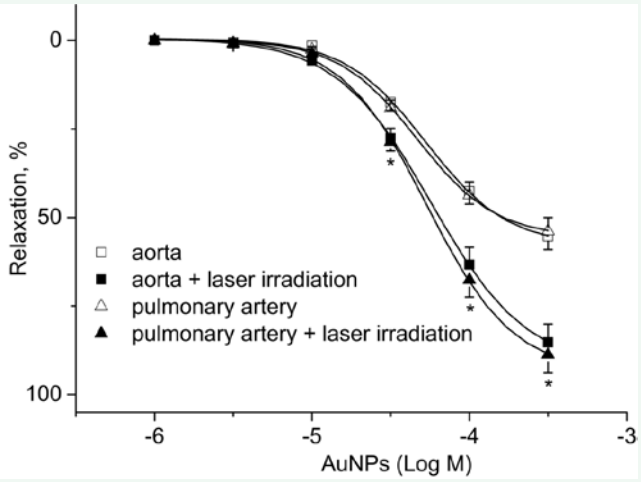

Figure 2 Concentration-relaxation curves for AuNPs ≈ 5nm core size and for AuNPs combined with 5 W/532 nm, green laser irradiation measured in intact rat thoracic aorta and pulmonary artery rings pre-constricted with 10^{-6} M norepinephrine.

in the electrochemical driving force for permeate ions at different test potentials, this way revealing the fundamental variables-maximal conductance (Gmax), the potential of half-maximal activation ($V_{1/2}$) and the Boltzmann slope factor (k). The latter two parameters characterize channel voltage dependence, while Gmax relates to the number of available for activation ion channels, their single-channel conductance and their maximal open probability. Thus, activation curve reflects the nature of ion channel gating, namely, the movement of charges in the voltage sensitive part of the channel protein in the membrane voltage field. Moreover, maxi-K channels are known to be both Ca^{2+} and voltage-sensitive, and channel activity is controlled by both voltage and intracellular free Ca^{2+} concentration in a mutually dependent manner.

Our previous study [2] found that in rat aortic myocytes AuNPs or AuNPs/GLI mainly activated BK_{Ca} channels and that the potentiating effect was not due to a common mechanism involving a negative shift of the activation curve (i.e. altered voltage sensitivity produced by the rise in free intracellular Ca^{2+} concentration). To the contrary, the activation curve of BK_{Ca} conductance shifted somewhat positively ($V_{1/2}$ was 8.5±0.5, 14.0 ± 0.8 mV, and 16.0 ± 0.8 mV in control, in the presence of AuNPs and with AuNPs/GLI, respectively; n=10). We then proposed that whole-cell conductance increase was most likely related to the changes of channel conductance (e.g. increase of unitary current amplitude) or single channel open probability (Po).

In rat pulmonary artery myocytes, we have now examined the possible role of calcium in more detail by comparing the effects of AuNPs and AuNPs/GLI in cells, whereby $[Ca^{2+}]_i$ was either allowed to vary (weak $[Ca^{2+}]_i$ buffering using 0.3 mM EGTA) (Figure 3) or it was "clamped" at 100 nM using 10 mM BAPTA/4.6 mM Ca^{2+} mixture (Figure 4). As shown in Figure (3A), with weak Ca^{2+} buffering, Maxi-K current density at 70 mV was increased by AuNPs applied at 10^{-4} M from 50 ± 5 pA/pF to 130 ± 6 pA (P<0.001; n=6), and further increased to 205 ± 8 pA (n=6; P<0.001) by green laser illumination. The potentiating effect of

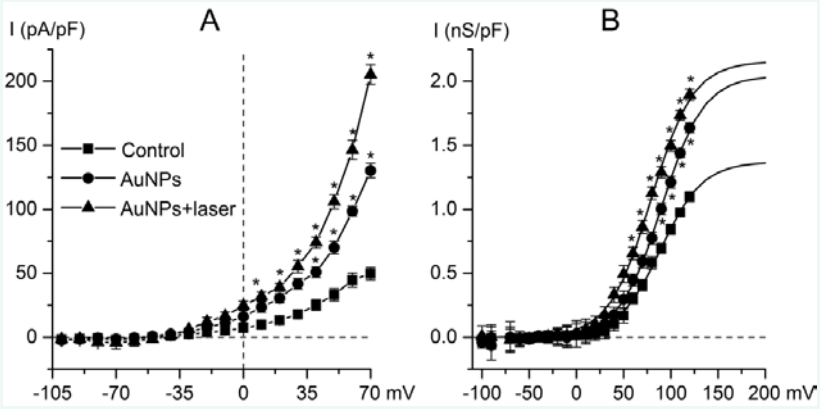

Figure 3 Effects of AuNPs alone and in combination with green laser irradiation on whole-cell outward potassium currents in isolated rat pulmonary artery smooth muscle cells under conditions of weak intracellular Ca2+ buffering (0.3 mM EGTA in the pipette solution).

A- mean current-voltage 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 with mean parameters described in the text. Data shown are mean ± SEM (n=10).

*- P<0.05 vs control.

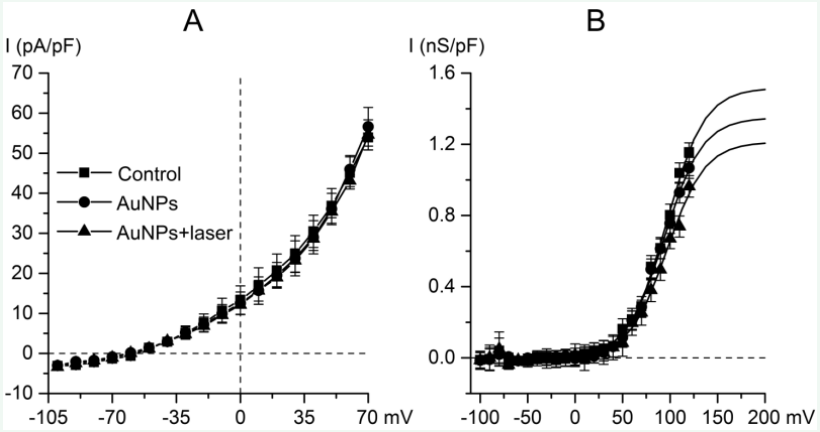

Figure 4 Effects of AuNPs alone and in combination with green laser irradiation on whole-cell outward potassium currents in isolated rat pulmonary artery smooth muscle cells under conditions of strong intracellular Ca^{2+} buffering (10 mM BAPTA/4.6 mM Ca^{2+} mixture was used in the pipette solution 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 with mean parameters described in the text. Data shown are mean ± SEM (n= 6).

AuNPs alone was due to an increase in the maximal conductance (Gmax) by about 50% without any shift of the activation curve of K^+ conductance (the potential of half-maximal activation $V_{1/2}$ was 89.6 ± 2.4 mV and 91.0 ± 2.0 mV in control and in the presence of AuNPs, respectively, n=6). Interestingly, green laser illumination (5 mW, 532 nm) had no significant additional effect on the maximal K^+ conductance (2.09 ± 0.08 and 2.18 ± 0.06 nS/pF before and after laser illumination, n=6), but instead shifted the $V_{1/2}$ value negatively to 79.6 ± 1.5 mV (n=6; P<0.05). In contrast, under conditions of strong intracellular Ca^{2+} buffering, no effect of AuNPs on current density was observed (54 ± 4 pA/pF in control *vs* 55 ± 4 in the presence of 10^{-4} M AuNPs; p>0.05, n=6) (Figure 3A). Gmax and the $V_{1/2}$ values also remained largely unchanged (Figure 4B).

Analysis of AuNPs and AuNPs/GLI effects at the single-channel level

To investigate the phenomenon further we have performed experiments using single channel recordings [1,2] using on-cell patches formed on rat pulmonary artery and mouse ileal myocytes (Figure 5 and 6, respectively). Application of AuNPs to the external solution potentiated Maxi-K activity with a

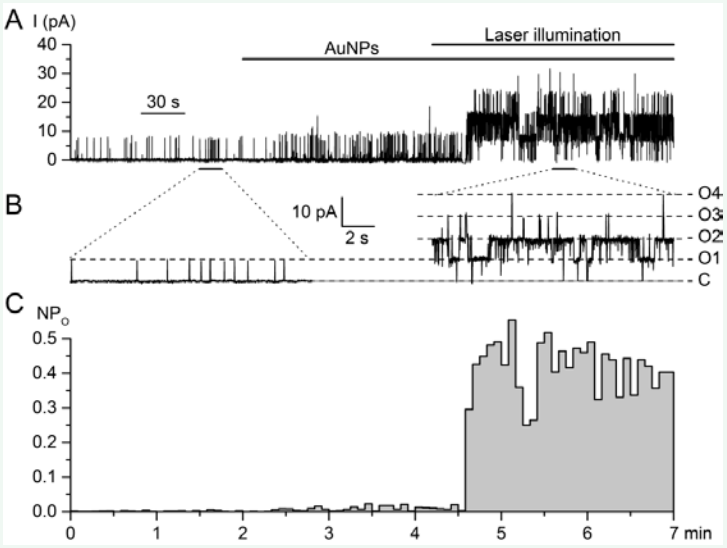

Figure 5 AuNPs activate single BK_{Ca} channels in rat pulmonary artery myocytes.

A- representative single-channel recording of BK_{Ca} channel activity in a cell-attached membrane patch held at 60 mV (n=3). The periods AuNPs application (10^{-4} M) followed by 5 mW/532 nm laser irradiation are indicated by the horizontal bars.

B- expended segments of the trace shown in A with the dashed horizontal lines indicating closed (C) and open (O) levels. Simultaneous openings of up to 4 channels (denoted O1-O4) of identical conductance were observed when BK_{Ca} channels were strongly activated by AuNPs irradiated by green laser.

C- time course of BK_{Ca} channel activity expressed as NP_0 measured in consecutive segments of 5 s duration. Time scale is the same as in panel A.

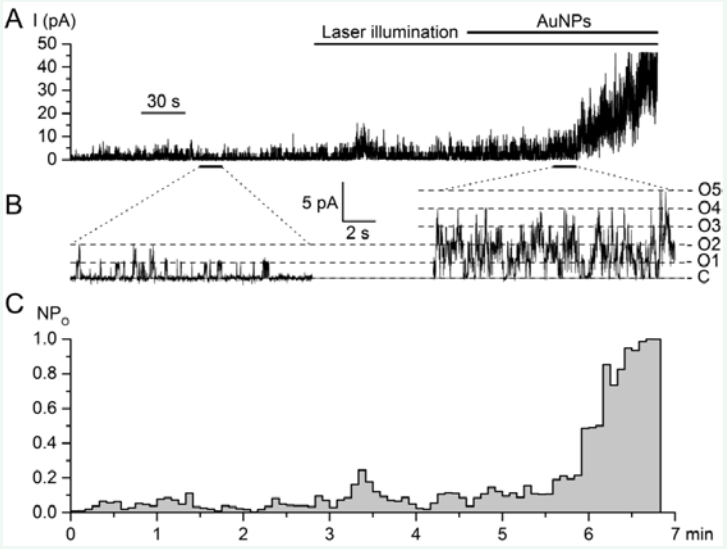

Figure 6 AuNPs activate single BK_{Ca} channels in mouse ileal myocytes.

A- representative single-channel recording of BK_{Ca} channel activity in a cell-attached membrane patch held at 0 mV (n=4). The periods of 5 mW/532 nm laser irradiation followed by application of AuNPs (10^{-4} M) are indicated by the horizontal bars.

B- expended segments of the trace shown in A with the dashed horizontal lines indicating closed (C) and open (O) levels. Simultaneous openings of up to 5 channels (denoted O1-O5) of identical conductance were observed when BK_{Ca} channels were strongly activated by AuNPs irradiated by green laser.

C- time course of BK_{Ca} channel activity expressed as NPo measured in consecutive segments of 5 s duration. Time scale is the same as in panel A.

delay of 1-2 min, as was evidenced initially by more frequent channel openings followed by the progressive appearance of additional open levels corresponding to multiple openings of channels that had identical single-channel conductance (Figure 5A,6A). Following green laser irradiation there was a significant increase in channel activity with multiple active channels evident (Figure 5A,B). Alternatively, green laser irradiation alone had no significant effect on channel activity unless AuNPs were

applied to the bath (Figure 6). Channel activity was quantified as NPo (where N is the maximal number of active channels and Po – channel open probability) calculated in consecutive time intervals of 5 s duration each and plotted in the bottom panels on the same time scale as current recordings (Figures 5C,6C).

These experiments have revealed that the potentiating effects of AuNPs were due to a significant increase in channel open probability (NPo), while unitary current amplitude at the same test potential remained constant. It is conceivable that AuNPs and the energy of their plasmonic resonance do not directly interfere with the ion conductance pathway, or channel pore, rather they act as gating modifiers that affect kinetics of transitions between closed and open states of the channel and, ultimately, channel open probability. Such mechanism of regulation is common for most pharmacological modulators of ion channels. However, in sharp contrast to other drugs, which typically access and similarly modulate the activity of all channels present, we have occasionally observed an unusual channel behavior during AuNPs/GLI, as illustrated in Figure (6). This phenomenon could be described as deviation from the principle of independent channel gating. Briefly, when multiple channels (N - their total number) are present in a membrane patch all having similar open probability P_0, the probability of seeing all channels closed or 1, 2, 3…X channels simultaneously open (P_X) is given by the binomial distribution:

$$P_X=\{N!/X!(N-X)!\}\ P_0^X\ (1-P_0)^{N-X}$$

In control, all channels gated independently as confirmed by closely similar measured and calculated according to the binomial distribution P_X values (Figure 7B, left panel). With AuNPs/GLI probability of seeing one channel open was unusually high (Figure 7A, right panel) as if only one out of four channels was activated. Indeed, the experimentally observed probabilities of seeing all channels closed or 1, 2, 3 or 4 channels simultaneously open did not confirm to the principle of independent channel gating (Figure 7B, right panel). The deviation was especially clear for $P_{X=0,}$ $P_{X=1}$ and $P_{X=2}$.

Taking the above described results together, we conclude that AuNPs activate Maxi-K channels via both Gmax increase and a negative $V_{1/2}$ shift. These processes are clearly calcium-dependent, as the potentiating effect of AuNPs could be completely abolished by "clamping" the intracellular Ca^{2+} concentration at 100 nM. Recordings of the activity of single Maxi-K channels expressed in rat pulmonary SM cells (Figure 5) and mouse ileal myocytes (Figure 6) clearly demonstrated that AuNPs act as BK_{Ca} "openers" both in vascular and visceral smooth muscles.

From the practical point of view it is important to emphasize that potassium channels contribute to the membrane potential regulation in all electrically excitable cells, including vascular SM. SM relaxation is known to be due to membrane hyperpolarization, which, in turn, is the result of K^+ efflux through K^+ channels. The final step of this series of events leading to vasorelaxation is the closure of voltage-dependent Ca^{2+} channels, which reduces Ca^{2+} entry.

The term "surface plasmon resonance" represents itself a collective charge density electron oscillation at the metal interface, propagating in a waveguide-like fashion along the surface [14]. AuNPs in core size of 5 nm having absorption maximum in the range of 510-570 nm may be excited even by natural tissue chemiluminescence initiated due to tissue oxidative reactions and normal reactive oxygen species production. Experimentally, this excitation can be significantly increased by 532 nm green laser illumination. It should be noted that AuNPs of ~5 nm core size have plasmon resonance of 532 nm.

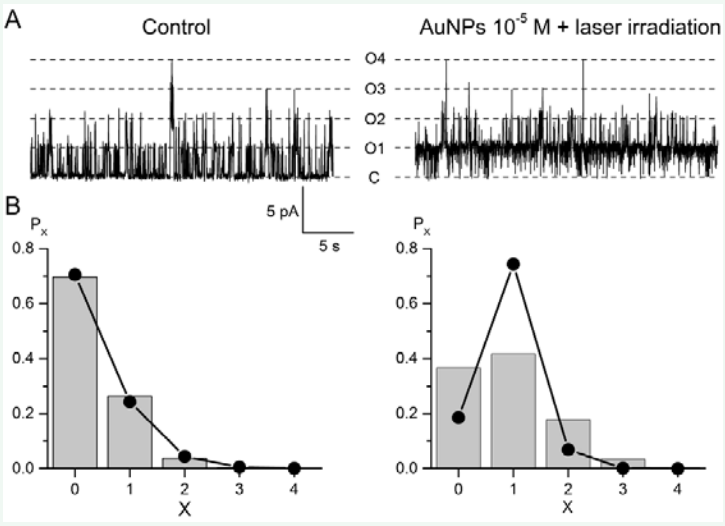

Figure 7 AuNPs-induced BK_{Ca} channel activation is associated with deviation from the principle of independent ion channel gating.

A- simultaneous openings of up to 4 BK_{Ca} channels were observed in a membrane patch both in control and with AuNPs/GLI. These current levels are denoted O1-O4, corresponding to probabilities $P_{X=1}$, $P_{X=2}$, $P_{X=3}$ and $P_{X=4}$ in panel B, while C denotes closed level, e.g. all channels are closed ($P_{X=0}$).

B- experimentally found P_X values (closed circles) and those predicted by binomial distribution (columns) assuming N=4, P_0=0.086 in control and N=4, P_0=0.222 in the presence of AuNPs/GLI. The P_0 values were obtained from the measured NP_0 values, 0.345 and 0.887 in control and with AuNPs/GLI, respectively.

It is very interesting to note that the origin of some novel properties of nano structured materials is different for metal nanoparticles compared to the semiconductor nanoparticles, i.e. a simple change in semiconductor size alters the optical properties of the nanoparticles - when metal (gold) nanoparticles are enlarged their optical properties change only slightly.

One plausible explanation for AuNPs interaction with potassium channels may be related to plasmon resonance phenomenon, i.e. excitation of surface plasmons on the AuNPs surface that may be increased due to GLI. It is known that AuNPs, as noble nanometer-sized structures, have their own inherent distinctive feature that collective electron charge oscillations associated with the surface plasmon resonance give rise to induced local electrical fields near the nanoparticle surface. In case of laser illumination, this local electrical field can be significantly enhanced due to local plasmon resonance that, in turn, interacts with the ion channel voltage sensor and this way increases outward currents leading to SM relaxation. In the absence of external irradiation, such plasmon may likely be excited by natural tissue chemoluminescence [15] with wavelength of 200-750 nm in the presence of nonlinear effects on AuNPs surface.

It is likely that the strength of the electrical field at the cell membrane depends on the concentration of AuNPs, and K^+ channel activation can be evoked when threshold AuNPs concentration takes place. It has been recently established that the plasmon excitations in metallic nanoparticleds can be propagated within the distance of the order of 1 μM [16].

It is known that the current density amplitude (or membrane conductance) depends on, at least, three main parameters: the number of channels available in the plasma membrane, single channel conductance and the open channel probability that, in turn, is a function of time and membrane potential. It has been firmly established that the main outward current in aortic smooth muscle under AuNPs treatment was carried through Maxi-K channels [2,6,7]. Conductance-voltage relationship studies demonstrated that the potential-dependent open probability of potassium channels appeared to be increased under AuNPs action. Nevertheless, one question remains considering the manner in which AuNPs produce this effect. One possibility is that AuNPs as well AuNPs combined with 5W/532 nm green laser irradiation could act primarily by increasing single channel conductance. From the analysis of whole-cell currents it is generally impossible to distinguish between changes in Po and single-channel conductance. That is why we have decided to investigate single-channel currents in order to address the exact mechanism of AuNPs action on Maxi-K channels.

Our single channel recordings using SM cells from different tissues provided a direct confirmation of Maxi-K activation by AuNPs at the single-channel level. GLI acted as an additional potentiating factor in the presence of AuNPs, but was inefficient by itself (compare Figures 5 and 6). Regarding the possible mechanism of AuNPs action, several observations in these experiments seem to be very pertinent.

First, the effect of AuNPs was observed in the cell-attached configuration, whereby the exterior of the membrane is not directly accessible to any molecules in the bath solution (Figure 1). For example, it is known that even small neurotransmitter molecules cannot act on ligand-gated ion channels, when they are isolated from the bath by the tight gigaseal. This indicates that either AuNPs can cross the membrane and act on the intracellular part of the channel, or that they can somehow alter membrane properties in such a way, that this signal (most likely surface plasmon resonance energy) can be transduced within the membrane plane by at least 1 mkm distance. The slow onset of AuNPs action seems to favor the former hypothesis, while the additional activation induced by laser illumination may work either way. Moreover, it is possible that AuNPs affect BK_{Ca} channels not directly, but instead they somehow alter channel interaction with the membrane lipids. Such scenario could also easily account for the distant regulation of the channel protein contained in the cell-attached membrane patch that is without any drug access to its extracellular domains.

Second, single-channel current amplitude during AuNPs application remained constant indicating that these nanoparticles do not interfere with the ion conductance pathway, or channel pore. Instead activation of the whole-cell currents at the single-channel level can be explained by the significant NP_0 increase as the main determinant.

Maxi-K channels are both Ca^{2+} and voltage-sensitive, hence we compared the effects of AuNPs on Maxi-K channel activity under weak (0.3 mM EGTA) or strong Ca^{2+} buffering (10 mM BAPTA/4.6 mM Ca^{2+}) to "clamp" the intracellular Ca^{2+} concentration at 100 nM. With weak Ca^{2+} buffering, Maxi-K current density was significantly increased by AuNCs and further increased by GLI while under conditions of strong intracellular Ca^{2+} buffering no effect of AuNCs on current density was observed. It became clear that these processes are calcium-dependent, as the potentiating effect of AuNCs could be completely abolished by "clamping" the intracellular Ca^{2+} concentration at 100 nM.

Third, although we could not analyse open and closed times, as multiple channels were present in all patches, at early times NP_0 increase by AuNPs seemed to be due to more frequent channel openings, rather than increase in channel open time. Understanding the exact mechanism of channel activation requires additional experiments, but it seems that AuNPs may modulate BK_{Ca} channels one by one and with variable time delays (Figure 7), which is different from the action of most other known channel chemical modulators. Such effect is clearly evident when one channel becomes already very active, yet double and triple openings still remain rare, clearly showing that other channels in this patch have not been yet potentiated at this point in time (Figure 7A). Indeed, measuring open probability for levels 1, 2, 3 and 4 (e.g., 1, 2, 3 or 4 simultaneously open channels) and comparing these values to those expected from the binomial distribution, which describes the discrete probability distribution for N independent events, confirmed that channels gated independently before, but not after AuNPs application (Figure 7B). With time, as more and more channels were activated by AuNPs, a significant increase in NP_0 occurred in all experiments. Within the technical limitations we encountered in single-channel recordings, the effect of AuNPs appears to be concentration-dependent.

For this effect to be of any practical usability, the real question concerns the delivery of light irradiation to a biological target. The depth of light penetration usually does not exceed several hundred micrometers. It is enough for isolated single cells and smooth muscle preparations *in vitro*, but is it plausible for some internal organs, for instance, heart or blood vessels? One approach consists in using pulse nanosecond irradiation in contrast to continued one, which enables irradiation energy to be enhanced while avoiding its side effect. Another possible approach proposes fiber-optic devices for endoscopic or intra-tissue delivery of radiation [14]. There is also an intriguing possibility that natural tissue chemiluminescence could be used to mediate AuNPs-induced activation of BK_{Ca} channels. This is a very attractive hypothesis to be tested since enhanced reactive oxygen species production associated with diseases such as diabetes mellitus could enhance tissue chemiluminescence thus facilitating BK_{Ca} activation and self-correctong blood flow (by relaxing blood vessels) in the affected tissues. In this connection it is important to note that Dykman and Klebtsov [14] postulated that AuNPs are being used with diagnostic aims and for cell phototermolysis mainly but rather seldomly used directly for therapeutic purposes. Namely, they were used for the treatment of rheumatoid arthritis due to AuNPs anti-angiogenic activity and as a vector for the delivery of tumor necrosis factor to solid tumors in rats but never used as a therapeutic agent for cardiovascular system treatment.

CONCLUSION

The relaxant effect of AuNPs of core size of 5 nm and its ability to open Maxi-K channels in a remote control manner in smooth muscle cells is now evident from series of our studies. To the best of our knowledge this is the first evidence that AuNPs possess the ability to control ion channel gaiting at a distance and this way it is likely to possess the ability to restore normal vascular contractility in a number of disease states related to the development of vascular SM hypercontractility.

This work confirms that this effect is not restricted to BK_{Ca} channels in rat thoracic aorta SM cells only and could be realized in other types of SM. We can now postulate the opening of Maxi-K channels under AuNPs treatment as a protype mode of regulation of voltage-gated ion channels and other type of voltage-gated ion channels could reproduce this effect. We suppose that AuNPs-induced vasorelaxation is due to BK_{Ca} channel activation which, in turn, is realized via interaction of its voltage sensor domain and surface plasmons on the AuNPs surface that may be increased due to green laser irradiation.

The new finding of this study is that AuNPs-induced ion channel activation appears to be very sensitive to Ca^{2+} homeostasis suggesting intimate calcium involvement in this process.

AuNPs may be considered as the potential K^+ opener because of their chemical stability and biocompatibility combined with their unique optical properties. Nevertheless, therapeutic usage of AuNPs as an effective potassium channel opener will become possible when the manner in which it functions as well as AuNPs toxicity are additionally and more precisely investigated. At this stage, it is clear that AuNPs-based control of voltage-dependent ion channels remains new, evolving technology for novel therapeutics. It remains an open question whether its activity can be realized in *in vivo* conditions or it is just one more tool of a narrow experimental utility. In any case, it may have a profound impact in non-invasive control of excitable tissues such as vascular and neural activity.

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