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PERSPECTIVES

Acoustic Waves in Axonal Membrane and Caveolins are the New Targets for Pain Treatment with High Frequency Ultrasound

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Abstract: Reciprocal interaction between electrical and mechanical waves observed in axonal membrane during its excitation leads to a paradigm shift in pain research making the uncoupling of electro-mechanical signals an interesting target in pain treatment. This uncoupling can be realized either through direct disturbance of the mechanical surface waves in axonal membrane or through shifting of the thermodynamic state of this membrane far from its phase transition point. Both effects can be effectively realized through application of the very high frequency ultrasound waves. Additional target for application of ultrasound in pain treatment is the caveolin-1, which is abundantly present in Schwann cells as well as in the non-axonal tissues. Both targets demonstrate frequency-dependent reactions, thus making a very high frequency ultrasound a promising treatment modality in pain treatment.

Keywords: chronic pain, axonal membrane, mechanical surface wave, caveolin, ultrasound

Introduction

For a long time, there was a broad consensus that the origin of peripheral pain is mainly connected to generation of action potentials (APs) and their propagation along the axonal membrane. APs were believed to have a pure electrical origin well described by a classical Hodgkin-Huxley model. According to this model, depolarization of the axonal membrane beyond a threshold potential leads to its local excitation and generation of spikes demonstrating some typical features such as all-or-none behavior, threshold stimulation and solitary character. Since AP generation was mainly connected to the functioning of the ion channels in axonal membrane, the primary attention in anesthesia was paid to the treatment methods influencing operation of proteins in these structures. This model remained predominant for a long time even despite the obvious contradiction with another cornerstone of anesthesiology – the Meyer-Overton correlation – stating that the effectiveness of applied anesthetics is positively connected to their lipophilicity. This correlation was observed over a very broad interval of solubilities and indicated that not the ion channels but the lipid components of the neuronal membrane should be the main site for anesthetic action.

However, some time ago it was revealed that axons during their excitation generate not merely electrical but also mechanical waves, which can propagate along the axonal membrane with a remarkably low attenuation. Electrical and mechanical waves interact with each other producing a reciprocal electro-mechanical excitation in axonal

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membrane. This finding caused a paradigm shift in neurostimulation and pain treatment – from a straightforward inhibition of electrical signals in axons through application of external electrical stimulation to more comprehensive treatment methods connected with "uncoupling" of electro-mechanical signals in axonal membrane. One of these methods is the application of ultrasound (US) waves, which received much attention during the recent past because of intensive research in the field of ultrasonic neuromodulation.[1]

While US was considered as a standard method for peripheral pain treatment, it is well-known that its applications demonstrate mixed outcomes even in the same pain conditions. Here we re-analyze the possible reasons for this diversity considering the new axonal targets for US revealed during recent years.

Generation and Propagation of Acoustic Waves in Axonal Membrane

Early investigations demonstrated that AP transmission modulates the viscosity of axonal membrane and generates an additional pressure of up to 10 mPa in axoplasm.[2,3] Much later, it was reported that the development of AP is accompanied by a modulation of the axonal diameter, which temporal course is very similar to that of AP.[4,5] These observations indicated that excitation of axonal membrane does not have a pure electric origin and should involve some mechanical and conformational changes. Such assumption led to the development of different electro-mechanical models describing mechanical surface waves induced in axonal membrane during AP propagation as well as an induction of APs by application of mechanical forces to this membrane.[4,6–8] These surface waves have a much lower attenuation than the normal bulk waves, which allows them to propagate far away from their place of origin.

It was also demonstrated that the non-linear surface acoustic waves can propagate in artificial lipid membranes if the thermodynamic state of these membranes is close to a corresponding phase transition point (melting from gel to liquid phase).[9] Under physiological conditions, the state of axonal membrane is indeed quite close to this point and thus surface acoustic waves can be easily stimulated by mechanical, thermic and chemical factors, which makes the initiation and propagation of the surface acoustic waves in these structures to a fundamental phenomenon. Among others, it was reported that such waves can be induced by electrical stimulation[7] and by a local threshold acidification of the interface.[10]

This means that mechanical surface waves can be induced both in the native and artificial membranes and have the universal mechanism of generation. Discovery of these waves had significant impact on the theory and practical applications in the field of central and peripheral neurostimulation. At the same time, it also allowed another insight into the axonal-US interaction between axons and US in pain treatment.

Radiation Force as the Main Factor in Direct US Modulation of AP

While the early research concerning the application of the focused US of different frequencies to the peripheral nerves revealed that increasing frequency causes an enhanced modulation of AP generation in axonal membrane, this phenomenon was then generally connected to the thermic effect of US and explained by a stronger absorption of the high frequency waves in the tissue. Recently, it was shown by different authors that US can modulate AP generation in neurons both in vitro and in vivo within a broad range of much lower intensities (0.1–10.0 W/cm^2).[11] This modulation occurred without any significant increase of the temperature, thus clearly indicating the primary role of mechanical effects in this phenomenon. Mechanical effects of US can be connected either with development of cavitation in the tissue or with a constitution of radiation pressure on the membranes. Investigation of the ganglion cell spiking activity by application of US with frequencies of 0.5–43.0 MHz revealed that the strength of axonal modulation increases with US frequency.[12] Also relevant to this discussion, the voltage-clamp experiments with lipid bilayers demonstrated that US with frequencies of 1 MHz and 43 MHz induce similar on/off currents.[11] Since the probability of cavitation quickly decreases with increasing US frequency, the radiation forces induced by US in axonal membrane must be the main factor responsible for this modulation.

Acoustic radiation force on the cell surface depends on different physical parameters and significantly varies for the targets of different geometries, e.g. spherical cells and cylindrical axons. This force also depends on the relationship between the wavenumber of the US wave (k) and the axonal radius (R), where $k = 2\pi f/c$, whereby f and c are the US frequency and velocity, respectively. Taking the typical

axon radius of 1 μm and the US velocity in the soft tissue of 1.500 m/s, condition $kR << 1$ will fulfill for $f << 240$ MHz and thus for all typical US frequencies applied for neuronal stimulation. In case of an US wave directed along the axis of a cylindrical axon, radiation force induced in axonal membrane will in this approximation be linearly proportional to the US frequency, f.[13]

The fluorescent investigations of the lipid membranes subjected to US pulses revealed sufficient conformational changes in these structures, which were especially pronounced as the membrane was thermodynamically in the vicinity of its phase transition point.[14] It is well-known that near this point the heat capacity and compressibility of the lipid phase reach their maximum values. Since the US-induced radiation force is proportional to the adiabatic compressibility of the target,[13] mechanical pressure induced by US in axonal membrane will also reach its maximum if the system is near its phase transition.

Altogether, US waves can induce mechanical perturbation in axonal membrane generating a propagating mechanical surface wave, which in its turn can cause generation of AP. This mechanism is actually considered an important pathway in neurostimulation.[1] On the other hand, mechanical stress induced in axonal membrane by acoustic radiation force can shift its thermodynamic state far away from a phase transition point, which will also suppress the AP generation. This should especially be the case if the US wave can produce a sufficient thermo-mechanical stress near axonal membrane. As we discussed previously, such stress quickly increases with increasing US frequency.[15]

Whereas a direct modulation of AP in axons is an important direct mechanism of US-axon interaction, another target connected with expression of caveolins in cell membranes should also be of significant importance at least in some types of pain.

Caveolin-1 as a Target for an Indirect Modulation of Pain

Caveolin-1 (Cav-1) is a principal structural component of the caveolae plasma membrane invaginations enriched in cholesterol and sphingolipids, which are present in different types of cells. Cav-1 is involved in rapid adaptation to cellular volume modifications, in multiple signal transduction processes as well as in the processes of endo- and exocytosis. Cav-1 demonstrates reciprocal interaction with oxidative stress: whereas induction of Cav-1 attenuates the oxidative stress induced by H_2O_2,[16] application of oxidative stress modulates Cav-1 expression in a dose-dependent and cell-type specific way.[17–19] This effect is directly connected to pain sensation: as it was demonstrated in mouse models, antioxidants attenuate constriction of the sciatic nerve indicating that oxidative stress should be directly involved in development of neuropathic pain.[20]

Caveolins were long believed to not be directly involved in neuronal signaling. The main reason for this was that these proteins were found only in glial cells and synapses, but not in axonal membranes.[21] However, the rapid propagation of action potentials along axonal fibers of motor and sensory neurons is enabled only through their myelination provided by Schwann cells wrapping the axons. Importantly, Cav-1 is highly abundant in the abaxonal myelin membranes of the Schwann cells and loss of Cav-1 compromises the functioning of these cells as well as decreases nerve regeneration after injury. On the other hand, Cav-1 expression significantly increases during myelination and strongly decreases after axotomy.[22]

Schwann cells react to oxidative stress induced by H_2O_2 with apoptotic cell death. This effect can be strongly attenuated by extracellular induction of the heat shock protein 72 (Hsp72).[23] Such Hsp72-mediated reduction of oxidative stress-induced injuries in Schwann cells has neuroprotective effect on peripheral nerves increasing their stress tolerance.[24] Remarkably, this protein demonstrates positive and almost linear correlation with Cav-1 level in human and mouse skin, indicating that induction of Hsp72 should lead to enhanced expression of Cav-1.[25]

Additionally, nociceptive pain is connected to a local inflammation in affected tissue and it is widely accepted that this pathway involves prostaglandin E_2 (PGE_2). PGE_2 is a cyclooxygenase (COX) product and a lipid mediator, and inhibitors of COX-1/COX-2 were reported to effectively suppress the inflammatory pain through reduction of PGE_2 expression.[26] Remarkably, COX-2 co-localizes and physically interacts with Cav-1.[27] Since the Cav-1-mediated suppression of COX-2 reduces PGE_2 expression,[28] Cav-1 can be considered as a potential target in treatment of inflammation-induced nociceptive pain.

Moreover, different transient receptor potential ion channels (TRPs) are known to be involved in pain sensation, among others, TRP ankyrin 1 (TRPA1). This TRP is expressed in nociceptive sensory neurons and epithelial cells, where it plays a key role in detection of chemical, thermal and mechanical noxious stimuli.[29–31] TRPA1 is

a common pathway for pro-nociceptive agonists generated in various pain conditions and thus a peripheral application of TRPA1 antagonists must be an effective approach for attenuation of primary hyperalgesia.[32] In mice, TRPA1 channel is regulated by the local lipid environment and it was recently suggested that targeting lipid-TRPA1 interactions may be a strategy for the treatment of pain and neurogenic inflammation.[31] Indeed, the amplitudes of the responses to such stimuli as cold and lipopolysaccharide (a toxic byproduct of bacterial lysis), were significantly lower in cells treated with the cholesterol scavenger methyl β-cyclodextrin deteriorating the lipid-TRPA1 interaction and reducing Cav-1 content in plasma membrane in a dose-dependent manner.[31] TRPA1 is expressed not only in nociceptors but also in Schwann cells. Whereas silencing of TRPA1 in nociceptors attenuated mechanical allodynia but did not affect the macrophage infiltration and oxidative stress, which led to development of pain sensation in injured area, silencing of these channels in Schwann cells reduced both allodynia and neuroinflammation.[33] Hence, it can be strongly supposed that modification of Cav-1 expression in Schwann cells can modulate the pain sensation.

Further, mechanical trauma of the soft tissue causing appearance of edema in the injured area is often accompanied by pain and inflammation. Pathophysiologically, edema is connected to excessive accumulation of the fluid in the tissue caused by capillary leakage. The fluid retention can take place intracellularly or extracellularly and leads to a local constriction of the nerves providing pain sensation followed by degeneration of myelinated axons. Cav-1 is strongly expressed in endothelial cells and regulates fluid exchange between circulation and peripheral tissues. Indeed, Cav-1$^{-/-}$ mice demonstrate pronounced abnormalities in vasoconstriction and vasorelaxation responses[34] as well as a microvascular hyperpermeability, which indicates that a microvascular leakage is mediated by Cav-1.[35] This effect was observed not only in an injured soft tissue but also in pulmonary and brain edema.[36,37] Overexpression of Cav-1 was reported to attenuate edema preventing degradation of intercellular junctions;[37] hence, Cav-1 can serve as a target in early stages of edema development.

Cav-1 is also involved in development of diabetic peripheral neuropathy. Diabetic Cav-1 KO mice exhibit much more severe decrease of motor nerve conduction velocity as well as of mechanical and thermal sensitivity than their non-diabetic counterparts.[38] It is well-known that myelinated Schwann cells undergo degeneration in diabetic neuropathy. Suppression of Cav-1 enhanced neuregulin-induced neuronal demyelination and this effect was not connected to neuronal loss since the axons remained intact.[39] Since induced expression of endogenous Cav-1 provides recovery of myelination, a proper Cav-1 modulation can be used to improve the neuropathic pain.

Osteoarthritis is generally connected to development of inflammation and chronic pain. Peroxisome proliferator-activated receptor gamma (PPARγ) demonstrates protective effect in osteoarthritis in vivo;[40] on the other hand, PPARγ and its agonists are the known positive regulators of Cav-1 in different types of cells.[41,42] Such interaction corresponds to a generally positive effect of Cav-1 induction in inflammatory conditions[43] and indicates that Cav-1 modulation can be a target in osteoarthritic pain.

Last but not least, muscle injury is a well-known source for acute and chronic pain. The process of muscle regeneration is substantially connected to modulation of Cav-1 expression in undifferentiated myogenic precursor (satellite) cells.[44] These cells quickly proliferate and fuse with damaged fibres after muscle injury. Under normal conditions, satellites cells are quiescent: they overexpress Cav-1 producing cellular senescence and cycle arrest, whereas suppression of Cav-1 induces their cycling. To initiate muscle regeneration, satellite cells must migrate to the wounded area shortly after injury. Simultaneously with this migration, Cav-1 level must be transiently downregulated. Recover of Cav-1 expression normally takes place three days after injury, which is a characteristic time for differentiation of precursors into myotubes. However, in a case of a high basal level of Cav-1 expression (e.g. in transgenic mice with overexpression of Cav-1) myogenic precursors are not able to reduce Cav-1 to a sufficiently low level allowing their migration, which can lead to impaired muscle regeneration.[44] From here, Cav-1 must also be an interesting target in muscle regeneration, and suppression of endogenous Cav-1 during the early stages of an acute muscle injury or in the case of delayed muscle recovery should provide improved healing.

US as a Local Modulator of Cav-1 Level in the Tissue

Caveolae are linked to the actin cytoskeleton and reorganization of this intracellular network can strongly modify their surface density.[45] Dependent on the amplitude of mechanical deformation, both stiffening and softening

(fluidization) of the cytoskeleton can occur.[46,47] Whereas mechanical forces at a frequency of 1 Hz fluidize the cytoskeleton and modify the microdomain structure of the plasma membrane at strains of about 10%, application of mechanical forces at frequency 1 MHz reduces the critical strain to about 10^{-5}.[48] Application of higher US intensity and higher US frequencies induces higher levels of strain in cells and thus can stronger modify the caveolae,[49] indicating that US of higher frequencies (very high frequency US, VHF-US) should be more effective in pain treatment. Of note, as discussed above, US waves demonstrate frequency-dependent modulation of generation and propagation of APs in axonal membrane. Also, US-induced expression of Hsp72, which expression proceeds almost parallel to expression of Cav-1,[25] is strongly frequency-dependent and increases with increasing US frequency.[50]

Analysis of expression profiles of US-induced genes revealed that the *CAV1* gene was indeed strongly upregulated after application of a low intensity US.[51] At the same time, stress-dependent modulation of Cav-1 demonstrates a biphasic behavior: a low-level stimulation generally provides an enhanced Cav-1 expression whereas application of a strong mechanical stress will cause Cav-1 degradation.[52] Additionally, US waves of different frequencies can induce very distinct values of mechanical stress in the cells, which can either properly modulate, not modulate or false modulate the local expression of Cav-1. Recent research also revealed that US can stimulate the mechanosensitive channels, thus directly influencing neurons and other excitable cells, and that the probability of such excitation strongly depends on the protocol of US application.[53] This can be an important reason for the mixed treatment outcomes observed after application of US for pain treatment. US intensity and frequency as well as the initial status of Cav-1 expression should be the main parameters defining direct and indirect interactions of US waves with axons and thus the observed pain release outcome.

Some Applications of VHF-US for Pain Treatment

From above, VHF-US should be effective in treatment of different types of peripheral pain. Applications of VHF-US with frequencies of 10 MHz and higher in different inflammatory and hyperproliferative conditions were discussed in our recent publications.[46,47,54–57] Here we shortly discuss some special clinical applications of VHF-US for pain reduction. All applications were provided by the emitter LDM®-MED (Wellcomet GmbH, Karlsruhe, Germany).

Effect of VHF-US in the form of dual US waves of 3 MHz and 10 MHz mixed in a special LDM® (Local Dynamical Micro-massage) mode on wound healing and pain reduction was investigated in a single-center, prospective pilot study on 10 patients with chronic venous leg ulcers.[58] In this mode, ultrasound waves are applied as short quickly oscillating pulse trains (in this study as 5 ms of 3 MHz and 5 ms of 10 MHz with spatial average temporal average ultrasound intensity (SATA) of 0.5–1.0 W/cm^2). All patients demonstrated complete wound closure and a significant reduction of self-reported pain of at least three points measured on the 5-point numerical rating scale (NRS) just after 3–4 treatments ($p < 0.01$). The effect of VHF-US technology on the pain in patients treated with injection lipolysis (IL) was investigated in a randomized, single-center, contralateral, controlled pilot study on seven female healthy subjects.[59] Pain is a well-known side-effect of IL predominantly connected to a strong inflammatory reaction in the tissue. Patients were treated with LDM® (5 ms of 3 MHz and 5 ms of 10 MHz with SATA of 1.0 W/cm^2) one time before and 10 times after injection lipolysis. Pain was assessed by patients using the 5-point NRS. Combinational IL +LDM® treatment provided not only a significant improvement of the fat tissue reduction compared to a pure IL, but also a significant reduction of pain ($p < 0.01$). A retrospective study on 58 patients who underwent breast reconstruction after mastectomy who complained about post-operative pain, which was not controllable with medication, was presented in Ahn et al.[60] Patients were treated with LDM® (10 MHz, SATA of 1.0 W/cm^2 and 1.5 W/cm^2 for 2 min each, then LDM® 3/10 MHz with SATA of 1.0 W/cm^2 and 2.0 W/cm^2 for 3 min each) every day or every other day. The degree of self-reported pain and discomfort with contracture were assessed by patients using the 10-point NRS. NRS score for pain was reduced from about 6.1 (pre-treatment) to about 3.8 (post treatment) ($p < 0.001$), corresponding NRS values for discomfort were 6.50 and 4.29, respectively ($p < 0.001$). Further, in a remarkable case report, the patient with radiation fibrosis and chronic pain induced by post-mastectomy radiotherapy was treated with LDM® (10 MHz, SATA of 1.0 W/cm^2 and 1.5 W/cm^2 for 2 min each, then LDM® 3/10 MHz with SATA of 1.0 W/cm^2 and 2.0 W/cm^2 for 3

min each).[61] Treatment begun about 3 years after radiotherapy. After the second LDM® treatment, patient reported a significant pain reduction, which decreased from 6 to 2 on the 10-point NRS. These examples demonstrate that VHF-US can indeed be effectively applied for treatment of different types of pain.

Conclusions

Recent results obtained in a rapidly growing field of ultrasound neurostimulation demonstrated the important role of mechanical surface waves in generation and propagation of action potentials. Reciprocal interaction between electrical and mechanical waves observed in axonal membrane during its excitation leads to a paradigm shift in the pain treatment making the "uncoupling" of electro-mechanical signals an interesting target in pain treatment. This uncoupling can be realized either through direct disturbance of the mechanical surface waves in axonal membrane or through shifting of the thermodynamic state of this membrane far from its phase transition point. Whereas the first effect can be of a pure mechanical nature, the second one will demand application of the thermo-mechanical stress. Both effects can be effectively realized through application of the very high frequency ultrasound waves. Additional target for application of ultrasound in pain treatment is the caveolin-1, which is abundantly present in Schwann cells as well as in the non-axonal tissues. Recent research demonstrated that ultrasound can significantly modulate caveolin-1 expression in frequency-dependent manner, thus regulating the processes involved in inflammation, building of edema and injury repair in the affected area. Both targets demonstrate frequency-dependent reaction, thus making a very high frequency ultrasound a promising treatment modality in pain treatment. Further research will be needed to elucidate the peculiarities of ultrasound interaction with these new treatment targets.

Abbreviations

AP, action potentials; Cav, caveolin; COX, cyclooxygenase; Hsp, heat shock protein; IL, injection lipolysis; LDM, local mechanical micro-massage; NRS, numerical rating scale; PGE_2, prostaglandin E_2; PPARγ, peroxisome proliferator-activated receptor gamma; TRPA1, receptor potential ion channel ankyrin 1; VHF-US, very high frequency ultrasound; US, ultrasound.

Disclosure

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