MECHANISMS OF CELL MEMBRANE PERMEABILIZATION WITH ULTRASOUND AND CONTRAST MICROBUBBLES

T.A. Tran[1-2-3], S. Roger[2-3], J.Y. Le Guennec[2-3], F. Tranquart[3-4] and A. Bouakaz[1-3]

1. Inserm U619, 37000 Tours, France
2. Inserm E0211, 37000 Tours, France
3. Université F. Rabelais, Tours, France
4. CIT, CHU Bretonneau, ours, France

Abstract - New clinical applications of ultrasound contrast agents extend beyond imaging and diagnostic towards therapeutic applications. A number of experimental findings have now demonstrated evidence of increased cell membrane permeability through sonoporation process. To explore the mechanisms by which the activation of microbubbles with ultrasound waves breach cell membranes, an electrophysiological experimental method is set up. The method consists of measuring the variations in membrane potential which directly indicates the modulation of ion exchange through the cell membrane and thus its conductance. For that purpose, patch clamp technique was used in the "whole cell" configuration where the membrane potential of a single cell is measured. Mammary cancer cells issued from MDA-MB-231 cell-lines were used. Sonovue microbubbles were continuously infused to the cells at a rate of 1ml/min. Ultrasound was applied using single element transducers of 1 and 2.25MHz, both focused at 14 mm. The microbubbles and cells were simultaneously monitored during ultrasound exposure using a video camera. The results revealed that during sonoporation, a marked hyperpolarization of the cell membrane potential occurs during the ultrasound excitation, indicating the triggering of specific ion channels while the cell and the bubble remain intact. At the highest acoustic exposure (pulses of 40 cycles at 1 MHz repeated every 100 μs during 20 sec); the membrane potential varied from about -30 mV (resting value) to -60 mV and this phenomenon was entirely reversible. This mechanism showed to be dependent on the number of contrast microbubbles in the close vicinity of the cell, but revealed that only cells in direct contact with the bubbles undergo membrane hyperpolarization. Smaller acoustic amplitudes or higher frequency induced only mild hyperpolarization (less than 20mV) while shutting off the ultrasound brings the potential to its resting value. However ultrasound alone does not affect the cell membrane potential.

In conclusion, the results demonstrate that microbubbles' oscillations under ultrasound activation entail modifications of the electrophysiological cell activities, by triggering the modulation of ionic transports through the plasmic cell membrane. However, only cells in a direct contact with the microbubbles are impacted. The involved mechanisms are related to activation of specific channels sensitive to mechanical stresses (stretch-activated channels) and possibly non-specific ion channels.

I. INTRODUCTION

Ultrasound contrast agents (UCA) consisting of encapsulated gas bubbles are nowadays used for imaging since gas bubbles are ideal reflectors of ultrasound waves. They are used for diagnostic purposes to opacify the intravascular space in various clinical applications such as liver, kidney and cardiac imaging [1]. UCA have demonstrated today utility in many applications by improving the accuracy and confidence of diagnosis, and play a decisive role in clinical decision-making. The future clinical applications of microbubbles are expanding beyond imaging and diagnosis. There has been considerable progress in the past few years in the development of drug and gene delivery systems using microbubbles and ultrasound.

Various research groups have shown that microbubbles under optimal ultrasound scanning conditions increase the permeability of cell membrane to external substances (drugs or genes) and enhance by that their uptake in a sonoporation process [2,3]. In addition to being a sonoporation promoter, microbubbles offer the possibility to be loaded with drugs or genes inside the encapsulating shell. Conceptually, ultrasound-mediated destruction of microbubble carrier will provide selective and local release of the therapeutic compound in the

targeted tissue. Despite the various progresses, the veritable mechanisms of interaction between ultrasound-bubbles and cells are still far from being understood. In addition, the effects by which ultrasound and gas bubbles augment cell permeability are not elucidated.

The purpose of our study is to investigate experimentally the effects of ultrasound and microbubbles on the cell membrane properties.

II. MATERIALS AND METHODS

To investigate the cellular response to ultrasound and microbubbles, an electrophysiological approach has been setup to detect and monitor the sonoporation process at the cellular level.

Mammary cancer cells:

Mammary cancer cells issued from MDA-MB-231 cell lines (American Type Culture Collection, Rockville, MD, USA) were used in the experiments. These cell lines were chosen since they represent a reference line for in-vitro studies of breast cancer mechanisms [4]. The cells were cultured in a DMEM culture medium, supplemented with 5% bovine serum, and then incubated at 37° C.

Ultrasound settings

The ultrasound waves were generated using two different single element transducers operating at 1 and 2.25 MHz, both were focused at 14mm. The transducers were excited using an electrical signal issued from a function generator and then amplified using a power amplifier (50 dB). The transmitted waveforms had acoustic pressures ranging from 50 kPa to 300 kPa, lengths of 5 to 40 cycles with exposure times extending from 2 to 20 seconds.

Contrast microbubbles

Sonovue microbulles generously provided by Bracco Research Geneva were used in our experiments. Sonovue was diluted to 1/250 and infused to the culture medium using a pump at a rate of 1ml per minute.

Proliferation and cell viability tests

We used the MTT tests to evaluate the proliferation and cell viability [5] in presence of ultrasound and microbubbles. The MTT test assesses quantitatively the cellular metabolism variations on a population of cells in response to external factors.

The proliferation and cell viability were evaluated using various ultrasound parameters and microbubble concentrations.

Electrophysiology protocol and patch-clamp technique

To assess the effects of ultrasound and microbubbles on cell membrane, patch clamp technique was used in the experiment. Patch clamp is a technique able to visualise and to quantify micro and macro ion currents through the cell membrane. We used in our study the patch clamp in a "whole cell" configuration [6, 7] which consists of applying gently a glass micropipette with a tip of only a few micrometers in diameter on the cell membrane to form a seal between the glass pipette and the membrane. Suction is then applied to the micropipette breaking a tiny section of the membrane and the solution inside the micropipette mixes up with the cytoplasm. When equilibrium is reached the ion activity can be recorded by measuring macroscopic currents, whole cell current and membrane potential.

III. RESULTS

Using ultrasound alone, Sonovue bubbles alone or their association did not show any effect on cell viability using acoustic pressures up to 300 kPa. Figure 1 shows the percentage of viable cells for control experiment compared to Sonovue bubbles alone, or ultrasound and Sonovue for 2 different acoustic pressures (150 kPa and 200 kPa). The results demonstrate no change in cell viability compared to the control.

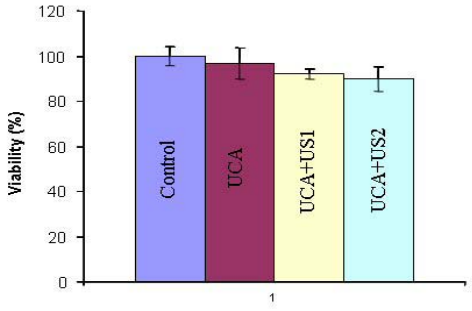

Figure 1. Cell viability for control, contrast bubbles alone, bubbles and ultrasound (1MHz, 150 kPa) and bubbles and ultrasound (1MHz, 200 kPa).

Effects of US and microbubbles on electrophysiological properties of cells

The results obtained from the patch clamp experiments showed that microbubbles in contact with cell membrane induce membrane potential hyperpolarization under ultrasound insonification. Microbubbles alone have no influence on the membrane potential as well as ultrasound alone as shown in Fig. 2. However ultrasound and microbubbles cause a membrane potential hyperpolarization (Fig. 3). The potential remains hyperpolarized as long as the ultrasound is transmitted and the microbubble in contact with the membrane. Therefore a direct contact between the microbubble and the cell is essential and plays a key role in modulating the hyperpolarization amplitude. The variations of the membrane potential extended up to 25± 1.4 mV (n=6). The hyperpolarization of the membrane potential seems to be likely induced by an excess inward flux of Chloride ions (Cl^-) into the cell or an outward flux of potassium ions (K^+) to the medium.

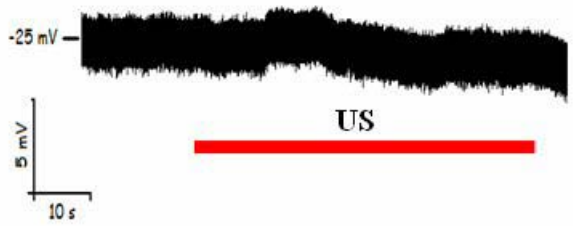

Figure 2. Cell membrane potential using ultrasound alone (1MHz, 200 kPa).

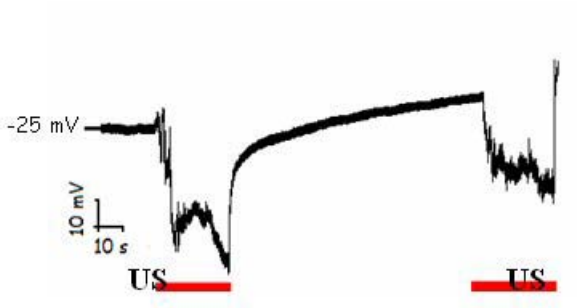

Figure 3. Cell membrane potential with ultrasound (1MHz, 200 kPa) and Sonovue microbubbles. Red lines correspond to ultrasound exposure.

We assumed that the membrane potential hyperpolarization is caused by a mechanical stress induced by the microbubble oscillations. We have tested this hypothesis by applying a mechanical stress using a glass rod pressed against the cell (Fig 4A). Similar hyperpolarization phenomena of the membrane potential have been observed with amplitudes up to -17 mV± 1,9 mV (n=5) following the application of mechanical pressure on the cell as shown in Fig. 4 B. The hyperpolarization was repeatedly observed when the mechanical pressure was applied. For the applied mechanical pressures, the amplitude of the membrane potential lies below -60 mV, which is above the equilibrium potential of Cl^-. Therefore, it is likely that the main known conductance involved with mechanical stresses would be related to K^+ and not to Cl^-. For the cells used in the experiments, MDA-MB-231, the main conductance is the large calcium-activated potassium conductance BK_{Ca} [8]. Therefore our results suggest that mechanical stress induced by a mechanical pressure or microbubble oscillations is responsible for triggering of the BK_{Ca} channel. To validate this finding, an inhibitor of the BK_{Ca} K^+ channel was introduced while applying the mechanical stress. Iberiotoxin, a known blocker of the large conductance Ca^+ - activated K^+ channel was used to interrupt the channel activity. As shown in Fig. 4C, in the presence of 100nM iberiotoxin, the application of the mechanical pressure does not activate any channel as appreciated from the membrane potential curve which remains stable around its initial value (lower panel).

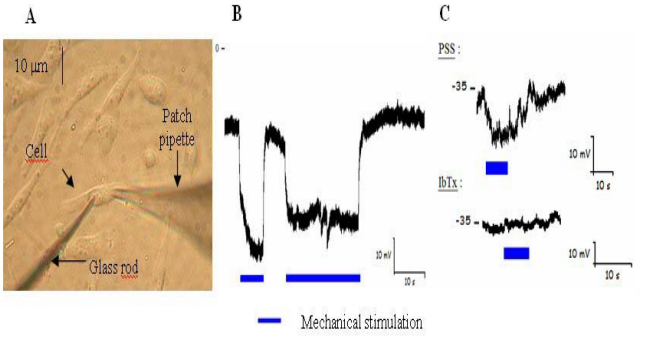

Figure 4. a): image showing patched cell in presence of mechanical stimulation using a glass rod; b): Cell membrane potential using mechanical stimulation; c): cell membrane potential without (top) and with Iberiotoxin blocker.

This result confirms that BK_{Ca} channels are directly involved in the cellular response to mechanical stresses and corroborates the hypothesis

that K+ ions are associated with the hyperpolarization induced by microbubble oscillations under ultrasound activation.

VI. DISCUSSIONS - CONCLUSIONS

The available literature has shown the possibility of associating gas microbubbles to ultrasound waves in order to achieve selective therapeutic benefits. Ultrasound contrast agents composed of gas microbubbles are capable to target specific cellular targets through ultrasound focusing and modify by that the electrophysiological properties of cells in correlation with transmembrane exchange.

While ultrasound alone and microbubbles alone have no detectable effects, a hyperpolarization of the membrane potential was measured when ultrasound waves are associated to microbubbles. The hyperpolarization has shown to be reversible and reproducible and can be associated with cell membrane deformation under the influence of microbubble oscillations. These variations induced by microbubbles and ultrasound observed in the membrane potential are similar to those induced by a mechanical pressure applied locally to the cell. They have shown also to be highly attenuated with Iberiotoxin, a specific blocker of potassium channels BK_{Ca}. This finding confirms a major implication of potassium current in the cell hyperpolarization.

The hyperpolarization does not seem to be the only consequence of microbubble oscillations but other effects such as potential depolarization might be triggered but still need to be elucidated. Therefore revealing the total action of ultrasound and microbubbles on the cell electrophysiological properties is a necessary step towards understanding mechanisms of cell membrane permeabilization with ultrasound and contrast microbubbles.

VII. REFERENCES

[1] B. Goldberg, J. Raichlen, and F. Forsberg, "Ultrasound Contrast Agents: Basic Principles and Clinical Applications.," M. Dunitz, Ed., 2nd ed, 2001.

[2] Y. Taniyama, K. Tachibana, K. Hiraoka, T. Namba, K. Yamasaki, N. Hashiya, M. Aoki, Y. Ogihara, K. Yasufumi. and R. Morishita. Local delivery of plasmid DNA into Rat carotid artery using ultrasound - Circulation. 105(10): 1233-1239, 2002.

[3] J. Christiansen, B. French, A. Klibanov, S. Kaul and J. Lindner. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles - Ultrasound Med Biol. 29(12): 1759-1767, 2003.

[4] H. Soule, J. Vasquez, A. Long, S. Albert and M. Brennan. A human cell line from a pleural effusion derived from a breast carcinoma - J Natl Cancer I. 51: 1409-1416, 1973.

[5] P. Twentyman and M. Luscombe. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity - Brit J Cancer. 56(3): 279-285, 1987.

[6] O. Hamill, A. Marty, E. Neher, B. Sakmann. and F. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches - Pflug Arch Eur J Phys. 391(2): 85-100, 1981.

[7] E. Neher and B. Sakmann. Single channel currents recorded from membrane of denervated frog muscle fiber - Nature. 260: 799-802, 1976.

[8] S. Roger, M. Potier, C. Vandier, JY. Le Guennec and P. Besson Description and role in proliferation of iberiotoxin-sensitive current in different human mammary epithelial normal and cancerous cells –Bioch Biophys Acta. 1667(2): 190-199, 2004.