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Blood brain barrier precludes the cerebral arteries to intravenously-injected antisense oligonucleotide.

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ABSTRACT

Alternative splicing of the ryanodine receptor subtype 3 (RyR3) produces a short isoform (RyR3S) able to negatively regulate the ryanodine receptor subtype 2 (RyR2), as shown in cultured smooth muscle cells from mice. The RyR2 subtype has a crucial role in the control of vascular reactivity via the fine tuning of Ca^{2+} signaling to regulate cerebral vascular tone. In this study, we have shown that the inhibition of RyR3S expression by a specific antisense oligonucleotide (asRyR3S) was able to increase the Ca^{2+} signals implicating RyR2 in cerebral arteries *ex vivo*. Moreover, we tried to inhibit the expression of RyR3S *in vivo*. The asRyR3S was complexed with JetPEI and injected intravenously coupled with several methods known to induce a blood brain barrier disruption. We tested solutions to induce osmotic choc (mannitol), inflammation (bacteria lipopolysaccharide and pertussis toxin), vasoconstriction or dilatation (sumatriptan, phenylephrine, histamine), CD73 activation (NECA) and lipid instability (Tween80). All tested technics failed to target asRyR3 in the cerebral arteries wall, whereas the molecule was included in hepatocytes or cardiomyocytes. Our results showed that the RyR3 alternative splicing could have a function in cerebral arteries *ex vivo*; however, the disruption of the blood brain barrier could not induce the internalization of antisense oligonucleotides in the cerebral arteries, in order to prove the function of RYR3 short isoform *in vivo*.

1. Introduction

The cerebrovascular network responds to the nervous system solicitations by modification of cerebral blood flow. The goal of this increase of functional hyperemia is to elevate the amount of glucose and oxygen. The vascular tone is due to vascular smooth muscle cells, and is in part encoded by Ca^{2+} signaling. The ryanodine receptors could be considered as the center of this signalisation. In fact, these Ca^{2+} channels, located in sarcoplasmic reticulum, encode several types of Ca^{2+} signals responsible for vasoconstriction (propagated Ca^{2+} waves), as well as vasodilatation (localized Ca^{2+} signals named Ca^{2+} sparks; for review Morel et al., 2007). As summarized in Fig. 1, ryanodine receptor subtypes (RyR1-3) are activated by an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). This first increase of $[Ca^{2+}]i$ is due to Ca^{2+} entry and/or previous Ca^{2+} release from intracellular Ca^{2+} store. Thus, the ryanodine receptors encode the Ca^{2+}-induced Ca^{2+} release mechanism (CICR) to amplify Ca^{2+} waves necessary for vasoconstriction.

It is well established that RyR1 and RyR2 subtypes are implicated in Ca^{2+} waves, as well as Ca^{2+} sparks (Coussin et al., 2000). Various functions for RyR3 subtype were suggested. It could be implicated in spontaneous Ca^{2+} signals, as shown in cerebral arteries from RyR3 knockout mice (Löhn et al., 2001), as well as in the regulation of sarcoplasmic reticulum Ca^{2+} loading, as shown by antisense oligonucleotides strategy *ex vivo* (Mironneau et al., 2001). Finally, the alternative splicing of RyR3 generates complete long (RyR3L) and short (asRyR3S) isoforms in smooth muscle (Jiang et al., 2003). The RyR3S isoform can inhibit the RyR2 function, which increases the ryanodine receptor-dependent Ca^{2+} signaling (Jiang et al., 2003; Dabertrand et al., 2006). The RyR3L isoform is able to encode Ca^{2+} signals and thus participates

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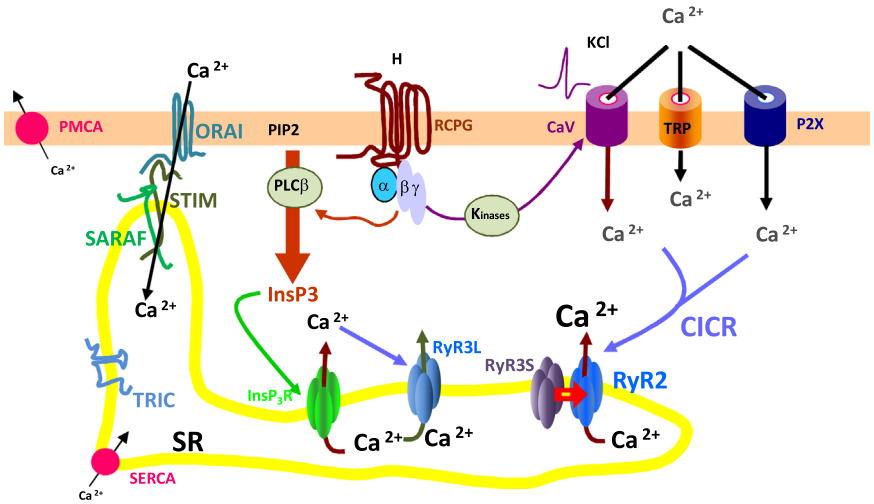

Fig. 1. Calcium signaling in vascular smooth muscle cells (VSMC). The Ca^{2+} entry (voltage-gated Ca^{2+} channels, ionotropic receptor as P2X, cationic channels as TRP) as well as InsP3-induced Ca^{2+} release are amplified by the CICR mechanism encoded by RyRs. Two different splice variant of RyR3 are co-expressed. The long isoform (RyR3L) is able to produce Ca^{2+} release whereas the short isoform (RyR3S) inhibits RyR2-dependent Ca^{2+} release. PMCA and SERCA encoded the extrusion of Ca^{2+} from cytoplasm to external compartment and sarcoplasmic reticulum (SR), respectively. FKBP12/12.6, sorcin were expressed and regulate RyR functions in vascular smooth muscle cell's Ca^{2+} signaling.

to the control of sarcoplasmic reticulum loading and modulation of spontaneous Ca^{2+} signaling (Dabertrand et al., 2008). Taken together, these data indicate that both isoforms of RyR3 are crucial for the regulation of Ca^{2+} signaling.

Firstly, all data concerning RyR3 isoforms were acquired in cultured cells (*ex vivo*) and their functions were never studied *in vivo*. Secondly, the fine regulation of Ca^{2+} signaling due to the splicing of RyR3 could participate in the regulation of functional hyperemia for the adaptation of the cerebral blood flow to neuronal activity.

Our goal was to study the RyR3 isoform functions *in vivo* by antisense oligonucleotide strategy to selectively decrease the expression of both isoforms in cerebral arteries in a mouse model.

Several techniques described below to address antisense oligonucleotide to cells into the cerebral artery wall were tested. Unfortunately, if the antisense oligonucleotide efficiency to inhibit RyR3 isoform was demonstrated in cerebral arteries in culture (*ex vivo*), the targeting of antisense oligonucleotide into cerebral arteries was not yet successful *in vivo*.

2. Materials and methods

2.1. Animals

The project was validated by the French ministry of research in accordance with European Community and French guiding principles. The principal investigator is authorized by French authorities to perform animal experiments (No C33-01-029). We have used 62 male C57BL/6J mice (Charles River, L'Arbresle, France). All animals were killed at the age of 4–6 months, by cervical dislocation or lethal injection of pentobarbital.

2.2. Treatments of mice to modulate the blood brain barrier (BBB) permeability

To induce the decrease of the expression of RyR3 splice variants *in vivo*, we injected intravenously a solution containing phosphorothioate antisense oligonucleotide (indicated with the prefix "as", for example asRYR3S targeted RYR3S isoform, asSCR is the scramble form of asRYR3 (Dabertrand et al., 2006)), coupled with JetPEI *in vivo* (Polyplus, Illkirsch, France) in a glucose solution (5%) following the recommendations of the supplier. Injections were performed via the retro-orbital way on anesthetized mice (Ketamine 1%, Xylazine 0.5% cocktail, 10 mL/Kg). The antisense oligonucleotide quantity was determined by the final volume unharmful for mice (200 μL, representing near 10% of blood volume).

We have also used the 2-O-methyl phosphorothioate oligoribonucleotide directed against exon 23 of the mouse dystrophin mRNA (asDYS) to test if the molecular structure is an important parameter.

Phosphorothioate antisense oligonucleotides were synthetized and coupled with Cy5 indomethacin at 5′ extremity (Eurogentec, Serain, Belgium). Therefore, cells containing antisense oligonucleotide were visualized by the fluorescence emitted at 680 nm.

The *in vivo* injection of antisense oligonucleotides was performed in association with a protocol inducing the temporary BBB disruption. The protocols were chosen and sometimes modified to be compatible with mouse survival during 4–5 days. Protocols are summarized in Table 1. Animals were killed by a lethal injection of pentobarbital.

Mannitol, pertussis toxin (PTx) (Sigma-Aldrich, St. Louis, MO) was diluted in 0.9% NaCl solution. NECA [1-(6-amino-9 *H*-purin-9-yl)-1-deoxy-*N*-ethyl-6-D-ribofuranuronamide], Tocris Biosciences, was dissolved in DMSO and before injection in 0.9% NaCl solution. Final concentration of DMSO was 0.5% (v/v).

2.3. Antisense oligonucleotide ex vivo

In this part of the study 25 mice were used. Cerebral arteries (anterior and middle cerebral artery trees) were discarded from brain, dissected and placed in vascular smooth muscle cell Lonza culture medium (Lonza, Levallois, France) containing antisense oligonucleotide (2×10^{-9} mol/well) and kept at 37 °C, 5% CO_2 during 2–4 days. Antisense oligonucleotides directed against RYR3 splice variants and RyR2 were previously described (Dabertrand et al., 2007; Dabertrand et al., 2008; Dabertrand et al., 2006). Phosphorothioate antisense oligonucleotides were synthetized and coupled with Cy3 indomethacin to visualize cells containing antisense oligonucleotide during Ca^{2+} measurement by the fluorescence at 568 nm. Animals were separated in several groups and treated as indicated in Table 2.