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Laminar shear stress modulates the activity of heterologously expressed $P2X_4$ receptors

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ABSTRACT

$P2X_4$ receptors are involved in mechanotransduction processes, but it is unknown whether or not $P2X_4$ receptors form mechanosensitive ion channels. This study questioned, whether laminar shear stress (LSS) can modulate $P2X_4$ receptor activity. Mouse $P2X_4$ receptor was cloned and heterologously expressed in *Xenopus laevis* oocytes. In two-electrode-voltage-clamp experiments the application of ATP (100 μM) produced a transient inward current that was decreased by about 50% upon a second ATP application, corresponding to the desensitization behavior of $P2X_4$ receptors. In $P2X_4$ expressing oocytes LSS (shear forces of ~5.1 dynes/cm^2) did not produce any effect. However, LSS modulated the response of $P2X_4$ to ATP. With LSS (~5.1 dynes/cm^2) the desensitization of the current due to the second ATP application was diminished. Ivermectin (IVM), a compound which stabilizes the open state of $P2X_4$ receptors, mimicked the effect of LSS (~5.1 dynes/cm^2), since there was no additional effect of LSS after pre-incubation with IVM detected. This indicates that LSS like IVM stabilizes the open state of the receptor, although the particular mechanism remains unknown. These data demonstrate that LSS modulates the activity of $P2X_4$ receptors by eliminating the desensitization of the receptors in response to ATP probably by stabilizing the open state of the channel.

1. Introduction

All organisms are exposed to mechanical forces and many physiological functions are associated with mechanical forces like tensile forces, compressive forces and shear forces [1,2]. The conversion of mechanical stimuli into biochemical signals is referred to as mechanotransduction. This process is of critical relevance for all organisms and cells to sense their environment and react to changes of their surrounding. For example, the detection of mechanical forces enables organisms to perceive touch [3] and is also crucial for the sensing of sound and gravity, which are essential prerequisites for hearing and the sense of balance [4–6]. In the cardiovascular system, the transduction of mechanical stimuli is associated with the regulation of vascular tone, angiogenesis, vascular remodeling and arteriogenesis [7,8]. Furthermore, mechanical stimuli play an important role in lung function and physiology, for example in the differentiation of alveolar cells [9] or in fetal growth and development of the lung [10].

Mechanosensitive ion channels are likely candidates for transducing mechanical stimuli into cellular signals [11–13]. Studies in *Caenorhabditis elegans* and *Drosophila melanogaster* were the basis for the identification of ion channels involved in mechanotransduction [11]. These studies identified members of the Degenerin/epithelial Na^+ channel (DEG/ENaC) protein family and Transient Receptor Potential (TRP) protein family to form mechanosensitive ion channels [11,14–16].

Another ion channel family, comprising members that have been recently identified to be associated with mechanotransduction processes, is the P2X receptor family [17,18]. The mammalian P2X receptor family consists of 7 different subtypes ($P2X_1$-$P2X_7$). These proteins form trimers that function primarily as ATP-gated cation channels, which are permeable for mono- and divalent cations [19]. Members that were identified to participate in mechanotransduction processes are the $P2X_3$ [17] and the $P2X_4$ subtype in particular [18,20].

The $P2X_4$ receptor subtype is expressed in many different tissues and cells, for example in the central nervous system [21], in immune cells like mast cells [22] or macrophages [23] and the urinary bladder [24]. It was also shown that the $P2X_4$ receptor subtype was by far the most expressed P2X subtype in endothelial cells and that this receptor plays a role in endothelial mechanotransduction processes [25,26,20]. This is of particular importance, since these cells are permanently exposed to mechanical forces, above all laminar shear stress, generated by the blood flow. Yamamoto et al. [20] described

Abbreviations: LSS, laminar shear stress; ATP, adenosine-5′-triphosphate; IVM, ivermectin; DEG/ENaC, Degenerin/epithelial Na^+ channel; TRP, transient receptor potential; ENaC, epithelial Na^+ channel; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; TEVC, two-electrode-voltage-clamp; ORi, oocyte Ringer's solution; DMSO, dimethyl sulfoxide; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid); TM, transmembrane domain

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that human endothelial cells respond to shear stress with an influx of Ca^{2+} ions via $P2X_4$ receptors. Further, it was also shown that endothelial cells of $P2X_4$ knockout mice did not react with an increase of intracellular calcium levels in response to shear forces [8]. This confirms the participation of $P2X_4$ receptors in mechanotransduction. It is suggested, that the shear stress mediated increase of intracellular calcium via $P2X_4$ receptors further leads to an increased production of NO, thus representing a crucial mechanism to regulate vasodilation and blood pressure [8].

However, the mechanism(s) how $P2X_4$ receptors participate in mechanical force transduction are unclear. There are two possibilities to explain the effect of shear stress on $P2X_4$ receptors: (1) Shear stress induces the release of ATP (via an unknown mechanism) and this subsequently binds and activates $P2X_4$ receptors. This was hypothesized by Yamamoto et al. [7] and is further supported by studies demonstrating that ATP can be released from endothelial cells in response to mechanical forces [27]. (2) Another possibility might be that $P2X_4$ receptors are *per se* mechanosensitive. The basic idea behind this concept derives from the structural similarities between P2X receptors and ENaC proteins and emerging evidence that ENaCs are mechanosensitive [20,28]. Thus, the aim of this study was to investigate whether or not $P2X_4$ receptors might directly respond to shear force, and laminar shear stress in particular.

2. Material and methods

2.1. Cloning and in vitro transcription of the $P2X_4$ receptor

The $P2X_4$ receptor was cloned from total RNA pools isolated from alveolar macrophages of C57/BL6 mice using a RNA mini isolation kit (RNeasy Mini Kit, Quiagen, Hilden, Germany). Genomic DNA was removed by treatment with DNase I (15 min in 37 °C, 1 U/reaction, Invitrogen, Darmstadt, Germany) and subsequently the RNA (using maximum 1 μg RNA) was reverse transcribed in cDNA using either Superscript II (Invitrogen) or iScript (BioRad, München, Germany) kits.

The cDNA was used as a template for a PCR reaction using Platinum Pfx DNA Polymerase (Invitrogen) and the following primers: forward primer: 5′-CTCGAGATGGCAGGCTGCTGCTCCGTGCTC–′3; reverse primer: 5′-CTCGAGTCACTGGTCCGTCTCTCCGGAAAG-′3.

The PCR reaction contained 8 μM forward and reverse primer, 300 μM dNTPs (Promega, Madison, USA), 1 mM $MgSO_4$ (Promega), 2 U Platinum Pfx DNA Polymerase (Invitrogen), 2.5 μl 10x Pfx amplification buffer, 1 μl cDNA and H_2O to a final volume of 25 μl. The PCR conditions were as follows: denaturation at 94 °C for 5 min, following 35 cycles of (1) 95 °C for 45 s, (2) annealing at 70 °C for 1 min, (3) extension at 68 °C for 2.5 min. The PCR was finished with a final extension at 68 °C for 10 min. The amplified PCR product was then analyzed on a 1% agarose gel and the amplicon of appropriate size was isolated from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega). This was followed with A-addition at 70 °C for 30 min using 1 μl Taq-Polymerase (Promega), 2 μl 5x Taq Buffer, 0.8 μl 25 mM $MgCl_2$, 0.2 μl 10 mM dATP, and 6 μl PCR amplification product.

The A-addition product was cloned into the pGEM-T Easy vector using the pGEM-T Easy vector system II (Promega) according to the manufacturer's instructions. These vectors were transformed into competent bacteria (*E. coli* JM109) and positive clones were detected via blue/white screening. The plasmids were isolated using a plasmid isolation kit (Wizard Plus SV Minipreps DNA purification System, Promega), and the amplicon was cut out using the restriction enzyme XhoI (Promega). The digestion product was separated via gel electrophoresis, which was followed by gel extraction (Wizard SV Gel and PCR Clean-Up System, Promega). For the functional expression of the receptor, the $P2X_4$ cDNA was subcloned into the pTNT expression vector (Promega) using Xho I sites and transformed into JM109 competent bacteria. The plasmids were isolated (Wizard Plus SV Minipreps DNA purification System, Promega or Perfect Prep Spin Mini Kit, 5 prime, Hamburg, Germany) and the resulting product was analyzed via sequencing. In order to obtain cRNA for oocyte injection, the plasmids were *in vitro* transcribed (RiboMax T7 transcription kit, Promega) according to a standard protocol. The cRNA was diluted with nuclease-free water.

2.2. Heterologous expression of $P2X_4$ receptors in Xenopus oocytes

Defolliculated *Xenopus laevis* oocytes of stage V and VI (Dumont, 1972) were injected with 23 nl or 50.6 nl of $P2X_4$ encoding RNA using a micro injector (Nanoject, Drummond Scientific, Broomall, USA). The injection of 23 nl RNA correlates to a concentration of 12 ng $P2X_4$ RNA for each oocyte. Injected oocytes were subsequently incubated in a culture solution containing (mM): 90 NaCl, 1 KCl, 2 $CaCl_2$, 5 HEPES (4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid), 2.5 pyruvate, 20 mg/l penicillin and 25 mg/ml Streptomycin; pH 7.4 at 16 °C. The oocytes were measured 1–4 days after injection. Control oocytes were injected with an identical volume of nuclease-free water.

2.3. Two-electrode-voltage-clamp experiments

Transmembrane currents of water- or RNA-injected oocytes were measured by the two-electrode-voltage-clamp technic (TEVC). Transmembrane currents of the oocytes were measured at −60 mV using a TEVC amplifier (Warner Instruments, Hamden, USA). The electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) and had a resistance of between 1 and 10 MΩ when filled with 1 M KCl solution. During measurements the oocytes were perfused with an oocyte Ringer's solution (ORi) containing (mM): 90 NaCl, 1 KCl, 2 $CaCl_2$, 5 HEPES, pH 7.4. All recordings were performed at room temperature.

For measurements the oocytes were placed in a perfusion chamber. This chamber contained a shield to avoid flow contamination due to the bath superfusion [28]. The bath superfusion was used to remove ATP from the measurement chamber or to incubate the oocytes with a drug. For the generation of laminar shear stress (LSS) at the surface of the oocyte a flow stream was applied via a Pasteur pipette (1 mm inner diameter) and the tip of the Pasteur pipette was placed in close proximity to the oocyte (~1 mm distance). Perfusion rates for the application of laminar shear stress due to the Pasteur pipette were 1 ml/min or 3 ml/min. This flow rates in combination with the inner diameter of the Pasteur pipette will produce shear forces of ~0.6 dynes/cm^2 (with 1 ml/min) and ~5.1 dynes/cm^2 (3 ml/min). Determination of shear forces was done in accordance to prior published procedures [28]. For the determination of the control ATP effect (without LSS), ATP was applied from a stock solution directly into the recording chamber by using a standard pipette. This was accomplished by dripping a drop of stock solution in close proximity of the oocyte. The final ATP concentration in the bath was 100 μM. During drug or LSS application via the pipette or via the Pasteur pipette the bath superfusion was switched off.

2.4. Chemicals

ATP (Adenosine-5′-triphosphate, Fluka, Steinheim, Germany) was generally applied in a concentration of 100 μM. Ivermectin (IVM, Sigma, Steinheim, Germany) was used at a concentration of 10 μM as a specific potentiator of the ATP-induced $P2X_4$ current and was pre-incubated 3 min before ATP application. In the appertaining controls DMSO (Dimethyl sulfoxide, Fluka), the solvent of IVM, was perfused in the same concentration (0.1% DMSO) as in the IVM measurements. PPADS (Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid), 1 mM, Sigma) was used as an antagonist of $P2X_4$ receptors. In these experiments, the oocytes were pre-incubated with PPADS 5 min before ATP application. All chemicals used for the preparation of

Ringer's solutions (storage and experiments) were purchased from Fluka expect for pyruvate and HEPES (Applichem, Darmstadt, Germany), Streptomycin (Sigma) and NaCl (Roth, Karlsruhe, Germany).

2.5. Statistics

All results are presented as means ± standard error of the mean (SEM). Due to the large variations obtained in response to the application of ATP the ATP-induced currents were normalized. Therefore, in each single experiment the ATP-induced current was estimated twice. The first ATP effect was defined as 1 (100%) and the second ATP effect was normalized with respect to the first ATP-induced current. The number of oocyte batches used is marked with "N" and the number of individual performed experiments with "n". Statistical evaluation was performed using the paired Student's *t*-test. Differences between means with a p-value <0.05 were considered as significantly different and marked with an asterisk (*).

3. Results

3.1. ATP activates heterologously expressed $P2X_4$ receptors

To determine the effect of LSS on $P2X_4$ receptor mediated currents, $P2X_4$ receptors were heterologously expressed in *Xenopus laevis* oocytes. 1–4 days after injection of $P2X_4$ encoding cRNA, two-electrode-voltage-clamp experiments were performed at a holding potential of −60 mV.

The application of 100 μM ATP led to a significant current increase of 2.3 ± 0.4 μA (n = 87, N = 12; Fig. 1A, B). This current increase was transient and desensitized within seconds during ATP exposure (Fig. 1A). Pre-incubation of the oocytes with the purinergic antagonist PPADS (1 mM) for 5 min prior to ATP application inhibited approximately 95% (n = 6, N = 3) of the ATP-induced current, compared to control conditions (without PPADS, Fig. 1B, C).

The increase of the membrane current due to 100 μM ATP as well as the desensitization profile (desensitization within seconds while ATP exposure) and the inhibition by only high concentrations of PPADS (low affinity to PPADS) are typical features of the $P2X_4$ receptors.

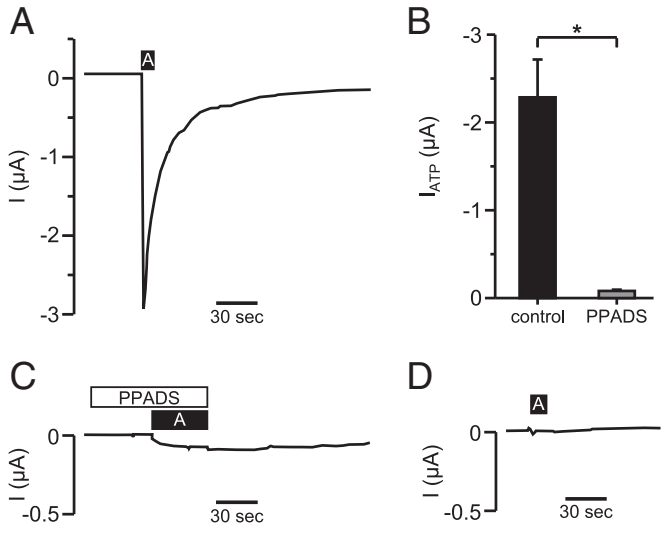

Fig. 1. Effect of ATP on heterologously expressed $P2X_4$ receptors. (A) Original recording illustrating the effect of 100 μM ATP (application marked with "A") on the $P2X_4$ receptor current. The application of ATP evoked a transient current increase (before ATP: 0.008 ± 0.014 μA, ATP peak current: −2.3 ± 0.4 μA; n = 87, N = 12; $p<0.05$). The current was measured at a holding potential of −60 mV. (B) Summarized results of the ATP-induced current (I_{ATP}) and its decrease via PPADS in $P2X_4$ expressing oocytes. ATP significantly increased the current of $P2X_4$ expressing oocytes and this effect was largely inhibited by PPADS (n = 6, N = 3, $p<0.05$). (C) Current trace depicting the inhibitory effect of PPADS (1 mM) on the ATP-induced current. (D) In control oocytes (injected with nuclease-free water) no ATP effect was detected.

To further ensure, that the ATP-induced current was mediated via $P2X_4$ receptors and not due to endogenous proteins of the *Xenopus laevis* oocytes, control oocytes (water-injected oocytes) were superfused with 100 μM ATP. In these oocytes the application of 100 μM ATP failed to affect the transmembrane current (Fig. 1D).

3.2. Effect of LSS on $P2X_4$ receptors

Different ion channels and the epithelial Na^+ channel in particular have been identified to respond to LSS and that this effect is independent from soluble mediators [28]. To investigate if LSS also activates $P2X_4$ receptors, $P2X_4$ expressing oocytes were exposed to LSS. Therefore a Pasteur pipette was placed in close proximity in front of the oocytes. A flow stream of ORi solution through this pipette generated LSS on the surface of the oocyte. The exposure to LSS, produced by a flow rate of 3 ml/min, had no effect on the transmembrane ion currents of $P2X_4$ expressing oocytes (n = 13, N = 3; Fig. 2A, B). Water injected control oocytes also did not respond to LSS (n = 11, N = 4; Fig. 2B, C). To ensure that LSS did not induce the release ATP from the oocytes and that this endogenous ATP causes activation of $P2X_4$ receptors experiments with ivermectin (IVM) and LSS were performed.

IVM is an allosteric modulator of $P2X_4$ receptors, known to stabilize the open state configuration of the pore. This alters the desensitization behavior of the receptor and leads to a potentiation of the ATP-induced current [29]. As observed with LSS alone the pre-incubation of the cells with IVM (10 μM, 3 min) and the subsequent application of LSS did not influence the membrane current of $P2X_4$ expressing oocytes (n = 6, N = 2; Fig. 2D, E). These results indicate that $P2X_4$ receptors are not directly activated by LSS alone.

3.3. Modulation of the ATP induced $P2X_4$ activation by LSS

Apart from a direct activation of $P2X_4$ receptors by LSS, it might be possible that LSS is able to modulate ATP-induced $P2X_4$ activity. To test this assumption experiments were performed in which 100 μM ATP was applied without LSS and under LSS with flow rates of 1 ml/min and 3 ml/min.

Control experiments were performed in which $P2X_4$ expressing oocytes were exposed twice to 100 μM ATP without applying LSS. Fig. 3A depicts a representative current trace of such an experiment. In both cases (first and second application) a transient current increase was observed. The comparison between the first and the second ATP effect showed, that the second ATP effect was approximately 50% smaller than the first ATP effect (n = 27, N = 7; Fig. 3A, B). This observation corresponds with the known desensitization behavior of $P2X_4$ receptors [19].

In another set of experiments $P2X_4$ expressing oocytes were again exposed twice to ATP but in these experiments the second ATP exposure was accompanied by the application of LSS. With a flow rate of 1 ml/min the effect in response to ATP and LSS was significantly smaller compared with the preceding ATP effect (without LSS; n = 10, N = 2; Fig. 3C, D). However, the application of ATP (100 μM) and LSS, using a flow rate of 3 ml/min, caused an ATP-induced current, which was not significantly different compared with the preceding first ATP effect (without LSS; n = 22, N = 7; Fig. 3E, F). Identical experiments were performed with 10 μM and 1 mM ATP. With 10 μM ATP LSS had no effect, while with 1 mM ATP LSS prevented the desensitizing effect that is observed during the second ATP exposure (data not shown). Additional experiments were performed in which ATP was applied two times under LSS with a flow rate of 3 ml/min. In these experiments the second ATP effect was also not significantly different from the first ATP effect (n = 12, N = 5; Fig. 3 G, H).

Taken together, these experiments demonstrate that LSS, with a flow rate of 3 ml/min, altered the response of the receptors to ATP.

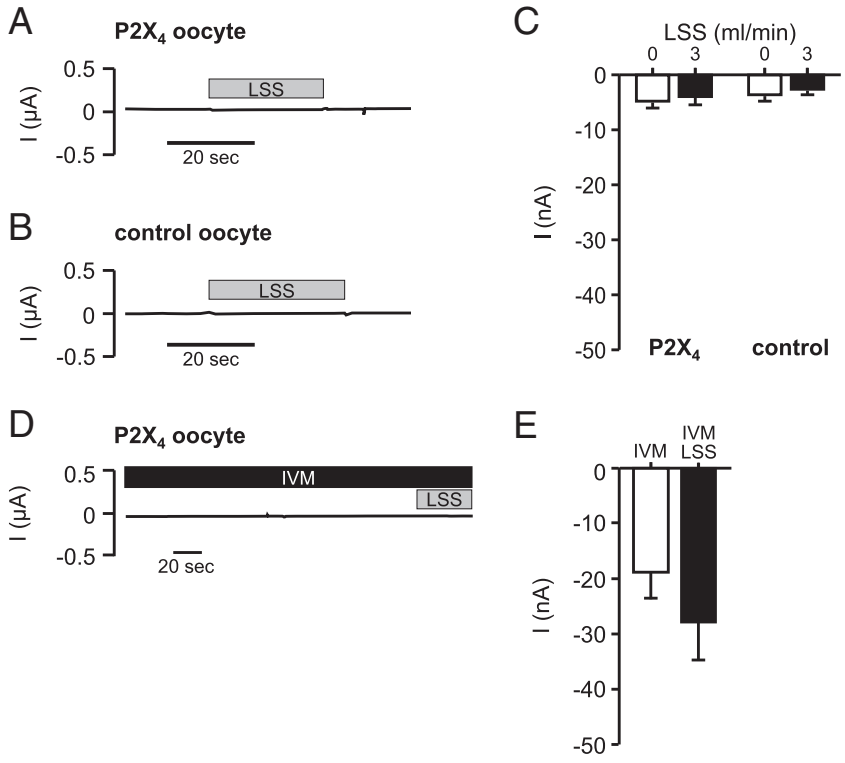

Fig. 2. Effect of laminar shear stress (LSS) on $P2X_4$ receptors expressed in *Xenopus* oocytes. (A) Representative current trace depicting the effect of LSS (application marked by a dashed bar) with a flow rate of 3 ml/min on $P2X_4$ receptors. The exposure of LSS did not change the current. (B) Similar experiment as shown in panel A, using nuclease-free water injected oocytes. (C) The exposure of LSS neither evoked an activation of the current in $P2X_4$ expressing oocytes (n = 13, N = 3) nor in control oocytes (n = 11, N = 4). Note that the magnitude scale of the ordinate is nA. (D) Original experiment showing the effect of LSS with a flow rate of 3 ml/min on the transmembrane current of $P2X_4$ expressing oocytes after pre-incubation with Ivermectin (10 μM, 3 min). (E) Statistical evaluation of the experiments shown in (D). The application of IVM and LSS together failed to influence the transmembrane current of $P2X_4$ expressing oocytes after pre-incubation the cells with IVM (10 μM, 3 min; n = 6, N = 2).

The observed effect of LSS is characterized by an increased ATP effect in response to the second ATP application, indicating an alteration of the desensitization behavior of the receptors.

3.4. Putative mechanism of the LSS-mediated effect

To determine whether or not LSS also prolongs the open state of $P2X_4$ receptors, experiments with IVM in combination with LSS were performed. Fig. 4A shows a representative current trace of a control experiment employing the ATP effect in absence and in the presence of DMSO (0.1 vol.%, pre-incubated for 3 min), which was used as solvent of IVM. As already demonstrated in the experiments depicted in Fig. 3, the second ATP effect was significantly reduced (by approx. 35%; n = 7, N = 3) compared with the first ATP effect (Fig. 4A, B). Similar experiments were performed with IVM (10 μM, dissolved in DMSO; Fig. 4C). Comparison of the first (no IVM) and the second ATP-induced current (IVM + DMSO) revealed, that the second ATP effect was not significantly different from the first ATP effect (n = 12, N = 3; Fig. 4D). This finding confirms the ability of IVM to potentiate $P2X_4$ receptor activity.

According to the same experimental procedure a third experiment was performed to assess whether potentiation of the ATP-induced current by IVM and LSS are additive. Therefore the second ATP application was also performed in the presence of IVM and LSS (flow rate of 3 ml/min; Fig. 4 E). Interestingly, no significant difference between the first and the second ATP-induced current was determined (n = 9, N = 3; Fig. 4F). This result is similar to the effect of IVM without LSS (Fig. 4D) and indicates, that LSS has probably no additive effect, when the open state is already pharmacologically prolonged by IVM.

4. Discussion

Due to the blood flow, endothelial cells are permanently exposed to shear forces. In the vascular system, the transduction of shear forces is associated with embryo genetic development, for example in the embryo genetic morphogenesis of heart and the embryo genetic remodeling of yolk sacs [30,31] and is also essential for different vascular functions like the production of vasodilator substances such as NO and prostacyclin [32,33], the reduction of oxidative stress [34] and blood vessel remodeling [35].

$P2X_4$ receptors were indicated as likely candidates for mechanotransduction in endothelial cells [20]. Since the mechanism of mechanotransduction via $P2X_4$ receptors is still unclear our aim was to investigate whether $P2X_4$ receptors are per se mechanosensitive or whether $P2X_4$ receptor activity can be directly modulated by laminar shear stress.

4.1. $P2X_4$ receptor activity is modulated by laminar shear stress

Since other ion channels were activated by laminar shear stress without involving soluble mediators [28], the present study investigated whether $P2X_4$ receptors might also be sensitive to laminar shear stress. In voltage-clamp measurements, the application of LSS did not influence the activity of $P2X_4$ receptors (Fig. 2). This observation indicates that $P2X_4$ receptors cannot be activated by LSS alone and is contrary to the activation properties of other mechanosensitive ion channels, like the ENaC [28].

Another possibility by which LSS might influence $P2X_4$ receptors is due to the modulation of their response to ATP. Interestingly, our results showed that LSS was able to increase the ATP-induced activation of $P2X_4$

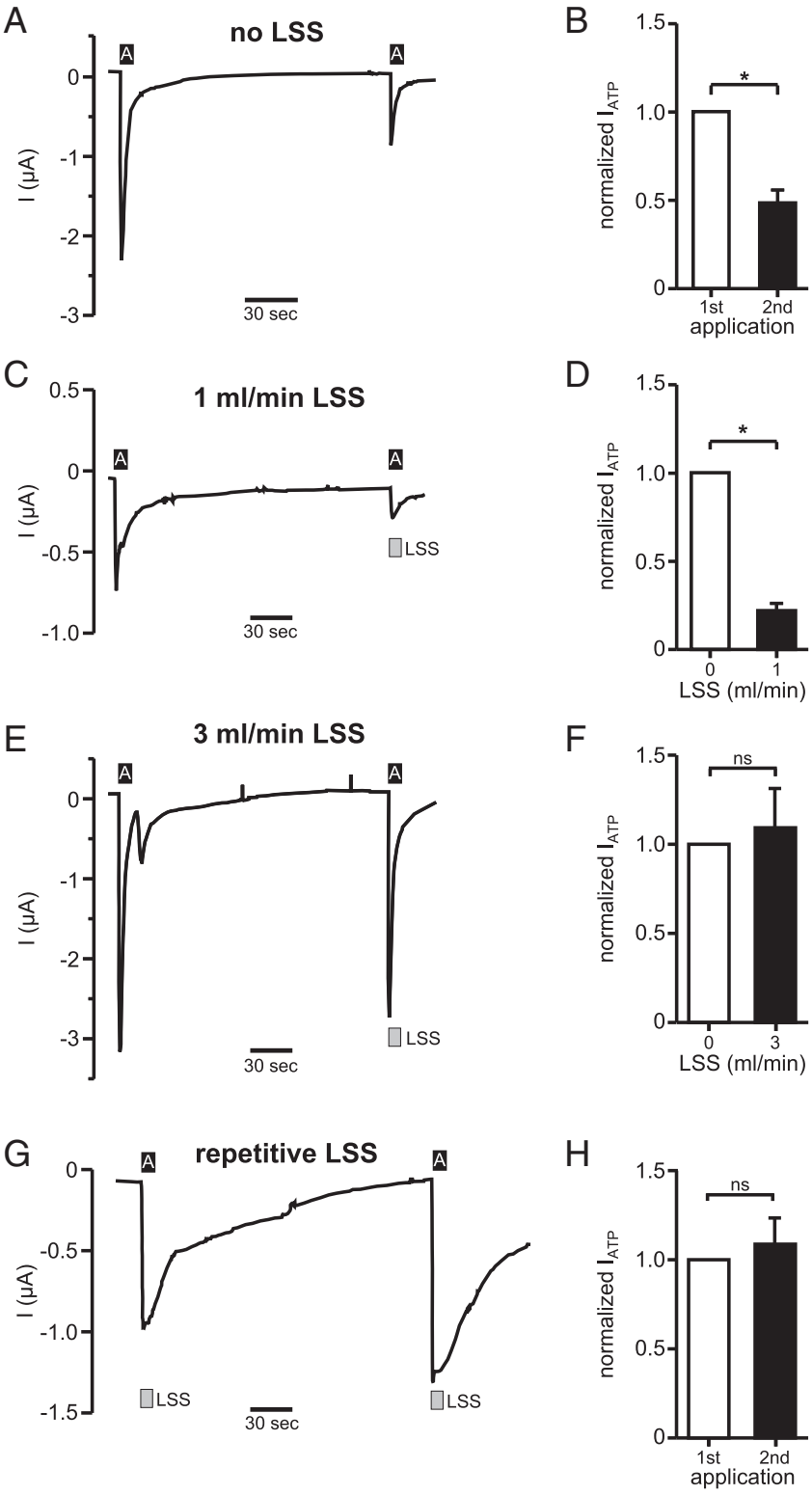

Fig. 3. Modulation of the ATP-induced current by LSS. (A) Recording of an experiment depicting the repeated application of 100 μM ATP on $P2X_4$ expressing oocytes. Between the first and the second ATP application, ATP was washed out 2 min with Ringer's solution. (B) Statistical evaluation of the experiments shown in A. Values represent normalized ATP-induced currents, where the first application (1st) is referred as 1 and the second ATP-induced current (2nd) was normalized to the first ATP effect. The second ATP-induced current was significantly smaller than the first ATP-induced current ($n = 27$, $N = 7$; $p < 0.05$). (C) Influence of LSS on the ATP-induced current. $P2X_4$ expressing oocytes were exposed twice to ATP (100 μM) — in the absence as well as with LSS with a flow rate of 1 ml/min. (D) The second ATP effect (with LSS) was also significantly smaller than the first ATP effect (without LSS; $n = 10$, $N = 2$; $p < 0.05$). (E) Current trace of an experiment as conducted in C, but with a LSS flow rate of 3 ml/min. (F) The effect of ATP under LSS with a flow rate of 3 ml/min was not significantly different compared with the preceding control ATP effect ($n = 22$, $N = 7$; $p = 0.66$). (G) Representative recording depicting the repeated application of 100 μM ATP together with LSS (flow rate of 3 ml/min). (H) Statistics comparing the means of the first effect evoked by ATP and LSS application with the second ATP and LSS application revealed that the means were not significantly different from each other ($n = 12$, $N = 5$).

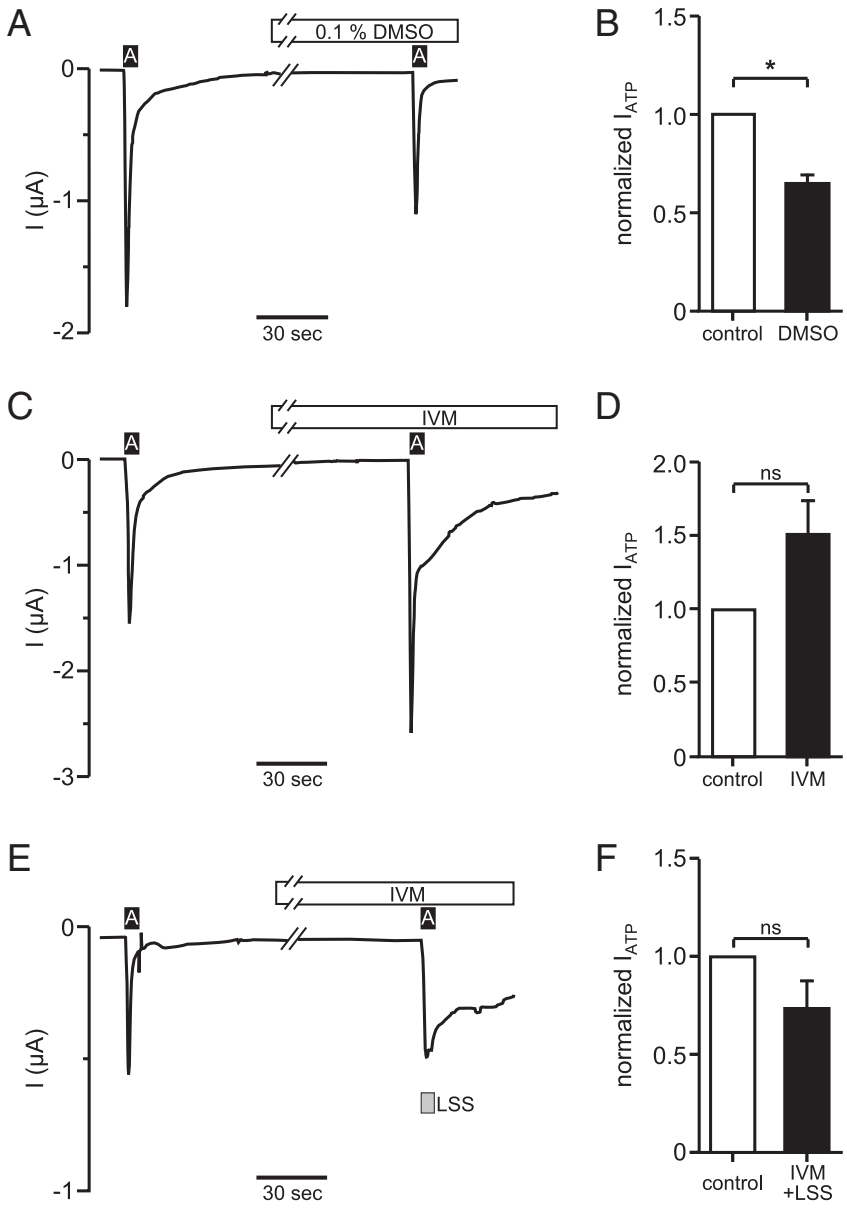

Fig. 4. Ivermectin (IVM) prevents the LSS-mediated effect. (A) Control experiment illustrating the ATP effect (100 μM) in the presence and the absence of DMSO (0.1 vol.%), the solvent of IVM. Oocytes were incubated for 3 min with DMSO until ATP was applied for a second time. (B) The second ATP-induced current (in the presence of DMSO) was significantly reduced compared with the first ATP-induced current (in the absence of DMSO; $n = 7$, $N = 3$; $p < 0.05$). (C) Original experiment evaluating the ATP effect in the absence and in the presence of IVM (10 μM, dissolved in DMSO). (D) Statistical evaluation of experiments presented in panel C. The second ATP application was not significantly different from the first application ($n = 12$, $N = 3$; $p = 0.06$). (E) Recording showing the effect of ATP in the absence and presence of IVM together with LSS (flow rate of 3 ml/min). (F) No significant difference between the first and the second ATP effect was detected when the second ATP application was performed in the presence of IVM and LSS (3 ml/min; $n = 9$, $N = 3$; $p = 0.24$).

receptors. This was due to preventing of desensitization upon a repetitive ATP exposure (Fig. 3). This observation clearly indicates, that the activity of $P2X_4$ receptors can be modulated by LSS. Generally this is in agreement with studies by Yamamoto et al. [20], describing that fluid shear stress activated a Ca^{2+} influx via $P2X_4$ receptors in human endothelial cells. Yamamoto and colleagues postulated that fluid shear stress stimulated ATP release from endothelial cells and this subsequently led to an activation of $P2X_4$ receptors. This proposed mechanism identifies $P2X_4$ receptors as a part of a mechanotransduction process. Activation of $P2X_4$ receptors in this case depends on the mechanically induced release of ATP from endothelial, which is well established [36,27], although the particular mechanism of ATP release is still under debate [37,38].

Our findings are in agreement with the mechanism that was postulated by Yamamoto and colleagues because LSS alone does not activate $P2X_4$ receptors. Nevertheless, our study provides for the first time evidence concerning the ability of LSS to modulate $P2X_4$ receptor activity and thereby that the receptors are able to detect/sense mechanical forces.

4.2. Putative mechanism of the LSS–mediated effect

In order to evaluate the putative mechanism by which LSS modulates the activity of the $P2X_4$ receptors experiments with ivermectin (IVM) were performed. IVM is a macrocyclic lactone that specifically enhances the ATP-induced current of $P2X_4$ receptors by interacting with transmembrane residues, which are close to the extracellular side of the membrane [39,40]. This interaction is characterized by an increase of the ATP-induced current amplitude due to reducing the desensitization of the channels and by slowing their deactivation [40]. As identified by

single channel recordings the reason for these effects could be attributed to the fact, that IVM stabilizes the open state of the receptors [40]. In accordance to these findings IVM abolished the desensitization of the ATP-induced activation as observed by the repetitive ATP application (Fig. 4C, D). Interestingly, the same effect on the ATP-induced current was observed by the application of LSS and IVM together (Fig. 4E, F), as well as with LSS alone (Fig. 3E, F). The lack of an additional effect of LSS in the presence of IVM could be a hint of a similar mechanism.

Therefore, it could be speculated that LSS – similar like IVM – stabilizes the open state of $P2X_4$ receptors. Further, it could be hypothesized that the IVM binding domain might be involved in LSS-mediated $P2X_4$ receptor gating. This domain could be responsible for the connection of the extracellular loops (that may function as the shear sensors) with the transmembrane domains. The motion of the transmembrane domain 2 (TM2) is considered to open the channel pore [41]. Opening of the channel definitely depends on ligand binding that provides the energy for the motion of TM2. Channels with bound ligands desensitize which means the transition from an open to a close state, while the ligand is bound. IVM is suggested to interfere with the transmembrane domains in the open state [42]. The potential mechanism how IVM stabilizes the open state of the receptors could be, that IVM binding/interference with the TM may provide free energy that prevents the transition to the closed state. Our data indicate that LSS can mimic the effect of IVM and this might be explained by the fact that LSS also provides energy that stabilized the open state similar to the effect that is caused by the binding of IVM.

5. Conclusions

This study provides evidence, that LSS is able to modulate the ATP-mediated response of $P2X_4$ receptors and the results indicate that $P2X_4$ receptors are able to detect mechanical forces. This property is of considerable importance for physiological processes and for the function of endothelial cells in particular. Endothelial cells are permanently exposed to laminar shear stress due to the blood flow and $P2X_4$ receptors are ubiquitously expressed in theses cells. Further, this property is important for the transduction of shear forces, since these forces have been identified as crucial triggers for many endothelial cell functions. These findings should be point of departure for further investigations, to clarify the mechanism(s) how $P2X_4$ receptors are able to sense laminar shear stress.

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