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Electrophysiological and functional effects of sphingosine-1-phosphate in mouse ventricular fibroblasts

Najate Benamer [a], Nassim Fares [b], Patrick Bois [a], Jean-François Faivre [a,*]

[a] UMR CNRS/Université de Poitiers No. 6187, Pôle Biologie Santé Bât B36, BP 633, 1 rue Georges Bonnet, 86022 Poitiers, France
[b] Laboratoire de Physiologie, Faculté de Médecine, Université Saint Joseph, Beyrouth, Lebanon

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ABSTRACT

The aim of this study was to characterize the effects of sphingosine-1-phosphate (S1P) on cardiac ventricular fibroblasts. Impacts of S1P on fibroblast excitability, cell migration, proliferation and secretion were characterized. The patch-clamp technique in the whole-cell configuration was used to study the S1P-induced current from mouse ventricular fibroblasts. The expression level of the S1P receptor during cell culture duration was evaluated by western-blot. Fibroblast proliferation and migration were quantified using the methylene blue assay and the Boyden chamber technique, respectively. Finally, fibroblast secretion properties were estimated by quantification of the IL-6 and collagen levels using ELISA and SIRCOL collagen assays, respectively. We found that S1P activated SUR2/Kir6.1 channel and that this effect was sensitive to specific inhibition of the S1P receptor of type 3 (S1P3R). In contrast, S1P1R receptor inhibition had no effect. Moreover, the S1P-induced current increased with cell culture duration whereas S1P3R expression level remained constant. The activation of SUR2/Kir6.1 channel by S1P *via* S1P3R stimulated cell proliferation and decreased IL-6 and collagen secretions. S1P also stimulated fibroblast migration *via* S1P3R but independently from SUR2/Kir6.1 channel activation. This study demonstrates that S1P, *via* S1P3R, affects cardiac ventricular fibroblasts function independently or through activation of SUR2/Kir6.1 channel. The latter effect occurs after fibroblasts differentiate into myofibroblasts, opening a new potential therapeutic strategy to modulate fibrosis after cardiac physiopathological injury.

1. Introduction

In pathological conditions, fibroblasts are submitted to a process of differentiation into myofibroblast, notably characterized by the expression of alpha smooth muscle actin (SMA) [1]. In response to pathological stress, myofibroblasts show altered proliferation, migration and secretion properties and modulate the ECM turnover through increase in protein synthesis such as collagen [2].

Recently, we have reported for the first time in these cells the functional expression of a potassium channel resulting from the association of SUR2 and Kir6.1 proteins [3]. We have also found that sphingosine-1-phosphate (S1P), a sphingolipid which is secreted by macrophages and platelets in response to cell damage activate this channel. Nevertheless, the mechanism involved in S1P-induced activation of SUR2/Kir6.1 channel as well as functional impacts of S1P on fibroblasts were not elucidated and represent the main objectives of the present study.

S1P receptors which are ubiquitously expressed, may modulate many cellular functions such as proliferation, differentiation and migration [4,5].

In the cardiovascular system, sphingolipids are considered as signaling mediators during pathological conditions [6]. In the heart, the presence of S1P receptors has been demonstrated [7,8], with a prevalence of isoform 1 in myocytes and isoform 3 in fibroblasts [9,10]. Functionally, activation of S1P receptors has been shown to protect cardiomyocytes from ischemia reperfusion damages *in vivo* [11]. For example, it has been reported that S1P *via* the S1P3 receptor significantly reduced infarction size in a model of myocardial ischemia/reperfusion by the inhibition of inflammatory neutrophil recruitment and cardiomyocyte apoptosis in the infarcted area [12]. Finally, S1P plays a key role in cardiac fibroblast proliferation and in the regulation of proinflammatory responses during hypoxia [13]. Whereas the S1P pathways are thus considered as determinant in cardiac physiopathology processes, the S1P effects on cardiac fibroblast remain poorly understood. The objectives were on the first hand to determine how S1P activated SUR2/Kir6.1 channel and on the second hand to evaluate whether S1P was able to affect proliferation, migration and secretion properties of cardiac fibroblasts.

* Corresponding author. Address: UMR CNRS/Université de Poitiers No. 6187, Pôle Biologie Santé Bât B36, BP 633, 1 rue Georges Bonnet, 86022 Poitiers, France. Fax: +33 5 49 45 40 14.
E-mail address: Jean-Francois.Faivre@univ-poitiers.fr (J.-F. Faivre).

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2. Materials and methods

2.1. Isolation and culture of adult mouse ventricular fibroblasts

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Cardiac fibroblasts were obtained from adult females Swiss mice and maintained in primary culture according to previously published methods [14]. This method resulted in a fibroblast enriched cell population which was not contaminated by endothelial and/or smooth muscle cells as controlled by immunolabelling studies performed in a previous study [14].

2.2. Western blotting

Western blotting were performed as previously described [3]. Briefly, fibroblasts were scraped on ice into 500 μL of lysis buffer containing protease inhibitors and phosphatase inhibitors. The suspension was sonicated for 30 s and centrifuged at 14,000 rpm for 5 min at 4 °C. Protein samples were then denatured for 3 min at 95 °C. Protein preparations were first separated on 12% SDS–polyacrylamide gels and then transferred to immunoblot membranes during 2 h. Membranes were blocked 1 h in TBS-Tween solution and incubated overnight at 4 °C with primary antibodies (anti-receptor of S1P 3 rabbit antibody (1/500 abcam) and the anti-GAPDH mouse antibody (1/1000 Invitrogen)) in TBS-Tween containing 2% BSA. Membranes were then incubated for 1 h at room temperature with Alexa Fluor 488 chicken anti-rabbit igG and Alexa Fluor 555 donkey anti-mouse (1/2000 Invitrogen).

2.3. Electrophysiological recordings

The whole-cell configurations of the patch-clamp technique was used to record currents *via* a patch-clamp amplifier (Axopatch 200B, Axon Instruments) interfaced to a Digidata acquisition system controlled by Clampex version 9.1 software (Axon Instruments). Patch electrodes were filled with an internal solution containing (in mM): 12 NaCl, 20 KCl, 110 K-aspartate, 1 $CaCl_2$, 1 $MgCl_2$, 2 K_2ATP, 10 EGTA, and 10 HEPES (pH adjusted to 7.2 with KOH). The extracellular solution was a modified Tyrode's solution containing (in mM): 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH adjusted to 7.4 with NaOH). All experiments were performed at room temperature (23–25 °C). Cell capacitance was measured by integrating the area under the capacitative transient elicited by 5 mV depolarizing steps from a holding potential of 0 mV. Data were analysed with pCLAMP software (Axon Instruments) and Origin 6.1 (OriginLab).

2.4. Cell proliferation assay

Fibroblast proliferation was evaluated every 48 h from 4 to 10 days after treatment following a procedure adapted from Oliver et al. [15]. After washing in PBS, cells were fixed in 0.5% glutaraldehyde and stained with a 1% methylene blue solution in borate buffer (pH 8.5) for 1 h. Then, cells were rinsed in distilled water and dried at room temperature overnight. Fixed methylene blue was removed by exposure to 0.1 N HCl for 1 h and OD (optical density) of supernatants was determined at 610 nm. All conditions were performed in triplicate.

2.5. IL-6 measurement by enzyme-linked immunosorbent assay (ELISA)

The DuoSet ELISA Development System (R and D Systems, UK) were used to quantify the IL-6 concentrations in fibroblast supernatant according to the manufacturers' protocol. All samples were measured in duplicate.

2.6. Collagen measurements by SIRCOL collagen kit assay

Total soluble collagen in cell culture supernatants was quantified using the SIRCOL collagen assay (Biocolor, Belfast, UK). Sirius Red (1 ml), an anionic dye that reacts specifically with $(Gly\text{-}X\text{-}Y)_n$ tripeptide in the triple-helix sequence of mammalian collagens under assay conditions, was added to 100 μL of supernatant and incubated under gentle rotation for 30 min at room temperature. After centrifugation for 10 min at 12,000*g*, the collagen-bound dye was redissolved with 1 ml of 0.5 M NaOH, and the absorbance was measured at 540 nm. All samples were measured in duplicate.

2.7. Cell migration assays

The ability of fibroblasts to migrate was investigated by the Boyden chamber technique. This was achieved with 24-well culture plate with insert containing an 8 μm pore size polycarbonate membrane (BD Biosciences). The membranes were coated before use with 25 μg/μL of collagen. After 8 days of culture, fibroblasts were trypsinized and resuspended in serum free DMEM before plating at 5×10^4 cells in the upper chamber. DMEM complemented with 10% of fetal calf serum was placed in the lower chamber. The fibroblast migration is allowed by the fetal calf serum gradient. After incubation during 4 h at 37 °C the suspension were aspirated and cells on the upper side of the filter were scraped off. Fibroblasts that had migrated to the lower side of the filter were fixed with paraformaldehyde 4% for 30 min and stained with Violet Crystal during 30 min. All conditions test were performed in triplicate.

2.8. Statistics

Results are presented as mean ± SEM. Statistical analysis was performed using repeated-measures analysis of variance followed by multiple comparisons using the Sidak procedure. A p value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Activation of a glibenclamide-sensitive current by S1P via the S1P3R

In our previous study, we reported that a pinacidil-activated SUR2/Kir6.1 channel was present in mouse cardiac fibroblasts [3] and we demonstrated that S1P was able to activate this channel at 20 nM. To characterize further this effect, different experiments have been conducted using the whole cell configuration of the patch clamp technique. Fig. 1A illustrates variation in steady-state current amplitude recorded at +50 mV in control condition and during the application of S1P. As expected, S1P (20 nM) activated an outward current at +50 mV. Kinetic analysis of this activation shows that current–density increased from 5.04 pA/pF ± 0.6 in control condition to 10.44 pA/pF ± 0.3 after about 2 min of S1P application. S1P-induced current was inhibited by the simultaneous application of glibenclamide (10 μM) in 2.5 min. The current–voltage relationship of the glibenclamide-sensitive current obtained after application of S1P at 20 nM (Fig. 1B) shows a reversal potential of −60 mV, which confirms that S1P can activate a potassium conductance sensitive to glibenclamide in mouse ventricular fibroblasts. To dissect out the effect of S1P on the SUR2/Kir6.1 channel, W146 and BML241, known as antagonists of the type 1 and type 3 of S1P receptors (S1PR), respectively, were tested at 10 μM. As shown in Fig. 1C, the S1P-induced current was not

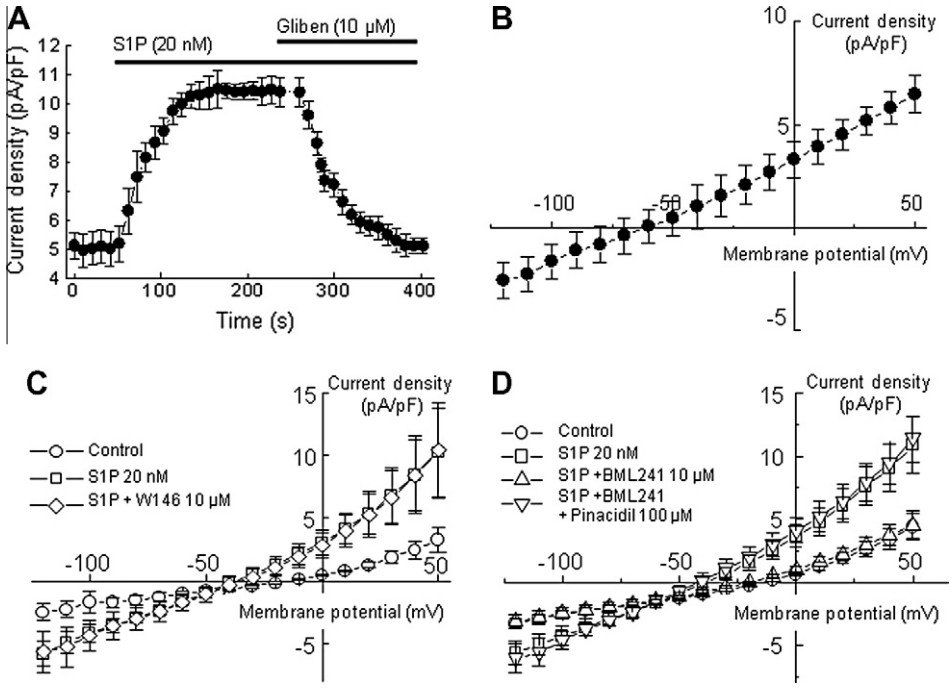

Fig. 1. Effect of S1P on whole-cell currents recorded from mouse ventricular fibroblasts. (A) Effects of S1P (20 nM) and S1P + glibenclamide (10 μM) on whole-cell currents recorded at a membrane potential of +50 mV (n = 7). Horizontal bars correspond to the external application of indicated compounds. (B) Current–voltage relationship of the glibenclamide-sensitive current measured after the application of S1P 20 nM. (C) I/V relationships obtained under control conditions, in the presence of S1P alone or supplemented with W146 10 μM (n = 4). (D) I/V relationships obtained under control conditions, in the presence of S1P 20 nM, in the presence of S1P + BML241 10 μM (n = 9) and in the presence of S1P + BML241 + pinacidil 100 μM (n = 4).

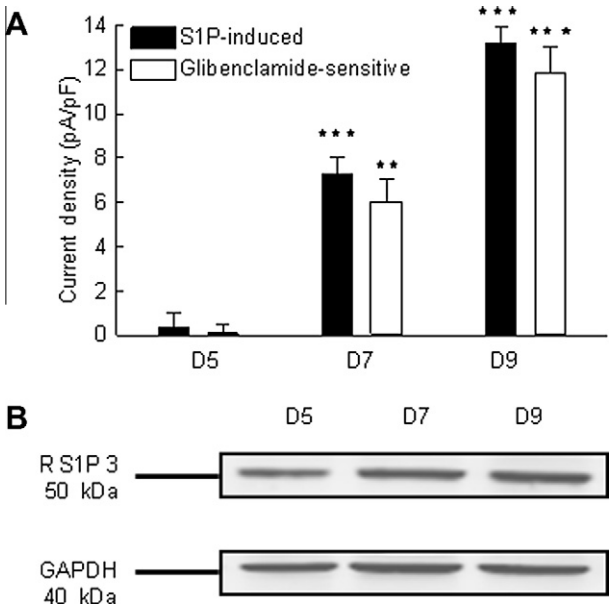

Fig. 2. Changes in S1P-induced current and in protein expression of S1P receptor of type 3 with culture duration in mouse ventricular fibroblasts. (A) S1P-induced and glibenclamide-sensitive current density at +50 mV were measured after 5 (n = 7), 7 (n = 7) and 9 days (n = 6) of cell culture. $^{**}p < 0.01$; $^{****}p < 0.001$ versus day 5 within each group. (B) Western blots were performed with protein preparations obtained from adult mouse ventricular fibroblasts at 5, 7 and 9 days of culture using the anti-receptor of S1P-3 rabbit antibody (1/500 abcam) and the anti-GAPDH mouse antibody (1/1000 Invitrogen) as an internal control (n = 2).

sensitive to W146. On the opposite, the current activated by S1P was completely inhibited by the simultaneous application of BML241 (Fig. 1D). Moreover, application of pinacidil after the inhibition of the S1P-induced current by BML241, activated a current with density similar to the S1P-induced current (Fig. 1D). All of these results suggest that S1P activates SUR2/Kir6.1 channel *via* the S1P3R, independently of S1P1R.

3.2. Analysis of the S1P-induced current and the S1P3R protein expression with cell culture duration

In Fig. 2A, we found that S1P was unable to activate currents before 7 days of culture. Afterwards, S1P-induced current increased significantly with time as shown by the comparison between the current recorded at 5, 7 and 9 days of culture. In all cases, the S1P-induced and the glibenclamide-sensitive currents were not significantly different, illustrating that the S1P-induced current was completely inhibited by glibenclamide. In agreement with the increase in expression of SUR2 and Kir6.1 proteins observed previously during cell culture [3], we show here that the S1P-induced current increases with cell culture duration. This result further confirms that S1P activates SUR2/Kir6.1 channel.

The potential change of the S1P3R protein expression with cell culture duration was also evaluated to estimate whether a progressive increase in S1P3R expression may contribute to the S1P-induced current increase (Fig. 2A). A western blot was stained with anti-S1P3R and GAPDH antibodies using in each well 100 μg of total protein preparations extracted at day 5, 7 and 9 days of fibroblast culture (Fig. 2B). Results show that the S1P3R is expressed in mouse ventricular fibroblasts and that the level of expression remained constant from 5 days up to 9 days of culture.

3.3. Impact of S1P on proliferation and migration

In response to pathological conditions, fibroblasts increase their proliferation and motility and become highly invasive. As illustrated in Fig. 3A, the number of fibroblasts increased with cell culture duration in control condition. Whereas ineffective from 0 to 5 days of culture, S1P significantly increased fibroblast prolifera-

tion when compared to control condition from D6 to D9. Simultaneous application of the S1P3R inhibitor BML241 completely abolished the effect of S1P on cell proliferation. A similar inhibitory effect was also obtained in the presence of glibenclamide, confirming the involvement of S1P3R and SUR2/Kir6.1 channel in this effect. These data strongly suggest that S1P modulates proliferation through the S1P3R and the activation of SUR2/Kir6.1 channel. To evaluate whether SUR2/Kir6.1 channel activation by S1P or pinacidil may influence fibroblast migration, we analyzed cellular migration at 8 days of culture in Boyden chamber (Fig. 3B). As expected, S1P stimulated fibroblast migration compared to control. In the presence of BML241, the effect of S1P was completely antagonised, suggesting that S1P effect on fibroblast migration occurred *via* S1P3R. On the opposite, inhibition of the SUR2/Kir6.1 channel by glibenclamide was ineffective, suggesting that S1P effect on fibroblast migration did not result from the activation of SUR2/Kir6.1 channel. Accordingly, modulation of SUR2/Kir6.1 channel by pinacidil with or without glibenclamide had no effect on fibroblast migration.

3.4. S1P effects on IL-6 and collagen secretion

In disease states, fibroblasts increase the secretion of pro-inflammatory and anti-inflammatory cytokines and amplify the ECM turnover by increasing synthesis of specific proteins including collagen. This rise in collagen secretion promotes fibrosis during cardiac remodeling [16]. The effects of S1P and SUR2/Kir6.1 channel activation on IL-6 and collagen secretion by ventricular fibroblasts have not yet been reported and were evaluated every 48 h from 4 to 10 days (Fig. 4A and B). We found that IL-6 and collagen secretion from cardiac fibroblasts increased with cell culture duration in control condition (Fig. 4A and B). In the presence of S1P, IL-6 secretion level was similar from 0 to 5 days of culture, but was significantly decreased from 6 days of culture when compared to control. Moreover, S1P effect on IL-6 secretion was completely inhibited by the S1P3R inhibitor BML241 and by the SUR2/Kir6.1 channel inhibitor glibenclamide.

As illustrated in Fig. 4B, S1P decreased collagen secretion from 6 days of culture. Inhibition of the S1P3R by BML241 antagonised the effect of S1P as well as the SUR2/Kir6.1 channel inhibitor glibenclamide. Similar results have been obtained when SUR2/Kir6.1 channels were activated by pinacidil (data not shown), i.e. pinacidil decreased collagen secretion from 6 days of culture and this effect was inhibited by glibenclamide.

4. Discussion

This study shows that the activation of SUR2/Kir6.1 channel by S1P occurs *via* the isoform 3 of the S1P receptor (S1P3R) and inde-

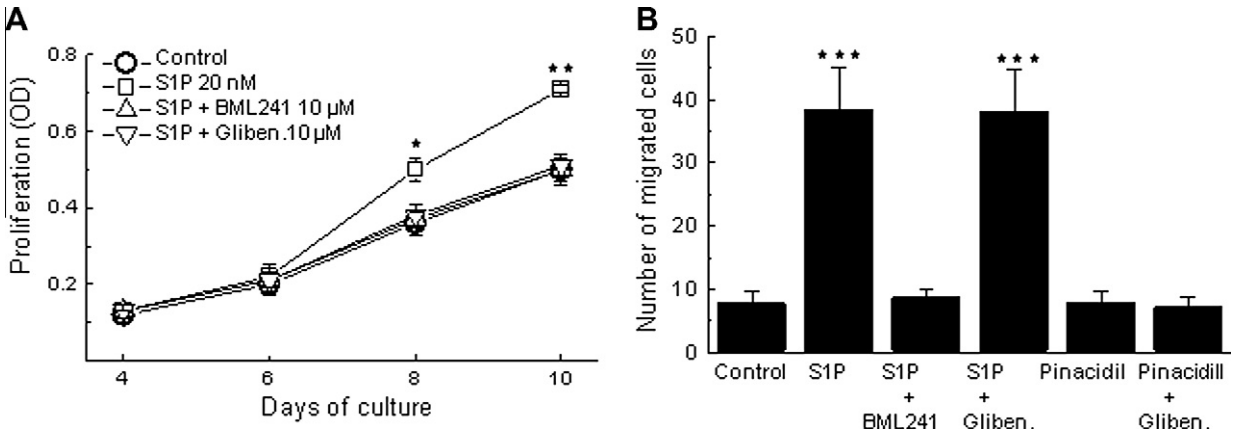

Fig. 3. Effects of S1P on proliferation and migration in mouse ventricular fibroblast. (A) The effects of S1P (20 nM), S1P + glibenclamide (10 µM) or +BML241 (10 µM) were evaluated on ventricular fibroblast proliferation by methylene blue assay during 10 days of culture every 48 h ($n = 4$). (B) The migration of ventricular fibroblast was measured at 8 days of culture in control condition or in the presence of S1P (20 nM), S1P (20 nM) + BML241 (10 µM) or +glibenclamide (10 µM) and in pinacidil (100 µM) alone or supplemented with glibenclamide (10 µM) ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ versus control.

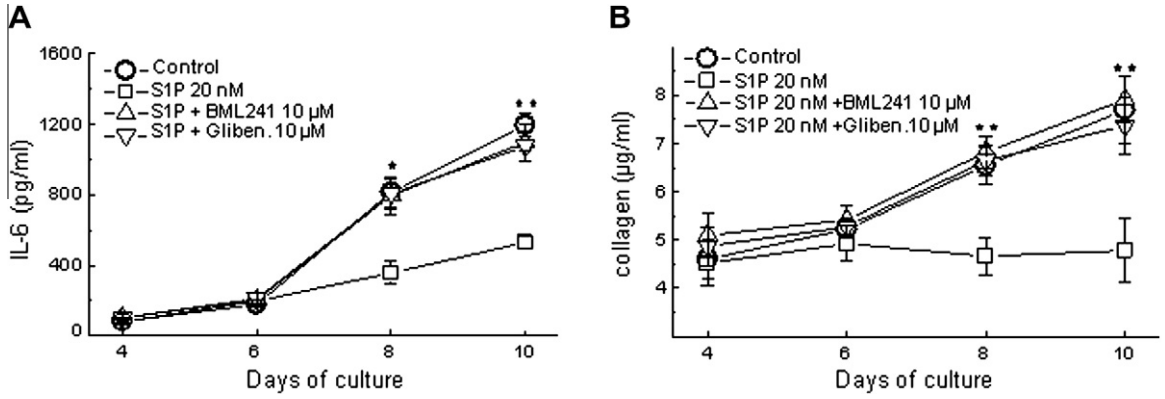

Fig. 4. Effects of S1P on Il-6 and collagen secretion. (A) The secretion of interleukin 6 was measured by ELISA from 4 to 10 days of culture in control condition, or in the presence of S1P 20 nM, S1P 20 nM + glibenclamide 10 µM or +BML241 (10 µM). (B) The secretion of collagen was measured from 4 to 10 days of culture in control, S1P 20 nM and S1P 20 nM + glibenclamide 10 µM or +BML241 (10 µM) ($n = 5$). ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ versus control.

pendently from the S1P1R in ventricular fibroblasts. During cell culture duration, the S1P-induced current–density increases progressively whereas S1P3R protein expression remains constant. Another major finding is that S1P affects proliferation, migration and secretion properties of ventricular fibroblasts. The effects observed with S1P on proliferation, IL-6 and collagen secretion occur *via* the S1P3R and are antagonised by glibenclamide, suggesting a major contribution of SUR2/Kir6.1 channel activation. On the opposite, S1P stimulates fibroblast migration *via* S1P3R, but independently from SUR2/Kir6.1 channel.

4.1. S1P effects via SUR2/Kir6.1 channel

We have previously reported that S1P was an activator of the SUR2/Kir6.1 channel and that pinacidil-induced and S1P-induced currents in mouse ventricular fibroblasts were similar [3]. The reversal potential of the glibenclamide-sensitive current obtained after the application of S1P is in accordance with strong potassium selectivity (Fig. 1B). In the literature, it has been shown that glibenclamide can inhibit not only SUR/Kir channels but also CFTR chloride channels. However, we have shown in a preliminary study that no CFTR current was present in ventricular fibroblasts. Indeed, forskolin was unable to induce any chloride flux from mouse ventricular fibroblasts [17]. On another hand, S1P has been demonstrated to activate 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS)-sensitive chloride channels in human lung fibroblasts [18]. The S1P-induced current recorded in our study was not sensitive to DIDS at concentrations up to 100 μM (data not shown). We thus conclude that the S1P-induced current in our conditions is mainly carried by potassium ions.

In the present work, one of our objectives was to determine the mechanism involved in SUR2/Kir6.1 channel activation by S1P. This effect is completely blocked by BML241 (an antagonist of S1P3R) but is not modified by W146 (an antagonist of S1P1R) suggesting that sphingosine-1-phosphate modulates the SUR2/Kir6.1 channel essentially *via* the S1P3R. After the inhibition of the S1P-induced current by BML241, pinacidil was still able to activate specifically SUR2/Kir6.1 (Fig. 1D), demonstrating that the SUR2/Kir6.1 channel and the S1P3R can be modulated independently.

Another interesting point is that the S1P-induced current increased with fibroblast culture duration (see Fig. 2A). In our previous study, SUR2/Kir6.1 expression in cardiac fibroblasts has also been reported to increase with time culture [3]. Because the S1P3R protein expression is constant with time, it can be extrapolated that the increase in the S1P-induced current exclusively results from the progressive rise in SUR2/Kir6.1 protein expression. These results further confirm that the electrophysiological consequences of S1P on fibroblast result from the activation of the SUR2/Kir6.1 channel.

Interestingly, SUR2/Kir6.1 protein expression, and the S1P-induced current increases with cell culture duration are concomitant with the differentiation of fibroblasts into myofibroblasts. These results suggest that SUR2/Kir6.1 channels activation by S1P occur only when fibroblasts differentiate into myofibroblasts. During physio-pathological stress, activation of SUR2/Kir6.1 channels by S1P in the infarct border zone where myofibroblasts are more numerous and where cell damages may contribute to an increase in S1P secretion may thus have an important impact on cardiac function.

S1P binding on the S1P3R is able to modulate fibroblast function including proliferation, IL-6 and collagen secretion. These effects are similar to those obtained with pinacidil and are antagonised by glibenclamide, suggesting that they occurred through SUR2/Kir6.1 channel activation. Moreover, all these S1P effects on fibroblast function increased with the duration of cell culture similarly with the S1P induced current. Proliferative effect of S1P in cardiac fibroblast was already described by Kacimi et al. [13] who reported that sphingosine-kinase1 (SK1) involved in S1P synthesis was required for optimal cardiac fibroblast proliferation during cardiac hypoxia [13].

During pathological injury, excessive collagen production and IL-6 secretion by cardiac fibroblast may promote cardiac fibrosis and may constitute a cardiomyocyte pro-hypertrophic signal [19,20]. The decrease in collagen and IL-6 secretion promoted by S1P *via* the SUR2/Kir6.1 channel might thus slow down this fibrotic process and reduce pro-hypertrophic signal. Nevertheless, stimulation of fibroblast proliferation by S1P may counterbalance these beneficial effects.

4.2. S1P effect on cell migration

S1P stimulated fibroblast migration *via* the S1P3R. This effect is in agreement with different reports from the literature. For example, S1P stimulates the migration of S1P3R transfected CHO cells and S1P3 antisense oligonucleotide impairs S1P-induced migration in HUVEC [21,22].

Contrary to S1P effects on proliferation and secretion properties, S1P modulation of fibroblast migration was not antagonised by glibenclamide, suggesting that this latter effect occurs independently from the activation of the SUR2/Kir6.1 channel. Accordingly, pinacidil was not able to mimic S1P. It may be suggested that the effect of S1P on SUR2/Kir6.1, proliferation and secretion occurred *via* signaling pathway independent from those involved in migration process.

4.3. S1P3R, a potential therapeutic target through SUR2/Kir6.1 channel in cardiac fibroblast

The present study shows that S1P modulates electrophysiological properties, cell proliferation, migration and excitation–secretion coupling in cardiac fibroblast. All these effects occurred *via* S1P3R. To understand further this mechanism, it would be important to characterize fully the signaling pathways involved in S1P effects observed on electrophysiological and functional properties of cardiac fibroblast.

S1P has already been reported to play an important role in the heart during cardiac remodeling, giving to sphingolipids a potential therapeutic role during myocardial injury. Majority of the S1P roles in the heart have been reported in cardiomyocytes and at concentrations much higher than those effective in fibroblasts (micromolar versus tens of nanomolar). To characterize better the effect of S1P on heart properties through cardiac fibroblast, an important perspective of the present work would consist in evaluating the effect of S1P *via* S1P3R in an *in vivo* physiopathological model of myocardial ischemia–reperfusion. Moreover, it seems important to test S1P on other fibroblast targets like those implied in Ca^{2+} signaling pathways in cultured human cardiac fibroblasts [23].

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