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Arachidonic acid effect on the allosteric gating mechanism of BK (Slo1) channels associated with the β1 subunit

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ABSTRACT

Arachidonic acid (AA) is a fatty acid involved in the modulation of several ion channels. Previously, we reported that AA activates the high conductance Ca^{2+}- and voltage-dependent K^+ channel (BK) in vascular smooth muscle depending on the expression of the auxiliary β1 subunit. Here, using the patch-clamp technique on BK channel co-expressed with β1 subunit in a heterologous cell expression system, we analyzed whether AA modifies the three functional modules involved in the channel gating: the voltage sensor domain (VSD), the pore domain (PD), and the intracellular calcium sensor domain (CSD). We present evidence that AA activates BK channel in a direct way, inducing VSD stabilization on its active configuration observed as a significant left shift in the Q-V curve obtained from gating currents recordings. Moreover, AA facilitates the channel opening transitions when VSD are at rest, and the CSD are unoccupied. Furthermore, the activation was independent of the intracellular Ca^{2+} concentration and reduced when the BK channel was co-expressed with the Y74A mutant of the β1 subunit. These results allow us to present new insigths in the mechanism by which AA modulates BK channels co-expressed with its auxiliary β1 subunit.

1. Introduction

Large conductance Ca^{2+}- and voltage-dependent channels (BK or Maxi K) are expressed in most of the mammalian cell types where are involved in different physiological processes such as smooth muscle cells relaxation, K^+ secretion in the kidney, action potential repolarization, among others [1,2]. The functional versatility of this channel is, in part, conferred for its gating operation since it can be independently activated by an increase in intracellular Ca^{2+} concentration, by membrane depolarization, or by a concerted activation of both stimuli. Moreover, it can also open in the absence of Ca^{2+} and membrane depolarization [1,3]. BK channels are tetramers of pore-forming α-subunits (encoded by the KCNMA1 gene, also named *Slo1*) and can be associated with one of several auxiliary subunits according to the specific cell type and tissues. Two classes of auxiliary subunits, with four members each one, have been identified so far: the β-subunits and the γ-subunits [4]. Each auxiliary subunit confers distinctive functional and pharmacological properties to the resulting channel complex [5,6]. In particular, the differences in pharmacological sensitivity provided by the co-expression of different auxiliary subunit emerged as a key factor for tissue-selective BK channel modulation using the auxiliary subunits as the molecular target.

Abbreviations: BK channel, large conductance Ca^{2+}- and voltage-dependent channels; SMC, smooth muscle cells; TRP channels, transient receptor potential channels; PIP2, phosphatidylinositol 4,5-bisphosphate; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; AA, arachidonic acid; ALA, α-linolenic acid; VSD, voltage sensor domain; PD, pore domain; CSD, calcium sensor domain; HA model, Horrigan and Aldrich model; BS, bath solution; COX, cyclooxygenase; LOX, lipoxygenase; CYP 450, cytochrome P450; CDC, α-cyanocinnamate; 17-ODYA, 17-Octadecynoic Acid; DMSO, dimethyl sulfoxide; TM, transmembrane domain.

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BK channel and its β1 modulatory subunit are highly expressed in smooth muscle cells (SMC) where is involved in the regulation of cell contraction. These channels are activated during cell contraction (by the increase in cytosolic intracellular Ca^{2+} concentration and/or by depolarization) and also by a local Ca^{2+} increase due to the Ca^{2+} sparks released by the opening of ryanodine receptors at the sarcoplasmic reticulum [1,3]. The activation of BK channels induces cell membrane hyperpolarization, which reduces the open probability of voltage operated Ca^{2+} channels (VOCC) decreasing the Ca^{2+} influx and producing SMC relaxation [3]. Alterations in BK channel activity have been reported in several pathologies that include hypertension, asthma, erectile dysfunction, and overactive bladder, and consequently have encouraged researchers to pursuit new BK channel modulators as a possible therapeutic intervention [1,7–9]. Moreover, the co-expression of the BK channel with the β1 subunit in smooth muscle cells allows a cell type-specific response and modulation [5,10,11].

The modulation of ion channels by lipids is a common feature in cell physiology. Voltage operated K^+, H^+, Na^+ and Ca^{2+} channels as well as voltage-independent K^+ channels and transient receptor potential (TRP) channels can be activated or inhibited by a broad variety of lipids including, fatty acids and their metabolites, phosphatidylinositol 4,5-bisphosphate (PIP2), cholesterol, hormones and its analogs [12–14]. Although the effect of lipids on ion channels activity could be explained as an unspecific modulation, due to a modification of the protein-lipid environment, several studies proved that some of these modulations are produced by specific interaction between the lipid and the ion channel protein [15,16]. In particular, in BK channels, different lipid binding sites have been probed for docosahexaenoic acid (DHA), PIP_2, cholesterol, 17-β-estradiol, sulfatides, and cholane steroids [17–20]. Interestingly, the cholane steroid binding site, located in the β1-subunit, can be stimulated by their nonsteroidal analogs (e.g.: HENA). This represents the first example of a synthetic drug acting specifically on a lipid binding site with a β-subunit selective effect [21].

Fatty acids are components of the phospholipids that constitute the plasma membrane but are also relevant messengers in cell signaling [22]. Numerous studies showed that fatty acids increase BK channel open probability [16,23–25]. Besides, this effect is higher when the fatty acid presents a negatively charged head group and a longer unsaturated tail [16,23–25]. However, regardless of this common chemical requirement, Hoshi and collaborators demonstrated that there is more than one binding site for fatty acids. They probed that DHA (a polyunsaturated fatty acid (PUFA), 22:6 Ω-3,) interacts with the C-terminal of the S6 segment of the α-subunit of the BK channel, being the Y318 residue essential for DHA induced activation. However, this site is not involved in BK activation induced by other PUFAs such as arachidonic acid (AA, 20:4 Ω-6) and α-linolenic acid (ALA, 18:3 Ω-3), suggesting the existence of other specific binding sites [17]. The reported differences in the effect of PUFAs on BK channel activity are also related to the presence of auxiliary β-subunits. AA activates the BK channel only when it is co-expressed with β1, β2, or β3 subunits [26,27] while DHA activates the homomeric BK channel formed only by α-subunits but shows a major effect when the BK channel is co-expressed with β1 or β4 subunits [6].

BK channel is a modular protein with three domains: the voltage sensor domain (VSD, S0-S4 transmembrane segments), the pore domain (PD, S5-S6 transmembrane segments), and the intracellular calcium sensor domain (CSD, RCK1, and RCK2). Allosteric interactions between these modules control BK channel gating [1], being principally modulated by the strength of the interaction between VSD and CSD [28]. This allosteric nature of the BK channel gating was explained by the Horrigan and Aldrich model (HA model) [29]. Thus, the pharmacological modulation of BK channel gating is possible by targeting one or more modules, and the resulting effect will have different consequences according to the affected domain. For instance, DHA induces BK channel activation through the facilitation of opening transitions by a mechanism independent of the voltage- and Ca^{2+}- sensors activation. Moreover, this mechanism of action suggests a modification in the gate of the channel, which is consistent with the loss of DHA activity in Y318 mutants [6,17].

In our previous work, we demonstrated that AA activates BK channels associated with β1-subunit in human vascular smooth muscle cells, inducing cell hyperpolarization [27]. In the present study, using heterologous expression systems, we evaluate the effect of AA on the three functional modules of BKα/β1 channels and show that AA decreases the free energy of the closed-open transition of the channel when the VSD are in the resting state, and the CSD are unoccupied. Furthermore, AA-induced BK channel activation also involves the stabilization of the VSD in its active configuration, while it is independent of the intracellular Ca^{2+} concentration.

2. Methods

2.1. Clones and transient transfection

HEK 293 cells were grown in DMEM high glucose and 10% Fetal Bovine Serum and split when reaching 70–80% confluence. Human Slo1 α-subunit (KCNMA1, U11058), the wild-type auxiliary subunit β1 (KCNMB1, AF035046) or one of the mutants [Y74A, S104A] β1 subunits and enhanced green fluorescent protein cDNAs (eGFP;) were transiently transfected into HEK 293 cells using FuGene 6. The α/β1 subunits were mixed at 1:3 M ratios to ensure an excess of β1-subunit over α-subunit, and eGFP was used as a reporter for transfection. After transfection, cells were kept in culture for 18 h. The electrophysiological measurements were performed 18 h after transfection. eGFP clone were kindly provided by Dr. J. Raingo (IMBICE, Argentina.).

2.2. Electrophysiology

Ionic currents were recorded in macropatches from eGFP-positive cells. The isolated cells were observed with a mechanically stabilized inverted epifluorescence microscope (Arcano, China) equipped with a 40× objective lens. The tested solutions were applied through a multi-barreled pipette positioned close to the target cell. After each experiment on a single cell, the experimental chamber was replaced by another one containing a new sample of cells. All experiments were performed at room temperature (~22 °C). The standard tight-seal inside-out configuration of the patch-clamp technique was used to record single-channel and macroscopic currents [30]. Glass pipettes were drawn from WPI PG52165-4 glass on a vertical micropipette puller (Pul-100, World Precision Instruments, Sarasota, USA), and pipette resistance ranged from 2 to 4 MΩ.

Currents were filtered with a 4-pole lowpass Bessel filter (Axopatch 200A amplifier) at 2 kHz and digitized (Digidata 1440, Molecular Devices) at a sampling frequency of 20 kHz. The pipette solution contained the following (in mM): 140 KCl, 0.5 $MgCl_2$, 10 HEPES, 6 glucose, 1 $CaCl_2$; pH was adjusted to 7.4 with KOH. The bath solution (BS) contained the following (in mM): 140 KCl, 0.5 $MgCl_2$, 10 HEPES, 6 Glucose, 1 EGTA, pH adjusted to 7.4 with KOH. Final free Ca^{2+} concentration was calculated using Maxchelator (software from Stanford University: http://maxchelator.stanford.edu). The effect of AA on BK channels was tested by adding the adequate quantity to the BS at 10 μM final concentration. Current stability was monitored by applying successive 180 ms voltage steps (from a holding potential of 0 mV to a test potential of +110 mV), discarding cells in which the current amplitude did not remain constant in time. The same voltage-clamp step protocol was applied in control conditions as in the presence of AA. After current stabilization, a voltage pulse protocol which consisted in a family of 180 ms voltage steps between −200 and 200 mV from a holding potential of 0 mV followed by a 50 ms voltage step to -50 mV, was applied both in control conditions and in the presence of AA, for further conductance-voltage relationship (G-V) analysis. The G-V curves were fitted using the GraphPad Prism (version 5.03) with a Boltzmann relationship as follows:

$$\frac{G}{G_{max}} = \frac{1}{1 + e^{\frac{zF\left(V_{\frac{1}{2}} - V\right)}{RT}}}$$

where $V_{1/2}$ is the potential at which $\frac{G}{G_{max}}$ is the half of the G_{max}, z describes voltage sensibility (steepness of the curve), F is the Faraday constant (96,500 *C/mol*), R is the gas constant (8.314 $J/(mol \times K)$), and T is room temperature in Kelvin (approximately 295 K). In the cases where G_{max} was not experimentally reached under control conditions, we constrained the G_{max} for Boltzmann fitting with the value corresponding to AA in the same patch.

The free energy required to activate the channel was calculated as $\Delta G = zFV_{1/2}$, with the z and $V_{1/2}$ values determined from G/G_{max}–V curves [31]. The change in ΔG value induced by AA was calculated as $\Delta\Delta G = F\left(z_{AA}V_{1/2\,AA} - z_c V_{1/2\,c}\right)$, where z_c and $V_{1/2\,c}$ correspond to the values for the control conditions and, z_{AA} and $V_{1/2\,AA}$ are the values in the presence of AA.

The relevance of the VSD and CSD in AA activation of the BK channel was evaluated by measuring the single channel activity in the inside-out configuration at −120 mV in a Ca^{2+} free solution [29]. Currents were filtered with a 4-pole low pass Bessel filter at 2 kHz and digitized at 16 kHz. Recordings were taken up to 30–60 s in the absence and presence of AA to obtain the probability to find the channel in the open state (NPo). The identification of BK channel presence in the patch was based on its unitary conductance value. Open probability was expressed as NPo below, where N is the number of channels present in each patch, and its value was calculated using the following expression:

$$NPo = \frac{\sum_{j=1}^{n} j t_j}{T}$$

where T is the recording duration, and t_j is the time spent with j = 1,2,3, …n channels open.

2.3. Gating currents recordings

Xenopus laevis oocytes were used as a heterologous system to express BK α and BK α/β1 channels. The cDNA coding for the human BK α-subunit (U11058) or BKβ1 (U25138) harbored in pcDNA3.1 plasmids kindly provided by L. Toro (University of California, Los Angeles, CA). mMESSAGE mMACHINE from Ambion (Waltham, MA, USA) was used for in vitro transcription of both subunits. The oocytes were injected in a proportion of 1:5 of α:β1 with 0.5 μg/μl of the respective RNA. *Xenopus laevis* oocytes were incubated in an ND96 solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4) at 18 °C for 4–8 days before electrophysiological recordings.

All recordings were made by using the patch-clamp technique in the inside-out configuration. Data were acquired with an Axopatch 200B (Molecular Devices) amplifier and the Clampex 10 (Molecular Devices) acquisition software. Gating current (I_G) records were elicited by 1 ms voltage steps from −90 to 350 mV in increments of 10 mV. Both the voltage command and current output were filtered at 20 kHz using an 8-pole Bessel low-pass filter (Frequency Devices). Current signals were sampled with a 16-bit A/D converter (Digidata 1550B; Molecular Devices), using a sampling rate of 500 kHz. Linear membrane capacitance and leak subtraction were performed based on a P/4 protocol [32].

Borosilicate capillary glasses (1B150F-4, World Precision Instruments) were pulled in a horizontal pipette puller (Sutter Instruments). After fire-polishing, pipette resistance was 0.5–1 MΩ. The external (pipette) solution contained (in mM): 110 tetraethylammonium (TEA)-$MeSO_3$, 10 HEPES, 2 $MgCl_2$; pH was adjusted to 7.0. The internal solution (bath) contained (in mM): *N*-methyl-D-glucamine (NMDG)-$MeSO_3$, 10 HEPES, and 5 EGTA. Channels were exposed to AA by perfusing excised patches with an internal solution with 10 μM AA, at least 5–10 times the volume of the chamber. An agar bridge containing 1 M NaMES connected the internal solution to a pool of the external solution grounded with an Ag/AgCl electrode. All experiments were performed at room temperature (20–22 °C).

All data analyses were performed with Clampfit 10 (Axon Instruments), and Excel 2013 (Microsoft, Redmont, WA, USA). Gating currents were integrated between 0 and 400 μs after the voltage step to obtain the net charge movement of the ON component of the recordings. Q-V relationships were fitted with a Boltzmann function:

$$\frac{Q}{Q_{max}} = \frac{1}{1 + e^{\frac{zF\left(V_{\frac{1}{2}} - V\right)}{RT}}}$$

where Q_{max} is the maximum charge, z is the voltage dependency of activation, $V_{1/2}$ is the half-activation voltage. Q_{max}, $V_{1/2}$, and z were determined by using the solver complement of Microsoft Excel. Data were aligned by shifting them along the voltage axis by the mean $\Delta V_{1/2} = (<V_{1/2}> - V_{1/2})$, then binning them in a range of 25 mV, between −100 mV and up to 350 mV to generate a mean curve that did not alter the voltage dependence. All error estimates are SEM.

2.4. Inhibition of AA metabolic enzymes

The BK channel modulation by AA was also evaluated in the presence of blockers of the 3 metabolic pathways of this PUFA, the cyclooxygenase (COX), lipoxygenase (LOX), and the cytochrome P450 (CYP 450) enzymes to discard any indirect activation of the channel through AA metabolites synthesized in situ. COX and LOX enzymes were inhibited with 10 μM indomethacin and 5 μM cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), respectively, which were applied to the bath solutions during the whole experiment. The CYP450 enzymes were irreversibly blocked by a 30 min cell incubation in the presence of 5 μM 17-Octadecynoic Acid (17-ODYA) before the current recording. Since CDC is a BK channel modulator, the time required to obtain current stability under control conditions was at least 30 min, and the G-V curves were shifted to the right respect to control conditions without the blocker. Thus, in most of the tested patches, the current did not reach G_{max} for this experimental condition. Therefore, current values were normalized to the control ones obtained at +170 mV to reduce the variability of the current introduced by different levels of channel expression.

2.5. Drugs and reagents used

AA, EGTA, HEPES, dimethyl sulfoxide (DMSO), and indomethacin were purchased from Sigma Chemical Co. CDC and 17-ODYA were obtained from Santa Cruz Biotechnology. All other reagents were of analytical grade and purchased from local suppliers as well as DMEM medium and FBS. AA, indomethacin, CDC, and 17-ODYA were prepared in DMSO. Fresh aliquots of stock solutions of AA were added to the bath solution on the day of the experiment. An appropriate amount of DMSO was added to all control solutions without AA. AA stock solution was stored at −20 °C in an N_2-atmosphere to prevent drug oxidation.

2.6. Statistics

The results are expressed as mean ± standard error of the mean. Paired or unpaired Student *t*-tests were used to compare control and AA groups. The *t*-test was used to compare AA activation at two different Ca^{2+} concentrations. Multiple groups were compared using a one-way ANOVA followed by a post hoc Holm-Sidak test for multiple comparisons. In all cases, a *P* value lower than 0.05 was considered for establishing statistically significant differences.

3. Results

In a previous study, we demonstrated that AA requires the co-expression of the β1 auxiliary subunit to activate the BK channel [27]. As shown in Fig. S1 (A–D) this fatty acid induced a significant left-shift in G-V relationships diminishing the $V_{1/2}$ value without any change in the z value. The AA effect was higher when its concentration increases (Fig. S1E–G; EC_{50}: 4.13 μM at +110 mV). The EC_{50} and the Hill slope were both voltage-independent (Fig. S1 H). Moreover, the effect of AA was independent of its metabolites (Fig. S1I and J), which could be produced by the membrane-bound enzymes located in the membrane portion attached to the pipette in the inside-out configuration [33,34].

Here, we aim to study the molecular mechanism by which AA activates the BK channel. To induce the BK channel opening, AA could affect one or more channel domains involved in the channel gating [1,35]. To know if AA is exerting its effect through changes in the VSD function, we measured the gating currents (I_g) of BK α/β1 channels in inside-out macropatches from *Xenopus laevis* oocytes membranes. Fig. 1A shows the representative traces of a family of I_g evoked as described in Methods. Fig. 1(B and C) shows that 10 μM AA induced a significant left-shift in the Q-V curves (Control: $V_{1/2} = 112$ mV ± 5 ($n = 7$); AA: $V_{1/2} = 89 \pm 4$ ($n = 4$), $p < 0.05$), with no appreciable changes in the voltage dependency of activation (Control: $z = 0.62 \pm 0.02$ (n = 7); AA: $z = 0.64 \pm 0.03$ (n = 4); NS, $p > 0.05$). Similarly to the observed in macroscopic ion currents [27], the gating currents of BK channels are unaffected by AA when there is no expression of auxiliary subunits (Fig. S2). It is known that β1 subunit stabilizes the VSD in the active configuration [36,37] and, our results show that AA enhances this stabilization by shifting the $V_{1/2}$ to the left with no change in the Q-V curve slope, suggesting that in BK α/β1 channels, AA can activate the channel changing the resting-active equilibrium of the VSD while the equivalent charge per voltage sensor is unaltered.

Could the AA effect on the VSD explain the increase in Po observed in α/β1 BK currents? To answer this question, we evaluate if the AA-induced changes in Q-V curves fully explain the AA-induced shift observed in G-V curves by using the HA model [29]. In this model, the VSD equilibrium between resting (*R*) - active (A) states is given by J, a voltage dependent parameter defined by the following equation:

$$J = \frac{A}{R} = J_0 e^{\frac{z_j F}{RT}} = e^{\frac{z_j F\left(V - V_{\frac{1}{2}}\right)}{RT}}$$

where J_0 is the equilibrium constant at 0 mV, z_j is the apparent number of gating charges per voltage sensor, V the membrane voltage, $V_{1/2}$ the half-activation voltage of the Q-V curve. Thus, the change in the Q-V curve promoted by AA is explained by an increase in J_0 since there was no change in z_j. Then, we introduce the function that describes the J parameter, with our experimental $V_{1/2}$ and z_j values, into the HA model equation to calculate the Po—V relationship before and after the addition of AA in a Ca^{2+}-free solution:

$$Po = \frac{L(1+JD)^4}{L(1+JD)^4 + (1+D)^4}$$

where L is the closed-open equilibrium constant, and D is an allosteric factor that couples voltage sensor activation with pore opening. Using the values of D and L parameters reported by Orio and Latorre [31], we obtained a predicted G-V shift value of −9 mV. This value, which is clearly lower than the observed experimentally (−50.5 mV, see Fig. S1), suggest that AA must modify other functional modules of the BK channel to reach its maximal effect.

Next, we evaluate the effect of AA on the BK channel when the two major physiological stimuli (Ca^{2+} and voltage) are absent. We measured the effect of AA on the open probability of the heteromeric BK α/β1 channel at the single-channel level with membrane potential held at −120 mV in a Ca^{2+}-free solution. Fig. 2A shows representative and consecutive traces of single channel activity recorded before (control, left panel) and after 10 μM AA perfusion (right panel). As is possible to

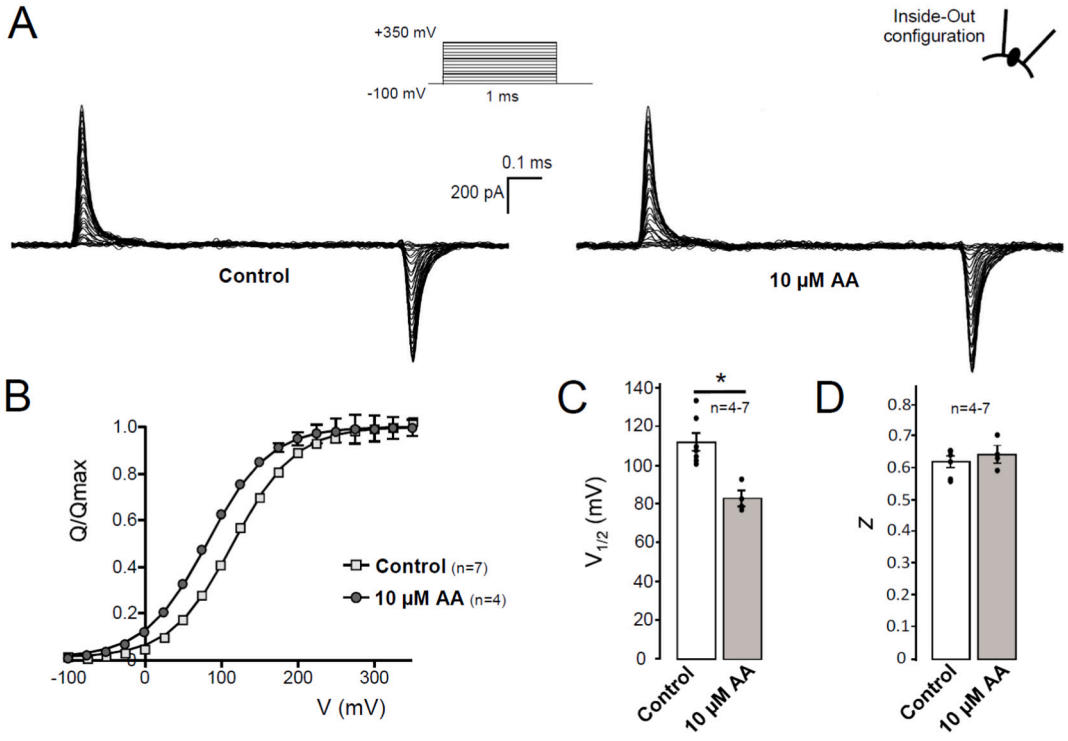

Fig. 1. *Arachidonic Acid (AA) modulates the BK channel voltage sensor domain in BK α/β1 channels.* **A**: Representative gating currents recordings from BK α/β1 channels expressed in *Xenopus laevis* oocytes before (left) and after 10 μM AA perfusion (right). **B**: Normalized ON gating charge vs. voltage (Q/Q_{max}-V) relationships in the absence or presence of 10 μM AA. Data points were fitted by a Boltzmann function (solid lines). **C**, **D**: Mean $V_{1/2}$ (C) and *z* (D) values obtained from the Boltzmann fitting of the Q-V curves under control conditions and 10 μM AA. The symbol * indicates a statistically significant difference from control (Student *t*-test,* $P < 0.05$).

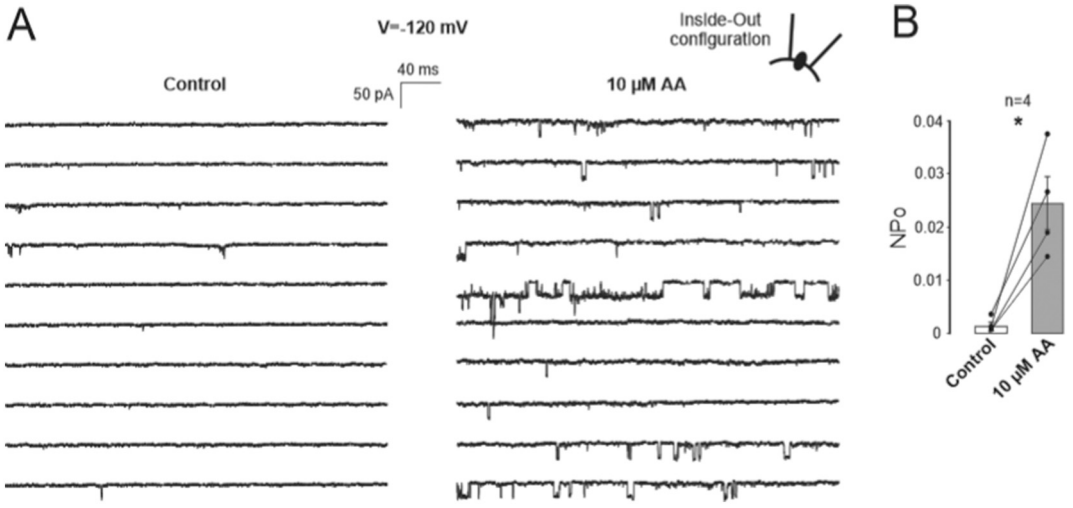

Fig. 2. *Arachidonic Acid (AA) promotes the BK channel opening independently of the voltage and Ca^{2+} sensor domains activation.* **A**: Typical recording of AA (10 μM) effect on the BK α/β1 channel open probability recorded in HEK293 cells in the virtual absence of intracellular Ca^{2+} (1 mM EGTA) and with membrane clamped at −120 mV. **B**: Mean probability (NPo) of BK channels recorded as shown in A in control conditions and with 10 μM AA (n = 4). The symbol * indicates a statistically significant difference from controls (paired *t*-test, $P < 0.05$).

observe, the AA produced a 20-fold increase in the BK open probability (NPo) (Fig. 2B, control: NPo = 0.0013 ± 0.0008; AA: NPo = 0.0245 ± 0.0051; $n = 4$, $p < 0.05$). This result suggests that AA promotes channel opening independently of the VSD and CSD.

Finally, we evaluated the relationship between the AA and the Ca^{2+} activation of the BK α/β1 channel. BK channel contains two intracellular Ca^{2+} binding sites with different affinities that promote its activation when the intracellular Ca^{2+} concentration rises [35]. In particular, β1 increases the apparent Ca^{2+} sensitivity of the BK channel, which is observed in channel activation by intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ higher than 1 μM [31]. To know if there is an interaction between the BK channel activation by AA and $[Ca^{2+}]_i$ increases, the activity of the BK α/β1 channels expressed in HEK293 cells was measured by exposing the intracellular leaflet of the membrane to a low Ca^{2+} concentration (Ca^{2+}-free solution), or a higher 1.2 μM free Ca^{2+} concentration, in the presence of AA. Figs. 3A and B show representative recordings of the BK channel currents induced by 10 μM AA in the absence and presence of Ca^{2+}. Steady-state G/G_{max}-V relationships analysis shows that, as expected, the increase in $[Ca^{2+}]_i$ shifts the curve to the left and, in addition, AA promotes an additional left shift of the G-V curves similar to the effect observed in Ca^{2+}-free condition (Fig. 3C and Table 1). As the figure shows, and according to the results presented in Fig. S1, the AA induced left-shift did not affect the voltage dependence in G/G_{max}-V curves (z parameter, Table 1). These results indicate that the AA activating effect is independent of the intracellular Ca^{2+} concentration and compatible with an additive effect of both activators.

Together, our results show that when the BK channel is co-expressed with the β1 subunit, AA promotes the channel opening in the absence of intracellular Ca^{2+} and when the VSD are resting and, also stabilizes the VSD in its active configuration under membrane depolarization. Moreover, Gruslova et al. in 2012 reported two β1 functional domains located in its extracellular loop (segments A and B), that are involved in the modulation of the channel function by the β1 subunit [38]. Therefore, we analyzed if these β1 domains are involved in AA-induced BK activation. So, we measured the effect of 10 μM AA on the BK channel currents expressed with the wild type (wt) β1 subunit and its Y74A and S104A mutants. Each mutated residue is located in one of the two segments of the extracellular loop of the β1 subunit postulated for the modulation of the BK channel (segments A and B, respectively). Fig. 4 shows the AA effect on the macroscopic currents of the BK channel expressed with the wt (Fig. 4A and B) and the mutated β1 subunits (Fig. 4C–F). The AA sustained the ability to activate all the resultant channel complexes; however, the induced left-shift in G/G_{max}-V curves was clearly decreased when the channel was co-expressed with the Y74A mutant (β1wt: $\Delta V_{1/2} = -43{,}37 \pm 5{,}75$ mV, $n = 6$; β1Y74A*: $\Delta V_{1/2} = -22{,}56$ mV ± 2,7, $n = 8$; β1S104A: $\Delta V_{1/2} = -43{,}77$ mV ± 5,81, n = 6; *$p < 0.05$, ANOVA post hoc Holm-Sidak test). These results indicate that the changes in BK channel function induced by the segment A of the β1 subunit are in part necessaries to promote the activating effect of AA.

4. Discussion

The present study shows the molecular mechanism involved in the AA-induced BK channel activation. We demonstrate that this endogenous PUFA can activate the BK channel associated with the β1 subunit, in a concentration-dependent mode and without requiring any metabolic modification. Moreover, we evidence that an increase in the opening transitions of the ion conduction gate of the channel and, stabilization of the VSD on its active configuration, appears to be involved in the AA effect on BK α/β1 channel activity. Additionally, we observed that intracellular Ca^{2+} concentration did not affect the AA-induced BK channel activation. Finally, the experiments performed with the Y74A β1 subunit mutant, suggest that AA modulation requires the functional coupling between α and β1 BK channel subunits.

AA is a relevant PUFA involved in a cell signaling complex pathway present in multiple physiological processes such as endothelial mediated vasodilation, platelet aggregation, gastric cytoprotection, regulation of renal blood flow, as wells as important in pathophysiological states like inflammation, algesia, and fever, among others. Initially, the biological effects of AA were mostly attributed to its metabolites (prostaglandins, leukotrienes, and epoxyeicosatrienoic acids), but subsequent studies showed that AA itself is also able to modulate several proteins, including ion channels [14]. In particular, the BK channel activation by AA, and other PUFAs, was early demonstrated in several smooth muscle cells where the channel is associated with the auxiliary β1 subunit. Later was reported that BK channel activation induced by AA is only observed when β1, β2, or β3 subunits are co-expressed with the α-channel subunit [26,27]. Sun et al. (2007) demonstrated that AA-activation of the BK channel co-expressed with β2 or β3 subunits is due to an inhibition of the inactivation process produced by these auxiliary subunits on the BK channel [26]. Here, we demonstrate a different mechanism of action responsible for the stimulatory effect of AA observed on non-inactivating BK channels co-expressed with the β1 auxiliary subunit.

Here, we fully characterized the AA effect on the BK current in

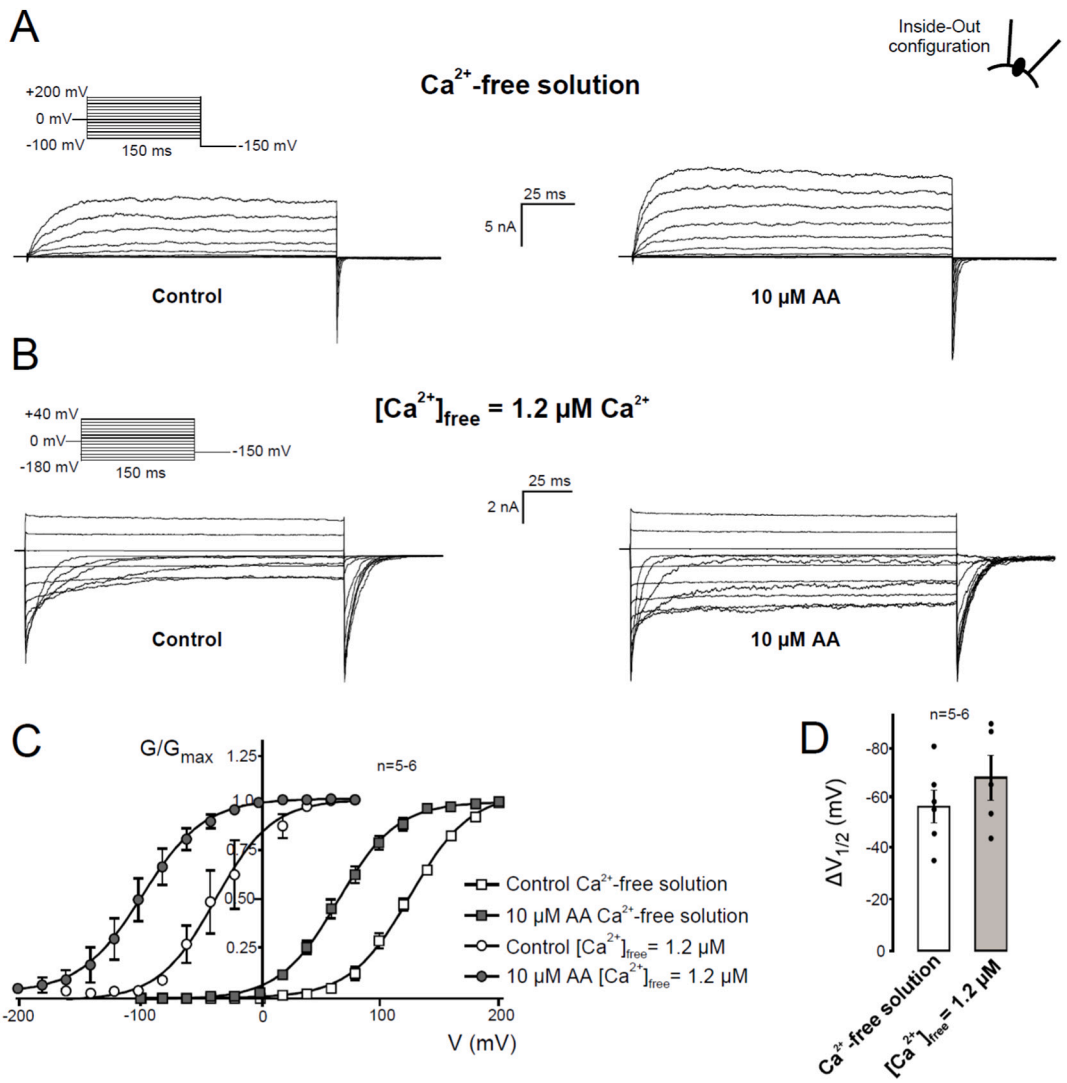

Fig. 3. *Arachidonic Acid (AA) activation of the BK channel is independent of intracellular Ca^{2+} concentration.* **A, B:** Superimposed macroscopic IO currents of BK channels expressed in HEK293 cells recorded in an intracellular Ca^{2+}-free solution (**A**) or at intracellular Ca^{2+} concentration of 1.2 µM (**B**), before (left) and after 5 min of 10 µM AA perfusion (right). **C:** Mean normalized conductance vs. voltage (G/G_{max}-V) curves corresponding to the control conditions and 5 min of 10 µM AA perfusion at the two intracellular Ca^{2+} conditions. Data points were fitted by a Boltzmann function (solid lines). **D:** Mean changes of $V_{1/2}$ values between control conditions and after 10 µM AA obtained from the Boltzmann fitting of the G-V curves shown in C, corresponding to each experimental condition. No statistically significant differences were observed (Student's *t*-test, $P > 0.05$).

Table 1
Mean $V_{1/2}$ and *z* values obtained from the Boltzmann fitting of the G/G_{max}-V curves under control conditions and after 10 µM AA obtained in an intracellular Ca^{2+}-free solution and at an intracellular Ca^{2+} concentration of 1.2 µM.

	$V_{1/2}$ (mV)		*z*	
	Control	10 µM AA	Control	10 µM AA
Ca^{2+}-free solution (*n* = 6)	125.1 ± 2.3	68.1 ± 4.4	1.08 ± 0.07	1.06 ± 0.0.06
$[Ca^{2+}]_{free}$ = 1.2 µM (*n* = 5)	−30.9 ± 13.0	−98.4 ± 11.5	1.30 ± 0.14	1.17 ± 0.09

HEK293 cells expressing α and β1 channel subunits. The AA produced a left-shift in G/G_{max}-V curves obtained from BK currents without modifying the steepness of the curve (Fig. S1), indicating that AA facilitates the activation of the channel induced by changes in membrane potential. This mechanism implies that the increase in cell membrane K^+ conductance, promoted by this PUFA, will be greater at more negative potentials when the channel open probability is usually low, as occurs in native cells. The voltage independence of the EC_{50} and Hill slope values suggest that AA binding to the BK protein is insensitive to the membrane voltage field. Moreover, the obtained EC_{50} value (4.15 µM) suggests that AA-induced BK channel activation can be physiologically and pathophysiologically relevant since it is near to the normal human plasma AA levels as free fatty acid [5.8 to 49.3 µM] [39,40] and, clearly below the plasmatic concentration of this PUFA in certain pathological conditions, such as preeclampsia or brain ischemia [41,42]. We also demonstrated that AA activates the channel by itself. First, we observed that AA activation persists in electrophysiological recordings performed in the cell-free inside-out configuration. Moreover, the activation still occurs when the three metabolic pathways were inhibited with indomethacin, CDC, and 17-ODYA to block the COX, LOX, and CYP450 enzymes, respectively.

Regarding the molecular mechanism of action of AA, the primary goal of this work was to analyze its pharmacological effect on the three functional modules of the BK channel. First, we demonstrated that AA affects VSD as the analysis of the gating currents proves. Specifically, we showed that AA induces a left-shift in Q-V relationships obtained

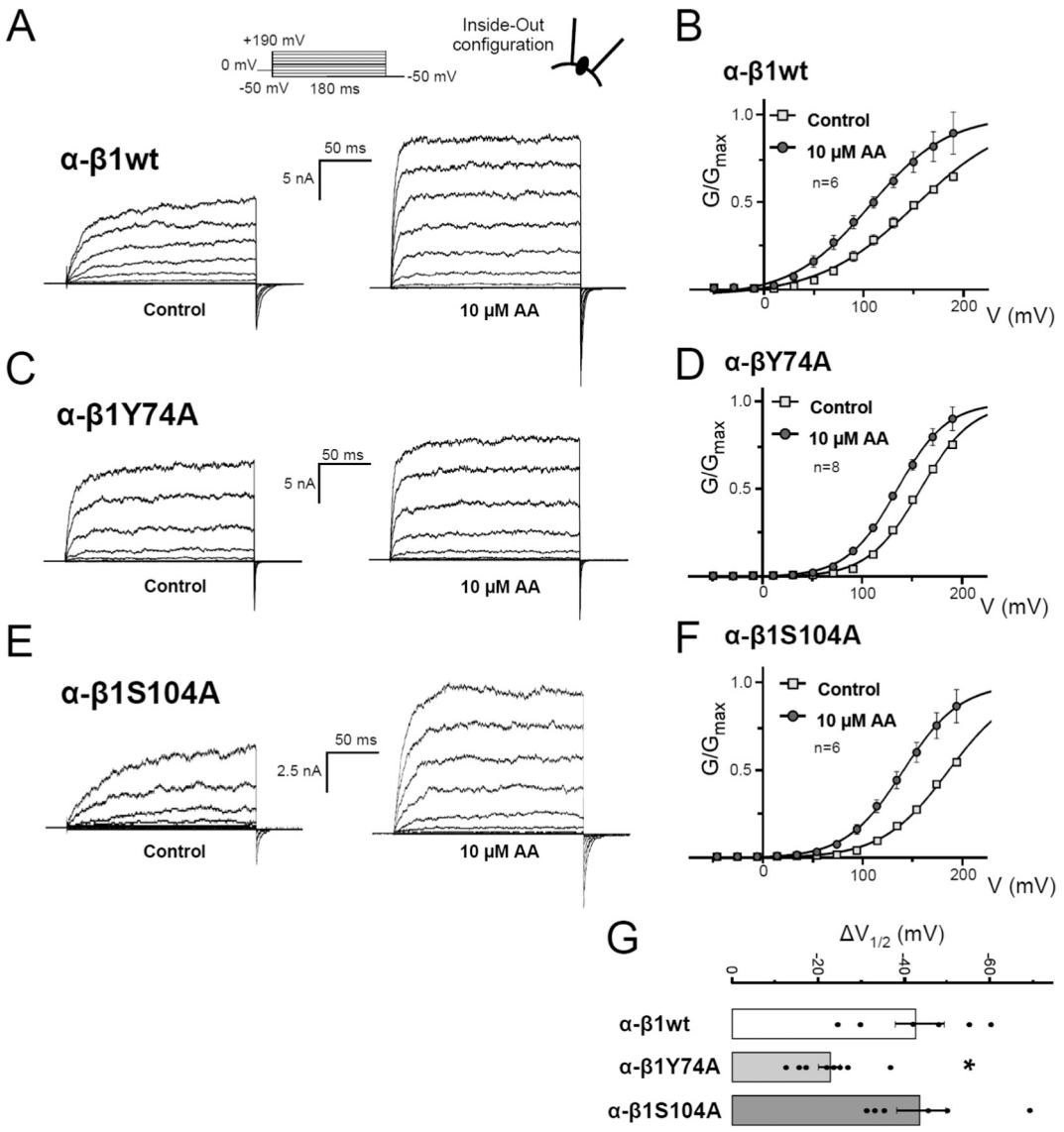

Fig. 4. *BK channel activation by Arachidonic Acid (AA) is reduced when the channel is co-expressed with the β1 Y74A mutant.* **A, C, E**: Superimposed macroscopic IO currents of BK channels expressed in HEK293 cells with the wild type (wt) β1 auxiliary subunit (A), and the mutants β1 Y74A (C) and β1 S104A (E), recorded before (left) and after 5 min of 10 μM AA perfusion (right). **B, D, F**: Mean normalized conductance vs. voltage (G/G_{max}-V) curves corresponding to the control conditions and 10 μM AA, for the three BK channel compositions indicated above for panels A, C, and E, respectively. Data points were fitted by a Boltzmann function (solid lines). **G**: Mean changes of $V_{1/2}$ values between control conditions and after 10 μM AA obtained from the Boltzmann fitting of the G-V curves corresponding to α-β1 wt, α-β1 Y74A, and α-β1 S104A. A statistically significant reduction in the $V_{1/2}$ changes was observed in the α-β1 Y74A group respect α-β1 wt and α-β1 S104A groups (ANOVA followed by a post hoc Holm-Sidak test, $p < 0.05$).

measuring the gating currents of α/β1 BK channels, indicating a VSD stabilization in the active configuration. Moreover, no changes in the slope of the Q-V curve suggest that the equivalent charge per voltage sensor was unaltered. Modulation of VSD by PUFAs was earlier described for the docosahexaenoic acid (DHA)-induced activation of the *Shaker* K^+ channel by the Elinder group [43]. The authors propose a mechanism called "lipolectric mechanism" where the fatty acid acts in the interface between the extracellular region of the ion channel and the outer leaflet of the lipid bilayer. The PUFA electrostatically interacts with the VSD, affecting the voltage sensor equilibrium. This mechanism could be shared between several voltage-gated channels that are modulated by fatty acids as the Ca_V, H_V, BK, and most of the K_V channels [13]. Our results are the first showing a change in the voltage sensor machinery of BK channels induced by a fatty acid in a similar way to the one described for the *Shaker* K^+ channel.

We also show that AA was able to increase the BK channel open probability when the VSD are mostly at rest and the CSD are unoccupied. Hoshi et al. (2013) showed that BK channel activation by DHA is mainly due to a similar effect on the opening and closing transitions of the ion conduction gate of the channel [44]. However, this DHA effect is lost when the tyrosine 318 is replaced for serine in the S6 segment of the BK α subunit while the AA activation of the BK channel fully persisted [17]. Thus, despite the fact that the effect of both fatty acids is similar, AA and DHA do not seem to be associated with a common binding site.

Finally, we demonstrated that the intracellular Ca^{2+} concentration does not influence the BK α/β1 channel activation induced by AA. The AA stimulatory effect was similar at intracellular Ca^{2+} concentration where the Ca^{2+} binding sites were unoccupied (Ca^{2+}-free solution) respect with the one observed when they were partially occupied ($[Ca^{2+}]_{free} = 1.2$ μM), suggesting an additive effect between AA and Ca^{2+} activations. The lack of a synergistic or an antagonist effect between BK channel activation induced by AA and by the intercellular

Ca^{2+} concentration implies that the mechanisms involved in both activations are independent. This result is in agreement with previous reports showing that the stimulatory effect of AA on the BK channel in retinal Müller glial cells [45] and coronary smooth muscle cell was Ca^{2+} independent [46].

Together, our results show that AA increases the L and the J parameters of the HA model, which reflect the closed–open equilibrium of the ion conduction gate and the resting-active equilibrium of the voltage sensor, respectively [29]. These changes produced a 1.2 kcal/mol decrease in the free energy needed to open the pore observed as the left-shift in macroscopic G/G_{max}-V in a Ca^{2+} independent manner. Remarkably, J and L parameters are modified in the BK channel function when it is co-expressed with the β1 subunit. Specifically, the presence of this auxiliary subunit produces a reduction in L_0, an increase in J_0, and also rises the allosteric factor between the voltage sensor activation and gating (the parameter D in the HA model) [31,35,38]. Several residues of the β1 subunit have been proposed to be involved in the modulation of the open probability of the BK channel [47,48]. In particular, Gruslova et al. (2011) [38] showed that single point mutations placed either at segment A (Y74) or segment B (S104A, Y105A, I106E) of the β1 subunit extracellular loop, partially reduce the β1 induced changes in G/G_{max}-V curves of BK channel expressed in HEK293 cells [38,49]. After an interpretation of these results, in the context of the HA gating model, they proposed that these domains are involved in the changes induced by the β1 subunit on both the VSD function and, the opening transitions at the ion conduction gate of the BK channel. However, the current available structural data, from cryo-EM structures of the BK channel in complex with the β4 subunit, published recently by Tao and MacKinnon [50], shows that the TM1 helix of β4 principally associates with the α subunit through hydrophobic interactions with S1sidechains and the pore helix. Moreover, lipid molecules are observed in the α/β4 interfaces at both, the inner and outer membrane leaflets, revealing a significant contribution to the channel complex structure. On the other hand, the lack of direct interactions between the β4 extracellular loop and the α subunit, suggests that the functional effects observed in the mutations and glycosylation at this region could be due to indirect effects. Despite there is no experimental structural information of the BK channel in complex with the β1 subunit, the sequence similarity with the β4 allows us to interpret our results with the structural information described above. Here, we observed that a punctual mutation in the β1 subunit (Y74A) significantly impairs the channel activation by the AA. So, based on the structural information, it is very unlikely that the AA effect on channel gating could be due to direct interaction with the Y74 of β1 subunits. So, a possible interpretation of our results could be attributed to a role of β1 extracellular loop in the spatial re-arrangements of both subunits that allow the AA binding to the channel. Furthermore, the Y74 could be important in the molecular transduction of the AA effect on the VSD, the BK channel gate, or the allosteric coupling between these domains. Alternatively, the reported presence of lipids in the interface between α and β subunits represent another possible site of AA interaction associated to its β-dependent BK channel modulation [50].

5. Conclusions

Our results provide new insights on the mechanism whereby AA modulates the α/β1 BK channel activity. These results become relevant in the physiological and pathophysiological context related to the function of smooth muscle cells (SMC), where the channel is associated with β1. The analysis of AA effect on each functional module of BK channel, allow proposing that AA (or any synthetic analog) will be able to produce an increase in BK channel activity at the resting membrane potential, hyperpolarizing the cell membrane and reducing its excitability. Moreover, this PUFA would be able to increase the activity of the channel when the SMCs are depolarized by an action potential (e.g.: detrusor or uterine smooth muscle cells) or by the stimulus of an agonist (e.g.: Angiotensin II or norepinephrine in vascular smooth muscle cells). Finally, due to its Ca^{2+} independent mechanism of action, AA would not modify the observed relaxing effect induced by BK activation in response to sarcoplasmic reticule released Ca^{2+} sparks [1]. This complete pharmacological profile would be useful to understand the benefits of searching for new compounds sharing with AA the molecular mechanism of action.

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Author contributions

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Acquisition, analysis or interpretation of data for the work: P.M., M. M., K.C., F.O., G.D.

Drafting the work or revising it critically for important intellectual content: P.M., K.C., J.M.F·F, V.M.

All authors approved the final version of the manuscript and are accountable for all aspects of the work.

Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2021.183550.

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