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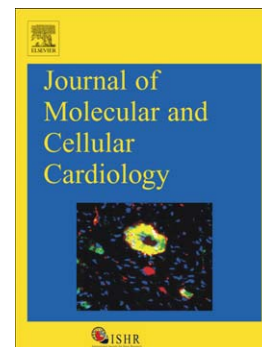
PII: S0022-2828(09)00286-7
DOI: doi:[10.1016/j.yjmcc.2009.07.011](https://doi.org/10.1016/j.yjmcc.2009.07.011)
Reference: YJMCC 6587

To appear in: *Journal of Molecular and Cellular Cardiology*

Received date: 27 February 2009
Revised date: 6 July 2009
Accepted date: 9 July 2009

Please cite this article as: Amorós Irene, Barana Adriana, Caballero Ricardo, Gómez Ricardo, Osuna Lourdes, Lillo M. Pilar, Tamargo Juan, Delpón Eva, Endocannabinoids and cannabinoid analogues block human cardiac Kv4.3 channels in a receptor-independent manner, *Journal of Molecular and Cellular Cardiology* (2009), doi:[10.1016/j.yjmcc.2009.07.011](https://doi.org/10.1016/j.yjmcc.2009.07.011)

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**Endocannabinoids and cannabinoid analogues
block human cardiac Kv4.3 channels in a
receptor-independent manner**

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Word count: 6228

1. Introduction

Endogenous cannabinoids (endocannabinoids), which are synthesized from lipid precursors in plasma membranes, are signaling lipids consisting of amides and esters of long chain fatty acids [1]. Some of these endocannabinoids are derivatives of arachidonic acid (AA), namely anandamide (N-arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG). Others, such as N-palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA), are synthesized from the palmitic (PA) and oleic (OA) fatty acids, respectively [1]. PEA and OEA are classified on the basis of their mode of action as endocannabinoids or cannabinoid analogues since they have been shown to potentiate AEA responses [1]. Endocannabinoids can be generated by virtually all cell types, including the cardiac muscle, and it is considered that they exert their broad range of biological effects mainly through their interaction with the G-protein-coupled receptors CB1, CB2, and GPR55, as well as additional, as yet unidentified, receptors [1,2].

It has been extensively demonstrated that endocannabinoids can produce biological effects which are not mediated by the interaction with receptors (i.e., the receptor-independent or direct effects of endocannabinoids) [1,3,4]. Indeed, endocannabinoids and cannabinoid analogues can modulate Na⁺ [5], K⁺ [6-8], and Ca²⁺ [9] channels in a receptor-independent manner [for review see 3,4]. However, almost all these studies analysed their effects on neuronal or smooth muscle tissues, whereas data on the putative direct effects of endocannabinoids on human cardiac channels are scarce or absent.

It has been described that AEA accelerated the inactivation of neuronal rapidly inactivating A-type (Kv3.4) channels, an effect that was independent of the endocannabinoid receptors activation [98]. In the human myocardium the most

important rapidly inactivating K^+ current is the Ca^{2+} -independent component of the transient outward K^+ current (I_{to1}), which is critical in determining the height and the duration of the plateau phase of the action potential (AP). I_{to1} is predominantly carried by Kv4.3 α -subunits, which assemble with KChIP2, DPP6, and MiRP ancillary subunits [10-13].

In the present paper we analyzed the direct effects of endocannabinoids and cannabinoid analogues such as AEA, its metabolically stable analogue, (R)-(+)-arachidonoyl-1'-hydroxy-2'-propylamide (MetAEA), 2-AG, PEA, OEA, and the endogenous lipid lysophosphatidylinositol (LPI) on human cardiac Kv4.3+KChIP2 current ($I_{Kv4.3}$). Moreover, the effects of the fatty acids from which some of them are endogenously synthesized have also been examined. The results demonstrated, for the first time, that among endocannabinoids and cannabinoid analogues, AEA and 2-AG are the more potent agents for inhibiting $I_{Kv4.3}$, an effect that was not mediated by either their interaction with cannabinoid receptors or the modification of the lipid order and microviscosity of the cell membrane.

2. Material and Methods

Chinese hamster ovary (CHO) cells stably transfected with hKv4.3-L/hKChIP2, that do not endogenously express any of the known cannabinoid receptors [2,9,14], were cultured as described [15,16]. In some experiments, CHO cells were transiently transfected with the cDNA encoding KCNE3 (1.6 μ g) or DPP6 (1 μ g) proteins together with the cDNA encoding the CD8 antigen (0.5 μ g) by using FUGENE6 [13,16]. I_{to1} was recorded on myocytes isolated from right atrial appendages of patients undergoing cardiac surgery [15]. The study conforms with the principles of Declaration of Helsinki. Currents were recorded using the whole-cell patch-clamp technique, sampled at 4 kHz, and filtered at half the sampling frequency. CHO cells were perfused with an external solution containing (mM): NaCl 136, KCl 4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). To record I_{to1} , the external solution contained (mM): NaCl 120, KCl 20, CaCl₂ 1, MgCl₂ 1, HEPES 10, glucose 10, 4-aminopyridine (50 μ M), nifedipine (1 μ M), and atropine (1 μ M) (pH 7.4 with NaOH). Recording pipettes were filled with an internal solution containing (mM): K-aspartate 80, KCl 42, KH₂PO₄ 10, MgATP 5, phosphocreatine 3, HEPES 5, and EGTA 5 (pH 7.2 with KOH). In some experiments, EGTA was replaced by 20 mM BAPTA in the internal solution. Steady-state and time-resolved anisotropy measurements were performed by using fluorescence depolarization techniques and a lipophilic fluorescent dye with an anionic polar head 2-carboxyethyl-1,6-diphenyl-1,3,5-hexatriene (PA-DPH) [17,18]. Complexity of each compound was calculated using the Bertz/Hendrickson/Ihlenfeldt equation [19]. AEA, MetAEA, LPI, AA, PA, stearic acid (SA), stearyl ethanolamide (SEA), OA [purchased from Sigma], 2-AG and OEA [purchased from Tocris] were dissolved in ethanol, whereas PEA (Sigma) was dissolved in dimethyl sulfoxide.

Data obtained in the absence and presence of each compound were compared in a paired manner. For comparisons at a single voltage, differences were analysed by using Student's t-test. To analyse block at multiple voltages, a two-way ANOVA was used, followed by Newman-Keuls test. Results were expressed as mean \pm s.e.m. A value of $P < 0.05$ was considered statistically significant. An expanded Materials and Methods section is available in the online data supplement.

3. Results

Fig. 1 shows the chemical structure of the endocannabinoids and cannabinoid analogues tested in the present study. Since most of them are ethanolamides of fatty acids, we also studied the effects of their corresponding fatty acids. Moreover, to gain more insight into the putative structure-effect relationship we also studied the effects of SA, an 18:0 fatty acid, and its ethanolamide (SEA). Fig. 2 shows $I_{Kv4.3}$ traces recorded by applying 250 ms-pulses to +50 mV from a holding potential of -80 mV under control conditions and in the presence of distinct endocannabinoids, AEA (A), its metabolically stable analogue MetAEA (B), 2-AG (C), OEA (D), LPI (E), and PEA (F) at a concentration of 1 μ M. Under control conditions currents rose rapidly to a peak ($\tau_{act}=1.5\pm0.2$ ms at +50 mV, $n=42$) and then inactivated according to a biexponential process, and τ_f and τ_s averaged 33.9 ± 2.1 ms and 92.4 ± 4.1 ms, respectively ($n=42$). AEA decreased the peak current elicited at +50 mV by $37.5\pm6.3\%$ ($n=5$, $P<0.05$) and accelerated the time course of the inactivation process, decreasing the τ_f and τ_s values to 3.7 ± 0.7 ms and 53.9 ± 10.6 ms ($P<0.05$), respectively (Figs. 2A, 3C, and online Table II). MetAEA, 2-AG, and OEA produced similar effects to those observed with AEA, i.e., they significantly reduced the peak $I_{Kv4.3}$ at +50 mV by $47.5\pm2.9\%$, $38.0\pm5.2\%$, and $28.8\pm7.6\%$, respectively, and accelerated the time course of current decay ($n=5$, $P<0.05$) (Figs. 2B-D, and 3C and online Table II). On the contrary, LPI and PEA slightly decreased the peak current amplitude ($6.2\pm2.0\%$ and $7.6\pm2.0\%$, respectively), without modifying the time course of current decay ($n=5$, $P>0.05$) (Figs. 2E, 2F, 3C, and online Table II).

Next, the effects of SEA and the fatty acids PA (16:0), SA, OA (18:1), AA (20:4), at 1 μ M were studied. AA (Fig. 3A), and OA significantly decreased $I_{Kv4.3}$ peak (by $39.6\pm5.6\%$ and $47.9\pm5.4\%$, respectively, $n=5$, $P<0.05$) and accelerated the time course of current decay (Fig.3D). In contrast, the effects of SA, SEA, and PA on both peak

current and time course of inactivation did not reach statistical significance (Fig. 3B, 3D and online Table II).

To better characterize the steady-state block produced by these agents, particularly of those that decreased the peak current and accelerated the inactivation kinetics of the current, the reduction of the total charge ($Q_{Kv4.3}$) crossing the membrane (calculated as current-time integrals at +50 mV) was measured. Consistent with the effects on the current decline kinetics described above, the highest reduction in $Q_{Kv4.3}$ was produced by AA ($73.3\pm 3.9\%$) and its derivatives AEA ($65.8\pm 3.5\%$), MetAEA ($70.0\pm 3.2\%$), and 2-AG ($81.6\pm 1.6\%$). Fig. 4 shows the concentration-response curves for the endocannabinoids (A), the ethanolamides (B), and the fatty acids (C) tested, obtained by plotting the reduction of $Q_{Kv4.3}$ at +50 mV as a function of the concentrations of each compound. The Hill equation was fitted to the data and the IC_{50} values were calculated (Table 1) for all the compounds but for PEA and PA, since they produced a blockade lower than 40% at the highest concentration tested. Among the endocannabinoids (Fig. 4A), the highest potency was exhibited by AEA and 2-AG, which yielded IC_{50} values of $0.4\pm 0.01 \mu\text{M}$ and $0.3\pm 0.01 \mu\text{M}$, respectively. When the potency of the fatty acids was compared (Fig. 4C) the results demonstrated that AA ($IC_{50} = 0.3\pm 0.02 \mu\text{M}$) is the most while PA is the least potent for inhibiting $I_{Kv4.3}$. In Fig. 4D the concentration dependence of the Kv4.3 block produced by the ethanolamides (either endocannabinoids or not) and their corresponding fatty acid is plotted together, for a better comparison among compounds. The results suggested that the number of carbons in the fatty acid chain, but not the presence or absence of the ethanolamide group, determines the potency of block, which increased when the number of carbons increased.

The time course of $I_{Kv4.3}$ inhibition produced by endocannabinoids and analogues was slow (around 12 min of drug perfusion) and no significant washout of the drug

effect was observed upon perfusion with drug-free solution ($Q_{\text{AEA}}/Q_{\text{Control}}=0.46\pm 0.04$ after >20 min of washout of AEA). Online Fig. I shows the time course of the onset and offset of AEA-induced block.

Fig. 5A shows representative $I_{\text{Kv4.3}}$ traces obtained in the absence and presence of 1 μM AEA with the protocol shown at the top. To determine the effects of endocannabinoids on the voltage dependence of steady-state inactivation the inactivation curves were constructed by plotting the peak current amplitude of the test pulse to +50 mV as a function of the voltage of the conditioning pulse (see online Methods) in the absence and presence of 1 μM of endocannabinoids and cannabinoid analogues together with the Boltzmann fit to the inactivation curves (Fig. 5B-G). The midpoint of inactivation (V_h) and slope values (k) obtained are summarized in the online Table II. AEA, MetAEA, 2-AG, and OEA significantly shifted the voltage dependence of inactivation to more negative potentials without modifying the slope factor. Indeed, in control conditions and in the presence of AEA, V_h averaged -23.8 ± 4.4 and -44.1 ± 6.0 mV, respectively ($n=6$, $P<0.01$). In contrast, neither LPI nor PEA modified the voltage dependence of inactivation. When analyzing the effects produced by the fatty acids as well as SEA, it can be appreciated that those that significantly inhibited the current (i.e., AA and OA) also modified the voltage dependence of inactivation by shifting the curve toward more negative potentials (online Fig. II and Table II). In contrast, PA, SA, and SEA did not modify the inactivation curve.

AEA significantly decreased the current amplitude at potentials negative to -10 mV and similar results were obtained with MetAEA, 2-AG, and OEA. As observed from the representation of the relative current (represented by squares in Fig. 5B-G), the endocannabinoid-induced blockade significantly increased in the voltage range

coinciding with that of channel inactivation, a result that suggests that endocannabinoids block Kv4.3 channels preferentially in the inactivated state.

3.1. Mechanism of endocannabinoid-induced block of Kv4.3 channels

It has been hypothesized that endocannabinoids and cannabinoid analogues can indirectly affect membrane proteins including ion channels by producing a perturbation in the cell membrane [20]. As an index of the liposolubility of each compound we used the partition ($\log P$) and the distribution ($\log D$ at $\text{pH}=7.4$) coefficients for the ethanolamides and the fatty acids, respectively. In Fig. 6A the $\log P$ or $\log D$ ($\log P/D$) values were plotted as a function of the IC_{50} of each compound tested. The results demonstrated that for both, fatty acids and their ethanolamides, there is no relationship between the liposolubility of the compound and its potency for blocking Kv4.3 channels. Results in Fig. 4D suggested that an increase in the number of C atoms in the fatty acid chain increased the potency of block. To further analyze this result, the IC_{50} of each compound was plotted against its *complexity* (Fig. 6B). This latter parameter considers the size and also the presence and nature of the reactive groups in the molecule and was calculated following the Bertz/Hendrickson/Ihlenfeldt equation [19] (See online methods). As can be observed for both ethanolamides and acids, the increase in the complexity was correlated with an increase in the potency of Kv4.3 blockade ($r^2= 0.9826$ for fatty acids and 0.8933 for ethanolamides).

We also compared the putative changes in fluorescence anisotropy of CHO cells stably expressing Kv4.3+KChIP2 channels induced by AEA, 2-AG, LPI, and PEA at the concentration of $1 \mu\text{M}$. Membrane fluidity was characterized by quantifying the rate and the range of the rotational motions of the lipophilic dye PA-DPH in the cell membrane [17,18]. PA-DPH is an approximately rod-shaped molecule with an anionic polar head

and dimensions similar to those of a lipid hydrocarbon chain. The time-scale of its fluorescence lifetimes coincides with the time-scale of interest for lipid rotational motions. PA-DPH is incorporated into the cell membrane, anchored to the bilayer surface, with the fluorescent chromophore (DPH) parallel to lipid hydrocarbon chains of neighbouring phospholipids.

The kinetics of the fluorescence of PA-DPH (2 μM) in control conditions was satisfactorily described by a major contribution ($\approx 92\%$ of the total intensity) with a lifetime of 6.8 ns and a short component of 2.1 ns. The fluorescence lifetimes of the probe incorporated into the cell membrane were not significantly modified by any of the compounds tested.

The steady-state fluorescence anisotropy (r_{ss}) of PA-DPH incorporated into CHO cells was measured under control conditions and after 20 min incubation with AEA, 2-AG, LPI, or PEA (1 μM). As shown in Fig. 6C, none of the compounds tested changed the r_{ss} which in control conditions averaged 0.281 ± 0.003 . It is known that r_{ss} is the sum of a structural (lipid order) and a dynamic (microviscosity) contribution. In order to quantify these components separately, we performed time-resolved anisotropy measurements. In Fig. 6D, the fluorescence anisotropy [$r(t)$] decay of PA-DPH in control conditions and in the presence of AEA or PEA is shown. As can be observed the $r(t)$ decayed in a few nanoseconds to a residual or limiting anisotropy (r_{∞}), which was calculated by fitting Eq. 8 to the $r(t)$ decay (see online Methods) and is directly related to the degree of molecular order imposed on the fluorophore by its microenvironment. The fit also yielded the rotational correlation time (ϕ) which is related with the microviscosity of the cell membrane. Incubation with AEA, 2-AG, LPI, or PEA for 20 min did not modify r_{∞} (0.26 ± 0.004) and ϕ (1.4 ± 0.2 ns) values (Fig. 6E and F). Similar results were obtained when these compounds were incubated for 120 min (data not shown). Overall these

results do not rule out that these compounds could produce a modification in the order and microviscosity of the cell membrane, but suggested that, if any, all of them produce similar unspecific perturbations of the lipid environment.

Cannabinoids have been shown to reduce [21] and to increase [2] Ca^{2+} release from ryanodine-sensitive stores in hippocampal neurones. The modification of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) could activate intracellular pathways that, in turn, could modify the $I_{\text{Kv4.3}}$. Therefore, to examine whether the observed effects were due to a cannabinoid-induced modification of the $[\text{Ca}^{2+}]_i$, we also tested the effects of AEA by using BAPTA as an intracellular chelator. The results demonstrated that, in cells dialyzed with BAPTA, AEA reduced the $Q_{\text{Kv4.3}}$ at +50 mV by $60.1 \pm 4.0\%$ ($n=5$), a reduction that was not statistically different from that produced by AEA in EGTA-dialyzed cells (Fig. 7A and B).

Cardiac I_{to1} is predominantly carried by Kv4.3 α -subunits, which assemble with KCHIP2, DPP6, and MiRP ancillary subunits [10-13]. Since previous studies demonstrated that effects produced by fatty acids on the K^+ channels might depend on the presence of ancillary subunits [22,23], in the next group of experiments we explored whether the presence of MiRP and DPP6 affects the AEA-induced block of Kv4.3 channels. In the human myocardium KCNE2 and KCNE3 are the most probable candidates to be involved in the I_{to1} channels [11,13]. Using coimmunoprecipitation techniques, it has been demonstrated that Kv4.3 and KCNE3 coassociate in the human heart [13]. Therefore, among MiRP proteins we selected MiRP2 (KCNE3), for which evidence for a functional role in the modulation of I_{to1} in the human heart has also been provided recently [13]. CHO cells stably expressing Kv4.3+KCHIP2 were transiently transfected with either MiRP2 or DPP6 encoding genes. Results in Fig. 7C demonstrated that the $Q_{\text{Kv4.3}}$ reduction produced by AEA (1 μM) was independent on the

presence or absence of MiRP2 or DPP6 in the channel complex. DPP6 accelerated and MiRP2 slowed Kv4.3+KChIP2 inactivation ($\tau_i = 17.1 \pm 1.4$ and 46.8 ± 3.4 ms, respectively). However, AEA significantly accelerated the current inactivation in both cases (4.0 ± 1.0 and 12.6 ± 2.7 ms, respectively, $n=5$). Further experimental support was obtained when analyzing the direct effects of AEA on human atrial I_{tot} , i.e., the current generated by channels with the native architecture. For this myocytes were perfused with rimonabant and AM630, selective antagonists of CB1 and CB2 receptors, respectively. Online Figure III demonstrates that the effects on I_{tot} (Q reduction at +50 mV = $62.2 \pm 2.5\%$, $n=5$) are identical to those produced on Kv4.3/KChIP2 channels.

In previous reports it has been demonstrated that in some cases endocannabinoid- and fatty acid-induced inhibition of ion channels was only evident when they are applied intra- or extracellularly [8,18,24]. To gain a deeper insight into the mechanism of Kv4.3 channels block, we compared the blockade produced by AEA (1 μ M) when it was added to the extracellular or to the intracellular solution (Fig.7D). The results demonstrated that intracellular application of AEA or its metabolically stable analogue MetAEA produced a $Q_{Kv4.3}$ reduction significantly lower than that produced when AEA was extracellularly applied. Similar results were obtained in AEA-dialyzed cells prior incubated for 20 min with URB-597 (0.1 μ M), a membrane permeable inhibitor of the main pathway of AEA degradation (the fatty acid amide hydrolase). Furthermore, in AEA-dialyzed cells, extracellularly applied AEA also reduced the $Q_{Kv4.3}$ in the same extent than in non-dialyzed cells. Overall these results, suggest that the blockade was produced as a consequence of the AEA interaction with the extracellular face of the channel.

4. Discussion

In the present paper the effects produced by endocannabinoids (AEA, 2-AG, and LPI) and cannabinoid analogues (PEA and OEA) on human cardiac Kv4.3 channels have been systematically analyzed for the first time. The results demonstrated that these lipid mediators, which are synthesized within the heart, inhibit $I_{Kv4.3}$ and I_{to1} currents by specifically interacting with the channel, AEA and 2-AG being the most potent for this effect. Furthermore, the effects produced by AEA and 2-AG on Kv4.3 channels appeared at identical concentrations to those responsible for their direct effects on other neuronal or vascular channels.

4.1. Endocannabinoids block cardiac Kv4.3 channels

Our results demonstrated, for the first time, that AEA and 2-AG block human cardiac Kv4.3 channels, effects that were independent of the CB1/CB2 receptor activation. Indeed, experiments were developed in CHO cells that do not endogenously express any of the known cannabinoid receptors [2,9,14]. Confirmative experiments were done by examining the effects of 1 μ M AEA in the presence of rimonabant (100 nM), a CB1 receptor antagonist [1]. As can be observed in online Figure III, AEA effects were identical in the absence or presence of rimonabant, a result which added further support to the hypothesis of a direct effect of the endocannabinoids on Kv4.3 channels. Direct effects of AEA on ionic channels have already been reported [3,4]. Indeed, AEA blocks neuronal Kv1.2 [6], TASK-1 [7], T-type Ca^{2+} [9], Kv3.4, Kv3.1, Kv1.1 [8], and Na^+ [5] channels.

The blockade produced by endocannabinoids and cannabinoid analogues of Kv4.3 channels was accompanied by a significant acceleration of the inactivation process. It has been previously demonstrated that AEA accelerates the inactivation of Na^+ , Kv1.2,

Kv3.4, Kv3.1, Kv1.1, and T-type Ca^{2+} channels [5-9], an effect that has been explained as a consequence of either an open channel block [6,9] or a conformational change in the selectivity filter region [8]. Our results also demonstrated that the blockade produced by endocannabinoids was accompanied by a hyperpolarizing shift in the voltage-dependence of inactivation of Kv4.3 channels. Again, similar effects were produced by AEA on the voltage dependence of Na^+ and Ca^{2+} channels [5,9].

Concerning the fatty acids, our results extend and confirm what was previously demonstrated, i.e., that fatty acids can modulate the activity of Ca^{2+} , K^+ , and Na^+ channels [18,23-27]. Furthermore, it has been previously demonstrated that AA inhibited the I_{to1} in rat cardiac myocytes [28]. Our results demonstrated that Kv4.3 channels are particularly sensitive to AA ($\text{IC}_{50}=0.4 \mu\text{M}$), being much more sensitive than, for instance, Kv1.5 channels ($\text{IC}_{50}=21 \mu\text{M}$) [24]. Furthermore, AA and OA also accelerated the inactivation and shifted to more negative potentials the voltage dependence of this process and similar effects were produced by AA and other fatty acids on other channels (for instance Kv1.1, Kv1.5, Kv4.1, Kv4.2, and L-type Ca^{2+} , and Na^+) [8,18,23-27].

Our results demonstrated that AEA ($\text{IC}_{50}=0.4 \mu\text{M}$) and 2-AG ($\text{IC}_{50}=0.3 \mu\text{M}$) inhibited cardiac $I_{\text{Kv4.3}}$ with a potency that is similar to that exhibited by AEA for blocking neuronal TASK-1 and T-type Ca^{2+} ($\text{IC}_{50}=0.33 \mu\text{M}$) channels [7,9], but greater than that exhibited for blocking Na^+ ($\text{IC}_{50}=5.5 \mu\text{M}$) channels in rat dorsal root ganglion neurons [5] or Kv1.2 ($\text{IC}_{50}=2.7 \mu\text{M}$) channels [7,8]. AA can be released by the hydrolysis of AEA and is a potent blocker of Kv4.3 channels. This raises the question of whether the AEA effects could be partially due to its metabolite. However, MetAEA, the metabolically stable analogue of AEA, also blocked Kv4.3 channels with identical

potency to that of AEA, which strongly suggests that the AEA effects were mainly attributable to AEA itself.

The time course of $I_{Kv4.3}$ inhibition produced by endocannabinoids and analogues was slow and no significant washout of the drug effect was observed upon perfusion with drug-free solution. This behaviour is similar to that exhibited by AEA when inhibiting Na^+ , Kv1.2, and TASK-1 channels [5,7,8]. It has been hypothesized that both endocannabinoids and fatty acids could modulate ion channel activity by altering the bulk lipid properties of the membrane as well as membrane fluidity, bilayer stiffness or membrane curvature [20]. In contrast, other reports demonstrated that lipid mediators, such as AA and some PUFA, did not modify the membrane properties at least at those concentrations at which they inhibit cardiac ionic channels [27,29]. For testing whether these unspecific effects can account for the blockade observed, we performed a structural and dynamical characterization of the cell membrane in the absence and in presence of the endocannabinoids. This analysis was done by means of a lipophilic fluorescent dye with an anionic polar head (PA-DPH). The measurement of the fluorescence anisotropy of PA-DPH provides a semiquantitative way of comparing the “fluidity” of the cell membrane under different experimental conditions [17,18]. With these experiments we cannot rule out that endocannabinoids, at the concentrations tested, modify the membrane order and microviscosity. However, the results demonstrated that, in any case, the effects produced by all of them in these membrane properties were almost identical and, thus, cannot account for the significant differences in Kv4.3 blocking potency between the most (AEA and 2-AG) and the least (LPI and PEA) potent endocannabinoids. Therefore, it could be possible that these lipid compounds were also specifically interacting with the channels. Indeed, the fact that AEA, MetAEA, 2-AG, and AA block Kv4.3 channels with a similar potency adds

further support to the hypothesis of the existence of a binding site on the channel that recognizes this type of molecules.

Human cardiac I_{to1} is predominantly carried by Kv4.3 α -subunits, assembled with KChIP2, DPP6, and probably also MiRP2 (KCNE3) ancillary subunits [10-13]. In neuronal Kv4.3 currents, the inactivation kinetics acceleration induced by AA was dependent on the presence of KChIP subunits [23]. Furthermore, effects produced by fatty acids on the cardiac slowly activating delayed rectifier current (I_{Ks}) are dependent on the presence of minK (KCNE1) [22]. Our results suggest that DPP6 and MiRP2 subunits do not modify the blockade produced by AEA on Kv4.3 channels. Moreover, direct effects produced by AEA on human atrial I_{to1} in the presence of CB1 and CB2-receptors antagonists are identical to those produced in transfected Kv4.3/KChIP2 channels. These results suggest that the AEA-interacting site is located in the Kv4.3 α -subunit.

Previous results demonstrated that AEA blocked Kv1.2 and Kv3.4 channels from the extracellular side [6,8], while it blocked T-type Ca^{2+} channels from the intracellular side of the membrane [9]. Our experiments in which cells were dialyzed with AEA or MetAEA demonstrated that they only blocked Kv4.3 channels from the outside, suggesting that the putative interacting site is in the extracellular surface of the channel. Direct interaction of ethanolamides or fatty acids with the channel proteins has been suggested previously [3-9,18,24-26]. Indeed, a single point mutation within the D1-S6 affects fatty acid block of human myocardial Na^+ channel α -subunit [26]. Assuming the existence of this external binding site for endocannabinoids at the Kv4.3 channels, it could be difficult to understand why the onset of block was so slow and the blockade so persistent after washout. However, endocannabinoids and cannabinoid analogues predominantly reside within the membrane bilayer and approach their sites of

action (even the CB receptors, which are oriented externally at the plasma membrane) by laterally diffusing within the membrane leaflet to the target protein [30]. The endocannabinoids slow diffusion until they reached the external face of the Kv4.3 channel and their residence in the lipid bilayer could account for the slow wash-in and washout kinetics.

For analyzing the putative structure-potency relationship, we also tested the effects of structurally related fatty acids and their corresponding ethanolamides. The results demonstrated that there were no differences in the potency of block between each fatty acid and its corresponding ethanolamides. Moreover, blockade seems not to be related with the liposolubility of the compound. In contrast, our results suggest that as the number of C in the fatty acid chain (at least between 16 and 20 atoms) and complexity increase, the potency of block increases. However, further experiments are needed for the molecular characterization of the putative binding site for lipid compounds at the human cardiac Kv4.3 channels.

4.2. Physiological relevance

Cardiovascular actions of endocannabinoids (including changes in arterial blood pressure, cardiac contractility and heart rate) are complex, involving effects on the vasculature and myocardium, as well as modulation of autonomic outflow [1,31]. These effects have been widely interpreted as being mediated by cannabinoid receptors [1,31]. However, several physiological roles for AEA and 2-AG, including modulation of neuronal excitability [32], pain [33], and cardiovascular functions, are independent of the cannabinoid receptors activation [1,31]. Indeed, AEA can directly activate VR-1 vallinoid receptors, modulate ion channels or act on as yet unidentified targets [1,3,31].

In the present study, we demonstrate for the first time that low micromolar concentrations of the two main endocannabinoids directly block human cardiac Kv4.3 channels and native I_{to1} . Radioligand studies indicate that the K_i values for AEA and 2-AG are in the range of 60-540 nM and 60-470 nM for CB1 and of 0.37-1.9 μ M and 0.15-1.4 μ M for CB2 receptors, respectively [1]. However, it is extremely difficult to establish a physiological range of concentrations for these lipidic compounds that are embedded and accumulated in the membranes.

Endocannabinoids and cannabinoid analogues are synthesized within the heart [1,31]. In other tissues these mediators are generated upon demand, their production being stimulated by various tissue insults or damages including inflammation, oxidative stress and apoptosis [1,31]. It seems reasonable to assume that this would also be the case in the myocardium. Furthermore, in neuronal tissues endocannabinoid synthesis is enhanced by increasing the $[Ca^{2+}]_i$ and at high-frequencies of stimulation [1], conditions both associated with cardiac tachyarrhythmias. Kv4.3 channels underlie the I_{to1} which plays a role in determining the height and the duration of phase 2 of the human cardiac AP [10]. The powerful direct inhibition of human $I_{Kv4.3}$ and I_{to1} produced by AEA and 2-AG, would increase the height and prolong the plateau duration of human AP. This direct effect would be of particular importance under those conditions which increase the endocannabinoid synthesis. The effects of these endogenous lipid mediators on cellular cardiac electrophysiology have never been tested. Therefore, further studies are needed to identify the putative endocannabinoid effects on human cardiac electrical activity as well as the presence of CB receptors in the human myocardium both in normal and pathological conditions.

Acknowledgments:

We thank Prof. Manuel Guzmán for his helpful suggestions. Supported by Fondo de Investigación Sanitaria (PI080665), Ministerio de Educación y Ciencia (SAF2008-04903), Instituto de Salud Carlos III (Red HERACLES RD06/0009), Fundación LILLY, Centro Nacional de Investigaciones Cardiovasculares (CNIC-13), Universidad Complutense de Madrid (UCM-4195), BFU2006-03905 (MPL) and Sociedad Española de Cardiología. Ricardo Gómez is a fellow of Comunidad Autónoma de Madrid.

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Table 1. IC₅₀ for Kv4.3 channel block obtained with each compound tested.

| Compound | IC₅₀ (μM) | Compound | IC₅₀ (μM) |
|-----------------|-----------------------------|-----------------|-----------------------------|
| AEA | 0.4 ± 0.01 | | |
| MetAEA | 0.6 ± 0.02 | AA | 0.3 ± 0.02 |
| 2-AG | 0.3 ± 0.01 | | |
| OEA | 1.6 ± 0.5 | OA | 2.2 ± 0.05 |
| SEA | 6.4 ± 2.1 | SA | 5.3 ± 0.8 |
| LPI | 8.0 ± 0.9 | | |

Figure Legends

Fig. 1. Chemical structure of the compounds tested.

Fig. 2. $I_{Kv4.3}$ traces elicited by 250 ms-pulses from -80 to +50 mV in the absence and presence of 1 μ M AEA (A), MetAEA (B), 2-AG (C), OEA (D), LPI (E), and PEA (F). Dashed lines represent zero current level.

Fig. 3. $I_{Kv4.3}$ traces elicited by 250 ms-pulses from -80 to +50 mV in the absence and presence of 1 μ M AA (A) and PA (B). Time constants of the fast component (τ_f) of current inactivation in the absence and presence of endocannabinoids and cannabinoid analogues (C) or fatty acids and ethanolamides (D). Each bar represents the mean \pm s.e.m. of ≥ 5 experiments. * $P < 0.05$ vs. control.

Fig. 4. Reduction of $Q_{Kv4.3}$ at +50 mV plotted as a function of the concentration of endocannabinoids (A), ethanolamides (B), fatty acids (C) and fatty acids and their corresponding ethanolamides (D). The Hill equation was fitted to the data fixing the top and the nH to 100% and the unity, respectively. Each point represents the mean \pm s.e.m. of ≥ 5 experiments.

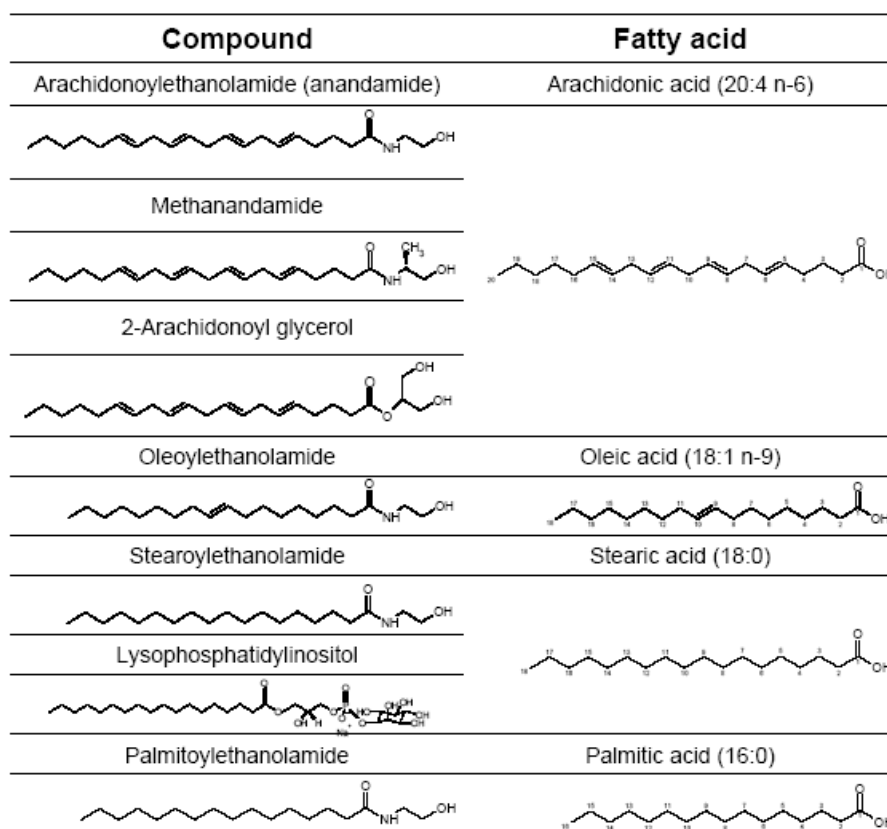
Fig. 5. (A) $I_{Kv4.3}$ traces in the absence and presence of 1 μ M AEA obtained when applying 250 ms-pulses to potentials ranging from -90 to +50 mV followed by a 250 ms-pulse to +50 mV. Inactivation curves in the absence (●) and presence (○) of 1 μ M AEA (B), MetAEA (C), 2-AG (D), OEA (E), LPI (F), and PEA (G). The squares represent the relative current as a function of the membrane potential. A Boltzmann equation was fitted to the data (continuous lines). The dashed lines represent the curve

in the presence of drug, normalized to the control amplitude. * $P < 0.05$ vs. control. # $P < 0.05$ vs. value at -90 mV. Each point represents the mean \pm s.e.m. of ≥ 5 experiments.

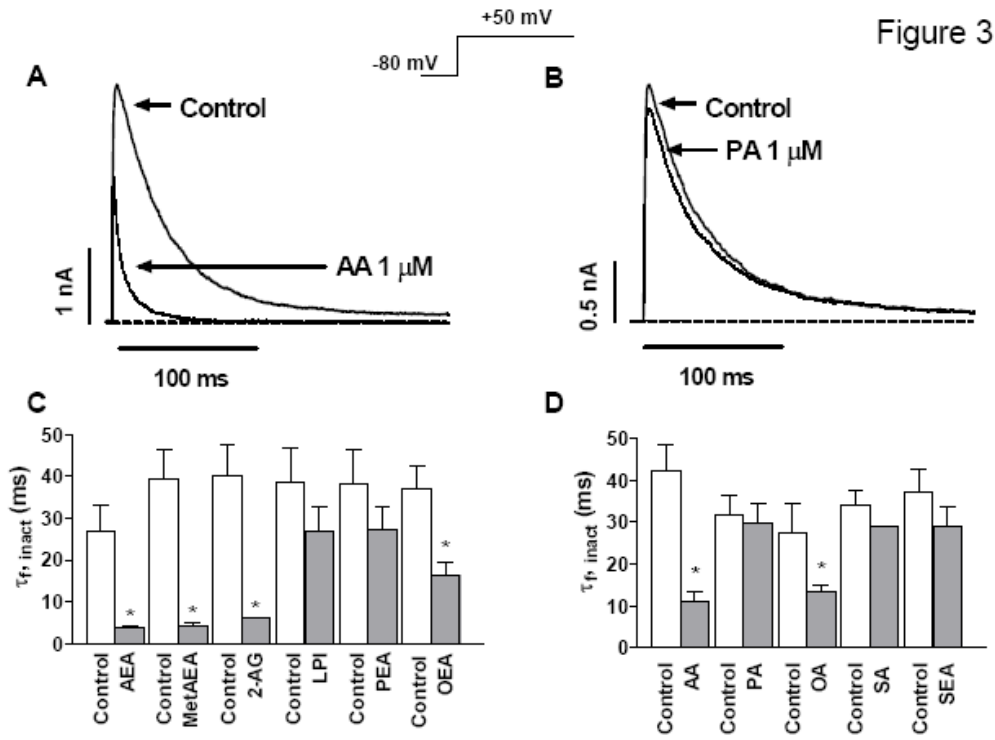
Fig. 6. (A) Partition (logP) or distribution (logD) coefficient of each compound plotted as a function of its corresponding IC_{50} . (B) Complexity of each compound plotted as a function of its corresponding IC_{50} . (C-F) Steady-state fluorescence anisotropy (r_{ss} , C), limiting anisotropy (r_{∞} , E) and correlation times (ns) (ϕ , F) values of PA-DPH ($2 \mu\text{M}$) incorporated in CHO cells measured in control conditions and after 20 min incubation with $1 \mu\text{M}$ AEA, 2-AG, LPI or PEA. (D) Fluorescence anisotropy decays of PA-DPH incorporated in CHO cells under control conditions and in the presence of $1 \mu\text{M}$ AEA or PEA. $\lambda_{ex} = 375$ nm. $\lambda_{em} = 450$ nm. The fit of Eq. 8 (see online supplemental data) to the decays is shown in solid lines. The randomly distributed weighted residuals are shown below.

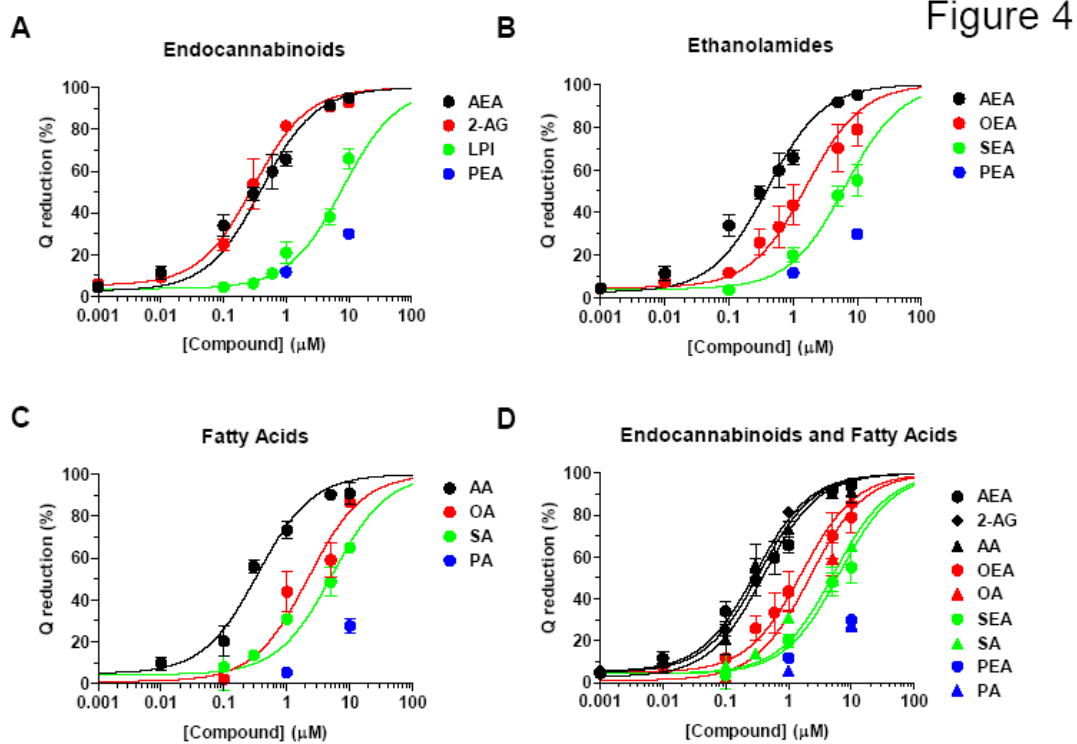
Fig. 7. (A) $I_{Kv4.3}$ traces elicited by 250 ms-pulses from -80 to $+50$ mV in the absence and presence of $1 \mu\text{M}$ AEA in a CHO cell dialyzed with BAPTA-containing internal solution. (B) $Q_{Kv4.3}$ reduction at $+50$ mV produced by $1 \mu\text{M}$ AEA in cells dialyzed either with EGTA- or BAPTA-containing internal solution. (C) $Q_{Kv4.3}$ reduction at $+50$ mV produced by $1 \mu\text{M}$ AEA in cells expressing Kv4.3+KChIP2, Kv4.3+KChIP2+KCNE3 or Kv4.3+KChIP2+DPP6 channels. (D) $Q_{Kv4.3}$ reduction at $+50$ mV produced by $1 \mu\text{M}$ AEA applied extracellularly, intracellularly or both, AEA applied intracellularly in the presence of URB-597, and MetAEA applied intracellularly. Each bar represents the mean \pm s.e.m. of ≥ 4 experiments.

Figure 1



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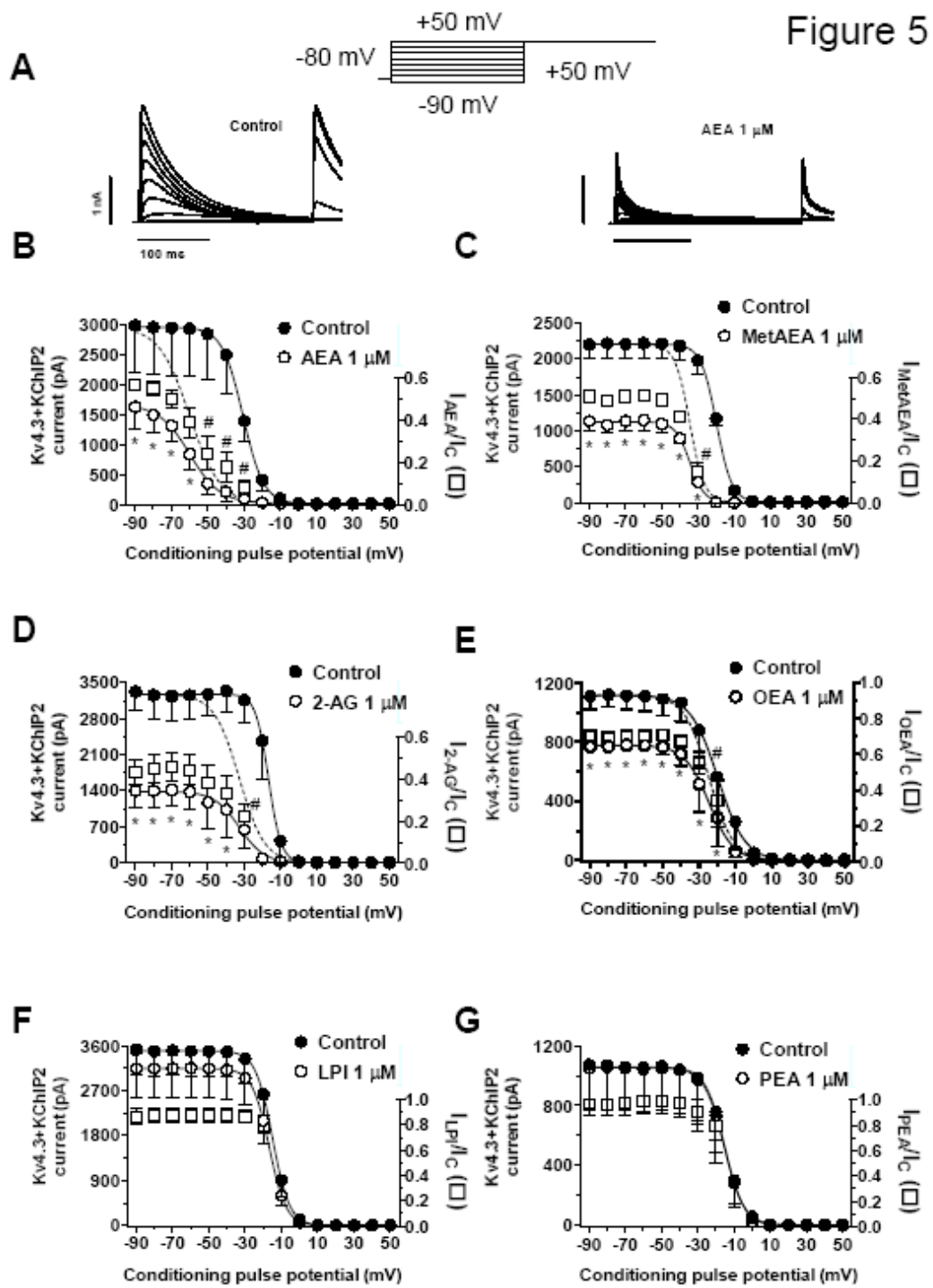
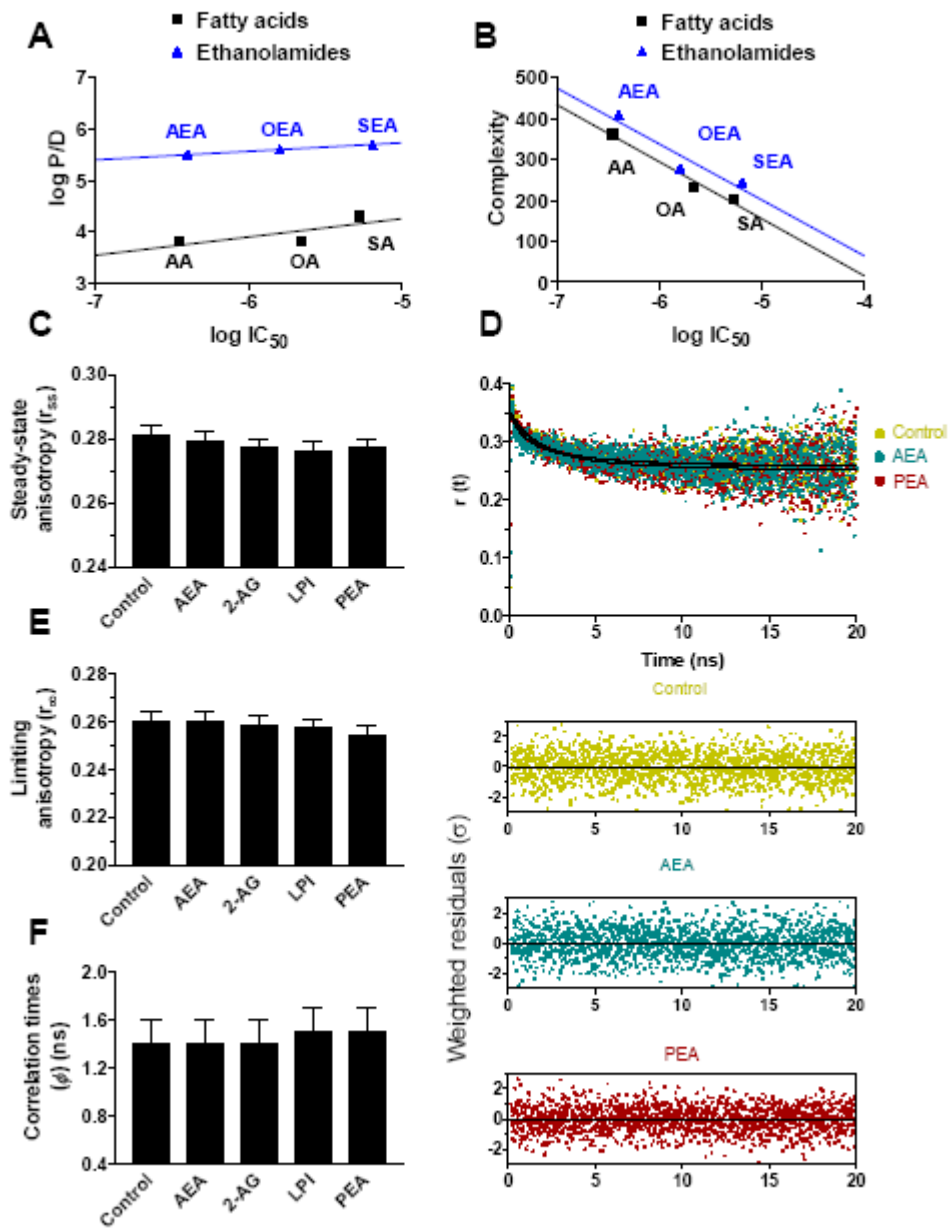
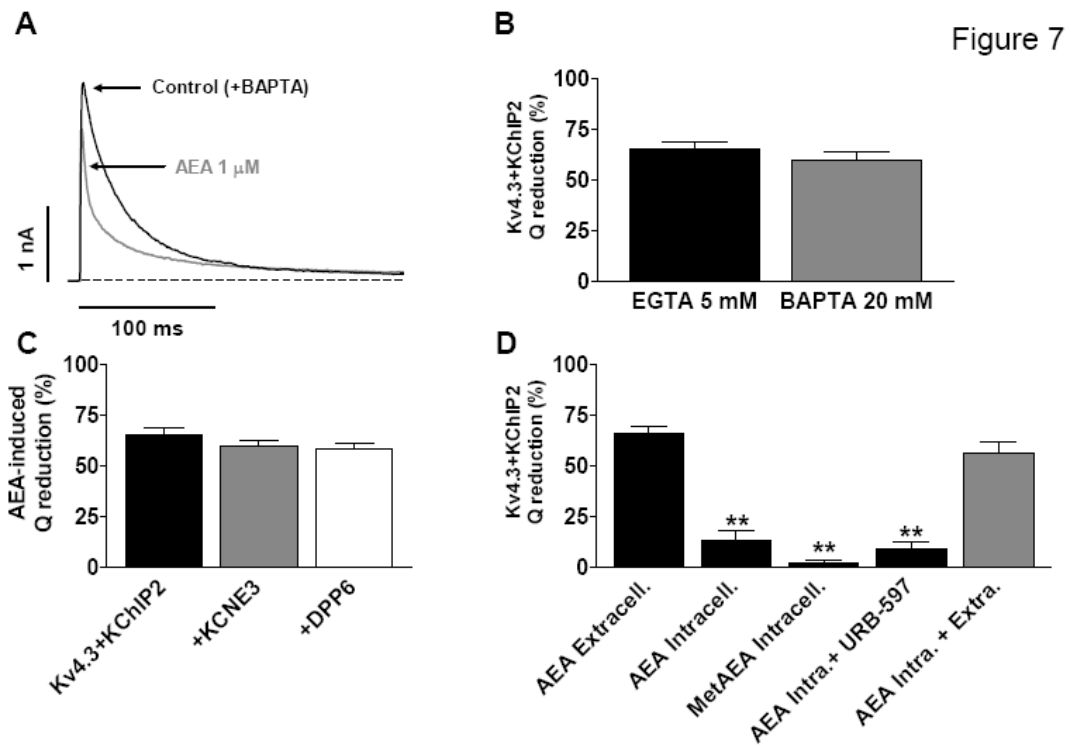


Figure 6





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