Endocannabinoids and cannabinoid analogues block cardiac hKv1.5 channels in a cannabinoid receptor-independent manner

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Aims
Endocannabinoids are synthesized from lipid precursors at the plasma membranes of virtually all cell types, including cardiac myocytes. Endocannabinoids can modulate neuronal and vascular ion channels through receptor-independent actions; however, their effects on cardiac K+ channels are unknown. This study was undertaken to determine the receptor-independent effects of endocannabinoids such as anandamide (N-arachidonoyl ethanolamine, AEA), 2-arachidonoylglycerol (2-AG), and endocannabinoid-related compounds such as N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA), the endogenous lipid lysophosphatidylinositol (LPI), and the fatty acids from which some of these compounds are endogenously synthesized, on human cardiac Kv1.5 channels, which generate the ultra-rapid delayed rectifier current (IKur).

Methods and results
hKv1.5 currents (IhKv1.5) were recorded in mouse fibroblasts (Ltk2 cells) by using the whole-cell patch-clamp technique. Most of these compounds inhibited IhKv1.5 in a concentration-dependent manner, the potency being determined by the number of C atoms in the fatty acyl chain. Indeed, AEA and 2-AG, which are arachidonic acid (20:4) derivatives, exhibited the highest potency (IC50 ~ 0.9–2.5 μM), whereas PEA, a palmitic acid (PA-16:0) derivative, exhibited the lowest potency. The inhibition was independent of cannabinoid receptor engagement and of changes in the order and microviscosity of the membrane. Furthermore, blockade induced by AEA and 2-AG was abolished upon mutation of the R487 residue, which determines the external tetraethylammonium sensitivity and is located in the external entryway of the pore. AEA significantly prolonged the duration of action potentials (APs) recorded in mouse left atria.

Conclusion
These results indicate that endocannabinoids block human cardiac Kv1.5 channels by interacting with an extracellular binding site, a mechanism by which these compounds regulate atrial AP shape.

Keywords
Anandamide • 2-arachidonoylglycerol • Kv1.5 • Endocannabinoids • Fatty acids • Arachidonic acid • Cardiac potassium channels

1. Introduction
hKv1.5 channels underlie the ultrarapid delayed rectifier current (IKur) which is critical for determining the height and duration of the human atrial action potential (AP).1 This current is absent in the human ventricle and hence represents a suitable target for selectively modulating atrial AP duration (APD).1
The endocannabinoids anandamide (N-arachidonoylthanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are arachidonic acid (AA) derivatives that exert a wide array of modulatory actions by engaging cannabinoid (CB1 and CB2) receptors. Very recently, the endogenous phospholipid 1-alpha-lyso phosphatidylcholine (LPI) has been identified as a ligand for a new CB receptor: the orphan G protein-coupled receptor 55 (GPR55). N-palmitoylethanolamine (PEA) and N-oleylethanolamine (OEA), which contain palmitic (PA) and oleic (OA) fatty acids as structural moieties, respectively, are usually considered on the basis of their structure and mode of action as cannabinoid analogues, as they do not bind with high affinity to CB receptors but potentiate AEA responses through various mechanisms.

Endocannabinoids and cannabinoid analogues are synthesized at the plasma membranes of virtually all cell types including the cardiac cells. Several physiological roles attributed to AEA, including modulation of neuronal excitability, pain, and cardiovascular functions, are at least in part independent of CB receptor activation. Thus, it has been demonstrated that endocannabinoids and cannabinoid analogues modulate functional properties of Na\(^+\), K\(^+\), and Ca\(^{2+}\) neuronal and/or vascular channels in a receptor-independent manner. However, data on the receptor-independent endocannabinoid effects in human cardiac channels are scarce.

Therefore, in the present study, we analysed the effect of endocannabinoids and cannabinoid analogues on the hKv1.5 current (\(I_{\text{hKv1.5}}\)) and found that AEA and 2-AG are the more potent agents at inhibiting \(I_{\text{hKv1.5}}\). This effect was: (i) independent of CB receptor engagement and of changes in cell membrane lipid order and microviscosity; (ii) exclusively apparent when the endocannabinoid was applied at the external surface of the cell membrane; and (iii) abolished upon mutation of the residue that determines K\(_{\text{V1.5}}\) channel external tetratetraethylammonium (TEA) sensitivity.

2. Methods

Mouse fibroblasts (Ltk\(^-\)) stably expressing hKv1.5 were cultured as described. Li\(_{\text{Na}}\) was recorded on myocytes isolated from right atrial appendages of patients undergoing cardiac surgery (Supplementary material online, Table S1). The study conforms with the principles of Declaration of Helsinki. Currents were recorded using the whole-cell patch-clamp technique. Ltk\(^-\) cells were perfused with an external solution containing (mM): NaCl 136, KCl 42, KH\(_2\)PO\(_4\) 10, MgATP 5, phospho-creatine 3, HEPES 5, and EGTA 5 (pH 7.4 with KOH). Recording pipettes were filled with an internal solution containing (mM): K-aspartate 80, KCl 42, KH\(_2\)PO\(_4\) 10, MgATP 5, phospho-creatine 3, HEPES 5, and EGTA 5 (pH 7.2 with KOH). Steady-state and time-resolved anisotropy measurements were performed by using fluorescence depolarization techniques and a lipophylic fluorescent dye 2-carboxyethyl-1,6-diphenyl-1,3,5-hexatriene (PA-DPH). Presence of CB1 and CB2 receptors in Ltk\(^-\) cells was analysed by western blot and real-time quantitative PCR assays. AEA, its non-hydrolysable analogue (R)-(±)-arachidonoyl-1'-hydroxy-Z-propylamide (MetAEA), LPI, AA, PA, stearic acid (SA), stearoylethanolamide (SEA), OA [Sigma], 2-AG and OEA [Tocris] were dissolved in ethanol, whereas PEA (Sigma) was dissolved in dimethylsulfoxide.

Results were expressed as mean ± SEM. For comparisons Student’s t-test or ANOVA were used as appropriate followed by Newman–Keuls test. A value of \(P < 0.05\) was considered statistically significant. An expanded Materials and Methods section is available in the Supplementary material online.

3. Results

Figure 1 shows the chemical structure of the endocannabinoids and cannabinoid analogues tested in this study. Since most of them are ethanolamides of fatty acids, we also analysed 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). Figure 2 shows \(I_{\text{hKv1.5}}\) traces recorded by applying 500 ms pulses from −80 mV to +60 mV and the tail currents recorded on return to −40 mV, in the absence and presence of AEA (Figure 2A), its non-hydrolysable analogue MetAEA (Figure 2B), 2-AG (Figure 2C), OEA (Figure 2D), LPI (Figure 2E), and PEA (Figure 2F) at 1 μM. AEA slightly decreased the peak current amplitude and induced a fast falling phase \((\tau = 19.4 ± 3.7 \text{ ms})\) of current decay without modifying the slow process of inactivation \((\tau_{\text{inact}} = 160 ± 17 \text{ ms})\) resulting in a reduction of the amplitude at the end of the pulse to +60 mV of 49.4 ± 4.1\%(\(P < 0.05\), \(n = 6\)). Moreover, AEA slowed the time course of tail current decay which was adjusted by a biexponential function, increasing the \(\tau_1\) and \(\tau_2\) values from 16.4 ± 2.9 and 98.1 ± 18.1 ms to 30.3 ± 6.5 and 173.9 ± 35.9 ms \((P < 0.05)\), respectively (Figure 2A and Supplementary material online, Table S2). Interestingly, this inhibition was reproduced in the presence of CB1 (rimonabant) and CB2 (AM630) selective antagonists (Supplementary material online, Figure S1). Moreover, western blot and real-time quantitative PCR analysis demonstrated that our Ltk\(^-\) cells do not express endogenous CB1 and CB2 receptors (Supplementary material online, Figure S1), supporting the hypothesis that the blockade was independent on the interaction with CB receptors. MetAEA, 2-AG, and OEA produced similar effects to those observed with AEA, i.e. they reduced \(I_{\text{hKv1.5}}\) by 39.0 ± 4.1\%, 46.1 ± 4.3\%, and 29.8 ± 4.7\%, respectively, and slowed the tail current decay \((n \geq 5, \ P < 0.05)\) (Figures 2B–D and Supplementary material online, Table S2). In contrast, LPI and PEA slightly decreased \(I_{\text{hKv1.5}}\) from 12.9 ± 0.8\% and 3.6 ± 1.2\%, respectively, without modifying the tail current kinetics \((n = 5, P > 0.05)\) (Figures 2E and F and Supplementary material online, Table S2). The time course of \(I_{\text{hKv1.5}}\) inhibition produced by endocannabinoids and cannabinoid analogues was slow (~10 min of drug perfusion) and no significant washout of the effect was observed upon perfusion with drug-free solution \((I_{\text{AEA/C24}} = 0.56 ± 0.06\) after 20 min of washout of AEA). Supplementary material online, Figure S2 shows the time course of the onset and offset of AEA-induced block.

Figure 3A shows \(I_{\text{hKv1.5}}\) traces recorded by applying the voltage-protocol at the top in the absence and presence of AEA, and Figures 3B–G current–voltage (IV) relationships constructed by plotting the current amplitude as a function of the membrane potential (500 ms isochronal). AEA, MetAEA, 2-AG, and OEA,
but not LPI and PEA, significantly inhibited the current at potentials between 0 and +60 mV. As inferred from the representation of the relative current (represented by squares in Figures 3B–G), the endocannabinoid-induced blockade steeply increased in the voltage range coinciding with that of channel opening (between -20 and 0 mV), remaining constant at more positive potentials.

Next, the effects of the fatty acids PA, SA, OA, and AA as well as that of SEA at 1 μM were studied (Supplementary material online, Figures S3 and S4). AA, OA, and SA decreased IhKv1.5 at the end of the pulses at +60 mV by 32.0 ± 2.1, 75.7 ± 1.6, and 31.9 ± 2.8%, respectively (ν = 4, P < 0.05) and slowed the time course of current deactivation (Supplementary material online, Table S2). In contrast, the effects of SEA and PA on the current amplitude and kinetics did not reach statistical significance (Supplementary material online, Figures S3 and S4 and Table S2).

Figure 4 shows the concentration-response curves for the endocannabinoids (Figure 5A), the ethanolamides (Figure 5B), and the fatty acids (Figure 5C) tested, obtained by plotting the reduction of IhKv1.5 at +60 mV as a function of the concentrations of each compound. The Hill equation was fitted to the data and the IC50 values were calculated (Supplementary material online, Table S3). Among the endocannabinoids (Figure 5A), the AA derivatives AEA and 2-AG exhibited the highest potency, yielding IC50 values of 0.9 ± 0.1 and 2.5 ± 0.2 μM, respectively. Blockade obtained in the presence of the highest concentration of PEA and LPI was below 40%, which impaired the fitting of the data to the Hill equation. When comparing the potency of the fatty acids (Figure 5C), the results demonstrated that OA and AA are

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fatty acid</th>
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<tr>
<td>N-Arachidonoyl ethanolamine (anandamide)</td>
<td>Arachidonic acid (20:4 n-6)</td>
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<tr>
<td>Methanandamide</td>
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<tr>
<td>2-Arachidonoyl glycerol</td>
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<tr>
<td>N-Oleylethanolamine</td>
<td>Oleic acid (18:1 n-9)</td>
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<tr>
<td>N-Stearoyl ethanolamine</td>
<td>Stearic acid (18:0)</td>
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<tr>
<td>Lysophosphatidylglycerol</td>
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<tr>
<td>N-Palmitoyl ethanolamine</td>
<td>Palmitic acid (16:0)</td>
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Figure 1 Chemical structures of the compounds tested.
the most potent while PA and SA are the least potent at inhibiting \( hKv1.5 \). In Figure 5D, the concentration dependence of the \( hKv1.5 \) channel 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 support that in all the cases, the ethanolamide is less potent than its corresponding fatty acid, excluding AEA and AA, which displayed the same potency. Moreover, with the exception of OA, when the number of carbons in the fatty acyl chain increases, the potency of block also increases.

3.1 Mechanism of endocannabinoid-induced block

Endocannabinoids and cannabinoid analogues are lipid mediators that could indirectly affect ion channels, by producing a perturbation in the cell membrane.\(^{15}\) As an index of the liposolubility of each compound, we used the partition (\( \log P \)) and the distribution (\( \log D \)) coefficient for the ethanolamides and the fatty acids, respectively. In Figure 6A, the logP or logD (logP/D) values were plotted as a function of the blockade produced by 1 \( \mu M \) of each compound. The results demonstrate that there is no relationship between the liposolubility of the compounds and their potencies for blocking \( hKv1.5 \) channels. To further analyse this issue, the blockade produced by each compound (at 1 \( \mu M \)) was plotted against its complexity (Figure 6B). This latter parameter considers not only the size but also the presence and nature of the reactive groups in the molecule and was calculated following the Bertz/Hendrickson/Ihlenfeldt equation\(^{16}\) (see Supplementary material online). For both ethanolamides and acids (with the exception of AA), the increase in complexity correlated with an increase in the potency of \( hKv1.5 \) blockade (\( r^2 = 0.9158 \) and 0.9685 for ethanolamides and acids, respectively).

We also compared the putative changes in fluorescence anisotropy of \( Ltk^- \) cells stably expressing \( hKv1.5 \) channels induced by AEA, 2-AG, LPI, PEA, AA, and PA at a concentration of 1 \( \mu M \). 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.\(^{13}\) The time-scale of PA-DPH 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 \( \mu M \)) in control conditions was satisfactorily described by a major contribution (\( \approx 90\% \) of the total intensity) with a lifetime of 5.7 ns and a short component of 2.4 ns. The fluorescence lifetimes were not significantly modified by any of the compounds tested.

The steady-state fluorescence anisotropy \( (r_{ss}) \) of PA-DPH incorporated into \( Ltk^- \) cells was measured under control conditions and after 20 min incubation with AEA, 2-AG, LPI, PEA, AA, and PA (1 \( \mu M \)). None of the compounds tested changed the \( r_{ss} \), which in control conditions averaged 0.282 ± 0.003 (Figure 6C). It is known that \( r_{ss} \) is the sum of a structural (lipid order) and a dynamic (microviscosity) contribution. To quantify these components separately, we performed time-resolved anisotropy measurements. In Figure 6D, the fluorescence anisotropy \( [r(t)] \) decay of PA-DPH in control conditions and in the presence of AEA or PEA is shown. The \( r(t) \) decayed in a few nanoseconds to a residual or limiting anisotropy \( (r_1) \), which was calculated by fitting Eq. (8) to the \( r(t) \) decay (see Supplementary material online) 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 \( (\phi) \)

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**Figure 2** \( hKv1.5 \) traces elicited by 500 ms pulses from \(-80\) to \(+60\) mV and tail currents recorded at \(-40\) mV in the absence and presence of 1 \( \mu M \) AEA (A), MetAEA (B), 2-AG (C), OEA (D), LPI (E), and PEA (F).
which is related with the microviscosity of the cell membrane. Incubation with AEA, 2-AG, LPI, PEA, AA, and PA for 20 min did not modify $r_m$ ($0.253 \pm 0.004$) and $\phi$ ($1.8 \pm 0.2$ ns) values (Figure 6E and F). Similar results were obtained when incubating for 120 min (data not shown). Overall these results do not completely rule out that these compounds might produce modifications in the order and microviscosity of the cell membrane, but support that, if any, all of them produce similar unspecific perturbations of the lipid environment.

Cannabinoids have been shown to modify Ca$^{2+}$ release from ryanodine-sensitive stores in hippocampal neurons.\(^4\) Changes in cytoplasmic-free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) modulate intracellular pathways that, in turn, could modify the $I_{hKv1.5}$. To examine whether the observed effects were due to a cannabinoid-induced modification of [Ca$^{2+}$], we tested the effects of AEA in the presence of the intracellular Ca$^{2+}$ chelator BAPTA. In BAPTA dialyzed cells, AEA reduced $I_{hKv1.5}$ amplitude at +60 mV by $48.3 \pm 2.7\%$ ($n = 5$), a reduction that was not statistically different from that produced by AEA in EGTA-dialyzed cells (Figure 7A and B).

It has been described that AEA can regulate protein kinase C (PKC) in rat brain.\(^18\) To determine whether the endocannabinoid-induced $I_{hKv1.5}$ inhibition is mediated by the stimulation of PKC, the effects of AEA in the presence of the PKC inhibitor staurosporine (0.1 μM) were analysed. Under these conditions, AEA induced a similar reduction ($38.4 \pm 10.2\%$ ...
at +60 mV) than that produced in the absence of staurosporine (P > 0.05, n = 4) (Figure 7C).

In some cases, endocannabinoid- and fatty acid-induced inhibition of ion channels is only evident when they are applied intra- or extracellularly.10,13,19 Therefore, we compared the effect produced by AEA (1 μM) when added to either the extracellular or the intracellular solution (Figure 7D). Addition of AEA to the intracellular solution produced a current reduction lower than that produced when it was added to the extracellular solution (6.2 ± 2.5 vs. 49.4 ± 4.1% at +60 mV, P < 0.05). Furthermore, in AEA-dialyzed cells, extracellularly applied AEA also reduced the current in the same extent as in non-dialyzed cells (Figure 7D). These results suggest that the AEA-induced block is produced as a consequence of its interaction with the extracellular face of the channel. Therefore, we next studied the putative competition between AEA and TEA for this extracellular binding site. TEAo (100 mM) inhibited hKv1.5 by 54.3 ± 3.4% at +60 mV and significantly reduced the blockade produced by AEA (15.5 ± 2.5%) (Figure 7E), suggesting that both TEAo and AEA compete for the same binding site on hKv1.5 channels. In these channels, TEAo sensitivity is determined by R487, a residue located at the external entryway of the pore.20 Thus, the AEA effects on R487Y channels stably expressed in Ltk− cells were also analysed (Figure 7F). AEA inhibited the current generated by R487Y channels only by 14.5 ± 6.4% at +60 mV, a reduction significantly lower than that produced in WT channels (P < 0.01, n = 6). Furthermore, R487Y mutation also significantly decreased the 2-AG blockade (Figure 7F, n = 5).
3.2 Effects of AEA on human atrial Isus and on mouse atrial APs

To test whether the effects of AEA so far described in cloned channels were reproduced in native currents, we next studied its direct effects on the Isus, recorded in enzymatically dissociated human atrial myocytes which is mainly carried by Kv1.5 channels.1,21 Experiments were done in the presence of rimonabant and AM630 (both at 1 μM).

Figure 8A shows Isus traces recorded at +50 mV (after a prepulse to +50 mV to inactivate the Ito) in the absence and presence of AEA 1 μM. The simultaneous presence of rimonabant and AM630 inhibited the current at the end of the pulse by a 22.9 ± 5.0%, and addition of AEA further decreased the current density by 48.6 ± 4.7% (n = 5, P < 0.05) (Figure 8B).

We next studied its effects on the AP characteristics recorded using microelectrode techniques in left mouse atria.12 Figure 8C shows APs in the presence of rimonabant (1 μM) and AM630 (1 μM) with and without 1 μM AEA. AEA did not modify the resting membrane potential (RPc = −87.0 ± 1.7 mV) or the AP amplitude (APAc = 114.4 ± 2.6 mV) (P > 0.05, n = 7), whereas it significantly lengthened the current density by 48.6 ± 4.7% (n = 5, P < 0.05) (Figure 8D).

4. Discussion

The results presented here demonstrate that the endocannabinoids AEA and 2-AG, as well as cannabinoid analogues such as OEA, inhibit human cardiac IhKv1.5. These effects were also produced with a similar potency by OA and AA. In contrast, SA, PA, their corresponding ethanolamides, and LPI were less potent at blocking hKv1.5 channels than the AA derivatives. Our results also support that AEA and 2-AG blockade occurs upon their specific interaction with the external face of the channel protein at the TEA binding site.

4.1 Endocannabinoids and cannabinoid analogues block hKv1.5 channels

AEA and 2-AG can be considered potent blockers of hKv1.5 channels since, in both cases, the IC50 values were in the low micromolar range. The fact that the AEA-induced effects on IKv1.5 and Isus were not abolished in the presence of CB1 or CB2 selective antagonists and that Ltk− cells do not express endogenous CB1 and CB2 receptors strongly support the hypothesis that the blockade is independent on the interaction with CB receptors. AEA and 2-AG exhibit a similar potency for blocking neuronal TASK-1 (IC50 = 0.7 μM),9 T-type Ca2+ (IC50 = 0.33 μM),11 Kv1.2 (IC50 = 2.7 μM),8 and cardiac Kv4.3 (IC50 = 0.4 μM)22 channels to that exhibited for blocking cardiac hKv1.5 channels. In contrast, AEA is more potent for blocking hKv1.5 than for blocking Na+ channels (IC50 = 5.5 μM) in rat dorsal root ganglion neurons.7

We also analysed the effects on hKv1.5 channels of some fatty acids (particularly those that constitute the structural scaffold of endocannabinoids and cannabinoid analogues) as tools to obtain...
more accurate information about a putative structure-effect relationship of endocannabinoid action. Our results demonstrate that OA and AA are potent hKv1.5 channel blockers, and extend previous reports demonstrating that fatty acids can modulate the activity of Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) channels.\(^{13,19,23,24}\) AA can be released by the hydrolysis of AEA. Therefore, it might be argued that the AEA-induced effects were due to its metabolite AA. However, MetAEA, a non-hydrolysable analogue of AEA, also blocked hKv1.5 channels with identical potency to that of AEA, which strongly suggests that the AEA effects are mainly attributable to AEA itself.

The results demonstrate that the fatty acids are more potent for blocking hKv1.5 channels than their corresponding ethanolamides, with the exception of AA and AEA. Moreover, blockade does not seem to be related with the liposolubility of the compound. In contrast, our results suggest that as the number of C atoms in the fatty acyl chain (at least between 16 and 20 atoms) and the complexity increase, the potency of block increases. A similar dependency on the chain length has been described for the blockade induced by alkanols of the I\(_a\) recorded in Aplysia neurons.\(^{25}\) The exception to this general rule is AA, which was less potent than predicted considering the length and the presence of four unsaturations in the chain.

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**Figure 6** (A and B) Partition (logP) or distribution (logD) coefficient (A) or complexity (B) of each compound plotted as a function of the blockade produced at 1 \(\mu\)M. (C–F) Steady-state fluorescence anisotropy \((r_{ss}, C)\), limiting anisotropy \((r_{lim}, E)\), and average correlation times \((\phi, F)\) values of PA-DPH incorporated in \(Ltk^-\) cells measured in control conditions and after 20 min incubation with 1 \(\mu\)M AEA, 2-AG, LPI, PEA, AA or PA. (D) Fluorescence anisotropy decays of PA-DPH incorporated in \(Ltk^-\) cells under control conditions and in the presence of 1 \(\mu\)M AEA or PEA. \(\lambda_{ex} = 375\) nm, \(\lambda_{em} = 450\) nm. The fit of Eq. (8) (Supplementary material online) to the decays is shown in solid lines. The randomly distributed weighted residuals are shown here.
Figure 7  (A) $I_{hKv1.5}$ traces elicited by 500 ms pulses from -80 to +60 mV in the absence and presence of 1 μM AEA in a BAPTA-dialyzed cell. (B) $I_{hKv1.5}$ inhibition at +60 mV produced by 1 μM AEA in cells dialyzed either with EGTA- or BAPTA-containing internal solution. (C) $I_{hKv1.5}$ inhibition at +60 mV produced by 1 μM AEA in the absence or presence of 0.1 μM staurosporine. The inset shows $I_{hKv1.5}$ traces at +60 mV in control conditions, in the presence of staurosporine alone, and plus AEA. (D) $I_{hKv1.5}$ inhibition at +60 mV produced by 1 μM AEA applied extracellularly, intracellularly, or both. The inset shows the time course of $I_{hKv1.5}$ induced inhibition by AEA when it is applied intracellularly (closed circles) or from both sides of the membrane (open circles). **$P < 0.01$ vs. block induced by AEA applied extracellularly. (E) $I_{hKv1.5}$ inhibition at +60 mV produced by 1 μM AEA, 100 mM TEA-o, and AEA in the presence of TEA-o. **$P < 0.01$ vs. block induced by AEA in the absence of TEA-o. (F) Current inhibition at +60 mV produced by 1 μM AEA and 2-AG in cells expressing WT or R487Y hKv1.5 channels. The inset shows R487Y currents at +60 mV in the absence and presence of 1 μM AEA. Each bar represents the mean ± SEM of ≥4 experiments. **$P < 0.01$ vs. block in WT channels.
Blockade, particularly that which is produced by fatty acids, could be an indirect action via fatty acid-sensitive subtypes of PKC, as described for an ATP-sensitive K_+ channel. This mechanism was discarded by analysing the effects of AEA in the presence of the PKC inhibitor staurosporine. Furthermore, 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. In contrast, other reports demonstrated that lipid mediators, such as AA and some polyunsaturated fatty acids, did not modify the membrane properties, at least at those concentrations at which they inhibit cardiac ionic channels. 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 presence of the endocannabinoids. The measurement of PA-DPH fluorescence anisotropy provides a semiquantitative way of comparing the ‘fluidity’ of the cell membrane under different experimental conditions. These experiments cannot rule out that endocannabinoids modify the membrane order and microviscosity. However, the results demonstrate that, in any case, the effects produced by all of them in these membrane properties are almost identical. In contrast, the differences in hKv1.5 blocking potency between the most (AEA and 2-AG) and the least (LPI and PEA) potent endocannabinoids were statistically significant. A similar finding has been demonstrated for the most (AA) and the least (PA) potent fatty acids. Therefore, and since differences in the effects on the membrane order and microviscosity do not account for the differences in the potency of block, it is conceivable that these lipid compounds specifically interact with the channels.

Previous results demonstrated that AEA blocks Kv1.2 and Kv3.4 channels from the extracellular side, while it blocks T-type Ca^{2+} channels from the intracellular side of the membrane. AEA blockade of hKv1.5 channels was only apparent when the compound was externally applied and was not modified by the presence of AEA in the intracellular medium. The finding that the combination of externally applied AEA and TEA produced less amount of block than either drug alone suggests that both compete and bind to the same or an overlapping external receptor site. The R487 residue on hKv1.5 channels is located at the external entryway of the pore and is equivalent to position 449 on Shaker channels which confers the TEA sensitivity. Our results demonstrate that AEA and 2-AG produce significantly less blockade in R487Y than in WT channels. This strongly suggests that the effects of AEA and 2-AG are the consequence of their binding to the external face of the hKv1.5 channels. Direct interaction of ethanolamides or fatty acids with the channel proteins have been suggested previously. Indeed, a single point mutation within the D1-S6 affects fatty acid block of human myocardial Na^+ channel α-subunit.

Assuming the existence of this external binding site for endocannabinoids at the hKv1.5 channels, it could be difficult to understand why the onset of block was so slow and the blockade so persistent even after long washout periods with drug-free solution. This behaviour is similar to that exhibited by AEA when inhibiting Na^+, Kv1.2, and TASK-1 channels. Endocannabinoids and

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**Figure 8** (A) Human atrial $I_{\text{Ks}}$ recorded by applying a 250 ms pulse to $+50 \text{ mV}$ after a 250 ms prepulse to $+50 \text{ mV}$ in ‘control’ conditions (in the presence of rimonabant and AM630) and in the presence of AEA (1 μM). (B) Mean $I_{\text{Ks}}$ density–voltage relationships in ‘control’ conditions and in the presence of AEA (250 ms isochronal). (C) APs recorded in atrial preparations in the absence and presence of AEA (1 μM). (D) Percentage of APD prolongation produced by AEA.
cannabinoid analogues predominantly reside within the membrane bilayers 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. Information on the AEA conformational properties in the membrane is controversial (hairpin vs. j-shaped, or extended). However, many studies locate the ethanolamide group near the lipid-water interface, thus allowing a critical interaction between this group and K192 at the CB1 receptor. Interestingly, for the AEA and 2-AG blockade of hKv1.5 channels, a positively charged residue (R487) is also critical.

The results suggest that the Kv1.5 channel transition into the open state promotes the AEA and 2-AG blockade. Indeed, blockade steeply increased in the voltage range coinciding with that of channel opening, AEA and 2-AG accelerated the current decline during the application of depolarizing pulses and slowed the deactivation of the tail currents. All these features of block perfectly fit with those of the open-state K+ channel blockers that access into the pore (from its intracellular entryway) when the channel opens. On the other hand, the results also suggest that the AEA- and 2-AG-binding site are the same or overlap the TEa binding site, which is located in the external entryway of the pore. In light of the data, it can be hypothesized that the apparent open-state blockade produced by endocannabinoids does not depend on their access to the receptor when the channel opens but on their preferential affinity for the receptor when the channel opens. However, further experiments would be necessary to accurately identify the endocannabinoid mechanism of block.

4.2 Physiological significance

Endocannabinoids and cannabinoid analogues can be synthesized within the heart.2,31 These mediators are generated upon demand, their production being stimulated by various tissue insults or damages.2 It seems reasonable to assume that this would also be the case in the myocardium in the context, for instance, of atrial tachyarrhythmias such as atrial fibrillation (AF), which produces inflammation, oxidative stress, myocyte degeneration,28 or other insults or damages.2 It seems reasonable to assume that this would also be the case in the myocardium in the context, for instance, of atrial tachyarrhythmias such as atrial fibrillation (AF), which produces inflammation, oxidative stress, myocyte degeneration, or other insults or damages.2 Moreover, endocannabinoid synthesis is enhanced in neuronal tissues by increasing calcium influx during neurotransmission.32 Furthermore, endocannabinoid synthesis is enhanced in neuronal tissues by increasing calcium influx during neurotransmission. Furthermore, cannabinoids are generated upon neuronal insults or damages.2 It seems reasonable to assume that this would also be the case in the myocardium in the context, for instance, of atrial tachyarrhythmias such as atrial fibrillation (AF), which produces inflammation, oxidative stress, myocyte degeneration, or other insults or damages.2

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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