Original article

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

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A B S T R A C T

Endocannabinoids are amides and esters of long chain fatty acids that can modulate ion channels through both receptor-dependent and receptor-independent effects. Nowadays, their effects on cardiac K+ channels are unknown even when they can be synthesized within the heart. We have analyzed the direct effects of endocannabinoids, such as anandamide (AEA), 2-arachidonoylglycerol (2-AG), the endogenous lipid lysophosphatidylinositol, and cannabinoid analogues such as palmitoylethanolamide (PEA), and oleoylethanolamide, as well as the fatty acids from which they are endogenously synthesized, on human cardiac Kv4.3 channels, which generate the transient outward K+ current (Ito1). Currents were recorded in Chinese hamster ovary cells, which do not express cannabinoid receptors, by using the whole-cell patch-clamp. All these compounds inhibited Ito4.3 in a concentration-dependent manner, AEA and 2-AG being the most potent (IC50 ~ 0.3–0.4 µM), while PEA was the least potent. The potency of block increased as the complexity of the number of C atoms in the fatty acyl chain increased. The effects were not mediated by modifications in the lipid order and microviscosity of the membrane and were independent of the presence of MiRP2 or DPP6 subunits in the channel complex. Indeed, effects produced by AEA were reproduced in human atrial Ito1 recorded in isolated myocytes. Moreover, AEA effects were exclusively apparent when it was applied to the external surface of the cell membrane. These results indicate that at low micromolar concentrations the endocannabinoids AEA and 2-AG directly block human cardiac Kv4.3 channels, which represent a novel molecular target for these compounds.

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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-arachidonoylthanolamide, AEA) and 2-arachidonoylglycerol (2-AG). Others, such as palmitoylethanolamide (PEA) and 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 analyzed 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 receptor activation [8]. In the human myocardium the most important rapidly inactivating K+ current is the Ca2+-independent component of the transient outward K+ current (Ito1), which is critical in determining the height and the duration of the plateau phase of the action potential (AP). Ito1 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 \(\mu\)g) or DPP6 (1 \(\mu\)g) proteins together with the cDNA encoding the CD8 antigen (0.5 \(\mu\)g) by using FUGENE6 [13,16]. \(I_{o1}\) was recorded on myocytes isolated from right atrial appendages of patients undergoing cardiac surgery [15]. The study conforms to 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, CaCl\(_2\) 1.8, MgCl\(_2\) 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). To record \(I_{o1}\) the external solution contained (mM): NaCl 120, KCl 20, CaCl\(_2\) 1, MgCl\(_2\) 1, HEPES 10, glucose 10, 4-aminopyridine (50 \(\mu\)M), nifedipine (1 \(\mu\)M), and atropine (1 \(\mu\)M) (pH 7.4 with NaOH). Recording pipettes were filled with an internal solution containing (mM): K-aspartate 80, KCl 42, KH\(_2\)PO\(_4\) 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-1,3,5-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), stearoyl-lethanolamide (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 analyzed by using Student’s t-test. To analyze 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fatty acid</th>
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<tbody>
<tr>
<td>Arachidonoylethanolamide (anandamide)</td>
<td>Arachidonic acid (20:4 n-6)</td>
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<tr>
<td>Methanandamide</td>
<td></td>
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<tr>
<td>2-Arachidonoyl glycerol</td>
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<tr>
<td>Oleylethanolamide</td>
<td>Oleic acid (18:1 n-9)</td>
</tr>
<tr>
<td>Stearoylthanolamide</td>
<td>Stearic acid (18:0)</td>
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<tr>
<td>Lysophosphatidylinositol</td>
<td></td>
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<tr>
<td>Palmitoylthanolamide</td>
<td>Palmitic acid (16:0)</td>
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\figure{Chemical structure of the compounds tested.}
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 \( I_{\text{Kv4.3}} \) traces elicited by 250 ms-pulses from −80 to +50 mV in the absence and presence of 1 μM AEA (A), MetAEA (B), 2-AG (C), OEA (D), LPI (E), and PEA (F). Dashed lines represent zero current level.

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 (\( Q_{\text{Kv4.3}} \)) 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 \( Q_{\text{Kv4.3}} \) was produced by AA (73.3±3.9%) and its derivatives AEA (65.8±3.5%), MetAEA (70.0±3.2%), and 2-AG (81.6±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 \( Q_{\text{Kv4.3}} \) 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±0.01 μM and 0.3±0.01 μM, respectively. When the potency of the fatty acids was compared (Fig. 4C) the results demonstrated that AA (IC\(_{50}\) = 0.3±0.02 μM) is the most while PA is the least potent for inhibiting \( I_{\text{Kv4.3}} \). In Fig. 4D the concentration dependencies of the Kv4.3 block produced by the ethanolamides (either endocannabinoids or not) and their corresponding fatty acid are 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 \( I_{\text{Kv4.3}} \) 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 (\( Q_{\text{AEA}} / Q_{\text{Control}} = 0.46±0.04 \) after >20 min of washout of AEA). Online Fig. 1 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 (Figs. 5B–G). The midpoint of inactivation (\( V_{50} \)) 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, \( V_{50} \) 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 Figs. 5B–G), the endocannabinoid-induced blockade significantly increased in the voltage range coinciding with that of channel inactivation, suggesting 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 (logP) and the distribution (logD at pH = 7.4) coefficients for the ethanolamides and the fatty acids, respectively. In Fig. 6A the logP or logD (logP/D) values were plotted as a function of the IC50 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 IC50 of each compound was plotted against its complexity (Fig. 6B). This latter parameter considered 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 by using AEA, 2-AG, LPI, and PEA at the concentration of 1 μM. 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 neighboring 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 ($r_{\infty}$) 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 ($\phi$) which is related with the microviscosity of the cell membrane. Incubation with AEA, 2-AG, LPI, or PEA for 20 min did not modify $r_{ss}$ (0.26 ± 0.004) and $\phi$ (1.4 ± 0.2 ns) values (Figs. 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 neurons. The modification of the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{i}$) could activate intracellular pathways that, in turn, could modify the $I_{K_{Na4.3}}$. Therefore, to examine whether the observed effects were due to a cannabinoid-induced modification of the [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 $Q_{K_{Na4.3}}$ at +50 mV by 60.1 ± 4.0% (n = 5), a reduction that was not statistically different from that produced by AEA in EGTA-dialyzed cells (Figs. 7A and B).

Cardiac $I_{Na}$ 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 Ito1 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 Ito1 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 demonstrate that the Q 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 (τf= 17.1 ± 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 Ito1, i.e., the current generated by channels with the native architecture. For this purpose, myocytes were perfused with rimonabant and AM630, selective antagonists of CB1 and CB2 receptors, respectively. Online Figure III demonstrates that the effects on Ito1 (Q reduction at +50 mV = 62.2 ± 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 μM) 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 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 μM), 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 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 Ito1 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 μM 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 Ca²⁺ [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 Ca²⁺ 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 Ca²⁺ channels [5,9].

Concerning the fatty acids, our results extend and confirm what was previously demonstrated, i.e., that fatty acids can modulate the

Fig. 5. (A) $I_{\text{Kv4.3}}$ traces in the absence and presence of 1 μM 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 μM 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±s.e.m. of ≥5 experiments.
activity of Ca\(^{2+}\), K\(^{+}\), and Na\(^{+}\) channels [18,23–27]. Furthermore, it has been previously demonstrated that AA inhibited the I_{\text{to1}} in rat cardiac myocytes [28]. Our results demonstrated that Kv4.3 channels are particularly sensitive to AA (IC\(_{50}\) = 0.4 \(\mu\)M), being much more sensitive than, for instance, Kv1.5 channels (IC\(_{50}\) = 21 \(\mu\)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 (IC\(_{50}\) = 0.4 \(\mu\)M) and 2-AG (IC\(_{50}\) = 0.3 \(\mu\)M) inhibited cardiac I_{Kv4.3} with a potency that is similar to that exhibited by AEA for blocking neuronal TASK-1 and T-type Ca\(^{2+}\) (IC\(_{50}\) = 0.33 \(\mu\)M) channels [79], but greater than that exhibited for blocking Na\(^{+}\) (IC\(_{50}\) = 5.5 \(\mu\)M) channels in rat dorsal root ganglion neurons [5] or Kv1.2 (IC\(_{50}\) = 2.7 \(\mu\)M) channels [78]. 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
Fig. 7. (A) $I_{\text{Kv4.3}}$ traces elicited by 250 ms-pulses from −80 to +50 mV in the absence and presence of 1 μM AEA in a CHO cell dialyzed with BAPTA-containing internal solution. (B) $Q_{\text{Kv4.3}}$ reduction at +50 mV produced by 1 μM AEA in cells dialyzed either with EGTA- or BAPTA-containing internal solution. (C) $Q_{\text{Kv4.3}}$ reduction at +50 mV produced by 1 μM AEA in cells expressing Kv4.3 + KChIP2, Kv4.3 + KChIP2 + KCNE3 or Kv4.3 + KChIP2 + DPP6 channels. (D) $Q_{\text{Kv4.3}}$ reduction at +50 mV produced by 1 μM AEA applied extracellularly, intracellularly or both, AEA applied intracellularly in the presence of URB-597, and MetAEA applied intracellularly. Each bar represents the mean±s.e.m. of ≥4 experiments. **P<0.01 vs. extracellularly applied AEA.

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

The time course of $I_{\text{Kv4.3}}$ 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 lipophylic 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 $I_{\text{Na}+}$ 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 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 $I_{\text{Na}+}$ in the presence of CB1 and CB2-receptor 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 Ca²⁺ 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 ethanolamide. 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 vanilloid receptors, modulate ion channels or act on as yet unidentified targets [1,31].

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 lr1a1. Radioligand studies indicate that the Kf 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 μM and 0.15–1.4 μM 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 [Ca2+]i, and at high-frequencies of stimulation [1], conditions both associated with cardiac tachyarrhythmias. Kv4.3 channels underlie the Ito current, 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 lr1a1 produces 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 (PI080065), Ministerio de Educación y Ciencia (SAF2008-04903), Instituto de Salud Carlos III (Red HERACLES RD06/0009), Fundación LILY, 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.

Appendix A. Supplementary data


References

[18] Guizy M, David M, Arias C, Zhang L, Cófin M, Ruiz-Gutierrez V, et al. Modulation of the atrial specific Kv1.5 channel by the n-3 polyunsaturated fatty acid, alpha-


ONLINE SUPPLEMENTAL DATA

Material and Methods

Cell Culture

Chinese hamster ovary (CHO) cells, which do not express cannabinoid receptors endogenously [1], stably transfected with hKv4.3-L/hKChIP2 were grown in Hams-F12 (Gibco, Grand Island, NY, USA) medium with 10% fetal bovine serum in a 5% CO$_2$ atmosphere [2,3]. The cultures were passaged every 4–5 days using a brief trypsin treatment. In some experiments, these CHO cells were transiently transfected with the cDNA encoding MiRP2 (KCNE3, 1.6 µg) or DPP6 (1 µg) proteins together with the cDNA encoding the CD8 antigen (0.5 µg) by using FUGENE6 (Roche Diagnostics; Barcelona, Spain) following manufacturer instructions [2,4]. For culture and transfections, 50 mm culture dishes were used. After 48 h, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450; Dynal, Norway). Most of the cells that were beaded also had channel expression. The cells were removed from the dish with a cell scraper and the cell suspension was stored at room temperature and used within 12 h for electrophysiological experiments.

Human atrial myocyte isolation

The study was approved by the Investigation Committee of the Hospital Universitario Gregorio Marañón (CNIC-13) and conforms the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent. Human atrial myocytes were enzymatically isolated following previously described methods [3,5]. Human right atrial specimens were obtained from patients (n=3) undergoing cardiac surgery (Table I). Samples were immediately placed into chilled Ca$^{2+}$-free Tyrode’s solution containing (mM): NaCl 100, KCl 10, KH$_2$PO$_4$ 1.2, MgSO$_4$ 5, taurine 50, MOPS 5, and glucose 20 (pH 7.0 with
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NaOH) and supplemented with 2,3-butanedione monoxime (30 mM), chopped into small pieces (≈1 mm³), and washed 3 times for 3 minutes with Ca²⁺-free Tyrode’s solution. Tissue pieces were then changed to Ca²⁺-free solution containing 254 U/mL collagenase type I (Worthington, Lakewood, NJ, USA) and 0.5 mg/mL protease type XXIV (Sigma Chemical Co, London, United Kingdom), and gently stirred for 15 minutes. Afterwards, the Ca²⁺ concentration was raised to 0.2 mM, and the tissue was stirred for another 30 minutes. Stirring was continued with Tyrode’s solution (0.2 mM Ca²⁺) containing only collagenase until rod-shaped striated myocytes were seen (≈35 minutes). During all these steps, the solutions were continuously oxygenated with 100% O₂ at 37°C. Myocytes were kept until use in a storage solution containing (mM): KCl 20, KH₂PO₄ 10, glucose 10, K-glutamate 70, 2 β-hidroxybutyrate 10, taurine 10, EGTA 10, and albumin 1 (pH 7.4 with KOH). Myocytes were used for electrophysiological recordings within 8 h.

Recording techniques [2-8]

A small aliquot of cell suspension was placed in a 0.5 ml chamber mounted on the stage of an inverted microscope (Nikon TMS, Nikon Co., Tokyo, Japan). After settling to the bottom of the chamber, cells were perfused at 1 ml/min with external solution (see composition below). Currents were recorded at room temperature (21-23°C) using the whole-cell patch-clamp technique with an Axopatch-200B patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Recording pipettes were pulled from 1.0 mm o.d. borosilicate capillary tubes (GD1; Narishige Co., Ltd Tokyo, Japan) using a programmable patch micropipette puller (Model P-2000 Brown-Flaming; Sutter Instruments Co., Novato, CA, USA) and were heat-polished with a microforge (Model MF-83, Narishige). Micropipette resistance was <3.5 MΩ when filled with the internal solution and immersed in the external solution. The capacitive transients elicited by symmetrical 10 mV steps from 0 mV were recorded at 50 kHz (filtered...
at 10 kHz) for subsequent calculation of capacitative surface area, access resistance and input impedance. In all the experiments, series resistance was manually compensated by using the series resistance compensation unit of the Axopatch amplifier, and ≥80% compensation was achieved. In CHO cells, mean uncompensated access resistance and cell capacitance were 1.6±0.2 MΩ, and 9.2±0.7 pF, respectively (n=42), whereas mean maximum I_{Kv4.3} amplitude at +50 mV was 2.4±0.2 nA. In human atrial myocytes mean maximum I_{to1} amplitude at +50 mV, uncompensated access resistance, and capacitance averaged 918.5±74.9 pA, 3.9±0.6 MΩ, and 81.3±17.9 pF (n=5), respectively.

Thus, under our experimental conditions no significant voltage errors (<5 mV) due to series resistance were expected with the micropipettes used. The current recordings were sampled at 4 kHz, filtered at half the sampling frequency and stored on the hard disk of a computer for subsequent analysis.

**Solutions and drugs**

To record I_{Kv4.3}, CHO cells were perfused with an external solution containing (mM): NaCl 136, KCl 4, CaCl$_2$ 1.8, MgCl$_2$ 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). To record human atrial I_{to1}, the external solution contained (mM): NaCl 120, KCl 20, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, glucose 10, 4-aminopyridine (4-AP, 50 µM), nifedipine (1 µM), and atropine (1 µM) (pH 7.4 with NaOH). Recording pipettes were filled with an internal solution containing (mM): K-aspartate 80, KCl 42, KH$_2$PO$_4$ 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. Anandamide (AEA), methanandamide (MetAEA), L-α-lysophosphatidylinositol (LPI), arachidonic acid (AA), palmitic acid (PA), stearic acid (SA), stearoylethanolamide (SEA), and oleic acid (OA) [all of them purchased from Sigma], 2-arachidonoylglycerol (2-AG) and oleoylethanolamide (OEA) [purchased from Tocris...
Bioscience, Ellisville, MO, USA] were dissolved in ethanol, whereas palmitoylethanolamide
(PEA, Sigma) was dissolved in dimethyl sulfoxide (DMSO). Rimonabant (kindly provided by
Sanofi-Aventis, France), AM630, and URB-597 (purchased from Tocris) were dissolved in
DMSO. In all the cases, 10 mM stock solutions in the appropriate solvent were initially
obtained and further dilutions were carried out in external or internal solution to obtain the
desired final concentration immediately before each experiment. Control solutions contained
the same solvent concentrations as the test solution.

Pulse protocols and analysis

The holding potential was maintained at -80 mV and the cycle time for any protocol
was 10 s to avoid accumulation of inactivation and/or block as previously described for Kv4.3
currents [2-4,7]. The protocol used to obtain current–voltage relationships consisted of 250
ms-pulses that were imposed in 10 mV steps between -90 and +50 mV. To obtain steady-state
inactivation curves, a two-step protocol was used: the first 250 ms-conditioning pulse from -
80 mV to potentials between -90 and +50 mV was followed by a test pulse (250 ms) to +50
mV. Steady-state inactivation curves were constructed by plotting the peak current amplitude
obtained with the test pulse as a function of the voltage command of the conditioning pulse
and a Boltzmann distribution of the form:

\[
y = \frac{A}{1 + \exp \left[ \frac{(V_h-V_m)}{k} \right]} \\
\]

where \( A \) is the amplitude term, \( V_h \) is the midpoint of activation or inactivation, \( V_m \) is the test
potential and \( k \) represents the slope factor of the curve, was fitted to the data.

To obtain the IC_{50} (concentration of compound that produces the half maximum blockade)
and the Hill coefficient, \( n_H \), a Hill equation was fitted to the percentage of block \( f \)
obtained at various drug concentrations \([D]\):

\[
f = \frac{1}{1+(IC_{50}/[D])^{n_H}} \\
\]

(2)
The Hill equation was fitted to the data fixing the top and the nH to 100% and the unity, respectively.

To describe the time course of current activation and inactivation upon depolarization an exponential analysis was used.

**Complexity index**

The complexity rating of the compounds, which is a rough estimate of how complicated a structure is, was calculated using the Bertz/Hendrickson/Ihlenfeldt equation [9]:

\[ C = C_\eta + C_E \]  

(3)

where \( C_\eta \) is the major term and measures the skeletal complexity as defined in the equation:

\[ C_\eta = 2\eta \log_2 \eta - \sum_i \eta_i \log_2 \eta_i \]  

(4)

The individual \( \eta_i \) values are calculated as:

\[ \eta_i = \frac{1}{2} (4-h) (3-h) \]  

(5)

where \( h \) is the number of hydrogens on atom \( i \).

\( \eta \) for the whole molecule can be computed with equation:

\[ \eta = \frac{1}{2} \sum_i (4-h_i)(3-h_i)-D-3T \]  

(6)

where \( h_i \) is the number of hydrogens on atom \( i \), \( D \) the number of double bonds and \( T \) the number of triple bonds in the molecule.

\( C_E \) is called the symmetry term and is a function of the diversity of the elements, and is calculated from:

\[ C_E = E \log_2 E - \sum_j E_j \log_2 E_j \]  

(7)

\( E \) being the total number of non-hydrogen atoms and \( E_j \), the number of atoms of element \( j \).

**Steady-state and time-resolved fluorescence anisotropy measurements**
A CHO cells suspension (1–3×10⁶ cells resuspended in 1 ml external solution) was incubated at 20 °C for 15 min with 0.5 µL of a stock solution (2 mM in DMSO) of 2-carboxyethyl-1,6-diphenyl-1,3,5-hexatriene (PA-DPH, Molecular Probes, Invitrogen, CA, USA) in the darkness. For testing the effects of AEA, 2-AG, LPI, and PEA on steady-state and time-resolved fluorescence anisotropy, cells were incubated for 20 or 120 min with the endocannabinoid at a concentration of 1 µM together with the fluorescent dye. Steady-state fluorescence anisotropy measurements were performed at 20° C on a photon counting ISS-PC1 spectrofluorimeter with Glan-Taylor polarizers in the excitation and emission channels, using 5×5 mm cuvettes and 2 nm/10 nm excitation/emission bandwidths (λ_exc = 375 nm, λ_em = 450 nm). The steady-state fluorescence anisotropy (r_{ss}) is defined as: r_{ss} = (I_{par}−I_{per}×G) / (I_{par} + 2×G×I_{per}) where I_{par} and I_{per} are vertical and horizontal polarized emission intensities elicited by vertically polarized excitation, respectively. G is a scaling factor that accounts for differences in the detection efficiency for the two polarized intensities. Total fluorescence intensity and r_{ss}, which was recorded as a function of time, reached plateau values in 3–5 min and remained constant for at least 1 h.

Time-resolved fluorescence intensity and anisotropy measurements were carried out using a modified version of the time-correlated single photon counting laser system previously described [10], with a PicoQuant 375 nm diode laser beam as the excitation source, with a time resolution of 12.2 ps/channel. The emission wavelength was 450 nm (8 nm bandwidth). An extra 418 nm cut-off filter was included in the emission side to minimize the scattering of the fluorescence signal. The fluorescence lifetimes were determined from the magic angle (relative to the plane of the vertically polarized excitation beam) decay I(m)(t) using non-linear least-squares global methods from the GLOBALS Unlimited (Urbana, IL, USA) general purposes program [10]. The fluorescence anisotropy decay, r(t), was determined by simultaneous analysis of the parallel, I_{par}(t), and perpendicular, I_{per}(t), emission intensity
components, assuming a biexponential function with a residual or limiting anisotropy \( (r_\infty) \), using the following equation \([11]\):

\[
r(t) = (r_o - r_\infty) \cdot \sum_i \beta_i \cdot \exp(-t/\phi_i) + r_\infty
\]

where \( \phi_i \) are relaxation times corresponding to the restricted rotational motion of the fluorescence probe in the membrane \([10,12-16]\), and the preexponential factors \( \beta_i \) are normalized amplitudes. The \( r_0 \) value, which is a spectroscopic property of the DPH derivatives, was taken as that determined experimentally \((0.390 \pm 0.003)\) \([15]\). The adequacy of the time-resolved anisotropy analyses was determined from the reduced weighted sum of squares of residuals and visual inspection of the distribution of weighted residuals.

**Statistical analysis**

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±s.e.m. A value of \( P<0.05 \) was considered statistically significant. To make comparisons between two concentration-response curves, an F-test was used.
**Results.**

**Online Figure I.** 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. Closed circles represent normalized charge crossing the membrane through Kv4.3 channels ($Q$) at $+50$ mV as a function of time for illustrating the stability of the current for at least 40 min. Open circles represent the normalized charge during control recordings, after beginning the AEA (1 µM) perfusion (arrow) and, finally, following the washout of the drug with drug-free solution. Each point represents the mean±s.e.m. of at least 4 cells. It can be observed that steady-state effects were reached after ≈12 min of perfusion with AEA and that current recovery after almost 20 min of washout was negligible ($Q_{AEA}/Q_{Control}=0.46±0.04$). The effects of AEA in Kv4.3 channels cannot be attributed to a time-dependent rundown of the current, since under our experimental conditions current amplitude remained unchanged during the time of recordings (closed circles).
Online Figure II. Steady-state inactivation curves of Kv4.3 channels obtained in the absence (●) and the presence (○) of 1 µM AA (A), OA (B), SA (C), SEA (D), and PA (E). Continuous lines represent the fit of a Boltzmann equation to the data. Dashed lines represent the curve in the presence of each drug, normalized to the control amplitude. Each data point represents the mean± s.e.m. of ≥4 experiments. *P<0.05 vs control. AA and OA decreased peak $I_{Kv4.3}$ recorded by applying a test pulse to +50 mV after depolarizing pulses ranging -90 and -30 mV and shifted the $V_h$ towards negative potentials (P<0.05) without modifying the slope ($k$) of the curve (Online Table II). Relative current (squares) was plotted as a function of the conditioning pulse, demonstrating that the inhibition induced by AA and OA remained unchanged at potentials between -90 mV and -50 mV and, thereafter, it progressively increased at more positive potentials reaching 67.5±8.1% for AA and 51.1±2.6% for OA at -30 mV (P<0.05 vs. inhibition at -90 mV). On the contrary, SA, SEA, and PA did not significantly modify $I_{Kv4.3}$ amplitude, the $V_h$ or the slope of the curves (Online Table II).
Online Figure II

A

B

C

D

E

Kv4.3+KChIP2 current (pA)

Conditioning pulse potential (mV)

Control

AA 1 µM

Control

OA 1 µM

Control

SA 1 µM

Control

SEA 1 µM

Control

PA 1 µM

Conditioning pulse potential (mV)
Online Figure III. (A) \( I_{Kv4.3} \) traces recorded by applying 250 ms-pulses from -80 mV to +50 mV under control conditions and in the presence of the CB1 receptor antagonist rimonabant (RMB, 100 nM) alone or plus AEA (1 µM). Superfusion with RMB alone reduced \( Kv4.3 \) charge by 23.5±3.4% (n=4), whereas superfusion of AEA plus RMB resulted in a further reduction (relative to the blockade obtained with RMB alone) of 67.8±4.3% (n=4), a reduction similar to that produced in the absence of RMB. (B) Human atrial \( I_{to1} \) traces recorded at +50 mV in the presence of 4-AP (50 µM), RMB (1 µM), and the CB2 receptor antagonist AM630 (1 µM) with and without AEA (1 µM). To record human atrial \( I_{to1} \), a 25 ms prepulse to -30 mV was applied to inactivate \( I_{Na} \), followed by a 250 ms pulse to potentials ranging -90 and +50 mV. The \( I_{to1} \) amplitude was measured as the difference between the peak and the steady-state current at the end of the 250 ms pulse. In the presence of 4-AP (to block the ultrarapid component of the delayed rectifier current, \( I_{Kur} \)), the simultaneous addition of RMB and AM630 decreased the peak and accelerated the fast phase of \( I_{to1} \) inactivation kinetics (from 37.9±3.8 to 21.3±3.1 ms, P<0.05, n=5), whereas it did not significantly modify the slow phase of inactivation (from 233.8±95.3 to 181.7±33.6 ms). These effects led to a charge decrease of 31.1±9.4%. Under these conditions, addition of AEA further decreased the peak amplitude and accelerated the \( I_{to1} \) inactivation kinetics (\( \tau_f=13.5±3.2 \) ms and \( \tau_s=132.0±32.4 \) ms, n=5, P<0.05 vs 4-AP+RMB+AM630). As a result, in the presence of CB1 and CB2 receptor antagonists, AEA reduced the charge crossing the membrane at +50 mV by 62.2±2.5%. (C) \( I_{to1} \) charge-voltage curves in the absence and presence of AEA. Each data point represents the mean±s.e.m. of 5 experiments. *P<0.05 vs 4-AP+RMB+AM630.
Online Table I. Patient characteristics

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
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<tbody>
<tr>
<td>Mean age (years)</td>
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</tr>
<tr>
<td>Female (n)</td>
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</tr>
<tr>
<td>Surgery</td>
<td></td>
</tr>
<tr>
<td>Valve surgery (n)</td>
<td>2/3</td>
</tr>
<tr>
<td>CABG surgery (n)</td>
<td>2/3</td>
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<td>Treatment</td>
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<tr>
<td>Beta blockers</td>
<td>2/3</td>
</tr>
<tr>
<td>ACE inhibitors/angiotensin receptor blockers</td>
<td>3/3</td>
</tr>
<tr>
<td>Statins</td>
<td>3/3</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>1/3</td>
</tr>
</tbody>
</table>

ACE, angiotensin converting enzyme; CABG, coronary artery bypass grafting.
Online Table II. Effects of endocannabinoids, cannabinoid analogues, fatty acids, and ethanolamides on the voltage-dependence and time-course of $I_{Kv4.3}$ inactivation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$V_h$ inact (mV)</th>
<th>$k$ inact (mV)</th>
<th>$\tau_s$ inact (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-23.8±4.4</td>
<td>4.9±0.1</td>
<td>95.6±12.1</td>
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<td>AEA</td>
<td>-44.1±6.0*</td>
<td>4.8±0.4</td>
<td>53.9±10.6*</td>
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<tr>
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<td>Met-AEA</td>
<td>-33.8±1.5*</td>
<td>3.8±0.1</td>
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<tr>
<td>Control</td>
<td>-16.8±1.3</td>
<td>4.3±1.1</td>
<td>95.2±10.7</td>
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<tr>
<td>2-AG</td>
<td>-28.7±0.1*</td>
<td>4.4±0.9</td>
<td>51.7±9.1*</td>
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<td>LPI</td>
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<td>97.9±6.3</td>
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<td>PEA</td>
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<td>4.5±0.9</td>
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<td>OEA</td>
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<td>SA</td>
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<td>83.4±12.0</td>
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<tr>
<td>Control</td>
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<td>4.4±0.2</td>
<td>96.0±17.6</td>
</tr>
<tr>
<td>SEA</td>
<td>-20.0±1.2</td>
<td>4.5±0.2</td>
<td>92.0±22.5</td>
</tr>
</tbody>
</table>

$V_h$ and $k$ are the midpoint and the slope values, respectively, yielded by the fit of a Boltzmann function to the inactivation curves. $\tau_s$ is the slow time constant of inactivation.

Each value represents the mean±s.e.m of ≥4 experiments. * P<0.05 vs. control values.
References


