Block of the background $K^+$ channel, TASK-1, contributes to the arrhythmogenic effects of platelet-activating factor

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Running Head: PAF induces arrhythmia by blocking TASK-1
Abstract

Platelet-activating factor (PAF), an inflammatory phospholipid, induces ventricular arrhythmia via an unknown ionic mechanism. We now link PAF-mediated cardiac electrophysiologic effects to inhibition of the two-pore domain K$^+$ channel, TASK-1. Superfusion of carbamyl-platelet-activating factor (C-PAF), a stable analogue of PAF, over murine ventricular myocytes causes abnormal automaticity, plateau phase arrest of the action potential and early afterdepolarizations in paced and quiescent cells from wild-type but not PAF receptor knockout mice. C-PAF-dependent currents are insensitive to Cs$^+$ and are outwardly rectifying with biophysical properties consistent with a K$^+$-selective channel. The current is blocked by TASK-1 inhibitors, including protons, Ba$^{2+}$, Zn$^{2+}$, and methanandamide, a stable analogue of the endogenous lipid ligand of cannabanoid receptors. In addition, when TASK-1 is expressed in CHO cells that express an endogenous PAFR, superfusion of C-PAF decreases the expressed current. Like C-PAF, methanandamide evoked spontaneous activity in quiescent myocytes. C-PAF- and methanandamide-sensitive currents are blocked by a specific PKC inhibitor, implying overlapping signaling pathways. In conclusion, C-PAF blocks TASK-1 or a closely related channel, the effect is PKC-dependent, and the inhibition alters the electrical activity of myocytes in ways that would be arrhythmogenic in the intact heart.

Key Words: two-pore domain potassium channels; ventricular myocytes; inflammatory lipids; mouse
Introduction

Lethal arrhythmias commonly occur after myocardial ischemia, especially when ischemic myocardium is reperfused. These arrhythmias are usually initiated by ectopic activity triggered by early and delayed afterdepolarizations (EADs and DADs) of the membrane potential. One consequence of ischemia and reperfusion is a rapid migration of polymorphonuclear leukocytes (PMNL) into the infarcted zone. Activated PMNL bind to activated myocytes and release several substances, including oxygen radicals, proteolytic enzymes and inflammatory lipids that increase the extent of myocardial injury (15). Depletion of circulating neutrophils or treatment with anti-inflammatory drugs effectively limits the size of the infarct zone and the extent of the damage in hearts from several species (15, 20, 22).

Hoffman et al. (4, 5) demonstrated that activation of PMNL bound to isolated canine myocytes dramatically changed the myocyte transmembrane action potential. These changes included prolongation of the action potential duration (APD), EADs and in some cases arrest during the plateau phase of the action potential. It was also shown that direct superfusion of myocytes with the inflammatory phospholipid, platelet-activating factor (PAF) mimicked the action of activated PMNL, and that under similar conditions PMNL produce significant levels of PAF. Furthermore, incubation of myocytes with the PAF receptor (PAFR) antagonist, CV-6209, prevented both PAF- and PMNL-induced changes in myocyte membrane potential. PAF also induces arrhythmias in mice that overexpress the PAFR when the lipid is administered at intravenous doses that have little effect on wild-type animals (7). These observations suggested that PMNL-derived PAF could induce triggered activity and thus ventricular arrhythmias.

There is considerable confusion regarding the molecular mechanisms by which PAF could alter the electrical activity of the heart. Although PAF binds to a cell-surface, G-protein-linked receptor and ultimately increases cytosolic Ca\(^{2+}\) levels (17, 19) little is known about PAF effects on membrane channels. Wahler et al. showed that subnanomolar concentrations of PAF markedly decreased the
inwardly rectifying potassium channel $I_{K1}$ in guinea pig ventricular myocytes (26), while Hoffman et al. suggested that depolarizing $Na^+$ current may play a role in the arrhythmogenic action of PAF (5).

Taking advantage of genetically modified mice in which PAFR have been knocked out (6), we have tested the ability of carbamyl-PAF (C-PAF), a non-metabolizable PAF analogue, to alter the membrane potential of isolated murine ventricular myocytes with the intent of clarifying the mechanisms determining the arrhythmogenic effects of this lipid.

**Methods**

*Cell Preparation*

Adult mice, 2-3 months old, were anesthetized with ketamine/xylazine and their hearts were removed according to protocols approved by the Columbia University IACUC. Experiments were performed on single rod-shaped, quiescent ventricular myocytes dissociated using a standard retrograde collagenase perfusion (11) from hearts of mice that were either wild-type (WT), or PAFR knockouts (KO). Both WT and KO mice were bred on a C57/Bl6 background. The derivation of the KO mice has been described previously (5).

*Heterologous Expression*

The TASK-1 clone (provided by Professor Y. Kurachi, Osaka University) was co-transfected in CHO cells with CD8 plasmid using Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. 48 h later cells were transferred to the electrophysiology chamber and anti-CD8 coated beads (Dynal Biotech) were added to identify CD8 expressing cells. Expressing cells were voltage clamped using a ramp clamp (see below). CHO cells were used in these experiments, in part, because they express endogenous PAFR.

*Buffers and Drugs*

Prior to electrophysiological measurements, cells were placed into the perfusion chamber and superfused at room temperature with Tyrode’s buffer (in
mM: NaCl, 140; KCl, 5.4; CaCl$_2$, 1; MgCl$_2$, 1; Hepes, 5; Glucose, 10; pH 7.4). The whole-cell patch clamp technique was used with pipettes having resistances of 1.5-3 M$\Omega$ (intracellular solution, in mM: aspartic acid, 130; KOH, 146; NaCl, 10; CaCl$_2$, 2; EGTA, 5; Hepes, 10; MgATP, 2; pH 7.2). Solutions of C-PAF, the PAFR antagonist, CV-6209 (Biomol) and the PKC inhibitor, bisindolylmaleimide I (BIM I; Calbiochem) were prepared in water and diluted in Tyrode’s before use. The inactive analog of BIM I (BIM V; Calbiochem), anandamide, its nonhydrolyzable analogue, methanandamide, and an inhibitor of anandamide hydrolysis, arachidonyltrifluoromethyl ketone (ATFK) (Biomol), were dissolved in DMSO then diluted in Tyrode’s. The final DMSO concentration did not exceed 0.1%. A custom-made fast perfusion device was used to exchange the solution around the cell within 1 s (2).

**Electrophysiological Recordings**

Current and voltage protocols were generated using Clampex 7.0 software applied by means of an Axopatch 200B amplifier and a Digidata 1200 interface (Axon Instruments). During voltage clamp, steady state current traces were acquired at 500 Hz and final filtered at 10 Hz. During current clamp, membrane voltage was acquired at 5 KHz and filtered at 1 KHz. Ramp clamps were conducted by imposing a voltage ramp (14 mV/s) at a 500 Hz acquisition rate with 1 kHz filtering. Data were analyzed using pCLAMP 8.0 (Axon) and Origin 6.0 (Microcal) and are presented as mean ± SEM. Steady-state current was determined by computer calculation of average current over a time period of at least 5 s. In all experiments, the n value indicates the number of myocytes studied, and represents pooled data from at least 2 (voltage clamp) or 3 (current clamp) animals. Student’s t-test, one-way ANOVA and $\chi^2$ tests were used; a value of p<0.05 was considered statistically significant. Records have been corrected for the junction potential, which was measured to be 9.8 mV.

**Results**

*C-PAF alters the rhythm of paced, wild-type, ventricular myocytes.*
Myocytes from WT mice were paced (cycle length 1000 ms) and monitored in current clamp mode to record action potentials. When the action potential duration was stable for 2 min, cells were superfused with C-PAF (185 nM, Figure 1), a concentration that elicited electrophysiologic effects in 9 of 11 cells. C-PAF-evoked responses occurred after a delay (94 ± 21 s; range 23 to 184 s), and typically included abnormal automaticity (Figure 1, 110 s) leading to a maintained depolarization (Figure 1, 111 s). In 8 of 9 cells, alteration of the membrane potential slowly returned to normal, presumably due to receptor desensitization and after 3 min of agonist perfusion was indistinguishable from control (Figure 1 inset).

C-PAF decreases an outward current that is $K^+$-selective and carried by TASK-1.

Cells were held at -10 mV and total steady state membrane currents were measured. The mean holding current was 133 ± 12 pA (n=24). WT myocytes responded to C-PAF with decreased net outward current that often began to reverse during the perfusion and recovered completely after wash out (Figure 2A). Since a depolarizing shift in steady state current can be caused by increased inward currents or decreased outward currents, we determined how C-PAF affected conductance. When a +10 mV step was applied during control and agonist superfusion, we found that C-PAF decreased conductance 17.5 ± 3.9% (n=5; p<0.05), indicating that the lipid inhibits outward current(s). The main conductance maintaining resting potential in the ventricle is $I_{K1}$, therefore, we tested whether this inwardly rectifying $K^+$ current was involved in the action of C-PAF. Cs$^+$ (5 mM), which largely blocks $I_{K1}$ under these conditions (data not shown), did not reduce the C-PAF-sensitive current in cells held at –70 mV. The average C-PAF-sensitive current density was 0.047 ± 0.01 pA/pF in control cells compared to 0.047 ± 0.03 pA/pF in cells in the presence of Cs$^+$ (n=6). By extending the voltage clamp study to other potentials, we obtained a nearly linear I-V relation for the C-PAF difference current (Figure 2B, filled squares). In KO myocytes the C-PAF-sensitive current was absent at all potentials tested (Figure 2B, filled circles).
We did not observe a clear reversal potential in physiologic K$^+$ over the voltage range tested. Therefore, we conducted additional experiments in elevated extracellular K$^+$ (50 mM with Na$^+$ reduced to 100 mM, plus Cs$^+$, 5 mM and TEA$^+$, 1 mM) designed to measure the reversal potential of the C-PAF-sensitive current. In elevated extracellular K$^+$, our results show a weakly outward rectifying current with an I-V relation that is consistent with that of a predominantly K$^+$-selective channel (Figure 2C). The calculated $E_K$ for these recording conditions is -27.6 mV and the observed reversal for the C-PAF-sensitive current occurred at -20.4 ± 3 mV (n=5).

The C-PAF-sensitive current was blocked by the PAFR antagonist, CV-6209 (100 nM; Figure 3). The lack of a C-PAF-dependent response in the presence of CV-6209 was identical to the results obtained in myocytes derived from KO mice (Figure 3). Taken together, these results confirm that the C-PAF effect is mediated by the PAFR and involves inhibition of an outward K$^+$ current distinct from $I_{K1}$.

These characteristics of the C-PAF-sensitive current suggested that it may be mediated by a member of the “two-pore domain” potassium channel family (13). TASK-1 is a member of this family that is expressed in mammalian heart (9, 10, 13, 14). In heterologous expression systems, this channel is outwardly rectifying and is blocked by H$^+$, Ba$^{2+}$, Zn$^{2+}$ and anandamide, an endogenous cannabinoid receptor ligand (9, 10, 13, 14, 16, 18, 24). Consistent with this, in isolated myocytes, when the external pH was lowered to 6.4 or when Ba$^{2+}$ (3 mM) or Zn$^{2+}$ (3 mM) were present, the C-PAF-sensitive current was significantly reduced (Figure 4, left panel). Methanandamide (10 µM), a stable analog of anandamide, also inhibited the C-PAF-sensitive current (Figure 4, right panel). In contrast, anandamide inhibition was only significant in the presence of ATFK (10 µM), an inhibitor of anandamide hydrolysis (Figure 4), suggesting rapid metabolism of anandamide by ventricular myocytes. ATFK alone had no effect (not shown).

CHO cells expressing TASK-1 exhibited a large outwardly rectifying current that was pH sensitive. The mean I-V relation at alkaline and acidic pH is
shown in Figure 5 (left panel) and demonstrates that the reduction of the external pH to 6 completely eliminated the outwardly rectifying current. Mean current density at +30 mV in cells expressing TASK-1 was 26 pA/pF compared to 0.6 pA/pF for non-transfected cells. When TASK-1 transfected CHO cells were superfused with C-PAF (185 nM), the expressed current was reduced (Figure 5, right panel) demonstrating the inhibitory effect of C-PAF on TASK-1-dependent current.

If both C-PAF and methanandamide block TASK-1, then methanandamide itself should cause a decreased net outward current. Thus, the methanandamide-sensitive current was measured (Figure 6). Since this current is comparable to the C-PAF-sensitive current, we also asked if the methanandamide-sensitive current was mediated by the PAFR and found that the lipid was fully effective in the presence of the PAFR antagonist, CV-6209 or when applied to myocytes from KO mice (Figure 6). Thus, the effect of methanandamide is not mediated by the PAFR.

**C-PAF action involves PKC-dependent block of TASK-1.**

In many cell-types, PAF initiates an intracellular pathway that results in activation of protein kinase C (PKC) (1, 17, 19, 23). To determine if C-PAF initiates this cascade in ventricular myocytes, we incubated cells with bisindolylmaleimide I (BIM I), a selective PKC inhibitor (25) ($K_i$, 14 nM) before applying C-PAF. The C-PAF-sensitive current was blocked in a dose-dependent manner (Figure 7A and B) by BIM I but was not altered by the addition of an inactive analogue, BIM V. The inhibition occurred in a voltage-independent manner (Figure 7C).

We next asked if the methanandamide-sensitive current also required PKC activity. BIM I (100 nM) significantly reduced the methanandamide-sensitive current in WT myocytes ($p<0.05$; $n=5$; data not shown).

**C-PAF and methanandamide induce spontaneous activity in quiescent myocytes.**
Because C-PAF and methanandamide affect net steady-state current at voltages near the resting potential, we asked if electrophysiologic effects occurred independent of pacing. Membrane potential was recorded from myocytes that remained quiescent for at least 2 min. Every WT quiescent myocyte tested was sensitive to C-PAF superfusion (11 of 11 cells; Figure 8A), typically responding with an action potential that arrested in the plateau phase (Figure 8A, inset) and exhibited many small fluctuations of the membrane potential and EAD. Eventually, the membrane repolarized. The duration of the effect was variable, but its appearance always followed an initial delay (96 ± 11 s). In contrast, when C-PAF was applied to ventricular myocytes isolated from PAFR KO mice, there was no response in most of the cells (7 of 9; Figure 8B). The responsiveness of WT and KO myocytes to C-PAF differed significantly (p<0.01; \( \chi^2 = 9.96 \)) although their resting potentials did not (-70.6 ± 1.1 mV versus -71.3 ± 1.5 mV). Finally, 6 of 8 quiescent wild-type cells failed to respond to C-PAF (185 nM) following BIM I treatment (100 nM). A comparison of BIM-treated to control myocytes indicated a significant reduction in susceptibility to spontaneous activity (p<0.01; \( \chi^2 = 8.84 \)).

If the decrease in outward current caused by blocking the TASK-1 channel is related to the arrhythmogenic effects of C-PAF, application of a TASK-1 inhibitor in current clamp mode should mimic the effects of C-PAF and evoke spontaneous activity. Accordingly, when methanandamide was applied to quiescent wild-type myocytes, spontaneous action potentials were observed (Figure 8C; 7 of 12 cells). Statistical analysis showed no difference in occurrence of spontaneous activity during methanandamide as compared to C-PAF superfusion.

**Discussion**

Inflammatory products released by PMNL can have negative effects on cardiac function and the survival of areas at risk following periods of ischemia and reperfusion (15). Our earlier studies, in isolated canine ventricular myocytes (4), demonstrated that PAF, a PMNL-derived inflammatory lipid, could alter
action potentials by prolongation of the APD, EADs and arrest at the plateau. The current study demonstrates that in murine ventricular myocytes C-PAF also triggers a series of alterations in the action potentials, including spontaneous beats, EADs and prolonged depolarization similar to those observed in canine myocytes (4, 5). This supports the validity of the mouse as a model in which to study the molecular basis of the arrhythmogenic effect of PAF.

We have measured changes in the membrane potential, spontaneous activity and in specific ion currents in myocytes as they are exposed to C-PAF. This lipid causes a small change in net current that develops over the first minute after application. Changes in the action potential (or appearance of spontaneous action potentials in quiescent cells) lag behind the peak current by approximately 20 s (at –70 mV the C-PAF-sensitive current peaked by 74 ± 13 s). The generation of spontaneous activity in quiescent myocytes implies that changes in membrane potential are not strictly dependent upon the stimulus or alterations in active currents but, rather, it is likely that the agonist perturbs the balance among those currents active at the resting membrane potential. Voltage clamp experiments measuring changes in conductance indicate that C-PAF effects are dependent on a decrease in outward current(s). In addition, the C-PAF-sensitive current, measured in elevated K⁺, showed weak outward rectification and had a reversal potential close to the calculated E_K. These data indicate that the C-PAF-sensitive current is largely carried by K⁺.

Since experiments utilizing Cs⁺ argue against the involvement of I_K1 in the ionic mechanism underlying the PAF-sensitive current, our attention shifted to other K⁺ channels that are active at rest. The two-pore domain K⁺ channels (13) are voltage and time-independent background channels having characteristics similar to the channel responsible for the C-PAF-sensitive current. Among this family, TASK-1 (TWIK related Acid-Sensitive K⁺ background channel; also referred to as cTBAK-1 (9) and Kcnk3 (14)) is expressed in the heart (10). TASK-1 is sensitive to small variations in external pH and is almost completely inhibited at pH 6.4. It is also blocked by Ba²⁺ or Zn²⁺ and by the putative endogenous lipid ligand of the cannabinoid receptors, anandamide (16). The C-
PAF-sensitive current in murine ventricular myocytes was sensitive to all these interventions suggesting that C-PAF-mediated effects are associated with inhibition of TASK-1 or a closely related channel. Confirmation that the TASK-1 channel is sensitive to C-PAF was obtained by expressing TASK-1 in CHO cells. When TASK-1 expressing CHO cells were superfused with C-PAF, the expressed current was reduced.

Since our data suggested that the C-PAF-sensitive current is due to TASK-1 blockade, we reasoned that anandamide treatment might prevent myocytes from responding to C-PAF. In fact, both anandamide in the presence of ATFK, an inhibitor of anandamide hydrolysis, and its nonhydrolyzable analogue, methanandamide, significantly reduced the C-PAF effect confirming our hypothesis. It follows that if C-PAF and methanandamide both inhibit TASK-1 and if this is the ionic basis for the C-PAF-sensitive effects, methanandamide should induce similar changes in myocyte physiology. As predicted, methanandamide causes both a decrease in net outward current and an increase in spontaneous activity in quiescent myocytes. Therefore, we conclude that both C-PAF and methanandamide exert their biological effects at least in part by inhibiting TASK-1 or a closely related channel.

In a heterologous expression system, Maingret et al. (16) found that anandamide inhibition of TASK-1 was not mediated by the known cannabinoid receptors and since the drug was effective on excised macropatches, they concluded that the lipid interacted directly with the channel. PAF, in contrast, is known to activate cells through a G-protein-linked receptor that initiates a signaling cascade involving activation of phospholipase C generating inositol phosphates and elevating intracellular calcium and diacylglycerol, ultimately activating PKC (1, 8, 17, 19). In our studies, the effect of C-PAF is clearly mediated by the PAFR since its activity can be blocked by the antagonist, CV-6209 and is absent in myocytes derived from mice in which the PAFR has been genetically deleted. In addition, we found that inhibition of PKC blocked the C-PAF-sensitive current. Although several reports suggest that TASK-1 is insensitive to PKC activators (3, 12), Lopes, et al. (14) found that PMA causes a
slowly developing block of TASK-1 current in an oocyte expression system. This further supports our hypothesis that C-PAF activity is mediated by activation of a PKC-dependent phosphorylation and although it does not resolve the mechanism behind the somewhat unexpected time course of the effect it is entirely consistent with our findings.

Interestingly, PKC inhibition also reduced the methanandamide-sensitive current suggesting that the two lipids share overlapping intracellular signaling pathways. Therefore, we tested whether methanandamide required the PAFR for its activity and found that it was fully functional in the presence of CV-6209 and in myocytes derived from KO mice. These data suggest that the methanandamide effect is dependent, at least in part, upon PKC activation. Alternatively the block of the TASK-1 channel by methanandamide may require a basal phosphorylation of the channel itself or an accessory protein and thus, ultimately depends upon but is not mediated by PKC. Such a scenario was recently described for a similar effect of anandamide on the VR1, vanilloid receptor, a non-selective cation channel. In this case, activation of the receptor by anandamide was significantly enhanced when the channel had been phosphorylated by PKC, and anandamide itself stimulated PKC (21).

These results for the first time suggest a role for the TASK-1 channel in PAF-mediated arrhythmias. However, additional questions remain. While block of TASK-1 channels could contribute to a longer APD and subsequent EADs, this does not preclude additional effects on other currents active during the action potential plateau, including Ca$^{2+}$, Na$^+$ and the delayed rectifier currents. In addition, the mechanism by which TASK-1 blockade might lead to initiation of spontaneous activity in a quiescent myocyte is not clear, since no measurable change in membrane potential was observed immediately preceding initiation of activity induced by either C-PAF or methanandamide. Additional mechanisms, either secondary to the block of TASK-1 or independent of this action, may occur after exposure to PAF. The murine model, and its amenability to genetic manipulations, should prove useful in the ultimate resolution of these remaining questions.
Legends

Figure 1. *C-PAF alters normal action potentials in mouse ventricular myocytes.* Paced action potentials (cycle length 1000 ms) were recorded in current clamp mode under control conditions (left trace, 0 s) and after perfusion of C-PAF (185 nM;). After a delay, C-PAF caused abnormal automaticity (trace 2, 110 s) and sustained depolarization (trace 3, 111 s). The action potential progressively shortened and normal rhythm was re-established, indicating desensitization of the receptor in continuous presence of drug (traces 4 and 5, 113 s and 140 s). The inset shows that traces during control perfusion and after recovery completely overlap. The data in this figure are derived from a single cell and are typical of 8 cells. The traces were recorded immediately before the application of C-PAF (trace 1) and 110, 111, 113, and 140 s after C-PAF (traces 2 through 5).

Figure 2. *Application of C-PAF causes a depolarizing shift in net membrane current in WT but not in KO myocytes.* Superfusion of C-PAF (185 nM) caused a transient decrease in the net outward current in a WT myocyte held at -10 mV (Panel A). In this trace the baseline outward holding current has been adjusted to zero to illustrate the C-PAF-sensitive current. The spontaneous reversal of the C-PAF effect probably indicates desensitization of the PAFR. The I-V relation of the C-PAF-difference current (control minus C-PAF) is plotted as a net outward current over a range of potentials in WT myocytes (Panel B, filled squares). In KO myocytes (filled circles) no C-PAF-sensitive current was detected at all potentials tested. Each data point is the mean ± SEM of data from at least 4 cells at each potential. The I-V relation was also measured using a ramp protocol in high extracellular K⁺ (50 mM) plus Cs⁺ (5 mM) and TEA⁺ (1 mM) to permit determination of the reversal potential (Panel C). Each data point is the mean ± SEM of data from at least 5 cells from 2 animals.

Figure 3. *The C-PAF-sensitive current is receptor-mediated.* The C-PAF-sensitive current was measured in WT myocytes held at –70 mV under various
conditions. The current under control conditions in wild-type myocytes disappeared in the presence of the PAFR antagonist, CV-6209 (100 nM; n=5). There was no C-PAF-sensitive current detected in myocytes from KO mice (n=3). *, p < 0.01.

**Figure 4.** Block of TASK-1 decreases the C-PAF-sensitive steady-state current. Wild-type myocytes were held at -10 mV and the C-PAF-sensitive current was measured at pH 7.4 (n=25). The change in net current elicited by C-PAF (185 nM) was significantly decreased in the presence of Tyrode’s at pH 6.4 (n=6), Ba$^{2+}$ (3 mM; n=6), or Zn$^{2+}$ (3 mM; n=8). The stable anandamide analogue, methanandamide (10 µM; n=12) also significantly reduced the C-PAF-sensitive current as did anandamide in the presence of ATFK, a drug that inhibits anandamide metabolism (10 µM; n=8). Anandamide alone did not significantly inhibit the current (10 µM; n=5) due to its rapid metabolic inactivation. *, p < 0.05 compared to control at pH 7.4.

**Figure 5.** TASK-1, heterologously expressed in CHO cells is sensitive to pH and to C-PAF. Net steady-state current was measured by a ramp clamp under alkaline (pH 8) and acidic (pH 6) conditions demonstrating the pH sensitivity of the expressed TASK-1 current. The I-V relation of each cell was normalized to the current at 30 mV to correct for cell-to-cell variability in expression levels and the mean normalized current density was plotted (left panel; n=13). In CHO cells exposed to C-PAF (185 nM) the expressed TASK-1 current was decreased (right panel). Representative I-V relations before (Control) and during drug treatment (C-PAF) were compared. This result is representative of 8 cells. On average, the I-V relation returned to within 5% of control value after washout of C-PAF.

**Figure 6.** The methanandamide-sensitive current is independent of the PAFR. WT cells held at -10 mV were superfused with methanandamide (10 µM) and the methanandamide-sensitive current was measured (WT Control; n=6). The methanandamide-sensitive current did not differ from control when WT cells were
incubated with the PAFR antagonist, CV-6209 (100 nM; n=3) or in myocytes derived from PAFR knockout mice (KO Control; n=6).

**Figure 7.** *The C-PAF-sensitive current is blocked by inhibition of PKC.*
The C–PAF-sensitive current is completely blocked in myocytes (held at -10 mV), exposed to BIM I, a specific PKC inhibitor (100 nM; Panel A). In this trace the baseline holding current has been adjusted to zero to illustrate the absence of a C-PAF-sensitive current. BIM I-mediated inhibition of the C-PAF-sensitive current is dose dependent (Panel B, 40 nM, n=7; 100 nM, n=11). An inactive BIM I analogue, BIM V does not block the C-PAF-sensitive current (Panel B, right; n=10). The inhibition of the C-PAF-sensitive current by BIM I is independent of voltage (Panel C; 100 nM BIM; n is at least 4 for each data point). *, p<0.05; **, p<0.001 versus control.

**Figure 8.** *C-PAF and methanadamide elicit spontaneous activity in quiescent WT myocytes.* Quiescent myocytes from WT and KO mice were studied in current clamp mode. C-PAF (185 nM) application elicited spontaneous activity in WT (Panel A) but not KO myocytes (Panel B). Superfusion of methanadamide (10 µM) over WT myocytes caused the same effect as C-PAF (Panel C). There was no measurable change in the resting potential prior to impulse initiation. These recordings are typical of 11 cells for Panel A, 7 cells for Panel B and 7 cells for Panel C.

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Reference List


18. **Millar JA, Barratt L, Southan AP, Page KM, Fyffe R, Robertson B, and Mathie A.** A functional role for the two-pore domain potassium channel


C-PAF-sensitive Current Density (pA/pF)

WT Control
WT + CV-6209
KO Control

* Statistically significant difference
Anandamide +

ATFK

Anandamide

Methanandamide

Zn$^{2+}$

Ba$^{2+}$

pH 6.4

pH 7.4

Current Density (pA/pF)

C-PAF-sensitive

Methanandamide

Anandamide

Anandamide +

ATFK
Methanandamide-sensitive Current Density (pA/pF)

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<tr>
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</tr>
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<td>KO Control</td>
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Methanandamide

A: WT
B: KO
C: WT

Membrane Potential (mV)

Time (s)

C-PAF

50 ms