Activation of Protein Kinase C ε Inhibits the Two-pore Domain K⁺ Channel, TASK-1, Inducing Repolarization Abnormalities in Cardiac Ventricular Myocytes

Alessandra Besana, Andrea Barbuti, Miyuki A. Tateyama, Aviva J. Symes, Richard B. Robinson, and Steven J. Feinmark
From the Center for Molecular Therapeutics, Department of Pharmacology, Columbia University, New York, New York 10032

Activation of the platelet-activating factor (PAF) receptor leads to a decrease in outward current in murine ventricular myocytes by inhibiting the TASK-1 channel. TASK-1 carries a background or “leak” current and is a member of the two-pore domain potassium channel family. Its inhibition is sufficient to delay repolarization, causing prolongation of the action potential duration, and in some cases, early after depolarizations. We set out to determine the cellular mechanisms that control regulation of TASK-1 by PAF. Inhibition of TASK-1 via activation of the PAF receptor is protein kinase C (PKC)-dependent. Using isoform-specific PKC inhibitor or activator peptides in patch clamp experiments, we now demonstrate that activation of PKCs is both necessary and sufficient to regulate murine TASK-1 current in a heterologous expression system and to induce repolarization abnormalities in isolated myocytes. Furthermore, site-directed mutagenesis studies have identified threonine 381, in the C-terminal tail of murine TASK-1, as a critical residue in this regulation.

Regulation of cardiac function depends on the appropriate cumulative activity of numerous ion channels in individual cardiomyocytes that are responsible for the sequential depolarization-repolarization cycle known as the action potential (AP). Although the major currents contributing to the AP have been described (1), additional small currents at specific points of low conductance in the AP cycle are sufficient to induce repolarization abnormalities in isolated cells (2, 3) and therefore arrhythmias in situ. These arrhythmias may contribute to the electrical abnormalities that lead to sudden death after myocardial infarction, which persists as the number one cause of death in the United States. We have focused on one channel that has been proposed to contribute to cardiac arrhythmias, TASK-1, a member of the recently described family of two-pore domain potassium channels (4).

The two-pore domain K⁺ channel family is composed of at least 15 different members. These channels are widely distributed in excitable tissue, primarily in the brain and heart, and in general are responsive to environmental cues such as temperature, pH, and stretch (5, 6). Several are also regulated by lipids such as arachidonic acid or platelet-activating factor (PAF) (7–9). PAF is an inflammatory phospholipid that has been linked to arrhythmogenesis in isolated canine ventricular myocytes (10). We have recently shown that PAF regulates the TASK-1 channel and determined that the arrhythmogenic effect of the stable PAF analog, carbamyl-platelet-activating factor (C-PAF) (7–9), is due to the inhibition of TASK-1 current in a protein kinase C (PKC)-dependent manner (2).

In this study, we elucidate the molecular mechanism of the C-PAF effect on TASK-1 current by identifying the ε isoform of PKC (PKCe) as a critical component in PAFR signaling. In addition, using site-directed mutagenesis, we have tentatively identified the critical residue that is the target for PKC in the murine channel.

EXPERIMENTAL PROCEDURES

Myocyte Preparation—Mouse ventricular myocytes were isolated using a retrograde coronary perfusion method published previously (11). All the experiments were carried out according to the guidelines issued by the IACUC of Columbia University. Adult mice, 2–3 months old, were anesthetized with a xylazine and ketamine mix and heparinized, the heart was quickly removed, and the ascending aorta was connected by the IACUC of Columbia University. Adult mice, 2–3 months old, were anesthetized with a xylazine and ketamine mix and heparinized, the heart was quickly removed, and the ascending aorta was connected to the outlet of a Langendorff column and perfused with 20–25 ml of a buffer solution (37 °C) containing (in mM): NaCl, 112; KCl, 5.4; NaHCO₃, 4.2; MgCl₂, 1.6; HEPES, 20; glucose, 5.4; NaH₂PO₄, 1.7; taunine, 10; t-glutamine, 4.1; minimum essential medium amino acids solution, 2%; minimum essential medium vitamin solution, 1%; adjusted to pH 7.4, and equilibrated with 100% O₂. Next, the heart was perfused with an enzyme solution containing collagenase (0.2 mg/ml; Worthington Type II) and trypsin (0.04 mg/ml) at 35 °C for 10–12 min. After this perfusion, the atria were removed, and the ventricles were minced and transferred to a 50-ml flask with an enzyme solution containing collagenase (0.45 mg/ml), trypsin (0.08 mg/ml), Ca²⁺ (0.75 mM), and bovine serum albumin (4.8 mg/ml). The flask was shaken vigorously for 5–10 min at 32 °C before the supernatant was removed and the cells were collected by centrifugation; this operation was repeated two or three times, and additional disaggregated cells were collected. After centrifugation, the myocytes were resuspended in the buffer solution containing Ca²⁺ (0.75 mM) and bovine serum albumin and stored at room temperature until use. Rod-shaped, Ca²⁺-tolerant
myocytes, obtained with this procedure, were used within 6 h of dissociation. Measurements were performed only on quiescent myocytes with clear striations. 

Plasmids—pCMV-TASK1 (cTBAK) consists of a 1.9-kb sequence of murine TASK-1 inserted in pcDNA3.1 (a kind gift of Dr. Yoshiiha Kurachi, University of Osaka, Osaka, Japan) and has been described previously (12). pEGFP-C1 and pIRES-EGFP were purchased from Clontech. pTIE (TASK1-IRES-EGFP) was constructed by inserting a 1.9-kb EcoR1 fragment from pCMV-TASK1 into EcoR1-digested pIRES-EGFP. Site-directed mutagenesis was performed on pTIE using the QuikChange kit (Stratagene) following the manufacturer’s instructions. Primers were designed to generate a mutation in pTIE in which threonine 381 was converted to alanine (T381A-pTIE): forward, 5'-TCG CAG GCT CCG GAG GCT GCG CCC GC ACG TCG-3'; and reverse, 5' -CGA GCT TAG ATG CCG ACG GGT CGC CCC GC ACG AAG-3'.

Cell Culture and Transfection—Chinese hamster ovary cells (CHO) were grown in F-12 medium supplemented with 10% fetal bovine serum. Twenty-four hours prior to transfection, cells were seeded into 6-well plates at 80–90% of confluence. Transfections were carried out with the GeneJammer transfection reagent (Stratagene) according to the manufacturer’s instructions. Briefly, cells were washed with phosphate-buffered saline, and their medium was replaced with supplemented F-12 medium (900 μl/well). For each well, GeneJammer (6 μl) was incubated with Opti-MEM (90 μl) followed by the addition of DNA (1 μg). This mixture was then added to the wells, and, after 1 h, an additional 2 ml of supplemented F-12 medium was added. After incubating overnight, the cells were washed, and their medium was replaced.

Cells were either co-transfected with pCMV-TASK1 together with pEGFP-C1 (1 μg total, 3:1) or transfected with pTIE or T381A-pTIE (1 μg). 48 h after the transfection, the cells were checked under the microscope for green fluorescence. Approximately 20% of the cells were positive for EGFP, and these were then used for patch clamp experiments. Due to the culture-to-culture variability in the expression of TASK-1 current, most comparisons were made on matched controls from the same transfection. Summary results were then obtained by pooling data from several different culture preparations.

Solutions and Recording Apparatus—The myocyte suspension or the coverslip with CHO cells was placed into a perfusion chamber, mounted on the stage of an inverted microscope. Unless otherwise indicated, CHO cells were superfused at room temperature with standard external Tyrode’s buffer, containing (in mM): NaCl, 140; KCl, 5.4; CaCl2, 1.8. This medium was then added to the wells, and, after 1200 interface (Axon Instruments). In current clamp mode, for recording action potentials, the signals were filtered at 1 kHz (low pass Bessel filter) and acquired at a sampling rate of 5 kHz. In voltage-clamp mode, the current signals were filtered at 1 kHz and acquired at 500 Hz.

Data Analysis and Statistics—Data were analyzed using pCLAMP 8.0 (Axon) and Origin 6.0 (Microcal) and are presented as mean ± S.E. Records have been corrected for the junction potential, which was measured to be −9.8 mV. Steady state currents were determined by computer calculation of average current over at least 1 min. Unless otherwise stated, current density comparisons were determined at a voltage of −30 mV. Current density changes are expressed as percentage of inhibition in CHO cell experiments in which TASK-1 is essentially the only current and a pretreatment baseline current can be readily recorded. In myocytes, TASK-1 is measured as the drug-sensitive current, and thus, it is not possible to measure a baseline current to decide whether the effect of C-PAF or PMA is on TASK-1. Therefore, changes in this current in myocytes are expressed in absolute values (pA/pF). Fisher’s exact test was used to test the significance of frequency data, and Student’s t test was used to compare paired or independent data; a value of p ≤ 0.05 was considered statistically significant.

RESULTS

C-PAF Inhibition of TASK-1 Current in CHO Cells Requires Activation of PKC—Untransfected CHO cells have no significant endogenous K+ currents (data not shown); thus, all of the current measured in transfected cells was carried by TASK-1. Therefore, we expressed TASK-1 in CHO cells to test the effect of C-PAF (185 nM) on the current in whole cell patch clamp experiments. During a slow ramp protocol (−110 mV to +30 mV in 6 s), C-PAF rapidly induced a reversible decrease in TASK-1 current that reached steady state within 2 min. When quantified at the maximal current (at +30 mV), this set of cells expressed 68.6 ± 16.4 pA/pF in control solution versus 60.2 ± 14.3 pA/pF in the presence of C-PAF, a 12% decrease in the mean current density (Fig. 1A; n = 9, p = 0.01). We next tested whether the effect of C-PAF on TASK-1 current was due to PKC activation by perfusing the cells with BIM-I (100 nM), a nonisoform-specific PKC inhibitor for 2 min before applying C-PAF. In the presence of BIM-I, there was no measurable C-PAF-sensitive current (Fig. 1B, n = 12).

To determine whether activation of PKC alone was sufficient to reduce TASK-1 current, we treated CHO cells expressing TASK-1 with a non-specific activator of PKC, PMA (100 nM). PMA significantly inhibited TASK-1 current in a manner that was similar to the effect of C-PAF (Fig. 1C; n = 11, p < 0.01). The specificity of the PMA effect was verified by exposing cells to an inactive PMA analogue, 4α-phorbol 12-myristate 13-acetate (α-PMA; 100 nM). α-PMA had no detectable effect on TASK-1 current expressed in CHO cells (Fig. 1D). In all TASK-1-expressing cells tested, the mean control current was 71.8 ± 12.3 pA/pF, whereas in the presence of PMA, the current fell to 59.2 ± 10.1 pA/pF. The PMA inhibition (19.8 ± 2.7%, n = 17) was significantly greater than that of C-PAF (12.1 ± 1.0%, n = 20; p < 0.01) when measured at the maximum test voltage of +30 mV, and was irreversible.

The Activation of PKCe Decreases TASK-1 Current in CHO Cells—Having shown that the activation of PKC by either C-PAF or PMA was sufficient to cause a decrease of the TASK-1 current, we next asked whether one specific isoform of PKC was responsible for this effect. We initially discounted the role of the classical PKC isoforms since preliminary studies had suggested that the PKC effect on TASK-1 was not calcium-dependent (data not shown). Given the prominent role of PKCe in cardiac physiology, we tested the ability of a PKCe-specific inhibitor peptide to block the drug-induced reduction in TASK-1 current. A scrambled peptide was used as a control (13). The peptides were introduced to the cells by dialysis through the patch pipette at a final concentration of 100 nm, and recordings were initiated 8–10 min after the rupture of the membrane to allow the peptide to equilibrate in the cell. C-PAF failed to inhibit TASK-1 current in the presence of the PKCe
inhibitor peptide (25.6 ± 12.2 pA/pF before C-PAF versus 25.4 ± 12.4 pA/pF after C-PAF, n = 8, not significant; Fig. 2A).

On the contrary, in the presence of the scrambled peptide, C-PAF-induced inhibition of TASK-1 (8.4 ± 1.5%, n = 10) did not differ from control trials in the absence of any peptide (data not shown). Similarly, the addition of the PKCe inhibitor peptide to the pipette completely blocked the PMA-sensitive current in CHO cells expressing TASK-1 (Fig. 2B; 42.4 ± 12.7 pA/pF before PMA versus 41.2 ± 12.3 pA/pF after PMA, n = 10, not significant), whereas the PMA effect was still present with the scrambled peptide (45.1 ± 7.0 pA/pF before PMA versus 36.6 ± 6.2 pA/pF after PMA, n = 11, p < 0.01). Summary data for C-PAF and PMA are shown in Fig. 2C.

Does C-PAF Inhibition of TASK-1 Current in Ventricular Myocytes Depend upon Activation of PKCe?—We next asked whether the C-PAF-sensitive current in murine ventricular myocytes, defined previously as a TASK-1 current (2), was also mediated by activation of PKCe. Recordings were done either with the PKCe inhibitor peptide or with the scrambled peptide in the patch pipette while cells were held at −10 mV. Ten to twelve min after the rupture of the membrane and when the holding current was stable for at least 1 min, C-PAF (185 nM) was superfused over the myocytes. In the presence of the scrambled peptide, C-PAF caused a decrease in outward current that was indistinguishable from the effect of C-PAF in the absence of peptide (a typical trace is shown in Fig. 3A). The effect of C-PAF was absent, however, when the PKCe inhibitor peptide was included in the patch pipette (a typical trace is shown Fig. 3B). Results from numerous trials showed that the inhibitor peptide significantly inhibited the ability of C-PAF to reduce TASK-1 current in isolated mouse ventricular myocytes, whereas the scrambled peptide had no effect (Fig. 3C).

To further verify that the C-PAF-sensitive current identified in voltage clamp studies was carried by the TASK-1 channel, the I-V relation in myocytes was studied with a slow ramp protocol (−50 mV to +30 mV over 6 s) in the presence of modified Tyrode’s. These conditions minimize the contamination of the TASK-1 current by other K+ currents and should allow the calculation of the C-PAF-sensitive current over a wide voltage range by minimizing the outward rectification. To confirm this, we first examined the expressed TASK-1 current in CHO cells in modified Tyrode’s. As expected, the I-V relation was markedly less rectifying (data not shown), and the reversal potential was less negative (−24.4 ± 1.5 mV, as compared with a calculated value of −27.5 mV in modified Tyrode’s for a K+-selective current). The C-PAF inhibition in the presence of elevated K+ (10.2 ± 1.8% inhibition, n = 16) was indistinguishable from the previously reported effect of the lipid on TASK-1 in CHO cells recorded in normal Tyrode’s (p = 0.33).

In modified Tyrode’s solution, myocytes exposed to the scrambled peptide in the patch pipette had a significant decrease in net current in response to C-PAF (a typical cell is shown in Fig. 4A; n = 8; p < 0.01) that was essentially identical to the effect measured in the absence of peptide in the pipette (data not shown). Typical of TASK-1 in high K+, the
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C-PAF-sensitive current is nearly linear and has a reversal potential of $-26.1 \pm 1.9$ mV (Fig. 4A2). In the presence of the inhibitor peptide, however, C-PAF had virtually no effect on net current (Fig. 4B1), and the C-PAF-sensitive current was abolished (Fig. 4B2), indicating that PKC\textsubscript{e} also plays a crucial role in the regulation of TASK-1 current by PAFR in myocytes. Summary data are shown in Fig. 4C.

Does PKC\textsubscript{e} Play a Role in C-PAF-induced Repolarization Abnormalities in Isolated Myocytes?—We previously showed that C-PAF induced abnormal automatcity in paced ventricular mouse myocytes and elicited spontaneous activity in quiescent myocytes (2). Now, we asked whether this abnormal automatcity could be due to PKC\textsubscript{e} activation. To test this, action potential recordings were done on mouse ventricular myocytes paced at 1 Hz with either the PKC\textsubscript{e}-specific inhibitor peptide or an inactive scrambled peptide in the pipette (100 nM). Action potentials were continuously monitored, from the rupture of the membrane until the end of the protocol. C-PAF was applied 10–12 min after the rupture. When the scrambled peptide was in the pipette, C-PAF-induced abnormalities during repolarization in 14 of 19 cells (Fig. 5A; not different from the response of cells treated with C-PAF in the absence of any peptide). In contrast, C-PAF failed to induce repolarization abnormalities in any of the eight cells that were exposed to the PKC\textsubscript{e}-specific inhibitor peptide (Fig. 5B). The difference in observed responses was significant ($p < 0.001$, Fisher’s exact test).

Further confirming that activation of PKC\textsubscript{e} is sufficient to alter the electrical activity of the myocyte, we observed that a specific activator peptide of this kinase included in the patch pipette induced prolongation of repolarization, early after depolarizations, and additional spontaneous beats in eight of nine cells tested in the absence of any added C-PAF. In these trials, recordings were begun immediately after the rupture of the membrane, and abnormal rhythm occurred 5–6 min later. Under similar conditions but with the scrambled peptide in the pipette, abnormal automatcity was observed in only 2 of 10 cells tested (Fig. 6; $p < 0.006$, Fisher’s exact test).

An analysis of the murine TASK-1 sequence revealed a single PKC consensus site that included threonine (residue 381) as the kinase target. Therefore, we constructed a site-directed mutant at this site, converting Thr-381 to alanine. The mutant construct, named T381A-pTIE, was expressed in CHO cells, and when tested by our typical ramp protocol, it demonstrated activity that was comparable with the wild-type channel. However, the mutant channel was no longer sensitive to C-PAF inhibition (maximal current recorded at $+30$ mV in the absence of C-PAF was $45.5 \pm 7 \text{ pA/pF}$ versus in the presence of C-PAF, $44.2 \pm 7 \text{ pA/pF}$; $n = 10$; not significant, Fig. 7). Similar results were obtained when mutant TASK-1 current was tested in the presence of PMA (Fig. 7C, right).

DISCUSSION

Previous studies have shown that the abnormalities of repolarization induced by PAF in ventricular myocytes are due to alterations of the background potassium current carried by TASK-1 (2). Shortly after the channel was cloned, heterologous expression studies showed that TASK-1 was inhibited by PMA and that the inhibition could be blocked by BIM-I (16), suggesting a role for PKC in the regulation of channel function. We now show that both overexpressed and native TASK-1 are inhibited by activation of the PAFR and that this inhibition is dependent upon the activation of the \( \epsilon \)-isoform of PKC. The activation of PKC\textsubscript{e} is not only necessary but also sufficient to alter repolarization in isolated myocytes. This sufficiency is evident both by the ability of PMA to inhibit TASK-1 current in CHO cells and by the ability of a PKC\textsubscript{e} activator peptide to induce abnormal automatcity in myocytes in the absence of added PAF. The results obtained when the TASK-1 channel is overexpressed in a heterologus system support the myocyte data by confirming that PAF inhibits TASK-1 in a PKC\textsubscript{e}-dependent manner. Furthermore, in the heterologous system, PKC\textsubscript{e} appears to be the only PKC isoform involved in the regulation of murine TASK-1 since blocking PKC\textsubscript{e} is sufficient to block the PMA effect on the channel.

Murine TASK-1 has a single consensus PKC site, which is threonine-381, a residue in the C-terminal cytoplasmic tail. Using site-directed mutagenesis, we mutated this site, replacing threonine with the nonphosphorylatable residue, alanine. The T381A mutant expresses normally in CHO cells but is not inhibited by the addition of C-PAF, nor is it sensitive to PMA treatment. Although we have yet to show direct biochemical evidence of channel phosphorylation at this site, the mutagen-
The C-PAF-dependent inhibition of TASK-1 current in mouse ventricular myocytes requires activation of PKCe, current-voltage relation. C-PAF-sensitive current was recorded in whole cell configuration using a ramp protocol (−50 to +30 mV) in modified Tyrode’s solution. The recordings started 10–12 min after the C-PAF application (A). The recordings were started 10–12 min after the current was stable for at least 1 min. C-PAF-sensitive current was obtained as the difference between the mean current (average of four successive ramps) at steady state in control and in the presence of C-PAF; the current was normalized by the capacitance of the cell and expressed as current density (pA/pF). A1 depicts the net current from a typical cell before and after C-PAF treatment in the presence of scrambled peptide. A2 depicts the mean C-PAF-sensitive current recorded from myocytes in the presence of scrambled peptide (100 nM in the pipette; n = 8). B1 depicts the net current from a typical cell before and after C-PAF treatment in the presence of scrambled peptide. B2 illustrates that in the presence of the inhibitor peptide, the mean C-PAF-sensitive current was abolished (100 nM in the pipette, n = 7; * p < 0.05). The mean C-PAF-sensitive current quantified at +30 mV is summarized in C.

The role of PKCe in cardiac function is complicated by observations that this isoform can mediate the cardioprotective events of ischemic preconditioning (Ref. 24, and reviewed in).
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Ref. 25), and under other conditions, plays a lead role in the development of hypertrophy and failure (26). Some of the explanations for these dichotomous results may lie in the variability of the level of expression of the kinase and in the subsequent control of its subcellular localization and formation of signaling complexes. For example, it has been shown that PKCe localizes in complexes at mitochondrial membranes after brief repeated episodes of ischemia. Could this sequester enough of the kinase to prevent its association with TASK-1 in the plasma membrane and thereby prevent the arrhythmogenic reduction in this background K+ current? Pharmacological antagonism of the PAFR or ischemic preconditioning are both able to significantly reduce the occurrence of ventricular ectopic beats after coronary occlusion (27) but likely work by different mechanisms. The effect of the PAF antagonist is calculated as the difference between mean current (average of four successive ramps) at steady state in control and in the presence of C-PAF or PMA as noted. C-PAF was applied for 2 min after the current was stable for at least 1 min. PMA was applied for 6 min after the current was stable for at least 1 min. The current was normalized by cell capacitance and expressed as current density (pA/pF). The percentage of control TASK-1 current was calculated, and the data were summarized (C). *

plateau represents a period of high membrane resistance in which even small currents can exert a significant effect. It is well recognized that reduction in net outward current during the action potential plateau can lead to action potential prolongation and subsequent arrhythmias through the activation of other currents (3). Further, in the setting of cardiac disease, down-regulation of outward K+ currents can result in the reduction of "repolarization reserve" (28) such that even a small further decrease in net outward current can lead to marked action potential prolongation and arrhythmogenesis. In our experiments, it is likely that there is a progressive inhibition of TASK-1 current either by C-PAF or by the activator peptide activating PKCe. However, due to the repolarization reserve, a marked failure of repolarization and subsequent arrhythmias does not occur until the current is reduced beyond a critical threshold level. This accounts for the delay in the onset of arrhythmias during C-PAF superfusion and suggests that PAF-induced inhibition of TASK-1 current is likely to be particularly arrhythmogenic in the context of cardiac disease, in which other K+ currents are already compromised.

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