The inhibition of the potassium channel TASK-1 in rat cardiac muscle by endothelin-1 is mediated by phospholipase C

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Aims The two-pore-domain potassium channel TASK-1 is robustly inhibited by the activation of receptors coupled to the Gq subgroup of G-proteins, but the signal transduction pathway is still unclear. We have studied the mechanisms by which endothelin receptors inhibit the current carried by TASK-1 channels (I\textsubscript{TASK}) in cardiomyocytes.

Methods and results Patch-clamp measurements were carried out in isolated rat cardiomyocytes. I\textsubscript{TASK} was identified by extracellular acidification to pH 6.0 and by the application of the TASK-1 blockers A293 and A1899. Endothelin-1 completely inhibited I\textsubscript{TASK} with an EC\textsubscript{50} of <10 nM; this effect was mainly mediated by endothelin-A receptors. Application of 20 nM endothelin-1 caused a significant increase in action potential duration under control conditions; this was significantly reduced after pre-incubation of the cardiomyocytes with 200 nM A1899. The inhibition of I\textsubscript{TASK} by endothelin-1 was not affected by inhibitors of protein kinase C or rho kinase, but was strongly reduced by U73122, an inhibitor of phospholipase C (PLC). The ability of endothelin-1 to activate PLC-mediated signalling pathways was examined in mammalian cells transfected with TASK-1 and the endothelin-A receptor using patch-clamp measurements and total internal reflection microscopy. U73122 prevented the inhibition of I\textsubscript{TASK} by endothelin-1 and blocked PLC-mediated signalling, as verified with a fluorescent probe for phosphatidylinositol-(4,5)-bisphosphate hydrolysis.

Conclusion Our results show that I\textsubscript{TASK} in rat cardiomyocytes is controlled by endothelin-1 and suggest that the inhibition of TASK-1 via endothelin receptors is mediated by the activation of PLC. The prolongation of the action potential observed with 20 nM endothelin-1 was mainly due to the inhibition of I\textsubscript{TASK}.

Keywords Cardiomyocytes • Phospholipase C • U73122 • K\textsubscript{2P} channels • Cardiac action potential

1. Introduction

It has long been suspected that the two-pore-domain potassium channel (K\textsubscript{2P} channel) TASK-1 contributes to the steady-state outward current in rat cardiac ventricular muscle.\textsuperscript{1,2} Recently, we have given a quantitative description of the current flowing through TASK-1 channels (I\textsubscript{TASK}) in cardiomyocytes of rat and mouse ventricular muscle and in human atrium using the specific inhibitor A293.\textsuperscript{3–6} The activation of G-protein-coupled receptors that signal through G\textsubscript{q} subunits causes the inhibition of I\textsubscript{TASK} in cardiomyocytes,\textsuperscript{7,8} neurons,\textsuperscript{7,8} and other cell types.\textsuperscript{9} However, the mechanisms by which TASK-1 (and TASK-3) channels are inhibited via Gq-coupled receptors are still controversial. It has been proposed that the inhibition of the channels is due to (i) the activation of protein kinase C (PKC),\textsuperscript{10} (ii) direct interaction of the G\textsubscript{q} subunit with the channel,\textsuperscript{11} or (iii) depletion of phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P\textsubscript{2}].\textsuperscript{12} In contradiction to the latter hypothesis, a recent study has shown that the activity of I\textsubscript{TASK} is independent of phosphoinositide concentrations in the physiological range.\textsuperscript{13}

In the present study, we examined the mechanisms underlying the signal transduction between G\textsubscript{q}-coupled receptors and I\textsubscript{TASK} using two complementary approaches: On the one hand, we studied the effects of the signalling peptide endothelin-1 (ET-1) on I\textsubscript{TASK} in isolated rat cardiomyocytes, on the other hand we reconstituted the signal transduction pathway by co-transfecting the endothelin-A (ETA) receptor and TASK-1 in a mammalian cell line (CHO cells).
In both systems, the activation of ET<sub>A</sub> receptors inhibited TASK-1 currents. Our results suggest that the activation of phospholipase C (PLC) is the major upstream mechanism leading to the inhibition of I<sub>TASK</sub>.

ET-1 is a potent vasoconstrictor and modulates action potential duration (APD) and contractility of cardiac muscle cells. The circulating levels of ET-1 are elevated in congestive heart failure, myocardial ischaemia, and hypertension. High levels of ET-1 lead to the alteration of cardiac gene expression, but also have immediate arrhythmogenic effects. Thus, the investigation of the molecular mechanisms by which ET-1 alters the electrical activity of the heart may be of pathophysiological relevance.

2. Methods

More detailed methods are provided in Supplementary material online, Methods.

2.1 Patch-clamp experiments with isolated rat cardiomyocytes

Rats weighing 200–300 g were anaesthetized by evaporating a lethal concentration of sevoflurane in a closed cage (2 mL liquid sevoflurane/L air). When the righting reflex had subsided and nociceptive withdrawal reflexes could no longer be elicited by pinching the forepaws, the animals were decapitated and the heart was quickly excised. The experimental procedures were approved by the animal protection committee of Marburg University and by the Regierungspäsidium Giessen; the investigation conforms with the Directive 2010/63/EU of the European Parliament. The isolation of cardiomyocytes and patch-clamp experiments were performed as described previously. In brief, the aorta was attached to a cannula and the heart was perfused for 10 min with HEPES-buffered physiological salt solution (PSS) at pH 7.4; the flow rate was 6–8 mL/min, the temperature was 37°C. Subsequently, the heart was perfused for 5 min with a nominally Ca<sup>2+</sup>-free PSS and for 9 more min with Ca<sup>2+</sup>-free PSS to which collagenase (Type II, Worthington; 70–90 mg/50 mL) was added. Then the heart was incubated in ‘recovery solution’, minced and dispersed with a glass pipette.

Steady-state current–voltage relationships of isolated cardiomyocytes were determined with slow voltage ramps (~15 mV s<sup>−1</sup>; see Supplementary material online, Figure S1). Membrane capacitance was measured with fast voltage ramps (500 mV s<sup>−1</sup>; see Supplementary material online, Figure S2). Action potentials were elicited by brief current pulses (50% above the threshold for the initiation of an action potential) of 1 ms duration at a frequency of 4 Hz. Data acquisition was performed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), an A/D converter (PCI-MIO 16-XE-10, National Instruments), and software developed in our laboratory (PC-DAQ1.1). The sampling rate was 2 or 5 kHz. To separate I<sub>TASK</sub> from other current components, the cardiomyocytes were superfused with a blocker cocktail that eliminates the transient outward current (I<sub>KATP</sub>: 2 mM 4-aminopyridine, the ATP-sensitive K<sup>+</sup> current (I<sub>KATP</sub>: 2 µM glibenclamide), the L-type Ca<sup>2+</sup> current (I<sub>L</sub>: 10 µM nifedipine), the rapid voltage activated K<sup>+</sup> current (I<sub>RK</sub>: 1 µM E-4031), and the slow voltage activated K<sup>+</sup> current (I<sub>Ks</sub>: 2 µM HMR1556). The resting potential of the cardiomyocytes under these conditions was ~−68 ± 0.3 mV (n = 152). The average membrane capacitance was 123 ± 2.7 pF (n = 152). All experiments were performed at room temperature (~22°C).

2.2 Patch clamp and total internal reflection fluorescence microscopy

Cell culture, transfection, and measurements with Chinese hamster ovary (CHO) cells were performed as described in detail elsewhere. Briefly, CHO/diff- cells (from American Type Culture Collection) were transiently transfected with the ET<sub>A</sub> receptor (in pcDNA3.1; Invitrogen) and with either TASK-1 (in pCDNA3.1; Invitrogen), Kv7.2 or PH<sub>PLCβ5</sub>-GFP (pEGFP-N1) using jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). Whole-cell recordings were performed with an Axopatch 200B amplifier; data were sampled with an ITC-16 interface (Instrutech, HEKA, Lambrecht, Germany) controlled by PatchMaster software (HEKA). Total internal reflection fluorescence (TIRF) microscopy was performed using an upright microscope (BX51WI, Olympus) equipped with a TIRF condenser (numerical aperture, 1.45) and a 488 nm laser (20 mW; Picarro, Sunnyvale, CA, USA). Image acquisition was performed with an IMAGO-QE cooled CCD camera (TILL Photonics, Gräfelfing, Germany) and TILLvision software (TILL Photonics).

2.3 Chemicals

U73122 (Sigma Aldrich) and U73343 (Calbiochem) were dissolved in DMSO as 5 mM stock solutions; the final concentration was 5 µM in PSS. Measurements were done within 1 h after diluting the stock solution in PSS. A293 [2-(butane-1-sulfonylamino)-N-[1-(R)-(6-methoxy-pyridin-3-yl)-propyl]-benzamide] and A1899 were gifts from Sanofi Aventis (Frankfurt, Germany). The rho kinase inhibitor Y27632 (BioVision) was dissolved in water as a 10 mM stock solution.

2.4 Statistics

Data are reported as means ± SEM. Statistical significance was determined using Student’s t-test. Significant differences to control values are marked by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant, P > 0.05).

3. Results

3.1 Isolation and characterization of the TASK-1-mediated current in cardiomyocytes

The current flowing through I<sub>TASK</sub> is maximally activated at pH 8.0 and completely inhibited at pH 6.0. To determine the maximal amplitude of I<sub>TASK</sub>, we measured steady-state current–voltage relationships in rat cardiomyocytes before and after a switch from pH 8.0 to 6.0 in the presence of the blocker cocktail designed to eliminate I<sub>KATP</sub>, I<sub>Ks</sub>, I<sub>RK</sub> and I<sub>P</sub> (see Section 2). The change to pH 6.0 caused a reduction in the outward currents in the range ~50 to +40 mV (Figure 1A). The current change was complete in ~30 s (Figure 1B), which corresponds to the time required for a complete exchange of the bath solution. When the extracellular solution was switched back to pH 8.0, the decrease in outward current was reversed; and a small transient overshoot was observed (Figure 1B). The changes in extracellular pH could be repeated several times, indicating that the recording configuration was stable enough to record small changes in steady-state currents over 30 min. The mean current amplitude at +30 mV in the presence of the blocker cocktail was 1.12 ± 0.05 pA/pF (n = 30 cells). The mean TASK-1 current, defined as the current component blocked by extracellular acidification, was 0.35 ± 0.03 pA/pF at +30 mV (n = 30; Figure 1C).

The identity of the acid-sensitive current was confirmed by superfusing the cardiomyocytes with the specific TASK-1 blocker A293. Again we first equilibrated the cells (for at least 5 min) at pH 8.0 and then applied 10 µM A293. The effects of A293 on the steady-state current–voltage relationship were similar to those of extracellular acidification. The mean current change measured at +30 mV after the application of A293 was 0.27 ± 0.03 pA/pF (n = 10). This was
slightly smaller but did not differ significantly \((P > 0.05)\) from the current change produced by acidification (Figure 1C).

### 3.2 Inhibition of \(I_{\text{TASK}}\) by endothelin-1

Next, we studied the inhibition of \(I_{\text{TASK}}\) via endothelin receptors. Superfusion of the cardiomyocytes with 200 nM ET-1 produced a similar change in steady-state outward current as the jump in extracellular pH (Figure 2A). The effects of ET-1 reached a steady state within 40 s but were poorly reversible (Figure 2B). The mean current change produced by 200 nM ET-1 was \(0.30 \pm 0.02\) pA/pF \((n = 24;\) Figure 1C and inset of Figure 2A). The \(\alpha_1\)-adrenergic agonist methoxamine (100 μM), acting through another Gq-coupled receptor, produced a similar current change as 200 nM ET-1 at pH 8.0 (Figure 1C).

To obtain an estimate of the EC\(_{50}\) of ET-1, we repeated these experiments with lower concentrations of ET-1. The current change produced by 200 nM ET-1 was

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\frac{\text{Current change} \pm \text{SEM}}{\text{pA/pF}} = 0.30 \pm 0.02 \quad (n = 24; \text{Figure 1C and inset of Figure 2A})
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\text{Figure 1} \quad \text{The acid-sensitive steady-state potassium current. (A) Representative steady-state current–voltage relationship measured with slow voltage ramps (see Supplementary material online, Figure S1A) at pH 8.0 (black trace) and at pH 6.0 (red trace); the difference curve is shown in green. The inset shows the averaged difference curve from 30 cardiomyocytes in the voltage range \(-30\) to \(+30\) mV. The currents from one cell were averaged at 10 mV intervals (for example, from \(-5\) to \(+5\) mV) and the corresponding values for all cardiomyocytes were used to calculate the mean \(\pm \text{SEM}\). (B) The time course of the currents measured at \(+30\) mV during changes of extracellular pH from 8.0 to 6.0. (C) Mean difference currents measured at \(+30\) mV after application of 200 nM endothelin-1 (ET-1), 100 μM methoxamine (MTX), 10 μM A239 or pH 6.0. The control solution was buffered to a pH of 8.0 to maximize \(I_{\text{TASK}}\). The current amplitude was related to the cell size (pA/pF) by determining the membrane capacitance of each cardiomyocyte (see Supplementary material online, Figure S1B). The number of cardiomyocytes from which the data were obtained is indicated in brackets.}

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\text{Figure 2} \quad \text{Effects of ET-1 on the steady-state current–voltage relationship. (A) Representative steady-state current–voltage relationship measured at pH 8.0 before (black trace) and after (blue trace) application of 200 nM ET-1; the difference curve is shown in green. The inset gives the mean values of the difference current measured in 24 cells. (B) The time course of the current change at \(+30\) mV. (C) Effects of different concentrations of ET-1 on the steady-state outward current at \(+30\) mV.}
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change produced by application of 50 or 20 nM ET-1 was not significantly different from that produced by 200 nM ET-1. Application of 10 nM and 5 nM ET-1 gave rise to the mean current changes of $0.19 \pm 0.03 \text{ pA/pF}$ and $0.12 \pm 0.03 \text{ pA/pF}$, respectively (Figure 2C). These data suggest that the EC$_{50}$ for ET-1 under our experimental conditions was between 5 and 10 nM.

To confirm that the outward currents inhibited by ET-1 and by the TASK blocker A293 are identical, we carried out experiments with sequential application of both substances (see Supplementary material online, Figure S2). Application of 200 nM ET-1 in the presence of the TASK-1 blocker A293 (10 μM) produced no or extremely small additional current changes (see Supplementary material online, Figure S2A and B). Similarly, when applied at pH 6.0 (which blocks TASK-1 completely), 200 nM ET-1 had almost no effect on the measured outward current (see Supplementary material online, Figure S2B).

In conclusion, extracellular acidification, application of the TASK-1 blocker A293, application of ET-1, and application of methoxamine (100 μM) all inhibited a current component that displayed the characteristic outwardly rectifying current–voltage relationship of TASK-1. The reductions in outward current measured after these interventions did not differ significantly (Figure 1C). These findings suggest that application of 20, 50, or 200 nM ET-1 caused complete inhibition of $I_{\text{TASK}}$ in rat cardiomyocytes and that the amplitude of $I_{\text{TASK}}$ at $+30 \text{ mV}$ was $\approx 0.30 \text{ pA/pF}$ at pH 8.0.

**Figure 3** Effects of 20 nM ET-1 on action potential duration. (A) Typical action potentials recorded at a stimulation frequency of 4 Hz with a sampling rate of 5 kHz; black trace: control; blue trace: after application of ET-1. (B) The statistical evaluation of the relative changes in APD measured 8 min after application of ET-1 under control conditions (blue bars) and after pre-incubation with 200 nM A1899 (orange bars). Under control conditions, both APD$_{50}$ and APD$_{90}$ were significantly prolonged after application of ET-1 ($P < 0.01$). In the presence of A1899, ET-1 did not produce any measurable change in APD$_{50}$ and a small but statistically significant ($P < 0.05$) increase in APD$_{90}$. Comparison of the change in APD observed after application of 20 nM ET-1 with and without pre-incubation with A1899 gave a significant difference using Student’s $t$-test ($P < 0.01$ for APD$_{50}$; $P < 0.05$ for APD$_{90}$). (C and D) The time course of the change in APD$_{50}$ and APD$_{90}$ during application of 20 nM ET-1 under control conditions (C) and after pre-incubation with 200 nM A1899 (D). (E) The time course of the whole-cell current measured at $+30 \text{ mV}$, derived from current–voltage relationships. First, a brief pH pulse was applied (switching from pH 7.4 to 6.0 for 90 s) and then the cardiomyocytes were superfused with 200 nM A1899.
3.3 Effects of endothelin-1 on action potential duration

To assess the functional significance of the endothelin-mediated inhibition of \( I_{\text{TASK}} \), we studied the effects of ET-1 on APD in rat cardiomyocytes at a stimulation frequency of 4 Hz in the absence of any ion channel blockers. Application of 20 nM ET-1 increased APD\(_{50} \) (the time at which the action potential reaches 50% repolarization) by 17.1 ± 3.4% and APD\(_{90} \) by 16.0 ± 2.2% (Figure 3A and B). The prolongation of the action potential was preceded by a transient decrease in APD (Figure 3C); the reason for the transient shortening is not yet clear. To clarify to what extent the prolongation of the action potential was attributable to the inhibition of \( I_{\text{TASK}} \), we repeated the application of ET-1 in the presence of the TASK-1 blocker A1899 (Figure 3B and D). This drug is more specific for TASK-1 than A293 and has a higher affinity with an IC\(_{50} \) value of ~7 nM in mammalian cells. In these experiments, we first switched to an extracellular solution with a pH of 6.0 for 90 s to determine the amplitude of \( I_{\text{TASK}} \) in this particular cell and then applied 200 nM A1899. Figure 3E shows that the drug blocked approximately the same current as the transient acidification, but with a much slower time course. When a steady state was reached (after ~8 min), we switched to the current clamp mode and applied 20 nM endothelin.

We found that after pre-incubation with the TASK-1 blocker, application of ET-1 increased APD\(_{90} \) by ~7% but no longer caused any measurable change in APD\(_{50} \) (Figure 3B and D). We conclude from these findings that the increase in APD\(_{50} \) produced by application of 20 nM ET-1 was to a large extent mediated by the inhibition of \( I_{\text{TASK}} \). However, ET-1 also had some effects on other channels in rat cardiomyocytes, as indicated by the small but significant (\( P = 0.012 \)) increase in APD\(_{90} \) in the presence of A1899 (Figure 3B). The effects of ET-1 on other K\(^+ \) channels were more pronounced at higher concentrations of ET-1 (200 nM), where we observed a 30% increase in APD\(_{90} \) that was blocked only partially by pre-incubation with TASK channel blockers (not illustrated).

3.4 Analysis of the signal transduction pathway in rat cardiomyocytes

Cardiomyocytes express both ET-1 receptor subtypes, \( \text{ET}_{\text{A}} \) and endothelin-B (\( \text{ET}_{\text{B}} \)). To analyse the relative contributions of these receptors, we pre-incubated the cardiomyocytes for 5 min with the specific \( \text{ET}_{\text{A}} \) antagonist BQ-123 (1 \( \mu \)M) and/or with the specific \( \text{ET}_{\text{B}} \) antagonist BQ-788 (1 \( \mu \)M) and measured the effects of 200 nM ET-1. In the absence of the \( \text{ET}_{\text{A}} \) antagonist, application of ET-1 blocked only a minor fraction of \( I_{\text{TASK}} \) (Figure 4A). In the presence of the \( \text{ET}_{\text{B}} \) antagonist, application of ET-1 blocked an outward current of ~0.23 pA/pF, which corresponds to ~77% of the current measured under control conditions (Figure 4A). After pre-incubation with both antagonists, application of 200 nM ET-1 produced no measurable current change (Figure 4A). The endothelin receptor antagonists alone had no significant effect. These data suggest that the major effect of ET-1 was mediated by \( \text{ET}_{\text{A}} \) receptors and that \( \text{ET}_{\text{B}} \) receptors made a small but significant contribution to the overall effect of ET-1.

Since previous studies had implicated a role of PKC in the inhibition of \( I_{\text{TASK}} \), we tested the effects of two inhibitors of PKC, bisindolylmaleimide (BIM), which inhibits conventional and novel PKCs with the same potency, and staurosporine, an unspecific inhibitor of PKC and other kinases. We pre-incubated the cardiomyocytes with 1 \( \mu \)M BIM for at least 5 min and then applied 200 nM ET-1. We found that pre-incubation with BIM did not significantly alter the difference current observed after application of ET-1 (Figure 4B). Similarly, pre-incubation (≥5 min) with 1 \( \mu \)M staurosporine had no effect on \( I_{\text{TASK}} \) current inhibition by ET-1 (Figure 4B). Application of BIM or staurosporine alone had no effect on the outward current measured at positive potentials. These findings suggest that in rat cardiomyocytes PKCs are not involved in the inhibition of \( I_{\text{TASK}} \) via G\(_{q}\)-coupled receptors.

Next, we tested the effect of the PLC inhibitor U73122 on the signal transduction between the endothelin receptor and \( I_{\text{TASK}} \) (Figure 5A). In these experiments, we first switched from pH 8.0 to pH 6.0 for 2 min to quantify \( I_{\text{TASK}} \) and then incubated the cardiomyocytes with 5 \( \mu \)M U73122 for 3 min before applying ET-1 (200 nM). Under these conditions, application of 200 nM ET-1 (on average)
inhibited only ~28% of the residual $I_{\text{TASK}}$ (estimated as the difference between the current measured at pH 6.0 and the current measured immediately before application of ET-1; Figure 5A, red arrow). This differs significantly ($P > 0.001$) from the effect of ET-1 observed under control conditions in the same series of experiments (Figure 5C). Since prolonged application of U73122 produces unwanted side effects,\(^25\) we did not attempt to block PLC completely by increasing drug concentration or the duration of pre-incubation.

We carried out further control experiments with U73343, a widely used inactive analogue of U73122. Surprisingly, we found that U73343 caused a decrease in steady-state outward current in cardiomyocytes at positive potentials (Figure 5B). This was most likely due to a direct blocking effect of U73343 on $I_{\text{TASK}}$ (see Supplementary material online, Figure S3A). However, the residual TASK-1 current remaining after application of U73343 was inhibited by ET-1 to the same extent as under control conditions (Figure 5C).

### 3.5 Analysis of the signal transduction pathway in an expression system

We next examined the inhibition of TASK-1 currents by the endothelin receptor in a heterologous expression system. For these experiments, the TASK-1$^{\text{ETAR}}$ mutant ($N\Omega$-TASK-1) was co-expressed with ETAR receptors in CHO cells. $N\Omega$-TASK-1 has an enhanced surface expression and thus yields larger currents.\(^21\) Transfected cells displayed characteristic TASK-mediated steady-state currents (Figure 6A–C) with a mean amplitude of $331 \pm 53$ pA at $+50$ mV ($n = 36$).

When ET-1 (200 nM) was applied to the cells, the currents rapidly decreased to $25 \pm 7\%$ of control amplitude (Figure 6A and D). This inhibition was largely irreversible when ET-1 was washed out. The small residual currents were predominantly intrinsic background currents of the CHO cells as indicated by amplitudes comparable with non-transfected cells (not shown), by a linear $I$–$V$ relationship, and by reversal potentials close to 0 mV. Thus, $N\Omega$-TASK-1 currents were essentially fully inhibited by the activation of ETAR receptors. Similar results were obtained with wild-type TASK-1 currents (see Supplementary material online, Figure S3B). Pre-incubation with the PLC inhibitor, U73122 (5 $\mu$M; 3 min), immediately before application of ET-1 abolished the endothelin-induced inhibition of TASK-1 currents (Figure 6D–F); the current measured after application of endothelin was $108 \pm 13\%$ of the current prior to endothelin application. Pre-application of the inactive analogue, U73343, produced a partial and reversible block of TASK-1 currents (Figure 6C and D, see Supplementary material online, Figure S3A), which may be attributable to a direct effect on the channel. However, U73343 had no effect on ET-1-mediated inhibition of residual TASK currents (Figure 6C–F).

For comparison, we also examined the inhibition of a bona fide PLC-regulated $K^+$ channel, Kv7.2 (KCNQ2),\(^26\) via the activation of the co-expressed ETAR receptor (see Supplementary material online, Figure S4). As in the case of TASK-1, application of ET-1 rapidly and completely inhibited the Kv7.2 currents, and this inhibition was abolished by pre-incubation with U73122 (5 $\mu$M; 3 min) but not by the inactive analogue, U73343. In contrast to $I_{\text{TASK}}$ (see Supplementary material online, Figure S3A), the current carried by Kv7.2 channels was not significantly inhibited by pre-incubation with U73343 (see Supplementary material online, Figure S4).

The ability of the ETAR receptor to activate PLC-mediated signalling pathways was further examined by using a fluorescent probe for PLC activity, PH$_{\text{PLC}}$-GFP (Figure 6G–I). PH$_{\text{PLC}}$-GFP specifically binds to the substrate of PLC, PI(4,5)P$_2$, such that the degree of binding of the probe to the plasma membrane is a direct measure of the concentration of PI(4,5)P$_2$.\(^27\) We measured the membrane association of this probe using TIRF microscopy\(^13,27\) (see Supplementary material online, Figure S4).
Figure 6 U73122 disrupts ET<sub>α</sub>-receptor-mediated inhibition of I<sub>TASK</sub> in an expression system. (A–C) TASK-1 currents measured during voltage ramps from −100 to +50 mV in CHO cells co-transfected with NQTASK-1 and ET<sub>α</sub> receptor. In each panel, current traces shown were obtained (a) 1 min after whole-cell formation; (b) after subsequent application of either standard extracellular solution (A), 5 μM U73122 (B) or 5 μM U73343 (C) for 3 min and (c) after additional application of 200 nM ET-1 for 1 min. (D) Averaged time courses of TASK-1 currents, measured as illustrated in (A–C). Light grey shading indicates application of control solution, U73122, or U73343, respectively; dark grey indicates application of ET-1. The time points corresponding to individual current traces shown in (A)–(C) are indicated (a, b and c). Current amplitudes were measured at +50 mV and were normalized to the amplitude immediately before application of ET-1 to account for current fluctuation during baseline measurements and for partial and reversible block of TASK-1 by U73343 (see Supplementary material online, Figure S3). (E) The fraction of whole-cell current (I/I<sub>0</sub>) remaining after 1 min of ET-1 treatment from the set of experiments shown in (D). (F) Endothelin-sensitive current after pre-treatment with U73122 or U73343 relative to the endothelin-inhibited current in control cells. (G) TIRF measurements of relative fluorescence changes in CHO cells co-transfected with the ET<sub>α</sub> receptor and PH<sub>PLCδ1</sub>−GFP, indicating membrane association of PH<sub>PLCδ1</sub>−GFP. (H) The mean remaining PH<sub>PLCδ1</sub>−GFP membrane fluorescence after application of ET-1. (I) The mean change in PH<sub>PLCδ1</sub>−GFP membrane fluorescence after application of ET-1. In (D)–(I) data from >16 cells from three independent experiments were averaged for each experimental condition.
online, Figure S5). In cells co-expressing the ET\textsubscript{A} receptor and PH\textsubscript{PLC\_GFP}, application of ET-1 triggered a robust and largely irreversible translocation of the probe from the membrane to the cytoplasm (Figure 6G–I), indicating the depletion of PI(4,5)P\textsubscript{2} and thus receptor-induced activation of PLC. Pre-incubation with U73122 (5 \textmu M; 3 min) prior to the administration of ET-1 fully blocked the translocation of PH\textsubscript{PLC\_GFP}, whereas U73343 was without effect (Figure 6G–I). These findings confirm the activation of PLC by the ET\textsubscript{A} receptor and the inhibition of PLC activity by U73122 at the concentration used and support the idea that the activation of PLC plays an essential role in the receptor-mediated inhibition of TASK channels.

Since a recent report suggested that \textit{I}\textsubscript{TASK} in pulmonary artery smooth muscle cells might be inhibited by ET-1 via a rho kinase-dependent pathway,\textsuperscript{28} we studied the effect of the rho kinase inhibitor Y27632 on the inhibition of heterologously expressed TASK-1 currents via the ET\textsubscript{A} receptor. We found that pre-incubation with Y27632 (5 or 10 \textmu M; 20 min; 37°C) had no influence on the inhibition of TASK-1 currents via co-expressed ET\textsubscript{A} receptors: ET-1 reduced the current to 14.4 \pm 7.0% of its initial amplitude in the presence of 5 \textmu M Y27632 (n = 5) and to 9.8 \pm 4.7% in the presence of 10 \textmu M Y27632 (n = 5; see Supplementary material online, Figure S6A).

We also tested the effects of the rho kinase inhibitor on Gq-coupled signal transduction in rat cardiomyocytes. We first applied a transient pH pulse to pH 6.0 and then measured the fraction of the pH sensitive current inhibited by ET-1 with and without pre-application of Y27632 (5 \textmu M; 30 min; 22°C). We found that the inhibition of \textit{I}\textsubscript{TASK} by ET-1 was not affected by pre-incubation with Y27632 (see Supplementary material online, Figure S6B). These results argue against an involvement of rho kinase in endothelin receptor-mediated inhibition of TASK-1 current.

4. Discussion

The inhibition of TASK-1 and TASK-3 channels via Gq-coupled receptors has been investigated extensively,\textsuperscript{7 – 13,28 – 30} both in native cells and in heterologous expression systems, but the underlying molecular mechanisms are still unclear. We have studied the signal transduction between endothelin receptors and TASK-1 channels in rat cardiomyocytes. We optimized the signal to noise ratio by measuring the inhibition of \textit{I}\textsubscript{TASK} at pH 8.0, where the channels are fully activated, and by blocking and/or inactivating most of the other channels that might be open in the potential range \(-40 \text{ to } +30\text{ mV}\). We monitored the change in steady-state outward current produced by (i) extracellular acidification to pH 6.0, (ii) block of \textit{I}\textsubscript{TASK} with A293, or (iii) application of ET-1. We found that all three interventions caused a reduction in outward current by \(-0.30\text{ pA/pF at } +30\text{ mV}\). These observations suggest that ET-1 can completely inhibit \textit{I}\textsubscript{TASK}. Inhibition of a steady-state outward current in rat cardiomyocytes by ET-1 has been observed previously\textsuperscript{31} using a somewhat different approach without any pharmacological tools.

At a stimulation frequency of 4 Hz, which is near the physiological heart rate at rest (7 – 8 Hz), application of 20 nM ET-1 caused a significant increase in both APD\textsubscript{DS} and APD\textsubscript{D0} (\(-17\%\)). After pre-incubation of the cardiomyocytes with the TASK-1 blocker A1899, ET-1 produced no significant change in APD\textsubscript{DS} and only a small (\(-7\%\)) increase in APD\textsubscript{D0}. These findings support the idea that the increase in APD\textsubscript{D0} elicited by ET-1 was mainly due to the inhibition of \textit{I}\textsubscript{TASK}. The small, persistent increase in APD\textsubscript{DS} observed after pre-incubation with A1899 suggests that other cardiac potassium channels may also be inhibited by ET-1. The prolongation of the action potential may contribute to the pro-arrhythmic effect of ET-1.\textsuperscript{18,19}

The concentration dependence of the effects of ET-1 on \textit{I}\textsubscript{TASK} indicates that the IC\textsubscript{50} was between 5 and 10 nM. In trying to identify the receptors responsible for the inhibition of \textit{I}\textsubscript{TASK}, we used specific ET\textsubscript{A} and ET\textsubscript{B} antagonists. In the presence of the ET\textsubscript{A} antagonist BQ-123, the current change produced by ET-1 was reduced to \(-30\%\) of that observed under control conditions; in the presence of the ET\textsubscript{B} antagonist BQ-788 the current change was reduced to \(-77\%\) of control. In the presence of both antagonists, the response to ET-1 was abolished. These findings suggest that the effects of ET-1 on \textit{I}\textsubscript{TASK} were mainly mediated by ET\textsubscript{A} receptors and are consistent with earlier studies indicating that in rat cardiomyocytes ET\textsubscript{A} receptors are more highly expressed than ET\textsubscript{B} receptors.\textsuperscript{17}

The major aim of our study was to determine whether PLC was involved in the signal transduction pathway. For this purpose, we employed the PLC inhibitor U73122, which has been used previously to assess the signal transduction pathway of G\textsubscript{q}-mediated inhibition of TASK-1 in neurons,\textsuperscript{29,30} and in heterologous expression systems.\textsuperscript{29,30} In neurons, U73122 failed to modulate the inhibition of a TASK-like current,\textsuperscript{29} whereas in Xenopus oocytes\textsuperscript{29} and in COS-7 cells\textsuperscript{30} cells U73122 reduced the inhibition via Gq-coupled receptors. In the two latter studies, the downstream intracellular messengers liberated by PLC (inositol trisphosphate, high intracellular Ca\textsuperscript{2+}, and diacylglycerol) were found to have no detectable effect on \textit{I}\textsubscript{TASK}, and it was speculated that TASK-1 current was inhibited via the depletion of PI(4,5)P\textsubscript{2}.

Since a recent study\textsuperscript{11} in a heterologous expression system reported that the inhibition of \textit{I}\textsubscript{TASK} via Gq-coupled receptors may be mediated by direct interaction of G\textsubscript{q\_subunits with the channel, and another recent study\textsuperscript{13} showed that the inhibition of \textit{I}\textsubscript{TASK} is not caused by the depletion of PI(4,5)P\textsubscript{2}, we initially considered it unlikely that PLC was involved in the inhibition of \textit{I}\textsubscript{TASK} in cardiomyocytes. Nevertheless, we found that after pre-incubation with the PLC inhibitor U73122 the effect of ET-1 on \textit{I}\textsubscript{TASK} was substantially reduced: only \(-28\%\) of the residual \textit{I}\textsubscript{TASK} could be inhibited by 200 nM ET-1 (Figure 5C).

To test whether under these conditions PI(4,5)P\textsubscript{2} hydrolysis by PLC was indeed inhibited by U73122, we reconstituted the signal transduction pathway in CHO cells. We found that the inhibition of TASK-1 current via ET\textsubscript{A} receptors was almost completely abolished after exposing the cells to 5 \textmu M U73122 for 3 min, and our TIRF microscopy measurements indicated that PI(4,5)P\textsubscript{2} hydrolysis was also abolished under these conditions. In contrast, normal inhibition of the residual TASK-1 current by ET-1 and normal PI(4,5)P\textsubscript{2} hydrolysis was observed after pre-incubation with the inactive analogue U73343.

To further confirm the efficacy of U73122 in inhibiting PLC-mediated signalling, we used the voltage-activated potassium channel Kv7.2 (KC\textsubscript{NQ2}) as a biosensor: PI(4,5)P\textsubscript{2}, the substrate of PLC, is required for Kv7 channel activity, and the activation of PLC via Gq-coupled receptors inhibits Kv7.2 by the depletion of PI(4,5)P\textsubscript{2}.\textsuperscript{36} We co-transfected ET\textsubscript{A} receptors and Kv7.2 channels in CHO cells and superfused the cells with ET-1. Application of ET-1 rapidly and completely inhibited Kv7.2 channels. Pre-incubation with U73122 (5 \textmu M) prevented endothelin-induced inhibition. In contrast, the inactive analogue U73343 had no effect on ET\textsubscript{A}-mediated inhibition of Kv7.2. Taken together, our TASK-1 measurements in CHO cells, our TIRF microscopy data, and our control experiments using
Kv7.2 channels demonstrate an efficient activation of PLC-mediated signalling under the same experimental conditions as used for studying TASK-1 regulation and confirm an efficient inhibition of PLC activity by U73122 in our experimental setting. On the basis of the results obtained in cardiomyocytes and our control experiments with the heterologous expression system, we conclude that the activation of PLC is the major upstream mechanism leading to the inhibition of $I_{\text{TASK}}$.

However, the downstream mechanisms leading to the inhibition of $I_{\text{TASK}}$ following the activation of PLC remain unclear. It has been proposed that in isolated cardiomyocytes and pulmonary artery smooth muscle cells inhibition of $I_{\text{TASK}}$ via Gq-coupled receptors is mediated by PKC.10,24 which acts as a downstream effector of PLC. In our experiments, application of BIM or staurosporine had no effect on the inhibition of $I_{\text{TASK}}$ by ET-1. Thus, we consider it unlikely that the inhibition of TASK-1 in rat cardiomyocytes is mediated by PKC. This conclusion contradicts some previous studies, but is in agreement with others.29,30 Another possibility is that the inhibition of $I_{\text{TASK}}$ via Gq-coupled receptors may be mediated by the depletion of P(4,5)P2. However, in a previous study we have clearly shown that this is not the case. Furthermore, our experiments also show that rho kinase, another proposed effector of endothelin receptors, is not involved in the inhibition of TASK-1 in cardiomyocytes and in CHO cells. Perhaps a metabolite of diacylglycerol is involved in the effects of ET-1 on $I_{\text{TASK}}$.

In conclusion, we have shown that ET-1 inhibits $I_{\text{TASK}}$ in rat cardiac muscle mainly via ETA receptors and subsequent activation of PLC, but the downstream mechanisms leading to the inhibition of $I_{\text{TASK}}$ via Gq-coupled receptors remain unclear. The inhibition of $I_{\text{TASK}}$ leads to the prolongation of the action potential. This may contribute to the pro-arrhythmic effect of ET-1.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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