Platelet-activating factor stimulates sodium-hydrogen exchange in ventricular myocytes

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Ajiro Y, Saegusa N, Giles WR, Stafforini DM, Spitzer KW. Platelet-activating factor stimulates sodium-hydrogen exchange in ventricular myocytes. Am J Physiol Heart Circ Physiol 301: H2395–H2401, 2011. First published September 23, 2011; doi:10.1152/ajpheart.00607.2011.—Sodium-hydrogen exchanger (NHE), the principal sarcolemmal acid extruder in ventricular myocytes, is stimulated by a variety of autocrine/paracrine factors and contributes to myocardial injury and arrhythmias during ischemia-reperfusion. Platelet-activating factor (PAF; 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent proinflammatory phospholipid that is released in the heart in response to oxidative stress and promotes myocardial ischemia-reperfusion injury. PAF stimulates NHE in neutrophils and platelets, but its effect on cardiac NHE (NHE1) is unresolved. We utilized quiescent guinea pig ventricular myocytes bathed in bicarbonate-free solutions and epifluorescence to measure intracellular pH (pHi). Methylcarbamyl-PAF (C-PAF; 200 nM), a metabolically stable analog of PAF, significantly increased steady-state pH. The alkalosis was completely blocked by the NHE inhibitor, cariporide, and by sodium-free bathing solutions, indicating it was mediated by NHE activation. C-PAF also significantly increased the rate of acid extrusion induced by intracellular acidosis. The ability of C-PAF to increase steady-state pH, was completely blocked by the PAF receptor inhibitor WEB 2086 (10 μM), indicating the PAF receptor is required. A MEK inhibitor (PD98059; 25 μM) also completely blocked the rise in pH, induced by C-PAF, suggesting participation of the MAP kinase signaling cascade downstream of the PAF receptor. Inhibition of PKC with GF109203X (1 μM) and chelerythrine (2 μM) did not significantly affect the alkalosis induced by C-PAF. In summary, these results provide evidence that PAF stimulates cardiac NHE1, the effect occurs via the PAF receptor, and signal relay requires participation of the MAP kinase cascade.

guinea pig cardiomyocytes; intracellular pH; ischemia reperfusion injury

INTRACELLULAR pH (pHi) is closely regulated in ventricular myocytes by a well-defined system of sarcolemmal acid extruder and loader proteins (56). Sodium-hydrogen exchanger (NHE1) is the most important acid extruder, activated principally by intracellular acidosis. Numerous extracellular stimuli including neurohumoral mediators and growth factors can also stimulate NHE1 activity by binding to G protein-coupled membrane receptors (5, 12). In addition to its important role in mediating acid extrusion, NHE1 activation contributes to injury and arrhythmias during ischemia-reperfusion (I/R) by promoting calcium overload via the sodium-calcium exchanger (21).

Platelet-activating factor (PAF; 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a proinflammatory phospholipid with a number of physiological and pathophysiological actions (40, 50). PAF and structurally related phospholipids are released by a variety of cell types including neutrophils, mast cells, platelets, endothelial cells, and cardiac myocytes in response to various stimuli including oxidative stress (32, 49, 50). In the heart, synthesis and release of PAF is normally very low but can be increased markedly after I/R (33). This occurs mainly through the remodeling pathway in which hydrolysis of membrane phospholipids by phospholipase A2 is followed by acetylation by an acyltransferase (45) to yield PAF (32, 50). The actions of PAF and PAF-like molecules generated from oxidative fragmentation of membrane phospholipids (27) are mediated through a G protein-coupled receptor, which is linked to a variety of intracellular signaling pathways (18, 19). PAF and PAF-like phospholipids are hydrolyzed by PAF acetylhydrolases (PAF-AHs). The plasma form of PAF-AH is also known as lipoprotein-associated phospholipase A2 and PLA2G7 (49).

The cardiac actions of PAF are diverse and include induction of arrhythmias in canine ventricular myocytes (17) and negative inotropic effects on rat ventricular myocytes (29, 39). At the whole heart level, considerable evidence indicates that PAF promotes myocardial I/R injury (32, 52). However, it has recently been proposed that PAF can have cardioprotective effects at very low concentrations (37).

Given the importance of PAF as a G protein-linked signaling molecule and its multiple actions on the heart, we sought to determine whether signaling through this pathway modulates NHE1 activity since this is a well-characterized, key contributor to myocardial injury and arrhythmia during I/R. PAF has been shown to stimulate NHE in neutrophils and platelets (16, 43). However, to our knowledge no studies have addressed whether PAF-mediated signaling regulates cardiac NHE activity. In this study we present evidence that PAF stimulates NHE1 in ventricular myocytes from adult hearts and provide mechanistic evidence suggesting that the MAP kinase signaling axis participates in this response.

MATERIALS AND METHODS

Myocyte isolation. The experiments were performed using adult ventricular myocytes isolated from healthy guinea pigs by enzymatic digestion, as described (61). All procedures involving animals were approved by the Animal Care and Use Committee of the University of Utah and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, guinea pigs were anesthetized with sodium pentobarbital (50 mg/kg ip), and then the excised heart was attached to an aortic cannula and perfused with solutions gassed with 100% O2 and held at 37°C, pH 7.2. Perfusion with a Ca2+-free solution for 5 min was followed by 14 min of

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perfusion with the same solution containing 0.1 mg/ml collagenase P (Roche Diagnostic, Manheim, Germany), 0.01 mg/ml protease (type XIV; Sigma-Aldrich, St. Louis, MO), and 0.05 mM CaCl2. The heart was then perfused for 5 min with the same solution containing no enzymes. The ventricle was isolated and minced, shaken for 10 min, and then filtered through a nylon mesh. Cells were stored at room temperature in normal HEPES-buffered solution. All cells used in this study were rectangular, had well-defined striations, and did not spontaneously contract. All experiments were conducted within 10 h of isolation.

Cell superfusion chamber. Lipid-free bathing solutions were held in glass reservoir bottles and delivered by gravity to the cell bath. Bathing solutions containing 1-O-alkyl-2-N-methylcarbamyl-sn-glycero-3-phosphocholine (C-PAF; Enzo Life Science, Plymouth Meeting, PA) or 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POV-PC; Cayman Chemical, Ann Arbor, MI) were held in plastic reservoir bottles and delivered by gravity. The temperature of the solutions in the superfusion chamber was 36 ± 0.3°C. The 1-mL Plexiglas cell bath had a clear glass bottom and was mounted on the stage of an inverted microscope (Diaphot; Nikon, Japan). Bathing solutions flowed continuously through the bath at 3 to 4 ml/min, and solution depth was held at ~1 mm. Exchange of the bath solution required ~5 s. The bottom of the bath was coated with laminin (Collaborative Research, Bedford, MA) to improve cell adhesion.

Bathing solution and drugs. Myocytes were continuously superfused with normal HEPES-buffered solution containing (in mM) 126.0 NaCl, 11.0 dextrose, 4.4 KCl, 1.0 MgCl2, 2.0 CaCl2, and 24.0 HEPES titrated to pH 7.4 with 1 M NaOH. In some experiments, NHE was blocked with either 60 μM cariporide (Sanofi-Aventis, Frankford, Germany) or by replacing external NaCl with an equimolar concentration of N-methyl-n-glucamine and adjusting the pH to 7.4 with 1.04 M HCl. Selective blockade of PAF receptors was accomplished using WEB 2086 (10 μM HEPES-buffered solution containing no added CO2 or bicarbonate. As noted above, these experimental conditions increase the likelihood of detecting changes in pHi result from the calibration curves from several myocytes to calculate pHi of the cells used in this study.

To insure that overall myocyte function was preserved during C-PAF exposure, we measured myocyte shortening during field stimulation (cycle length = 2 s). Changes in cell length were measured with an intensified charge-coupled device camera coupled to a video edge-detector device (53). Individual cells without SNARF loading were first paced in control solution until shortening stabilized (~1 min). The stimulus was shut off, and C-PAF (200 nM) was applied to the quiescent cells for 15 min at the end of which time pacing was resumed. The results were analyzed as shortening before and after C-PAF. The cells were not continuously paced during the entire 15 min of C-PAF exposure to mimic the quiescent conditions of the pH experiments.

Determination of sarcolemmal acid efflux [JH]. Intracellular acid loading was achieved using the ammonium prepulse (15 mM NH4+) technique (25, 61). As noted above, the use of HEPES-buffered cell bathing solutions ensured inactivation of NBC (25, 51). The rate of acid efflux (JH) was evaluated as the indicator of NHE activity and plotted as a function of pHi. JH9 was calculated as 9/9/9/9/9 nM H+ at "/". Changes in field stimulation (cycle length = 2 s). Changes in cell length were measured with an intensified charge-coupled device camera coupled to a video edge-detector device (53). Individual cells without SNARF loading were first paced in control solution until shortening stabilized (~1 min). The stimulus was shut off, and C-PAF (200 nM) was applied to the quiescent cells for 15 min at the end of which time pacing was resumed. The results were analyzed as shortening before and after C-PAF. The cells were not continuously paced during the entire 15 min of C-PAF exposure to mimic the quiescent conditions of the pH experiments.

RESULTS

Effect of C-PAF and POV-PC on steady-state pH. In the first series of experiments we examined the effect of C-PAF and POV-PC (PAF analog) on steady-state pH in quiescent myocytes bathed in HEPES-buffered solution containing no added CO2 or bicarbonate. As noted above, these experimental conditions increase the likelihood of detecting changes in pH attributable to NHE activity. C-PAF induced a dose-dependent rise in steady-state pH, an example of which is shown in Fig. 1Aa. By 15 min the mean increase in pH in 200 nM C-PAF was 0.15 ± 0.02 units (Fig. 1Ab). Repaplication of the control solution for 5 min following 15 min of C-PAF typically attenuated or blocked the rise in pH, but did not restore it to its control value (not shown), perhaps reflecting the low level of metabolic acid production and absence of Cl–HCO3– exchange. Recovery did occur in response to short periods (~5 min) of C-PAF exposure (not shown). Smaller increases in pH,
occurred when the concentration of C-PAF was reduced (Fig. 1A). Because 200 nM C-PAF gave a maximum pH response, this concentration was used in all subsequent experiments.

Ten minutes of superfusion with a PAF analog (200 nM), which is recognized by the PAF receptor in human macrophages (36), also elicited a significant increase in steady-state pHi of 0.08 ± 0.01 units (n = 4, paired, P < 0.01), demonstrating that bioactive phospholipids other than C-PAF can induce this effect.

There was no evidence that superfusion with either C-PAF or POV-PC degraded cell viability during the course of the experiments. The myocytes remained quiescent and rod-shaped in appearance with well-defined striations and without spontaneous contractions or the appearance of blebs. Further evidence that C-PAF did not affect cell function is the absence of any significant changes in myocyte shortening following 15 min of superfusion with 200 nM C-PAF (n = 5, paired).

Effect of cariporide and external sodium on C-PAF-induced changes in steady-state pHi. To identify the mechanism responsible for the intracellular alkalosis produced by C-PAF, we performed experiments with myocytes bathed in solutions containing either 60 μM cariporide, a highly selective NHE1 inhibitor (28), or no external sodium, to inhibit sodium-hydrogen exchange. NHE1 blockade was initiated at least 5 min before exposure to 200 nM C-PAF and continued throughout the experiment. Both interventions completely inhibited the rise in pHi, strongly suggesting it is mediated by acid extrusion via NHE1 (Fig. 2, A and B).

Effect of C-PAF on pHi recovery from intracellular acidosis. Intracellular acidosis is the major activator of NHE1, and pHi recovery from ammonia prepulses is mediated entirely by NHE1 in guinea pig ventricular myocytes bathed in HEPES-buffered solutions (25, 61). Figure 3 shows that C-PAF (200 nM) significantly increased the rate of pHi recovery from acid loading producing an upward shift in the JH versus pHi curve at all values of pHi. Thus C-PAF stimulates NHE1-mediated acid extrusion over a wide range of pHi values.

Effect of WEB 2086 on C-PAF-induced rise in steady-state pHi. In many cells and organs, PAF transduces signals through a G protein-coupled receptor, the PAF receptor (18, 19). To determine whether the effect of C-PAF on steady-state pHi required engagement of the PAF receptor, we preincubated ventricular myocytes with WEB 2086 (5 min, 10 μM), a highly specific PAF receptor antagonist (8). The cells were then exposed to C-PAF (200 nM) in the continued presence of receptor antagonist. We found that WEB 2086 completely inhibited the ability of C-PAF to raise pHi (Fig. 4), indicating that C-PAF-mediated stimulation of NHE1 requires functional PAF receptors.

Role of MAP kinase and PKC signaling in C-PAF-induced alkalosis. Previous work has shown that phosphorylation of cardiac NHE1 by various agonists involves several protein
kinases, including members of the MAP kinase signaling axis such as MEK, MAP kinase/ERK, and p90RSK, as well as PKC (5, 12, 34). To assess possible involvement of the MAP kinase pathway in the pHi response to C-PAF, we performed experiments in the presence of PD98059 (25 μM), a MEK inhibitor (11). As shown in the example record in Fig. 5A and summarized in Fig. 5B, PD98059 completely blocked the rise in pHi elicited by 200 nM C-PAF, suggesting that signals downstream from MEK, perhaps MAP kinase (ERK) and RSK, are involved.

Although alkalosis induced by 200 nM C-PAF was somewhat reduced by PKC blockade with GF109203X (1 μM) and chelerythrine (2 μM), the effect was not statistically significant (Fig. 5B), suggesting that PKC activation does not play a major role in the stimulation of NHE1 by C-PAF.

**DISCUSSION**

The present work demonstrates that C-PAF stimulates NHE1 in ventricular myocytes at both normal resting pHi and during intracellular acidosis. A classical PAF receptor mediates these effects. The downstream signaling appears to involve primarily the MAP kinase pathway with little or no contribution by PKC activation. Earlier work reported stimulation of NHE1 in neutrophils and platelets by PAF (16, 43), but to our knowledge this is the first report of PAF-induced modulation of NHE1 in myocytes from adult mammalian heart.

**Modulation of NHE1 activity by endogenous ligands.** Under normal conditions [pHi ~7.2, pHo ~7.4] the rate of acid extrusion via cardiac NHE1 is low. However, it is markedly increased by a fall in pHi (56). This stimulation is attributed to allosteric control of carrier activity by proton occupancy of the cytosolic proton sensor on the transporter domain of NHE1 (59). Transport activity is also significantly increased in ventricular myocytes by a variety of paracrine and autocrine factors, including angiotensin (14, 30), endothelin (24), thrombin (62), and phenylephrine (47). These agents act through G protein-coupled receptors to phosphorylate the cytoplasmic COOH-terminal regulatory domain of the transporter and increase the affinity of the proton sensor (5, 12, 59). Phosphorylation of NHE1 by these agents involves several protein kinases, including key members of the MAP kinase signaling axis (i.e., MAP kinase/ERK and p90RSK), as well as PKC (5, 12, 34). MAP kinase/ERK and p90RSK have been shown to directly phosphorylate myocardial NHE1 (4, 9, 34). In contrast, PKC apparently does not have this effect, even though it has a significant influence on transporter activity (13). Our data are consistent with participation of the MAP kinase signaling pathway on PAF-induced activation of NHE and suggest little, if any, role for PKC in this process.

Occupancy of PAF receptors in noncardiac cells activates numerous signal transduction pathways that vary among cell types and include members of the MAP kinase axis, PKCs, protein tyrosine kinases, and phosphatidylinositol 3-kinases (19, 50). PAF-induced signaling in cardiac cells includes PKC activation in cultured neonatal rat ventricular myocytes (29) and adult rat heart (38), p38 MAP kinase activation in H9c2 cultured cardiomyocytes (64), and phosphatidylinositol 3-kinase activation leading to nitric oxide (NO) production in adult mice atrium (1). PAF-induced NO production has also been reported in adult guinea pig papillary muscle (2). Interestingly, we have found that NO appears to inhibit NHE1 in adult rat ventricular myocytes (20).

**Fig. 3. Effect of C-PAF on the relationship between net acid efflux via NHE1 (JH) and pHi.** **C-PAF** was significantly increased by C-PAF (200 nM) at all values of pHi (**P < 0.01, paired). N values for each point range from 7 to 10 myocytes.
Our results do not provide detailed information concerning the intracellular signaling pathways responsible for stimulation of NHE1 by C-PAF. However, the ability of PD98059, a MEK inhibitor (11), to completely block the effect (Fig. 5) suggests that signals downstream from MEK, perhaps MAP kinase/ERK and p90RSK, are involved. PD98059 also inhibits the stimulation of NHE1 in rat ventricular myocytes by hydrogen peroxide (46), phenylephrine (47), and angiotensin II via the AT1 receptor (14). Our results further suggest that PKC may not be involved in the stimulation of NHE1 since two different PKC inhibitors, GF109203X (55) and chelerythrine (15), did not significantly reduce the alkalosis elicited by 200 nM C-PAF (Fig. 5). It is important to note, however, that although PD98059, GF109203X and chelerythrine, are widely used signaling pathway blockers, there are issues concerning the selectivity of each. For example, PD98059 has been shown to inhibit both MEK and cyclooxygenase 2 (6). In addition, GF109203X at concentrations higher than −3 μM has been shown to also inhibit p90RSK (42). However, our use of 1 μM GF109203X decreases the likelihood of this being an issue in the present study.

**Possible clinical implications of PAF-induced stimulation of NHE1.** Under normal conditions the synthesis and release of PAF in the heart is very low but is markedly increased following I/R (22, 23, 33). Sources of myocardial PAF production are thought to include endothelial cells, neutrophils, platelets, and myocytes (52). Although the role of PAF in myocardial I/R pathophysiology is not completely understood, numerous studies have documented its negative inotropic and arrhythmogenic actions (32, 52). PAF has also been proposed to have cardioprotective properties, but these effects are thought to occur under conditions of very slow rates of release, as occurs following brief ischemic periods (37).

Our results suggest that NHE1 can be stimulated significantly by conditions that promote the synthesis and release of myocardial PAF and PAF analogs. NHE1 is an established contributor to calcium overload and consequent myocardial damage and arrhythmogenesis during ischemia, and especially

![Fig. 4. Effect of PAF receptor inhibition on C-PAF-induced stimulation of NHE1. A: example pH signals from 2 myocytes illustrating the ability of PAF receptor blockade with WEB 2086 (10 μM) to inhibit the stimulatory effect of C-PAF (200 nM) on NHE1. B: summarized results of ΔpH_i induced by 10 min superfusion with 200 nM C-PAF under control conditions (n = 16) and in the presence of 10 μM WEB 2086 (n = 5). **P < 0.01, unpaired control vs. WEB 2086.](image)

![Fig. 5. Effects of PD98059 (MEK inhibitor), GF109203X (PKC inhibitor), and chelerythrine (PKC inhibitor) on C-PAF-induced stimulation of NHE1. A: example pH signals from 2 myocytes illustrating the ability of PD98059 (25 μM) to completely block the stimulatory effect of 200 nM C-PAF on NHE1. B: summarized results of ΔpH_i induced by 10 min superfusion with C-PAF (200 nM) under control conditions (n = 16) and in the presence of either 25 μM PD98059 (n = 6), 1 μM GF109203X (n = 4), 2 μM chelerythrine (n = 7). In contrast with PKC inhibition, MEK blockade significantly reduced the stimulatory effect of C-PAF on NHE1. **P < 0.01, unpaired control vs. PD98059.](image)
reperfusion due to its ability to raise intracellular sodium during acid extrusion and thus reduce Ca\(^{2+}\) influx and or increase influx via sodium-calcium exchanger (21, 31). Thus it seems likely that release of PAF and PAF analogs in the ischemic and surrounding myocardium may have the undesirable effect of further stimulating NHE1 and exacerbating I/R injury. Thus our results may help explain some of the known beneficial effects of PAF receptor antagonists and PAF-AH in mitigating the effects of I/R on infarct size and arrhythmias (3, 22, 26, 33, 35, 41, 44, 51).

In addition to PAF, oxidative stress also promotes production of several other oxidized phospholipids such as POV-PC (10). POV-PC is reported to bind to PAF receptors (36) and, as with C-PAF, it increased steady-state \(pH_i\) at a concentration of 200 nM, perhaps by stimulating NHE1. Taken together, our present findings combined with earlier work help clarify the role of PAF-like oxidized phospholipids in myocardial I/R injury and further define the cardioprotective mechanism of NHE blockade.

In summary, our results demonstrate for the first time that PAF promotes acid extrusion from guinea pig ventricular myocytes at both normal steady-state values of \(pH_i\) and in response to intracellular acid loading. This effect is mediated by stimulation of NHE1 and requires occupancy of the PAF receptor. Inhibition of C-PAF-induced acid extrusion by a MEK blocker suggests that signaling via the MAP kinase axis is involved. The action of PAF and PAF-analogs to stimulate NHE may help explain some of the beneficial effects of PAF receptor blockade in reducing injury to the ventricular mycardium in the setting of I/R.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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