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A Cardioprotective Role for Platelet Activating Factor Through NOS Dependent S-Nitrosylation

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Abstract

Controversy exists as to whether platelet activating factor (PAF), a potent phospholipid mediator of inflammation, can actually protect the heart from post-ischemic injury. To determine whether endogenous activation of the PAF receptor is cardioprotective we examined post-ischemic functional recovery in isolated hearts from wild type and PAF receptor knock-out mice. Post-ischemic function was reduced both in hearts with targeted deletion of the PAF receptor and in wild type hearts treated with a PAF receptor antagonist. Furthermore, perfusion with picomolar concentrations of PAF improved postischemic function in hearts from wild type mice. To elucidate the mechanism of a PAF-mediated cardioprotective effect we employed a model of intracellular Ca^{2+} overload and loss of function in non-ischemic ventricular myocytes. We found that PAF receptor activation attenuates both the time-dependent loss of shortening and increases in intracellular Ca^{2+} transients in Ca^{2+} overloaded myocytes. These protective effects of PAF depend on NO, but not activation of cyclic guanosine monophosphate. In addition, we found reversible S-nitrosylation of myocardial proteins must occur in order for PAF to moderate Ca^{2+} overload and loss of myocyte function. Thus, our data are consistent with the hypothesis that low level PAF receptor activation initiates NO-induced S-nitrosylation of Ca^{2+} handling proteins, for example L-type Ca^{2+} channels, to attenuate Ca^{2+} overload during ischemic-reperfusion in the heart. Since inhibition of the PAF protective pathway reduces myocardial postischemic function, our results raise concern that clinical therapies for inflammatory diseases that lead to complete blockade of the PAF receptor may eliminate a significant, endogenous cardioprotective pathway.

INTRODUCTION

Platelet Activating Factor (PAF) is a phospholipid best known for its ability to mediate proinflammatory effects. PAF can also induce systemic hypotension, coronary vasoconstriction, and cardiac arrhythmias (17). Furthermore, ischemic hearts exposed to nanomolar concentrations of PAF during reperfusion have decreased myocardial functional recovery and an increase in infarct size (7). Consistent with this is the observation made by some studies that PAF receptor antagonists protect the heart from ischemic-reperfusion (I/R) damage (1, 3, 7). However, one study demonstrates hearts pretreated with picomolar concentrations of PAF have reductions in I/R-induced dysfunction and infarct size (18). This

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is consistent with the observation that PAF receptor antagonists do not always reduce myocardial infarct size (4, 18). Thus, the first aim of the present study was to use PAF receptor agonists and antagonists, and hearts with a targeted deletion of the PAF receptor to definitively establish whether low levels of PAF receptor activation attenuate I/R-induced damage in the heart.

Most of PAF effects are mediated through a dedicated G-protein coupled receptor. PAF-receptor activation has been linked to increases in phosphatidylinositol (3,4,5)-triphosphate and production of NO (1, 2, 24). Previous studies have also shown increased NO production alters intracellular Ca^{2+} handling in cardiomyocytes, and NO-dependent decreases in L-type Ca^{2+} channel activity correlate to a decrease in I/R injury in hearts (6, 10, 29). These observations led us to hypothesize that a cardioprotective effect of PAF may be mediated through an NO induced reduction in intracellular Ca^{2+} overload during and after myocardial I/R. To test for a relationship between PAF and intracellular Ca^{2+} homeostasis, we employed a non-ischemic, cardiac myocyte model of calcium overload and contractile dysfunction. This allowed us to address the second aim of the present study which was to determine if PAF protects cells from contractile dysfunction through an NO-dependent decrease in intracellular Ca^{2+} overload.

NO effects can be cGMP-dependent or cGMP-independent. One of the increasingly studied alternatives to the NO-cGMP pathway is post-translational S-nitrosylation of proteins. For example, knocking out neuronal NO synthase (NOS1) reduces S-nitrosylation of the ryanodine receptor on the sarcoplasmic reticulum, which leads to diastolic Ca^{2+} leak in cardiomyocytes (9). Further, NO induced S-nitrosylation of the L-type Ca^{2+} channel correlates with gender dependent reduction in I/R injury in ventricular myocytes (29). Thus, the third aim of the present study was to investigate the role of cGMP and S-nitrosylation in PAF-dependent cardioprotection from I/R injury.

If low level release of PAF acts in a paracrine or autocrine fashion to activate PAF receptors on cardiac myocytes and initiate an endogenous protective pathway during I/R, then a better understanding of such a pathway may ultimately lead to the development of novel clinical therapies. Further, if such a pathway exists, consideration needs to be given to the possibility that current therapies used to dramatically reduce PAF in inflammatory diseases may have unintended consequences to the heart. With this in mind, the present study explores a possible protective role for PAF receptor activation in limiting myocardial I/R-induced dysfunction, looks at changes in calcium handling as one potential end-target of a PAF-mediated cardioprotection, and characterizes the relative contribution of cGMP and S-nitrosylation in PAF-dependent NO effects.

MATERIALS and METHODS

Animal procedures used were approved by the Animal Care and Use Committees at the University of Tennessee Health Science Center and St. Jude Children's Research Hospital. Male and female, 12 -16 wk old C57BL/6 wild type mice or PAF receptor knock-out mice were utilized. Generation of mice lacking the PAF receptor is extensively described in Radin et al (21). PAF receptor knock-out was confirmed using PCR (21). Male and female Wistar rats were purchased from Harlan. All animals were allowed free access to standard lab chow and water.

Pharmacologic Agents

PAF was used at a concentration of 0.1 μM since this concentration does not alter myocardial contractility (18). The PAF receptor antagonist BN52021 was used at 1 μM as a similar concentration of BN52021 blocks the PAF-induced release of atrial natriuretic factor

from rat hearts (22). Rp-8-bromoguanosine-3',5'-cyclic monophosphorothioate (Rp-8-Br-cGMPs), a cell permeable cGMP antagonist, and 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase, were used at a concentration of 50 μ M and 0.4 μ M respectively. Control experiments, see Results, were done to insure these concentrations of Rp-8-Br-cGMP and ODQ inhibit known cGMP effects in ventricular myocytes. Nitro-L-arginine (L-NNA), a competitive inhibitor of nitric oxide synthase (NOS) with selectivity for the neuronal and endothelial isoforms, was used at a concentration of 0.1 μ M. The L-NNA K_i value for nNOS is 0.09 μ M, for eNOS is 0.02 μ M, and for iNOS is >25 μ M (23). 2-(N,N-Diethylamino)-diazene-2-oxide diethylammonium (DEA/NO), an NO donor, was used at 1 nM since at this concentration it has no effect on twitch amplitude (15). DEA/NO was also used at a concentration of 100 μ M to inhibit shortening of cardiac myocytes as described by Layland *et al* (15). A stock of DEA/NO was kept in aliquots at -80°C and brought to room temperature 5 minutes before use. Sodium ascorbate, a reducing agent for S-nitrosylation, was used at 1 mM as this concentration is not toxic to cells over a 1 hour exposure time (26). Ascorbate was made immediately prior to use. Agents were purchased from Sigma Chemical or BIOMOL International.

Langendorff-Perfused Hearts

Mice were anesthetized with sodium pentobarbital and the excised heart perfused in a retrograde fashion with a modified Krebs bicarbonate buffer. Krebs buffer contained in mM: NaCl 119, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11, CaCl₂ 2.5, pyruvate 0.5, EDTA 0.5. Krebs was maintained at 37°C and pH 7.4 by bubbling with 95% O₂ / 5% CO₂. Perfusion was at a constant pressure of 80 mm Hg and constant temperature of 37°C. Hearts were immersed in a water-jacketed perfusate bath maintained at 37°C. Aortic pressure was monitored using a pressure transducer (CAPTO SP844, AD Instruments) in-line with the aortic cannula. A 0.6 mm diameter cellophane balloon was inserted into the left ventricle and connected to a pressure transducer to monitor contractile function. Balloon volume was adjusted to maintain a left ventricular diastolic pressure of 2-5 mm Hg. External pacing at 7 Hz was set at a voltage 20% greater than threshold. Systolic pressure, end diastolic pressure (EDP), temperature, $\pm$ dP/dt, and aortic pressure were constantly recorded (Power Lab, AD Instruments). Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures. Coronary effluent rate was measured every 10 minutes.

Following instrumentation, baseline measurements were obtained for 30 minutes. Hearts which were unable to maintain an LVDP greater than 90 mm Hg during this equilibration period were excluded from data analysis. After equilibration, global ischemia for 15 or 20 minutes was produced by clamping the aortic cannula and simultaneously bubbling 95% N₂-5% CO₂ into the organ bath. Pacing was discontinued during global ischemia. At the end of ischemia hearts were reperfused for 30 min. In some experiments hearts were treated with 0.1 pM PAF or 1 μ M BN 52021 during equilibration and reperfusion. PAF and BN 52021 were delivered to the perfusate immediately above the aorta at a rate of 0.02 ml/min by a syringe pump.

Detection of S-Nitrosylation

A separate set of Langendorff perfused hearts were used to detect S-nitrosylation. Wild type mouse hearts were initially perfused with 100 μ M L-NNA for 30 min, 10 μ M ODQ for 10 min, or Vehicle. Subsequently, hearts were perfused for 10 min with 0.1 pM PAF, 100 μ M sodium ascorbate, PAF + L-NNA, PAF + ODQ, PAF + ascorbate, or Vehicle. Hearts then underwent 20 minutes of ischemia followed by 5 minutes of reperfusion, and ventricles were homogenized in 1 ml of HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine). Homogenates were then centrifuged at 2000g for 10 minutes at 4°C and 250

μL of the supernatant mixed with 800 μL of HEN buffer containing freshly prepared *S*-methyl methanethiosulfonate (10% v/v in *N,N*-dimethylformamide) and SDS (25% v/v) to final concentrations of 0.1 and 2.5%, respectively. This mixture was incubated at 50 °C for 20 min. Proteins were then precipitated with 10 mls of chilled acetone at -20 °C for 15 min. After centrifuging at 2000 g for 10 minutes, the pellet was carefully separated and resuspended in 180 μL of HEN buffer containing 1% SDS. To biotinylate cysteines, 60 μL of biotin-HPDP (2.5 mg/ml in Me_2SO) and 10 μL of freshly prepared sodium ascorbate in HEN buffer was added and incubated for 1 hour. Samples were stored with an equal volume of Laemmli buffer without dithiothreitol (DTT) at -20 °C. Processing of the tissue and labeling reactions were conducted in the dark. This protocol was based on that of Jaffrey et al (11). For detection of protein biotinylation, protein concentration in stored samples was equalized to 2.5 $\mu\text{g}/\mu\text{L}$ and 20 μL of each sample was run on an 8 % polyacrylamide gel. This was followed by immunoblotting with an anti-biotin antibody (1:4000).

Isolation of Rat Ventricular Myocytes

Ventricular myocytes were isolated using a modified procedure of Liu and Hofmann (16). In brief, hearts were cannulated and perfused at 37°C with Ca^{2+} -free Ringer solutions containing 0.5 mM EDTA, for a 2 minute perfusion, followed by Ringers containing 0.001g / ml Type II Collagenase (Worthington). Collagenase perfusion continued until the initial coronary effluent drip rate doubled (about 25 minutes). Ventricles were then chopped into 2 pieces and sequentially incubated with Ca^{2+} -free Ringer containing: (1) 0.05% BSA with 0.0005 g/ml collagenase for five minutes, and (2) 0.1% BSA for five minutes. Any remaining pieces of tissue were triturated, and released cells were transferred to a Ca^{2+} -free Ringer containing 0.1% BSA for 10 minutes. Cells were then exposed to Ca^{2+} -Ringer's containing 1.25 mM CaCl_2 and 0.1% BSA, and photomicrographs obtained. Isolations containing a <50% yield of rectangular-shaped myocytes relative to total myocytes plus myoballs were not used. Ringer's solution contained in mM: NaCl 118, KCl 4.8, KH_2PO_4 2, Pyruvate 5, HEPES 25, pH 7.4, MgCl_2 3.4, glucose 7.5.

Unloaded Shortening of Ventricular Myocytes

Cells in Ringer's solution containing 1.25 mM CaCl_2 and 0.1% BSA were placed in a field stimulation chamber mounted on an inverted microscope. In the absence of electrical stimulation, non-contracting myocytes with a minimum cell length of 100 μm and a length to width ratio > 5 were selected for use. Cells were then stimulated with a voltage $1.5 \times$ threshold at 0.5 Hz. A Javelin JE7326 CCD video camera was used to collect images with the output displayed on a monitor and stored. Cell shortening was measured using a video edge detector (Crescent Electronics, Crescent, CO). Unloaded fractional shortening was determined by averaging peak change in cell length, relative to total cell length at rest, from a train of 5 contractions at the time point indicated. Fractional shortening measurements were all done at room temperature.

A PAF dose-response curve was obtained by increasing PAF concentration every 5 minutes and measuring peak fractional shortening. No more than 4 different PAF concentrations were tested / cell in order to avoid rundown in fractional shortening. These experiments were done in a Ringer's solution containing 1.25 mM CaCl_2 . Data were expressed as percent change in peak fractional shortening (PS) as compared to fractional shortening obtained in the absence of PAF.

High extracellular calcium challenge experiments were done by pretreating cells in 1.25 mM CaCl_2 Ringers with 0.1 pM PAF, 1 μM BN52021, 0.4 μM ODQ, 50 μM Rp-8-Br-cGMPs, 0.1 μM L-NNA, 1 nM DEA/NONOate, 5 mM sodium ascorbate, 0.1% ethanol (Vehicle), or combination of the above. The pretreatment period was for five minutes with pacing, and

cells demonstrating greater than 10% variability of PS over this period were discarded. After a 5 minute exposure, baseline PS was recorded for use in the normalization of data. Bathing Ca^{2+} was then increased to 6 mM CaCl_2 for 10 minutes with pacing and PS recorded only at the 1st, 5th, and 10th minute. At the end of 10 minutes of 6 mM CaCl_2 , continuous pacing was resumed and the bath extracellular Ca^{2+} was reduced to 2.1 mM CaCl_2 without a change in the concentration of the pharmacologic agent(s). Cell PS was monitored over the subsequent 20 minutes. Data are presented as percent change in peak fractional shortening (PS) as compared to fractional shortening obtained immediately prior to exposure to high Ca^{2+} . It should be noted after PAF's protection was established using PAF and the PAF receptor antagonist BN52021, cells from isolations that did not demonstrate a robust protective effect of PAF were not used (3 out of a total of 24 isolations).

Measurement of Intracellular Ca^{2+}

Myocytes were loaded with 5 μM fura-2/AM (Invitrogen, Carlsbad, CA) in a Ringers solution for 15 min at room temperature. Following loading, cells were washed 3 times in Ringer's with 1.25 mM CaCl_2 and 0.1% BSA, and allowed to sit for at least 20 minutes to insure deesterification. Cells were then stored on ice to lessen compartmentalization.

Fura loaded cells were incubated for 5 min with 0.1 μM L-NNA, 0.4 μM ODQ, 5 mM ascorbate or Vehicle prior to being placed in a low volume chamber on the stage of an inverted microscope and paced. Fluorescence was recorded at 510 nm following excitation from light passed through a 340 or 380 nm filter using a dual-excitation fluorescence photomultiplier system (IonOptix, Milton, MA). Fluorescence ratios were compared to give relative calcium changes between groups (described below). All measurements were conducted at room temperature. Fluorescent measurements were obtained prior to and after PAF or Vehicle exposure, and after a high Ca^{2+} challenge. To do this the Ca^{2+} transients of cells paced at 0.5 Hz in a 1.25 mM Ca^{2+} containing solution were recorded. Pacing was then stopped and 0.1 pM PAF or vehicle was added to the bath for a 5 minute period of equilibration. Pacing was resumed and Ca^{2+} transients were again recorded. Cells were then challenged by an immediate increase in extracellular Ca^{2+} to 4 mM.

To insure consistency of response over the period of data collection, the first and last cell of the day were exposed to 20 mM caffeine. The peak amplitude of the Ca^{2+} transient induced by caffeine changed by less than 10% from first to last cell in all isolations. Further, fluorescence adjacent to cells was monitored and found to be consistent and minimal between isolations over the entire period of data collection, i.e. none of the isolations had cells with a higher than normal release of Fura into the bathing solution. Finally, neither the addition of caffeine or high extracellular Ca^{2+} altered the extracellular bathing media fluorescence.

Statistical Analysis

All values are presented as mean $\pm$ standard error. Statistical significance was assumed for $p < 0.05$. Data were analyzed by a standard analysis of variance (ANOVA) followed by a Student t-test.

RESULTS

PAF Effects on Isolated Heart Post-Ischemic Recovery

Isolated hearts from wild type mice were equilibrated with 0.1 pM PAF or Vehicle for 30 minutes, and measurements of LVDP and EDP obtained (Figure 1). PAF had no effect on pre-ischemic LVDP or EDP. Following the 30 minute equilibration period, hearts underwent 20 minutes of global ischemia and 30 minutes of reperfusion. Post-ischemic LVDP was

significantly greater in PAF treated compared to Vehicle treated hearts, $74.6 \pm 4.0\%$ and $46.1 \pm 6.2\%$ respectively (Figure 1A). Consistent with improved post-ischemic LVDP, EDP was lower in PAF treated compared to Vehicle treated hearts (Figure 1B).

Hearts from wild type mice were equilibrated with $1 \mu\text{M}$ BN52021, a PAF receptor antagonist, or Vehicle for 30 minutes, and measurements of LVDP and EDP obtained (Figure 2). BN52021 had no effect on pre-ischemic LVDP or EDP. Following the 30 minute equilibration, hearts underwent 15 minutes of global ischemia and 30 minutes of reperfusion. Post-ischemic LVDP was significantly reduced in BN52021 treated compared to Vehicle treated hearts, $42.0 \pm 4.7\%$ and $71.0 \pm 4.6\%$ respectively (Figure 2A). Consistent with impaired post-ischemic LVDP, EDP was significantly higher in BN52021 treated compared to Vehicle treated hearts (Figure 2B).

Functional recovery from global ischemia was evaluated in hearts from PAF receptor knock-out mice (Figure 3). Deletion of the PAF receptor did not significantly alter pre-ischemic LVDP. Pre-ischemic LVDP in hearts from wild type mice was $127.3 \pm 2.5 \text{ mm Hg}$ (n of 8) and in PAF receptor knock-out hearts was $133.3 \pm 10.4 \text{ mm Hg}$ (n of 8). However, following 20 minutes of global ischemia and 30 minutes of reperfusion LVDP was significantly reduced and end-diastolic pressure significantly higher in PAF receptor knock-out hearts as compared to hearts from wild type mice (Figure 3). Further, post-ischemic TnI content in the myocardial effluent was significantly greater from PAF receptor knock-out hearts as compared to hearts from wild type mice (Figure 3C). Prior to ischemia TnI released into the myocardial effluent was not different, $10.1 \pm 1.9 \text{ ng TnI / gram heart}$ (n of 6) for wild type and $6.8 \pm 1.3 \text{ ng TnI / gram heart}$ (n of 7) for PAF receptor knock-out hearts.

PAF Effects on Ventricular Myocyte Shortening

Isolated ventricular myocytes were exposed to increasing concentrations of PAF and the peak shortening determined. Concentrations of $0.01 - 1 \text{ pM}$ PAF had no effect on peak shortening (Figure 4A). Exposure to concentration 10 pM PAF lead to a progressive decrease in peak shortening. To minimize the potentially confounding influence of this negative inotropic effect on data interpretation, all subsequent experiments were done using 0.1 pM PAF.

Isolated ventricular myocytes were subjected to Ca^{2+} overload by exposing cells to 6 mM extracellular Ca^{2+} for 10 minutes followed by a return to a physiologic Ca^{2+} (Figure 4B). This was done to mimic the rise in intracellular Ca^{2+} that occurs during and after I/R. Cells were pretreated with Vehicle, PAF and /or the PAF receptor antagonist BN52021. During the 10 minutes of high extracellular Ca^{2+} exposure the functional response of the cells was highly variable and did not reach a statistically significant difference between groups. At 20 minutes post-calcium challenge the Vehicle treated cells had a $64.3 \pm 4.1\%$ decrease in peak shortening. PAF reduced myocyte dysfunction with a loss in peak shortening of only $14.6 \pm 2.4\%$ at 20 minutes. Co-exposure to PAF and the PAF receptor antagonist BN52021 blocked the PAF protective effect with a $77.4 \pm 6.5\%$ decrease in peak shortening at 20 minutes post-calcium challenge. In the absence of PAF, BN52021 treated cells had a significantly greater loss in peak shortening as compared to Vehicle, $83.4 \pm 10.6\%$ vs $64.3 \pm 4.1\%$ decrease in peak shortening at 20 minutes post-calcium challenge respectively.

NO, but not cGMP, Involvement in PAF Dependent Protection

The protective effect of PAF was blocked by the addition of L-NNA, a competitive arginine inhibitor of NOS (Figure 5A). PAF treated cells had a decrease in peak shortening of $19.4 \pm 4.5\%$ while L-NNA and L-NNA plus PAF treated cells had a loss in peak shortening of 82.0

$\pm 4.6\%$ and $76.3 \pm 5.7\%$ respectively. DEA/NO, an NO donor, mimicked the protective effect of PAF to Ca^{2+} overload (Figure 5B). Exposure of cells to 1 nM DEA/NO prior to Ca^{2+} overload led to a $43.1 \pm 4.0\%$ decrease in peak shortening as compared to Vehicle treated cells which lost $81.4 \pm 4.9\%$ in peak shortening at 20 minutes post-calcium challenge. Previous studies have demonstrated that 1 nM DEA/NO is below the concentration of the negative inotropic effect of DEA/NO (15).

The PAF and NO protective effects from Ca^{2+} overload appear to be independent of cGMP. The DEA/NO protective effect was not blocked by pretreatment with ODQ, an inhibitor of soluble guanylyl cyclase (Figure 5B). The PAF-induced protection against loss of peak shortening following a Ca^{2+} challenge was also not effected by ODQ (Figure 5C). Decay in peak shortening in cells treated with PAF or PAF plus ODQ was not statistically different, $23.3 \pm 3.0\%$ and $13.5 \pm 7.3\%$ respectively. Finally, the PAF-induced protection against loss of peak shortening was not effected by Rp-8-Br-cGMPs, a cell permeable cGMP antagonist (Figure 5D). Decay in peak shortening in cells treated with PAF or PAF plus Rp-8-Br-cGMPs was not statistically different, $15.0 \pm 3.7\%$ and $2.0 \pm 4.2\%$ respectively.

Control experiments were done to insure ODQ and Rp-8-Br-cGMPs were inhibiting cGMP (as in Figure 5). Previous studies have demonstrated that cGMP activation accounts for the negative inotropic effect of 100 μM DEA/NO (15). As such, we first confirmed 100 μM DEA/NO reduces shortening of ventricular myocytes bathed in a Ringers solution containing 1.25 mM CaCl_2 . In this non-calcium overloaded control, DEA/NO treatment alone caused an immediate decrease in peak shortening by $69.5 \pm 11.1\%$ (n of 6) as compared to Vehicle treated myocytes (n of 4). This effect was abolished by a 4 min pretreatment with 0.4 μM ODQ such that ODQ plus DEA/NO treated cells had only a $3.7 \pm 6.9\%$ decrease in peak shortening (n of 6). A similar ability to block the negative inotropic effect of DEA/NO using 50 μM Rp-8-Br-cGMPs was seen in 3 cells (data not shown).

NO Dependent S-Nitrosylation as a Mechanism of PAF Protection

NO-dependent S-nitrosylation of proteins was examined as a possible mechanism of PAF induced protection. To do this hearts from wild type mice were perfused with PAF, L-NNA, ODQ, and/or the S-nitrosylating reducing agent sodium ascorbate. All hearts then underwent 20 minutes of ischemia followed by 5 minutes of reperfusion. To monitor the S-nitrosylation of cysteine moieties on proteins, nitrosylated groups were converted to stable biotinylations (see Methods). A protein with a molecular mass of ~ 220 kD, consistent with the mass of the α -1 subunit of the L-type Ca^{2+} channel, was biotinylated /nitrosylated by PAF (Figure 6A). L-NNA, an inhibitor of NOS, reduced PAF-dependent biotinylation/nitrosylation of the 220 kD protein (Figure 6A). ODQ, an inhibitor of guanylyl cyclase, did not reduce PAF-dependent biotinylation/nitrosylation of the 220 kD protein (Figure 6A). Sodium ascorbate blocked PAF dependent biotinylation/nitrosylation of the 220 kD protein (Figure 6A). Further, non-ischemic ventricular myocytes pretreated with ascorbate followed by PAF exposure demonstrated a loss in PAF-induced protection in the high extracellular Ca^{2+} challenge model (Figure 6B). Ascorbate plus PAF treatment led to an $84.7 \pm 4.1\%$ decrease in peak shortening as compared PAF treatment alone which caused a $26.6 \pm 5.0\%$ decrease in peak shortening at 20 minutes post-calcium challenge.

Attenuation of the Ca^{2+} Transient as a Mechanism of PAF Dependent Protection

A PAF-dependent improvement in intracellular Ca^{2+} handling was examined as a possible mechanism of PAF-induced protection. To do this, we determined basal and peak Ca^{2+} transients in rat ventricular myocytes treated with PAF or Vehicle and then challenged with an increase in extracellular Ca^{2+} from 1.25 mM to 4 mM (Figure 7). PAF reduced the rise in basal Ca^{2+} and the increase in peak Ca^{2+} transient following exposure to 4 mM CaCl_2 as

compared to Vehicle treated myocytes (Figure 7). Inhibition of NOS, using L-NNA, blocked the ability of PAF to attenuate Ca^{2+} overload during exposure to a high extracellular Ca^{2+} (Figure 7). However, inhibition of guanylyl cyclase, using ODQ, did not block the ability of PAF to reduce Ca^{2+} overload (Figure 8). Further, de-nitrosylation using ascorbate blocked the ability of PAF to reduce Ca^{2+} overload (Figure 8). These data are consistent with PAF-dependent attenuation of Ca^{2+} overload being mediated by a cGMP independent, NO-induced nitrosylation of a Ca^{2+} handling protein(s).

DISCUSSION

The present study supports the hypothesis that low level activation of the PAF receptor reduces injury to the heart due to ischemia-reperfusion. This effect appears to occur through an NO-dependent decrease in intracellular Ca^{2+} overload in ventricular myocytes. Further, our studies indicate the PAF-NO protection from Ca^{2+} overload induced damage is mediated by an increase in S-nitrosylation of protein(s) responsible for Ca^{2+} homeostasis.

Various cell types within the heart release PAF during myocardial I/R (27). Prolonged ischemia and the resulting high concentrations of PAF are thought to promote post-ischemic injury through a direct negative inotropic effect of PAF on the heart, and PAF-induced coronary vasoconstriction as well as arrhythmogenic effects (27). Consistent with the idea that PAF is harmful to the heart, PAF receptor antagonists have been shown to reduce myocardial postischemic contractile dysfunction and infarct size (1, 3, 7, 27). However, not all studies demonstrate PAF receptor antagonists reduce myocardial infarct size (4, 18). Further, Penna *et al* (18) showed that pre-ischemic treatment with 20 pM PAF protects the heart from reperfusion damage, and PAF released during brief cycles of I/R improves post-ischemic recovery. This suggests concentrations of PAF in the pM range, such as seen with brief periods of ischemia, exercise, or at the onset of inflammation, may initiate cellular protective pathways. In the present study, we confirmed that pM concentrations of PAF improve myocardial post-ischemic function. We went on to demonstrate that PAF receptor antagonists given prior to a brief period of ischemia increase myocardial post-ischemic dysfunction. Furthermore, we showed that targeted deletion of the PAF receptor reduces the recovery of hearts from I/R. Thus, basal to low levels of PAF receptor activation are cardioprotective. This finding suggest a cautious approach may be needed in the clinical use of PAF inhibitors and PAF receptor antagonists in inflammatory diseases (8, 13, 20, 30). Complete inhibition of PAF receptor activation may block endogenous PAF-protective mechanism(s) rendering the heart susceptible to ischemic damage.

The mechanism(s) by which low concentrations of PAF improve post-ischemic myocardial recovery have not previously been studied. However, studies by Pietsch *et al* (19) demonstrated that low concentrations of PAF decrease intracellular peak Ca^{2+} concentration in ventricular myocytes. Since decreasing Ca^{2+} overload during I/R reduces injury to the heart (14, 28), we hypothesized that PAF-dependent protection was due to attenuation of Ca^{2+} overload associated with I/R. To address this hypothesis we induced Ca^{2+} overload by exposing non-ischemic myocytes to a high extracellular Ca^{2+} concentration. The resulting acute increase in intracellular Ca^{2+} led to a rapid loss in function of ventricular myocytes. Utilizing this model we demonstrated that PAF protects from Ca^{2+} -dependent loss in function and that the PAF receptor antagonist BN52021 blocks this protection in ventricular myocytes. Consistent with these observations, PAF significantly reduced basal Ca^{2+} and peak intracellular Ca^{2+} in the calcium overload model. From these studies we concluded that cardioprotective effects of PAF are likely mediated through Ca^{2+} homeostatic mechanisms.

To further explore the basis of PAF-protection we next tested the hypothesis that PAF-dependent protection from the loss of function in Ca^{2+} -overload was due to NOS activation

in myocytes. PAF stimulation was previously shown to induce phosphorylation and activation of NOS3 in endothelial cells and ventricular myocytes (1, 24). In addition, cardiac specific NOS3 overexpression improves post-ischemia function in I/R hearts (5) while targeted deletion of NOS3 or pharmacologic inhibition of NOS3 increases the area of infarct and reduces LVDP in ischemia-reperfused hearts (12, 29). In our studies we found inhibition of NOS1 and NOS3 blocked PAF-dependent protection, and increasing NO production mimicked the PAF protection from Ca^{2+} -overload induced loss of function in myocytes. Further, inhibition of NOS blocked the PAF-dependent decrease in resting and peak intracellular Ca^{2+} levels in the Ca^{2+} overload model. We also demonstrated that cGMP was not involved in the PAF-NO dependent protection from loss of function or the PAF-induced decrease in resting Ca^{2+} . Thus, PAF is protective through NO production and a cGMP-independent decrease in intracellular Ca^{2+} overload.

One cGMP-independent pathway that can mediate NO effects is reversible S-nitrosylation of cysteines. NO has been shown to S-nitrosylate cysteines on the ryanodine receptor (9, 32) and the L-type Ca^{2+} channel in ventricular myocytes (29). S-nitrosylation of the ryanodine receptor sensitizes the cell to Ca^{2+} -induced Ca^{2+} release (32) while hyponitrosylation of the ryanodine receptor leads to sarcoplasmic reticular leak of Ca^{2+} during diastole (9). S-nitrosylation of the L-type Ca^{2+} channel has been shown to decrease I_{Ca} and the Ca^{2+} transient (29). In our studies we found PAF receptor activation consistently increased the S-nitrosylation of a protein with a molecular mass of ~220 kD. This is similar to the reported ~210 kD (apparent mass on SDS polyacrylamide gels) to 240 kD (calculated mass) of the pore forming α -1 subunit of L-type Ca^{2+} channels (25). However, the present study does not prove the S-nitrosylated protein of ~220 kD is the L-type Ca^{2+} channel, but rather that S-nitrosylation is an important PAF mechanism of action. To this end we demonstrated PAF-dependent nitrosylation of the ~220 kD protein was blocked by NOS inhibition, but not through inhibition of soluble guanyl cyclase. Brief treatments with ascorbate, known to reduce S-nitrosylated cysteines to thiols, blocked PAF-dependent nitrosylation. Furthermore, ascorbate blocked the protection afforded by PAF with respect to the Ca^{2+} -overload induced loss of function increase in resting Ca^{2+} levels in myocytes. These data are consistent with the hypothesis that PAF-dependent nitrosylation of Ca^{2+} handling proteins is cardioprotective.

Limitations of the present study should be noted. One potential limitation is that targeted deletion of the PAF receptor (Fig 3) could have unidentified consequences during embryonic development that reduce the ability of the adult heart to recover from I/R. However, hearts with the PAF receptor deletion and wild type hearts treated with a PAF receptor antagonist both had increased I/R induced dysfunction. This suggests the poor performance in these two independent models is due to the shared absence of PAF receptor responsiveness in the heart. Another concern is the comparison of data from hearts with different levels of ischemic insult. This is a concern as the pathways activated in control hearts with an 80% post-ischemic recovery (Fig 3) may not be identical to that activated in hearts with a 40% post-ischemic recovery. However, findings between these studies are consistent with the overall hypothesis that activation of the PAF receptor is cardioprotective. It should also be noted that Ca^{2+} overload is but one mechanism responsible for injury in I/R (Figs 4-7). Thus our studies can not exclude the possibility that PAF-dependent protection in I/R may also attenuate damage due to other effects such as a decrease in free radical production. Finally, the biotin switch method of determining nitrosylation and use of ascorbate to block nitrosylation has limits (Fig 6). The biotin assay is useful when nmol/mg protein levels of S-nitrosylation are present (33). Thus S-nitrosylated proteins expressed in low levels or with low levels of nitrosylation would not be identified by this technique. Further, ascorbate is a broad spectrum reducing agent. However, ascorbate efficiently

reduces S-nitrosylated groups, and no other non-toxic, cell permeable agent is currently available with a higher degree of selectivity.

The present study demonstrates that picoMolar concentrations of PAF are protective from myocardial dysfunction, and PAF-induced cardioprotection likely involves an NO-dependent nitrosylation of Ca^{2+} handling proteins, such as L-type Ca^{2+} channels, to attenuate Ca^{2+} overload during and after ischemia. Complete inhibition or antagonism of this PAF protective pathway reduces myocardial post-ischemic functional recovery. This raises concerns that clinical treatments which either reduce circulating PAF concentrations to undetectable levels or elicit complete PAF receptor antagonism, such as called for in recent human studies on inflammation and anaphylaxis (31), may unintentionally lead to the loss of a PAF-dependent endogenous protective mechanism in the heart.

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References

1. Alloatti G, Levi R, Malan D, Del Sorbo L, Bosco O, Barberis L, Marcantoni A, Bedendi I, Penna C, Azzolino O, Altruda F, Wymann M, Hirsch E, Montrucchio G. Phosphoinositide 3-kinase gamma-deficient hearts are protected from the PAF-dependent depression of cardiac contractility. *Cardiovasc Res.* 2003; 60:242–9. [PubMed: 14613853]
2. Alloatti G, Penna C, De Martino A, Montrucchio G, Camussi G. Role of nitric oxide and platelet-activating factor in cardiac alterations induced by tumor necrosis factor-alpha in the guinea-pig papillary muscle. *Cardiovasc Res.* 1999; 41:611–9. [PubMed: 10435033]
3. Auchampach JA, Pieper GM, Caverio I, Gross GJ. Effect of the platelet-activating factor antagonist RP 59227 (Tulopafant) on myocardial ischemia/reperfusion injury and neutrophil function. *Basic Res Cardiol.* 1998; 93:361–71. [PubMed: 9833148]
4. Black SC, Driscoll EM, Lucchesi BR. Inhibition of platelet-activating factor fails to limit ischemia and reperfusion-induced myocardial damage. *J Cardiovasc Pharmacol.* 1992; 20:997–1005. [PubMed: 1282605]
5. Brunner F, Maier R, Andrew P, Wölkart G, Zechner R, Mayer B. Attenuation of myocardial ischemia/reperfusion injury in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Cardiovasc Res.* 2003; 57:55–62. [PubMed: 12504814]
6. Campbell DL, Stamler JS, Strauss HC. Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and S-nitrosothiols. *J Gen Physiol.* 1996; 108:277–93. [PubMed: 8894977]
7. Chakrabarty S, Thomas P, Sheridan DJ. Contribution of platelets and platelet-activating factor (PAF) to the arrhythmogenic, haemodynamic and necrotic effects of acute myocardial ischaemia. *Eur Heart J.* 1991; 12:583–9. [PubMed: 1874258]
8. Evans L, Aarons L, Brearley C. A pharmacokinetic/pharmacodynamic model for a platelet activating factor antagonist based on data arising from Phase I studies. *J Pharm Pharmacol.* 2005; 57:183–9. [PubMed: 15720781]
9. Gonzalez DR, Beigi F, Treuer AV, Hare JM. Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in cardiomyocytes. *Proc Natl Acad Sci U S A.* 2007; 104:20612–7. [PubMed: 18077344]
10. Hare JM. Nitric oxide and excitation-contraction coupling. *J Mol Cell Cardiol.* 2003; 35:719–29. [PubMed: 12818561]
11. Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STK.* 2001; 86:PL1.

12. Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourdain D, Huang PL, Lefer DJ. Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. *Am J Physiol*. 1999; 276:H1567–73. [PubMed: 10330240]
13. Karasawa K. Clinical aspects of plasma platelet-activating factor-acetylhydrolase. *Biochim Biophys Acta*. 2006; 1761:1359–72. [PubMed: 17049457]
14. Kusuoka H, Porterfield JK, Weisman HF, Weisfeldt ML, Marban E. Pathophysiology and pathogenesis of stunned myocardium. Depressed Ca^{2+} activation of contraction as a consequence of reperfusion-induced cellular calcium overload in ferret hearts. *J Clin Invest*. 1987; 79:950–61. [PubMed: 3818956]
15. Layland J, Li JM, Shah AM. Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes. *J Physiol*. 2002; 540:457–67. [PubMed: 11956336]
16. Liu Q, Hofmann PA. Modulation of protein phosphatase 2a by adenosine A1 receptors in cardiomyocytes: role for p38 MAPK. *Am J Physiol Heart Circ Physiol*. 2003; 285:H97–103. [PubMed: 12649078]
17. Montrucchio G, Alloati G, Camussi G. Role of platelet-activating factor in cardiovascular pathophysiology. *Physiol Rev*. 2000; 80:1669–99. [PubMed: 11015622]
18. Penna C, Alloati G, Cappello S, Gattullo D, Berta G, Moggetti B, Losano G, Pagliaro P. Platelet-activating factor induces cardioprotection in isolated rat heart akin to ischemic preconditioning: role of phosphoinositide 3-kinase and protein kinase C activation. *Am J Physiol Heart Circ Physiol*. 2005; 288:H2512–20. [PubMed: 15637120]
19. Pietsch P, Hunger T, Braun M, Roediger A, Baumann G, Felix SB. Effects of platelet-activating factor on intracellular Ca^{2+} concentration and contractility in isolated cardiomyocytes. *J Cardiovasc Pharmacol*. 1998; 31:758–63. [PubMed: 9593076]
20. Poeze M, Froon AH, Ramsay G, Buurman WA, Greve JW. Decreased organ failure in patients with severe SIRS and septic shock treated with the platelet-activating factor antagonist TCV-309: a prospective, multicenter, double-blind, randomized phase II trial. TCV-309 Septic Shock Study Group. *Shock*. 2000; 14:421–8. [PubMed: 11049104]
21. Radin JN, Orihuela CJ, Murti G, Guglielmo C, Murray PJ, Tuomanen EI. beta-Arrestin 1 participates in platelet-activating factor receptor-mediated endocytosis of *Streptococcus pneumoniae*. *Infect Immun*. 2005; 73:7827–35. [PubMed: 16299272]
22. Rayner TE, Menadue MF, Oliver JR. Platelet-activating factor stimulates the release of atrial natriuretic factor from the rat heart. *J Endocrinol*. 1991; 130:281–8. [PubMed: 1833501]
23. Reif DW, McCreedy SA. N-nitro-L-arginine and N-monomethyl-L-arginine exhibit a different pattern of inactivation toward the three nitric oxide synthases. *Arch Biochem Biophys*. 1995; 320:170–6. [PubMed: 7540822]
24. Sánchez FA, Savalia NB, Durán RG, Lal BK, Boric MP, Durán WN. Functional significance of differential eNOS translocation. *Am J Physiol Heart Circ Physiol*. 2006; 291:H1058–64. [PubMed: 16679407]
25. Shistik E, Keren-Raifman T, Idelson GH, Blumenstein Y, Dascal N, Ivanina T. The N terminus of the cardiac L-type Ca^{2+} channel $\alpha_1\text{C}$ subunit. The initial segment is ubiquitous and crucial for protein kinase C modulation, but is not directly phosphorylated. *J Biol Chem*. 1999; 274:31145–9. [PubMed: 10531304]
26. Song JH, Shin SH, Wang W, Ross GM. Involvement of oxidative stress in ascorbate-induced proapoptotic death of PC12 cells. *Exp Neurol*. 2001; 169:425–37. [PubMed: 11358456]
27. Stangl V, Baumann G, Stangl K, Felix SB. Negative inotropic mediators released from the heart after myocardial ischaemia-reperfusion. *Cardiovasc Res*. 2002; 53:12–30. [PubMed: 11744010]
28. Strömer H, de Groot MC, Horn M, Faul C, Leupold A, Morgan JP, Scholz W, Neubauer S. $\text{Na}^{+}/\text{H}^{+}$ exchange inhibition with HOE642 improves postischemic recovery due to attenuation of Ca^{2+} overload and prolonged acidosis on reperfusion. *Circulation*. 2000; 101:2749–55. [PubMed: 10851214]
29. Sun J, Picht E, Ginsburg KS, Bers DM, Steenbergen C, Murphy E. Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca^{2+} channel α_1 subunit and reduced ischemia/reperfusion injury. *Circ Res*. 2006; 98:403–11. [PubMed: 16397145]

30. Taggart DP, Browne SM, Wade DT, Halligan PW. Neuroprotection during cardiac surgery: a randomised trial of a platelet activating factor antagonist. *Heart*. 2003; 89:897–900. [PubMed: 12860868]
31. Vadas P, Gold M, Perelman B, Liss GM, Lack G, Blyth T, Simons FE, Simons KJ, Cass D, Yeung J. Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. *N Engl J Med*. 2008; 358:28–35. [PubMed: 18172172]
32. Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science*. 1998; 279:234–7. [PubMed: 9422697]
33. Zhang J, Jin B, Li L, Block ER, Patel JM. Nitric oxide-induced persistent inhibition and nitrosylation of active site cysteine residues of mitochondrial cytochrome-c oxidase in lung endothelial cells. *Am J Physiol Cell Physiol*. 2005; 288:C840–9. [PubMed: 15561762]

Figure 1.

Effect of 0.1 pM PAF on isolated heart left ventricular developed pressure (LVDP; A) and end-diastolic pressure (EDP; B) prior to and after 20 minutes of global ischemia. LVDP is expressed relative to the preischemic baseline. Data are an average $\pm$ SE of 8 hearts / group.

*Denotes $p < 0.05$.

Figure 2.

Effect of 1 μ M BN52021, a PAF receptor antagonist, on isolated heart left ventricular developed pressure (LVDP; A) and end-diastolic pressure (EDP; B) prior to and after 15 minutes of global ischemia. LVDP is expressed relative to the preischemic baseline. Data are an average $\pm$ SE of 8 hearts / group. *Denotes $p < 0.05$.

Figure 3.

Effect of deletion of the PAF receptor on isolated heart left ventricular developed pressure (LVDP; A), end-diastolic pressure (EDP; B), and post-ischemic cardiac effluent TnI content (C). Following equilibration, hearts from PAF receptor knock-out and wild type mice underwent 20 minutes of global ischemia and 30 minutes of reperfusion. Functional data are the average $\pm$ SE of 8 hearts / group while post-ischemic TnI values were obtained from 6-7 hearts / group. *Denotes $p < 0.05$.

Figure 4.

Ventricular myocyte shortening as a function of extracellular PAF concentration (A), or following Ca²⁺ overload in myocytes treated with a PAF receptor agonist and/or antagonist (B). A. For a given PAF concentration the change in peak shortening was normalized to peak shortening in the absence of PAF. Data are the average $\pm$ SE from 4-5 cells. †Denotes $p < 0.05$ as compared to shortening in the absence of PAF. B. Ventricular myocytes were pretreated with vehicle, 0.1 pM PAF, 1 μ M BN52021 (PAF receptor antagonist), or a combination of PAF and BN52021. Myocyte shortening was continually monitored during a 10 min exposure to Ringer's solution containing 6 mM Ca²⁺ (High Calcium; 0-10 min) and upon return to Ringer's containing 2 mM Ca²⁺ (10-30 min). Peak shortening at a given time

was normalized to the peak shortening immediately prior to the high Ca^{2+} exposure (time 0) for each cell. Data are expressed as the average $\pm$ SE. *Denotes $p < 0.05$ as compared to Vehicle. Significant differences were also observed in the 15-30 minute range between BN52021 treated and Vehicle.

Figure 5.

Effect of various inhibitors on PAF and NO donor-induced protection from Ca^{2+} overload. A. Effect of pretreatment with Vehicle, 0.1 pM PAF, and/or 0.1 μM L-NNA (inhibitor of NOS1 and NOS3) on peak shortening in Ca^{2+} challenged ventricular myocytes. B. Effect of pretreatment with Vehicle, 1 nM DEA (NO donor), and/or 0.4 μM ODQ (inhibitor of soluble guanyl cyclase) on peak shortening in Ca^{2+} challenged ventricular myocytes. C. Effect of pretreatment with Vehicle, 0.1 pM PAF, and/or 0.4 μM ODQ on peak shortening in Ca^{2+} challenged ventricular myocytes. D. Effect of pretreatment with Vehicle, 0.1 pM PAF, and/or 50 μM Rp-cGMP (cGMP antagonist) on peak shortening in Ca^{2+} challenged ventricular myocytes. Shortening was monitored during a 10 min exposure to a Ringer's solution containing 6 mM Ca^{2+} (High Calcium; 0-10 min) and after return to Ringer's containing 2 mM Ca^{2+} (10-30 min). Peak shortening at a given time was normalized to the peak shortening immediately prior to the high Ca^{2+} exposure (time 0) for each cell and data are expressed as the average $\pm$ SE. *Denotes $p < 0.05$ as compared to Vehicle.

Figure 6.

Demonstration of protein nitrosylation in I/R hearts pretreated with PAF and inhibitors to purported mediators of PAF (A), and effect of ascorbate-induced denitrosylation on PAF protection in myocytes (B). A. To determine the extent of protein nitrosylation, isolated mouse hearts were perfused with 0.1 pM PAF, 100 μ M L-NNA, 10 μ M ODQ, 100 μ M sodium ascorbate, a combination of these agents, or untreated (Control), and underwent 20 minutes of global ischemia and 5 minutes of reperfusion. Proteins from heart homogenates were then processed to convert nitrosylated cysteines into biotinylated cysteines (see Methods). A biotin containing/nitrosylated protein of approximate molecular mass of 220 kD is shown. Numbers in the treatment group label denote unique hearts. For all groups

similar results were obtained in 3 or more hearts. B. To determine the effect of de-nitrosylation on the protective effect of PAF, ventricular myocytes were pretreated with vehicle, 0.1 pM PAF, and/or 5 mM ascorbate. Peak shortening was continually monitored during a 10 min exposure to a Ringer solution containing 6 mM Ca^{2+} (High Calcium; 0-10 min) and upon return to Ringer's containing 2.1 mM Ca^{2+} (10-30 min). Peak shortening at a given time was normalized to the peak shortening immediately prior to the high Ca^{2+} exposure (time of 0) for each cell and data are expressed as the average $\pm$ SE. *Denotes $p < 0.05$ as compared to Vehicle.

Figure 7.

Effect of high extracellular Ca^{2+} on intracellular Ca^{2+} in adult rat ventricular myocytes pretreated with Vehicle, 0.1 pM PAF, and/or 0.1 μM L-NNA. Shown are representative Fura ratio tracings (A) and cumulative data of baseline and peak amplitude intracellular Ca^{2+} (B). Extracellular Ca^{2+} was increased from 1.25 to 4 mM (arrow). Values obtained in high extracellular Ca^{2+} solution were normalized to the Fura ratio of the cell bathed in the lower Ca^{2+} Ringer's solution and data are expressed as the average $\pm$ SE. *Denotes $p < 0.05$ as compared to Vehicle.

Figure 8.

Effect of high extracellular Ca^{2+} on intracellular free Ca^{2+} in adult rat ventricular myocytes pretreated with 0.4 μM ODQ, ODQ+0.1 pM PAF, 5 mM Ascorbate, or Ascorbate+PAF. Shown are representative Fura ratio tracings (A) and cumulative data of baseline and peak amplitude intracellular Ca^{2+} (B). Extracellular Ca^{2+} was increased from 1.25 to 4 mM (arrow). Values obtained in high extracellular Ca^{2+} solution were normalized to the Fura ratio of the cell bathed in the lower Ca^{2+} Ringer's solution and data are expressed as the average $\pm$ SE.