British Journal of Pharmacology (2004) 142, 797–807
www.nature.com/bjp

Redox modulation of basal and β-adrenergically stimulated cardiac L-type Ca^{2+} channel activity by phenylarsine oxide

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1 Phenylarsine oxide (PAO) is commonly used to inhibit tyrosine phosphatase activity. However, PAO can affect a variety of different processes because of its ability to promote sulfhydryl oxidation. In the present study, we investigated the effects that PAO has on basal and β-adrenergically stimulated L-type Ca^{2+} channel activity in isolated cardiac myocytes.

2 Extracellular application of PAO transiently stimulated the basal L-type Ca^{2+} channel activity, whereas it irreversibly inhibited protein kinase A (PKA)-dependent regulation of channel activity by isoproterenol, forskolin and 8-CPT-cAMP (8-*p*-chlorophenylthioadenosine 3′,5′-cyclic monophosphate). PAO also inhibited channel activity irreversibly stimulated in the presence of adenosine 5′-(3-thiotriphosphate) tetralithium salt.

3 Neither the stimulatory nor the inhibitory effects of PAO were affected by the tyrosine kinase inhibitor lavendustin A, suggesting that tyrosine phosphorylation is not involved.

4 Extracellular application of the sulfhydryl-reducing agent dithiothreitol (DTT) antagonized both the stimulatory and inhibitory effects of PAO. Yet, following intracellular dialysis with DTT, only the inhibitory effect of PAO was antagonized.

5 The inhibitory effect of PAO was mimicked by intracellular, but not extracellular application of the membrane impermeant thiol oxidant 5,5′-dithio-*bis*(2-nitrobenzoic acid).

6 These results suggest that the stimulatory effect of PAO results from oxidation of sulfhydryl residues at an extracelluar site and the inhibitory effect is due to redox regulation of an intracellular site that affects the response of the channel to PKA-dependent phosphorylation. It is concluded that the redox state of the cell may play a critical role in modulating β-adrenergic responsiveness of the L-type Ca^{2+} channel in cardiac myocytes.
British Journal of Pharmacology (2004) **142,** 797–807. doi:10.1038/sj.bjp.0705845

Keywords: Redox modulation; β-adrenergic; cardiac; L-type Ca^{2+} channel

Abbreviations: ATPγS, adenosine 5′-(3-thiotriphosphate) tetralithium salt; 8-CPT-cAMP, 8-*p*-chlorophenylthioadenosine 3′,5′-cyclic monophosphate; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); DTT, dithiothreitol; Iso, isoproterenol; PAO, phenylarsine oxide; PKA, protein kinase A

Introduction

A variety of cardiac ion channels are regulated by the signaling cascade initiated by the activation of β_1-adrenergic receptors (β_1-ARs) (Hartzell, 1988). It has been established that this type of regulation involves the production of cAMP and subsequent phosphorylation by protein kinase A (PKA). Tyrosine kinases have also been reported to modulate the activity of cardiac ion channels (Shuba & McDonald, 1997; Zhou *et al*., 1997; Wang & Lipsius, 1998; Hool *et al*., 1998; Maier *et al*., 1999; Mason *et al*., 2002; Tiran *et al*., 2003). We previously demonstrated that genistein, a tyrosine kinase inhibitor, can increase the sensitivity of cardiac ion channels to β_1-AR stimulation, suggesting that basal tyrosine kinase activity inhibits β-adrenergic responsiveness in cardiac myocytes (Hool *et al*., 1998). Consistent with this idea, we found that vanadate derivatives that inhibit tyrosine phosphatase activity can also inhibit β-adrenergic responses, including β-adrenergic stimulation of the L-type Ca^{2+} current ($I_{Ca\text{-}L}$) (Sims *et al*., 2000; Belevych *et al*., 2001). Vanadate can act as a phosphate analog, binding to and inhibiting phosphoryl transfer enzymes like tyrosine phosphatases (Shaver *et al*., 1995). Phenylarsine oxide (PAO) is an arsenic-based compound that inhibits tyrosine phosphatase activity by modifying cysteine residues at the active site of the enzyme (Garcia-Morales *et al*., 1990; Hecht & Zick, 1992). A membrane-permeable sulfhydryl oxidant, PAO, preferentially reacts with vicinal cysteine residues to form stable ring structures (Webb, 1966). Furthermore, PAO has been reported to stimulate the $I_{Ca\text{-}L}$ in noncardiac preparations (Pafford *et al*., 1995; Wijetunge *et al*., 1998). However, in addition to inhibiting tyrosine phosphatase activity, PAO also inhibits NADPH oxidase and PI4 kinase activities, as well as unrelated processes involved in glucose transport, receptor internalization, and mitochondrial function (Knutson *et al*., 1983; Yang *et al*., 1992; Le & Maridonneau-Parini, 1995; Wiedemann *et al*., 1998; Korge *et al*., 2001). Hence, PAO is a potent thiol oxidant that is capable of altering the function of many different proteins. Therefore, it is conceivable that PAO might affect L-type Ca^{2+} channel function by redox mechanisms that do not involve the inhibition of tyrosine phosphatase

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Advance online publication: 1 June 2004

activity. In fact, oxidizing and reducing agents have been reported to have varied effects on the basal L-type Ca^{2+} current in cardiac myocytes (Chiamvimonvat *et al*., 1995; Lacampagne *et al*., 1995; Campbell *et al*., 1996; Fearon *et al*., 1999; Yamaoka *et al*., 2000). However, the effect that changes in the redox state of the cell might have on β-adrenergic regulation of the L-type Ca^{2+} current have not been thoroughly investigated. The present study was undertaken to determine what effects PAO might have on the cardiac $I_{Ca\text{-}L}$ and its regulation by β-AR stimulation. We found that while PAO transiently stimulates the basal Ca^{2+} channel activity, it irreversibly inhibits channels stimulated by PKA-dependent phosphorylation. Although our observations are consistent with the conclusion that PAO exerts its actions through sulfhydryl oxidation, no evidence was found linking these effects to the inhibition of tyrosine phosphatase activity. Our results suggest that the redox state of the cell is another important factor affecting β-adrenergic regulation of cardiac Ca^{2+} channel function.

Methods

Cell isolation

Single ventricular myocytes were isolated from adult Hartley guinea-pigs as described previously (Hool *et al*., 1998). Experiments were performed on the day of cell isolation only. The methods used in this procedure are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Data acquisition

The L-type Ca^{2+} current was recorded using the whole-cell configuration of the patch-clamp technique (Hamill *et al*., 1981). Borosilicate glass (Corning 7052, Garner Glass, Claremont, CA, U.S.A.) microelectrodes (0.5 and 1.5 MΩ) were filled with an intracellular solution containing (in mM): 130 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 5 MgATP, 5 EGTA, 0.1 Tris-GTP and 5 HEPES (pH 7.2). When adenosine 5′-(3-thiotriphosphate) tetralithium salt (ATPγS) was used in the patch pipette, cells were dialyzed with an intracellular solution containing (in mM): 120 CsCl, 20 TEA-Cl, 5 EGTA, 5 Li_4-ATPγS, 5 $MgCl_2$, 0.1 GTP, 5 HEPES (pH 7.2). Currents recorded using an Axopatch 200 voltage-clamp amplifier were filtered at 5 kHz and sampled at 10 kHz using a Pentium-based computer with a Digidata 1200a interface and pCLAMP (v 8.1) software (Axon Instruments, Foster City, CA, U.S.A.).

Myocytes were placed in a 0.5 ml chamber on the stage of an inverted microscope and bathed in a K^+-free extracellular solution containing (in mM): 140 NaCl, 5.4 CsCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 11 glucose and 5.5 HEPES (pH 7.4) flowing at a rate of 1–2 ml min^{-1}. The time course of changes in the size of the Ca^{2+} current was monitored by measuring the absolute magnitude of the total peak inward current recorded during 100 ms voltage-clamp steps to 0 mV applied following a 50 ms prepulse to −30 mV from a holding potential of −80 mV once every 6 s. All experiments were performed at room temperature. The L-type Ca^{2+} current was isolated by blocking K^+ channels with Cs^+ and TEA^+, inactivating Na^+ channels with a voltage clamp prepulse step to −30 mV and eliminating the driving force for Cl^- currents by measuring the Ca^{2+} current close to the predicted Cl^- equilibrium potential (0 mV). Owing to variable expression of CFTR Cl^- channels in guinea-pig ventricular myocytes, a time-independent Cl^- current can be observed at prepulse and holding potentials following the activation of PKA in some experiments.

The voltage dependence of channel activation and inactivation was determined as described previously (Belevych *et al*., 2002). For these experiments, voltage-clamp protocols were repeated in the presence and absence of 100 μM $CdCl_2$, and the L-type Ca^{2+} current was defined as the Cd^{2+}-sensitive difference current. PAO-induced changes in activation and inactivation were determined after the response to the drug had reached steady state.

Chemicals

Isoproterenol (Iso), 8-*p*-chlorophenylthioadenosine 3′,5′-cyclic monophosphate (8-CPT-cAMP), ATPγS and dithiothreitol (DTT) were prepared as aqueous stock solutions and later diluted with extracellular or intracellular solutions to achieve the desired final concentration. Ascorbic acid (50 μM) was added to all Iso-containing solutions. Forskolin, PAO, lavendustin A and 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB) were prepared as stock solutions in either polyethylene glycol (MW 400), dimethyl sulfoxide or ethanol. Diluent alone never exceeded 0.1% (v/v), a concentration at which none of these solvents affected either basal or Iso-stimulated currents. DTNB was added to solutions prior to adjusting the pH. All drugs were obtained from Sigma-Aldrich, St Louis, MO, U.S.A., except where noted.

Data analysis

Parameters describing the voltage-dependent properties of the channel were obtained by fitting normalized data to Boltzman equations using a nonlinear curve fitting routine (SigmaPlot, SPSS Inc., Chicago, IL, U.S.A.).

Responses to Iso, forskolin, 8-CPT-cAMP and ATPγS were measured at steady state. Responses to PAO and DTNB were measured at time points indicated. Results are reported as the mean ± s.e. of at least three or more independent experiments. Statistical comparisons between two groups of experimental data were performed using the paired Student's two-tailed *t*-test where indicated. Statistical comparisons between multiple groups were performed using the Holm–Sidak method.

Results

PAO transiently stimulates the basal L-type Ca^{2+} channel activity

Previous studies have demonstrated that the inhibition of tyrosine phosphatase activity by vanadate derivatives has little or no effect on the basal L-type Ca^{2+} current in cardiac myocytes (Wang & Lipsius, 1998; Ogura *et al*., 1999; Sims *et al*., 2000; Belevych *et al*., 2001). On the other hand, inhibition of tyrosine phosphatase activity by PAO, an arsenic-based compound, has been reported to regulate the basal

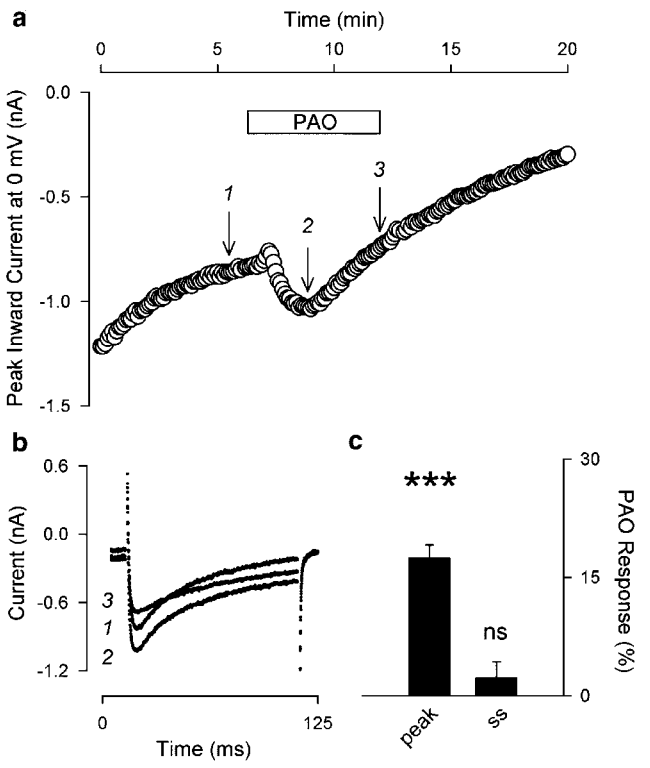

Figure 1 PAO transiently stimulates the basal L-type Ca^{2+} current. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied every 6 s, following exposure to 100 μM PAO. (b) Individual current traces recorded at time points indicated in panel a. (c) Cumulative responses normalized to the magnitude of the basal Ca^{2+} current recorded under control conditions. ***The magnitude of the current measured after 1.5–2 min of exposure to PAO ($n=30$) was significantly larger than that measured under control conditions ($P<0.001$). NS, the magnitude of the current measured following 3–5 min of exposure to PAO ($n=16$) was not significantly different than that measured under control conditions.

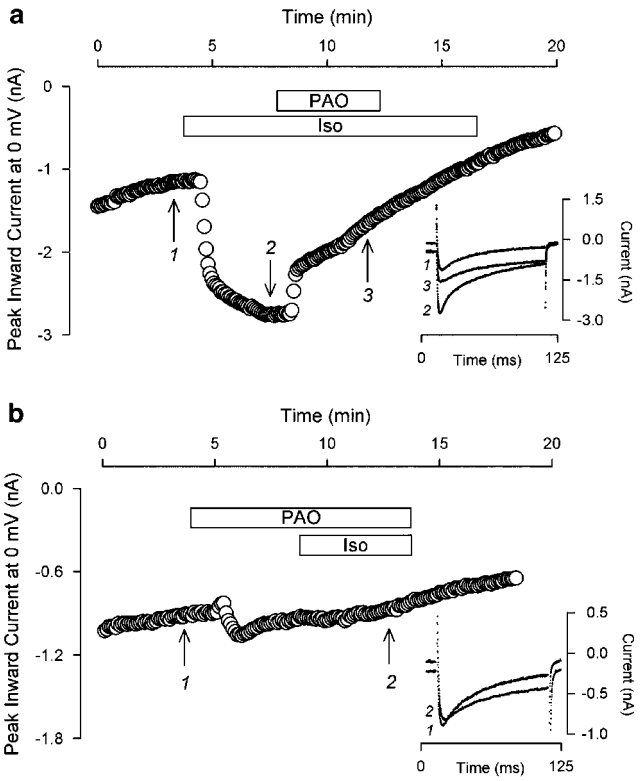

Figure 2 PAO antagonizes stimulation of the L-type Ca^{2+} current by the β-adrenergic agonist Iso. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied every 6 s, following exposure to 30 nM Iso alone and Iso plus 100 μM PAO. (b) Time course of changes in peak Ca^{2+} current following exposure to 100 μM PAO alone and PAO plus 30 nM Iso. *Insets*, individual current traces recorded at time points indicated.

L-type Ca^{2+} channel activity in vascular smooth muscle cells and neurons (Pafford *et al*., 1995; Wijetunge *et al*., 1998). To further evaluate the role of tyrosine kinase-dependent mechanisms in modulating cardiac L-type Ca^{2+} channel activity, we first examined the effect of PAO on the basal current in guinea-pig ventricular myocytes. As demonstrated in Figure 1, exposure of myocytes to 100 μM PAO alone transiently increased the Ca^{2+} current on an average of $17\pm1.7\%$ ($n=30$, $P<0.001$) over the baseline. The stimulatory effect of PAO peaked within 1.5–2 min, after which the magnitude of the Ca^{2+} current returned toward the baseline levels. No obvious steady-state effects of PAO were observed, as the magnitude of the current following 3–5 min of exposure to PAO was only $2.3\pm2.0\%$ ($n=16$) over the baseline, and washout was not associated with any obvious change in current magnitude.

PAO antagonizes β-adrenergic regulation of the Ca^{2+} current

Although our previous studies found that vanadate-based inhibitors of tyrosine phosphatase activity have no effect on the basal Ca^{2+} current in cardiac myocytes, these compounds selectively and reversibly antagonized β-adrenergic stimulation of the L-type Ca^{2+} current. Therefore, to further assess the potential role of a tyrosine kinase-dependent mechanism in modulating cAMP-dependent cardiac signaling, we characterized the effect of PAO on β-adrenergic regulation of L-type Ca^{2+} channel activity. This was accomplished by first exposing myocytes to the β-AR agonist Iso, and then adding PAO in the continued presence of Iso. The exposure of myocytes to 30 nM Iso stimulated the Ca^{2+} current by $146\pm16.0\%$ ($n=6$) over the baseline. Subsequent exposure to 100 μM PAO in the continued presence of Iso resulted in a biphasic, irreversible inhibition of the Ca^{2+} current (Figure 2a). The first phase of the response was rapid, occurring on a time scale of seconds, while the second phase was slower, occurring on a time scale of minutes. After exposure to 100 μM PAO for 5 min, the magnitude of the Iso-stimulated Ca^{2+} current decreased to only $46\pm14\%$ ($n=5$, $P<0$ 0.01) above the baseline. This suggests that PAO inhibited the magnitude of the Ca^{2+} current response to Iso by 69%. The continuation of the slow phase of inhibition of the Iso response even following PAO washout suggests that this compound permanently alters the response of the Ca^{2+} current to β-adrenergic stimulation.

It cannot be ruled out that the apparent inhibitory effect of PAO on the Iso-stimulated L-type Ca^{2+} current illustrated in Figure 2a may, in fact, be due to a direct inhibitory effect on Ca^{2+} channels that was not obvious in the absence of β-adrenergic stimulation (see Figure 1). To determine if PAO directly antagonizes β-adrenergic regulation of the L-type

Ca^{2+} current, unpaired experiments were conducted. In myocytes first treated with 100 μM PAO for 4 min, subsequent exposure to 30 nM Iso produced no obvious stimulatory response (Figure 2b). The magnitude of the current actually decreased by 13±1.4% ($n=6$) during the time these cells were exposed to Iso, a change that is consistent with current rundown (Belles *et al*., 1988). This response is significantly different from the 165±20.0% ($n=7$) increase over the baseline observed in control cells exposed to 30 nM Iso at an equivalent point in time after beginning dialysis. These results suggest that PAO completely blocks β-adrenergic stimulation of the Ca^{2+} current and that the inhibitory effects of PAO cannot be attributed to rundown of the response to Iso.

PAO alters the voltage-dependent properties of the Ca^{2+} channel

Inspection of individual current traces in both Figures 1 and 2 reveals that the rate and magnitude of L-type Ca^{2+} current inactivation is significantly altered when myocytes are exposed to PAO. This led us to speculate that changes in the voltage-dependent properties of the channel might contribute to the inhibitory effects of PAO observed in the presence of β-adrenergic stimulation. Therefore, we measured the voltage dependence of channel activation and inactivation under control conditions and following the exposure of myocytes to 100 μM PAO, 30 nM Iso and Iso in the continued presence of PAO. The results are presented in Table 1. PAO alone caused a small, but significant ($P<0.02$) hyperpolarizing shift in the voltage dependence of Ca^{2+} channel activation. Yet, PAO did not significantly affect the voltage dependence of activation when added following the exposure to Iso. PAO also had no significant effect on the voltage dependence of channel inactivation, either alone or in the presence of Iso, although it did significantly increase the fraction of the total current that was resistant to inactivation in both the presence and absence of Iso. However, none of the PAO-induced changes in channel voltage dependence can explain the inhibitory effect this compound has on the response to β-AR stimulation.

PAO regulation of Ca^{2+} channel activity and the role of tyrosine phosphorylation

If the effects of PAO on both the basal and Iso-stimulated L-type Ca^{2+} current are due to inhibition of tyrosine phosphatase activity and subsequent increase in tyrosine phosphorylation, it should be possible to block or prevent such effects by inhibiting basal tyrosine kinase activity. To test this hypothesis, we evaluated the effect of the tyrosine kinase inhibitor lavendustin A (Onoda *et al*., 1989). Previous studies from our laboratory have demonstrated that while lavendustin A does not affect the response of the Ca^{2+} current to 30 nM Iso, it can antagonize the ability of vanadate derivatives to inhibit β-AR regulation of L-type Ca^{2+} channel activity (Belevych *et al*., 2001). To determine if PAO is acting by a similar mechanism, we compared the effects that this arsenic-based compound had on the Ca^{2+} current in control and lavendustin A-treated myocytes. In myocytes that had been preincubated for 5 min with 5 μM lavendustin A, PAO still produced a transient stimulation of the Ca^{2+} current (Figure 3). The peak stimulatory effect was 13±4.3% ($n=12$) above the baseline. While the magnitude of this transient stimulatory response was smaller than that observed in control myocytes, the difference was not statistically significant ($P>0.6$). Lavendustin A also had no effect on the ability of PAO to antagonize the response of the L-type Ca^{2+} current to β-adrenergic stimulation. In the presence of 5 μM lavendustin A and 100 μM PAO, the Ca^{2+} current was still unable to respond to β-adrenergic stimulation. The magnitude of the current decreased by 16±2.4% ($n=11$) during the time these cells were exposed to 30 nM Iso, which is not significantly different ($P>0.3$) from what was observed when cells were exposed to Iso in the presence of PAO alone (see Figure 2b). These results suggest that PAO may modulate the cardiac L-type Ca^{2+} current by a mechanism that is independent of its ability to inhibit tyrosine phosphatase activity.

DTT blocks PAO-induced stimulatory effects

PAOs capacity to react with vicinal cysteine residues in general contributes to its ability to produce many effects not

Table 1 Effects of PAO on voltage dependence of $I_{Ca\text{-}L}$ activation and inactivation

	Activation				*Inactivation*			
	V_r (mV)	$V_{0.5}$ (mV)	*Slope*	n	$V_{0.5}$ (mV)	*Slope*	% I_{ir}	n
Control	57±1.7	−4.4±0.87	6.8±0.10	4	−28±1.3	−4.6±0.18	10±0.70	4
PAO	59±3.4	−8.0±0.7**	6.9±0.28	5	−26±1.0	−5.7±0.5	30±2.3**	6
Iso	55±1.0	−12±0.62	6.1±0.26	4	−31±2.0	−4.2±0.14	9±1.3	5
PAO+Iso	54±0.32	−9.3±1.6	7.3±0.25	4	−31±1.7	−6.9±0.82	30±2.5**	4

The voltage dependence of Ca^{2+} channel activation and inactivation was determined before and during exposure to 100 μM PAO, 30 nM isoproterenol (Iso) and PAO plus Iso. Parameters for the voltage dependence of activation were obtained from the least-squares fit of data points to the equation: $g/g_{max}=1/1+\exp-((V_T-V_{0.5})/b))$, where g/g_{max} represents the normalized Ca^{2+} conductance, V_T represents test potentials from −30 to 30 mV, $V_{0.5}$ is the potential at which activation was half-maximal and b is the slope. The conductance at each test potential was calculated using the reversal potential (V_r) obtained from the current–voltage relationship measured under each set of conditions. Parameters for the voltage dependence of inactivation were obtained from the equation: $I=I_{ir}+(1-I_{ir})/1+\exp-((V_c-V_{0.5})/b))$, where I is the normalized magnitude of the peak inward current measured during a test pulse to 0 mV following a 5 s conditioning pulse (V_c) to membrane potentials between −50 and 20 mV, I_{ir} is the inactivation resistant current, $V_{0.5}$ is the potential at which inactivation was half maximal and b is the slope. The current elicited during the test pulse was normalized to the magnitude of the current recorded during a pretest pulse to 0 mV, which preceded each conditioning pulse. This corrected for changes in current magnitude due to rundown (Belevych *et al*., 2002). **Significantly different when compared to the absence of PAO ($P<0.01$).

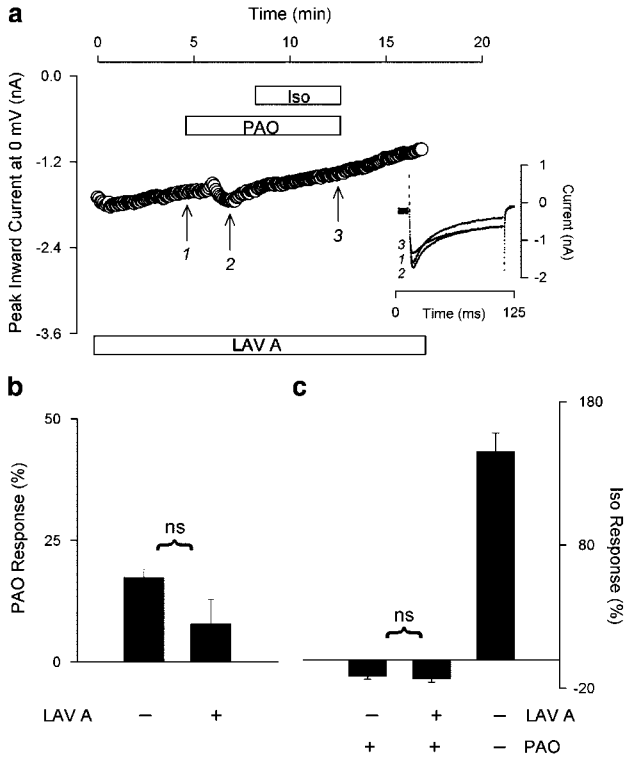

Figure 3 Lavendustin A does not alter the effect of PAO on the basal L-type Ca^{2+} current or the L-type Ca^{2+} current response to Iso. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied every 6 s, following exposure to 100 μM PAO alone and PAO plus 30 nM Iso in cells that were pre-exposed to 5 μM lavendustin A. *Inset,* individual current traces recorded at time points indicated. (b) Cumulative responses to PAO in control cells ($n=30$) and in cells pretreated with lavendustin A ($n=12$). (c) Cumulative responses to Iso alone ($n=6$) and PAO plus Iso in control cells ($n=6$) and in cells pretreated with 5 μM lavendustin A ($n=11$). NS, the magnitude of the response to PAO or Iso following exposure to PAO was not significantly different in control and lavendustin A-treated cells.

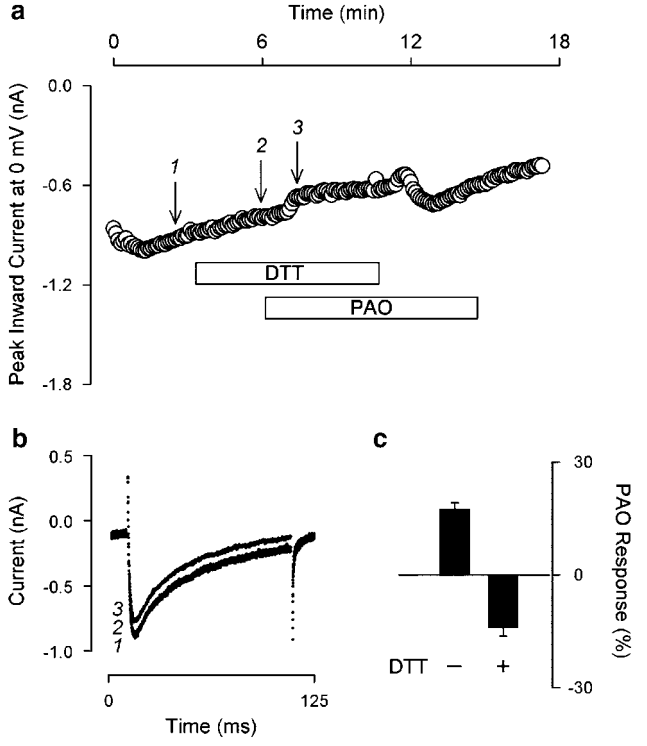

Figure 4 DTT blocks PAO-mediated transient stimulation of the basal Ca^{2+} current. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied every 6 s, following exposure to 1 mM DTT and DTT plus 100 μM PAO. (b) Individual current traces recorded at time points indicated in panel a. (c) Cumulative responses in cells exposed to 100 μM PAO in the absence ($n=30$) and presence ($n=14$) of 1 mM DTT.

specifically related to tyrosine phosphatase inhibition (Knutson *et al*., 1983; Yang *et al*., 1992; Le & Maridonneau-Parini, 1995; Wiedemann *et al*., 1998; Korge *et al*., 2001). Exposure to PAO results in the formation of stable disulfide complexes at cysteine residues of proteins (Webb, 1966). Although these complexes are not readily reversible upon washout, they can be removed by the addition of small dithiols like DTT (Zahler & Cleland, 1968; Schaefer *et al*., 1994). To determine if the stimulatory response of PAO on the basal L-type Ca^{2+} channel activity is mediated by this type of oxidative mechanism, we monitored the effect of PAO on the Ca^{2+} current in the presence and absence of DTT. The exposure of myocytes to 1 mM DTT alone had no apparent effect on the basal Ca^{2+} channel activity (Figure 4). The magnitude of the Ca^{2+} current following exposure to 1 mM DTT for 2 min decreased by $4.0\pm1.8\%$ ($n=12$) from the baseline. However, subsequent exposure to PAO in the continued presence of 1 mM DTT did not produce any obvious stimulatory effect. In fact, it appeared to cause a small, yet nonsignificant decrease in basal Ca^{2+} channel activity (Figure 4). The magnitude of the Ca^{2+} current recorded after exposure to PAO for 1.5 min, the time when PAO produced its peak stimulatory response in the absence of DTT, actually decreased by $14\pm2.4\%$ ($n=14$, $P<0.001$). However, there was a transient increase in the magnitude of the Ca^{2+} current upon washout of DTT in the continued presence of PAO. These results suggest that PAO-mediated transient stimulation of basal Ca^{2+} channel activity occurs by a mechanism involving sulfhydryl oxidation.

DTT reverses PAO-induced inhibitory effects

We next attempted to determine whether the ability of PAO to antagonize β-adrenergic stimulation of L-type Ca^{2+} channel activity is also due to cysteine modification. In these experiments, cells were exposed to 100 μM PAO for 4 min. After washing out PAO, cells were then exposed to 30 nM Iso (Figure 5). Despite the fact that PAO was no longer present, the response to Iso was completely inhibited. During a 5 min exposure to Iso, the Ca^{2+} current actually decreased by $13\pm5.2\%$ ($n=7$) compared to the magnitude of the current measured upon PAO washout. This result demonstrates that that the ability of PAO to inhibit the Ca^{2+} current in the presence of Iso (see Figure 2) was not due to oxidation of the β-adrenergic agonist itself. It also demonstrates that the inhibitory effect of PAO is not reversible upon washout. More importantly, subsequent exposure to 1 mM DTT in the continued presence of Iso produced a dramatic stimulatory response, suggesting that it had reversed the inhibitory effect of PAO. Under these conditions, the magnitude of the Ca^{2+} current increased by $82\pm23\%$ ($n=7$, $P<0.01$) following exposure to DTT. This result is consistent with the idea that PAO antagonizes the Ca^{2+} current response to Iso by a mechanism involving sulfhydryl oxidation of cysteine residues.

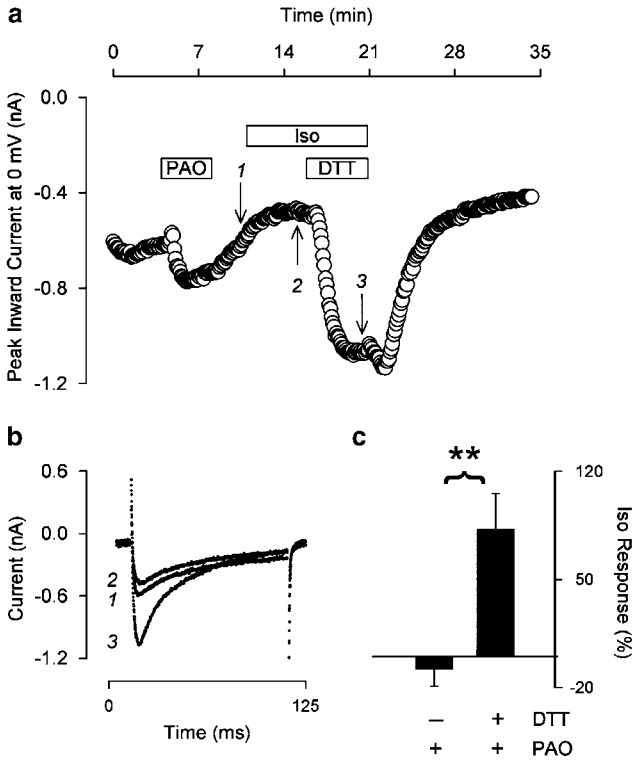

Figure 5 DTT reverses PAO-induced inhibition of the L-type Ca^{2+} current response to the β-adrenergic agonist Iso. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied once every 6 s, following exposure to 100 μM PAO alone, subsequent exposure to 30 nM Iso alone, and Iso plus 1 mM DTT. (b) Individual current traces recorded at time points indicated in panel a. (c) Effect of 100 μM PAO on the response to 30 nM Iso before and after exposure to 1 mM DTT ($n=7$). **Magnitude of the Ca^{2+} current response to Iso was significantly larger following exposure to DTT ($P<0.01$).

We also evaluated alternative explanations as to why, in PAO-treated myocytes, DTT might produce a stimulatory response in the presence of Iso. One possibility is that DTT increases myocyte sensitivity to β-AR stimulation independent of PAO-treatment. However, this does not seem to be the case, since exposure to 1 nM Iso stimulated the Ca^{2+} current by $74\pm11\%$ ($n=4$) and $59\pm5.4\%$ ($n=4$) ($P>0.1$) in the absence and presence of 1 mM DTT, respectively. Another possibility is that DTT reverses an inhibitory effect that PAO has on Ca^{2+} channel activity that is independent of β-AR stimulation. This also does not seem likely, since PAO alone does not exhibit any obvious inhibitory effects (see Figure 1). However, in cells pretreated with 100 μM PAO, subsequent exposure to 1 mM DTT alone did stimulate the Ca^{2+} current, but only by $19\pm3.6\%$ ($n=8$), which is significantly smaller than the response to DTT observed in the presence of Iso ($P<0.05$). These results support the conclusion that PAO affects the Ca^{2+} channel response to β-AR stimulation through a mechanism involving sulfhydryl oxidation and that DTT is able to reverse this effect partially.

Cytosolic cysteine residues regulate Ca^{2+} channel responses to Iso

Although our data suggest that PAO affects cardiac L-type Ca^{2+} channel function by sulfhydryl modification of cysteine residues, this compound is membrane permeable. Therefore, it is uncertain as to whether PAO is acting at residues accessible from the extracellular and/or cytosolic compartments. To confirm that the responses to PAO do involve thiol oxidation and to determine the sidedness of these effects, we conducted experiments with DTNB, a membrane-impermeable, sulfhydryl-oxidizing agent (Campbell *et al.*, 1996). First, we examined the Ca^{2+} current response to extracellular application of DTNB. Exposure to DTNB alone had no effect on the basal Ca^{2+} current. The magnitude of the Ca^{2+} current decreased by $7.5\pm3.5\%$ ($n=4$) during exposure to 200 μM DTNB for 5 min. This small change was presumably due to rundown. Extracellular application of DTNB also had no significant effect on Iso-stimulated Ca^{2+} channel activity (Figure 6a). The exposure of myocytes to 30 nM Iso stimulated the L-type Ca^{2+} current by $163\pm31.3\%$ ($n=5$) over the baseline. The addition of 200 μM DTNB decreased this to $157\pm23.9\%$ ($P>0.8$). The lack of effect upon extracellular application of DTNB on either basal or Iso-stimulated L-type Ca^{2+} channel activity suggests that this compound does not alter the channel protein by acting at cysteine residues accessible from the outside of the cell.

We next examined the effect of intracellular application of DTNB. In cells that were dialyzed with a pipette solution containing 200 μM DTNB for 10 min, subsequent exposure to 30 nM Iso stimulated the L-type Ca^{2+} current by $65\pm12\%$ ($n=10$) over the baseline (Figure 6b). This represents a 60% reduction in the magnitude of the Iso response ($P<0.01$). This result is consistent with the idea that sulfhydryl oxidation of cysteine residues accessible only from the cytosolic compartment is responsible for the inhibition of the Ca^{2+} current response to β-adrenergic stimulation.

Based on the results presented so far, it is unclear as to whether the transient stimulatory effect that PAO has on basal Ca^{2+} channel activity is due to oxidation of sulfhydryl residues accessible from the cytosolic or extracellular compartment. Extracellular application of the membrane-permeable reducing agent DTT completely blocked the transient stimulatory effect of PAO on the basal Ca^{2+} current (see Figure 4). However, neither cytosolic nor extracellular application of DTNB produced any obvious stimulatory effect on basal Ca^{2+} channel activity. To further elucidate the membrane sidedness of the effects of PAO, we dialyzed myocytes with a pipette solution containing 1 mM DTT. Even though DTT is membrane permeable, one might predict that this approach would minimize its action at extracellular sites. The presence of DTT inside the cell did not alter the transient stimulatory effect that exposure to extracellular PAO had on basal Ca^{2+} channel activity. In myocytes that were dialyzed with 1 mM DTT for 5 min, exposure to 100 μM PAO still produced a transient stimulatory effect. The peak response increased the magnitude of the L-type Ca^{2+} current by $26\pm4.4\%$ ($n=10$) over the baseline (Figure 7), which is not significantly different ($P>0.2$) from the 17% increase produced by PAO alone. This suggests that the transient stimulatory effect of PAO results from oxidation of cysteine residues accessible from the extracellular surface of the membrane.

Dialysis of myocytes with DTT allowed us to further substantiate the conclusion that oxidation of cytosolic residues affects the ability of the Ca^{2+} channel to respond to

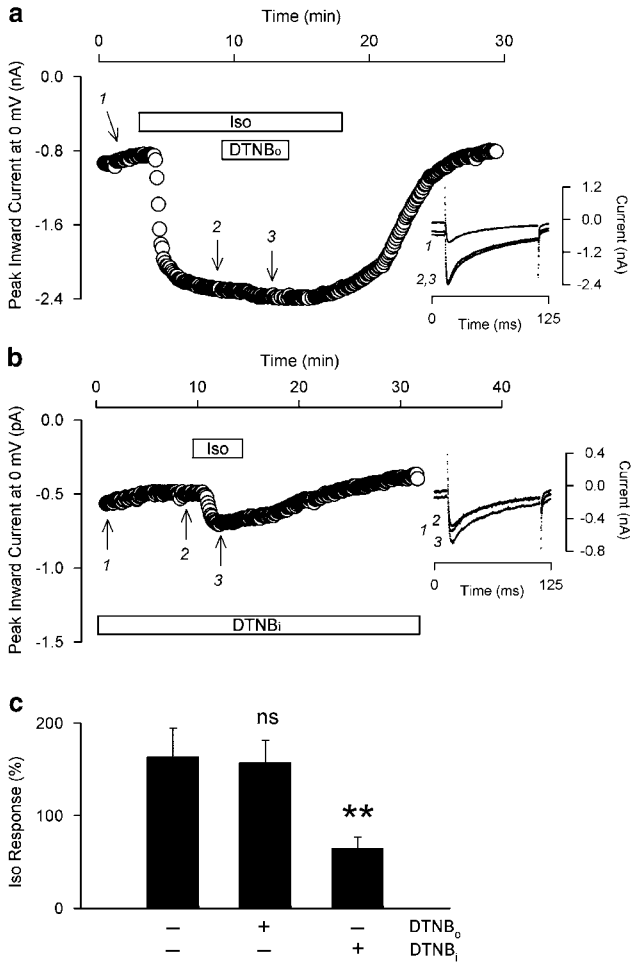

Figure 6 Intracellular but not extracellular application of DTNB attenuates the L-type Ca^{2+} current response to Iso. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied once every 6 s, following exposure to 30 nM Iso alone, Iso plus 200 μM extracellular DTNB ($DTNB_o$), and $DTNB_o$ alone. *Inset*, individual current traces recorded at time points indicated. (b) Time course of changes in peak Ca^{2+} current recorded from a myocyte dialyzed with a pipette solution containing 200 μM DTNB ($DTNB_i$) following exposure to 30 nM Iso alone. *Inset*, individual current traces recorded at time points indicated. (c) Response to 30 nM Iso before and following exposure to 200 μM $DTNB_o$ ($n=5$) and in cells dialyzed with 200 μM $DTNB_i$ ($n=10$). NS, the magnitude of the response to Iso alone or Iso following exposure to $DTNB_o$ was not significantly different. **Magnitude of the current measured in the presence of $DTNB_i$ plus Iso was significantly smaller than the magnitude of the current measured following exposure to Iso in the absence of $DTNB_i$ ($P<0.01$). Statistical comparisons were performed using the Holm–Sidak method for multiple comparisons.

β-adrenergic stimulation. As demonstrated in Figure 7, in cells dialyzed with 1 mM DTT, pre-exposure to 100 μM PAO did not completely block the Ca^{2+} current response to β-AR stimulation. Under these conditions, 30 nM Iso stimulated the Ca^{2+} current by 37±8.0% over the baseline ($n=10$). This is significantly different ($P<0.001$) from the complete lack of stimulatory response observed in the absence of DTT (see Figure 5). Taken together, these data suggest that the sulfhydryl-oxidizing properties of PAO inhibit β-adrenergically regulated channels by targeting cytosolic cysteine residues.

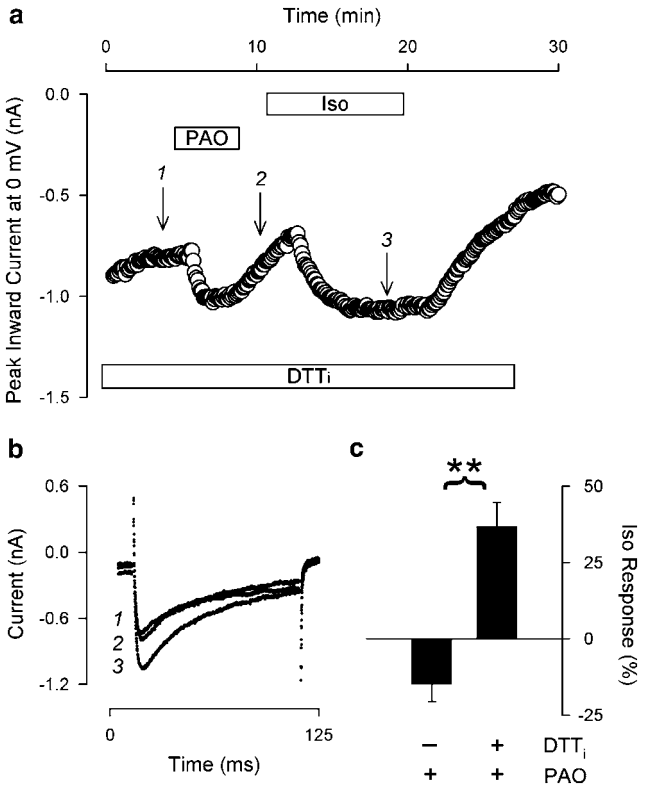

Figure 7 Intracellular dialysis of myocytes with dithiothreitol (DTT_i) attenuates the ability of PAO to block the L-type Ca^{2+} current response to Iso. (a) Time course of changes in peak Ca^{2+} current recorded from a myocyte dialyzed with a pipette solution containing 1 mM DTT following exposure to 100 μM PAO and subsequent exposure to 30 nM Iso alone. (b) Individual current traces recorded at time points indicated in panel a. (c) Effect of 100 μM PAO on the L-type Ca^{2+} current response to 30 nM Iso in control cells ($n=6$) and cells dialyzed with 1 mM DTT ($n=10$). **Ability of PAO to inhibit the response to Iso was significantly altered in cells dialyzed with DTT ($P<0.01$).

PAO inhibits the phosphorylated Ca^{2+} channel

The ability of PAO to inhibit the L-type Ca^{2+} current stimulated by Iso suggests that it exerts an effect at some point in the β-adrenergic signaling pathway. To assess where the effect of PAO is mediated, we examined its capacity to modulate the response to direct stimulation of adenylyl cyclase with forskolin. Exposure to 3 μM forskolin enhanced the L-type Ca^{2+} current 156±21.0% ($n=4$) over the baseline. Similar to the effect it had on the response to Iso, subsequent application of 100 μM PAO resulted in a biphasic, irreversible inhibition of the forskolin-stimulated Ca^{2+} current. After exposure to 100 μM PAO for 5 min, the magnitude of the forskolin-stimulated current decreased to 40±20% ($n=4$) above the baseline (see Figure 8c). This represents a 75% decrease in the magnitude of the forskolin response. These results are consistent with PAO acting either at the level of or distal to adenylyl cyclase in the signaling pathway.

We next evaluated the ability of PAO to modulate stimulation of L-type Ca^{2+} channel activity by the membrane-permeable cAMP analog 8-CPT-cAMP. Exposure to 200 μM 8-CPT-cAMP stimulated the Ca^{2+} current by 151±8.30% ($n=6$) over the baseline (Figure 8a). Subsequent

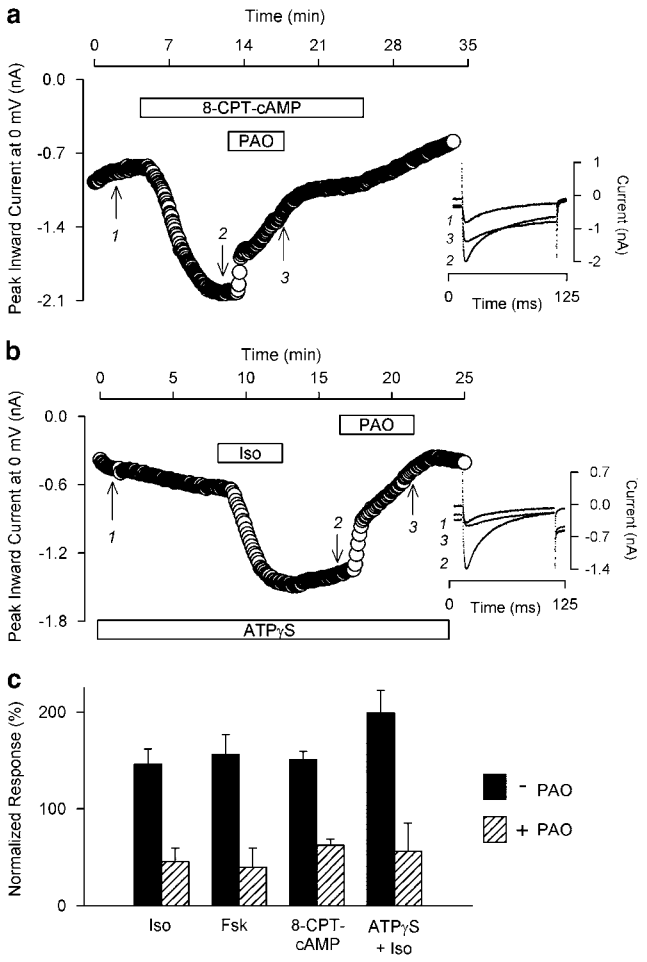

Figure 8 PAO irreversibly inhibits the L-type Ca^{2+} current stimulated by Iso, forskolin, 8-CPT-cAMP and Iso in the presence of ATPγS. (a) Time course of changes in peak Ca^{2+} current recorded during depolarizing voltage-clamp steps to 0 mV, applied once every 6 s, following exposure to 200 μM 8–CPT-cAMP alone and 8-CPT-cAMP plus 100 μM PAO. *Inset*, individual current traces recorded at time points indicated. (b) Time course of changes in peak Ca^{2+} current recorded from a myocyte dialyzed with a pipette solution containing 5 mM ATPγS following exposure to 30 nM Iso alone, washout of Iso and subsequent exposure to 100 μM PAO alone. *Inset*, individual current traces recorded at time points indicated. (c) Cumulative responses to 30 nM Iso ($n=6$), 3 μM forskolin ($n=4$), 200 μM 8-CPT-cAMP ($n=6$) and 30 nM Iso in the presence of 5 mM ATPγS ($n=4$) before and after exposure to 100 μM PAO.

application of 100 μM PAO again resulted in an irreversible, biphasic inhibition of the stimulatory response. Under these conditions, exposure to PAO for 5 min reduced the magnitude of the 8-CPT-cAMP-stimulated current to $63\pm6.2\%$ ($n=6$) above the baseline. This represents a 59% decrease in the magnitude of the 8-CPT-cAMP response. This result suggests that PAO exerts its inhibitory effect by acting distal to cAMP production in the β-adrenergic signaling pathway.

To further distinguish the point in the cAMP signaling cascade at which PAO exerts its inhibitory effect, we monitored the ability of PAO to antagonize the Ca^{2+} current stimulated in the presence of ATPγS. This compound is a slowly hydrolyzable ATP analog that can substitute for ATP in kinase reactions. In the presence of ATPγS, kinase reactions generate thiophosphorylated proteins that are resistant to phosphatase activity. Experiments were conducted by including 5 mM ATPγS in the pipette solution. After establishing the whole-cell configuration, ATPγS was allowed to dialyze into the cell for 5–7 min. This procedure resulted in a gradual increase in the Ca^{2+} current, presumably due to thiophosphorylation produced by basal PKA activity (Kameyama *et al.*, 1986). The magnitude of the Ca^{2+} current following this initial dialysis with ATPγS increased $30\pm13\%$ ($n=4$) (Figure 8b). Subsequent exposure to 30 nM Iso resulted in a further increase in current magnitude that was not reversed upon washout. The magnitude of the Ca^{2+} current measured following washout of Iso was $199\pm23.2\%$ ($n=4$) over that observed at the onset of cell dialysis with ATPγS. Subsequent exposure to 100 μM PAO resulted in the same irreversible, biphasic inhibition of the stimulated Ca^{2+} current. Following exposure to PAO for 5 min, the Iso/ATPγS-stimulated current was reduced to $56\pm29\%$ ($n=4$, $P<0.001$) over the level observed at the onset of dialysis with ATPγS. This represents a 73% decrease in the Iso/ATPγS-stimulated current. These data suggest that PAO antagonizes β-adrenergic stimulation of L-type Ca^{2+} channel activity by directly affecting the phosphorylated channel protein.

Discussion

In the present study, we demonstrate that PAO transiently stimulates the basal Ca^{2+} current and irreversibly inhibits the current enhanced by PKA-dependent phosphorylation. These effects can be blocked and/or reversed by the membrane-permeable reducing agent DTT, suggesting that the mechanism involves sulfhydryl oxidation of cysteine residues. The stimulatory effect of PAO was blocked by extracellular but not intracellular application of DTT, suggesting that modification of sulfhydryl residues accessible from the extracellular surface of the plasma membrane is involved. The inhibitory effect of PAO was antagonized by both extracellular and intracellular application of DTT. Furthermore, intracellular but not extracellular application of the membrane-impermeable thiol oxidant DTNB inhibited β-adrenergic regulation of the Ca^{2+} current, suggesting that oxidation of sulfhydryl residues accessible from the cytosolic surface of the plasma membrane are involved in mediating this response.

PAO and inhibition of tyrosine phosphatase activity

PAO has been demonstrated to affect numerous physiologically significant processes by modifying proteins through sulfhydryl oxidation. Perhaps, the best documented example is PAO's ability to inhibit protein tyrosine phosphatase activity (Garcia-Morales *et al.*, 1990; Hecht & Zick, 1992). Therefore, an important question is whether the results observed in the current study can be attributed to such an effect. Lavendustin A inhibits both receptor and nonreceptor tyrosine kinase activity. However, when used at the same concentration previously found to antagonize vanadate-mediated inhibition of ion channel responses to β-adrenergic stimulation (Belevych *et al.*, 2001), lavendustin A did not block the effects of PAO. This suggests that the responses to PAO observed in the present study do not involve the inhibition of tyrosine phosphatase activity. Although we cannot exclude the

possibility that PAO may be acting by preventing dephosphorylation of a site that is phosphorylated by a lavendustin A-insensitive kinase, this is unlikely to explain the stimulatory response produced by PAO acting at an extracellular site. This then supports the notion that PAO is capable of producing effects independent of tyrosine phosphatase inhibition in cardiac myocytes.

PAO regulation of basal Ca^{2+} channel activity

More than 40 cysteine residues span the length of the α_{1C} subunit of the guinea-pig L-type Ca^{2+} channel (Ding *et al*., 1999). This provides many potential targets for redox regulation of the channel protein, and redox modulation of the basal L-type Ca^{2+} current has been reported by several groups (Chiamvimonvat *et al*., 1995; Lacampagne *et al*., 1995; Campbell *et al*., 1996; Fearon *et al*., 1999; Yamaoka *et al*., 2000). However, there is no unifying explanation for the diversity of results that have been obtained. In studies using recombinant pore-forming α_{1C} subunits from rabbit pulmonary smooth muscle and human heart, the thiol oxidants 2,2′-dithiodipyridine, thimerosal and *p*-chloromercuribenzene sulfonic acid were found to inhibit the basal Ca^{2+} current, and these effects were reversed by DTT (Chiamvimonvat *et al*., 1995; Fearon *et al*., 1999). Although these results differ from what we observed, this could be attributable to the presence of auxiliary channel subunits in native cardiac myocytes, which may modify and/or prevent redox responses. In an investigation of the native L-type Ca^{2+} channel in guinea-pig ventricular myocytes, another sulfhydryl oxidant, *p*-hydroxymercuric-phenylsulfonic acid (PHMPS), also inhibited the basal L-type Ca^{2+} current (Lacampagne *et al*., 1995). However, PHMPS, unlike PAO, can only form a single covalent bond with free -SH groups, which interferes with the formation of disulfide linkages (Lacampagne *et al*., 1995). Variations in the sensitivity of specific redox sites to different thiol-modifying agents highlights another possible explanation for the diversity of cardiac Ca^{2+} channel responses (Kohr *et al*., 1994).

Effects similar to those observed in the present study have been reported in frog and ferret ventricular myocytes (Nakajima *et al*., 1990; Campbell *et al*., 1996; Yamaoka *et al*., 2000). In each of these studies sulfhydryl oxidants stimulated the basal Ca^{2+} current. In ferret myocytes, this stimulatory effect was postulated to arise from modulation of an extracellular redox switch on the L-type Ca^{2+} channel, which is consistent with our observations (Campbell *et al*., 1996). In frog myocytes, the stimulatory effect has been attributed either to a direct redox effect on the channel (Yamaoka *et al*., 2000) or enhancement of cAMP production in combination with a direct effect on the channel (Nakajima *et al*., 1990). However, Yamaoka *et al*. (2000) proposed that the PKA-independent stimulatory response involves thiol oxidation of sites accessible only from the cytosolic side of the membrane. Our results suggest that the basal current is stimulated by the action of PAO on extracellular cysteine residues.

β-*adrenergic response/cAMP signaling*

To our knowledge the present study is the first to demonstrate that sulfhydryl-modifying agents can antagonize the cardiac Ca^{2+} channel response to β-AR stimulation. The ability of DTT to reverse this inhibitory effect suggests that it is due to sulfhydryl oxidation. Evidence for redox modulation of the β-adrenergic response was further supported when intracellular application of DTNB, a membrane-impermeable sulfhydryl-oxidizing agent, blunted the response of the Ca^{2+} current to Iso. Consistent with this, we found that intracellular dialysis of myocytes with DTT antagonized the ability of PAO to inhibit the β-adrenergic response. However, DTT, whether applied internally or externally, did not restore the full magnitude of the Iso response. One potential explanation for this is that as PAO complexes with vicinal sulfhydryl groups to form stable ring structures, it may alter the conformation of the protein, limiting access to DTT. Redox sites modified by PAO may also vary in their ability to be reduced by DTT (Kohr *et al*., 1994; Omerovic *et al*., 1994; 1995; Sullivan *et al*., 1994). Another possible explanation is that DTT was unable to reverse completely the extent of modification produced by the amount of PAO used in the present study. Consistent with this idea, we found that lower concentrations of PAO still produced an inhibitory effect, yet subsequent application of DTT was able to produce a more complete restoration of the Ca^{2+} current response to β-adrenergic stimulation (C. Sims and R.D. Harvey, unpublished observations). The fact that DTT appeared to be less effective at blocking the ability of PAO to antagonize β-adrenergic responses when it was introduced *via* the pipette solution could be explained if the actual concentration of DTT inside the cell was limited by dialysis and/or reduced by the capacity of DTT to diffuse across the plasma membrane into a rapidly exchanging bath solution.

The ability of PAO to inhibit the L-type Ca^{2+} current stimulated by Iso suggests that it exerts an effect at some point in the β-adrenergic signaling pathway. To assess where the effect of PAO is mediated, we examined its capacity to modulate cAMP-dependent responses at specific points in this pathway. PAO inhibited the response to Iso, forskolin, 8-CPT-cAMP and ATPγS alike. The fact that PAO inhibited channels irreversibly activated by thiophosphorylation in the presence of ATPγS indicates that this effect is at the level of channel protein. However, PAO does not appear to exert a significant inhibitory effect on the basal channel activity. This suggests that rather than affecting the β-adrenergic signaling pathway itself, PAO alters a redox-sensitive site that influences the ability of the channel to respond to PKA-dependent phosphorylation.

The binding of Iso to β-ARs in cardiac myocytes initiates a signaling cascade that results in the activation of PKA-dependent phosphorylation events that modulate a variety of cardiac ion channels. We previously demonstrated that the response of the L-type Ca^{2+} current to β-adrenergic stimulation is inhibited by a tyrosine kinase-dependent mechanism acting selectively at the level of the β-AR (Sims *et al*., 2000; Belevych *et al*., 2001). This is supported by the observation that vanadate compounds inhibited the L-type Ca^{2+} current stimulated by Iso, but not the basal current or the current stimulated by forskolin (Sims *et al*., 2000). This suggests that inhibition of tyrosine phosphatase activity affects β-AR-dependent responses by blocking cAMP production. In the present study, we found that PAO-mediated sulfhydryl oxidation can alter the ability of the L-type Ca^{2+} channel to respond to PKA-dependent phosphorylation, independent of cytosolic cAMP levels. Although there is no direct evidence

that PAO affects more proximal elements of the β-adrenergic signaling cascade, the possibility that it may also inhibit cAMP production cannot be ruled out.

This study presents evidence that the redox state of the cell may modulate the L-type Ca^{2+} channel response to PKA-dependent phosphorylation. This highlights the crucial significance of cysteine residues in maintaining normal physiological responses in the heart. The redox state of cardiac myocytes can be altered by pathological conditions such as ischemia and subsequent reperfusion. This might then be expected to reduce Ca^{2+} entry in response to sympathetic stimulation, which could be a cardioprotective response to limit Ca^{2+} overload (Goldhaber & Qayyum, 2000). However, unless there are parallel changes in the β-adrenergic response of other ion channels, such effects could also lead to changes in the action potential duration that are potentially arrhythmogenic.

We thank M. Sanders for expert technical assistance, and A. Belevych, S. Warrier and I. Juranek for helpful discussions. This work was supported by a grant from the National Institutes of Health (HL 68170).

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(Received January 12, 2004
Revised February 23, 2004
Accepted February 25, 2004)