Thiol-Modifying Phenylarsine Oxide Inhibits Guanine Nucleotide Binding of Rho but Not of Rac GTPases

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ABSTRACT

Phenylarsine oxide (PAO) is a phosphotyrosine phosphatase inhibitor that cross-links vicinal thiol groups, thereby inactivating phosphatases possessing XCysXXCysX motifs. The RhoA-GTPase, but not the Rac1-GTPase, also possesses vicinal cysteines within the guanine nucleotide-binding region (aa 13–20) and the phosphohydrolase activity site. Treatment of Caco-2 cells with PAO showed a dose-dependent reorganization of the actin cytoskeleton, indicating involvement of Rho GTPases. As tested by pull-down experiments, RhoA, but not Rac1, from cell lysates was inactivated by PAO in a concentration-dependent manner. Modification of RhoA by PAO resulted in altered mobility on SDS-polyacrylamide gel electrophoresis, and PAO-modified RhoA was no longer substrate for C3-catalyzed ADP-ribosylation. Furthermore, RhoA treated with PAO, but not Rac1 treated with PAO, lost its property to bind to guanine nucleotides. Matrix-assisted laser desorption ionization-mass analysis of PAO-modified RhoA showed a mass shift according to an adduction of a single PAO molecule per molecule RhoA. Further analysis of Glu-C-generated RhoA peptides confirmed binding of PAO to a peptide harboring the guanine nucleotide binding region. Thus, PAO does not exclusively inhibit phosphotyrosine phosphatases but also inactivates RhoA by alteration of nucleotide binding.

Phenylarsine oxide (PAO) is a trivalent arsenical compound covalently binding to vicinal thiol groups of proteins that are in suitable proximity. PAO is commonly used in cell biological research as an inhibitor of phosphotyrosine phosphatases (PTPases); in particular, PTPases HA1, HA2 (Liao et al., 1991), and SH-PTP2 (Noguchi et al., 1994) have been identified as substrates for PAO. Covalent binding of PAO to cysteinyl residues residing in the catalytic sites causes inactivation of PTPases. PAO also acts on other phosphatases that harbor vicinal thiol groups [i.e., inositol 1,2,3,5,6-pentakisphosphate 5-phosphatase or calcineurin, a Ca2+/calmodulin-dependent serine/threonine phosphatase (Bandopadhyay et al., 1997; Bogumil et al., 2000)]. Also, NADPH oxidase and mitochondrial adenine nucleotide transporter were reported to be inhibited by PAO (Costantini et al., 2000). Furthermore, steric inhibition of PAO-linked NFeB was suggested to be responsible for its decreased binding to DNA (Oda et al., 2000).

The ability of PAO to cross-link cysteinyl residues of any protein, if these residues are exposed at the surface of the molecule, makes PAO a less specific agent. For its usage as specific PTPase inhibitor in cellular processes, it is necessary to meet a well-defined concentration. In some studies, PAO was applied as a specific PTPase inhibitor to examine the participation of tyrosine phosphorylation in cell adhesion and in the regulation of tight junctions, respectively (Staddon et al., 1995; Retta et al., 1996; Rao and Li, 1999), all actin cytoskeleton-dependent processes. However, reorganization of the actin cytoskeleton implicates involvement of Rho GTPases, which are key regulators of stress fiber formation and membrane ruffling (Hall, 1998; Kaibuchi et al., 1999). Moreover, the subtypes RhoA, -B, and -C possess vicinal cysteinyl residues within their guanine nucleotide binding region to be possibly affected by PAO. Therefore, Rho GTPases have to be considered possible targets for PAO. This study reveals that signaling activity of RhoA, but not of Rac is inhibited by PAO in micromolar concentrations, whereby guanine nucleotide binding to loop I is altered. The inactivation of RhoA by PAO causes reorganization of the cytoskeleton, which differs from changes induced by vanadate, a PTPase inhibitor that does not interfere with the nucleotide binding of RhoA.

Materials and Methods

Materials. Caco-2 cells were from Cell Line Service, (Heidelberg, Germany). Cell culture media and supplements were supplied from Clostridium limosum exoenzyme C3; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; GTP[S], guanosine 5’-O-(3-thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; JNK, c-Jun N-terminal kinase.

ABBREVIATIONS: PAO, phenylarsine oxide; PTPase, phosphotyrosine phosphatase; PBS, phosphate-buffered saline; C3imm, Clostridium limosum exoenzyme C3; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; GTP[S], guanosine 5’-O-(3-thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; JNK, c-Jun N-terminal kinase.
Biochrom (Berlin, Germany). Phenylarsine oxide, Na3VO4, α-cyano-4-hydroxycinnamic acid, and l-fucose came from Sigma-Aldrich (Steinheim, Germany). Glutathione-Sepharose was from Amersham Biosciences (Freiburg, Germany). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, OR). RhoA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rac1 antibody was from BD Transduction Laboratories (Heidelberg, Germany). [α-32P]GTP and [32P]NAD were from PerkinElmer (Boston, MA). All other substances were of highest purity available.

**Cell Culture.** Caco-2 cells were cultured under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were subcultured every week. For pull-down experiments, cells were seeded on Petri dishes (10 cm in diameter) and were grown until they reached confluence.

**Rhodamine-Phalloidin Staining of Actin Filaments.** Cells on cover slips were incubated in culture medium containing 5 or 50 μM phenylarsine oxide and 1 mM sodium ortho-vanadate for 45 min. Thereafter, cells were rinsed with PBS and incubated in 3% formaldehyde solution containing 0.05% Triton X-100 for 10 min at room temperature. The cells were washed three times with PBS and incubated with 15 ng/ml rhodamine-phalloidin. After three washes with PBS, coverslips were mounted on slides for fluorescence microscopy.

**Purification of Recombinant Proteins.** Clostridium limosum exoenzyme C3 (C3lim) was prepared as described by Böhmer et al. (1996). Expression and purification of recombinant RhoA and Rac1 was performed after standard protocol for GST-fusion proteins. Expression of the GST-fusion proteins of the Rho-binding domain of rhotekin (C-21) and the Rac-binding domain of Pak (Crib domain) in BL21 (DE3) (Novagen) was induced at 37°C was added by inducing 0.1 mM isopropyl β-D-thiogalactoside (final concentration) at OD600 of 1.0. Two hours after induction, cells were collected and lysed by sonication in lysis buffer (50 mM NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 2 mM dithiothreitol, 10% glycerol, and 1 mM PMSF). The lysate was centrifuged at 10,000g, and the supernatant was used for purification of GST-fusion proteins by affinity purification with glutathione-Sepharose. Beads loaded with GST-fusion proteins were washed three times with PBS and were used immediately for GTPase pull-down experiments.

**Pull-Down Experiments with GST-C21 and GST-Pak Crib Domain.** Pull-down experiments were performed as described by Reid et al. (1996). After incubation with PAO or vanadate, the culture medium was removed and cells were rinsed twice with PBS. Cells were lysed by addition of 500 μl of ice-cold lysis buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 1% Nonidet P40, 0.25% Triton X-100, 5 mM dithiothreitol, and 100 μM PMSF). Cells were then scraped from the Petri dishes, transferred into Eppendorf vials, and rotated for 5 min at 4°C. The lysates were centrifuged at 14,000 rpm and the supernatant was used for pull-down experiments. Pull-down assay with recombinant GTPγS-Rhoa (1 μg) was performed in lysis buffer without dithiothreitol. To this end, beads in 20 μl of slurry of the Rho binding domain GST-C21 from Rhotekin or the Rac binding domain GST-Pak-Crib domain bearing approximately 30 or 10 μg, respectively, of fusion protein, were added to each sample and rotated at 4°C for 30 min. The beads were collected by centrifugation at 10,000 rpm and washed twice with lysis buffer. To each sample, 20 μl of Laemmli buffer was added to the beads and 12% SDS-PAGE (with subsequent transfer of proteins onto nitrocellulose) was performed. Rhoa and Rac1 were detected by Western blot using specific antibody.

**Exoenzyme C3-Catalyzed [32P]ADP-Ribosylation of Rhoa.** For ADP-ribosylation studies, cells were lysed in 500 μl of ice-cold lysis buffer (50 mM NaCl, 20 μM HEPES, pH 7.4, 4 mM MgCl2, and 10 μM PMSF) and briefly sonicated on ice. Cell lysates (50 μl; 100 μg of protein) were subjected to subsequent [32P]ADP-ribosylation assay by adding 15 μl of ribosylation mix to a final concentration of 50 mM HEPES, pH 7.4, 10 mM thymidine, 5 mM MgCl2, 2.5 mM dithiothreitol, and 2.5 mM NAD. To start ADP-ribosylation, 0.5 μCi of [32P]NAD and 50 ng of C. limosum exoenzyme C3lim were added. For ADP-ribosylation of recombinant proteins 0.5 μg of Rhoa were incubated in 15 μl of ribosylation buffer containing 0.1% dimethyl sulfoxide with or without 200 μM PAO. Exoenzyme C3lim and [32P]NAD were added to a final concentration of 0.75 μg/ml and 0.1 μCi per sample, respectively. The samples were incubated for 30 min at 37°C and the reaction was stopped by addition of 20 μl of Laemmli sample buffer. Proteins were separated on 12.5% SDS-PAGE, and [32P]ADP-ribosylated proteins were analyzed by filmless autoradiographic analysis.

**Mant-GDP Exchange.** This method was performed after a method described by Sehr et al. (1998). In brief, recombinant RhoA or Rac1 was incubated in 50 mM Tris HCl, pH 7.5, 5 mM EDTA, and 10 μM GDP for 15 min on ice. After this, the Rho/Rac GDP-bound form was stabilized by adding 10 μM MgCl2. Before starting the mant-GDP exchange, 5 μg of Rhoa or 50 μg of Rac1 were allowed to equilibrate in a cuvette in 500 μl of triethanolamine buffer (50 μM triethanolamine, 5 μM MgCl2) for 5 min at 37°C. The assay was started by adding mant-GDP to a final concentration of 10 mM, and the fluorescence was measured every 20 s (excitation, 357 nm; emission, 444 nm).

**Guanine Nucleotide Release Assay.** The release of the guanine nucleotide from Rhoa by treatment with PAO was measured by release of bound [α-32P]GTP from the GTPases. Therefore, 100 μg of RhoA in 1 ml of triethanolamine buffer (50 μM triethanolamine and 2 mM MgCl2) were incubated at 30°C. The uptake of GTP was started by adding [α-32P]GTP to the Rho GTPases to a final concentration of 200 mM. At indicated time points, 50 μl was taken from the solution and transferred onto a nitrocellulose filter. The filters were washed twice with 3 ml of buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 2 mM MgCl2) to remove all bound [α-32P]GTP. After 30 min of loading with [α-32P]GTP, PAO was added to a final concentration of 50 μM, and the release of [α-32P]GTP from RhoA was measured for additional 40 min. The RhoA-bound [α-32P]GTP was measured in a scintillation counter.

**Gel Shift Assay.** To remove excessive dithiothreitol (DTT), recombinant RhoA in 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 1 mM DTT was filtrated using microcon (10-kDa cut off) and was washed three times with 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 1 μM DTT. Rhoa was incubated with or without 100 μM PAO for further 60 min at 4°C. Laemmli sample buffer (5 μl [5-fold]) with or without 10 mM DTT was given to a 25-μl sample, and 12% SDS-PAGE was performed.

**Mass Determination By Matrix-Assisted Laser Desorption Ionization-Mass Spectrometry.** Enzymatic protein cleavage: 10 μl of buffered solutions of Rho A and its PAO derivative (2 mg/ml in 0.15 M NaCl, 2 mM MgCl2, 20 mM Tris-HCl, 1 mM glutathione, and 0.2 mM GDP, pH 7.0) were mixed with 30 μl of PBS (0.05 M NaH2PO4, 0.15 M NaCl, pH 7.7) before the addition of 6 μl of an endoproteinase Glu-C solution (1 mg/ml; EC 3.4.21.19, Roche Diagnostics, Mannheim, Germany). The incubation was performed overnight under gentle stirring at 25°C followed by measuring the mass spectra by MALDI-MS.

MALDI mass spectra from the intact and PAO-modified Rho A and their proteolytic cleavage products were obtained on a Voyager DE Pro mass spectrometer (Applied Biosystems, Weiterstadt, Germany). Data were acquired in the positive linear operation mode after external calibration using a matrix of α-cyano-4-hydroxycinnamic acid mixed with l-fucose according to the dried drop technique. Data acquisition and analyses were performed using the Voyager control software and Data Explorer version 4.0 software supplied by the manufacturer. Samples of proteolytic incubation mixtures were desalted using ZipTips (Millipore, Bedford, MA), and intact proteins were initially diluted 1:10 in acetonitrile/0.1% trifluoroacetic acid before MALDI-MS measurements.

**Amino Acid Sequence Analysis.** N-terminal sequencing of HPLC-purified peptides was performed on a Procise 494 sequencer (Applied
Results

PAO-Induced Reorganization of the Actin Cytoskeleton. The actin cytoskeleton of confluent human colonic Caco-2 cells (Fig. 1A) is characterized by prominent actin bundles (stress fibers) and a thin perijunctional actin ring (Fig. 1A, arrows). The phosphatase inhibitor vanadate (1 mM) induced an overall increase in actin polymerization (Fig. 1B), characterized by an increased formation of stress fibers and an increase in actin filaments at the cell contact sites. Cells that were incubated with PAO exhibited dose-dependent changes in the organization of the actin cytoskeleton. Low concentration of PAO (5 μM) induced reduction of stress fibers, whereas short actin filaments at cell contact sites seemed to be increased (Fig. 1C). Higher concentration of PAO (50 μM) further reduced stress fibers and decreased actin polymerization at the cell contact sites and the perijunctional region (Fig. 1D). Instead, short actin filaments assembled in the perinuclear region of the cell.

Inhibition of Rho GTPases by PAO-Treatment of cells. The Rho- and Rac-binding domains of the effector proteins rhotekin (C21) and PAK, respectively, were used to precipitate the active, GTP-bound forms of GTPases from cell lysates (pull-down assay). The Western blot analysis of the precipitates of PAO-treated cells showed a dose-dependent decrease in active RhoA compared with control cells. Incubation of cells with 5 μM PAO for 45 min reduced the amount of RhoA binding to C21, whereas 50 μM completely abolished binding of RhoA to C21 (Fig. 2A). In contrast, vanadate did not affect, or even slightly increased, the binding of RhoA to C21. The pull-down experiments using GST-Pak crib domain to study activation of Rac proteins revealed no effect of either PAO (5 and 50 μM) or vanadate (1 mM) on Rac activation (Fig. 2A). In accordance with cellular RhoA, recombinant RhoA activated by GTP[γS] also showed reduced binding to C21 when incubated with 50 μM PAO (Fig. 2B). To exclude nonspecific effects of PAO, we preincubated GST-C21 beads (three cysteines within C21) with 10 μM PAO, which is the maximum final concentration of residual PAO in pull-down assays. Preincubation did not disturb interaction with RhoA-GTP[γS], suggesting a specific effect of PAO on Rho.

PAO Alters Conformation of RhoA, -B, and -C. To exclude intracellular degradation of RhoA after PAO modification, RhoA content was checked by Western blot analysis. As shown in Fig. 3A, PAO (50 μM) treatment did not alter the RhoA content of the cells within the observed period of 90 min. However, PAO led to an altered migration behavior of RhoA on SDS-PAGE, indicating a direct covalent modification of RhoA by PAO. The changed migration behavior of RhoA was also tested with recombinant RhoA; to this end, recombinant RhoA (10 μM) incubated with and without PAO (100 μM) was analyzed by SDS-PAGE. In fact, PAO-modified RhoA migrated faster than unmodified RhoA (Fig. 3B), corroborating the data obtained with cellular Rho. This effect was reversed when PAO-modified RhoA was incubated with 10 mM dithiothreitol before electrophoresis.

Fig. 1. PTPase inhibitors induce reorganization of the actin cytoskeleton. Caco-2 cells were either untreated (A) or treated with 1 mM sodium ortho-vanadate (B), 5 μM PAO (C), or 50 μM PAO (D) for 45 min. After fixation, actin filaments were stained with rhodamine-phalloidin and cells were subjected for fluorescence microscopy.
To test the influence of PAO on the structure of RhoA, RhoA was subjected to \( \text{C}_{3}^{\text{lim}} \) catalyzed \([^{32}\text{P}]\text{ADP-ribosylation} \), because \( \text{C}_{3}^{\text{lim}} \) exclusively modifies native RhoA (Habermann et al., 1991). RhoA from cells, treated with 50 \( \mu \text{M} \) PAO (Fig. 3C), was not substrate for \( \text{C}_{3}^{\text{lim}} \). The same was true for recombinant RhoA. An effect of PAO on the transference \( \text{C}_{3}^{\text{lim}} \) can be excluded, because \( \text{C}_{3}^{\text{lim}} \) does not contain any cysteinyl residue.

**Effect of PAO on the Guanine Nucleotide Binding of Rho GTPases.** The direct effect of PAO on RhoA was tested by the mant-GDP binding assay. After addition of fluorescent mant-GDP to RhoA, a rapid increase in fluorescence could be observed. This increase indicated an exchange of guanine nucleotide accompanied by binding of mant-GDP to RhoA. After 20 min, the exchange reached a plateau, and no further increase of fluorescence was detected (Fig. 4). Preincubation of RhoA with 50 \( \mu \text{M} \) PAO prevented an increase in fluorescence, indicating that PAO inhibited the exchange or a binding of nucleotides. As shown in Fig. 4, bottom, addition of PAO to RhoA-mantGDP induced a rapid decrease in fluorescence, caused by the release of mant-GDP from RhoA.

In contrast to RhoA, binding of mant-GDP to Rac1 was not affected by PAO (Fig. 4, right). Nontreated as well as PAO-treated Rac1 showed a rapid increase in fluorescence after addition of mant-GDP, which was saturated after 10 min. The maximum fluorescence of PAO-treated sample is marginally lower than that of control Rac1 and was always within variation of separate experiments. In contrast to RhoA, addition of PAO to mant-GDP loaded Rac1 did not decrease fluorescence, indicating still-bound mant-GDP and lack of interference of PAO with mant-GDP.

**PAO Releases Guanine Nucleotides from Rho GTPases.** The GTP/GDP release from RhoA is shown in Fig. 5. After addition of \([\alpha^{32}\text{P}]\text{GTP} \) to the samples, there was a time-dependent increase in binding of \([\alpha^{32}\text{P}]\text{GTP} \) to RhoA, which was saturated after 30 min; controls showed stable nucleotide binding for a further 40 min. Intrinsic GTPase activity of Rho GTPases leads to an equilibrium of GTP and GDP binding to RhoA in this assay. Thus, labeled nucleotide can be GTP as well as GDP. Release of GTP from RhoA was also indicated by pull-down assay as shown in Fig. 2B. Addition of PAO (50 \( \mu \text{M} \)) to the sample resulted in a time-dependent decrease of bound nucleotide, leading to an almost complete release from RhoA within 40 min. In contrast to RhoA, the GTP binding to Rac1 remained unaffected by PAO. Rac1 showed a time-dependent loading with \([\alpha^{32}\text{P}]\text{GTP} \), which was saturated after 60 min. Addition of PAO did not interfere with the nucleotide binding to Rac1. A statistical analysis showed a significant reduction in the amount of bound nucleotide in PAO-treated samples compared to controls.

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**Fig. 2. Effect of PTPase inhibitor on Rho GTPases.** A, after treatment of Caco-2 cells with different concentrations of PAO or vanadate for 45 min, pull-down assays for active, GTP-bound Rho and Rac from cell lysates were performed to estimate the activity state of Rho GTPases. Precipitated RhoA and Rac1 were detected by Western blot. B, RhoA pull-down using recombinant Rac1. RhoA-GTP\([\gamma^{32}\text{P}] \) was incubated without (lane 1) or with (lane 2) PAO and subjected to pull down assay. To exclude direct PAO effect on C21, GST-C21 was preincubated with PAO before RhoA-pull down (lane 3).

**Fig. 3.** PAO alters conformation of RhoA. A, Caco-2 cells were treated with 50 \( \mu \text{M} \) PAO for the indicated time. The incubation was stopped by addition of Laemmli buffer for SDS-PAGE, and Western blot was performed to detect the amount of RhoA in cell lysates. B, Coomassie stained gel of a gel shift assay of PAO-modified and unmodified recombinant RhoA. Reversibility of PAO modification by DTT is shown. C, the effect of PAO on the structure of RhoA was proven by exoenzyme C3lim-catalyzed ADP-ribosylation, because C3lim recognizes only native RhoA as substrate. Lysates from cells treated with PAO for 45 min were subjected to \([^{32}\text{P}]\text{ADP-ribosylation} \) followed by SDS-PAGE. \([^{32}\text{P}]\text{ADP-ribosylated Rho was visualized by filmless autoradiographic analysis.} \)
Crosslinking of Cysteinyl Residues by PAO. Separated mass determinations of RhoA (~22,164 Da) and its PAO-treated derivative (~22,325 Da) by MALDI-MS resulted in a mass difference of about 161 Da. N-terminal sequence analyses by Edman degradation revealed the first 12 amino acids (GPLGSMMAIRKK), confirming the expected recombinant RhoA sequence (Fig. 6). With regard to the accuracy of the determination of high molecular mass compounds (0.1%), the expected mass difference of 150 Da was found. Additionally, enzymatic cleavage and mass determinations of both substrates by endoproteinase Glu-C allowed high sequence recovery and assignment of the pure and cross-linked N terminus as indicated in Fig. 6. Mass spectra of the desalted incubation mixtures of RhoA and its PAO product showed many distinct signals in the mass range between 1,000 and 4,500 Da. Most of these signals were found in both digests, except the signals at m/z 3859.9 (only detected in the Rho A digest) and m/z 4011.9 (only detected in the Rho A-PAO digest). The assigned sequences could also be confirmed by Edman degradation after HPLC separation of the incubation mixture. These results underline the assumed quantitative cross-link between the two cysteinyl residues located within the guanine nucleotide-binding loop. Signals that could not be assigned to the expected cleavage products of Rho A or endoproteinase Glu-C itself (C-terminally at glutamic acid and aspartic acid) may be caused by initial peptidic or proteinaceous impurities.

Discussion

In the present study, we describe the effect of the phosphotyrosine phosphatase inhibitor PAO on Rho GTPases, the master regulators of the actin cytoskeleton. The effect of PAO on the cytoskeleton of Caco-2 cells is concentration-dependent; lower micromolar concentrations lead to an increase in cortical actin filaments, whereas higher concentrations result in a general fragmentation of actin filaments and their perinuclear localization. The reorganization of the cytoskeleton differs from that induced by ortho-vanadate, a different phosphatase inhibitor. This difference may be explained by other phosphatases inhibited by PAO in comparison with ortho-vanadate. However, further findings, such as inactivation of RhoA but not of Rac1, were not in line with the expected effects of the phosphatase inhibitor. This led to the

Fig. 4. Guanine nucleotide exchange of RhoA is affected by PAO. The nucleotide exchange was fluorimetrically measured using mant-GDP. Binding of mant-GDP to GTPases results in an increase in fluorescence. The nucleotide exchange was determined with 5 μg of RhoA or 50 μg of Rac1 that were either untreated or treated with 50 μM PAO (top). Bottom, addition of PAO (50 μM final concentration) to RhoA/Rac1 after the equilibrium of mant-GDP binding was reached.

Fig. 5. PAO induces guanine nucleotide release from RhoA. A filter assay was used to determine the GTP/GDP release of Rho GTPases by PAO. 0.5 μCi of [α-32P]GTP (final concentration, 200 μM GTP) were given to 7 μg of RhoA or Rac1, and aliquots of 0.5 μg were taken at indicated times. The aliquots were given on nitrocellulose filters, and free [α-32P]–labeled nucleotide was removed by washing the filter twice. RhoA/Rac1-bound [α-32P]GTP/GDP was measured in a scintillation counter. After 30-min loading with [α-32P]GTP, 50 μM final concentration of PAO (□) or 0.1% dimethyl sulfoxide (■) were added. Inserted into the time lapse are bar charts of statistical analyses, using t test, of values 30 min after PAO addition. Maximum α-32P–labeled nucleotide binding before PAO addition was taken as 100% (mean ± S.D., n = 5, p < 0.000001; ■, RhoA control; □, RhoA PAO-modified).
working hypothesis that PAO also acts on cellular targets other than phosphotyrosine phosphatases.

Based on our results, we concluded that PAO acts directly on the signal transduction level of RhoA. A first hint of a modification of RhoA by PAO was given by gel shift assay. The downward gel shift of PAO-treated RhoA was detected with endogenous as well as with recombinant RhoA. The downward shift is not in accordance with an increase in molecular mass because of addition of PAO; rather, it indicates conformational changes of RhoA. Such a reversed effect can also be detected by cytotoxic-necrotizing factor-induced deamidation of RhoA (Gerhard R. et al., 1998). A lower molecular mass induces an upward shift to higher apparent molecular masses on SDS-PAGE. Additional evidence of a covalent binding of PAO to RhoA was expected by MALDI-MS analysis (Kussmann and Przybylski, 1995). The comparison of the complete modified and unmodified proteins, as well as their endoproteinase Glu-C-generated peptides, confirmed the hypothesis of an PAO-adduct. The covalent modification of RhoA by PAO raised the question of its functional effects. The finding that PAO binds to the N-terminal region of RhoA, where the GTP-binding domain resides, suggests interference with the nucleotide exchange or binding. In fact, the modification of Rho by PAO alters guanine nucleotide binding. The effect of PAO on the nucleotide binding was investigated by GDP-exchange assay and additional GTP-release assay. To exclude false results in GDP-exchange assay caused by quenching effects of PAO on the mant-GDP fluorescence, we also assayed GTP-release from RhoA by filtration technique. The release of [$^{32}$P]GTP from preloaded RhoA in combination with the mant-GDP fluorescence experiments showed that binding of PAO to RhoA is dominant compared with guanine nucleotide binding. Binding of PAO occurs to GDP-bound as well as GTP-bound RhoA. Because the loss of nucleotide causes inactivation of GTPases, the activity state of endogenous RhoA was tested with pull-down assay. In accordance with the observed nucleotide release, PAO-treatment led to inactivation of cellular RhoA. In general, liberation of guanine nucleotide leads to conformational changes, or even to denaturation of the GTPases. Accordingly, PAO-treated, nucleotide-free recombinant RhoA and cellular RhoA from PAO-treated cells were no longer substrate for exoenzyme C3. Thus, PAO, which binds to the nucleotide-binding region, does not substitute the nucleotide in its ability to maintain conformational features of RhoA. Nevertheless, we did not observe a degradation of modified RhoA within the cells over a period of 90 min. So far, it is unclear whether addition of phenylarsine prevents RhoA from being degraded or modified RhoA is sequestered by regulatory proteins, such as guanlate nucleotide-exchange factors. As can be deduced from our results, RhoA modified by PAO loses its ability to interact with the Rho-binding domains of effector proteins, resulting in inhibition of downstream signaling (Bishop and Hall, 2000).

The finding that RhoA is functionally inactivated by PAO contradicts the reported activation of RhoA by inhibition of tyrosine phosphatases (Schoenwaelder and Burridge, 1999). This activation was explained as an effect upstream in the Rho signaling pathway. In the present study, we cannot exclude similar regulatory effects of PAO at lower concentrations that were caused by inhibition of tyrosine phosphatases. Nevertheless, we provide profound evidence that even micromolar concentrations of PAO result in inactivation of RhoA.

PAO that cross-links vicinal thiol groups affects RhoA in vitro and in vivo, but not Rac1. The inactivation of RhoA and its isotypes RhoB and C is caused by binding of PAO to cysteinyl residues 16 and 20 within the guanine nucleotide-binding region (Fig. 7). Rac GTPases that only possess one cysteinyl residue in position 20 (according to RhoA sequence) would be protected from PAO modification by interchain disulfide bonds.

**Fig. 6.** MALDI-MS and amino acid sequence of PAO derivatized RhoA. A, MALDI-MS was measured after desalting the incubation mixtures of PAO-derivatized RhoA (solid line) and underivatized RhoA (dotted line; shown as excerpt) with endoproteinase Glu-C. signals detected in both digests that are assigned to cleavage products of RhoA. signals detected in both digests that are not identified. B, assigned masses are indicated by the under- and overlined amino acids specified by their corresponding theoretical masses. The phenylarsine (PA) cross-linked N terminus was assigned to m/z 4011.9, whereas the underivatized segment was assigned to m/z 3859.9, m/z 3105.0 and m/z 3534.0 could not be assigned.

**Fig. 7.** Schematic phenylarsine-modified RhoA peptide and alignment of loop I from RhoGTPases. Shown are the amino acid sequences of the GTP binding region in loop I of Rho and Ras. Positions are labeled according to RhoA amino acid sequence.
are unaffected by PAO regarding mant-GDP exchange and GDP-release. Furthermore, endogenous Rac1 was not affected by PAO in the pull-down assay. Inactivation of RhoA by PAO, but not of Rac1, interferes with the cross talk between Rho and Rac, altering the balance between Rho and Rac functions. This alteration leads to a prevalence of cellular Rac functions (Van Aelst and D’Souza-Schorey, 1997). Considering this notion, the effects of Rac on the cytoskeleton, such as increase in cortical actin filaments, may be pronounced even without increased activation of Rac. This effect may also contribute to the reorganization of the actin cytoskeleton induced by lower PAO concentrations.

Rho GTPases not only regulate the organization of the actin cytoskeleton, but also can govern the c-Jun N-terminal kinase (JNK) signaling pathway (Teramoto et al., 1996a,b). It is known that arsenical compounds are able to induce activation of the JNK via Rho and Rac in human embryonic kidney 293 cells (Porter et al., 1999). In that study, activation of JNK was dose-dependent and was maximal at 300 μM arsenite/arsenate. Expression of inactive Rho mutants could abolish the arsenite/arsenate-induced JNK activation. We found similar effects; 5 μM PAO led to activation of JNK (data not shown). Interestingly, 50 μM PAO, which led to total inactivation of RhoA in Caco-2 cells, totally inhibited JNK activity. Further experiments must characterize the specificity of inorganic arsenical compounds compared with phenylarsine oxide regarding their ability to inactivate Rho GTPases and their effects on the signal transduction downstream of Rho.

In summary, PAO—in addition to its property to block phosophatases—inhibits cellular functions of RhoA by covalent binding to the guanine nucleotide-binding region, resulting in altered nucleotide binding. The overall effect of PAO is the inhibition of RhoA downstream signaling. Because of its specificity toward GTPases, PAO may be a model compound to develop cell-permeable selective inhibitors of cellular Rho function.

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Inhibition of Rho GTPases by Phenylarsine Oxide 1355

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