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Properties of Pervanadate and Permolybdate

CONNEXIN43, PHOSPHATASE INHIBITION, AND THIOL REACTIVITY AS MODEL SYSTEMS

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Properties of Pervanadate and Permolybdate are irreversible protein-tyrosine phosphatase inhibitors, with IC50 values of 0.3 and 20 μM, respectively, in intact cells. Maximal inhibition was obtained within 1 min at higher concentrations of the compounds. They induced prominent changes in the phosphorylation status of the gap junction protein, connexin43. These effects were utilized as model systems to assess the stability and inactivation of the compounds. Although the concentrated stock solutions were relatively stable, the diluted compounds were unstable. The biological activity had decreased to 20–30% after 6 h of incubation in a phosphate buffer, 1 h in phosphate buffer with 10% fetal calf serum, and 1–3 minutes in culture medium. Thiols reacted rapidly with the compounds and inactivated them (initial reaction rates with cysteine: pervanadate > pervanadate > H2O2). Catalase inactivated the compounds, and permolybdate was the more sensitive. The cells inactivated permolybdate faster than pervanadate. Cellular inactivation of permolybdate, and to a lesser degree pervanadate, appeared to be partly dependent on catalase and thiols. However, a general decrease in cellular thiols was not the mediator of the biological effects of pervanadate or permolybdate. Mathematical modeling of the thiol reactivity suggested that monoperoxovanadate at maximum could possess 20% of the biological activity of diperoxovanadate.

Tyrosine phosphorylation is a central regulatory mechanism with numerous participating tyrosine kinases and counteracting protein-tyrosine phosphatases (PTPases)1 (1). Metal ions like vanadate and molybdate are PTPase inhibitors (2–4) that probably mimic inorganic phosphate. Many of the PTPase inhibitors are of low potency, especially in intact cells (5–8). Interestingly, the combination of vanadate and H2O2, two PTPase inhibitors, with IC50 values of 5–8, was a much more potent inhibitor called pervanadate (PV) (9). PV may be somewhat unstable under certain conditions (9). The procedure for generating PV and PMo can have a significant effect on the stabilities, and thereby also the potencies, of the compounds (7, 16). However, there are no studies that have been fully dedicated to this problem. Because they are potent and useful experimental compounds with some potential for clinical therapies (15, 19), it was of interest to perform such an investigation. We have here studied some factors that influence the stability and inactivation of PV and PMo. We show that the compounds are rapidly inactivated under normal cell culture conditions and that several factors are involved in the inactivation.

MATERIALS AND METHODS

Chemicals—Catalase (2× crystallized), N-ethylmaleimide (NEM), sodium orthovanadate (Na3VO4), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 3-amino-1,2,4-triazole (amitrole) and para-nitrophenol phosphatase (pNP) were purchased from Sigma. H2O2 and sodium molybdate (Na2MoO4 × 2H2O) were obtained from Aldrich.

Preparation of Peroxocompounds—The concentration of the parent metal salt was always considered as the concentration of the peroxo-compound. Throughout this work we have based the concentrations of vanadate on e265 = 2925 m−1cm−1 (20), although other values can also be found in the literature (e265 = 3550 m−1cm−1 (21); in this case the concentrations will be 18.5% lower than shown). PV was made as a 30 mM stock solution using 60 mM H2O2 (7). PMo was made as a 100 mM stock solution. HCl (120 mM) was added before 200 mM H2O2 (16). The mixtures were incubated for 15 min in the dark at room temperature before use. The compounds were often diluted in Ca2+/Mg2+-supplemented phosphate buffered saline (PBSS) (137 mM NaCl, 2.7 mM KCl, 1.45 mM KH2PO4, 6.45 mM Na2HPO4, 0.5 mM CaCl2, 0.8 mM MgCl2, pH 7.2) or in Ca2+/Mg2+-supplemented Hepes-buffered saline (HBSS) (10 mM Hepes, 140 mM NaCl, 0.5 mM CaCl2, 0.8 mM MgCl2, pH 7.2).

Cell Cultures—Primary cell cultures from Syrian hamster (Wright, Chelmsford, Essex, UK) embryos were prepared as described (7, 12). The cells were used between passages 2 and 10. No differences in responses were found between early and later passages. The growth medium was Dulbecco’s modified Eagle’s medium (DMEM) (Bio-Whittaker, Walkersville, NY) with 10% fetal calf serum (FCS) (Life Technologies, Inc.) and no antibiotics. The cells were maintained at 37°C in a humidified 10% CO2 atmosphere. When the cells were incubated in PBSS or HBSS, no CO2 was added to the atmosphere.

Western Blotting—The cells were seeded in 35-mm dishes. They reached confluence 2 days later. The cells were then exposed as indi-
cated. They were rinsed in phosphate-buffered saline, scraped into electrophoresis sample buffer, and sonicated. Five μl (8–10 μg of protein) of the homogenate were used for electrophoresis and Western blotting as described previously (7, 12), using rabbit anti-Cx43 antisera (7, 12) and an anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology, Lake Placid, NY) as primary antibodies. The band shift of Cx43 induced by PV (7) or PMo (16) is fully developed after 15 min of exposure. The standard exposure time in this work, unless otherwise indicated, was therefore 15 min. Densitometry of blots were performed using NIH Image software on scans obtained with an Agfa Arcus scanner.

**Phosphatase (pNPPase) Assay—**pTPases hydrolyze pNPP efficiently (4, 15, 22). pNPPase activity was measured as described (4, 22), with some modifications to achieve measurements on cells grown in 96-well plates. Cells (15,000 per well) were grown overnight. They were then exposed to the compounds in HBSS or PBSS (usually for 15 min) and rinsed in HBSS before the enzyme assay. Phosphatase assay buffer (50 μl of 37.5 mM sodium acetate, pH 5.0, 30% (w/v) glycerol, 1.5 mM EDTA, 0.15% Triton X-100, 5 mM freshly added DTT) was added directly to each well followed by 25 μl of 16 μM pNPP. Triton X-100 was included to obtain a rapid lysis of the cells. The detergent did not affect the pNPPase activity. DTT was increased to 5 mM to quench any remaining PV or PMo (see below). The plates were incubated at 37 °C for 1 h. The reaction was stopped by adding 50 μl of 3 mM unbuffered Tris. The reaction mixtures were linear with regard to time and number of cells. The absorbance was read at 405 nm. The pNPPase data are shown as mean ± S.D. for n = 3–4, each in four parallel measurements, except for the kinetics data, which are shown as mean ± S.D. of 4 single independent measurements.

**Determination of Remaining Biological Activity—**The remaining biological activities were determined by the dose-dependent shift in alterations of the Cx43 band pattern or by the dose-dependent shift in IC50 values of pNPPase. In both cases, the dose-response curve of test-treated (e.g. PV preincubated with in PBSS for 6 h) cultures were compared with the dose-response curve from control-treated cultures, i.e. PV or PMo diluted in PBSS and immediately added to the cells. Note that the 0 h incubation period in reality is a 1–3-min incubation due to the handling of dilutions and cells. The Western blot data shown are taken from one of several similar experiments.

**Measurement of Nonprotein Thiols—**DTNB was used to measure cysteine in a cell-free reaction mixture and nonprotein thiols in cells (23). For the former, 0.5 ml of 0.2 M phosphate buffer (pH 7.5), 420 μl of 0.5% Triton X-100, 5 ml of DTNB (60 mM in Me2SO) were mixed. The samples were made by mixing PV, PMo, H2O2, or the parent compounds. PV inhibited pNPPase activity, with IC50 = 0.3 μM; for PMo, IC50 = 20 μM (Fig. 1). Around 20–25% of the pNPPase activity remained at high concentrations of PV or PMo, similar to previous observations with PV (24). Similar results were also obtained when the cells were exposed in PBSS.

The pNPPase assay is simple and rapid, but it depends on the amount of cellular protein. On the other hand, induction of altered phosphorylation status of the gap junction protein Cx43, as evaluated by Western blot (7, 8, 12, 16), is less sensitive to variations in the amount of protein. Cx43 was therefore employed to investigate the latter possibility. One dish was exposed for 15 min to the compounds diluted in HBSS before assaying the pNPPase activity. The activity in unexposed cells was defined as 100% activity (corresponds to 1.35 nmol μg protein; n = 24). The data are shown as mean ± S.D. (n = 3–4, each in four parallel measurements). ☐, vanadate; *, molybdate; †, H2O2; ○, PMo; □, PV. Dotted lines, cells were also exposed to NEM in growth medium for 15 min followed by a 15 min incubation in HBSS (☉), 30 μM NEM in growth medium for 15 min followed by PMo in HBSS for 15 min (☉), or 30 μM NEM in growth medium for 15 min followed by PV in HBSS for 15 min (□).

**RESULTS AND DISCUSSION**

**Inhibition of Phosphatases by PV and PMo**

PV and PMo inhibit cytosolic PTPase with IC50 values of 3.3 and 10 μM, respectively, in a cell-free system (15). However, PMo was probably generated in a suboptimal way by Li et al. (15). It was therefore of interest to investigate the relative potencies of phosphatase inhibition of the compounds in intact cells. The cells were exposed to PV, PMo, and the parent compounds vanadate, molybdate, and H2O2 during 15-min exposures in HBSS. Vanadate or molybdate slightly inhibited pNPPase at very high concentrations (Fig. 1), indicating reversibility of inhibition and/or low permeability into cells. H2O2 caused an inhibition of pNPPase activity, with IC50 = 1000 μM (Fig. 1). PV and PMo were significantly more potent than their parent compounds. PV inhibited pNPPase activity, with IC50 = 0.3 μM; for PMo, IC50 = 20 μM (Fig. 1). Around 20–25% of the pNPPase activity remained at high concentrations of PV or PMo, similar to previous observations with PV (24). Similar results were also obtained when the cells were exposed in PBSS.

The pNPPase assay is simple and rapid, but it depends on the amount of cellular protein. On the other hand, induction of altered phosphorylation status of the gap junction protein Cx43, as evaluated by Western blot (7, 8, 12, 16), is less sensitive to variations in the amount of protein. Cx43 was therefore used as the principal model system to study the stability and inactivation of PV and PMo. The inhibition of pNPPase was used as a complementary approach.

**Inactivation under Normal Cell Culture Conditions**

In control cells, Cx43 showed a pattern of three major and some minor bands (Fig. 2). The lower major band is the non-photophosphorylated (NP) form of Cx43, and the two upper major bands are phosphorylated forms (P1 and P2) (12). The P2 band was sometimes split into a double band (e.g. Fig. 2D), and a faint band (P*) often appeared immediately above the NP band. Cells exposed to PV or PMo for 15 min showed prominent changes in the phosphorylation status (Fig. 2A, B, and C; see also densitometric scans in Fig. 3C), characterized by decreased intensity of the NP band, a smear in the P1-P2 area, and the appearance of a band (P*) immediately below P1. The intensity of the P* band could vary somewhat between the experiments (compare Figs. 2 and 3).

At least some of the PV- and PMo-induced cellular changes are reversible during continuous exposure (7, 16). This could be due to regeneration of phosphatase activity in the cells (see below) and/or a decrease in concentration of the biologically active compounds in the medium. A transfer protocol was employed to investigate the latter possibility. One dish was exposed, and after 15 min, the spent medium was transferred to previously unexposed cells, and so on. PV (10 μM) was inactivated within 45–60 min, i.e. the active concentration of PV in the medium had become <2 μM (Fig. 2C). The biological activity of PMo (300 μM) decreased to below detectable level, i.e. <70 μM, within 60–75 min (Fig. 2D). Thus, the concentration of the biologically active compounds decreased during normal cell
FIG. 2. Inactivation of PV and PMo under normal cell culture conditions. A, dose response to PV. NP and phosphorylated (P', P, P1, and P2) forms of Cx43 are marked on the right. B, dose response to PMo. C, cells were exposed to 10 μM PV in growth medium. Every 15 min, the medium was transferred to another dish, and samples for Western blotting were immediately prepared from the previous dish. The numbers indicate the time from addition of PV to the medium. D, the cells were treated as in C, but 300 μM PMo was used.

FIG. 3. Dose responses for PV and PMo in cells incubated in PBSS. The compounds were diluted in PBSS before addition to the cells. The exposure period was 15 min. Cx43 band pattern was detected as described under “Materials and Methods.” A, dose response to PV. B, dose response to PMo. C, densitometric scans of the blots shown in A and B. The short lines under the start of each curve correspond to the base line of the densitogram. The curve for 30 μM PMo is dashed to better distinguish it from the neighboring curve.

Properties of Pervanadate and Permolybdate

Catalase has traditionally been added to the PV stock solutions to break down excess H₂O₂. PV has been claimed to be rather resistant against catalase (10, 17). Our preliminary observations did not fully support this interpretation. It was first necessary to investigate the stability of the compounds in a buffer without catalase. We chose PBS as this is a buffer that our cells tolerate well during incubations of up to 30 min and because phosphate is present in the medium. Both PV and PMo were relatively stable (no measurable breakdown) for at least 6 h when kept in stock solution at room temperature and protected from light as measured by the Cx43 and pNPPase assays (not shown). When incubated in diluted solutions (0.15–20 μM PV or 6–300 μM PMo), the compounds showed an evident time-dependent breakdown (Fig. 4A). After 6 h of incubation, around 20–30% of the original potencies were present as indicated by both Cx43 and pNPPase assays (Fig. 4A).

PV and PMo were clearly sensitive to catalase. The compounds lost their ability to induce the characteristic band shift of Cx43 in a manner dependent on both the incubation time with catalase and the amount of catalase, but PMo was 100-fold more sensitive to catalase (Fig. 4B). The reason for the higher sensitivity to catalase of PMo is uncertain, but in crystals, the Mo–O₅ bonds are 0.1 Å longer than the V–O₅ bonds (25). There may, therefore, be less steric hindrance involved in catalase approaching the peroxy-groups in PMo. The inactivation of PV by exogenous catalase is apparently in contrast to previous results (17), in which a rapid inactivation of PV was found when catalase was added simultaneously with the mixing of vanadate and H₂O₂. The inactivation was slower when catalase was added 10 min or more after mixing (17). When catalase was added in the present experiments, it was done 15 min after mixing of metal salts and H₂O₂. The catalase-induced inactivation is dependent on the amount of added enzyme (Fig. 4B). By extension, the inactivation also depend on the specific activity of the enzyme. Our results are consistent with analytical work showing that PV is degraded by catalase at a rate of 1–2% of that of H₂O₂ (26). Interestingly, high concentrations of PV partly inhibit the catalase action on H₂O₂ (26).

Inactivation by FCS or DMEM

Various concentrations of PV or PMo were incubated in PBSS with 10% FCS or in DMEM (without FCS) for periods between 0 and 6 h before exposure to the cells for 15 min. The mixing of the compounds with PBSS/FCS rapidly (1–3 min, see “Materials and Methods”) inactivated around 30% of the activity in the diluted solutions (Fig. 5A). Thereafter followed a slower breakdown, leaving 5–10% of the activity after 6 h of incubation (Fig. 5A).

DMEM was an efficient inactivator of the diluted compounds. By 1–3 min after mixing, 70–80% of the biological
FIG. 4. Breakdown of PV and PMo in PBSS and by catalase. A, spontaneous breakdown. The compounds were diluted in PBSS to concentrations of 0.2–30 μM for PV (□) and 6–600 μM for PMo (▵), incubated at 37 °C in the dark for the given periods, and then added to the cells. The remaining biological activity was determined by Cx43 band changes (□) or pNPPase activities (▵ dotted curve). The symbol might be slightly displaced to avoid overlapping. B, inactivation by catalase. The compounds were diluted to 1 mM in PBSS and added catalase. The mixtures were incubated at 37 °C in the dark for the given periods and then added in various concentrations to the cells in PBSS for 15 min to determine the remaining biological activity as described. ■, PV and 2 μg/ml catalase; □, PV and 20 μg/ml catalase; ◆, PMo and 0.02 μg/ml catalase; ○, PMo and 0.2 μg/ml catalase.

activity had disappeared. After 1 h of preincubation, less than 5% of the activity remained (Fig. 5B). Indeed, in contrast, 1 mM PV was preincubated in DMEM for 1 h and then diluted and assayed in PBSS, around 70% of the activity remained (not shown). This suggests that there are ingredients at relatively low concentrations in the DMEM that rapidly inactivate the compounds. A practical implication is that the potencies of PV and PMo are partly dependent on the exact procedure during the exposure.

Reaction of Peroxocompounds with Cysteine

The reaction of thiols with PV and PMo is of interest for two reasons. First, cysteine is the reactive part of glutathione, a major detoxification system in cells. Second, PV and PMo are PTPase inhibitors. All PTPases have a cysteine residue in the active site, which could be a participant in the inhibitory effect of the peroxocompounds. We have therefore used cysteine as the major model compound in studies of the reaction between thiol and peroxocompounds. It has been reported that glutathione could react with PV and PMo in a 1:1 molar ratio (15).

The consumption of cysteine was measured during the reaction with the peroxocompounds by a spectrophotometric assay (Fig. 6). We also determined the remaining biological activity after such reactions (see Fig. 11). H2O2 caused 5–15% of the cysteine to be consumed within 5 s. The curves approximately followed one-phase exponential decay irrespective of whether 1, 2, or 3 molar equivalents of cysteine were added. There was no remaining cysteine after 15 min of reaction time (Fig. 6A).

Vanadate needed about 2 h to consume an equimolar amount of cysteine, and the curve followed an approximately linear decay (Fig. 6B). PV showed a more complex consumption curve. There was a very rapid initial decrease in cysteine, corresponding to approximately one consumed cysteine during the first 5 s, and then another cysteine consumed at 120 s (Fig. 6B, inset). The subsequent decrease was approximately linear with time, paralleling that of vanadate-induced removal of cysteine (Fig. 6B).

Indeed, UV spectrophotometric measurements showed that the reaction product of PV and thiol possessed a peak at the same wavelength (265 nm at pH 11.0) and height as expected for vanadate, but with higher absorbance at lower wavelengths (not shown). The peak was fully developed at 1.5–2 molar equivalents of cysteine added.

Molybdate reacted very slowly with cysteine. After 24 h of reaction, 60% of the cysteine still remained (Fig. 6C). As a control, reaction mixtures with only cysteine contained 81 ± 6% (mean ± S.D.; n = 11) of free SH groups after 24 h (Fig. 6C). PMo consumed approximately two cysteines during the first 5–10 s and another cysteine within 30 s (Fig. 6C). The fourth cysteine was consumed within 5 min. UV spectrophotometric measurements showed that the reaction product of PV and thiol possessed a peak at the nearly the same wavelength as molybdate (250 versus 253 nm, measured in 1:1 mixture of 50 mM glycine, pH 3.0, and PBSS) but 5–10% higher than molybdate (not shown). The peak was fully developed at 3.5–4 molar equivalents of cysteine added.

The results shown in Fig. 6 are in contrast to results obtained by Li et al. (15). They found both PV and PMo to consume thiol at a 1:1 ratio. We therefore measured the consumption of thiols using a procedure that more closely approached their conditions (Hepes buffer, constant concentration of thiol, and varying the concentration of PV or PMo). We still found the consumption of thiol to be 2:1 and 4:1 (thiol: peroxocompound) for PV and PMo, respectively (not shown).
respectively. The line metal salts, vanadate and molybdate, are shown in reactions between the 1:1 ratio of cysteine and the parent (f), heteroliganded compounds may therefore be less cystine during a 4-h period than does vanadate, but less than (29), heteroliganded PV compounds oxidize more cysteine to H$_2$O$_2$. This can be explained by the more strained, and therefore more reactive, bonds in the triangular V/Mo structure than in the H$_2$O$_2$ molecule. According to Shaver et al. (29) lend support to the concept that the bio-logical effects of peroxocompounds are due to reaction with the attacking thiol, or the thiol is attacking via the position occupied by water in PV but by the heteroligand in the heteroliganded compounds. Both our observations and the results of Shaver et al. (29) lend support to the concept that the bi- logical effects of peroxocompounds are due to reaction with the active site cysteine in PTPases (30) and other enzymes. PMo and H$_2$O$_2$ probably act through a similar mechanism.

The difference may partly be explained by their suboptimal generation of PMo and their use of catalase to remove excess H$_2$O$_2$.

The initial rate of reaction with cysteine was PMo > PV > H$_2$O$_2$. This can be explained by the more strained, and therefore more reactive, bonds in the triangular V/Mo—O$_{peroxo}$ structure than in the H$_2$O$_2$ molecule. According to Shaver et al. (29), heteroliganded PV compounds oxidize more cysteine to cystine during a 4-h period than does vanadate, but less than H$_2$O$_2$. The heteroliganded compounds may therefore be less reactive. Thus, the heteroligand may cause sterical hindrance for the attacking thiol, or the thiol is attacking via the position occupied by water in PV but by the heteroligand in the heteroliganded compounds. Both our observations and the results of Shaver et al. (29) lend support to the concept that the bi- logical effects of peroxocompounds are due to reaction with the active site cysteine in PTPases (30) and other enzymes. PMo and H$_2$O$_2$ probably act through a similar mechanism.

It was previously observed that PMo induced a faster accumulation of phosphotyrosine in cells than did PV (7, 16). We asked whether this was reflected in a faster inhibition of pNPPase activity in intact cells due to the faster reaction with thiols. Concentrations that resulted in 60–70% inhibition of pNPPase activity were chosen. PMo (30 μM) caused a near immediate 50% inhibition of pNPPase activity (Fig. 7). PV (1 μM) gave a near immediate 20% inhibition, followed by a slow increase in the inhibition up to 15 min (Fig. 7A). A higher concentration of PV (30 μM) caused a near immediate 45% inhibition of pNPPase, and maximal inhibition was obtained after 1 min (Fig. 7A). The difference is therefore apparently caused by a larger concentration gradient over the cell membrane.

The reversibility of pNPPase inhibition was investigated. PV oxidizes the cysteine in the active site of PTPases to its -SO$_3$H derivative (30) and is therefore assumed to be an irreversible inhibitor. However, the cellular tyrosine phosphorylation, changes in Cx43 band pattern, and the decreased gap junctional intercellular communication are readily reversible for the lower concentrations of the compounds (7, 16). Cycloheximide (10–100 μM) did not appreciably affect pNPPase activity during 4-h exposures (not shown), suggesting a low turnover of pNPPases. PV and PMo caused inhibition of pNPPase activity that only slightly and slowly recovered during continuous exposure in growth medium (Fig. 7B). Very similar curves were obtained if the cells were exposed to the compounds for 15 min, washed, and allowed to recover for periods between 15 min and 4 h (not shown). Two explanations can be offered for the seem-
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Enzymes Inhibited by Amitrole—From the results above, endogenous catalase could be one candidate for an inactivation system of the compounds. Catalase (and some other peroxidases) is inhibited by amitrole (see Ref. 33). Incubation overnight with 6 mM amitrole decreased catalase activity to about 20% of normal (33, 34). Amitrole had no effect on Cx43 band pattern alone, and there were minimal changes in the dose response of Cx43 to PV or PMo in cells pre-exposed to amitrole (not shown).

A transfer protocol was used to evaluate the ability of cells to inactivate PV and PMo. The cells were exposed to the compounds in PBSS for 30 min before transfer of the buffer to the next dish. PMo (100 μM) was inactivated by nonpretreated cells after two 30-min incubations (Fig. 8, A and B, left panels). In amitrole-treated cells, PMo was inactivated substantially slower, needing four or five 30-min incubations before decreasing below detectable levels (Fig. 8, A and B, right panels). More than 6 h is needed to spontaneously inactivate 100 μM PMo (not shown, but compare Figs. 3B and 4A). Thus, PMo is at least partly inactivated by enzymes inhibited by amitrole.

When the cells were exposed to 5 μM PV, the Cx43 band pattern normalized after two or three transfers, i.e. 1–1.5 h of incubation (not shown). More than 6 h is needed to spontaneously inactivate 5 μM PV (not shown, but compare Figs. 3A and 4A). Thus, the cells inactivated PMo around 20-fold faster than PV. Amitrole-treated cells lagged only slightly behind the inactivation of PV by the control cells (not shown). This suggested that cellular catalase (or other amitrole-inhibited peroxidases) is only of minor importance for the inactivation of PV. The cells used in the present work are embryonic fibroblasts with a relatively low content of catalase (34, 35). Endogenous catalase may be of greater importance in cells with high catalase content. For example, rat hepatocytes have approximately 400-fold higher activity of catalase than Syrian hamster embryo cells (35). The difference in sensitivity of PV and PMo to endogenous catalase is consistent with the sensitivity of the compounds for exogenous catalase (Fig. 4B).

Cellular Thiols—NEM was used to deplete the cells for thiols. Nonprotein thiols (i.e. mainly glutathione) were measured in cells exposed to NEM and peroxocompounds for 15 min. Unexposed cells contained 3.54 ± 0.74 nmol of nonprotein thiols/10⁶ cells (Table I). This corresponds to an intracellular concentration of approximately 2 mM, assuming a cellular volume corresponding to a radius of 7.5 μm. NEM (30 μM) significantly depleted the nonprotein thiols in cells (Table I). After removal of NEM (30 μM), the level of cellular nonprotein thiols remained at the same low level for more than 30 min (not shown). The lower biologically active concentrations of PV (up to 30 μM) or PMo (up to 300 μM) did not significantly affect the level of cellular nonprotein thiols (Table I), whereas a high concentration of H₂O₂ (10 mM) did. Thus, the biological effect of PV and PMo is not mediated through a general decrease in glutathione.

### Table I

| Exposure | 1 μM | 3 μM | 10 μM | 30 μM | 100 μM | 300 μM | 1 mM | 3 mM | 10 mM |
|----------|------|------|-------|-------|--------|--------|------|------|-------|
| NEM      | 88 ± 17 | 67 ± 22 | 59 ± 27 | 25 ± 9a | 24 ± 11a |
| PV       | 103 ± 21 | 101 ± 10 | 113 ± 27 | 104 ± 25 |
| PMo      | 99 ± 7 | 92 ± 10 | 88 ± 10 | 84 ± 6 |
| H₂O₂     | 96 ± 10 | 90 ± 7 | 82 ± 6 | 67 ± 8a |

* a p < 0.05 calculated on the basis of the amount of non protein thiols/10⁶ cells (analysis of variance with Dunn’s multiple comparisons).
NEM at 100–300 μM showed some phosphotyrosine-inducing effect (Fig. 9A, upper panel), and it inhibited pNPPase activity with IC$_{50}$ = 30 μM (Fig. 1). This is consistent with previous observations (36). Cells exposed to PV or PMo in PBSS for 15 min strongly increased the amount of phosphotyrosine at concentrations at and above 1 and 20 μM, respectively (Fig. 10A and data not shown).

Interestingly, the sequential exposure to NEM (30 μM, giving approximately 50% inhibition of pNPPase activity) and PV or PMo did not increase the maximal inhibition of pNPPase (Fig. 1). Furthermore, the pre-exposure to NEM did not appreciably affect the IC$_{50}$ values of PV or PMo (calculated from the difference between the inhibition by 30 μM NEM and the maximal inhibition; Fig. 1). The sequential exposure to NEM (30 μM for 15 min in growth medium) and peroxocompounds (various concentrations for 15 min in PBSS) caused a slight increase in cellular phosphotyrosine relative to peroxocompounds alone at low concentrations (compare Fig. 10, A and B). The maximal amount of phosphotyrosine obtained at higher concentrations of the peroxocompounds after the sequential exposure was not substantially affected (Fig. 10, A and B).

NEM affected the Cx43 band pattern only at high concentration (300 μM) (Fig. 9A, lower panel, and B). The sequential exposure to NEM (30 μM for 15 min) followed by PMo did not change the concentration of PMo where the first alterations in the Cx43 band pattern started to occur, but the response pattern of Cx43 was different (compare Figs. 2B, 3B, and 10C). The P'$^*$ band, or a band very close to this position, became much more evident in the NEM/PMo exposed cells relative to other exposures. Furthermore, the smear in the P1-P2 area was

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Fig. 9. NEM affects cellular tyrosine phosphorylation and Cx43. Cells were exposed to various concentrations of NEM in growth medium for 15 min before samples were made. A, upper panel, blot developed with anti-phosphotyrosine as the primary antibody; lower panel, the same samples developed with the anti-Cx43 antiserum. B, densitometric scans of the Cx43 blot from A. Only the control and the 100 and 300 μM NEM lanes are shown. Note the changes occurring from 100 to 300 μM NEM in the P'$^*$ area.

Fig. 10. NEM pre-exposure affects the responses to PMo. Cells were treated with PMo in PBSS (A) or pre-exposed to 30 μM NEM in growth medium before the exposure to PMo in PBSS (B and C). The blots were developed for detection of phosphotyrosine (A and B) or Cx43 (C). The phosphotyrosine blots have been relatively weakly developed to avoid saturation, causing bands in the control lanes not to be visible. D, cells were pre-exposed to 30 μM NEM in growth medium for 15 min, rinsed, and then exposed to 100 μM PMo in PBSS. Every 30 min, the PMo/PBSS was transferred to next dish (also pre-exposed to NEM), and the previous dish was sampled for Western blotting. The blot was developed for detection of Cx43. The corresponding control (no preincubation to NEM) is shown in Fig. 8 (left panel). E, densitometric scans of key lanes from C and D. Note that the Cx43 P'$^*$ is very prominent in cells sequentially exposed to NEM and PMo. Note also that the normalization of band pattern is delayed in cells sequentially exposed to NEM and PMo (compare with Fig. 8).
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Thiol-induced Inactivation of PV and PMo: Mathematical Model

The remaining biological activity by the peroxocompounds was studied after the reaction with thiols. Thiols (cysteine or DTT) were added to PV (1 mM in PBSS) in ratios of 0.5:1, 0.75:1, or 1:1 (molar equivalents of thiol groups: PV). After an incubation period of 20 min, the mixture was added to cells in PBSS to obtain concentrations of 0.15 to 100 μM PV (in PBSS) using small increments in concentration, especially below 1 μM. Although 1.5 mM cysteine nearly abolished the biological activity of PV (>1–3% left), around 10, 20, 40, and 50% activity was present after incubation with 1.25, 1, 0.75, and 0.5 molar ratios of thiols, respectively (Fig. 11A). Similar to the reactions described in Fig. 6, PMo needed double the amount of thiols relative to PV for the inactivation (Fig. 11B).

We first considered PV as the model compound. PV was assumed to possess two initially equal peroxy groups per metal ion (7). The thiol compound was assumed to be inactivated by the reaction. The nonperoxocompound (presumably vanadate, see Fig. 6B and the corresponding text) was assumed to be biologically inactive in the present system (7) and not to interact with thiol groups over the time scale of interest (see Fig. 6B). We assumed the following reactions:

\[ \text{Diperoxon} \xrightarrow{k_2} \text{Monoperoxon} \xrightarrow{k_1} \text{Nonperoxon} \]

\[ \text{SH} \xrightarrow{k_5} \text{SH} \]

Some simple models can easily be rejected or are not likely. For example, suppose that thiols react much more efficiently with the diperoxocompound than with the monoperoxocompound, i.e., \( k_2 \gg k_5 \). Then all diperoxocompound molecules will react with thiols before any reaction between thiols and the monoperoxocompounds occur. In this case, the best fit is obtained when monoperoxovanadate has a biological activity of 16 ± 5% (mean ± S.D.) relative to diperoxovanadate. This model gives a break in the curvature at 1 thiol added, with

\[^{3}\text{S.-O. Mikalsen, unpublished observations.}\]
linear curves between 0 and 1, and between 1 and 2 added thiols (not shown). This appears not to fit well with the measured biological activity. Conversely, when $k_1 \gg k_2$, the monoperoxocompound will only exist in negligible amounts, and the biological activity should decrease linearly with the amount of thiols added up to 2. Thus, $k_1 \gg k_2$ can be dismissed, and it is not likely that $k_2 \gg k_1$.

We assumed that the concentrations of the various peroxidedation states $C_0$ (nonperoxovanadate), $C_1$ (monoperoxovanadate), and $C_2$ (diperoxovanadate), and the concentration of thiol groups (as cysteine or DTT), $C_{SH}$, were governed by the following reaction equations.

$$\frac{dC_2}{dt} = -k_2 \cdot C_2 \cdot C_{SH} \quad \text{(Eq. 1)}$$

$$\frac{dC_1}{dt} = k_2 \cdot C_2 \cdot C_{SH} - k_1 \cdot C_1 \cdot C_{SH} \quad \text{(Eq. 2)}$$

$$\frac{dC_{SH}}{dt} = -(k_2 \cdot C_2 + k_1 \cdot C_1) \cdot C_{SH} \quad \text{(Eq. 3)}$$

As expected, combining Equations 1–3 gave the equation,

$$\frac{d[C_1 + C_2]}{dt} = \frac{dC_{SH}}{dt} \quad \text{(Eq. 4)}$$

because the consumption of reducing and oxidizing equivalents must be equal. It can be seen from Equation 4 that if the biological activity of the diperox state is twice that of the monoperox state, the biological activity would decrease linearly with the amount of thiol used. The data in Fig. 11 show a curve convex toward the thiol axis, indicating that the diperox state has more than twice the biological activity of the monoperox state.

If we assumed that the diperox state alone has biological activity, the curve could be found when we solved for $C_2$ as a function of $C_{SH}$. The solution was particularly simple when $k_{rel} = k_2/k_3 = \frac{1}{2}$, i.e. when the thiol reacted independently and with the same rate with each peroxo group. We assumed a prior complete peroxidation, i.e. the initial concentration of the monoperox state was zero ($C_1 = 0$). This is in agreement with the published reaction constants (37), giving an equilibrium concentration of $C_2 = 98\%$ and $C_1 = 0.2\%$ of the vanadate supplied. We further assumed that the reaction with thiol was complete, i.e. the final concentration of thiol was zero ($C_{SH} = 0$). The final concentration of the diperox state ($C_{dip}$) was then shown by the equation,

$$C_d = C_2 \cdot (1 - (C_{SH}/C_{dip})^{1/4}) \quad \text{(Eq. 5)}$$

where $C_{2i}$ and $C_{SHi}$ are the initial concentrations. In Fig. 11A, the curve corresponding to Equation 5 is depicted. It can be seen that this curve indicates a biological activity slightly higher than the measured biological activity. This suggested that $k_{rel} < \frac{1}{2}$.

When $k_{rel} \neq \frac{1}{2}$, the inverse solution was as follows,

$$C_{SH} = C_2 \cdot (2 - (2 - \ln(C_d/C_2)) \cdot C_d/C_2) \quad \text{(Eq. 6)}$$

for $k_1 = k_2$, and

$$C_{SH} = C_2 \cdot (2 - (1 - k_2/k_1) \cdot C_d/C_2 - k_2/k_1 \cdot (C_d/C_2)^{1/k_2}) \quad \text{(Eq. 7)}$$

for $k_1 \neq k_2$.

From the discussion above, it was unlikely that $k_1 = k_2$. Using Equation 7, the numerical solutions gave the best fit to the measured biological data when $k_{rel} = k_1/k_2 = 0.31 \pm 0.07$. (Note that if the alternative value for the extinction coefficient, $e_{380} = 3550 \text{ M}^{-1} \text{cm}^{-1}$, was used, a reasonable fit was achieved when $k_{rel} = 1.02 \pm 0.30$ if monoperoxovanadate has no biological activity. On the other hand, a better fit was produced if we assumed that $k_{rel} = 0.6$. Then, monoperoxovanadate would have a biological activity of $14 \pm 3\%$ relative to diperoxovanadate.)

For PMo, a similar model was employed, with the exception that four reducing equivalents of thiols were used to completely reduce the highest peroxidation state. The biological activities and the theoretical curves corresponding to Equation 5 are shown in Fig. 11, A and B. The best fit was obtained at $k_{rel} = 0.33 \pm 0.06$. We are presently not able to explain why PMo can consume four thiols versus two thiols for PV, but we note that measurements by three methods (thiol consumption, UV spectrophotometry, and biological activities) are consistent with respect to this difference.

From the above results, it seems likely that the monoperox state of PV has a low biological activity, at maximum 20% relative to diperoxovanadate. Both diperoxovanadate and monoperoxovanadate are assumed to possess a seven-coordinated pentagonal bipyramidal geometry (25, 38, 39). Because of the reactivity of the peroxo group with thiols, we had expected the monoperoxovanadate to have a considerable biological activity. In support, some heteroliganded monoperoxovanadates show PTPase inhibitory effects of the same degree as the corresponding heteroliganded diperoxovanadates, as determined by insulin receptor dephosphorylation in rat liver endosomes (40). In contrast, Huyer et al. (30) used the previously calculated equilibrium constants for the peroxo forms of vanadate (37) to conclude that the monoperoxovanadate is a less potent inhibitor of PTP1B, adding that the monoperox form may not be inhibitory at all. We note, however, that Huyer et al. (30) added 0.4 $\mu$M of peroxovanadates to a reaction mixture containing 10 $\mu$g/ml catalase, 5 mM DTT, and 200 ng/ml PTP1B. Thus, there appear to be ample possibilities for adverse reactions in their assay. Therefore, it is still premature to conclude that monoperoxovanadate is devoid of all biological activity. To this end, more analytical methods, e.g. NMR, must be used together with measurements of biological activity.

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