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# ACTIVATION OF GLYCOGEN PHOSPHORYLASE IN RAT PHEOCHROMOCYTOMA PC12 CELLS AND ISOLATED HEPATOCYTES BY ORGANOPHOSPHATES

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**Abstract**—Several organophosphates including diisopropylfluorophosphonate (DPF) and a variety of compounds used as chemical warfare agents produced dose- and time-dependent increases in phosphorylase-*a*, the phosphorylated form of glycogen phosphorylase in rat pheochromocytoma cells, PC12, and isolated hepatocytes. Increases in phosphorylase-*a* did not occur in cells exposed to the carbamates, physostigmine or pyridostigmine, or to *O*-ethyl *S*-2-diisopropylaminoethylmethyl-phosphonathiolate (VX), an organophosphate which is protonated at physiological pH. When extracellular pH was increased to pH 8, VX acted like the other organophosphates and increased phosphorylase-*a* activity. The possibility that organophosphates increase phosphorylase-*a* in intact cells by releasing $Ca^{2+}$ from intracellular binding sites is supported by the following findings: organophosphate-induced increases in phosphorylase-*a* did not correlate with changes in cyclic AMP in the two cell types studied; in PC12 cells, increases in this activity occurred in the absence of extracellular calcium and were not inhibited by the calcium channel blocker, verapamil; fluorescence of the calcium sensitive dye, Quin-2, in PC12 cells preloaded with the acetoxymethyl ester of the dye was increased by soman; finally, addition of the calcium ionophore, A23187, to PC12 cells maintained in calcium-free medium caused sarin-stimulated phosphorylase-*a* activity to return rapidly to basal levels. Collectively, these data argue strongly that organophosphates increase phosphorylase-*a* activity in intact cells via a novel mechanism involving release of calcium from intracellular binding sites.

The possibility that organophosphates enhance $Ca^{2+}$-dependent events in intact cells is suggested by a number of recent findings. Release of histamine from mast cells [1] and acetylcholine from presynaptic elements of the neuromuscular junction [2] is increased after exposure of these cells to organophosphates. Organophosphates have also been found to stimulate calcium-dependent population spikes of electrical activity in hippocampal slices [3] and to enhance calcium-induced calcium release in muscle fibers [4]. Because of the important function of calcium as a second messenger within cells [5] and its role in cellular toxicity [6, 7], we evaluated the actions of organophosphates on the activity of phosphorylase-*a* in rat pheochromocytoma PC12 cells and isolated hepatocytes, two distinct cell types used widely in studies of intracellular calcium homeostasis [8–11]. Conversion of phosphorylase-*b* to phosphorylase-*a* provides an indirect measure of changes in cytosolic $[Ca^{2+}]$ in intact cells since phosphorylase-*b* kinase is stimulated by increases in cytosolic free $[Ca^{2+}]$ over the range of 10 nM to about 2 $\mu$M [12–14], concentrations known to exist in most cells under physiological conditions [5, 15]. Quin-2, a calcium-sensitive dye that allows the continuous monitoring of $[Ca^{2+}]$ in cell suspensions [16], was also employed as an independent index of the action of organophosphates on cytosolic calcium.

Results reported below indicate that a number of organophosphates, including DFP (diisopropylfluorophosphonate), soman (*O*-pinacolyl methylphosphonofluoridate), sarin (*O*-isopropyl methylphosphonafluoridate), tabun (*O*-ethyl *N,N*-dimethylphosphoramid), and VX (*O*-ethyl *S*-2-diisopropylaminoethylmethyl-phosphonathiolate), increase phosphorylase-*a* activity in PC12 cells and hepatocytes. In contrast, two carbamates, physostigmine and pyridostigmine, did not increase the activity of this enzyme in the two cell types. Increases in phosphorylase-*a* activity triggered by organophosphates did not correlate with changes in cyclic AMP (cAMP), occurred in the absence of extracellular calcium, and were not inhibited by the calcium channel blocking agent verapamil.

## MATERIALS AND METHODS

*Cells.* Undifferentiated PC12 cells orginally isolated by Greene and Tischler [17] were maintained in 75 $mm^2$ flasks containing Dulbecco's Minimum Essential Medium (high glucose) supplemented with 10% fetal calf serum and 5% horse serum. Prior to treatment with organophosphates, cells were harvested and resuspended to a cell density of 1–1.5 $\times$ $10^6$ cells/mL in Krebs–Henseleit bicarbonate buffer. Aliquots (0.5 mL) of the cell suspension were transferred to 1.5-mL plastic microfuge tubes and equilibrated at 37° for 10 min in an incubator gassed with 5% $CO_2$ and air prior to addition of organophosphates.

Hepatocytes were isolated from adult male rats

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(150–225 g), anesthetized with sodium pentobarbital (75 mg/kg), according to established methods [18, 19]. Viable hepatocytes were isolated by centrifugation through a 30% Percoll gradient [20] and were resuspended ($1 \times 10^7$ cells/mL) in Krebs–Henseleit bicarbonate buffer containing 2% bovine serum albumin. Suspensions of hepatocytes were kept on ice and gently bubbled with a mixture of 95% $O_2$:5% $CO_2$ until used for experiments. Viability of isolated cells was greater than 95% as indexed by trypan blue exclusion. Isolated hepatocytes were preincubated in Krebs–Henseleit bicarbonate buffer for 30 min at 37° under an atmosphere of 95% $O_2$:5% $CO_2$ prior to the addition of organophosphates to allow cells to recover basal phosphorylase-*a* activity.

*Phosphorylase determinations.* Cells were quickly sedimented by brief (30 sec) centrifugation at 8000 *g* in an Eppendorf microfuge, and supernatant fractions were removed and the cell pellets frozen immediately by immersing the microfuge tubes in a dry ice/methanol bath. After collection of all experimental samples, the cells were resuspended in 50 μL of 10 mM sodium phosphate and lysed by sonication followed by addition of 100 mM NaF and 20 mM EDTA to inhibit phosphatase and phosphorylase kinase as described previously [21]. Phosphorylase-*a* activity in PC12 cells and hepatocytes was determined fluorometrically (340 → 420 nm) by transferring 10 μL of the cell lysate to 1 mL of reagent containing: 0.02 M imidazole-HCl, pH 7.0, 2 mM $MgCl_2$, 0.1 mM $NADP^+$, 0.02% bovine serum albumin, 0.5 mM EDTA, 50 mM NaF, 0.5 μM glucose-1,6-diphosphate, 5 mM $K_2HPO_4$, 0.5 mm dithiothreitol, 0.08% rabbit muscle glycogen, 1.6 μg/mL glucose-6-phosphate dehydrogenase and 0.5 μg/mL phosphoglucomutase [22]. After recording a linear rate of phosphorylase-*a* activity, total phosphorylase in PC12 cells was determined by adding 1 mM AMP to the reaction mixture and recording the increased rate of NADPH production.

*Assay of biochemical intermediates.* Cyclic AMP was measured using a radiochemical method based on the displacement of [$^3$H]cAMP from a cAMP binding protein [23]. Adenine nucleotides were measured fluorometrically using coupled enzymatic techniques [24, 25]. Protein was determined according to Lowry *et al.* [26] using bovine serum albumin as a standard.

*Measurement of cytosolic calcium using Quin-2.* PC12 cells ($4 \times 10^6$) were incubated for 5 min with 60 μM Quin-2 acetoxymethyl ester in 2 mL of Krebs–Henseleit bicarbonate buffer containing 2 mM glutamine at 37° in 25-mL plastic Erlenmeyer flasks. After the 5-min loading period, cell suspensions were diluted 8-fold in buffer and resedimented by centrifugation for 5 min at 200 *g*. The cells were suspended and washed twice more with 15 mL buffer. The washed cells were suspended ($2 \times 10^6$ cells/mL) in Krebs–Henseleit bicarbonate buffer and equilibrated for 30 min at 37° in air:$CO_2$ (5%) before being dispersed in 10 × 75 mm culture tubes and treated with organophosphates. Quin-2 containing cells were exposed to organophosphates for 10 min at 37° in a shaking water bath before fluorescence (340 → 490) of the cells was determined using a Farrand filter fluorometer equipped with narrow band interference filters. Maximal fluorescence was obtained by permeabilization of the cells with a small amount of digitonin (about 3 nmol/mg protein). Minimal fluorescence was recorded in the presence of excess ethyleneglycolbis (aminoethylether)tetraacetate (EGTA) (2–5 mM). Cytosolic [$Ca^{2+}$] was calculated from determinations of maximal and minimal fluorescence assuming a $K_d$ for the Quin-2 calcium complex of 115 nM according to Tsien *et al.* [16].

*Reagents.* Organophosphates including soman, sarin, tabun, and VX were provided by the U.S. Army Biomedical Research Laboratory (Aberdeen Proving Ground, MD). Stock solutions (1 mg/mL) of the organophosphates distributed in 100 or 200 μg quantities were kept frozen in sealed vials at −70°. There was no change in the capacity of these stock solutions to inhibit the activity of a standard eel cholinesterase preparation; thus, the stored organophosphates appeared relatively stable over the course of this work. All procedures involving the handling of organophosphate stock solutions were carried out in a hood specially equipped with a charcoal filter system. Diisopropylfluorophosphonate (DFP), atropine, physostigmine and pyridostigmine were obtained from the Sigma Chemical Co. (St Louis, MO). All other reagents were of analytical grade from standard sources.

## RESULTS

Exposure of PC12 cells to various concentrations of DFP, sarin, and soman increased phosphorylase-*a* activity in a dose-dependent manner (Fig. 1). Under the conditions of this experiment, sarin appeared more potent than the other organophosphates with respect to activation of phosphorylase in PC12 cells. Activity of phosphorylase-*a* noted with the highest dose of sarin represented about 85% of the total phosphorylase present in PC12 cells. VX, at concentrations ranging up to 500 μM, did not increase phosphorylase-*a* activity in PC12 cells incubated in Krebs–Henseleit bicarbonate buffer at pH 7.4. Dose-dependent increases in phosphorylase-*a* similar to those seen in PC12 cells occurred in isolated hepatocytes treated with soman and DFP (Fig. 2). Data for PC12 cells and liver are expressed on the basis of phosphorylase-*a* rather than as a percent of total phosphorylase activity because phosphorylase-*b* in liver, unlike the activity in neural tissue, is not activated by AMP [27]. As in PC12 cells, VX did not increase the activity of phosphorylase-*a* in isolated hepatocytes.

The time course of action of various organophosphates and two carbamates on phosphorylase-*a* activity in PC12 cells is illustrated in Fig. 3. Maximal increases in phosphorylase-*a* activity occurred about 5 min after exposure of the cells to soman, sarin and DFP. Activity noted at 10 min in cells treated with each of the organophosphates was about 60% of total phosphorylase activity. VX had no effect on enzyme activity over the entire time course of the experiment. Activities of phosphorylase-*a* in PC12 cells treated with soman, sarin, tabun and DFP remained elevated for at least 30 min. In contrast to

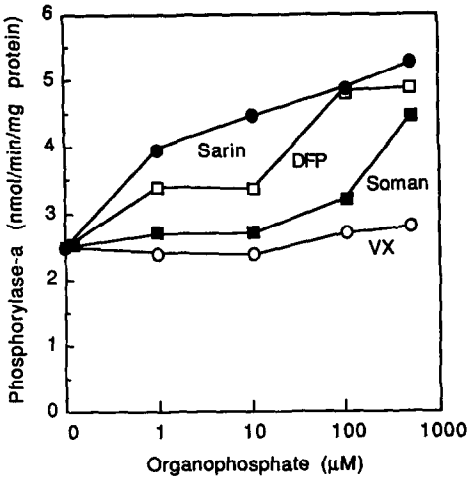


Fig. 1. Phosphorylase-*a* activity in PC12 cells treated with various concentrations of organophosphates. PC12 cells (1–1.5 × $10^6$ cells/mL) suspended in Krebs–Henseleit bicarbonate buffer and equilibrated for 10 min at 37° under a 5% $CO_2$ atmosphere were exposed various amounts of organophosphates [sarin (●), DFP (□), soman (■), and VX (○)] for 5 min. Cells were sampled and assayed for phosphorylase-*a* as described in Materials and Methods. Each point is the average of three replicate samples obtained in a single experiment. Each experiment was repeated three times with essentially the same results. Average phosphorylase-*a* and total phosphorylase activity (mean ± SEM) measured in the absence of organophosphates was 1.9 ± 0.2 and 6.2 ± 1.2 nmol/mg protein/min, respectively, for the three experiments.

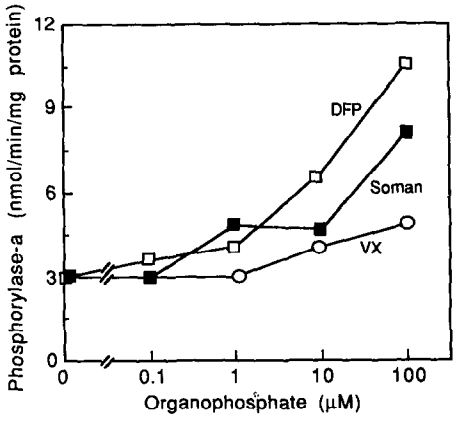


Fig. 2. Phosphorylase-*a* activity in hepatocytes treated with various concentrations of organophosphates. Hepatocytes (1 × $10^6$ cells/mL) were preincubated in Krebs–Henseleit bicarbonate buffer under an atmosphere of 95% $O_2$:5% $CO_2$ at 37° for 30 min prior to addition of various amounts of organophosphates [DFP (□), soman (■), and VX (○)]. Cells were exposed to the organophosphates for 5 min prior to being sampled and assayed for phosphorylase-*a* activity as described in Materials and Methods. Values are averages of three replicate samples from a single experiment. The coefficient of variations between replicate samples was less than 5%. Typical experiment.

the organophosphates, physostigmine and pyridostigmine did not increase the activity of phosphorylase-*a* in PC12 cells (Fig. 3).

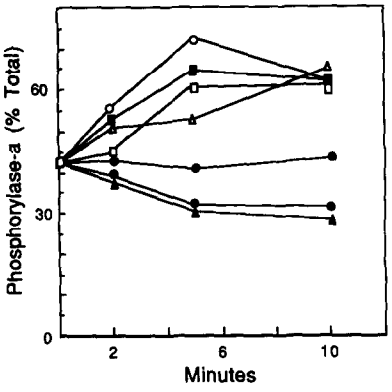


Fig. 3. Phosphorylase-*a* as a percent of total phosphorylase activity in PC12 cells at various intervals in the presence of organophosphates and carbamates. PC12 cells were equilibrated in Krebs–Henseleit bicarbonate buffer as described in the legend to Fig. 1 before additions of organophosphates, 100 μM [soman (○), sarin (□), DFP (■), tabun (△) and VX (●)] or the two carbamates, 100 μM [physostigmine (▲), pyridostigmine (◉)]. Each value is the average of three replicate samples obtained from a single experiment. Experiments were repeated with the same results three times.

The actions of organophosphates on phosphorylase-*a* in hepatocytes as a function of time are shown in Fig. 4. Maximal increases in phosphorylase-*a* activity occurred at about 5 min after treatment of hepatocytes with soman and sarin followed by a gradual decline toward basal activity at 30 min. Phosphorylase-*a* activity in untreated hepatocytes and those exposed to VX remained essentially the same

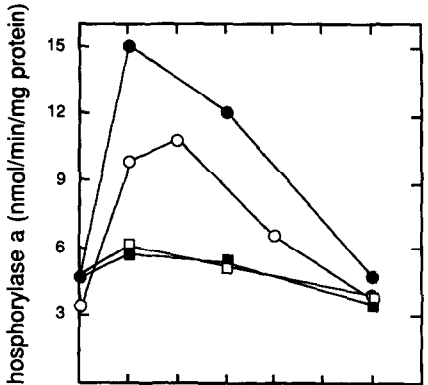


Fig. 4. Effect of organophosphates on phosphorylase-*a* activity in isolated hepatocytes as a function of time. Hepatocytes isolated from rat liver were preincubated in Krebs–Henseleit bicarbonate buffer as described in the legend to Fig. 2 prior to addition of organophosphates, 100 μM [soman (●), sarin (○) and VX(■)] or vehicle (□). Each point is the average of two replicate samples obtained at the various time intervals. A second experiment yielded the same results.

Table 1. Effect of pH on activation of glycogen phosphorylase in PC12 cells by VX

| | Phosphorylase-*a* (% total phosphorylase) | |
|---|---|---|
| pH | Control | VX (100 μM) |
| 7.4 | 41.1 ± 0.7 | 40.3 ± 0.9 |
| 8.0 | 41.1 ± 1.1 | 53.2 ± 2.2* |

PC12 cells ($1 \times 10^6$ cells/mL) were equilibrated in Krebs–Henseleit bicarbonate buffer, pH 7.4, for 10 min prior to addition of VX (100 μM) or VX plus a calculated amount of 0.1 N NaOH to adjust the incubation medium to pH 8.0. Cells were sampled 5 min after the addition of VX and assayed for phosphorylase-*a* and total phosphorylase as described in Materials and Methods. Each value is the mean of four incubations ± SEM. Total phosphorylase activity at pH 7.4 and 8.0 was 4.4 ± 0.3 and 4.2 ± 0.2 nmol/min/mg protein respectively.

* $P < 0.01$ (VX vs control).

Table 2. Effects of atropine, pyridostigmine and physostigmine on activation of phosphorylase in hepatocytes by soman

| Additions | Phosphorylase-*a* (nmol/min/mg protein) |
|---|---|
| None | 2.99 ± 0.24 |
| Soman (10 μM) | 4.77 ± 0.62 |
| +Pyridostigmine (100 μM) | 5.32 ± 0.57 |
| +Physostigmine (100 μM) | 5.51 ± 0.90 |
| +Atropine (2 μM) | 6.13 ± 0.30 |

Hepatocytes were preincubated in Krebs–Henseleit bicarbonate buffer for 30 min prior to additions of soman and other agents as described in Materials and Methods. Carbamates and atropine were added to cell suspensions immediately before the addition of soman. Cells were incubated for 5 min in the presence and absence of the various agents before being assayed for phosphorylase-*a* as described in Materials and Methods. Values are means ±SEM of three preparations.

over the entire time course or the experiment (Fig. 4). The possibility that the organophosphates increased phosphorylase-*a* by interacting with an intracellular site is suggested by data with VX. VX is a tertiary amine and is protonated at physiological pH and thus would not enter cells as readily as the other organophosphates. When the pH of the incubation medium was increased from 7.4 to 8.0, VX acted like the other organophosphates and increased phosphorylase-*a* activity (Table 1).

It is unlikely that increases in activity of phosphorylase-*a* produced by the organophosphates in the two cell types are related to their anticholinesterase activities. Concentrations of organophosphates required to increase phosphorylase-*a* were well above those needed to inhibit cholinesterase which are on the order of 10 nM [28]. Neither pyridostigmine nor physostigmine increased phosphorylase-*a* in PC12 cells (Fig. 3). Moreover, addition of these carbamates as well as atropine, at a concentration that totally inhibits muscarinic binding, did not inhibit the activity of phosphorylase-*a* in hepatocytes treated with soman (Table 2).

Table 3. Effect of organophosphates on cAMP concentrations in PC12 cells and isolated hepatocytes

| | Cyclic AMP (pmol/mg protein) | |
|---|---|---|
| Treatment | PC12 cells | Hepatocytes |
| Control | 10.47 ± 0.71 (8) | 1.67 ± 0.20 (7) |
| Sarin, 100 μM | 12.89 ± 0.90 (3) | |
| Soman, 100 μM | 14.16 ± 1.97 (3) | 2.09 ± 0.20 (5) |
| VX, 100 μM | 13.19 ± 1.28 (6) | 1.92 ± 0.22 (5) |
| DFP, 100 μM | 14.64 ± 1.82 (6) | |
| Adenosine, 100 μM | 16.28 ± 2.16* (6) | |

PC12 cells and isolated hepatocytes were equilibrated and incubated in the presence of various organophosphates or adenosine for 5 min as described in Materials and Methods. Incubations were terminated by addition of 0.3 M $HClO_4$ to the cells. cAMP was measured in neutralized perchloric acid extracts, using a protein binding assay as described in Materials and Methods. Values are means ± SEM of the number of experiments with cell suspensions indicated in parentheses.

* $P < 0.05$ (treated vs control).

The increases in phosphorylase-*a* noted above could occur secondarily to increases in either cytosolic free $Ca^{2+}$, which directly activates phosphorylase kinase [29], or in cAMP, which phosphorylases this kinase via cAMP-dependent protein kinase [19, 30]. The effects of the various organophosphates on cAMP in PC12 cells and isolated hepatocytes are shown in Table 3. Organophosphates, which increased phosphorylase-*a* in PC12 cells, did not increase cAMP in these cells. Adenosine, which is known to stimulate adenylcyclase in a number of cell types [31] increased cAMP significantly ($P < 0.05$) in PC12 cells. Neither soman, which elevated phosphorylase-*a*, nor VX increased cAMP in isolated hepatocytes.

Since phosphorylase-*a* may also increase secondarily to inhibition of cellular energy metabolism and non-specific increases in cytosolic $Ca^{2+}$, we examined the effects of soman on adenine nucleotides in PC12 cells and hepatocytes (Table 4). An effect of the organophosphate on cellular energy metabolism is suggested by the profiles of adenine nucleotides. Although soman did not alter ATP content in PC12 cells, decreases in the ATP/ADP ratios did occur. Soman decreased both the ATP content and the ATP/ADP ratio in hepatocytes.

The effects of soman on phosphorylase-*a* and on cytosolic $Ca^{2+}$ calculated from Quin-2 fluorescence in PC12 cells are shown in Table 5. Measurements were made precisely 10 min after exposure of the cells to organophosphates or vehicle. Soman increased Quin-2 fluorescence. With each pair of matched samples, Quin-2 fluorescence was higher in cell suspensions treated with soman compared to cells treated with vehicle alone. Increases in calcium measured by Quin-2 fluorescence corresponded with increases in phosphorylase-*a* and these increases were not inhibited by the calcium channel blocker verapamil (Table 5).

The possibility that organophosphates increase phosphorylase-*a* by releasing $Ca^{2+}$ from intracellular

Table 4. Effect of soman on adenine nucleotides in PC12 cells and rat hepatocytes

| | ATP | ADP | AMP | ATP/ADP |
|---|---|---|---|---|
| | (nmol/mg protein) | | | |
| PC12 cells | | | | |
| Control (3) | 19.9 ±0.4 | 1.4 ± 0.1 | 0.5 ± 0.04 | 51.0 ± 1.5 |
| Soman | | | | |
| 5 min (3) | 19.0 ± 0.4 | 1.8 ± 0.1 | 0.5 ± 0.01 | 10.8 ± 0.8* |
| 10 min (3) | 18.2 ± 0.8 | 2.0 ± 0.4 | 0.6 ± 0.05 | 9.7 ± 1.6* |
| Hepatocytes | | | | |
| Control (2) | 7.7 | 11.6 | 5.1 | 0.7 |
| Soman | | | | |
| 5 min (2) | 4.8 | 11.6 | 6.6 | 0.4 |

Values are presented as averages or as means ± SEM of the number of preparations shown in parentheses. Cells were equilibrated and incubated in Krebs–Henseleit bicarbonate buffer at 37° in the presence and absence of soman (100 μM) for the times indicated. Incubations were terminated with 0.3 M $HClO_4$, and adenine nucleotides were measured as described in Materials and Methods.

* $P < 0.05$ (soman vs control).

Table 5. Phosphorylase-*a* and [$Ca^{2+}$] in PC12 cells exposed to soman in the presence and absence of verapamil

| Additions | Control | Soman (100 μM) |
|---|---|---|
| | Phosphorylase-*a* (nmol/min/mg protein) | |
| None | 0.44 ± 0.05 | 0.75 ± 0.03* |
| Verapamil, 10 μM | 0.40 ± 0.01 | 1.05 ± 0.20† |
| | [$Ca^{2+}$] (nM) | |
| None | 96.9 ± 28 | 136.9 ± 27.1† |
| Verapamil, 10 μM | 45.9 ± 12.5 | 115.2 ± 12.4* |

Values are means ± SEM of four matched pairs of control and soman-treated cell suspensions. PC12 cells were loaded with Quin-2 acetoxymethyl ester and washed as described in Methods. Quin-2 loaded cells were preincubated in Krebs–Henseleit bicarbonate buffer for 30 min prior to addition of the organophosphates. Cells were exposed to the organophosphate or vehicle for 10 min before measurement of Quin-2 flourescence or phosphorylase-*a*. Data were analyzed statistically using a two-tailed matched pair *t*-test.

* $P < 0.01$ (soman vs control).

† $P < 0.05$ (soman vs control).

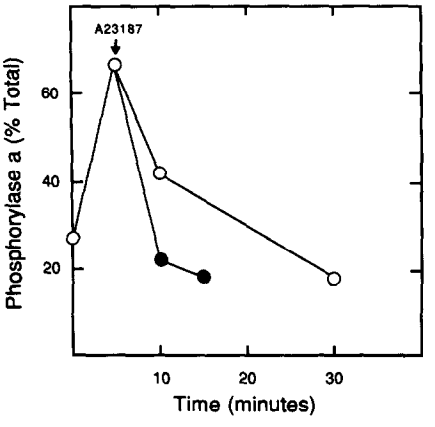


Fig. 5. Effect of sarin on phosphorylase-*a* activity in PC12 cells maintained in calcium-free medium. PC12 cells ($1 \times 10^6$ cells/mL) were preincubated in 1.5-mL plastic microfuge tubes in a calcium-free Krebs–Henseleit bicarbonate buffer containing 0.1 mM EGTA for 10 min prior to the addition of 100 μM sarin. Duplicate sets of incubation tubes were sampled at each time interval and assayed for phosphorylase-*a* and total phosphorylase as described in Materials and Methods. A23187 (1 μM) was added to a second group of cells 5 min after the addition of sarin and at the peak of phosphorylase-*a* activity. Key: sarin alone (○), and sarin + A23187 (●). Values are averages of duplicate samples.

binding sites was studied by employing cells maintained in calcium-free medium. When sarin was added to PC12 cells maintained in $Ca^{2+}$-free medium, the increase in phosphorylase-*a* differed markedly from that produced in cells incubated in $Ca^{2+}$-containing medium (Fig. 5). In contrast to cells maintained in $Ca^{2+}$-containing medium, phosphorylase-*a* reached a peak at about 2 min after addition of the organophosphate and then returned to baseline activity over 30 min of incubation (Fig. 5). Addition of the calcium ionophore, A23187, to these cells at the peak of phosphorylase-*a* activity greatly accelerated the return of activity to baseline values at about 10 min. Addition of A23187 to cells maintained in normal Krebs–Henseleit bicarbonate buffer containing 1.2 mM calcium had an opposite effect to that noted in Fig. 5 and increased phosphorylase-*a* to near maximal activity.

## DISCUSSION

Increases in phosphorylase-*a* noted in PC12 cells and hepatocytes exposed to organophosphates represent a novel action that is not related to their anti-esteratic activity. Relatively high concentrations of organophosphates were required to increase

phosphorylase-*a* activity in intact PC12 cells and rat hepatocytes. For example, 1 μM DFP was required to elevate phosphorylase-*a* in PC12 cells (Fig. 1). Thus, amounts of organophosphates needed to increase phosphorylase-*a* in the two cell types greatly exceeded those required to inhibit cholinesterase which are on the order of 10 nM [28]. Moreover, organophosphate-induced increases in phosphorylase-*a* were not inhibited by atropine or by preincubation with physostigmine or pyridostigmine, two carbamates that would be expected to compete for organophosphate binding sites on cholinesterase (Table 5).

Increases in phosphorylase-*a* activity in PC12 cells and hepatocytes treated with organophosphates most likely occur via activation of phosphorylase kinase by increases in cytosolic $Ca^{2+}$. The delta subunit of phosphorylase kinase is calmodulin [32, 33]. Although cAMP also stimulates phosphorylase kinase via cAMP-dependent protein kinase [30], increases in cAMP did not correlate with increases in phosphorylase-*a* noted in the two cell types (Table 3). Involvement of calcium in organophosphate-induced increases in phosphorylase-*a* is further supported by the action of sarin on phosphorylase-*a* activity in PC12 cells maintained in calcium-free medium. In cells maintained in calcium-containing medium, phosphorylase-*a* declined after reaching a peak at about 5 min (Fig. 5). The decline in activity of this enzyme in cells kept in calcium-free medium was most likely due to extrusion of $Ca^{2+}$ from intracellular sites into the extracellular medium. Acceleration in the decline in sarin-stimulated phosphorylase-*a* activity after addition of the calcium-ionophore A23187 to cells incubated in calcium-free medium (Fig. 5) is consistent with this possibility. In the absence of extracellular $Ca^{2+}$, the ionophore would be expected to facilitate an exchange of protons for intracellular $Ca^{2+}$ across the plasma membrane and cause a rapid decrease in calcium-activated phosphorylase kinase activity.

Phosphorylase-*a* was elevated significantly 5 min after exposure of hepatocytes or PC12 cells to organophosphates; however, the time course of change in activity of this enzyme differed in the two cell types. Phosphorylase-*a* remained elevated in PC12 cells for at least 30 min after addition of organophosphates but declined in hepatocytes after reaching a peak at about 5 min. Differencds in the time course of action of organophosphates on phosphorylase-*a* in the two cell types may be related to different patterns of metabolism of organophosphates in the two cell types. Liver contains very high amounts of carboxylesterase, a 60 kD protein that binds organophosphates with a relatively high affinity [34] and limits their time course of action.

The possibility that the organophospates act at intracellular binding sites to increase phosphorylase-*a* is supported by results obtained with VX. Under normal incubation conditions at pH 7.4 this organophosphate, which is a protonated tertiary amine, did not increase phosphorylase-*a* activity. When the pH of the incubation medium was increased to 8.0, VX acted like the other organophosphates and increased phosphorylase-*a* (Table 1). Experiments with Quin-2 and the calcium channel inhibitor verapamil (Table 5) lend further support to the release of $Ca^{2+}$ from intracellular binding sites. Increases in cytosolic $Ca^{2+}$, calculated from changes in Quin-2 fluorescence, corresponded with increases in phosphorylase-*a* triggered by soman. Addition of the calcium channel blocker did not prevent elevations in phosphorylase-*a* activity and Quin-2 fluorescence (Table 5).

Organophosphates could act at a variety of sites to increase intracellular free $Ca^{2+}$ including phospholipids having a high affinity for $Ca^{2+}$ [35], various high-affinity calcium binding proteins such as calmodulin [36], voltage-dependent [37, 38] and -independent calcium channels, plasma membrane $Na^+/Ca^{2+}$ exchanges [39] and $Ca^{2+}$-dependent ATPases on the plasma membrane and subcellular organelles [40]. Increases in calcium brought about by organophosphates may also occur secondarily to an action of these agents on the inositol phosphate pathway [41]. Several of these sites may be ruled out as possible targets for the organophosphates. Since $Ca^{2+}$ stimulated phosphorylase kinase via interaction with the calmodulin subunit of this enzyme [32, 33], it is unlikely that organophosphates increase cytosolic $Ca^{2+}$ by displacement from high-affinity sites on calmodulin. It is also unlikely that organophosphates interact with voltage-dependent calcium channels because increases in phosphorylase-*a* and Quin-2 fluorescence in PC12 cells treated with soman were not inhibited by verapamil (Table 5). Interaction of organophosphates with the inositol phosphate pathway cannot be ruled out. High concentrations of ethanol have been reported recently to mobilize $Ca^{2+}$ in hepatocytes by activation of phosphoinositide-specific phospholipase C [42]. The possibility that the organophosphates interfered with calcium transport ATPases is also a possibility because declines in ATP/ADP ratios occurred in PC12 cells and hepatocytes treated with soman (Table 4). This result was unexpected since exposure of explants of the rat superior cervical ganglion to soman did not decrease ATP or phosphocreatine [43]. Additional studies are required to determine whether changes in the ATP/ADP ratios noted above precede the increases in phosphorylase-*a* or occur as a consequence of increased energy demands due to elevation of cytosolic $Ca^{2+}$ within organophosphate-treated cells.

Collectively, data presented above argue strongly that increases in phosphorylase-*a* brought about by exposure of PC12 cells and hepatocytes to organophosphates are due to release of $Ca^{2+}$ from intracellular binding sites. Increases in cytosolic calcium may occur secondarily to the actions of a variety of toxic chemicals. For example, there is growing evidence indicating that agents which undergo redox cycling or modify membrane sulfhydryl groups increase cytosolic $Ca^{2+}$ via their capacity to interfere with calcium translocation across the plasma membrane and endoplasmic reticulum [7, 44]. Recently organochlorine insecticides have been reported to increase free calcium within synaptosomes isolated from rat brain [45]. The observed increases in phosphorylase-*a* brought about by exposure of PC12 cells and hepatocytes to organophosphates raise the possibility that elevation of cytosolic calcium may occur in a variety of cell types exposed to high

concentrations of organophosphates. Release of histamine from mast cells exposed to high concentrations of organophosphates (500 $\mu$M) [1] and acetylcholine from presynaptic elements [2] may well be related to the release of calcium from intracellular binding sites [3]. Although the concentrations of organophosphates used above exceed those required for lethality *in vivo*, further consideration of the mechanisms(s) underlying organophosphate-induced increases in phosphorylase-*a* activity may provide further insight into events regulating cytosolic $Ca^{2+}$.

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