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STIMULATION OF PHOSPHOLIPID HYDROLYSIS AND CELL DEATH BY MERCURIC CHLORIDE: EVIDENCE FOR MERCURIC ION ACTING AS A CALCIUM-MIMETIC AGENT

W.T. Shier and D.J. DuBourdieu

Department of Pharmaceutical Cell Biology, College of Pharmacy
University of Minnesota, Minneapolis, Minnesota 55455

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SUMMARY: Mercuric chloride stimulates phospholipid hydrolysis and prostaglandin release in 3T3 mouse fibroblasts. This response is distinctly different from that stimulated by other sulfhydryl-reactive agents, but it exhibits a variety of characteristics similar to the phospholipid hydrolysis response stimulated by Ca^{2+} plus ionophore A23187. Also, the additivity of phospholipid hydrolytic responses stimulated by Hg^{2+}, Ca^{2+} and A23187 is consistent with Hg^{2+} interacting with a Ca^{2+}-dependent enzyme(s). These results are consistent with Hg^{2+} acting by a novel, Ca^{2+}-mimetic mechanism; i.e., with it entering cells and activating cell processes that are activated by Ca^{2+} in calcium-dependent cell death.

Ingestion of acute toxic doses of ionizable mercuric salts such as mercuric chloride ($HgCl_2$) causes immediate necrosis in the mouth, throat and stomach(1). In view of the tissue destructive effects of $HgCl_2$ we have investigated its effects on stimulatable phospholipid hydrolyzing activity in cultured 3T3 mouse fibroblasts. We have observed that $HgCl_2$ in a narrow concentration range induces high levels of phospholipid hydrolysis with concomitant synthesis of prostaglandins. The hydrolytic activity stimulated by $HgCl_2$ exhibits properties unlike those of any other stimulatable phospholipid hydrolytic activity (2-6) except that activated by Ca^{2+} in the presence of ionophore A23187 (7,8). Extracellular Ca^{2+} plus A23187 causes cell killing (9), presumably by altering intracellular Ca^{2+} concentrations in an example of what Farber and associates (10,11) have proposed is a final common pathway in toxic cell death. We have presented evidence (7) that the phospholipid hydrolysis stimulated by extracellular Ca^{2+} plus A23187 plays an important role in triggering calcium-dependent cell killing. The results obtained with Hg^{2+} in this study are consistent with it entering cells and activating the same cellular processes that are activated by Ca^{2+} in calcium-dependent cell death.

MATERIALS AND METHODS: [5,6,8,9,11,12,14,15(n)-^{3}H]Arachidonic acid (83.7 Ci/mmole) was obtained from New England Nuclear (Boston, MA). Ionophore A23187 was purchased from Calbiochem Corp. (San Diego, CA). Verapamil hydrochloride was the gift of G.D. Searle Co. (Chicago, IL). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Swiss mouse 3T3 fibroblasts (clone KJ) were a subclone of cells obtained from R.W. Holley (Salk Institute) and maintained at 37° in a humidified 15% CO_2:85% air atmosphere in Dulbecco's modified Eagle's medium (DME) and 10% calf serum (Grand Island Biological Co., Grand

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Island, NY). Trypsin (0.05%) was used to subculture cells. The cells were free of mycoplasma as judged by autoradiography with Kodak nuclear track emulsion type NTB-2 after tritiated thymidine incorporation.

Phospholipid hydrolysis assay conditions. Phospholipid hydrolysis assays were carried out in triplicate as described previously (5,12) by using 3T3 cells cultured at 1.2×10^5 cells per 34 mm Falcon plastic dishes for 24 h in 2 ml of medium containing 0.25% calf serum and 0.5 μCi of tritiated arachidonic acid. Under these conditions the label is incorporated predominantly into phospholipids (~94%) with ~3% as triglycerides and ~3% as free fatty acid. For assay the cells were washed three times on the dish with 2 ml of medium prepared without cystine and incubated the required time under normal culture conditions with 0.6 ml of the same medium. The radioactivity released into the culture medium has been shown to reflect hydrolysis of labeled phospholipids in the cells (5,12). The medium from the cultures was extracted and analysed for lipid composition of radioactivity as described (5,12). The radioactivity released as free arachidonic acid and known metabolites was calculated as a percent of total incorporated radioactivity in the cells at the beginning of the assay. The data are presented as the mean ± SEM. A23187 and p-chloromercuribenzoic acid were added to cultures in sufficient dimethylsulfoxide to give a final concentration of 0.05% (v/v); control cultures received an equal amount of dimethylsulfoxide.

Assay of acyltransferase activity. 3T3 fibroblasts were cultured as described for the phospholipid hydrolysis assay except that no tritiated arachidonic acid was used. For assay parallel cultures of the cells were processed and set up as described for the phospholipid hydrolysis assay except that 0.3 μCi of tritiated arachidonate (0.3 μCi, 3.1×10^{-12} mole) was included. Analysis of the phospholipid distribution of radio-activity incorporated during the assay period was carried out on lipids extracted from cells on the plastic dishes by using a modified Folch extraction procedure (13). The extracts were evaporated *in vacuo* and fractionated by thin layer chromatography on silica gel (Macherey-Nagel Co., Postfach, Germany) with the solvent system chloroform:methanol:water:acetic acid 25:15:2:4 at 4°. Authentic lipid standards were co-applied, and radioactivity co-migrating with the standards was determined as described for phospholipid hydrolysis assays.

Assay of cell killing. 3T3 fibroblasts were cultured as described for the assay of phospholipid hydrolysis except that no tritiated arachidonic acid was used. Cell killing was assessed by failure to exclude 0.023% trypan blue dye for 2 min at room temperature by using a Nikon inverted microscope with phase optics.

RESULTS: $HgCl_2$ stimulates the hydrolysis and release of up to 30% of the [^{3}H] arachidonic acid biosynthetically incorporated into the phospholipids of a selected clone of 3T3 Swiss mouse fibroblasts (3T3-KJ) (Fig. 1A). Other clones of 3T3 cells exhibited a similar but quantitatively lower response. Optimal stimulation occurred in a narrow concentration range (20 to 50 μM) with sharply reduced stimulation at higher concentrations ($\geq$ 100 μM). Concomitant release of chromatographically identifiable prostaglandins E_2 and $F_{2\alpha}$ occurred, representing conversion of up to 52.6% and 21.8% respectively, of the released [^{3}H]arachidonic acid (Fig. 1B, 2A).

Release of phospholipid hydrolysis products from cells can in principle be achieved either by stimulating phospholipid hydrolysis or by inhibition of the reacylation part of the normal phospholipid turnover processes. The major pathway for phospholipid turnover has been suggested (14) to be the phosphoglyceride deacylation-reacylation cycle involving a coenzyme A-dependent two-step process for reincorporation of free fatty acids into phospholipids. This process would

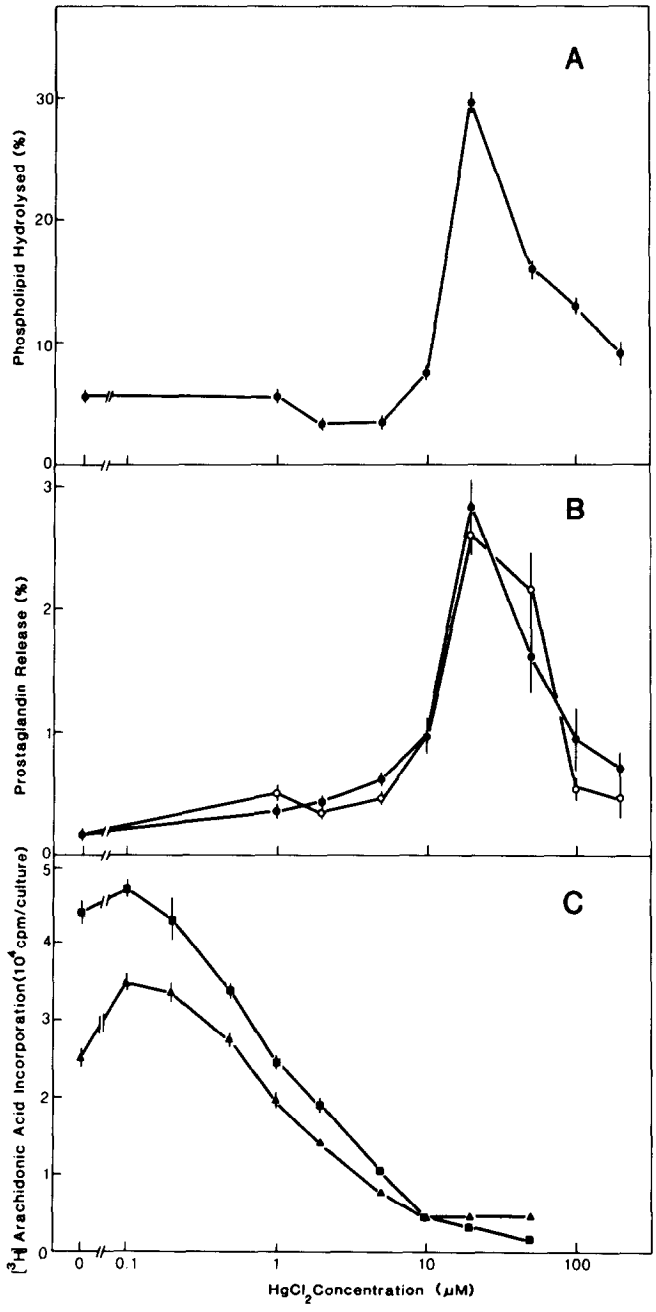

Fig. 1. Effect of a range of concentrations of $HgCl_2$ on (A) phospholipid hydrolysis, (B) prostaglandin synthesis and (C) fatty acid acyltransferase activity. A. Free arachidonic acid and known metabolites (expressed as a percent of total incorporated radioactivity in 3T3 cells biosynthetically labeled with [^{3}H]arachidonate) released during 20 min exposure to the indicated concentrations of $HgCl_2$. B. Release of chromatographically identifiable prostaglandins E_2 (●) and $F_{2\alpha}$ (○) from the same cultures expressed in the same way. C. The incorporation of [^{3}H]arachidonic acid into phosphatidylcholine (■) and phosphatidylethanolamine (▲) in 60 min in unlabeled parallel cultures of 3T3 cells exposed to the indicated concentrations of $HgCl_2$. Cultures were established and labeled and assays carried out as described in Materials and Methods.

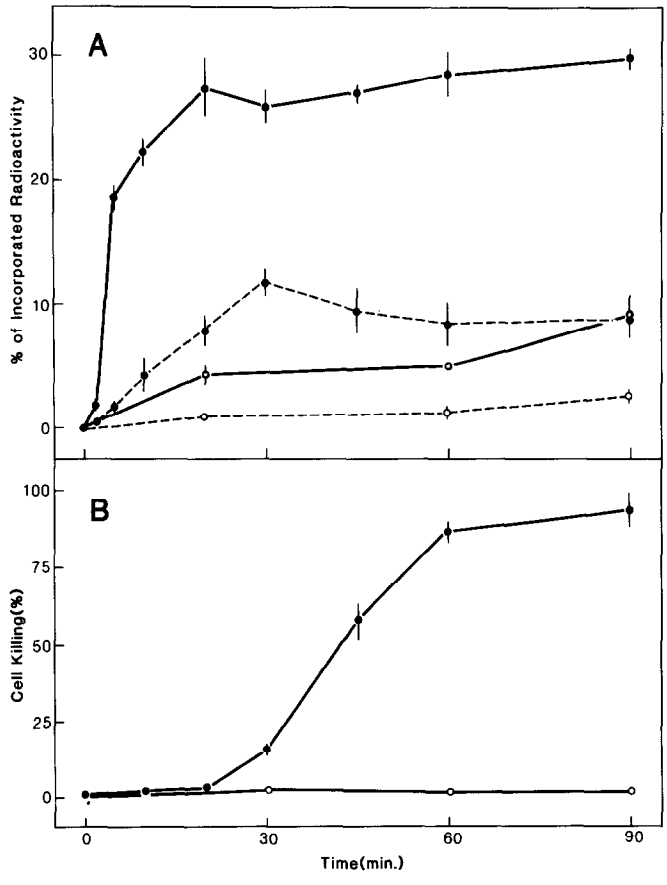

Fig. 2. Time course for $HgCl_2$ effects on (A) phospholipid hydrolysis and (B) cell killing. A. Release of total labeled phospholipid hydrolysis products (—) and prostaglandin E_2 (---) from biosynthetically labeled 3T3 cells following treatment with 20 μM $HgCl_2$ in cystine-free medium (●) or with medium alone (○). B. Cell killing following treatment of unlabeled parallel cultures of 3T3 cells with 20 μM $HgCl_2$ in cystine-free medium (●) or with medium alone (○). Cultures were established, labeled and assayed as described in Materials and Methods.

be expected to be inhibited by depletion of the free coenzyme A pool by reaction of its functional sulfhydryl group with agents such as $HgCl_2$. Acyltransferase activity was studied by monitoring the rate of incorporation of [^{3}H]arachidonic acid under the conditions of the phospholipid hydrolysis assay. [^{3}H]Arachidonic acid was incorporated into phospholipids in a biphasic manner with an initial rapid incorporation phase complete in 20 min followed by a slower rate of incorporation. At 20 min approximately 80% of the label is incorporated into phosphatidylcholine, 12.5% into phosphatidylinositol plus phosphatidylserine and 7% into phosphatidylethanolamine. As shown in Fig. 1C, $HgCl_2$ inhibits reacylation of phospholipids under the experimental conditions used, but the inhibition occurs in a different concentration range (half-maximal at 1 μM) than stimulation of the major phospholipid hydrolyzing activity (half-maximal at >10 μM).

Other sulfhydryl-reactive agents. Additional evidence against inhibition of reacylation as the mechanism for stimulation of phospholipid hydrolysis is provided by comparing the rates of release of free arachidonic acid and metabolites stimulated by $HgCl_2$ with that stimulated by other agents known to react with sulfhydryl groups. $HgCl_2$ stimulates rapid release of arachidonic acid from phospholipids and concomitant synthesis and release of prostaglandins (Fig. 2A) with maximum release being achieved by 20 min, after which little net release of [^{3}H]arachidonic acid and metabolites occurs. As observed with Ca^{2+} plus A23187 (7), cytotoxicity caused by 20 μM $HgCl_2$ occurs later (20 to 60 min, Fig. 2B) suggesting that the phospholipid hydrolysis process is not itself responsible for the membrane permeabilization detected by failure to exclude trypan blue dye, although it may be initiating the process(es) which are. In contrast, a series of other sulfhydryl-reactive agents (N-ethylmaleimide, iodoacetamide, p-chloromercuribenzoic acid) under the same conditions induce a slow release of phospholipid hydrolysis products from 3T3 cells that persists in a near linear fashion for at least 3 h (Fig. 3A). Cell death begins when > 10% hydrolysis of labeled phospholipids has been achieved and occurs concomitant with additional phospholipid hydrolysis.

Other metal ions. Lead diacetate (0.2 to 1000 μM), which does not cause tissue distruction, did not stimulate phospholipid hydrolysis alone or in the presence of 5 μM ionophore A23187. Similarly, $CdCl_2$ (0.5 to 1000 μM) and $MnCl_2$ (3 to 1000 μM) in the presence or absence of 5 μM A23187 and $CuCl_2$ (0.5 to 2000 μM) did not stimulate phospholipid hydrolysis in 3T3 cells. $TbCl_3$ (1 μM to 10 mM), $BaCl_2$ (1 μM to 1 mM) and $SrCl_2$ (1 μM to 1 mM) neither activated phospholipid hydrolysis alone nor inhibited hydrolysis stimulated by A23187 and 1.8 mM $CaCl_2$. Studies with $BaCl_2$ and $SrCl_2$ were carried out in medium prepared without sulfate ions.

Inhibitors. Stimulation of phospholipid hydrolysis and prostaglandin synthesis by 20 μM $HgCl_2$ is inhibited by 10 mM EDTA and by British anti-Lewisite (BAL, 2,3-dimercapto-1-propanol; a standard therapeutic agent for $HgCl_2$ intoxication (1)) at concentrations > 10 μM. However, BAL added 2 to 10 min after $HgCl_2$ did not inhibit the phospholipid hydrolysis response which had already begun. Stimulation of phospholipid hydrolysis and prostaglandin synthesis by 20 μM $HgCl_2$ is inhibited by $MnCl_2$ at concentrations > 3 μM in medium prepared without $CaCl_2$. It is partially inhibited in regular medium by 0.1 mM p-bromophenacyl bromide, but not by N-ethylmaleimide (5 mM) or verapamil hydrochloride (2.2 μM). Indomethacin (1 μM) inhibited prostaglandin release without inhibiting phospholipid hydrolysis.

Comparison with phospholipid hydrolysis stimulated by Ca^{2+} plus A23187. The phospholipid hydrolysis stimulated by $HgCl_2$ exhibits a variety of characteristics similar to the phospholipid hydrolysis response stimulated in 3T3 cells by Ca^{2+}

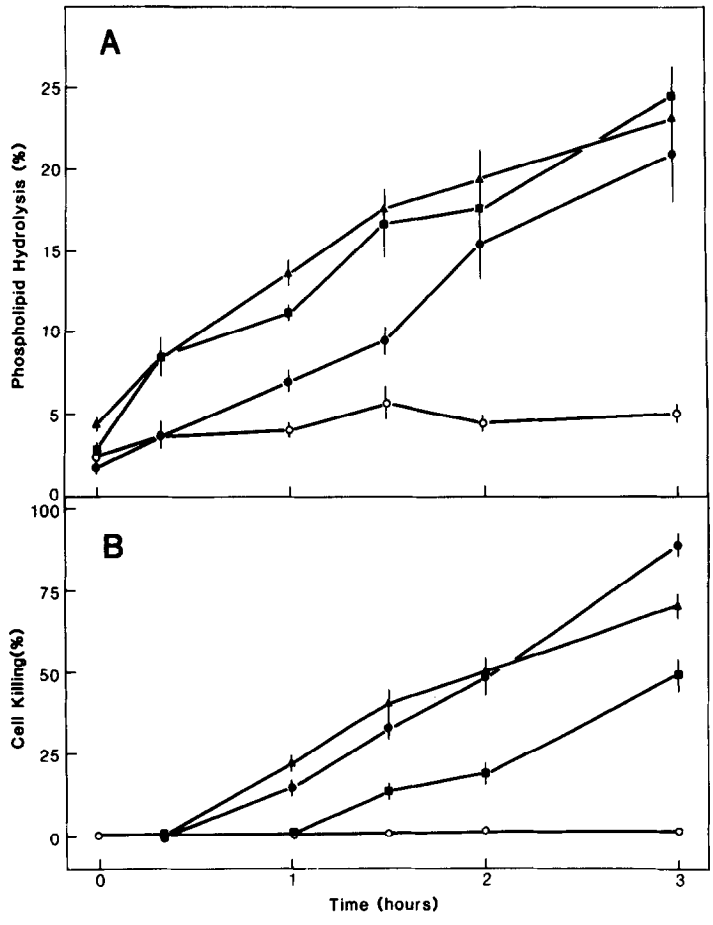

Fig. 3. Time course of the effect of other sulfhydryl-reactive agents on (A) phospholipid hydrolysis and (B) cell killing. A. Release of total labeled phospholipid hydrolysis products from biosynthetically labeled cells following treatment with cystine-free medium alone (○) or containing 50 μM N-ethylmaleimide (■), 200 μM p-chloromercuribenzoic acid (▲), or 500 μM iodoacetamide (●). B. Cell killing following treatment of unlabeled parallel cultures of 3T3 cells with the same agents. Cultures were established, labeled and assayed as described in Materials and Methods.

plus ionophore A23187 (7). These characteristics include: (i) both responses are rapid and complete in 20 min; (ii) the time courses of cytotoxicity caused by the two treatments differ from the time courses of phospholipid hydrolysis in similar ways; (iii) both responses are effectively inhibited by $MnCl_2$ but not by a series of agents which inhibit phospholipid hydrolysis responses stimulated by toxins and other agents (2,3); (iv) the arachidonic acid released from phospholipids in response to both agents is converted to prostaglandins in high yield; and (v) both responses exhibit a strong specificity for phospholipids containing [^{3}H]arachidonate relative to [^{3}H]oleate and [^{3}H]palmitate (15, data not shown). The interaction between Hg^{2+} and Ca^{2+} was examined directly (Fig. 4) by comparing the phospholipid hydrolysis response stimulated in 10 min in 3T3 cells by a range

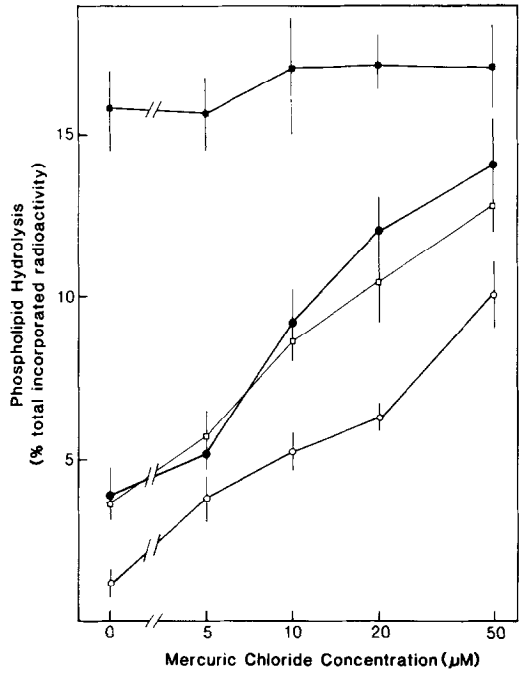

Fig. 4. Effect of Ca^{2+} and divalent cation ionophore A23187 on phospholipid hydrolysis stimulated by $HgCl_2$. 3T3 cells were cultured, labeled and assayed as described in Materials and Methods except that Ca^{2+}-free DME prepared without $CaCl_2$, calcium pantothenate or cystine was used for washing the cells. The cultures were assayed for stimulation of phospholipid hydrolysis in 10 min by the indicated concentrations of $HgCl_2$ under four sets of conditions designed to alter Ca^{2+} levels in different parts of the cell: (i) Ca^{2+}-free DME (○), which should effectively lower the extracellular Ca^{2+}; (ii) the same medium with 1.8 mM $CaCl_2$ added (●), which provides the extracellular concentration of Ca^{2+} found in DME; (iii) the medium in (i) plus 5 μM ionophore A23187 (□), which mobilizes intracellular Ca^{2+} throughout the cells and medium; and (iv) the medium in (i) supplemented with both 1.8 mM $CaCl_2$ and 5 μM A23187 (■), which should admit high Ca^{2+} concentrations to all parts of the cell.

of $HgCl_2$ concentrations under four sets of conditions designed to alter Ca^{2+} levels in different parts of the cell (5). In the absence of $HgCl_2$ and extracellular Ca^{2+} (Fig. 4, at 0 μM $HgCl_2$) A23187 stimulates low levels of arachidonic acid release, presumably due to mobilization of intracellular Ca^{2+} pools, although the possibility of a direct effect of A23187 on a membrane enzyme cannot be excluded. Raising the extracellular Ca^{2+} concentration in the absence of A23187 or adding A23187 in the absence of extracellular Ca^{2+} stimulates only a modest amount of phospholipid hydrolysis. $HgCl_2$ induces an additive dose-dependent stimulation of phospholipid hydrolysis independent of the presence or absence of either A23187 or extracellular Ca^{2+}, indicating that $HgCl_2$ does not act by inhibiting Ca^{2+} transport out of the cell. Raising the Ca^{2+} concentration in the presence of A23187 (which also elevates intracellular Ca^{2+} concentrations) stimulates much greater amounts of phospholipid hydrolysis. $HgCl_2$ stimulates no significant additional phospholipid hydrolysis beyond that stimulated by the near-optimal (7) concentrations of extracellular Ca^{2+} used in Fig. 4.

DISCUSSION: The characteristics of the phospholipid hydrolysis stimulated in 3T3-KJ by $HgCl_2$ serve to differentiate it from the phospholipid hydrolysis responses stimulated by all cytolytic toxins studied to date (2,3), by cell growth factors (4,5) or by sulfhydryl-reactive agents. However, all of the observed characteristics are shared with the phospholipid hydrolysis response stimulated by A23187 in the presence of extracellular Ca^{2+} (7,15). These results and studies on the additivity of the hydrolytic responses (Fig. 4) are consistent with the same hydrolytic enzyme system being activated by $HgCl_2$ or by Ca^{2+} plus A23187. The simplest mechanism for achieving this would be interaction of Hg^{2+} with the same binding site used by Ca^{2+} for cofactor activation of a rate-limiting enzyme in the phospholipid hydrolyzing system. The interaction of other metal ions (e.g. La^{3+}, Ru^{3+}, Tb^{3+}) with Ca^{2+}-binding sites of proteins to either inhibit or induce the Ca^{2+}-stimulated function has been well-documented (16). The apparent competitive inhibition of Ca^{2+}-stimulated phospholipid hydrolysis in 3T3 cells by Mn^{2+} (7) is also consistent with the Ca^{2+}-binding site exhibiting a less than absolute specificity for Ca^{2+} ions. Since this phospholipid hydrolytic process appears to trigger the toxic cell death induced by Ca^{2+} plus A23187 (7), Hg^{2+} may be inducing cell death by the same mechanism. Chronic mercury toxicity is generally thought to involve different mechanism(s) than acute toxicity (1). Since Ca^{2+} affects many processes in cells, it is plausible that other toxic effects of mercury could involve interaction of Hg^{2+} with other Ca^{2+}-mediated cellular processes.

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REFERENCES

1. Berlin, M. (1979) in Handbook on the Toxicology of Metals (Friberg, L., Norberg, G.F. and Vouk, V.B., eds) pp. 503-530, Elsevier/North Holland, New York.
2. Shier, W.T. (1980) in Natural Toxins (Eaker, D. and Wadstrom, T., eds.) pp. 193-200, Pergamon Press, New York.
3. Shier, W.T. (1982) J. Toxicol.-Toxin Reviews, 1,1.
4. Levine, L. and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 76, 1181-1187.
5. Shier, W.T. (1980) Proc. Natl. Acad. Sci. USA 77, 137-141.
6. Levine, L. and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 79, 477-483.
7. Shier, W.T. and DuBourdieu, D.J. (1982) Biochem. Biophys. Res. Commun. 109, 106-112.
8. Hong, S.L. and Deykin, D. (1979) J. Biol. Chem. 254, 11463-11466.
9. Durant, S., Homo, F., and Duval, D. (1980) Biochem. Biophys. Res. Commun. 93, 385-391.
10. Farber, J.L. (1981) Life Sciences 29, 1289-1295.
11. Schanne, F.A.X., Kane, A.B., Young, F.E., and Farber, J.L. (1979) Science 206, 700-702.
12. Shier, W.T. and Durkin, J.P. (1982) J. Cell. Physiol. 112, 171-181.
13. Shier, W.T. (1977) Biochem. Biophys. Res. Commun. 78, 1168-1174.
14. Resch, K. and Ferber, E. (1975) in Immune Recognition (Rosenthal, A.S., ed) pp. 281-312, Academic Press, New York.
15. Hong, S.L. and Deykin, D. (1979) J. Biol. Chem. 254, 11463-11466.
16. Kretsinger, R.H. (1980) CRC Crit. Rev. Biochem. 8, 119-174.