HgCl₂-induced Interleukin-4 Gene Expression in T Cells Involves a Protein Kinase C-dependent Calcium Influx through L-type Calcium Channels

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Mercuric chloride (HgCl₂) induces T helper 2 (Th2) autoreactive anti-class II T cells in Brown Norway rats. These cells produce interleukin (IL)-4 and induce a B cell polyclonal activation that is responsible for autoimmune disease. In Brown Norway rats, HgCl₂ triggers early IL-4 mRNA expression both in vivo and in vitro by T cells, which may explain why autoreactive anti-class II T cells acquire a Th2 phenotype. The aim of this study was to explore the transduction pathways by which this chemical operates. By using two murine T cell hybridomas that express IL-4 mRNA upon stimulation with HgCl₂, we demonstrate that: 1) HgCl₂ acts at the transcriptional level without requiring de novo protein synthesis; 2) HgCl₂ induces a protein kinase C-dependent Ca²⁺ influx through L-type calcium channels; 3) calcium/calcineurin-dependent pathway and protein kinase C activation are both implicated in HgCl₂-induced IL-4 gene expression; and 4) HgCl₂ can activate directly protein kinase C, which might be one of the main intracellular targets for HgCl₂. These data are in agreement with an effect of HgCl₂ which is independent of antigen-specific recognition. It may explain the T cell polyclonal activation in the mercury model and the expansion of pathogenic autoreactive anti-class II Th2 cells in this context.

HgCl₂, and gold salts induce in Brown Norway (BN)1 rats and in susceptible mice a T helper 2 (Th2) cell-dependent B cell polyclonal activation responsible for an increase in serum IgE concentration and for the production of various autoantibodies (1, 2). Anti-laminin autoantibodies are associated in BN rats with the development of a glomerulopathy (1) which resembles the one observed in some patients exposed to mercurials or gold salts. Autoreactive anti-class II T cell lines have been derived from diseased BN rats. These T cell lines produce interleukin (IL)-4 and may transfer autoimmunity in CD8⁻ cell-depleted BN rats (3) by stimulating B cells polyclonally.

CD4⁺ T cells are divided into at least two subsets, Th1 and Th2, which differ by their functions and the profile of cytokines they produce (4). Th1 cells produce IL-2 and interferon-γ and are responsible for cell-mediated immune reactions; Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and are implicated mainly in B cell help for IgG1 and IgE production. In addition, each cell subset antagonizes the other.

It is well known that IL-4 is crucial for the differentiation of naive T cells into Th2 cells. Th2 cells, once activated, produce IL-4. However, the nature of the cell that initially produces IL-4 and allows the differentiation into Th2 cells is a matter of debate. Candidates include natural killer 1.1⁺ T cells (5), mast cells, basophils, and eosinophils (6). It is also possible that IL-6, which is produced by antigen-presenting cells, initiates IL-4 production by naive CD4⁺ T cells (7).

Our previous results (8) demonstrate that CD4⁺ T cells from BN rats produce in vitro IL-4 when cultured in the presence of HgCl₂. This suggests that some stimuli may induce an early production of IL-4 by CD4⁺ T cells and that T cells themselves may condition the differentiation of Th2 cells. Interestingly, HgCl₂ also induces IL-4 gene expression in BN mast cells (9).

It is currently admitted that ligation of the extracellular domains of the TCR activates a cascade of protein tyrosine kinases including p56lik, p59yn, and ZAP-70, which leads to phosphorylation and activation of the γ isoform of phospholipase C (10–12). Inositol 1,4,5-trisphosphate and diacylglycerol are produced, resulting in release of stored intracellular Ca²⁺ and protein kinase C (PKC) activation, respectively. Both IL-2 and IL-4 promoters bind NF-AT and AP-1 nuclear factors, which integrate Ca²⁺ and PKC-dependent signaling pathways in T cells (13–15). In fact, Ca²⁺- and PKC-dependent pathways have been explored mainly in the context of TCR-dependent IL-2 production, and much less is known about IL-4 production.

It has been put forward that the variation of [Ca²⁺], required to induce IL-4 in Th2 cells is lower than the one required to induce IL-2 in Th1 cells (16, 17). It has also been proposed that IL-4 production does not involve the classical protein tyrosine kinases associated with the TCR or phospholipase C activation (18, 19).

The aim of this work was to understand the mechanisms of HgCl₂-induced IL-4 mRNA expression by T cells. Using two murine T cell hybridomas that express IL-4 mRNA upon stimulation with HgCl₂, we show that HgCl₂ induces a PKC-dependent calcium influx through L-type calcium channels and that the Ca²⁺-dependent pathway and PKC activation are both required for HgCl₂-induced IL-4 gene expression. PKC might
be therefore one of the main target of HgCl₂, in this cell model because chemical can activate PKC in a cell-free system.

**EXPERIMENTAL PROCEDURES**

**Mouse T Cell Hybridomas**—The following mouse T cell hybridomas were used in this study: H-2<sup>b</sup>-restricted SM1.27.9, specific for the Myo102–118s peptide derived from myelin (20); I-E<sup>k</sup>-restricted 1H11.3, specific for the peptide 108–116 derived from hen egg lysosome (21); I-A<sup>k</sup>-restricted 2G12.1, specific for the peptide 26–39 derived from β<sub>2</sub>-microglobulin (22); I-E<sup>k</sup>-restricted 2B7.1, specific for peptide 1–18 derived from hen egg lysosome (22); and I-A<sup>k</sup>-restricted B31.1, specific for peptide 34–45 of hen egg lysosome (23). Hybridomas were grown in RPMI containing 10% FCS (Life Technologies, Inc., Cergy Pontoise, France) nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), penicillin (100 units/ml)/streptomycin (100 μg/ml), and 1-glutamine (2 mM; Biochrom, KG Germany).

*Stimulation Assay—*Four protocols were used.

In the first protocol, T cell hybridomas were cultured in RPMI plus 10% FCS in the absence or in the presence of HgCl₂ or iodomyocin. HgCl₂ (10<sup>−2</sup> M; Sigma) was prepared as a stock solution in 0.9% NaCl, and iodomyocin (1 μM; Sigma) was initially dissolved in dimethyl sulfoxide at a concentration of 2 mM. Further dilutions were done in FCS-free medium.

In the second protocol, T cell hybridomas were preincubated for 30 min with the various inhibitors listed below, and HgCl₂ was added for another 4 h before RNA extraction. These inhibitors have been used: cycloheximide (Sigma), as an inhibitor of protein synthesis; actinomycin D (Sigma), as an inhibitor of transcription; cyclosporin A (Sandimmune, Sandoz, Rueil Malaunais, France), as an inhibitor of calciunin; U-73122 (1-[2-(4-1H-indol-3-yl)

**mRNA Detection and IL-4 Enzyme-linked Immunoassay Assay—**RNA extraction was done after 4 h of culture by using the TRIZol procedure (Life Technologies, Inc.). Semiquantitative reverse transcriptase polymerase chain reaction (PCR) was performed as described previously (26). Briefly, RNA was reverse transcribed to cDNA using poly(dT) as primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a final volume of 40 μl (28). The following primers were used: β-actin sense, 5'-TCA AAT CCT GGT GCC TCA TCC AGT AAA C-3'; β-actin antisense, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'; mouse IL-4 sense, 5'-AAC ACC ACA GAG AGT GAG CTC GTC T-3'; mouse IL-4 antisense, 5'-TGG ACT CAT TCA TGG TGC AGC TTA T-3'; rat IL-4 sense, 5'-TGA TGG GTG TCA GCC CAC ACC TCC C-3'; rat IL-4 antisense, 5'-CTT TCA GGT GTG TGA GGC TGG ACT C-3'. Primers were designed to amplify cDNA fragments representing mature mRNA transcripts for β-actin, 178 base pairs for mouse IL-4, and 378 base pairs for rat IL-4. cDNAs were amplified in a 50-μl reaction volume containing 0.6 mM concentration of each dNTP (dATP, dGTP, dCTP, and dTTP) (Phar-macia, Uppsala, Sweden), 1 μg/ml of each oligonucleotide primer, 2.5 mM MgCl₂, 1 unit of Taq-DNA polymerase (Boehringer Mannheim, Germany), and, for IL-4, 5 μl of PCR buffer (10 μi x (Boehringer). Reactions were performed in a DNA thermal cycler (Perkin-Elmer) for 20 cycles (β-

**IL-4 Production**—IL-4 production was quantified by using two-color sandwich enzymelinked immunosorbent assay (ELISA) purchased from Pharmingen (27). Briefly, 11B11 anti-IL-4 monoclonal antibody was used for coating. After three washes with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-Tween), undiluted culture supernatants (100 μl/well) were incubated overnight at 4 °C. Plates were then washed three times and incubated with biotinylated BV6D-24G2 anti-IL-4 monoclonal antibody in PBS-Tween containing 1% bovine serum albumin (PBS-Tween-bovine serum albumin). After washing, the bound biotinylated monoclonal antibody was visualized by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson, Immunoresearch Laboratories, Avondale, PA) diluted 1/5,000 in PBS-Tween-bovine serum albumin. After washing, the plates were incubated with the substrate p-nitrophenyl phosphate disodium (Sigma) in diethanolamine buffer, pH 9.6. The reaction was stopped by the addition of 0.2 mg/ml sodium carbonate washed twice at 450 nm. Cytokines were quantified from standard curves generated by various concentrations of recombinant mouse IL-4 diluted in PBS containing 1% FCS and 0.1% phenol. The detection limit was 15 pg/ml.

**PCR-based Nuclear Run-on Assay—**1H11.3 cells were incubated with HgCl₂ (20 μM) for 1 h 30 min or 3 h. Cells were harvested, and nuclei were prepared as described by Rolfe and Deacon (28). Nuclei were split into two aliquots of 100 μl and incubated for 30 min at 30 °C in 20% glycerol, 30 μM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl, 1 mM dithiothreitol, and 40 units of RNasin. A 0.5 mM concentration of each rNTP (dATP, dCTP, dGTP, and dTTP) was added to each aliquot. No rNTPs were added to the second aliquot. After 30 min, nuclei were lysed. RNA extraction, reverse transcription, and PCR were performed as described above. Results were expressed by using the IL-4:β-actin ratio as described above.

**Protein Kinase C (PKC) Assay—**Purified rat brain PKC (enriched in α, β, and γ isoforms; 5 ng/well; Calbiochem) was incubated or not with HgCl₂ (5, 10, 25, or 100 μM) in the presence of EGTA (20 μM). We also tested the effect of Ca<sup>2+</sup> (10 or 100 μM) in the presence or in the absence of EGTA. The capacity of PKC to phosphorylate a specific substrate was assessed by using a specific PKC kit assay (Calbiochem). This assay was performed in the presence of phosphatidylinerine and ATP but in the absence of diacylglycerol.

**Analysis of Intracellular Ca<sup>2+</sup> Concentration—**Measurement of [Ca<sup>2+</sup>]i, was performed by emission microspectrophotometry as described previously (29). Cells were incubated with 5 μM fluo3/AM (Molecular Probes) for 30 min at 37 °C. [Ca<sup>2+</sup>], was measured in T cells stimulated by HgCl₂ (15 or 20 μM), iodomyocin (1 μM), phorbol 12-myristate 13-acetate (PMA; 10 ng/ml), Sigma), and S<subichert</sub>-Bay K8644 as an agonist of the L-type calcium channel (6 μM). Iodomyocin, PMA, and S<subichert</sub>-Bay K8644 were dissolved initially in dimethyl sulfoxide at concentrations of 2 mM, 0.2 mg/ml, and 5 mM, respectively. We also checked the effect of Ro 31-8220. In some experiments, cells were stimulated by HgCl₂ in Hanks’ balanced saline solution without calcium and magnesium and without phenol red (Life Technologies, Inc.) supplemented with 5 mM EGTA (20 μM). Cell preparation was then placed on the stage of an inverted microscope (Diaphot, Nikon) and observed with an objective (× 40). Excitation light was 490 nm with a 525 nm barrier filter. Fluorescence was detected by a CCD camera intensified (Hamamatsu C2400-80). With the magnification used (× 40) a field of 200 x 200 μm was recorded by the camera. Three to five fields were observed for each experiment, and in each field 12 windows (9 μm) were measured on different microsomes cells transferred for fluorescence were captured at intervals of 10 s and processed with the Argus 50 processing image system (Hamamatsu Photonics, Hamamatsu, Japan). Time courses of Ca<sup>2+</sup> signals in cells were analyzed with the Argus 50 software. Data are presented as the ratio of fluorescence (F<sub>T</sub>) in stimulated cells to fluorescence (F<sub>B</sub>) at the resting level. Cells were scored as positive if the fluorescence intensity variation was 5% above the resting level.
**RESULTS**

**Effect of HgCl₂ on the Induction of the IL-4 Gene in Mouse T Cell Hybridomas—**Two T cell hybridomas (SM1.27.9 and 1H11.3) out of the five tested were selected because they expressed IL-4 mRNA upon stimulation with nontoxic amounts of HgCl₂. The experiments herein reported have been performed on both hybridomas. Results were similar whatever the hybridoma tested. HgCl₂ induced IL-4 mRNA expression in a dose-dependent manner in both hybridomas (Fig. 1A and not shown). The effect was optimal when 1H11.3 T cells and SM1.27.9 T cells were incubated with 20 and 15 μM HgCl₂, respectively. In these conditions and in six independent experiments, the IL-4:β-actin ratio was 9.2 ± 4.5 in SM1.27.9 T cells stimulated with HgCl₂ versus 1.2 ± 2 in unstimulated cells and was 14.2 ± 8.7 in 1H11.3 T cells stimulated with HgCl₂ versus 1.8 ± 1.5 in unstimulated cells. A semiquantitative assay in which serial dilutions of cDNA were performed confirms that HgCl₂ induces IL-4 mRNA expression as ionomycin does (Fig. 1B and not shown). As soon as 2 h after stimulation with HgCl₂, IL-4 mRNA was observed, with a peak at 4 h and a decline at 6 h (not shown). Actinomycin D, an inhibitor of transcription, abolished HgCl₂-induced IL-4 gene expression in SM1.27.9 (Fig. 1C) and 1H11.3 (not shown) T cell hybridomas, whereas cycloheximide, an inhibitor of protein synthesis, had no effect (Fig. 1D and not shown).

To demonstrate that HgCl₂ actually induced IL-4 gene transcription, a PCR-based run-on assay was performed because it has indeed been shown previously that a classical run-on assay may be not sensitive enough to detect cytokine gene transcription (28). As shown in Fig. 2, no expression of IL-4 mRNA was observed after a 1-h 30-min stimulation with HgCl₂ in the absence of rNTPs, whereas the addition of rNTPs to the isolated nuclei allowed detection of IL-4 messenger. In contrast, 3 h after stimulation with HgCl₂, IL-4 mRNA was detected in nuclei whether rNTPs were added or not. Altogether, these results show that HgCl₂ induces IL-4 gene and that this effect does not require de novo protein synthesis.

IL-4 was not detected by enzyme-linked immunosorbent assay when 5 x 10⁵ cells/ml were cultured in the presence of HgCl₂ because the IL-4 assay is not sensitive enough. Indeed, when the cell density was increased (5 x 10⁶/ml), although mortality was high (around 40% in both stimulated and unstimulated cultures), IL-4 was detected (144 ± 41 pg/ml, n = 4 in HgCl₂-stimulated cells versus < 15 pg/ml in control cultures). This shows that HgCl₂ induces not only IL-4 mRNA expression but also IL-4 production.

HgCl₂ Induces a PKC-dependent Influx of Ca²⁺ in T Cell Hybridomas—It is well known that the Ca²⁺-dependent pathway is important for IL-4 gene induction. Therefore, we first checked whether HgCl₂ was able to increase [Ca²⁺] in 1H11.3 T cell hybridoma. As shown by microspectrofluorometry, in 58 out of 60 cells HgCl₂ induced a transient increase in fluorescence.
HgCl₂-induced Ca²⁺-dependent IL-4 Gene Expression

Because HgCl₂ induced a PKC-dependent Ca²⁺ influx, we tested the effects of an inhibitor of PKC and of an intracellular Ca²⁺ chelator on HgCl₂-induced IL-4 gene expression. The specific PKC inhibitor (Ro 31-8220) suppressed, in a dose-dependent manner, HgCl₂-induced IL-4 gene expression (Fig. 5A). BAPTA/AM, a chelator of intracellular Ca²⁺, abolished the effect of HgCl₂ on IL-4 gene expression (Fig. 5B). Cyclosporin A, which inhibits calcineurin phosphatase (31), also inhibited HgCl₂-induced IL-4 gene transcription (Fig. 5C), supporting a role for the calcium/calmodulin/calcineurin-dependent pathway. Because there is some evidence that H11.3 T cells express L-type calcium channels, we have tested the effect of an L-type calcium channel blocker. R(+)-Bay K8344 abolished HgCl₂-induced IL-4 gene induction (Fig. 5D), suggesting that HgCl₂ induces a Ca²⁺ influx through L-type calcium channels which leads to IL-4 gene transcription.

It has been shown recently that an increase in extracellular Ca²⁺ concentration amplified calcium-dependent pathways including NF-AT nuclear translocation (32). In our system, increasing medium Ca²⁺ concentration from 1 to 10 mM resulted in a marked increase in IL-4 gene expression (Fig. 6A) and IL-4 production (Fig. 6B). These results reinforce the role of a calcium influx in HgCl₂-mediated IL-4 induction.

HgCl₂ Activates PKC—To assess whether PKC activation implies phospholipase C-mediated pathway, we used U-73122 as an inhibitor of phospholipase C. By itself this agent increased IL-4 gene expression in a H11.3 T cell hybridoma (Fig. 7A). Moreover, not only it did not decrease but it enhanced the effect of HgCl₂ on IL-4 gene expression (Fig. 7A). Similar results were observed with herbimycin A, a protein tyrosine kinase inhibitor (Fig. 7B).

Because PKC activation plays a major role in HgCl₂-induced IL-4 gene expression and because upstream activation pathways for PKC activation did not seem to be involved, the

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**Fig. 2.** PCR-based nuclear run-on assay. 1H11.3 T cells (10⁶ cells) were unstimulated (Ctr) or were stimulated with HgCl₂ (20 μM) for 1 h 30 min or for 3 h. Nuclei were prepared and incubated in presence or absence of each rNTP for 30 min. RNA was extracted, and reverse transcriptase PCR for IL-4 and for β-actin was performed as described under “Experimental Procedures.” Results are representative of two independent experiments.

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**Fig. 3.** HgCl₂ induces a PKC-dependent calcium influx. Panel A, HgCl₂ (20 μM) was added to 1H11.3 T cells, and the variation of [Ca²⁺]i was measured. Panel B, depletion of culture medium in Ca²⁺ abolished the HgCl₂-induced increase in [Ca²⁺]i. Panel C, pretreatment of 1H11.3 T cells with the PKC inhibitor Ro 31-8220 (5 μM) abolished the HgCl₂-induced increase in [Ca²⁺]i.

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**Fig. 4.** Intracellular Ca²⁺ concentration variation in 1H11.3 T cells stimulated with HgCl₂, PMA, or an agonist of L-type calcium channels. 1H11.3 T cells were stimulated with HgCl₂, PMA (an activator of PKC; 10 ng/ml), S(-)-Bay K8344 (an activator of L-type Ca²⁺ channels; 6 μM). We have also checked the effect of Ca²⁺-free medium and of the inhibitor of PKC (Ro 31-8220) on HgCl₂-induced [Ca²⁺]i, variation (HgCl₂, Ca₀ and HgCl₂+Ro, respectively). Results are expressed as mean ± 1 S.D. of experiments performed on 36–60 cells.
question was addressed as to whether HgCl₂ could activate PKC directly. As shown in Fig. 8, HgCl₂ activated rat brain PKC in a dose-dependent manner even in the virtual absence of Ca²⁺, as the experiments were done in the presence of 20 mM EGTA, a concentration that abolished Ca²⁺-dependent PKC activation.

**Fig. 5. Role of PKC activation and Ca²⁺-dependent pathway in HgCl₂-induced IL-4 gene expression.** Panel A, 1H11.3 T cell hybridoma (5 × 10⁵ cells) was preincubated with medium (−) or with the inhibitor of PKC, Ro 31-8220 (RO) at the indicated concentrations (1, 2.5, or 5 μM); 30 min later, 20 μM HgCl₂ was (+) or not (−) added, and the cells were incubated for an additional 4 h. Results are representative of three experiments. Panel B, SM1.27.9 T cell hybridoma (5 × 10⁵ cells) was preincubated or not with the intracellular Ca²⁺ chelator BAPTA/AM (BPT; 30 μM) for 1 h. Then cells were washed twice, and HgCl₂ (20 μM) was added or not for another 4 h. Ctr represents cells cultured in medium alone. Panel C, 1H11.3 T cell hybridoma (5 × 10⁵ cells) was preincubated with medium (Ctr) or cyclosporin A (CsA; 0.1 μg/ml); 30 min later, 20 μM HgCl₂ was added or not, and the cells were incubated for another 4 h. RNA extraction and reverse transcriptase PCR were performed as described under "Experimental Procedures." Results are representative of three independent experiments (panels B and C). Panel D, inhibition of HgCl₂-induced IL-4 gene expression by a blocker of L-type Ca²⁺ channels. 1H11.3 T cell hybridoma (5 × 10⁵ cells) was preincubated with medium (−) or R(+)-Bay K, an L-type Ca²⁺ channel blocker at the indicated concentrations (Bay K⁺; 1 or 10 μM). 30 min later, 20 μM HgCl₂ was (+) or not (−) added, and the cells were incubated for an additional 4 h. RNA extraction and reverse transcriptase PCR were performed. Results are representative of three independent experiments.

**Fig. 6. An increase in extracellular Ca²⁺ concentration enhances HgCl₂-induced IL-4 mRNA expression and IL-4 production.** Panel A, 1H11.3 T cell hybridoma was incubated with medium or HgCl₂ (20 μM) in a normal (1 mM) or 10 mM Ca²⁺-containing medium. RNA extraction and reverse transcriptase PCR were performed; results are representative of three independent experiments. Panel B, 1H11.3 T cells (5 × 10⁵ cells) were cultured for 24 h in a 10 mM Ca²⁺-containing medium in the absence (Ctr) or in the presence of HgCl₂ (15 or 25 μM). The presence of IL-4 was assessed by enzyme-linked immunosorbent assay. One experiment among four is presented.

HgCl₂ Triggers IL-4 mRNA Expression in a Ca²⁺- and PKC-Dependent Manner in BN Spleen Cells—To demonstrate that the Ca²⁺- and PKC-dependent pathways were also implicated in the effect of HgCl₂ on IL-4 expression in BN T cells, we tested the effects of cyclosporin A and of the inhibitor of PKC (Ro 31-8220). As shown in Fig. 9, HgCl₂ induced IL-4 mRNA
expression in BN spleen cells, and this effect was abolished both by cyclosporin A and by the PKC inhibitor.

**DISCUSSION**

Previous results indicated that the ability of HgCl₂ to induce IL-4 gene expression and IL-4 production by rat T cells directly (8) was of major importance in an understanding of why BN rats develop Th2-mediated autoimmunity after HgCl₂ exposure. The mechanisms of HgCl₂-induced IL-4 gene expression were explored by using two mouse T cell hybridomas that responded to HgCl₂. Using these T cell hybridomas, we showed that 1) HgCl₂ induced IL-4 gene transcription without de novo protein synthesis; 2) HgCl₂ induced a PKC-dependent Ca²⁺ influx through L-type calcium channels; 3) PKC activation and Ca²⁺-dependent pathways were both required for HgCl₂-induced IL-4 gene expression; and that 4) HgCl₂ was able to activate PKC directly in a cell-free system.

Others have shown that HgCl₂ induces expression of the mercuric ion reductase gene (33) and metallothionein gene (34) by interacting directly with DNA or transcription factors, respectively. The fact that a chelator of intracellular calcium and inhibitors of PKC both abolished IL-4 gene expression ruled out a role for an interaction of HgCl₂ with DNA or transcription factors. This indicates that HgCl₂, which has the ability to enter the cell (35), has different intracellular targets. PKC and Ca²⁺-dependent pathways were implicated in HgCl₂-induced IL-4 gene transcription since an inhibitor of PKC (Ro 31-8220), the chelator of intracellular calcium BAPTA/AM and cyclosporin A abolished the induction of IL-4 gene by HgCl₂. H-7, another inhibitor of PKC which is different chemically from Ro 31-8220, also abolished HgCl₂-induced IL-4 gene expression (not shown), confirming that PKC was implicated. An elevation in the extracellular calcium concentration, which increases calcium-dependent NF-AT translocation in the nucleus (32), enhanced the effect of HgCl₂ on IL-4 mRNA expression and increased the production of IL-4, thus confirming the importance of the Ca²⁺-dependent pathway.

Our results are in agreement with numerous reports that demonstrate the involvement of both PKC and calcium-dependent pathways in IL-4 production upon stimulation through TCR or after ionomycin plus PMA treatment (15, 36). However, whether Ca²⁺ influx is inositol 1,4,5-trisphosphate-dependent or not is a matter of debate. On the one hand, it is generally accepted that T cell activation through TCR induces inositol 1,4,5-trisphosphate-dependent or not is a matter of debate. On the one hand, it is generally accepted that T cell activation through TCR induces inositol 1,4,5-trisphosphate-dependent mobilization of calcium stores and secondarily an influx of Ca²⁺ from the external medium (for review, see Ref. 37). On the other hand, depletion of extra-cellular Ca²⁺ inhibited both the initial and the sustained Ca²⁺ elevation induced by TCR-mediated stimulation in some T cells, which rules out a role for an initial Ca²⁺ mobilization from the stores (38). Gajewski et al. (17) also reported that signaling in IL-4-producing Th2 clones was associated with weak variations in [Ca²⁺], in the absence of inositol 1,4,5-trisphosphate production. The fact that HgCl₂ induced a cal-

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**FIG. 7.** Effect of an inhibitor of phospholipase C and of an inhibitor of protein tyrosine kinases on HgCl₂-induced IL-4 gene expression. Panel A, 1H11.3 T cell hybridoma (5 × 10⁵ cells) was preincubated or not with a phospholipase C inhibitor, U-73122 (1 μM) for 30 min. Then HgCl₂ (Hg; 20 μM) was added or not for 4 h more. Panel B, 1H11.3 T cell hybridoma (5 × 10⁵ cells) was preincubated or not for 30 min with herbimycin A (herb; 250 ng/ml) used as an inhibitor of protein tyrosine kinases. Then HgCl₂ (20 μM) was added not for another 4 h. RNA extraction and reverse transcriptase PCR were performed; results are representative of three independent experiments.

**FIG. 8.** Dose-dependent stimulation of protein kinase C by HgCl₂. Rat brain PKC was incubated in the presence of EGTA (20 mM) without or with various doses of HgCl₂. As controls PKC was incubated with Ca²⁺ in the presence or in the absence of EGTA. The ability of PKC to phosphorylate a specific substrate was assessed by using a nonradioactive PKC kit assay. Results are expressed as: ([PKC stimulated/ PKC unstimulated] - 1) × 10⁶. Results are the mean ± 1 S.D. of four experiments. Only one experiment was done with 10 μM Ca²⁺ and with 10 μM HgCl₂.

**FIG. 9.** Role of PKC activation and Ca²⁺-dependent pathway in HgCl₂-induced IL-4 gene expression in BN spleen cells. BN spleen cells (8 × 10⁵ cells) were preincubated with medium (Ctr), with the inhibitor of PKC, Ro 31-8220 (RO) at the indicated concentrations (2.5 or 10 μM) or with cyclosporin A (CsA at 1 μg/ml); 30 min later, 20 μM of HgCl₂ was added (Hg) or not, and the cells were incubated for another 4 h. RNA extraction and reverse transcriptase PCR were performed as described under “Experimental Procedures.” Results are representative of three independent experiments.
cium influx in the absence of an initial mobilization of Ca\(^{2+}\) stores could be related to the fact that the T cell hybridomas used in this study resemble Th2 cells. Indeed, they produced IL-4 but no interferon-\(\gamma\) upon stimulation via TCR (not shown). Alternatively, HgCl\(_2\) is able to induce Ca\(^{2+}\) entry in brain and renal cells without mobilization of Ca\(^{2+}\) stores (39), and it might have the same effect on T cells.

We then investigated the relationship that could exist between PKC and the Ca\(^{2+}\)-dependent pathway. Ro 31-8220, a PKC inhibitor, suppressed HgCl\(_2\)-induced Ca\(^{2+}\) entry, but it had no effect on an ionomycin-induced (Ca\(^{2+}\)) increase (not shown). In addition, PMA, an activator of PKC, also triggered an entry of calcium in CH11.3 T cell hybridoma. Dihydropyridine-sensitive L-type Ca\(^{2+}\) channels, known to be a target for PKC (30), have already been described in T cells (40, 41). \((S\rightarrow)-\text{Bay K8344, an agonist of L-type Ca}^{2+}\) channels, induced an entry of Ca\(^{2+}\), indicating that these channels were expressed by CH11.3 T cell hybridoma. These channels are likely to be implicated in HgCl\(_2\)-induced IL-4 gene expression because an L-type Ca\(^{2+}\) channel blocker, \((S\rightarrow)-\text{Bay K8344, abolished the induction of IL-4 gene by HgCl\(_2\). Thus, our experiments are in agreement with a pathway in which PKC activation is responsible for an influx of calcium through L-type Ca\(^{2+}\) channels. An ability of PKC to activate L-type or other Ca\(^{2+}\) channels has not been described in T cells to the best of our knowledge, but it is widely accepted in other cell types (29, 42, 43).

To answer the question of how HgCl\(_2\) may activate PKC, we used in a cell-free system rat brain PKC that contains predominantly Ca\(^{2+}\)-dependent \(\alpha, \beta,\) and \(\gamma\) isoforms (44). In this system we show that Hg was as efficient as Ca\(^{2+}\) in activating rat brain PKC. That relatively high concentrations of Hg\(^{2+}\) and Ca\(^{2+}\) were required for PKC activation is probably because the test was performed in the absence of diacylglycerol, which dramatically increases the affinity of the enzyme for Ca\(^{2+}\) (45). Interestingly, lead also activates rat brain PKC (46) and favors IL-4 production \textit{in vivo} and \textit{in vitro} (47). Which PKC isoform(s) is(are) activated by HgCl\(_2\) as well as the site of interaction of protein tyrosine kinase and phospholipase C \((PKC)_{\text{in}}\) the study were in a toxic range, the cell types used were different, and it might have the same effect on T cells. Alternatively, HgCl\(_2\) is able to induce Ca\(^{2+}\) entry in brain and renal cells without mobilization of Ca\(^{2+}\) stores (39), and it might have the same effect on T cells.

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