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# Heptachlor epoxide induces a non-capacitative type of $Ca^{2+}$ entry and immediate early gene expression in mouse hepatoma cells

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**Abstract**

The effects of the organochlorine (OC) liver tumor promoter heptachlor epoxide (HE) and a related non-tumor promoting OC, delta-hexachlorocyclohexane (δ-HCH), on the dynamics of intracellular calcium ($Ca^{2+}$) were investigated in mouse 1c1c7 hepatoma cells. HE induced a non-capacitative, $Ca^{2+}$ entry-like phenomenon, which was transient and concentration-dependent with 10 and 50 μM HE. The plasma membrane $Ca^{2+}$ channel blocker SKF-96365 antagonized this HE-induced $Ca^{2+}$ entry. δ-HCH failed to induce $Ca^{2+}$ entry, rather it antagonized the HE-induced $Ca^{2+}$ entry. Both HE and δ-HCH induced $Ca^{2+}$ release from endoplasmic reticulum (ER) at treatment concentrations as low as 10 μM; at 50 μM, the former induced 5× as much $Ca^{2+}$ release as the latter. The HE-induced $Ca^{2+}$ release from the ER was antagonized using the $IP_3$ receptor/channel blocker xestospongin C, suggesting that HE induces ER $Ca^{2+}$ release through the $IP_3$ receptor/channel pore. These results show that the effect of HE on cellular $Ca^{2+}$ mimics that of mitogens such as epidermal and hepatocyte growth factors. They also provide insight into the similarities and differences between tumorigenic and non-tumorigenic OCs, in terms of the mechanisms and the extent of the $[Ca^{2+}]_i$ increased by these agents.


*Keywords:* $Ca^{2+}$ entry; Hepatoma cells; Heptachlor epoxide; Delta-HCH; Intracellular $Ca^{2+}$; Liver cancer

## 1. Introduction

Heptachlor epoxide (HE) is a highly persistent and lipophilic cyclodiene organochlorine (OC) compound and metabolite of the insecticide heptachlor, which bioconcentrates in the liver to induce a number of chronic effects. One of these chronic effects is hepatic tumor promotion (NCI, 1977; WHO, 1984; Williams and Numoto, 1984) for which the mechanism of action is poorly understood. On the other hand, a physicochemically very similar agent, δ-HCH, a hexachlorocyclohexane compound and isomer of lindane (γ-hexachlorocyclohexane), shows little or no tumorigenic activity.

Previously, it has been reported from this laboratory (Hansen and Matsumura, 2001a) that HE, given in diet at 20 ppm, causes down-regulation of the enzymatic activities of protein kinase C that were stimulated by exogenuously added $Ca^{2+}$ in liver homogenates prepared from treated male B6C3F1 mouse livers. Up-regulation of AP-1 proteins was found to accompany this phenomenon. This observation lends support to the notion that the ability of HE to increase intracellular $Ca^{2+}$ concentrations may play a role in its tumor promoting action. Elevated levels of intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) is

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one of the earliest effects induced by certain OC compounds (Yamaguchi et al., 1980; Madhukar et al., 1983; Suzaki et al., 1988; Criswell et al., 1994). $Ca^{2+}$ is critical for many cellular signal transduction processes. Following cell-surface-receptor activation by agonists like bradykinin, angiotensin II, or peptide growth factors, for instance, the phosphoinositide (PI) signal transduction cascade is initiated. Among the earliest events in PI signaling is the release of $Ca^{2+}$ from stores in the endoplasmic reticulum (ER), followed by $Ca^{2+}$ influx across the plasma membrane, a process termed capacitative or depletion-activated $Ca^{2+}$ entry (Parekh and Putney, 2005). Spatially and temporally defined changes in $[Ca^{2+}]_i$ provide essential secondary signals necessary for cell activation and proliferation. Mitochondria also play a role in PI signaling, accumulating $Ca^{2+}$ released from nearby ER and activating a number of $Ca^{2+}$-responsive metabolic enzymes (Hoek et al., 1995; Jouaville et al., 1995).

Altered regulation of $Ca^{2+}$ signaling events have been shown to be an important component of the tumor promotion process in several cases. For instance, elevation of $[Ca^{2+}]_i$ is associated with decreased gap junctional intercellular communication (GJIC) and closure of gap junctions, effects used to document the tumor-promoting potential of a compound (Rose et al., 1977; Peracchia and Peracchia, 1980; Matesic et al., 1994). A number of chemicals that primarily affect $[Ca^{2+}]_i$ such as thapsigargin (TG) and ionomycin are also well-documented tumor promoters (Rose and Loewenstein, 1975; Thastrup et al., 1987; Perchellet et al., 1990; Iijima et al., 1991; Lazrak and Peracchia, 1993; Crow et al., 1994), whereas compounds that inhibit the actions of $Ca^{2+}$ such as calmodulin inhibitors (e.g., flunarizine) and $Ca^{2+}$-channel blockers (e.g., verapamil) are known to inhibit tumor development (McGaughey and Jensen, 1980, 1982; Sezzi et al., 1985; Simpson, 1985; Tsuruo et al., 1985; Hait, 1987). Non-pathophysiological $Ca^{2+}$ signals are typically characterized by their temporal transience. In hepatocytes, when intracellular $[Ca^{2+}]_i$ remains elevated for prolonged periods, adverse cellular responses can result such as oxidative stress and release of inflammatory cytokines and formation of ER stress response proteins like glucose-related-protein 78 and CHOP (C/EBP homologous protein), leading to decreased GJIC. Furthermore, mobilization of intracellular $Ca^{2+}$ is associated with cellular changes occurring at the level of the gene, for example, up-regulation of the immediate-early gene product and protooncogene c-FOS (Morgan and Curran, 1986; Bandyopadhyay and Bancroft, 1989; Diliberto et al., 1990).

OC tumor promoters such as DDT, chlordane, and HE have been reported to increase $[Ca^{2+}]_i$ (Yamaguchi et al., 1980; Madhukar et al., 1983; Suzaki et al., 1988) and decrease GJIC (Ruch et al., 1990; Matesic et al., 1994) in a number of cell types. Matesic et al. (1994), for example, using moderate OC concentrations (25.7 μM HE or 26.3 μM dieldrin) found that the OCs HE and dieldrin inhibited GJIC in liver cell cultures within 2 min of exposure. Though some recovery followed, GJIC was still significantly inhibited 24 h post-treatment. Additionally, Suzaki et al. (1988) showed that heptachlor, HE, and chlordane elevated $[Ca^{2+}]_i$ in leukocytes by inducing $Ca^{2+}$ influx, release from intracellular stores, and $Ca^{2+}$ release from unspecified membrane sites. These observations indicate that OCs as a group generally appear to affect cellular $Ca^{2+}$ regulation, but they do not offer information on a precise cellular target or mechanisms of action. Therefore, a substantial amount of additional work is needed to clarify the specificity of action of individual OCs. The present work examines the mechanisms by which HE and δ-HCH alter intracellular $Ca^{2+}$ and how this deregulation correlates to their tumor promoting activity. In the studies described herein, HE and δ-HCH were employed as representative tumorigenic and non-tumorigenic agents in an attempt to decipher the mechanistic differences between the two OCs related to OC-induced $Ca^{2+}$ deregulation and AP-1 activation. In order to distinguish each source of calcium input that is affected by these pesticides and thereby contributes to the eventual rise in its intracellular concentration from others in the current study, we have relied on the use of well accepted diagnostic agents as shown in Fig. 1. The major new findings documented here include the observation that HE induces a pattern of $Ca^{2+}$ signaling similar to that induced by PI cascade agonists - that is $Ca^{2+}$ release from ER stores followed by $Ca^{2+}$ entry. Also important are our findings that $Ca^{2+}$ influx appears to be through SKF-96365-sensitive, plasma membrane, $Ca^{2+}$ channels, while the ER $Ca^{2+}$ release induced by HE-treatments appears to be through xestospongin C-sensitive, $IP_3$ receptor, $Ca^{2+}$ channels. The temporal persistence of HE-mediated $Ca^{2+}$ signals appears to be sufficient by itself to significantly enhance AP-1 activation and DNA binding.

## 2. Material and methods

### 2.1. Chemicals

HE was obtained from Dow Chemical (Midland, MI). Fura-2/AM and pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Dialyzed calf serum (10 kDa max pore size), oligomycin, thapsigargin, 1× trypsin/EDTA

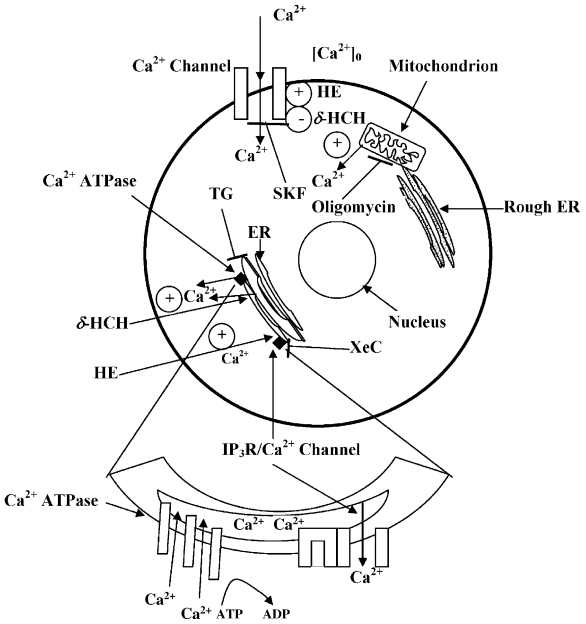


Fig. 1. Schematic diagram showing the target sites for heptachlor epoxide (HE) and δ-HCH, related to modulation of intracellular free $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$), as well as diagnostic agents used in this work in 1c1c7 hepatoma cells (large circle). A "+" sign indicates that the agent increases $Ca^{2+}$ mobilization or ($[Ca^{2+}]_i$), while a "−" sign indicates that the agent decreases it. A blow-up of the ER is included below the cell to show the $Ca^{2+}$ ATPase and $IP_3R/Ca^{2+}$ channel in greater detail. Generally speaking, HE mobilizes $Ca^{2+}$ from the ER and promotes $Ca^{2+}$ influx through plasma membrane $Ca^{2+}$ channels. δ-HCH, on the other hand, inhibits or blocks $Ca^{2+}$ influx through the plasma-membrane-incorporated $Ca^{2+}$ channels, but induces $Ca^{2+}$ mobilization from an unknown target in the ER. SKF96365 blocks $Ca^{2+}$ channels in the plasma membrane, preventing $Ca^{2+}$ influx; TG inhibits the ER $Ca^{2+}$ ATPase, which pumps $Ca^{2+}$ into the lumen of the ER against a gradient, thereby causing $Ca^{2+}$ to leak out of the ER; oligomycin inhibits ATP synthase, causing $Ca^{2+}$ to leak out of the mitochondria; and XeC blocks $Ca^{2+}$ from passing through the $IP_3R$ associated $Ca^{2+}$ channel from the lumen to the ER into the cytosol. Abbreviations used are: ER (endoplasmic reticulum), $IP_3R/Ca^{2+}$ channel (inositol-1,4,5-triphosphate receptor/$Ca^{2+}$ channel), TG (thapsigargin), XeC (Xestospongin C), and SKF (SKF96365).

medium, (±)-sulfinpyrazone, δ-HCH, and other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Piperonyl butoxide was purchased from Aldrich Chemical (Milwaukee, WI). Ionomycin (free acid) and SKF-96365 HCl (1-(β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole HCl) were purchased from Calbiochem–Novabiochem Corporation (La Jolla, CA). α-Minimum essential medium (α-MEM) and antibiotic/antimycotic solution were purchased from Life Technologies Inc. (Gibco BRL, Grand Island, NY). The $IP_3$ (inositol triphosphate) receptor/ion channel blocker xestospongin C, isolated from a marine sponge, was generously provided by Drs. Isaac Pessah and Tadeusz Molinski, University of California, Davis (Gafni et al., 1997; Miyamoto et al., 2000).

### 2.2. Cell culture

Mouse 1c1c7 hepatoma cells were a gift from Dr. Harkinson (University of California, Los Angeles; Hankinson et al., 1985; Watson and Hankinson, 1992). Human HepaG2 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in 100 mm diameter plastic cell culture dishes, containing 6–10 ml of "culture medium" (α-MEM supplemented with 26.2 mM sodium bicarbonate, 10 % FBS, and 1% antibiotic-antimycotic solution, pH 7.4, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B in 0.85% NaCl) depending on cell density. Media was changed every 2 or 3 days, and cells were subcultured following trypsinization every 1–2 weeks.

### 2.3. Cell preparation

Hepatoma cells were grown to confluence, rinsed with 3 ml of PBS (phosphate-buffered saline; 154 mM NaCl, 1 mM $KH_2PO_4$; 3 mM $Na_2HPO_4{\cdot}7H_2O$, pH 7.4), then detached with 1× trypsin/EDTA (Sigma Chemical Co.). Cells were diluted 1:1 in culture medium, and sedimented at 50 × *g* in an IEC model Centra-7R™ refrigerated centrifuge (International Equipment Company; Needham Heights, MA) for 5 min at room temperature. The supernatant was removed, and the cells were resuspended in culture medium and stored at 37 °C/5% $CO_2$ until use within 6 h.

### 2.4. Loading intact hepatoma cells with fura-2/AM

Hepatoma cells were loaded for 30 min at 37 °C with 5 μM fura-2/AM (20% (w/v) pluronic F-127 in DMSO, 5 μl/ml media) in the presence of 1% dialyzed (10 kDa maximum pore size) FBS (fetal bovine serum), 1% antibiotic-antimycotic solution, 250 μM sulfinpyrazone (from DMSO stock), and 100 μM piperonyl butoxide (from ethanol stock). Cells were then sedimented at 4 °C for 3 min. (1156 × $g_{max}$), rinsed with 4 ml of the "physiologic $Ca^{2+}$ medium" (140 mM NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4), and re-sedimented. Cells were then resuspended in 3 ml of the appropriate isotonic buffer (physiologic or low $Ca^{2+}$ medium), containing 250 μM sulfinpyrazone and 1% dialyzed FBS (10 kDa maximum pore size), 1% antibiotic–antimycotic solution and placed in a disposable methacrylate fluorometry cuvette.

### 2.5. Intracellular $Ca^{2+}$ measurements

Intracellular $Ca^{2+}$ was measured with cells suspended in a 4 ml disposable methacrylate cuvette using a computer controlled PTI (Photon Technology International Inc., South Brunswick, NJ) fluorimeter with a lens-based sample com-

partment, single excitation and emission monochrometers, LPS-220 arc lamp supply, SC-550 shutter control, and MD-5020 motor driver. The excitation wavelengths of 340 and 380 nm were used to monitor the $Ca^{2+}$ bound and unbound forms of fura-2, respectively, and the emission wavelength utilized was 510 nm. For a given group of experiments, a representative fluorescence maximum ($R_{max}$) was obtained by adding the $Ca^{2+}$ ionophore ionomycin (20 μM in DMSO, final conc.) in the presence of at least 1 mM $Ca^{2+}$. A fluorescence minimum ($R_{min}$) of 0.99 (340/380 nm) was obtained by treating cells suspended in "$Ca^{2+}$-free isotonic medium" (140 mM NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 1 mM MgSO4, pH 7.4) with 20 μM ionomycin, followed by 20 mM EGTA. $[Ca^{2+}]_i$ was determined using the following equation: $[Ca^{2+}] = K_d \times (R - R_{min})/(R_{max} - R) \times Sf2/Sb2$ (Sb2 = 294,705), according to the method of Grynkiewicz et al. (1985), with $K_d = 224$ nM ($K_d$ in presence of $Mg^{2+}$). In all cases, rates were determined for $[Ca^{2+}]_i$ as follows: for a given trace ($[Ca^{2+}]$ in nM versus time in minutes), a straight portion of the steepest portion of the curve was analyzed via linear regression. The slope of the linear regression equation was taken as the rate value expressed in nM/min.

### 2.6. Determination of the effect of HE and δ-HCH on $[Ca^{2+}]_i$ in intact cells

Fura-2/AM loaded mouse hepatoma cells were prepared as described above. Cells were suspended in physiologic $Ca^{2+}$ medium, containing 1 mM extracellular $Ca^{2+}$, and treated with HE (1–50 μM in ethanol) or δ-HCH (1–50 μM in DMSO). For all of the test compound containing assays, appropriate controls were performed using the largest volume of solvent used for the HE and δ-HCH assays.

### 2.7. Determination of $Ca^{2+}$ source(s) for HE and δ-HCH induced $[Ca^{2+}]_i$ elevations in intact cells

The sources of OC-mobilized free $Ca^{2+}$ were investigated using the following approach. The extracellular $Ca^{2+}$ pool was partially isolated by suspending cells in physiologic $Ca^{2+}$ medium (~1 mM free $Ca^{2+}$) and depleting ER and mitochondrial, oligomycin-sensitive $Ca^{2+}$ stores (Huang and Chueh, 1996; Cho et al., 1997) with the ER, $Ca^{2+}$-ATPase inhibitor thapsigargin (TG; 500 nM; in ethanol) and the mitochondrial inhibitor oligomycin (6 μM; in DMSO), respectively. These concentrations were deemed sufficient since additional TG (1 μM), oligomycin (12 μM), and/or the mitochondrial inhibitor rotenone (2 μg/ml) did not result in release of additional $Ca^{2+}$.

The ER $Ca^{2+}$ store was isolated as a $Ca^{2+}$ source by suspending the fura-2/AM loaded hepatoma cells in a "low $Ca^{2+}$ medium" (physiologic $Ca^{2+}$ medium, buffered with 1.69 mM EGTA to yield ~100 nM free $Ca^{2+}$), thus eliminating the extracellular $Ca^{2+}$ as a potential source of free $Ca^{2+}$, and depleting the oligomycin-sensitive, mitochondrial-$Ca^{2+}$ store with oligomycin.

### 2.8. Determination of the effect of SKF-96365 and δ-HCH on HE-induced $Ca^{2+}$ influx

To further confirm whether HE was affecting the extracellular $Ca^{2+}$ pool, cells were treated with the receptor-mediated, $Ca^{2+}$-channel blocker SKF-96365 (Cabello and Schilling, 1993), to antagonize $Ca^{2+}$ influx through receptor-mediated $Ca^{2+}$ channels, or δ-HCH, which inhibits $Ca^{2+}$ entry by an unknown mechanism (Mohr et al., 1995).

### 2.9. AP-1 transcription factor binding

AP-1 transcription factor binding experiments were performed as described by Hansen and Matsumura (2001b). Protein extracts (10 μg) were incubated for 20 min at 25 °C in "gel mobility shift incubation buffer" (80 mM KCl, 10 mM HEPES, 4% Ficoll, 1 mM EDTA, pH 7.9) with 0.2–0.6 ng/lane radioactively labelled AP-1-responsive element oligonucleotide. Poly (dI-dC) [1 μg/0.1 ng oligonucleotide] and acetylated BSA (5 μg/lane) were incubated to inhibit nonspecific binding. Specificity was determined by preincubated selected samples with a 250-fold molar excess of cold, AP-1-responsive element. Samples were separated on a 6% non-denaturing, polyacrylamide gel for 2–4 h at 30 mA/gel. Gels were dried and incubated with X-ray film, and bands were quantified using densitometry.

### 2.10. Statistical analysis

Statistical significance was determined via a two-tailed student's *t*-test for paired comparisons, for appropriate data, with difference judged significant if $P \leq 0.05$. Each data point was expressed as a mean ± standard error of the mean.

## 3. Results

### 3.1. HE rapidly increases $[Ca^{2+}]_i$

The effects of various HE and δ-HCH concentrations on $[Ca^{2+}]_i$ were tested in intact mouse hepatoma cells. Fig. 2 shows that 1 μM HE produced a slight increase in $[Ca^{2+}]_i$, characterized by a slow rate. Upon addition of 5 μM HE, hepatoma cells responded with an initial rapid rise in $[Ca^{2+}]_i$ that progressed with a more gradual second phase (Fig. 2A, second trace). With higher HE concentrations (10, 30, and 50 μM), the mean maximal rates increased in a concentration-dependent manner. With 50 μM HE a very robust rise in $[Ca^{2+}]_i$ was observed (Fig. 2A, bottom trace), with the mean maximal rates of the HE-induced rise in $[Ca^{2+}]_i$ summarized in Fig. 2B. The mean maximal rate of $Ca^{2+}$ elevation seen with 50 μM HE was 45.3 nM/min. In $Ca^{2+}$-replete hepatoma cells (containing ~1 mM $Ca^{2+}$), δ-HCH (50 μM), given by itself, rapidly elevated $[Ca^{2+}]_i$ much like HE,

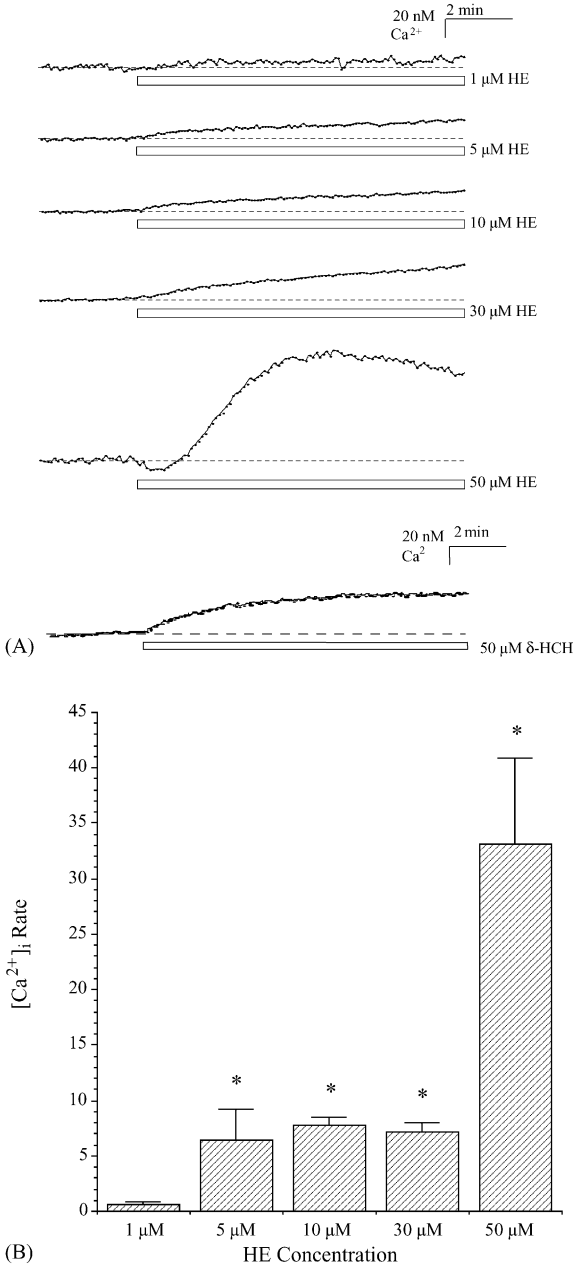


Fig. 2. HE concentration–response experiments in intact hepatoma cells in physiologic $Ca^{2+}$ medium. Mouse hepatoma cells were loaded with 5 μM fura-2/AM suspended in 3 ml of physiologic $Ca^{2+}$ medium containing approximately 1 mM free $Ca^{2+}$, as described in Section 2. HE additions of 1, 5, 10, 30, and 50 μM were made 3 min after beginning fluorimetric monitoring and presented (A) as real time intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$). Each trace represents a mean trace composed of combined traces from three or four separate assays, plotted as mean maximal rates of increased $[Ca^{2+}]_i$ (B.). Bars represent mean rates ± standard errors of the mean. Control rate = 0. *: Statistically significant at $P \leq 0.05$.

but to a much lesser extent (Fig. 2A). δ-HCH also elicited concentration-dependent increases in $[Ca^{2+}]_i$, although to a lesser extent than induced by HE (data not shown).

### 3.2. Effect of HE and δ-HCH on AP-1 transcription factor binding

Experiments were also performed to determine if HE influences activator protein 1 (AP-1) transcription factor (TF) DNA binding, an interaction known to be enhanced by certain $Ca^{2+}$ signals. AP-1 like other primary-response (or immediate-early) TFs are the first TFs activated in response to growth factors and other mitogens (Mohn et al., 1990). Nuclear extracts were isolated from cells treated with 0, 10, or 50 μM HE for 1 h, conditions similar to those utilized to assess $[Ca^{2+}]_i$. AP-1 DNA binding was increased by 112.6 and 155.8% over that of vehicle-treated, control cells (Fig. 3A and B). The results showed that HE (10 μM), a compound that induces $Ca^{2+}$ entry, had a prolonged effect on AP-1 DNA binding, significantly increasing DNA binding at 1 and 3 h, with binding leveling off to the control level at 6 h (Fig. 3C). In contrast, δ-HCH also increased AP-1 DNA binding only with a 1 h treatment but down-regulated binding at 3 and 6 h. Therefore, activation of AP-1 DNA binding correlated well with HE-induced changes in $Ca^{2+}$ handling in terms of both treatment concentration and time course.

### 3.3. Differential mechanisms for HE- and δ-HCH-induced $Ca^{2+}$ entry

In the experiments shown in Fig. 4, an attempt was made to determine if the action of HE on $[Ca^{2+}]_i$ was due solely to release from an intracellular $Ca^{2+}$ store or from $Ca^{2+}$ influx, as well. As expected, sequential addition of 6 μM oligomycin and 500 nM thapsigargin (TG) caused sharp rises in $[Ca^{2+}]_i$, ascribable to emptying of mitochondrial and ER stores, respectively. In the hepatoma cell line used here, as is common with other cells, the ER appears to represent the major $Ca^{2+}$ store, judging by the larger increase in $[Ca^{2+}]_i$ induced by TG versus oligomycin. The reduction in $[Ca^{2+}]_i$ subsequent to the TG addition is likely due to unidirectional efflux out of the cells, since in this experiment, both of the major $Ca^{2+}$ sequestering organelles have been already inhibited. The prolonged $[Ca^{2+}]_i$ elevation commonly seen after depletion of stores has been ascribed to a small component of depletion-activated $Ca^{2+}$ entry (Llopis et al., 1992). Interestingly, exposure of the $Ca^{2+}$-depleted cells to HE resulted in a sharp, concentration-dependent rise in $[Ca^{2+}]_i$, which is likely to be attributable to the ability

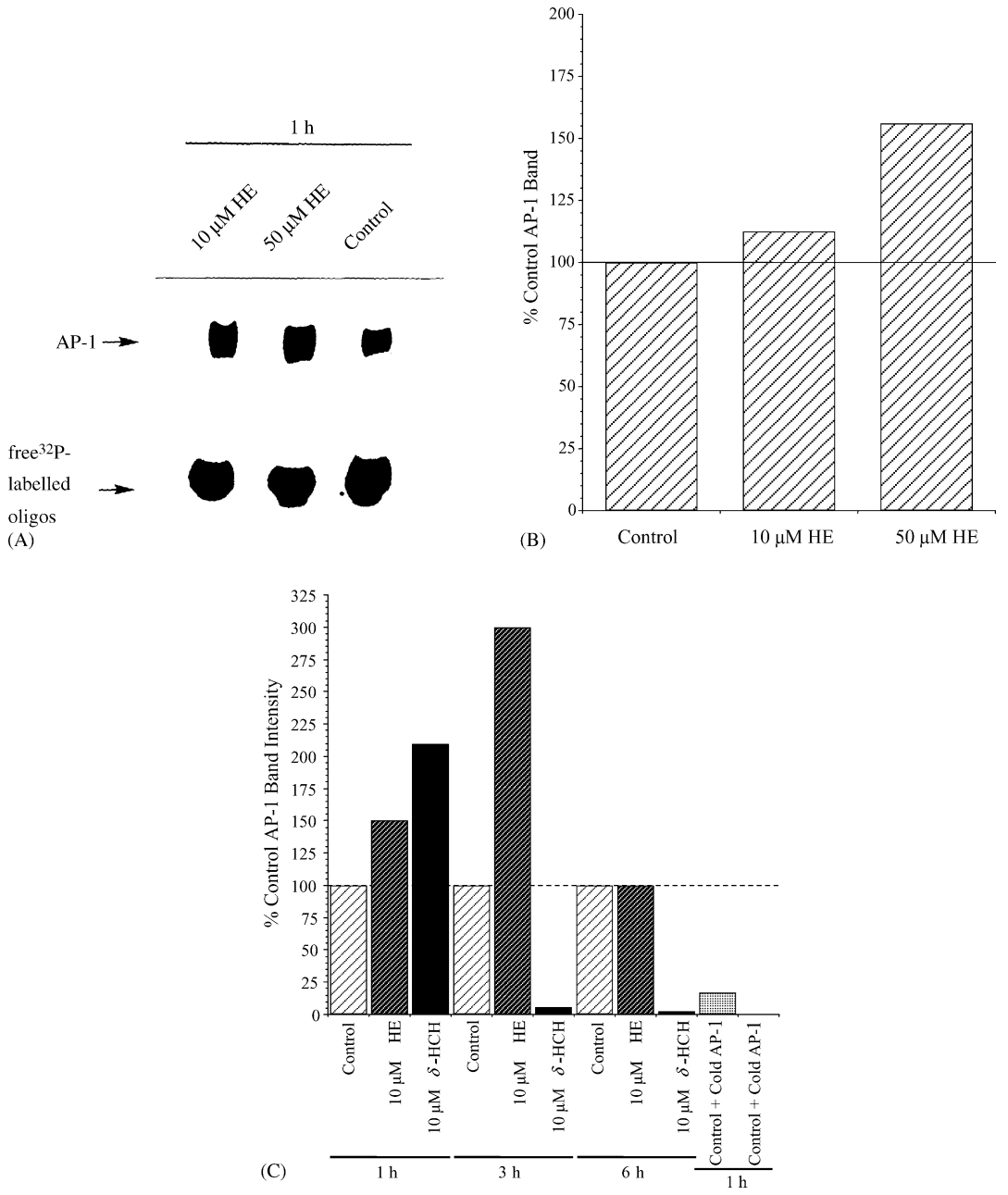


Fig. 3. Effect of HE and δ-HCH on AP-1 nuclear transcription factor binding. (A) and (B) Nuclear extracts were isolated from 1c1c7 cells exposed to 0, 10, or 50 μM HE for 1 h. AP-1 binding to its DNA response element was performed as described in Section 2. (C) Shows a time-course effect of HE and δ-HCH on human HepaG2 cells for 1, 3, and 6 h. AP-1 bands (not shown) of interest were plotted following densitometer analysis of each band expressed as a percent of control AP-1 DNA binding activity (control = 100%) from single values. A large excess of cold AP-1 was used to show specificity of the DNA binding effect for AP-1.

of this OC to induce $Ca^{2+}$ entry. The mean maximal rates for $Ca^{2+}$ influx were $3.9 \pm 5.9$ and $13.9 \pm 7.2$ nM/min for 10 and 50 μM HE, respectively (Fig. 4B).

To test this possibility, the effects of δ-HCH on hepatoma cells $[Ca^{2+}]_i$ were studied. Although δ-HCH mobilizes $Ca^{2+}$ from stores in RBL cells, it has also been reported to concomitantly inhibit $Ca^{2+}$ entry in these cells (Mohr et al., 1995). Hence, both HE and δ-HCH elevate $[Ca^{2+}]_i$ as has been previously reported in other cell types (Yamaguchi et al., 1980; Suzaki et al., 1988; Mohr et al., 1995 for δ-HCH). However, an important difference was observed here in the response of hep-

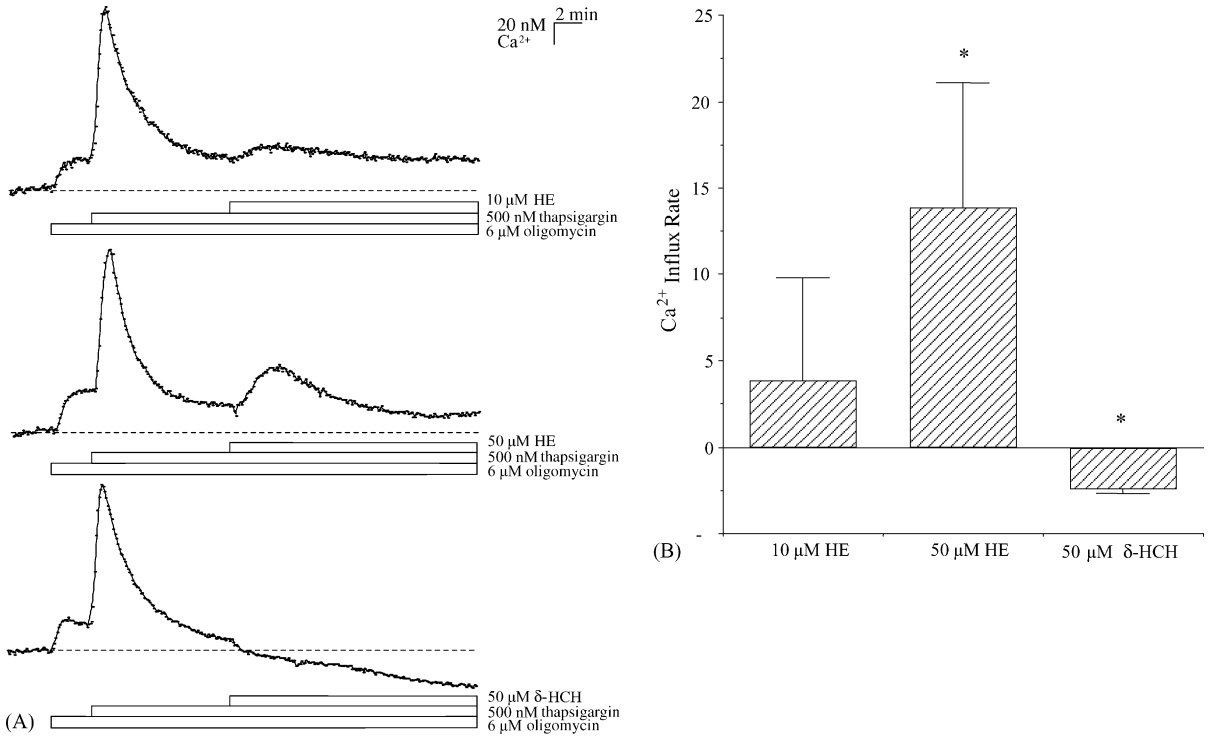


Fig. 4. Differential effects of HE and δ-HCH on $Ca^{2+}$ entry. Experiments were performed essentially as described in Fig. 2 and Section 2. The extracellular $Ca^{2+}$ pool was partially isolated (A) by depleting the oligomycin-sensitive mitochondrial $Ca^{2+}$ pool with 6 μM oligomycin 3 min after beginning fluorimetric monitoring and the ER $Ca^{2+}$ pool with 500 nM thapsigargin 6 min following initiation of the experiment. Cells were then treated 13 min after beginning experiment with 10 or 50 μM HE or 50 μM δ-HCH to determine their effects on the extracellular $Ca^{2+}$ pool. The concentrations of oligomycin and thapsigargin used were found to completely deplete the oligomycin-sensitive mitochondrial and ER $Ca^{2+}$ pools, respectively. The vehicles (ethanol for HE and DMSO for δ-HCH) had no effect on $[Ca^{2+}]_i$ (data not shown). Each trace represents a mean trace composed of combined traces from three or four separate assays, plotted as mean maximal rates of $Ca^{2+}$ influx (B). Bars represent mean rates ± standard errors of the mean. Control rate = 0. *: Statistically significant at $P \leq 0.05$.

atoma cells to these two OC compounds. As Fig. 4A (bottom trace) shows, δ-HCH introduced to oligomycin- and TG-pretreated cells, did not stimulate $Ca^{2+}$ uptake, unlike the case of HE (Fig. 4A top and center traces). Instead, δ-HCH reduced the internal $Ca^{2+}$ concentration beyond the resting level (i.e., below the dotted line) under this experimental condition.

The HE-induced, $Ca^{2+}$-entry phenomenon was studied more intensively in hepatoma cells. As illustrated in Fig. 5A (second trace), SKF-96365, an agent shown to attenuate $Ca^{2+}$ influx through plasma membrane, receptor-mediated, $Ca^{2+}$ channels (Cabello and Schilling, 1993), enhanced the $Ca^{2+}$-extrusion process in $Ca^{2+}$-depleted hepatoma cells (compare first and second traces). Subsequent addition of 50 μM HE after 50 μM SKF-96365 dramatically reduced HE-induced $Ca^{2+}$ influx (second trace). Furthermore, pretreatment with 100 μM SKF-96365 completely eliminated HE-induced $Ca^{2+}$ influx (third trace). HE induced a mean rise of $[Ca^{2+}]_i$ of $36.3 \pm 8.5^*$ nM ($^*$statistical significance at $P \leq 0.05$ compared to control), with a mean maximal influx rate of $17.7 \pm 6.5$ nM/min. The respective HE-induced increases in $[Ca^{2+}]_i$ after 50 and 100 μM SKF-96365 were 18.3 and 14.1 nM, respectively, with corresponding maximal $Ca^{2+}$-entry rates of 6.4 and $2.3 \pm 5.2$ nM/min.

Likewise, 50 μM δ-HCH substantially inhibited HE-induced $Ca^{2+}$ entry, while 100 μM δ-HCH almost completely inhibited HE-induced $Ca^{2+}$ influx (Fig. 5A, fourth and fifth traces). Hence, HE induces $Ca^{2+}$-entry through the plasma membrane, whereas δ-HCH attenuates $Ca^{2+}$ entry even in the presence of HE in mouse hepatoma cells.

### *3.4. HE Mobilizes $Ca^{2+}$ from the ER Store by a xestospongin C-sensitive mechanism*

The mechanism by which HE and δ-HCH mobilize $Ca^{2+}$ from the ER $Ca^{2+}$ store was evaluated under conditions which minimize $Ca^{2+}$ entry into intact mouse

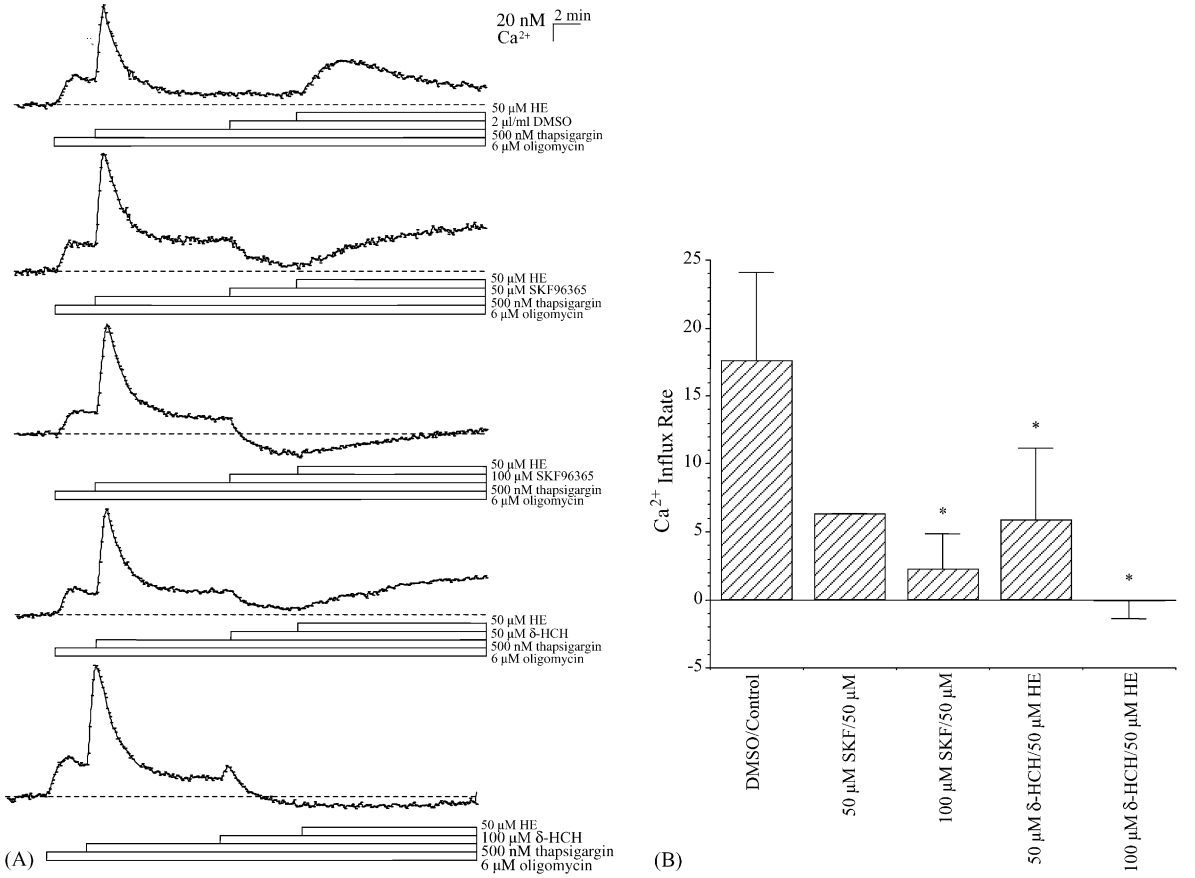


Fig. 5. Inhibitory effect of SKF-96365 and δ-HCH on HE-induced extracellular $Ca^{2+}$ influx. Experiments were performed essentially as described in Fig. 2 and Section 2. The extracellular $Ca^{2+}$ pool was studied in partial isolation in intact hepatoma cells as described in Fig. 3. Cells were treated (A) 16 min into the experiment with vehicle, 50 or 100 μM SKF-96365, or 50 or 100 μM δ-HCH to determine their effect on HE-induced $Ca^{2+}$ entry. HE was added 21 min after initiating fluorimetric monitoring. Each trace represents a mean trace composed of combined traces from three or four separate assays, plotted as mean maximal rates of $Ca^{2+}$ influx (B.). Bars represent mean rates ± standard errors of the mean. Control rate = 0. *: Statistically significant at $P \leq 0.05$.

hepatoma cells by buffering the extracellular $Ca^{2+}$ concentration to ∼100 nM free $Ca^{2+}$ and depleting the mitochondrial $Ca^{2+}$ store with oligomycin (6 μM; Fig. 6). Under these conditions, 10 and 50 μM HE elevated $[Ca^{2+}]_i$ by $32.3 \pm 1.6^*$ and $106.5 \pm 27.3^*$ nM (*statistical significance at $P \leq 0.05$) over control $[Ca^{2+}]_i$, respectively (Fig. 6 top two traces). The corresponding mean maximal rates of ER $Ca^{2+}$ release were $3.6 \pm 0.9$ and $21.0 \pm 4.4$ nM/min (Fig. 6B). In these experiments, TG (500 nM) was subsequently added to demonstrate that elevated $[Ca^{2+}]_i$ induced by HE corresponded to ER-store depletion (Fig. 6, compare traces one and two).

As shown in Fig. 6A (third trace) 10 μM δ-HCH induced a slight, $6.3 \pm 0.9^*$ nM (*statistical significance at $P \leq 0.05$; mean maximal rate of ER $Ca^{2+}$ efflux of $1.0 \pm 0.5$ nM/min), but measurable release of ER $Ca^{2+}$. Upon addition of 500 nM TG, a further, $17.8 \pm 2.7^*$ nM mobilization of $Ca^{2+}$ from the ER was observed. A $21.4 \pm 2.2^*$ nM increase in $[Ca^{2+}]_i$ was observed with 50 μM δ-HCH (Fig. 6, bottom trace), with a mean maximal rate of ER $Ca^{2+}$ efflux of $7.5 \pm 0.5$ nM/min. In cells depleted of their extracellular and oligomycin-sensitive, mitochondrial $Ca^{2+}$ stores, the δ-HCH/TG-induced portions of the $Ca^{2+}$ curve were less steep and less robust than were the HE/TG induced $Ca^{2+}$ curves. This may be due to decreased quantities of sequestered $Ca^{2+}$ in these δ-HCH treated cells compared to cells used in the HE/ER $Ca^{2+}$ store experiments. Therefore, it is reasonable to conclude that while HE and δ-HCH both induce ER $Ca^{2+}$ release as is the case for other tumor promoters such as TG and ionomycin, the latter compound (δ-HCH) appears to reduce the capacity of ER to sequester $Ca^{2+}$.

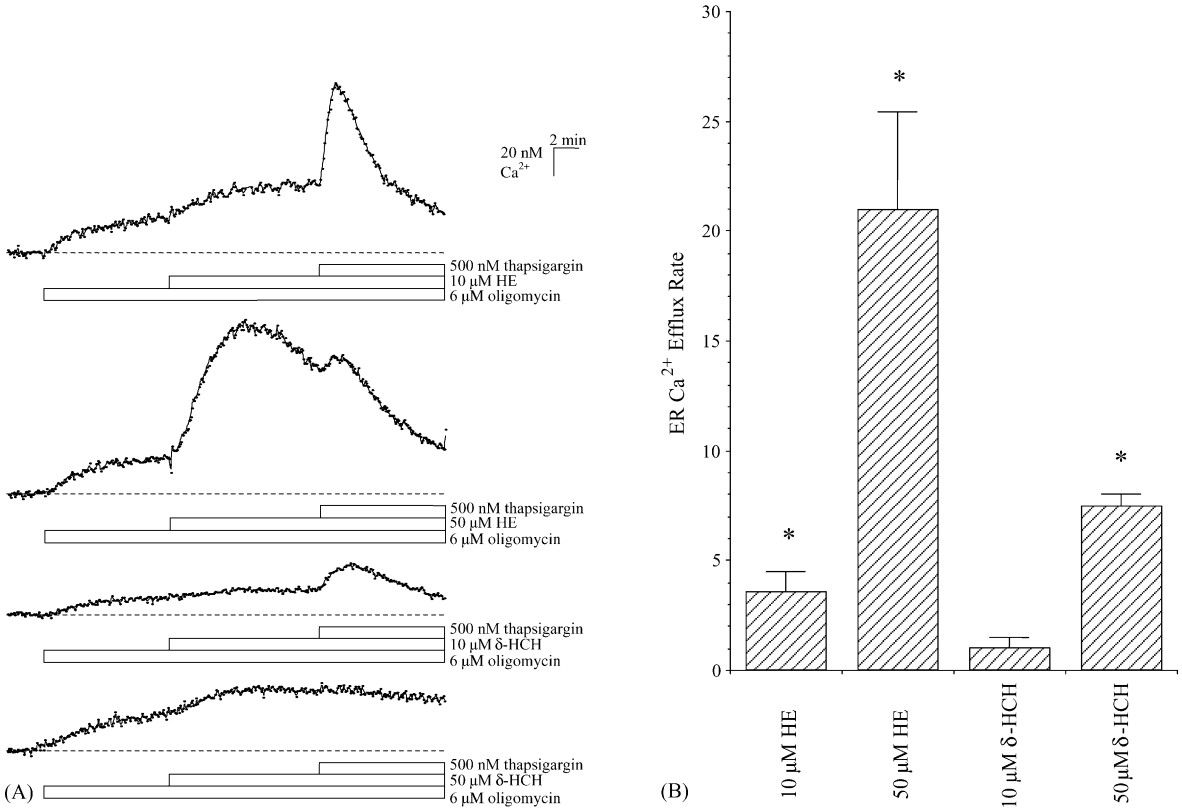


Fig. 6. Studies on the $Ca^{2+}$ mobilizing actions of HE and δ-HCH from ER $Ca^{2+}$ store under conditions minimizing $Ca^{2+}$ entry from outside and $Ca^{2+}$ release from mitochondria in hepatoma cells. The ER $Ca^{2+}$ pool was partially isolated in mouse hepatoma cells loaded with 5 μM fura-2/AM suspended in 3 ml of Low $Ca^{2+}$ Medium to deplete the extracellular $Ca^{2+}$ pool. The mitochondrial $Ca^{2+}$ pool was depleted with 6 μM oligomycin 3 min after beginning fluorimetric monitoring. Cells were then treated with 10 (1st trace) or 50 (2nd trace) μM HE or 10 (3rd trace) or 50 (4th trace) μM δ-HCH 13 min after beginning experiments. The ER $Ca^{2+}$ pump inhibitor thapsigargin (500 nM) was added at 25 min to determine the extent of ER-$Ca^{2+}$-pool depletion. Each trace represents a mean trace composed of combined traces from three or four separate experiments. *: Statistically significant at $P \leq 0.05$.

To further elucidate the mechanism by which HE mobilizes $Ca^{2+}$ from ER stores, experiments were performed with xestospongin C, which interacts with the $IP_3R/Ca^{2+}$ channel complex to prevent ER $Ca^{2+}$ release, (Gafni et al., 1997), which has been extensively studied to date. As shown in Fig. 7A, 10 μM xestospongin C significantly attenuated the HE-mediated elevation in $[Ca^{2+}]_i$, decreasing the average maximal rate of ER $Ca^{2+}$ efflux from 10.8 to 5.4 nM/min ($n = 2$). Further, 20 mM xestospongin C completely inhibited ER $Ca^{2+}$ mobilization, but left the TG-induced release of ER $Ca^{2+}$ unaffected. The maximal rates of ER $Ca^{2+}$ efflux were 38.1 nM/min ($n = 2$) for the control and 1.3 nM/min ($n = 1$) for the 20 μM xestospongin C treated cells (Fig. 7B), following HE application. These data strongly suggest that HE mobilizes $Ca^{2+}$ through the $IP_3$ receptor/$Ca^{2+}$ channel.

## 4. Discussion

An important question to address when studying the effects of extremely lipophilic organochlorine (OCs) compounds on cell $Ca^{2+}$ is whether the increased $[Ca^{2+}]_i$ induced by these agents is due to a specific or non-specific mechanism. The results obtained in the current work provide persuasive evidence that these agents modulate $Ca^{2+}$ in liver cells via specific mechanisms, rather than non-specific ones such as cell-membrane damage. For instance, HE drastically increased $Ca^{2+}$ influx from the extracellular pool while another equally lipophilic OC, δ-HCH, antagonized $Ca^{2+}$ influx. On the other hand, both of these agents were found to act on common targets in this study, both affecting the ER to deplete its $Ca^{2+}$ store. In these cells, however, HE produced a higher maximum level of $Ca^{2+}$ release from either site than did δ-HCH.

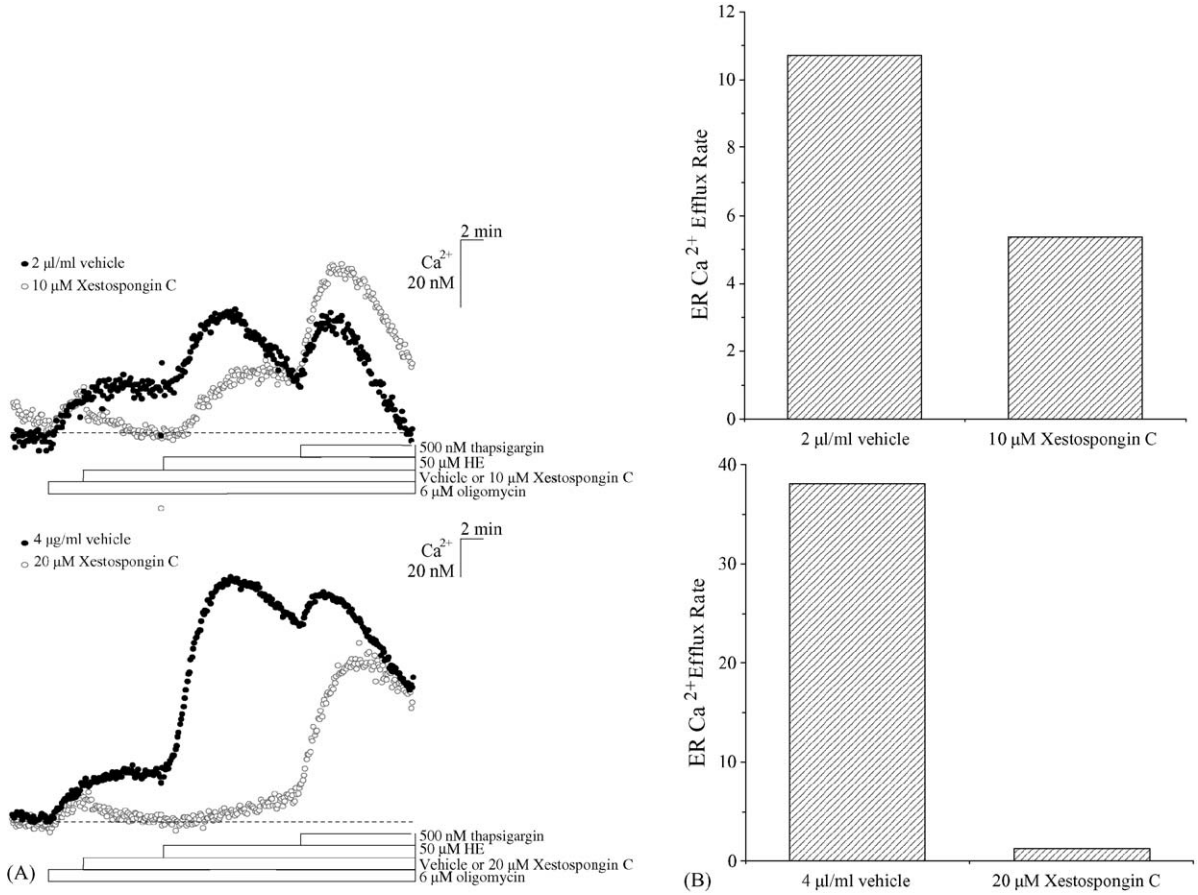


Fig. 7. Effect of Xestospongin C on HE induced ER $Ca^{2+}$ release. Mouse hepatoma cells were prepared as described in Fig. 2 and were suspended in 3 ml of low $Ca^{2+}$ medium, containing approximately 100 nM free $Ca^{2+}$ to deplete the extracellular pool. The oligomycin-sensitive, mitochondrial-$Ca^{2+}$ pool was depleted by oligomycin (6 μM) addition 3 min after beginning fluorimetric monitoring (A) followed at 6 min by vehicle or 10 or 20 μM xestospongin C, the $IP_3$ receptor/channel pore blocker. Addition of 50 μM HE was at 13 min and 500 nM thapsigargin was at 25 min. Each trace represents an average of two assays, plotted as mean maximal rates of ER $Ca^{2+}$ efflux (B.). Bars represent mean rates ± standard errors of the mean. The 20 μM xestospongin C-treated curve represents a single assay.

A second question to address is whether agents utilized to inactivate certain components of the cell's calcium regulatory machinery indeed performed as expected in hepatoma cells. In this work two agents critical for our analysis were oligomycin (6 μM) and TG (500 nM), which have been definitively shown by other scientists to incapacitate mitochondrial ATP synthetase and inactivate the ER $Ca^{2+}$ pump, respectively, in various cell types including those in the liver (Brustovetsky et al., 1993; Wolvetang et al., 1994). Furthermore, their actions on cell $Ca^{2+}$ in this cell line are quite distinctive and completely independent of each other (e.g., see Fig. 4). Their actions were also found to be distinct from two other agents employed here: the receptor-mediated, $Ca^{2+}$-channel blocker, SKF-96365 (Fig. 5), and the $IP_3$-receptor/$Ca^{2+}$-channel blocker, xestospongin C (Fig. 7). SKF-96365 decreases $Ca^{2+}$ influx from the extracellular pool by affecting plasma membrane $Ca^{2+}$ channels, an effect that has been documented in many cell types, including non-excitable cells like those in the liver (Cabello and Schilling, 1993; Lenz and Kleineke, 1997). Furthermore, its close analog verapamil has been used extensively in hepatocytes (Lenz and Kleineke, 1997; Yeo and Mugiya, 1997). Therefore, all these observations support the notion that the actions of these agents are indeed specific. The $Ca^{2+}$-entry-inhibiting action of δ-HCH is also well known (Mohr et al., 1995) and appears to be analogous to the effect of SKF-96365. In our hands, both compounds were effective at substantially decreasing HE-induced-$Ca^{2+}$ entry in a concentration-dependent manner. That these agents performed in precisely the manner expected in oligomycin and TG-pretreated cells further supports the notion that HE was targeting a specific cellular site

to promote $Ca^{2+}$ entry. The fact that verapamil, a $Ca^{2+}$-channel blocker like SKF-96365, similarly decreased $Ca^{2+}$ influx induced by HE provides additional supportive evidence (data not shown). Among standard diagnostic agents used, xestospongin C recently has been extensively utilized as a diagnostic research tool (Gafni et al., 1997; Oka et al., 2002). Criteria supporting the idea that xestospongin C produced a specific action on its intended target, the $IP_3$ receptor/$Ca^{2+}$ channel, are that its action was: (1) independent of those of TG and oligomycin, and (2) concentration-dependent.

In this study, HE was found to induce $Ca^{2+}$ influx in treated cells. Experiments employing cells treated with the receptor-mediated, $Ca^{2+}$-channel blocker SKF-96365 (Fasolato et al., 1990; Cabello and Schilling, 1993) also support this finding. Having established that a primary action of HE on cell $Ca^{2+}$ is induction of $Ca^{2+}$ entry from the external $Ca^{2+}$ pool, a critical question needing to be addressed is whether HE induces "capacitative" $Ca^{2+}$ entry or a "non-capacitative" type of $Ca^{2+}$ entry. The former is characterized by its coupling to ER $Ca^{2+}$ store filling, while the latter is not (Parekh and Putney, 2005). In hepatocytes, it appears that both of these processes are operating independently (Llopis et al., 1992). Based on the observation that HE stimulates $Ca^{2+}$ entry in cells depleted of their $Ca^{2+}$-storage capacity by pretreatment with TG, HE appears to cause a non-capacitative type $Ca^{2+}$ influx. The fact that this HE-induced-$Ca^{2+}$-entry phenomenon was transient in nature (as opposed to a long-lasting effect seen after inhibition of an enzyme, transporter, or ion pump) further suggests that this was $Ca^{2+}$ entry through a $Ca^{2+}$ channel. Additionally, it was demonstrated here that δ-HCH, which has previously been reported to inhibit $Ca^{2+}$ entry in rat basophilic leukemia (RBL) cells (Mohr et al., 1995), inhibited $Ca^{2+}$ entry in 1c1c7 hepatoma cells exposed to HE. Thus, there is a good possibility that in 1c1c7 cells, these two sources of $Ca^{2+}$ (i.e., extracellular $Ca^{2+}$ influx store and ER $Ca^{2+}$ store) are not coupled. If so, HE must be acting on these two sites independently from each other.

HE and δ-HCH were both found to induce ER $Ca^{2+}$ release here, as has been reported previously in other cell types (Suzaki et al., 1988; Pessah et al., 1992; Mohr et al., 1995; Buck et al., 1999; Buck and Pessah, 1999). Additionally, HE was found in the current work to induce ER $Ca^{2+}$ mobilization from ER by acting on the $IP_3$-sensitive, $Ca^{2+}$ store, possibly through the $IP_3$ receptor/channel. This was based on data showing that HE-induced $Ca^{2+}$ release from the ER was inhibited by the $IP_3$-receptor/$Ca^{2+}$-channel, pore blocker xestospongin C (Gafni et al., 1997). In contrast, δ-HCH has been shown to affect the ryanodine receptor (RyR) in cardiac myocytes (Buck et al., 1999) and in cardiac sarcoplasmic reticulum (Pessah et al., 1992) and perhaps an additional ionophore-like mechanism (Buck and Pessah, 1999).

Our interpretation of the data on the overall actions of δ-HCH (see Fig. 5) is, therefore, that this compound blocks $Ca^{2+}$ entry through the plasma membrane $Ca^{2+}$-channel based on its similarity to SKF96365 (Fig. 5A compare the second and the fourth tracing from the top), and at the same time depletes the ER $Ca^{2+}$ store (Fig. 6A bottom tracing). The action of this compound to increase modestly the internal $Ca^{2+}$ was not sufficiently evaluated in this work to define the mechanism. An important observation made here is that pretreatments with 20 μM xestospongin C (Fig. 7) almost completely antagonized the HE-induced ER $Ca^{2+}$ release, while TG (500 nM) addition after HE, resulted in $Ca^{2+}$ mobilization at levels comparable to those observed with the same treatment in control (no xestospongin C) or 10 μM xestospongin C exposed cells. This suggests that the action of HE on ER $Ca^{2+}$ is not mediated by effects on the ER $Ca^{2+}$ pump.

Another aspect of this work was to contrast the effects of a tumorigenic OC, HE, to an OC with weak or no tumorigenic activity, δ-HCH, on cellular $Ca^{2+}$, because $Ca^{2+}$ appears to play a role in the tumor promotion process (Verma and Boutwell, 1981; Perchellet et al., 1990; Dwivedi et al., 1994; Battalora et al., 1995; Tannheimer et al., 1997), at least in certain cases. The fact that the $Ca^{2+}$ modulating agents TG and ionomycin are tumor promoters supports the notion that $Ca^{2+}$ can be important in tumor promotion (Rose and Loewenstein, 1975; Thastrup et al., 1987; Perchellet et al., 1990; Iijima et al., 1991; Lazrak and Peracchia, 1993; Crow et al., 1994). Further, experiments have shown that compounds that increase $[Ca^{2+}]_i$ enhance cancer induction, while $Ca^{2+}$ antagonists decrease it (McGaughey and Jensen, 1980, 1982; Sezzi et al., 1985; Simpson, 1985; Tsuruo et al., 1985; Hait, 1987).

Therefore, a compound that induces $Ca^{2+}$ release from two stores, like HE, would be a more potent promoter than one that induces release a single store, like δ-HCH, assuming that increased $[Ca^{2+}]_i$ is important in tumor promotion and that the threshold for cytotoxicity is not exceeded. Further, HE, in contrast to δ-HCH, was found to mimic the action of PI agonist mitogens like epidermal and hepatocyte growth factors by inducing ER $Ca^{2+}$ release followed by a $Ca^{2+}$-entry phase. This ability of HE to mimic mitogens may be an important factor in explaining the greater potency of HE as a tumor promoter. Finally, we would like to point out that the main objective of this study has been to compare HE and δ-HCH from the viewpoint of

calcium-induced, liver tumorigenesis, along the lines of the thapsigargin example (e.g., Thasrup et al., 1990). The question whether there could be other types of activities of HE promoting liver tumorigenesis was not addressed here.

Having clarified this point, however, we also would like to point out that, as in the case of thapsigargin and phorbol esters, $Ca^{2+}$ is known to activate AP-1. There is substantial evidence demonstrating that the ability of a compound to promote $Ca^{2+}$ entry, as is the case with HE, is an important mechanistic requirement linking altered $Ca^{2+}$ signaling and activation of AP-1 binding to target genes. This is significant since AP-1 has been shown to be a critically important factor in chemical-induced tumor promotion (Bernstein and Colburn, 1989; Ben-Ari et al., 1992; Dong et al., 1994, 1995; Li et al., 1996). It must be stated that, although the extent of stimulation of AP-1 by HE appears to be modest (50% to three-fold, see Fig. 3), there are precedents in literature that $Ca^{2+}$-stimulated up-regulation of AP-1 activities are in the same order of magnitude (e.g., Ng et al., 2000; Hanley et al., 2000) as our observation. Knowing the importance of AP-1 protein in tumorigenesis, our observation demonstrates the functional consequence of the rise in $[Ca^{2+}]_i$ induced by HE.

In summary, the most conspicuous difference between the effects of HE and δ-HCH on calcium homeostasis in 1c1c7 cells is that HE induces $Ca^{2+}$ influx from the extracellular pool, while δ-HCH blocks it. Whether this accounts for the difference in the liver cancer potential of each compound will require future studies. An additional finding of note is that the $Ca^{2+}$ influx observed here appears to be through SKF-96365-sensitive, plasma membrane, $Ca^{2+}$ channels, while the ER $Ca^{2+}$ release induced by HE-treatments appears to be through xestospongin C-sensitive, $IP_3$ receptor/$Ca^{2+}$ channels (see Fig. 1). Our current research findings, however, provide solid experimental evidence that $Ca^{2+}$ entry is differentially affected by these two OCs with very similar chemical properties providing a future research avenue for studies into the role of $Ca^{2+}$ regulation in hepatic carcinogenesis.

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