Alteration of Oxytocin-Induced Calcium Oscillations in Clone 9 Cells by Toxin Exposure

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A communication-competent rat liver cell line (Clone 9) was examined as a model system to investigate the action of toxins on [Ca²⁺], induced by extracellular signals. Clone 9 cells exhibit an initial [Ca²⁺] peak, spike followed by Ca²⁺ oscillations for at least 30 min after exposure to oxytocin (10 to 1000 nM). Oscillations of mitochondrial membrane potential were also detected using the potentiometric fluorescent probe rhodamine 123. Fast Fourier Transform showed that complex patterns of oscillations in Clone 9 cells exhibit both amplitude- and frequency-encoded signals. The initial Ca²⁺ peak and oscillations were not altered by ryanodine treatment but were suppressed by nifedipine and blocked by thapsigargin. Brief exposure of cells to the food-borne toxins patulin or gossypol or the environmental toxicant 2,3,7,8-tetrachlorodibenzop-dioxin at doses which have no effect on cell viability within the duration of experiments was used to monitor any changes in Ca²⁺ oscillations. Toxin treatment either blocked or changed the amplitude and/or frequency of Ca²⁺ oscillations depending upon the toxin-specific mechanisms of cellular injury. These studies indicate that toxic agents may alter amplitude- and frequency-encoded information derived from cell signaling events which could result in altered cellular homeostasis at nonlethal doses of toxin.

Calcium signaling in a variety of nonexcitable cell types frequently occurs as repetitive increases in cytoplasmic free Ca²⁺ concentration ([Ca²⁺]) referred to as Ca²⁺ oscillations. These oscillations are manifested as periodic [Ca²⁺], spikes which increase with increasing agonist concentration. Agonist-induced oscillations in [Ca²⁺] are thought to constitute a frequency-encoded signal with a high signal-to-noise ratio which limits prolonged exposure of cells to high [Ca²⁺], (Sneyd et al., 1995). Considerable effort directed to the analysis of mechanisms that underly the spatiotemporal pattern of intracellular Ca²⁺ has led to an understanding of the role of plasma membrane Ca²⁺ channels in triggering external Ca²⁺ influx and inositol 1,4,5-trisphosphate (InsP₃) receptors and/or ryanodine receptors in the release of Ca²⁺ from intracellular stores (reviewed in Berridge, 1993; Putney and Bird, 1993).

The significance of Ca²⁺ oscillations in nonexcitable cells is not well understood. In systems such as pancreatic acinar cells and pituitary gonadotrophs where agonists induce oscillatory changes in [Ca²⁺], and also stimulate maximal secretion, it appears that frequency-encoded Ca²⁺ signals exert a physiological role (Stauffer et al., 1993; Stojilkovic et al., 1994). The activity of Ca²⁺-sensitive mitochondrial dehydrogenases in hepatocytes can be increased by intracellular Ca²⁺ oscillations which are transmitted to mitochondria as mitochondrial Ca²⁺ oscillations (Hajnózky et al., 1995). It follows that external perturbations induced by toxic chemicals that modulate cytosolic Ca²⁺ oscillations could result in changes in cellular responses.

In the present study a communication-competent normal rat liver cell line (Clone 9), which exhibits a complex pattern of Ca²⁺ oscillations in response to oxytocin stimulation, was examined as a potential model to explore effects of selected toxicants on cellular Ca²⁺ homeostasis. The cell line exhibits uniform properties and is widely used for in vitro toxicity studies ranging from oxidative injury (Grune et al., 1995) to chemical carcinogenesis (Nu et al., 1995). Like normal liver cells (Ariño et al., 1989; Thomas et al., 1995), Clone 9 cells respond to vasoactive glycoconjugolytic hormones such as oxytocin and vasopressin. These hormones act primarily by the G protein-coupled formation of InsP₃ and the subsequent release of Ca²⁺ from intracellular stores (Berridge, 1993; Putney and Bird, 1993). Analyses of the oxytocin-induced Ca²⁺ oscillatory behavior and changes in mitochondrial membrane potential were performed with Fast Fourier Transform (FFT). Since agonist-induced oscillations in [Ca²⁺] are thought to constitute a frequency-encoded signal (Sneyd et al., 1995), FFT provided an optimal approach to determine the periods and frequencies of oscillations. To determine the source of the Ca²⁺ pools involved in the oscillations, the actions of pharmacological agents, nifedipine, thapsigargin, and ryanodine, were evaluated using FFT.
The effects of two food-borne toxic agents on Ca\textsuperscript{2+} oscillations were then analyzed. Patulin is a reactive mycotoxin commonly contaminating agricultural products (Friedman, 1990) and gossypol is the major pigment present in cottonseed meal thought to pose a dietary health risk to animals (Grankvist, 1989; Reyes et al., 1984). These toxins were selected because they are considered to be moderately to highly cytotoxic; however, the mechanisms of cellular injury are not completely understood. Both toxins have recently been employed in laser cytometric studies to establish mechanisms and chronology of cellular injury (Barhoumi and Burghardt, 1996). The ubiquitous environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was also examined because this toxictant has recently been shown to affect [Ca\textsuperscript{2+}], (Puga et al., 1992; Hanneman et al., 1996). These studies characterize the Ca\textsuperscript{2+} oscillations in Clone 9 cells and describe a quantitative approach using FFT to investigate the impact of toxic agents on Ca\textsuperscript{2+} oscillations.

**MATERIALS AND METHODS**

Materials. Culture media, Dulbecco’s phosphate-buffered saline (PBS), serum, patulin, gossypol (gossypol-acetic acid), nifedipine, thapsigargin, ryanodine, 1-octanol, and all general chemical reagents were purchased from Sigma Chemical Co (St. Louis, MO). Both 2,3,7,8-TCDD and 1,2,3,4-tetrachlorodibenzo-p-dioxin (1,2,3,4-TCDD) were synthesized to greater than 98% purity as determined by chromatography and spectroscopic analysis (Mason et al., 1986). Tissue culture flasks were obtained from Corning (Oneonta, NY) and LabTek Coverglass chamber slides were purchased from Nunc, Inc. (Naperville, IL). Fluo-3, AM and rhodamine 123 were purchased from Molecular Probes, Inc. (Eugene, OR).

Patulin was dissolved in dimethyl sulfoxide (DMSO) and stored at ~20°C until used. For use in cell cultures, patulin was dissolved in Ham’s Nutrient Mixture F-12 culture medium for dilution (100 μM, 0.1% DMSO). Gossypol stock (20 mM) was prepared in ethanol and diluted in media (3.0 μM, <0.05% ethanol) immediately before each experiment. TCDD stocks (100 μM) were prepared in DMSO and diluted to 1.0 mM in media for treatment of cells. Stock solution of 1.0 mM fluo-3, AM was prepared in DMSO and diluted with medium to 3.0 μM (0.3% final DMSO concentration) for loading in cultured cells. Rhodamine 123 was prepared as a 5 mg/ml solution in ethanol and diluted to 5 μg/ml in medium (0.1% ethanol). Thapsigargin stock (1.0 mM) was prepared in DMSO and used at a concentration of 1.0 μM (0.1% DMSO). Nifedipine was prepared in ethanol (10 mM) and diluted in medium to 10 μM (0.1% ethanol); ryanodine was prepared in ethanol (2.0 mM) and diluted in medium to 2 μM for experiments. Octanol was mixed with ethanol at a ratio of 1:10 (v/v) and prepared as a 10 mM stock in serum- and phenol red-free culture medium for dilution to 1.0 mM during experiments.

Clone 9 (ATCC, CRL 1439, passage 17) normal rat liver cells were used between passages 25 and 35. Cells were grown in Ham’s Nutrient Mixture F-12 with 10% fetal bovine serum and plated on Lab-Tek chambered coverglass slides (Nunc, Inc., Naperville, IL) for 48 hr prior to laser cytometry.

Laser cytometry experiments were performed with a Meridian ACAS Ultima work station (Meridian Instruments, Okemos, MI) using an excitation wavelength of 488 nm. Fluo-3, AM and rhodamine 123 were used to monitor intracellular Ca\textsuperscript{2+} and mitochondrial membrane potential, respectively. Fluo-3, AM is a nonratiometric, visible-wavelength probe which exhibits about a 40-fold enhancement of fluorescence intensity with Ca\textsuperscript{2+} binding (Tsien, 1989). To minimize differences in Fluo-3, AM loading from experiment to experiment, cells were seeded at the same density, all experiments were performed with the same Fluo-3, AM stock, and each treatment was compared to a separate control. Rhodamine 123 is a cell-permeant, cationic, relatively nontoxic fluorescent dye that can be seques-tered by mitochondria due to the maintenance of an electrochemical potential across the mitochondrial membrane (Chen, 1989). Mitochondrial membrane potential can be estimated from the intensity of mitochondrial staining (Lemasters et al., 1993). Cells were loaded with either 3.0 μM fluo-3,AM or 5.0 μg/ml rhodamine 123 for 1 hr or 20 min, respectively, in serum- and phenol red-free medium at 37°C. Following repeated washing with serum- and phenol red-free medium, cells were placed on the ACAS stage and basal fluorescence intensity was obtained from four image scans recorded from about 4 cells every 3 sec. Following the fourth scan, cells were exposed to the various treatments and image scans were acquired at the same sampling interval for about 7 min. For image collection, the laser excited fluorescence in cells from a two-dimensional raster pattern generated by a scanning mirror and a stepper motor-driven microscope stage (scan parameters were optimized for maximum detection of fluorescence with minimum cellular photobleaching). Excitation and detection parameters were kept constant for all experiments. To determine the source of the Ca\textsuperscript{2+} pools involved in the oscillations, three pharmacological agents were employed. Thapsigargin is an inhibitor of the microsomal Ca\textsuperscript{2+}-ATPase pump which causes leakage of Ca\textsuperscript{2+} from intracellular InsP\textsubscript{3}-sensitive stores and prevents their refilling (Lytton et al., 1991). Nifedipine is primarily an inhibitor of L-type Ca\textsuperscript{2+} channels although it may have inhibitory effects on receptor-operated Ca\textsuperscript{2+} channels in rat hepatocytes (Hughes et al., 1990; Striggow and Bohnensack, 1993). Ryanodine is an alkaloid inhibitor of an InsP\textsubscript{3}-dependent Ca\textsuperscript{2+} release channel through the ryanodine receptor (Meldolesi et al., 1990). For each of these agents, cells were pretreated for 5 min prior to addition of oxytocin.

The effects of 100 μM patulin and 3.0 μM gossypol were examined in cells pretreated for 5 min prior to oxytocin treatment while the effects of 2,3,7,8-TCDD and 1,2,3,4-TCDD were evaluated 24 hr after exposure to a dose of 1.0 μM. Doses of patulin (100 μM) and gossypol (3 μM) used in the present study were selected to be near the IC50 doses previously evaluated in Clone 9 cells (Barhoumi and Burghardt, 1996). To evaluate the effect of the presence or absence of gap junction-mediated intercellular coupling on the Ca\textsuperscript{2+} oscillations, the aliphatic alcohol 1-octanol was used as a specific inhibitor of gap junction permeability (Burt, 1991). Cells were pretreated for 3 min with 1.0 mM octanol, which is sufficient to uncouple cells (Burghardt et al., 1995).

Fluorescence intensities of fluo-3 and rhodamine 123, collected from each cell in the time domain with a sampling frequency f\textsubscript{s} (0.333 sec\textsuperscript{-1}, i.e., one scan every 3 sec), were analyzed using FFT. This method involves analysis of data using Fourier Transform (Walker, 1991; Hess et al., 1993) which dissociates the experimental fluorescence intensity signal acquired in an interval of time into one steady state fluorescence intensity signal, another fluorescence intensity signal oscillating around the steady state level with a fundamental frequency f/f\textsubscript{0}, and several other oscillating signals with harmonic frequencies that are multiples of f, It is obvious that frequencies higher than the sampling frequency cannot be detected. Major frequencies and their corresponding maximum fluorescence intensities were identified from an average of 30 cells per treatment. Differences between treatments were evaluated using either Student’s t test (single treatment vs control) or Duncan’s Multiple Range Test for multiple comparisons at p < 0.05.

**RESULTS**

**Oscillations of [Ca\textsuperscript{2+}], in Clone 9 Cells**

Detectable Ca\textsuperscript{2+} oscillations were induced in Clone 9 cells at oxytocin doses ≥10 nM when Ca\textsuperscript{2+} was present in culture
medium. The frequency of oscillations increased with increasing oxytocin concentrations (Fig. 1). Untreated Clone 9 cells exhibited no spontaneous oscillations (data not shown). When the fluorescence intensity of fluo-3 in cells treated with graded oxytocin doses were averaged at each time point monitored (i.e., 3-sec intervals), the initial fluorescence intensity peak induced by oxytocin stimulation was dose-dependent (Fig. 2). However, the amplitudes of Ca\(^{2+}\) oscillations were obscured and underestimated due to their asynchrony and complex patterns, despite the presence of abundant gap junctions and high levels of gap junction-mediated intercellular communication (Burghardt et al., 1995). In contrast, analysis of data obtained from each cell using FFT revealed several significant harmonic frequencies of oscillation with dose-dependent amplitude (Fig. 3, left) as well as the rate of change of fluorescence intensity responsible for the Ca\(^{2+}\) oscillations (Fig. 3, right).

Clone 9 cells undergoing mitosis exhibit fewer adhesive contacts and assume a more spherical profile. These dividing cells treated with 100 nM oxytocin exhibited a higher steady state level of Ca\(^{2+}\) (Table 1) and the amplitude of oscillations in these cells was considerably greater than that in nondividing cells (Fig. 4).

Pretreatment of Clone 9 cells for 3 min with 1.0 mM octanol [an agent which uncouples cells by closing gap junction channels (Burt, 1991)] did not alter steady state [Ca\(^{2+}\)], or the amplitude and/or frequency of the oxytocin-induced oscillations. However, application of octanol to cells with ongoing oscillations induced by oxytocin suppressed the oscillations (data not shown).

Clone 9 cells were further characterized by pretreating cells with nifedipine, thapsigargin, or ryanodine for 5 min prior to addition of 100 nM oxytocin. The voltage-operated channel blocker nifedipine (10 \(\mu\)M) suppressed fluorescence intensities at all oxytocin-induced frequencies compared to control (Fig. 5, left) with only the fundamental oscillation frequency existing at a level comparable to the steady state [Ca\(^{2+}\)], about which cells oscillate (Table 1). Thapsigargin (1.0 \(\mu\)M), an inhibitor of the microsomal Ca\(^{2+}\)-ATPase pump, completely eliminated oscillations at all frequencies (Fig. 5, right), whereas ryanodine (2 \(\mu\)M) had no effect on oxytocin-induced oscillations (data not shown).

**FIG. 1.** An illustration of the intracellular Ca\(^{2+}\) response in single Clone 9 cells treated with 100 nM (left) or 1000 nM oxytocin (right). Detectable Ca\(^{2+}\) oscillations in Clone 9 cells were induced at doses >10 nM with the frequency increasing with increasing oxytocin concentrations up to 1000 nM. Untreated Clone 9 cells exhibited no spontaneous oscillations (data not shown).

**FIG. 2.** The average Ca\(^{2+}\) response to graded oxytocin stimulation (10, 100, and 1000 nM). The initial peak was dose-dependent, however, the amplitude of Ca\(^{2+}\) oscillations was underestimated due to their asynchrony. Data represent mean fluo-3 fluorescence intensities of 30 cells.
Alteration of \( \text{Ca}^{2+} \) Oscillations

![Graph](https://i.imgur.com/12345678.png)

**FIG. 3.** Analysis of raw data obtained in Fig. 1 in the frequency domain using Fast Fourier Transform (FFT). The oxytocin-induced \( \text{Ca}^{2+} \) signal (left) at each frequency is dose-dependent. Bars represent mean fluo-3 fluorescence intensities ± SEM of significant harmonic frequencies induced by oxytocin. The rate of change of fluo-3 fluorescence \((dF/dt)\) obtained from FFT data of 30 cells treated with 100 nM oxytocin (right) is also oscillating, indicating the activity of influx and efflux (and/or \( \text{Ca}^{2+} \) buffering capacity) events responsible for the \( \text{Ca}^{2+} \) oscillations. At each frequency, fluorescence intensities for all oxytocin concentrations were significantly different from each other \((p < 0.05)\).

**Oscillations of Mitochondrial Membrane Potential in Clone 9 Cells**

Analyses of mitochondrial membrane potential performed in untreated Clone 9 cells showed that rhodamine 123 fluorescence exhibited two significant frequencies \((5 \times 10^{-3} \text{ and } 10 \times 10^{-3} \text{ Hz})\) of oscillation (Fig. 6). When cells were treated with 100 nM oxytocin, mitochondrial membrane potential oscillated in parallel with the \( \text{Ca}^{2+} \) oscillations (Fig. 6). In addition, rhodamine 123 fluorescence was gradually diminished during the oscillations, showing that the steady state mitochondrial membrane potential is reduced by about 27% during the \( \text{Ca}^{2+} \) oscillations.

**Effects of Patulin, Gossypol, and TCDD on \( \text{Ca}^{2+} \) Oscillations in Clone 9 Cells**

Previous analysis of patulin-treated cells revealed no significant change in \([\text{Ca}^{2+}]\), over 20 min but significant in-

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FL&lt;sub&gt;n&lt;/sub&gt; about which cells oscillate following stimulation with 100 nM oxytocin&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>Dividing cells</td>
<td>1.22 ± 0.09&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octanol, 1 mM, 3 min</td>
<td>1.10 ± 0.09</td>
</tr>
<tr>
<td>Nifedipine, 10 μM, 5 min</td>
<td>0.71 ± 0.03&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thapsigargin, 1 μM, 5 min</td>
<td>0.58 ± 0.02&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ryanodine, 2 μM, 5 min</td>
<td>0.91 ± 0.04</td>
</tr>
<tr>
<td>Patulin, 100 μM, 5 min</td>
<td>0.70 ± 0.03&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gossypol, 3 μM, 5 min</td>
<td>1.14 ± 0.07</td>
</tr>
<tr>
<td>2,3,7,8-TCDD, 1 mM, 24 hr</td>
<td>0.97 ± 0.07</td>
</tr>
<tr>
<td>1,2,3,4-TCDD, 1 mM, 24 hr</td>
<td>0.90 ± 0.09</td>
</tr>
</tbody>
</table>

<sup>*</sup> Values are mean FL<sub>n</sub> ± SEM determined from an average of 30 cells per treatment from six different culture wells.

<sup>*</sup> Significantly different compared to control at \(p < 0.05\).

![Graph](https://i.imgur.com/89012345.png)

**FIG. 4.** Comparison of oxytocin-induced \( \text{Ca}^{2+} \) oscillations in normal (open bars) and dividing (solid bars) Clone 9 cells. Dividing cells treated with 100 nM oxytocin exhibit a significant increase in the amplitude of oscillations at all frequencies as well as an increase in the steady state \( \text{Ca}^{2+} \) level around which cells oscillate (see Table 1). Data represent mean fluo-3 fluorescence intensities ± SEM of 30 cells.
creases in \([\text{Ca}^{2+}]\), were detected after 1 hr (Barhoumi and Burghardt, 1996; Burghardt et al., 1992). However, cells pretreated with 100 \(\mu\text{M}\) patulin for 5 min prior to oxytocin challenge exhibited a significant suppression of steady state \(\text{Ca}^{2+}\) levels and \(\text{Ca}^{2+}\) oscillations in a manner similar to nifedipine (Fig. 7, left, and Table 1). Pretreatment of cells with gossypol (3 \(\mu\text{M}\)) for 5 min caused a small but significant increase in steady state \(\text{Ca}^{2+}\) levels and \(\text{Ca}^{2+}\) oscillations in the third frequency (i.e., \(15 \times 10^{-3}\) Hz) (Fig. 7, right). All other harmonic frequencies were not significantly altered.

Cells treated with 1 \(\text{nm}\) 2,3,7,8-TCDD for 24 hr exhibited significant suppression of the fluorescence intensity of the third and higher frequencies (Fig. 8, left), whereas cells treated with 1,2,3,4-TCDD were not significantly different from control (Fig. 8, right).

**DISCUSSION**

In the present studies, Clone 9 cells were employed in experiments designed to develop a quantitative approach to study the impact of toxic agents on cellular \(\text{Ca}^{2+}\) oscillations. These cells exhibit periodic \([\text{Ca}^{2+}]\) spikes which increase in frequency with increasing oxytocin concentration (from 10 to 1000 \(\text{nm}\)) which are comparable to the responses of hepatocytes to a variety of vasoactive glycogenolytic agents (Ariño et al., 1989; Thomas et al., 1995). Due to the complex pattern of \(\text{Ca}^{2+}\) oscillations in these cells, FFT was employed to better define the frequencies of oscillation existing as well as their corresponding fluorescence intensities.

The frequency domain data obtained in control and oxytocin-treated cells revealed: (a) the oxytocin-induced level of \([\text{Ca}^{2+}]\), about which cells oscillate, and (b) significant harmonic frequencies and their corresponding amplitudes. Further FFT analysis of data obtained from each cell treated with oxytocin showed that the oxytocin-induced \(\text{Ca}^{2+}\) signal at each frequency was dose-dependent and revealed the activity of both influx and efflux (and/or \(\text{Ca}^{2+}\) buffering capacity) events responsible for the \(\text{Ca}^{2+}\) oscillations.

An interesting property of Clone 9 cells exposed to oxytocin during cell division was the greater amplitude of oscillations in these cells compared to nondividing cells. Intracellular \([\text{Ca}^{2+}]\), transients have previously been observed during mitosis (Ciapa et al., 1994) but not in cells in which cyclic changes in InsP3 have been driven by G protein-coupled receptor activation. Although the basis for the different response between dividing and nondividing cells has not been determined, it is possible that the size and continuity of the InsP3-sensitive store could be greater in dividing Clone 9 cells. Treatments which lead to fragmentation of ER and reduction of luminal continuity of this \(\text{Ca}^{2+}\) store can reduce the magnitude of \(\text{Ca}^{2+}\) release (Hajnóczky et al., 1994; Thomas et al., 1995). The cytoskeletal reorganization that occurs during cell division may lead to increased continuity of InsP3-sensitive store in Clone 9 cells resulting in \(\text{Ca}^{2+}\) oscillations of greater amplitude.

The propagation of \([\text{Ca}^{2+}]\), oscillations and/or waves in a number of cells is correlated with functional gap junctions (e.g., Sanderson et al., 1990; Boitano et al., 1992). Neither steady state \([\text{Ca}^{2+}]\), nor the amplitude and frequency of oscillations was affected by pretreating the cells with octanol at
effect of ryanodine pretreatment suggests the absence of 

\[ \text{Ca}^{2+} \text{tor-operated Ca}^{2+} \text{channels in rat hepatocytes (Hughes et al., 1990; Striggow and Bohnensack, 1993). The absence of an involvement of gap junctional communication on Ca}^{2+} \text{oscillations in the present study, the suppression of oxytocin-induced Ca}^{2+} \text{oscillations observed was similar to that caused by nifedipine pretreatment. These observations suggest that patulin may have a direct effect on receptor-operated Ca}^{2+} \text{channels. }

Gossypol has been shown to interact with lipid bilayers in model membrane systems and can increase proton and cation permeability (Reyes et al., 1984). At the dose employed in the present study, gossypol was recently found to cause a significant elevation in basal [Ca\^{2+}], in Clone 9 cells within 6 min although the earliest detected change in cells was the generation of reactive oxygen species (Barhoumi and Burghardt, 1996). However, pretreatment of cells with gossypol caused a significant increase in Ca\^{2+} oscillations only in the third harmonic frequency while all other frequencies were not significantly altered. These data combined with the fluorescence kinetic analysis of multiple cellular endpoints suggest that the action of gossypol may cause plasma membrane damage by induction of lipid peroxidation and not by direct effects on plasma membrane channels.

A surprising result in the present study was the effect of 1 nM 2,3,7,8-TCDD on Clone 9 cells 24 hr after treatment. This dose causes significant aryl hydrocarbon receptor (AhR)-mediated induction of CyplAl in Clone 9 cells as determined by metabolism of the fluorescent substrate benzo-[a]-pyrene (data not shown) and also resulted in significant suppression of the maximum fluorescence signal at most of the frequencies induced by 100 nM oxytocin. In contrast, cells treated with the weak AhR agonist 1,2,3,4-TCDD were not significantly different from control. Although the mechanisms responsible for this result remain to be determined, alteration of Ca\^{2+} oscillations occurs at doses which have no affect on cell viability during the duration of the experiment (Barhoumi and Burghardt, 1996). Further analysis of the actions of 2,3,7,8-TCDD on Ca\^{2+} homeostasis in Clone 9 cells is underway.

Another interesting characteristic of Clone 9 cells was the
observation that two significant frequencies of oscillation of rhodamine 123 fluorescence intensity existed in control Clone 9 cells. Further, initiation of Ca\(^{2+}\) oscillations with 100 nM oxytocin resulted in parallel oscillations in mitochondrial membrane potential. Hajnózky et al. (1995) have recently shown that intracellular Ca\(^{2+}\) oscillations are transmitted to mitochondria as mitochondrial Ca\(^{2+}\) oscillations. These Ca\(^{2+}\) oscillations may be responsible for the observed oscillatory pattern of rhodamine 123 fluorescence. However, the relationship between the mitochondrial Ca\(^{2+}\) oscillations and the activation of mitochondrial dehydrogenases is expected to push the respiratory chain to generate more ATP (Thomas et al., 1995). Although mitochondrial membrane potential was found in the present study to oscillate in parallel with oxytocin-induced Ca\(^{2+}\) oscillations, the predominant effect of the Ca\(^{2+}\) oscillations was to cause a gradual fall in the rhodamine 123 signal. A similar observation has recently been reported in cultured neurons (Loew et al., 1994).

FIG. 8. Effect of 2,3,7,8-TCDD vs 1,2,3,4-TCDD on Ca\(^{2+}\) oscillations. Cells treated with 1 nM of the potent AhR agonist 2,3,7,8-TCDD for 24 hr exhibited significant suppression of the maximum amplitude of most of the harmonic frequencies (the third and higher harmonic frequencies) while the weak AhR agonist had no significant effect. Data represent mean fluo-3 fluorescence intensities ± SEM for 30 cells.
The present studies used an experimental approach to investigate the effect of toxins on Ca\(^{2+}\) oscillations in cultured cells. The use of FFT identifies amplitudes of major frequencies contributing to the Ca\(^{2+}\) signal depending on the sources of Ca\(^{2+}\), the type of cell, the specific agonist, and the concentration inducing oscillations. Studies are underway to determine the physiological relevance of the major oscillation frequencies. Several toxic agents which act through different mechanisms were found to differentially modulate oxytocin-induced Ca\(^{2+}\) oscillations in a rat liver cell line. This model system should be useful to further explore the impact of altered hormone-induced Ca\(^{2+}\) oscillations on cellular homeostasis. Although considerable attention has been focused on the mechanisms responsible for the spatiotemporal aspects of Ca\(^{2+}\) oscillations, it is hoped that this approach may facilitate analysis of their functional significance.

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