Elevated Ca\textsuperscript{2+}\textsubscript{i} transients induced by trimethyltin chloride in HeLa cells: types and levels of response

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Abstract

Humans are exposed to organotins, like trimethyltin (TMT) chloride via air, water and food, and intoxication might result in severe health complications. Toxic effects of organotin compounds are well documented, but possible mechanisms remain unclear and only little information is available how organometallic species interact with calcium controlling mechanisms. Therefore, the aim of this work was to investigate the effects of TMT on calcium homeostasis in HeLa S3 cells. Dynamic changes of cytosolic calcium (Ca\textsuperscript{2+}\textsubscript{i}) were monitored using laser-scanning microscopy and fluo-4 loaded cells. Application of TMT resulted in sustained as well as in transient elevations of Ca\textsuperscript{2+}\textsubscript{i}. The number of reacting cells was directly correlated to the concentration of TMT used: with 500\textmu{}M TMT all cells reacted, with 50\textmu{}M TMT 80% and with 5\textmu{}M 74%. The fast Ca\textsuperscript{2+}\textsubscript{i}-transients (spikes), measured in single cells, occurred even with 0.25\textmu{}M TMT and varied in size and duration. The sustained increase of Ca\textsuperscript{2+}\textsubscript{i}, measured as the average over all cells, was dose dependent with an ∼8% increase for 5\textmu{}M TMT, ∼12.3% for 50\textmu{}M and ∼145% for 500\textmu{}M TMT. Moreover, this effect was partly reversible. A second application resulted in a similar sustained rise of Ca\textsuperscript{2+}\textsubscript{i} compared to the first application of TMT, there was also no difference when no calcium was added to the external solution (151±10% compared to 145±15%; 500\textmu{}M TMT). This rise of Ca\textsuperscript{2+}\textsubscript{i} was highly reduced (<10% increase) when the internal calcium stores were depleted before TMT (500\textmu{}M) was applied. Our data suggest that TMT influences Ca\textsuperscript{2+}\textsubscript{i}-homeostasis of HeLa S3 cells, which might be related to its toxicity in this cell line.

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1. Introduction

Intracellular calcium (Ca\textsuperscript{2+}) is a universal messenger in excitable and non-excitable cells. It regulates physiological processes as gene expression, fertilisation, proliferation, differentiation and cellular ageing, development, learning, memory, contraction and secretion. Moreover, calcium is involved in pathological processes, e.g. cell injury/death. A Ca\textsuperscript{2+} overload or a perturbation of its compartmentalisation may trigger acute or chronic cell injury, as well as accidental or programmed cell death [1–9]. Ca\textsuperscript{2+} elevations are also involved in activation of oncogenes, initiation, wound repair, and tumour promotion [5,8]. Moreover, the interest in the role of internal calcium stores (mitochondria, endo-sarcoplasmatic reticulum) in signal transduction and cell death has grown over the last decade. Intracellular membrane receptors such as inositol triphosphate (IP3) or ryanodine receptors (Ryr) are related to calcium release channels that control calcium uptake and release from the calcium stores. They are found in different cell types, playing an essential role in calcium signalling especially for shaping fast as well as sustained calcium transients (for review see [3,4,9]).

Numerous metallic entities—like lead, mercury, methyl-mercury or arsenite—have been shown to perturb physiological processes including calcium homeostasis [8,10–20]. Organometal(loid)s released from natural or anthropogenic sources are contaminants of air and water and are widely en-
Compounds is triggered by an increase of Ca$^{2+}$ and continues. Narita et al. [55] suggested that apoptosis induced by organotin metabolism [23,35,46]. Therefore, it is not surprising, that oxidative phosphorylation, resulting in a suppressed energy ATP-synthase and the hydroxide/chloride-antiport [44,45]. In addition, organotin compounds are inhibitors of oxidase phosphorylation, resulting in a suppressed energy metabolism [23,35,46]. Therefore, it is not surprising, that after treatment of different cell lines with organotins cell death via apoptosis or necrosis has been reported [47–56]. Gennari et al. [55] suggested that apoptosis induced by organotin compounds is triggered by an increase of Ca$^{2+}$ and continues by a release of ROS and cytochrome c from mitochondria as well as by the activation of caspases which finally results in DNA fragmentation.

Such a Ca$^{2+}$ increase might result from changes of calcium influx, redistribution from intracellular stores or from both sources. Such changes of Ca$^{2+}$ may affect the plasma membrane, the mitochondria and the endo-/ sarcoplasmatic reticulum [8,57,58]. The localised fast calcium signalling between endoplasmatic reticulum (ER) and mitochondria is a widely accepted concept [59]. Generally, fast calcium transients are difficult to detect since calcium spikes are not synchronised and are of short duration [60]. Surprisingly, very little is known how organometals interfere with calcium signals and their specific functional role. However, this is an important aspect, while perturbation of calcium homeostasis can alter cells fate from promoting proliferation to triggering of cell death.

To determine and understand the mechanisms of trimethyltin (TMT) induced calcium perturbations in HeLa cells, we investigated sustained and transient changes of intracellular calcium during the application of trimethyltin.

2. Materials and methods

2.1. Cell culture

HeLa S3 cells were purchased from American Type Culture Collection (ATCC, CCL-22). This cell line is derived from human cervix adenocarcinoma and was maintained in Ham’s F12 media (Sigma) supplemented with 10% heat-inactivated foetal calf serum (Gibco), 100 IU/ml penicillin and streptomycin. Cells were incubated at 37 °C under an atmosphere of 5% CO$_2$. For imaging studies these cells were sub-cultured and seeded on “easy-grip” culture dishes (Falcon). For experiments, non-confluent monolayer cells were used.

2.2. Chemicals and application

Fluo-4/AM (fluor-4) was purchased at Molecular Probes (OR, USA). Trimethyltin chloride (Me$_3$SnCl; TMT) was ordered from Sigma (Taufkirchen, Germany), dissolved in methanol and then in Ca$^{2+}$/Mg$^{2+}$ free phosphate buffer saline (PBS). The stock solution was stored at −20 °C and final concentrations where dissolved in Tyrodes buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, 10 mM HEPES, pH 7.2). For experiments, this buffer was used with no calcium added or with the addition of 2 mM calcium (all chemicals from Sigma). The application of TMT was made with a flow system (flow-rate: 60 ml in 20 min). Up to 150 ml trimethyltin chloride (0.25–500 μM) were applied in each experiment. For reversibility-tests a minimum of 50 ml Tyrodes buffer was flushed through the system. The dose response studies were carried out with: (1) additive and increasing concentrations of TMT in one experiment, or (2) single applications of one concentration in one experiment.

2.3. Confocal laser-scanning microscopy

Fluo-4/AM was used to monitor changes of the Ca$^{2+}$ level in cytosol. Fluor-4 (50 μg) was dissolved in DMSO (20 μl) and 2 μl of this stock solution was added to each culture dish in 2 ml Tyrodes buffer (for 30 min). After washing of the remaining dye, cells were incubated in Tyrodes buffer and changes in cellular free Ca$^{2+}$ were measured after TMT application using a Zeiss 510 confocal imaging system with a water immersion lens (C Apochromat 40×; num. app.: 1.2). The Argon laser was used at 25% from full laser and 3–5% of this was used for the Ca$^{2+}$ determination in the full screen. The excitation wavelength was 488 nm and emission channel 2 was used with a band pass filter of 505–530 nm. Fluorescence images were collected at room temperature. No photo bleaching was observed for up to several hours with the settings used, even when images were taken every 30 s for up to 4 h. Two scanning methods were applied for these experiments: line scanning over several cells and full screen imaging. For the line scan the laser was passing over selected cells. Scans were obtained every 30 s for up to 8000 s. For the full images, the content of the scanned images were stored on the computer hard drive. At the end of the experiments the images were analysed over time, using regions of interest (ROI’s). The change of intensity of the dye within the selected ROI’s was measured and plotted over time.

The experiments were repeated at least twice. For analysing the data the percent of intensity was standardised to baseline intensity before the application of TMT. The formula applied was [(100/baseline control) × exposure]. Blank
experiments were performed additionally using HeLa cells loaded with fluo-4, but no significant modification in the dye intensity was observed over time. Also, the interaction of TMT with fluo-4 using inert material was tested and no modification in the dye intensity as a reaction with TMT has been observed. Cells were considered to be “reacting cells” when they showed a continuous increase of at least 20 units of relative intensity in comparison to noise level and no rundown was observed.

3. Results

After 30 min loading of HeLa cells with fluo-4 and an additional resting time (at least 15 min) a stable fluorescence signal was established. Occasionally, “hot spots” with higher fluorescence intensity were observed in the cells. Such incorporations in the calcium stores were rarely seen but sometimes the cell nucleus showed higher affinity for fluo-4. With initial experiments no interaction of TMT with fluo-4 was detected (data not shown).

To study the characteristics of calcium elevation induced by TMT, we tested if (1) TMT modifies fluo-4 intensity in HeLa cells, (2) the effect is reversible and (3) the effect depends on the presence of calcium in the external solution (by application of TMT in PBS to which no calcium was added). Furthermore, we investigated (4) the dose dependency of the effect, applying a large range of TMT concentrations varying from 0.25 to 500 μM. Finally, we investigated (5) whether calcium is taken up from the external solution, by using a nominal calcium free solution or (6) whether it is released from the internal stores by depleting calcium from stores with caffeine before applying TMT.

Our results demonstrate that trimethyltin chloride modifies the intracellular calcium level of HeLa cells and the increase of the fluorescence signal depends on a calcium release from the stores but not on the existence of calcium in the external solution.

With TMT (500 μM), the intracellular calcium level increased until a plateau was reached after 5–8 min. An example, how the fluorescent signal increased in 12 different cells (marked by colour coded ROI’s) under the application of 500 μM TMT, is illustrated in Fig. 1A. Fig. 1B shows a line scan experiment, averaging the signal over four different cells. It demonstrates that the effect of TMT at the concentration used is partly reversible (≈70%) and that the increase as well as the reversibility of the fluorescence is repeatable in the same group of cells.

The time course of recovery was approximately two times slower than the rising time (Fig. 1B). An elevation of the fluorescent signal reached a similar intensity as seen for the first application of the same concentration when TMT was applied subsequently a second time. Generally, the change of the fluorescence signal observed in single cells followed a similar time course for application and for withdrawal of TMT as when the data were averaged over a group of cells.

The increase of the calcium signal was dependent on the TMT concentration applied. In Fig. 1C (black bars) the fluorescent signal was averaged over all cells of three independent experiments. The calculated increase of fluorescence after TMT application was ≈8% for 5 μM, ≈12.3% for 50 μM and ≈145% with 500 μM. A subsequent application of TMT—as
Table 1: Number of total cells tested in correlation to the concentration of TMT and the number (as well as the percentage) of non-reaction cells in relation to the TMT concentration

<table>
<thead>
<tr>
<th>Concentration TMT (µM)</th>
<th>Total cells</th>
<th>Non-reacting</th>
<th>Percentage of non-reacting cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>75</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>149</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>50</td>
<td>93</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>500</td>
<td>45</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

performed in some experiments—resulted in a similar rise of calcium as when a single application was given in one experiment (data not shown). In addition, with increasing concentrations the number of reacting cells was rising with higher concentrations of TMT. While at a concentration of 500 µM TMT all cells expressed a clear increase of fluorescence, the number of reacting cells within one experiment dropped with decreasing concentrations as shown in Table 1. When selecting only such reacting cells and measuring the increase of the signal only in those cells, a clear increase in the fluorescence signal even in the low concentration range was observed. For example, choosing 29 reacting cells (out of a total of 75 cells analysed) at a concentration of 0.25 µM TMT, the cytosolic fluorescent signal increased ∼15% (Fig. 1C, white bars) while the average increase over all cells was less than 0.5% at this concentration (Fig. 1C, black bars).

In addition to the sustained increase of the fluorescent signal, transient elevations (spiking) were observed (Fig. 2). After the application of TMT such fast calcium transients were elicited from the sustained (increased) calcium level as well as from the initial (baseline) level. These fast calcium elevations occurred within 30 s of the scan cycle and were completely recovered within another 30 s in most cases. The maximum increase might have been even larger and faster, but due to the limitations of the scanning interval used (30 s) in our experiments (with a faster scan interval bleaching of the dye occurred) it was not possible to analyse faster events. However, the occurrence of such calcium transient elevations was clearly dependent on TMT application. This fast rise of the intracellular signal did not depend on the external calcium concentration. As shown in part A of Fig. 3 they also appeared when no calcium was added to the external solution. This is a clear indication that such a fast rise of the calcium signal is a result of a calcium release from the internal calcium stores.

These transient increases (spikes) were observed at all concentrations of TMT used in this study (0.25–500 µM). The calcium spikes in the single cells were independent of each other. No synchronisation was seen in any of the experiments or with any of the TMT concentrations used in this study.

In general, the spike phenomena were found more often at low concentrations (e.g. 0.25 µM) whereas at higher concentrations the sustained increase of the signal dominates. Nevertheless, spikes that were also detected at higher concentrations (e.g. 500 µM, Fig. 3A) lasted generally longer than spikes elicited at lower concentrations of TMT (e.g. 0.25 µM).

In order to investigate whether the sustained increase—like the transient increase—is also independent of the external calcium concentration, TMT (500 µM) was applied to HeLa S3 cells in a solution containing 2 mM calcium and to a solution where no calcium was added (Fig. 3B). While the baseline fluorescence increased when calcium was added to the external solution, there was a clear rise of the calcium signal when TMT was added under both conditions. Averaging the data of three independent experiments with 23 different cells the calculated relative increase of dye fluorescence from the baseline was not significantly different (n = 3) (151 ± 10% versus 145 ± 15%) whether calcium was present in the external solution or whether it was not present.

This indicates that the rise of the calcium-induced by TMT in HeLa cells does not depend on the calcium in the extracellular matrix. Therefore, we considered that the rise is a result of a calcium release from internal stores. To confirm this conclusion, we used a calcium free external solution and released calcium from the internal stores (mitochondria, ER) by a pre-incubation of cells with 10 mM caffeine. In this case an increase in the cytosolic calcium was observed which was reversible during the application of caffeine (Fig. 3C). After this treatment 500 µM TMT was added to the solution. No
significant rise of calcium was found, which is an additional indication that the calcium increase seen after application of TMT is caused by a release from the internal stores.

To conclude, TMT reacts within HeLa cells modulating calcium signalling. Following uptake, TMT reacts intracellularly shown here as (1) the interaction with the internal calcium stores and by (2) incomplete recovery after removal of TMT in solution. However, these effects might also result from interaction of TMT with membrane receptors, which in turn trigger the release from intracellular stores indicated. This possibility is indicated by (1) the fast calcium elevation (immediately after TMT application) and (2) by the (partially) reversibility of the effect (when TMT was removed) (Fig. 4).

4. Discussion

The aim of this study was to investigate the possible effects of TMT on calcium homeostasis of HeLa cells. Our results illustrate that application of TMT modifies the intracellular calcium level of HeLa cells by releasing calcium mainly from the internal calcium stores. Thereby, two types of calcium changes could be distinguished: a fast transient increase of calcium and a sustained rise of the cytosolic calcium to a plateau. The sustained increase of the intracellular calcium was TMT–concentration dependent and independent on the external Ca\(^{2+}\)–concentration. In addition, the number of reacting cells decreased in a concentration-dependent manner, when the TMT concentration was reduced. Furthermore, the sustained increase of the signal was partly reversible and did not depend on the presence of calcium in the external solution but on a calcium release from the stores, since the rise of the fluorescence was abolished when the calcium stores were depleted before TMT was applied.

The data presented here demonstrate the actual change of the fluorescent signal, which is in a direct correlation to the intracellular calcium concentration. However, the correlation between these two parameters is not linear but sigmoid when using a logarithmic scale for the calcium concentration [61,62]. Especially calcium changes in the range below 0.10 μM and above ~5 μM will be underestimated by taking just the pure fluorescent signal. Therefore, especially the small-sustained increase of the fluorescent signal seen with low concentrations of TMT (0.25–5 μM) could in fact reflect a larger change of the calcium signal within the cell.

Other organotin compounds (tributyltin (TBT), dibutyltin (DBT)) increased cytosolic free Ca\(^{2+}\) [48,53,55,63] and such a perturbation of the Ca\(^{2+}\) signal could be an important factor to explain the toxicity of these substances, especially since a sustained increase in Ca\(^{2+}\) activates cytotoxic mechanisms and results in perturbation of cellular structure and function [6,8]. This could also lead to cell death via apoptosis or necrosis [48,53]. Oyama et al. [63] showed that the Ca\(^{2+}\) increase induced by application of TBT was reduced under Ca\(^{2+}\)-free conditions, suggesting its dependence on the external calcium concentration. The same group found that lower trialkyltins, such as triethyltin and trimethyltin exerted little or no changes on Ca\(^{2+}\). Therefore, they speculated that the cytotoxic action of TBT may be different from those of lower trialkyltins [63]. These findings are not in line with our own findings. Moreover, in our study, there was a clear dose dependent increase...
Fig. 4. Possible sides of action for TMT at the cell, which results in a rise of the intracellular calcium concentration. At the extra-cellular side of the membrane TMT could interact with membrane channels (Ch.) or with diverse receptor sides (R.). Such receptors could trigger intracellular pathways, which result in a release of Ca²⁺ from the endo-/sarcoplasmatic reticulum (ER) or from mitochondria (Mito). TMT passing the cell membrane (most likely less than 1%) could directly change the calcium homeostasis by action at the stores (release as well as inhibition of re-uptake) or at the nucleus (Nuc.).

of the intracellular calcium level. A possible explanation for these contradictory findings could be the difference of normal compared to tumour cells used in the two studies.

4.1. Possible sites of TMT action

TMT releases calcium from the intracellular stores. The question, whether TMT acts via a receptor activation at the cell membrane or acts directly intracellularly cannot be finally answered by our experiments.

A calcium release from intracellular stores by TMT could possibly be explained by an activation of membrane-bound receptors, which in turn trigger the release of calcium from intracellular stores. While we have no prove for such an action, this possibility is underlined by two observations: (1) the fast action of TMT and (2) the (partial) reversibility of the effect. Other authors have reported an activation of ligand gated membrane receptors, e.g. TNF-alpha by organotin [6,64] and, moreover this activation was strongly related with intracellular calcium rise and apoptosis [60,64] (Fig. 4).

As an indicator that TMT directly acts within the cells the incomplete reversibility of the calcium rise could be taken. Several authors have shown interactions of organotins with intracellular calcium stores [47,48,64] and clearly under the influence of TMT, the cytosolic rise of the fluorescence signal in our experiments was not due to a calcium uptake from the extra-cellular matrix. In addition we previously affirmed that there is some TMT uptake in vitro [21,34,65]. While the amount is very low, reaching less than 1% of the incubation substrate after 1h of exposure, this could be of enormous importance.

With our experiments we still do not know whether TMT acts directly at the stores or via a transduction pathway triggered by the activation of a receptor at the cell membrane (compare Fig. 4). However, the dynamic interactions between calcium stores and cytosolic calcium play a critical role for the control of cellular functions (for review see [9]). Such mechanisms for the regulation of calcium homeostasis are very complicated. Additionally, it has to be considered that some toxic stimuli—like TMT—might converge here and interact with a major signalling pathway.

Finally, it also has to be considered that TMT could interact at the ATP-dependent calcium pumps at the membranes of the calcium stores. Such an action could modify the re-uptake of the calcium after it has been released. Unfortunately, our results were not designed to prove such an interaction. Nevertheless, our observation suggests that the modulation of Ca²⁺, by TMT could participate in calcium related steps of the signal transduction pathway.

4.2. Types of response

In our study, fast calcium spikes were detected using a time resolution of 2 frames/min or 2 lines/min in line scan mode. These spikes were not synchronised in the different cells, which was also observed by Pu et al. [60]. Most likely intracellular membrane receptors (e.g. Ryr, IP₃), which were found in mitochondria and the endo-/sarcoplasmatic reticulum (ER), are involved in shaping of calcium signals [4,9,66,67]. Mitochondrial calcium dynamics might occur due to (1) an ATP dependent uptake of Ca²⁺ from the cytosol into the ER; (2) a Ca²⁺ release from the ER through channels following a calcium-induced calcium release mechanism, or (3) a potential-dependent Ca²⁺ leak flux out of the ER [68]. Substantial evidence shows that the accumulation of calcium in calcium stores plays a key role as a trigger of cell death and for additional stressors (e.g. oxidative stress) [66,69].

The fact that not all cells gave calcium spikes in the presence of TMT in our study, and that a sustained increase of the fluorescence signal in some cases occurred simultaneously with the transient response, makes a possible explanation even more challenging. An easy explanation would be...
that the different responses of cells even under the same cul-
ture conditions could be an indication that different calcium
release mechanisms are triggered and overlap in the calcium
signal measured. Another possibility is that these differences
reflect variations in the cell cycle of the cells.

Despite the renewal interest in the role of calcium stores in
signaling calcium signals, the molecular mechanisms that allow
these organelles to quickly accumulate and release large cal-
cium quantities are still not completely elucidated. However,
umerous studies suggested that the rapid Ca\textsuperscript{2+}
spikes regulate fast responses, whereas repetitive global Ca\textsuperscript{2+}
transients control slower responses [4]. These sustained increases
in Ca\textsuperscript{2+} activate death mechanisms [6] or accelerates degener-
avative processes that could lead to cell death when feed back
mechanisms fail [8]. The interaction of TMT with these pro-
cesses is possibly the key to explain its toxicity.

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