Effect of triethyltin on Ca$^{2+}$ movement in Madin–Darby canine kidney cells

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The effects of the environmental toxicant, triethyltin, on Ca$^{2+}$ mobilization in Madin–Darby canine kidney (MDCK) cells have been examined. Triethyltin induced an increase in cytosolic free Ca$^{2+}$ levels ([Ca$^{2+}$]) at concentrations larger than 2 μM in a concentration-dependent manner. Within 5 min, the [Ca$^{2+}$] signal was composed of a gradual rise and a sustained phase. The [Ca$^{2+}$] signal was partly reduced by removing extracellular Ca$^{2+}$. In Ca$^{2+}$-free medium, pretreatment with thapsigargin (1 μM), an endoplasmic reticulum Ca$^{2+}$ pump inhibitor, reduced 50 μM triethyltin-induced [Ca$^{2+}$] increase by 80%. Conversely, pretreatment with triethyltin abolished thapsigargin-induced Ca$^{2+}$ release. Pretreatment with U73122 (2 μM) to inhibit phospholipase C-coupled inositol 1,4,5-trisphosphate formations failed to alter 50 μM triethyltin-induced Ca$^{2+}$ release. Incubation with triethyltin at a concentration (1 μM) that did not increase basal [Ca$^{2+}$], for 3 min did not alter ATP (10 μM)- and bradykinin (1 μM)-induced [Ca$^{2+}$] increases. Collectively, this study shows that triethyltin altered Ca$^{2+}$ movement in renal tubular cells by releasing Ca$^{2+}$ from multiple stores in an inositol 1,4,5-trisphosphate-independent manner, and by inducing Ca$^{2+}$ influx. Human & Experimental Toxicology (2002) 21, 457–462.

Key words: Ca$^{2+}$ signaling; fura-2; MDCK cells; thapsigargin; triethyltin

Introduction

Organotins have been used in plastic industries and as agricultural chemicals, and are considered as environmental pollutants.¹ The toxic effect of organotins has been intensely studied in vivo and in vitro. Organotins were shown to disrupt components of glutamate homeostasis in rat astrocyte cultures² and activation of mitogen-activated protein kinases,³ induce spontaneous noradrenaline release from rat hippocampal slices,⁴ interfere with Ca$^{2+}$ signaling, potentiate norepinephrine release in PC12 cells,⁵ and cause rapid cytoskeleton modification.⁶ Furthermore, organotins have been shown to induce apoptosis.⁷ In vivo, both acute and long-term exposures of triethyltin in rat resulted in accumulation of tin in the brain, liver, kidney, heart, and blood.⁸,⁹ Evidence shows that organotins are toxic to cell membrane functions, which are intimately involved in the movement of electrolytes in renal tubular-like tissues.⁹

Ca$^{2+}$ plays a pivotal role in signal transduction in all cells.¹⁰–¹² Abnormal elevations in intracellular free Ca$^{2+}$ levels ([Ca$^{2+}$]) often lead to cell injury and apoptosis.¹³ A [Ca$^{2+}$] increase can occur by Ca$^{2+}$ store release and/or extracellular Ca$^{2+}$ entry. In Madin–Darby canine kidney (MDCK) cells, the inositol 1,4,5-trisphosphate-sensitive Ca$^{2+}$ store is an important Ca$^{2+}$ store that releases Ca$^{2+}$ into the cytosol when cells are stimulated by transmitters like ATP¹⁴ and bradykinin.¹⁵ Inhibitors of the endoplasmic reticulum Ca$^{2+}$ pump, such as thapsigargin and tert-butylhydroquinone, can release Ca$^{2+}$ in an inositol 1,4,5-trisphosphate-independent manner.¹⁶,¹⁷ The Ca$^{2+}$ release may induce Ca$^{2+}$ influx across the plasma membrane via the process of store-operated Ca$^{2+}$ entry.¹⁸

The present study explored the effect of triethyltin on [Ca$^{2+}$] in MDCK cells, using fura-2 as a fluorescent Ca$^{2+}$ indicator. It has been found that triethyltin induced a significant increase in [Ca$^{2+}$]. The time course and the concentration–response relationship were evaluated. The Ca$^{2+}$ sources of the Ca$^{2+}$ signal
and the role of phospholipase C-coupled inositol 1,4,5-trisphosphate formation in the signal have been explored.

**Methods**

**Cell culture**

MDCK cells obtained from American Type Culture Collection (Manassas, VA, USA) (CRL-6253) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were kept at 37°C in 5% CO₂-containing humidified air.

**Solutions used in [Ca²⁺]ᵢ measurements**

The Ca²⁺-containing medium had 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 5 mM glucose, pH 7.4. In Ca²⁺-free medium, Ca²⁺ was substituted with 1 mM EGTA. Triethyltin was dissolved in dimethyl sulfoxide and kept at −20°C as a 0.1-M stock, and was diluted to the final concentration before the experiments. Other drugs were dissolved in water, ethanol, or dimethyl sulfoxide as stock solutions. The concentration of the organic solvent(s) (ethanol and/or dimethyl sulfoxide) in the [Ca²⁺]ᵢ measurements did not exceed 0.1% and did not alter basal [Ca²⁺]ᵢ (n=4).

**[Ca²⁺]ᵢ measurements**

Trypsinized cells (10⁶/mL) were allowed to recover in serum-free culture medium for 1 hour before loading with 2 μM fura-2/AM for 30 min at 25°C in the same medium. Cells were washed and resuspended in Ca²⁺-containing medium. The fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 mL of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) by continuously recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 10 mM CaCl₂) and 20 mM EGTA sequentially at the end of each experiment. [Ca²⁺]ᵢ was calculated as described previously, assuming a Kᵣ of 155 nM.

**Chemicals**

All chemicals used in culture were obtained from Gibco (Grand Island, NY). The other chemicals were obtained from Sigma (St. Louis, MO).

**Statistics**

The data are presented as the mean ± SEM of four to six replicates. Statistical analysis was made by Student’s t-test. P<0.05 was considered significant.

**Results**

The effect of triethyltin on basal [Ca²⁺]ᵢ was explored. Figure 1A shows that in Ca²⁺-containing medium, triethyltin at concentrations between 2 and 50 μM increased [Ca²⁺]ᵢ in a concentration-dependent manner. Over a time period of 250 s, the [Ca²⁺]ᵢ signals induced by triethyltin expressed an initial rise followed by a slower rise. For example, at a concentration of 50 μM (trace a), triethyltin induced a [Ca²⁺]ᵢ increase that reached a net (baseline subtracted) maximum of 231±3 nM at the time point of 250 s (n=5). The Ca²⁺ signal saturated at 50 μM triethyltin because 100 μM triethyltin produced similar responses. At a concentration of 1 μM, triethyltin had no effect (trace d). Figure 1C shows the concentration-response curve of the triethyltin-induced Ca²⁺ increases (filled circles). The curve suggests an EC₅₀ value of about 10 μM.

Experiments were performed to examine the relative role of extracellular Ca²⁺ influx and Ca²⁺ store release in triethyltin-induced [Ca²⁺]ᵢ increase. Figure 1B shows that removal of extracellular Ca²⁺ reduced triethyltin-induced [Ca²⁺]ᵢ increase. The Ca²⁺ signal induced by 50 μM triethyltin (trace a) was composed of an initial rise that reached a net maximum of 151±3 nM after a latency time of 101±4 s (n=6). The Ca²⁺ signal remained elevated in the following 270 s (n=6). The concentration-response relationship was shown in Figure 1C (open circles). Figure 1C shows that Ca²⁺ removal reduced 30–50% (depending on the concentration) of triethyltin-induced [Ca²⁺]ᵢ increases.

Effort was made to explore the stores from which triethyltin released Ca²⁺. Figure 2A shows that in Ca²⁺-free medium, thapsigargin (1 μM), an inhibitor of the endoplasmic reticulum Ca²⁺ pump that passively and selectively depletes Ca²⁺ from the endoplasmic reticulum, induced a [Ca²⁺]ᵢ increase with a net maximum of 141±3 nM (n=6). The Ca²⁺ signal gradually decayed to a sustained level of a net value of 5±1 nM in 10 min. Subsequently added triethyltin (50 μM) induced a [Ca²⁺]ᵢ increase with a net maximum of 35±1 nM, which was 25±4% of the control 50 μM triethyltin-induced [Ca²⁺]ᵢ increase (Figure 2C; n=5, P<0.05). This suggests that triethyltin released Ca²⁺ from the endoplasmic reticulum and other stores. Experiments in Figure 2B were conducted to test whether the Ca²⁺ store in mitochondria was involved in triethyltin-induced Ca²⁺...
Figure 1  Effect of triethyltin on $[Ca^{2+}]_i$ in MDCK cells. (A) Triethyltin-induced $[Ca^{2+}]_i$ increases in $Ca^{2+}$-containing medium. The concentrations of triethyltin were 50 (trace a), 20 (trace b), 2 (trace c), and 1 (trace d) μM. The chemical was added at 20 s. (B) Effect of removing extracellular $Ca^{2+}$ on triethyltin-induced $[Ca^{2+}]_i$ increases. The experiments were performed in $Ca^{2+}$-free medium. The concentrations of triethyltin were 50 (trace a), 20 (trace b), and 2 (trace c) μM. (C) The concentration–response plot of triethyltin-induced $Ca^{2+}$ signals in $Ca^{2+}$-containing medium (solid circles) and $Ca^{2+}$-free medium (open circles). The y-axis was the percentage of control. Control was the net (baseline subtracted) maximum of 50 μM triethyltin-induced $[Ca^{2+}]_i$ increase in $Ca^{2+}$-containing medium. Data were mean ± SEM of four to six replicates. *P < 0.05 between filled circles and open circles.

The experiments were performed at PENNSYLVANIA STATE UNIV on May 12, 2016.

Figure 2  Intracellular $Ca^{2+}$ stores of triethyltin-induced $[Ca^{2+}]_i$ increase. The experiments were performed in $Ca^{2+}$-free medium. The chemicals were added at the time points indicated by arrows. The concentrations of chemicals were: thapsigargin 1 μM, CCCP 2 μM, triethyltin 50 μM. Data were mean ± SEM of four to six replicates.
release. Pretreatment with 2 μM carbonylcyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler that had been shown to cause [Ca\(^{2+}\)]\(i\) increases in MDCK cells previously,22 induced an immediate [Ca\(^{2+}\)]\(i\) increase, suggesting that mitochondrial Ca\(^{2+}\) was depleted. However, CCCP pretreatment did not alter subsequently added thapsigargin (1 μM)- and triethyltin (50 μM)-induced [Ca\(^{2+}\)]\(i\) increases (n=5, P>0.05). Conversely, pretreatment with 50 μM triethyltin for 700 s abolished 1 μM thapsigargin- and 2 μM CCCP-induced [Ca\(^{2+}\)]\(i\) increases (n=4–6; Figure 2C).

The role of phospholipase C-coupled inositol 1,4,5-trisphosphate formation in triethyltin-induced Ca\(^{2+}\) release was evaluated. U73122 is an effective inhibitor of phospholipase C in many cell types.23 Figure 3A shows that in Ca\(^{2+}\)-free medium, the inositol 1,4,5-trisphosphate-dependent Ca\(^{2+}\) mobilizer ATP induced a [Ca\(^{2+}\)]\(i\) increase with a net maximum of 121±5 nM (n=4). This ATP response was abolished by pretreatment with 2 μM U73122 for 6 min (Figure 3B; n=4). U73122 did not alter basal [Ca\(^{2+}\)]\(i\). The inactive U73122 analogue U7331323 did not alter ATP-induced [Ca\(^{2+}\)]\(i\) increase, suggesting that U73122 effectively inhibited phospholipase C activity. Figure 3B further shows that addition of 50 μM triethyltin subsequently to U73122 and ATP pretreatment induced a [Ca\(^{2+}\)]\(i\) increase indistinguishable from the control triethyltin response shown in Figure 2C (n=4, P>0.05).

In MDCK cells, it has been shown previously that drugs may alter other agonists-induced [Ca\(^{2+}\)]\(i\) increases at a concentration that does not change basal [Ca\(^{2+}\)]\(i\).24–26 Thus, the effect of a low concentration of triethyltin on the [Ca\(^{2+}\)]\(i\) increases induced by ATP and bradykinin, two robust Ca\(^{2+}\) mobilizers in MDCK cells14,15 was investigated. Pretreatment with triethyltin for 3 min at a concentration (1 μM) that did not increase basal [Ca\(^{2+}\)]\(i\) (shown in Figure 1A, trace d) did not alter the [Ca\(^{2+}\)]\(i\) increases induced by 10 μM ATP and 1 μM bradykinin (not shown; n=6, P>0.05).

**Discussion**

The present study has examined the effect of triethyltin on Ca\(^{2+}\) movement in renal tubular cells. The data suggest that triethyltin induced a concentration-dependent [Ca\(^{2+}\)]\(i\) increase above 2 μM with an EC\(_{50}\) of 10 μM. The Ca\(^{2+}\) signal was contributed by both Ca\(^{2+}\) influx and Ca\(^{2+}\) release because it was reduced by removing extracellular Ca\(^{2+}\). For comparison, in PC12 cells, triethyltin at a single concentration of 10 μM was shown to cause a slow increase in basal [Ca\(^{2+}\)]\(i\), which was attributed to Ca\(^{2+}\) release alone since removing extracellular Ca\(^{2+}\) did not alter the signal.8 Thus, the mechanism of triethyltin-induced [Ca\(^{2+}\)]\(i\) increases varies in different cell types.

It appears that in MDCK cells, triethyltin is able to deplete thapsigargin-sensitive endoplasmic reticulum Ca\(^{2+}\) stores and to release Ca\(^{2+}\) from other stores, based on the result that depleting the endoplasmic reticulum Ca\(^{2+}\) stores with 1 μM thapsigargin reduced triethyltin-induced Ca\(^{2+}\) release, whereas pretreatment with triethyltin abolished thapsigargin-induced [Ca\(^{2+}\)]\(i\) increases. Mitochondrial Ca\(^{2+}\) probably did not play a role in the triethyltin-induced Ca\(^{2+}\) release because depleting mitochondrial Ca\(^{2+}\) with a mitochondrial uncoupler did not augment the inhibitory effect of thapsigargin on triethyltin-induced Ca\(^{2+}\) release. Triethyltin was also shown to cause Ca\(^{2+}\) release from sarcoplasmic reticulum vesicles.27 The thapsigargin-insensitive compartment of the triethyltin-sensitive store is unclear due to the lack of selective pharmacological tools. It is unlikely that inositol 1,4,5-trisphosphate was involved in triethyltin-induced Ca\(^{2+}\) release because the release was not altered by inhibiting phospholipase C activity with U73122. How triethyltin releases stored Ca\(^{2+}\) is unclear, but it may act in a manner similar to thapsigargin given the evidence that it can inhibit cardiac sarcoplasmic reticulum Ca\(^{2+}\) transport in rat.28,29

In MDCK cells, several Ca\(^{2+}\)-mobilizing compounds at concentrations that are not high enough to increase basal [Ca\(^{2+}\)]\(i\) have been shown to alter the Ca\(^{2+}\) signals induced by other Ca\(^{2+}\) mobilizers. Examples are propranolol,24 zinc,25 and sevoflurane.26 Our data show that at a lower concentration (1 μM) that did not increase basal [Ca\(^{2+}\)]\(i\), triethyltin also did not modify ATP- and bradykinin-induced [Ca\(^{2+}\)]\(i\) increases.

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**Figure 3** Effect of U73122 on triethyltin-induced [Ca\(^{2+}\)]\(i\) increase. The experiments were performed in Ca\(^{2+}\)-free medium. (A) ATP (10 μM) was added at 260 s. (B) U73122 (2 μM), ATP (10 μM), and triethyltin (50 μM) were added at time points indicated by arrows. Data were mean ± SEM of four to six replicates.
increases, and to decrease the decay rate of ATP (but not bradykinin)-induced \([\text{Ca}^{2+}]_i\), increases.\textsuperscript{5}

Together, this study shows that triethyltin induces prolonged \([\text{Ca}^{2+}]_i\) increase in renal tubular cells. Triethyltin acts in a concentration-dependent manner by causing phospholipase C-independent \(\text{Ca}^{2+}\) release from thapsigargin-sensitive endoplasmic reticulum stores and other stores, and by causing \(\text{Ca}^{2+}\) entry. Furthermore, triethyltin at lower concentrations does not modulate the \([\text{Ca}^{2+}]_i\) increases induced by physiological agonists. Because lasting increases in \([\text{Ca}^{2+}]_i\) induced by physiological agonists. Because lasting increases in \([\text{Ca}^{2+}]_i\) often interfere with diverse cellular processes, such as protein activation, gene expression, ion transport, and transmitter secretion, and may lead to apoptosis.\textsuperscript{30} the \(\text{Ca}^{2+}\)-elevating effect of triethyltin may play a significant role in this compound’s cytotoxic action. This is consistent with the evidence that triethyltin-induced \(\text{Ca}^{2+}\) overload was implicated in apoptosis in PC12 cells.\textsuperscript{30}

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References

21. Thastrup O, Cullen PT, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular \(\text{Ca}^{2+}\) stores by specific inhibition of the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase. Proc Natl Acad Sci USA 1990; 87: 2466–70.
25. Jan CR, Wu SN, Tseng CJ. \(\text{Zn}^{2+}\) increases resting cytosolic \(\text{Ca}^{2+}\) levels and abolishes capacitative
Ca\textsuperscript{2+} entry induced by ATP in MDCK cells. *Naunyn-Schmiedeberg’s Arch Pharmacol* 1999; **360**: 249–55.


29. Kodavanti PR, Cameron JA, Yallapragada PR, Vig PJ, Desaiah D. Inhibition of Ca\textsuperscript{2+} transport associated with cAMP-dependent protein phosphorylation in rat cardiac sarcoplasmic reticulum by triorganotins. *Arch Toxicol* 1991; **65**: 311–17.