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La^{3+} Inhibits the UTP-induced Ca^{2+} Mobilization in MDCK Cells

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Abstract

We have studied the effects of La^{3+} on UTP-induced rises in intracellular calcium levels ($[Ca^{2+}]_i$) measured by fura-2 fluorimetry in Madin Darby canine kidney (MDCK) cells. UTP induced $[Ca^{2+}]_i$ rises dose-dependently with an EC_{50} of 1 mM. The Ca^{2+} signal was triggered by a Ca^{2+} release from the inositol 1,4,5-trisphosphate (IP_3)-sensitive pool because the signal was completely blocked by pretreatment of the endoplasmic reticulum (ER) Ca^{2+} pump inhibitor thapsigargin (TG) or the phospholipase C (PLC) inhibitor U73122. Both the peak height and under-curve area of 10 μM UTP-induced Ca^{2+} signal was reduced by approximately 40% by extracellular Ca^{2+} removal, suggesting that UTP induced capacitative Ca^{2+} entry. La^{3+} inhibited the UTP-induced Ca^{2+} signal dose-dependently when added before or after UTP. Pretreatment of 0.1 mM La^{3+} inhibited the UTP response more than Ca^{2+} removal did. The mechanisms underlying the La^{3+} inhibition appear to involve not only block of capacitative Ca^{2+} entry.

Key Words: UTP, La^{3+}, MDCK cells, Ca^{2+} signaling, fura-2

Introduction

Extracellular nucleotides (such as UTP, ATP, and ADP) are physiologically potent Ca^{2+}-mobilizing agents via activating P_2 receptors (2). P_2 receptors have been subdivided into three ATP-sensitive (P_{2x}, P_{2y}, and P_{2z}), one ATP- and UTP-sensitive (P_{2u}) and one platelet-selective, ADP-sensitive (P_{2t}) subtypes (2). Depending on the cell type, nucleotides could elevate $[Ca^{2+}]_i$ at least via two primary pathways: 1] mobilization of internal Ca^{2+} from the G-protein coupled, IP_3-sensitive pool in the cases of P_{2u}/P_{2y} receptor activation; and 2] by opening plasmalemmal Ca^{2+}-permeable channels as in the cases of P_{2z}/P_{2y} or may be P_{2t} purinocepter activation (5). Several P_2 receptor subtypes have been described in the kidney microvasculature (8). Several studies also indicate the existence of P2 receptors on renal epithelial cells in culture. Extracellular ATP and UTP raise $[Ca^{2+}]_i$ in A6 cells, which express morphological characteristics of amphibian distal nephron, and Madin-Darby canine kidney (MDCK) cells, a cell line derived from the distal tubule of canine kidney (13, 16). There is evidence suggesting the presence of both P_{2u} and P_{2y} receptors on MDCK cells based on rank order of potency of nucleotides on transmembrane ion transport and arachidonic acid release (6, 25).

In addition to elevation of $[Ca^{2+}]_i$ (5), in MDCK cells, UTP has been shown to activate a short-circuit current (25), release of arachidonic acid (6), and hyperpolarization of cell membrane (10). The mechanism underlying the UTP-induced $[Ca^{2+}]_i$ rises has not been studied previously.

La^{3+} interacts strongly with Ca^{2+}-binding sites and therefore affects most membrane transport processes involving Ca^{2+} (4). For example, La^{3+} can inhibit plasma membrane Ca^{2+} pumps (14), capacitative Ca^{2+} entry (3), Na^+/Ca^{2+} exchange (24) and voltage-dependent Ca^{2+} currents (15). In contrary

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to its Ca^{2+}-blocking effects, La^{3+} has been reported to elevate $[Ca^{2+}]_i$ in rat osteoclasts (21) and release intracellular Ca^{2+} stores by acting on a presumed "polyvalent cation receptor" in bovine anterior pituitary cells (22). La^{3+} can be transported by the Na^+/Ca^{2+} exchange pathway and directly triggers catecholamine release from bovine chromaffin cells (18).

In this study we have elucidated the mechanism of rise of the UTP-induced Ca^{2+} signal in MDCK cells. We have found that La^{3+} potently inhibited the Ca^{2+} signal by blocking capacitative Ca^{2+} entry and possibly interfering with UTP binding to its receptors.

Materials and Methods

Cell Culture

MDCK cells obtained from American Type Culture Collection (CRL-6253, MD, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air. Only cells from passages 70-80 were used.

Solutions

Normal buffer (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1.8; Hepes 5; glucose 5. Ca^{2+}-free buffer contained no added Ca^{2+} plus 0.1 mM EGTA. Nominal Ca^{2+}-free buffer contained no added Ca^{2+} and no EGTA. The experimental solution contained less than 0.1% of solvent (DMSO or ethanol) which did not affect $[Ca^{2+}]_i$ (n=3).

Optical Measurements of $[Ca^{2+}]_i$

Loading with 2 μM fura-2/AM was performed in trypsinized cells (10^6/ml) for 30 min at 25°C in DMEM. Cells were washed and resuspended in normal buffer and were washed again before each experiment to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring, which normally contained 1 ml of buffer and 0.5 million of cells unless otherwise stated. Fluorescence was monitored with a Hitachi F-4500 spectrofluorophotometer (Japan) by continuously collecting excitation signals at 340 nm and 380 nm and emission signal at 510 nm in 1-s intervals. Maximal and minimal fluorescences were obtained by adding TX-100 (0.1%) and EGTA (20 mM) sequentially at the end of the experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously (7) assuming a K_d of 155 nM.

Chemical Reagents

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). All other reagents were from Sigma (St. Loius, MO, USA).

Statistical Analysis

All values were reported as the mean±SE (n=3). Statistical comparisons were determined using Student's *t* test, and significance was accepted when $p < 0.05$.

Results

Effects of UTP on $[Ca^{2+}]_i$

UTP elevated $[Ca^{2+}]_i$ in a dose-dependent manner (Figure 1A). The $[Ca^{2+}]_i$ transients evoked by 1 μM-0.1 mM UTP (traces *a-c*) consisted of an initial rapid peak followed by a gradual decay phase (traces *a*, *b*, *c*). The response saturated at an UTP concentration of 0.1 mM which elevated $[Ca^{2+}]_i$ up to ~700 nM. 0.1 μM UTP was nearly ineffective (trace *d*). The relationship between the UTP concentration and the net peak height (baseline subtracted) of the $[Ca^{2+}]_i$ transient was plotted as a curve and was shown in Figure 2A (control). The half maximum effect was obtained at 1 μM UTP (EC_{50}; calculated by fitting a Boltzmann distribution to the data).

UTP-induced $[Ca^{2+}]_i$ Signal was Triggered by Internal Release

We next examined whether the UTP-induced $[Ca^{2+}]_i$ signal was triggered by a Ca^{2+} release from the endoplasmic reticulum (ER) Ca^{2+} pool, by using thapsigargin (TG) which inhibits the ER Ca^{2+} pump permitting Ca^{2+} to diffuse from the ER pool (23). Figure 1B, trace a, shows that TG (0.1 μM) induced a $[Ca^{2+}]_i$ transient which reached a peak (423±23 nM; n=3) in ~ 1 min followed by a gradual decline to pre-stimulatory baseline in ~ 6 min. This Ca^{2+} signal should reflect at least partial depletion of the ER Ca^{2+} pool. The UTP-induced Ca^{2+} signal was apparently triggered by a Ca^{2+} release from the ER Ca^{2+} pool since application of UTP (0.1 mM) at 10 min did not evoke a significant $[Ca^{2+}]_i$ rise. We also found that UTP failed to elevate $[Ca^{2+}]_i$ after pretreatment of 10 μM of the phospholipase C (PLC) inhibitor U73122

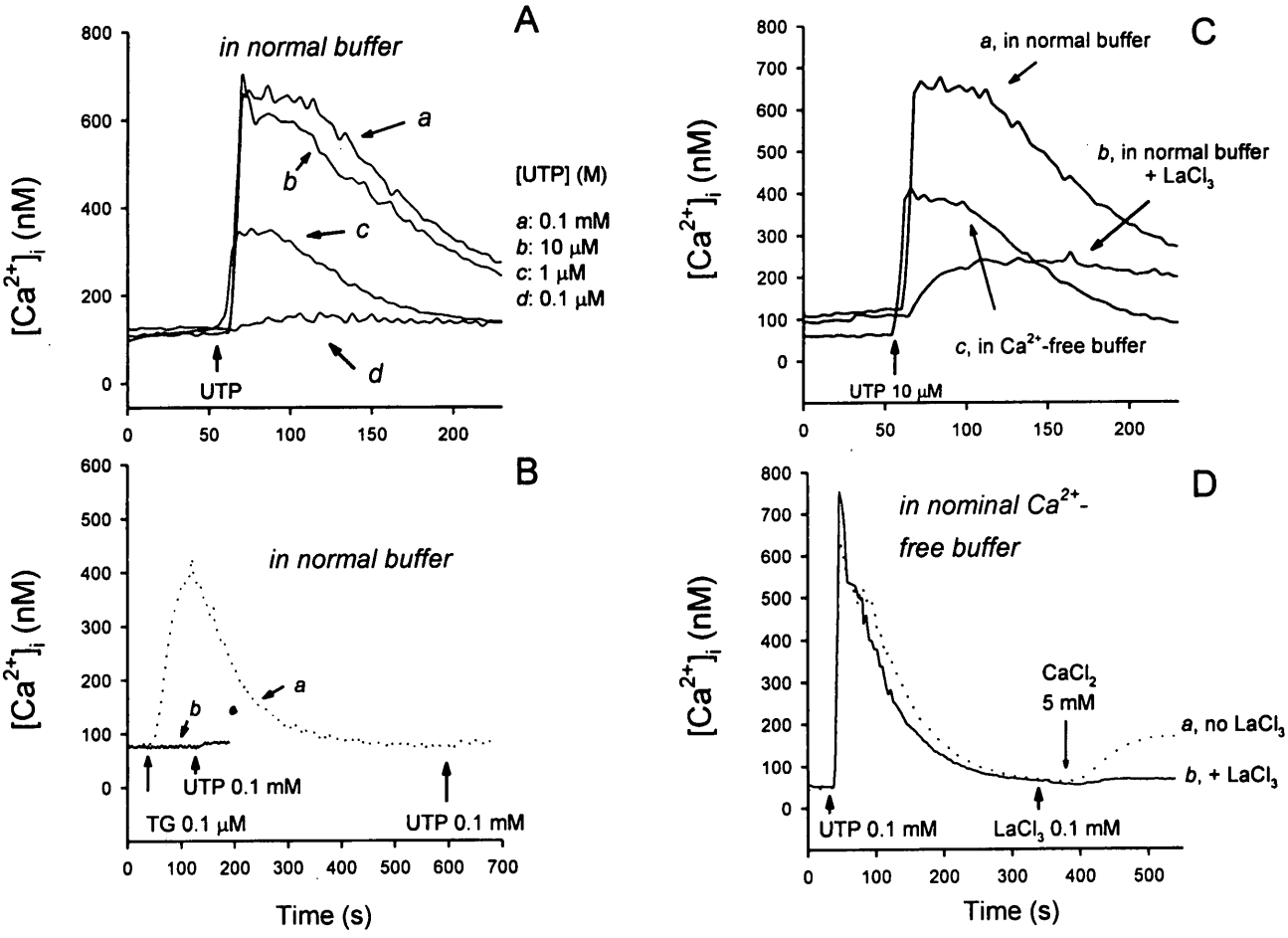

Fig. 1. Effects of UTP on $[Ca^{2+}]_i$ in fura-2-loaded MDCK cells. **A**, Dose-response relationship determined in normal buffer. UTP was added at the indicated time at concentrations of 0.1 mM (trace *a*), 10 μM (trace *b*), 1 μM (trace *c*), and 0.1 μM (trace *d*). **B**, Trace *a*, in normal buffer, 0.1 μM thapsigargin (TG) was added at the indicated time to mobilize the endoplasmic reticulum (ER) Ca^{2+} store. 0.1 mM UTP was added afterward. Trace *b*, 0.1 mM UTP was added at the indicated time when cells had been pretreated with 10 μM U73122 for 20 min (the experiment started after 18 min of U73122 pretreatment). **C**, Comparison of UTP-induced $[Ca^{2+}]_i$ rises in normal buffer (trace *a*), normal buffer containing 0.1 mM $LaCl_3$ (trace *b*), and Ca^{2+}-free buffer (no added Ca^{2+} plus 0.1 mM EGTA; trace c). UTP (10 μM) was added as indicated. $LaCl_3$ was added 20 s prior to UTP addition. **D**, UTP-induced capacitative Ca^{2+} entry. In nominal Ca^{2+}-free buffer (no added Ca^{2+} and EGTA), cells were stimulated with 0.1 mM UTP at the indicated time. 5 mM $CaCl_2$ was added at 380 s. Trace *a*, control without $LaCl_3$ addition; trace *b*, 0.1 mM $LaCl_3$ was added at 335 s followed by addition of $CaCl_2$. The traces are typical of three experiments.

(1) for 20 min (trace b).

We next explored whether Ca^{2+} influx contributed to the UTP-induced Ca^{2+} signal, by measuring the effects of UTP on $[Ca^{2+}]_i$ in Ca^{2+}-free buffer (no added Ca^{2+} plus 0.1 mM EGTA). As shown in Figure 1C, resting $[Ca^{2+}]_i$ was lowered to ~65 nM from ~100 nM by Ca^{2+} removal, and that the $[Ca^{2+}]_i$ transient induced by UTP (10 μM) was considerably reduced by ~ 40% in the peak height and under-curve area. Thus, it is obvious that internal release and external influx both contributed to the UTP-induced Ca^{2+} signal.

Capacitative Ca^{2+} Entry underlay the UTP-induced Ca^{2+} Influx

It has been widely established that mobilization of internal Ca^{2+} could trigger Ca^{2+} influx via capacitative Ca^{2+} entry (19). To determine whether the UTP-induced Ca^{2+} influx was mediated by capacitative Ca^{2+} entry, we performed the following experiments and the results were shown in Figure 1D. In nominal Ca^{2+}-free buffer (no added Ca^{2+} and no EGTA), UTP (0.1 mM) induced a $[Ca^{2+}]_i$ rise of a peak of 750±23 nM (n=3). After ~ 6 min, 5 mM $CaCl_2$ was added which induced a significant gradual rise in $[Ca^{2+}]_i$(trace *a*; 185±19 nM; n=3) which was greater than control (without UTP pre-stimulation; not shown; 51±5 nM; n=3; $p < 0.05$). This result suggested that UTP induced capacitative Ca^{2+} entry. Thus, it is clear that in MDCK cells the UTP-induced Ca^{2+} rises result from mobilization of the IP_3-sensitive ER Ca^{2+} pool followed by a Ca^{2+} influx via capacitative Ca^{2+} entry.

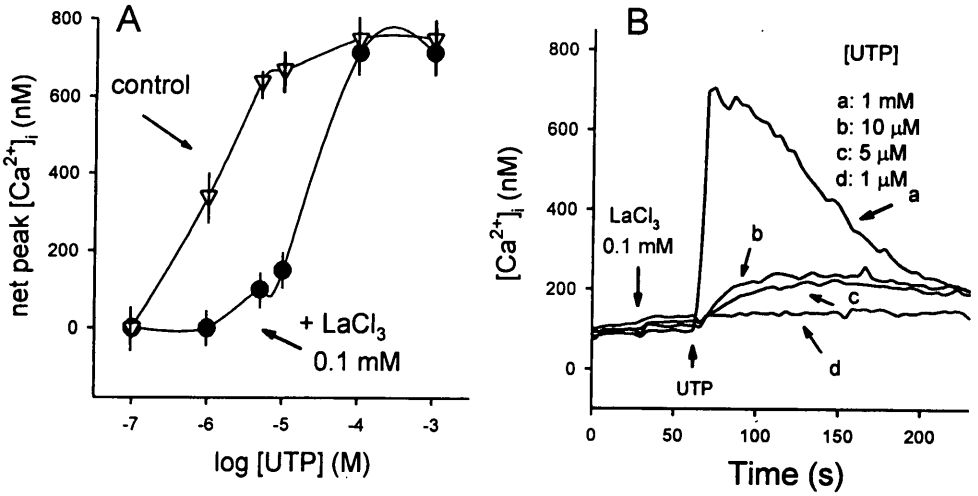

Fig. 2. Effects of $LaCl_3$ pretreatment on the UTP-induced Ca^{2+} signal. The experiments were performed in normal buffer. **A**, Dose-response relationship of UTP-induced Ca^{2+} signal in the absence (*open triangle*) and presence (*filled circle*) of $LaCl_3$ pretreatment. The y axis represents the net peak height of the $[Ca^{2+}]_i$ transient after subtracting the baseline. The x axis represents the UTP concentration ranging from 10^{-7} M to 10^{-3} M. 0.1 mM $LaCl_3$ was added 20 s prior to UTP addition. Data are mean±SE of three experiments. **B**, Representative traces of experiments in **A**. The traces are typical of three experiments.

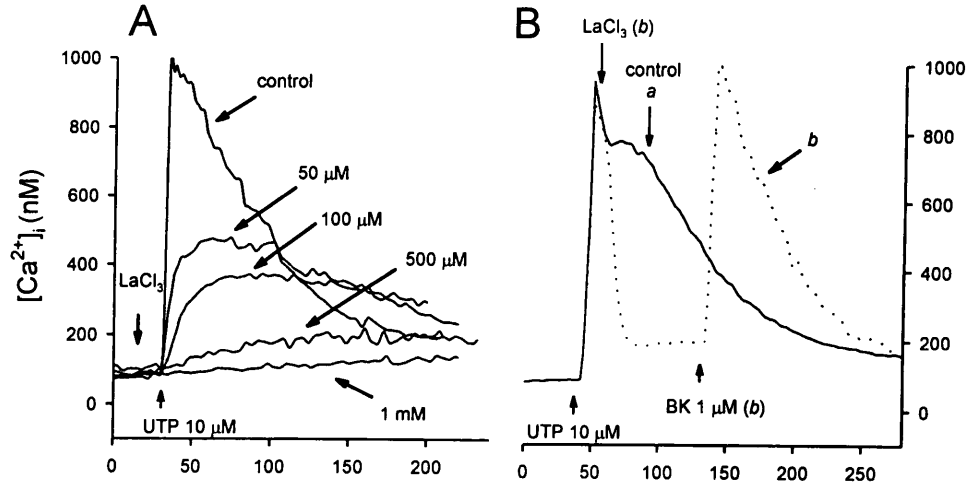

Fig. 3. A, Dose-dependent effects of $LaCl_3$ pretreatment on the UTP-induced $[Ca^{2+}]_i$ transients. $LaCl_3$ was added 20 s prior to addition of 10 μM UTP at concentrations of zero (control), 50 μM, 100 μM, 500 μM, and 1 mM. **B**, Effects of $LaCl_3$ added during the decay of 10 μM UTP-induced $[Ca^{2+}]_i$ transient. UTP was added at 40 s. Trace *a*, control without $LaCl_3$ addition; trace *b*, with 0.1 mM $LaCl_3$ added at 50 s. The traces are typical of three experiments. In trace *b* (but not in *a*), 1 μM of bradykinin (BK) was added at 130 s. The traces are typical of three experiments.

$LaCl_3$ Pretreatment Potently Inhibited the UTP-Induced $[Ca^{2+}]_i$ Rises

In the next series of experiments, we examined the effects of La^{3+} on the UTP-induced $[Ca^{2+}]_i$ rises. Trace *b* in Figure 1C shows that in normal buffer containing 0.1 mM $LaCl_3$, the $[Ca^{2+}]_i$ transient induced by 10 μM UTP (trace *a*) was markedly reduced into a small gradual rise with a maximum height of 225±25 nM (n=3). $LaCl_3$ was added 20 s prior to addition of UTP and did not alter resting $[Ca^{2+}]_i$.

Because La^{3+} has been shown to inhibit TG-induced capacitative Ca^{2+} entry in MDCK cells (3), we examined whether La^{3+} could inhibit the UTP-induced capacitative Ca^{2+} entry. Figure 1D shows that pretreatment of 0.1 mM $LaCl_3$ for 30 s prior to addition of $CaCl_2$ abolished the UTP-induced capacitative Ca^{2+} entry. In Figure 1D, the experiments were conducted in nominal Ca^{2+}-free buffer without EGTA because EGTA is known to have a higher affinity for La^{3+} than for Ca^{2+} (12).

Effects of $LaCl_3$ on the Dose-Response Curve of the UTP-Induced $[Ca^{2+}]_i$ Rises

Pretreatment of $LaCl_3$ (0.1 mM) reduced the potency of UTP without altering its efficacy in elevating $[Ca^{2+}]_i$ (Figure 2A). The baseline of ~100

nM was subtracted from the data. The EC_{50} of UTP was increased more than 10-fold. Figure 2B illustrates the $[Ca^{2+}]_i$ transients induced by 1 μM-1 mM UTP with pretreatment of 0.1 mM $LaCl_3$ for 25 s.

$LaCl_3$ Inhibited the UTP-Induced $[Ca^{2+}]_i$ Rises Dose-Dependently

Pretreatment of $LaCl_3$ for 20 s dose-dependently inhibited the $[Ca^{2+}]_i$ rises induced by 10 μM UTP (Figure 3A). $LaCl_3$ acted by decreasing the peak value and delaying the peaking time. The control (without $LaCl_3$ pretreatment) peak response in this set of experiments was 990±25 nM (n=3), which was reduced to 460±19 nM (52% inhibition), 378±15 nM (60% inhibition), and 201±10 nM (79% inhibition) by 50 μM, 100 μM, and 500 μM $LaCl_3$, respectively. 1 mM $LaCl_3$ completely blocked the UTP-induced Ca^{2+} signal.

$LaCl_3$ Inhibited the UTP Response When Added During the Decay of the Signal

Our data have shown that pretreatment of $LaCl_3$ potently inhibited the UTP-induced Ca^{2+} signal. An interesting question was whether $LaCl_3$ added during the decay of the UTP response could have an inhibition. Figure 3B illustrated that UTP (10 μM) applied at 40 s induced a $[Ca^{2+}]_i$ transient (trace *a*). When $LaCl_3$ (0.1 mM) was added at 50 s (trace *b*), at which time the UTP response was at its peak, the $[Ca^{2+}]_i$ signal was almost immediately brought down to a plateau which was only slightly higher than the pre-stimulatory baseline. This potent inhibition of $LaCl_3$ was not an artifact because trace *b* shows that bradykinin (BK, 1 μM) added at 135 s still induced a $[Ca^{2+}]_i$ transient similar to control (without $LaCl_3$ pretreatment; not shown).

Discussion

We have examined the effects of La^{3+} on the UTP-induced $[Ca^{2+}]_i$ rises in populations of MDCK cells loaded with the Ca^{2+}-sensitive fluorescent dye fura-2. We report here that La^{3+} potently inhibited the UTP-induced $[Ca^{2+}]_i$ rises when added before or after application of UTP.

In order to interpret the effects of La^{3+}, it was necessary to understand the mechanism underlying the rise of the UTP response. We found that UTP elevated $[Ca^{2+}]_i$ in a dose-dependent manner over a concentration range of 1 μM-0.1 mM with an EC_{50} of 1 μM. 0.1 μM UTP was nearly ineffective. This UTP response was apparently triggered by PLC-IP_3-dependent Ca^{2+} release from the ER Ca^{2+} pool because it was abolished by pretreatment of the inhibitor of the ER Ca^{2+} pump TG and the PLC inhibitor U73122. However, internal Ca^{2+} release cannot fully explain the source for the Ca^{2+} signal because the signal was significantly reduced by external Ca^{2+} removal. For example, Figure 1C shows that the response induced by UTP (10 μM) was reduced by ~ 40% in the peak height and under-curve area, implying that ~ 40% of the response was due to Ca^{2+} influx. Capacitative Ca^{2+} entry underlies the Ca^{2+} influx because: 1. UTP could not trigger any Ca^{2+} influx when the ER Ca^{2+} had been mobilized by TG (Figure 1B); and 2. Figure 1D demonstrated that UTP induced significant capacitative Ca^{2+} entry. Thus, our data lead to the conclusion that the UTP response was triggered by a Ca^{2+} release from the ER pool followed by a Ca^{2+} influx mediated by capacitative Ca^{2+} entry. This conclusion is consistent with the recent reports showing that MDCK cells express P_{2u} and P_{2y} receptors (6, 25), which are known to be coupled to the PLC-IP_3 system (5).

The most important finding in this study was that La^{3+} potently inhibited the UTP response. When added before UTP, 0.1 mM La^{3+} increased the EC_{50} of the dose-response curve of the UTP response by 10-fold without altering the maximum response. La^{3+} appeared to act competitively with UTP because when UTP concentration was 10-fold higher than that of La^{3+}, La^{3+} failed to exert its inhibition; in contrast, when La^{3+} concentration was 10-fold higher than that of UTP, the UTP-induced $[Ca^{2+}]_i$ rises were dramatically reduced. Since we have elucidated the Ca^{2+} sources responsible for the UTP response, the mechanisms that might underlie the La^{3+}-induced inhibition of the UTP response include: 1. La^{3+} blocks capacitative Ca^{2+} entry; 2. La^{3+} interferes with UTP binding to its receptors; and 3. La^{3+} enters cells and quench fura-2 fluorescence or inhibit Ca^{2+} release from the ER via an unknown mechanism.

It is clear that La^{3+} abolished the UTP-induced capacitative Ca^{2+} entry as demonstrated in Figure 1D. However, as depicted in Figure 1C, block of capacitative Ca^{2+} entry, which was the only Ca^{2+} influx mechanism in the UTP response, could not explain why Ca^{2+} removal had a less effect on the Ca^{2+} signal than La^{3+} did. In addition to block of capacitative Ca^{2+} entry, a possible explanation for the inhibition of La^{3+} is that La^{3+} interferes with UTP binding to the UTP receptors. Two lines of evidence supported this hypothesis: 1. La^{3+} inhibited the Ca^{2+} signal evoked by UDP, ATP, the ATP analogue ATP-γ-S, and also ADP in a fashion similar to its inhibition of the UTP response (not shown); 2. When added at the peak of the Ca^{2+} signal evoked by 10 μM UTP, 0.1 mM La^{3+} immediately and dramatically blunted the signal (Figure 3B). La^{3+} may act by binding UTP molecules and/or directly inhibiting the UTP receptors.

If La^{3+} acts by binding UTP, then it would imply that La^{3+} could potently bind UDP, ATP, ATP-γ-S, and ADP because La^{3+} also strongly inhibited the Ca^{2+} signals evoked by these nucleotides (not shown). Our hypothesis is possible since La^{3+} was found to modulate the binding activity of NMDA receptors on brain membranes (20). However, because direct evidence is lacking, whether La^{3+} acts by interfering UTP binding to its receptors awaits further studies.

The possibility that La^{3+} exerted its inhibition by entering cells is unlikely because La^{3+} is generally considered to be cell impermeant (11, 22). Additionally, La^{3+} is known to bind the Ca^{2+} chelation site of fura-2 and results in an increase in 340 nm excitation and a decrease in 380 nm excitation in a fashion similar to Ca^{2+} (9). If La^{3+} could enter MDCK cells, one would expect to see a rise in resting $[Ca^{2+}]_i$, however we found that La^{3+} did not significantly affect the 340 nm and 380 nm excitation fluorescence of fura-2-loaded cells. Thus, La^{3+} is more likely to act extracellularly.

Lastly, the possibility that La^{3+} interferes with the PLC-IP_3 system and/or inhibits Ca^{2+} release from the ER was excluded since bradykinin, another potent agonist which mobilizes IP_3-sensitive internal Ca^{2+} (17), evoked $[Ca^{2+}]_i$ rises normally in the presence of La^{3+} under the same conditions when the UTP-induced Ca^{2+} signal was abolished (Figure 3B).

To conclude, we have found that La^{3+} inhibits the UTP-induced $[Ca^{2+}]_i$ rises in MDCK cells. Our results suggest that La^{3+} acts by inhibiting capacitative Ca^{2+} entry and possibly UTP binding to its receptors. This possible direct inhibition of La^{3+} on UTP binding to its receptors has not been proposed previously, and it would be interesting to see if similar phenomena could be observed in other cell types.

Acknowledgments

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