Reactive Blue 2 Induces Calcium Oscillations in HeLa Cells

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Abstract: Reactive Blue 2 (RB), which is used as an ATP receptor antagonist, induced Ca^{2+} oscillations in HeLa cells. RB-induced Ca^{2+} oscillations were abolished in Ca^{2+} -free solution. RB, however, did not affect Ca^{2+} influx measured by Mn^{2+} quenching. The PLC cascade and intracellular Ca^{2+} release were involved as U73122 and

thapsigargin inhibited RB-induced Ca^{2+} oscillations. RB enhanced a Ca^{2+} response to histamine that is linked to the PLC cascade. RB may activate the PLC cascade in an extracellular Ca^{2+} -dependent manner and induce Ca^{2+} oscillations. [Japanese Journal of Physiology, 51, 389–393, 2001]

Key words: Reactive Blue 2, calcium oscillations, HeLa cells.

Calcium ion is an intracellular second messenger important for cellular functions in a variety of cells. In many cell types, transient and repetitive increases in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) are observed upon stimulation by agonists. These phenomena are called Ca^{2+} oscillations. They are thought to substitute for the prolonged rise of $[\text{Ca}^{2+}]_i$, which is harmful to cells, and to work as frequency-modulation (FM) signals [1]. Ca^{2+} oscillations have been implicated in the control of various cellular functions such as gene expression [2, 3], enzyme activity in hepatocytes [4], and ciliary activity in airway epithelia [5]. However, the details of these mechanisms and functions are still unclear.

HeLa cells, a non-excitabile epithelial cell line, are often used to investigate the mechanisms of intracellular Ca^{2+} signaling such as Ca^{2+} responses to hormones and Ca^{2+} oscillations. Histamine induces a Ca^{2+} response followed by Ca^{2+} oscillations in HeLa cells via the activation of G-protein, PLC (phospholipase C) cascade, and IP_3 receptors on the endoplasmic reticulum (ER) [6–8]. Histamine-induced Ca^{2+} oscillations are primarily caused by Ca^{2+} release from the ER, but they also require Ca^{2+} entry to maintain or modulate Ca^{2+} oscillations [7, 8].

Reactive Blue 2 (RB) has been used as an antagonist of ATP receptors (P2-type purinergic receptors) [9, 10]. RB, however, is a histochemical dye containing anthraquinone and has been reported to have other effects on cells and enzymes. RB inhibited GABA, glutamate [11], and NMDA [12] receptor channels; volume sensitive Cl^- channels [13]; a thylakoid protein kinase [14]; and a certain dehydrogenase [15].

Here we found that the application of RB induced Ca^{2+} oscillations in HeLa cells. We also found that RB-induced Ca^{2+} oscillations had unique characteristics different from the other receptor-activated Ca^{2+} oscillations reported in HeLa cells [6–8].

Materials and Methods

HeLa cells were grown in DME/F12 (SIGMA, MO, USA) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. Cells were cultured on glass coverslips (25-mm diameter, MATSUNAMI #1) in a humidified atmosphere (5% CO_2 , 95% air) at 37°C.

To introduce fura-2 into the cells, they were incubated with 2 μM fura-2 AM (acetoxymethyl ester; Dojindo, Kumamoto, Japan) and 0.2% cremophor EL (SIGMA) for 50 min at 37°C. Coverslips bearing the

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cells were then placed in a chamber on the stage of an inverted epifluorescence microscope (Olympus IMT2-RFL) to which was attached a xenon lamp. The chamber was continuously perfused. The normal medium consisted of (in mM) NaCl 152, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.8, glucose 5.6, HEPES 10 (pH 7.4). The composition of the Ca²⁺-free solution was (in mM) NaCl 152, KCl 5.4, MgCl₂ 0.8, EGTA 0.5, glucose 5.6, HEPES 10 (pH 7.4). As stock solutions (100 mM), Reactive Blue 2 (RBI, MA, USA), histamine (SIGMA), LaCl₃ and MnCl₂ were dissolved in water, and thapsigargin (SIGMA), U73122 (RBI), and U73343 (RBI) in DMSO. Each drug was diluted with the corresponding medium. Before the solution of the stimulants (1–2 ml) was applied to the chamber, perfusion was stopped and the medium was aspirated so that only a thin layer of the medium (less than 100 μ l) remained in the bath chamber. Restarting perfusion washed out the stimulants.

Changes in [Ca²⁺]_i were measured with an image analysis system, ARGUS-50/CA (Hamamatsu Photonics), described previously [16]. All experiments were done at room temperature (about 24°C).

Results and Discussion

Properties of RB-induced Ca²⁺ oscillations.

Within a few minutes after the application of Reactive Blue 2 (RB; 100 μ M), Ca²⁺ oscillations were induced in HeLa cells (Fig. 1, A and B). Usually, agonist-induced Ca²⁺ responses consist of two phases, a first transient response and a second sustained increase. In many cases, Ca²⁺ oscillations occur at the second phase after the first transient response. However, RB-induced Ca²⁺ oscillations occurred without any first transient response or any sustained increase in [Ca²⁺]_i (Fig. 1B). The interval of the oscillations was a few minutes. In some cases, Ca²⁺ oscillations persisted for over 30 min. The oscillations were terminated by washout of RB. Ca²⁺ oscillations occurred in individual cells and did not propagate to neighboring cells (Fig. 1A).

The induction of Ca²⁺ oscillations by RB was dose-dependent. The percentage of cells that showed oscillations increased with the concentration of RB (Fig. 1C). At 100 μ M, almost all cells exhibited Ca²⁺ oscillations in response to RB. The amplitude of the oscillations did not change remarkably with the dose, while the number of oscillating cells increased and the interval of the oscillations was shortened as the dose was increased. At higher doses (300 to 500 μ M), RB induced a sustained increase in [Ca²⁺]_i in some cells. We used 100 μ M RB to stimulate Ca²⁺ oscillations in subsequent experiments.

Fig. 1. Ca²⁺ oscillations induced by RB in HeLa cells. Reactive Blue 2 (RB; 100 μ M) induced Ca²⁺ oscillations in almost all cells we measured (878 of 1,008 cells from 9 experiments). **A:** Spatial changes of Ca²⁺ oscillations. The top-left frame shows a phase contrast image of a monolayer of HeLa cells. Images of the fura-2 fluorescence ratio (F_{340}/F_{360}) at 7 s intervals after the stimulation of RB are arranged from left to right and top to bottom. **B:** Temporal changes of Ca²⁺ oscillations. Typical traces of Ca²⁺ oscillations induced by RB (100 μ M) in 3 cells are shown. **C:** Dose response relationship of RB. The percentage of cells that responded to RB was plotted against each concentration of RB. Each data point was the average of over 224 cells from 2 or 3 experiments.

Effects of extracellular Ca²⁺. In Ca²⁺-free solution (CFS), the application of RB induced no Ca²⁺ response and a re-addition of Ca²⁺ initiated Ca²⁺ oscillations (Fig. 2A). RB-induced Ca²⁺ oscillations

Fig. 2. Effects of extracellular Ca^{2+} on the RB-induced Ca^{2+} oscillations. **A:** RB-induced Ca^{2+} oscillations were suppressed in Ca^{2+} -free solution (CFS). Extracellular solution was changed to CFS at 5 min before the application of 100 μM RB. Three typical traces observed from 336 cells in 3 different experiments are shown. A re-addition of Ca^{2+} induced Ca^{2+} oscillations. **B:** Ca^{2+} entry, measured as quenching speed of fura-2 fluorescence on addition of 100 μM MnCl_2 , did not increase with the RB-induced Ca^{2+} oscillations. However, histamine increased the Ca^{2+} entry. Two fura-2 fluorescence, F_{340} (upper trace; 4 typical cells), and F_{360} normalized at time 0 (lower trace; average of 24 cells) are shown. Similar results were obtained in 4 different experiments.

ceased when CFS was perfused (data not shown). These findings suggest that Ca^{2+} entry is necessary for Ca^{2+} oscillations induced by RB.

So we first considered that RB might activate a Ca^{2+} entry pathway. We examined whether the Ca^{2+} influx was increased by RB, using Mn^{2+} quenching technique. Even in a resting condition, fura-2 fluorescence excited at 360 nm gradually quenched after 100

μM Mn^{2+} was added in the solution (Fig. 2B). This indicates the existence of Ca^{2+} influx in the resting condition in HeLa cells. The quenching speed did not change during Ca^{2+} oscillations induced by RB (Fig. 2B). The slopes of quenching were -0.029 ± 0.0056 before the application of RB and -0.031 ± 0.0041 during Ca^{2+} oscillations induced by RB (mean $\pm$ standard error, $n=4$). On the other hand, an application of histamine (100 μM) induced additional Ca^{2+} responses and increased the quenching speed significantly ($-0.111 \pm 0.0051^*$ at the initial phase, and $-0.041 \pm 0.0063^*$ at the steady phase; *significant with Student's t test, $p < 0.05$) (Fig. 2B). This increase in Ca^{2+} influx may be due to the increase of capacitative Ca^{2+} entry after the histamine response. There remains a possibility that Mn^{2+} permeability is too low to detect by the quenching method, but the basal Ca^{2+} level is unaltered by the application of RB and the Ca^{2+} oscillations start without any basal Ca^{2+} increase (e.g., Fig. 1B). These findings suggested that RB-induced Ca^{2+} oscillations required Ca^{2+} entry but RB did not increase Ca^{2+} influx.

La^{3+} is an inhibitor of a certain Ca^{2+} entry pathway. RB-induced Ca^{2+} oscillations were not inhibited by an application of 1 μM LaCl_3 (3 cases out of 3 experiments; not shown).

Contribution of PLC cascade. U73122 (an inhibitor of PLC; 3 μM) inhibited the occurrence of Ca^{2+} oscillations induced by RB (Fig. 3A), but U73343 (an inactive analogue of U73122; 3 μM) did not (Fig. 3B). Thapsigargin (an inhibitor of Ca^{2+} pump of the ER membrane; 1 μM) also inhibited Ca^{2+} oscillations (Fig. 3C). These findings indicate that not only extracellular Ca^{2+} entry but also Ca^{2+} release from the ER is necessary for RB to induce Ca^{2+} oscillations.

We tested the possibility that RB could affect an intracellular pathway leading to $[\text{Ca}^{2+}]_i$ increase. Histamine is known to induce a Ca^{2+} response followed by Ca^{2+} oscillations through the activation of G-protein, PLC, and IP_3 -induced Ca^{2+} release [6–8]. So we used histamine as a tool to activate intracellular pathways. To avoid the effects of Ca^{2+} entry, Ca^{2+} responses to histamine were examined in CFS before and after the RB treatment. The Ca^{2+} response to histamine increased in the presence of RB and returned to the control level after washout of RB (Fig. 4, A and B). This indicates that the treatment with RB enhances Ca^{2+} responses to histamine.

In conclusion, a remarkable characteristic of RB-induced Ca^{2+} oscillations is primarily necessary for Ca^{2+} influx, although the activation of PLC cascades is also included. The Ca^{2+} influx seems to work as a

Fig. 3. Involvement of PLC cascade in RB-induced Ca²⁺ oscillations. **A:** U73122 (3 μM) suppressed RB-induced Ca²⁺ oscillations. U73122 was added at 3 min before the application of RB (100 μM). Three typical traces observed from 224 cells in 2 different experiments are shown. **B:** U73343 (3 μM) had no effect on RB-induced Ca²⁺ oscillations. **C:** Thapsigargin (TG; 1 μM) suppressed RB-induced Ca²⁺ oscillations. TG was added at 15 min before the application of RB (100 μM).

trigger of the Ca²⁺ oscillations. This is different from Ca²⁺ oscillations induced by ATP or histamine, where the Ca²⁺ oscillations occurred even in CFS. In these cases, the Ca²⁺ influx works to maintain Ca²⁺ oscillations by replenishing the intracellular stores. In both cases, Ca²⁺ oscillations themselves are caused by Ca²⁺ release from intracellular stores. So where is the action site of RB?

We first thought that RB might affect the Ca²⁺ influx pathway because RB-induced Ca²⁺ oscillations required extracellular Ca²⁺. However, this is unlikely because Ca²⁺ influx measured with the Mn²⁺ quenching method did not increase and no basal increase of Ca²⁺ was observed on RB treatment.

Fig. 4. Enhancement of PLC pathway by RB. Treatment with RB (100 μM) enhanced the Ca²⁺ response to histamine (100 μM). Responses to histamine were measured at 5 to 6 min after CFS perfusion and at 4 min after RB treatment. **A:** Data are shown by a typical trace. **B:** Changes of peak increase of Ca²⁺ on average (84 cells) with RB (100 μM) treatment and after washout. Error bars indicate standard error. The increase induced by RB treatment was significant with a *p*-value of less than 0.001 by Student's *t* test. Similar results were obtained in 3 different experiments.

We next considered that RB might affect the PLC pathway because U73122 and thapsigargin inhibited Ca²⁺ oscillations. In fact, RB enhanced Ca²⁺ responses to histamine. Histamine induces a Ca²⁺ response through the activation of the H₁-receptor, G protein, PLC, and IP₃ receptor [6–8]. Our data suggest that RB acts upstream of PLC, i.e., on receptors, G protein, or PLC. However, RB did not activate these processes like the agonists did, because RB-induced Ca²⁺ oscillations required Ca²⁺ influx to trigger, while the Ca²⁺ oscillations induced by receptor activation of histamine or ATP could occur even in CFS. Among these processes, PLC is known to be regulated by Ca²⁺ [17]. Actually in HeLa cells, extracellular Ca²⁺ affected the frequency of Ca²⁺ oscillations through Ca²⁺-stimulated activation of PLC [8]. Although the

mechanism of Ca^{2+} -regulation of PLC is not well understood, an isoenzyme PLC δ was reported to be activated by Ca^{2+} [18].

So a plausible action site of RB to induce Ca^{2+} oscillations in HeLa cells is the PLC. RB may enhance the Ca^{2+} sensitivity of PLC, making it possible to activate PLC with a small influx of Ca^{2+} that exists even in the resting condition. The activation of PLC causes IP_3 production and release of Ca^{2+} from intracellular stores. Further investigations, such as direct measurements of PLC activity or fluorescent analysis using the PH domain of PLC, should help to confirm our model.

RB has been used as a blocker of ATP receptors [9, 10], but there are also reports that RB blocks some channels and receptors [11–15]. Besides these inhibitory actions of RB, we here demonstrated that RB had a stimulatory action on the PLC cascade. Our results show not only that one should be careful when using RB as a blocker of ATP receptors, but also that it might be interesting to use RB as a tool for an investigation of the 'endogenous' activation of Ca^{2+} oscillations.

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