Depression of ATP-Induced Ca^{2+} Signalling by High K^+ and Low Cl^- Media in Human Aortic Endothelial Cells

K. YUMOTO, H. YAMAGUCHI, and R. OCHI*

*Cardiovascular Division of Internal Medicine and *Department of Physiology,
Juntendo University School of Medicine, Tokyo, 113 Japan*

Abstract We studied the contribution of the Cl^- channel as well as K^+ channel in the regulation of Ca^{2+} signalling in fura-2-loaded cultured human aortic endothelial cells. Low Cl^- (20 mM) superfusion did not affect the ATP (10 μM)-induced $[\text{Ca}^{2+}]_i$ increase at the initial peak (control 309 ± 30 nM (mean $\pm$ SD, $n = 17$) versus 20 mM $[\text{Cl}^-]_o$ 308 ± 40 nM ($n = 8$)) but depressed it at the sustained phase (at 5 min, 170 ± 29 nM versus 85 ± 10 nM). Similar selective depression of the sustained phase occurred also in Ca^{2+} -free and 140 mM K^+ solutions and in the presence of niflumic acid (300 μM), a blocker of the Cl^- channel and Ca^{2+} permeable cation channel. After application of ATP, the increase of $[\text{Cl}^-]_o$ from 20 to 146 mM resulted in a Ca^{2+} overshoot. Both Cl^- and K^+ channels play an important role in the regulation of Ca^{2+} influx presumably by controlling the membrane potential in vascular endothelial cells.

Key words: endothelium, ATP, Ca^{2+} signalling, Cl^- channel, K^+ channel.

Stimulation of endothelial cells with vasoactive agents (e.g. bradykinin, histamine, ATP, and acetylcholine) causes an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) as a key signal triggering several cellular responses, such as the formation of the two autacoids, prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF, NO) [1, 2]. The Ca^{2+} signal consists of an initial transient component reflecting the release of Ca^{2+} from intracellular stores by a second messenger, inositol 1,4,5-trisphosphate (IP_3), and subsequent sustained component resulting from influx of Ca^{2+} from the extracellular space [3]. The initial transient $[\text{Ca}^{2+}]_i$ increase is accompanied by the production of PGI_2 [4], while the sustained phase is related to NO synthesis [5]. The transmembrane Ca^{2+} influx occurs through voltage-independent Ca^{2+} -permeable channels activated by: 1) inositol-tetrakisphosphate, a product of phosphoinositides hydrolysis initiated by the stimulation of phospholipase C by agonists [6], 2) shear stress [7], and 3) the depletion

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* To whom correspondence should be addressed.

of intracellular Ca^{2+} stores [8–10]. According to the capacitative Ca^{2+} entry model in the third activation mechanism, the extent of depletion of the stores by Ca^{2+} release during the initial transient phase influences subsequent Ca^{2+} influx [8].

The fact that depolarization induced by high K^+ depresses the agonist-induced sustained $[\text{Ca}^{2+}]_i$ increase [11–13] indicates that the Ca^{2+} influx through the voltage-independent Ca^{2+} -permeable channels is regulated by the resting membrane potential, which determines the electromotive force for ion permeation through the channels. Accordingly, activation of ion channels such as Ca^{2+} -activated K^+ channels [14] and ATP-sensitive K^+ channels [12] increases the Ca^{2+} influx by inducing hyperpolarization. Recent studies have shown that Cl^- channels exist in endothelial cells [15–18]. In mast cells, activation of Cl^- channels produces an increase in $[\text{Ca}^{2+}]_i$ via hyperpolarization [19]. The endothelial Cl^- channels may thus play a role in the voltage-dependent regulation of $[\text{Ca}^{2+}]_i$. ATP elicits a biphasic increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) as a signal for synthesis of vasoactive autacoids in endothelial cells. We report here that the reduction of $[\text{Cl}^-]_o$ as well as an increase in $[\text{K}^+]_o$ markedly depresses the ATP-induced increase of $[\text{Ca}^{2+}]_i$ at the sustained phase in human aortic endothelial monolayers. Some of the present results have been reported previously in abstract form [20, 21].

MATERIALS AND METHODS

Culture of endothelial cells. Human aortic endothelial cells (HAEC) were purchased at passage number 4 (Clonetics Corporation, San Diego, California, U.S.A.) and grown in E-GM UV culture medium adapted from MCDB 113 (Kurabo Industries, Osaka) supplemented with growth factor from bovine cerebrum (Kurabo Industries) and 2% fetal bovine serum in 5% CO_2 containing air at 37°C . The cells were transferred to new flasks before the confluent growth stage and then used during passages 5 and 7. For the experiment, cells were grown until confluency on coverslips ($9\text{ mm} \times 9\text{ mm}$) in tissue culture dishes, changing the culture medium every 3 d. The confluent cells exhibited a typical monolayer with a cobblestone-like morphology and showed uptake of DiI-Ac-LDL, a marker of endothelial cells.

Fluorometry of $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was measured using fura-2/AM (Dojin Chemicals, Kumamoto). Confluent monolayers of cells on glass coverslips were soaked in the culture medium containing $3\text{ }\mu\text{M}$ fura-2/AM for 60 min at room temperature, washed in normal Tyrode's solution and then incubated at 37°C for 15 min. The fura-2-loaded monolayers were placed in a perfusion chamber (SC-03, Jasco, Tokyo) and perfused at 3 ml/min. A fluorescence spectrophotometer (CAF-110, Jasco) was used to record the fura-2 fluorescence with alternating excitation at 340 and 380 nm and emission at 500 nm. The ratio signal (R) obtained from the two outputs allows more sensitive determination of $[\text{Ca}^{2+}]_i$ than the original ones. The light signals at 340 and 380 nm excitations and R were registered on a thermal

array chart recorder (RTA-1100M, Nihon Kohden, Tokyo). Conversion of R to $[Ca^{2+}]_i$ was achieved by a standard calibration technique [22]. Briefly, fluorescence was measured in ionomycin (10 μ M)-treated cells exposed to absolutely Ca²⁺-free (no added Ca²⁺, 4.0 mM EGTA) and Ca²⁺-saturated solutions to yield the 340/380 minimum ratio (R_{min}) and maximum ratio (R_{max}), respectively. $[Ca^{2+}]_i$ was then calculated from the equation

$$[Ca^{2+}]_i = K_d [(R - R_{min}) / (R_{max} - R)] (S_{f_2} / S_{b_2}),$$

where K_d was taken to be 224 nM [22] and S_{f_2}/S_{b_2} is the ratio of fluorescence intensity at 380 nm for 0 Ca²⁺ versus that for saturating Ca²⁺.

Solutions and drugs. The composition of the superfusates were as follows (in mM): normal Tyrode's solution, NaCl 135, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, and glucose 10, buffered by NaOH to pH 7.4; Ca²⁺-free solution, NaCl 139, KCl 5.4, MgCl₂ 1, EGTA 1, HEPES 5, and glucose 10 (pH 7.4); 20 mM Cl⁻ solution, NaCl 6.6, Na aspartate 127, KCl 5.4, CaCl₂ 3, MgCl₂ 1, HEPES 5, and glucose 10 (pH 7.4). The CaCl₂ concentration in the 20 mM Cl⁻ solution was increased to 3 mM to maintain a free Ca²⁺ concentration equivalent to that in the normal Tyrode's solution, since Ca²⁺ is chelated by aspartate. High K⁺ solution (140 mM K⁺) was made by substituting NaCl in normal Tyrode's with isomolar KCl. The experiments were performed at room temperature (22–25°C).

Numerical values are presented as mean $\pm$ SD. Statistical significance of differences was evaluated using Student's *t*-test for non-paired samples.

RESULTS

The minimal concentration of ATP for inducing a visible increase in $[Ca^{2+}]_i$ was about 0.2 μ M. Maintained responses were elicited at low concentrations and their amplitude increased steeply at around 1 μ M. With further increases in the ATP concentration, the ATP-induced $[Ca^{2+}]_i$ response increased by a small amount at the initial phase, and a maximum response was obtained at around 100 μ M (Fig. 1A). Figure 1B shows a typical response obtained with 10 μ M ATP: the application produced a sudden increase in $[Ca^{2+}]_i$, which reached a peak at around 10 s and then decreased slowly, resulting in a sustained phase that persisted for as long as 10 min [23, 24]. Several ATP-induced responses could be induced in each monolayer by interposing 3–10 min superfusion of agonist-free normal Tyrode's solution.

Effect of $[Ca^{2+}]_o$ reduction

$[Ca^{2+}]_o$ was reduced by superfusion of Ca²⁺-free Tyrode's solution containing 1 mM EGTA. It produced a slight decrease in the basal $[Ca^{2+}]_i$ level and that of the initial phase of the ATP-induced $[Ca^{2+}]_i$ increase. In the absence of extracellular Ca²⁺, the Ca²⁺ signal decayed rapidly as reported in other endothelial cells [12–15] to the concentration below the basal level (Fig. 1C) ($n = 6$). Subsequent recovery

Fig. 1. ATP-induced $[Ca^{2+}]_i$ increase and its reduction by Ca^{2+} -free solution. A: ATP-induced $[Ca^{2+}]_i$ increase produced by 1, 10, and 100 μ M ATP. B: Time course of $[Ca^{2+}]_i$ increase induced by 10 μ M ATP. C: Effect of Ca^{2+} -free solution on the ATP-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ with calibration was obtained from the ratio signal of the emission light at 500 nm by excitation at 340 and 380 nm.

of $[Ca^{2+}]_o$ from Ca^{2+} -free to 1.8 mM after the ATP-induced transient $[Ca^{2+}]_i$ increase produced a large increase in $[Ca^{2+}]_i$ (Ca^{2+} overshoot) [8].

Effect of $[K^+]_o$ increase

The effect of external K^+ was examined by increasing $[K^+]_o$ from 5.4 to 140 mM, producing an 83-mV positive shift of the K^+ equilibrium potential (E_K). In the presence of 140 mM $[K^+]_o$, ATP induced a rapid increase in $[Ca^{2+}]_i$ with an amplitude as large as that in normal Tyrode's solution during the initial phase (Fig. 2A), and this was followed by accelerated decay to a low level. In seven paired experiments, the maximal increase in amplitude of $[Ca^{2+}]_i$ at 140 mM $[K^+]_o$ was $98 \pm 16\%$ of the control with 5.4 mM $[K^+]_o$ (mean $\pm$ SD), whereas that in the sustained phase at 5 min was $30 \pm 25\%$ of the control. After depression of the sustained phase, reduction of $[K^+]_o$ from 140 to 5.4 mM in the presence of ATP recovered the $[Ca^{2+}]_i$ to a level equivalent to that in the sustained phase in normal Tyrode's solution (Fig. 2A) ($n=6$).

Effect of $[Cl^-]_o$ reduction

The contribution of the Cl^- current to regulation of the sustained $[Ca^{2+}]_i$ increase was examined by reducing $[Cl^-]_o$ from 146 to 20 mM with a 51-mV shift

Fig. 2. Depression of ATP-induced $[Ca^{2+}]_i$ increase by $[K^+]_o$ increase and $[Cl^-]_o$ reduction. ATP (10 μ M) was applied in the presence of 140 mM $[K^+]_o$ (A) and 20 mM $[Cl^-]_o$ (B). Decrease in $[K^+]_o$ and increase in $[Cl^-]_o$ in the presence of ATP increased $[Ca^{2+}]_i$.

of the Cl⁻ equilibrium potential (E_{Cl}) in the positive direction. To examine the effect of low Cl⁻ solution on the transient phase, 20 mM $[Cl^-]_o$ solution was introduced shortly before the application of ATP. ATP (10 μ M) caused a rapid and large $[Ca^{2+}]_i$ increase in the low Cl⁻ solution with an amplitude similar to that in normal Tyrode's solution, and thereafter $[Ca^{2+}]_i$ decreased rapidly to a small sustained level (Figs. 2B, 3A). Also the introduction of 20 mM $[Cl^-]_o$ during the sustained phase of the ATP-induced $[Ca^{2+}]_i$ increase rapidly decreased $[Ca^{2+}]_i$, and a subsequent increase in $[Cl^-]_o$ 146 mM reversibly increased $[Ca^{2+}]_i$ to the preceding sustained level (second challenge in Fig. 3A). In the presence of ATP, the recovery of $[Cl^-]_o$ from 20 to 146 mM produced a large increase in $[Ca^{2+}]_i$ to concentrations higher than that in the sustained phase in normal Tyrode's solution, and was almost comparable with the preceding transient phase (Figs. 2B, 3A). In the presence of 10 μ M ATP, an increase of $[Cl^-]_o$ from 20 to 140 mM increased $[Ca^{2+}]_i$ to 1.08 ± 0.47 ($n=6$) of the preceding transient peak. Thus, the Ca²⁺ overshoot was elicited by an increase in $[Cl^-]_o$ after the occurrence of ATP-induced Ca²⁺ signalling in low $[Cl^-]_o$.

When ATP was applied twice in 20 mM $[Cl^-]_o$, the second challenge of ATP

Fig. 3. Effect of 20 mM Cl^- superfusion on the ATP-induced $[Ca^{2+}]_i$ increase and Ca^{2+} overshoot. A: Reduction of $[Cl^-]_o$ from 146 to 20 mM before and during ATP ($10 \mu M$) application. Note a large increase (overshoot) in $[Ca^{2+}]_i$ by the first increase in $[Cl^-]_o$. The second reduction of $[Cl^-]_o$ during the sustained phase produced a rapid decrease in $[Ca^{2+}]_i$. B: Application of ATP with a long interval in 20 mM $[Cl^-]_o$. From a preparation different to that in A. Note the marked depression of the ATP-induced $[Ca^{2+}]_i$ increase at the second challenge. The arrow shows the increase in $[Cl^-]_o$ from 20 to 146 mM in the absence of ATP, which resulted in a slow increase in $[Ca^{2+}]_i$.

elicited a much smaller increase in $[Ca^{2+}]_i$ (Fig. 3B). Assuming that the mechanism of the ATP-induced Ca^{2+} release was not affected by the reduction of $[Cl^-]_o$, this indicates that internal Ca^{2+} stores were not sufficiently replenished by Ca^{2+} due to a decrease in the Ca^{2+} influx. Subsequently, an increase in $[Cl^-]_o$ to 146 mM, after washout of ATP in the presence of 20 mM $[Cl^-]_o$, induced a $[Ca^{2+}]_i$ increase beyond the basal level before the ATP application ($n=4$). This indicated that the presence of ATP was not a prerequisite for the Ca^{2+} overshoot.

Figure 4 compares the ATP-induced $[Ca^{2+}]_i$ increase in 20 mM $[Cl^-]_o$ ($n=8$) and that in normal Tyrode's solution ($n=17$). The $[Ca^{2+}]_i$ in normal Tyrode's solution versus that in 20 mM $[Cl^-]_o$ solution was (in nM): before ATP application, 70 ± 17 (control) versus 65 ± 6 (low Cl^-); peak value, 309 ± 30 versus 308 ± 40 ; at 2 min, 233 ± 36 versus 139 ± 28 ($p < 0.01$); at 5 min, 170 ± 29 versus 85 ± 10 ($p < 0.01$). In other words, the reduction in $[Cl^-]_o$ did not affect the initial phase but depressed the late phase of the ATP-induced $[Ca^{2+}]_i$ increase.

Fig. 4. Comparison of the ATP (10 μ M)-induced $[Ca^{2+}]_i$ increase in the presence of 20 and 146 mM $[Cl^-]_o$. Hollow circles, 146 mM $[Cl^-]_o$; filled circles 20 mM $[Cl^-]_o$. Mean $\pm$ SD from 17 (control) and 8 (20 mM $[Cl^-]_o$) experiments. * Indicates statistical significance between the two values ($p < 0.01$).

Effect of niflumic acid

Niflumic acid blocks both the Ca²⁺-activated Cl⁻ channel [25] and Ca²⁺-permeable cation channel [26]. The application of 100 μ M ATP in the presence of 300 μ M niflumic acid produced a rapid increase in $[Ca^{2+}]_i$ with an amplitude similar to that in normal Tyrode's solution (Fig. 5A, C). However, the Ca²⁺ increase decayed rapidly to the basal level. Washout of niflumic acid in the presence of ATP produced an increase in $[Ca^{2+}]_i$ to a level beyond that of the sustained phase in normal Tyrode's solution ($n=5$) (Fig. 5C). Simultaneous washout of ATP and niflumic acid with normal Tyrode's solution produced a transient increase in $[Ca^{2+}]_i$ with a much faster time course than that of the above Ca²⁺ overshoots obtained by an increase in $[Ca^{2+}]_o$ and $[Cl^-]_o$ (Fig. 5A) ($n=7$). Figure 6 summarizes the ATP (100 μ M)-induced $[Ca^{2+}]_i$ increase in the presence and absence of 300 μ M niflumic acid. $[Ca^{2+}]_i$ for control ($n=10$) versus niflumic acid ($n=8$) was (in nM): before application of ATP, 58 ± 9 versus 63 ± 7 ; peak value, 366 ± 24 versus 350 ± 64 ; 3 min, 194 ± 15 versus 66 ± 10 ($p < 0.01$); 5 min, 168 ± 16 versus 69 ± 14 ($p < 0.01$). Thus, niflumic acid selectively depressed the sustained phase of the ATP-induced $[Ca^{2+}]_i$ increase.

DISCUSSION

The reduction in $[Cl^-]_o$ and the increase in $[K^+]_o$ depressed the sustained phase of the ATP-induced $[Ca^{2+}]_i$ increase with little change in the initial transient phase in cultured human aortic endothelial monolayers. Similar depression of the sustained phase of agonist-induced $[Ca^{2+}]_i$ increase has been demonstrated by

Fig. 5. Effect of niflumic acid on the ATP ($100\text{ }\mu\text{M}$)-induced $[\text{Ca}^{2+}]_i$ increase. A: ATP-induced $[\text{Ca}^{2+}]_i$ increase in the presence of $300\text{ }\mu\text{M}$ niflumic acid (NFA) and transient increase in $[\text{Ca}^{2+}]_i$ by simultaneous washout of niflumic acid and ATP. B: Control $[\text{Ca}^{2+}]_i$ increase induced by $100\text{ }\mu\text{M}$ ATP. A and B: from the same preparation, in the order of the experiments. C: Depression of the sustained phase of the ATP-induced $[\text{Ca}^{2+}]_i$ increase and Ca^{2+} overshoot produced by washing out of niflumic acid.

Ca^{2+} -free superfusion (Fig. 1C) in various endothelial monolayers, e.g. from piglet aorta with ATP [23], human umbilical vein with ATP [4, 24] and with histamine [9], bovine aorta with bradykinin [27], and bovine atria with bradykinin [13]. As Ca^{2+} -free superfusion should abolish the Ca^{2+} influx immediately, similar depression of the sustained phase by 20 mM $[\text{Cl}^-]_o$ and 140 mM $[\text{K}^+]_o$ suggests that they induce a reduction of the agonist-induced Ca^{2+} influx.

In single endothelial cells, external agonists often produce oscillatory Ca^{2+} signals [28, 29]. If such fluctuating Ca^{2+} signals occurred without synchronization, they would have been recorded as a maintained elevation of $[\text{Ca}^{2+}]_i$ in the present preparation using monolayers. If so, the selective depression of the sustained phase by low Cl^- solution, suggests that the oscillatory signals reflect Ca^{2+} entry (cf. Laskey *et al.* [30]) and can be also depressed by the reduction of $[\text{Cl}^-]_o$.

In endothelial cells, Ca^{2+} influx occurs not through voltage-gated Ca^{2+} channels but through voltage-independent Ca^{2+} -permeable non-specific cation channels [31]. The Ca^{2+} influx through Ca^{2+} -permeable channels is given by the macroscopic

Fig. 6. Comparison of ATP (100 μ M)-induced $[Ca^{2+}]_i$ increase in the presence and absence of niflumic acid (300 μ M); hollow circles, control from 10 experiments; solid circles, in the presence of niflumic acid from 8 experiments. Bars, SD. *Statistical significance ($p < 0.01$).

ic Ca²⁺ conductance and by the electromotive force. Therefore, the Ca²⁺ influx should be regulated by the modulation of the conductance and the resting membrane potential. The voltage-dependent regulation of Ca²⁺ influx has been demonstrated by selective depression of the sustained $[Ca^{2+}]_i$ increase with little change in the transient increase upon high K⁺ superfusion (Fig. 2) [12, 13]. The resting potential of endothelial cells is considerably more positive than E_K [21, 32, 33]. Therefore, an increase in Ca²⁺-activated K⁺ conductance by an agonist-induced $[Ca^{2+}]_i$ increase hyperpolarizes the membrane [14, 21] to increase the Ca²⁺ influx [12].

The Cl⁻ conductance is increased by depolarization [17], hypoosmolarity [18], and an increase of $[Ca^{2+}]_i$ in endothelial cells [34]. During stimulation by ATP and other agonist, the Ca²⁺-activated K⁺ current develops, and decreases the ratio of Cl⁻ permeability (P_{Cl}) to K⁺ permeability (P_K) [14, 21, 35]. However, agonist-induced enhancement of the Cl⁻ current gradually develops [20, 21, 35]. The marked depression of the sustained phase of the $[Ca^{2+}]_i$ increase by reduction of $[Cl^-]_o$ can be well explained by the depolarization produced by a large P_{Cl}/P_K . The agonist-induced $[Ca^{2+}]_i$ increase measured from individual human aortic endothelial cells is dependent exclusively on $[Cl^-]_o$ without any dependence on $[K^+]_o$ [29], in contrast to the clear depression by high $[K^+]_o$ in our monolayer study. This suggests that the P_{Cl}/P_K may vary among cell populations. The comparison of the contribution of P_{Cl} and P_K in the regulation of the Ca²⁺ influx awaits further study. However, as Ca²⁺-activated K⁺ channels are activated immediately during the initial phase of the agonist-induced $[Ca^{2+}]_i$ increase [14], it should play the major role in the early phase of the response.

The suppression of the sustained phase of the ATP-induced $[Ca^{2+}]_i$ increase by niflumic acid does not provide conclusive proof that the Cl^- current is indispensable for Ca^{2+} influx, since the drug blocks non-specific cation channels [26] as well as Cl^- channels [25]. Also, *N*-phenylanthranilic acid used as a selective Cl^- channel blocker to block the sustained Ca^{2+} increase [29], can suppress these non-specific cation channels [36]. The transient $[Ca^{2+}]_i$ increase induced by simultaneous washout of ATP and niflumic acid (Fig. 6) is a new observation. This may be related to the increase in Ca^{2+} influx due to store depletion in the presence of niflumic acid, as the drug is known to increase the release of Ca^{2+} from intracellular stores [37]. A more potent and selective Cl^- channel blocker showing no absorption of light or emission of fluorescence is highly desirable to evaluate the contribution of the Cl^- channel to $[Ca^{2+}]_i$ regulation under normal Cl^- distribution. However, the similarity of 20 mM $[Cl^-]_o$ to niflumic acid in suppressing the sustained phase of the ATP-induced $[Ca^{2+}]_i$ increase is further evidence suggesting that low Cl^- superfusion suppresses the influx of Ca^{2+} into endothelial cells.

The depletion of Ca^{2+} stores by application of the agonists in Ca^{2+} -free media is followed by a Ca^{2+} overshoot on a subsequent increase in $[Ca^{2+}]_o$ [8]. A Ca^{2+} influx factor (CIF) produced by the Ca^{2+} depleted Ca^{2+} stores is considered to increase the conductance of Ca^{2+} -permeable channels [38], resulting in the overshoot. Here, we obtained a Ca^{2+} overshoot by recovering $[Cl^-]_o$ in the presence (Fig. 3A) and absence (Fig. 3B) of ATP after its sustained reduction in the presence of ATP. The low Cl^- solution did not affect the Ca^{2+} release triggered by the second messenger IP_3 , since it did not change the initial phase of the ATP-induced $[Ca^{2+}]_i$ increase. The depletion of Ca^{2+} stores by application of ATP in the low Cl^- solution, evidenced by a much smaller $[Ca^{2+}]_i$ increase upon the second application of ATP, would produce the CIF. In the presence of the CIF, when $[Cl^-]_o$ is recovered, the membrane is repolarized by the Cl^- current or could be even hyperpolarized as $[Cl^-]_i$ had been decreased by low $[Cl^-]_o$ superfusion, resulting the Ca^{2+} overshoot (Fig. 3B). Furthermore, since Ca^{2+} stores remain depleted in 20 mM Cl^- solution, refilling of the stores requires Ca^{2+} influx through channels which sense the change in membrane potential. Therefore, the Cl^- channels together with K^+ channels, via the membrane potential, could regulate the initial transient as well as the sustained phase of the agonist-induced $[Ca^{2+}]_i$ increase.

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