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# REFILLING STATE OF INTERNAL $Ca^{2+}$ STORES IS NOT THE ONLY INTRACELLULAR SIGNAL STIMULATING $Ca^{2+}$ INFLUX IN HUMAN ENDOTHELIAL CELLS

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**Abstract**—To further analyse the role of the refilling state of internal $Ca^{2+}$ pools in the stimulation of $Ca^{2+}$ influx in human endothelial cells, we investigated the combined effect of thapsigargin (TG) and histamine on cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and inositol polyphosphate production. At normal extracellular $Ca^{2+}$ levels, TG induced a progressive and sustained elevation in $[Ca^{2+}]_i$ which was dose-dependently prevented by pretreatment with 1–10 $\mu$M histamine. Similarly, pretreatment with 0.1 and 1 $\mu$M TG suppressed histamine-induced $Ca^{2+}$ transients partially and totally, respectively. TG pretreatment did not alter the inositol triphosphate ($IP_3$) level liberated by histamine, but modified $IP_3$ metabolism by decreasing inositol biphosphate ($IP_2$) and increasing inositol monophosphate ($IP_1$) contents. In the absence of $Ca^{2+}$ influx, 1 $\mu$M TG only induced a small transient increase in $[Ca^{2+}]_i$ whereas the $Ca^{2+}$ mobilization evoked by 10 $\mu$M histamine was unchanged. In both cases, the absence of any additional effect of either TG, histamine or 2 $\mu$M ionomycin indicated the complete depletion of $Ca^{2+}$ stores. The re-establishment of the transmembrane $Ca^{2+}$ gradient induced a transient rise in $[Ca^{2+}]_i$. Its amplitude differed between histamine- and TG-treated cells. It was imposed by cell pretreatment and was selectively affected by changes in the membrane potential. At 5 mM external $K^+$, the transient rise in $[Ca^{2+}]_i$ was more marked in histamine- than in TG-stimulated cells; this difference was suppressed by TG pretreatment. The presence of 130 mM external $K^+$ increased $Ca^{2+}$ entry in TG-treated cells but reduced it in histamine-stimulated cells. These results indicate that the refilling state of internal $Ca^{2+}$ stores does not constitute the single regulator of $Ca^{2+}$ influx. TG and histamine seem to activate $Ca^{2+}$ influx through distinct but interdependent pathways regulated by membrane potential.

*Key words:* human endothelial cell; cytosolic $Ca^{2+}$ concentration; inositol polyphosphates; thapsigargin, histamine

Vascular endothelial cells respond to various neurohumoral and physical stimuli by releasing substances such as endothelium-derived relaxing factor [1], prostacyclin [2, 3] and endothelins [4–6], which regulate smooth muscle cell tone and interactions between platelets and vessel wall [1, 7].

In endothelial cells, an increase in cytosolic-free $Ca^{2+}$ concentration is required for numerous cell functions such as synthesis and secretion of vasoactive agents [1, 2] and changes in cell shape and permeability [8–10]. Various compounds, such as histamine, thrombin, bradykinin and ATP, which are released or locally produced in the blood, evoke a well-characterized biphasic rise in $[Ca^{2+}]_i$† [2, 3, 9–14]. The initial transient increase in $[Ca^{2+}]_i$ reflects $Ca^{2+}$ mobilization from internal stores triggered by $IP_3$ [3, 10, 11] whereas the sustained component of the $[Ca^{2+}]_i$ response reflects the agonist-induced activation of bivalent cation influx [12–14].

In his original version of the capacitative $Ca^{2+}$ entry model, Putney proposed that the decrease in $Ca^{2+}$ content within intracellular stores would lead to the opening of plasma membrane channels, thus causing a continuous inward flux of $Ca^{2+}$ to refill the pool [15]. This hypothesis has been confirmed in endothelial cells [16–20]. However, if depletion of the internal $Ca^{2+}$ pool provides a signal for activating $Ca^{2+}$ entry, the nature of such a signal has not been clearly defined. Two possibilities are generally considered: (1) the signal could be a soluble messenger called $Ca^{2+}$ influx factor (CIF), generated or released from an intracellular store when it is depleted of $Ca^{2+}$ or (2) the signal could be a conformational or structural event transmitted to the plasma membrane thereby activating a $Ca^{2+}$ current, the $Ca^{2+}$-release activated $Ca^{2+}$ current (CRAC) [15, 21].

The present study was designed to further investigate the participation of the $Ca^{2+}$ store refilling state in the control of $Ca^{2+}$ influx. To this end, the stores were depleted either by a receptor-dependent stimulus, histamine, or by TG, which specifically inhibits the $Ca^{2+}$ pump of endoplasmic reticulum

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† Abbreviations: $[Ca^{2+}]_i$, cytosolic $Ca^{2+}$ concentration; $[Ca^{2+}]_o$, external $Ca^{2+}$ concentration; $IP_1$, inositol monophosphate; $IP_2$, inositol biphosphate; $IP_3$, inositol triphosphate; TG, thapsigargin; HUVEC, human umbilical vein endothelial cell; ROC, receptor operated channel; CIF; $Ca^{2+}$ influx factor; CRAC, $Ca^{2+}$-release, activated $Ca^{2+}$ current.

membranes [22, 23] and mobilizes sequestered $Ca^{2+}$ without inositol polyphosphate production [17]. We thus investigated $[Ca^{2+}]_i$ and inositol polyphosphates in human umbilical vein endothelial cell (HUVEC) monolayers treated with histamine or TG, alone and combined, in the absence and presence of $Ca^{2+}$ influx. Our results demonstrate that the refilling state of internal $Ca^{2+}$ stores is not the only regulator for stimulation of $Ca^{2+}$ influx, but that other intracellular signals may also play an important role.

## MATERIALS AND METHODS

*Chemicals.* Histamine dihydrochloride, EGTA, HEPES, nigericin and thapsigargin were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Collagenase A and ionomycin were from Boehringer (Mannheim, Germany). Medium 199 was obtained from Eurobio (Les Ulis, France). RPMI 1640 medium, Hank's balanced salt solution (HBSS), foetal calf serum, L-glutamine, penicillin/streptomycin, fungizone and PBS were obtained from Gibco BRL (U.S.A.). Fura-2 acetoxymethyl ester (fura-2AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.).

*Cell culture.* Endothelial cells were isolated from human umbilical cord vein as previously described by Jaffe *et al.* [24]. Briefly, the cells were detached by incubation for 10 min at 37° with 10–20 mL PBS containing 0.1 mg/mL collagenase A, then collected by centrifugation at 100 *g* for 10 min and resuspended in medium 199 (40%) and RPMI 1640 (40%) containing 20% foetal calf serum, penicillin/streptomycin (100 U/mL/100 μg/mL), L-glutamine (2 mM) and fungizone (2.5 μg/mL). The cells were plated into 25 $cm^2$ flasks (Corning, New York, NY, U.S.A.) and incubated at 37° in a humidified atmosphere of air/$CO_2$ (19:1). The medium was renewed the following day and then every second day. Confluent cells (4–5 days) were detached by incubation with 0.05% trypsin and 0.02% EDTA for 1–3 min at room temperature, washed with the culture medium, centrifuged and reseeded onto 9 × 35 mm glass coverslips in Leighton tubes (Costar, Cambridge, MS, U.S.A.). Confluent cells (3–5 days) from the first passage were used for $[Ca^{2+}]_i$ and inositol polyphosphate determinations.

*Measurements of* $[Ca^{2+}]_i$. Cytosolic free $Ca^{2+}$ concentration was determined in cell monolayers using fura-2. Cells were first incubated for 30 min in buffer A (136 mM NaCl, 5 mM KCl, 2 mM $NaH_2PO_4$, 0.4 mM $Mg_2SO_4$, 4 mM $NaHCO_3$, 8 mM glucose, 25 mM hepes pH 7.4, at 37°) complemented with 20% foetal calf serum, amino acids similar to those of medium 199, and 2 mM glutamine, then incubated for 25 min at 37° with 2 μM fura-2AM and transferred to fresh buffer A for a further 25 min incubation at 37° to complete de-esterification. Cells were washed twice with buffer A containing only 1% serum and placed in a 2 mL quartz suprasil cuvette thermostated at 37°, specially designed to allow frontal fluorescence measurements [25]. Fura-2 was excited at 340 nm and 380 nm and emission was recorded at 505 nm on a spectrofluorimeter SPEX CMIII (ISA-Jobin-Yvon, Longjumeau, France). All measurements were corrected for autofluorescence of unloaded cells. Calibration of fura-2 fluorescence intensities in saline buffers was performed as previously described [26], and $[Ca^{2+}]_i$ was calculated according to the equation established by Grynkiewicz *et al.* [27].

To suppress the transmembrane $Ca^{2+}$ gradient, experiments were performed in the presence of 50 nM external $Ca^{2+}$ ($[Ca^{2+}]_o$) obtained by adding 0.1 mM EGTA and 42 μM $CaCl_2$ to buffer A. 1 mM $[Ca^{2+}]_o$ was then re-established by adding 1.1 mM $CaCl_2$.

In some experiments, the cell membrane potential was modified by varying the extracellular $K^+$ concentration (0 or 130 mM KCl).

*Determination of inositol polyphosphate production.* Cells obtained by trypsin treatment as described above were seeded onto multiwell plates and grown to confluence. They were then incubated for 48 hr with 4 μCi/mL myo-[$^3$H]inositol (Amersham, Les Ulis, France). Labeled cells were washed twice with buffer A, incubated for 30 min at 37° with 20 mM LiCl and exposed to histamine, TG, or both combined. The reaction was stopped by incubating cells with 4.5% perchloric acid at 4°. [$^3$H]inositol polyphosphates were then extracted by using anion-exchange columns as previously described, and counted [28].

*Statistical analysis.* Results are expressed as means ± SEM. Multiple comparisons and dose–response effects were examined by one-way analysis of variance with *post hoc* Fisher's test. When two groups were compared, significance of differences was assessed by two-tailed unpaired Student's *t* test. P values $< 0.05$ were considered as significant.

## RESULTS AND DISCUSSION

To investigate the role of the internal $Ca^{2+}$ store refilling state in the control of $Ca^{2+}$ entry, the $Ca^{2+}$ pools were mobilized either by TG and/or histamine.

### *Thapsigargin effects on* $[Ca^{2+}]_i$ *in histamine-stimulated cells*

We first studied the effects of increasing TG doses on $[Ca^{2+}]_i$ in unstimulated HUVEC to determine the maximal concentration required to totally empty the internal $Ca^{2+}$ pools. After 30–40 sec delay, TG increased $[Ca^{2+}]_i$ in a time- and concentration-dependent manner (Fig. 1). At 0.5 μM and above, $[Ca^{2+}]_i$ reached steady-state values 2-fold higher than those observed in the absence of TG (225 ± 13 vs 108 ± 10 nM, N = 17). The apparent $EC_{50}$ for the initial rate of $[Ca^{2+}]_i$ rise and the sustained increases were 520 and 220 nM, respectively (Fig. 1). They were in the same range as those determined in bovine endothelial cells and platelets [17, 22].

The effects of TG were then evaluated after $Ca^{2+}$ mobilization from intracellular stores by histamine. As previously described [16], histamine induced, without detectable latency, a biphasic $[Ca^{2+}]_i$ response: a transient $Ca^{2+}$ peak followed by a sustained elevation (Figs 2 and 3, trace a). In cells prestimulated with a low dose of histamine (1 μM), 1 μM TG was still able to increase $[Ca^{2+}]_i$ up to 235 ± 24 nM (N = 4) (Fig. 2, trace b). In contrast, pretreatment by 10 μM histamine abolished the response to 1 μM TG (Fig. 2, trace c). Our results

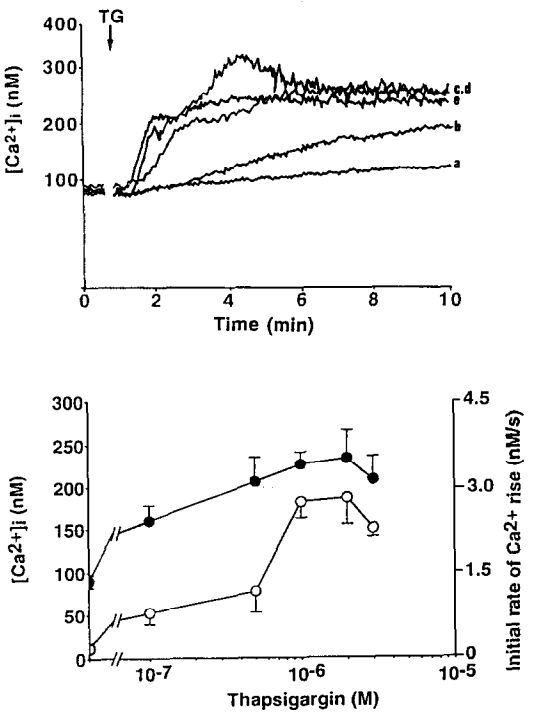


Fig. 1. Dose-dependent effect of TG on $[Ca^{2+}]_i$ in human endothelial cells. $[Ca^{2+}]_i$ was determined in fura-2 loaded cells incubated in the presence of 1 mM $[Ca^{2+}]_o$. Upper panel: the arrow indicated addition of vehicle (a) or TG at concentrations 0.1 (b), 0.5 (c), 1 (d), or 3 $\mu$M (e). A representative trace is shown. Lower panel: dose-dependent action of TG on the apparent initial rates of $[Ca^{2+}]_i$ rise calculated from the linear portions of the time-course trace (○) ($F_{5,25} = 13.5$, $P < 0.001$) and on steady-state $[Ca^{2+}]_i$ values evaluated between 8 and 10 min (●) ($F_{5,25} = 7.7$, $P < 0.001$). Data are from 4 to 10 independent experiments from different cultures.

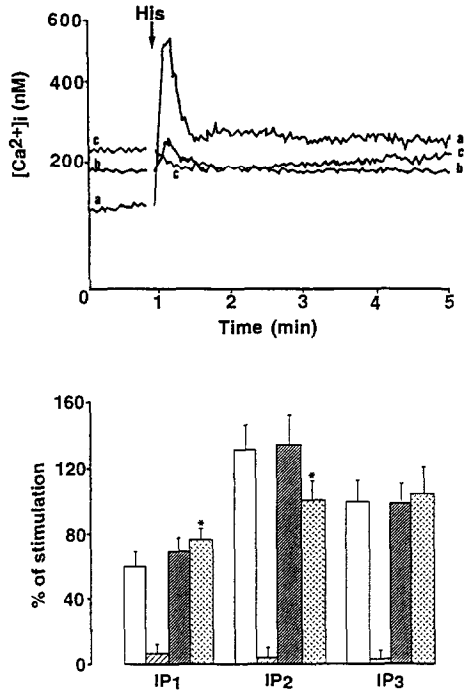


Fig. 3. Effects of TG pretreatment on histamine-induced $[Ca^{2+}]_i$ variations and inositol polyphosphate production. $[Ca^{2+}]_i$ and inositol polyphosphates were determined in the presence of 1 mM $[Ca^{2+}]_o$. Upper pannel: cells were preincubated for 6 min in the absence (a) and presence of 0.1 (b) or 1 $\mu$M TG (c). The arrow indicated the addition of 10 $\mu$M histamine (His). Traces are representative of 4–5 independent experiments. Lower panel: cells were preincubated in the absence (□) and presence of 0.1 (▨) of 1 $\mu$M TG (▨, ▩), then incubated for 2 min with 10 $\mu$M histamine (□, ■, ▩). Values are means ± SEM of 8 independent experiments.

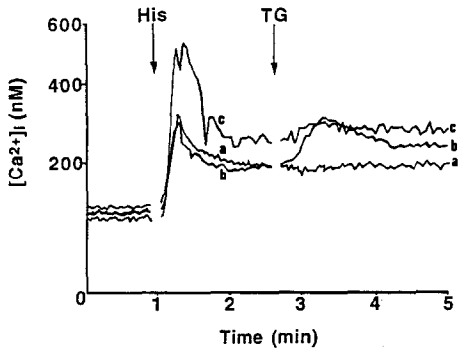


Fig. 2. Effects of histamine pretreatment on TG-induced $[Ca^{2+}]_i$ variations. $[Ca^{2+}]_i$ was determined in the presence of 1 mM external $Ca^{2+}$. The endothelial cells were stimulated by 1 $\mu$M (traces a, b) or 10 $\mu$M (trace c) histamine (His) then 1 $\mu$M TG was added (traces b, c). Traces are representative of 4–6 independent experiments.

agree with the observation that TG was still able to further activate inward $K^+(Ca^{2+})$ current after cell stimulation by a low dose of bradykinin in cultured bovine endothelial cells [19]. In single HUVEC, TG increased $[Ca^{2+}]_i$ after stimulation with 100 $\mu$M histamine followed by washout of the agonist [20], which allowed $Ca^{2+}$ stores to be replenished [13, 16]. This suggests that TG did not stimulate $Ca^{2+}$ influx when it could not further mobilize $Ca^{2+}$ within internal stores.

*Effect of histamine on* $[Ca^{2+}]_i$ *and inositol polyphosphates in TG-treated cells*

Pretreatment of HUVEC with increasing TG concentrations also progressively reduced histamine-induced $Ca^{2+}$ mobilization. When compared to the $[Ca^{2+}]_i$ rise induced by 10 $\mu$M histamine in untreated cells (Fig. 3a, trace a), preincubation for 6 min with a low dose of TG (100 nM) reduced but did not suppress the $[Ca^{2+}]_i$ response to histamine (Fig. 3, trace b). $[Ca^{2+}]_i$ increased from 185 ± 21 to 235 ± 23 nM (N = 5), then returned to pre-histamine values. In contrast, when cells were pretreated with 1 $\mu$M TG, the subsequent addition of 10 $\mu$M

histamine caused a transient decrease in $[Ca^{2+}]_i$ from 237 ± 35 to 195 ± 31 (P = 0.004, N = 9) (Fig. 3a, trace c). This could not be overcome by increasing the histamine concentration (decrease from 232 ± 38 to 169 ± 27 nM in the presence of 100 μM histamine, P = 0.02, N = 5). A similar phenomenon has recently been described in bovine endothelial cells where bradykinin reduced $[Ca^{2+}]_i$ below prestimulation levels when internal stores were depleted either by pretreatment with 1 μM TG or by two previous exposures to bradykinin [29]. These authors proposed that a non-calcium signal associated with receptor activation stimulates $Ca^{2+}$ extrusion.

To determine whether TG modified inositol polyphosphate production induced by 10 μM histamine, we incubated [$^3$H]inositol-labeled cells with TG, histamine or both together. Preincubation of cells for 6 min with 0.1 or 1 μM TG did not modify $IP_3$ production elicited by 10 μM histamine although the $[Ca^{2+}]_i$ response was nearly or totally suppressed (Fig. 3a and b). Interestingly, pretreatment with 1 μM TG altered the content of $IP_3$ degradation products, decreasing $IP_2$ and increasing $IP_1$ (P = 0.039 and 0.022, respectively, N = 8) (Fig. 3b). This indicates that in TG-treated cells, histamine was still able to activate phospholipase C and produce $IP_3$, but that secondary effects of TG may disturb $IP_3$ metabolism in HUVEC.

*Influence of external $Ca^{2+}$ concentration on the combined effect of TG and histamine*

The reduction of $Ca^{2+}$ movements in TG- or histamine-pretreated cells, in spite of an unchanged $IP_3$ content, suggests that the $Ca^{2+}$ level within the $IP_3$-sensitive internal store directly controls the stimulation of $Ca^{2+}$ entry in HUVEC. To determine the role of $Ca^{2+}$ influx in $[Ca^{2+}]_i$ changes, the transmembrane $Ca^{2+}$ gradient was suppressed by reducing $[Ca^{2+}]_o$ to 50 nM just before depleting internal stores by 1 μM TG or 10 μM histamine; $Ca^{2+}$ entry was then initiated by increasing $[Ca^{2+}]_o$ from 50 nM to 1 mM. In the absence of $Ca^{2+}$ influx, the effect of 1 μM TG still appeared with a lag time similar to that in 1 mM $[Ca^{2+}]_o$ medium, but the amplitude of the $[Ca^{2+}]_i$ rise was reduced by 60% (54 ± 14 vs 133 ± 13 nM, N = 6, P = 0.006). In contrast, as previously described [16], the amplitude of $Ca^{2+}$ release elicited by 10 μM histamine was similar in low and normal $Ca^{2+}$ media (350 ± 33 and 395 ± 25 nM, respectively, N = 24 for both). This highlights the difference in $[Ca^{2+}]_i$ responses evoked by TG and histamine. The $Ca^{2+}$ influx largely accounts for the initial $[Ca^{2+}]_i$ rise evoked by TG whereas the initial $[Ca^{2+}]_i$ signals induced by histamine are independent of external $Ca^{2+}$.

To verify that $Ca^{2+}$ stores were indeed void of cells treated with 1 μM TG or 10 μM histamine in low $Ca^{2+}$ medium, 2 μM ionomycin, 10 μM histamine ot 1 μM TG were subsequently added. Under each condition, basal $[Ca^{2+}]_i$ values did not increase further (85 nM on average), indicating a complete discharge of the $Ca^{2+}$ pool. This also demonstrates that TG and histamine mobilize $Ca^{2+}$ from the $IP_3$-sensitive store. These results agree with the observations that in $Ca^{2+}$-free medium, mobilization of internal $Ca^{2+}$ pools with TG greatly reduced or abolished subsequent depletion of the $IP_3$-sensitive pool by bradykinin [17, 29]. In contrast, mobilization of intracellular $Ca^{2+}$ stores evoked by maximal bradykinin concentration only partially reduced the subsequent $[Ca^{2+}]_i$ response to TG, suggesting the presence of an $IP_3$-insensitive $Ca^{2+}$ pool in bovine aortic endothelial cells [17].

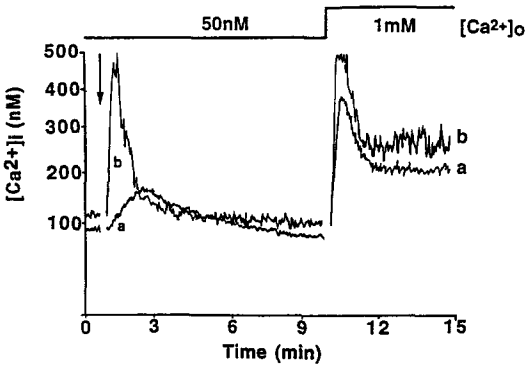


Fig. 4. Influence of $[Ca^{2+}]_o$ on TG- and histamine-induced $[Ca^{2+}]_i$ variations. Cells were incubated for 1 min at 37° in 50 nM $[Ca^{2+}]_o$ buffer and 1 μM TG (trace a) or 10 μM histamine (trace b) was added as indicated by the arrow. Cells were first suspended in 50 nM $[Ca^{2+}]_o$ buffer, then the $Ca^{2+}$ gradient was re-established by increasing $[Ca^{2+}]_o$ to 1 mM. Traces are representative of 5 independent experiments from different cultures.

In both TG- and histamine-treated cells, the re-establishment of the transmembrane $Ca^{2+}$ gradient brought about a large and transient increase in $[Ca^{2+}]_i$ followed by a return towards steady-state values similar to those obtained in 1 mM $[Ca^{2+}]_o$ (Fig. 4). This confirms that the sustained $[Ca^{2+}]_i$ elevation elicited by TG corresponds to an activation of $Ca^{2+}$ entry from the extracellular space. This also agrees with previous studies performed in bovine endothelial cells, where the mobilization of intracellular $Ca^{2+}$ stores by TG or 2′,5′-di(*tert*-butyl)-1,4-benzohydroquinone (BuBHQ) stimulated $^{45}Ca^{2+}$ influx [17, 18]. Electrophysiological studies demonstrated that both TG and histamine open non-selective cation channels as the result of the discharge of $Ca^{2+}$ stores in single bovine and human endothelial cells [14, 19, 20, 30]. In the present study, when we compared the $Ca^{2+}$ responses induced by the re-establishment of the transmembrane $Ca^{2+}$ gradient, we observed that the initial rate of $[Ca^{2+}]_i$ rise and the peak $[Ca^{2+}]_i$ values were significantly higher in histamine- than in TG-treated HUVEC (Table 1). Interestingly, the subsequent addition of histamine after TG did not increase the $Ca^{2+}$ response induced by the re-establishment of the transmembrane $Ca^{2+}$ gradient, in spite of the higher response induced by 10 μM histamine alone (Table 1). This indicates that TG pretreatment partially prevented a subsequent stimulation of $Ca^{2+}$ influx by histamine. The addition of TG after $Ca^{2+}$ mobilization by histamine also did not change the amplitude of $[Ca^{2+}]_i$ movements elicited by the increase in $[Ca^{2+}]_o$. These results suggest either the selective regulation of one $Ca^{2+}$ channel by an intracellular messenger such as CIF

Table 1. $[Ca^{2+}]_i$ changes induced by re-establishment of transmembrane $Ca^{2+}$ gadient in histamine and/or thapsigargin-treated human endothelial cells

| Treatment 1<br>Treatment 2 | Histamine | TG | Histamine<br>TG | TG<br>Histamine |
|---|---|---|---|---|
| Initial rate (nM/sec) | 19.2 ± 2.3 | 8.4 ± 1.3* | 17.4 ± 3.4 | 7.6 ± 2.4† |
| Peak $[Ca^{2+}]_i$ (nM) | 538 ± 63 | 337 ± 26* | 463 ± 43 | 302 ± 52* |
| Plateau $[Ca^{2+}]_i$ (nM) | 265 ± 19 | 232 ± 26 | 255 ± 26 | 187 ± 26 |

The $[Ca^{2+}]_i$ changes induced by increasing external $Ca^{2+}$ concentration from 50 nM to 1 mM were measured as the apparent initial rates of $[Ca^{2+}]_i$ rise and $[Ca^{2+}]_i$ values at peak and plateau, in cells treated by 10 $\mu$M histamine or 1 $\mu$M TG alone and in combination. Results are means ± SEM of 5–10 independent experiments for each group.

* $P < 0.01$ when compared to values of histamine-treated cells.

† $P < 0.05$ when compared to values of histamine-pretreated, TG-treated cells.

or the participation of at least two types of $Ca^{2+}$ channels, ROC and CRAC, opened by histamine and by discharge of internal $Ca^{2+}$ stores, respectively.

*Influence of external $K^+$ concentration on $Ca^{2+}$ influx activated by TG and histamine*

To investigate whether only one channel was activated after stimulation with both TG and histamine, $Ca^{2+}$ store depletion and re-establishment of the $Ca^{2+}$ gradient were performed in the presence of three extracellular $K^+$ concentrations ($[K^+]_o = 0$, 5 and 130 mM). The amplitude of the $Ca^{2+}$ release evoked by 1 $\mu$M TG in the absence of $Ca^{2+}$ influx was increased at 0 and 130 mM $[K^+]_o$ (110 ± 16 and 137 ± 26 nM, N = 5) compared to 5 mM (62 ± 10 nM, N = 14, P = 0.02 and 0.004, respectively). In contrast, the amplitude of $Ca^{2+}$ mobilization evoked by histamine did not increase at 0 and 130 mM $[K^+]_o$ (266 ± 22 and 359 ± 78 nM, N = 6) compared to 5 mM (396 ± 71 nM, N = 6).

As indicated in Fig. 5, the $[Ca^{2+}]_i$ response to the increase in $[Ca^{2+}]_o$ from 50 nM to 1 mM varied with $[K^+]_o$ in both TG- and histamine-treated cells. It is noteworthy that the response of TG-treated cells to various $[K^+]_o$ was different from that of histamine-stimulated cells. In the latter, the removal of extracellular $K^+$ abolished the transient increase in $[Ca^{2+}]_i$ induced by the re-establishment of $Ca^{2+}$ gradient thereby decreasing the initial rate of $[Ca^{2+}]_i$ rise (Table 2). In contrast, removal of external $K^+$ changed neither the initial rate of $[Ca^{2+}]_i$ rise nor the peak $[Ca^{2+}]_i$ values in TG-treated cells (Table 2). In endothelial cells from various origins, various agonists induced a hyperpolarization which parallels the transient rise in $[Ca^{2+}]_i$ [31]. In HUVEC, Nilius *et al.* observed a voltage-dependent latency between the application of histamine and the current onset [14]. They suggested a voltage-dependent step in the signal transduction between $H_1$-receptor and G-protein. The removal of external $K^+$ may have disturbed intracellular events responsible for the opening of histamine-activated non-selective cation channels.

When cell membranes were depolarized by increasing $[K^+]_o$ to 130 mM, the $Ca^{2+}$ entry elicited by the re-establishment of transmembrane $Ca^{2+}$ gradient also differed between TG- and histamine-stimulated cells (Fig. 5, trace c). In high $K^+$ buffer, both the initial rate of $[Ca^{2+}]_i$ rise and peak $[Ca^{2+}]_i$ values were decreased in histamine-stimulated cells. This agrees with previous studies which reported that membrane depolarization decreased the sustained $[Ca^{2+}]_i$ rise evoked by agonists under normal

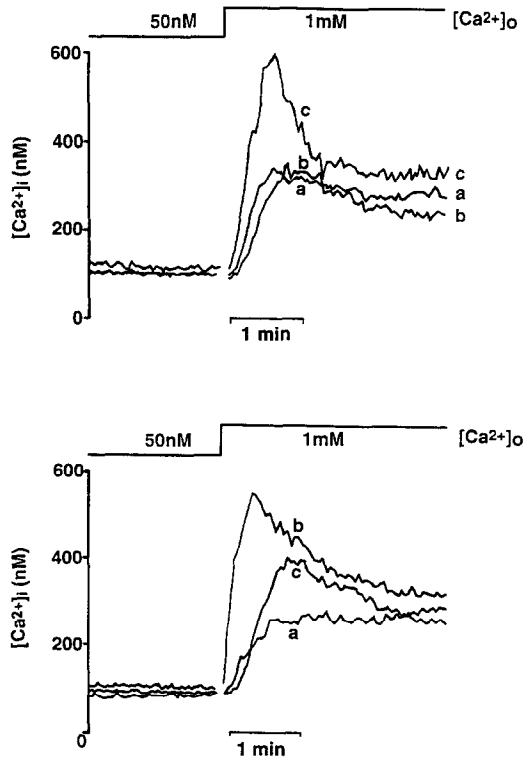


Fig. 5. Influence of extracellular $K^+$ concentration on $[Ca^{2+}]_i$ variations evoked by re-establishment of $Ca^{2+}$ gradient in TG- and histamine-treated cells. Cells incubated for 1 min at 37° in buffer A containing 50 nM $[Ca^{2+}]_o$ and either 0 (a), 5 (b) or 130 mM KCl (c) were treated by 1 $\mu$M TG (upper panel) or 10 $\mu$M histamine (lower panel) as shown in Fig. 4, then $[Ca^{2+}]_o$ was increased to 1 mM. Traces are representative of 5–6 independent experiments.

Table 2. Influence of external $K^+$ on $[Ca^{2+}]_i$ changes induced by re-establishment of transmembrane $Ca^{2+}$ gradient in histamine- and thapsigargin-treated cells

| | Initial rate (nM/sec) | | Peak $[Ca^{2+}]_i$ (nM) | | Plateau $[Ca^{2+}]_i$ (nM) | |
|---|---|---|---|---|---|---|
| $[K^+]_o$ (mM) | Histamine | TG | Histamine | TG | Histamine | TG |
| 0 | 5.2 ± 1.0‡ | 7.15 ± 0.8 | (284 ± 39†) | 335 ± 29 | 297 ± 29 | 284 ± 28 |
| 5 | 19.2 ± 2.3 | 8.4 ± 1.3* | 538 ± 63 | 337 ± 26§ | 265 ± 19 | 232 ± 24 |
| 130 | 9.1 ± 0.9‡ | 10.4 ± 2.8 | 383 ± 31† | 589 ± 84§† | 282 ± 20 | 334 ± 31† |

The $[Ca^{2+}]_i$ changes evoked by increasing $[Ca^{2+}]_o$ from 50 nM to 1 mM were measured as the apparent initial rates of $[Ca^{2+}]_i$ rise and $[Ca^{2+}]_i$ values at peak and plateau. Values in brackets indicated that $[Ca^{2+}]_i$ was evaluated under similar conditions but in the absence of measurable peaks. Results are means ± SEM from 5–10 independent experiments for each group.

§ * $P < 0.05$ and 0.01 when compared to values of histamine-treated cells.

† ‡ $P < 0.05$ and 0.01 when compared to values determined in the presence of 5 mM $[K^+]_o$.

$[Ca^{2+}]_o$ conditions [32–34]. In contrast, membrane depolarization increased peak and steady-state $[Ca^{2+}]_i$ values in TG-treated cells (Table 2). Under these conditions, peak $[Ca^{2+}]_i$ values were significantly higher in TG- than in histamine-treated cells although the initial rate of $[Ca^{2+}]_i$ rise did not differ (Table 2). This indicates that the $Ca^{2+}$ pathways gated by TG could have been activated by membrane depolarization whereas those opened by histamine were partially inactivated under these conditions. Our results supported the proposal that two types of channels participate in activation of $Ca^{2+}$ influx in stimulated HUVEC. However, the regulation of a putative intracellular CIF by extracellular $K^+$ cannot be ruled out.

In conclusion, the refilling state of internal $Ca^{2+}$ stores is not the only factor responsible for activating $Ca^{2+}$ influx. Intracellular signals elicited by the increase in $[Ca^{2+}]_i$ but also by other TG-induced metabolic events, such as those associated with the altered $IP_3$ metabolism, seem to have an important role. We propose that in human endothelial cells, the pathways involved in the stimulation of $Ca^{2+}$ influx evoked by TG and histamine are distinct but mutually regulated and dependent on membrane potential.

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