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Suppression of Ca^{2+} influx by unfractionated heparin in non-excitable intact cells via multiple mechanisms☆

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Abstract

Effect of unfractionated heparin (UFH), described as a cell-impermeant IP3 receptor antagonist, was studied on the capacitive Ca^{2+} entry in non-permeabilized, intact cells, measuring the intracellular Ca^{2+} levels using fluorescence microplate technique. Ca^{2+} influx induced via Ca^{2+} mobilization by histamine in Hela cells or evoked by store depletion with thapsigargin in RBL-2H3 cells was dose-dependently suppressed by UFH added either before or after the stimuli. UFH also prevented the spontaneous Ba^{2+} entry indicating that the non-capacitive Ca^{2+} channels may also be affected. In addition, UFH caused a significant and dose-dependent delay in Ca^{2+}, and other bivalent cation inflow after treatment of the cells with Triton X-100, but it did not diminish the amount of these cations indicating that UFH did not act simply as a cation chelator, but modulated the capacitive Ca^{2+} entry possibly via store operated Ca^{2+} channels (SOCCs). Inhibitory activities of UFH and 2-aminoethyl diphenyl borate on the capacitive Ca^{2+} influx was found reversible, but the time courses of their actions were dissimilar suggesting distinct modes of action. It was also demonstrated using a fluorescence potentiometric dye that UFH had a considerable hyperpolarizing effect and could alter the changes of membrane potential during Ca^{2+} influx after store depletion by thapsigargin. We presume that the hyperpolarizing property of this agent might contribute to the suppression of Ca^{2+} influx. We concluded that UFH can negatively modulate SOCCs and also other non-capacitive Ca^{2+} channels and these activities might also account for its multiple biological effects.

Keywords: Unfractionated heparin; Intracellular Ca^{2+}; Store operated calcium channels; Membrane potential; 2-Aminoethyl diphenyl borate; Bivalent cation entry

Although at first place heparin is noted as an anti-coagulant, it possesses a wide range of other pharmacological and biological activities including anti-inflammatory, anti-tumor, anti-asthmatic and anti-proliferative effects [1,2]. Positively charged clusters, which can form specific binding sites for heparin have been found in many different proteins including growth factors, cell adhesion molecules, cytokines, enzymes and receptors [3,4]. Interactions via these binding sites are thought to be responsible for the diverse biological effects of heparin.

Heparin binding proteins are also implicated in some aspects of calcium homeostasis and regulation. It has been known for about 20 years, that heparin binds to IP3 receptors, acting as intracellular Ca^{2+} channels on endoplasmic reticulum membrane, and is an effective competitive antagonist of these receptors not only in isolated membrane preparations but also in living cells [5–8]. Heparin is also a modulator of ryanodine receptor, another type of intracellular Ca^{2+} channels, it has been shown to act as a receptor activator in planar lipid bilayer experiments, but this activity has not been confirmed in cells yet [9]. Heparin binding sites on L-type Ca^{2+} channels have been also characterized. Heparin and related polyanions may interact with basic amino acids in the large extracellular domain connecting the fifth and sixth transmembrane segment in the first motif of the ionic pore-forming $\alpha 1$ subunit, thereby inhibiting the binding of their ligands to the plasma membrane Ca^{2+} channel of myocytes [10,11]. Direct suppressive effect of heparin on L-type Ca^{2+} chan-

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Abbreviations: 2-APB, 2-aminoethyl-diphenyl-borate; CRAC, Ca^{2+} release activated Ca^{2+} channel; IP3, inositol-triphosphate; SOCC, store operated Ca^{2+} channel; TG, thapsigargin; TRP proteins, transient receptor potential proteins; UFH, unfractionated heparin

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nels has been demonstrated in cardiac myocytes isolated from several mammalian species, detecting the Ca^{2+} current with whole-cell patch clamp method [12]. In spite of the detailed investigations, the exact mode of action of heparin on L-type Ca^{2+} channels has remained largely unknown.

Heparin is considered to be a cell-impermeant agent. When heparin is used as a conventional IP3 receptor antagonist either to study the signal transduction pathways of various receptors or to explore the role of IP3 receptors on the regulation of the store operated Ca^{2+} channels (SOCCs) [13–19], it is usually applied in direct microinjection into the cytoplasm [19–21], examined on permeabilized cells [22–23] or the agent is introduced through patch pipette [13–18]. The store depletion mediated Ca^{2+} influx through SOCCs referred to as capacitive Ca^{2+} entry, was unaffected when heparin or heparin conjugated to albumin was microinjected into the cytoplasm of the cells and the intracellular Ca^{2+} levels were monitored with fluorescence Ca^{2+} indicators [19]. In most studies on Ca^{2+} release activated Ca^{2+} channel (CRAC), which is the best characterized type of SOCCs, heparin was directly introduced into the cytoplasm for blocking the IP3 receptors, but it was unable to modify the Ca^{2+} current (Icrac) measured by pacth clamp technique [13–18]. In contrast, the direct connection of IP3 receptors with the activation mechanism has been revealed in cells transfected with a recombinant transient receptor potential (TRP) protein, TRPC3, which operates as SOCC [15]. The conflicting findings obtained from the studies utilizing intracellular heparin for blocking the IP3 receptor function support that the conformational coupling theory is not generally valid for the activation mechanism of different SOCCs [13–19] and indeed, the exact mechanisms of activation of SOCCs with diverse molecular and pharmacological properties have not been clarified yet [24,25].

Only few examinations related to SOCCs have been published where the direct effect of heparin was studied after extracellular application in intact cells. It has been demonstrated that Icrac is unaffected by heparin used extracellularly. Although changes in intracellular Ca^{2+} levels were not examined in parallel with these patch clamp recordings conducted mainly in RBL cells, it has been concluded that heparin has no direct effect on CRACs [14,18]. Furthermore, there are no biochemical and functional evidences for the existence of heparin binding structural elements on any members of TRP protein family having SOCC activity.

This study is the first detailed investigation on unfractionated heparin (UFH), which examines its effect on the net changes in the intracellular Ca^{2+} levels after activating the capacitive Ca^{2+} influx and applying this agent exclusively in extracellular route in intact non-excitable cells. The action of UFH on the capacitive Ca^{2+} entry was further characterized with Sr^{2+}, Ba^{2+} and Mn^{2+} influxes, and its effect was compared with that of 2-aminoethyl diphenyl borate (2-APB) known as an inhibitor of SOCCs. Changes in the membrane potential were also examined during the action of these Ca^{2+} channels.

1. Materials and methods

1.1. Materials

Fura-2-acetoxymethylester (Fura-2/AM) and Fluo-4-acetoxymethylester (Fluo-4/AM) in solutions, sulfinpyrazone and bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4(3)) were purchased from Molecular Probes (Eugene, OR, USA). Unfractionated heparin sodium salt, 2-aminoethyl diphenyl borate, histamine, thapsigargin, ionomycin and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RBL-2H3, rat basophilic leukemia cell line and HeLa, human cervix epitheloid carcinoma cell line were originated from American Type Culture Collection (Rockville, MD, USA).

1.2. Loading the cells with fluorescence Ca^{2+} indicators

RBL-2H3 and HeLa cells were maintained in monolayer cultures in Dulbecco's modified essential medium supplemented with 10% fetal calf serum. After harvesting the cells with 10 mM EDTA in phosphate buffered saline, 2×10^4 or 4×10^4 cells/well were plated into 96-well tissue culture plates and cultured for 48–72 h to grow to confluency. Cells were loaded with 5 μM of Fura-2/AM or Fluo-4/AM in Tyrode's salt buffer (137 mM NaCl, 3 mM KCl, 0.4 mM NaH_2PO_4, 5.6 mM glucose, 10 mM Hepes, 2 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4) containing 0.1 mg/ml sulfinpyrazone for 45 min at 37 °C. The plates were washed twice with the buffer and the cells were allowed to recover for 15 min at room temperature before measurements. For Ba^{2+} and Sr^{2+} influxes and Mn^{2+} quench experiments, Fura-2/AM was used for the loading of cells because Fluo-4 is unable to form complexes with these cations.

1.3. Measurement of intracellular Ca^{2+} mobilization and Ca^{2+} influx

After equilibration of the fluorescence to a stable baseline, cells were stimulated with thapsigargin (1 μM) or histamine (100 μM) or ionomycin (10 μM) and the changes in fluorescence intensity were monitored by fluorescence microplate reader (Fluostar Galaxy X, BMG Labtechnologies GmbH, Offenburg, Germany) at a single (492 nm) excitation wavelength and at the emission wavelength of 510 nm. In Ca^{2+} mobilization experiments, the extracellular medium was nominally Ca^{2+} free Tyrode's buffer (without $CaCl_2$ and supplemented with 0.1 mM EGTA), whereas the calcium influx was studied in the

presence of 2 mM $CaCl_2$. 10 mM of $CaCl_2$ was also used in some experiments. The cytosolic Ca^{2+} concentrations were calculated from the fluorescence intensity values measured for Fluo-4–Ca^{2+} complexes according to the equation $[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$ [26]. The fluorescence levels were calibrated in terms of $[Ca^{2+}]_i$ determining the F_{min} and F_{max} values in the absence or in the presence of extracellular Ca^{2+} after the lysis of the cells with 0.1% Triton X-100. K_d value for Fluo-4 was 345 nM.

1.4. Measurement of Ba^{2+} and Sr^{2+} influxes and Mn^{2+} quench

Ba^{2+} and Sr^{2+} influxes and Mn^{2+} quench was studied in Ca^{2+} free Tyrode's buffer containing 0.2 mM EGTA. One millimolar of $BaCl_2$ or $SrCl_2$ or $MnCl_2$ were used without stimulating the cells for measuring spontaneous cation entry and after the stimulation with 1 μM thapsigargin for the measurement of stimulated cation entry. Ten millimolars of these cations was also applied in some experiments. Sr^{2+} and Ba^{2+} ions form fluorescence complexes with Fura-2, which were measured at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm [27,28]. Data expressed in fluorescence ratios at 340/380 nm. Mn^{2+} quenches the fluorescence intensity of Fura-2, which was monitored at the isobestic point of the dye (360 nm) [27,29].

1.5. Measurement of ionomycin and Triton X-100 triggered divalent cation entry

10 μM ionomycin or 0.1% Triton X-100 was added to Fluo-4 or Fura-2 loaded cells after treatment with heparin or 2-APB in the presence of extracellular Ca^{2+} or Sr^{2+} or Ba^{2+} or Mn^{2+} ions. In some experiments, Triton X-100 was added before treatment with UFH and some cases EGTA was also used. Fluorescence was measured as described above.

1.6. Measurement of membrane potential using the potentiometric dye DiBAC4(3)

Cells grown to confluency were incubated with 200 nM DiBAC4(3) in Tyrode's buffer for 10–15 min. The fluorescence intensity of DiBAC4(3) was measured at an excitation of 492 nm and at an emission of 520 nm. Increase in DiBAC4(3) fluorescence means membrane depolarization, whereas the fluorescence intensity decreases during hyperpolarization [30]. The basal fluorescence intensity was determined before the addition of the agents. The changes in basal traces were continuously detected during these experiments. Results were expressed in Δ Fluorescence units (FU) calculated after the subtraction of the basal values from the values measured at different time points.

1.7. Treatment of the cells with UFH and 2-APB

UFH and 2-APB was extracellularly added to intact cells before the inducers or the Ca^{2+} surrogates or after the activation of cation influx during the plateau phase. Concentration of UFH was expressed in milligram per milliliter as indicated on the figures. In the reversibility experiment, the agents were washed out and replaced with the appropriate extracellular buffers as described above. Experiments were conducted at least in three independent measurements, using three separate cell cultures, and three or four parallels were run in each experiment. Results are shown as mean of these experiments.

2. Results

2.1. Effect of UFH on Ca^{2+} mobilization and Ca^{2+} influx

Two classical models were employed for studying the effect of UFH on the capacitive Ca^{2+} entry; thapsigargin induced Ca^{2+} influx in RBL-2H3 cells and histamine stimulated Ca^{2+} entry in HeLa cells [31,32]. Thapsigargin, the most potent and specific irreversible inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA pumps) evokes intracellular Ca^{2+} release in a large scale of cells [33,34]. Increase in intracellular Ca^{2+} is due to the passive Ca^{2+} release from the stores through a leakage mechanism in the endoplasmic reticulum membrane (Fig. 1A). HeLa cells mobilize intracellular Ca^{2+} in response to stimulation with histamine because these cells express H1 receptors, and the activation of the signal transduction cascade leads to the production of IP3 and the emptying of the IP3-sensitive Ca^{2+} stores [31] (Fig. 1B). Cytosolic Ca^{2+} returned to baseline rapidly in Ca^{2+} free medium in both cases, but the difference in the duration of the Ca^{2+} transient reflects two types of mechanism by which intracellular Ca^{2+} mobilization takes place. Neither the IP3 receptor mediated Ca^{2+} mobilization nor the thapsigargin stimulated passive Ca^{2+} release were affected by UFH up to 6 mg/ml (Fig. 1A and B). These observations indicate that UFH does not act as an IP3 receptor antagonist when it is added extracellularly to intact cells. We also found that UFH did not affect the basal intracellular Ca^{2+} levels in cells in the concentration range examined in our experiments. This finding is in a good accordance with the observations on native heparin and some heparin derivatives with high molecular weights as described previously [35].

Increase in intracellular Ca^{2+} levels due to the depletion of internal stores with either histamine or thapsigargin activates the SOCCs in the plasma membrane to refill the empty stores [33,34]. When extracellular Ca^{2+} was present a massive and rapid Ca^{2+} influx through these Ca^{2+} channels was observed, which lasted for a substantial period of time with a plateau phase. The plateau phase

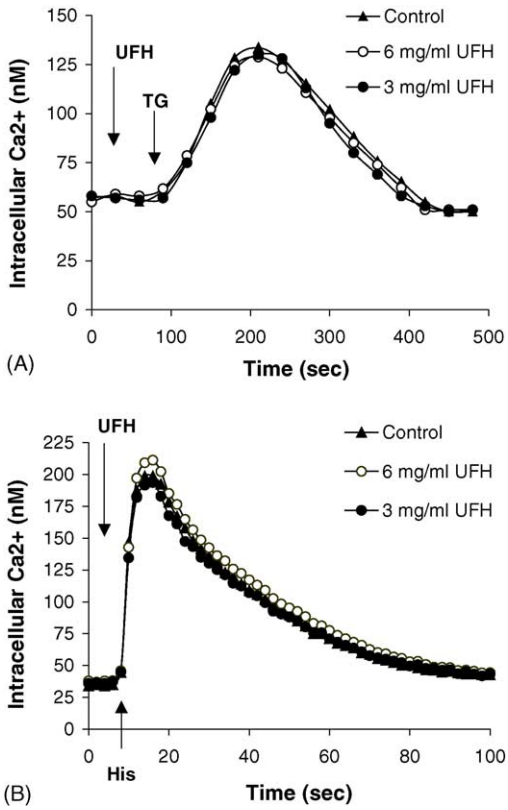

Fig. 1. Effect of UFH on thapsigargin and histamine induced Ca^{2+} mobilization in RBL-2H3 and HeLa cells. Intracellular Ca^{2+} release from stores were measured in nominally Ca^{2+} free buffer after stimulation of Fluo-4 loaded cells with 1 μg/ml thapsigargin (RBL-2H3 cells) (A) or with 100 μM histamine (HeLa cells) (B). Data represent means of 12 parallels from three separate cell cultures.

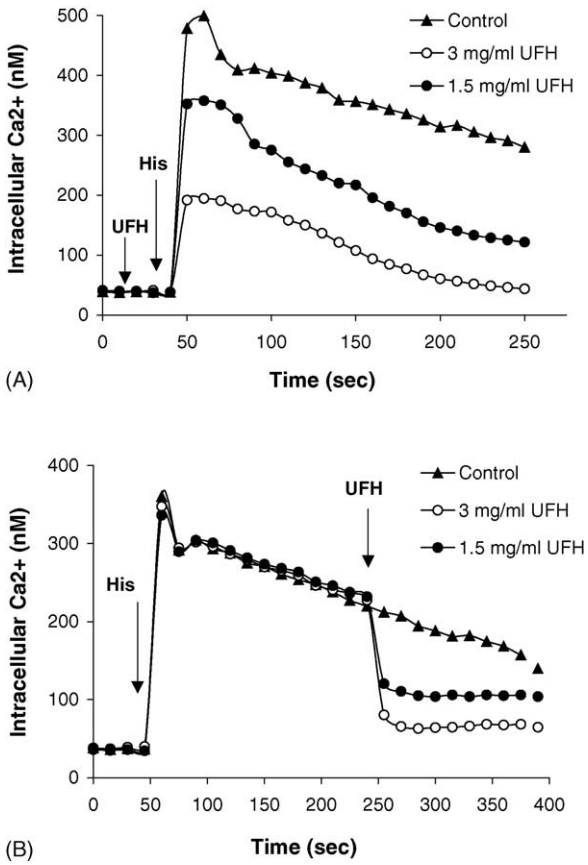

Fig. 2. Effect of UFH on histamine induced Ca^{2+} influx in HeLa cells. UFH was added to Fluo-4 loaded cells before the inducer (A), or at the plateau phase of Ca^{2+} influx (B), measured in 2 mM $CaCl_2$ containing buffer. Data represent means of 9 parallels from three separate cell cultures.

in thapsigargin stimulated RBL-2H3 cells was extremely long likely as a consequence of the irreversible block of SERCAs, when the stores cannot be filled with Ca^{2+} any more, and probably because the membrane Ca^{2+}-ATPases are not so active in these cells as in HeLa cells. Ca^{2+} influx decreases in time after normal physiological stimulus in HeLa cells indicating that the Ca^{2+} is pumped into the stores and the excess is extruded from the cells via plasma membrane Ca^{2+}-ATPase [31,32]. The effect of UFH on the influx and on the plateau phase of histamine or thapsigargin induced Ca^{2+} entry was examined separately. UHF dose-dependently suppressed the influx phase independently of the stimulus for the opening of SOCCs in HeLa and RBL-2H3 cells at concentrations equal to or higher than 0.75 mg/ml as seen on Fig. 2A and Fig. 3A. UFH dose-dependently and instantaneously inhibited the Ca^{2+} entry at the sustained phase of Ca^{2+} influx after SOCCs having been fully activated by store depletion with thapsigargin or histamine (Fig. 2B and Fig. 3B). This immediate decrease in intracellular Ca^{2+} levels in response to UFH suggested that it was possibly due to the activation of the plasma membrane Ca^{2+}-ATPases together with the inactivation of Ca^{2+} channels. However, as UFH did not modify the rate of Ca^{2+} extrusion observed in study on Ca^{2+} mobilization (Fig. 1A and B), we believe that the rapid suppressive effect would be more likely mediated by the inactivation of the SOCCs apparently from the extracellular site.

Furthermore, we found that the suppressive effect of UFH in thapsigargin stimulated THP 1 cells, a human monocytic cell line, was similar to that observed in RBL-2H3 and HeLa cells, but thapsigargin triggered Jurkat cells were less sensitive to UFH than the former ones (data not shown) indicating that the number and the composition of the ion channels in the cell membranes of these cell types are likely to be different.

2.2. *Effect of UFH on Ba^{2+} and Sr^{2+} influxes and Mn^{2+} quench*

The influence of UFH on the Ca^{2+} channels was further investigated by studying the effect of the agent on Ba^{2+}, Sr^{2+} and Mn^{2+} entry, which takes place via plasma membrane Ca^{2+} channels [27,28,29]. Unidirectional cation influx can be studied with Ba^{2+}, because this cation is trapped in the cytoplasm and not pumped out of the cells or into the internal stores [27,28]. We observed that both RBL-2H3 cells and HeLa cells were highly permeable to Ba^{2+} without any stimulation, but after triggering the cells

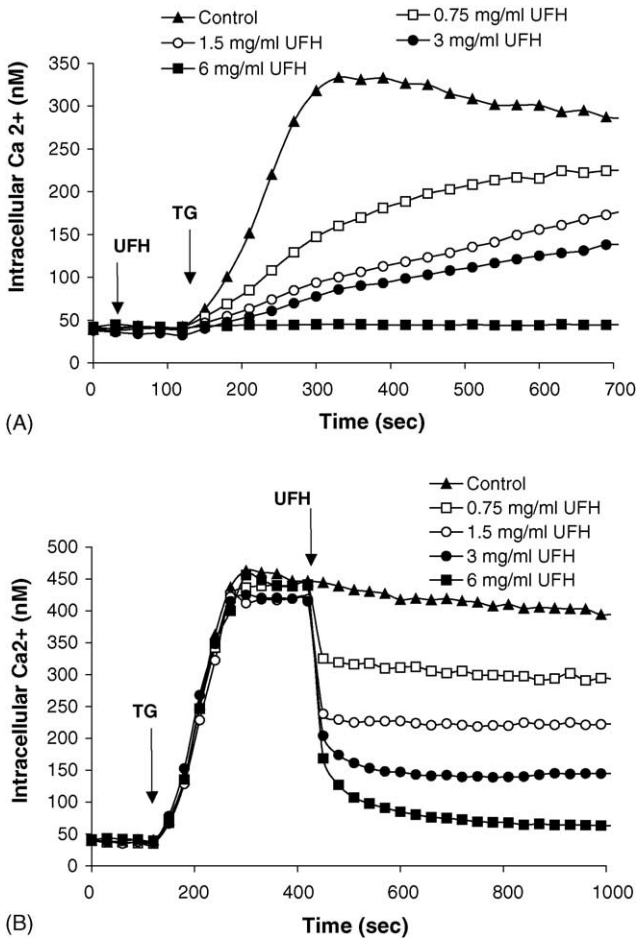

Fig. 3. Effect of UFH on thapsigargin induced Ca^{2+} influx in RBL-2H3 cells. UFH was added to Fluo-4 loaded cells before the addition of thapsigargin (A) or at the plateau phase of Ca^{2+} influx (B), measured in 2 mM $CaCl_2$ containing buffer. Data represent means of 12 parallels from three separate cell cultures.

with thapsigargin, the permeability to Ba^{2+} further increased. UFH dose-dependently inhibited both the spontaneous and the stimulated Ba^{2+} influx (Fig. 4A) indicating that other Ca^{2+} channels than SOCCs were also influenced by this agent.

Unlike spontaneous Ba^{2+} influx the spontaneous Sr^{2+} entry was very low in RBL-2H3 cells and also in HeLa cells (Fig. 4B). Thapsigargin induced a strong and fast Sr^{2+} inflow indicating that it is attributed mainly to SOCCs activation. As for Ca^{2+}, Sr^{2+} is also extruded from the cells via plasma membrane Ca-ATPase [27]. Thus, the effect of UFH could be also studied on the plateau phase. Sr^{2+} entry induced by store depletion was dose-dependently reduced by UFH at both phases of this cation influx (Fig. 4B), but the spontaneous entry remained to be unaffected. The intracellular Ca^{2+} release from internal stores triggered by thapsigargin could be also seen during these experiments and it was absolutely unaffected by UFH when it was added before the stimulus for store depletion reaffirming that UFH had no effect on Ca^{2+} mobilization.

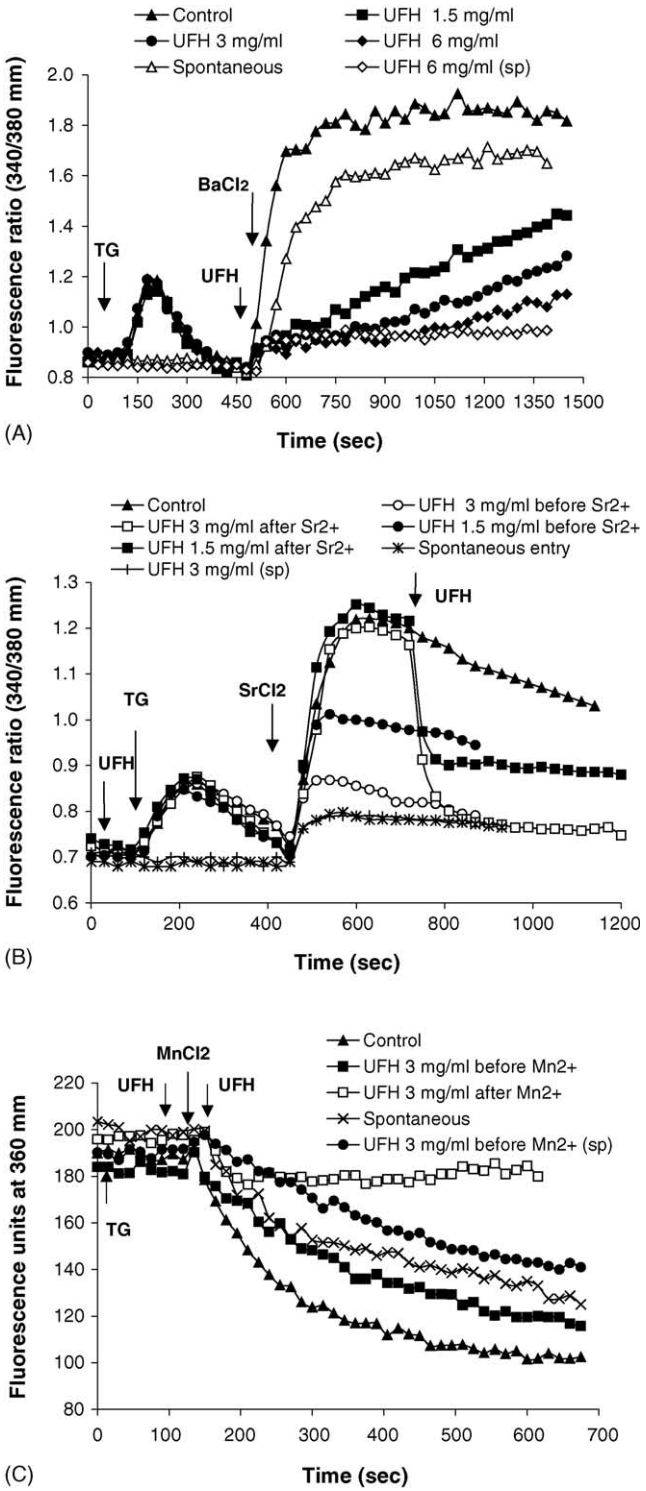

Fig. 4. Effect of UFH on thapsigargin induced Ba^{2+}, Sr^{2+} and Mn^{2+} influxes in RBL-2H3 cells. Ba^{2+} entry (A), Sr^{2+} entry (B) and Mn^{2+} quench (C) were measured in Fura-2 loaded cells using 1 mM of these cations in the extracellular buffers and 1 μM thapsigargin. UFH was added before or after the stimulus for store depletion or applied at the plateau phase of Sr^{2+} influx. Spontaneous Ba^{2+}, Sr^{2+} and Mn^{2+} influxes were studied without any addition of thapsigargin. Mn^{2+} quench was studied on UFH pretreated cells or UFH was added immediately after the injection of 1 mM $MnCl_2$. Data represent means of 9 or 12 parallels from three separate cell cultures.

Another technique commonly used to measure Ca^{2+} channel activity is to monitor the quenching of Fura-2 by Mn^{2+} [29]. Spontaneous Mn^{2+} inflow was found both in HeLa and RBL-2H3 cells, but histamine or thapsigargin caused a rise in the rate of Mn^{2+} quench (Fig. 4C). UFH was able to prevent the Mn^{2+} entry when it was applied immediately after admixing $MnCl_2$ to RBL-2H3 cells. Its inhibitory activity was not so strong when the cells were pretreated with UFH before the addition of thapsigargin, but even in this setup it decreased the rate of Mn^{2+} quench. The spontaneous Mn^{2+} entry was relatively high and UFH also reduced it. Suppressive effect on Mn^{2+} quench seemed to be independent of the stimuli used for the activation of SOCCs because it was also observable in HeLa cells after induction with histamin or thapsigargin.

The results with Ca^{2+} surrogates also support that UFH may interact with SOCCs, but these experiments indicated that UFH might also influence other non-store operated Ca^{2+} channels in the plasma membranes when this agent is applied extracellularly.

2.3. Reversible action of 2-APB and UFH

To explore the possible mechanism of inhibition, the effect of UFH was compared with that of 2-APB, known as a SOCC blocker on thapsigargin stimulated Ca^{2+} influx. Many different agents can inhibit the SOCC activity, but selective blockers for these Ca^{2+} channels have not been developed yet [36]. 2-APB was originally described as a membrane permeant IP3 receptor antagonist for some cell types, but recently, based on several findings, it is considered to be an universal and non-specific inhibitor of SOCCs with extracellular site of action in most cell types [19,21,23,27,34–39]. The inhibitory action of UFH and 2-APB was examined at the plateau phase of Ca^{2+} influx. The suppressive activity of UFH was broken down within 1–2 min when the agent was washed out of the cells and was replaced with fresh UFH-free medium. Inhibitory effect of 2-APB was also reversible, but the suppressive effect lasted for a prolonged time after withdrawal of this agent (Fig. 5A). This slowly reversible action of 2-APB has

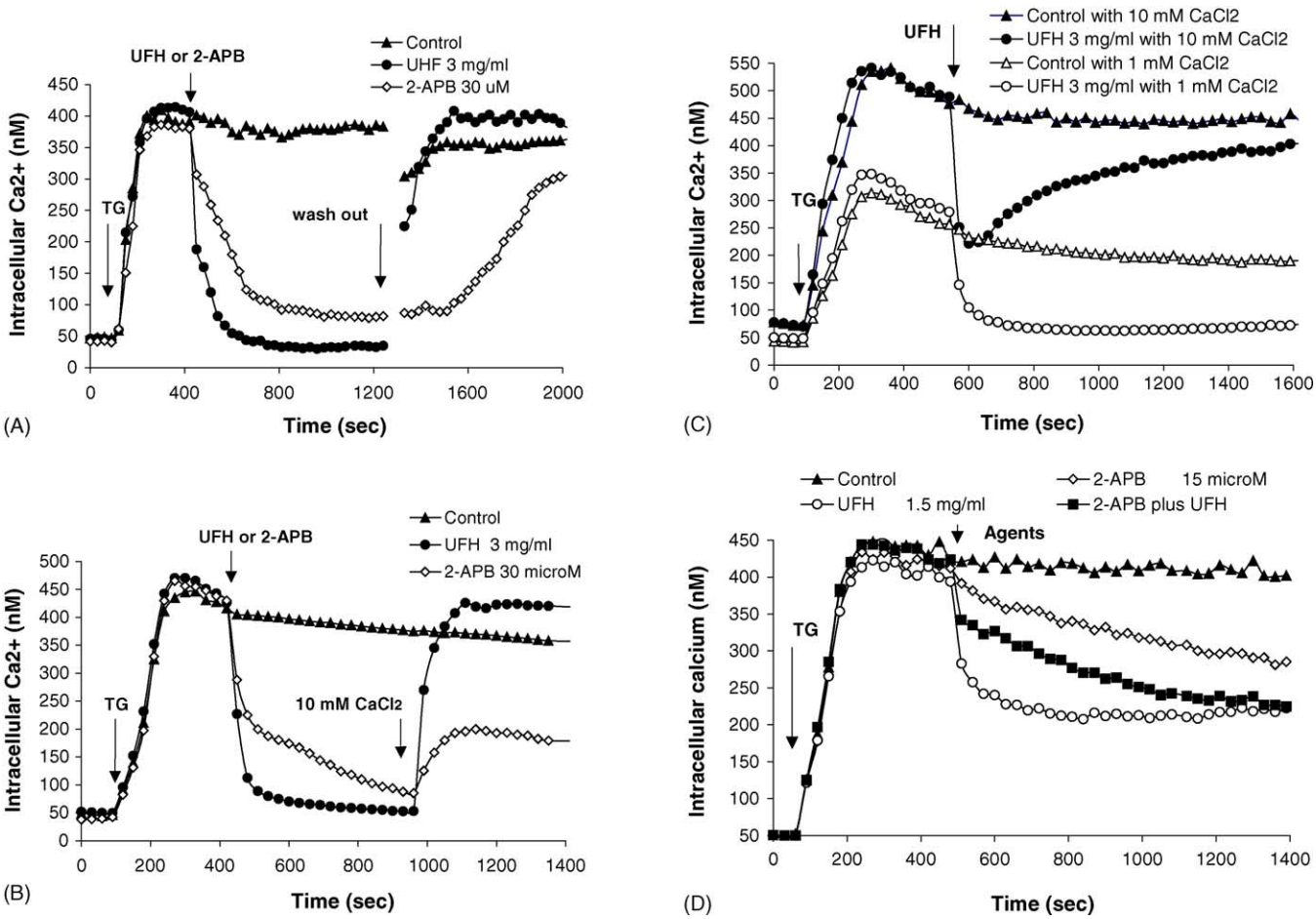

Fig. 5. Examination of different variables on the action of UFH and 2-APB to the thapsigargin induced Ca^{2+} influx in RBL-2H3 cells. Thapsigargin induced Ca^{2+} influxes were measured in RBL-2H3 cells under the following conditions. 30 μM 2-APB and/or 3 mg/ml UFH were added in the sustained phase of Ca^{2+} influx evoked by thapsigargin in Fluo-4 loaded cells. The agents were washed out and fresh buffer was added to the cells (A). High extracellular $CaCl_2$ (10 mM) was injected into the wells when the maximal, steady state suppression of Ca^{2+} influx by the agents has been reached (B). Inhibitory effect of 3 mg/ml UHF was studied in buffers containing low (1 mM) and high (10 mM) concentrations of $CaCl_2$ (C). 15 μM 2-APB and 1.5 mg/ml UFH were added to the cells separately or in combination (D). Data represent means of 12 parallels from three separate cell cultures.

been demonstrated previously [23,37], however depending on its concentrations and the cell types on which it was examined, irreversible action has been also observed [38]. When 10 mM $CaCl_2$ was added to the buffer at the late plateau phase, the inhibitory activity of UFH was totally abrogated whereas 2-APB retained its suppressive effect only its efficacy diminished (Fig. 5B). When high extracellular Ca^{2+} (10 mM) was present in the extracellular buffer during the whole experiment, the inhibitory activity of UFH reduced (Fig. 5C). 2-APB was also less potent in high extracellular concentrations of $CaCl_2$. Similar results were obtained in Ba^{2+} and Sr^{2+} influxes experiments when 10 mM $SrCl_2$ or $BaCl_2$ was used instead of the commonly applied 1 mM (data not shown). Our results indicate that the binding of UFH may be very weak at the extracellular site and its efficacy seems to be highly dependent on extracellular concentrations of the bivalent cations. As UFH has been described as a mild bivalent cation chelator in solutions [12], it cannot be ruled out that this cation chelating activity might have contributed to the outcomes of these experiments. Independently of this fact, however our results also point out that the permeability of SOCCs abnormally increases when Ca^{2+} or other bivalent cations are present in high concentrations, and these Ca^{2+} channels might be disengaged from the normal regulation existing under physiological circumstances.

UFH was also examined in combination with 2-APB and they showed non-additive suppressive effect on thapsigargin induced Ca^{2+} influx (Fig. 5D). Similarly, non-additive suppression of Sr^{2+} influx triggered by thapsigargin was observed (data not shown). The difference between the time courses of the reversibility and the non-additive effect of these agents suggest two distinct modes of action at least in part on the same Ca^{2+} channels.

2.4. Effect on Ionomycin induced Ca^{2+} inflow and divalent cation inflow triggered by Triton X-100

To examine the specificity of the action of UFH on capacitive Ca^{2+} entry, we studied its effect on the ionomycin stimulated Ca^{2+} entry. Ionomycin driven Ca^{2+} inflow is a complex process. Ionomycin has a passive ionophoric effect and it also causes an active Ca^{2+} mobilization from the stores, therefore its action is in part an independent process of the Ca^{2+} influx via SOCCs, and probably its ionophoric action at a concentration above 5 μM may predominate over its Ca^{2+} channel activating character [40]. UFH dose-dependently reduced the Ca^{2+} entry triggered by ionomycin (Fig. 6A). Furthermore, the suppressive effect could be partially abolished by addition of high $CaCl_2$ (10 mM) as it was observed with the Ca^{2+} chelator EGTA. This observation and the results obtained from the reversibility experiments would favor a direct interaction between UFH and the divalent cations in the extracellular buffer, but UFH also inhibited the ionomycin induced Ca^{2+} mobilization in the absence of extracellular

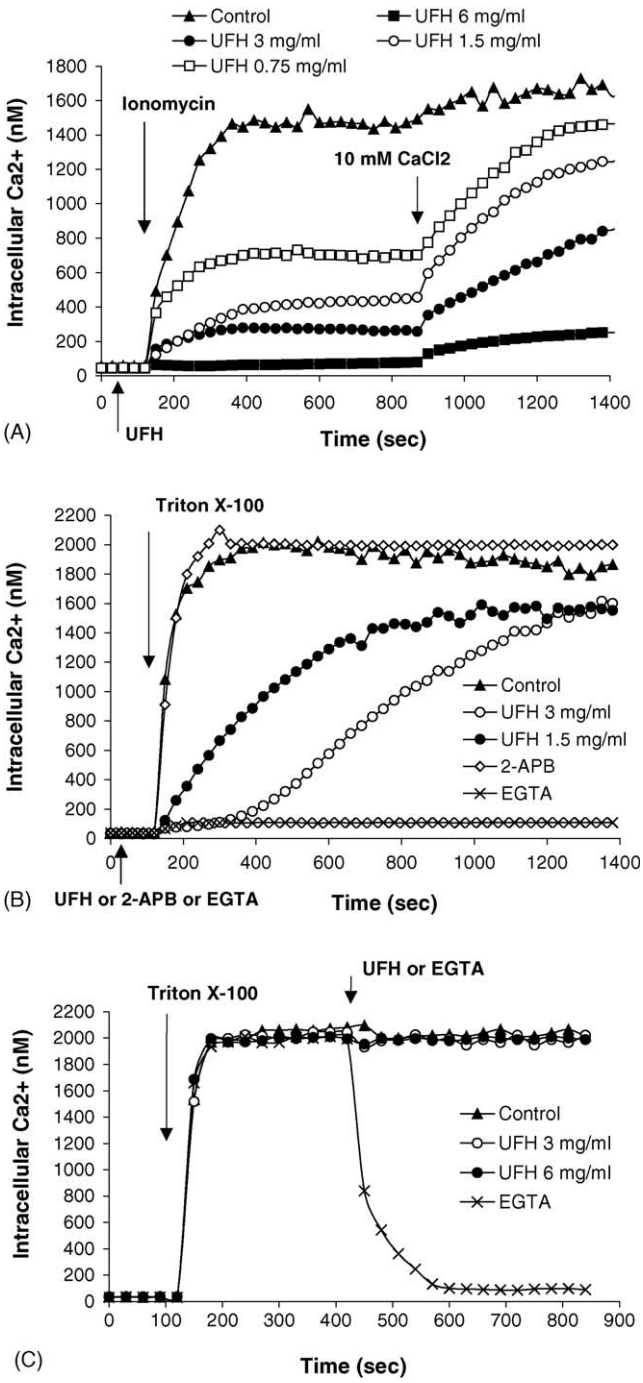

Fig. 6. Effect of UFH on ionomycin and Triton X-100 triggered Ca^{2+} entry in RBL-2H3 cells. Ca^{2+} entry was evoked by 10 μM ionomycin in UFH pretreated cells (A). At the plateau phase 10 mM $CaCl_2$ was added to the cells. Data represent means of 12 parallels from three separate cell cultures. Ca^{2+} entry triggered by 0.1% Triton X-100 was studied on Fluo-4 loaded cells pretreated with different concentrations of UFH as indicated on the figures or 5 mM EGTA or 30 μM 2-APB (B). UFH or 5 mM EGTA were added to cells after permeabilization with Triton X-100 (C). Measurements were done in extracellular buffer containing 2 mM $CaCl_2$. Data represent means of 9 or 12 parallels from three separate cell cultures.

Ca^{2+} (data not shown). We suggest that UFH may interfere either with the ionomycin itself at the extracellular site or more likely it blocks the interaction of ionomycin with the plasma membrane of the cells.

Accordingly, additional experiments were carried out for the purpose to ascertain the cation chelating effect of UFH. We examined Triton X-100 triggered bivalent cation inflow in RBL-2H3 cells. When Triton X-100 was added to cells pretreated with UFH, the Ca^{2+} entry slowed down and this effect was dependent on the concentration of UFH (Fig. 6B). Similar effect of UFH was observed with Sr^{2+}, Mn^{2+} and Ba^{2+} entry after permeabilization of cells with Triton X-100 (data is not shown). However, UFH only temporally reduced the amounts of Ca^{2+} and other bivalent cations compared to the controls and gradually their detected amount ascended close to the control level. Unlike UFH EGTA caused a significant and immediate decline in the intracellular Ca^{2+} levels during Triton X-100 triggered Ca^{2+} inflow (Fig. 6B). When UFH was added to cells pretreated with Triton X-100, the Ca^{2+} concentrations were not changed whereas EGTA, an authentic Ca^{2+} chelator was able to reduce the amounts of this cation immediately (Fig. 6C). These observations indicate that even if UFH can capture Ca^{2+} or other bivalent cations to some extent, its robust suppressive effect on capacitive Ca^{2+} entry cannot be simply explained with its cation chelating activity, which however may play a minor role in suppressing Ca^{2+} entry. 2-APB had no effect on Triton X-100 triggered divalent cation inflow as seen in Fig. 6B.

2.5. Effect of UFH on membrane potential

The prompt suppression of Ca^{2+} influx by UFH and the fast reactivation of SOCCs upon removing the agent imply on a mechanism that may be in close relation with the process taking place on the plasma membrane. Involvement of the membrane potential on the action of SOCCs has been reported in several investigations [41–44] and driving force of Ca^{2+} movement is influenced by the changes in the membrane potential. We examined the effect of UFH and 2-APB on the membrane potential in unstimulated cells and during thapsigargin induced Ca^{2+} entry using a fluorescence potenciometric dye, DiBAC4(3). UFH by itself dose-dependently decreased the fluorescence of the dye indicating that it shifted the resting potential of RBL-2H3 cells to a hyperpolarizing state (Fig. 7A). Membrane hyperpolarizing effect in unstimulated RBL-2H3 cells seemed to be independent of the concentrations of the extracellular Ca^{2+}. As described above, UFH causes neither increase nor decrease in the intracellular Ca^{2+} levels in unstimulated cells indicating that its membrane hyperpolarization effect may not evoke any changes in the basal levels of intracellular Ca^{2+}. Thapsigargin elicited an increase in fluorescence trace in the presence of extracellular Ca^{2+}, which reflected its membrane depolarizing effect. In some investigations with different cell types, thapsigargin usually induced a biphasic change in membrane potential with a fast hyperpolarization followed by a sustained depolarization [41–43]. We could not observe any hyperpolarization after stimulation

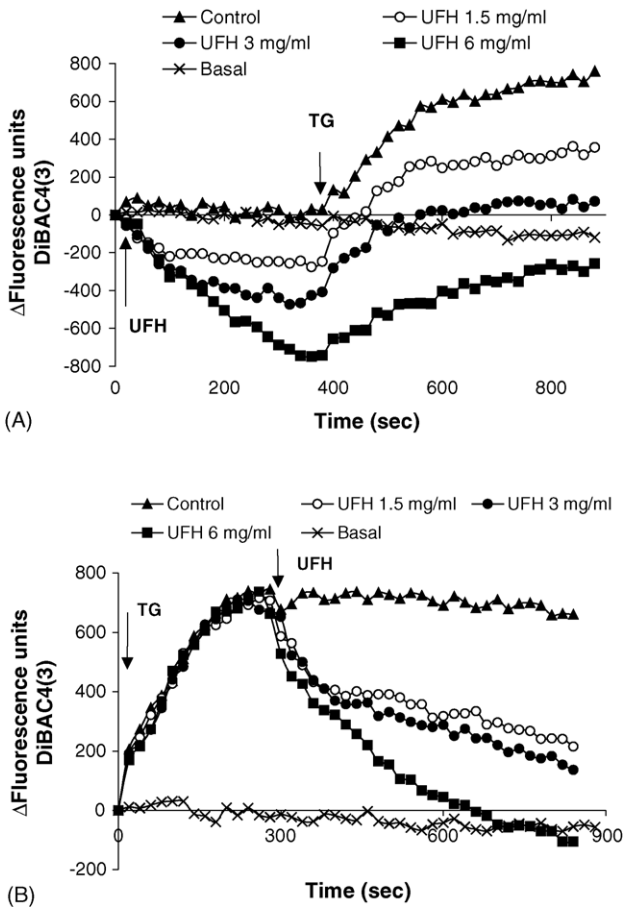

Fig. 7. Effect of UFH on the membrane potential in untriggered and thapsigargin induced RBL-2H3 cells. UFH alone was added to untriggered cells which were preincubated with DiBAC4(3) for 10–15 min, and 1 μM thapsigargin was injected after then (A). UFH was added after the induction of the cells with thapsigargin (B). Traces represent means of 12 parallels from three separate cell cultures.

of RBL-2H3 cells with thapsigargin, but thapsigargin induced depolarization was found to be a Ca^{2+} dependent process. When Ca^{2+} free medium was used the membrane depolarization was lower than that measured under physiological circumstances. When UFH was added immediately before the stimulation with thapsigargin, the membrane depolarization also reduced (data not shown). UFH fully abolished also the sustained membrane depolarization (Fig. 7B). When high extracellular Ca^{2+} (10 mM) was used, the changes in membrane potential detected in UFH treated and thapsigargin stimulated cells were smaller compared to those samples which were run in 2 mM $CaCl_2$ (data not shown). This Ca^{2+} dependent action of UFH seems to be partly compatible with that seen in Ca^{2+} influx experiments because the depression of depolarization at the plateau phase was not reverted by high extracellular Ca^{2+}. It appears that UFH can set the membrane potential to more negative value than the normal, can prevent and destroy the membrane depolarization after store depletion with thapsigargin at physiological concentrations of extracellular Ca^{2+}. Contrary to this, 2-APB did

not alter the resting potential and it only slightly attenuated the thapsigargin induced membrane depolarization (data not shown).

3. Discussion

In the present study, the effect of UFH was examined on the capacitive Ca^{2+} entry in two different in vitro models, namely the thapsigargin induced store depletion mediated Ca^{2+} influx in RBL-2H3 cells and the histamine induced IP3 mediated Ca^{2+} entry in HeLa cells [31,32]. Although the measurement of the intracellular Ca^{2+} levels by fluorescence Ca^{2+} indicators only indirectly shows the action of plasma membrane Ca^{2+} channels, such as SOCCs and other non-store operated Ca^{2+} channels that are used to be detected directly by patch clamp technique, monitoring the net change in the Ca^{2+} concentrations within the cells by fluorescence indicators is a reliable and widely used technique to follow the physiological consequences of both the activities of these channels and the action of the intracellular Ca^{2+} channels.

We have demonstrated for the first time that the extracellularly applied UFH has a significant suppressive effect on the capacitive Ca^{2+} entry without any influence on Ca^{2+} mobilization and the basal Ca^{2+} levels in intact, non-excitable cells. We have shown that UFH dose-dependently and significantly reduces the intracellular Ca^{2+} levels at both the initiative and the plateau phase of Ca^{2+} influx after store depletion with either histamine or a SERCA pump inhibitor, thapsigargin (Figs. 2 and 3). Contrary to this, UFH does not act on the process of Ca^{2+} mobilization indicating that this agent exert its suppressive effect on the intracellular Ca^{2+} levels via inhibition of Ca^{2+} influx from the outside and it has no IP3 receptor antagonistic effect after extracellular usage (Figs. 1–3). The entry of the Ca^{2+} surrogates, Ba^{2+}, Sr^{2+} and Mn^{2+} were also suppressed by UFH after store depletion similarly to the Ca^{2+} influx experiments (Fig. 4A–C). The differences perceived in the spontaneous permeability of RBL-2H3 and HeLa cells to Ba^{2+}, Sr^{2+} and Mn^{2+} ions show that many different Ca^{2+} channels are present on these cells. The spontaneous Sr^{2+} influx was very low in these cells and it was not affected by UFH. However, a significant spontaneous Mn^{2+} and a relatively high Ba^{2+} inflow was observed in these cells which both were reduced by UFH. As the spontaneous Ba^{2+} influx was very high compared to that seen in store depleted cells, this Ba^{2+} influx seems to be almost completely independent of the capacitive Ca^{2+} entry and unrelated to SOCC activity. Inhibition of spontaneous Ba^{2+} and Mn^{2+} entry by UFH indicates that other non-capacitive Ca^{2+} channels are also affected by this agent and they may take part in the suppression of intracellular Ca^{2+} levels. High spontaneous Ba^{2+} entry has been found in unstimulated human platelets that was inhibitable by 2-APB, a SOCC blocker. This finding was interpreted by the authors as a result of the existence of SOCCs in a basal activity [27]. SOCCs are a heterogenic population of Ca^{2+} channels with distinct selectivity for the various cations as described in many investigations [24,25]. As indicated above, in our experiments, Sr^{2+} seemed to be the best Ca^{2+} surrogate for the examination on SOCCs because it showed low spontaneous entry in unstimulated cells and it was inducible by store depletion with thapsigargin or histamine, furthermore it had bidirectional movement. The inhibitory activity of UFH on the capacitive Ca^{2+} influx was reversible and seemed to be Ca^{2+} dependent similarly to a non-specific SOCC blocker, 2-APB, but the kinetics of their actions were different (Fig. 5A–C). Their usage in combination resulted in a non-additive interaction between these agents on the capacitive Ca^{2+} entry suggesting that their molecular targets may be only partly identical (Fig. 5D).

We have examined the bivalent cation chelating activity of UFH, which is a polysulphatated glycosaminoglycan molecule with multiple negative charges [3,4]. Experiments on ionomycin triggered Ca^{2+} entry after the first observation suggested that UFH similarly to EGTA, was a Ca^{2+} chelator, but interestingly, UFH inhibited also Ca^{2+} mobilization during addition of ionomycin (Fig. 6A). We would explain these findings that UFH can interact with ionomycin itself and it impedes the ionophoric effect of the latter. Contrary to results obtained from Triton X-100 triggered Ca^{2+} and other bivalent cation influx experiments, and a comparative study with an authentic Ca^{2+} chelator, EGTA suggested that UFH cannot be a strong cation chelator (Fig. 6B and C). Interestingly, UFH slowed down the process of Triton X-100 triggered Ca^{2+} and other bivalent cation inflow. It appears that the cell membrane becomes transiently resistant against the non-ionic detergent, Triton X-100 after UFH pretreatment indicating that this agent may have a membrane protective or stabilizing effect probably via its binding to the cell surface structures. Although we cannot completely exclude that UFH is able to capture bivalent cations in solutions to some extent, but its robust suppressive effect cannot be attributed to this property.

We have found that UFH can hyperpolarize the membrane both in untriggered cells and the thapsigargin induced cells (Fig. 7A and B). It has been revealed that 2-APB did not generate substantial changes in the membrane depolarization induced by thapsigargin. In the present study, the mechanism by which UFH evokes membrane hyperpolarization has not been examined further. With regard to the role of membrane hyperpolarization on Ca^{2+} influx upon store depletion, there is an apparent contradiction between the hyperpolarizing effect of UFH and its suppressive influence on the Ca^{2+} influx because the hyperpolarization can augment the electrochemical gradient that facilitates the Ca^{2+} movement into the cytoplasm when Ca^{2+} channels are open. Although, it is conceivable that the unusual combination of these bio-

Table 1
Multiple influences of extracellularly applied UFH on Ca^{2+} homeostasis in intact cells

Experimental findings	Likely target	Note
Suppression of Ca^{2+} and Sr^{2+} entry after store depletion in non-excitable cells (this study)	Capacitive Ca^{2+} entry via SOCCs	UFH's minor Ca^{2+} chelating activity may influence it; no direct evidence for binding of UFH to SOCCs
Suppression of spontaneous Ba^{2+} entry and Mn^{2+} quench in non-excitable cells (this study)	Other non-capacitive Ca^{2+} channels	UFH's minor Ca^{2+} chelating activity may influence it; no direct evidence for binding of UFH to non-capacitive Ca^{2+} channels
Membrane hyperpolarization in non-excitable cells (this study)	Different ion channels n the plasma membrane (SOCCs, other non-capacitive Ca^{2+}, K^+ or Cl^- channels?)	May be a cause of UFH-mediated suppression of capacitive and voltage operated Ca^{2+} channels
No significant suppression, but a delay of Triton-X-100 triggered bivalent cation influx in non-excitable cells (this study)	Ca^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+} ions in the extracellular buffer; plasma membrane	UFH's minor chelating activity cannot be completely excluded; UFH may have a transient membrane stabilizing effect
Suppression of voltage dependent Ca^{2+} current and Ba^{2+} current in myocytes [12]	L-type Ca^{2+} channels	UFH's minor Ca^{2+} chelating activity may influence it; direct evidence for binding of UFH to L-type Ca^{2+} channels [10,11]

chemical activities may be in close association because UFH shows both effects at the same concentration range. Many different anion or cation channels would take part in the maintenance of the membrane potential. Inwardly rectifying K^+ channel has an important role in setting the resting membrane potential to a normal value in mast cells and other cells as well [45]. K^+ efflux or Cl^- influx may lead to membrane hyperpolarization. Ca^{2+} activated Cl^- and K^+ channels have been also demonstrated in most cells [42,45], which are activated by the increase in intracellular Ca^{2+} during store depletion or after induction with Ca^{2+} ionophore. K^+ channel blockers can decrease the Ca^{2+} influx through SOCCs [42,43,46] because they depolarize the membrane and diminish the main driving force of Ca^{2+} entry. Furthermore, it has been reported that blocking of outwardly rectifying Cl^- channels inhibits the Ca^{2+} influx through SOCCs, and inversely store depletion mediated Ca^{2+} influx blocks this channel function [46]. Thus, a very complex link between the actions of SOCCs and other ion channels may exists, and probably it has various influences on the cell calcium homeostasis. According to many observations conducted in non-excitable cells like RBL-1 and Jurkat, it has generally been accepted that CRACs operated with store depletion are not voltage-dependent Ca^{2+} channels [24,25], although a voltage-dependent conductance change in Icrac was also demonstrated, where the amplitude of Icrac at hyperpolarized potentials was lower than that seen at depolarized potentials [44]. The mechanism of this hyperpolarization induced inhibition of CRACs is unknown, a protein has been hypothetized by the authors that binds to or unbinds to these Ca^{2+} channels depending on the actual membrane potential. Although, the background of the effect of UFH on the membrane potential is not clear, we presume that this hyperpolarization induced inhibition of SOCCs was observed in our experiments. It has been described previously that UFH inhibited the L-type Ca^{2+} channel function in myocytes [12]. Its action was reversible, fast, Na^+ and Mg^{2+} dependent but not Ca^{2+} dependent, in addition it was also capable of shifting the steady-state inactivation toward negative potentials. This latter finding also suggests that UFH might have a membrane hyperpolarizing effect.

Summarizing our results and findings reported by other authors, UFH might have multiple effects on Ca^{2+} homeostasis of intact cells (see on Table 1). These activities are apparently independent of its IP3 antagonistic effect when UFH is applied in an extracellular route. However, the direct binding of UFH molecules was not examined in our experiments and there is no evidence for its binding to SOCCs in the literature, but it has been published by many investigators that UFH is able to interact with lots of proteins located in the plasma membranes [4]. We propose that UFH indirectly act on these Ca^{2+} channels probably as a result of its interaction with other membrane proteins, which may have a regulatory function on SOCCs. However, we do not exclude the possibility that UFH can exert its Ca^{2+} chelating effect at the site of Ca^{2+} inflow near the mouth of Ca^{2+} channels in all cases. Altogether, direct relationship between the membrane hyperpolarizing character and the suppressive effect of UFH on SOCCs or the voltage operated L-type Ca^{2+} channels is ambiguous, and its biochemical background remaines an open question. Finally, we demonstrated that UFH and 2-APB represent two non-specific SOCC blockers with different mode of action. Effect of both agents may be mediated by diverse molecular targets reported by many authors [6,9,10–12,16,27,36–39]. 2-APB can exert its activity via direct interaction with SOCCs as described in many cells but the exact molecular mechanism is unclear [16,27,36–39].

By all means, a significant and long-lasting decrease in intracellular Ca^{2+} by blocking of SOCCs with any agents obviously leads to the loss of some cell function. Nevertheless, the physiological and pharmacological relevance of our findings is uncertain because of the high concentrations of UFH examined in our experiments. The therapeutic concentration of UFH in biological fluids has never been estimated for more than 1 IU/ml that corresponds to 6 μg/ml during anti-thrombotic therapy [47], but UFH may

be present in much higher concentrations at least locally, accumulated on cell membranes via its coupling to various proteins bearing heparin binding sites or on the surface fluid of the cells when it is administered, e.g. in inhalation therapy of asthma [48]. Many different types of TRP channel proteins have been partially identified on airways epithelial cells, lymphocytes, neutrophils, mast cells and smooth muscle cells in the lung [49]. It has been described that some of these channel proteins such as TRPC1, TRPC4 and TRPV6 are important components of CRACs functionating as SOCCs. Under these circumstances, the suppressive effect of UFH either on the capacitive Ca^{2+} entry via SOCCs or on other Ca^{2+} channels, present in many immuno-competent and airway structural cells might also contribute to its anti-inflammatory action in inhalation therapy of asthma.

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