Store-operated Ca^{2+}-channels are sensitive to changes in extracellular pH

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Abstract

The sensitivity of store-operated Ca^{2+}-entry to changes in the extra- and intracellular pH (pH_o and pH_i, respectively) was investigated in SH-SY5Y human neuroblastoma cells. The intracellular Ca^{2+}-stores were depleted either with 1 mM carbachol (CCH) or with 2 μM thapsigargin (TG). Extracellular acidification suppressed both the CCH- and TG-mediated Ca^{2+}-entry while external alkalinization augmented both the CCH- and the TG-induced Ca^{2+}-influx. Mn^{2+}-quenching experiments revealed that the rates of Ca^{2+}-entry at the thapsigargin- or carbachol-induced plateau were both accelerated at pH_o 8.2 and slowed down at pH_o 6.8 with respect to the control at pH_o 7.4. Alteration of pH_o between 6.8 and 8.2 did not have any significant prompt effect on pH_i and changes in pH_i left the CCH-induced Ca^{2+}-entry unaffected. These findings demonstrate that physiologically relevant changes in pH_o affect the store-operated Ca^{2+}-entry in SH-SY5Y cells and suggest that endogenous pH_o shifts may regulate cell activity in situ via modulating the store-operated Ca^{2+}-entry.
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Extracellular pH (pH_o) is a well-known modulator of plasma membrane ion channels [1–3]. Ca^{2+}-channels are a diverse class of ion channels. Interestingly, however, the different types of Ca^{2+}-channels examined to date appear to share a common pattern in terms of their responsiveness to changes in pH_o: external acidification has been found to impair the functioning of Ca^{2+}-channels in different cell types, e.g., tunicate egg cells [4], guinea pig ventricular myocytes [5], hybridoma cells [6], human mesenteric arterial cells [7], rat hippocampal CA1 neurons [8], whereas external alkalinization has been observed to stimulate them. In neural cells, pH_o is of particular importance as pH_o can affect neuronal activity and the effects of H^+ on neuronal activity have been attributed to the modulation of a variety of different neuronal ion channel types [8]. Also, dynamic changes occur during periods of physiolog-ical neuronal activity, such as synaptic activation [9]. Changes in pH_o also occur during pathological events falling to below pH 6.5 during ischemia [11,12] and rising by several tenths of pH units during seizure activity and spreading depression [10,13].

Store-operated Ca^{2+}-channels (SOCs) constitute a relatively new group of plasma membrane Ca^{2+}-channels. These channels open following depletion of intracellular (mainly ER or SR) Ca^{2+}-stores [14–17]. Depletion of these intracellular Ca^{2+}-stores is a part of the physiological sequence of events of the signal transduction cascade elicited by hormones [18] or neurotransmitters [19]. In addition, activation of SOCs can be achieved in vitro in a receptor-dependent process by muscarinic agonists like carbachol [20] and in a receptor-independent process by the Ca^{2+}-ATPase inhibitor thapsigargin [21]. SH-SY5Y human neuroblastoma cells have been found responsive to treatments with both carbachol [20] and thapsigargin [19,22], indicating that these cells possess SOCs. In our experiments, we wanted to test the sensitivity of SOCs to changes in pH_o

and to see whether their sensitivity shows a pattern similar to that described for other types of Ca^{2+}-channels.

Materials and methods

Cell culture and treatments. All chemicals were from Sigma except otherwise stated. SH-SY5Y human neuroblastoma cells were a kind gift from Dr. H. De Smedt (Laboratory of Physiology, K.U. Leuven, Leuven, Belgium). The cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM, from Gibco), supplemented with 10% (v/v) heat-inactivated fetal calf serum (from Invitrogen N.V., Merelbeke, Belgium), 4 mM L-glutamine, 4.5 mM glucose, 0.005 mM pyridoxine–HCl, 0.1 mM hypoxanthine, 400 nM aminopterin, 0.016 mM thymidine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere with 5% (v/v) CO_2. The cells were subcultured twice weekly. For fluorescence measurement, the cells were seeded on glass coverslips and were allowed to grow until at least 80% of the coverslip was populated with cells. This usually required 1–2 days. Then the culture medium was replaced by a physiological salt solution containing 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 20 mM TES, pH 7.4. The Ca^{2+}-free medium had the same composition but $CaCl_2$ was replaced by 1 mM EGTA. The coverslips with the cells were mounted as the bottom plate of a small bath and placed on the stage of an inverted epifluorescence microscope (TMD35; Nikon, Tokyo, Japan). For measurements of the cytoplasmic Ca^{2+}-concentration, the cells were loaded with 10 μM Fura-2 AM (from Molecular Probes, Eugene, Oregon, USA) for 30 min at 37 °C and were washed three times with the physiological saline prior to the fluorescence measurements. For assessing any changes in cytoplasmic pH, the cells were loaded with 10 μM 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, from Molecular Probes, Eugene, Oregon, USA) for 40 min at 37 °C and were washed three times with the physiological saline prior to the fluorescence measurements.

Fluorescence measurements. Fluorescence was elicited by illumination with a 100 W Xe lamp (Nikon). All optical filters and dichroic mirrors were obtained from Omega Optical (Brattleboro, Vermont, USA). To avoid bleaching of the probe and photodamage of the cells, the illumination was restricted to the periods when the images were taken. The excitation light was directed to the sample by a dichroic mirror and a Zeiss objective LD Achroplan (40×/0.6 corr.). The fluorescence collected by the objective was transmitted through the dichroic mirror and a bandpass emission filter to a Quantix CCD camera (Photometrics, Tucson, Arizona, USA). The exposure time for each image was 2 s. The signals were obtained by integrating spatially the pixels over the cells. The background image was automatically subtracted, pixel-by-pixel, from the image of the loaded cells. The fluorescence measurements were performed in dual excitation ratiometric mode at 37 °C. In the experiments with Fura-2 AM, the samples were excited at 340 and 380 nm (filters 340DF10 and 380DF13) and the emission was monitored at 510 nm (filter 510WB40). In the experiments with BCECF-AM, the samples were excited at 495 and 440 nm (filters 495DF20 and 440DF20) and the emission was monitored at 535 nm (filter 535DF25). Fluorescence excitation ratio value calibration in terms of absolute Ca^{2+} concentration was performed according to the protocol described by [23] (using 10 μM ionomycin, 2 mM EGTA, and 50 μM BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl)) ester). $[Ca^{2+}]_i$ was determined from the relationship $[Ca^{2+}] = K_d((R - R_{min})/(R_{max} - R))\beta$, where K_d (the dissociation constant for the Fura-2-Ca^{2+} complex) is taken to be 220 nM [23], R is the actual fluorescence excitation ratio F_{340}/F_{380}, R_{min}, and R_{max} are the minimal and maximal fluorescence excitation ratios measured in a nominally Ca^{2+}-free medium and in a medium with saturating Ca^{2+}-concentrations, respectively, and β is the ratio of the fluorescence intensities excited at 380 nm measured in a nominally Ca^{2+}-free medium and in a medium with saturating Ca^{2+}-concentration.

The Mn^{2+}-quenching experiments were performed in Ca^{2+}-containing media (1 mM $CaCl_2$) with 200 μM $MnCl_2$. The cells were challenged with thapsigargin or carbachol and when the plateau was attained, $MnCl_2$ was added to the cells and the fluorescence intensities at 510 nm, excited at 340 and 380 nm, respectively, were monitored over a time period of 5 min. The $[Ca^{2+}]_i$-independent fluorescence (F_i) values were calculated using the equation $F_i = F_{340} + \alpha F_{380}$ [24]. The values of α were determined as the absolute values of the slopes of the best fits of F_{340} versus F_{380} during the recovery following challenging the cells with either carbachol or thapsigargin. Even in the absence of the quencher, the data obtained for F_i exhibited a time-dependent decay, probably due to dye leakage and/or photobleaching. This initial decay was corrected for before applying $MnCl_2$ and the initial slopes of the quencher-induced fluorescence decay curves ($\Delta F/s$) were computed using the Microsoft Excel program LINEST.

For monitoring any changes in cytoplasmic pH (pH_i), the fluorescence excitation ratios (F_{495}/F_{440}) of the cells labeled with BCECF-AM were converted to pH_i values using the high-K^+/nigericin technique [25]. To do this, at the end of each experiment the physiological salt solution was consecutively replaced by media containing 140 mM KCl, 1 mM $CaCl_2$, 10 mM Hepes, and 13 μM nigericin, with pH of 6.0, 7.0, and 8.0, respectively, and the fluorescence excitation ratios were recorded. A calibration curve was drawn to express the fluorescence excitation ratios in terms of pH_i values. In the NH_4Cl-pulse experiments, the physiological salt solution was replaced by a solution containing 20 mM NH_4Cl which was an equimolar replacement for NaCl.

Statistical analysis of the data. The numerical results of the experiments are expressed as means ± SD (standard deviation) throughout the manuscript, with "n" referring to the number of experiments, i.e., the number of coverslips, upon which individual measurements were made. Independent data sets were compared by using Student's t test. Reported data marked with "∗∗∗" are significantly different at $p < 0.01$.

Results

Effect of changes in pH_o on the carbachol-induced Ca^{2+}-signal in SH-SY5Y cells

Challenge of SH-SY5Y cells with 1 mM carbachol brought about a biphasic pattern of the Ca^{2+}-signal, starting with a sharp rise and followed by a plateau. The $[Ca^{2+}]_i$ in these cells was found to be 92 ± 11 nM ($n = 45$). The $[Ca^{2+}]_i$ level at the carbachol-induced peak was 835 ± 48 nM ($n = 32$), whereas that of the plateau was 223 ± 14 nM ($n = 32$). The trace of a representative experiment is shown in Fig. 1. Once a stable level of plateau was established, the pH_o was altered by changing the medium of the cells with a Hepes-buffered saline solution of the same ionic composition except for the pH, still in the presence of 1 mM carbachol. Decreasing the pH_o to 6.8 induced a fast (complete in ≈1 min) decrease in the plateau value to 98 ± 7 nM ($n = 20$), which was fully reversible in ≈1 min to 219 ± 11 nM, $n = 20$, upon setting pH_o back to 7.4 (Fig. 1). On the other hand, raising the pH_o to 8.2 induced a slower (complete in ≈2 min) increase in the plateau to 406 ± 22 nM ($n = 25$), which recovered to 233 ± 18 nM ($n = 25$) in about 2 min upon setting pH_o back to 7.4 (Fig. 1).

The same experiments were repeated in nominally Ca^{2+}-free media. Under these conditions the basal $[Ca^{2+}]_i$ was found to be 63 ± 6 nM ($n = 20$). Addition of 1 mM carbachol to the cells at pH 7.4 brought about a sharp rise to 374 ± 16 nM ($n = 12$) followed by a recovery to 69 ± 8 nM ($n = 12$). In Ca^{2+}-free media there was no plateau and the fluorescence fully recovered to the basal value.
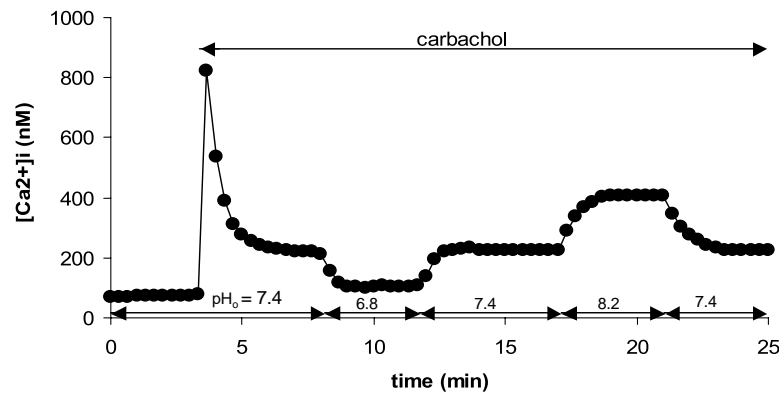
Fig. 1. Representative experiment of the effect of pH_o on the carbachol-mediated Ca^{2+}-plateau in SH-SY5Y cells in the presence of 1 mM $[Ca^{2+}]_o$. The cells were challenged with 1 mM carbachol at pH_o 7.4. After the first peak of the Ca^{2+}-signal was completed and a steady-state plateau was attained, changes of the external medium of the cells were carried out in Hepes-buffered media of identical composition, except for the pH values indicated. Carbachol (1 mM) was present throughout the whole experiment.

We also studied the pH_o-dependence of the recovery from the Ca^{2+}-peak and found no significant difference in the rate of recovery when pH_o was varied between 6.8 and 8.2 (data not shown). Once a stable level of $[Ca^{2+}]_i$ was established, the pH_o was altered by changing the medium of the cells with a Hepes-buffered saline solution of the same ionic composition except for the pH, still in the presence of 1 mM carbachol. No detectable change in $[Ca^{2+}]_i$ was observed at any pH_o investigated (data not shown).

The pH_o-sensitivity of the carbachol-induced Ca^{2+}-peak in the presence of external Ca^{2+} was also studied. As compared to the pattern obtained at pH_o 7.4 (see also Fig. 1), external acidification did not significantly affect the value of the Ca^{2+}-peak (835 ± 48 nM ($n = 32$) at pH_o 7.4; 828 ± 17 nM ($n = 7$) at pH_o 6.8). However, it affected the rate and extent of recovery as $[Ca^{2+}]_i$ fully recovered in 1 min to a level of 97 ± 8 nM ($n = 7$) which was indistinguishable from the basal $[Ca^{2+}]_i$ (i.e., the plateau was completely eliminated). Representative experiments are shown in Fig. 2. External alkalinization raised the basal $[Ca^{2+}]_i$ to 188 ± 17 nM ($n = 7$), and it raised the carbachol-induced Ca^{2+}-peak to 942 ± 34 nM ($n = 7$), so the relative magnitude of the peak remained practically unchanged (Fig. 2). Additional experiments revealed that lowering of the pH_o decreased $[Ca^{2+}]_i$ at the carbachol-induced plateau from 222 ± 11 nM (at pH_o 7.4) to $156 \pm 6^{***}$ nM (at pH_o 7.2), $130^{***} \pm 5$ nM (at pH_o 7.0), and $103^{***} \pm 6$ nM (at pH_o 6.8) (Fig. 3). On the other hand, increasing of the pH_o increased $[Ca^{2+}]_i$ at the carbachol-induced plateau from 224 ± 13 nM (at pH_o 7.4) to $276 \pm 14^{***}$ nM (at pH_o 7.6), $331 \pm 12^{***}$ nM (at pH_o 7.9), and $407 \pm 15^{***}$ nM (at pH_o 8.2, Fig. 4).

Effect of changes in pH_o on the thapsigargin-induced Ca^{2+}-transient in SH-SY5Y cells

In the absence of extracellular Ca^{2+} 2 μM thapsigargin induced a fast (complete in ≈ 20 s) increase in $[Ca^{2+}]_i$ from 65 ± 7 nM ($n = 12$) to 152 ± 11 nM ($n = 12$), which proved
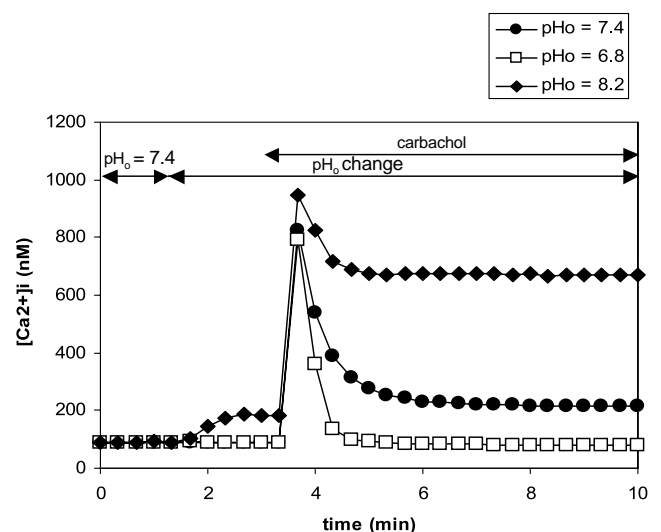

Fig. 2. Traces of representative experiments of the effect of pH_o on the carbachol-induced Ca^{2+}-peak in SH-SY5Y cells in the presence of 1 mM $[Ca^{2+}]_o$. First, the external medium of the cells was changed to Hepes-buffered media of identical composition. Then the cells were challenged with 1 mM carbachol and the pattern of the Ca^{2+}-peak was recorded in the continued presence of 1 mM carbachol.

to be transient in nature and the $[Ca^{2+}]_i$ was restored to 67 ± 9 nM ($n = 12$) in about 3 min. A representative experiment is shown in Fig. 5. Re-addition of 1 mM $[Ca^{2+}]_o$ in the continued presence of 2 μM thapsigargin raised $[Ca^{2+}]_i$ first to 214 ± 10 nM ($n = 12$) in 60–80 s, followed by a decline to a stable plateau of 141 ± 8 nM ($n = 12$, Fig. 5). Once this stable level of plateau was reached, changes in pH_o to 6.8 or 8.2 were induced. These changes significantly altered the level of the plateau. Alkaline external pH (pH_o 8.2) induced a significant increase of $[Ca^{2+}]_i$ to 217 ± 8 nM*** ($n = 8$) in 2 min whereas acidic external pH (pH_o 6.8) caused a marked decrease of $[Ca^{2+}]_i$ to $69 \pm 9^{***}$ nM ($n = 8$) in about 4 min (Fig. 5).

Similar results were obtained in the continued presence of 1 mM $[Ca^{2+}]_o$. In this case, the addition of 2 μM thapsigargin at pH_o 7.4 induced a slow rise in $[Ca^{2+}]_i$ from
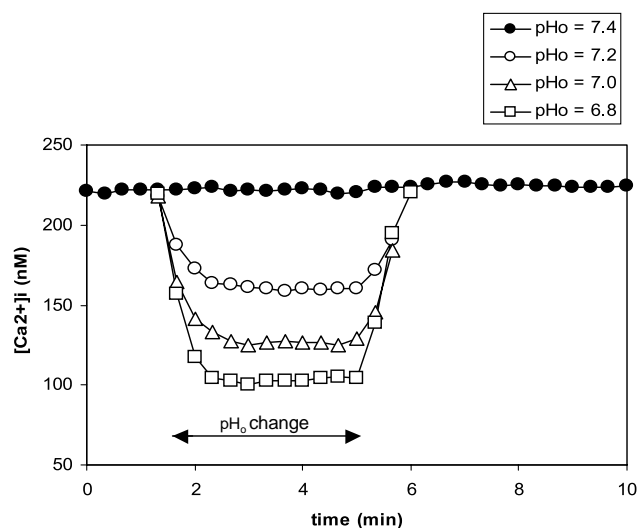
Fig. 3. Traces of representative experiments of the effect of decreasing pH_o on the carbachol-mediated Ca^{2+}-plateau in SH-SY5Y cells ($n = 5$). The cells were challenged with 1 mM carbachol at pH_o 7.4. After the first peak of the Ca^{2+}-signal was completed and a steady-state plateau was attained, the external medium of the cells was changed to Hepes-buffered media of identical composition, except for the pH values indicated. Carbachol (1 mM) was present throughout the whole experiment.
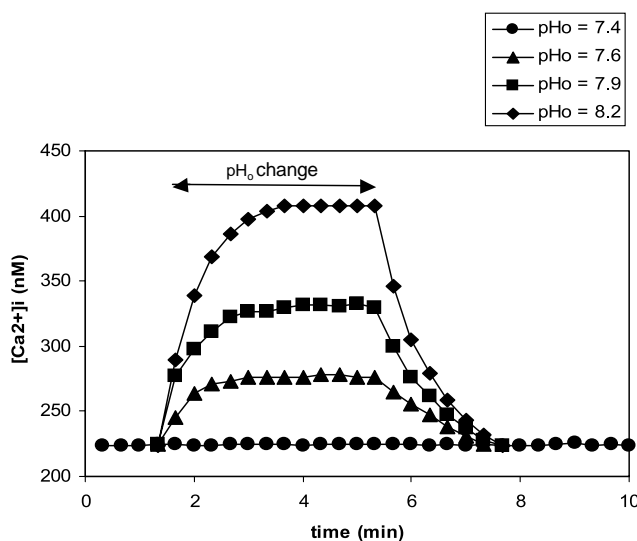

Fig. 4. Traces of representative experiments of the effect of increasing pH_o on the carbachol-mediated Ca^{2+}-plateau in SH-SY5Y cells ($n = 5$). The cells were challenged with 1 mM carbachol at pH_o 7.4. After the first peak of the Ca^{2+}-signal was completed and a steady-state plateau was attained, the external medium of the cells was changed to Hepes-buffered media of identical composition, except for the pH values indicated. Carbachol (1 mM) was present throughout the whole experiment.

Fig. 5. Representative experiment of the effect of pH_o on the thapsigargin-mediated Ca^{2+}-plateau in SH-SY5Y cells in a nominally Ca^{2+}-free medium. The cells were challenged with 2 μM thapsigargin at pH_o 7.4 in the absence of extracellular Ca^{2+} and in the presence of 1 mM EGTA. After the first peak of the Ca^{2+}-signal was completed, 1 mM $[Ca^{2+}]$ was introduced into the extracellular medium of the cells. This led to a second wave of Ca^{2+}-signal comprising a peak and a plateau. When this steady-state plateau was attained, the external medium of the cells was changed to Hepes-buffered media of identical composition, except for the pH values indicated. Thapsigargin (2 μM) was present throughout the whole experiment.
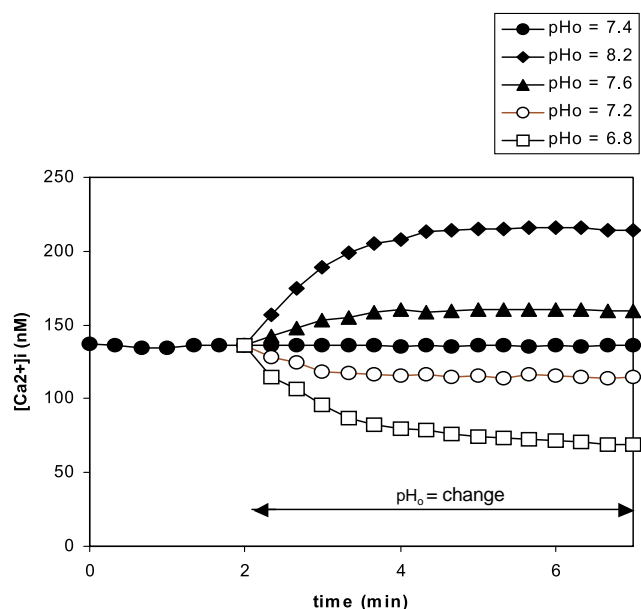
Fig. 6. Effect of pH_o on the thapsigargin-mediated Ca^{2+}-plateau in SH-SY5Y cells ($n = 5$). The cells were challenged with 2 μM thapsigargin at pH_o 7.4 in a nominally Ca^{2+}-free medium but in the presence of 1 mM EGTA. After the first peak of the Ca^{2+}-signal was completed, 1 mM $[Ca^{2+}]$ was reintroduced into the extracellular medium of the cells. This led to a second wave of Ca^{2+}-signal comprising a peak and a plateau. When this steady-state plateau was attained, the external medium of the cells was changed to Hepes-buffered media of identical composition, except for the pH values indicated. Thapsigargin (2 μM) was present throughout the whole experiment.

71 ± 8 nM ($n = 7$) to 146 ± 10 nM ($n = 7$). Changes in pH_o to 6.8 or 8.2 significantly altered the level of the plateau. Alkaline external pH (pH_o 8.2) induced a significant increase of $[Ca^{2+}]_i$ to $234 \pm 10^{***}$ nM ($n = 5$) in 2 min whereas acidic external pH (pH_o 6.8) caused a marked decrease of $[Ca^{2+}]_i$ to $65 \pm 8^{***}$ nM ($n = 5$) in about 2 min (data not shown). Additional experiments revealed that consecutive changes
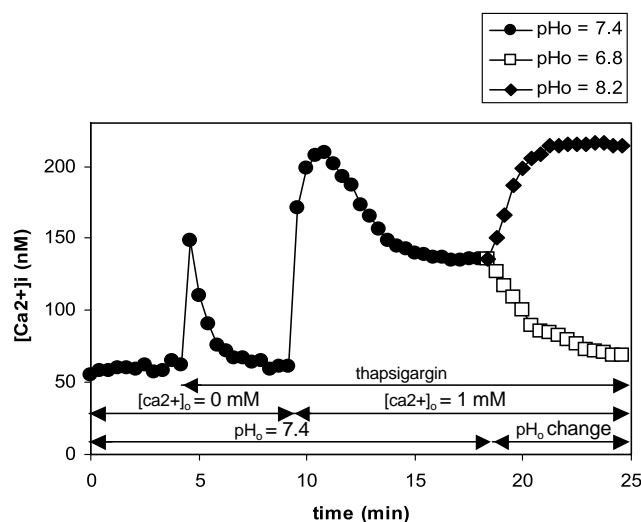
in pH_o induced a change in $[Ca^{2+}]_i$ at the thapsigargin-induced Ca^{2+} plateau from 134 ± 8 (at pH_o 7.4) to $214 \pm 10^{***}$ (at pH_o 8.2), $160 \pm 7^{***}$ (at pH_o 7.6), $115 \pm 9^{***}$ (at pH_o 7.2), and $71 \pm 6^{***}$ (at pH_o 6.8, Fig. 6).

Effect of pH_o on the rate of Ca^{2+}-entry as revealed by Mn^{2+}-quenching experiments

In order to demonstrate that changes in $[Ca^{2+}]_i$ during the plateau phase are indeed—at least partially—due to Ca^{2+} entry, and that the Ca^{2+} entry shows pH-dependence, we performed Mn^{2+}-quenching experiments at various pH_o values. As a measure of the fluorescence quenching, we computed the initial slopes of the decay curves of the calcium-insensitive fluorescence (F_i). F_i values were determined as described in Materials and methods. These values indeed proved to be insensitive to even significant changes in $[Ca^{2+}]_i$, e.g., when the cells were challenged by 1 mM carbachol (Fig. 7). The rate of calcium-independent fluorescence (F_i) quenching induced by Mn^{2+} (Fig. 8) was found
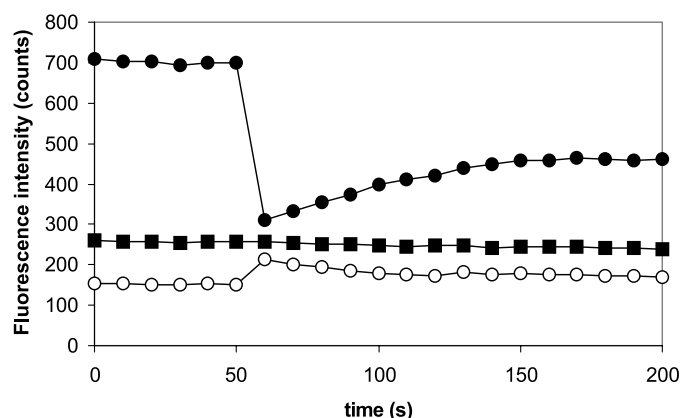
Table 1
Initial slopes ($\Delta F/s$) of the calcium-independent fluorescence (F_i) decay curves of Fura-2 quenched by Mn^{2+} following treatment of the cells with 2 μM thapsigargin or 1 mM carbachol at various pH_o

Treatment	pH_o	$\Delta F_i/s$[a,b]
Thapsigargin	6.8	$-0.22 \pm 0.02^{***}$
Thapsigargin	7.4	-0.43 ± 0.02
Thapsigargin	8.2	$-0.71 \pm 0.07^{***}$
Carbachol	6.8	$-0.32 \pm 0.03^{***}$
Carbachol	7.4	-0.53 ± 0.04
Carbachol	8.2	$-0.85 \pm 0.08^{***}$

[a] Mean $\pm$ SD, $n = 3$.
[b] The expression for F_i is given in Materials and methods.
*** Significantly different from the control (pH_o 7.4) ($p < 0.01$ by Student's t test).

to depend on pH_o for both thaspigargin and carbachol (Table 1).

Effect of changes in pH_i on Ca^{2+}-entry

pH_i might play a role in the observed effects. First of all we investigated whether the changes applied in pH_o affected pH_i. In the pH_o range used in the course of the experiments (between 6.8 and 8.2), change in pH_o had a very slight effect on pH_i. The basal pH_i of SH-SY5Y cells at 37 °C was found to be 6.91 ± 0.06 ($n = 5$). Prolonged (20-min) exposure to external media with pH_o 6.8 and 8.2, respectively, yielded pH_i 6.90 ± 0.05 ($n = 5$) and 6.93 ± 0.04 ($n = 5$, data not shown), respectively.

Second, the effect of sudden changes in pH_i on the level of the plateau of the carbachol-induced Ca^{2+}-signal was investigated by exposing the cells to 20 mM NH_4Cl to induce cytoplasmic alkalinization. After about 5 min, the NH_4Cl was washed out to induce cytoplasmic acidification. As seen in Fig. 9, neither alkalinization nor acidification of the cytoplasm caused any significant change in the carbachol-induced plateau, suggesting that the Ca^{2+}-entry channels studied exhibit no expressed sensitivity to changes in pH_i in these cells. In order to see whether pH_i was indeed altered by the NH_4Cl load and washout method, the effect of addition and removal of 20 mM NH_4Cl on pH_i of the cells was measured with BCECF-AM. As seen in Fig. 10, NH_4Cl load indeed brought about a significant cytoplasmic alkalinization raising pH_i to $7.82 \pm 0.11^{***}$ ($n = 5$) and the following NH_4Cl washout induced a marked cytoplasmic acidification reducing pH_i to $6.31 \pm 0.15^{***}$ ($n = 5$).

Discussion

In this paper, we describe that Ca^{2+}-entry seems to be responsible for the carbachol-induced sustained Ca^{2+}-plateau in SH-SY5Y cells and for the thapsigargin-induced increase in $[Ca^{2+}]_i$. We suggest that Ca^{2+}-entry occurs through SOCs and this Ca^{2+}-entry is sensitive to changes

Fig. 7. Effect of 1 mM carbachol on the values of F_{380} (filled circles), F_{340} (open circles), and F_i (filled squares). F_i was calculated as described in Materials and methods.
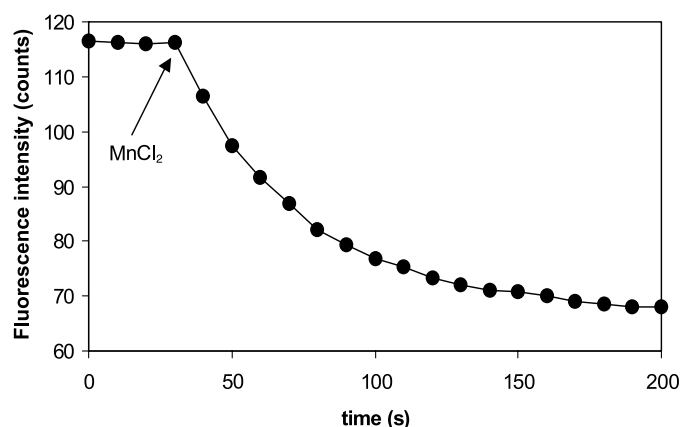

Fig. 8. Effect of 200 μM $MnCl_2$ on F_i. F_i was calculated as described in Materials and methods.
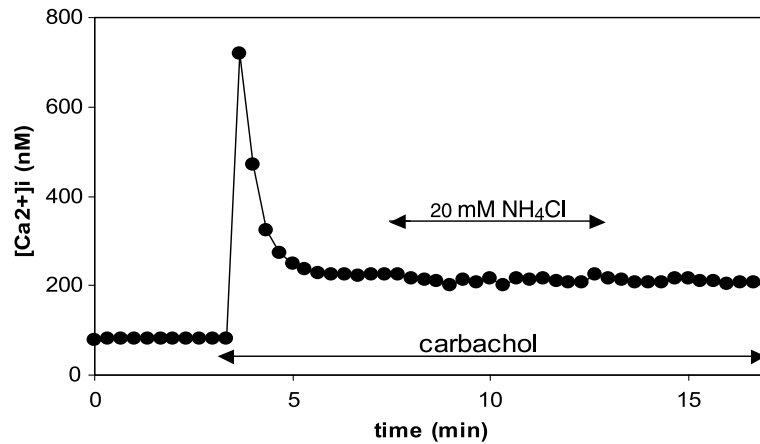
Fig. 9. Representative experiment of the effect of changes in pH_i (addition and removal of 20 mM NH_4Cl) on the carbachol-mediated Ca^{2+}-plateau in SH-SY5Y cells. The cells were challenged with 1 mM carbachol at pH_o 7.4. After the first peak of the Ca^{2+}-signal was completed and a steady-state plateau was attained, 20 mM NH_4Cl was added to the cells to induce intracellular alkalinization. After about 5 min, the medium was changed to NH_4Cl-free medium to induce intracellular acidification. Carbachol (1 mM) was present throughout the whole experiment.
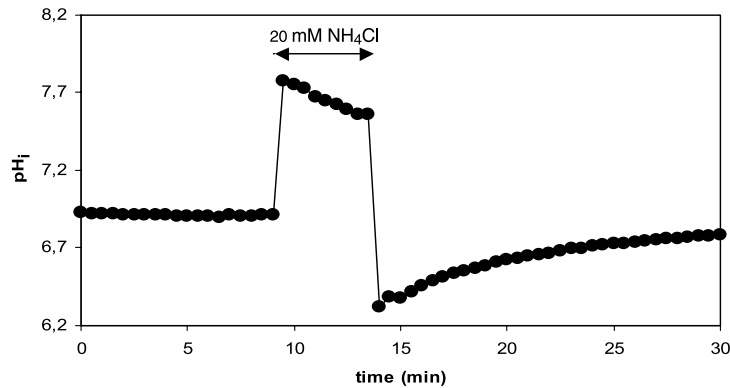

Fig. 10. Representative experiment of the effect of the addition and removal of 20 mM NH_4Cl on intracellular pH (pH_i) in SH-SY5Y cells loaded with 10 μM BCECF-AM. The cells were challenged with 20 mM NH_4Cl to induce intracellular alkalinization. After about 5 min, the medium was changed to NH_4Cl-free medium to induce intracellular acidification.

in extracellular pH. Our results are consistent with earlier observations that the carbachol-induced plateau requires the previous depletion of the intracellular Ca^{2+}-stores, which is evident by the significant rise in $[Ca^{2+}]_i$ ("first" Ca^{2+}-peak) upon binding carbachol to its receptor. Similarly, we have seen that the plateau requires the presence of Ca^{2+} in the extracellular space, so it is probably associated with plasma membrane Ca^{2+}-channels. The Ca^{2+}-channels responsible for the Ca^{2+}-entry following store-depletion (sometimes referred to as "capacitative" Ca^{2+}-entry), e.g., [16,17] have been designated store-operated Ca^{2+}-channels (SOCs, e.g., [26]). Binding of carbachol to its receptors, however, activates Ca^{2+}-entry via various receptor-associated mechanisms of which receptor-operated Ca^{2+}-channels (ROCs) should be taken into consideration. Therefore, we attempted to activate SOCs with thapsigargin, which bypasses the receptor-operated events and activates SOCs by inhibiting ER Ca^{2+}-ATPase [21]. The fact that the Ca^{2+}-channels activated by

both agents (carbachol and thapsigargin) showed a similar pattern of pH_o-dependence in our experiments serves as a strong argument to claim that we have been dealing with SOCs.

The main conclusion of our results is therefore that SOCs in SH-SY5Y cells are sensitive to physiologically relevant changes in pH_o. The arguments supporting this conclusion are the following: (1) the magnitude of the carbachol-associated plateau exhibited profound sensitivity to pH_o, whereas the first carbachol-induced Ca^{2+}-peak proved to be relatively insensitive to changes in pH_o. (2) The plateau was absent in Ca^{2+}-free media at any pH_o value investigated. (3) The thapsigargin-induced Ca^{2+}-entry exhibited a similar pH_o-sensitivity as the carbachol-induced plateau. (4) The rates of Ca^{2+}-entry at the plateau induced by both thapsigargin and carbachol, investigated by Mn^{2+}-quenching experiments, were found to be significantly higher at pH_o 8.2 and lower at pH_o 6.8 than at pH_o 7.4.

Agonist-induced entry of Ca^{2+} into cells often exhibits a biphasic pattern: binding of the agonist to its cell surface receptor initiates a sharp rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a recovery to a level which is higher than that of the original level and is therefore referred to as a "plateau." This pattern of Ca^{2+}-signal has been observed in a variety of different cell types (both excitable and non-excitable cells) following stimulation with a variety of agents, e.g., endothelin-1 in aortic smooth muscle cells [18], thrombin in platelets [27], or activation of muscarinic acetylcholine receptors in neuroblastoma cells with carbachol [20,28]. It has been established that the sharp rise is attributed to the opening of inositol-1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+}-channels on the endoplasmic reticulum [24] membrane, whereas the plateau is the result of the opening of SOCs present in the plasma membrane [29–31]. The features we have observed are consistent with the characteristics of SOCs, which have been described in a variety of different cell types including excitable and non-excitable cells [14–16,32]. On the other hand, thapsigargin has been shown to activate SOCs [21].

The carbachol-induced plateau can be considered as a new steady state of $[Ca^{2+}]_i$ in which the interplay between the Ca^{2+}-entry (e.g., SOCs) and the Ca^{2+}-extruding mechanisms attained a new equilibrium. In excitable cells, one of the principal Ca^{2+}-extruding mechanisms is the Na^+/Ca^{2+} exchanger. It is well documented that the Na^+/Ca^{2+} exchanger is very sensitive to changes in pH_i, which can explain why this exchanger is dramatically down-regulated during ischemic conditions [33]. However, its sensitivity to pH_o is controversial and appears to be dependent on the cell type. Both moderate external acidification and alkalinization reduced the rate of Na^+/Ca^{2+} exchange in guinea pig ventricular myocytes [34]. If a similar effect had been exerted on the Na^+/Ca^{2+} exchange in SH-SY5Y cells, then the plateau would have been increased in acidic pH_o as the rate of Ca^{2+}-efflux is lower. We observed the opposite. On the other hand, in MDCK cells high pH_o stimulated the Na^+-H^+ antiport, and the subsequent rise in $[Na^+]_i$ decreased the Na^+ electrochemical potential, thereby activating the reverse mode of Na^+/Ca^{2+} exchange (Ca^{2+} influx vs. Na^+ efflux) which led to the rise in $[Ca^{2+}]_i$ [35]. If this had been the case in SH-SY5Y cells, we would have been able to detect intracellular alkalinization at high pH_o due to the activation of the Na^+-H^+ antiport. However, no significant change in pH_i was detectable following exposure of the cells to pH_o values ranging from 6.8 to 8.2. This leaves the possibility that external alkalinization might reduce the rate of Na^+/Ca^{2+} exchange in SH-SY5Y cells, which could contribute to the observed elevation of the plateau. However, in neural cells, at least in dialyzed squid axons, the rate of Na^+/Ca^{2+} exchange was found to be insensitive to external alkalinization between 7.3 and 9.0 [36].

Another important Ca^{2+}-extruding mechanism is the plasma membrane Ca^{2+}-ATPase, which is known to be a Ca^{2+}/H^+ exchanger [33] and mediates either electrogenic [34] or electroneutral [35] Ca^{2+}/H^+ exchange and has also been shown to be sensitive to changes in both pH_i and pH_o [36]. Our observation that raising of pH_o induced an increase in $[Ca^{2+}]_i$ in untreated cells (Fig. 2) might be explained by the inhibition of the plasma membrane Ca^{2+}-ATPase by pH_o 8.2 and therefore we cannot rule out the participation of Ca^{2+}/H^+ exchanger in establishing the new equilibrium between Ca^{2+}-entry and -efflux characteristic of the plateau phase. However, we have several arguments, which favor the idea that this exchange mechanism is probably of minor significance with respect to our results. (1) We did not see any change in $[Ca^{2+}]_i$ when pH_o was lowered to 6.8 (Fig. 2). Under these conditions the Ca^{2+}-ATPase is stimulated and this should have resulted in lower $[Ca^{2+}]_i$. (2) Although an increase in pH_o caused an increase in steady-state $[Ca^{2+}]_i$ in Ca^{2+}-containing media (Fig. 2), we did not see any similar increase in Ca^{2+}-free media, suggesting that the pH-elevation-induced Ca^{2+}-rise could be the consequence of stimulation of the entry rather than inhibition of the efflux. (3) The rate of recovery following the carbachol-induced Ca^{2+}-peak in Ca^{2+}-free media did not show any pH_o-dependence. If the Ca^{2+}-ATPase was playing an important role, then the rate of recovery should be faster at pH_o 6.8 and slower at pH_o 8.2. (4) We demonstrated that in SH-SY5Y cells neither the intracellular acidification nor intracellular alkalinization (brought about by NH_{4+}-pulse experiments) affected $[Ca^{2+}]_i$ (Figs. 9 and 10). If the the Ca^{2+}-ATPase was playing an important role in these cells, its rate should be sensitive to changes in pH_i and consequently $[Ca^{2+}]_i$ should change. The effect of thapsigargin-sensitive sarco/endoplasmic reticulum Ca^{2+}-pumps in the removal of cytoplasmic Ca^{2+} under the challenges considered in this study is minimal as this removal is essentially the same in the presence of thapsigargin.

SOCs were originally described in non-excitable cells and were regarded as one of the possible ways whereby these cells, which, unlike excitable cells, lack voltage-gated Ca^{2+}-channels, can mediate Ca^{2+}-entry from the extracellular environment [14,16]. Contrary to the previous expectations, however, neural cells [37], astrocytes [38,39] or cells that have innervation and respond to neural stimuli, e.g., pancreatic acinar cells [40], also exhibit store-operated Ca^{2+}-entry. In these cells, voltage-gated and store-operated Ca^{2+}-channels apparently co-exist, rendering them capable of carrying out a fine-tuned regulation of cytoplasmic Ca^{2+} homeostasis.

In this report, we provide evidence suggesting that SOCs are present in human neuroblastoma cells and they are sensitive to physiologically relevant changes in pH_o. This strong pH_o-sensitivity might be of physiological significance as it can be directly or indirectly associated with

pathogenic factors that induce cellular degeneration in the nervous system.

Acknowledgment

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