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Extracellular pH Determines the Rate of Ca^{2+} Entry into Madin-Darby Canine Kidney-Focus Cells

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Abstract. We investigated the relationship between intracellular Ca^{2+} and pH homeostasis in Madin-Darby canine kidney-focus (MDCK-F) cells, a cell line exhibiting spontaneous oscillations of intracellular Ca^{2+} concentration (Ca^{2+}_i). Ca^{2+}_i and intracellular pH (pH_i) were measured with the fluorescent dyes Fura-2 and BCECF by means of video imaging techniques. Ca^{2+} influx from the extracellular space into the cell was determined with the Mn^{2+} quenching technique. Cells were superfused with HEPES-buffered solutions. Under control conditions (pH 7.2), spontaneous Ca^{2+}_i oscillations were observed in virtually all cells investigated. Successive alkalinization and acidification of the cytoplasm induced by an ammonia ion prepulse had no apparent effect on Ca^{2+}_i oscillations. On the contrary, changes of extracellular pH value strongly affected Ca^{2+}_i oscillations. Extracellular alkalinization to pH 7.6 completely suppressed oscillations, whereas extracellular acidification to pH 6.8 decreased their frequency by 40%. Under the same conditions, the respective pH_i changes were less than 0.1 pH units. However, experiments with the Mn^{2+} quenching technique revealed that extracellular alkalinization significantly reduced Ca^{2+} entry from the extracellular space. Large increases of Ca^{2+}_i triggered by the blocker of the cytoplasmic Ca^{2+}-ATPase, thapsigargin, had no effect on pH_i. We conclude: intracellular Ca^{2+} homeostasis in MDCK-F cells is pH dependent. pH controls Ca^{2+} homeostasis mainly by effects on the level of Ca^{2+} entry across the plasma membrane. On the contrary, the intracellular pH value seems to be insensitive to rapid changes of Ca^{2+}_i.

Key words: MDCK-F cells — Ca^{2+} entry — pH — Video imaging — Oscillations

Introduction

Both intracellular Ca^{2+} ions and intracellular H^+ ions are widely believed to act as second messengers in response to cell stimulation with a variety of physiological stimuli. With respect to intracellular Ca^{2+}, cell stimulation often leads to a Ca^{2+} release from intracellular Ca^{2+} store(s), and/or to Ca^{2+} entry into the cytoplasm from the extracellular space [1, 2, 17]. The subsequent Ca^{2+} increase modifies such diverse cellular functions as cell contraction [29], cell cycle events [15] and gene expression [11]. On the other hand, numerous hormones and growth factors are known to alter the intracellular pH value. Changes of intracellular pH value are most often due to alteration of the activity of the Na^+/H^+ exchanger, which is a common transport protein regulating the intracellular pH value [5, 9, 10, 12, 13, 16, 22]. Similar to intracellular Ca^{2+} concentration, intracellular pH controls the activity of different cellular processes. Among others, cytoplasmic alkalinization is a prerequisite or an associated event for the stimulation of transepithelial reabsorption [13], platelet stimulation [22] and gene expression [5]. Moreover, intracellular alkalinization is frequently linked to the tumor cell phenotype [3, 12, 19].

Several lines of evidence suggest that intracellular pH value and Ca^{2+} concentration may be related to

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each other. So far, no general model of relationship between Ca^{2+} and pH can be derived from the available literature. Indeed, intracellular Ca^{2+} was reported both to alter and to have no effect on intracellular pH [25, 32]. On the other hand, intracellular Ca^{2+} responses could be evoked either by cell acidification or cell alkalinization [26, 27, 32]. Obviously, this diversity of cellular Ca^{2+} and pH interactions reflects different cell functions which are specific to a given cell type. Nevertheless, there is established evidence that alteration of intracellular or extracellular pH changes the activity of Ca^{2+} pumps in the plasma membrane and in the endoplasmic reticulum [6]. Moreover, the open probability of Ca^{2+} channels [20] and generation of Ca^{2+}-releasing second messenger $InsP_3$ have been reported to be dependent on the proton concentration [24]. The effect of intracellular Ca^{2+} concentration changes on intracellular pH value may be mediated by a Ca^{2+}-dependent protein kinase C which controls the activity of the Na^+/H^+ exchanger. The exchanger activity is also dependent on the second messenger diacylglycerol which is released in parallel with the Ca^{2+}-releasing second messenger $InsP_3$ following the activation of the phosphoinositide pathway [7]. Thus, activation of this pathway may modulate both intracellular Ca^{2+} concentration and the cytoplasmic pH value.

In this study, we investigated the relationships between Ca^{2+} and pH homeostasis in MDCK-F cells [14]. MDCK-F cells are a novel cell line exhibiting spontaneous oscillations of intracellular Ca^{2+} concentration which are generated in a cytoplasmic, $InsP_3$- and thapsigargin-sensitive Ca^{2+} store [30]. Cytoplasmic Ca^{2+} and proton concentrations were monitored with Fura-2 and BCECF video imaging, and Ca^{2+} entry from the extracellular space was monitored by means of the Mn^{2+} quenching technique. We present evidence that the activity of the intracellular Ca^{2+} oscillator is dependent on extracellular rather than on intracellular pH value. Thus, pH controls Ca^{2+} homeostasis at the level of Ca^{2+} entry across the plasma membrane.

Materials and Methods

Cell Culture

Transformed MDCK cells (MDCK-F cells, registered at the American Type Culture Collection, Bethesda, MD) [14] were grown in poly-L-lysine coated plastic culture dishes in Minimum Essential Medium with Earle's salts, nonessential amino-acids and L-glutaminic acid (MEM-medium, Biochrom, Berlin, FRG). The MEM-medium was supplemented with 10% fetal calf serum (FCS) and 26 mmol/liter $NaHCO_3$ (Biochrom). Cells were grown at 37°C in a 5% CO_2 in air, humidified atmosphere.

Intracellular Ca^{2+} and pH Imaging

Cells grown on poly-L-lysine-coated glass coverslips were loaded for 30 min with the Ca^{2+}-sensitive dye Fura-2 (2 μmol/liter) or with the pH-sensitive dye BCECF (1 μmol/liter). Dye loading resulted in apparently uniform distribution of fluorescence in the cytoplasm. Loaded cells were mounted into a heating chamber thermostatically controlled at 37°C. The chamber was installed on a Nikon Diaphot microscope equipped with a 40× quartz oil immersion objective operating in epifluorescence mode. The volume of the buffer in the chamber was 1,000 μl. Buffer changes and drug applications were performed by simple pipetting of 2–5 ml of the appropriate, preheated solution into the incubation chamber. The solution excess was sucked via a suction pipette mounted over the chamber. The buffer used was a standard Ringer solution containing (in mmol/liter): 122 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.0 Na_2HPO_4/NaH_2PO_4, 1.2 $CaCl_2$, 5.5 glucose, 10 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); adjusted to various pH values (*see below*) with NaOH.

Dynamic video imaging of intracellular Ca^{2+} concentration and of intracellular pH was carried out with MagiCal hardware and TARDIS software (Applied Imaging, Sunderland, UK). Cells were imaged with an intensified CCD camera operating at video frame rate (Photonics Science, Robertsbridge, UK). In experiments with Fura-2, the cells were illuminated alternatively at 340 and 380 nm by means of a rotating filter wheel and the emitted light was collected through a dichroic mirror at 510 nm. For pH measurements with BCECF, dye-loaded cells were alternatively excited at 440 and 490 nm, and the emitted light was collected through a 520-nm-long pass filter. The filter wheel was controlled by the computer. Video frames were averaged four to eight times with an analog hardware averager to reduce noise from the camera.

Background subtraction was performed on-line in all experiments before ratioing operations were carried out. Background fluorescence after excitation at 340 and 380 nm (Fura-2) or 440 and 490 nm (BCECF) was determined in a visual field with no cells. Using the wavelengths of excitation and emission noted here and with similar gain settings of the camera and intensifier, no autofluorescence was detected from cells that had not been loaded with dye.

After background subtraction, 340/380 or 440/490 ratios were calculated on average video frames on a pixel-by-pixel basis. Calculated ratios were computed against look-up tables to yield a ratio grey level representative of calibrated Ca^{2+} concentration or of pH value. Pseudocolor images were produced by applying a false color table to the grey ratio images on a pixel-by-pixel basis. Software-based image analysis allowed quantitation of Ca^{2+} or pH value in the whole visual field, or in a selected cell or region of a cell *vs.* time. Calibration and calculation of intracellular Ca^{2+} and pH values were performed as described previously. Briefly, for Ca^{2+} calibration the minimal and maximal fluorescence values were obtained after cell permeabilization with 5 μmol/liter ionomycin in the presence of solutions containing either no Ca^{2+} (substitution with EGTA) or 10 mmol/liter Ca^{2+}. The apparent dissociation constant K_d of Fura-2 was taken to be 225 nmol/liter. Calibration of pH measurements was performed with HEPES-buffered solutions containing 130 mmol/liter K^+ and adjusted to various pH values from 5.5 to 8.0. Equalization of intracellular and extracellular pH values was obtained with the K^+/H^+ exchanger nigericin (5 μmol/liter). Individual data were exported to an ASCII file which was eventually incorporated into a SigmaPlot 5.0 spreadsheet for further calculations.

Monitoring of Ca^{2+} Influx Using Manganese

Measurements of Ca^{2+} entry into the cytoplasm were performed as described previously [31]. The manganese quenching technique is based on two unique properties of the Mn^{2+} ion. First, Mn^{2+} ions compete with Ca^{2+} ions for entry into the cell via many Ca^{2+} channels. Second, Mn^{2+} ions bind to fluorescent Ca^{2+} indicators such as

Fura-2 and Quin-2 with a higher affinity than Ca^{2+} and quench their fluorescence. Hence, when Mn^{2+} is present in the extracellular medium, a decrease in intracellular fluorescence will reflect the Mn^{2+} entry via Ca^{2+} channels [4, 8].

To monitor Mn^{2+} entry, the Fura-2 fluorescence was followed at 510 nm when cells were excited at 360 nm. Emission at this excitation wavelength (so-called isosbestic point of the Fura-2 spectrum) is insensitive to changes in Ca^{2+} concentration. Thus, changes in the 360 nm emission reflects exclusively Mn^{2+} entry from the extracellular space into the cytoplasm. The Mn^{2+} concentration in the extracellular solution was 0.2 to 0.5 mmol/liter.

The monitoring of Mn^{2+} was performed by means of the Magi-Cal system. In these experiments, the cells were excited with a 360 nm filter which was mounted in the filter wheel. The image collection, background subtraction and averaging parameters were the same as for wavelengths used to monitor intracellular Ca^{2+} and pH values (*see above*).

Results

Effect of Intracellular pH Changes on Ca^{2+} Oscillations

Figures 2, 4, 5, 6 and 9 show examples of spontaneous Ca^{2+} oscillations in MDCK-F cells. Spontaneous oscillatory activity was observed in 80–95% of investigated cells. Individual spikes started from a basal Ca^{2+}_i concentration of about 100 nmol/liter reaching the peak value in the range of 400–700 nmol/liter. Oscillations were observed with a frequency of 0.5–2 spikes/min.

In the first experimental approach, changes of intracellular pH value (pH_i) were induced by means of an ammonia prepulse technique [18]. A 5-min exposure of the cells to 20 mmol/liter NH_4Cl increased intracellular pH by 0.37 ± 0.04 (n = 7) due to the trapping of H^+ ions by NH_3 diffusing into the cell (Fig. 1). Subsequent NH_4Cl removal caused a fall of pH_i by 0.40 ± 0.06 (n = 7) below the initial pH_i because of the NH_4^+ dissociation into NH_3 and H^+ ions. As shown in Fig. 2, neither intracellular alkalinization nor acidification induced by NH_4Cl pulse affected significantly amplitude or frequency of oscillations.

Figure 3 shows the effect of substitution of 100 mmol/liter Na^+ ion with choline$^+$ ion on pH_i. Na^+ removal resulted in a cell acidification, by 0.47 ± 0.08 pH units (n = 6), most likely due to lowering of the electrochemical Na^+ potential which is the driving force for Na^+/H^+ exchange. As shown in Fig. 4, Na^+ removal-induced cell acidification was paralleled by a change of oscillatory pattern. The individual spikes seemed wider and spiking activity reduced, compared to the control period. Strikingly, restoration of control Na^+ concentration immediately normalized both frequency and shape of Ca^{2+} spikes (Fig. 3), whereas pH returned to its initial value after 5–7 min (Fig. 4).

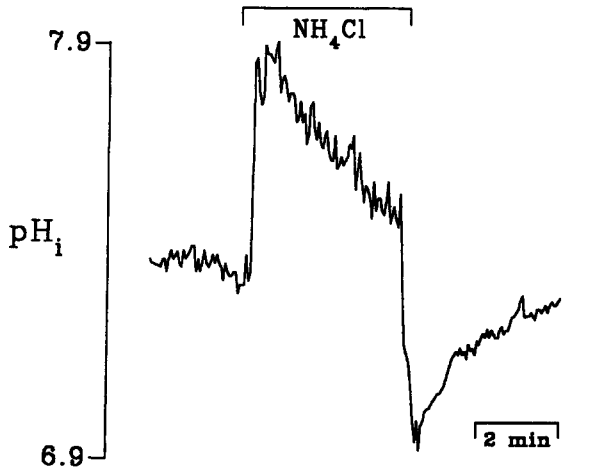

Fig. 1. Effect of extracellular NH_4Cl application (20 mmol/liter; isosmotic NaCl removal) on intracellular pH value (pH_i) in a MDCK-F cell. Trace shown represents seven experiments.

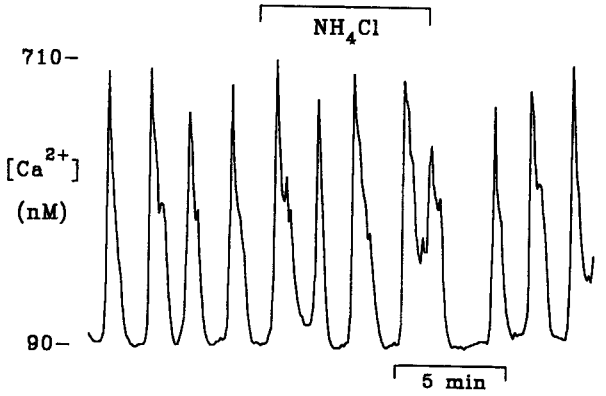

Fig. 2. Effect of extracellular NH_4Cl application (20 mmol/liter; isosmotic NaCl removal) on spontaneous Ca^{2+} oscillations in a MDCK-F cell. Trace shown represents seven experiments.

Effect of Extracellular pH Changes on Ca^{2+} Oscillations

The effect of extracellular pH increase from 7.2 to 7.6 on spontaneous Ca^{2+} oscillations in a MDCK-F cell is shown in Fig. 5. Extracellular alkalinization completely blunted oscillations in a fully reversible fashion. The influence of extracellular pH decrease from 7.2 to 6.8 was less pronounced (Fig. 6). The frequency of oscillations decreased by 40 ± 7% (n = 10) and a moderate widening of spikes was observed (Fig. 6). Both effects were reversible. Interestingly, neither extracellular alkalinization nor extracellular acidification significantly modified intracellular pH (Fig. 7). The respective pH_i changes in all cells were less than 0.1 pH unit.

As shown above, extracellular alkalinization abolished Ca^{2+} oscillations without apparent changes of cytoplasmic pH. However, the effect of alkalinization

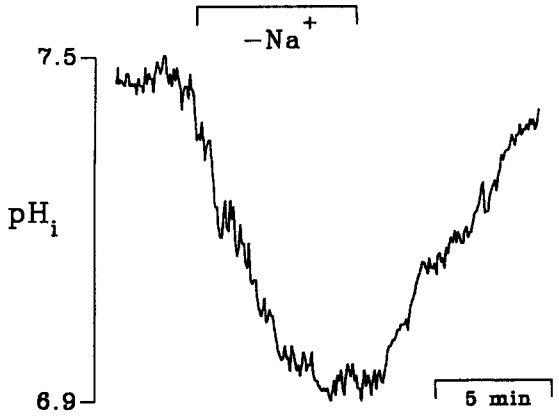

Fig. 3. Effect of removal of 100 mmol/liter Na^+ (substitution with 100 mmol/liter choline$^+$) on intracellular pH value (pH_i) in a MDCK-F cell. Trace shown represents six consistent experiments.

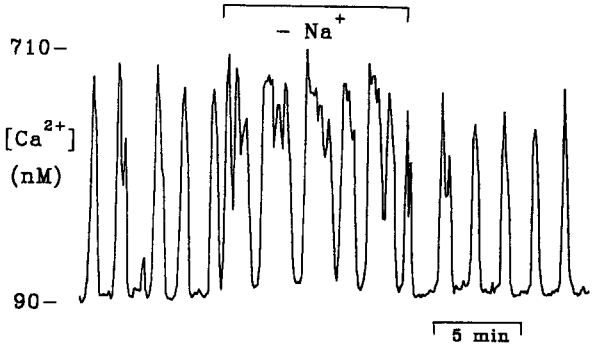

Fig. 4. Effect of removal of 100 mmol/liter Na^+ (substitution with 100 mmol/liter choline$^+$) on spontaneous Ca^{2+} oscillations in a MDCK-F cell. Trace shown represents seven experiments.

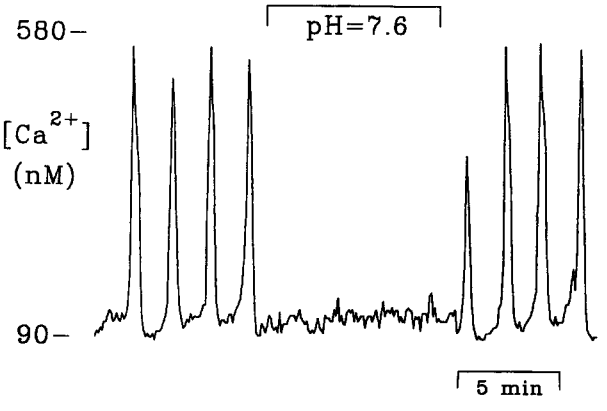

Fig. 5. Effect of extracellular alkalinization (pH = 7.6) on spontaneous Ca^{2+} oscillations in a MDCK-F cell. Trace shown represents 12 experiments.

could be due to an inhibition of Ca^{2+} entry into MDCK-F cells which determines the frequency of Ca^{2+} oscillations. Ca^{2+} entry in these cells can be monitored as quenching of Fura-2 fluorescence by Mn^{2+} ions entering the cell via Ca^{2+} channels in the plasma membrane. Therefore, we investigated the effect of extracellular alkalinization on Ca^{2+} entry from the extracellular space. Figure 8 shows an original recording of Ca^{2+} influx monitored as Mn^{2+} quenching of Fura-2 at 360 nm. In the presence of Mn^{2+} (0.2–0.5 mmol/liter), a rapid and constant decrease of intracellular fluorescence was observed. An increase of extracellular pH to 7.6 significantly ($P < 0.005$) inhibited the rate of Mn^{2+} entry into the cell. Thus, extracellular alkalinization apparently inhibits Ca^{2+} entry from the extracellular space.

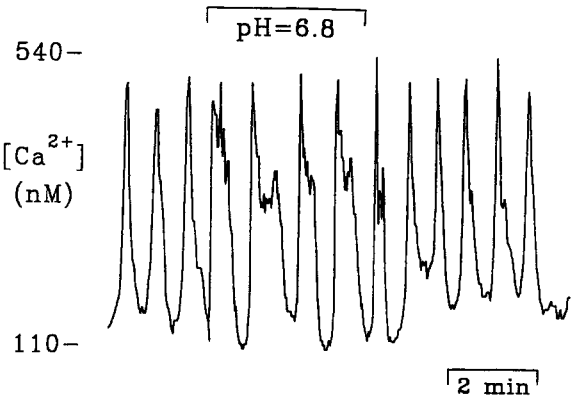

Fig. 6. Effect of extracellular acidification (pH = 6.8) on spontaneous Ca^{2+} oscillations in a MDCK-F cell. Trace shown represents 10 experiments.

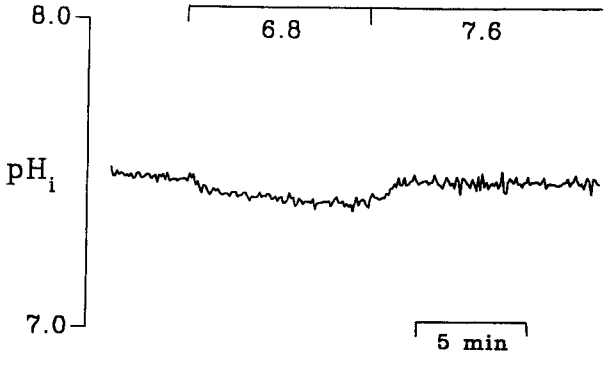

Fig. 7. Effect of successive extracellular acidification (pH = 6.8) and alkalinization (pH = 7.6) on intracellular pH value (pH_i) in a MDCK-F cell. Trace shown represents eight experiments.

Effect of Thapsigargin on Intracellular Ca^{2+} and pH

In the final experimental series, we investigated the effect of changes of Ca^{2+} homeostasis on intracellular pH value. The tumor promoter thapsigargin is a selective (though irreversible) inhibitor of a Ca^{2+}-ATPase [23] localized in the $InsP_3$-sensitive Ca^{2+} pool of MDCK-F cells [30]. As shown in Fig. 9, thapsigargin (100 nmol/

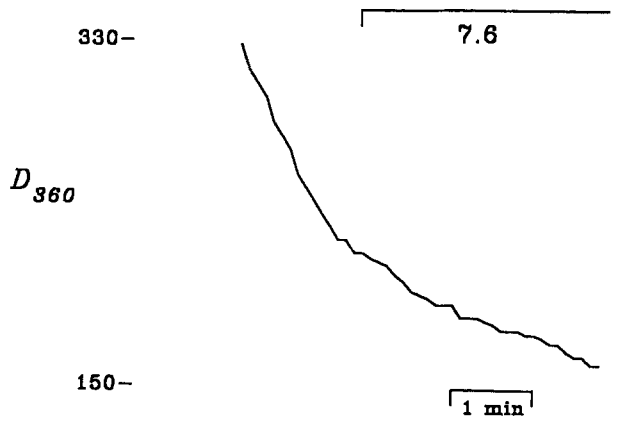

Fig. 8. Effect of extracellular alkalinization (pH = 7.6) on quenching of Fura-2 fluorescence at 360 nm (arbitrary units) by Mn^{2+} ions applied extracellularly. Trace shown represents five separate experiments. Mn^{2+} concentration was 0.2 mmol/liter.

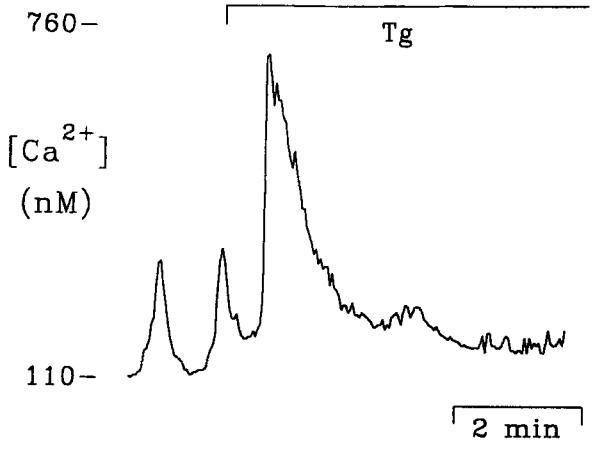

Fig. 9. Effect of thapsigargin (Tg; 100 nmol/liter) on spontaneous Ca^{2+} oscillations in a MDCK-F cell. Trace shown represents eight experiments.

liter) completely abolished oscillations. The inhibitory effect was preceded by a large, transient Ca^{2+} spike due to Ca^{2+} release from the thapsigargin-sensitive Ca^{2+} pool. A representative pH_i measurement under the same conditions is shown in Fig. 10. Apparently, the thapsigargin-induced changes of cytoplasmic Ca^{2+} concentration had no effect on intracellular pH.

Discussion

MDCK-F cells are a novel cell line derived from parent epithelial MDCK cells by exposure to chronic alkaline stress [14]. The most striking features of MDCK-F cells are continuous and spontaneous oscillations of intracellular Ca^{2+} concentration. Ca^{2+} spikes are generated in an intracellular Ca^{2+} store which is sensitive both to the Ca^{2+}-releasing second messenger $InsP_3$ and to selective blockers of the cytoplasmic Ca^{2+}-ATPase [30]. The mechanism underlying the generation of Ca^{2+} oscillations and their functional role(s) remain unknown. Nevertheless, due to spontaneous Ca^{2+} oscillations, MDCK-F cells are a useful and sensitive model for studying relationships between Ca^{2+} metabolism and other signaling pathways.

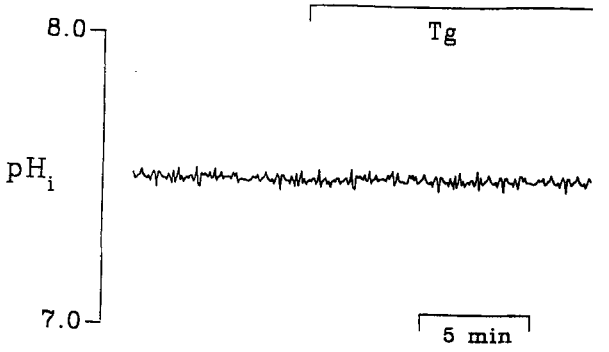

Fig. 10. Effect of thapsigargin (Tg; 100 nmol/liter) on intracellular pH value (pH_i) in a MDCK-F cell. Trace shown represents seven consistent experiments.

The most interesting finding of this study is the strong dependence of intracellular Ca^{2+} oscillations in MDCK-F cells on the extracellular pH. Previously, we reported that the frequency of membrane potential oscillations in MDCK-F cells is very sensitive to changes of the extracellular pH with an optimal pH of about 7.2 [28]. Oscillations of membrane potential in MDCK-F cells are a parallel but secondary event to Ca^{2+} oscillations and their molecular basis is the Ca^{2+}-sensitivity of plasma membrane K^+ channels [21]. Ca^{2+} measurements presented in this study confirm fully those previous findings. Both extracellular acidification and alkalinization decreased the frequency of oscillations. Interestingly, the pH dependence of oscillations seems to be more pronounced in the alkaline range since an extracellular pH of 6.8 decreased oscillatory frequency only by about 40%, whereas oscillations completely disappeared at a pH 7.6.

Surprisingly, these profound effects of extracellular pH changes on spiking activity of MDCK-F cells were not paralleled by any significant change of intracellular pH. This was most likely due to the activity of cellular pH-regulating transport systems. On the other hand, profound changes of intracellular pH brought about by successive alkalinization and acidification of the cytoplasm induced by an ammonia pulse had no apparent effect on Ca^{2+} oscillations. These findings suggested that extracellular pH changes may exert their action on Ca^{2+} metabolism via an external part of the cell, i.e., most likely on the plasma membrane. This hypothesis was confirmed by means of the manganese quenching technique. In an earlier paper, we have re-

ported that Mn^{2+} influx into MDCK-F cells parallels Ca^{2+} influx across the plasma membrane. The latter was fundamental for triggering of oscillations [31]. In the present experiments, extracellular alkalinization significantly reduced the rate of Mn^{2+} influx into the cytoplasm. Thus, it appears that extracellular pH value may control the rate of Ca^{2+} entry into MDCK-F cells and in this manner influence their oscillatory activity. Taking into consideration the lack of significant changes of intracellular pH under these experimental conditions, the action of extracellular pH changes on Ca^{2+} metabolism in MDCK-F cells seems to be restricted exclusively to the plasma membrane. Similar sensitivity of plasma membrane Ca^{2+} permeability to extracellular pH was recently described in acinar cells of the pancreas [26].

The effect of Na^+ removal on Ca^{2+} oscillations appears to disagree with the conclusions above. In these experiments, a profound cytoplasmic acidification induced by Na^+ removal was paralleled by a decrease of oscillatory frequency and by a widening of Ca^{2+} spikes. However, besides cytoplasmic acidification, these effects may be due to a separate effect of Na^+ removal, i.e., to the reduction of the electrochemical Na^+ gradient across the plasma membrane which is a driving force for the Na^+/Ca^{2+} exchange. A thorough comparison of Ca^{2+}_i and pH_i time courses under these experimental conditions supports this hypothesis (Figs. 3 and 4). After restoration of control Na^+ concentration, cytoplasmic pH increases slowly and reaches the initial value after a period of 5–7 min. On the contrary, the frequency and shape of Ca^{2+} spikes normalize immediately, despite the persistent cytoplasmic acidification. This finding indicates that the effect of Na^+ removal on Ca^{2+} oscillations is most likely due to the altered activity of a Na^+-dependent, Ca^{2+}-transporting mechanism of the plasma membrane. This explanation, however, is rather speculative at the present time and will require further studies to resolve.

Whereas pH may influence intracellular Ca^{2+} metabolism in MDCK-F cells, there is obviously no reciprocal relationship between those parameters. Indeed, changes of cytoplasm Ca^{2+} concentration induced by thapsigargin had no significant effect on the intracellular pH value. This was quite surprising, since Ca^{2+} ions are known as a potent agonist of protein kinases C, and the latter are believed to control the function of a plasma membrane Na^+/H^+ exchanger [7]. However, it is possible that the Na^+/H^+ exchanger in MDCK-F cells is controlled by a Ca^{2+}-independent isoform of protein kinase C [7].

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