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Modulation of Ca^{2+} Influx in Leech Retzius Neurons. I. Effect of Extracellular pH

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Abstract. We investigated the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) of leech Retzius neurons in situ while varying the extracellular and intracellular pH as well as the extracellular ionic strength. Changing these parameters had no significant effect on $[Ca^{2+}]_i$ when the membrane potential of the cells was close to its resting value. However, when the cells were depolarized by raising the extracellular K^+ concentration or by applying the glutamatergic agonist kainate, extracellular pH and ionic strength markedly affected $[Ca^{2+}]_i$, whereas intracellular pH changes appeared to have virtually no effect. An extracellular acidification decreased $[Ca^{2+}]_i$, while alkalinization or reduction of the ionic strength increased it. Correspondingly, $[Ca^{2+}]_i$ also increased when the kainate-induced extracellular acidification was reduced by raising the pH-buffering capacity. At low extracellular pH, the membrane potential to which the cells must be depolarized to evoke a detectable $[Ca^{2+}]_i$ increase was shifted to more positive values, and it moved to more negative values at high pH. We conclude that in leech Retzius neurons extracellular pH, but not intracellular pH, affects $[Ca^{2+}]_i$ by modulating Ca^{2+} influx through voltage-dependent Ca^{2+} channels. The results suggest that this modulation is mediated primarily by shifts in the surface potential at the extracellular side of the plasma membrane.

Key words: Ca^{2+} channels — Intracellular Ca^{2+} — Fura-2 — Ion-sensitive microelectrodes — Surface potential

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Introduction

In addition to changes in the activity of several other ion species (*see* Walz, 1989), neuronal excitation causes significant shifts in the intracellular and extracellular pH (pH_i, pH_o; Chesler & Kaila, 1992). These pH changes may modulate cellular metabolism including energy transduction (Roos & Boron, 1981) as well as neuronal excitability by affecting ion channels (e.g., Konnerth, Lux & Morad, 1987; Traynelis & Cull-Candy, 1990). A modulation of Ca^{2+} channel function by pH has been demonstrated in various cell types, such as tunicate egg cells (Ohmori & Yoshii, 1977), muscle cells (Prod'hom, Pietrobon & Hess, 1989; Klöckner & Isenberg, 1994), mouse hybridoma cells (Iijima, Ciani & Hagiwara, 1986), vertebrate neurons (Ou-Yang et al., 1994; Zhou & Jones, 1996; Tombaugh & Somjen, 1997; Church, 1999), and cone photoreceptors (Barnes & Bui, 1991). In most studies the pH effects have been attributed to a modulation of channel gating due to changes in the surface potential of the cell membrane, but there is also evidence for other mechanisms such as proton block of the permeation pathway or allosteric effects (*see* Iijima et al., 1986; Prod'hom et al., 1989; Chen, Bezprozvanny & Tsien, 1996).

The Retzius neurons of the leech central nervous system possess slowly inactivating voltage-dependent Ca^{2+} channels (Stewart, Nicholls & Adams, 1989; Gottmann, Dietzel & Lux, 1989; Bookman & Liu, 1990). The activation of these channels by depolarizing the cell membrane was found to induce an increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$), which was significantly larger if the depolarization was caused by raising the extracellular K^+ concentration ($[K^+]_o$) than by activating the glutamate receptors of the cells by the specific agonist kainate (Dierkes, Hochstrate & Schlue, 1996). This discrepancy might be explained by various mechanisms, one of which is the modulation of Ca^{2+} influx by pH. Both raising $[K^+]_o$ and kainate application

have been shown to induce an intracellular acidification (Dörner, 1991; Frey & Schlue, 1993; Kilb & Schlue, 1999), but kainate also evokes a marked extracellular acidification (Dörner, 1991; Rose & Deitmer, 1995b), which might not occur upon raising $[K^+]_o$.

To demonstrate the putative feedback between changes in pH and Ca^{2+} influx we investigated the effect of pH_o and pH_i on $[Ca^{2+}]_i$ and on the membrane potential (E_m) of leech Retzius neurons in situ under different experimental conditions. In situ experiments avoid possible changes in cellular parameters due to cell isolation or culturing, and hence allow for a better comparison with the effects of neuronal stimulation in the intact nervous system (*see* Rose & Deitmer, 1995a, b). The results strongly suggest that pH_o, but not pH_i, affects $[Ca^{2+}]_i$ by modulating the activity of the voltage-dependent Ca^{2+} channels, which appears to be primarily due to changes in the surface potential at the outer side of the plasma membrane. In particular, it is shown that the kainate-induced extracellular acidification quantitatively explains the discrepancy between the $[Ca^{2+}]_i$ increases that are caused by raising $[K^+]_o$ and those caused by the activation of the glutamate receptors.

Materials and Methods

Preparation and Dye Injection

The experiments were done on Retzius neurons in intact segmental ganglia of the leech *Hirudo medicinalis* that were prepared from adult animals (*see* Muller, Nicholls & Stent, 1981). Except for ganglia 5 and 6, all segmental ganglia were used for the experiments. The ganglia were fixed at their connectives in a special holder by means of fine steel clips. The preparation and the experimental setup have been described in detail in previous papers (Hochstrate, Piel & Schlue, 1995; Dierkes, Hochstrate & Schlue, 1997).

The Retzius neurons (∅ = 79 ± 8 μm, n = 28) were iontophoretically loaded with Fura-2 (Molecular Probes, Eugene, OR, USA) by using single-barrelled microelectrodes filled at their tip with the pentapotassium salt of Fura-2 dissolved in distilled water (100 mM; electrode resistance 50 to 130 MΩ, injection current: –20 nA for 30 sec). The intracellular Fura-2 concentration was 200 μM, as estimated by comparing the fluorescence of Fura-2-loaded cells with that of similar-sized droplets of aqueous Fura-2 solutions in oil. Dye injection had no significant effect on the resting potential or on frequency and amplitude of the spontaneous action potentials. Control experiments in which the amount of injected Fura-2 was doubled or reduced to half or less gave essentially the same results.

Experimental Setup

About 2 min after dye injection the preparation was transferred into a flow chamber mounted on the stage of an inverted microscope (Diaphot-TMD; Nikon), which was part of a commercial microspectrofluorimeter (Deltascan 4000; Photon Technology International, Wedel, Germany). The chamber was perfused with a flow rate of 4 ml min^{-1}, which exchanged the chamber volume about 15 times per min. The fluorescence was alternately excited by light of 340 and 380 nm wavelength, which was guided to the preparation via a 40× objective with high numerical aperture (Fluor 40 Ph3DL; Nikon). The fluorescence light was collected by the same objective, filtered through a 510/540 nm barrier filter and measured by a photon-counting photomultiplier tube with a data acquisition rate of 1 sec^{-1}. The object area was limited to a rectangular field by means of a variable diaphragm (10 to 50 μm edge length).

Calculation of $[Ca^{2+}]_i$

In order to obtain the fluorescence of the injected Fura-2 (F_{340}, F_{380}), the corresponding autofluorescence of the preparation was subtracted from the raw data. $[Ca^{2+}]_i$ was calculated from the ratio $R = F_{340}/F_{380}$, according to the equation given by Grynkiewicz, Poenie & Tsien (1985): $[Ca^{2+}]_i = K_d \cdot ((R - R_{min})/(R_{max} - R)) \cdot (F_f/F_s)$, where R_{min} designates the minimum R, which is measured in the absence of Ca^{2+}, and R_{max} the maximum R at saturating Ca^{2+} concentrations. F_f/F_s is the fluorescence ratio of the Ca^{2+}-free and Ca^{2+}-saturated form of Fura-2 upon 380 nm excitation; K_d is the apparent dissociation constant of the Ca^{2+} · Fura-2 complex.

The parameters R_{min}, R_{max}, and F_f/F_s were determined by use of solutions of 30 to 300 μM Fura-2 in 100 mM KCl buffered to pH 7.4 with 50 mM HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]), to which either 10 mM $CaCl_2$ or 10 mM EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid) had been added. For K_d a value of 135 nM was used; this value was measured under similar conditions by Grynkiewicz et al. (1985) and closely corresponds to K_d values obtained by in situ calibrations in exposed leech neuropile glial cells (Munsch & Deitmer, 1992; Munsch, Nett & Deitmer, 1994). The vertical scaling of the recordings is linear in R(340nm/380nm), because this is the experimentally relevant parameter. Mean values of $[Ca^{2+}]_i$ were determined by averaging the ratios R(340nm/380nm) measured in the single experiments (*see* Hochstrate et al., 1995).

In vitro the apparent K_d of the Ca^{2+} · Fura-2 complex was found to be pH-dependent (Lattancio, 1990; Martinez-Zaguilan et al., 1991; Uto, Arai & Ogawa, 1991). Therefore, a $[Ca^{2+}]_i$ increase that is paralleled by a drop in pH_i might not be detected by Fura-2 due to a corresponding increase in the apparent K_d. Within the pH_i range covered by applying NH_4^+ or propionate in the resting state, possible undetected $[Ca^{2+}]_i$ changes should be small, since $[Ca^{2+}]_i$ is low and the apparent K_d changes by less than 10%. However, following kainate application or $[K^+]_B$ elevation, pH-induced changes in the apparent K_d and hence undetected changes in $[Ca^{2+}]_i$ might be larger, since $[Ca^{2+}]_i$ is raised and pH_i reduced. A perfect compensation of pH-induced shifts in the apparent K_d by corresponding $[Ca^{2+}]_i$ changes seems unlikely and is possible only if the pH sensitivity of putative intracellular Ca^{2+} binding sites or stores is identical with that of Fura-2. However, it seems possible that $[Ca^{2+}]_i$ was underestimated at more acid pH_i.

Electrophysiological Recordings

The electrophysiological recordings were performed in a different setup. The exchange rate of the bath medium in the perfusion chamber was similar to that used in the fluorescence measurements. pH_o, pH_i, and E_m were measured by using double-barrelled ion-sensitive microelectrodes, the fabrication and calibration of which has been described previously in detail (Schlue & Thomas, 1985; Schlue, 1987). The pH electrodes were pulled from theta-type capillaries (TGC 200-15; Clark Electromedical Instruments, Pangbourne, England). After silanization of the prospective ion-sensitive barrel, the tip was filled either with H^+ ionophore I-cocktail A (Fluka, Buchs, Switzerland) followed by 0.1 M

sodium citrate, or with H^+ ionophore II-cocktail A followed by 0.1 M potassium citrate. The electrodes were calibrated in solutions which had the same ionic composition as the physiological solution, except that the pH was changed. The E_m was also recorded by the use of conventional single-barrelled microelectrodes filled with 3 M KCl. The pH recordings were low-pass filtered with 0.5 Hz, the E_m recordings with 5 Hz.

pH_o was recorded by inserting a pH-sensitive microelectrode between the cell bodies of the two Retzius neurons about halfway between the outer and inner capsule of the segmental ganglion. During pH_o recordings the pH-sensitive channel often became clogged, as indicated by a sudden shift in the electrode potential that was paralleled by an increase in electrode resistance. The risk of clogging was reduced by withdrawing the electrode by a few μm after the insertion, thereby possibly creating a small artificial cavern. During pH_i recordings electrode clogging usually did not occur. After cell impalement pH_i reached a steady state within 5 to 10 min; shortly after the impalement pH_i was slightly lower than the steady-state pH_i.

Solutions

The physiological solution had the following composition (in mM): 85 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.40 with NaOH, which increased the Na^+ concentration by 4 mM. Since different amounts of NaOH had to be added to obtain test solutions with different pH, the amount of NaCl was adjusted to keep the Na^+ concentration constant. We used only HEPES as pH buffer to avoid possible side effects of other pH-buffering substances (Robinson & Davies, 1987; Raval, Carter & Fairbanks, 1989; Schmidt, Mangold & Deitmer, 1996). To increase the extracellular pH-buffering capacity, the HEPES concentration was raised to 50 mM. To mimic the concomitant rise in osmolarity and ionic strength, control experiments were performed using solutions to which 30 mM sucrose plus 15 mM NaCl were added. In solutions with elevated K^+ concentration, equimolar amounts of NaCl were replaced by KCl. In Na^+-free solutions, NaCl was replaced by the chloride salt of N-methyl-D-glucamine (NMDG). The glutamatergic agonist kainate was added from a 50 mM stock solution without osmotic compensation. In solutions with 20 mM sodium propionate the equivalent amount of NaCl was omitted to keep the Na^+ concentration constant. NH_4Cl was added in solid form shortly before use without osmotic compensation. The osmolarity and ionic strength of the solutions were varied by reducing or increasing the NaCl concentration and/or adding carbohydrates such as sucrose, glucose, or mannose. If not otherwise stated the pH of the experimental solutions was adjusted to 7.40. Kainate was obtained from Sigma (Deisenhofen, Germany); sodium propionate and NH_4Cl were from Merck (Darmstadt, Germany), and HEPES was from Roth (Karlsruhe, Germany).

Quantification of the Observed Effects

We investigated the effect of various experimental manipulations on different cellular parameters. Unfortunately, most parameters were usually drifting and, in general, it was not feasible to wait for a stabilization before the manipulations were performed. Since amplitude and time course of the drift varied from experiment to experiment, it was impossible to construct reference lines by applying a standardized procedure. Nevertheless, to summarize observations made in identically performed experiments, we made a quantitative estimation by using reference lines that were adapted visually with the example of experiments in which the respective manipulation was omitted (*see* Figs. 1*A*, 6, 9). Certainly, this procedure is not free of arbitrariness, but the analyzed effects were relatively large, so that their quantitative estimation did not critically depend on the exact positioning of the reference line.

In contrast to the other parameters investigated, the $[Ca^{2+}]_i$ increase caused by a moderate rise in the extracellular K^+ concentration was on average constant. Therefore, changes in the K^+-induced $[Ca^{2+}]_i$ increase could be quantified reasonably by using reference lines which were obtained by linear interpolation (*see* Fig. 1A). In a single experiment, this straight line may deviate markedly from the visually adapted reference line; however, such deviations occurred in both directions. The mean changes in $[Ca^{2+}]_i$ determined by using straight reference lines were very similar to those obtained by using visually adapted reference lines (Figs. 1*B*, 2).

Results

Effect of Bath pH on $[Ca^{2+}]_i$ at Normal and Increased $[K^+]_B$

The effect of varying the pH of the bathing solution (pH_B) on the $[Ca^{2+}]_i$ of Retzius neurons is shown in Fig. 1. In physiological solution, a change in pH_B had no effect on $[Ca^{2+}]_i$, but it caused marked changes in $[Ca^{2+}]_i$ when the bathing solution had an increased K^+ concentration ($[K^+]_B$), which induced an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Hochstrate et al., 1995; Dierkes et al., 1997). A reduction of pH_B evoked a decrease in $[Ca^{2+}]_i$, while raising pH_B caused a $[Ca^{2+}]_i$ increase. The $[Ca^{2+}]_i$ decrease after reducing pH_B required several minutes, and in 3 out of 5 experiments a steady state was not reached within 5 min. In contrast, the $[Ca^{2+}]_i$ increase upon raising pH_B was almost complete within 2 min and often showed a transient maximum. The recovery of $[Ca^{2+}]_i$ after returning to physiological solution took 5 to 10 min and was not affected by changing pH_B (Fig. 1*A*). The pH_B-induced changes in $[Ca^{2+}]_i$ were unaffected by dinitroquinoxaline (DNQX) and d-tubocurarine (100 μM each), which excludes an involvement of excitatory glutamatergic or cholinergic synaptic transmission (*see* Rose & Deitmer, 1995a, b).

The effect of pH_B on $[Ca^{2+}]_i$ was quantified as illustrated in Fig. 1*A*. At $[K^+]_B = 30$ mM, the $[Ca^{2+}]_i$ varied between about 200 nM at $pH_B = 6.2$ and 600 nM at $pH_B = 8.6$ (Fig. 1*B*). At low pH_B, $[Ca^{2+}]_i$ was certainly overestimated, because in most experiments a steady state was not reached. The $[Ca^{2+}]_i$ recorded in different preparations at a given $[K^+]_B$ were rather variable, but the changes in $[Ca^{2+}]_i$ caused by shifting pH_B were fairly linearly correlated with the $[Ca^{2+}]_i$ increase that was measured before the pH_B shift, at the normal pH_B of 7.4 (Fig. 2*A*). Therefore, the modulatory effect of pH_B could be quantified by relating the pH_B-induced $[Ca^{2+}]_i$ changes to the corresponding $[Ca^{2+}]_i$ increase at $pH_B = 7.4$ (relative $[Ca^{2+}]_i$ change; Fig. 2*B*).

Effect of Bath pH on pH_o, pH_i, and E_m

Changing pH_B will shift the extracellular pH (pH_o), and this might also affect the intracellular pH (pH_i) and/or

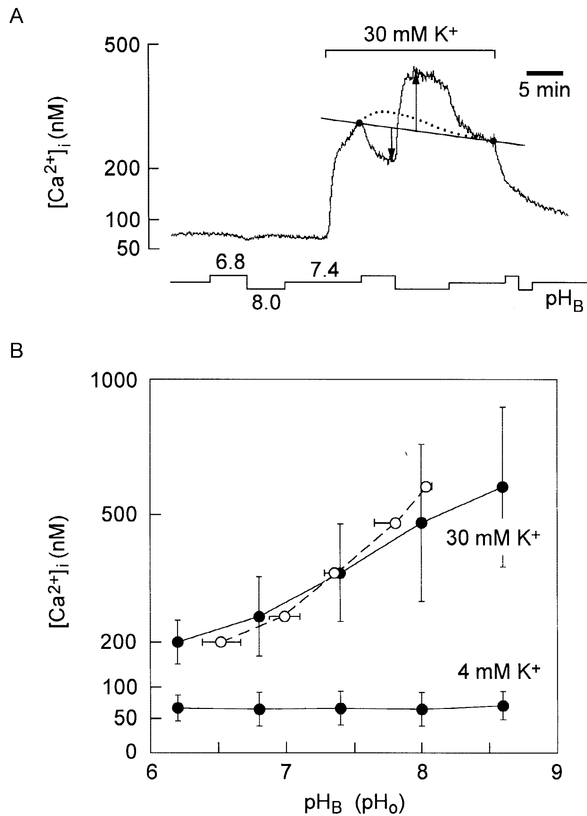

Fig. 1. Effect of pH_B on the $[Ca^{2+}]_i$ of leech Retzius neurons. (*A*) Effect of shifting pH_B to 6.8 or 8.0 on $[Ca^{2+}]_i$ before, during and after raising $[K^+]_B$ to 30 mM. The pH_B shifts evoked significant changes in $[Ca^{2+}]_i$ when $[K^+]_B$ was raised but not in physiological solution. At $[K^+]_B$ = 30 mM, E_m was −23.4 ± 5.7 mV (n = 21; resting E_m −47.8 ± 4.1 mV); pH_B had virtually no effect on E_m, either at normal or at increased $[K^+]_B$. To quantify the effect of pH_B on $[Ca^{2+}]_i$ a reference line was constructed by linear interpolation between the $[Ca^{2+}]_i$ values recorded at the beginning and at the end of an experiment after 5 min incubation at pH_B = 7.4. The $[Ca^{2+}]_i$ at altered pH_B was determined at the moment of maximum deflection of the recorded trace from the reference line. The coordinate points on the reference line were taken as a measure for $[Ca^{2+}]_i$ at pH_B = 7.4. Dotted line gives the putative course of the trace without shifting pH_B (*see* Materials and Methods). (*B*) Relationship between $[Ca^{2+}]_i$ and pH_B in physiological solution ($[K^+]_B$ = 4 mM) and at $[K^+]_B$ = 30 mM. Data points are the mean (± SD) of n = 5 to 13 experiments performed like that in shown in *A*. In a single experiment, pH_B was changed in the order 7.4, 6.8, 8.0, 7.4 or 7.4, 8.0, 6.8, 7.4, and correspondingly for pH_B = 7.4, 6.2 and 8.6. Open circles joined by broken lines give the relationship between $[Ca^{2+}]_i$ and the pH_o that was measured by pH-sensitive microelectrodes between the cell bodies of the two Retzius neurons (*see* Fig. 3*A*).

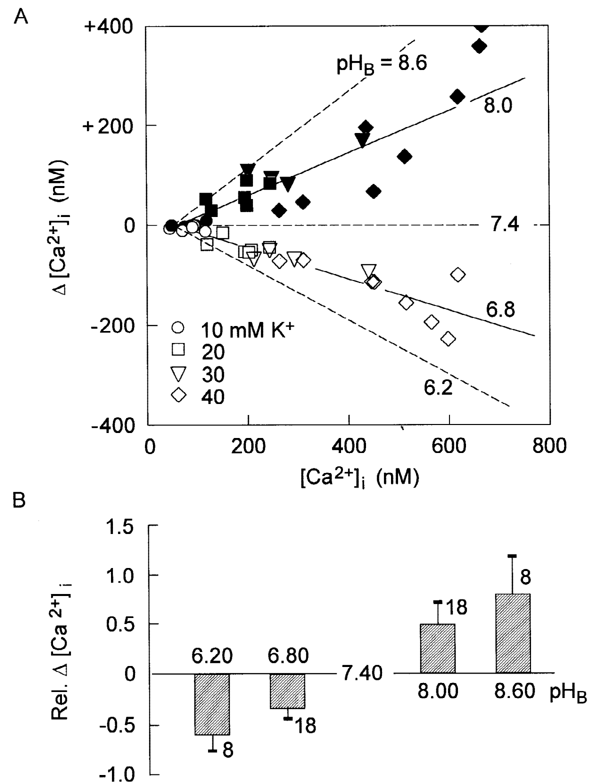

Fig. 2. Modulatory effect of pH_B in relation to $[Ca^{2+}]_i$. (*A*) Changes in $[Ca^{2+}]_i$ caused by shifting pH_B to 6.8 (open symbols) or 8.0 (filled symbols) in relation to the $[Ca^{2+}]_i$ reached after raising $[K^+]_B$ to 10, 20, 30, or 40 mM at pH_B = 7.4. The experiments were performed and evaluated as illustrated in Fig. 1*A*. Solid lines were calculated by linear regression of the presented data points; broken lines give the linear regression of data obtained at pH_B = 6.2 or 8.6. Note that the regression lines cross the broken horizontal line marked "7.4" close to the basal $[Ca^{2+}]_i$ measured in physiological solution. (*B*) pH_B-induced changes in $[Ca^{2+}]_i$ at $[K^+]_B$ = 20, 30, or 40 mM, normalized to the $[Ca^{2+}]_i$ increase at pH_B = 7.4. Bars give the mean ± SD of n = 8 or 18 experiments.

E_m. It has been shown that even in physiological solution pH_B and pH_o are not identical (Frey & Schlue, 1993), and marked changes in pH_o are induced at constant pH_B by applying weak acids (Schlue et al., 1991), the glutamatergic agonist kainate (Dörner, 1991) or by electrical stimulation (Rose & Deitmer, 1995b). To gain insight into the mechanism by which pH_B affects the K^+-induced $[Ca^{2+}]_i$ increase we investigated the effect of pH_B on pH_o, pH_i, and E_m.

In physiological solution, pH_o was slightly lower than pH_B. pH_o followed pH_B at both normal and increased $[K^+]_B$, but a steady state was often not reached within the 5-min period during which pH_B was changed. Raising $[K^+]_B$ sometimes caused a slight drop in pH_o (Fig. 3*A*), but normally it had no effect (*see* Fig. 6C). pH_i also followed pH_B but the rate was considerably lower, and within 5 min a steady state was never reached (Fig. 3*B*; *compare* Deitmer, 1991). The effect of raising $[K^+]_B$ on pH_i varied considerably from preparation to preparation. In some cells, pH_i remained unaffected, but mostly it was reversibly reduced (Figs. 3*B*, 6*B*). On average, pH_i dropped by 0.17 ± 0.18 pH units (n = 7), which is somewhat less than found by Frey & Schlue (1993).

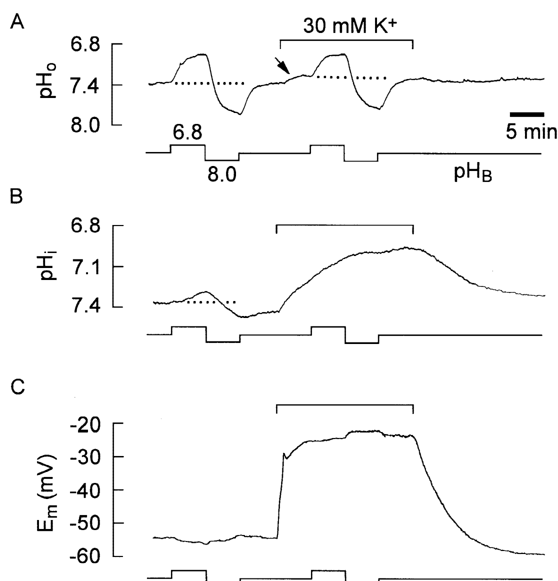

Fig. 3. Effect of changing pH_B and $[K^+]_B$ on pH_o, pH_i, and E_m. Traces recorded in two different preparations by the use of double-barrelled, pH-sensitive microelectrodes. In *A,* the tip of the electrode was positioned extracellularly between the cell bodies of the two Retzius neurons; in *B* and *C,* the electrode was inserted into one of the two Retzius neurons. Dotted lines extrapolate baselines before the first pH_B shift. Arrow in *A* marks drop in pH_o after raising $[K^+]_B$ (*compare* Fig. 6*C*).

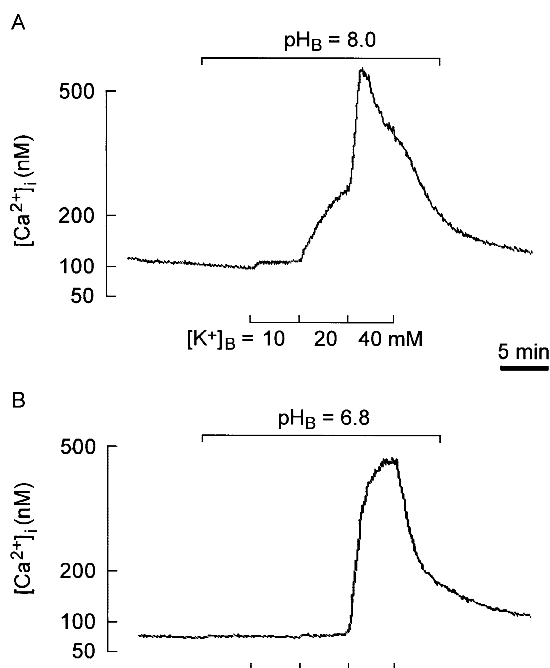

Fig. 4. Effect of raising $[K^+]_B$ at $pH_B = 8.0$ (*A*) and 6.8 (*B*). At $pH_B = 8.0$, $[Ca^{2+}]_i$ was significantly increased upon raising $[K^+]_B$ to 10 mM, whereas even an elevation to 20 mM was ineffective at $pH_B = 6.8$. In *A,* the partial recovery of $[Ca^{2+}]_i$ at 40 mM K^+ is probably due to a shift of E_m into the negative direction, as it was often observed in electrophysiological recordings at high $[K^+]_B$.

pH_B had virtually no effect on E_m, either at normal or at increased $[K^+]_B$ (Fig. 3*C*), which excludes E_m shifts as the cause of the pH_B-induced changes in $[Ca^{2+}]_i$. After long-lasting superfusion with acidified solutions, E_m was shifted to more negative values, but the effect was small and not significant (*see* Fig. 5). A comparison of the recordings in Figs. 1 and 3 shows that the pH_B-induced changes in pH_o and $[Ca^{2+}]_i$ occurred within a similar time scale, whereas the changes in pH_i proceeded more slowly. This result suggests that the modulations of the K^+-induced $[Ca^{2+}]_i$ increase were predominantly due to shifts in pH_o.

Effect of Bath pH on the Relationship Between $[Ca^{2+}]_i$ and E_m

pH_B affected the $[K^+]_B$ necessary to evoke a definitive increase in $[Ca^{2+}]_i$ (Figs. 4, 5). In 12 cells, raising $[K^+]_B$ to 10 mM at $pH_B = 6.8$ never had a detectable effect on $[Ca^{2+}]_i$, whereas a $[Ca^{2+}]_i$ increase was seen in 5 out of 11 cells at $pH_B = 8.0$. At $pH_B = 6.8$, even raising $[K^+]_B$ to 20 mM evoked a $[Ca^{2+}]_i$ increase in only 5 out of the 12 cells, but a marked effect always occurred at $pH_B = 8.0$. From the plot of $[Ca^{2+}]_i$ against E_m it can be estimated that the threshold E_m for the K^+-induced $[Ca^{2+}]_i$ increase was shifted by about 10 mV in the positive direction upon changing pH_B from 8.0 to 6.8 (Fig. 5*B*; compare Hochstrate et al., 1995). This shift of the $[Ca^{2+}]_i/E_m$ relation is quantitatively similar to that found in other preparations (Iijima et al., 1986; Krafte & Kass, 1988; Barnes & Bui, 1991), as well as to predictions from model calculations (Hille, Woodhull & Shapiro, 1975). The data also show that the steady-state $[Ca^{2+}]_i$ was not dependent on the order in which $[K^+]_B$ and pH_B were changed.

Effect of Changing pH_i

The possible role of pH_i in the regulation of $[Ca^{2+}]_i$ was investigated by extracellular application of propionate or NH_4^+ (*see* Roos & Boron, 1981). In physiological solution, propionate caused a slight increase in $[Ca^{2+}]_i$ or was ineffective, but a marked reversible $[Ca^{2+}]_i$ increase was evoked when $[K^+]_B$ was raised (Fig. 6*A*). The recovery of $[Ca^{2+}]_i$ after changing to physiological solution was not affected by propionate. In addition to an intracellular acidification that changed to an alkalinization after washout (Fig. 6*B*), propionate also caused transient shifts in pH_o: an increase upon its application and a decrease after its removal, both shifts having a similar amplitude and time course (Fig. 6*C*). Propionate had no significant effect on E_m; some cells slightly de- or hyperpolarized

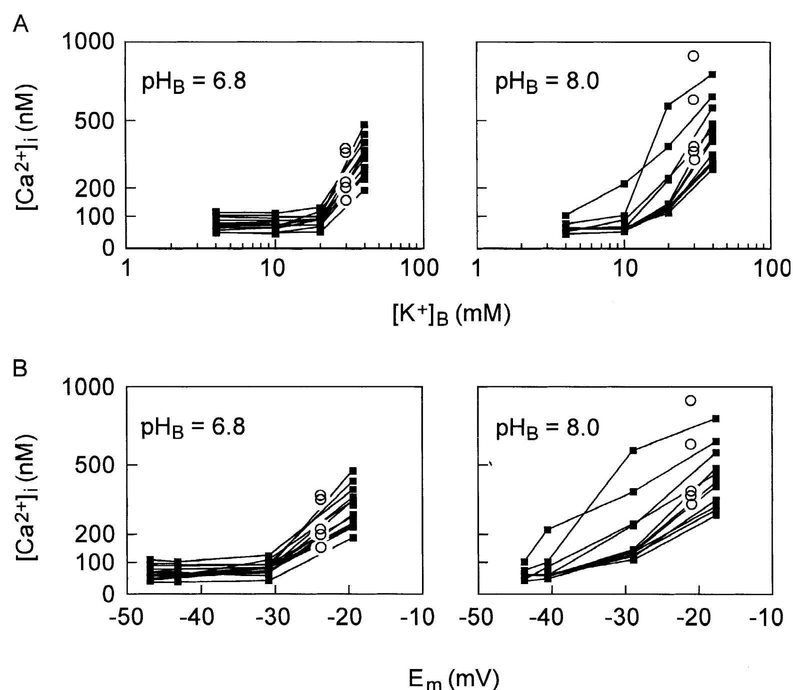

Fig. 5. Relationship between $[Ca^{2+}]_i$ and $[K^+]_B$ (*A*) and between $[Ca^{2+}]_i$ and E_m (*B*) at $pH_B = 6.8$ and 8.0. $[Ca^{2+}]_i$ data obtained from experiments as shown in Fig. 4, each measured 5 min after changing $[K^+]_B$. Diagrams in *B* constructed by plotting the $[Ca^{2+}]_i$ values in *A* versus the corresponding mean E_m ($n = 7$ to 16 E_m recordings per data point; single-barrelled microelectrodes). Open circles are from experiments in which pH_B was varied at $[K^+]_B = 30$ mM (*see* Figs. 1, 2). Note that the E_m at the different $[K^+]_B$ were slightly more positive at $pH_B = 8.0$ than at $pH_B = 6.8$.

(Fig. 6*D*), whereas others were unaffected (*see* Schlue & Thomas, 1985; Frey & Schlue, 1993). The propionate-induced changes in pH_o and $[Ca^{2+}]_i$ but also those in pH_i were significantly smaller when the HEPES concentration of the bathing solution was increased in order to augment the extracellular pH-buffering capacity (Fig. 7*A*). Since raising the HEPES concentration also increased the osmolarity and ionic strength of the superfusion solution, we applied propionate after adding equivalent amounts of sucrose and NaCl to the 30 mM K^+ solution (*see* Materials and Methods). Under this condition, the propionate-induced changes in $[Ca^{2+}]_i$, pH_i, and pH_o were like those in normal 30 mM K^+ solution. Raising the HEPES concentration or addition of sucrose plus NaCl had no significant effect on E_m.

The effects of NH_4^+ on $[Ca^{2+}]_i$, pH_o, and pH_i were essentially the mirror image of the propionate effects (Fig. 7*B*). NH_4^+ evoked a drop in $[Ca^{2+}]_i$ when $[K^+]_B$ was raised, while it was virtually ineffective in physiological solution. Amplitude and time course of the changes in pH_i and pH_o were similar to those caused by propionate, but of opposite sign (*see* Schlue & Thomas, 1985; Schlue et al., 1991). In physiological solution, NH_4^+ induced a reversible membrane depolarization by 3.7 ± 0.9 mV ($n = 7$), and by 2.2 ± 0.4 mV ($n = 5$) with increased $[K^+]_B$. In the presence of 50 mM HEPES, the NH_4^+-induced changes in pH_o and $[Ca^{2+}]_i$ were markedly reduced; the changes in pH_i were slightly but not significantly attenuated, and those in E_m were unaffected. After adding sucrose plus NaCl the NH_4^+-induced shifts in pH_o, pH_i, and E_m were unchanged, but the drop in $[Ca^{2+}]_i$ was also significantly reduced.

Effect of Extracellular Ionic Strength

The pH-induced changes in $[Ca^{2+}]_i$ may be attributed to shifts in the membrane surface potential (*see* Hille, 1992), which could also explain the effect of reducing the extracellular ionic strength on the K^+-induced $[Ca^{2+}]_i$ increase. After exchanging extracellular NaCl for sucrose, $[Ca^{2+}]_i$ increased markedly, passed through a maximum and then began to recover (Fig. 8, left traces). The recovery was accelerated after readdition of NaCl, and subsequently $[Ca^{2+}]_i$ undershot the level reached before the exchange. The exchange of NaCl caused a membrane depolarization which recovered within 5 min, and after restoration of the initial conditions, E_m was slightly shifted in the negative direction. The changes in $[Ca^{2+}]_i$ and E_m were qualitatively similar when glucose or mannose was added to the superfusion medium, but both $[Ca^{2+}]_i$ increase and membrane depolarization were more pronounced. When NaCl was omitted from the superfusion medium without osmotic compensation, the changes in $[Ca^{2+}]_i$ were similar to those following NaCl replacement by sucrose, except that the amplitude was slightly smaller and $[Ca^{2+}]_i$ recovery was less pronounced (Fig. 8, middle traces). E_m appeared to be unaffected; following NaCl omission the cells were often lost, but after re-impalement E_m was virtually the same as in Na^+-containing solution. Readdition of NaCl caused a transient E_m shift in the negative direction. The replacement of NaCl by the chloride salt of N-methyl-D-glucamine caused a small decrease in $[Ca^{2+}]_i$, but did not affect E_m (Fig. 8, right traces). The results show that the effect on $[Ca^{2+}]_i$ of removing extracellular NaCl is not Na^+-

specific and probably due to the reduced ionic strength of the superfusion medium. At normal $[K^+]_B$, neither the addition of carbohydrates nor omission or replacement of NaCl had an effect on $[Ca^{2+}]_i$.

Enhancement of Kainate-induced $[Ca^{2+}]_i$ Increase by Raising the Extracellular pH-buffering Capacity

In Retzius neurons, the glutamatergic agonist kainate evokes a membrane depolarization due to the activation of nonselective cation channels (Dörner, Ballanyi & Schlue, 1990; Dörner, 1991; Dörner, Zens & Schlue, 1994). Furthermore, kainate induces an increase in $[Ca^{2+}]_i$, which is exclusively mediated by voltage-dependent Ca^{2+} channels, because $[Ca^{2+}]_i$ is completely unaffected in the presence of Ca^{2+} channel blockers (Hochstrate & Schlue, 1994; Dierkes et al., 1996). Finally, kainate causes an intracellular and extracellular acidosis (Dörner, 1991; Schlue et al., 1991; Rose & Deitmer, 1995b). The results presented so far strongly suggest that the kainate-induced pH shifts feed back on the kainate-induced $[Ca^{2+}]_i$ increase.

Raising the HEPES concentration and hence the extracellular pH-buffering capacity reduced the kainate-induced extracellular acidification by about 40 % and enhanced the $[Ca^{2+}]_i$ increase by 60 % (Figs. 9, 10). These effects were paralleled by an augmented membrane depolarization. The addition of sucrose plus NaCl during kainate exposure did not affect the kainate-induced extracellular acidification, but it also caused an additional increase in $[Ca^{2+}]_i$ as well as a membrane depolarization. However, $[Ca^{2+}]_i$ increased significantly less (+ 25 %) than after raising the HEPES concentration, while the membrane depolarization was even slightly larger. Both HEPES and sucrose plus NaCl left pH_i virtually unaffected. The results strongly suggest that raising the HEPES concentration enhanced the kainate-induced $[Ca^{2+}]_i$ increase predominantly by reducing the extracellular acidification.

Discussion

Modulation of Ca^{2+} Influx by pH_o

The results show that pH_B markedly affected the $[Ca^{2+}]_i$ of leech Retzius neurons, provided that the cells were depolarized by raising $[K^+]_B$ or by applying the glutamatergic agonist kainate. In the depolarized state, $[Ca^{2+}]_i$ is increased, which is exclusively due to the activation of voltage-dependent Ca^{2+} channels (Hochstrate & Schlue, 1994; Hochstrate et al., 1995; Dierkes et al., 1996, 1997). Since $[Ca^{2+}]_i$ approaches a new steady state far below the electrochemical equilibrium, the continuous influx of Ca^{2+} must be compensated by active Ca^{2+} extrusion. A modulation of Ca^{2+} extrusion by pH (*see* Xu & Roufogalis, 1988; Milanick, 1990) is unlikely to contribute to the observed $[Ca^{2+}]_i$ changes, because the recovery from a K^+-induced $[Ca^{2+}]_i$ increase, which essentially reflects Ca^{2+} extrusion, was unaffected by pH_B (Fig. 1*A*). Furthermore, a contribution of cytosolic Ca^{2+} buffering or sequestration by intracellular compartments appears to be unlikely, because shifts in pH_i caused by changing pH_B or application of propionate or NH_4^+ had virtually no effect on $[Ca^{2+}]_i$ in physiological solution (Figs. 1, 6) and probably also at raised $[K^+]_B$ (*see* below). We therefore conclude that the changes in $[Ca^{2+}]_i$ caused by varying pH_o are mainly due to a modulation of Ca^{2+} influx through the voltage-dependent Ca^{2+} channels. This conclusion is supported by the result that the modulatory effect of pH_B was the larger, the more $[Ca^{2+}]_i$ was increased, i.e., the more Ca^{2+} channels were activated (Fig. 2*A*).

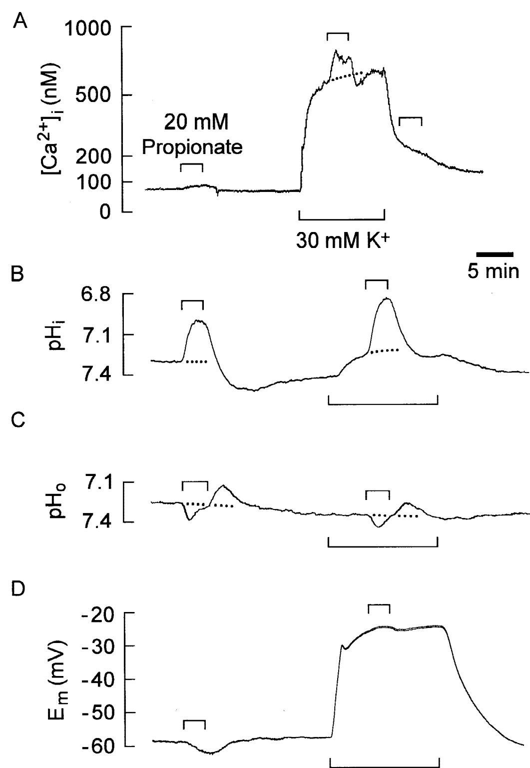

Fig. 6. Effect of propionate on $[Ca^{2+}]_i$, pH_o, pH_i, and E_m in physiological solution and after raising $[K^+]_B$. pH_i and E_m were recorded simultaneously in the same cell by using a double-barrelled pH-sensitive microelectrode; the recordings of $[Ca^{2+}]_i$ and pH_o were performed in different preparations. Dotted lines give the putative course of the traces without propionate application. These lines were constructed with the example of recordings in which the application of propionate was omitted and were used for a quantitative estimation of the propionate effect shown in Fig. 7 (*see* Materials and Methods).

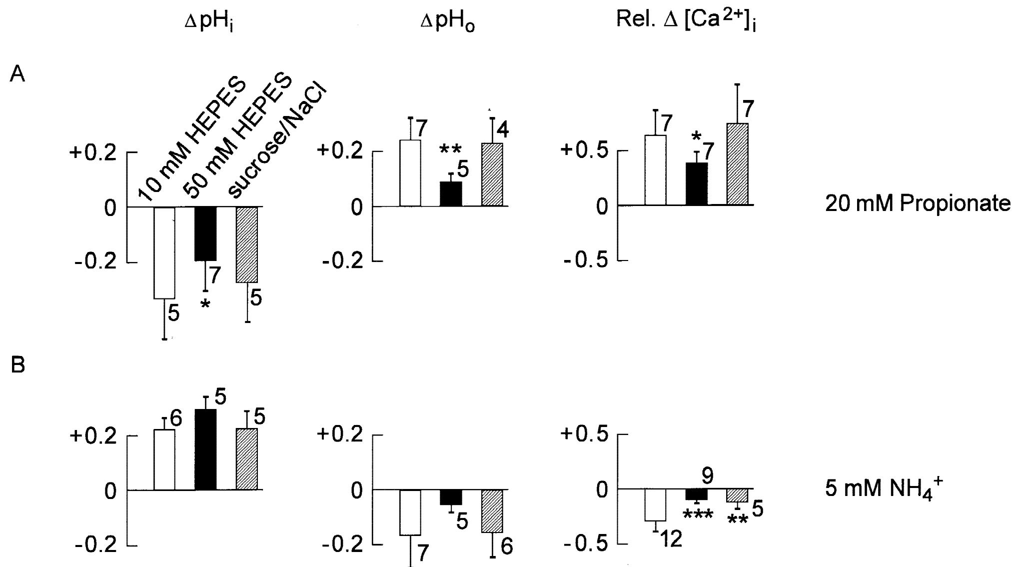

Fig. 7. Effect of the extracellular pH-buffering capacity on the action of propionate (*A*) and NH_4^+ (*B*) on pH_i, pH_o, and $[Ca^{2+}]_i$ at $[K^+]_B = 30$ mM. Open bars: normal 30 mM K^+ solution (10 mM HEPES); black bars: 5 min after raising the extracellular pH-buffering capacity (50 mM HEPES); hatched bars: 5 min after adding 30 mM sucrose plus 15 mM NaCl. Data are the mean (± SD) of $n = 4$ to 7 experiments; asterisks mark data that deviate significantly from those obtained in normal 30 mM K^+ solution ($p < 0.05$, two-tailed *t*-test; 2 or 3 asterisks, $p < 0.01$ or 0.001).

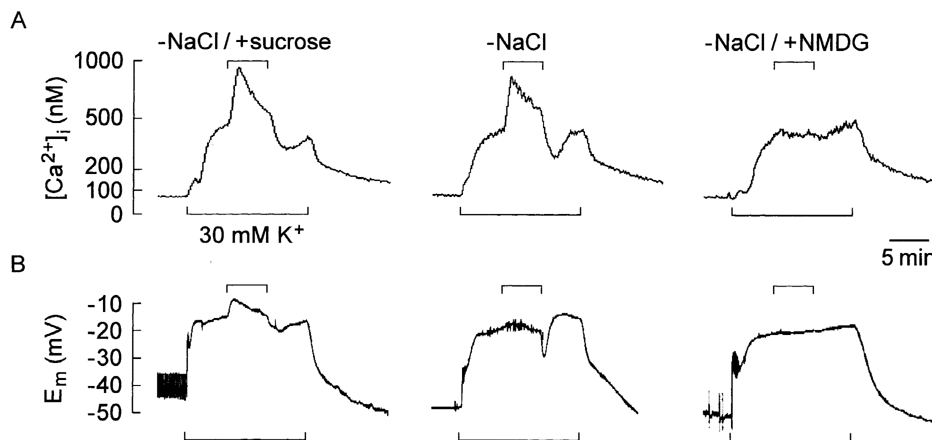

Fig. 8. Effect of reducing ion strength and/or osmolarity on the K^+-induced $[Ca^{2+}]_i$ increase (*A*) and on the K^+-induced membrane depolarization (*B*). Left: replacement of extracellular NaCl by sucrose (118 mM); middle: omission of extracellular NaCl (59 mM); right: replacement of extracellular NaCl by the chloride salt of N-methyl-D-glucamine.

Role of pH_i in the Control of Ca^{2+} Influx

The application of propionate or NH_4^+ caused marked changes in $[Ca^{2+}]_i$, which were strongly reduced by raising the extracellular pH-buffering capacity and thus attenuating the concomitant shifts in pH_o (Figs. 6, 7). However, this result does not exclude a role of pH_i in the control of Ca^{2+} influx, because raising the extracellular pH-buffering capacity also reduced the propionate-induced intracellular acidification. Furthermore, the reduction of the NH_4^+-induced drop in $[Ca^{2+}]_i$ after raising the pH-buffering capacity was mimicked by raising extracellular osmolarity and ionic strength (Fig. 7). An effect of pH_i is suggested by the result that the $[Ca^{2+}]_i$ changes per pH_o unit evoked by propionate were considerably larger than those caused by varying pH_B, as shown in Fig. 11*A* which summarizes the data presented in Figs. 2, 7, and 10. Nevertheless, three observations argue against a significant role of pH_i in the control of Ca^{2+} influx, at least within the range that was covered experimentally. First, when the pH-buffering capacity was raised, the propionate-induced shift in pH_i was markedly reduced (Fig. 7*A*), but the change in $[Ca^{2+}]_i$ relative to that in pH_o was unaffected (Fig. 11*A*). Second,

the $[Ca^{2+}]_i/pH_o$ relationship was also relatively steep when the pH_o changes were caused by application of NH_4^+. Since NH_4^+ and propionate shift pH_i in the opposite direction, it appears improbable that the steepness of the $[Ca^{2+}]_i/pH_o$ relationships is due to changes in pH_i. Third, raising the pH-buffering capacity during kainate exposure left pH_i almost unaffected (Figs. 9, 10), but the $[Ca^{2+}]_i$ increase per pH_o unit was similar to that caused by propionate or NH_4^+ (Fig. 11).

The apparently different effect of pH_o on Ca^{2+} influx depending on the experimental conditions might be explained by a different relationship between the measured pH_o and the pH close to the cell membrane (pH_M), which is relevant to membrane transport. As illustrated in Fig. 11*B,* the changes in pH_M caused by shifting pH_B are expected to be smaller than the changes in pH_o. Conversely, the pH_M changes evoked by propionate, NH_4^+, or kainate, which are due to the transport of acid-base equivalents across the plasma membrane, should be larger than the changes in pH_o. Consequently, the modulatory effect of extracellular pH on Ca^{2+} influx is larger than estimated from shifting pH_B and smaller than suggested by the pH_o changes caused by propionate, NH_4^+, or kainate.

That pH_o but not pH_i modulates the Ca^{2+} influx through voltage-dependent Ca^{2+} channels has also been demonstrated in various vertebrate neurons (Ou-Yang et al., 1994; Zhou & Jones, 1996; Tombaugh & Somjen, 1996; Church, 1999), but there are also reports demonstrating a modulatory effect of pH_i (Mironov & Lux, 1991; Takahashi et al., 1993; Tombaugh & Somjen, 1997; Kiss & Korn, 1999). The different sensitivity to pH_i may be cell-specific and depend on the prevailing type of Ca^{2+} channel. Thus, N-type Ca^{2+} channels are particularly pH_i-sensitive, while the pH_i sensitivity of L-type channels is moderate and that of T-type channels is virtually absent (Tombaugh & Somjen, 1997; Kiss & Korn, 1999). The Ca^{2+} channels of leech neurons seem to be akin to vertebrate L-type channels (Hochstrate et al., 1995; Dierkes, 1998), and hence a pronounced pH_i dependence is not to be expected.

Possible Mechanism of Ca^{2+} Channel Modulation

The pH effects on Ca^{2+} channel function observed in various preparations have often been attributed to shifts in the surface potential of the cell membrane (McLaughlin, 1977; 1989), which affect channel gating by changing the potential drop across the channels' voltage sensor. Since extracellular acidification shifts the surface potential at the outer membrane side in the positive direction, the cells must become more depolarized to achieve channel gating, and at a given E_m fewer Ca^{2+} channels may be activated. Conversely, extracellular alkalinization shifts the surface potential in the negative direction, thereby facilitating Ca^{2+} channel activation. This mechanism would explain the effect of pH_B on $[Ca^{2+}]_i$ (Figs. 1, 2, 11), as well as the shift of the $[Ca^{2+}]_i/E_m$ relationship (Figs. 4, 5). The significance of the extracellular surface potential is supported by the effect of reducing the extracellular ionic strength, which shifts the surface potential in the negative direction and hence facilitates Ca^{2+} influx (Fig. 8). It is noted that the charges generating the surface potential need not be restricted to the head groups of the membrane lipids but may also be located on the channel protein (*see* Hille, 1992). The absence of an effect of pH_i on Ca^{2+} influx implies that in Retzius neurons the charge density at the inner membrane surface is much lower than at the outer surface. Other mechanisms might participate in the modulation of Ca^{2+} influx, such as proton block by interaction with a site along the permeation pathway (Chen et al., 1996) or proton binding to a regulatory binding site affecting channel gating or ion permeation (Prod'hom et al., 1989; Klöckner & Isenberg, 1994). In particular, the modulatory effect of pH_i on Ca^{2+} influx found in various vertebrate neurons seems to be due to allosteric effects,

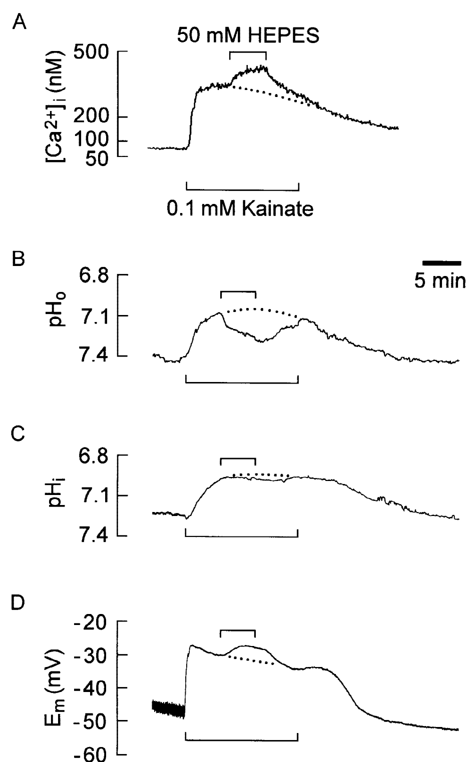

Fig. 9. Effect of raising the extracellular pH-buffering capacity (50 mM HEPES) on the changes in $[Ca^{2+}]_i$, pH_o, pH_i, and E_m caused by 0.1 mM kainate. Recordings of pH_o, pH_i, and E_m were performed in the same ganglion by using double-barrelled pH-sensitive microelectrodes; the $[Ca^{2+}]_i$ recording was made in a different preparation. Dotted lines give the putative course of the traces without raising the HEPES concentration (see Fig. 6).

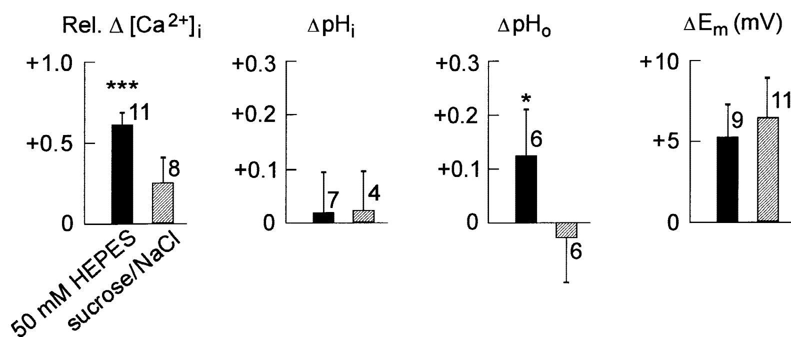

Fig. 10. Changes in $[Ca^{2+}]_i$, pH_i, pH_o, and E_m caused by raising the extracellular pH-buffering capacity during kainate exposure (0.1 mM). Black bars: effect of raising the extracellular pH-buffering capacity (50 mM HEPES); hatched bars: effect of adding 30 mM sucrose plus 15 mM NaCl. Data are the mean (± SD) of $n = 4$ to 11 experiments; asterisks mark significant differences (*see* Fig. 7).

because the voltage dependence of channel activation was not or only slightly changed (Mironov & Lux, 1991; Tombaugh & Somjen, 1997; Kiss & Korn, 1999). Furthermore, the extracellular pH may affect the Ca^{2+} concentration near the mouth of the channel pore and hence the ion flux through the pore (*see* Green & Andersen, 1991; Zhou & Jones, 1996). However, such changes in Ca^{2+} permeation appear to be overwhelmed by surface potential effects, since raising the Ca^{2+} concentration of the bathing solution did not increase $[Ca^{2+}]_i$ but reduced it (Hochstrate et al., 2001; *see* Baldridge, Kurennyi & Barnes, 1998).

The traces in Figs. 1*A,* 6, and 8 show that $[Ca^{2+}]_i$ was persistently increased at raised $[K^+]_B$, which indicates that the voltage-dependent Ca^{2+} channels were constantly activated over many minutes (*see* Hochstrate et al., 1995, Dierkes et al., 1997). This persistent activity does not exclude changes in channel activity due to Ca^{2+}-dependent or voltage-dependent inactivation, but these changes occur within the subsecond scale and were not resolved under the experimental conditions used here (*see* Stewart et al., 1989; Bookman & Liu, 1990).

Virtually all voltage-dependent ion channels are likely to be affected by the surface potential, although the effect on Na^+ and K^+ channels is probably much smaller (*see* Tombaugh & Somjen, 1997). Furthermore, the modulation of the Ca^{2+} channels will presumably affect Ca^{2+}-dependent ion channels (Yang et al., 1987; Johansen & Kleinhaus, 1988; Stewart et al., 1989). Therefore, changes in the extracellular pH will alter the flux of many ion species across the plasma membrane. However, the overall ion selectivity of the plasma membrane seemed to be not significantly changed, because E_m was hardly affected by pH_B.

NEGATIVE FEEDBACK OF KAINATE-INDUCED ACIDOSIS ON Ca^{2+} INFLUX

In Retzius neurons, both kainate and elevation of $[K^+]_o$ induce an increase in $[Ca^{2+}]_i$ due to the activation of voltage-dependent Ca^{2+} channels (Hochstrate & Schlue, 1994; Hochstrate et al., 1995). However, the $[Ca^{2+}]_i$ increase caused by 0.1 mM kainate was only about half as large as that caused by raising $[K^+]_B$ to 30–40 mM, although the membrane depolarization was the same (Dierkes et al., 1996). The results presented here strongly suggest that this discrepancy is due to the different effect of kainate and extracellular K^+ on pH_o.

Both kainate and extracellular K^+ induce a comparable intracellular acidification by about 0.2 pH units (Figs. 3*B*, 6*B*, 9*C;* Dörner, 1991; Frey & Schlue, 1993; Kilb & Schlue, 1999). However, kainate also induces a marked extracellular acidification by about 0.3 pH units (Figs. 9*B*, 11; Dörner, 1991; Rose & Deitmer, 1995b), whereas pH_o was hardly affected by raising $[K^+]_B$ (Figs. 3*A*, 6*C*). The kainate-induced intracellular acidification is triggered by the increase in the cytosolic Na^+ concentration (Kilb & Schlue, 1999); the mechanism of the K^+-induced acidification is unknown (*see* Frey & Schlue, 1993). The kainate-induced extracellular acidification may result from H^+ transport via Na^+-H^+ exchange as well as from the generation of CO_2 (*see* Rose & Deitmer, 1995b). That pH_o was virtually unaffected by raising $[K^+]_o$ although the cytosolic acidification of the Retzius neurons was similar as upon kainate application, may have two reasons: 1) pH_o is not controlled primarily by the neurons but by the glial cells. Leech glial cells acidify upon kainate application (Dörner, 1991; Deitmer & Schneider, 1997), but become alkaline upon raising $[K^+]_o$ (Ballanyi & Schlue, 1989; Deitmer & Szatkowski, 1990). Consequently, in the presence of kainate, but not upon raising $[K^+]_o$, the glial cells would extrude H^+ and hence cause an extracellular acidification. 2) Due to the marked Na^+ influx metabolic activation and hence CO_2 generation might be more pronounced upon kainate application than upon raising $[K^+]_o$.

The data in Fig. 10 suggest that about two-thirds of the $[Ca^{2+}]_i$ increase caused by raising the extracellular pH-buffering capacity during kainate exposure was due to the reduction of the extracellular acidification, while one-third was caused by the enhanced membrane depolarization. The extracellular acidification was reduced by 40%, and this reduction enhanced the kainate-induced $[Ca^{2+}]_i$ increase by 40%, as estimated by correcting the

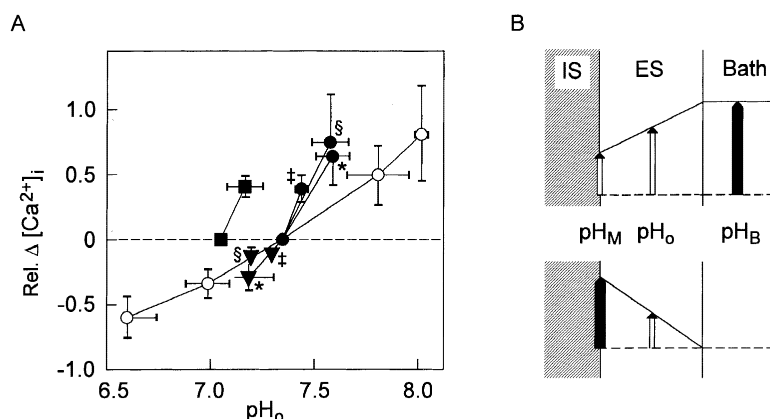

Fig. 11. Relationship between the changes in $[Ca^{2+}]_i$ and those in pH_o measured under different experimental conditions. (*A*) Open circles: pH_o changed by shifting pH_B to 6.2, 6.8, 8.0, or 8.6 at $[K^+]_B$ = 20, 30, or 40 mM (same data as in Fig. 2). Filled circles: application of 20 mM propionate at $[K^+]_B$ = 30 mM, either at normal (*) or at raised HEPES concentration (50 mM;|), or after adding 30 mM sucrose plus 15 mM NaCl (§); filled triangles: analogous experiments performed with 5 mM NH_4^+ (same data as in Fig. 7). The pH_o before shifting pH_B or application of propionate or NH_4^+ was taken to be identical with that upon superfusion with physiological solution (7.36 ± 0.07, n = 23; *see* Fig. 1*B*). Black squares identify the $[Ca^{2+}]_i$ increase upon raising the HEPES concentration to 50 mM in the presence of 0.1 mM kainate. During kainate exposure, pH_o dropped by 0.30 ± 0.10 pH units (n = 11), and this acidification was partially reversed by raising the HEPES concentration. The concomitant $[Ca^{2+}]_i$ increase was corrected for the depolarization-induced portion in order to obtain the pH_o-induced effect (*see* Fig. 10). (*B*) Schematic drawing illustrating the putative relationship between the pH_o measured under different experimental conditions by pH-sensitive microelectrodes and the pH close to the cell membrane (pH_M; ES, IS: extracellular, intracellular space). *Top:* changing pH_B (black arrow) causes a shift in pH_o which is smaller than that in pH_B (*see* Fig. 1), and hence the shift in pH_M will be smaller than that in pH_o. *Bottom:* the shift in pH_M caused by transport of acid/base equivalents across the cell membrane upon application of propionate, NH_4^+, or kainate is expected to be larger than that in pH_o. For the sake of simplicity it was assumed that there was no pH difference between bath and extracellular space in the resting state (broken line) and that the experimentally induced pH gradients within the extracellular space were linear.

total HEPES-induced $[Ca^{2+}]_i$ increase for the depolarization-induced portion. Correspondingly, the complete suppression of the kainate-induced extracellular acidification is expected to double the kainate-induced $[Ca^{2+}]_i$ increase and hence to abolish the discrepancy between kainate-induced and K^+-induced $[Ca^{2+}]_i$ increase.

Physiological Implications

The modulation of Ca^{2+} influx by pH_B may be functionally significant, since it occurs within the physiological pH range (Fig. 2). Furthermore, changes in pH_o (and pH_i) have been recorded in the leech nervous system upon neuronal activity evoked either by application of neurotransmitters or by electric stimulation (Dörner, 1991; Rose & Deitmer, 1995a, b). Neuronal excitation in the leech nervous system is accompanied by a sudden alkalinization of the extracellular space, which changes to an acidification that declines within a few minutes after stimulus cessation. Correspondingly, the influx of Ca^{2+} should be enhanced at the onset of neuronal activity, and then decrease. In the neuropil, the pH_o changes recorded during electrical stimulation were 0.3 pH units at maximum (Rose & Deitmer, 1995a), which is sufficient to affect Ca^{2+} influx significantly (*see* Figs. 1, 2), but these changes may be underestimated due to the limited time resolution of the pH-sensitive microelectrodes, the distance of the electrode tip from the membrane surface, and the putative perturbation of the tissue structure. The physiological changes in pH_o might be considerably larger and faster than experimentally recorded (*see* Gottfried & Chesler, 1996) and hence contribute strongly to the control of Ca^{2+} influx during neuronal activity.

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